PALACKÝ UNIVERSITY IN OLOMOUC
FACULTY OF SCIENCE
DEPARTMENT OF BIOPHYSICS
CENTRE OF THE REGION HANÁ FOR BIOTECHNOLOGICAL AND AGRICULTURAL RESEARCH

EFFECT OF CYTOKININS ON MAINTENANCE OF PHOTOSYSTEM II FUNCTION DURING INDUCED SENESCENCE

Autoreferát

Helena Janečková

Olomouc 2019

Meno a priezvisko studenta:	Mgr. Helena Janečková (rod. Melkovičová)
Oddelenie:	Katedra biofyziky, Přírodovědecká fakulta Univerzity Palackého v Olomouci
Študijní program:	Fyzika – obor Biofyzika
Názov práce slovensky:	Vplyv cytokinínov na udržanie funkcie fotosystému II v priebehu indukovanej senescencie
Školiteľ:	RNDr. Martina Špundová, Ph.D.
Oponenti práce:	**prof. RNDr. Břetislav Brzobohatý, CSc.** Ústav molekulární biologie a radiobiologie Mendelova univerzita v Brně **prof. Ing. Miloš Barták, CSc.** Ústav experimentální biologie Přírodovědecká fakulta, Masarykova univerzita
Miesto a termín obhajoby:	**.....**

S dizertačnou prácou a posudkami je možné sa oboznámiť na: Katedra Biofyziky, Přírodovědecká fakulta Univerzity Palackého v Olomouci.

Declaration

I hereby declare that I have written this text independently as the orginal work, with the help of my supervisor RNDr. Martina Špundová, Ph.D. The complete list of used literature and other information sources is included in the section “References”.

Olomouc, Czech Republic, 2019 Author

Abstract

Leaf senescence, the final stage of leaf development, is a complex process that involves numerous physiological and biochemical changes. Senescence is usually accompanied by a loss of chlorophyll (Chl) and by inhibition of photosynthetic processes, including photosystem (PSII) photochemistry. Plant hormones cytokinins (CKs) are negative regulators of senescence. It is well known that in detached leaves, both exogenously applied CKs and light retard senescence, however, the mechanism of their mutual interaction is not clear. We have analysed changes in content of endogenous CKs and individual CK forms in detached leaves of *Arabidopsis thaliana* kept under different light conditions and we have correlated these changes with changes in chlorophyll content, efficiency of PSII photochemistry, and lipid peroxidation. We have shown that the content of endogenous CKs and individual CK forms in detached *Arabidopsis* leaves differs significantly under various light conditions. In leaves kept in darkness, we have observed decreased content of the most abundant CK free bases and ribosides. We have suggested that the light-mediated retardation of senescence in detached *Arabidopsis* leaves is related to light-dependent persistence of N^6-(Δ^2-isopentenyl)adenine CK biosynthesis during senescence. We have also found that light was able to partially compensate the disrupted CK signalling caused by the loss-of-function mutation in CK receptors during senescence of detached leaves of *Arabidopsis* mutants. Recent studies have shown that Chl *b* is important for the regulation of senescence. We have found that the deficiency of Chl *b* in barley mutant significantly accelerates the inhibition of PSII photochemistry during dark-induced senescence of detached leaves. We suppose that the stronger impairment of PSII function is associated with a more pronounced damage of reaction centre of PSII. Exogenously applied CKs were able to eliminate the acceleration of PSII impairment, probably by the stabilization of reaction centres of PSII.

Key words: cytokinins, light, photosynthesis, photosystem II, senescence

Abstrakt

Listová senescencia, konečné štádium vývoja listov, je komplexný proces, ktorý zahŕňa množstvo fyziologických a biochemických zmien. Senescencia je zvyčajne sprevádzaná poklesom obsahu chlorofylu a inhibíciou fotosyntetických procesov, vrátane fotochémie fotosystému II (PSII). Rastlinné hormóny cytokiníny (CK) sú známe ako negatívne regulátory starnutia. V oddelených listoch je dobre známe, že exogénne aplikované CK ako i svetlo spomaľujú priebeh senescencie, avšak mechanizmus ich vzájomnej interakcie pri sprostredkovaní tohto účinku zostáva nejasný. Analyzovali sme zmeny v obsahu endogénnych CK a jednotlivých CK foriem v oddelených listoch *Arabidopsis thaliana* inkubovaných v rôznych svetelných podmienkach a tieto zmeny sme korelovali so zmenami v obsahu chlorofylu, účinnosti fotochémie PSII a peroxidácii lipidov. Ukázali sme, že v oddelených listoch *Arabidopsis* inkubovaných pod rôznymi svetelnými podmienkami sa obsah endogénnych CK a ich jednotlivých foriem výrazne líši. V listoch inkubovaných v tme sme pozorovali znížený obsah najhojnešie sa vyskytujúcich CK báz a ribosidov. Navrhli sme, že spomalenie senescencie vyvolaná svetlom u oddelených listov *Arabidopsis* môže súvisieť s pretrvávajúcou biosyntézou N^6-(Δ^2-isopentenyl)adenínu. Ukázali sme, že počas senescencie oddelených listov *Arabidopsis* je svetlo schopné nahradiť CK signál v prípade porušenej signálnej dráhy kvôli nefunkčnosti CK receptorov. Nedávne štúdie ukázali, že chlorofyl (Chl) *b* má tiež dôležitú úlohu v regulácii senescencie. Zistili sme, že deficit Chl *b* u mutanta jačmeňa výrazne zrýchľuje inhibíciu fotochémie PSII počas indukovanej senescencie oddelených listov inkubovaných v tme. Predpokladáme, že silnejšie poškodenie funkcie PSII súvisí s výraznejším poškodením reakčných centier PSII. Exogénne aplikovaný CK eliminoval urýchlenie poškodenia PSII, pravdepodobne stabilizáciou reakčných centier PSII.

Kľúčové slová: cytokininy, fotosyntéza, fotosystém II, senescence, svetlo

Contents

List of Publications

This thesis is based on two papers, referred in the text by name of the first author.

I. **Janečková H (29 %)**, Husičková A, Ferretti U, Prčina M, Pilařová E, Plačková L, Pospíšil P, Doležal K, Špundová M. 2018. The interplay between cytokinins and light during senescence in detached Arabidopsis leaves. Plant Cell and Environment 41: 1870–1885. IF (2017): 6.173
(*Janečková H designed the final experiments, performed chlorophyll content assay and chlorophyll fluorescence measurement and wrote the manuscript*)

II. **Janečková H (30 %)**, Husičková A, Lazár D, Ferretti U, Pospíšil P, Špundová M. 2019. Exogenous application of cytokinin during dark senescence eliminates the acceleration of photosystem II impairment caused by chlorophyll b deficiency in barley. Plant Physiology and Biochemistry 136: 43–51. IF (2017): 2.718
(*Janečková H designed and performed the experiments, analyzed the data, interpreted results and wrote the manuscript*)

Paper by the same author that is not included in the thesis:

III. Ilík P, Špundová M, Šicner M, **Melkovičová H (5 %)**, Kučerová Z, Krchňák P, Fürst T, Večeřová K, Panzarová K, Benediktyová Z, Trtílek M. 2018. Estimating heat tolerance of plants by ion leakage: A new method based on gradual heating. New Phytologist 218: 1278–1287.IF (2017): 7.43

Abbreviations

AHK - Arabidopsis histidine kinase
AHP - Arabidopsis histidine phosphotransfer protein
ARR - Arabidopsis response regulator
BA - N^6-benzylaminopurine
car - carotenoids
Chl - chlorophyll
clo - *chlorina f2*^{f2} (barley mutant deficient in Chl *b*)
CK(s) - cytokinin(s)
CKX - cytokinin oxidase/dehydrogenase
*c*Z - *cis*-zeatin
F_V/F_M - maximal quantum yield of photosystem II photochemistry in the dark-adapted state
iP - N^6-(Δ^2-isopentenyl)adenine
IPT - isopentenyltransferase
Lhca - light-harvesting chlorophyll *a/b*-binding protein of photosystem I
Lhcb - light-harvesting chlorophyll *a/b*-binding protein of photosystem II
LHC(s) - light harvesting complex(es)
MDA - malondialdehyde
*m*T - *meta*-topolin
PSI - photosystem I
PSII - photosystem II
RCI - reaction center of photosystem I
RCII - reaction center of photosystem II
ROS - reactive oxygen species
Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase
*t*Z - *trans*-zeatin
WT - wild-type
$\Phi_{f,D}$ - quantum yield of constitutive non-regulatory dissipation processes in the light-adapted state
Φ_{NPQ} - quantum yield of regulatory non-photochemical quenching in the light-adapted state
Φ_P - effective quantum yield of photosystem II photochemistry in the light-adapted state
Φ_{PSII} - maximal quantum yield of photosystem II photochemistry in the light-adapted state

1 INTRODUCTION

The main topic of the dissertation thesis is leaf senescence, a very complex developmental process characterized by numerous alterations of cell structure, metabolism and gene expression. In particular, the thesis is focused on the involvement of plant hormones cytokinins and light in the regulation of this process. During leaf senescence, the nutrients are remobilized from mature leaves to other developing parts of the plant (such as seeds or young leaves), which ensures either the survival of the plant itself (polycarpic plants), or the survival of next plant generations (monocarpic plants). However, premature senescence, induced for example by stress conditions, can limit the growth phase of plants and is therefore connected with reduced yield in crops. It can also cause post-harvest problems, such as leaf yellowing or nutrient loss in vegetable crops. Thus, understanding the proper mechanism of leaf senescence and its regulation has big potential implications for agriculture.

1.1 Leaf senescence

Leaf senescence involves inhibition of various cellular processes, decrease of photosynthetic pigment contents and hydrolysis of macromolecules such as proteins, lipids etc. (e.g. Buchanan-Wollaston et al., 2003). Chloroplast are the first organelles to show the symptoms of senescence. During leaf senescence, the amount and size of chloroplasts become reduced and their shape is changed from nearly oval to more spherical. The inner components are degraded, thylakoid membranes are disintegrated and chloroplasts gradually transform into gerontoplasts (Biswal et al., 2012). Membrane disorganization in chloroplasts is sequential and starts with the unstacking of granal thylakoids, followed by the formation of loose and elongated lamellae. These loose lamellae subsequently undergo massive degradation with concomitant formation of plastoglobules (e.g. Biswal, 1997; Biswal et al., 2012).

1.1.1 Protein degradation and chlorophyll breakdown pathway during leaf senescence

The chloroplast proteins are degraded through the action of proteolytic enzymes (proteases) (e.g. Biswal et al., 2012) and the degradation may occur via chloroplastic or/and extra-chloroplastic pathways (for a review, see e.g. Martínez et al., 2008).

Pigment-protein complexes gradually degrade during leaf senescence (e.g. Mae et al., 1993; Tang et al., 2005; Nath et al., 2013), whereas the rate of the degradation of individual complexes is different. Compared to the proteins in stroma, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the membrane proteins localized in thylakoid membranes are more difficult targets for degradation (Grover, 1993; Biswal, 1997; Biswal et al., 2012). Thus, the decrease in the activity and content of Rubisco usually precedes the inhibition of primary photosynthetic reactions and loss of thylakoid proteins (Camp et al., 1982; Grover 1993; Mae et al., 1993; Sobieszczuk-Nowicka et al., 2018).

Various studies indicate that during leaf senescence, proteins of light harvesting complexes (LHCs) are more stable than the proteins of reaction centres (RCs) (e.g. Park et al., 2007; Nath et al., 2013; Sakuraba et al., 2013). As chlorophyll (Chl) *a* is associated with both RC and LHCs in higher plants, whereas Chl *b* is specific only to LHCs, the changes in Chl *a/b* ratio can be used as a simple estimate of the changes in the relative amount of LHCs and RCs (Leong and Anderson, 1984; Tanaka and Tanaka, 2011). In numerous studies, leaf senescence in darkness was reported to be accompanied by a decrease in this ratio, indicating that RCs are degraded faster than LHCs (e.g. Hidema et al., 1991; Kusaba et al., 2007, Nath et al., 2013; **Janečková et al., 2019**).

Chl degradation is carried out by specific enzymes and involves several steps (for review see e.g. Kuai et al., 2018). Chl degradation is a prerequisite for LHC degradation (e.g. Tanaka and Tanaka, 2011; Kuai et al., 2018). The gradual loss of Chl during leaf senescence, as well as the degradation of structural components of photosynthetic apparatus, is generally accompanied by a decrease in photosynthetic activity. Photochemical efficiency of photosystem II (PSII) (e.g. Špundová et al., 2003; 2005*a*; Nath et al., 2013; Krieger-Liszkay et al., 2015; **Janečková et al., 2018, 2019**) as well as photosystem I (PSI) (Nath et al., 2013; Krieger-Liszkay et al., 2015; **Janečková et al. 2019**) declines during leaf senescence. However, from the data presented in the literature, it is not clear whether the decrease in photosynthetic activity of PSII precedes the inhibition of PSI or vice versa (e.g. Nath et al., 2013; Krieger-Liszkay et al., 2015).

Due to the impairment of photosynthetic apparatus during leaf senescence, the light energy cannot be properly utilized by photochemistry and the activity of non-photochemical processes increases (e.g. Lu and Zhang, 1998; Lu et al., 2001; Wingler et al., 2004; **Janečková et al., 2019**). The excess light energy is dissipated via regulatory non-photochemical quenching processes, which protect photosynthetic apparatus against photooxidative damage and/or non-regulatory quenching processes. The regulatory non-photochemical quenching processes are related to the activation of the xanthophyll cycle, which dissipates excess of light energy as heat in antenna complexes before it reaches RCs (Demmig-Adams et al., 2014). Violaxanthin (V) is converted to zeaxanthin (Z) by de-epoxidation catalyzed by violaxanthin de-epoxidase, through the intermediate antheraxanthin (A) (e.g. Lu et al., 2001; Demmig-Adams, 2014). Z then stimulates non-photochemical quenching within LHC. The extent of the de-epoxidation is expressed as the de-epoxidation state of xanthophylls (DEPS = (A + Z)/(A + Z + V)), which usually increases in senescing leaves (Lu et al., 2001; Špundová et al., 2005*b*; Vlčková et al., 2006).

To evaluate the partitioning of absorbed light energy for photochemical and non-photochemical processes in the light-adapted state, the following parameters can be used: the effective quantum yield of PSII photochemistry (Φ_P), quantum yield of constitutive non-regulatory dissipation processes ($\Phi_{f,D}$) and quantum yield of regulatory non-photochemical quenching (Φ_{NPQ}) (for a review, see Lazár, 2015). $\Phi_{f,D}$ represents quantum yield of constitutive (basal) energy dissipation, whereas Φ_{NPQ} is quantum yield of regulatory quenching, which is

induced by illumination to protect the photosynthetic apparatus against excess light and consequent accumulation of reactive oxygen species and oxidative damage (Demmig-Adams et al., 2014). Together, the sum of these quantum yields equals unity (Lazár, 2015).

Unlike in non-senescing leaves, where the majority of absorbed light energy utilized by PSII photochemistry, in dark-senescing leaves the absorbed light energy is, to a great extent, allocated into non-photochemical quenching processes (**Janečková et al., 2019**; Fig. 1). In senescing leaves of WT, energy non-utilized by PSII photochemistry was dissipated in both regulatory and non-regulatory processes to a similar extent (comparable Φ_{NPQ} and $\Phi_{f,D}$). However, in senescing leaves of *chlorina f2*^{f2} mutant (*clo*) (see 1.1.3 Chlorophyll *b* and leaf senescence), which had greatly impaired PSII photochemistry, the non-regulatory processes ($\Phi_{f,D}$) pronouncedly prevailed (Fig. 1). Thus, the ability to dissipate energy via regulatory processes of non-photochemical quenching declined during later stages of senescence, and when the photosynthetic apparatus was almost completely inactivated, the non-regulatory processes prevailed (**Janečková et al., 2019**).

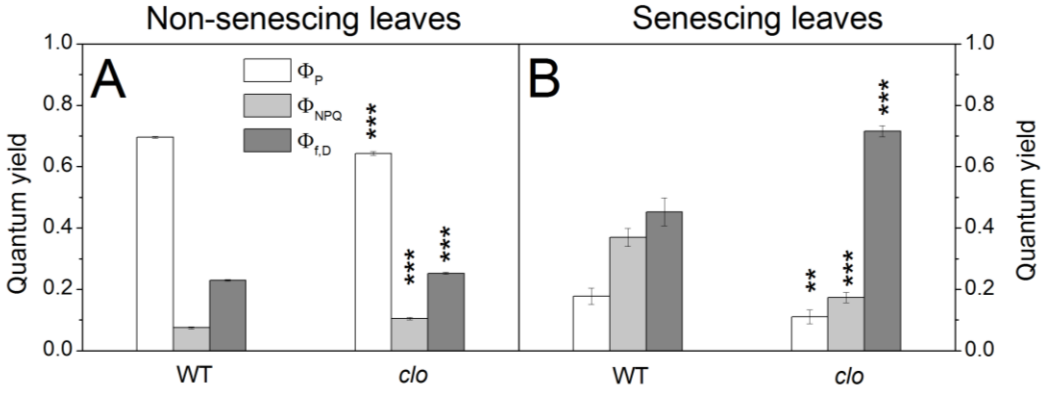

Fig. 1: Alterations in partitioning of absorbed light energy for photochemical and non-photochemical processes in (A) non-senescing and (B) dark senescing leaves of barley (wild-type, WT and *chlorina f2*^{f2} mutant, *clo*). The effective quantum yield of PSII photochemistry (Φ_P), quantum yield of regulatory non-photochemical quenching (Φ_{NPQ}) and quantum yield of constitutive non-regulatory dissipation processes ($\Phi_{f,D}$) in the light-adapted state. Means and SD are shown, n = 6. Asterisks indicate statistically significant difference (Student´s t-test) between WT and *clo* (**, P < 0.01; ***, P < 0.001). Adapted from **Janečková et al. (2019)**.

1.1.2 Light and senescence

Light plays an essential role in the regulation of senescence and its effect depends on intensity, length of photoperiod and spectral quality (e.g. Biswal and Biswal, 1984; Noodén et al., 1996; Marchetti et al., 2018).

Light deprivation, severe shade or darkness usually enhance senescence (Weaver and Amasino, 2001; Špundová et al., 2005*a*; Liebsch and Keech, 2016; **Janečková et al., 2018**). Leaf senescence of darkened detached leaves is characterized by extensive loss of photosynthetic

pigments (e.g. Oh et al., 2003; Vlčková et al., 2006; Kusaba et al., 2007; Park et al., 2007; **Janečková et al., 2019**) and pigment-protein complexes (e.g. Kusaba et al., 2007; Park et al., 2007), as well as by a decrease in photosynthetic activity (Oh et al., 2003; Špundová et al., 2003; Vlčková et al., 2006; Kusaba et al., 2007; **Janečková et al., 2019**).

In light, senescence-induced changes are usually reduced when compared to darkness (Biswal and Biswal, 1984; Okada et al., 1992; Klerk et al., 1993; Weaver et al., 1998; Špundová et al., 2003; 2005*a*; **Janečková et al., 2018**). Detached leaves senescing under light usually maintain higher content of photosynthetic pigments (e.g. Thimann et al., 1985; **Janečková et al., 2018**) and proteins (Okada et al., 1992; Klerk et al., 1993; Chang and Kao, 1998), as well as higher photosynthetic activity (Špundová et al., 2003; **Janečková et al., 2018**). However, when the light intensity is too high (HL), an opposite effect is observed, i.e. the progress of senescence is faster (Biswal and Biswal, 1984; Mae et al., 1993; Prochazková and Wilhelmová, 2004). During senescence, the activity of photosynthetic apparatus is inhibited and energy transfer is limited. The elevated supply of excitations due to HL might lead to an imbalance between the generation and demand of electrons in electron transport chain (Vlčková et al., 2006), which results in undesirable formation of ROS. PSII proteins and lipids might be oxidized by ROS and higher levels of malondialdehyde (MDA), a product of lipid peroxidation, can be detected (Chang and Kao, 1998; Pospíšil, 2016).

1.1.3 Chlorophyll *b* and leaf senescence

Recent studies have shown that Chl *b* has an important role in the regulation of leaf senescence. Chl *b* is required for the stabilization of LHCII complexes and their proper assembly in thylakoid membrane (Bellemare et al., 1982; Tanaka and Tanaka, 2011; Voitsekhovskaja and Tyutereva, 2015). Mutants with higher Chl *b* content appear to have a slower senescence-related degradation of Chl, LHCs and thylakoid membranes (Kusaba et al., 2007; Sato et al., 2009; Sakuraba et al., 2012; Voitsekhovskaja and Tyutereva, 2015). On the other hand, studies dealing with leaf senescence in plants lacking Chl *b* suggest that Chl *b* deficiency is accompanied by faster senescence-associated changes (Kusaba et al., 2007; Yang et al., 2016). A study with *pgl* rice mutant has shown that Chl *b* deficiency is associated with increased Chl degradation, accumulation of ROS and electrolyte leakage during both natural senescence of flag leaves and dark-induced senescence of detached leaves (Yang et al., 2016). The faster Chl degradation was observed also in dark-incubated detached leaves of *cao-2* rice mutant deficient in Chl *b* (Kusaba et al., 2007). Besides these findings, any information about senescence-induced decrease in PSII and PSI function in such plants was missing. Therefore, we have focused our research on the progress of leaf senescence in plants lacking Chl *b* and we tried to clarify whether the deficiency of Chl *b* accelerates senescence-induced impairment of PSII and PSI activities (**Janečková et al., 2019**).

1.2 Cytokinins

Plant hormones cytokinins (CKs) play key roles in numerous processes of plant growth and development, including embryogenesis, activity of root and shoot meristems, vascular system development, leaf senescence and the response to biotic and abiotic stresses (for a review, see e. g. Kieber and Schaller, 2014).

Naturally occurring CKs are adenine derivatives, which, based on the configuration of their side chain at the N^6 position, can be classified into two classes. The first class is represented by isoprenoid CKs, which contain an isoprenoid side chain and inclused N^6-(Δ^2-isopentenyl)adenine (iP) or zeatin. In higher plants, zeatin is present as two isomers, the *trans*-(*t*Z) and the *cis*-form (*c*Z) (Kieber and Schaller, 2014). Both iP and *t*Z occur with high abundance in *Arabidopsis thaliana* (Osugi and Sakakibara, 2015). The second class of CKs is a group of aromatic CKs, which have an aromatic benzyl or hydroxybenzyl group at the N^6-position, this includes for example 6-benzylaminopurine (BA) or *meta*-topoline (*m*T) (Strnad, 1997).

CKs can be present in plants in different forms, namely in a form of free bases, ribosides, ribotides or glucosides (Mok and Mok, 2001; Sakakibara, 2006; Fig. 4). As the CK activity of various forms differs, the interconversion among the forms is an important regulatory mechanism of CK homeostasis (Sakakibara, 2006). CK ribosides have a ribose sugar attached at the N^9 position of the purine ring (Fig. 4) and in ribotides, the ribose moiety contains a phosphate group. Ribosides and ribotides represent the main transport form of CKs and can be found in xylem and phloem (for a review, see e.g. Kieber and Schaller, 2014). CKs can also be glycosylated (i.e. conjugated with sugar molecule, usually glucose) to form *O*- and *N*-glycosides (Fig. 4), which cannot bind to *Arabidopsis* CK receptors and were shown to be inactive in bioassays (Spíchal et al., 2004). The *O*-glycosylation is reversible and these forms of CKs can be converted by β-glucosidases into the active CK form (Brzobohatý et al., 1993; Kieber and Schaller, 2014). On the other hand, the *N*-glycosylation is irreversible and leads to complete biological inactivation of CKs (Sakakibara, 2006; Kieber and Schaller, 2014).

Free CK bases are usually the most active CK forms in bioassays (for a review, see e.g. Spíchal, 2012). Besides the free bases, it was shown that CK ribosides are also active and that they are able to interact tightly with CK receptors (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004, Heyl et al., 2012). In 2015, however, Lomin et al. (2015) had found out that compared to free bases, CK ribosides have a strongly reduced affinity for the CK receptors in planta, indicating that ribosides have no or very weak CK activity. Despite the finding by Lomin et al. (2015), the results from our correlation analysis (**Janečková et al. 2018**) indicate that CK ribosides could play an active role in the regulation of leaf senescence. This seeming discrepancy could be explained by possible fast conversion of CK ribosides to their bases, which was suggested also by Lomin et al. (2015). Hence, besides being the transport CK form, CK ribosides could be considered also as a kind of storage pool of CKs, which are converted into active CKs after reaching the target tissue.

1.2.1 Cytokinin perception and signalling

The CK signal is transmitted to target genes by two-component signalling pathway by His-Asp phosphorelay of particular components (e.g. Hwang et al., 2012). In Arabidopsis, CK binds to histidine kinase (AHK) receptor, which is localized at the plasma membrane or membrane of endoplasmic reticulum. This activates the signalling pathway via a cascade of phosphorelays. Signal is transmitted via histidine phosphotransfer proteins (AHP) to nuclear response regulators (ARRs) of type-B or type-A. Activation of ARRs type-B leads to transcription of CK inducible genes, including ARRs type-A, which mediate a negative feedback loop (for a review, see e.g. Kieber and Schaller, 2014 Fig. 2).

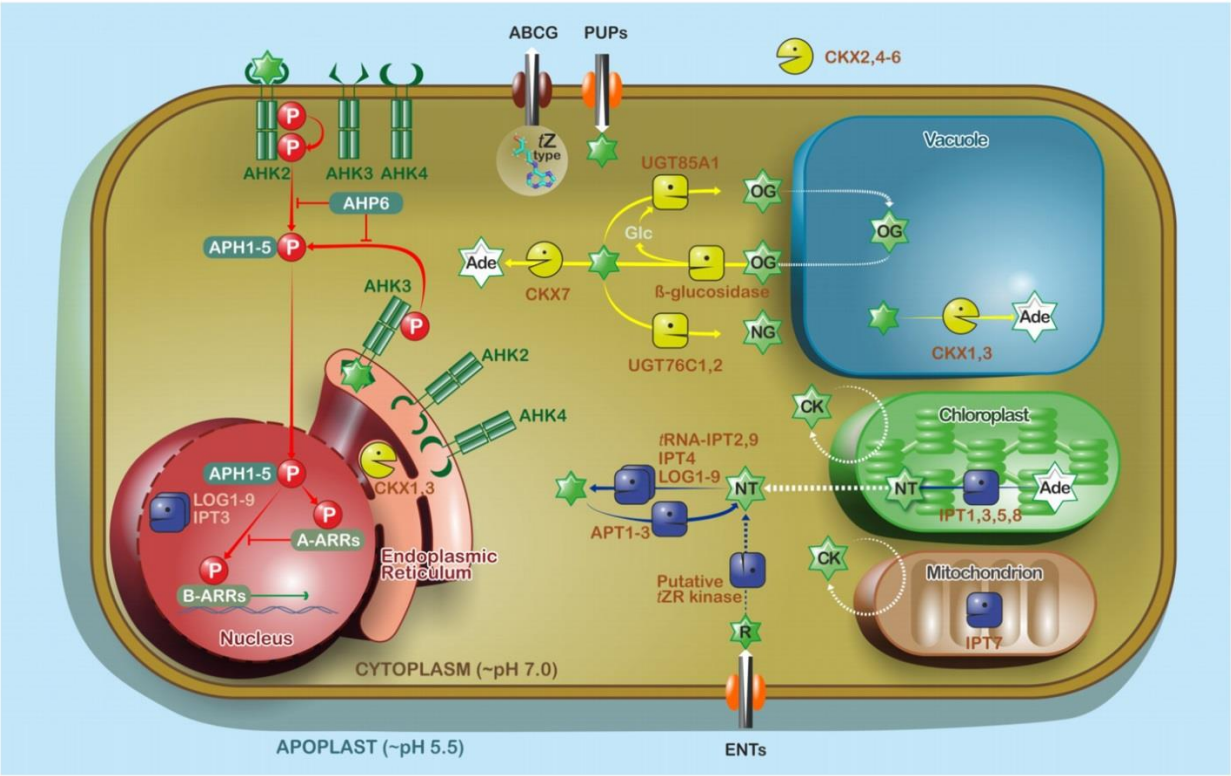

Fig. 2: Model of cellular and subcellular CK homeostasis and signalling in *Arabidopsis*. In CK signalling pathways (marked by red arrows), CK binds to AHK receptor, which is localized at the plasma membrane or membrane of endoplasmic reticulum. Signal is transmitted via AHP to nuclear ARRs of type-B or type-A. Activation of ARRs type-B leads to transcription of CK inducible genes, including ARRs type-A, which mediate a negative feedback loop. CKs homeostasis is achieved by its biosynthesis, transport, degradation and inactivation. CKs biosynthesis is catalysed by isopentenyl transferases (IPT) located mainly in chloroplasts, but also in mitochondria, cytosol and nucleus. CKs can be modified by uridine diphosphate glycosyltransferases (UGT) or β-glucosidase (yellow arrows). CKs degradation is catalysed by CK dehydrogenases/oxidases (CKX). Transport (white arrows) of CK free bases and corresponding ribosides is mediated by purine permeases (PUPs) and equilibrative nucleoside transporters (ENTs). Green stars indicate CK; Glc, glucose; NT, CK nucleotides; NG, *N*-glucosides; OG, *O*-glucosides; P, phosphate moiety; R, riboside. Solid arrows indicate known, well-described

pathways, dashed arrows indicate not well-defined pathways (Skalický et al., 2018; open access). For a more detailed description of the model see Skalický et al. (2018).

Three CK receptors are known to be present in *Arabidopsis thaliana* - AHK2, AHK3 and AHK4 (for a review, see e.g. Heyl et al., 2012; Kieber and Schaller, 2014). Using *ahk* mutant plants, it has been shown that CK-dependent regulation of senescence is mediated mainly by the AHK3 receptor, coupled with the phosphorylation/activation of the B-type ARR, ARR2 (Kim et al., 2006; Riefler et al., 2006; Hwang et al., 2012). The specific AHK3–ARR2 phosphorelay plays a major regulatory role in the CK-dependent leaf longevity by modulating downstream targets implicated in the senescence programme (Kim et al., 2006; Hwang et al., 2012). Besides AHK3, the AHK2 receptor is considered to have a redundant function in the senescence regulation, whereas the AHK4 receptor is thought to have only minor relevance (Riefler et al., 2006). During high light stress the role of AHK3 and AHK2 in mantainenance of PSII function has been also shown (Cortleven et al., 2014). We showed that the presence of functional AHK3 and AHK2 led to retained Chl content and preserved PSII function during dark-induced senescence. Moreover, we have suggested AHK4 to have the main responsibility for CK-induced inhibition of lipid peroxidation during dark-induced senescence (**Janečková et al., 2018**).

1.2.2 Effect of cytokinins on photosynthetic performance during induced senescence

Today it is well known that CKs are involved in regulation of leaf senescence (for a review, see e.g. Hönig et al., 2018). The onset of leaf senescence is usually connected to a decrease in the content of active CK forms (e.g. Singh et al., 1992; Gan and Amasino, 1995). This can be the result of inhibition of CK biosynthesis (Perilli et al., 2010), result of faster CK degradation by cytokinin oxidase/dehydrogenase (CKX) (Perilli et al., 2010; Werner et al., 2006) and/or the result of irreversible inactivation of CKs by uridine diphosphate glycosyltransferases (Šmehilová et al., 2016). However, the progress of leaf senescence can be delayed both by exogenous application of active CKs and by increase in their endogenous content.

In detached senescing leaves, CKs have been shown to retain content of photosynthetic pigments, such as Chl, carotenoids (car) and xanthophylls (e.g. Zavaleta-Mancera et al., 2007; Oh et al., 2005; Vlčkovae et al., 2006; **Janečková et al., 2019**), to mitigate lipid peroxidation (Dhindsa a kol., 1982; Vlčková et al., 2006; Liu et al., 2016), and to prevent the formation of plastoglobules and modification of chloroplast shape (Hudák et al., 1996; Vlčková et al., 2006; Zavaleta-Mancera et al., 2007). The CK-mediated mitigation of lipid peroxidation could be linked to the ability of CKs to activate xanthophyll cycle (e.g. Vlčková et al., 2006) or antioxidant enzymes, such as catalase and ascorbate peroxidase (Dhinsa et al., 1982; Zavaleta-Mancera et al., 2007), which all pronouncedly contribute to the CK-mediated protection of photosynthetic apparatus against oxidative damage during senescence.

In senescing leaves, CKs help to preserve photosynthetic activity (e.g. Vlčková et al., 2006, Zubo et al., 2008, Talla et al., 2016; Vylíčilová et al., 2016; **Janečková et al., 2019**). They were found to maintain the content and activity of Rubisco (e.g. Ohya and Suzuki, 1991; Zavaleta-Mancera et al., 2007) and CO_2 assimilation (Vlčková et al., 2006) in senescing leaves. With respect to the primary phase of photosynthesis, CKs are known to delay the inhibition of PSII photochemistry during senescence (Oh et al., 2005; Vlčková et al., 2006; Vylíčilová et al., 2016; **Janečková et al., 2019**). The function of PSII photochemistry, estimated from Chl fluorescence parameters (F_V/F_M and Φ_P, maximal and effective quantum yield of PSII photochemistry in the dark- and in the light-adapted state, respectively), was shown to be highly maintained in dark senescing CK-treated leaves (Oh et al., 2005; Vlčková et al., 2006; Talla et al., 2016; **Janečková et al., 2019**). Besides PSII photochemistry, CKs were also able to delay the loss of oxygen evolution activity during dark-induced senescence of rice leaves (Talla et al., 2016).

Senescence-induced alterations in the structure of pigment-protein complexes are suppressed in the presence of CKs. CKs were shown to stabilize LHCII as well as RCII in dark-senescing leaves (Jackowski 1996; Oh et al., 2005; Zavaleta-Mancera et al., 2007; Talla et al., 2016; **Janečková et al., 2019**). The effect of CKs on LHCII stability may be possibly related to the CK-dependent retention of Chl *b*, as this pigment is preferentially bound by LHCII. The preferential retention of Chl *b* accompanied by higher stability of LHCII proteins was reported for example by Zavaleta-Mancera et al. (2007) in dark-senescing detached leaves of wheat treated with BA. As mentioned in previous chapters, Chl *b* is important for the stabilization of LHCII complexes (see 1.1.3) and its presence has pronounced impact on the progress of leaf senescence (e.g. Kusaba et al., 2007; Voitsekhovskaja and Tyutereva, 2015; Yang et al., 2016; **Janečková et al., 2019**).

The question is, whether and to what extent are CKs able to suppress senescence-associated changes in mutant that is deficient in Chl *b*. We thus decided to also study the effect of exogenously applied CK (BA) on senescence-induced changes in Chl *b*-deficient mutant of barley (*Hordeum vulgare* L.), *clo* (**Janečková et al., 2019**) (see part 3.2).

1.2.3 Interplay between cytokinins and light

The effects of light and CKs on plant growth and development are similar and influence each other in many aspects (e.g. Chory et al., 1994; Zubo et al., 2008; Cortleven et al., 2016). It has been shown that light- and CK-dependent signalling pathways share a number of common components, for example, ARR4 and HY5, AHP6 etc. (Sweere et al., 2001; Vandenbussche et al., 2007; Argueso et al., 2010; Zdarska et al., 2015). Furthermore, both light and CKs influence photosynthesis - CKs affect the function and structure of the photosynthetic apparatus (e.g. Kusnetsov et al., 1998; Synková et al., 1999; Cortleven and Valcke, 2012) and influence photosynthesis-related genes (Talla et al., 2016; Vylíčilová et al., 2016).

It is obvious that there is a close interaction between light and CKs, therefore it is natural to assume that the light-mediated retardation of leaf senescence interferes with CK signal and vice versa. However, a detailed investigation of the interaction between these two factors during senescence is missing. One aspect of the interaction between light and CKs in the delay of leaf senescence can be light-induced changes in the content of endogenous CKs, as light participates in the regulation of CK biosynthesis, degradation and transport (Qamaruddin and Tillberg, 1989; Ananieva et al., 2008; Zubo et al., 2008; for review see e.g. Zdarska et al., 2015).

Despite the obviously important role of light in the regulation of CK content, it is not clear if and how endogenous CK level is changed after the detachment of leaves and how it is affected by light conditions. There are only a few studies describing changes in the content of endogenous CKs in detached leaves under different light conditions and their results are contradictory (Zubo et al., 2008; Causin et al., 2009; Roberts et al., 2011). Zubo et al. (2008) have shown that the content of zeatin derivatives increased in detached leaves of barley both in the dark and under continuous light (270 µmol photons m^{-2} s^{-1}), with more pronounced increase in light. Decrease in isopentenyladenosine (iPA) content was observed in detached wheat leaves exposed to white light (260 µmol photons m^{-2} s^{-1}) (Causin et al., 2009). On the contrary, Roberts et al. (2011) found a pronounced decrease in isopentyladenine (iP) and iPA content in detached senescing leaves of wheat kept in darkness (Roberts et al., 2011). A more complex analysis of endogenous CK content was performed by Marchetti et al. (2018) in detached wheat leaves kept under shade with or without blue light. The study shows that the presence of blue light eliminates increase in the activity of CKX and maintains content of free CKs in wheat leaves senescing under shade. It implies that not only changes in light intensity, but also changes in spectral quality of light have effect on CK level in detached senescing leaves (Marchetti et al., 2018).

In our study, we have analyzed changes in the content of various CK types and forms in detached *Arabidopsis* leaves senescing under different light conditions (**Janečková et al., 2018**). To investigate the effect of light on plants with impaired CK signalling, we have also used three CK receptor double mutants of *Arabidopsis* (see part 3.1).

2 Aims of research

Plant hormones CKs are important endogenous factors that are involved in the regulation of senescence. It is well known that in detached leaves, senescence is retarded by both exogenously applied CKs and light, however, the mechanism of their mutual interaction is not clear. As light has been shown to influence CK biosynthesis, transport and degradation, the light-mediated delay of leaf senescence could be associated with changes in endogenous CK levels. However, it is not clear if and how endogenous CK level is modified in leaves after their detachment and how it is affected by light conditions. Recent studies have shown that Chl *b* has also an important role in the regulation of leaf senescence. However, there is only limited information about senescence of plants lacking Chl *b* and senescence-induced decrease in photosynthetic function has not even been investigated in such plants. The aims of our investigations were as follows:

I. to find out whether endogenous CK content is modified in detached *Arabidopsis* leaves senescing under various light conditions,
II. to study the connection between the changes in endogenous CK levels and effect of light on senescence-induced changes (Chl content, efficiency of PSII photochemistry, and lipid peroxidation) in detached leaves of *Arabidopsis*,
III. to evaluate the role of individual CK receptors and role of light in leaf senescence of *Arabidopsis* plants with impaired CK signalling,
IV. to verify whether Chl *b* deficiency accelerates dark-induced senescence of detached leaves and to describe the related senescence-induced changes in the function of PSII,
V. to find out whether and to what extent exogenously applied CKs are able to suppress the supposedly accelerated senescence in leaves of mutant deficient in Chl *b*.

3 Results

3.1 The interplay between cytokinins and light during senescence in detached Arabidopsis leaves

In the paper **Janečková et al., 2018**, we estimated changes in the content of different CK forms and types under different light conditions, i.e. in the dark and under growth (GL; 150 µmol photons m^{-2} s^{-1}) and high (HL; 400 µmol photons $m^{-2}s^{-1}$) light intensity in detached *Arabidopsis* leaves and compared them with senescence-induced changes in Chl content, efficiency of PSII photochemistry and lipid peroxidation. To clarify the role of individual CK receptors (AHK2, AHK3, and AHK4) and to evaluate the role of light in the regulation of senescence in plants with impaired CK signalling, three *Arabidopsis* AHK double mutants were used in our study, each of them with only one functional CK receptor (*ahk2 ahk3*, *ahk2 ahk4* and *ahk3 ahk4*; Fig. 3).

Fig. 3: Phenotype of *Arabidopsis leaves* of wild-type (WT) and receptor mutants immediately after the detachment (control) and after 6 days under dark, growth light (120 µmol photons m^{-2} s^{-1}), or higher light (400 µmol photons m^{-2} s^{-1}) conditions

Leaves of mutants with mutation in AHK3 (i.e. *ahk2 ahk3* and *ahk3 ahk4*) were characterized by constitutively lower Chl content (by about 15 - 20 % compared to WT; Fig. 4A), whereas PSII photochemistry and the level of lipid peroxidation were unaffected (Fig. 4B-D). These mutants had approximately two-fold higher overall CK content in comparison to WT (Fig. 5A). The content of isopentenyl adenine (iP) and *trans*-zeatin (*t*Z) forms, both absolute and relative levels of iP and *t*Z precursors, their free bases and their ribosides were increased pronouncedly in *ahk2 ahk3* and *ahk3 ahk4* (the sum of iP and *t*Z was increased even nine-fold

and five-fold, respectively), implying stimulation of biosynthesis of both these CK groups. On the other hand, the content of *c*Z CKs was the lowest in both of these mutants.

The increased CK level of iP and *t*Z metabolites in double mutants with non-functional AHK3 can be interpreted as an attempt of the plant to compensate the insufficient CK output. Qualitatively same (but smaller) changes of the CK contents were found in the *ahk2 ahk4* mutant.

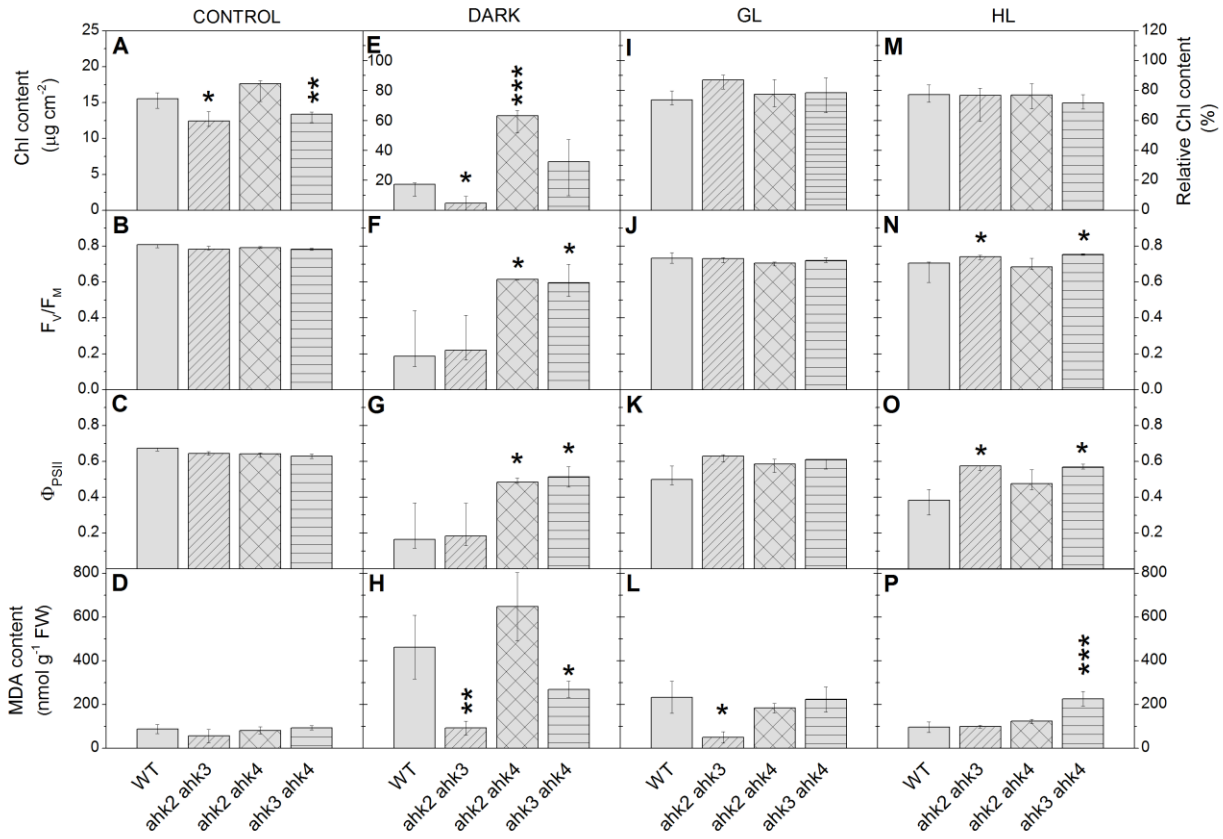

Fig. 4: Chlorophyll (Chl) content, F_V/F_M (the maximal quantum yield of photosystem II photochemistry in dark-adapted state), Φ_{PSII} the maximal quantum yield of photosystem II photochemistry in light-adapted state), and malondialdehyde (MDA) content estimated by HPLC related to fresh weight (FW) in control leaves (A–D) and detached leaves incubated for 6 days under dark (E–H), growth light (GL; 120 µmol photons m^{-2} s^{-1}) (I–L), or higher light (HL; 400 µmol photons m^{-2} s^{-1}) (M–P). Chl content of detached leaves is expressed in % of the content in control leaves. Medians and quartiles are presented for Chl content, F_V/F_M, and Φ_{PSII}, n = 5 – 15. For MDA content, means and SD are presented, n = 4–5. Asterisks indicate statistical significance (Student's t test) of the difference between values measured in receptor mutants and WT under particular conditions: * P < 0.05; ** P < 0.01; *** P < 0.001.

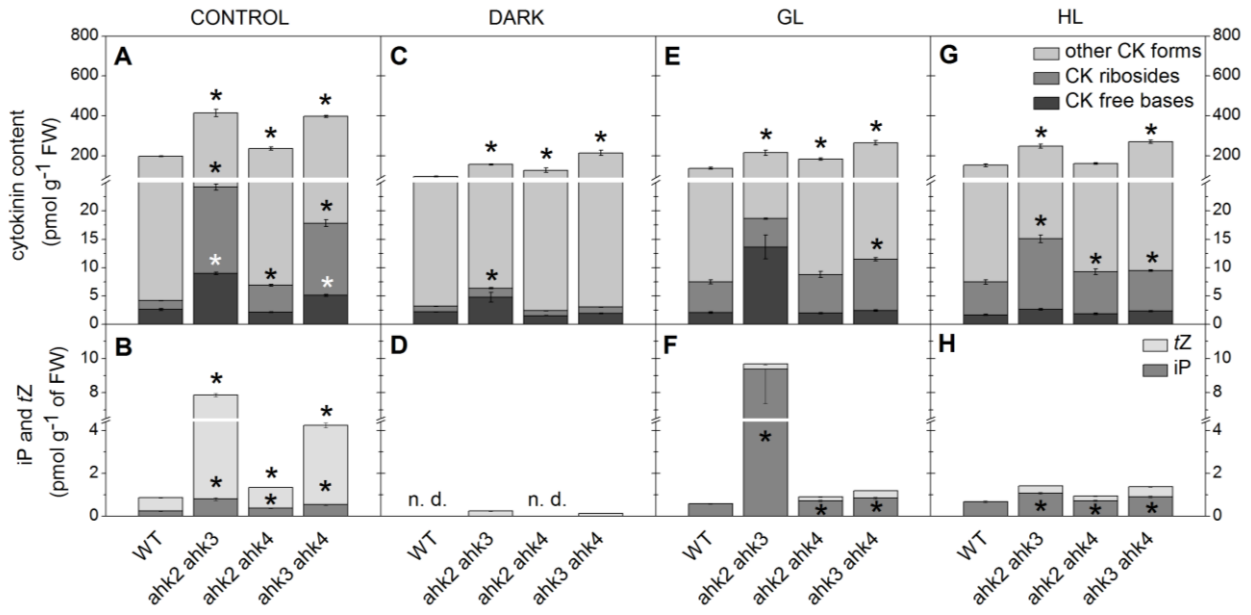

Fig. 5: Endogenous content of total cytokinin (CK) free bases (iP, *t*Z, *c*Z, and *m*T; black), cytokinin ribosides (iPR, *t*ZR, *c*ZR, DHZR and *m*TR; dark grey), and other cytokinin forms (iP7G, iP9G, iPR5'MP, *t*ZOG, *t*ZROG, *t*Z7G, *t*Z9G, *t*ZR5'MP, *c*ZOG, *c*ZROG, *c*Z7G, *c*Z9G, *c*ZR5'MP, DHZOG, DHZ7G, DHZ9G, and DHZR5'MP; light grey) (A,C,E,G), and the content of iP and tZ (B,D,F,H) related to fresh weight (FW) in control leaves (A,B) and detached leaves incubated for 6 days under dark (C,D), growth light (GL; 120 µmol photons m^{-2} s^{-1}) (E,F, or higher light (HL; 400 µmol photons m^{-2} s^{-1}) (G,H). Means and SD (n = 3) are presented; n. d., under detection limit. Asterisks indicate statistical significance (Student's t-test) of the difference between values measured in receptor mutants and WT under particular conditions: * $P < 0.05$.

After 6 days in darkness, detached leaves of all genotypes had decreased Chl content and PSII function and increased lipid peroxidation (Figs. 3 and 4E-H). These changes were accompanied by a marked decrease in total CK content (Fig. 5C). The content of free bases, ribosides and monophosphates of *t*Z and iP markedly decreased as well (Fig. 5D); after 6 days no iP and only negligible residues of *t*Z were detected. These changes indicated that CK inactivation and degradation prevailed over their biosynthesis. On the contrary, increase in *c*Z and *m*T content was found in dark-senescing leaves of all genotypes compared with the mature leaves indicating possible stimulation of *c*Z and *m*T biosynthesis in darkness. The pool of missing (most abundant) free bases (i.e. iP and *t*Z) seems to be thus partially or completely replenished by these CK forms. This finding supports a hypothesis that *c*Z plays a role in the ensuring of minimal CK activity necessary for the maintenance of essential physiological processes under conditions characterized by the lack of energy sources that lead to down-regulation of the main active CKs (Gajdošová et al., 2011). Higher *c*Z content has been repeatedly observed under growth-limiting conditions induced by various external or internal factors (for a review, see e.g. Schäfer et al.,

2015) or has been found in senescing leaves (Gajdošová et al., 2011; Šmehilová et al., 2016). We also proposed that AHK3 is the main CK receptor mediating this *c*Z action.

In the case of dark senescing leaves of mutant where both receptors AHK3 and AHK2 were non-functional (*ahk2 ahk3*), total free bases content decreased pronouncedly more than in WT or than in mutant where AHK3 was functional (*ahk2 ahk4*) (Fig. 5C), even though the (initial) CK content was the highest in *ahk2 ahk3* (Fig. 5A). In this mutant the most pronounced decrease in Chl content and PSII function was observed (Fig. 4E). This could be explained by the role of AHK3 and AHK2 in CK-mediated Chl retention (Kim et al., 2006; Riefler et al., 2006) and in the maintenance of PSII function (Cortleven et al., 2014) (see part 1.2.1). As AHK4 did not have sufficient promoting effect on Chl content and PSII function, CK-dependent protection of photosynthesis during senescence was lost. This mutant had the lowest MDA content (Fig. 4H), which could be explained by a combination of high initial content of CK and functional AHK4 receptor that we suggested to be the main receptor for CK-dependent suppression of lipid peroxidation. The opposite effect was observed in a mutant lacking functional AHK2 and AHK4 (*ahk2 ahk4*). In this mutant, the higher initial CK content (Fig. 5A) together with the presence of functional AHK3 led to highly maintained photosynthetic performance (Fig. 4E-G), whereas the absence of functional AHK4 and AHK2 contributed to the highest lipid peroxidation (Fig. 4H). In the *ahk3 ahk4* mutant, the content of CK as well as the maintenance of Chl content, PSII function and low lipid peroxidation (Figs. 4E-H, 5C), was between *ahk2 ahk3* and *ahk2 ahk4*, indicating that AHK2 can partially take over the role of both AHK3 and AHK4 (**Janečková et al., 2018**).

Under both light conditions (GL and HL, 120 and 400 µmol photons m^{-2} s^{-1}, respectively, with photoperiod 8 h light/16 h dark), the detached leaves of all genotypes were characterized by highly maintained Chl content and PSII function and by reduced lipid peroxidation compared to situation in darkness. Surprisingly, under HL, both mutants without functional AHK3 receptor had markedly better PSII function than WT. The disrupted CK signalling via AHK3 thus led to lower Chl content and impaired PSII function in darkness (Fig. 4E-G), however, under GL, the CK-signalling deficiency was apparently compensated by light (Fig. 4I-K, M-O). Even more so, the photosynthetic performance of leaves of AHK3 deficient mutant senescing under HL was even better than in WT. Light thus seems to have the ability to somehow substitute the insufficient CK signalling, and under HL conditions, it was probably able to even reverse the negative effect of AHK3 dysfunction. The overall CK level decreased under both light conditions as well as in the dark (Fig. 5E, G), however, the total content of iP forms was highly maintained under light in all mutants (Fig. 5F, H). The increased content of iP indicated that the biosynthesis of iP is functional (or even stimulated) in detached leaves. The pronounced promoting effect of light (both GL and HL) on the maintenance of high photosynthetic parameters was thus associated with higher level of iP and its riboside (iPR). On the other hand, light did not have any significant effect on the content of *t*Z forms in the detached leaves (Fig. 5F, H). In illuminated leaves, the *t*Z

biosynthesis seemed to be inhibited in a similar manner as in the leaves kept in the dark, even though the decrease in total content of *t*Z forms was slightly smaller. This supports the hypothesis that *t*Z-type CKs are predominantly synthetized in roots (Hirose et al., 2008; Frébort et al., 2011).

To summarize, our results show that the content of CKs and their various forms differs significantly in detached *Arabidopsis* leaves kept under various light conditions (Fig. 5). While light did not have any significant effect on the metabolism of *t*Z CKs in the detached leaves, in the case of iP light stimulated its accumulation (Fig. 5F, H). We suggest that the light-mediated maintenance of photosynthesis in detached leaves could be thus related to persisted biosynthesis of iP. And when comparing the senescence-induced changes in the CK receptor double mutants, light appears to be able to compensate disrupted CK signalling caused by loss-of-function mutation in AHK receptors (**Janečková et al., 2018**).

There is obviously an extensive crosstalk between light and CKs and this crosstalk seems to be very important also for the regulation of plant senescence. However, the molecular mechanism of this interaction remains to be clarified in the future.

3.2 Exogenous application of cytokinin during dark senescence eliminates the acceleration of photosystem II impairment caused by chlorophyll b deficiency in barley

In this paper **Janečková et al., 2019**, the changes in photosynthetic pigment content and PSII and PSI activities associated with dark-induced senescence were studied in a Chl *b*-deficient barley mutant. We used chlorina $f2^{f2}$ (*clo*) barley mutant, which is deficient in Chl *b* due to the mutation in chlorophyll(id) *a* oxygenase, a key enzyme of Chl *b* biosynthesis (Mueller et al., 2012). The *clo* mutant has also lower content of Chl *a* and car compared to WT (Štroch et al., 2004; 2008;). The mutant is deficient in the light-harvesting complexes Lhcb1, Lhcb6 and Lhca4 and has reduced amount of Lhcb2, Lhcb3 and Lhcb4 (Bossmann et al., 1997).

After four days in darkness, we have observed that although the relative decrease in Chl content was similar to WT (Fig. 6), the absolute Chl content was pronouncedly lower in *clo* leaves. This was caused by the fact that even before the induction of senescence, the Chl content in the leaves of *clo* was lower than in WT. On the other hand, the relative decrease in the content of car and xanthophylls in *clo* mutant leaves was lower than in WT (Fig. 6).

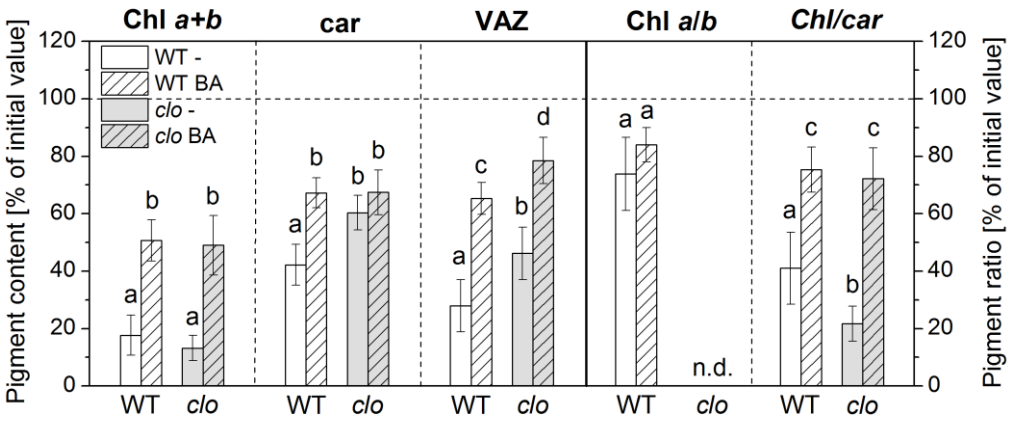

Fig. 6: Chlorophyll (Chl *a*+*b*), carotenoid (car) and xanthophyll (VAZ) content related to leaf area and Chl *a*/*b* and Chl/car ratios in detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (-) or with 6-benzylaminopurine (BA). Relative values (% of the initial values before senescence induction) are presented. Means and SD are shown, n = 6. Except of the Chl *a/b* ratio (analyzed by the t-test, P = 0.139), all other data were analyzed by ANOVA test (P < 0.001 in all cases) and statistically significant differences in following *post hoc* statistical testing (Holm-Sidak test) at P < 0.05 are indicated by different letters.

In comparison to WT, the senescing *clo* leaves had pronouncedly lower both the maximal quantum yield of PSII photochemistry in the dark-adapted state (F_V/F_M; Fig. 7) and the effective quantum yield of PSII photochemistry in the light-adapted state (Φ_P; Fig. 1), which indicates that PSII photochemistry was markedly impaired. Unlike in WT, regulatory quenching processes were almost inactive in senescing leaves of *clo* and dissipation via non-regulatory processes prevailed (Fig. 1).

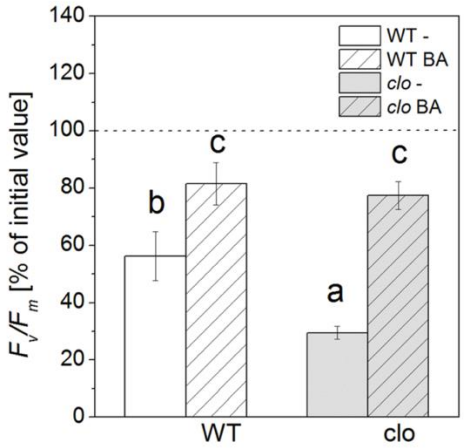

Fig. 7: The maximal efficiency of PSII photochemistry in dark-adapted state (F_V/F_M, % of the initial values before senescence induction) in detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (-) or with 6-benzylaminopurine (BA), means and SD estimated from measurable leaves are shown. Data were analyzed by ANOVA test (P < 0.001) and statistically significant difference in following *post hoc* statistical testing (Holm-Sidak test) at P < 0.05 are indicated by different letters.

Pronounced changes in Chl fluorescence parameters (Fig. 8) indicated that RCII was damaged to a greater extent than LHCII in both WT and *clo*, whereas the RCII damage was more pronounced in *clo*.

In senescing WT leaves, the OJIP curve was more flat than in non-senescing ones due to the pronounced increase in the height of the O-step and decrease in the height of the P-step (Fig. 8A). Additionally, the normalized curve showed a relative increase in the J-step (Fig. 8B), reflected also in the increased parameter V_J (1.5-times when compared to leaves before senescence induction; Fig. 8C). The $(dV/dt)_0$ parameter also increased, but more (2.5-times) than V_J, thus ABS/RC proportional to their ratio increased more pronouncedly (4-times). The increase of ABS/RC suggests increase in apparent antenna size of active RCII, which in turn indicates preferential impairment of RCII compared to LHCII. This resulted in increased supply of excitations to remaining active RCII and thus a pronounced Q_A reduction could be observed in these RCII. We proposed that the preferential RCII impairment was caused by their degradation, as the Chl *a*/*b* ratio decreased in the WT senescing leaves (Fig. 6). Since Chl *b* occurs mainly in LHCII, the decrease in the Chl *a*/*b* ratio reflects a relative decrease in RCII abundance (Leong and Anderson, 1984). In senescing WT leaves, RE_0/ABS as well as δ_{R0} decreased by about 80 % and 45 %, respectively (Fig. 8D). These changes indicate that the electron transport was reduced more within than behind the PSII. This was accompanied by lower relative amount of $P700^+$ (Fig. 8D).

In the senescing leaves of *clo*, changes in the OJIP curve and in fluorescence parameters were much more profound than in WT (Fig. 8), indicating that the impairment of RCII during dark-senescence was much more pronounced in *clo* than in WT. The greater damage of RCII could be related to effect of Chl *b*/LHC deficiency, as the proper assembly of LHCII seems to stabilize the structure of PSII complex and their function (Havaux and Tardy, 1997). Interestingly, despite the markedly impaired PSII photochemistry in *clo*, the activity of PSI in this mutant remained higher than in WT, indicating that the missing Lhca4 did not decrease the stability of PSI during senescence. We assume that the acceleration of PSII impairment in *clo* was caused by the Chl *b* deficiency associated with the lover LHCII content and consequent destabilization and damage of RCII.

As cytokinins are known decelerators of senescence, we also wanted to find out whether and to what extent exogenously applied CKs will be able to suppress the pronounced senescence in *clo* mutant. We thus decided to study the effect of exogenously applied CK (BA) on senescence-induced changes in this mutant. BA treatment slowed down the degradation of photosynthetic pigments (Fig. 6) and suppressed the senescence-induced impairment of PSII photochemistry in both *clo* and WT leaves (Fig. 7), but in *clo* the effect was more pronounced.

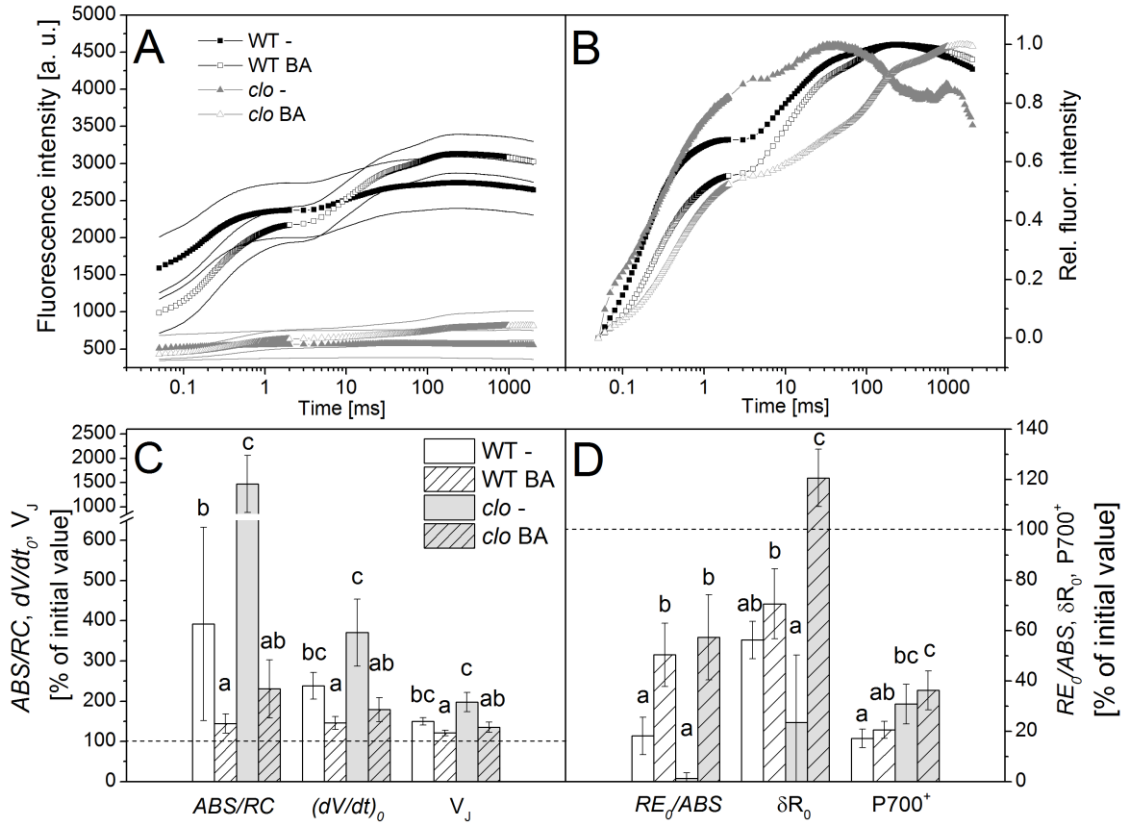

Fig. 8: Chl fluorescence induction transient (OJIP curves), related fluorescence parameters and changes in the PSI activity of detached WT and clo leaves kept for 4 days in the dark in 0.2% DMSO solution without (-) or with 6-benzylaminopurine (BA). A, OJIP curves; B, the normalized OJIP curves; C, the relative values of the specific energy flux ABS/RC, the initial slope of the O-J fluorescence raise $(dV/dt)_0$, and the relative variable fluorescence at the J-step (V_J); D, quantum yield of electron transport from reduced QA to final acceptors of photosystem I (RE_0/ABS); the efficiency of electron transport from reduced plastoquinone to final acceptors of photosystem I (δ_{R0}) and the relative amount of oxidized primary electron donor of photosystem I, $P700^+$, expressed as % of the initial values before senescence induction. Means and SD are shown, n = 8 – 12. Data were analyzed by Kruskal-Wallis ANOVA on Ranks test ($P < 0.001$) and statistically significant differences in following post hoc statistical testing (Dunn's test) at $P < 0.05$ are indicated by different letters.

The protective effect of BA on PSII function in senescing leaves was also reflected in less pronounced changes in the shape of OJIP curve (Fig. 8A) and smaller changes in corresponding parameters. In leaves undergoing senescence in the presence of BA, we have observed a smaller increase in relative height of the J-step (i.e., V_J) (Fig. 8B) and also the increase in ABS/RC and $(dV/dt)_0$ parameters was considerably smaller compared to leaves senescing in the absence of BA (Fig. 8C). In both genotypes, BA suppressed the decrease in RE_0/ABS and δ_{R0} (Fig. 8D). In *clo* the BA application even increased δ_{R0} by about 20 % (Fig. 8D). These changes were accompanied by less decrease in the relative amount of $P700^+$ (Fig. 8D). Taken all together our results indicate

that in the BA treated leaves of both genotypes the electron transport from PSII to final PSI acceptors was more maintained.

The changes in measured parameters indicate that BA suppressed the senescence-induced impairment of PSII photochemistry in both WT and *clo*. Interestingly, in the presence of BA, the progress of senescence in *clo* became more similar to WT (Figs. 6-8), although in the absence of exogenous cytokinin the senescence-induced impairment of PSII function was much more pronounced in *clo*. The stronger effect of BA in the case of *clo* is further apparent from the significantly lower increase in ABS/RC and V_J (Fig. 8C).

The delay of senescence-induced changes in *clo* was observed also for other CKs (iP and TDZ, Fig. 9; unpublished results). In these experiments, all CKs suppressed (even though to a different extent) the senescence-induced decrease in relative Chl content as well as a decrease in efficiency of PSII photochemistry in detached leaves of both WT and *clo* (Fig. 9).

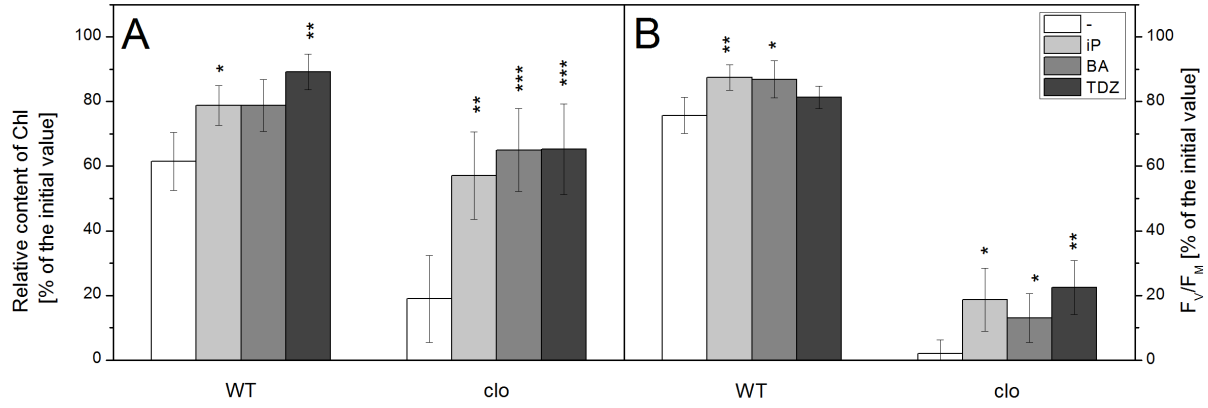

Fig. 9: Relative content of chlorophyll (Chl) (A) and the maximal efficiency of PSII photochemistry in dark-adapted state (F_V/F_M) (B) in detached WT and *clo* leaves kept for 3 days in the dark in 0.2% DMSO solution without (-) or with 100 µM of isopentenyladenine (iP), 6-benzylaminopurine (BA) or thidiazuron (TDZ). Relative values (% of the initial values before senescence induction) are presented. Means and SD are shown, n = 6. Asterisks indicate statistically significant difference (Student´s t-test) between DMSO solution without CKs and with CKs (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

It seems that exogenous CK application eliminates the destabilizing effect of Chl *b*/LHC deficiency on PSII function in the dark-senescing *clo* leaves. As the impairment of PSII function seems to be related to more pronounced damage of RCII, we assume that the protective effect of CK on PSII function in WT as well as in *clo* is based mainly on a suppression of this impairment.

4 Experimental approach

4.1 Plant materials and growth conditions

For the study of **Janečková et al. (2018)**, *Arabidopsis thaliana* plants (wild-type [WT] Columbia-0 and three CK receptor double mutants - *ahk2 ahk3*, *ahk2 ahk4*, and *ahk3 ahk4*; Riefler et al., 2006) were grown in a growth chamber on a commercial substrate (Potgrond H, Klasmann-Deilmann Substrate, Germany) under short-day conditions: 8 h of white light (120 µmol photons m^{-2} s^{-1})/16 h dark, at 22/20 °C and relative air humidity 60 %. Seeds were kept at 4 °C for 3 days in the dark before sowing. After 5 weeks, mature leaves (seventh leaf on average) were cut off from plants and placed into a 0.2% solution of dimethyl sulfoxide. In a separate experiment, we have verified that this solution did not affect senescence of detached Arabidopsis leaves in comparison with distilled water. The detached leaves floated on the solution in a closed six-hole macrotiter plates and were kept under three different light conditions - dark, growth light (GL; 120 µmol photons m^{-2} s^{-1}), and higher light (HL; 400 µmol photons m^{-2} s^{-1} from the same light source as GL; 8 h light/16 h dark). Measurements were performed immediately after leaf detachment ("control") and on the second, fourth, sixth and twelfth day after detachment. Data presented in Janečková et al. (2018) are from the day of the detachment ("control") and from the sixth day after detachment.

For the study of **Janečková et al. (2019)**, seeds of wild-type barley (*Hordeum vulgare* L. cv. Bonus; WT) and chlorina $f2^{f2}$ (*clo*) mutant were soaked in deionized water for 24 h before sowing and then transferred into pots containing perlite with Hoagland solution. Pots were placed in a growth chamber under controlled conditions of 16 h light (150 µmol of photons m^{-2} s^{-1})/8 h dark, 22/20 °C and 60 % relative air humidity. Eight days after the sowing, 4-cm segments were cut off from the primary leaves. The leaf segments were placed either into a 0.2% solution of dimethylsulfoxide (DMSO) or into a 10^{-5} mol l^{-1} solution of 6-benzylaminopurine (BA) in 0.2% DMSO (BA-treated leaves). The leaf segments were then kept in the dark (other conditions were same as during plant growth). Measurements were performed immediately after the leaf detachment and on the 4th day after detachment.

4.2 Pigment analyses

4.2.1 Chlorophyll content in *Arabidopsis* leaves

In **Janečková et al. (2018),** Chl content was measured using a chlorophyll meter SPAD-502 (Minolta Sensing Konica, Osaka, Japan) after 30-min dark adaptation for elimination of the effect of chloroplast movement (Nauš et al., 2010). To obtain the absolute Chl content, a calibration of the SPAD values was performed for the individual genotypes by simultaneous analytical determination of Chl content.

For the analytical determination, the leaves were homogenized in a chilled mortar with $MgCO_3$ and 80% acetone. The homogenate was then centrifuged using a centrifuge 3-30KS Sigma (SIGMA Laborzentrifugen, Germany) at 4,000*g* for 5 min and at 10 °C. After centrifugation, the absorbance (A) of the supernatant was measured at the wavelengths of 646.8, 663.2, and 750 nm using a spectrophotometer Unicam UV550 (ThermoSpectronic, UK). Total Chl concentration was calculated according to Lichtenthaler (1987).

The Chl content was related to a leaf area (μg cm^{-2}). The Chl content of the leaves measured on the sixth day after detachment was expressed as percent of the values of the corresponding control leaves.

4.2.2 Chlorophyll and xanthophyll content in barley leaves

For the determination of the content of pigments in **Janečková et al. (2019)**, the area of leaf samples was estimated and then the leaves were homogenized in liquid nitrogen, with $MgCO_3$ and 80% acetone. The homogenates were centrifuged at 4,000*g* and 4 °C for 10 min. The supernatant was used for the spectrophotometric estimation of Chl and total car contents (a sum of carotenes and xanthophylls) according to Lichtenthaler (1987) by a spectrophotometer Unicam UV550 (Thermo Spectronic, United Kingdom) and also for the quantification of individual xanthophylls (violaxanthin, V; antheraxanthin, A; zeaxanthin, Z) by high performance liquid chromatography (HPLC).

For the estimation of xanthophyll content (VAZ) by an HPLC system (Alliance e 2695 HPLC System, Waters, USA), the supernatant was filtered through 0.45 μm polytetrafluoroethylene membrane (Acrodisc, Waters, USA) into dark vials. The 100 μl amount was injected into the HPLC system. A LiChroCAR TRP-18 (5 μm; 4.6 x 250 mm) column (Merck & Co., USA) was used. The analysis was performed by a gradient reverse-phase analysis (1.5 ml min^{-1} at 25 °C). The analysis started with isocratic elution using the mobile phase composed of acetonitrile, methanol and 0.1 mol l^{-1} Tris (pH 8) in the ratio 87:10:3 (v:v:v) for 10 min and was followed by a 2-min linear gradient using mobile phase composed of a mixture of methanol and n-hexane in the ratio 4:1 (v:v). Absorbance was detected at 440 nm using a UV/VIS detector. The amount of pigments in samples was determined using their conversion factors (Färber and Jahns, 1998). The de-epoxidation state of xanthophylls (DEPS) was calculated according to Gilmore and Björkman (1994) as (A+Z)/(V+A+Z)× 100 (%).

4.3 Chl fluorescence

4.3.1 Fluorescence measurement with FluorCam 700 MF imaging system

In **Janečková et al. (2018)**, Chl fluorescence parameters were measured from the adaxial leaf side at room temperature by FluorCam 700 MF imaging system (Photon Systems Instruments, Czech Republic).

At the beginning, the minimum Chl fluorescence (F_0) was determined after 30 min of dark-adaptation of the sample by application of the measuring flashes (red light) which intensity was low enough to avoid the closure of RCII. For the determination of maximum fluorescence (F_M), a saturating pulse of 1.6 s (white light, 500 µmol m^{-2} s^{-1}) was applied on the dark-adapted sample. After dark-relaxation (2 min), when the measured Chl fluorescence signal dropped to F_0, the leaf sample was exposed to actinic light (red light, 120 µmol of photons m^{-2} s^{-1}, the same intensity as used for plant growth). To determine the maximum Chl fluorescence during light-adaptation (F_M'), a set of the saturating pulses was applied. The first saturation pulse (white light, 500 µmol photons m^{-2} s^{-1}) was applied 3 s after the actinic light was switched on and was followed by 6 pulses in 23 s intervals, 3 pulses in 47 s intervals and the last 2 pulses in 70 s intervals.

The maximal quantum yield of PSII photochemistry in the dark-adapted state (F_V/F_M) was calculated as $(F_M - F_0)/F_M$, where F_M is maximal fluorescence and F_0 is minimal fluorescence in the dark-adapted leaf sample. In the light-adapted leaf samples (after 7 min of actinic light), the maximal quantum yield of PSII photochemistry (Φ_{PSII}) was calculated as $(F_M' - F_0')/F_M'$, where F_M' is maximal fluorescence for the light-adapted state and F_0' represents the minimal fluorescence for light-adapted state calculated as $F_0/(F_V/F_M + F_0/F_M')$.

4.3.2 Chl fluorescence mesurements with Plant Efficiency Analyser and PlantScreen phenotyping platform

In **Janečková et al. (2019)**, the Chl fluorescence induction transient (OJIP curves) and the quenching analysis were measured at room temperature on adaxial side of barley leaf samples. Freshly detached leaves (i.e., leaves before senescence induction) were dark-adapted for 25 min before the measurement. The OJIP curves were measured in the middle of leaf segments by Plant Efficiency Analyser (Hansatech Instruments, United Kingdom) for 2 s with excitation light intensity of 1100 µmol photons m^{-2} s^{-1}. The initial slope of the O-J Chl fluorescence rise $(dV/dt)_0$, the relative variable fluorescence at the J step (V_J), and the specific energy flux ABS/RC were evaluated as follows (see Stirbet et al., 2018). The $(dV/dt)_0 = 4(F_{300\mu s} - F_{50\mu s})/F_V$, where $F_{300\mu s}$ and $F_{50\mu s}$ are fluorescence intensities at the indicated times and F_V is variable fluorescence ($F_V = F_P - F_0$; F_0 is a minimal fluorescence and F_P is fluorescence at the P step). The $(dV/dt)_0$ parameter, defined as the maximal rate of the accumulation of the fraction of closed RCII (Strasser et al., 2000), reflects the rate of excitation supply into the RCII and subsequently the rate of Q_A reduction. Parameter V_J, reflecting the fraction of reduced Q_A, was calculated as $(F_J - F_0)/F_V$, where F_J is fluorescence intensity at 2 ms. ABS/RC was calculated as $((dV/dt)_0/V_J) \times F_P/F_V$ and reflects apparent antenna size of active RCII (Strasser et al., 2000). Further, the quantum yield of electron transport from reduced Q_A to final acceptors of PSI (RE_0/ABS) and the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI (δR_0) were estimated as follows: $RE_0/ABS = F_V/F_P \times (1 - V_I)$ and $\delta R_0 = (1 - V_I)/(1 - V_J)$, where V_I is the relative variable

fluorescence at the I step (Stirbet et al., 2018). The measured OJIP curves as well as curves normalized to F_V are presented.

The quenching analysis was performed using PlantScreen (Photon Systems Instruments, Czech Republic) phenotyping platform (Humplík et al., 2015) according to the following protocol. At the beginning, the minimal fluorescence F_0 was determined using measuring flashes (duration of 10 μs) of red light (650 nm), which did not cause any closure of RCII. Then a saturating pulse (white light, 1900 μmol photons m^{-2} s^{-1}, duration of 800 ms) was applied to measure maximal fluorescence F_M. After 90 s of dark-relaxation, when the measured fluorescence signal dropped to F_0, the leaf sample was exposed to actinic light for 25 min (red light, 150 μmol of photons m^{-2} s^{-1}, the same intensity as used for plant growth). To determine the maximal fluorescence during the actinic light exposition (F_M'), a set of the saturating pulses was applied. The first pulse was applied 10 s after the actinic light was switched on and was followed by 9 pulses in 20 s intervals and then by 22 pulses in 59 s intervals.

The maximal quantum yield of PSII photochemistry in the dark-adapted state was estimated as $F_V/F_M = (F_M - F_0)/F_M$. The maximal quantum yield of PSII photochemistry in the light-adapted state was calculated as $F_V'/F_M' = (F_M' - F_0')/F_M'$, where F_0' is minimal fluorescence for the light-adapted state, which was calculated as $F_0/(F_V/F_M + F_0/F_M')$. The effective quantum yield of PSII photochemistry in the light-adapted state was calculated as $\Phi_P = (F_M' - F_t)/F_M'$, where F_t is fluorescence at time t measured immediately prior to the application of the saturating pulse. The quantum yield for regulatory non-photochemical quenching was calculated as $\Phi_{NPQ} = (F_t/F_M') - (F_t/F_M)$ and the quantum yield for constitutive non-regulatory dissipation processes was calculated as $\Phi_{f,D} = F_t/F_M$. The sum of Φ_P, Φ_{NPQ} and $\Phi_{f,D}$ equals unity (for a review, see Lazár, 2015). In the case of F_V'/F_M', Φ_P, Φ_{NPQ}, and $\Phi_{f,D}$, values obtained at the end of the actinic light exposition are presented.

4.3.3 Measurement of P700 oxidation

For estimation of light-induced oxidation of P700 (the primary electron donor of PSI) in **Janečková et al. (2019)**, the I830 signal as a difference of transmittance at 875 nm and 830 nm was determined using Dual PAM 100 (Walz, Germany), see, e.g. Lazár (2013). The methodology assumes that P700 is fully reduced in the dark-adapted leaf and thus the I830 signal is zero. During illumination of the leaf, the I830 signal rises to a peak level reflecting an equilibrated maximal $P700^+$ level as a result of P700 oxidation by the charge separation and $P700^+$ reduction by plastocyanin. In both WT and *clo* leaves before senescence induction, the I830 signal reached the peak level at 17 ms. In senescing leaves, the level of $P700^+$ at 17 ms of illumination was expressed in % of the peak level observed in the leaves before senescence induction.

4.4 Determination of malondialdehyde content

In **Janečková et al. (2018)**, the content of malondialdehyde (MDA) was measured using HPLC. The isolation and derivatization of MDA using 2,4-dinitrophenylhydrazine (DNPH) was performed as described in Pilz et al. (2000) with some modifications. Leaves were homogenized in 0.11% butylhydroxytoluene dissolved in 100% methanol. This step was followed by 10 min centrifugation at 2,000*g*. 125 µl of supernatant was poured into an eppendorf tube and 25 µl of 6 mol l^{-1} aqueous NaOH was added to achieve alkaline hydrolysis of protein bounded MDA. Samples were incubated for 30 min in dry bath at 60°C (Thermo-Shaker TS100, Biosan, Latvia). To reach the precipitation of proteins in samples, 62.5 µl of 35% (v:v) perchloric acid was added to the sample, vortexed and centrifuged at 16,000*g* for 10 min. 125 µl of supernatant was poured into a vial, stirred up with 1 µl of 50 mmol l^{-1} DNPH dissolved in 50% sulphuric acid and incubated in dark at room temperature for 30 min to obtain MDA-DNPH adducts. Then 25 µl of the solution was injected into an HPLC system (Alliance e 2695 HPLC System, Waters, Milford, MA, USA). A Symmetry C18 (3.5 µm; 4.6 x 75 mm) Column (Waters, Milford, MA, USA) was used, the elution was performed isocratically (1 mL/min at 35°C), using a mixture of 25 mmol l^{-1} triethylamine (pH 3.5) and acetonitrile in the ratio 50:50 (v:v). MDA was detected at 310 nm using a UV/VIS detector.

4.5 Identification and quantification of endogenous cytokinins

To analyse the endogenous CKs content in study of **Janečková et al. (2018)**, leaves from 5-week-old *A. thaliana* plants were harvested, frozen in liquid nitrogen, and stored at –80°C. Three replicates were measured, each consisted of 440 ± 10 mg of leaf mass (6^{th}, 7^{th} and 8^{th} true leaves; an average number of leaves per sample was 12 for WT, 24 for *ahk2 ahk3*, 11 for *ahk2 ahk4* and 10 for *ahk3 ahk4*). The procedure used for CK purification was a combination of the methods described by Dobrev and Kamínek (2002) and Svačinová et al. (2012). Isotope-labelled CK internal standards (Olchemim, Czech Republic) were added (0.25 pmol per sample of B, 7G and 0.5 pmol per sample of OG, NT) to check the recovery during purification and to validate the determination (Novák et al., 2008). The samples were purified using C18 and MCX cartridges (Dobrev and Kamínek, 2002). Eluates were evaporated to dryness using a SpeedVac concentrator and dissolved in 40 µl of 10% methanol. Ten µl of each sample were then analysed by an ultra-performance liquid chromatograph (Acquity UPLC System; Waters, USA) coupled to a triple quadrupole mass spectrometer equipped with an electrospray interface (Xevo TQ-S, Waters, Manchester, UK) by a method utilized on the StageTips technology (Svačinová et al., 2012). Quantification was obtained by multiple reaction monitoring of $[M+H]^+$ and the appropriate product ion. Optimal conditions, dwell time, cone voltage, and collision energy in the collision cell, corresponding to the exact diagnostic transition, were optimized for each CK for selective MRM experiments (Novák et al., 2008). Quantification was performed by Masslynx software using a standard isotope dilution method (Novák et al., 2003).

4.6 Correlation and statistical analysis

In **Janečková et al. (2018)**, correlation between the contents of different CK forms in detached leaves and the relative Chl content, the maximal quantum yield of PSII photochemistry in dark-adapted state, and MDA content was estimated using the Pearson's correlation coefficient. Statistical analysis was performed using Student's t-test, in which the values of parameters measured with receptor mutants were compared with corresponding values in WT. The significant differences are marked by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

In all statistical tests of Janečková et al. (2019), related data sets were first tested for normality (Kolmogorov-Smirnov test with Lilliefors' correction) and equality of variances (Levene Median test). If fulfilled, the Student's t-test or ANOVA test (with all pairwise multiple comparison by Holm-Sidak *post hoc* test) were used and if not fulfilled, the Mann-Whitney Rank Sum test or Kruskal-Wallis ANOVA on Ranks test (with all pairwise multiple comparison by Dunn's *post hoc* test) were used. The critical level of 0.05 was chosen for all tests (the P-value of the test is marked by *). If the P-value of a test was even lower than 0.01 or even lower than 0.001, the results are marked by ** or ***, respectively. All testing was performed using SigmaPlot version 11 (Systat Software, USA).

5 Conclusions and Future Perspectives

Leaf senescence is a complex developmental process, characterized by numerous physiological and biochemical changes. An important role of senescence is the remobilization and transport of nutrients, especially nitrogen, into storage and growing tissues and organs. Senescence is usually accompanied by a loss of Chl content, by inhibition of photosynthetic processes including PSII photochemistry and by higher lipid peroxidation. Plant hormones CKs are known as negative regulators of senescence, they preserve photosynthetic structures and function, especially PSII, during leaf senescence.

It is well known that in detached leaves, both exogenously applied CKs and light retard senescence, however, the mechanism of their mutual interaction is not clear. In our work, we have shown that the content of endogenous CKs and individual CK forms differs significantly in detached *Arabidopsis* leaves kept under various light conditions. In detached leaves kept in darkness, senescence-induced decrease in Chl content and PSII function and increase in lipid peroxidation were accompanied by significantly lower content of endogenous CKs. The content of free bases, ribosides and monophosphates of *t*Z and iP significantly decreased, indicating that inactivation and degradation of these CKs prevailed over their biosynthesis. On the other hand, we have found increased content of *m*T and *c*Z free bases, possibly indicating stimulation of their biosynthesis in darkness. Our results contribute to the clarification of the role of *c*Z in plants and at the same time support the hypothesis that *c*Z maintains the basal viability of leaves under growth-limiting conditions. Furthermore, we have shown that functional CK receptors AHK3 and AHK2 are important for CK-dependent maintenance of Chl content and PSII function in detached leaves during dark-induced senescence. We have proposed that AHK4 receptor plays a major role in CK-mediated protection against senescence-associated lipid peroxidation in detached *Arabidopsis* leaves.

In detached leaves kept in light, the Chl content and PSII function were highly maintained and increase in lipid peroxidation was lower compared to dark-senescing leaves. These changes were accompanied by higher content of free bases of iP. It has been suggested that the light-mediated maintenance of photosynthesis in detached leaves could be related to persisted biosynthesis of iP. In addition, from the comparison of senescence-induced changes in the CK receptor double mutants, it appears that light is able to compensate the disrupted CK signalling caused by loss-of-function mutation in AHK receptors.

Recent studies have shown that Chl *b* has also an important role in the senescence regulation. However, information about senescence of plants lacking Chl *b* is limited and senescence-induced decrease in photosynthetic activity has not even been investigated in such plants. We have observed pronouncedly faster inhibition of PSII photochemistry during dark-induced senescence of detached leaves of Chl *b* deficient barley mutant. We suppose that the stronger impairment of PSII function is related to a more pronounced damage of reaction

centres of PSII, probably due to destabilizing effect of Chl *b*/LHC deficiency. Exogenously applied CKs were able to eliminate this effect, probably via the stabilization of reaction centres of PSII.

Although a significant progress was made over the last two decades in our understanding of CK involvement in senescence regulation, many questions still remain unanswered. The mechanism of light and CK interaction during senescence remains ambiguous and also the molecular mechanism of protective effect of CKs on photosynthesis during senescence remains to be clarified. Another unresolved issue is the mechanism of the CK-mediated acceleration of leaf senescence. It has been shown that in some cases, CKs act as positive regulators of leaf senescence. This usually happens when high concentrations of exogenous CK are applied or when the exogenous CK treatment is combined with higher light intensity.

Elucidation of the mechanism of CK-mediated regulation of senescence will contribute to our understanding of the process itself and can also provide important information for genetic manipulations leading to the improvement of agronomic traits, such as crop yields and post-harvest quality of plants.

6 References

Ananieva KT, Ananiev ED, Doncheva S, Georgieva KM, Tzvetkova N, Kamínek M, Motyka V, Dobrev PI, Gajdošová S, Malbeck J. 2008. Senescence progression in a single darkened cotyledon depends on the light status of the other cotyledon in *Cucurbita pepo* (zucchini) seedlings: potential involvement of cytokinins and cytokinin oxidase/dehydrogenase activity. Physiologia Plantarum 134: 609–623.

Argueso CT, Raines T, Kieber JJ. 2010. Cytokinin signaling and transcriptional networks. Current Opinion in Plant Biology 13: 533–539.

Bellemare G, Bartlett SG, Chna NH. 1982. Biosynthesis of chlorophyll *a/b*-binding polypeptides in wild type and the Chlorina f2 mutant of barley. The Journal of Biological Chemistry 257: 7762–7767.

Biswal B. 1997. Chloroplast Metabolism during Leaf Greening and Degreening. In: Pessarakali M. (ed) Handbook of Photosynthesis, Marcel Dekker Inc, New York pp. 71–81.

Biswal UC, Biswal B. 1984. Photocontrol of Leaf Senescence. Photochemistry and Photobiology 39: 875–879.

Biswal B, Mohapatra PK, Biswal UC, Raval MK. 2012. Leaf Senescence and Transformation of Chloroplasts to Gerontoplasts. In: Eaton-Rye J, Tripathy B, Sharkey T. (eds) Photosynthesis. Advances in Photosynthesis and Respiration, Springer, Dordrecht pp. 217–230.

Bossmann B, Knoetzel J, Jansson S. 1997. Screening of *chlorina* mutants of barley (*Hordeum vulgare* L.) with antibodies against light-harvesting proteins of PSI and PSII: Absence of specific antenna proteins. Photosynthesis Research 52: 127–136.

Brzobohatý B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K. 1993. Release of active cytokinin by a β-glucosidase localized to maize root meristem. Science 262: 1051–1054.

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D. 2003. The molecular analysis of leaf senescence – a genomic approach. Plant Biotechnology Journal 1: 3–22.

Camp PJ, Huber SC, Burke JJ, Moreland DE. 1982. Biochemical changes that occur during senescence of wheat leaves. Plant Physiology 70: 1641–1646.

Causin HF, Roberts IN, Criado MV, Gallego SM, Pena LB, Ríos MD, Barneix AJ. 2009. Changes in hydrogen peroxide homeostasis and cytokinin levels contribute to the regulation of shade induced senescence in wheat leaves. Plant Science 177: 698–704.

Chang CJ, Kao CH. 1998. H_2O_2 metabolism during senescence of rice leaves: changes in enzyme activities in light and darkness. Plant Growth Regulation 25: 11–15.

Chory J, Reinecke D, Sim S, Washburn T, Brenner M. 1994. A role for cytokinins in de-etiolation in *Arabidopsis*. Plant Physiology 104: 339–347.

Cortleven A, Marg I, Yamburenko MV, Schlicke H, Hill K, Grimm B, Schaller GE, Schmülling T. 2016. Cytokinin regulates the etioplast-chloroplast transition through the two-component signaling system and activation of chloroplast-related genes. Plant Physiology 172: 464–478.

Cortleven A, Nitschke S, Klaumünzer M, AbdElgawad H, Asard H, Grimm B, Riefler M, Schmülling T. 2014. A novel protective function for cytokinin in the light stress response is mediated by the ARABIDOPSIS HISTIDIN KINASE2 and ARABIDOPSIS HISTIDIN KINASE3 receptors. Plant Physiology 164: 1470–1483.

Cortleven A, Valcke R. 2012. Evaluation of the photosynthetic activity in transgenic tobacco plants with altered endogenous cytokinin content: lessons from cytokinins. Plant Physiology 144: 394–408.

Dhindsa RS, Plumb-Dhindsa P, Reid DM. 1982. Leaf senescence and lipid peroxidation: effect of some phytohormones, and scavengers of free radicals and singlet oxygen. Physiologia Plantarum 56: 453–457.

Demmig-Adams B, Stewart JJ, Adams WW III. 2014. Chloroplast photoprotection and the trade-off between abiotic and biotic defense. In: Demmig-Adams B, Garab G, Adams WW III, Govindjee (eds). Non-photochemical quenching and energy dissipation in plants, algae and cyanobacteria. Springer, Netherlands. pp. 631–643.

Dobrev PI, Kamínek M. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed mode solid-phase extraction. Journal of Chromatography A 950: 21–29.

Färber A, Jahns P. 1998. The xanthophyll cycle of higher plants: influence of antenna size and membrane organization. Biochimica et Biophysica Acta 1363: 47–58.

Frébort I, Kowalska M, Hluska T, Frébortová J, Galuszka P. 2011. Evolution of cytokinin biosynthesis and degradation. Journal of Experimental Botany 62: 2431–2452.

Gajdošová S, Spíchal L, Kamínek M, Hoyerová K, Novák O, Dobrev PI, Galuszka P, Klíma P, Gaudinova A, Žižková E, Hanuš J, Dančák M, Trávníček B, Pešek B, Krupička M, Vaňková R, Strnad M, Motyka V. 2011. Distribution, biological activities, metabolism, and the conceivable function of *cis*-zeatin-type cytokinins in plants. Journal of Experimental Botany 62: 2827–2840.

Gan S, Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270: 1986–1988.

Gilmore AM, Björkman O. 1994. Adenine nucleotides and the xanthophyll cycle in leaves. I. Effects of CO_2- and temperature-limited photosynthesis on adenylate energy charge and violaxanthin deepoxidation. Planta 192: 526–536.

Grover A. 1993. How do senescing leaves lose photosynthetic activity? Current Science 64: 22–234.

Havaux M, Tardy F. 1997. Thermostability and photostability of photosystem II in leaves of the *chlorina-f2* barley mutant deficient in light-harvesting chlorophyll *a/b* protein complexes. Plant Physiology 113: 913–923.

Heyl A, Riefler M, Romanov GA, Schmülling T. 2012. Properties, functions and evolution of cytokinin receptors. European Journal of Cell Biology 91: 246–256.

Hidema J, Makino A, Mae T, Ojima K. 1991. Photosynthetic characteristics of rice leaves aged under different irradiances from full expansion through senescence. Plant Physiology 97: 1287–1293.

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H. 2008. Regulation of cytokinin biosynthesis, compartmentalization and translocation. Journal of Experimental Botany 59: 75–83.

Hönig M, Plíhalová L, Husičková A, Nisler J, Doležal K. 2018. Role of cytokinins in senescence, antioxidant defence and photosynthesis. International Journal of Molecular Sciences 19: 4045.

Hudák J, Vizárová G, Šikulová J, Ovečková O. 1996. Effect of cytokinins produced by strains of Agrobacterium tumefaciens with binary vectors on plastids in senescent barley leaves. Acta Physiologie Plantarum 18: 205–210.

Hwang I, Sheen J, Müller B. 2012. Cytokinin signaling networks. Annual Review of Plant Biology 63: 353–380.

Jackowski G. 1996. Senescence-related changes in the subcomplex organization of the major light-harvesting chlorophyll *a/b*-protein complex of photosystem II (LHCII) as influenced by cytokinin. Zeitschrift für Naturforschung C 51: 464–472.

Janečková H, Husičková A, Ferretti U, Prčina M, Pilařová E, Plačková L, Pospíšil P, Doležal K, Špundová M. 2018. The interplay between cytokinins and light during senescence in detached *Arabidopsis* leaves. Plant, Cell & Environment 41: 1870–1885.

Janečková H, Husičková A, Lazár D, Ferretti U, Pospíšil P, Špundová M. 2019. Exogenous application of cytokinin during dark senescence eliminates the acceleration of photosystem II impairment caused by chlorophyll *b* deficiency in barley. Plant Physiology and Biochemistry 136: 43-51.

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, Kamiya Y, Yamaguchi S, Sakakibara H. 2004. Distinct isoprenoid origins of *cis*- and *trans*-zeatin biosyntheses in *Arabidopsis*. The Journal of Biological Chemistry 279: 14049–14054.

Kieber JJ, Schaller GE. 2014. Cytokinins. The Arabidopsis Book 12: e0168.

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I. 2006. Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proceedings of the National Academy of Sciences of The United States of America 103: 814– 819.

Klerk HC, Rebers M, Loon LC. 1993. Effects of light and regulators on senescence-related changes in soluble proteins in detached oat (*Avena sativa* L.) leaves. Plant Growth Regulation 13: 137–145.

Krieger-Liszkay A, Trösch M, Krupinska K. 2015. Generation of reactive oxygen species in thylakoids from senescing flag leaves of the barley varieties Lomerit and Carina. Planta 241: 1497–1508.

Kuai B, Chen J, Hörtensteiner S. 2018. The biochemistry and molecular biology of chlorophyll breakdown. Journal of Experimental Botany 69: 751–767.

Kusaba M, Ito H, Morita R, Iida S, Sato Y, Fujimoto M, Kawasaki S, Tanaka R, Hirochika H, Nishimura M, Tanaka A. 2007. Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. The Plant Cell 19: 1362–1375.

Kusnetsov VV, Herrmann RG, Kulaeva ON, Oelmüller R. 1998. Cytokinin stimulates and abscisic acid inhibits greening of etiolated *Lupinus luteus* cotyledons by effecting the expression of the light-sensitive protochlorophyllide oxidoreductase. Molecular &General Genetics 259: 21–28.

Lazár D. 2013. Simulations show that a small part of variable chlorophyll *a* fluorescence originates in photosystem I and contributes to overall fluorescence rise. Journal of Theoretical Biology 335: 249–264.

Lazár D. 2015. Parameters of photosynthetic partitioning. Journal of Plant Physiology 175: 131–147.

Leong TM, Anderson JM. 1984. Adaptation of the thylakoid membranes of pea chloroplasts to light intensities. II. Regulation of electron transport capacities, electron carriers, coupling factor (CF1) activity and rates of photosynthesis. Photosynthesis Research 5: 117–128.

Liebsch D, Keech O. 2016. Dark-induced leaf senescence: new insights into a complex light-dependent regulatory pathway. New Phytologist 212: 563–570.

Liu L, Li HR, Zeng H, Cai Q, Zhou X, Yin C. 2016. Exogenous jasmonic acid and cytokinin antagonistically regulate rice flag leaf senescence by mediating chlorophyll degradation, membrane deterioration, and senescence-associated genes expression. Journal of Plant Growth Regulation 35: 366–376.

Lomin SN, Krivosheev DM, Steklov MY, Arkhipov DV, Osolodkin DI, Schmülling T, Romanov GA. 2015. Plant membrane assays with cytokinin receptors underpin the unique role of free cytokinin bases as biologically active ligands. Journal of Experimental Botany 66: 1851–1863.

Lu C, Lu Q, Zhang J, Kuang T. 2001. Characterization of photosynthetic pigment composition, photosystem II photochemistry and thermal energy dissipation during leaf senescence of wheat plants grown in the field. Journal of Experimental Botany 52: 1805–1810.

Lu C, Zhang JA. 1998. Changes in photosystem II function during senescence of wheat leaves. Physiologia Plantarum 104: 239–247.

Mae T, Thomas H, Gay AP, Makino A, Hidema J. 1993. Leaf development in *Lolium temulentum*: photosynthesis and photosynthetic proteins in leaves senescing under different irradiances. Plant & Cell Physiology 34: 391–399.

Marchetti CF, Škrabišová M, Galuszka P, Novák O, Causin HF. 2018. Blue light suppression alters cytokinin homeostasis in wheat leaves senescing under shading stress. Plant Physiology and Biochemistry 130: 647–657.

Martínez DE, Costa ML, Guiamet JJ. 2008. Senescence-associated degradation of chloroplast proteins inside and outside the organelle. Plant Biology 10: 15–22.

Mok DWS, Mok MC. 2001. Cytokinin metabolism and action. Annual Review of Plant Physiology and Plant Molecular Biology 52: 89–118.

Mueller AH, Dockter C, Gough SP, Lundqvist U, von Wettstein D, Hansson M. 2012. Characterization of mutations in barley *fch2* encoding chlorophyllide *a* oxygenase. Plant & Cell Physiology 53: 1232–1246.

Nath K, Phee B-K, Jeong S, Lee SY, Tateno Y, Allakhverdiev SI, Lee C-H, Nam HG. 2013. Age-dependent changes in the functions and compositions of photosynthetic complexes in the thylakoid membranes of *Arabidopsis thaliana*. Photosynthesis Research 117: 547–556.

Nauš J, Prokopová J, Řebíček J, Špundová M. 2010. SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. Photosynthesis Research 105: 265–271.

Noodén LD, Hillsberg JW, Schneider MJ. 1996. Induction of leaf senescence in *Arabidopsis thaliana* by long days through a light-dosage effect. Physiologia Plantarum 96: 491–495.

Novák O, Hauserová E, Amakorová P, Doležal K, Strnad M. 2008. Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. Phytochemistry 69: 2214–2224.

Novák O, Tarkowski P, Tarkowská D, Doležal K, Lenobel R, Strnad M. 2003. Quantitative analysis of cytokinins in plants by liquid chromatography-single-quadrupole mass spectrometry. Analytica Chimica Acta 480: 207–218.

Oh M, Moon Y, Lee C. 2003. Increased stability of LHCII by aggregate formation during dark-induced leaf senescence in the *Arabidopsis* mutant, *ore10*. Plant & Cell Physiology 44: 1368–1377.

Oh MH, Kim JH, Zulfugarov IS, Moon Y-H, Rhew T-H, Lee C-H. 2005. Effects of benzyladenine and abscisic acid on the disassembly process of photosystems in an Arabidopsis delayed-senescence mutant, *ore9*. Journal of Plant Biology 48: 170–177.

Ohya T, Suzuki H. 1991. The effects of benzyladenine on the accumulation of messenger RNAs that encode the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase and light-harvesting chlorophyll *a/b* protein in excised cucumber cotyledons. Plant & Cell Physiology 32: 577–580.

Okada K, Inoue Y, Satoh K, Katoh S. 1992. Effects of light on degradation of chlorophyll and proteins during senescence of detached rice leaves. Plant & Cell Physiology 33: 1183–1191.

Osugi A, Sakakibara H. 2015. Q&A: How do plants respond to cytokinins and what is their importance? BMC Biology 13: 102.

Park SY, Yu JW, Park JS, Li J, Yoo SC, Lee NY, Lee SK, Jeong SW, Seo HS, Koh HJ, Jeon JS, Park YI, Paek NC. 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. The Plant Cell 19: 1649–1664.

Perilli S, Moubayidin L, Sabatini S. 2010. The molecular basis of cytokinin function. Current Opinion in Plant Biology 13: 21–26.

Pilz J, Meineke I, Gleiter CH. 2000. Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. Journal of Chromatography B 742: 315–325.

Pospíšil P. 2016. Production of reactive oxygen species by photosystem II as a response to light and temperature stress. Frontiers in Plant Science. 7: 1950

Qamaruddin M and Tillberg E. 1989. Rapid effects of red light on the isopentenyladenosine content in Scots pine seeds. Plant Physiology 91: 5–8.

Riefler M, Novák O, Strnad M, Schmülling T. 2006. Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism: The Plant Cell 18: 40–54.

Roberts IN, Caputo C, Kade M, Criado MV, Barneix AJ. 2011. Subtilisin-like serine proteases involved in N remobilization during grain filling in wheat. Acta Physiologiae Plantarum 33: 1997–2001.

Sakakibara H. 2006. Cytokinins: activity, biosynthesis, and translocation. Annual Review of Plant Biology 57: 431–449.

Sakuraba Y, Balazadeh S, Tanaka R, Mueller-Roeber B, Tanaka A. 2012. Overproduction of Chl *b* retards senescence through transcriptional reprogramming in Arabidopsis. Plant & Cell Physiology 53: 505–17.

Sakuraba Y, Kim Y, Yoo S, Hörtensteiner S, Paek N. 2013. 7-Hydroxymethyl chlorophyll *a* reductase functions in metabolic channeling of chlorophyll breakdown intermediates during leaf senescence. Biochemical and Biophysical Research Communications 430: 32–37.

Sato Y, Morita R, Katsuma S, Nishimura M, Tanaka A, Kusaba M. 2009. Two short-chain dehydrogenase/reductases, NON-YELLOW COLORING 1 and NYC1-LIKE, are required for chlorophyll *b* and light-harvesting complex II degradation during senescence in rice. The Plant Journal 57: 120–131.

Schäfer M, Brütting C, Meza-Canales ID, Grosskinsky DK, Vankova R, Baldwin IT, Meldau S. 2015. The role of *cis*-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. Journal of Experimental Botany 66: 4873–4884.

Singh S, Letham DS, Palni LMS. 1992. Cytokinin biochemistry in relation to leaf senescence. VIII. Translocation, metabolism and biosynthesis of cytokinins in relation to sequential leaf senescence of tobacco. Physiologia Plantarum 86: 398–406.

Skalický V, Kubeš M, Napier R, Novák O. 2018. Auxins and cytokinins – The role of subcellular organization on homeostasis. International Journal of Molecular Sciences. 19: 3115.

Sobieszczuk-Nowicka E, Wrzesinski TM, Bagniewska-Zadworna A, Kubala S, Rucińska-Sobkowiak R, Polcyn W, Misztal LH, Mattoo AK. 2018. Physio-genetic dissection of dark-induced leaf senescence and timing its reversal in barley. Plant Physiology 178: 654–671.

Spíchal L. 2012. Cytokinins – Recent news and views of evolutionally old molecules. Functional Plant Biology 39: 267–284.

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T. 2004. Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. Plant & Cell Physiology 45: 1299–1305.

Strnad M. 1997. The aromatic cytokinins. Physiologia Plantarum 101: 674–688.

Svačinová J, Novák O, Plačková L, Lenobel R, Holík J, Strnad M, Doležal K. 2012. A new approach for cytokinin isolation from *Arabidopsis* tissues using miniaturized purification: Pipette tip solid-phase extraction. Plant Methods 8: 17.

Sweere U, Eichenberg K, Lohrmann J, Mira-Rodado V, Bäurle I, Kudla J, Nagy F, Schäfer E, Harter K. 2001. Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. Science 294: 1108–1111.

Synková H, Van Loven K, Pospíšilová J, Valcke R. 1999. Photosynthesis of transgenic *Pssu-ipt* tobacco. Journal of Plant Physiology 155: 173–182.

Šmehilová M, Dobrůšková J, Novák O, Takáč T, Galuszka P. 2016. Cytokinin-specific glycosyltransferases possess different roles in cytokinin homeostasis maintenance. Frontiers in Plant Science 7: 1264.

Špundová M, Popelková H, Ilík P, Skotnica J, Novotný R, Nauš J. 2003. Ultra-structural and functional changes in the chloroplasts of detached barley leaves senescing under dark and light conditions. Journal of Plant Physiology 160: 1051–1058.

Špundová M, Slouková K, Hunková M, Nauš J. 2005*a*. Plant shading increases lipid peroxidation and intensifies senescence-induced changes in photosynthesis and activities of ascorbate peroxidase and glutathione reductase in wheat. Photosynthetica 43: 403–409.

Špundová M, Strzałka K, Nauš J. 2005*b*. Xanthophyll cycle activity in detached barley leaves senescing under dark and light. Photosynthetica 43: 117–124.

Štroch M, Čajánek M, Kalina J, Špunda V. 2004. Regulation of the excitation energy utilization in the photosynthetic apparatus of *chlorina f2* barley mutant grown under different irradiances. Journal of Photochemistry and Photobiology B Biology 75: 41–50.

Štroch M, Lenk S, Navrátil M, Špunda V, Buschmann C. 2008. Epidermal UV-shielding and photosystem II adjustment in wild type and *chlorina f2* mutant of barley during exposure to increased PAR and UV radiation. Environmental and Experimental Botany 64: 271–278.

Talla SK, Panigrahy M, Kappara S, Nirosha P, Neelamraju S, Ramanan R. 2016. Cytokinin delays dark-induced senescence in rice by maintaining the chlorophyll cycle and photosynthetic complexes. Journal of Experimental Botany 67: 1839–1851.

Tanaka R, Tanaka A. 2011. Chlorophyll cycle regulates the construction and destruction of the light-harvesting complexes. Biochimica et Biophysica Acta 1807: 968–976.

Tang Y, Wen X, Lu C. 2005. Differential changes in degradation of chlorophyll-protein complexes of photosystem I and photosystem II during flag leaf senescence of rice. Plant Physiology and Biochemistry 43: 193–201.

Thimann KV. 1985. The senescence of detached leaves of *Tropaeolum*.Plant Physiology 79: 1107–1110.

Vandenbussche F, Habricot Y, Condiff AS, Maldiney R, Van der Straeten D, Ahmad M. 2007. HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*. The Plant Journal 49: 428–441.

Vlčková A, Špundová M, Kotabová E, Novotný R, Doležal K, Nauš J. 2006. Protective cytokinin action switches to damaging during senescence of detached wheat leaves in continuous light. Physiologia Plantarum 126: 257–267.

Voitsekhovskaja OV, Tyutereva EV. 2015. Chlorophyll *b* in angiosperms: Functions in photosynthesis, signaling and ontogenetic regulation. Journal of Plant Physiology 189: 51–64.

Vylíčilová H, Husičková A, Spíchal L, Srovnal J, Doležal K, Plíhal O, Plíhalová L. 2016. C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus. Phytochemistry 122: 22–33.

Weaver LM, Amasino RM. 2001. Senescence is induced in individually darkened *Arabidopsis* leaves, but inhibited in whole darkened plants. Plant Physiology 127: 876–886.

Weaver LM, Gan S, Quirino B, Amasino RM. 1998. A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatments. Plant Molecular Biology 37: 455–469.

Werner T, Köllmer I, Bartrina I, Holst K, Schmülling T. 2006. New insights into the biology of cytokinin degradation. Plant Biology 8: 371–381.

Wingler A, Marès M, Pourtau N. 2004. Spatial patterns and metabolic regulation of photosynthetic parameters during leaf senescence. New Phytologist 161: 781-789.

Yang Y, Xu J, Huang L, Leng Y, Dai L, Rao Y, Chen L, Wang Y, Tu Z, Hu J, Ren D, Zhang G, Zhu L, Guo L, Qian Q, Zeng D. 2016. *PGL*, encoding chlorophyllide *a* oxygenase 1, impacts leaf senescence and indirectly affects grain yield and quality in rice. Journal of Experimental Botany 67: 1297–1310.

Yonekura-Sakakibara K, Kojima M, Yamaya T, Sakakibara H. 2004. Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to *cis*-zeatin. Plant Physiology 134: 1654–1661.

Zavaleta-Mancera HA, López-Delgadob H, Loza-Taverac H, Mora-Herrerab M, Trevilla-García C, Vargas-Suárezc M, Oughame H. 2007. Cytokinin promotes catalase and ascorbate peroxidase activities and preserves the chloroplast integrity during dark-senescence. Journal of Plant Physiology 164:1572–1582.

Zdarska M, Dobisová T, Gelová Z, PernisováM, Dabravolski S, Hejátko J. 2015. Illuminating light, cytokinin, and ethylene signalling crosstalk in plant development. Journal of Experimental Botany 66: 4913–4931.

Zubo YO, Yamburenko MV, Selivankina SY, Shakirova FM, Avalbaev AM, Kudryakova NV, Zubkova NK, Liere K, Kulaeva ON, Kusnetsov VV, Börner T. 2008. Cytokinin stimulates chloroplast transcription in detached barley leaves. Plant Physiology 148: 1082–1093.

7 Appendix (Curriculum Vitae)

Profile

Name	Helena Janečková
Maden name	Melkovičová
Citizenship	Slovakia
Date of Birth	01.01.1990
Permanent Address	Dobrá 1192, Trenčianska Teplá 914 01, Slovakia
Temporary Address	Dukelská 557, Stará Ves n. Ondřejnicí 739 23, Czech republic
Mobile	+420732648657
E-mail	melkovicovahelena@gmail.com

Education

Period	2012- 2014
Organization	Palacký University Olomouc, Faculty of Science
Degree	Mgr.
Field of Study	Biophysics
Diploma thesis	Concentration-dependent effect of cytokinins on plant senescence and effect of light conditions

Period	2014 - present
Organization	Palacký University Olomouc, Faculty of Science
Degree	Ph.D.
Field of Study	Biophysics

Position

Period	2014 - present
Position	Research Scientist
Organization	Centre of Region Haná for Biotechnological and Agricultural Research, Department of Biophysics, Palacký University, Olomouc
Research Fields	cytokinins and their influence on leaf senescence interaction of light and cytokinins photosynthesis of higher plants

Conference presentations

Conference	Green for Good Conference, Olomouc
Date	05/2015
Presentation	Poster
Theme	Effect of 6-benzylaminopurine on induced senescence of cytokinin receptor mutants of *Arabidopsis thaliana* under dark and light

Conference	13th Student Days of Experimental Plant Biology, Brno

Date	09/2015
Presentation	Oral presentation
Theme	Effect of 6-benzylaminopurine on induced senescence of cytokinin receptor mutants of *Arabidopsis thaliana* under dark and light
Conference	14th Student Days of Experimental Plant Biology, Bratislava
Date	09/2017
Presentation	Oral presentation
Theme	The Interplay between Cytokinins and Light during Senescence in Detached Arabidopsis Leaves

Skills and trainings

Traineeship	Department of Plant Physiology, Umeå Plant Science Centre, Sweden supervisor: Dr. Olivier Keech four months, 2017
Teaching experience	Trainings in microscopy and spectroscopy – Single beam spectrophotometry summer semester, 2016
Measuring skills	Chlorophyll fluorescence *in vivo* (imaging, PSII efficiency, Chlorophyll Fluorescence Quenching), Fluorescence temperature curves
Training school	Plant stress biology: Introduction and Challenges, in Brno with dr. Marcel A.K. Jansen (University College Cork, Ireland) 2014
Language Skills	Slovak - Native Language English – actively (B2 level) German – passively
Computer skills	Windows MS Office Origin 8.5
Another skills	License driver Fitness trainer (Fitness Academy Ronnie)

PALACKÝ UNIVERSITY IN OLOMOUC
FACULTY OF SCIENCE
DEPARTMENT OF BIOPHYSICS
CENTRE OF THE REGION HANÁ FOR BIOTECHNOLOGICAL AND AGRICULTURAL RESEARCH

EFFECT OF CYTOKININS ON MAINTENANCE OF PHOTOSYSTEM II FUNCTION DURING INDUCED SENESCENCE

Ph.D. thesis

Helena Janečková
Ph.D. program Physics - Biophysics

Supervisor: Martina Špundová

Olomouc 2019

Bibliographical identification

Author's first name and surname: Mgr. Helena Janečková (maiden name: Melkovičová)

Title: Effect of cytokinins on maintenance of photosystem II function during induced senescence

Type of thesis: Ph.D. thesis

Department: Department of Biophysics

Supervisor: RNDr. Martina Špundová, Ph.D.

Consultant: Mgr. Alexandra Husičková, Ph.D.; Mgr. Karel Doležal, Dr., DSc.

The year of presentation: 2019

Abstract: Leaf senescence, the final stage of leaf development, is a complex process that involves numerous physiological and biochemical changes. Senescence is usually accompanied by a loss of chlorophyll (Chl) and by inhibition of photosynthetic processes, including photosystem (PSII) photochemistry. Plant hormones cytokinins (CKs) are negative regulators of senescence. It is well known that in detached leaves, both exogenously applied CKs and light retard senescence, however, the mechanism of their mutual interaction is not clear. We have analysed changes in content of endogenous CKs and individual CK forms in detached leaves of *Arabidopsis thaliana* kept under different light conditions and we have correlated these changes with changes in chlorophyll content, efficiency of PSII photochemistry, and lipid peroxidation. We have shown that the content of endogenous CKs and individual CK forms in detached *Arabidopsis* leaves differs significantly under various light conditions. In leaves kept in darkness, we have observed decreased content of the most abundant CK free bases and ribosides. We have suggested that the light-mediated retardation of senescence in detached *Arabidopsis* leaves is related to light-dependent persistence of N^6-(Δ^2-isopentenyl)adenine CK biosynthesis during senescence. We have also found that light was able to partially compensate the disrupted CK signalling caused by the loss-of-function mutation in CK receptors during senescence of detached leaves of *Arabidopsis* mutants. Recent studies have shown that Chl *b* is important for the regulation of senescence. We have found that the deficiency of Chl *b* in barley mutant significantly accelerates the inhibition of PSII photochemistry during dark-induced senescence of detached leaves. We suppose that the stronger impairment of PSII function is associated with a more pronounced damage of reaction centre of PSII. Exogenously applied CKs were able to eliminate the acceleration of PSII impairment, probably by the stabilization of reaction centres of PSII.

Key words: cytokinins, light, photosynthesis, photosystem II, senescence

Number of pages including references: 76

Number of appendices: 2

Language: English

Bibliografická identifikácia

Meno a priezvisko autora: Mgr. Helena Janečková (rod. Melkovičová)

Názov práce: Vplyv cytokinínov na udržanie funkcie fotosystému II v priebehu indukovanej senescencie

Typ práce: doktorská

Oddelenie: Katedra biofyziky

Vedúci práce: RNDr. Martina Špundová, Ph.D.

Konzultanti: Mgr. Alexandra Husičková, Ph.D.; Mgr. Karel Doležal, Dr., DSc.

Rok obhajoby práce: 2019

Abstrakt: Listová senescencia, konečné štádium vývoja listov, je komplexný proces, ktorý zahŕňa množstvo fyziologických a biochemických zmien. Senescencia je zvyčajne sprevádzaná poklesom obsahu chlorofylu a inhibíciou fotosyntetických procesov, vrátane fotochémie fotosystému II (PSII). Rastlinné hormóny cytokiníny (CK) sú známe ako negatívne regulátory starnutia. V oddelených listoch je dobre známe, že exogénne aplikované CK ako i svetlo spomaľujú priebeh senescencie, avšak mechanizmus ich vzájomnej interakcie pri sprostredkovaní tohto účinku zostáva nejasný. Analyzovali sme zmeny v obsahu endogénnych CK a jednotlivých CK foriem v oddelených listoch *Arabidopsis thaliana* inkubovaných v rôznych svetelných podmienkach a tieto zmeny sme korelovali so zmenami v obsahu chlorofylu, účinnosti fotochémie PSII a peroxidácii lipidov. Ukázali sme, že v oddelených listoch *Arabidopsis* inkubovaných pod rôznymi svetelnými podmienkami sa obsah endogénnych CK a ich jednotlivých foriem výrazne líši. V listoch inkubovaných v tme sme pozorovali znížený obsah najhojnešie sa vyskytujúcich CK báz a ribosidov. Navrhli sme, že spomalenie senescencie vyvolaná svetlom u oddelených listov *Arabidopsis* môže súvisieť s pretrvávajúcou biosyntézou N^6-(Δ^2-isopentenyl)adenínu. Ukázali sme, že počas senescencie oddelených listov *Arabidopsis* je svetlo schopné nahradiť CK signál v prípade porušenej signálnej dráhy kvôli nefunkčnosti CK receptorov. Nedávne štúdie ukázali, že chlorofyl (Chl) *b* má tiež dôležitú úlohu v regulácii senescencie. Zistili sme, že deficit Chl *b* u mutanta jačmeňa výrazne zrýchľuje inhibíciu fotochémie PSII počas indukovanej senescencie oddelených listov inkubovaných v tme. Predpokladáme, že silnejšie poškodenie funkcie PSII súvisí s výraznejším poškodením reakčných centier PSII. Exogénne aplikovaný CK eliminoval urýchlenie poškodenia PSII, pravdepodobne stabilizáciou reakčných centier PSII.
Kľúčové slová: cytokininy, fotosyntéza, fotosystém II, senescence, světlo

Počet strán vrátane zoznamu literatúry: 76

Počet príloh: 2

Jazyk: Anglický

Declaration

I hereby declare that I have written this thesis independently as the orginal work, with the help of my supervisor RNDr. Martina Špundová, Ph.D. The complete list of used literature and other information sources is included in the section “References”.

Olomouc, Czech Republic, 2019 Author

Contents

List of Publications

This thesis is based on two papers, referred in the text by name of the first author. These research papers are enclosed at the end of the thesis.

I. **Janečková H (29 %)**, Husičková A, Ferretti U, Prčina M, Pilařová E, Plačková L, Pospíšil P, Doležal K, Špundová M. 2018. The interplay between cytokinins and light during senescence in detached Arabidopsis leaves. Plant, Cell &Environment 41: 1870–1885. IF (2017): 6.173
(*Janečková H designed the final experiments, performed chlorophyll content assay and chlorophyll fluorescence measurement and wrote the manuscript*)

II. **Janečková H (30 %)**, Husičková A, Lazár D, Ferretti U, Pospíšil P, Špundová M. 2019. Exogenous application of cytokinin during dark senescence eliminates the acceleration of photosystem II impairment caused by chlorophyll b deficiency in barley. Plant Physiology and Biochemistry 136: 43–51. IF (2017): 2.718
(*Janečková H designed and performed the experiments, analyzed the data, interpreted results and wrote the manuscript*)

Paper by the same author that is not included in the thesis:

III. Ilík P, Špundová M, Šicner M, **Melkovičová H (5 %)**, Kučerová Z, Krchňák P, Fürst T, Večeřová K, Panzarová K, Benediktyová Z, Trtílek M. 2018. Estimating heat tolerance of plants by ion leakage: A new method based on gradual heating. New Phytologist 218: 1278–1287. IF (2017): 7.43

Abbreviations

AHK	-	Arabidopsis histidine kinase
AHP	-	Arabidopsis histidine phosphotransfer proteins
ARR	-	Arabidopsis response regulator
ATP	-	adenosine triphosphate
BA	-	N^6-benzylaminopurine
CAO	-	chlorophyllid *a* oxygenase
car	-	carotenoids
Chl	-	chlorophyll
clo	-	*chlorina f2*^{f2}(barley mutant deficient in Chl *b*)
CK(s)	-	cytokinin(s)
CKX	-	cytokinin oxidase/dehydrogenase
Cyt b_6/f	-	cytochrome b_6/f
*c*Z	-	*cis*-zeatin
DHZ	-	dihydrozeatin
DMAPP	-	dimethylallyl pyrophosphate
DMSO	-	dimethylsulfoxide
F_V/F_M	-	maximal quantum yield of photosystem II photochemistry in the dark-adapted state
HMBDP	-	hydroxymethylbutenyl disphosphate
HmChl *a*	-	7-hydroxymethyl chlorophyll *a*
HPLC	-	high performance liquid chromatography
iP	-	N^6-(Δ^2-isopentenyl)adenine
iPR	-	iP-riboside
iPRMP/iPRDP/iPRTP	-	isopentenyladenosine-5´-monophosphate/-diphosphate/-triphosphate
IPT	-	isopentenyltransferase
Lhca	-	light-harvesting chlorophyll *a/b*-binding protein of photosystem I
Lhcb	-	light-harvesting chlorophyll *a/b*-binding protein of photosystem II
LHCII	-	light-harvesting complex of photosystem II
LHC(s)	-	light harvesting complex(es)
LOG	-	lonely guy
MDA	-	malondialdehyde
MEP	-	methylerythritol phosphate pathway
*m*T	-	*meta*-topolin
MVA	-	mevalonate pathway
NOL	-	non-yellow coloring1-like
NYC1	-	non-yellow coloring1
ORE1	-	ORESARA1
PAO	-	pheophorbide *a* oxygenase
*p*FCC	-	primary fluorescent Chl catabolite
phy	-	phytochrome(s)
phyA	-	phytochrome A

phyB	-	phytochrome B
PIF(s)	-	phytochrome-interacting trancription factor(s)
PPH	-	pheophytin pheophorbide hydrolase
PQ	-	plastoquinone
PSI	-	photosystem I
PSII	-	photosystem II
RCI	-	reaction center of photosystem I
RCII	-	reaction center of photosystem II
RCC	-	red chlorophyll catabolite
RC(s)	-	reaction center(s)
ROS	-	reactive oxygen species
RRs	-	response regulators
Rubisco	-	ribulose-1,5-bisphosphate carboxylase/oxygenase
SAG(s)	-	senescence-associated gene(s)
TDZ	-	thidiazuron
TF(s)	-	transcription factors
*t*Z	-	*trans*-zeatin
UGTs	-	uridine diphosphate glycosyltransferases
WT	-	wild-type
$\Phi_{f,D}$	-	quantum yield of constitutive non-regulatory dissipation processes in the light-adapted state
Φ_{NPQ}	-	quantum yield of regulatory non-photochemical quenching in the light-adapted state
Φ_{P}	-	effective quantum yield of photosystem II photochemistry in the light-adapted state
Φ_{PSII}	-	maximal quantum yield of photosystem II photochemistry in the light-adapted state

1 INTRODUCTION

The main topic of the presented dissertation thesis is leaf senescence, a very complex developmental process characterized by numerous alterations of cell structure, metabolism and gene expression. In particular, the thesis is focused on the involvement of plant hormones cytokinins and light in the regulation of this process.

During leaf senescence, the nutrients are remobilized from mature leaves to other developing parts of the plant (such as seeds or young leaves), which ensures either the survival of the plant itself (polycarpic plants), or the survival of next plant generations (monocarpic plants). However, premature senescence, induced for example by stress conditions, can limit the growth phase of plants and is therefore connected with reduced yield in crops. It can also cause post-harvest problems, such as leaf yellowing or nutrient loss in vegetable crops. Thus, understanding the proper mechanism of leaf senescence and its regulation has big potential implications for agriculture.

The first chapter of the thesis deals with different changes associated with leaf senescence, in particular with the senescence-related degradation of photosynthetic proteins and loss of photosynthetic function. The second part of the thesis is focused on plant hormones cytokinins, which are well known to delay leaf senescence. Their effect on the stru cture as well as function of photosynthetic apparatus, especially photosystem II photochemistry, during leaf senescence is discussed here. In the last chapter, I summarize all the methods used during my research.

The thesis is written in a form of a mini-review, summarizing the current knowledge related to the topic of leaf senescence and cytokinins. The results of my research published in Janečková et al. (2018) and (2019) are discussed in the context these up-to-date findings and are referred in the text by bold green letters. The publications themselves are enclosed in the Appendix of the thesis.

1.1 Leaf senescence

Leaf senescence (senescence from Latin “senēscere” meaning “to grow old”) is a highly regulated process that involves coordinated dismantling of cellular components and remobilization of valuable compounds. Senescing cell undergoes changes in structure, metabolism and gene expression. Senescence can be followed by leaf death, but the death is actively delayed until all nutrients are removed (Buchanan-Wollaston et al., 2003).

To study the processes of leaf senescence, many approaches have been developed. One of the most common ones, used to initiate a synchronized and fast senescence, is based on the detachment of a leaf or a leaf segment, which is then kept in the dark (Weaver and Amasino, 2001). Although it is now known that developmental senescence differs from the senescence induced in detached darkened leaves (Buchanan-Wollaston et al., 2005), some of the senescence-associated genes (SAGs) have been found to function in both of these processes, suggesting a partially shared mechanism between them (see e.g. Woo et al., 2001; Guo and Gan, 2006; Kim et al., 2009). Other systems that have been used to study senescence include darkening of individual leaves that are attached to otherwise illuminated plant (see e.g. Brouwer et al., 2012) or darkening of the whole plant (see e.g. Weaver and Amasino, 2001) (Fig. 1).

Chloroplasts are the first organelles to show the symptoms of senescence. These organelles, besides providing energy for plant organism, represent an important storage site of total leaf nitrogen, as about 70 – 80 % of the total leaf nitrogen is located here (Makino and Osmond, 1991; Smart, 1994; Ishida et al., 2014). Around 70 % of chloroplast nitrogen is present in the stroma (Makino and Osmond, 1991) and the rest is in the thylakoid membrane (Ishida et al., 2014). The chloroplast enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which catalyzes the CO_2 assimilation and photorespiration, comprises up to 50 % of the total chloroplast nitrogen (Smart, 1994) and around 30 % of the leaf nitrogen (Makino et al., 2003). The apoproteins of photosystems and light-harvesting complexes store about 30 % of the total chloroplast nitrogen (Smart, 1994) and around 7 % of leaf nitrogen is found in the light-harvesting chlorophyll *a/b*-

binding protein complexes of photosystem II (LHCII) in thylakoid membrane (Makino et al., 2003).

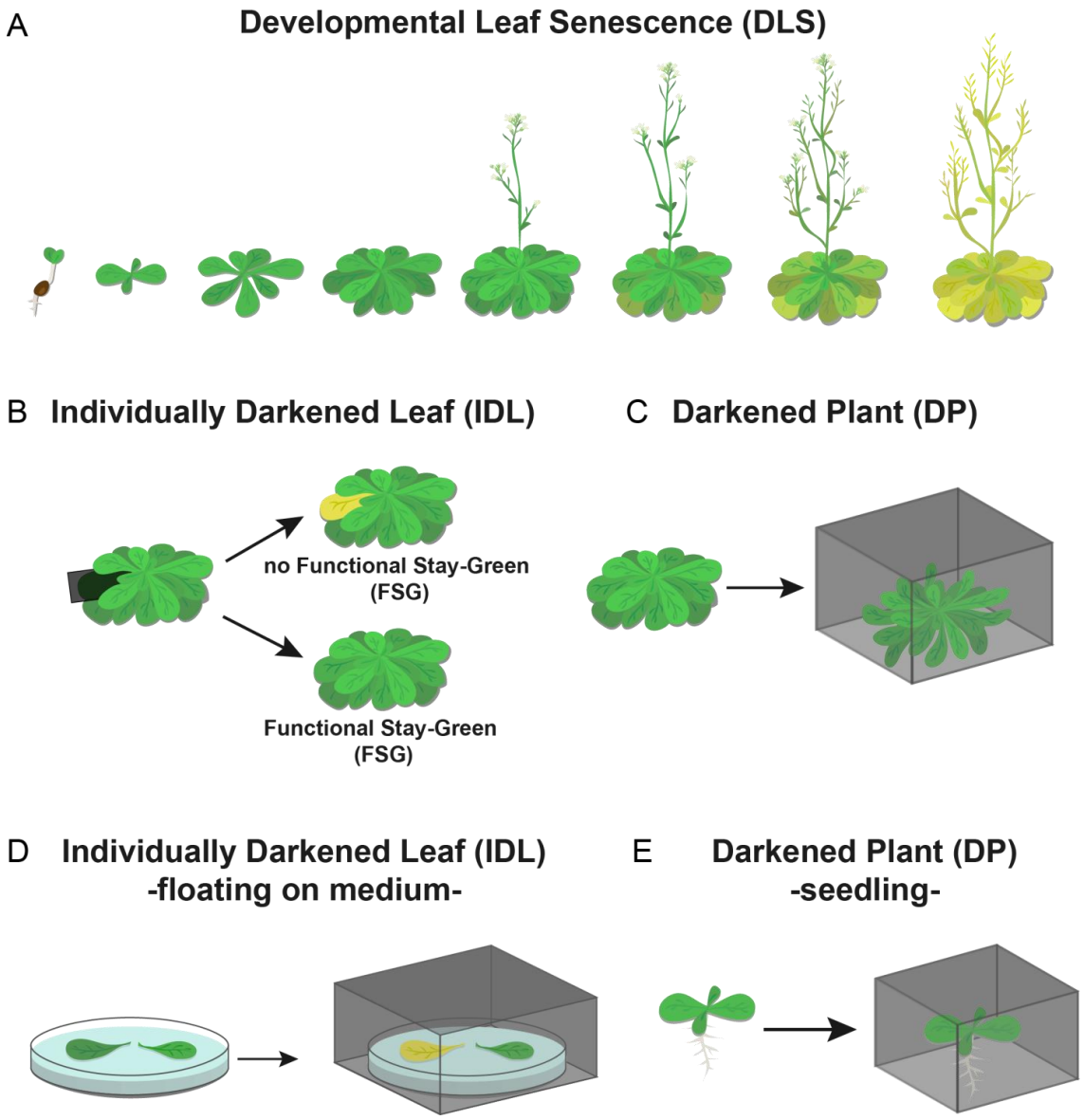

Fig. 1: Various approaches to study senescence. (A) Developmental leaf senescence, (B) individually darkened leaf on an illuminated plant, (C) whole plant kept in darkness, (D) detached leaf/leaves floating on a medium and kept in darkness or (E) darkened seedlings. Illustrated and kindly provided by Daria Chrobok.

During leaf senescence, the amount and size of chloroplasts become reduced and their shape is changed from nearly oval to more spherical. Some of the inner components

are degraded, thylakoid membranes are disintegrated and chloroplasts gradually transform into gerontoplasts (for a review, see e.g. Biswal et al., 2012). Membrane disorganization in chloroplasts is sequential and starts with the unstacking of granal thylakoids, followed by the formation of loose and elongated lamellae (for a review, see e.g. Biswal, 1997; Khanna-Chopra, 2012). These loose lamellae subsequently undergo massive degradation with concomitant formation of plastoglobules. Plastoglobules serve as storage of senescence-associated catabolic products and they even participate in the transformation of these products into storage compounds (for a review, see e.g. Rottet et al., 2015). In chloroplasts of senescing soybean leaves, Guiamét et al. (1999) observed that plastoglobules carrying photosynthetic components (such as chlorophyll derivatives) protruded through the chloroplast envelope and emerged into the cytoplasm, where these globules received a polygonal coat and eventually disintegrated.

Structural disorganization of chloroplast membranes, connected with the degradation of lipids, proteins and/or chlorophylls, is an enzyme-regulated process. Lipase, acyl hydrolase, phosphatidic acid phosphatase, lipoxygenase and/or phospholipase-D are suggested to play a role in senescence-induced loss of lipids (Buchanan-Wollaston et al., 2003; Biswal et al., 2012). Galactolipids of thylakoid membranes, namely monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), the major lipids in photosynthetic membrane, are degraded by various phospolipases, releasing polyunsaturated fatty acids (Smart, 1994; Biswal et al., 2012). It seems that fatty acids become an important source of energy in senescent leaves, which are depleted of carbohydrate. This suggestion is supported by senescence-enhanced expression of genes associated with peroxisomal β-oxidation, glyoxylate pathway and gluconeogenesis (Buchanan-Wollaston et al., 2003; Biswal et al., 2012; for a detailed information about fatty acid catabolism, see e.g. Graham and Eastmond, 2002). For example, the acetyl coenzym A derived from fatty acid degradation can be used directly for respiration or via the glyoxylate pathway and gluconeogenesis for the synthesis of sugars (Buchanan-Wollaston et al., 2003).

1.1.1 Protein degradation and chlorophyll breakdown pathway during leaf senescence

During leaf senescence, the loss of proteins is significant (Smart, 1994; Biswal, 1997; Martínez et al., 2008*b*; Biswal et al., 2012). The chloroplast proteins are degraded through the action of proteolytic enzymes (proteases) and the degradation may occur via chloroplastic or/and extra-chloroplastic pathways (for a review, see e.g. Martínez et al., 2008*b*). Proteases are either present in the cell (in chloroplast or/and in cytoplasm) or are synthesized *de novo* during senescence (Biswal et al., 2012; Khanna-Chopra, 2012). Increased activity of proteases has been observed during leaf senescence of cowpea (Srivalli et al., 2001), wheat (Martínez et al., 2007) or *Arabidopsis* and soybean (Otegui et al., 2005).

Different protease families were shown to participate in the degradation of chloroplast proteins, including Clp (clip proteases), FtsH (Filamentous temperature sensitive H), DegP (degradative protease) or aspartic proteases. Some of the chloroplast proteases, such as Clp or FtsH, are ATP-dependent, i.e. energy requiring (Sakamoto, 2006; Biswal et al., 2012). The pathways of chloroplast protein degradation outside the chloroplast can include the role of central vacuole in cell, autophagy or/and involvement of small senescence-associated vacuoles (for a review, see e.g. Martínez et al., 2008*b*). The senescence-associated vacuoles (SAVs) as an alternative extra-chloroplastic pathway has been shown to operate, for instance, in senescent leaves of soybean, *Arabidopsis* or tobacco (Otegui et al., 2005; Martínez et al., 2008*a*, 2008*b*; Ishida et al., 2014). These vacuoles can be found only in senescing photosynthetic tissues and contain a cysteine protease SAG12 (senescence-associated gene 12) (Otegui et al., 2005; Ishida et al., 2014). It has been suggested that stromal proteins might cross the chloroplast envelope and then can be directly transferred to SAVs via yet unknown mechanism (Ishida et al., 2014). Besides SAVs, autophagy via Rubisco-containing bodies for degradation in central vacuole has been reported as another extra-chloroplastic pathway in the degradation of stromal proteins, with the involvement of the authophagy-related gene (ATGs) (Ishida et al., 2014).

The stromal proteins can be degraded also non-enzymatically, by the action of reactive oxygen species (ROS) (Ishida et al., 1999; 2002; Buchanan-Wollaston et al., 2003; Biswal et al., 2012; Khanna-Chopra, 2012). It has been proposed that ROS generated during

senescence in the chloroplast can initiate the degradation of proteins. Subsequently, the breakdown products of proteins may be released to cytoplasm and the final proteolysis may be performed by vacuolar proteases (Biswal et al., 2003; Buchanan-Wollaston et al., 2003; Biswal et al., 2012). However, the increase in ROS levels during senescence is likely to be the result of macromolecule degradation and thus ROS accumulation occurs after protein and lipid degeneration. This discrepancy clearly demonstrates that the exact mechanism of ROS production in senescing chloroplast has not been elucidated so far and that the precise regulatory mechanism of protein degradation is not yet clearly understood (for a review, see e.g. Buchanan-Wollaston et al., 2003; Biswal et al., 2012; Krieger-Liszkay et al., 2019).

Protein complexes that participate in light-driven electron transport in the primary phase of photosynthesis are localized in the thylakoid membrane. The photosynthetic apparatus consists of photosystem II (PSII) and photosystem I (PSI), two multi-subunit pigment-binding protein complexes associated with their respective light-harvesting complexes (LHCs), the cytochrome b_6/f (Cyt b_6/f) complex and ATP synthase. PSII and PSI are organized into large supercomplexes with variable amounts of membrane-bound peripheral antenna complexes. PSII complexes are predominantly located in grana thylakoids, whereas PSI complexes are located in stroma lamellae (for a review, see e.g. Dekker and Boekema, 2005).

The photochemical reaction center complex of PSII (RCII) includes D1 (PsbA) and D2 (PsbD) proteins, together with cytochrome b_{559}, which associates with CP47 (PsbB) and CP43 (PsbC) subunits. CP47 and CP43 have a light-harvesting function, i.e. they absorb light and transfer the excitation energy (also excitation energy from the peripheral antennae) to the RCII. Peripheral light-harvesting complex of PSII (LHCII) is formed by trimers, consisting of proteins Lhcb1, Lhcb2 and Lhcb3. In addition, monomeric complexes CP29 (Lhcb4), CP26 (Lhcb5) and CP24 (Lhcb6) located between the core complexes of PSII (i.e. subunits PsbA – PsbD) and the LHCII trimers and serve as minor light-harvesting antennae of PSII. The oxygen-evolving complex is localized on the lumenal (donor) side of PSII (see e.g. Jansson, 1994, 1999 or Dekker and Boekema, 2005).

The primary electron donor, chlorophyll *a* special pair (P680), passes electrons via pheophytin to the primary electron acceptor of PSII, Q_A. Another quinone molecule (denoted as Q_B) is transiently bound at the acceptor side of PSII and accepts electrons from Q_A. After two-electron reduction, Q_B binds two protons from the stroma and is released from PSII into the plastoquinone pool. Reduced plastoquinol transfers electrons to Cyt b_6/f complex, from which the electrons are transferred further to PSI.

The PSI reaction center (RCI) is formed by PsaA and PsaB proteins, which associate with additional proteins PsaG, PsaH, PsaO and PsaN. The main antenna complex of PSI (LHCI) consists of Lhca1, Lhca2, Lhca3 and Lhca4 proteins, which are assembled in dimeric forms (Jansson, 1999). In addition, there are also Lhca5 and Lhca6 proteins, which are however present at much lower amounts than the major proteins Lhca1 – 4 (Jansson, 1999; Klimmek et al., 2006). The primary PSI electron donor (Chl *a,* P700) passes electrons to acceptor molecules. At the acceptor side of PSI, ferredoxin is reduced and electrons are subsequently transferred to ferredoxin-$NADP^+$ reductase (FNR), which reduces $NADP^+$ to NADPH. In addition, ferredoxin may donate electrons to other pathways, such as cyclic electron transfer and/or sulfur and nitrogen assimilation. The electron transfer reactions are coupled with proton pumping into the thylakoid lumen, and the resulting electrochemical gradient of protons is harnessed to produce ATP. The ATP and NADPH from the primary photosynthetic reactions are then used as a fuel for the Calvin-Benson cycle to fixate CO_2 and for other assimilatory processes (for more information about PSI, see e.g. Nelson and Junge, 2015).

All Lhcb and Lhca proteins bind various numbers of pigment molecules - chlorophylls (Chl) *a* and *b* and xanthophylls (lutein, violaxanthin and/or and neoxanthin) (see e.g. Jansson, 1994; Jansson, 1999). Chl *b* stabilizes the final conformation of LHC. It has been shown that Chl *b* molecules bind to LHC more tightly than molecules of Chl *a* and that the absence of Chl *b* leads to the destabilization of LHC apoproteins (Tanaka and Tanaka, 2011).

Pigment-protein complexes are known to gradually degrade during leaf senescence (Mae et al., 1993; Humbeck et al., 1996; Miersch et al., 2000; Tang et al., 2005; Nath et al., 2013; Żelisko and Jackowski, 2004), but the rate of the degradation of individual complexes

is different. Compared to the proteins in stroma, such as Rubisco, the membrane proteins localized in thylakoid membranes are more difficult targets for degradation (Grover, 1993; Biswal, 1997; Khanna-Chopra, 2012). Thus, the decrease in the activity and content of Rubisco usually precedes the inhibition of primary photosynthetic reactions and loss of thylakoid proteins (Camp et al., 1982; Grover 1993; Mae et al., 1993; Sobieszczuk-Nowicka et al., 2018). The loss of thylakoid membrane proteins was observed during both developmental (Humbeck et al., 1996; Miersch et al., 2000; Tang et al., 2005; Nath et al., 2013) and induced senescence (Oh et al., 2003, Żelisko and Jackowski 2004; Oh et al., 2005; Kusaba et al., 2007; Park et al., 2007; Zavaleta-Mancera et al., 2007; Sato et al., 2009; Sakuraba et al., 2013) of various plant species.

The loss of individual thylakoid membrane proteins during developmental senescence of *Arabidopsis* leaves has been described in detail by Nath et al. (2013). They have shown that the senescence is accompanied by a loss of D1, PsaA and PsaB proteins and Cyt b_6/f complex as well as by a loss of some LHC proteins. They observed that Lhcb4, the minor antenna protein in LHCII, showed the highest rate of degradation, whereas the amounts of Lhcb1, Lhcb2 and Lhcb3 seemed to be rather stable during senescence, indicating that major antenna proteins are preferentially retained in the thylakoid membranes. In the case of LHCI, Lhca1 and Lhca2 remained rather stable during senescence, followed by Lhca4, then Lhca3 (Nath et al., 2013). These observations confirm that the degradation rate can vary even between different members of the LHC protein family.

In detached leaves of rice senescing in the dark, the degradation of LHCI and LHCII proteins as well as components of core complexes of both photosystems was observed by Kusaba et al. (2007). They observed pronounced decrease in the amount of CP47, a PSII core antenna, and PsaF, a component of RCI. Moreover, their results also suggest that the major trimeric LHCII proteins, Lhcb1 and Lhcb2, are more stable than the monomeric proteins (Lhcb4 and Lhcb6) (Kusaba et al., 2007).

Various studies indicate that during leaf senescence, LHC proteins are more stable than the proteins of RC (Humbeck et al., 1996; Miersch et al., 2000; Tang et al., 2005; Park

et al., 2007; Nath et al., 2013; Sakuraba et al., 2013). For example, Park et al. (2007) have shown that LHCI and LHCII proteins are retained in the dark-senescing leaves of rice (*Oryza sativa* L.), while D1 protein is progressively degraded (Park et al., 2007). As Chl *a* is associated with both RC and LHCs in higher plants, whereas Chl *b* is specific only to LHCs, the changes in Chl *a/b* ratio can be used as a simple estimate of the changes in the relative amount of LHC and RC (Leong and Anderson, 1984; Tanaka and Tanaka, 2011). In numerous studies, leaf senescence in darkness was reported to be accompanied by a decrease in this ratio, indicating that RCs are degraded faster than LHCs (e.g. Hidema et al., 1991; Kusaba et al., 2007, Nath et al., 2013; **Janečková et al., 2019**). In our study (**Janečková et al. 2019**) we have observed a decrease in the Chl *a/b* ratio in detached barley leaves (*Hordeum vulgare* L.) senescing in darkness, indicating preferential decrease in the amount of RCII in comparison to LHCII. Similarly, Nath et al. (2013) reported that the amount of LHCII was rather stable during developemntal senescence of *Arabidopsis* leaves, whereas the amount of RCII proteins decreased, which was consistent with the observed decrease in the Chl *a/b* ratio (Nath et al., 2013). The decreased Chl *a/b* has been reported also during dark-induced senescence of rice (*Oryza sativa* L.) leaves by Kusaba et al. (2007). However, Krupinska et al. (2012) have shown that the Chl *a/b* ratio increased during senescence of flag leaves of barley (*Hordeum vulgare* L.) under field conditions, and that LHCII proteins were degraded faster than RCII during senescence. The increased Chl *a/b* ratio was also reported in detached *Arabidopsis* leaves during dark induced senescence by Pružinská et al. (2007). The above mentioned studies also imply that the order of senescence-associated degradation of the complexes may be different under different conditions of senescence and also in different species.

It has been assumed that chloroplastic proteases are involved in the degradation of pigment-binding proteins during senescence, however, the exact proteases that participate in this process are still largely unknown. The only exception is the degradation of the D1 protein, which is known to be degraded via chloroplast-localized DegP and FtsH proteases (Adam and Clarke 2002; Khanna-Chopra, 2012). Serine protease DegP2, localized on the stroma side of the thylakoid membrane, was shown to be responsible for the initial

cleavage of damaged D1 protein before its complete degradation by FtsH proteases (Haussühl et al., 2001). The FtsH metalloprotease was proposed to be involved in the secondary proteolysis of the D1 protein (Lindahl et al., 2000). In 2005, Żelisko et al. reported that during dark-induced senescence of *Arabidopsis* plants, the degradation of LHCII, namely Lhcb3, involves the action of FtsH6 protease. In addition, the involvement of DegP2 protease in senescence program has been confirmed also by the finding that leaf senescence is delayed in plants with repressed activity of this protease (Lucińsky et al., 2011).

Before the degradation of LHCs, Chl molecules have to be removed from these complexes (Hörtensteiner and Feller, 2002; Buchanan-Wollaston et al., 2003; Martínez et al., 2008*b*; Tanaka et al., 2011; Hörtensteiner, 2013). The breakdown of Chl *b* is required for LHCII degradation, while the degradation of LHCII seems to be necessary for the breakdown of Chl *a* and some carotenoids (car) contained within LHCII (Kusaba et al., 2007; Sato et al., 2009). Chl degradation (Fig. 2) is carried out by specific enzymes and involves several steps (for a review, see e.g. Hörtensteiner, 2013; Kuai et al., 2018).

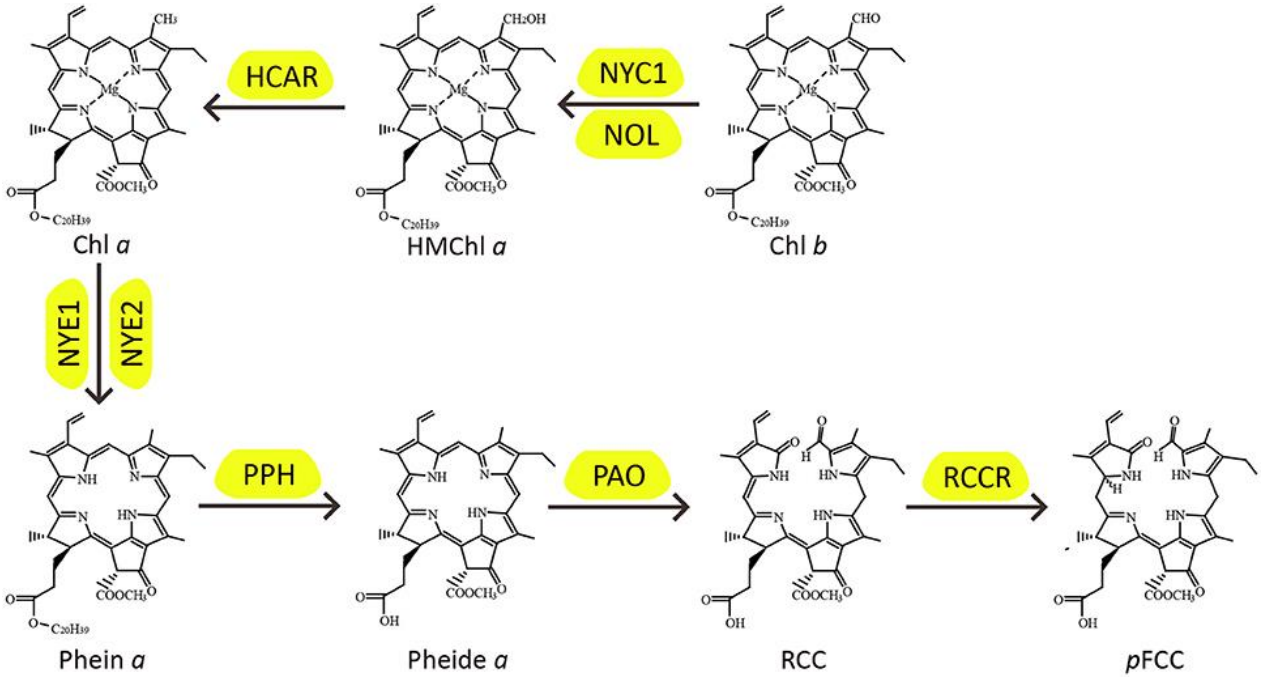

Fig. 2: Chlorophyll degradation pathway. Products: HmChl *a*, 7-hydroxymethyl Chl *a*; Chl *a*, chlorophyll *a*; Phein *a*, pheophytin *a*; Pheide *a*, pheophorbide *a*; RCC, red Chl catabolite; *p*FCC, primary fluorescent Chl catabolite. Enzymes: NYC1, non-yellow coloring1; NOL, NYC1-LIKE; HCAR, 7-

hydroxymethyl Chl *a* reductase; NYE, non-yellowing; PPH, pheophytinase; PAO, pheophorbide *a* oxygenase; RCCR, red Chl catabolite reductase (Zhu et al., 2017; open access).

Firstly, Chl *b* must be converted to Chl *a*. The reason is the specifity of the downstream catabolic enzymes, as the respective "*b*" pigments are not recognized as substrates (Hörtensteiner et al., 1995, Shimoda et al., 2016). The conversion proceeds via two-step process, which is a part of so-called Chl cycle , and includes two enzymes - Chl *b* reductase, which requires NADPH as electron source and is localized in the thylakoid membrane, and hydroxymethyl Chl *a* reductase (HCAR), which is localized in stroma (Hörtensteiner, 2013). The first step is the reduction of Chl *b* into 7-hydroxymethyl chlorophyll *a* (HmChl *a*) by Chl *b* reductase, followed by a reduction of HmChl *a* to Chl *a* by HCAR (Meguro et al., 2011; Hörtensteiner, 2013). The Chl *b* reductase is encoded by two genes, *NON-YELLOW COLORING1* (*NYC1*) and *NYC1-like* (*NOL*) (Kusaba et al., 2007; Horie et al., 2009; Sato et al., 2009). The proper function of both subunits NYC1 and NOL of Chl *b* reductase is required for the degradation of LHCII during leaf senescence (Kusaba et al., 2007).

The next step in the Chl degradation pathway is Mg-dechelation and subsequent dephytylation (Schelbert et al., 2009; Hörtensteiner, 2013; Kuai et al., 2018). Not long time ago, uncertainty existed about the order of these two reactions. It had been suggested that phytol removal precedes Mg-dechelatation. In agreement with it, the chlorophyllase enzyme, which hydrolyzes phytol from Chl, was believed to be active during senescence-related Chl breakdown. However, some contradictory results were obtained in experiments with deregulated expression of chlorophyllase, as the absence or silencing of its genes had little effect on senescence-related Chl breakdown (e.g. Schenk et al., 2007). In 2009, Schelbert et al. identified a phytol hydrolase in *Arabidopsis thaliana*, named pheophytinase (pheophytin pheophorbide hydrolase; PPH), which specifically hydrolyzes pheophytin, is chloroplast-localized and senescence-induced. Its respective mutants were unable to degrade Chl and exhibited stay-green phenotype (Schelbert et al., 2009). Thus PPH specifically dephytylates pheophytin, yielding the respective pheophorbide (Schelbert et al., 2009; Hörtensteiner, 2013; Kuai et al., 2018). This was confirmed by results of two other research groups in *Arabidopsis* (Ren et al., 2010) and rice (Morita et al., 2009).

The Mg-dechelation of Chl *a* is catalysed by Mg-dechelatase, which is encoded by Mendel's green cotyledon genes, NON-YELLOWINGs/STAY-GREENs (NYEs/SGRs) (Shimoda et al., 2016; Wu et al., 2016; Fig. 2). After removal of the central Mg atom, dephytylation of pheophytin *a* by PPH occurs, resulting in pheophorbide *a* (Schelbert et al., 2009; Hörtensteiner, 2013). Pheophorbide *a* is then converted into a red Chl catabolite (RCC) by pheophorbide *a* oxygenase (PAO) that opens the ring structure (Pružinská et al., 2003; Hörtensteiner, 2013). The activity of PAO was shown to be pronouncedly increased during senescence (Hörtensteiner et al., 1995; Pružinská et al., 2005). RCC is further converted by RCC reductase (RCCR) to primary fluorescent Chl catabolite (*p*FCC), leading to the loss of green color (Pružinská et al., 2007; Hörtensteiner, 2013; Fig. 2). As converted to *p*FCC, the potential phototoxicity of Chl is abolished (Kuai et al., 2018).

All the above-described reactions take place within the chloroplast, whereas the following reactions of Chl catabolism occur outside this organelle (Hörtensteiner, 2013; Kuai et al., 2018). The *p*FCC is transported to the cytoplasm and then to vacuoles. Here, the role of a catabolite transporter and ATP-binding cassette transporter (ABC transporter) is considered. In vacuoles, *p*FCC is probably converted to respective non-fluorescent Chl catabolites (NCCs) (Hörtensteiner, 2009; Biswal et al., 2012; Hörtensteiner, 2013). This conversion is driven by acidic pH, which further supports the vacuolar localization of this process (Oberhuber et al., 2003; Kuai et al., 2018).

As the degradation of Chl is a prerequisite for the degradation of LHCs in senescent leaves, it is very important for accessing this second-largest pool of chloroplast nitrogen (Hörtensteiner and Feller, 2002; Wu et al., 2016; Kuai et al., 2018). The Chl breakdown has to be quick, as illuminated free Chl is a source of toxic free radicals and can induce photooxidative damage of cells (Biswal et al., 2003). By-products of Chl degradation can be further re-used for biosynthetic purposes, particularly phytol, which was shown to be incorporated into tocopherol during leaf senescence (Kuai et al., 2018).

The gradual loss of Chl during leaf senescence, as well as the degradation of structural components of photosynthetic apparatus, is generally accompanied by a decrease in photosynthetic activity. The activity of the primary photosynthetic reactions,

including PSII photochemistry, is usually inhibited later than the activity of Calvin-Benson cycle (e.g. Camp et al., 1982; Grover, 1993; Špundová et al., 2005*a*; Vlčková et al., 2006). Photochemical efficiency of PSII (Oh et al., 1996; Špundová et al., 2003; 2005*b*; Vlčková et al., 2006; Nath et al., 2013; Krieger-Liszkay et al., 2015; **Janečková et al., 2018, 2019**) as well as PSI (Nath et al., 2013; Krieger-Liszkay et al., 2015; **Janečková et al., 2019**) declines during leaf senescence. However, from the data presented in the literature, it is not clear whether the decrease in photosynthetic activity of PSII precedes the inhibition of PSI or vice versa (e.g., Nath et al., 2013; Krieger-Liszkay et al., 2015). In our study (**Janečková et al., 2019**) we have observed that the activity of PSII in dark-senescing leaves of barley (*Hordeum vulgare* L.) was more impaired than the activity of PSI. On the other hand, Krieger-Liszkay et al. (2015) observed that PSI activity declined earlier than PSII activity during senescence of flag barley leaves of cv. Carina. Similar trend was observed by Tang et al. (2005), who reported that PSII appears to remain more functional than PSI during flag leaf senescence of rice (Tang et al., 2005).

Due to the impairment of photosynthetic apparatus during leaf senescence, the light energy cannot be properly utilized by photochemistry and the activity of non-photochemical processes increases (e.g. Lu and Zhang, 1998; Lu et al., 2001; Wingler et al., 2004; **Janečková et al., 2019**). The excess light energy is dissipated via regulatory non-photochemical quenching processes, which protect photosynthetic apparatus against photooxidative damage and/or non-regulatory quenching processes. The regulatory non-photochemical quenching processes are related to the activation of the xanthophyll cycle, which dissipates excess of light energy as heat in antenna complexes before it reaches RCs. Violaxanthin (V) is converted to zeaxanthin (Z) by de-epoxidation catalyzed by violaxanthin de-epoxidase, through the intermediate antheraxanthin (A) (e.g. Lu et al., 2001; Demmig-Adams, 2014). Z then stimulates non-photochemical quenching within LHC. The extent of the de-epoxidation is expressed as the de-epoxidation state of xanthophylls (DEPS = (A + Z)/(A + Z + V)), which usually increases in senescing leaves (Lu et al., 2001; Špundová et al., 2005*b*; Vlčková et al., 2006).

To evaluate the alterations in partitioning of absorbed light energy for photochemical and non-photochemical processes in the light-adapted state, the following parameters can be used: the effective quantum yield of PSII photochemistry (Φ_P), quantum yield of constitutive non-regulatory dissipation processes ($\Phi_{f,D}$) and quantum yield of regulatory non-photochemical quenching (Φ_{NPQ}) (for a review, see Lazár, 2015). $\Phi_{f,D}$ represents quantum yield of constitutive (basal) energy dissipation, whereas Φ_{NPQ} is quantum yield of regulatory quenching, which is induced by illumination to protect the photosynthetic apparatus against excess light and consequent accumulation of ROS and oxidative damage (Demmig-Adams et al., 2014). Together, the sum of these quantum yields equals unity (Lazár, 2015).

Unlike in non-senescing leaves, where the majority of absorbed light energy utilized by PSII photochemistry, in dark-senescing leaves the absorbed light energy is, to a great extent, allocated into non-photochemical quenching processes (**Janečková et al., 2019**; Fig. 3). In senescing leaves of WT, energy non-utilized by PSII photochemistry was dissipated in both regulatory and non-regulatory processes to a similar extent (comparable Φ_{NPQ} and $\Phi_{f,D}$). However, in senescing leaves of *chlorina f2*^{f2} mutant (see 1.1.3 Chlorophyll *b* and leaf senescence), which had greatly impaired PSII photochemistry, the non-regulatory processes ($\Phi_{f,D}$) pronouncedly prevailed (Fig. 3). The energy dissipation via regulatory processes of non-photochemical quenching increases during senescence and in the later stages the ability to dissipate in regulatory processes decreases. And when the photosynthetic apparatus is almost completely inactivated, the non-regulatory processes begin to prevail (**Janečková et al., 2019**).

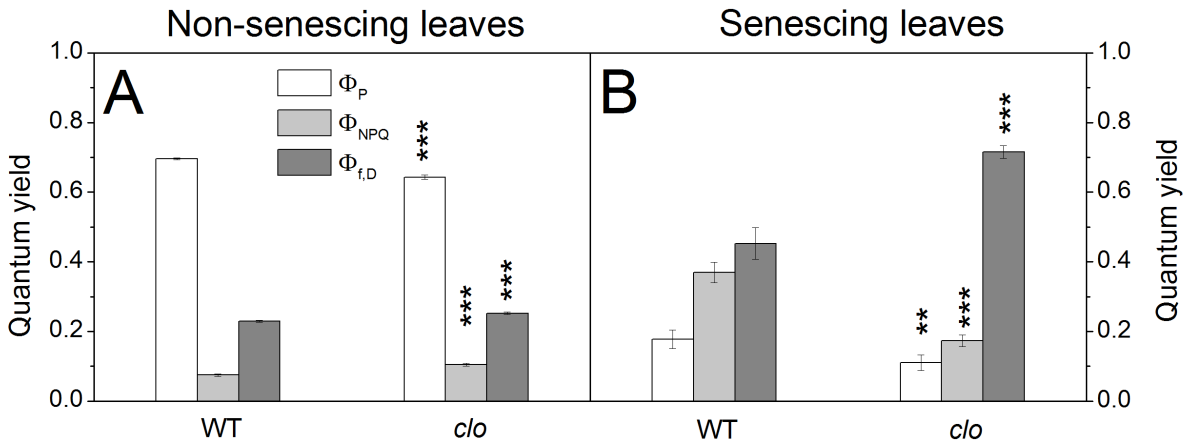

Fig. 3: Alterations in partitioning of absorbed light energy for photochemical and non-photochemical processes in (A) non-senescing and (B) dark senescing leaves of barley (wild-type, WT and *chlorina* $f2^{f2}$ mutant, *clo*). The effective quantum yield of PSII photochemistry (Φ_P), quantum yield of regulatory non-photochemical quenching (Φ_{NPQ}) and quantum yield of constitutive non-regulatory dissipation processes ($\Phi_{f,D}$) in the light-adapted state. Means and SD are shown, n = 6. Asterisks indicate statistically significant difference (Student´s t-test) between WT and *clo* (**, P < 0.01; ***, P < 0.001). Adapted from **Janečková et al. (2019)**.

1.1.2 Regulation of leaf senescence

Leaf senescence is regulated at the level of cell nucleus and its onset is genetically controlled (for a review, see e.g. Kim et al., 2018). However, the initiation as well as the progress of leaf senescence may be influenced by diverse environmental (external) and endogenous (internal) factors. The environmental factors include various abiotic (e.g. drought, extreme temperatures, nutrition deficiency or limitation, shading) and biotic (e.g. pathogen infection) stresses (Smart, 1994; Lim et al., 2007; Khanna-Chopra, 2012). The endogenous factors include phytohormones, different transcription factors or developmental age (Smart, 1994; Lim et al., 2007). Up to date, numerous transcription factors (TFs) have been described as important players in modulating senescence, including PIFs (phytochrome-interacting factors), ORE1 (ORESARA1) or WRKY TFs (Kim et al., 2009; Balazadeh et al., 2010; Hornitschek et al., 2012; Sakuraba et al., 2014; for a review, see e.g. Liebsch and Keech, 2016).

Recent studies have also stressed the effect of the presence of Chl *b* on the progression of senescence (Kusaba et al., 2007; Yang et al., 2016; Sakuraba et al., 2012;

Voitsekhovskaja and Tyutereva, 2015; **Janečková et al., 2019**). The role of Chl *b* and light in leaf senescence is discussed in detail in the following chapters. Plant hormones cytokinins are reviewed in the rest of the thesis, where their role in senescence is thoroughly discussed.

1.1.3 Light and senescence

Light plays an essential role in the regulation of senescence and its effect depends on intensity, length of photoperiod and spectral quality (Biswal and Biswal, 1984; Smart, 1994; Noodén et al., 1996; Lepistö and Rintamäki, 2012; Marchetti et al., 2018).

Light deprivation, severe shade or darkness usually enhance senescence (Weaver and Amasino, 2001; Špundová et al., 2005*a*; Liebsch and Keech, 2016; **Janečková et al., 2018**). Leaf senescence of darkened detached leaves is characterized by extensive loss of photosynthetic pigments (Okada et al., 1992; Oh et al., 2003; Vlčková et al., 2006; Kusaba et al., 2007; Park et al., 2007; **Janečková et al., 2019**) and pigment-protein complexes (Okada et al., 1992; Kusaba et al., 2007; Park et al., 2007), as well as by a decrease in photosynthetic activity (Oh et al., 2003; Špundová et al., 2003; Vlčková et al., 2006; Kusaba et al., 2007; **Janečková et al., 2019**). In addition, chloroplast degradation is accompanied by the formation of plastoglobules (Hurkman, 1979; Vlčková et al., 2006, Park et al., 2007), increased activity of PAO (Pružinská et al., 2005, see chapter 1.1.1) or by the induction of SAGs (Weaver et al., 1998).

However, senescence-associated changes typically observed in the dark can differ, depending on the form of dark treatment (e.g. individual leaves or whole plants). It has been shown that the progress of senescence in individual leaves is affected by light status of the entire plant (Weaver and Amasino, 2001). When the whole plant is darkened, leaf senescence of older leaves is delayed (Weaver and Amasino, 2001), whereas in individually darkened leaves the senescence is strongly enhanced (Hidema et al., 1991; Mae et al., 1993; Ono et al., 1996; Weaver and Amasino, 2001).

In light, senescence-induced changes are usually reduced when compared to darkness (Biswal and Biswal, 1984; Okada et al., 1992; Klerk et al., 1993; Park et al., 1998; Weaver et al., 1998; Špundová et al., 2003; 2005*a*; **Janečková et al., 2018**). Detached leaves

senescing under light usually maintain higher content of photosynthetic pigments (e.g. Thimann et al., 1985; **Janečková et al., 2018**) and proteins (Okada et al., 1992; Klerk et al., 1993; Chang and Kao, 1998), as well as higher photosynthetic activity (Špundová et al., 2003; **Janečková et al., 2018**). However, when the light intensity is too high (HL), an opposite effect is observed, i.e. the progress of senescence is faster (Biswal and Biswal, 1984; Mae et al., 1993; Procházková and Wilhelmová, 2004). During senescence, the activity of photosynthetic apparatus is inhibited and energy transfer is limited. The elevated supply of excitations due to HL might lead to an imbalance between the generation and demand of electrons in electron transport chain (Vlčková et al., 2006), which results in undesirable formation of ROS. PSII proteins and lipids might be oxidized by ROS and higher levels of malondialdehyde (MDA), a product of lipid peroxidation, can be detected (Chang and Kao, 1998; Pospíšil, 2016).

Light regulates senescence not only by acting as a driving force of photosynthesis, but also via signalling pathways (Biswal and Biswal, 1984; Barber and Andersson, 1992; Brouwer et al., 2014). A light signalling-dependent retardation of senescence is mediated through phytochromes (phy), red/far-red light receptors of plants (Biswal and Biswal, 1984; Brouwer et al., 2012, 2014; Sakuraba et al., 2014; Liebsch and Keech, 2016). Phy are converted to their active form (Pfr) by absorbing red light and effectively reverted to the inactive form (Pr) by absorbing far-red light (Liebsch and Keech, 2016).

In mediation of senescence retardation, phytochrome B (phyB) seems to be the main photoreceptor. While phyB mutants of *Arabidopsis thaliana* are insensitive to continuous and pulsed red light with respect to the delay in senescence (Brouwer et al., 2014; Sakuraba et al., 2014), phyB overexpression lines display delayed dark-induced senescence. Senescence is also affected by phytochrome A (phyA), which can transduce signal of far-red light and which has been suggested to be involved in response to low light intensities (Rousseaux et al., 1997; Brouwer et al., 2012).

Depending on its state, phyB interacts with phytochrome-interacting transcription factors (PIFs) and thus activates and/or inhibits the expression of target genes (Liebsch and Keech, 2016). In the light, PIFs are degraded through a phyB-dependent mechanism, thus

light suppresses PIF-dependent senescence activation. At the molecular level, active phy directly interacts with PIFs to induce their detachment from DNA (Park et al., 2012). In shaded plants, the phyB-mediated PIF degradation is inhibited, while phyA may contribute to senescence suppression by regulating photosynthesis-associated genes involved e.g. in Chl biosynthesis (Liebsch and Keech, 2016). In darkness, both phyA and phyB become inactive and PIFs can activate the expression of various transcription factors. PIFs activate expression of the senescence regulator *ORE1*. ORE1, together with PIFs and other TFs, upregulate genes of Chl degradation (such as *stay-green1* (*SGR1*) and *NYC1;* Song et al., 2014; Zhang et al., 2015) and, together with PIFs, represses the chloroplast maintenance regulator (*golden2-like*, *GLK;* Waters et al., 2009). ORE1 also activates other SAGs required for downstream senescence processes, e.g for protein and nucleic acid breakdown (Liebsch and Keech, 2016). PIFs can also activate the expression of other senescence regulators, e.g. WRKY transcription factors (Liebsch and Keech, 2016). It has been shown that among the group of PIFs, PIF4, PIF5 and PIF3 play prominent roles in promoting dark-induced and partially developmental senescence in *Arabidopsis* (Sakuraba et al., 2014; Song et al., 2014; Zhang et al., 2015). Overexpression of these PIFs led to an acceleration of dark-induced senescence symptoms, whereas their mutant combinations displayed a delayed senescence phenotype (Leivar et al., 2008; Liebsch and Keech, 2016).

Information about the involvement of other light receptors (such as cryptochromes or phototropins) in the light-retardation of senescence is limited. In soybean, a role of cryptochrome2 receptor in delay of leaf senescence was reported (Meng et al., 2013). However, in *Arabidopsis,* the involvement of cryptochrome signal in regulation of senescence was not observed (Weaver and Amasino, 2001; Sakuraba et al., 2014). Whether also other light receptors are involved in light-dependent regulation of senescence remains to be clarified.

Light signalling pathways share common intermediates with signalling pathways of plant hormones, such as ethylene or cytokinins (see, e.g., Zdarska et al., 2015) and some light-regulated processes can be modulated by plant hormones and vice versa. However, the question how and to what extent light and hormones interact together in leaf

senescence regulation is yet to be answered. The interplay between cytokinins and light is discussed in the part 1.2.5.

1.1.4 Chlorophyll *b* and leaf senescence

Chl *b* is synthetized from Chl *a* by the oxidation of a methyl group of Chl *a* to a formyl group by chlorophyllide *a* oxygenase (CAO; Tanaka and Tanaka, 2011). The reverse reaction is catalysed by two enzymes, Chl *b* reductase (NYC1 and NOL) and HCAR (see chapter 1.1.1). Chl *b* is required for the stabilization of LHCII complexes and their proper assembly in thylakoid membrane (Bellemare et al., 1982; Voitsekhovskaja and Tyutereva, 2015).

Recent studies have shown that Chl *b* has an important role in the regulation of leaf senescence (Kusaba et al., 2007; Sakuraba et al., 2012; Voitsekhovskaja and Tyutereva, 2015; Yang et al., 2016). It has been shown that mutants without Chl *b* reductase retain Chl content in senescent leaves, in particular Chl *b*, and show higher stability of some LHCII proteins (Kusaba et al., 2007; Sato et al., 2009). Mutants of *nyc1* were characterized by suppressed reduction of Chl *b* content and showed higher stability of Lhcb1, Lhcb2, Lhcb4 and Lhcb6 in senescent leaves compared to WT (Kusaba et al., 2007). Similar phenotype as in *nyc1* was observed also in *nol* mutant plants, which retained more Chl *a* and *b* and car, maintained Lhcb1 and Lhcb2, and inhibited deterioration of grana stack in senescing leaves (Sato et al., 2009). Overexpression of gene in *Arabidopsis* led to overproduction of Chl *b*, increased size of LHCII and their higher stability (Tanaka et al., 2011; Sakuraba et al., 2012). These transgenic plants exhibited a significant delay in both developmental and dark-induced leaf senescence. The loss of Chl, LHCII, Rubisco and chloroplast structure together with photosynthetic function was observed in senescing WT, while transgenic plants retained them longer than WT. Senescence-related TFs were down-regulated, indicating that overproduction of Chl *b* modifies transcription of genes involved in senescence (Sakuraba et al., 2012). Higher Chl *b* content associated with higher stability of LHCII seems to slow down the progress of leaf senescence. Voitsekhovskaja and Tyutereva (2015) suggested that stabilization of LHCII complexes during senescence by excess Chl *b* and thus the maintenance of their function can probably change the level of some signalling molecules that are necessary to "switch between the ontogenetic programs”

(Voitsekhovskaja and Tyutereva, 2015). However, which signal molecules are the right candidates remains to be elucidated in the future.

Studies dealing with leaf senescence in plants deficient in Chl *b* are scarce and suggest that plants lacking Chl *b* are characterized by faster senescence-associated changes (Kusaba et al., 2007; Yang et al., 2016). A recent study with *pgl* rice mutant has shown that Chl *b* deficiency is associated with increased Chl degradation, accumulation of ROS and electrolyte leakage during both developmental senescence of flag leaves and dark-induced senescence of detached leaves (Yang et al., 2016). The faster Chl degradation was observed also in dark-incubated detached leaves of *cao-2* rice mutant deficient in Chl *b* (Kusaba et al., 2007). Besides these findings, any information about senescence-induced decrease in PSII and PSI function in such plants was missing. Therefore, we have focused our research on the progress of leaf senescence in plants lacking Chl *b* and we tried to clarify whether the deficiency of Chl *b* accelerates senescence-induced impairment of PSII and PSI activities (**Janečková et al., 2019**). We used chlorina $f2^{f2}$ (*clo*) barley mutant, which is deficient in Chl *b* due to the mutation in CAO (Mueller et al., 2012). The *clo* mutant has also lower content of Chl *a* and car compared to WT (Štroch et al., 2004; 2008; **Janečková et al., 2019**). The mutant is deficient in the light-harvesting complexes Lhcb1, Lhcb6 and Lhca4 and has reduced amount of Lhcb2, Lhcb3 and Lhcb4 (Bossmann et al., 1997).

After four days in darkness, we have observed that although the relative decrease in Chl content was similar to WT, the absolute Chl content was pronouncedly lower in *clo* leaves. This was caused by the fact that even before the induction of senescence, the Chl content in the leaves of *clo* was lower than in WT. On the other hand, the relative decrease in the content of car and xanthophylls in *clo* mutant leaves was lower than in WT. In comparison to WT, the senescing *clo* leaves had pronouncedly lower both the maximal quantum yield of PSII photochemistry in the dark-adapted state (F_V/F_M) and the effective quantum yield of PSII photochemistry in the light-adapted state (Φ_P), which indicates that PSII photochemistry was markedly impaired. Unlike in WT, regulatory quenching processes were almost inactive in senescing leaves of *clo* and dissipation via non-regulatory processes prevailed. Pronounced changes in Chl fluorescence parameters indicated that RCII was

damaged to a greater extent than LHCII, and at the same time the RCII damage was even more pronounced in comparison to WT. The greater damage of RCII could be related to effect of Chl *b*/LHC deficiency, as the proper assembly of LHCII seems to stabilize the structure of PSII complex and their function (Havaux and Tardy, 1997). Interestingly, despite the markedly impaired PSII photochemistry in *clo*, the activity of PSI in this mutant remained higher than in WT, indicating that the missing Lhca4 did not decrease the stability of PSI during senescence (**Janečková et al., 2019**). Our study showed that the leaf senescence of Chl *b* deficient mutant of *clo* is accompanied by a substantial acceleration of the inhibition of PSII photochemistry, which is supposedly related to more pronounced damage of RCII (**Janečková et al., 2019**).

From the presented results it is obvious that the presence of Chl *b* has considerable impact on the progress of leaf senescence. While higher Chl *b* content was accompanied by slower senescence-associated changes and higher stability of LHCII (Kusaba et al., 2007; Sakuraba et al., 2012; Voitsekhovskaja and Tyutereva, 2015), its deficiency led to accelerated inhibition of PSII photochemistry during dark-induced senescence (**Janečková et al., 2019**). As cytokinins are known decelerators of senescence, we also wanted to find out whether and to what extent exogenously applied CKs will be able to suppress the pronounced senescence in Chl *b*-deficient *clo* mutant (see chapter 1.2.3).

1.2 Cytokinins

Cytokinins (CKs) represent a class of plant hormones that were identified in the 1950s as the factors promoting cell division and growth. The first discovered CK was 6-furfurylaminopurine (kinetin), which was isolated from autoclaved products of herring sperm DNA (Miller et al., 1955; Mok and Mok, 2001). Two decades later, the first CK was obtained from plants. It was named zeatin and was isolated from immature maize (*Zea mays* L.) endosperm (Letham, 1973). Since then, CKs have been discovered in many plant species and their importance for plants became obvious (for a review, see e.g. Kieber and Schaller, 2014). CKs were found to play key roles in numerous processes of plant growth and development, including embryogenesis, activity of root and shoot meristems, vascular system development, leaf senescence and the response to biotic and abiotic stresses (for a review, see e. g. Kieber and Schaller, 2014). CKs were shown to play roles also in different cell cycle phases, including the G1/S transition, S phase and the G2/M transition (Riefler et al., 2006; Werner et al., 2008; for a review, see e.g. Kieber and Schaller, 2014).

Chloroplasts are one of the main target organelles of CKs, as these hormones affect both chloroplast development and its function (for a review, see e. g. Cortleven and Schmülling, 2015). CKs stimulate the transition of etioplasts to chloroplasts (e.g. Chory et al., 1994; Cortleven et al., 2016), control chloroplast division and increase their number (e.g. Okazaki et al., 2009) and stimulate Chl biosynthesis (e.g. Yaronskaya et al., 2006; Cortleven et al., 2016). Regarding Chl biosynthesis, CKs were shown to increase the activities and levels of various enzymes, such as Mg-chelatase and NADPH-protochrolorophyllide oxidoreductase (POR; Kusnetsov et al., 1998; Yaronskaya et al., 2006; for a review, see Cortleven and Schmülling, 2015). CKs affect the expression of nuclear genes encoding chloroplast proteins (Chory et al., 1994; Kusnetsov et al., 1994; Rashotte et al., 2003; Brenner et al., 2005; Kiba et al., 2005) as well as the expression of chloroplast genes within chloroplast genome (Cortleven and Schmülling, 2015).

Naturally occurring CKs are adenine derivatives, which, based on the configuration of their side chain at the N^6 position, can be classified into two classes (Fig. 4). The first class is represented by isoprenoid CKs, which contain an isoprenoid side chain with or without a

double bond. Isoprenoid CKs with double bond include N^6-(Δ^2-isopentenyl)adenine (iP) or zeatin (Fig. 4). In higher plants, zeatin is present as two isomers, the *trans*- (*t*Z) and the *cis*-form (*c*Z) (Mok and Mok, 2001; Sakakibara, 2006; Kieber and Schaller, 2014). Both iP and *t*Z occur with high abundance in *Arabidopsis thaliana* (Kieber and Schaller, 2014; Osugi and Sakakibara, 2015). Isoprenoid CKs without a double bond are represented for example by dihydrozeatin (DHZ) (for a review, see e.g. Mok and Mok, 2001; Kieber and Schaller, 2014). The second class of naturally occurring CKs is a group of aromatic CKs, which have an aromatic benzyl or hydroxybenzyl group at the N^6-position. Typical representatives of this class are 6-benzylaminopurine (BA), kinetin or *meta*-topoline (*m*T) (Strnad, 1997; for a review, see e.g. Mok and Mok, 2001; Kieber and Schaller, 2014).

Besides the naturally occurring CKs, there is also a group of synthetic CKs. These are compounds with significant CK activity that are not normally present in plant tissues. This group involves phenylurea derivatives, such as thidiazuron (TDZ) or *N*-phenyl-*N*´-[2-chloro-4-pyridyl]urea (CPPU) (Nisler et al., 2018; for a review, see e.g. Mok and Mok, 2001).

As the adenine ring or N^6-isoprenoid side chain can be chemically modified in various ways, CKs can be present in plants in different forms, namely in a form of free bases, ribosides, ribotides or glucosides (Mok and Mok, 2001; Sakakibara, 2006; Fig. 4). As the CK activity of various forms differs, the interconversion between the forms is an important regulatory mechanism of CK homeostasis (Sakakibara, 2006). CK ribosides have a ribose sugar attached at the N^9 position of the purine ring (Fig. 4) and in ribotides, the ribose moiety contains a phosphate group. Ribosides and ribotides represent the main transport form of CKs and can be found in xylem and phloem (for a review, see e.g. Kieber and Schaller, 2014). Transport of CK free bases and their ribosides to cytoplasm is mediated by purine permeases (PUPs) (Bürkle et al., 2003; for a review, see e.g. Sakakibara, 2006; Skalický et al., 2018) and equilibrative nucleoside transporters (ENTs) (Sun et al., 2005; Hirose et al., 2008; for a review, see e.g. Sakakibara, 2006; Skalický et al., 2018), respectively (Fig. 5). Lomin et al. (2018) proposed that ENTs are involved in the transport of *t*ZR to cytosol, where *t*ZR is subsequently converted via a putative kinase and LOG into an active CK base form, which enters the endoplasmic reticulum and triggers signalling.

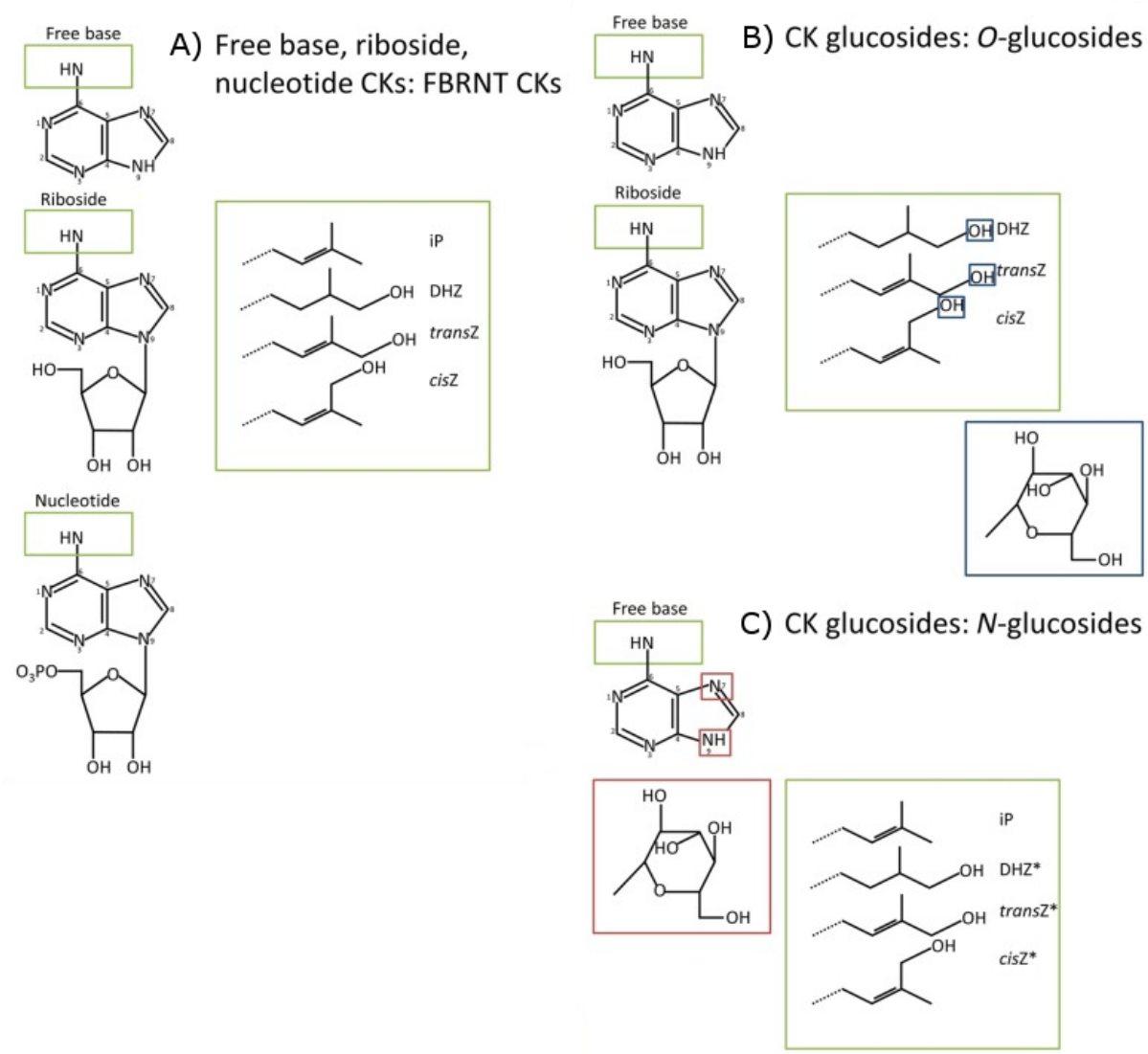

Fig. 4: Representative structures of CKs from maize. (A) Free base, riboside, nucleotide CKs (B) CK glucosides including *O*-glucosides and (C) N-glucosides. Smaller green boxes indicate addition of side chains; large green boxes show different side chains. Blue boxes indicate the structure of glucose and location of glucosylation for *O*-glucosides. Red boxes indicate the structure of glucose and location of glucosylation for *N*-glucosides. Adapted from Morrison et al. (2015); open access.

CKs can also be glycosylated (i.e. conjugated with sugar molecule, generally glucose) to form *O*- and *N*-glucosides (Fig. 4), which cannot bind to *Arabidopsis* CK receptors and were shown to be inactive in bioassays (Spíchal et al., 2004). The conjugation of CK with a sugar moiety is mediated by uridine diphosphate glycosyltransferases (UGTs) (Šmehilová et

al., 2016). Glycosylation of CKs may occur at the N^3, N^7 and N^9 position of the purine ring (*N*-glucosides), or at the hydroxyl group of the side chains of zeatin or DHZ (*O*-glucosides or *O*-xylosides) (Sakakibara, 2006; Kieber and Schaller, 2014). The *O*-glycosylation is reversible and these forms of CKs can be converted by β-glucosidases into the active CK form. Therefore the *O*-glycosylated CKs are thought to serve as storage forms of CKs (Brzobohatý et al., 1993; Sakakibara, 2006; Kieber and Schaller, 2014). On the other hand, the *N*-glycosylation is irreversible and leads to complete biological inactivation of CKs (Sakakibara, 2006; Kieber and Schaller, 2014).

Free CK bases are usually the most active CK forms in bioassays (for a review, see e.g. Spíchal, 2012). Besides the free bases, it was shown that CK ribosides are also active and that they are able to interact tightly with CK receptors (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004, Heyl et al., 2012). In 2015, however, Lomin et al. (2015) had found out that compared to free bases, CK ribosides have a strongly reduced affinity for the CK receptors in planta, indicating that ribosides have no or very weak CK activity. Despite the finding by Lomin et al. (2015), the results from our correlation analysis (**Janečková et al. 2018**) indicate that CK ribosides could play an active role in the regulation of leaf senescence. This seeming discrepancy could be explained by possible fast conversion of CK ribosides to their bases, which was suggested also by Lomin et al. (2015). Hence, besides being the transport CK form, CK ribosides could be considered also as a kind of storage pool of CKs, which are converted into active CKs after reaching the target tissue.

1.2.1 Cytokinin biosynthesis and degradation

Originally, CKs were thought to be synthesized only in roots and then transported into shoots, but later studies revealed that CKs are synthesized throughout the plant, including shoots (Myiawaki et al., 2004; for a review, see e.g, Sakakibara, 2006; Hirose et al., 2008; Kieber and Schaller, 2014). Genes involved in CK biosynthesis were shown to be expressed in diverse cellular locations in *Arabidopsis*. For example, four out of seven isopentenyl transferase (IPT) enzymes were found in chloroplasts, while other IPTs were located in mitochondria, cytosol and nucleus (Kasahara et al., 2004; for a review, see e.g. Frébort et al., 2011; Cortleven and Schmülling, 2015; Fig. 5).

Precursors of CKs in biosynthetic pathways are dimethylallyl pyrophosphate (DMAPP) and hydroxymethylbutenyl diphosphate (HMBPP). The precursor HMBDP is synthesized by methylerythritol phosphate (MEP) pathway, which is functional in bacteria and in plastids. DMAPP is synthesized via the MEP pathway as well as via the mevalonate (MVA) pathway, which is commonly found in the cytosol of eukaryotes (for a review, see e.g. Sakakibara, 2006; Frébort et al., 2011; Spíchal, 2012; Kieber and Schaller, 2014). Isoprenoid side chains of iP and *t*Z predominantly originate from the MEP pathway, whereas a large fraction of the *c*Z side chain is derived from the MVA pathway (Kasahara et al., 2004; for a review, see e.g. Sakakibara, 2006).

In the first step of CK biosynthesis, IPT catalyzes the formation of iP ribonucleotides (iP-riboside 5'-monophosphate iPRMP, diphosphate iPRDP or triphosphate iPRTP) from DMAPP and adenine nucleotides, with preferential utilization of ATP or ADP. Further, the end of the prenyl side chain of iP ribonucleotides is hydroxylated by cytochrome P450 monooxygenase 735A (CYP735A) to produce *t*Z ribotides (Takei et al., 2004; for a review, see e.g. Sakakibara, 2006; Osugi and Sakakibara, 2015). Finally, a LONELY GUY (LOG) enzyme converts the precursors iPRMP and *t*Z-riboside 5'-monophosphate (*t*ZRMP) to their active forms, iP and *t*Z, respectively (Kurakawa et al., 2007; for a review, see e.g. Osugi and Sakakibara, 2015).

In parallel with the above described pathway, the metabolic pool of iPRMP and iPRDP is also created by dephosporylation of iPRTP and iPRDP by phosphatase, by phosphorylation of iPR by adenosine kinase or by conjugation of phosphoribosyl moieties to iP by adenine phophoribosyltransferase (APRT). APRT utilizes not only iP, but also other CK nucleobases (Sakakibara, 2006).

The degradation of CKs involves cytokinin oxidase/dehydrogenase (CKX) enzyme, which catalyzes the cleavage of CKs into adenine/adenosine and the corresponding side chain aldehyde (for a review, see e.g. Mok and Mok, 2001; Frébort et al., 2011; Fig. 5). CKX irreversibly inactivates CKs and causes complete loss of their biological activity. In *Arabidopsis thaliana,* the CKX gene family has seven members, *AtCKX1* to *AtCKX7* (for a review, see e.g. Schmülling et al., 2003).

It has been shown that higher activity of CKX lowers the endogenous content of CKs (Kamínek et al., 1997). Transgenic plants with overexpressed CKX show a "CK deficiency syndrome" phenotype (Werner et al., 2003; 2008). The main features of these plants include the formation of slow-growing, stunted shoots with small leaves and an enhanced root system. The shoot tissue has markedly reduced content of sugars, decreased activity of vacuolar and cytosolic invertases and reduced ATP content.

1.2.2 Cytokinin perception and signalling

CKs trigger physiological response by changing gene expression (Osugi and Sakakibara, 2015). The CK signal is transmitted to target genes by two-component signalling pathway by His-Asp phosphorelay of particular components (for a review, see e.g. Hwang et al., 2012; Kieber and Schaller, 2014; Osugi and Sakakibara, 2015).

First, CK has to be perceived by its corresponding receptor (Fig. 5). CK receptors belong to a family of sensor histidine kinases and are multi-domain transmembrane proteins. Receptors contain CHASE (cyclases/histidine kinases-associated sensor extracellular) domain, cytoplasmic histidine kinase (HK) domain and receiver domain. The CHASE domain is responsible for binding and recognition of particular CK. Binding of CK to the receptor induces conformational change of the receptor´s cytoplasmic HK domain, which results in autophosphorylation of the histidine residue. This activates the signalling pathway via a cascade of phosphorelays. Phosphate is transferred via the aspartate residue of the receptor´s C-terminal receiver domain to histidine phosphotransfer proteins (HPt; in *Arabidopsis* AHP), which are located in cytoplasma. Phosphorylated HPts are further translocated into the nucleus, where they activate response regulators (RRs; in *Arabidopsis* ARRs) (for a review, see e.g. Kieber and Schaller, 2014). There are two types of ARRs, type-B and type-A. It was shown that ARRs of type-B work as DNA-binding transcription regulators and regulate CK responsive genes, including ARRs type-A. The type-A ARRs are negative regulators as they can inhibit the CK signalling by competing with type-B ARRs for phosphate (To et al., 2007; Argyros et al., 2008; for review, see e.g. Hwang et al., 2012; Osugi and Sakakibara, 2015).

Three CK receptors are known to be present in *Arabidopsis thaliana* - AHK2 (Arabidopsis histidine kinase2), AHK3 and AHK4/CRE1/WOL1 (CRE1 as Cytokinin response1; WOL1 as Woodenleg1) (Mähönen et al., 2000; Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001; for a review, see e.g. Heyl et al., 2012). AHK receptors were originally reported to be localized in plasma membrane (Inoue et al., 2001; Ueguchi et al., 2001; Fig. 5). However, a few years ago this finding was questioned by several studies, which show that CK receptors of *Arabidopsis* (also of maize) are localized predominantly inside the cell in the membrane of endoplasmic reticulum (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011). The exact subcelullar localization of CK receptors as well as the participation of differently localized receptors in CK signalling is still under extensive research. For up-to-date review on this topic, see for example a recent review by Romanov et al. (2018).

Although the proteins of AHK receptors show a high degree of sequence identity, the receptors have different ligand preferences (Spíchal et al., 2004; Romanov et al., 2006; Stolz et al., 2011; Lomin et al., 2015; for a review, see, e.g., Heyl et al., 2012). The receptors were shown to bind isoprenoid and aromatic CKs with different affinities (Yamada et al., 2001; Spíchal et al., 2004; Romanov et al., 2006; Stolz et al., 2011; Spíchal, 2012). The results from the bacterial assay systems suggest that AHK3 has the highest affinity for *t*Z, lower affinity for iP and the lowest affinity for *c*Z and BA (Romanov et al., 2006; Heyl et al., 2012). For AHK4, *t*Z and iP are the most preferred ligands, while DHZ and *c*Z were far less effective (Yamada et al., 2001; Spíchal et al. 2004; Romanov et al., 2006). The ligand preference of AHK2 is closer to AHK4 than to AHK3. AHK2 has the highest affinities for iP and *t*Z, followed by BA and *c*Z (Stolz et al., 2011; Heyl et al., 2012). Similar profiles of ligand specifity of AHK3 were obtained by Lomin et al. (2015) in plant membrane assay system. They have observed the highest affinity of AHK3 to *t*Z, followed by DHZ and iP, while *c*Z and BA showed the lowest affinity to AHK3. The interaction between receptors and CKs was shown to be pH-, salt- and temperature-dependent and with K_d in the nanomolar range (Romanov et al., 2006; Lomin et al., 2015; Spíchal et al., 2012).

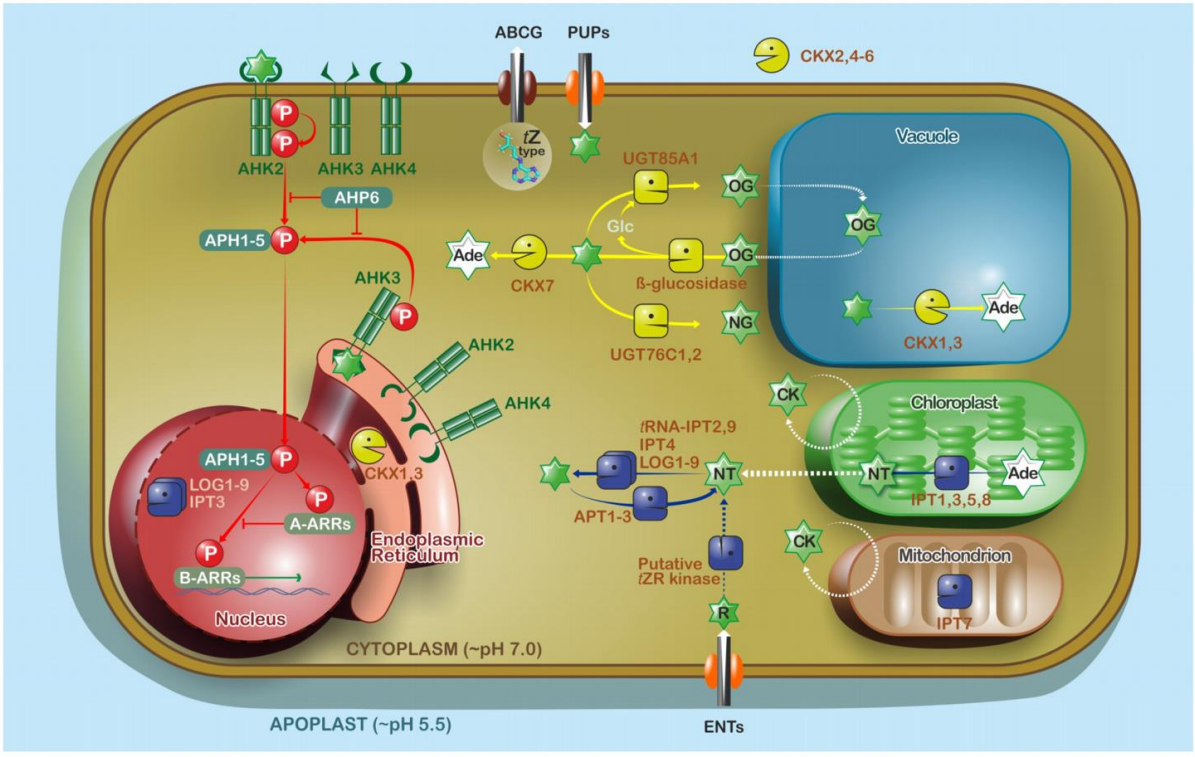

Fig. 5: Model of cellular and subcellular CK homeostasis and signalling in *Arabidopsis*. In CK signalling pathways (red arrows), CK binds to AHK receptor, which is localized at the plasma membrane or membrane of endoplasmic reticulum. Signal is further transmitted via AHP to nuclear ARRs of type-B or type-A. Activation of ARRs type-B leads to transcription of CK inducible genes, including ARRs type-A, which mediate a negative feedback loop. CKs homeostasis is achieved by its biosynthesis, transport, degradation and inactivation. CKs biosynthesis is catalysed by isopentenyl transferases (IPT) located mainly in chloroplasts, but also in mitochondria, cytosol and nucleus. Most of the active CKs can be modified by uridine diphosphate glycosyltransferases (UGTs) or β-glucosidase (yellow arrows). CKs degradation is catalysed by CK dehydrogenases/oxidases (CKX). Transport (white arrows) of CK free bases and corresponding ribosides is mediated by purine permeases (PUPs) and equilibrative nucleoside transporters (ENTs). Green stars indicate CK; Glc, glucose; NT, CK nucleotides; NG, *N*-glucosides; OG, *O*-glucosides; P, phosphate moiety; R, riboside. Solid arrows indicate known, well-described pathways, dashed arrows indicate not well-defined pathways (Skalický et al., 2018; open access). For a more detailed description of the model see Skalický et al. (2018).

AHK receptors were shown to play different roles in various CK-regulated processes during plant development (Higuchi et al., 2004; Riefler et al., 2006; Kim et al., 2006; Cortleven et al., 2014; Cortleven et al., 2016). It has been shown that there is a high degree of functional redundancy among these receptors (Riefler et al., 2006; Stolz et al., 2011; Heyl et al., 2012). Thus, unambiguous phenotypic differences were not observed in plants

carrying single mutations in genes for individual AHK receptors. Distinct phenotypes can be seen only in multiple receptor mutants, which indicates that only a few traits depend on one single CK receptor (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Heyl et al., 2012).

In 2006, a comprehensive analysis of single, double and triple mutants in the *AHK* genes was published by Riefler et al. (2006). They have shown that although functions of AHK receptors partially overlap, each receptor contribute to the CK response in various assays to a different extent (Riefler et al., 2006).

Mutations in AHK3 receptor and to a lower extent also in AHK2 cause a reduction in the Chl content (Riefler et al., 2006). The Chl content was reduced to 75 % of WT levels in *ahk3* and *ahk3 ahk4* mutants and to only about 60 % in *ahk2 ahk3* mutants. Except of lower Chl content, these mutants have higher endogenous CK content. Plants with the mutation in AHK3, *ahk2 ahk3* and *ahk3 ahk4*, showed similar increase in CK content, while in *ahk2 ahk4,* the content of CKs (except of some zeatin compounds) was basically unchanged. In *ahk2 ahk3* and *ahk3 ahk4* mutants, a strong increase in the content of *t*Z and its metabolites has been described. The mutant *ahk2 ahk3* had about 3.5-fold higher content of *t*Z compared to WT; however, its iP content was only half of the WT value (Riefler et al., 2006).

We have performed a detailed study of changes in endogenous content of different CK forms and types in the *ahk* double receptor mutants (*ahk2 ahk3*, *ahk2 ahk4* and *ahk3 ahk4*) (**Janečková et al., 2018**). Leaves of mutants with mutation in AHK3 (i.e. *ahk2 ahk3* and *ahk3 ahk4*) were characterized by constitutively lower Chl content (by about 15 - 20 % compared to WT), whereas PSII photochemistry and the level of lipid peroxidation were unaffected. These mutants had approximately two-fold higher overall CK content in comparison to WT (**Janečková et al., 2018).** The content of iP and *t*Z forms, both absolute and relative levels of iP and *t*Z precursors, their free bases and their ribosides were increased pronouncedly in these mutants, implying stimulation of biosynthesis of both these CK groups. On the other hand, the content of *c*Z CKs was the lowest in both of these mutants. The increased CK level of iP and *t*Z metabolites in double mutants with non-

functional AHK3 can be interpreted as an attempt of the plant to compensate the insufficient CK output (**Janečková et al., 2018**).

According to our results, the increase in the content of *t*Z and iP free bases in *ahk2 ahk3* was significantly higher compared to what was reported earlier by Riefler et al. (2006). The *t*Z level was 11-fold higher and iP content was increased 3.5-fold above the levels of these free bases in WT (**Janečková et al., 2018**). This discrepancy could be explained by different light conditions during plant growth, as we used short-day conditions, whereas Riefler et al. (2006) used long-day conditions. It seems that CK biosynthesis in the receptor double mutants is markedly more stimulated under short-day conditions. The other explanation can be different age of used plant material. We have analysed CK content in leaves of 35 days-old plants, while Riefler et al. (2006) determined CK endogenous content in shoots of 22-day old mutant plants.

1.2.3 Effect of cytokinins on photosynthetic performance during induced senescence

Today it is well known that CKs are involved in regulation of leaf senescence (for a review, see e.g. Zwack and Rashotte, 2013; Hönig et al., 2018). The onset of leaf senescence is usually connected to a decrease in the content of active CK forms (Tao et al., 1983; Singh et al., 1992; Gan and Amasino, 1995, 1996, 1997; Ananieva et al., 2008; Hönig et al., 2018). This can be the result of inhibition of CK biosynthesis (Perilli et al., 2010), result of faster CK degradation by CKX (Perilli et al., 2010; Werner et al., 2006; see 1.2.1 Cytokinins biosynthesis and degradation) and/or the result of irreversible inactivation of CKs by UGTs (Šmehilová et al., 2016). However, it should be noted that a low CK content is not the trigger of senescence, but is one of the factors that enable the senescence to proceed (Werner et al., 2003). The progress of leaf senescence can be delayed both by exogenous application of active CKs and by increase in their endogenous content (Smart et al., 1991; Gan and Amasino, 1995; Cortleven and Valcke, 2012; for a review, see e.g. Zwack and Rashotte, 2013; Hönig et al., 2018).

The first evidence of the antisenescent activity of CKs was given by Richmond and Lang (1957), who have observed higher retention of Chl and proteins in detached leaves of cocklebur plants (*Xanthium pennsylvanicum* L.) treated exogenously with kinetin. The

retention of Chl content in CK treated detached senescing leaves has since been shown in many other plant species such as wheat (Holub et al., 1998; Pavalan-Ünsal et al., 2002; Vlčková et al., 2006; Zavaleta-Mancera et al., 2007), barley (Selivanka et al., 2001; **Janečková et al., 2019**), *Arabidopsis* (Oh et al., 2005) or rice (Kao, 1980; Talla et al., 2016) and even re-greening of yellowing leaves has been reported (Zavaleta-Mancera et al., 1999). Talla et al. (2016) have reported that the retention of Chl content during dark-induced senescence of BA treated leaves of rice was accompanied by accumulation of HmChl *a*, an intermediate of Chl cycle (see chapter 1.1.1). This finding suggests that CKs could delay senescence-induced Chl loss by regulating the conversion of Chl *b* to Chl *a* (Talla et al., 2016). The CK-mediated retention of Chl during leaf senescence could also be related to the reduction of activity of enzymes involved in the Chl degradation, as BA was shown to reduce activity of Mg-dechelatase and Chl degrading peroxidase during post-harvest senescence of broccoli florets (Costa et al., 2005).

In detached senescing leaves, CKs have been also shown to delay the decrease in the content of car and xanthophylls (Zavaleta-Mancera et al., 2007; Vylíčilová et al., 2016; **Janečková et al., 2019**), to mitigate lipid peroxidation (Dhindsa a kol., 1982; Huang et al., 1997; Vlčková et al., 2006; Liu et al., 2016), to inhibit protease activity (Pavalan-Ünsal et al., 2002), and to prevent the formation of plastoglobules and modification of chloroplast shape (Hudák et al., 1996; Vlčková et al., 2006; Zavaleta-Mancera et al., 2007). The CK-mediated mitigation of lipid peroxidation could be linked to the ability of CKs to activate xanthophyll cycle (e.g. Vlčková et al., 2006) or antioxidant enzymes, such as catalase and ascorbate peroxidase (Dhindsa et al., 1982; Zavaleta-Mancera et al., 2007), which all pronouncedly contribute to the protection of photosynthetic apparatus against oxidative damage during senescence.

In senescing leaves, CKs help to preserve photosynthetic activity (e.g. Vlčková et al., 2006, Zubo et al., 2008, Talla et al., 2016; Vylíčilová et al., 2016; **Janečková et al., 2019**). They were found to maintain the content and activity of Rubisco (Weidhase et al., 1987; Ohya and Suzuki, 1991; Zavaleta-Mancera et al., 2007) and CO_2 assimilation (Vlčková et al., 2006) in senescing leaves. With respect to the primary phase of photosynthesis, CKs are

known to delay the inhibition of PSII photochemistry during senescence (Oh et al., 2005; Vlčková et al., 2006; Vylíčilová et al., 2016; **Janečková et al., 2019**). The function of PSII photochemistry, estimated from Chl fluorescence parameters (F_V/F_M and Φ_P, maximal and effective quantum yield of PSII photochemistry in the dark- and in the light-adapted state, respectively), was shown to be highly maintained in dark senescing CK-treated leaves of *Arabidopsis* (Oh et al., 2005), wheat (Vlčková et al., 2006), rice (Talla et al., 2016) or barley (**Janečková et al., 2019**). Besides PSII photochemistry, CKs were also able to delay the loss of oxygen evolution activity during dark-induced senescence of rice leaves (Talla et al., 2016).

Senescence-induced alterations in the structure of pigment-protein complexes are suppressed in the presence of CKs. CKs were shown to stabilize LHCII as well as RCII in dark-senescing leaves (Jackowski 1996; Oh et al., 2005; Zavaleta-Mancera et al., 2007; Talla et al., 2016; **Janečková et al., 2019**). The stabilizing effect of CKs on photosynthetic complexes has been shown also under stress conditions (e.g. Monakhova and Chernyad'ev, 2004). The effect of CKs on LHCII stability may be possibly related to the CK-dependent retention of Chl *b*, as this pigment is preferentially bound by LHCII. The preferential retention of Chl *b* accompanied by higher stability of LHCII proteins was reported for example by Zavaleta-Mancera et al. (2007) in dark-senescing detached leaves of wheat (*Triticum aestivum* L.) treated with BA. As mentioned in previous chapters, Chl *b* is important for the stabilization of LHCII complexes (see 1.1.3 Chlorophyll *b* and leaf senescence) and its presence has pronounced impact on the progress of leaf senescence (Kusaba et al., 2007; Sakuraba et al., 2012; Voitsekhovskaja and Tyutereva, 2015; Yang et al., 2016; **Janečková et al., 2019**).

The question is, whether and to what extent are CKs able to suppress senescence-associated changes in mutant that is deficient in Chl *b*. We thus decided to study the effect of exogenously applied CK (BA) on senescence-induced changes in Chl *b*-deficient mutant of barley (*Hordeum vulgare* L.), *clo* (**Janečková et al., 2019**). BA treatment slowed down the degradation of photosynthetic pigments and suppressed the senescence-induced impairment of PSII photochemistry in both *clo* and WT leaves, but in *clo* the effect was more pronounced. The progress of senescence in *clo* became similar to WT, although in the

absence of exogenous CKs the senescence-induced impairment of PSII function was more marked in this mutant. As the impairment of PSII function seems to be related to more pronounced damage of RCII, we assume that the protective effect of BA on PSII function in WT as well as in *clo* is based mainly on a suppression of this impairment (**Janečková et al., 2019**).

The delay of senescence-induced changes in *clo* was observed also for other CKs (iP and TDZ, Fig. 6; unpublished results). In these experiments, all CKs suppressed (even though to a different extent) the senescence-induced decrease in relative Chl content as well as a decrease in efficiency of PSII photochemistry in detached leaves of both WT and *clo* (Fig. 6; unpublished results).

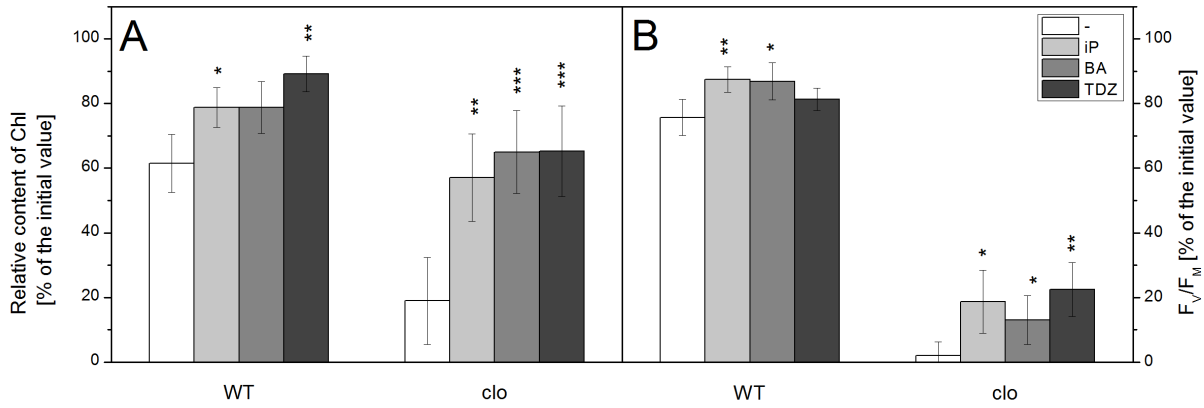

Fig. 6: Relative content of chlorophyll (Chl) and the maximal efficiency of PSII photochemistry in dark-adapted state (F_V/F_M) in detached WT and *clo* leaves kept for 3 days in the dark in 0.2% DMSO solution without (-) or with 100 µM of isopentenyladenine (iP), 6-benzylaminopurine (BA) or thidiazuron (TDZ). Relative values (% of the initial values before senescence induction) are presented. Means and SD are shown, n = 6. Asterisks indicate statistically significant difference (Student´s t-test) between DMSO solution without CKs and with CKs (*, P < 0.05; **, P <0.01; ***, P < 0.001).

The photochemistry of PSII in *chlorina* mutant has been previously reported to be more sensitive to stress and this sensitivity was attributed to reduced amount of LHCs (Havaux and Tardy, 1997). The destabilizing effect of Chl *b*/LHC deficiency on PSII function in *clo* mutant seems to be suppressed by CKs (**Janečková et al., 2019**; Fig. 6). We may also say that the presence of Chl *b* is probably not decisive for the protective CK effect on PSII photochemistry in dark-senescing leaves (**Janečková et al., 2019**).

The understanding of CK regulation of leaf senescence was improved by gene expression studies. It has been shown that CKs inhibit transcription of SAGs (e.g. Gan and Amasino, 1997) and that they also alter the expression of genes related to photosynthesis in senescing leaves (Zubo et al., 2008; Talla et al., 2016; Vylíčilová et al., 2016). In dark-senescing leaves of *Arabidopsis,* the expression of various genes related to RC and LHC of both PSII and PSI was highly retained by CKs, including *Lhcb2.2*, *Lhcb3*, *Lhcb5*, *Lhcb6*, *Lhca1* or *PsaK* (subunit K of PSI) (Vylíčilová et al., 2016). In dark senescing leaves of rice, CK-dependent retention of higher expression was observed for *Lhcb4* and *Lhcb6* genes, encoding Chl *a/b*-binding proteins of the LHC, and for oxygen-evolving enhancer genes *PsbO* and *PsbP* (Talla et al., 2016). An increase in gene expression of *CAO,* involved in biosynthesis of Chl *b,* and of *NOL* and *HCAR*, involved in the conversion of Chl *b* to Chl *a,* were reported (Talla et al., 2016). Mantained expression of genes related to Calvin cycle, such as *RBCS* (small subunit of Rubisco), Rubisco methyltransferase-like protein or fructose-bisphosphate aldolase, and genes coding the subunits of the Cyt b_6/f, was found as well (Ohya and Suzuki, 1991; Talla et al., 2016; Vylíčilová et al., 2016).

To sum up, the CK-dependent mantainance of the expression of PSII related genes might contribute to higher stability of LHCII during leaf senescence. This effect could be complemented by the ability of CKs to modulate the gene expression and activity of Chl degradation enzymes, which leads to slower degradation of Chl during leaf senescence. Higher residual Chl content could then contribute to the overall protective effect of CKs on photosynthesis during leaf senescence. However, our understanding of the precise mechanism of CK-dependent protection of photosynthetic activity during leaf senescence is still limited. For example, data describing the effect of CKs on the activity of PSI during senescence are very scarce. In our study, BA did not exhibit any significant effect on PSI activity during dark-induced senescence of barley leaves (**Janečková et al., 2019**).

Besides exogenous CK treatments, leaf senescence is delayed also by the elevation of endogenous CK content (e.g., Gan and Amasino, 1995; Lara et al., 2004). This can be achieved by the overexpression of the *IPT* gene, encoding the key enzyme that catalyses the rate-limiting step of CK biosynthesis (see 1.2.1 Cytokinin biosynthesis and degradation).

Mutants overexpressing the *IPT* gene under a control of various promoters have been constructed. However, the expected protective effect of CKs has not always been achieved and in some studies, no change or even accelerated leaf senescence was observed (e.g. Li et al., 1992; Gan and Amasino, 1996). The expression of the *IPT* gene has to be properly regulated to obtain "beneficial" effects of increased CK levels (e.g. increased yield of seeds). Early studies used strong constitutive promoters (Smigocki and Owens, 1988; 1989; Li et al., 1992), which resulted in abnormally high endogenous CK levels and plants exhibited morphological and physiological abnormalities. Later, it has been shown that a minimal increase in IPT expression is sufficient to obtain the beneficial effects on plant development (Faiss et al., 1997).

Gan and Amasino (1995) developed transgenic tobacco plants (*Nicotiana tabacum* L.) with expression of *IPT* gene of *Agrobacterium tumefaciens* under the control of senescence-associated gene promoter (P_{SAG12}). The *SAG12* gene encodes a cysteine protease, which is expressed in senescing leaves (Noh and Amasino, 1999). The P_{SAG12} promoter activates the *IPT* expression at the onset of senescence in leaf cells of tobacco plants, which results in higher endogenous CK content. The higher content of CKs then inhibits senescence, which in turn attenuates P_{SAG12}-*IPT* activity and prevents the system from overproduction of CKs. This system is thus autoregulated (Gan and Amasino, 1995). The oldest leaves of P_{SAG12}-*IPT* plants were shown to have retained higher Chl, Rubisco and total protein content and maintained higher rate of CO_2 assimilation (Wingler et al., 1998; Jordi et al., 2000; McCabe et al., 2001) and higher antioxidant concentrations compared to WT (Dertinger et al., 2003). In addition, transgenic P_{SAG12}-*IPT* plants have significantly reduced susceptibility to pathogen infection (McCabe et al., 2001).

It has been found that the delay of leaf senescence in P_{SAG12}-*IPT* transgenic plants correlates with the increase in the activity of extracellular invertase (Lara et al., 2004). Activity of this enzyme is essential for the mobilization of nutrients and for slowing down senescence (Lara et al., 2004). To establish a relationship between CKs and extracellular invertase in the delay of senescence, Lara et al. (2004) employed transgenic plants that allowed inhibition of extracellular invertase in the presence of CKs. It has been shown that

the protective effect of CKs is lost in the presence of invertase inhibitor. This enzyme thus can be considered as a key element of CK-dependent regulation of senescence (Lara et al., 2004). These observations demonstrate that the regulation of leaf senescence is accompanied by changes in source-sink relations and that there is a direct link between CK effect and primary metabolism (Lara et al., 2004).

Another system of transgenic plants overexpressing *IPT* is represented by P_{SARK}*::IPT* plants, where the expression of IPT is under the control of senescence-associated receptor kinase (SARK), a stress- and senescence-associated promoter (Rivero et al., 2007). These plants have better drought tolerance due to suppressed drought-induced leaf senescence (Rivero et al., 2007). During stress, these plants have higher photochemical and lower non-photochemical quenching and higher rate of electron transport compared to WT plants (Rivero et al., 2010). It has been reported that they also have elevated rate of CO_2 assimilation (Rivero et al., 2007) and higher expression of genes and higher content of proteins related to PSII, Cyt b_6/f, PSI, NADH oxidoreductase and the ATP synthase complex. Enhanced CK content in P_{SARK}*::IPT* plants thus prevented the loss of photosynthetic function and proteins during stress (Rivero et al., 2010). Higher drought tolerance induced by increased CK levels was accompanied by enhanced photorespiration and prevention of degradation of photosynthetic protein complexes during periods of water stress (Rivero et al., 2009, 2010).

There are also other systems overexpressing the *IPT* gene under the control of various promoters and displaying delayed leaf senescence, for example, P_{SEE1}*IPT* or transgenic plants (Robson et al., 2004), where *SEE1* is a maize senescence-enhanced protease from the same cysteine family as SAG12. Older leaves of P_{SEE1}*IPT* plants had higher Chl content and photosynthetic activity than leaves of corresponding WT plants (Robson et al., 2004).

Despite the relatively large number of various studies of transgenic plants expressing *IPT*, there is still a lack of detailed information about the changes in the function or structure of the photosynthetic apparatus in such plants during senescence. Comprehensive investigation of photosynthetic activity of transgenic plants expressing *IPT* was done by

Synková group (1999; 2003; 2006*a*; 2006*b*) and by Cortleven and co-workers (see Cortleven et al., 2011; Cortleven and Valcke, 2012), but these studies were not focused on senescence. These studies have been using Pssu-*ipt*, transgenic tobacco plants expressing *IPT* under control of a light-inducible promoter of the Rubisco enzyme of *Pisum sativum*.

It has been found that the chloroplasts of transgenic Pssu-*ipt* tobacco plants exhibited an amoeboid shape with a presence of peripheral reticulum, large crystaloids and globular bodies (Synková et al., 2003; Synková et al., 2006*a*). Synková et al. (2006*a*) suggested that the crystaloids are formed by aggregated LHCII proteins. Such aggregates of LHCII were previously reported during senescence of *Cucumis sativus* cotyledons by Prakash et al. (2001). The detection of CKs in the chloroplasts of these transgenic plants indicates that CKs can be transported from the cytoplasm to the chloroplasts (Synková et al., 2006*a*). These plants have also higher Chl content than WT (Synková et al., 1999; Cortleven and Valcke, 2012) and in later stages of their development they have higher activity of antioxidant enzymes (such as catalase and gluthathione reductase) (Synková et al., 2006*b*). Stromal proteins or protein complexes of thylakoid membrane do not exhibit any significant changes in Pssu-*ipt* plants (Synková et al., 1999; Cortleven et al., 2011). Synková et al. (1999) have found that PSII activity was slightly reduced while PSI activity was inhibited by 70 % in mature Pssu*:ipt* plants compared to corresponding WT plants. The lower quantum yield of primary photochemistry in light-adapted state in these plants was later reported by Cortleven and Valcke (2012).

In addition to the above described transgenic lines with increased endogenous content of CKs, there are also plants where the CK content is artificially lowered. This is achieved by the overexpression of *CKX* genes, coding the enzyme that irreversibly degrades CKs (Werner et al., 2003; 2006). Interestingly, the transgenic tobacco plants with increased CKX activity and thus significantly reduced CK content did not show any signs of accelerated senescence (Werner et al., 2003; Mýtinová et al., 2006). Leaves of 35S:*AtCKX* transgenic tobacco plants remained green longer and the senescence of its detached leaves was not accelerated compared to WT (Werner et al., 2003). This finding supports the hypothesis

that lower concentration of CKs does not act as a trigger of leaf senescence (Werner et al., 2003).

The regulatory role of CKs in senescence can be studied also with the help of different transgenic plants with modified/damaged CK signalling pathway, including *Arabidopsis* CK receptor mutants (*ahk* mutant plants; see 1.2.5 Interplay between cytokinins and light). Using *ahk* mutant plants, it has been shown that CK-dependent regulation of senescence is mediated mainly by the AHK3 receptor, coupled with the phosphorylation/activation of the B-type ARR, ARR2 (Kim et al., 2006; Riefler et al., 2006; Hwang et al., 2012). The specific AHK3–ARR2 phosphorelay plays a major regulatory role in the CK-dependent leaf longevity by modulating downstream targets implicated in the senescence programme (Kim et al., 2006; Hwang et al., 2012). Besides AHK3, the AHK2 receptor is considered to have a redundant function in the senescence regulation, whereas the AHK4 receptor is thought to have only minor relevance (Riefler et al., 2006). During high light stress the role of AHK3 and AHK2 in mantainenance of PSII function has been also shown (Cortleven et al., 2014). We showed that the presence of functional AHK3 and AHK2 led to retained Chl content and preserved PSII function during dark-induced senescence in *Arabidopsis* leaves. Moreover, we have suggested AHK4 to have the main responsibility for CK-induced inhibition of lipid peroxidation during dark-induced senescence (**Janečková et al., 2018**).

1.2.4 Senescence-accelerating effect of cytokinins

Besides the generally observed and widely described delay of senescence by CKs, there is also evidence that when CKs are applied at high concentration, they can have an opposite effect – they are able to accelerate it (Pospíšilová et al., 1993; Genkov et al., 1997; Carimi et al., 2004; Vescovi et al., 2012). We have also observed this effect earlier and in our experiments it is became obvious that the final effect of CKs (delay/acceleration) is dependent on their concentration (e. g. Melkovičová 2012, 2014). Although the acceleration of senescence observed under exogenous treatments with high CK concentration has been described in the literature, its exact mechanism has not yet been elucidated. Possible explanation of this effect could be linked to CK-dependent induction of oxidative stress

observed by Mlejnek et al. (2003) in the suspension of tobacco cells. It is thereby important to have in mind that the final effect of exogenous CK treatment on senescence-associated changes depends on the concentration we apply.

The effect of CKs on senescence is also affected by light conditions (Vlčková et al., 2006; Prokopová et al., 2010; Melkovičová, 2012; for more information, see chapter 1.2.5 Interplay between cytokinins and light). Vlčková et al. (2006) have observed a protective effect of *m*T treatment on detached wheat leaves senescing in darkness, while under continuous light (370 µmol photons m^{-2} s^{-1}), this protective *m*T effect switched to detrimental in a few days after the detachment (Vlčková et al., 2006). Further, Prokopová et al. (2010) observed that lettuce leaf discs, which were treated by BA (200 µM) and incubated under growth light (100 µmol photons m^{-2} s^{-1}), had impaired PSII function. However, when decreased light intensity (25 µmol photons m^{-2} s^{-1}) was applied, the negative effect of CKs on PSII activity was clearly diminished (Prokopová et al., 2010). Similarly, in my bachelor thesis (Melkovičová, 2012) I have shown that the final effect of CK treatment on senescence-induced changes is dependent on applied light conditions. We have analysed the effect of three different concentrations of *m*T (10, 100 and 300 µM) on the senescence of detached barley leaves. After 6 days in darkness, we observed positive effect of *m*T (higher Chl content, better PSII function) at all applied concentrations. When the detached leaves were kept under moderate light (periodic or continual; 70 µmol photons m^{-2} s^{-1}), the positive effect was observed only for two lower *m*T concentrations. In the case of high light (370 µmol photons m^{-2} s^{-1}), the highest *m*T concentration accelerated the senescence-induced changes (Melkovičová, 2012). Thus the positive CK effect on senescence can change to detrimental when higher light intensity or light dose is used.

1.2.5 Interplay between cytokinins and light

The effects of light and CKs on plant growth and development are similar and influence each other in many aspects (Chory et al., 1994; Zubo et al., 2008; Cortleven and Schmülling, 2015; Cortleven et al., 2016). It has been shown that light- and CK-dependent signalling pathways share a number of common intermediates at multiple levels (Sweere et al., 2001;

Vandenbussche et al., 2007; Oh et al., 2009; for a review, see e.g. Argueso et al., 2010; Zdarska et al., 2015).

A direct link between the CK and light signalling is represented by the RR of type A, *ARR4*, whose gene expression is induced by both white and red light (Sweere et al., 2001). ARR4 interacts specifically with the N-terminal part of phyB, forming a phyB-ARR4 complex. ARR4 thereby stabilizes the active (Pfr) form of phyB by retarding its dark conversion (Sweere et al., 2001). CKs are known to control gene expression of members of the phy signalling pathway, upregulating the genes for phyA, *PAT1* (*PHYTOCHROME-A SIGNAL TRANSDUCTION*), *PKS1 (PHYTOCHROME KINASE SUBSTRATE 1*), *SPA1* (*SUPPRESSOR OF PHYA-105 1*) and *COP1* (*CONSTITUTIVE PHOTOMORPHOGENIC 1*) (Bolle et al., 2000; Brenner et al., 2005, 2012; Brenner and Schmülling, 2012). PAT1 and PKS1 act as a positive and negative regulator of phy signalling pathway, respectively (Fankhauser et al., 1999; Bolle et al., 2000; Zdarska et al., 2015). The SPA1 and COP1 proteins form a complex and contribute to a rapid degradation of active phyA (Brenner and Schmülling, 2012). The signalling pathways of CK and phyA are interconnected also via *CKI1* (*CYTOKININ INDEPENDENT1*) gene, which is under the control of phyA and encodes a sensor His kinase of CK signalling pathway (Dobisova et al., 2017).

Another possible connection between the CK and light signalling may be the basic leucine zipper (bZIP) transcription factor LONG HYPOCOTYL 5 (HY5), which promotes photomorphogenesis (Ang et al., 1998; Vandenbussche et al., 2007) and is involved in the response to blue light in cryptochrome (cry) signalling pathway. Cry increase the stability of HY5 by preventing its ubiquitination and thus HY5 protein is accumulated under blue light. CK signal coming from ARR4 also prevents the degradation of HY5 (Vandenbussche et al., 2007; for review, see e.g. Argueso et al., 2010). Indirect evidence suggests that the inhibition of *HY5* expression might contribute to the control of reorganization and damage of microtubules during dark-induced senescence (Keech et al., 2010).

Marchadier and Hetherington (2014) have reported that CK signalling components are involved in the regulation of stomatal aperture by light in *Arabidopsis*. The AHP2 (Arabidopsis histidine phophotransferase2) protein, which is involved in CK signal

transduction, was found to be expressed in guard cells of *Arabidopsis*. Deficiency in AHP2 results in much narrower stomatal aperture after the light stimulus compared to WT. In contrast, overexpression of AHP2 in plants leads to wider stomatal aperture. Functional *AHP2* gene is thus required for full light-stimulated stomatal opening. Analysis of AHK mutants suggested that even AHK2 and AHK3 receptors of CKs are involved in the control of light-induced stomatal opening (Marchadier and Hetherington, 2014).

Both light and CKs influence photosynthesis. As mentioned previously, CKs affect the function as well as structure of the photosynthetic apparatus (Kusnetsov et al., 1998; Synková et al., 1999; Yaronskaya et al., 2006; Cortleven and Valcke, 2012) and influence photosynthesis-related genes (Talla et al., 2016; Vylíčilová et al., 2016). It has been shown that CKs also act as signals for photosynthetic acclimation to canopy light gradients (Boonman et al., 2007).

It is obvious that there is a close interaction between light and CKs, therefore it is natural to assume that the light-mediated retardation of leaf senescence interferes with CK signal and vice versa. However, a detailed investigation of the interaction between these two factors during senescence is missing. One aspect of the interaction between light and CKs in the delay of leaf senescence can be light-induced changes in the content of endogenous CKs, as light participates in the regulation of CK biosynthesis, degradation and transport (Qamaruddin and Tillberg, 1989; Ananieva et al., 2008; Zubo et al., 2008; Boonman et al., 2007; Marchetti et al., 2018; for review see e.g. Kurepin and Pharis, 2014; Zdarska et al., 2015).

Stimulating effect of light on endogenous CK content has been described in many various studies (e.g. Mizuno et al., 1971; Qamaruddin and Tillberg, 1989; Kraepiel et al., 1994; Nováková et al., 2005). Kurepin et al. (2007) investigated effect of light with different far-red ratios and two different intensities on the elongation of sunflower (*Helianthus annuus* L.) internodes. They reported markedly increased content of CK free bases and ribosides under low far-red ratio in combination with higher light intensity (421 µmol photons m^{-2} s^{-1}) (Kurepin et al., 2007). On the other hand, markedly decreased content of

zeatin was found in *pew1* and *pew2* mutants of tobacco (*Nicotiana plumbaginifolia* L.), which are deficient in phy (Kraepiel et al., 1995).

The activity of CKX and thus the degradation of CKs appears to be affected by light conditions as well, however, contradictory results were observed. Schlüter et al. (2011) have found that CKX activity in senescing detached barley (*Hordeum vulgare* L.) leaf segments increased much more under light/dark conditions than in complete darkness. Although the senescence of leaf segments kept under the light/dark regime was slower than of those kept in the dark, their CKX activity increased much faster. A possible explanation could be that light not only increased CK degradation, but also stimulated the formation of active CKs in senescing detached leaves (Schlüter et al., 2011) However, this was not proved by analyse of the endogenous CK content. Further, Ananieva et al. (2008) reported lowered CKX activity in cotyledons kept in the dark, but when one of the cotyledon leaves was illuminated and the other kept in the dark, CKX activity increased. Interestingly, Marchetti et al. (2018) found out that CKX activity in detached leaves depends on spectral quality of light, where blue light was able to suprress an increase in CKX activity in shaded leaves by shading

Despite the obviously important role of light in the regulation of CK content, it is not clear if and how endogenous CK level is changed after the detachment of leaves and how it is affected by light conditions. There are only a few studies describing changes in the content of endogenous CKs in detached leaves under different light conditions and their results are contradictory (Zubo et al., 2008; Causin et al., 2009; Roberts et al., 2011).

Zubo et al. (2008) have shown that the content of zeatin derivatives increased in detached leaves of barley (*Hordeum vulgare* L.) both in the dark and under continuous light (270 µmol m^{-2} s^{-1}), with more pronounced increase in light. A decrease in isopentenyladenosine (iPA) content was observed in detached wheat leaves (*Triticum aestivum* L.) exposed to white light (260 µmol m^{-2} s^{-1}) (Causin et al., 2009). On the contrary, Roberts et al. (2011) found a pronounced decrease in isopentyladenine (iP) and iPA content in detached senescing leaves of wheat (*Triticum aestivum* L.) kept in darkness (Roberts et al., 2011). A more complex analyses of endogenous CK content was performed by Marchetti

et al. (2018) in detached wheat (*Triticum aestivum* L.) leaves kept under shade with a suppression or a presence of blue wavelengths. When blue light was filtered out, senescence of detached wheat leaves was accelerated. The content of *t*Z and *c*Z free bases decreased in detached leaves and the content of glycosylated CK forms was elevated. This was accompanied by increased CKX activity. On the contrary, when blue light was present, a significantly higher endogenous level of *t*Z and *c*Z was maintained and accumulation of glycosylated CK forms and increase of CK degradation was delayed. No significant differences in the endogenous content of iP were found. The study shows that the presence of blue light maintains free CKs content and eliminates increase in the activity of CKX in wheat leaves senescing under shade and it further demonstrates that not only changes in light intensity, but also changes in spectral quality of light have effect on CK level in detached senescing leaves (Marchetti et al., 2018).

We have analyzed changes in the content of various CK types and forms in detached *Arabidopsis* leaves senescing under different light conditions, i.e. in the dark and under growth (GL; 150 µmol photons m^{-2} s^{-1}) and high (HL; 400 µmol photons $m^{-2}s^{-1}$) light intensity (**Janečková et al., 2018**). To investigate the effect of light on plants with impaired CK signalling, we have also used three CK receptor double mutants of *Arabidopsis*.

After 6 days in darkness, detached leaves of WT had lower Chl content, impaired PSII function and increased lipid peroxidation. These changes were accompanied by a marked decrease in endogenous CK content. The content of free bases, ribosides and monophosphates of *t*Z and iP markedly decreased, indicating that inactivation and degradation of these CKs prevailed over their biosynthesis. In general, the decrease of overall CK content and of iP and *t*Z forms was observed also in other genotypes kept in the dark. In the case of dark senescing leaves of mutant where both receptors AHK3 and AHK2 were non-functional (*ahk2 ahk3*), total free bases content decreased pronouncedly more than in WT or than in mutant where AHK3 was functional (*ahk2 ahk4*), even though the (initial) CK content was the highest in *ahk2 ahk3*. In this mutant the most pronounced decrease in Chl content and PSII function was observed (**Janečková et al., 2018**). This could be explained by the role of AHK3 and AHK2 in CK-mediated Chl retention (Kim et al., 2006;

Riefler et al., 2006) and in the maintenance of PSII function (Cortleven et al., 2014) (see part 1.2.3). As AHK4 did not have sufficient promoting effect on Chl content and PSII function, CK-dependent protection of photosynthesis during senescence was lost. This mutant had the lowest MDA content, which could be explained by a combination of high initial content of CK and functional AHK4 receptor that we suggested to be the main receptor for CK-dependent suppression of lipid peroxidation**.** The opposite effect was observed in a mutant lacking functional AHK2 and AHK4 (*ahk2 ahk4*). In this mutant, the higher initial CK content together with the presence of functional AHK3 led to highly maintained photosynthetic performance, whereas the absence of functional AHK4 and AHK2 contributed to the highest lipid peroxidation. In the *ahk3 ahk4* mutant, the content of CK as well as the maintenance of Chl content, PSII function and low lipid peroxidation, was between *ahk2 ahk3* and *ahk2 ahk4*, indicating that AHK2 can partially take over the role of both AHK3 and AHK4 (**Janečková et al., 2018**).

Interestingly, leaves of all genotypes senescing in the dark were characterized by an increase in *c*Z and *m*T content. The pool of missing (most abundant) free bases (i.e. iP and *t*Z) seems to be partially or completely replenished by these CK forms, indicating possible stimulation of *c*Z and *m*T biosynthesis in darkness (**Janečková et al., 2018**). This is in agreement with previous suggestions that *c*Z can have a role in the ensuring of minimal CK activity necessary for the maintenance of essential physiological processes under conditions characterized by the lack of energy sources that lead to down-regulation of the main active CKs. (Gajdošová et al., 2011). Higher *c*Z content has been repeatedly observed under growth-limiting conditions induced by various external or internal factors (Prerostova et al., 2018; for a review, see e.g. Schäfer et al., 2015) or has been found in senescing leaves (Gajdošová et al., 2011; Šmehilová et al., 2016).

Under both light conditions (GL or HL), the detached leaves of all genotypes were characterized by highly maintained Chl content and PSII function and by reduced lipid peroxidation compared to situation in darkness. Surprisingly, under HL, both mutants without functional AHK3 receptor had markedly better PSII function than WT. The disrupted CK signalling via AHK3 thus led to lower Chl content and impaired PSII function in darkness,

however, under GL, the CK-signalling deficiency was apparently compensated by light. Even more so, the photosynthetic performance of leaves of AHK3 deficient mutant senescing under HL was even better than in WT. Light thus seems to have the ability to somehow substitute the insufficient CK signalling, and under HL conditions, it was probably able to even reverse the negative effect of AHK3 dysfunction (**Janečková et al., 2018**). The overall CK level decreased under both light conditions as well as in the dark, however, the total content of iP forms was highly maintained under light in all mutants. Moreover, the content of iP forms even increased in the detached WT leaves when kept in light. This may indicate that the biosynthesis of iP is functional or even stimulated in detached leaves. The pronounced promoting effect of light (both GL and HL) on the maintenance of high photosynthetic parameters was thus associated with higher level of iP and its riboside (iPR) (**Janečková et al., 2018**). On the other hand, light did not have any significant effect on the content of *t*Z forms in the detached leaves. In illuminated leaves, the *t*Z biosynthesis seemed to be inhibited in a similar manner as in the leaves kept in the dark, even though the decrease in total content of *t*Z forms was slightly smaller (**Janečková et al., 2018**). This supports the hypothesis that *t*Z-type CKs are predominantly synthetized in roots (Hirose et al., 2008; Frébort et al., 2011).

To summarize, our results show that the content of CKs and their various forms differs significantly in detached *Arabidopsis* leaves kept under various light conditions. While light did not have any significant effect on the metabolism of *t*Z CKs in the detached leaves, in the case of iP light stimulated its accumulation. We suggest that the light-mediated maintenance of photosynthesis in detached leaves could be thus related to persisted biosynthesis of iP. And when comparing the senescence-induced changes in the CK receptor double mutants, light appears to be able to compensate disrupted CK signalling caused by loss-of-function mutation in AHK receptors (**Janečková et al., 2018**). There is obviously an extensive crosstalk between light and CKs and this crosstalk seems to be very important also for the regulation of plant senescence. However, the molecular mechanism of this interaction remains to be clarified in the future.

2 Aims of research

Plant hormones cytokinins (CKs) are important endogenous factors that are involved in the regulation of senescence. It is well known that in detached leaves, senescence is retarded by both exogenously applied CKs and light, however, the mechanism of their mutual interaction is not clear. As light has been shown to influence CK biosynthesis, transport and degradation, the light-mediated delay of leaf senescence could be associated with changes in endogenous CK levels. However, it is not clear if and how endogenous CK level is modified in leaves after their detachment and how it is affected by light conditions. Recent studies have shown that chlorophyll (Chl) *b* has also an important role in the regulation of leaf senescence. However, there is only limited information about senescence of plants lacking Chl *b* and senescence-induced decrease in photosynthetic function has not even been investigated in such plants. The aims of our investigations were as follows:

I. to find out whether endogenous CK content is modified in detached *Arabidopsis* leaves senescing under various light conditions,
II. to study the connection between the changes in endogenous CK levels and effect of light on senescence-induced changes (Chl content, efficiency of photosystem II (PSII) photochemistry, and lipid peroxidation) in detached leaves of *Arabidopsis*,
III. to evaluate the role of individual CK receptors and role of light in leaf senescence of *Arabidopsis* plants with impaired CK signalling,
IV. to verify whether Chl *b* deficiency accelerates dark-induced senescence of detached leaves and to describe the related senescence-induced changes in the function of PSII,
V. to find out whether and to what extent exogenously applied CKs are able to suppress the supposedly accelerated senescence in leaves of mutant deficient in Chl *b*.

3 Experimental approach

3.1 Plant materials and growth conditions

For the study of Janečková et al. (2018), *Arabidopsis thaliana* plants (wild-type [WT] Columbia-0 and three CK receptor double mutants - *ahk2 ahk3*, *ahk2 ahk4*, and *ahk3 ahk4*; Riefler et al., 2006) were grown in a growth chamber on a commercial substrate (Potgrond H, Klasmann-Deilmann Substrate, Germany) under short-day conditions: 8 h of white light (120 µmol photons m^{-2} s^{-1})/16 h dark, at 22/20 °C and relative air humidity 60 %. Seeds were kept at 4 °C for 3 days in the dark before sowing. After 5 weeks, mature leaves (seventh leaf on average) were cut off from plants and placed into a 0.2% solution of dimethyl sulfoxide. In a separate experiment, we have verified that this solution did not affect senescence of detached Arabidopsis leaves in comparison with distilled water. The detached leaves floated on the solution in a closed six-hole macrotiter plates and were kept under three different light conditions - dark, growth light (GL; 120 µmol photons m^{-2} s^{-1}), and higher light (HL; 400 µmol photons m^{-2} s^{-1} from the same light source as GL; 8 h light/16 h dark). Measurements were performed immediately after leaf detachment ("control") and on the second, fourth, sixth and twelfth day after detachment. Data presented in Janečková et al. (2018) are from the day of the detachment ("control") and from the sixth day after detachment.

For the study of Janečková et al. (2019), seeds of wild-type barley (*Hordeum vulgare* L. cv. Bonus; WT) and chlorina $f2^{f2}$ (*clo*) mutant were soaked in deionized water for 24 h before sowing and then transferred into pots containing perlite with Hoagland solution. Pots were placed in a growth chamber under controlled conditions of 16 h light (150 µmol of photons m^{-2} s^{-1})/8 h dark, 22/20 °C and 60 % relative air humidity. Eight days after the sowing, 4-cm segments were cut off from the primary leaves. The leaf segments were placed either into a 0.2% solution of dimethylsulfoxide (DMSO) or into a 10^{-5} mol l^{-1} solution of 6-benzylaminopurine (BA) in 0.2% DMSO (BA-treated leaves). The leaf segments were then kept in the dark (other conditions were same as during plant growth). Measurements

were performed immediately after the leaf detachment and on the 4th day after detachment.

Exogenous CKs can be applied in different ways. In our experiments we either let the leaves or leaf segments float in CK solution (experiments with *Arabidopsis* or tobacco leaves) or kept the base of detached leaves in CK solution placed in microplates (experiments with crops) (Fig. 7).

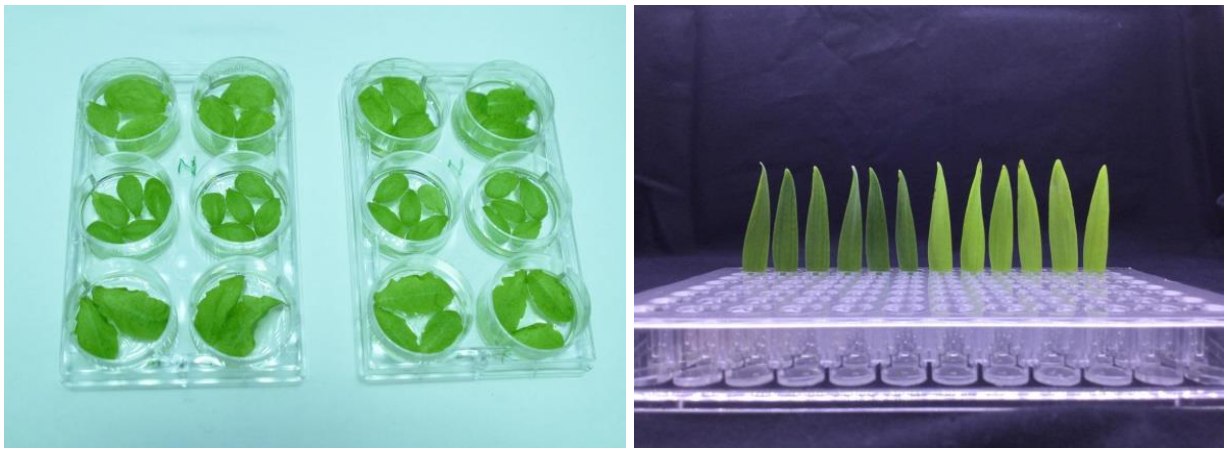

Fig. 7: Experimental set-up of exogenous CK treatment. Left: floating leaves in CK solution, right: keeping the base of detached barley leaves in CK solution placed in microplates.

3.2 Pigment analyses

3.2.1 Chlorophyll content in *Arabidopsis* leaves

In Janečková et al. (2018), Chl content was measured using a chlorophyll meter SPAD-502 (Minolta Sensing Konica, Osaka, Japan) after 30-min dark adaptation for elimination of the effect of chloroplast movement (Nauš et al., 2010). To obtain the absolute Chl content, a calibration of the SPAD values was performed for the individual genotypes by simultaneous analytical determination of Chl content.

For the analytical determination, the leaves were homogenized in a chilled mortar with $MgCO_3$ and 80% acetone. The homogenate was then centrifuged using a centrifuge 3-30KS Sigma (SIGMA Laborzentrifugen, Germany) at 4,000*g* for 5 min and at 10 °C. After centrifugation, the absorbance (A) of the supernatant was measured at the wavelengths of 646.8, 663.2, and 750 nm using a spectrophotometer Unicam UV550 (ThermoSpectronic, UK). Total Chl concentration was calculated according to Lichtenthaler (1987).

The Chl content was related to a leaf area (μg cm^{-2}). The Chl content of the leaves measured on the sixth day after detachment was expressed as percent of the values of the corresponding control leaves.

3.2.2 Chlorophyll and xanthophyll content in barley leaves

For the determination of the content of pigments in Janečková et al. (2019), the area of leaf samples was estimated and then the leaves were homogenized in liquid nitrogen, with $MgCO_3$ and 80% acetone. The homogenates were centrifuged at 4,000*g* and 4 °C for 10 min. The supernatant was used for the spectrophotometric estimation of Chl and total car contents (a sum of carotenes and xanthophylls) according to Lichtenthaler (1987) by a spectrophotometer Unicam UV550 (Thermo Spectronic, United Kingdom) and also for the quantification of individual xanthophylls (violaxanthin, V; antheraxanthin, A; zeaxanthin, Z) by high performance liquid chromatography (HPLC).

For the estimation of xanthophyll content (VAZ) by an HPLC system (Alliance e 2695 HPLC System, Waters, USA), the supernatant was filtered through 0.45 μm polytetrafluoroethylene membrane (Acrodisc, Waters, USA) into dark vials. The 100 μl amount was injected into the HPLC system. A LiChroCAR TRP-18 (5 μm; 4.6 x 250 mm) column (Merck & Co., USA) was used. The analysis was performed by a gradient reverse-phase analysis (1.5 ml min^{-1} at 25 °C). The analysis started with isocratic elution using the mobile phase composed of acetonitrile, methanol and 0.1 mol l^{-1} Tris (pH 8) in the ratio 87:10:3 (v:v:v) for 10 min and was followed by a 2-min linear gradient using mobile phase composed of a mixture of methanol and n-hexane in the ratio 4:1 (v:v). Absorbance was detected at 440 nm using a UV/VIS detector. The amount of pigments in samples was determined using their conversion factors (Färber and Jahns, 1998). The de-epoxidation state of xanthophylls (DEPS) was calculated according to Gilmore and Björkman (1994) as (A+Z)/(V+A+Z)× 100 (%).

3.3 Chl fluorescence

3.3.1 Fluorescence measurement with FluorCam 700 MF imaging system

In Janečková et al. (2018), Chl fluorescence parameters were measured from the adaxial leaf side at room temperature by FluorCam 700 MF imaging system (Photon Systems Instruments, Czech Republic).

At the beginning, the minimum Chl fluorescence (F_0) was determined after 30 min of dark-adaptation of the sample by application of the measuring flashes (red light) which intensity was low enough to avoid the closure of RCII. For the determination of maximum fluorescence (F_M), a saturating pulse of 1.6 s (white light, 500 µmol m^{-2} s^{-1}) was applied on the dark-adapted sample. After dark-relaxation (2 min), when the measured Chl fluorescence signal dropped to F_0, the leaf sample was exposed to actinic light (red light, 120 µmol of photons m^{-2} s^{-1}, the same intensity as used for plant growth). To determine the maximum Chl fluorescence during light-adaptation (F_M'), a set of the saturating pulses was applied. The first saturation pulse (white light, 500 µmol photons m^{-2} s^{-1}) was applied 3 s after the actinic light was switched on and was followed by 6 pulses in 23 s intervals, 3 pulses in 47 s intervals and the last 2 pulses in 70 s intervals.

The maximal quantum yield of PSII photochemistry in the dark-adapted state (F_V/F_M) was calculated as $(F_M - F_0)/F_M$, where F_M is maximal fluorescence and F_0 is minimal fluorescence in the dark-adapted leaf sample. In the light-adapted leaf samples (after 7 min of actinic light), the maximal quantum yield of PSII photochemistry (Φ_{PSII}) was calculated as $(F_M' - F_0')/F_M'$, where F_M' is maximal fluorescence for the light-adapted state and F_0' represents the minimal fluorescence for light-adapted state calculated as $F_0/(F_V/F_M + F_0/F_M')$.

3.3.2 Chl fluorescence mesurements with Plant Efficiency Analyser and PlantScreen phenotyping platform

In Janečková et al. (2019), the Chl fluorescence induction transient (OJIP curves) and the quenching analysis were measured at room temperature on adaxial side of barley leaf samples. Freshly detached leaves (i.e., leaves before senescence induction) were dark-

adapted for 25 min before the measurement. The OJIP curves were measured in the middle of leaf segments by Plant Efficiency Analyser (Hansatech Instruments, United Kingdom) for 2 s with excitation light intensity of 1100 μmol photons m^{-2} s^{-1}. The initial slope of the O-J Chl fluorescence rise $(dV/dt)_0$, the relative variable fluorescence at the J step (V_J), and the specific energy flux ABS/RC were evaluated as follows (see Stirbet et al., 2018). The $(dV/dt)_0 = 4(F_{300\mu s} - F_{50\mu s})/F_V$, where $F_{300\mu s}$ and $F_{50\mu s}$ are fluorescence intensities at the indicated times and F_V is variable fluorescence ($F_V = F_P - F_0$; F_0 is a minimal fluorescence and F_P is fluorescence at the P step). The $(dV/dt)_0$ parameter, defined as the maximal rate of the accumulation of the fraction of closed RCII (Strasser et al., 2000), reflects the rate of excitation supply into the RCII and subsequently the rate of Q_A reduction. Parameter V_J, reflecting the fraction of reduced Q_A, was calculated as $(F_J - F_0)/F_V$, where F_J is fluorescence intensity at 2 ms. ABS/RC was calculated as $((dV/dt)_0/V_J) \times F_P/F_V$ and reflects apparent antenna size of active RCII (Strasser et al., 2000). Further, the quantum yield of electron transport from reduced Q_A to final acceptors of PSI (RE_0/ABS) and the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI (δR_0) were estimated as follows: $RE_0/ABS = F_V/F_P \times (1 - V_I)$ and $\delta R_0 = (1 - V_I)/(1 - V_J)$, where V_I is the relative variable fluorescence at the I step (Stirbet et al., 2018). The measured OJIP curves as well as curves normalized to F_V are presented.

The quenching analysis was performed using PlantScreen (Photon Systems Instruments, Czech Republic) phenotyping platform (Humplík et al., 2015) according to the following protocol. At the beginning, the minimal fluorescence F_0 was determined using measuring flashes (duration of 10 μs) of red light (650 nm), which did not cause any closure of RCII. Then a saturating pulse (white light, 1900 μmol photons m^{-2} s^{-1}, duration of 800 ms) was applied to measure maximal fluorescence F_M. After 90 s of dark-relaxation, when the measured fluorescence signal dropped to F_0, the leaf sample was exposed to actinic light for 25 min (red light, 150 μmol of photons m^{-2} s^{-1}, the same intensity as used for plant growth). To determine the maximal fluorescence during the actinic light exposition (F_M'), a set of the saturating pulses was applied. The first pulse was applied 10 s after the actinic light was

switched on and was followed by 9 pulses in 20 s intervals and then by 22 pulses in 59 s intervals.

The maximal quantum yield of PSII photochemistry in the dark-adapted state was estimated as $F_V/F_M = (F_M - F_0)/F_M$. The maximal quantum yield of PSII photochemistry in the light-adapted state was calculated as $F_V'/F_M' = (F_M' - F_0')/F_M'$, where F_0' is minimal fluorescence for the light-adapted state, which was calculated as $F_0/(F_V/F_M + F_0/F_M')$. The effective quantum yield of PSII photochemistry in the light-adapted state was calculated as $\Phi_P = (F_M' - F_t)/F_M'$, where F_t is fluorescence at time t measured immediately prior to the application of the saturating pulse. The quantum yield for regulatory non-photochemical quenching was calculated as $\Phi_{NPQ} = (F_t/F_M') - (F_t/F_M)$ and the quantum yield for constitutive non-regulatory dissipation processes was calculated as $\Phi_{f,D} = F_t/F_M$. The sum of Φ_P, Φ_{NPQ} and $\Phi_{f,D}$ equals unity (for a review, see Lazár, 2015). In the case of F_V'/F_M', Φ_P, Φ_{NPQ}, and $\Phi_{f,D}$, values obtained at the end of the actinic light exposition are presented.

3.3.3 Measurement of P700 oxidation

For estimation of light-induced oxidation of P700 (the primary electron donor of PSI) in Janečková et al. (2019), the I830 signal as a difference of transmittance at 875 nm and 830 nm was determined using Dual PAM 100 (Walz, Germany), see, e.g. Lazár (2013). The methodology assumes that P700 is fully reduced in the dark-adapted leaf and thus the I830 signal is zero. During illumination of the leaf, the I830 signal rises to a peak level reflecting an equilibrated maximal $P700^+$ level as a result of P700 oxidation by the charge separation and $P700^+$ reduction by plastocyanin. In both WT and *clo* leaves before senescence induction, the I830 signal reached the peak level at 17 ms. In senescing leaves, the level of $P700^+$ at 17 ms of illumination was expressed in % of the peak level observed in the leaves before senescence induction.

3.4 Determination of malondialdehyde content

In Janečková et al. (2018), the content of malondialdehyde (MDA) was measured using HPLC. The isolation and derivatization of MDA using 2,4-dinitrophenylhydrazine (DNPH) was performed as described in Pilz et al. (2000) with some modifications. Leaves were

homogenized in 0.11% butylhydroxytoluene dissolved in 100% methanol. This step was followed by 10 min centrifugation at 2,000*g*. 125 µl of supernatant was poured into an eppendorf tube and 25 µl of 6 mol l^{-1} aqueous NaOH was added to achieve alkaline hydrolysis of protein bounded MDA. Samples were incubated for 30 min in dry bath at 60°C (Thermo-Shaker TS100, Biosan, Latvia). To reach the precipitation of proteins in samples, 62.5 µl of 35% (v:v) perchloric acid was added to the sample, vortexed and centrifuged at 16,000*g* for 10 min. 125 µl of supernatant was poured into a vial, stirred up with 1 µl of 50 mmol l^{-1} DNPH dissolved in 50% sulphuric acid and incubated in dark at room temperature for 30 min to obtain MDA-DNPH adducts. Then 25 µl of the solution was injected into an HPLC system (Alliance e 2695 HPLC System, Waters, Milford, MA, USA). A Symmetry C18 (3.5 µm; 4.6 x 75 mm) Column (Waters, Milford, MA, USA) was used, the elution was performed isocratically (1 mL/min at 35°C), using a mixture of 25 mmol l^{-1} triethylamine (pH 3.5) and acetonitrile in the ratio 50:50 (v:v). MDA was detected at 310 nm using a UV/VIS detector.

3.5 Identification and quantification of endogenous cytokinins

To analyse the endogenous CKs content in study of Janečková et al. (2018), leaves from 5-week-old *A. thaliana* plants were harvested, frozen in liquid nitrogen, and stored at –80°C. Three replicates were measured, each consisted of 440 ± 10 mg of leaf mass (6^{th}, 7^{th} and 8^{th} true leaves; an average number of leaves per sample was 12 for WT, 24 for *ahk2 ahk3*, 11 for *ahk2 ahk4* and 10 for *ahk3 ahk4*). The procedure used for CK purification was a combination of the methods described by Dobrev and Kamínek (2002) and Svačinová et al. (2012). Isotope-labelled CK internal standards (Olchemim, Czech Republic) were added (0.25 pmol per sample of B, 7G and 0.5 pmol per sample of OG, NT) to check the recovery during purification and to validate the determination (Novák et al., 2008). The samples were purified using C18 and MCX cartridges (Dobrev and Kamínek, 2002). Eluates were evaporated to dryness using a SpeedVac concentrator and dissolved in 40 µl of 10% methanol. Ten µl of each sample were then analysed by an ultra-performance liquid chromatograph (Acquity UPLC System; Waters, USA) coupled to a triple quadrupole mass spectrometer equipped with an electrospray interface (Xevo TQ-S, Waters, Manchester, UK)

by a method utilized on the StageTips technology (Svačinová et al., 2012). Quantification was obtained by multiple reaction monitoring of $[M+H]^+$ and the appropriate product ion. Optimal conditions, dwell time, cone voltage, and collision energy in the collision cell, corresponding to the exact diagnostic transition, were optimized for each CK for selective MRM experiments (Novák et al., 2008). Quantification was performed by Masslynx software using a standard isotope dilution method (Novák et al., 2003).

3.6 Correlation and statistical analysis

In Janečková et al. (2018), correlation between the contents of different CK forms in detached leaves and the relative Chl content, the maximal quantum yield of PSII photochemistry in dark-adapted state, and MDA content was estimated using the Pearson's correlation coefficient. Statistical analysis was performed using Student's t-test, in which the values of parameters measured with receptor mutants were compared with corresponding values in WT. The significant differences are marked by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

In all statistical tests of Janečková et al. (2019), related data sets were first tested for normality (Kolmogorov-Smirnov test with Lilliefors' correction) and equality of variances (Levene Median test). If fulfilled, the Student's t-test or ANOVA test (with all pairwise multiple comparison by Holm-Sidak *post hoc* test) were used and if not fulfilled, the Mann-Whitney Rank Sum test or Kruskal-Wallis ANOVA on Ranks test (with all pairwise multiple comparison by Dunn's *post hoc* test) were used. The critical level of 0.05 was chosen for all tests (the P-value of the test is marked by *). If the P-value of a test was even lower than 0.01 or even lower than 0.001, the results are marked by ** or ***, respectively. All testing was performed using SigmaPlot version 11 (Systat Software, USA).

4 Conclusions and Future Perspectives

Leaf senescence is a complex developmental process, characterized by numerous physiological and biochemical changes. An important role of senescence is the remobilization and transport of nutrients, especially nitrogen, into storage and growing tissues and organs. Senescence is usually accompanied by a loss of Chl content, by inhibition of photosynthetic processes including PSII photochemistry and by higher lipid peroxidation. Plant hormones CKs are known as negative regulators of senescence, they preserve photosynthetic structures and function, especially PSII, during leaf senescence.

It is well known that in detached leaves, both exogenously applied CKs and light retard senescence, however, the mechanism of their mutual interaction is not clear. In our work, we have shown that the content of endogenous CKs and individual CK forms differs significantly in detached *Arabidopsis* leaves kept under various light conditions. In detached leaves kept in darkness, senescence-induced decrease in Chl content and PSII function and increase in lipid peroxidation were accompanied by significantly lower content of endogenous CKs. The content of free bases, ribosides and monophosphates of *t*Z and iP significantly decreased, indicating that inactivation and degradation of these CKs prevailed over their biosynthesis. On the other hand, we have found increased content of *m*T and *c*Z free bases, possibly indicating stimulation of their biosynthesis in darkness. Our results contribute to the clarification of the role of *c*Z in plants and at the same time support the hypothesis that *c*Z maintains the basal viability of leaves under growth-limiting conditions. Furthermore, we have shown that functional CK receptors AHK3 and AHK2 are important for CK-dependent maintenance of Chl content and PSII function in detached leaves during dark-induced senescence. We have proposed that AHK4 receptor plays a major role in CK-mediated protection against senescence-associated lipid peroxidation in detached *Arabidopsis* leaves.

In detached leaves kept in light, the Chl content and PSII function were highly maintained and increase in lipid peroxidation was lower compared to dark-senescing leaves. These changes were accompanied by higher content of free bases of iP. It has been suggested that the light-mediated maintenance of photosynthesis in detached leaves could

be related to persisted biosynthesis of iP. In addition, from the comparison of senescence-induced changes in the CK receptor double mutants, it appears that light is able to compensate the disrupted CK signalling caused by loss-of-function mutation in AHK receptors.

Recent studies have shown that Chl *b* has also an important role in the senescence regulation. However, information about senescence of plants lacking Chl *b* is limited and senescence-induced decrease in photosynthetic activity has not even been investigated in such plants. We have observed pronouncedly faster inhibition of PSII photochemistry during dark-induced senescence of detached leaves of Chl *b* deficient barley mutant. We suppose that the stronger impairment of PSII function is related to a more pronounced damage of reaction centres of PSII, probably due to destabilizing effect of Chl *b*/LHC deficiency. Exogenously applied CKs were able to eliminate this effect, probably via the stabilization of reaction centres of PSII.

Although a significant progress was made over the last two decades in our understanding of CK involvement in senescence regulation, many questions still remain unanswered. The mechanism of light and CK interaction during senescence remains ambiguous and also the molecular mechanism of protective effect of CKs on photosynthesis during senescence remains to be clarified. Another unresolved issue is the mechanism of the CK-mediated acceleration of leaf senescence. It has been shown that in some cases, CKs act as positive regulators of leaf senescence. This usually happens when high concentrations of exogenous CK are applied or when the exogenous CK treatment is combined with higher light intensity.

Elucidation of the mechanism of CK-mediated regulation of senescence will contribute to our understanding of the process itself and can also provide important information for genetic manipulations leading to the improvement of agronomic traits, such as crop yields and post-harvest quality of plants.

5 Acknowledgements

I would like to express my deepest gratitude to Dr. Martina Špundová for her supporting supervision during my doctoral studies. I thank her for her guidance, all the valuable advices and the time dedicated to helping me.

I extend my gratitude also to prof. Petr Ilík for giving me the opportunity to pursue my doctoral studies at the Department of Biophysics. I want to thank also to all the other members of the department, which with certain extent gave me the environment to pass my studies. Thanks all for their collaborations, help and support. My thanks then belong also to Dr. Iva Ilíková for the critical reading of my thesis and all our publications. Especially, I express my most sincere gratitude to Dr. Alexandra Husičková for her continuous help, endless support, and encouraging belief that everything will finish successfully. I also take this opportunity to thank Bára Baránková, Ursula Ferretti and Lukáš Nosek for their special friendship, which our studies brought into our ways.

I am also truly grateful to Dr. Olivier Keech for the opportunity to be a part of his research group and to be involved in their projects during my four months research stay at the Department of Plant Physiology at Umeå University, Sweden. I thank to all the members of Olivier's group who guided me and helped me during this stay.

I thank from the bottom of my heart to my husband, who stood behind me for the whole studies and went through all the good and bad moments with me. I thank my grandmother, sister and brother for their love and persistent support and encouragement. And last but not least, thank You mom! I am convinced that you are looking at me from the heaven.

Projects represented in this thesis were supported by Grant No. LO1204 (Sustainable Development of Research in the Centre of the Region Haná) from the National Program of Sustainability I, Ministry of Education, Youth and Sports, Czech Republic and by ERDF project "Plants as a tool for sustainable global development" (No. CZ.02.1.01/0.0/0.0/16_019/0000827). And my four months research stay was supported by the funds from Erasmus plus program.

6 References

Adam Z, Clarke AK. 2002. Cutting edge of chloroplast proteolysis. Trends in Plant Science 7: 451–456.

Ananieva KT, Ananiev ED, Doncheva S, Georgieva KM, Tzvetkova N, Kamínek M, Motyka V, Dobrev PI, Gajdošová S, Malbeck J. 2008. Senescence progression in a single darkened cotyledon depends on the light status of the other cotyledon in *Cucurbita pepo* (zucchini) seedlings: potential involvement of cytokinins and cytokinin oxidase/dehydrogenase activity. Physiologia Plantarum 134: 609–623.

Ang LH, Chattopadhyay S, Wei N, Oyama T, Okada K, Batschauer, A, Deng, XW. 1998. Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of Arabidopsis development. Molecular Cell 1: 213–222.

Argueso CT, Raines T, Kieber JJ. 2010. Cytokinin signaling and transcriptional networks. Current Opinion in Plant Biology 13: 533–539.

Argyros RD, Mathews DE, Chiang Y-H, Palmer CM, Thibault DM, Etheridge N, Argyros DA, Mason MG, Kieber JJ, Schaller GE. 2008. Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. The Plant Cell 20: 2102–2116.

Balazadeh S, Siddiqui H, Allu AD, Matallana-Ramirez LP, Caldana C, Mehrnia M, Zanor MI, Kohler B, Mueller-Roeber B. 2010. A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. The Plant Journal 62: 250–264.

Barber J, Andersson B. 1992. Too much of a good thing: light can be bad for photosynthesis. Trends in Biochemical Sciences 17: 61–66.

Bellemare G, Bartlett SG, Chna NH. 1982. Biosynthesis of chlorophyll *a/b*-binding polypeptides in wild type and the Chlorina f2 mutant of barley. The Journal of Biological Chemistry 257: 7762–7767.

Biswal B. 1997. Chloroplast Metabolism during Leaf Greening and Degreening. In: Pessarakli M. (ed.) Handbook of Photosynthesis, Marcel Dekker Inc, New York pp. 71–81.

Biswal UC, Biswal B. 1984. Photocontrol of Leaf Senescence. Photochemistry and Photobiology 39: 875–879.

Biswal UC, Biswal B, Raval MK. 2003. Chloroplast Biogenesis: from Proplastid to Gerontoplast. Dordrecht, The Netherlands: Kluwer- Academic Publishers.

Biswal B, Mohapatra PK, Biswal UC, Raval MK. 2012. Leaf Senescence and Transformation of Chloroplasts to Gerontoplasts. In: Eaton-Rye J, Tripathy B, Sharkey T. (eds) Photosynthesis. Advances in Photosynthesis and Respiration, Springer, Dordrecht, pp. 217–230.

Bolle C, Koncz C, Chua N-H. 2000. PAT1, a new member of the GRAS family, is involved in phytochrome A signal transduction. Genes & Development 14: 1269–1278.

Boonman A, Prinsen E, Gilmer F, Schurr U, Peeters AJM, Voesenek LAC, Pons TL. 2007. Cytokinin import rate as a signal for photosynthetic acclimation to canopy light gradients. Plant Physiology 143: 1841–1852.

Bossmann B, Knoetzel J, Jansson S. 1997. Screening of *chlorina* mutants of barley (*Hordeum vulgare* L.) with antibodies against light-harvesting proteins of PSI and PSII: Absence of specific antenna proteins. Photosynthesis Research 52: 127–136.

Brenner WG, Ramireddy E, Heyl A, Schmülling T. 2012. Gene regulation by cytokinin in Arabidopsis. Frontiers in Plant Science 3: 8.

Brenner WG, Romanov GA, Köllmer I, Bürkle L, Schmülling T. 2005. Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. The Plant Journal 44: 314–333.

Brenner WG, Schmülling T. 2012. Transcript profiling of cytokinin action in Arabidopsis roots and shoots discovers largely similar but also organs specific responses. BMC Plant Biology 12: 112.

Brouwer B, Gardeström P, Keech O. 2014. In response to partial plant shading, the lack of phytochrome A does not directly induce leaf senescence but alters the finetuning of chlorophyll biosynthesis. Journal of Experimental Botany 65: 4037–4049.

Brouwer B, Ziolkowska A, Bagard M, Keech O, Gardeström P. 2012. The impact of light intensity on shade-induced leaf senescence. Plant, Cell & Environment 35: 1084–1098.

Brzobohatý B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K. 1993. Release of active cytokinin by a β-glucosidase localized to maize root meristem. Science 262: 1051–1054.

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D. 2003. The molecular analysis of leaf senescence – a genomic approach. Plant Biotechnology Journal 1: 3–22.

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, Leaver CJ. 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. The Plant Journal 42: 567–585.

Bürkle L, Cedzich A, Döpke C, Stransky H, Okumoto S, Gillissen B, Kühn C, Frommer WB. 2003. Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of Arabidopsis. The Plant Journal 34: 13–26.

Caesar K, Thamm AMK, Witthöft J, Elgass K, Huppenberger P, Grefen C, Horak J, Harter K. 2011. Evidence for the localization of the Arabidopsis cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. Journal of Experimental Botany 62: 5571–5580.

Camp PJ, Huber SC, Burke JJ, Moreland DE. 1982. Biochemical changes that occur during senescence of wheat leaves. Plant Physiology 70: 1641–1646.

Carimi F, Terzi M, De Michele R, Zottini M, Lo Schiavo F. 2004. High levels of the cytokinin BAP induce PCD by accelerating senescence. Plant Science 166: 963–969.

Causin HF, Roberts IN, Criado MV, Gallego SM, Pena LB, Ríos MD, Barneix AJ. 2009. Changes in hydrogen peroxide homeostasis and cytokinin levels contribute to the regulation of shade induced senescence in wheat leaves. Plant Science 177: 698–704.

Chang CJ, Kao CH. 1998. H_2O_2 metabolism during senescence of rice leaves: changes in enzyme activities in light and darkness. Plant Growth Regulation 25: 11–15.

Chory J, Reinecke D, Sim S, Washburn T, Brenner M. 1994. A role for cytokinins in de-etiolation in *Arabidopsis*. Plant Physiology 104: 339–347.

Cortleven A, Marg I, Yamburenko MV, Schlicke H, Hill K, Grimm B, Schaller GE, Schmülling T. 2016. Cytokinin regulates the etioplast-chloroplast transition through the two-component signaling system and activation of chloroplast-related genes. Plant Physiology 172: 464–478.

Cortleven A, Nitschke S, Klaumünzer M, AbdElgawad H, Asard H, Grimm B, Riefler M, Schmülling T. 2014. A novel protective function for cytokinin in the light stress response is mediated by the ARABIDOPSIS HISTIDIN KINASE2 and ARABIDOPSIS HISTIDIN KINASE3 receptors. Plant Physiology 164: 1470–1483.

Cortleven A, Noben JP, Valcke R. 2011. Analysis of the photosynthetic apparatus in transgenic tobacco plants with altered endogenous cytokinin content: a proteomic study. Proteome Science 9: 33.

Cortleven A, Schmülling T. 2015. Regulation of chloroplast development and function by cytokinin. Journal of Experimental Botany 66: 4999–5013.

Cortleven A, Valcke R. 2012. Evaluation of the photosynthetic activity in transgenic tobacco plants with altered endogenous cytokinin content: lessons from cytokinins. Plant Physiology 144: 394–408.

Costa ML, Civello PM, Chaves AR, Martínez GA. 2005. Effect of ethephon and 6-benzylaminopurine on chlorophyll degrading enzyme and a peroxidase-linked chlorophyll bleaching during post-harvest senescence of broccoli (*Brassica oleraceae* L.) at 20 degrees C. Postharvest Biology and Technology 35: 191–199.

Dertinger U, Schaz U, Schulze E-D. 2003. Age-dependence of the antioxidative system in tobacco with enhanced glutathione reductase activity or senescence-induced production of cytokinins. Physiologia Plantarum 119: 19–29.

Dhindsa RS, Plumb-Dhindsa P, Reid DM. 1982. Leaf senescence and lipid peroxidation: effect of some phytohormones, and scavengers of free radicals and singlet oxygen. Physiologia Plantarum 56: 453–457.

Dekker JP, Boekema EJ. 2005. Supramolecular organization of thylakoid membrane proteins in green plants. Biochimica et Biophysica Acta 1706: 12–39.

Demmig-Adams B, Stewart JJ, Adams WW III. 2014. Chloroplast photoprotection and the trade-off between abiotic and biotic defense. In: Demmig-Adams B, Garab G, Adams WW III, Govindjee (eds). Non-photochemical quenching and energy dissipation in plants, algae and cyanobacteria. Springer, Netherlands. pp. 631–643.

Dobisova T, Hrdinova V, Cuesta C, Michlickova S, Urbankova I, Hejatkova R, Zadnikova P, Pernisova M, Benkova E, Hejatko J. 2017. Light controls cytokinin signaling via transcriptional regulation of constitutively active sensor histidine kinase CKI1. Plant Physiology 174: 387–404.

Dobrev PI, Kamínek M. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed mode solid-phase extraction. Journal of Chromatography A 950: 21–29.

Faiss M, Zalubílová J, Strnad M, Schmülling T. 1997. Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. The Plant Journal 12: 401–415.

Fankhauser C, Yeh KC, Lagarias JC, Zhang H, Elich TD, Chory J. 1999. PKS1, a substrate phosphorylated by phytochrome that modulates light signaling in Arabidopsis. Science 284: 1539–1541.

Färber A, Jahns P. 1998. The xanthophyll cycle of higher plants: influence of antenna size and membrane organization. Biochimica et Biophysica Acta 1363: 47–58.

Frébort I, Kowalska M, Hluska T, Frébortová J, Galuszka P. 2011. Evolution of cytokinin biosynthesis and degradation. Journal of Experimental Botany 62: 2431–2452.

Gajdošová S, Spíchal L, Kamínek M, Hoyerová K, Novák O, Dobrev PI, Galuszka P, Klíma P, Gaudinova A, Žižková E, Hanuš J, Dančák M, Trávníček B, Pešek B, Krupička M, Vaňková R, Strnad M, Motyka V. 2011. Distribution, biological activities, metabolism, and the conceivable function of *cis*-zeatin-type cytokinins in plants. Journal of Experimental Botany 62: 2827–2840.

Gan S, Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270: 1986–1988.

Gan S, Amasino RM. 1996. Cytokinins in plant senescence: from spray and pray to clone and play. BioEssays 18: 557–565.

Gan S, Amasino RM. 1997. Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). Plant Physiology 113: 313–319.

Genkov T, Tsoneva P, Ivanova I. 1997. Effects of cytokinins on photosynthetic pigments and chlorophyllase activity in vitro cultures of axiliary buds of *Dianthus caryophyllus* L. Journal of Plant Growth Regulation 16: 169–172.

Gilmore AM, Björkman O. 1994. Adenine nucleotides and the xanthophyll cycle in leaves. I. Effects of CO_2- and temperature-limited photosynthesis on adenylate energy charge and violaxanthin deepoxidation. Planta 192: 526–536.

Graham IA, Eastmond PJ. 2002. Pathways of straight and branched chain fatty acid catabolism in higher plants. Progress in Lipid Research 41: 156–181.

Grover A. 1993. How do senescing leaves lose photosynthetic activity? Current Science 64: 22–234.

Guiamét JJ, Pichersky E, Nooden LD. 1999. Mass exodus from senescing soybeans chloroplasts. Plant and Cell Physiology 40: 986–992.

Guo Y, Gan S. 2006. AtNAP, a NAC family transcription factor, has an important role in leaf senescence. The Plant Journal 46: 601–612.

Haussühl K, Andersson B, Adamska I. 2001. A chloroplast DegP2 protease performs the primary cleavage of the photodamaged D1 protein in plant photosystem II. The EMBO Journal 20: 713–722.

Havaux M, Tardy F. 1997. Thermostability and photostability of photosystem II in leaves of the *chlorina-f2* barley mutant deficient in light-harvesting chlorophyll *a/b* protein complexes. Plant Physiology 113: 913–923.

Heyl A, Riefler M, Romanov GA, Schmülling T. 2012. Properties, functions and evolution of cytokinin receptors. European Journal of Cell Biology 91: 246–256.

Hidema J, Makino A, Mae T, Ojima K. 1991. Photosynthetic characteristics of rice leaves aged under different irradiances from full expansion through senescence. Plant Physiology 97: 1287–1293.

Higuchi M, Pischke MS, Mähönen AP, Miyawaki K, Hashimoto Y, Seki M, Kobayashi M, Shinozaki K, Kato T, Tabata S, Helariutta Y, Sussman MR, Kakimoto T. 2004. In planta functions of the Arabidopsis cytokinin receptor family. Proceedings of the National Academy of Sciences The United States of America 101: 8821–8826.

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H. 2008. Regulation of cytokinin biosynthesis, compartmentalization and translocation. Journal of Experimental Botany 59: 75–83.

Holub J, Hanuš J, Hanke DE, Strnad M. 1998. Biological activity of cytokinins derived from ortho- and meta-hydroxybenzyladenine. Plant Growth Regulation 26: 109–115.

Hönig M, Plíhalová L, Husičková A, Nisler J, Doležal K. 2018. Role of cytokinins in senescence, antioxidant defence and photosynthesis. International Journal of Molecular Sciences 19: 4045.

Horie Y, Ito H, Kusaba M, Tanaka R, Tanaka A. 2009. Participation of chlorophyll *b* reductase in the initial step of the degradation of light-harvesting chlorophyll *a/b*-protein complexes in *Arabidopsis*. The Journal of Biological Chemistry 284: 17449–17456.

Hornitschek P, Kohnen MV, Lorrain S, Rougemont J, Ljung K, López-Vidriero I, Franco-Zorrilla JM, Solano R, Trevisan M, Pradervand S, Xenarios I, Fankhauser C. 2012. Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. The Plant Journal 71: 699–711.

Hörtensteiner S. 2009. Stay-green regulates chlorophyll and chlorophyll-binding protein degradation during senescence. Trends in Plant Science 14: 155–162.

Hörtensteiner S. 2013. Update on the biochemistry of chlorophyll breakdown. Plant Molecular Biology 82: 505-517.

Hörtensteiner S, Feller U. 2002. Nitrogen metabolism and remobilization during senescence. Journal of Experimental Botany 53: 927–937.

Hörtensteiner S, Vicentini F, Matile P. 1995. Chlorophyll breakdown in senescent cotyledons of rape, *Brassica napus* L.: enzymatic cleavage of phaeophorbide *a in vitro*. New Phytologist 129: 237–246.

Huang FY, Philosoph-Hadas S, Meir S, Callaham DA, Sabato R, Zelcer A, Hepler PK. 1997. Increases in cytosolic Ca^{2+} in parsley mesophyll cells correlate with leaf senescence. Plant Physiology 115: 51–60.

Hudák J, Vizárová G, Šikulová J, Ovečková O. 1996. Effect of cytokinins produced by strains of Agrobacterium tumefaciens with binary vectors on plastids in senescent barley leaves. Acta Physiologie Plantarum 18: 205–210.

Humbeck K, Quast S, Krupinska K. 1996. Functional and molecular changes in the photosynthetic apparatus during senescence of flag leaves from field-grown barley plants. Plant, Cell & Environment 19: 337–344.

Humplík JH, Lazár D, Fürst T, Husičková A, Hýbl M, Spíchal L. 2015. Automated integrative high-throughput phenotyping of plant shoots: a case study of the cold tolerance of pea (*Pisum sativum* L.). Plant Methods 11: 20.

Hurkman WJ. 1979. Ultrastructural changes of chloroplasts in attached and detached, aging primary wheat leaves. American Journal of Botany 66: 64–70.

Hwang I, Sheen J, Müller B. 2012. Cytokinin signaling networks. Annual Review of Plant Biology 63: 353–380.

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T. 2001. Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature 409: 1060–1063.

Ishida H, Anzawa D, Kokubun N, Makino A, Mae T. 2002. Direct evidence for non-enzymatic fragmentation of chloroplastic glutamine synthetase by a reactive oxygen species. Plant, Cell & Environment 25: 625–631.

Ishida H, Izumi M, Wada S, Makino A. 2014. Roles of autophagy in chloroplast recycling. Biochimica et Biophysica Acta Bioenergetics 1837: 512–521.

Ishida H, Makino A, Mae T. 1999. Fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase by reactive oxygen species occurs near Gly-329. The Journal of Biological Chemistry 274: 5222–5226.

Jackowski G. 1996. Senescence-related changes in the subcomplex organization of the major light-harvesting chlorophyll *a/b*-protein complex of photosystem II (LHCII) as influenced by cytokinin. Zeitschrift für Naturforschung C 51: 464–472.

Janečková H, Husičková A, Ferretti U, Prčina M, Pilařová E, Plačková L, Pospíšil P, Doležal K, Špundová M. 2018. The interplay between cytokinins and light during senescence in detached *Arabidopsis* leaves. Plant, Cell & Environment 41: 1870–1885.

Janečková H, Husičková A, Lazár D, Ferretti U, Pospíšil P, Špundová M. 2019. Exogenous application of cytokinin during dark senescence eliminates the acceleration of photosystem II impairment caused by chlorophyll *b* deficiency in barley. Plant Physiology and Biochemistry 136: 43–51.

Jansson S. 1994.The light-harvesting chlorophyll *a/b*-binding proteins. Biochimica et Biophysica Acta 1184: 1–19.

Jansson S. 1999. A guide to the Lhc genes and their relatives in *Arabidopsis*. Trends in Plant Science 4: 236–240.

Jordi W, Schapendonk A, Davelaar E, Stoopen GM, Pot CS, De Visser R, Van Rhijn JA, Gan S, Amasino RM. 2000. Increased cytokinin levels in transgenic P_{SAG12}-*IPT* tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. Plant, Cell & Environment 23: 279–289.

Kamínek M, Motyka V, Vaňková R. 1997. Regulation of cytokinin content in plant cells. Physiologia Plantarum 101: 689–700.

Kao CH. 1980. Senescence of rice leaves. IV. Influence of benzyladenine on chlorophyll degradation. Plant Cell and Physiology 21: 1255–1262.

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, Kamiya Y, Yamaguchi S, Sakakibara H. 2004. Distinct isoprenoid origins of *cis*- and *trans*-zeatin biosyntheses in *Arabidopsis*. The Journal of Biological Chemistry 279: 14049–14054.

Keech O, Pesquet E, Gutierrez L, Ahad A, Bellini C, Smith SM, Gardeström P. 2010. Leaf senescence is accompanied by an early disruption of the microtubule network in *Arabidopsis*. Plant Physiology 154: 1710–1720.

Khanna-Chopra R. 2012. Leaf senescence and abiotic stresses share reactive oxygen species-mediated chloroplast degradation. Protoplasma 249: 469–481.

Kiba T, Naitou T, Koizumi N, Yamashino T, Sakakibara H, Mizuno T. 2005. Combinatorial microarray analysis revealing *Arabidopsis* genes implicated in cytokinin responses through the His->Asp Phosphorelay circuitry. Plant and Cell Physiology 46: 339–355.

Kieber JJ, Schaller GE. 2014. Cytokinins. The Arabidopsis Book 12: e0168.

Kim J, Kim JH, Lyu JI, Woo HR, Lim PO. 2018. New insights into the regulation of leaf senescence in *Arabidopsis*. Journal of Experimental Botany 69: 787–799.

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I. 2006. Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proceedings of the National Academy of Sciences of The United States of America 103: 814– 819.

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG. 2009. Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in *Arabidopsis*. Science 323: 1053–1057.

Klerk HC, Rebers M, Loon LC. 1993. Effects of light and regulators on senescence-related changes in soluble proteins in detached oat (*Avena sativa* L.) leaves. Plant Growth Regulation 13: 137–145.

Klimmek F, Sjödin A, Noutsos C, Leister D, Jansson S. 2006. Abundantly and rarely expressed Lhc protein genes exhibit distinct regulation patterns in plants. Plant Physiology 140: 793–804.

Kraepiel Y, Jullien M, Cordonnier-Pratt MM, Pratt L. 1994. Identification of two loci involved in phytochrome expression in *Nicotiana plumbaginifolia* and lethality of the corresponding double mutant. Molecular & General Genetics 242: 559–565.

Kraepiel Y, Marree K, Sotta B, Camoche M, Migniac E. 1995. In vitro morphogenic characteristics of phytochrome mutants in *Nicotiana plumbaginifolia* are modified and correlated to high indole-3-acetic acid levels. Planta 197: 142–146.

Krieger-Liszkay A, Krupinska K, Shimakawa G. 2019. The impact of photosynthesis on initiation of leaf senescence. Physiologia Plantarum. doi:10.1111/ppl.12921.

Krieger-Liszkay A, Trösch M, Krupinska K. 2015. Generation of reactive oxygen species in thylakoids from senescing flag leaves of the barley varieties Lomerit and Carina. Planta 241: 1497–1508.

Krupinska K, Mulisch M, Hollmann J, Tokarz K, Zschiesche W, Kage H, Humbeck K, Bilger W. 2012. An alternative strategy of dismantling of the chloroplasts during leaf senescence observed in a high-yield variety of barley. Physiologia Plantarum 144: 189–200.

Kuai B, Chen J, Hörtensteiner S. 2018. The biochemistry and molecular biology of chlorophyll breakdown. Journal of Experimental Botany 69: 751–767.

Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, Kyozuka J. 2007. Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature 445: 652–655.

Kurepin LV, Emery RJN, Pharis RP, Reid DM. 2007. Uncoupling light quality from light irradiance effects in *Helianthus annuus* shoots: putative roles for plant hormones in leaf and internode growth. Journal of Experimental Botany 58: 2145–2157.

Kusaba M, Ito H, Morita R, Iida S, Sato Y, Fujimoto M, Kawasaki S, Tanaka R, Hirochika H, Nishimura M, Tanaka A. 2007. Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. The Plant Cell 19: 1362–1375.

Kusnetsov VV, Herrmann RG, Kulaeva ON, Oelmüller R. 1998. Cytokinin stimulates and abscisic acid inhibits greening of etiolated *Lupinus luteus* cotyledons by effecting the expression of the light-sensitive protochlorophyllide oxidoreductase. Molecular &General Genetics 259: 21–28.

Kusnetsov VV, Oelmüller R, Sarwat M, Porfirova SA, Cherepneva GN, Herrmann RG, Kulaeva ON. 1994. Cytokinins, abscisic acid and light affect accumulation of chloroplast proteins in *Lupinus luteus* cotyledons, without notable effect on steady-state mRNA levels. Planta 194: 318–327.

Lara MEB, Gonzalez Garcia M-C, Fatima T, Ehness R, Lee TK, Proels R, Tanner W, Roitsch T. 2004. Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. The Plant Cell 16: 1276–1287.

Lazár D. 2013. Simulations show that a small part of variable chlorophyll *a* fluorescence originates in photosystem I and contributes to overall fluorescence rise. Journal of Theoretical Biology 335: 249–264.

Lazár D. 2015. Parameters of photosynthetic partitioning. Journal of Plant Physiology 175: 131–147.

Leivar P, Monte E, Oka Y, Liu T, Carle C, Castillon A, Huq E, Quail PH. 2008. Multiple phytochrome-interacting bHLH transcription factors repress premature seedling photomorphogenesis in darkness. Current Biology 18: 1815–1823.

Leong TM, Anderson JM. 1984. Adaptation of the thylakoid membranes of pea chloroplasts to light intensities. II. Regulation of electron transport capacities, electron carriers, coupling factor (CF1) activity and rates of photosynthesis. Photosynthesis Research 5: 117–128.

Lepistö A, Rintamäki E. 2012. Coordination of plastid and light signaling pathways upon development of *Arabidopsis* leaves under various photoperiods. Molecular Plant 4: 799-816.

Letham DS. 1973. Cytokinins from *Zea mays*. Phytochemistry 12: 2445–2455.

Li Y, Hagen G, Guilfoyle TJ. 1992. Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Developmental Biology 153: 386–395.

Lichtenthaler HK. 1987. Chlorophylls and carotenoids - Pigments of photosynthetic biomembranes. Methods in Enzymology 148: 350–382.

Liebsch D, Keech O. 2016. Dark-induced leaf senescence: new insights into a complex light-dependent regulatory pathway. New Phytologist 212: 563–570.

Lim PO, Kim HJ, Nam HG. 2007. Leaf senescence. Annual Review of Plant Biology 58: 115–136.

Liu L, Li HR, Zeng H, Cai Q, Zhou X, Yin C. 2016. Exogenous jasmonic acid and cytokinin antagonistically regulate rice flag leaf senescence by mediating chlorophyll degradation, membrane deterioration, and senescence-associated genes expression. Journal of Plant Growth Regulation 35: 366–376.

Lindahl M, Spetea C, Hundal T, Oppenheim AB, Adam Z, Andersson B. 2000. The thylakoid FtsH protease plays a role in the light-induced turnover of the photosystem II D1 protein. The Plant Cell 12: 419-431.

Lomin SN, Krivosheev DM, Steklov MY, Arkhipov DV, Osolodkin DI, Schmülling T, Romanov GA. 2015. Plant membrane assays with cytokinin receptors underpin the unique role of free cytokinin bases as biologically active ligands. Journal of Experimental Botany 66: 1851–1863.

Lomin SN, Yonekura-Sakakibara K, Romanov GA, Sakakibara H. 2011. Ligand-binding properties and subcellular localization of maize cytokinin receptors. Journal of Experimental Botany 62: 5149–5159.

Lomin SN, Myakushina YA, Arkhipov DV, Leonova OG, Popenko VI, Schmülling T, Romanov GA. 2018. Studies of cytokinin receptor–phosphotransmitter interaction provide evidences for the initiation of cytokinin signalling in the endoplasmic reticulum. Functional Plant Biology 45: 192.

Lu C, Lu Q, Zhang J, Kuang T. 2001. Characterization of photosynthetic pigment composition, photosystem II photochemistry and thermal energy dissipation during leaf senescence of wheat plants grown in the field. Journal of Experimental Botany 52: 1805–1810.

Lu C, Zhang JA. 1998. Changes in photosystem II function during senescence of wheat leaves. Physiologia Plantarum 104: 239–247.

Luciński R, Misztal LH, Samardakiewicz S, Jackowski GR. 2011. The thylakoid protease Deg2 is involved in stress-related degradation of the photosystem II light-harvesting protein Lhcb6 in *Arabidopsis thaliana*. New Phytologist 192: 74–86.

Mae T, Thomas H, Gay AP, Makino A, Hidema J. 1993. Leaf development in *Lolium temulentum*: photosynthesis and photosynthetic proteins in leaves senescing under different irradiances. Plant and Cell Physiology 34: 391–399.

Mähönen AP, Bonke M, Kauppinen L, Riikonen M, Benfey PN, Helariutta Y. 2000. A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. Genes and Development 14: 2938–2943.

Makino A, Osmond B. 1991. Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. Plant Physiology 96: 355–362.

Makino A, Sakuma H, Sudo E, Mae T. 2003. Differences between maize and rice in N-use efficiency for photosynthesis and protein allocation. Plant and Cell Physiology 44: 952–956.

Marchadier E, Hetherington A. 2014. Involvement of two-component signalling systems in the regulation of stomatal aperture by light in *Arabidopsis thaliana*. New Phytologist 203: 462–468.

Marchetti CF, Škrabišová M, Galuszka P, Novák O, Causin HF. 2018. Blue light suppression alters cytokinin homeostasis in wheat leaves senescing under shading stress. Plant Physiology and Biochemistry 130: 647–657.

Martínez DE, Bartoli CG, Grbic V, Guiamet JJ. 2007. Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors. Journal of Experimental Botany 58: 1099–1107.

Martínez DE, Costa ML, Gomez FM, Otegui MS, Guiamet JJ. 2008*a*. 'Senescence-associated vacuoles' are involved in the degradation of chloroplast proteins in tobacco leaves. The Plant Journal 56: 196–206.

Martínez DE, Costa ML, Guiamet JJ.2008*b*. Senescence-associated degradation of chloroplast proteins inside and outside the organelle. Plant Biology 10: 15–22.

Mccabe MS, Garratt LC, Schepers FM, Jordi W, Stoopen GM, Davelaar EJ, Rhijn JA, Power J, Davey MR. 2001. Effects of P_{SAG12}-*IPT* gene expression on development and senescence in transgenic lettuce. Plant Physiology 127: 505–516 .

Meguro M, Ito H, Takabayashi A, Tanaka R, Tanaka A. 2011. Identification of the 7-hydroxymethyl chlorophyll *a* reductase of the chlorophyll cycle in *Arabidopsis*. The Plant Cell 23: 3442–3453.

Melkovičová H. 2012. Koncentračná závislosť účinkov cytokinínov na indukovanú senescenciu rastlín. Concentration-dependent effect of cytokinins on induced plant senescence. Bachelor thesis. Palacký University Olomouc.

Melkovičová H. 2014. Koncentračná závislosť účinkov cytokinínov na senescenciu rastlín a vplyv svetelných podmienok. Concentration-dependent effect of cytokinins on plant senescence and effect of light conditions. Master thesis. Palacký University Olomouc.

Meng Y, Li H, Wang Q, Liu B, Lin C. 2013. Blue light-dependent interaction between cryptochrome2 and CIB1 regulates transcription and leaf senescence in soybean. Plant Cell 25: 4405–4420.

Miersch I, Heise J, Zelmer I, Humbeck K. 2000. Differential degradation of the photosynthetic apparatus during leaf senescence in barley (*Hordeum vulgare* L.). Plant Biology 2: 618–623.

Miller CO, Skoog F, von Saltza MH, Strong M. 1955. Kinetin, a cell division factor from deoxyribonucleic acid. Journal of the American Chemical Society 77: 1329–1334.

Miyawaki K, Matsumoto-Kitano M, Kakimoto T. 2004. Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. The Plant Journal 37: 128–138.

Mizuno K, Shimokor M, Komamine A. 1971. Vessel element formation in cultured carrot-root phloem slices. Plant and Cell Physiology 12: 823.

Mlejnek P, Doležal P, Procházka S. 2003. Intracellular phosphorylation of benzyladenosine is related to apoptosis induction in tobacco BY-2 cells. Plant, Cell & Environment 26: 1723–1735.

Mok DWS, Mok MC. 2001. Cytokinin metabolism and action. Annual Review of Plant Physiology and Plant Molecular Biology 52: 89–118.

Monakhova OF, Chernyad'ev II. 2004. Effects of cytokinin preparations on the stability of the photosynthetic apparatus of two wheat cultivars experiencing water deficiency. Applied Biochemistry and Microbiology 40: 573–580.

Morrison EN, Emery RJ, Saville BJ, Wang W. 2015. Phytohormone involvement in the *Ustilago maydis*–Zea mays pathosystem: relationships between abscisic acid and cytokinin levels and strain virulence in infected cob tissue. PloS One 10: e0130945.

Morita R, Sato Y, Masuda Y, Nishimura M, Kusaba M. 2009. Defect in non-yellow coloring3, an a/b hydrolase-fold family protein, causes a stay-green phenotype during leaf senescence in rice. The Plant Journal 59: 940–952.

Mueller AH, Dockter C, Gough SP, Lundqvist U, von Wettstein D, Hansson M. 2012. Characterization of mutations in barley *fch2* encoding chlorophyllide *a* oxygenase. Plant and Cell Physiology 53: 1232–1246.

Mýtinová Z, Haisel D, Wilhelmová N. 2006. Photosynthesis and protective mechanisms during ageing in transgenic tobacco leaves with over-expressed cytokinin oxidase/dehydrogenase and thus lowered cytokinin content. Photosynthetica 44: 599–605.

Nath K, Phee B-K, Jeong S, Lee SY, Tateno Y, Allakhverdiev SI, Lee C-H, Nam HG. 2013. Age-dependent changes in the functions and compositions of photosynthetic complexes in the thylakoid membranes of *Arabidopsis thaliana*. Photosynthesis Research 117: 547–556.

Nauš J, Prokopová J, Řebíček J, Špundová M. 2010. SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. Photosynthesis Research 105: 265–271.

Nelson N, Junge W. 2015. Structure and energy transfer in photosystems of oxygenic photosynthesis. Annual Review of Biochemistry 84:659–683.

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C. 2004. Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. The Plant Cell 16: 1365–1377.

Nisler J, Zatloukal M, Sobotka R, Pilný J, Zdvihalová B, Novák O, Strnad M, Spíchal L. 2018. New urea derivatives are effective anti-senescence compounds acting most likely via a cytokinin-independent mechanism. Frontiers in Plant Science. doi.org/10.3389/fpls.2018.01225

Noh Y, Amasino RM. 1999. Identification of a promoter region responsible for the senescence-specific expression of SAG12. Plant Molecular Biology 41: 181–194.

Noodén LD, Hillsberg JW, Schneider MJ. 1996. Induction of leaf senescence in *Arabidopsis thaliana* by long days through a light-dosage effect. Physiologia Plantarum 96: 491–495.

Novák O, Hauserová E, Amakorová P, Doležal K, Strnad M. 2008. Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. Phytochemistry 69: 2214–2224.

Novák O, Tarkowski P, Tarkowská D, Doležal K, Lenobel R, Strnad M. 2003. Quantitative analysis of cytokinins in plants by liquid chromatography-single-quadrupole mass spectrometry. Analytica Chimica Acta 480: 207–218.

Nováková M, Motyka V, Dobrev PI, Malbeck J, Gaudinová A, Vanková R. 2005. Diurnal variation of cytokinin, auxin and abscisic acid levels in tobacco leaves. Journal of Experimental Botany 56: 2877–2883.

Oberhuber M, Berghold J, Breuker K, Hörtensteiner S, Kräutler B. 2003. Breakdown of chlorophyll: a nonenzymatic reaction accounts for the formation of the colorless “nonfluorescent” chlorophyll catabolites. Proceedings of the National Academy of Sciences of The United States of America 100: 6910–6915.

Oh E, Kang H, Yamaguchi S, Park J, Lee D, Kamiya Y, Choi G. 2009. Genome-wide analysis of genes targeted by PHYTOCHROME INTERACTING FACTOR 3-LIKE5 during seed germination in Arabidopsis. The Plant Cell 21: 403–419.

Oh M, Moon Y, Lee C. 2003. Increased stability of LHCII by aggregate formation during dark-induced leaf senescence in the Arabidopsis mutant, *ore10*. Plant and Cell Physiology 44: 1368–1377.

Oh MH, Kim JH, Zulfugarov IS, Moon Y-H, Rhew T-H, Lee C-H. 2005. Effects of benzyladenine and abscisic acid on the disassembly process of photosystems in an Arabidopsis delayed-senescence mutant, *ore9*. Journal of Plant Biology 48: 170–177.

Oh S, Lee S, Chung I, Lee C, Nam H. 1996. A senescence-associated gene of Arabidopsis thaliana is distinctively regulated during natural and artificially induced leaf senescence. Plant Molecular Biology 30: 739–754.

Ohya T, Suzuki H. 1991. The effects of benzyladenine on the accumulation of messenger RNAs that encode the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase and light-harvesting chlorophyll *a/b* protein in excised cucumber cotyledons. Plant and Cell Physiology 32: 577–580.

Okada K, Inoue Y, Satoh K, Katoh S. 1992. Effects of light on degradation of chlorophyll and proteins during senescence of detached rice leaves. Plant and Cell Physiology 33: 1183–1191.

Okazaki K, Kabeya Y, Suzuki K, Mori T, Ichikawa T, Matsui M, Nakanishi H, Miyagishima S. 2009. The PLASTID DIVISION1 and 2 components of the chloroplast division machinery determine the rate of chloroplast division in land plant cell differentiation. The Plant Cell 21: 1769–1780.

Ono K, Terashima I, Watanabe A. 1996. Interaction between nitrogen deficit of a plant and nitrogen content in the old leaves. Plant and Cell Physiology 37: 1083–1089.

Osugi A, Sakakibara H. 2015. Q&A: How do plants respond to cytokinins and what is their importance? BMC Biology 13: 102.

Otegui MS, Noh Y-S, Martínez DE, Petroff MGV, Staehelin LA, Amasino RM, Guiamet JJ. 2005. Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean. The Plant Journal 41: 831–844.

Park E, Park J, Kim J, Nagatani A, Lagarias JC, Choi G. 2012. Phytochrome B inhibits binding of phytochrome-interacting factors to their target promoters. The Plant Journal 72: 537–546 .

Park JH, Oh SA, Kim YH, Woo HR, Nam HG. 1998. Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in Arabidopsis. Plant Molecular Biology 37: 445–454.

Park SY, Yu JW, Park JS, Li J, Yoo SC, Lee NY, Lee SK, Jeong SW, Seo HS, Koh HJ, Jeon JS, Park YI, Paek NC. 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. The Plant Cell 19: 1649–1664.

Pavalan-Ünsal N, Çağ S, Çetin E, Büyüktunçer D. 2002. Retardation of senescence by *meta*-topolin in wheat leaves. Journal of Cell and Molecular Biology 1: 101–108.

Perilli S, Moubayidin L, Sabatini S. 2010. The molecular basis of cytokinin function. Current Opinion in Plant Biology 13: 21–26.

Pilz J, Meineke I, Gleiter CH. 2000. Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. Journal of Chromatography B 742: 315–325.

Pospíšil P. 2016. Production of reactive oxygen species by photosystem II as a response to light and temperature stress. Frontiers in Plant Science. 7: 1950

Pospíšilová J, Čatský J, Synková H, Macháčková I, Solárová J. 1993. Gas exchange and *in vivo* chlorophyll fluorescence in potato and tobacco plantlets *in vitro* as affected by various concentrations of 6-benzylaminopurine. Photosynthetica 29: 1–12.

Prakash JSS, Baig MA, Mohanty P. 2001. Senescence induced structural reorganization of thylakoid membranes in *Cucumis sativus* cotyledons; LHCII involvement in reorganization of thylakoid membranes. Photosynthesis Research 68: 153–161.

Prerostova S, Dobrev PI, Gaudinova A, Knirsch V, Körber N, Pieruschka R, Fiorani F, Brzobohatý B, Černý M, Spichal L, Humplik JF, Vanek T, Schurr U, Vankova R. 2018. Cytokinins: Their impact on molecular and growth responses to drought stress and recovery in Arabidopsis. Frontiers in Plant Science 9: 655.

Procházková D, Wilhelmová N. 2004. Changes in antioxidative protection in bean cotyledons during natural and continuous irradiation-accelerated senescence. Biologia Plantarum 48: 33–39.

Prokopová J, Špundová M, Sedlářová M, Husičková A, Novotný R, Doležal K, Nauš J, Lebeda A. 2010. Photosynthetic responses of lettuce to downy mildew infection and cytokinin treatment. Plant Physiology and Biochemistry 48: 716–723.

Pružinská A, Anders I, Aubry S, Schenk N, Tapernoux-Lüthi E, Müller T, Kräutler B, Hörtensteiner S. 2007. *In vivo* participation of red chlorophyll catabolite reductase in chlorophyll breakdown. The Plant Cell 19:369-387.

Pružinská A, Tanner G, Anders I, Roca M, Hörtensteiner S. 2003. Chlorophyll breakdown: pheophorbide *a* oxygenase is a Rieske-type iron–sulfur protein, encoded by the accelerated cell death 1 gene. Proceedings of the National Academy of Sciences of The United States of America 100: 15259–15264.

Pružinská A, Tanner G, Aubry S, Anders I, Moser S, Müller T, Ongania KH, Kräutler B, Youn JY, Liljegren SJ, Hörtensteiner S. 2005. Chlorophyll breakdown in senescent *Arabidopsis* leaves. Characterization of chlorophyll catabolites and of chlorophyll catabolic enzymes involved in the degreening reaction. Plant Physiology 139: 52–63.

Pružinská A, Anders I, Aubry S, Schenk N, Tapernoux-Lüthi E, Müller T, Kräutler B, Hörtensteiner S. 2007. *In vivo* participation of red chlorophyll catabolite reductase in chlorophyll breakdown. The Plant Cell 19: 369–387

Qamaruddin M and Tillberg E. 1989. Rapid effects of red light on the isopentenyladenosine content in Scots pine seeds. Plant Physiology 91: 5–8.

Rashotte AM, Carson SDB, To JPC, Kieber JJ. 2003. Expression profiling of cytokinin action in Arabidopsis. Physiologia Plantarum 132: 1998–2011.

Ren G, Zhou Q, Wu S, Zhang Y, Zhang L, Huang J, Sun Z, Kuai B. 2010. Reverse genetic identification of CRN1 and its distinctive role in chlorophyll degradation in *Arabidopsis*. Journal of Integrative Plant Biology 52, 496–504.

Richmond AE, Lang A. 1957. Effect of kinetin on protein content and survival of detached Xanthium leaves. Science 125: 650–651.

Riefler M, Novák O, Strnad M, Schmülling T. 2006. Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism: The Plant Cell 18: 40–54.

Rivero RM, Gimeno J, Van Deynze A, Walia H, Blumwald E. 2010. Enhanced cytokinin synthesis in tobacco plants expressing P_{SARK}*::IPT* prevents the degradation of photosynthetic protein complexes during drought. Plant and Cell Physiology 51: 1929–1941.

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E. 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proceedings of the National Academy of Sciences of The United States of America 104: 19631–19636.

Rivero RM, Shulaev V, Blumwald E. 2009. Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant Physiology 150: 1530–1540.

Roberts IN, Caputo C, Kade M, Criado MV, Barneix AJ. 2011. Subtilisin-like serine proteases involved in N remobilization during grain filling in wheat. Acta Physiologiae Plantarum 33: 1997–2001.

Robson PR, Donnison IS, Wang K, Frame BR, Pegg SE, Thomas AE, Thomas H. 2004. Leaf senescence is delayed in maize expressing the *Agrobacterium IPT* gene under the control of a novel maize senescence-enhanced promoter. Plant Biotechnology Journal 2: 101–112.

Romanov GA, Lomin SN, Schmülling T. 2006. Biochemical characteristics and ligand binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. Journal of Experimental Botany 57: 4051–4058.

Romanov GA, Lomin SN, Schmülling T. 2018. Cytokinin signaling: from the ER or from the PM? That is the question! New Phytologist 218: 41–53.

Rottet S, Besagni C, Kessle F. 2015. The role of plastoglobules in thylakoid lipid remodeling during plant development. Biochimica et Biophysica Acta 1847: 889–899.

Rousseaux MC, Ballare CL, Jordan ET, Vierstra RD. 1997. Directed overexpression of PHYA locally suppresses stem elongation and leaf senescence response to far-red radiation. Plant Cell Environ 20, 1551–1558.

Sakakibara H. 2006. Cytokinins: activity, biosynthesis, and translocation. Annual Review of Plant Biology 57: 431–449.

Sakamoto W. 2006. Protein degradation machineries in plastids. Annual Review of Plant Biology 57: 599–621.

Sakuraba Y, Balazadeh S, Tanaka R, Mueller-Roeber B, Tanaka A. 2012. Overproduction of chl b retards senescence through transcriptional reprogramming in Arabidopsis. Plant and Cell Physiology 53: 505–17.

Sakuraba Y, Jeong J, Kang MY, Kim J, Paek NC, Choi G. 2014. Phytochrome interacting transcription factors PIF4 and PIF5 induce leaf senescence in Arabidopsis. Nature Communications 5: 4636.

Sakuraba Y, Kim Y, Yoo S, Hörtensteiner S, Paek N. 2013. 7-Hydroxymethyl chlorophyll *a* reductase functions in metabolic channeling of chlorophyll breakdown intermediates during leaf senescence. Biochemical and Biophysical Research Communications 430: 32–37.

Sato Y, Morita R, Katsuma S, Nishimura M, Tanaka A, Kusaba M. 2009. Two short-chain dehydrogenase/reductases, NON-YELLOW COLORING 1 and NYC1-LIKE, are required for chlorophyll *b* and light-harvesting complex II degradation during senescence in rice. The Plant Journal 57: 120–131.

Schäfer M, Brütting C, Meza-Canales ID, Grosskinsky DK, Vankova R, Baldwin IT, Meldau S. 2015. The role of *cis*-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. Journal of Experimental Botany 66: 4873–4884.

Schelbert S, Aubry S, Burla B, Agne B, Kessler F, Krupinska K, Hörtensteiner S. 2009. Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in *Arabidopsis*. The Plant Cell 21: 767–785.

Schenk N, Schelbert S, Kanwischer M, Goldschmidt EE, Dörmann P, Hörtensteiner S. 2007. The chlorophyllases AtCLH1 and AtCLH2 are not essential for senescence-related chlorophyll breakdown in *Arabidopsis thaliana*. FEBS Letters 581: 5517–5525.

Schlüter T, Leide J, Conrad K. 2011. Light promotes an increase of cytokinin oxidase/dehydrogenase activity during senescence of barley leaf segments. Journal of Plant Physiology 168: 694–698.

Schmülling T, Werner T, Riefler M, Krupková E, Manns IB. 2003. Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. Journal of Plant Research 116: 241–252.

Selivankina SY, Karavaiko NN, Kuiper DG, Novikova GV, Kulaeva O. 2001. Cytokinin activity of zeatin allylic phosphate, a natural compound. Plant Growth Regulation 33: 157–164.

Shimoda Y, Ito H, Tanaka A. 2016. Arabidopsis STAY-GREEN, Mendel's green cotyledon gene, encodes magnesium-dechelatase. The Plant Cell 28: 2147–2160.

Singh S, Letham DS, Palni LMS. 1992. Cytokinin biochemistry in relation to leaf senescence. VIII. Translocation, metabolism and biosynthesis of cytokinins in relation to sequential leaf senescence of tobacco. Physiologia Plantarum 86: 398–406.

Skalický V, Kubeš M, Napier R, Novák O. 2018. Auxins and cytokinins - The role of subcellular organization on homeostasis. International Journal of Molecular Sciences. 19: 3115.

Smart CM. 1994. Gene expression during leaf senescence. New Phytologist 126: 419–448.

Smart CM, Scofield SR, Bevan MW, Dyer TA. 1991. Delayed leaf senescence in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium*. The Plant Cell 3: 647–656.

Smigocki AC, Owens LD. 1988. Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. Proceedings of the National Academy of Sciences of The United States of America 85: 5131–5135.

Smigocki AC, Owens LD. 1989. Cytokinin-to-auxin ratios and morphology of shoots and tissues transformed by a chimeric isopentenyl transferase gene. Plant Physiology 91: 808-811.

Sobieszczuk-Nowicka E, Wrzesinski TM, Bagniewska-Zadworna A, Kubala S, Rucińska-Sobkowiak R, Polcyn W, Misztal LH, Mattoo AK. 2018. Physio-genetic dissection of dark-induced leaf senescence and timing its reversal in barley. Plant Physiology 178: 654–671.

Song Y, Yang C, Gao S, Zhang W, Li L, Kuai B. 2014. Age-triggered and dark induced leaf senescence require the bHLH transcription factors PIF3, 4, and 5. Molecular Plant 7: 1776–1787.

Spíchal L. 2012. Cytokinins - Recent news and views of evolutionally old molecules. Functional Plant Biology 39: 267–284.

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T. 2004. Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. Plant and Cell Physiology 45: 1299–1305.

Srivalli B, Bharti S, Khanna-Chopra R. 2001. Vacuolar cysteine proteases and ribulose-1,5-bisphosphate carboxylase/oxygenase degradation during monocarpic senescence in cowpea leaves. Photosynthetica 39: 87–93.

Stirbet A, Lazár D, Kromdijk J, Govindjee. 2018. Chlorophyll *a* fluorescence induction: Can just a one-second measurement be used to quantify abiotic stress responses? Photosynthetica 56: 86–104.

Stolz A, Riefler M, Lomin SN, Achazi K, Romanov GA, Schmülling T. 2011. The specificity of cytokinin signalling in *Arabidopsis thaliana* is mediated by differing ligand affinities and expression profiles of the receptors. The Plant Journal 67: 157–168.

Strasser RJ, Srivastava A, Tsimilli-Michael M. 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Mohanty, Yunusa and Pathre (eds) Probing Photosynthesis: Mechanism, Regulation & Adaptation: pp. 443-480. Taylor & Francis, London.

Strnad M. 1997. The aromatic cytokinins. Physiologia Plantarum 101: 674–688.

Sun J, Hirose N, Wang X, Wen P, Xue L, Sakakibara H, Zuo J. 2005. Arabidopsis SOI33/AtENT8 gene encodes a putative equilibrative nucleoside transporter that is involved in cytokinin transport in planta. Journal of Integrative Plant Biology 47: 588–603.

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizumo T. 2001. The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. Plant and Cell Physiology 42: 107–113.

Svačinová J, Novák O, Plačková L, Lenobel R, Holík J, Strnad M, Doležal K. 2012. A new approach for cytokinin isolation from *Arabidopsis* tissues using miniaturized purification: Pipette tip solid-phase extraction. Plant Methods 8: 17.

Sweere U, Eichenberg K, Lohrmann J, Mira-Rodado V, Bäurle I, Kudla J, Nagy F, Schäfer E, Harter K. 2001. Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. Science 294: 1108–1111.

Synková H, Pechová R, Valcke R. 2003. Changes in chloroplast ultrastructure in Pssu-*ipt* tobacco during plant ontogeny. Photosynthetica 41: 117–126.

Synková H, Schnablová R, Polanská L, Husák M, Siffel P, Vácha F, Malbeck J, Macháčková I, Nebesárová J. 2006*a*. Three-dimensional reconstruction of anomalous chloroplasts in transgenic ipt tobacco. Planta 223: 659–671.

Synková H, Semorádová Š, Schnablová R, Witters E, Hušák M, Valcke R. 2006*b*. Cytokinin-induced activity of antioxidant enzymes in transgenic *Pssu-ipt* tobacco during plant ontogeny. Biologia Plantarum 50: 31–41.

Synková H, Van Loven K, Pospíšilová J, Valcke R. 1999. Photosynthesis of transgenic *Pssu-ipt* tobacco. Journal of Plant Physiology 155: 173–182.

Šmehilová M, Dobrůšková J, Novák O, Takáč T, Galuszka P. 2016. Cytokinin-specific glycosyltransferases possess different roles in cytokinin homeostasis maintenance. Frontiers in Plant Science 7: 1264.

Špundová M, Popelková H, Ilík P, Skotnica J, Novotný R, Nauš J. 2003. Ultra-structural and functional changes in the chloroplasts of detached barley leaves senescing under dark and light conditions. Journal of Plant Physiology 160: 1051–1058.

Špundová M, Slouková K, Hunková M, Nauš J. 2005*a*. Plant shading increases lipid peroxidation and intensifies senescence-induced changes in photosynthesis and activities of ascorbate peroxidase and glutathione reductase in wheat. Photosynthetica 43: 403–409.

Špundová M, Strzałka K, Nauš J. 2005*b*. Xanthophyll cycle activity in detached barley leaves senescing under dark and light. Photosynthetica 43: 117-124.

Štroch M, Čajánek M, Kalina J, Špunda V. 2004. Regulation of the excitation energy utilization in the photosynthetic apparatus of *chlorina f2* barley mutant grown under different irradiances. Journal of Photochemistry and Photobiology B Biology 75: 41–50.

Štroch M, Lenk S, Navrátil M, Špunda V, Buschmann C. 2008. Epidermal UV-shielding and photosystem II adjustment in wild type and *chlorina f2* mutant of barley during exposure to increased PAR and UV radiation. Environmental and Experimental Botany 64: 271–278.

Takei K, Yamaya T, Sakakibara H. 2004. Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-zeatin. The Journal of Biological Chemistry 279: 41866–41872.

Talla SK, Panigrahy M, Kappara S, Nirosha P, Neelamraju S, Ramanan R. 2016. Cytokinin delays dark-induced senescence in rice by maintaining the chlorophyll cycle and photosynthetic complexes. Journal of Experimental Botany 67: 1839–1851.

Tanaka R, Kobayashi K, Masuda T. 2011. Tetrapyrrole metabolism in *Arabidopsis thaliana*. Arabidopsis Book 9: e0145.

Tanaka R, Tanaka A. 2011. Chlorophyll cycle regulates the construction and destruction of the light-harvesting complexes. Biochimica et Biophysica Acta 1807: 968–976.

Tang Y, Wen X, Lu C. 2005. Differential changes in degradation of chlorophyll-protein complexes of photosystem I and photosystem II during flag leaf senescence of rice. Plant Physiology and Biochemistry 43: 193–201.

Tao G-Q, Letham DS, Palni LMS, Summons RE. 1983. Cytokinin biochemistry in relation to leaf senescence I.The metabolism of 6-benzylaminopurine and zeatin in oat leaf segments. Journal of Plant Growth Regulation 2: 89–102.

Thimann KV. 1985. The senescence of detached leaves of *Tropaeolum*. Plant Physiology 79: 1107–1110.

To JPC, Deruère J, Maxwell BB, Morris VF, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ. 2007. Cytokinin regulates type-A Arabidopsis response regulator activity and protein stability via two-component phosphorelay. The Plant Cell 19: 3901–3914.

Ueguchi C, Sato S, Kato T, Tabata S. 2001. The AHK4 gene involved in the cytokinin signalling pathway as a direct receptor molecule in *Arabidopsis thaliana*. Plant and Cell Physiology 42: 751–755.

Vandenbussche F, Habricot Y, Condiff AS, Maldiney R, Van der Straeten D, Ahmad M. 2007. HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*. The Plant Journal 49: 428–441.

Vescovi M, Riefler M, Gessuti M, Novák O, Schmülling T, Lo Schiavo F. 2012. Programmed cell death induced by high levels of cytokinin in *Arabidopsis* cultured cells is mediated by the cytokinin receptor CRE1/AHK4. Journal of Experimental Botany 63: 2825–2832.

Vlčková A, Špundová M, Kotabová E, Novotný R, Doležal K, Nauš J. 2006. Protective cytokinin action switches to damaging during senescence of detached wheat leaves in continuous light. Physiologia Plantarum 126: 257–267.

Voitsekhovskaja OV, Tyutereva EV. 2015. Chlorophyll *b* in angiosperms: Functions in photosynthesis, signaling and ontogenetic regulation. Journal of Plant Physiology 189: 51–64.

Vylíčilová H, Husičková A, Spíchal L, Srovnal J, Doležal K, Plíhal O, Plíhalová L. 2016. C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus. Phytochemistry 122: 22–33.

Waters MT, Wang P, Korkaric M, Capper RG, Saunders NJ, Langdale JA. 2009. GLK transcription factors coordinate expression of the photosynthetic apparatus in Arabidopsis. The Plant Cell 21: 1109–1128 .

Weaver LM, Amasino RM. 2001. Senescence is induced in individually darkened *Arabidopsis* leaves, but inhibited in whole darkened plants. Plant Physiology 127: 876–886.

Weaver LM, Gan S, Quirino B, Amasino RM. 1998. A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatments. Plant Molecular Biology 37: 455–469.

Weidhase RA, Lehmann J, Kramell H, Sembdner G, Parthier B. 1987. Degradation of ribulose-1,5-bisphosphate carboxylase and chlorophyll in senescing barley leaf segments triggered by jasmonic acid methylester, and counteraction by cytokinin. Physiologia Plantarum 69: 161–166.

Werner T, Holst K, Pörs Y, Guivarc'h A, Mustroph A, Chriqui D, Grimm B, Schmülling T. 2008. Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. Journal of Experimental Botany 59: 2659–2672.

Werner T, Köllmer I, Bartrina I, Holst K, Schmülling T. 2006. New insights into the biology of cytokinin degradation. Plant Biology 8: 371–381.

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T. 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. The Plant Cell 15: 2532–2550.

Wingler A, von-Schaewen A, Leegood RC, Lea PJ, Quick WP. 1998. Regulation of leaf senescence by cytokinin, sugars, and light. Effects on NADH-dependent hydroxypyruvate reductase. Plant Physiology 116: 329–335.

Wingler A, Marès M, Pourtau N. 2004. Spatial patterns and metabolic regulation of photosynthetic parameters during leaf senescence. New Phytologist 161: 781-789.

Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG. 2001 ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. Plant Cell 13: 1779– 1790.

Wu S, Li Z, Yang L, Xie Z, Chen J, Zhang W, Liu T, Gao S, Gao J, Zhu Y, Xin J, Ren G, Kuai B. 2016. NON-YELLOWING2 (NYE2), a close paralog of NYE1, plays a positive role in chlorophyll degradation in *Arabidopsis*. Molecular Plant 9: 624–627.

Wulfetange K, Lomin SN, Romanov GA, Stolz A, Heyl A, Schmülling T. 2011. The cytokinin receptors of Arabidopsis are located mainly to the endoplasmic reticulum. Physiologia Plantarum 156: 1808–1818.

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T. 2001. The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant and Cell Physiology 42: 1017– 1028.

Yang Y, Xu J, Huang L, Leng Y, Dai L, Rao Y, Chen L, Wang Y, Tu Z, Hu J, Ren D, Zhang G, Zhu L, Guo L, Qian Q, Zeng D. 2016. *PGL*, encoding chlorophyllide *a* oxygenase 1, impacts leaf senescence and indirectly affects grain yield and quality in rice. Journal of Experimental Botany 67: 1297–1310.

Yaronskaya E, Vershilovskaya I, Poers Y, Alawady AE, Averina N, Grimm B. 2006. Cytokinin effects on tetrapyrrole biosynthesis and photosynthetic activity in barley seedlings. Planta 224: 700–709.

Yonekura-Sakakibara K, Kojima M, Yamaya T, Sakakibara H. 2004. Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to *cis*-zeatin. Plant Physiology 134: 1654–1661.

Zavaleta-Mancera HA, Franklin KA, Ougham HJ, Thomas H, Scott IM. 1999. Regreening of senescent *Nicotiana* leaves. I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll *a/b* binding protein. Journal of Experimental Botany 50: 1677–1682.

Zavaleta-Mancera HA, López-Delgadob H, Loza-Taverac H, Mora-Herrerab M, Trevilla-García C, Vargas-Suárezc M, Oughame H. 2007. Cytokinin promotes catalase and ascorbate peroxidase activities and preserves the chloroplast integrity during dark-senescence. Journal of Plant Physiology 164: 1572–1582.

Zdarska M, Dobisová T, Gelová Z, Pernisová M, Dabravolski S, Hejátko J. 2015. Illuminating light, cytokinin, and ethylene signalling crosstalk in plant development. Journal of Experimental Botany 66: 4913–4931.

Żelisko A, García-Lorenzo M, Jackowski G, Jansson S, Funk C. 2005. AtFtsH6 is involved in the degradation of the light-harvesting complex II during high-light acclimation and senescence. Proceedings of the National Academy of Sciences of The United States of America 102: 13690–13704.

Żelisko A, Jackowski GR. 2004. Senescence-dependent degradation of Lhcb3 is mediated by a thylakoid membrane-bound protease. Journal of Plant Physiology 161: 1157–1170.

Zhang Y, Liu Z, Chen Y, He JX, Bi Y. 2015. PHYTOCHROME INTERACTING FACTOR 5 (PIF5) positively regulates dark-induced senescence and chlorophyll degradation in *Arabidopsis*. Plant Science 237: 57–68.

Zhu X, Chen J, Qiu K, Kuai B. 2017. Phytohormone and light regulation of chlorophyll degradation. Frontiers in Plant Science 8: 1911.

Zubo YO, Yamburenko MV, Selivankina SY, Shakirova FM, Avalbaev AM, Kudryakova NV, Zubkova NK, Liere K, Kulaeva ON, Kusnetsov VV, Börner T. 2008. Cytokinin stimulates chloroplast transcription in detached barley leaves. Plant Physiology 148: 1082–1093.

Zwack PJ, Rashotte AM. 2013. Cytokinin inhibition of leaf senescence. Plant Signaling and Behavior 8: e24737.

7 Appendix

I. **Janečková H**, Husičková A, Ferretti U, Prčina M, Pilařová E, Plačková L, Pospíšil P, Doležal K, Špundová M. 2018. The interplay between cytokinins and light during senescence in detached Arabidopsis leaves. Plant, Cell &Environment 41: 1870–1885.

II. **Janečková H**, Husičková A, Lazár D, Ferretti U, Pospíšil P, Špundová M. 2019. Exogenous application of cytokinin during dark senescence eliminates the acceleration of photosystem II impairment caused by chlorophyll b deficiency in barley. Plant Physiology and Biochemistry 136: 43–51.

Received: 13 July 2017 | Revised: 13 April 2018 | Accepted: 23 April 2018

DOI: 10.1111/pce.13329

ORIGINAL ARTICLE

WILEY Plant, Cell & Environment

The interplay between cytokinins and light during senescence in detached *Arabidopsis* leaves

Helena Janečková[1] | Alexandra Husičková[1] | Ursula Ferretti[1] | Maroš Prčina[1] | Eva Pilařová[2] | Lenka Plačková[2,3] | Pavel Pospíšil[1] | Karel Doležal[2,3] | Martina Špundová[1]

[1] Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Biophysics, Faculty of Science, Palacký University, 78371 Olomouc, Czech Republic

[2] Laboratory of Growth Regulators, Faculty of Science, Palacký University & Institute of Experimental Botany AS CR, 78371 Olomouc, Czech Republic

[3] Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Chemical Biology and Genetics, Faculty of Science, Palacký University, 78371 Olomouc, Czech Republic

Correspondence
A. Husičková, Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Biophysics, Faculty of Science, Palacký University, Olomouc, Czech Republic.
Email: alexandra.husickova@upol.cz

Funding information
National Programme of Sustainability I, Ministry of Education, Youth and Sports, Czech Republic, Grant/Award Number: LO1204

Abstract

Light and cytokinins are known to be the key players in the regulation of plant senescence. In detached leaves, the retarding effect of light on senescence is well described; however, it is not clear to what extent is this effect connected with changes in endogenous cytokinin levels. We have performed a detailed analysis of changes in endogenous content of 29 cytokinin forms in detached leaves of *Arabidopsis thaliana* (wild-type and 3 cytokinin receptor double mutants). Leaves were kept under different light conditions, and changes in cytokinin content were correlated with changes in chlorophyll content, efficiency of photosystem II photochemistry, and lipid peroxidation. In leaves kept in darkness, we have observed decreased content of the most abundant cytokinin free bases and ribosides, but the content of *cis*-zeatin increased, which indicates the role of this cytokinin in the maintenance of basal leaf viability. Our findings underscore the importance of light conditions on the content of specific cytokinins, especially N^6-(Δ^2-isopentenyl)adenine. On the basis of our results, we present a scheme summarizing the contribution of the main active forms of cytokinins, cytokinin receptors, and light to senescence regulation. We conclude that light can compensate the disrupted cytokinin signalling in detached leaves.

KEYWORDS

AHK, *ahk* mutants, chlorophyll, photosystem II, malondialdehyde

1 | INTRODUCTION

Senescence, the final stage of plant or leaf development, is a complex and highly regulated process that involves physiological and biochemical changes as well as changes at the level of gene expression (Gan & Amasino, 1997; Lim, Kim, & Nam, 2007; Smart, 1994; Zhang & Zhou, 2013). Precise regulation of senescence is crucial for a controlled breakdown of cellular components, enabling effective relocation of valuable nutrients to the rest of the plant. During the process of leaf senescence, the degradation of chlorophylls (Chls) and disintegration of photosynthetic apparatus result in the inhibition of photosynthesis (Buchanan-Wollaston, 1997; Hensel, Grbić, Baumgarten, & Bleecker, 1993; Lim et al., 2007; Noodén, Guiamét, & John, 1997). Although leaf senescence is primarily controlled by endogenous signals, it is also known to be affected by a number of environmental factors, which can trigger, accelerate, retard, or postpone the whole process (Gan & Amasino, 1997; Khanna-Chopra, 2012; Lim et al., 2007; Smart, 1994; Zhang & Zhou, 2013).

Important endogenous factors that are involved in the regulation of senescence are plant hormones, cytokinins (CKs). There are two classes of naturally occurring CKs—isoprenoid and aromatic CKs. Isoprenoid CKs, which represent the most abundant CK class, include isopentenyl (iP)-type CKs and zeatin-type CKs, which occur in either *cis* (*c*Z) or *trans* (*t*Z) form. The reduction of the double bond in the side chain of *t*Z results in dihydrozeatin (Mok & Mok, 2001). Aromatic CKs have a ring substitution at the N^6-position and include for instance N^6-benzylaminopurine or *meta*-topolin (*m*T). CKs are present in plants in various chemical forms. Free CK bases have the highest activity in

All listed authors confirm that they have no conflicts of interest.

bioassays and are thought to be the most active forms of CKs (Mok & Mok, 2001; Sakakibara, 2006; Spíchal et al., 2004). CK ribosides, as a transport form, were reported to be less active (Spíchal et al., 2004).

Today, it is well known that both exogenous applications of active CKs and increase in their endogenous content can delay senescence (Cortleven & Valcke, 2012; Gan & Amasino, 1995; Hwang, Sheen, & Müller, 2012; Smart, Scofield, Bevan, & Dyer, 1991; Zwack & Rashotte, 2013). During senescence, CKs have been widely reported to preserve Chl content and photosynthetic activity (Vlčková et al., 2006; Vylíčilová et al., 2016; Wingler, von Schaewen, Leegood, Lea, & Quick, 1998; Zacarias & Reid, 1990; Zubo et al., 2008) and to mitigate lipid peroxidation (Huang et al., 1997; L. Liu et al., 2016; X. H. Liu & Huang, 2002; Todorov, Alexieva, & Karanov, 1998). The onset of leaf senescence is usually connected to a decrease in the level of active CK forms (Ananieva et al., 2008; Gan & Amasino, 1995, 1996, 1997; Singh, Letham, & Palni, 1992; Tao, Letham, Palni, & Summons, 1983; Van Staden, Cook, & Noodén, 1988). This decrease can be a result of the inhibition of CK biosynthesis (Perilli, Moubayidin, & Sabatini, 2010), but it can be also caused by faster CK degradation or inactivation. The main degradation pathway involves CK oxidase/dehydrogenase (Perilli et al., 2010; Werner, Köllmer, Bartrina, Holst, & Schmülling, 2006). Irreversible inactivation, which is mediated by uridine diphosphate glycosyltransferases, results in the formation of CK *N*-glucosides (Šmehilová, Dobrůšková, Novák, Takáč, & Galuszka, 2016). *O*-glucosylation, on the other hand, serves as reversible CK inactivation, and the resulting *O*-glucosides are used as a storage form of CKs.

In the *Arabidopsis* CK signal transduction pathway, hybrid histidine protein kinases (AHKs) serve as CK receptors. Histidine phosphotransfer proteins (AHPs) transmit the signal from AHKs to nuclear response regulators (ARRs), which can activate or repress gene expression (Hwang et al., 2012). There are three known CK receptors in *Arabidopsis thaliana*—AHK2 (ARABIDOPSIS HISTIDINE KINASE2) and AHK3 and AHK4 (also known as CRE1 or WOL; Inoue et al., 2001; Mähönen et al., 2000; Suzuki et al., 2001; Ueguchi, Sato, Kato, & Tabata, 2001; Yamada et al., 2001). CK-dependent regulation of senescence is known to be mediated mainly by the AHK3 receptor, coupled with the phosphorylation/activation of the B-type response regulator ARR2 (ARABIDOPSIS RESPONSE REGULATOR2; Hwang et al., 2012; Kim et al., 2006; Riefler, Novák, Strnad, & Schmülling, 2006). The specific AHK3–ARR2 phosphorelay plays a major regulatory role in the CK-dependent leaf longevity by modulating downstream targets implicated in the senescence programme (Hwang et al., 2012; Kim et al., 2006). Besides AHK3, the AHK2 receptor is considered to have a redundant function in the senescence regulation, whereas the AHK4 receptor is thought to have only minor relevance (Riefler et al., 2006). Plants with the loss-of-function mutation in specific AHK receptors enabled to reveal the importance of individual CK receptors in numerous processes, including senescence. Such mutation resulted in an increase in endogenous CK levels, particularly in the *ahk3* mutants; nevertheless, this increase was reported not to be sufficient to compensate the lost receptor activity (Riefler et al., 2006).

One of the most important environmental factors affecting leaf senescence is light, and its effect depends on both intensity and length of the photoperiod (Biswal & Biswal, 1984; Lepistö & Rintamäki, 2012; Noodén, Hillsberg, & Schneider, 1996; Smart, 1994). Light regulates senescence through photosynthesis, but it has also a signalling role (Barber & Andersson, 1992; Biswal & Biswal, 1984; Brouwer, Gardeström, & Keech, 2014). Shading of a plant or a leaf (or shortening of the photoperiod) has been shown to promote senescence in general, including decrease in Chl content and photosynthetic activity and increase in oxidative stress and lipid peroxidation (Ananieva et al., 2008; Brouwer et al., 2014; Špundová, Slouková, Hunková, & Nauš, 2005; Weaver & Amasino, 2001).

The effects of light and CKs on plant growth and development are similar and influence each other in many aspects (Chory, Reinecke, Sim, Washburn, & Brenner, 1994; Cortleven et al., 2016; Cortleven & Schmülling, 2015; Zubo et al., 2008), which indicates their extensive crosstalk (Zdarska et al., 2015). It has been shown that light- and CK-dependent signalling pathways share a number of common intermediates at multiple levels (Argueso, Raines, & Kieber, 2010; Oh et al., 2009; Sweere et al., 2001; Vandenbussche et al., 2007). It is well known that light is an important factor in the control of endogenous CK levels, as it participates in the regulation of their biosynthesis, degradation, and transport (Ananieva et al., 2008; Boonman, Prinsen, Voesenek, & Pons, 2009; Zubo et al., 2008). Close relation between endogenous CK level and light was found in numerous studies (for a review, see, e.g., Kurepin & Pharis, 2014). Light regulation of senescence could therefore be associated with the light-induced changes in the contents of endogenous CKs. In detached leaves, a retarding effect of light on senescence is well known (Klerk, Rebers, & van Loon, 1993; Okada, Inoue, Satoh, & Katoh, 1992; Špundová et al., 2003; Thimann, 1985; Vlčková et al., 2006). However, it is not clear if and how endogenous CK level in leaves is modified after their detachment and how it is affected by light conditions. There are only few studies describing changes in the content of endogenous CKs in detached leaves under different light conditions, and the results are contradictory (Causin et al., 2009; Roberts, Caputo, Kade, Criado, & Barneix, 2011; Zubo et al., 2008).

The aim of our work was to analyze the changes in the content of 29 main CK forms during senescence in detached *Arabidopsis* leaves and to evaluate the correlation of their content with senescence-induced changes in Chl content, efficiency of photosystem II (PSII) photochemistry, and in lipid peroxidation. To clarify the role of individual CK receptors and to evaluate the role of light in the regulation of senescence in plants with impaired CK signalling, we have employed three *Arabidopsis* AHK double mutants, each of them with only one functional CK receptor. As light is able to modulate both the content and signalling pathways of CKs, we have also focused on the CK–light interaction.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

A. thaliana plants (wild-type [WT] Columbia-0 and three CK receptor double mutants—*ahk2 ahk3*, *ahk2 ahk4*, and *ahk3 ahk4*; Riefler et al., 2006) were grown in a growth chamber on a commercial substrate (Potgrond H, Klasmann-Deilmann Substrate, Germany) under short-

day conditions: 8 hr of white light (120 μmol photons m^{-2} s^{-1})/16 hr dark, at 22/20 °C and relative air humidity 60 %. Seeds were kept at 4 °C for 3 days in the dark before sowing.

After 5 weeks, mature leaves (seventh leaf on average) were cut off from plants and placed into a 0.2% solution of dimethyl sulfoxide. In a separate experiment, we have verified that this solution did not affect senescence of detached *Arabidopsis* leaves in comparison with distilled water. The detached leaves floated on the solution in a closed six-hole macrotiter plates and were kept under three different light conditions—dark, growth light (GL; 120 μmol photons m^{-2} s^{-1}), and higher light (HL; 400 μmol photons m^{-2} s^{-1} from the same light source as GL; 8 hr light/16 hr dark). Measurements were performed immediately after leaf detachment ("control") and on the sixth day after detachment.

2.2 | Chl content assay

Chl content was measured using a chlorophyll meter SPAD-502 (Minolta Sensing Konica, Osaka, Japan) after 30-min dark adaptation for elimination of the effect of chloroplast movement (Nauš, Prokopová, Řebíček, & Špundová, 2010). To obtain the absolute Chl content, a calibration of the SPAD values was performed for the individual genotypes by simultaneous analytical determination of Chl content. For the analytical determination, the leaves were homogenized in a chilled mortar with $MgCO_3$ and 80% acetone. The homogenate was then centrifuged using a centrifuge 3-30KS Sigma (SIGMA Laborzentrifugen, Germany) at 4,000 *g* for 5 min and at 10 °C. After centrifugation, the absorbance (A) of the supernatant was measured at the wavelengths of 646.8, 663.2, and 750 nm using a spectrophotometer Unicam UV550 (ThermoSpectronic, UK). Total Chl concentration was calculated according to Lichtenthaler (1987):

$$\text{Chl}\ (a+b) = 7.15\cdot[A\ (663.2) - A\ (750)] + 18.71\cdot[A\ (646.8) - A\ (750)].$$

The Chl content was related to a leaf area (μg/cm^2). The Chl content of the leaves measured on the sixth day after detachment was expressed as percent of the values obtained for the corresponding control leaves.

2.3 | Chl fluorescence measurement

Chl fluorescence parameters were measured from the adaxial leaf side at room temperature by FluorCam 700 MF imaging system (Photon Systems Instruments, Czech Republic) after 30-min dark adaptation. The measurement was performed as described in Takáč et al. (2014), with a slight modification in the series of light saturation pulses. First saturation pulse (white light, 500 μmol photons m^{-2} s^{-1}) was applied 3 s after the actinic light was switched on and was followed by six pulses in 23-s intervals, three pulses in 47-s intervals, and the last two pulses in 70-s intervals.

The maximal quantum yield of PSII photochemistry (F_V/F_M) was calculated as $(F_M - F_0)/F_M$, where F_M is maximal fluorescence and F_0 is minimal fluorescence in the dark-adapted leaf sample. In the light-adapted leaf samples (after 7 min of red actinic light, 120 μmol photons m^{-2} s^{-1}), the maximal quantum yield of PSII photochemistry (Φ_{PSII}) was calculated as $(F_M' - F_0')/F_M'$, where F_M' is maximal fluorescence for the light-adapted state and F_0' represents the minimal fluorescence for light-adapted state calculated as $F_0/(F_V/F_M + F_0/F_M')$.

2.4 | Determination of malondialdehyde content

The content of malondialdehyde (MDA) was measured using high-performance liquid chromatography (HPLC). The isolation and derivatization of MDA using 2,4-dinitrophenylhydrazine (DNPH) was performed as described in Pilz, Meineke, and Gleiter (2000) with some modifications. Leaves were homogenized in 0.11% butylhydroxytoluene dissolved in 100% methanol. This step was followed by 10-min centrifugation at 2,000 *g*. One hundred twenty-five microlitres of supernatant were poured into an Eppendorf tube, and 25 μl of 6-M aqueous NaOH was added to achieve alkaline hydrolysis of protein-bounded MDA. Samples were incubated for 30 min in dry bath at 60 °C (Thermo-Shaker TS100, Biosan, Riga, Latvia). To reach the precipitation of proteins in samples, 62.5 μl of 35% (*v*/*v*) perchloric acid was added to the sample, vortexed and centrifuged at 16,000 *g* for 10 min. One hundred twenty-five microlitres of supernatant were poured into a vial, stirred up with 1 μl of 50-mM DNPH dissolved in 50% sulfuric acid, and incubated in dark at room temperature for 30 min to obtain MDA–DNPH adducts. Then 25 μl of the solution was injected into an HPLC system (Alliance e2695 HPLC System, Waters, Milford, MA, USA). A Symmetry C18 (3.5 μm; 4.6 × 75 mm) Column (Waters, Milford, MA, USA) was used, the elution was performed isocratically (1 ml/min at 35 °C), using a mixture of 25-mM triethylamine (pH 3.5), and acetonitrile in the ratio 50:50 (v:v). MDA was detected at 310 nm using UV/VIS detector.

2.5 | Identification and quantification of endogenous CKs

Leaves from 5-week-old *A. thaliana* plants were harvested, frozen in liquid nitrogen, and stored at −80 °C. Three replicates were measured, each consisted of approx. 440 mg of leaf mass (sixth, seventh, and eighth true leaves; average number of leaves per sample was 12 for WT, 24 for *ahk2 ahk3*, 11 for *ahk2 ahk4*, and 10 for *ahk3 ahk4*). The procedure used for CK purification was a combination of the methods described by Dobrev and Kamínek (2002) and Svačinová et al. (2012). Isotope-labelled CK internal standards (Olchemim Ltd., Czech Republic) were added (0.25 pmol per sample of B, 7G and 0.5 pmol per sample of OG, NT) to check the recovery during purification and to validate the determination (Novák, Hauserová, Amakorová, Doležal, & Strnad, 2008). The samples were purified using C18 and MCX cartridges (Dobrev & Kamínek, 2002). Eluates were evaporated to dryness using a SpeedVac concentrator and dissolved in 40 μl of 10% methanol. Ten microlitres of each sample were then analysed by ultraperformance liquid chromatography (Acquity UPLC System; Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer equipped with an electrospray interface (Xevo TQ-S, Waters, Manchester, UK) by a method utilized on the StageTips technology (Svačinová et al., 2012). Quantification was obtained by multiple reaction monitoring of $[M + H]^+$ and the appropriate product ion. Optimal conditions, dwell time, cone voltage, and collision energy in the

collision cell, corresponding to the exact diagnostic transition, were optimized for each CK for selective multiple reaction monitoring experiments (Novák et al., 2008). Quantification was performed by Masslynx software using a standard isotope dilution method (Novák et al., 2003).

2.6 | Correlation and statistical analyses

Correlation between the contents of different CK forms in detached leaves and the relative Chl content, the maximal quantum yield of PSII photochemistry in dark-adapted state, and MDA content was estimated using the Pearson's correlation coefficient. Statistical analysis was performed using Student's *t* test, in which the values of parameters measured with receptor mutants were compared with corresponding values in WT. The significant differences are marked by * ($p < .05$), ** ($p < .01$), and *** ($p < .001$).

3 | RESULTS

3.1 | PSII function and lipid peroxidation were not changed pronouncedly in CK receptor double mutants, despite their generally high endogenous CK content

Phenotype of leaves of WT and CK receptor mutants is shown in Figure 1 ("control" leaves). Compared with WT plants, both CK receptor mutants with the loss-of-function mutation of the gene for CK receptor AHK3 had lower Chl content (by about 15–20 %, Figure 2a). However, no significant differences in F_V/F_M, Φ_{PSII}, and MDA content were found between WT and any of the AHK receptor double mutants (Figure 2b–d), which indicates that the function of PSII in both dark-adapted and light-adapted states of the leaves and the level of lipid peroxidation were not altered in the mutants.

A detailed analysis of endogenous CK levels showed differences among genotypes. When compared with WT, the *ahk2 ahk3* and *ahk3 ahk4* mutants had approximately two-fold overall content of CKs, sum of iP forms and the sum of *t*Z forms (Figures 3a, 4a–d, and 5a–d). The sum of all free bases was approximately three-fold in *ahk2 ahk3* and two-fold in *ahk3 ahk4* (Figure 3a), and the sum of iP and *t*Z was increased even nine-fold and five-fold in *ahk2 ahk3* and *ahk3 ahk4*, respectively (Figure 3b). Similar increase in the levels of the mentioned CK forms (with the exception of the level of total free bases) was observed also in the *ahk2 ahk4* mutant, even though the changes were smaller. The pronounced increase in both absolute and relative levels of iP and *t*Z precursors, free bases, and ribosides in *ahk2 ahk3* and *ahk3 ahk4* (and to the lower extent also in *ahk2 ahk4*; Figures 4b,d and 5b,d; Tables 1 and 2) indicates stimulation of biosynthesis of both these CK groups in the receptor mutants.

The overall content of dihydrozeatin CKs in *ahk2 ahk3* and *ahk3 ahk4* was also markedly higher than in WT (more than three-fold, Table S1). Interestingly, the content of *c*Z CKs was the lowest in both of these mutants, and all of the tested double mutants had lower content of *c*Z than WT (half or lower, Table 3). The level of *c*ZR was lowered four-fold in *ahk2 ahk3* and two-fold in both *ahk2 ahk4* and *ahk3 ahk4* (Table 3).

3.2 | PSII function in detached leaves kept in darkness was markedly more maintained in mutants with solely functional AHK3 or AHK2

A pronounced decrease in Chl content and impairment of PSII function (monitored as F_V/F_M and Φ_{PSII}) were observed in detached leaves of WT after 6 days in darkness. The Chl content decreased to about 20 % and F_V/F_M and Φ_{PSII} to about 25 % of the initial value (Figures 1 and 2e–g). In the *ahk2 ahk3* mutant, the Chl content was reduced more than in WT (to about 5 % of the initial content; Figures 1 and 2e), whereas the extent of decrease in PSII function was similar to WT (to about 25 %; Figure 2f–g). On the other hand, in the *ahk3 ahk4* mutant, the reduction of Chl content was similar to WT (to about

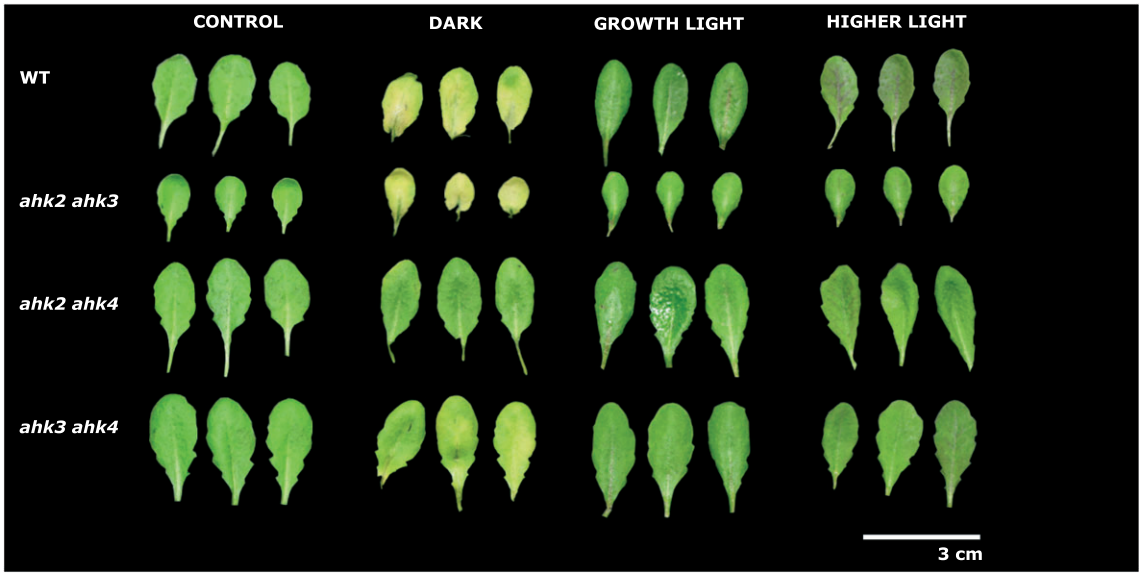

FIGURE 1 Phenotype of leaves of wild-type (WT) and receptor mutants immediately after the detachment (control) and after 6 days under dark, growth light (120 µmol photons m^{-2} s^{-1}), or higher light (400 µmol photons m^{-2} s^{-1}) conditions

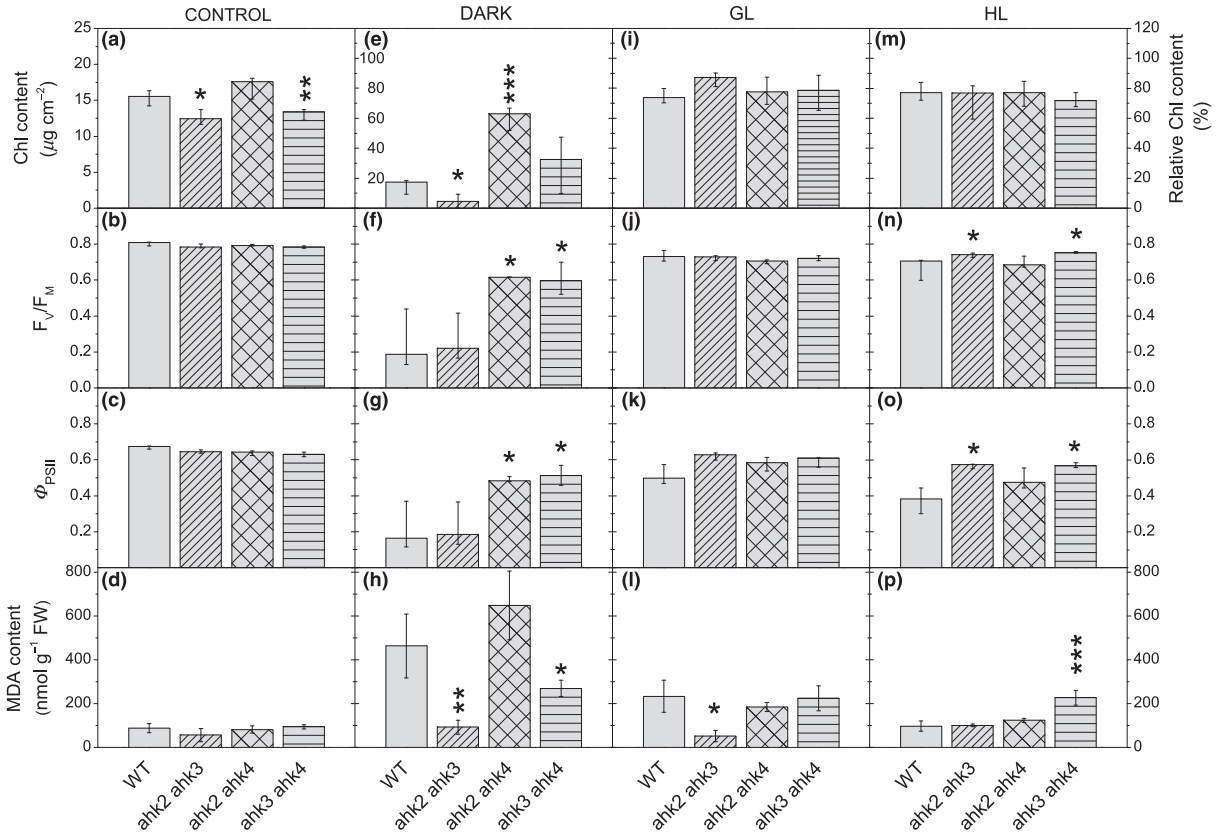

FIGURE 2 Chlorophyll (Chl) content, F_V/F_M (the maximal quantum yield of photosystem II photochemistry in dark-adapted state), Φ_{PSII} (the maximal quantum yield of photosystem II photochemistry in light-adapted state), and malondialdehyde (MDA–DNPH adduct) content estimated by high-performance liquid chromatography related to fresh weight (FW) in control leaves (a–d) and detached leaves incubated for 6 days under dark (e–h), growth light (GL; 120 μmol photons m^{-2} s^{-1}) (i–l), or higher light (HL; 400 μmol photons m^{-2} s^{-1}) (m–p). Chl content of detached leaves is expressed in % of the content in control leaves. Medians and quartiles are presented for Chl content, F_V/F_M, and Φ_{PSII}, n = 5–15. For MDA content, means and *SD* are presented, n = 4–5. Asterisks indicate statistical significance (Student's t test) of the difference between values measured in receptor mutants and WT under particular conditions: * $p < .05$; ** $p < .01$; *** $p < .001$

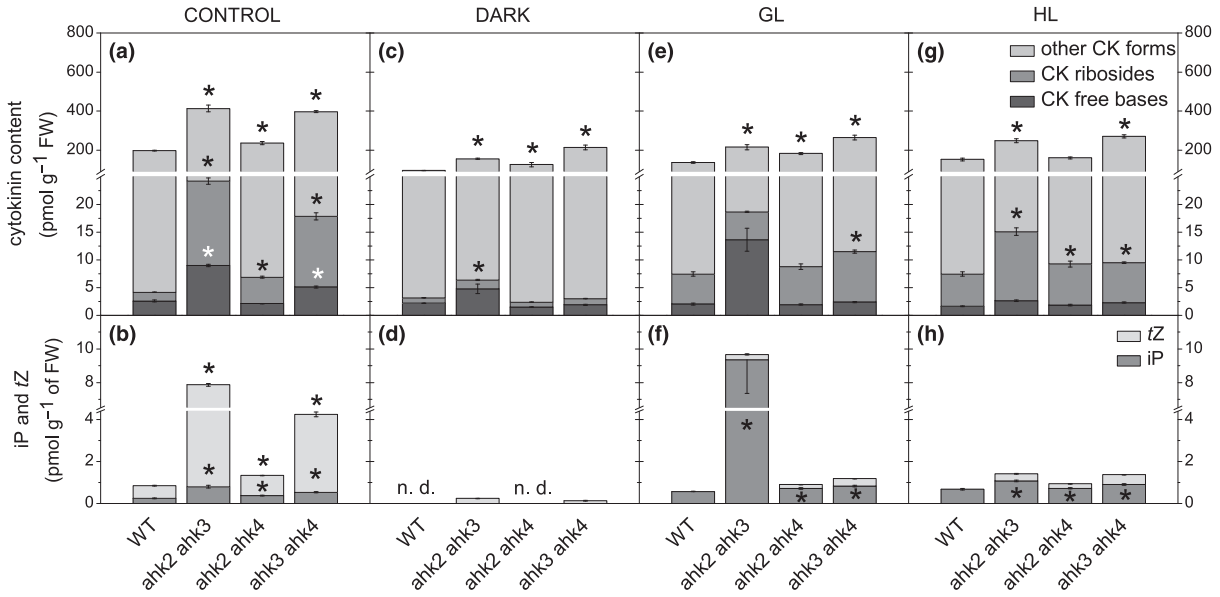

FIGURE 3 Endogenous content of total cytokinin (CK) free bases (iP, *t*Z, *c*Z, and *m*T; black), cytokinin ribosides (iPR, *t*ZR, *c*ZR, DHZR and *m*TR; dark grey), and other cytokinin forms (iP7G, iP9G, iPR5′MP, *t*ZOG, *t*ZROG, *t*Z7G, *t*Z9G, *t*ZR5′MP, *c*ZOG, *c*ZROG, *c*Z7G, *c*Z9G, *c*ZR5′MP, DHZOG, DHZ7G, DHZ9G, and DHZR5′MP; light grey) (a,c,e,g), and the content of iP and *t*Z (b,d,f,h) related to fresh weight (FW) in control leaves (a,b) and detached leaves incubated for 6 days under dark (c,d), growth light (GL; 120 μmol photons m^{-2} s^{-1}) (e,f), or higher light (HL; 400 μmol photons m^{-2} s^{-1}) (g,h). Means and *SD* (n = 3) are presented; n.d., under detection limit. Asterisks indicate statistical significance (Student's t test) of the difference between values measured in receptor mutants and WT under particular conditions: * $p < .05$

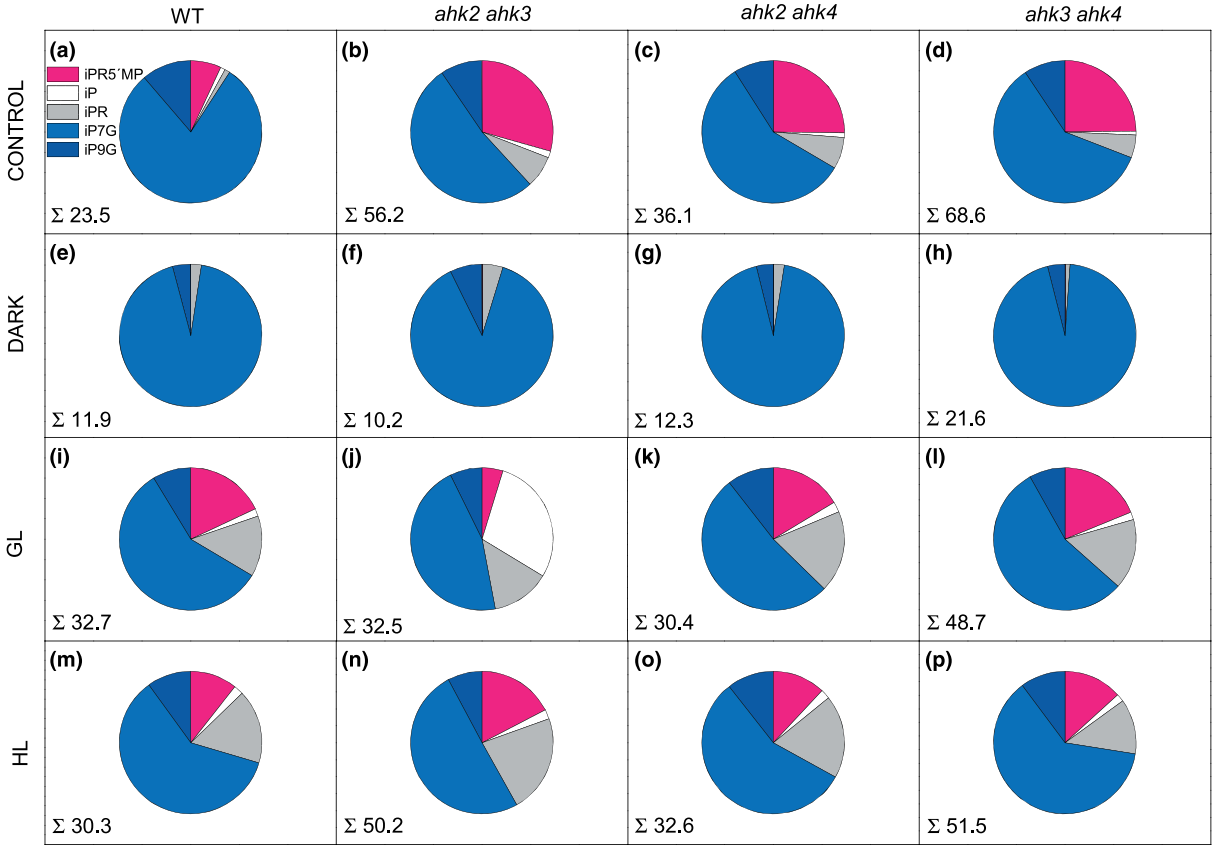

FIGURE 4 The overall content of iP forms (Σ; pmol/g of fresh weight) and relative amount of particular iP forms (iPR5′MP, N^6-(Δ^2-isopentenyl) adenosine 5′-monophosphate; iP, N^6-(Δ^2-isopentenyl)adenine; iPR, N^6-(Δ^2-isopentenyl)adenosine; iP7G, N^6-(Δ^2-isopentenyl)adenine 7-glucoside; iP9G, N^6-(Δ^2-isopentenyl)adenine 9-glucoside) in control leaves (a–d) and detached leaves incubated for 6 days under dark (e–h), growth light (GL; 120 µmol photons m^{-2} s^{-1}) (i–l), or higher light (HL; 400 µmol photons m^{-2} s^{-1}) (m–p) [Colour figure can be viewed at wileyonlinelibrary.com]

30 %), whereas F_V/F_M and Φ_{PSII} were reduced only slightly (to about 75 % and 80 %, respectively). The highest Chl content (about 60 % of the initial value) was observed in the *ahk2 ahk4* mutant (Figure 1), which had also well preserved PSII function (decrease to 75–78 %; Figure 2e–g). Thus, the senescence-induced decrease in PSII function was significantly lower in the *ahk2 ahk4* and *ahk3 ahk4* mutants, and the extent of its maintenance did not fully correspond to the remaining Chl content in the leaves.

The analysis of lipid peroxidation revealed the lowest (by about 60 %) increase in MDA content during dark senescence in the *ahk2 ahk3* mutant, which was the mutant with the most pronounced decrease in Chl content. Interestingly, the highest (eight-fold) increase in MDA content was observed in the *ahk2 ahk4* mutant, which maintained the highest Chl content during senescence. In WT and the *ahk3 ahk4* mutant, the MDA content increased almost five-fold and three-fold, respectively (Figure 2h).

3.3 | Overall CK level in detached leaves was markedly reduced in the dark

The overall CK level in detached leaves incubated 6 days in darkness decreased markedly (by about 50–60 %) in all genotypes (cf. Figure 3a and Figure 3c). The amount of total free bases also decreased in all genotypes—more in *ahk2 ahk3* and *ahk3 ahk4* (by about 50 % and 60 %, respectively) and less in WT and *ahk2 ahk4* (by about 15 % and 30 %, respectively; Figure 3c). The overall content of all iP and *t*Z forms decreased as well (Figures 4e–h and 5e–h). A huge decline was observed in the relative and absolute contents of free bases, ribosides, and their 5′-monophosphates of both iP and *t*Z (except iPR in WT; Figures 4e–h and 5e–h; Tables 1 and 2). Interestingly, iP was not detectable in any genotype and only a negligible residue of *t*Z was observed in *ahk2 ahk3* and *ahk3 ahk4* (Figure 3d). On the other hand, relative levels of glucoside forms iP7G, iP9G, *t*Z7G, and *t*Z9G increased (Figures 4e–h and 5e–h), although their absolute concentrations decreased (Tables 1 and 2). These changes indicate that the iP and *t*Z inactivation and degradation prevailed over their biosynthesis. However, despite the massive decrease in iP and *t*Z contents under dark conditions (Figure 3d), the pool of free bases was (partially or completely) replenished by elevated levels of *c*Z and *m*T in all genotypes (Figure 3c; Tables 3 and S2), indicating possible stimulation of *c*Z and *m*T biosynthesis in darkness. When compared with the initial values, the increase was also observed in the content of *c*ZR in all double mutants (Table 3).

3.4 | Chl content and PSII function were highly maintained in the detached leaves of all genotypes under GL and HL intensities

When the leaves were exposed to GL (120 µmol photons m^{-2} s^{-1}) or HL intensity (400 µmol photons m^{-2} s^{-1}) light for 6 days after

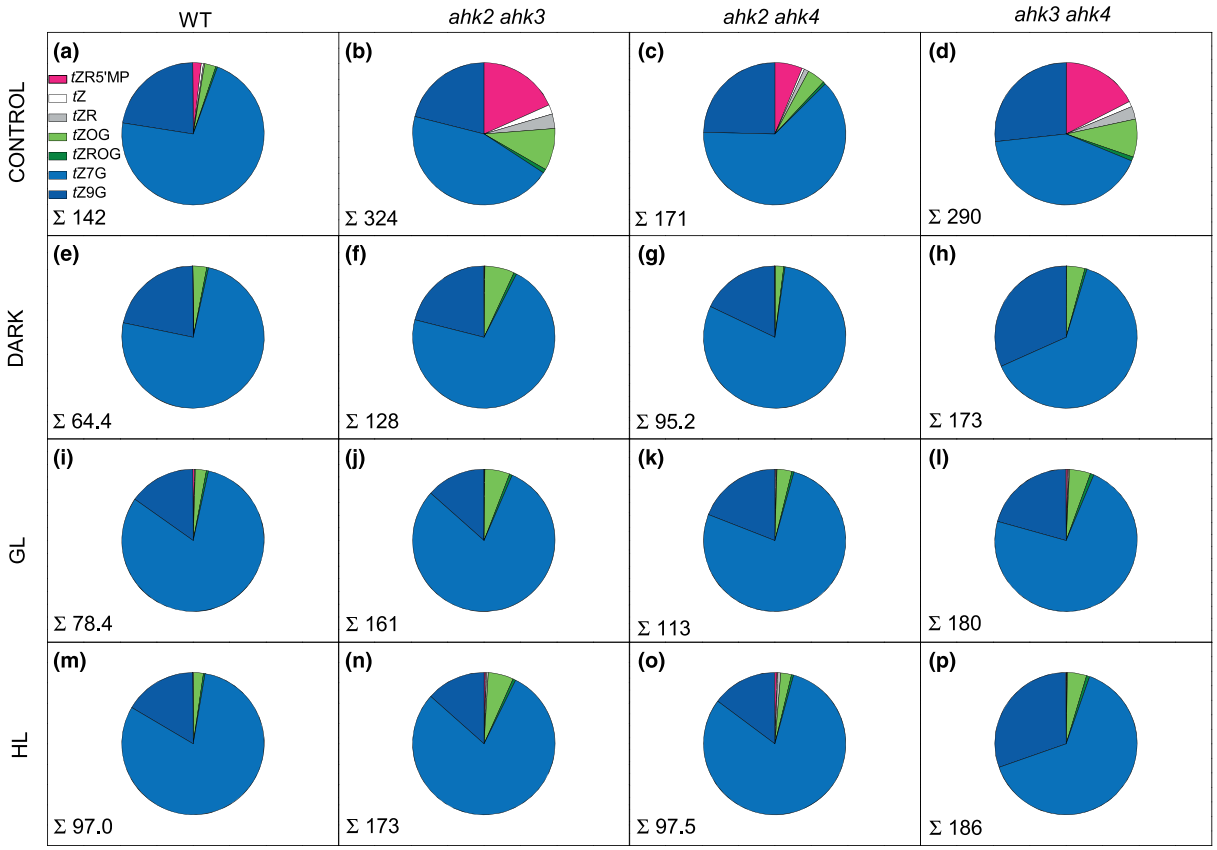

FIGURE 5 The overall content of *t*Z forms (Σ; pmol/g of fresh weight) and relative amount of particular *t*Z forms (*t*ZR5′MP, *trans*-zeatin riboside 5′-monophosphate; *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside; *t*ZOG, *trans*-zeatin *O*-glucoside; *t*ZROG, *trans*-zeatin riboside *O*-glucoside; *t*Z7G, *trans*-zeatin 7-glucoside; *t*Z9G, *trans*-zeatin 9-glucoside) in control leaves (a–d) and detached leaves incubated for 6 days under dark (e–h), growth light (GL; 120 µmol photons m^{-2} s^{-1}) (i–l), or higher light (HL; 400 µmol photons m^{-2} s^{-1}) (m–p) [Colour figure can be viewed at wileyonlinelibrary.com]

detachment, Chl content and PSII function were highly maintained in all genotypes (Figures 1 and 2). Compared with the control leaves, the most pronounced decrease in Chl content (by about 30 %) was observed under GL in WT leaves and under HL in *ahk3 ahk4*; however, these changes were substantially smaller compared with the decrease in Chl content in darkness. The smallest decrease (by about 15 %) was observed in *ahk2 ahk3* leaves under GL (Figure 2i). The most pronounced decrease in Φ_{PSII} was observed in WT leaves (Figure 2k,o). Under HL, the decrease in both of the parameters reflecting the function of PSII (F_V/F_M and Φ_{PSII}) was significantly smaller in *ahk2 ahk3* and *ahk3 ahk4* than in WT (Figure 2n,o).

3.5 | MDA content was influenced differently by GL and HL

As mentioned above, the decrease in Chl content and maximal quantum efficiency of PSII in detached leaves of all genotypes was suppressed similarly under both GL and HL. When compared with the senescence in darkness, the light of both intensities markedly reduced the increase in MDA level in WT and *ahk2 ahk4*. Their MDA content increased only about two-fold or less in comparison with the initial value in control (Figure 2h,l,p). The MDA content of the other two double mutants, however, exhibited contrasting responses to the two applied light conditions. The MDA content in the *ahk3 ahk4* was lower than in WT in darkness (Figure 2h); however, under GL, it reached the value comparable with WT (Figure 2l), and in leaves kept under HL, its content even exceed the value observed in WT (two-fold increase, Figure 2p). On the other hand, in the *ahk2 ahk3* mutant, the content of MDA was five-fold lower than in WT not only in darkness but also under GL (Figure 2h,l). HL treatment was necessary for *ahk2 ahk3* leaves to reach MDA level comparable with WT (Figure 2p).

3.6 | Light stimulated accumulation of iP in detached leaves of all genotypes, but the accumulation of *t*Z was affected only minimally

The total content of iP forms was increased in the detached leaves of WT and maintained at high levels in all mutants under both GL and HL (Figure 4i–l,m–p), possibly indicating functional biosynthesis of iP CKs. The level of iP increased in all genotypes compared with control values (Table 1). In WT, the increase in the total content of iP forms, together with the increase in the absolute content of iPR5′MP, iP, and iPR (Table 1), indicates that their biosynthesis is even stimulated. Although the absolute content of iP and iPR in the mutants increased as well, the absolute level of iPR5′MP decreased, which could be explained by active utilization of iPR5′MP for iP and iPR formation. In the *ahk2 ahk3* mutant, the contents of iP and total free bases under GL were much higher compared with HL (almost nine-fold and five-

TABLE 1 Content of isopentenyl (iP) forms (pmol/g of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL; 120 µmol photons m^{-2} s^{-1}), or higher light (HL; 400 µmol photons m^{-2} s^{-1})

Line	iP	iPR	iP7G	iP9G	iPR5′MP
Control					
WT	0.24 ± 0.02	0.28 ± 0.01	18.6 ± 0.2	2.66 ± 0.04	1.64 ± 0.08
ahk2 ahk3	**0.80 ± 0.06**	**4.12 ± 0.13**	**29 ± 1**	**5.32 ± 0.20**	**16.5 ± 0.2**
ahk2 ahk4	**0.38 ± 0.02**	**2.59 ± 0.12**	21 ± 1	**3.26 ± 0.20**	**9.12 ± 0.70**
ahk3 ahk4	**0.54 ± 0.03**	**3.62 ± 0.09**	**41 ± 1**	**6.42 ± 0.38**	**17.1 ± 0.3**
Dark					
WT	n.d.	0.29 ± 0.01	11.1 ± 0.4	0.49 ± 0.03	n.d.
ahk2 ahk3	n.d.	**0.49 ± 0.05**	**8.95 ± 0.37**	**0.74 ± 0.01**	n.d.
ahk2 ahk4	n.d.	0.31 ± 0.00	11.5 ± 0.8	0.47 ± 0.02	n.d.
ahk3 ahk4	n.d.	**0.25 ± 0.01**	**20 ± 1**	**0.84 ± 0.06**	n.d.
GL					
WT	0.57 ± 0.01	4.48 ± 0.36	18.9 ± 1.3	2.83 ± 0.24	5.91 ± 0.73
ahk2 ahk3	**9.37 ± 2.00**	4.32 ± 0.12	**14.8 ± 0.6**	2.37 ± 0.13	**1.56 ± 0.03**
ahk2 ahk4	**0.71 ± 0.04**	5.65 ± 0.52	15.9 ± 0.3	3.20 ± 0.09	4.99 ± 0.44
ahk3 ahk4	**0.84 ± 0.04**	**7.77 ± 0.28**	**27 ± 1**	**3.97 ± 0.19**	**9.20 ± 0.74**
HL					
WT	0.68 ± 0.04	5.08 ± 0.40	18.3 ± 0.3	3.03 ± 0.11	3.22 ± 0.16
ahk2 ahk3	**1.07 ± 0.03**	**11.2 ± 0.7**	**25 ± 1**	**3.91 ± 0.13**	**8.76 ± 0.37**
ahk2 ahk4	0.72 ± 0.03	6.15 ± 0.52	18.4 ± 0.5	**3.46 ± 0.15**	3.92 ± 0.11
ahk3 ahk4	**0.90 ± 0.04**	**6.42 ± 0.14**	**32 ± 3**	**5.23 ± 0.14**	**6.87 ± 0.72**

Note. Means and *SD* (n = 3) are presented; n.d., under detection limit. Statistically significant differences (compared with wild type [WT], $p < .05$) are indicated in bold.

TABLE 2 Content of *trans*-zeatin (*tZ*) forms (pmol/g of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL; 120 µmol photons m^{-2} s^{-1}), or higher light (HL; 400 µmol photons m^{-2} s^{-1})

Line	*tZ*	*tZOG*	*tZR*	*tZROG*	*tZ7G*	*tZ9G*	*tZR5′MP*
Control							
WT	0.62 ± 0.02	3.67 ± 0.20	0.47 ± 0.02	0.54 ± 0.01	102 ± 1	32 ± 2	2.68 ± 0.03
ahk2 ahk3	**7.07 ± 0.09**	**31 ± 0**	**10.7 ± 0.6**	**2.99 ± 0.03**	145 ± 17	**68 ± 5**	**59 ± 0**
ahk2 ahk4	**0.96 ± 0.01**	**7.14 ± 0.37**	**1.74 ± 0.10**	**1.03 ± 0.08**	108 ± 7	**42 ± 2**	**10.8 ± 0.3**
ahk3 ahk4	**3.70 ± 0.12**	**25 ± 1**	**8.58 ± 0.61**	**2.86 ± 0.09**	122 ± 1	**78 ± 4**	**51 ± 2**
Dark							
WT	n.d.	2.03 ± 0.07	n.d.	0.23 ± 0.01	48 ± 1	14.1 ± 0.5	n.d.
ahk2 ahk3	0.24 ± 0.01	**8.62 ± 0.57**	0.06 ± 0.00	**0.81 ± 0.01**	**91 ± 0**	**27 ± 3**	n.d.
ahk2 ahk4	n.d.	1.95 ± 0.01	n.d.	**0.31 ± 0.01**	76 ± 11	17.1 ± 0.2	n.d.
ahk3 ahk4	0.13 ± 0.01	**7.29 ± 0.30**	0.05 ± 0.00	**0.89 ± 0.07**	**110 ± 12**	**55 ± 2**	n.d.
GL							
WT	n.d.	1.97 ± 0.18	0.15 ± 0.00	0.33 ± 0.03	64 ± 4	11.9 ± 0.3	0.28 ± 0.05
ahk2 ahk3	0.30 ± 0.03	**9.20 ± 0.51**	0.10 ± 0.01	**1.00 ± 0.01**	**129 ± 13**	**22 ± 1**	n.d.
ahk2 ahk4	0.19 ± 0.01	**3.83 ± 0.05**	0.15 ± 0.03	**0.56 ± 0.04**	**86 ± 4**	**22 ± 2**	0.26 ± 0.05
ahk3 ahk4	0.35 ± 0.00	**8.71 ± 0.31**	**0.39 ± 0.02**	**1.39 ± 0.06**	**132 ± 12**	**37 ± 1**	0.75 ± 0.06
HL							
WT	n.d.	2.16 ± 0.22	0.10 ± 0.01	0.35 ± 0.01	78 ± 7	16.1 ± 1.8	n.d.
ahk2 ahk3	0.35 ± 0.01	**9.96 ± 0.26**	0.61 ± 0.04	**1.18 ± 0.02**	**137 ± 10**	**23 ± 1**	0.71 ± 0.03
ahk2 ahk4	0.22 ± 0.01	2.41 ± 0.13	**0.59 ± 0.06**	**0.40 ± 0.02**	79 ± 4	14.4 ± 0.6	0.52 ± 0.04
ahk3 ahk4	0.46 ± 0.01	**8.17 ± 0.26**	**0.15 ± 0.01**	**1.33 ± 0.10**	**119 ± 7**	**57 ± 2**	n.d.

Note. Means and *SD* (n = 3) are presented; n.d., under detection limit. Statistically significant differences (compared with wild type [WT], $p < .05$) are indicated in bold.

TABLE 3 Content of *cis*-zeatin (*c*Z) forms (pmol/g of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL; 120 µmol photons m^{-2} s^{-1}) or higher light (HL; 400 µmol photons m^{-2} s^{-1})

Line	*c*Z	*c*ZOG	*c*ZR	*c*ZROG	*c*Z7G	*c*Z9G	*c*ZR5′MP
Control							
WT	0.20 ± 0.01	0.71 ± 0.04	0.74 ± 0.03	1.86 ± 0.06	14.2 ± 0.7	0.24 ± 0.01	7.80 ± 0.18
ahk2 ahk3	**0.10 ± 0.00**	**0.94 ± 0.08**	**0.17 ± 0.01**	**2.04 ± 0.02**	**8.98 ± 0.11**	**0.17 ± 0.01**	**2.61 ± 0.23**
ahk2 ahk4	**0.08 ± 0.00**	**0.94 ± 0.06**	**0.35 ± 0.03**	2.10 ± 0.15	12.8 ± 0.3	0.21 ± 0.00	**6.16 ± 0.20**
ahk3 ahk4	**0.08 ± 0.01**	1.03 ± 0.06	**0.36 ± 0.02**	1.75 ± 0.05	**12.3 ± 0.4**	0.22 ± 0.01	**3.72 ± 0.07**
Dark							
WT	0.38 ± 0.03	1.21 ± 0.10	0.60 ± 0.04	2.75 ± 0.22	8.62 ± 0.68	0.08 ± 0.01	2.62 ± 0.63
ahk2 ahk3	**0.60 ± 0.04**	0.96 ± 0.07	**0.94 ± 0.10**	**2.01 ± 0.11**	**4.17 ± 0.28**	0.12 ± 0.01	1.13 ± 0.17
ahk2 ahk4	**0.20 ± 0.00**	1.06 ± 0.14	0.49 ± 0.05	2.70 ± 0.16	9.38 ± 0.05	0.09 ± 0.01	2.10 ± 0.07
ahk3 ahk4	0.33 ± 0.02	0.90 ± 0.00	0.75 ± 0.02	2.40 ± 0.16	6.67 ± 0.31	0.10 ± 0.00	2.13 ± 0.17
GL							
WT	0.18 ± 0.02	1.29 ± 0.02	0.67 ± 0.05	5.00 ± 0.50	13.0 ± 0.3	0.15 ± 0.00	3.14 ± 0.24
ahk2 ahk3	**0.24 ± 0.02**	1.21 ± 0.12	0.50 ± 0.03	2.97 ± 0.19	**6.90 ± 0.06**	0.18 ± 0.00	**1.42 ± 0.04**
ahk2 ahk4	**0.26 ± 0.02**	2.37 ± 0.25	**0.91 ± 0.02**	5.89 ± 0.57	**20.5 ± 1.5**	**0.26 ± 0.01**	**5.57 ± 0.52**
ahk3 ahk4	**0.25 ± 0.01**	1.48 ± 0.06	0.81 ± 0.05	5.50 ± 0.28	14.7 ± 0.4	0.19 ± 0.01	4.63 ± 0.09
HL							
WT	0.19 ± 0.00	1.09 ± 0.00	0.55 ± 0.02	2.62 ± 0.13	15.7 ± 1.4	0.27 ± 0.01	1.95 ± 0.08
ahk2 ahk3	**0.26 ± 0.01**	1.23 ± 0.09	0.61 ± 0.04	**3.65 ± 0.10**	**11.1 ± 0.2**	0.19 ± 0.01	1.98 ± 0.14
ahk2 ahk4	0.24 ± 0.01	1.31 ± 0.10	0.57 ± 0.02	**3.09 ± 0.04**	**19.3 ± 1.1**	**0.34 ± 0.02**	2.77 ± 0.10
ahk3 ahk4	**0.28 ± 0.02**	0.90 ± 0.06	0.54 ± 0.02	2.68 ± 0.17	17.0 ± 0.7	0.32 ± 0.00	3.26 ± 0.18

Note. Means and *SD* (n = 3) are presented. Statistically significant differences (compared with wild type [WT], $p < .05$) are indicated in bold.

fold, respectively; Figures 3e–h and 4j,n), whereas in other genotypes, there was basically no difference between the effect of GL and HL.

The decrease in the total content of *t*Z forms in all genotypes under both GL and HL conditions was slightly smaller than in the dark (Figure 5 and Table 2). The decrease in *t*ZR5′MP, *t*Z, and *t*ZR contents, followed by a corresponding increase in the content of glucoside forms *t*Z7G and *t*Z9G (Figure 5), indicates that *t*Z deactivation was partially stimulated in comparison with the control. From the decrease in the overall content of *t*Z forms (Figure 5), we can further deduce that *t*Z biosynthesis was inhibited in a similar manner as in the leaves kept in the dark. Thus, on the contrary to iP CKs, light did not have any significant effect on the metabolism of *t*Z CKs in the detached leaves, which supports the hypothesis that *t*Z-type CKs predominantly originate in roots (Frébort, Kowalska, Hluska, Frébortová, & Galuszka, 2011; Hirose et al., 2008).

3.7 | The best correlation between changes in physiological parameters and CK content was found when not only free bases but also their ribosides were considered

The connection between the senescence-induced changes in physiological parameters and the content of specific CK forms was evaluated via correlation analysis (Table 4). The positive correlation could be expected between CK content and Chl content and F_V/F_M (i.e., higher CK level corresponding to higher Chl content and better PSII function) and negative correlation between CK and MDA contents (i.e., higher CK level corresponding to lower lipid peroxidation). Interestingly, in most cases, the highest correlation coefficients were obtained when we took into consideration not only the sum of iP and *t*Z free bases (i.e., iP + *t*Z), but the sum of these free bases with their ribosides (i.e., iP + *t*Z + iPR + *t*ZR; Table 4). Overall, the strongest correlations were shown for iP + iPR and iP + *t*Z + iPR + *t*ZR. Despite the recent finding by Lomin et al. (2015) that CK ribosides *in planta* have no or minor affinity to AHK receptors, our results indicate that CK ribosides could play an active role in the regulation of senescence. This seeming discrepancy could be explained by possible fast conversion of CK ribosides to its bases.

Very unexpected is a strong negative correlation between Chl content and F_V/F_M and the content of *c*Z, which was found in all genotypes except *ahk2 ahk4*. In this mutant, we observed similarly strong but positive correlation (Table 4).

4 | DISCUSSION

Although CKs and light are both known to be crucial for the regulation of plant senescence, a detailed investigation of their interaction during senescence is missing. It has been well documented that light affects the content of endogenous CKs in leaves (Ananieva et al., 2008; Boonman et al., 2009; Kurepin & Pharis, 2014; Zdarska et al., 2015) and that it is able to slow down the progress of senescence (Okada et al., 1992; Špundová et al., 2003; Thimann, 1985; Vlčková et al., 2006). There are only a few studies dealing with the changes in the endogenous CK content in detached leaves kept under different light conditions, and their results are contradictory. Zubo et al. (2008) have shown that in detached barley leaves, the content of zeatin derivatives increased two-fold under continuous light, whereas in leaves kept in

TABLE 4 Pearson's correlation coefficient between physiological parameters (relative chlorophyll content [% of respective control], maximal quantum yield of photosystem II photochemistry in dark-adapted state [F_V/F_M], and content of malondialdehyde [MDA–DNPH adduct]) and contents of particular cytokinin forms (FB stands for sum of free bases and FB + R for sum of free bases with their ribosides) in detached leaves incubated for 6 days under dark, growth light (GL; 120 μmol photons m^{-2} s^{-1}), or higher light (HL; 400 μmol photons m^{-2} s^{-1}) conditions

	iP	iP + iPR	*t*Z	*t*Z + *t*ZR	iP + *t*Z	iP + *t*Z + iPR + *t*ZR	FB	FB + R	*c*Z	*c*Z + *c*ZR	*m*T
WT											
Chl	1.00	1.00	–	0.93	1.00	1.00	−0.76	1.00	−1.00	−0.91	−0.90
F_V/F_M	0.98	0.99	–	0.96	0.98	0.99	−0.69	1.00	−1.00	−0.87	−0.85
MDA	−0.99	−0.99	–	−0.83	−0.99	−0.99	0.87	−0.97	0.95	0.98	0.97
ahk2 ahk3											
Chl	0.68	1.00	0.85	0.52	0.68	1.00	0.44	0.99	−1.00	−1.00	−0.45
F_V/F_M	0.57	0.99	0.91	0.63	0.58	1.00	0.31	0.95	−1.00	−0.99	−0.57
MDA	−0.56	0.24	0.71	0.95	−0.56	0.28	−0.78	0.06	−0.29	−0.19	−0.97
ahk2 ahk4											
Chl	1.00	1.00	0.99	0.80	1.00	0.99	0.98	1.00	0.95	0.71	−0.98
F_V/F_M	0.97	0.96	0.94	0.67	0.97	0.94	1.00	0.96	0.99	0.84	−0.92
MDA	−1.00	−1.00	−0.99	−0.81	−1.00	−0.99	−0.98	−1.00	−0.95	−0.70	0.98
ahk3 ahk4											
Chl	0.98	1.00	0.89	1.00	0.96	1.00	1.00	1.00	−0.97	−0.44	−0.86
F_V/F_M	0.99	0.94	0.99	0.91	1.00	0.94	0.93	0.91	−0.84	−0.71	−0.98
MDA	0.24	0.03	0.49	−0.05	0.30	0.03	0.01	−0.05	0.20	−0.91	−0.54

Note. Red shades represent negative correlation; blue shades represent positive correlation.

darkness, the change was much smaller. Two other studies provided conflicting results about the changes in the iP content; Roberts et al. (2011) have reported a decrease in iP and iPR contents in detached leaves of wheat kept in the dark, whereas Causin et al. (2009) have found a reduced iPR content in detached leaves of wheat exposed to light.

4.1 | The loss-of-function mutation of AHK3 led to increase in iP and *t*Z contents

Double mutants *ahk2 ahk3* and *ahk3 ahk4* have constitutively lower Chl content in comparison with WT (Figure 2a), which is in agreement with the results of Riefler et al. (2006) and Danilova et al. (2014). The lower content of Chl in these mutants is a consequence of their dysfunctional AHK3 receptor, which is known to play a main role in CK-mediated stimulation of Chl biosynthesis (Kim et al., 2006).

In order to compensate the insufficient CK output, the biosynthesis of iP and *t*Z forms in these double mutants was stimulated when compared with WT (Figures 3b, 4a–d, 5a–d, and 6). Comparable increases in iP and *t*Z contents, caused by the loss-of-function mutation of AHK3, were observed by Vescovi et al. (2012) in cell cultures derived from the *ahk2 ahk3* double mutant. Only a modest increase in the CK level was observed in the *ahk2 ahk4* mutant, in which the AHK3 receptor is functional (Figures 3a, 4a–d, 5a–d, and 6).

A strong increase in the content of *t*Z and its metabolites in *Arabidopsis* double mutants *ahk2 ahk3* and *ahk3 ahk4* has been described also by Riefler et al. (2006). They have found that the *ahk2 ahk3* mutant had about 3.5-fold higher content of *t*Z compared with WT; however, its iP content was only half of the WT value. Under our conditions, the *t*Z level was 11-fold higher and iP content was increased 3.5-fold above the levels of these free bases in WT (Tables 1 and 2 and Figure 3b). This discrepancy could be explained by different light conditions during plant growth, as we used short-day conditions, whereas Riefler et al. (2006) used long-day conditions. It seems that the CK biosynthesis in the receptor double mutants was markedly more stimulated under short-day conditions.

4.2 | Higher constitutive iP and *t*Z contents in the presence of functional AHK3 and AHK2 receptors protected photosynthetic apparatus during dark senescence

A marked decrease in Chl content and PSII function, which are changes typical for dark senescence of detached leaves (Ananieva et al., 2008; Špundová et al., 2003; Thomas, 1978; Vlčková et al., 2006), was observed in all the tested genotypes after 6 days in darkness. Interestingly, the decrease in both parameters was significantly lower in the *ahk2 ahk4* and *ahk3 ahk4* mutants (Figures 1 and 2e–g). This could be explained by the fact that these mutants have high constitutive CK levels as well as functional receptors (AHK3 or AHK2) that are important for Chl retention (Kim et al., 2006; Riefler et al., 2006) and for the maintenance of PSII function (Cortleven et al., 2014). As the Chl content and PSII function best correlated with the sum of contents of iP, *t*Z, and their ribosides (Table 4), we hypothesize that these are the most important CK forms for the maintenance of the function of photosynthetic apparatus. The most pronounced increase in the total content of iP + iPR and *t*Z + *t*ZR was observed in *ahk2 ahk3* (Tables 1 and 2 and Figure 6); however, the high content of these CKs was not sufficient to maintain Chl content and PSII function due to the non-functional AHK3 and AHK2 receptors in this mutant. The proposed scheme of combined effects of iP + iPR and *t*Z + *t*ZR in the presence of particular receptors is summarized in Figure 6.

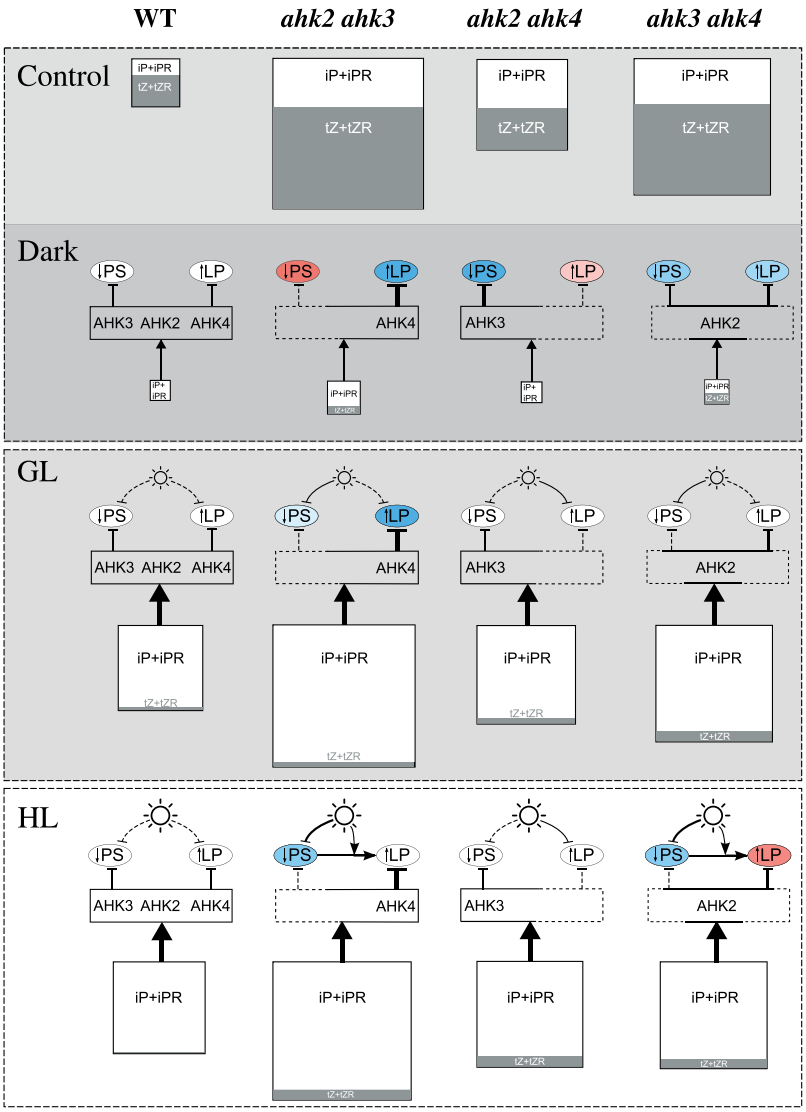

FIGURE 6 The proposed scheme of the interplay between cytokinins (CKs) and light during senescence of detached *Arabidopsis* leaves. Different genotypes are presented in columns, whereas horizontal sections represent situation after 6 days in darkness (dark), under growth light (GL; 120 µmol photons m^{-2} s^{-1}), or higher light (HL; 400 µmol photons m^{-2} s^{-1}); the uppermost section shows the initial content of CKs in leaves immediately after the detachment (control). The areas of the squares shown for particular variants are proportional to the summary content of iP, *t*Z, iPR, and *t*ZR. The area of the square for WT controls represents 1.61 pmol/g of fresh weight. The white parts of the squares represent the sum of iP and iPR; the dark grey parts of squares represent the sum of *t*Z and *t*ZR. CK receptors that are active in each genotype are indicated in the rectangles. A "strength" of the CK signalling output on the senescence-associated decrease in photosynthetic parameters (↓PS; chlorophyll content and photosystem II function) and increase in lipid peroxidation (↑LP; evaluated from the malondialdehyde content) is represented by a dashed or full line of various thickness. Background colour of ↓PS and ↑LP represents marked difference in comparison with the respective WT; red shades indicate promotion; blue shades indicate inhibition of the senescence-associated changes. DARK: In WT, the (initial) content of iP, *t*Z, and their ribosides was not sufficient to maintain the high PS and low LP even though all three CK receptors were active. In CK receptor double mutants, the (initial) summary contents of iP + iPR and *t*Z + *t*ZR were higher, the highest being in *ahk2 ahk3*. In this mutant, the pronouncedly increased level of iP + iPR and *t*Z + *t*ZR maintained the lowest LP because of the presence of AHK4 that is proposed to be the main receptor mediating CK effect on LP. However, the solely functional AHK4 did not have sufficient promoting effect on PS. The smallest increase in the content of iP + iPR and *t*Z + *t*ZR was found in *ahk2 ahk4*, but it was accompanied by the highly maintained PS due to the presence of the AHK3 receptor, the supposed main receptor mediating CK effect on PS during senescence, and by the highest LP. In the *ahk3 ahk4* mutant, the content of iP, *t*Z, iPR, and *t*ZR, as well as the maintenance of PS and LP, is between *ahk2 ahk3* and *ahk2 ahk4*, indicating that in the absence of AHK3 and AHK4, AHK2 can partially take over the role of both these receptors in the regulation of PS as well as LP. GL: The pronounced promoting effect of light (both GL and HL) on the maintenance of high PS was associated with the increased level of iP + iPR. The highest content of iP + iPR in *ahk2 ahk3*, together with functional AHK4, maintained the lowest LP. The elevation of the content of active CK forms, however, was not the only way how light inhibited senescence-associated changes, as the PS decrease in this mutant was small despite the poor effect of AHK4 on PS. The presence of light compensated also the insufficient effect of receptors in the *ahk2 ahk4* and *ahk3 ahk4* mutants, resulting in similar maintenance of LP and PS in comparison with WT. HL: The effect of light was dependent on light dose. The effect of HL contrasted to GL in the *ahk2 ahk3* and *ahk3 ahk4* mutants, which—due to the HL—maintained higher PS in spite of the inactive AHK3 receptor. The maintained PS, together with the elevated supply of excitations due to the increased light dose, resulted in the promotion of LP. In *ahk2 ahk3*, the LP increase was minimized by the AHK4 action, but in *ahk3 ahk4*, the protection mediated by AHK2 was poor [Colour figure can be viewed at wileyonlinelibrary.com]

4.3 | AHK4 seems to play a major role in the protection against lipid peroxidation in detached *Arabidopsis* leaves

Senescence is typically accompanied by the increase in the level of lipid peroxidation and its marker MDA (Huang et al., 1997; L. Liu et al., 2016; X. H. Liu & Huang, 2002; Todorov et al., 1998). After 6 days in darkness, the MDA content increased markedly in leaves of WT as well as in the *ahk2 ahk4* mutant. Smaller increase was observed in leaves of the *ahk3 ahk4* mutant, whereas no change in lipid peroxidation was found in the leaves of the *ahk2 ahk3* mutant (Figure 2h). The most pronounced MDA increase in *ahk2 ahk4* indicates that AHK2 and AHK4 receptors play an important role in CK-mediated protection against lipid peroxidation. This idea is supported by the finding that in the *ahk2 ahk3* and *ahk3 ahk4* mutants, where the CK levels were elevated (free bases + ribosides; Figure 3a,c) and either AHK2 or AHK4 was functional, lipid peroxidation was significantly lower than in WT (Figures 2h and 6). When the leaves were kept under GL, the suppression of lipid peroxidation in comparison with WT was detected only in the *ahk2 ahk3* mutant (Figure 2l), which suggests that AHK4 might be the main receptor mediating the effect of CK on lipid peroxidation. The reduced MDA content in the *ahk2 ahk3* mutant can be associated also with increased tocopherol concentration in this mutant (Cortleven et al., 2014), as tocopherols are known to protect cellular membranes against lipid peroxidation (Abbasi, Hajirezaei, Hofius, Sonnewald, & Voll, 2007; Maeda, Sakuragi, Bryant, & DellaPenna, 2005). The proposed effect of particular CK receptors on lipid peroxidation in combination with the effect of light is also summarized in the scheme presented in Figure 6.

It should be noted that Danilova et al. (2014), using spectrophotometric estimation of the MDA–TBA_2 adduct (MDA adducted to two molecules of thiobarbituric acid), found an opposite trend—increased level of MDA in the control leaves of *ahk2 ahk3* in comparison with WT. When we estimated the MDA–TBA_2 by analogous assay, we found similarly increased level of MDA–TBA_2 in the *ahk2 ahk3* in comparison with WT in control leaves as well as in leaves kept in darkness (data not shown). However, the presented specific HPLC estimation of MDA–DNPH adduct revealed lower level of the by-product of lipid peroxidation in *ahk2 ahk3* leaves in comparison with WT (Figure 2d, h). Differences between results of both assays were explained by Pilz et al. (2000), who reported that the spectrophotometric determination of MDA–TBA_2 adducts corresponds to general oxidative damage of proteins and other compounds rather than to lipid peroxidation and that the HPLC assay should be used for more accurate estimation of the level of lipid peroxidation.

4.4 | Light-mediated maintenance of photosynthesis in detached leaves could be related to persisted iP biosynthesis

Compared with dark-incubated detached leaves, both GL and HL suppressed the decrease in Chl content and PSII function in all genotypes (Figures 1 and 2). The light-dependent slowdown of senescence of detached leaves is well documented (Klerk et al., 1993; Špundová et al., 2003; Vlčková et al., 2006). The higher Chl content and F_V/F_M correlated well with the content of iP + iPR, whereas the correlation between *t*Z + *t*ZR and changes in physiological parameters was very weak, most probably due to its very low content in senescing leaves (Figure 6). The analysis of the contents of particular CK forms indicated that under light (GL as well as HL), the biosynthesis of iP was functional in leaves of all genotypes, and the data suggest that in WT, the iP biosynthesis was even stimulated (Figure 4i,m and Table 1). On the other hand, the biosynthesis of *t*Z seems to be markedly reduced in all genotypes (Figure 5 and Table 2). The importance of iP under light is also supported by a strong correlation between iP + iPR and Chl content or F_V/F_M (Table 4). Therefore, the promoting effect of light on the Chl content and PSII function could be connected with the maintenance of iP biosynthesis.

4.5 | Can light substitute CK signalling?

It has been well documented that there is an extensive crosstalk between light and CKs at multiple levels (Argueso et al., 2010; Oh et al., 2009; Sweere et al., 2001; Vandenbussche et al., 2007). Therefore, we have analysed how light can modify senescence in mutants where two out of three CK receptors are non-functional.

As expected, the dysfunction of AHK3 receptor led to constitutively lower Chl content (Danilova et al., 2014; Riefler et al., 2006) in both the *ahk2 ahk3* and *ahk3 ahk4* double mutants (Figure 2a). After 6-day-long incubation of detached leaves in darkness, the most pronounced decrease in Chl content was observed in mutant where both AHK3 and AHK2 were non-functional (Figures 1 and 2e). Surprisingly, this acceleration of Chl loss was not observed under GL treatment (Figures 1 and 2i). Even more surprisingly, under HL conditions, both mutants without functional AHK3 had markedly better PSII function in comparison with WT (Figure 2n,o). These results are even more interesting in the light of the detailed analysis of the CK content. Under HL conditions, the leaves of the *ahk2 ahk3* mutant contained 9 times lower amount of iP compared with GL conditions (Figure 3f, h); nevertheless, the photosynthesis was much better protected than in WT (Figure 2o). The disrupted CK signalling via AHK3 thus led to lower Chl content in darkness; nevertheless, under GL, it was compensated by light, and under HL, we could observe even improvement of the photosynthetic function in comparison with WT. Light thus seems to have the ability to substitute the insufficient CK signalling, and under HL conditions, it was able to even reverse the negative effect of AHK3 dysfunction. Possible explanation is offered by AHK-mediated activation of type A ARRs that negatively regulate response to CK as well as to light (Mira-Rodado et al., 2007; Sweere et al., 2001). The missing AHK3-mediated feedback inhibition of photosynthesis by CK and/or by light might explain the seemingly surprising finding that the mutants with non-functional AHK3 had more maintained photosynthesis during senescence in HL.

The ability of light to compensate the insufficient CK signalling would further explain the surprising drop in the level of iP under HL (but not GL) in the *ahk2 ahk3* mutants. The intensity of GL was supposedly not high enough to fully compensate the insufficient CK signalling in *ahk2 ahk3*, and the iP accumulation was thus markedly promoted (Figure 4j). However, in leaves exposed to HL intensity, the relative content of iP significantly dropped in favour of other, less biologically active

iP forms (Figure 4n). Under HL conditions, the light was probably strong enough to compensate the impaired CK signalling and as a result, iP could be converted to less active iP metabolites. Furthermore, this drop in iP content demonstrates that light is able to protect photosynthetic apparatus also by other ways than by increasing endogenous levels of active CKs. We have shown that HL was able to minimize the decrease in PSII efficiency in the *ahk2 ahk3* mutant leaves even though the content of CK active forms was lower than in leaves kept at GL (cf. Figure 3 f,h). In addition, we still have to keep in mind that this *ahk2 ahk3* mutant, in which we have observed the light-dependent maintenance of photosynthetic function, has inactive AHK3 and AHK2 receptors that are responsible for the CK-dependent modulation of photosynthetic activity.

Unlike WT and *ahk2 ahk4*, both the *ahk2 ahk3* and *ahk3 ahk4* mutants showed unexpected increase in lipid peroxidation under HL compared with GL (Figure 2l,p), which could be explained by the above-discussed strongly maintained photochemistry of PSII under HL (Figure 2n,o). Earlier, we have shown that in detached leaves with relatively well-preserved photosynthesis, the elevated supply of excitations due to HL dose might lead to an imbalance between the generation and demand of electrons in electron transport chain (Vlčková et al., 2006). This imbalance in turn increases excitation pressure on PSII (1-qP) and leads to higher production of reactive oxygen species and lipid peroxidation. Such situation probably occurred in detached leaves of both the *ahk2 ahk3* and *ahk3 ahk4* mutants exposed to HL, which were characterized by relatively high PSII efficiency (Figure 2n,o), and the measurement confirmed high excitation pressure under HL conditions (1-qP; data not shown). Although in the dark the MDA content in the leaves of these mutants was markedly lower than in WT (Figure 2h), under HL conditions, its content was similar (*ahk2 ahk3*) or even markedly higher (*ahk3 ahk4*) than in WT (Figure 2p). It seems that in *ahk2 ahk3*, the increase in lipid peroxidation was partially suppressed via the AHK4-mediated CK-dependent protection, making the MDA content comparable with WT. However, in *ahk3 ahk4*, the CK-dependent protection mediated by AHK2 was not sufficient for the suppression of lipid peroxidation and the MDA content rose pronouncedly.

Comparison of senescence in the dark and in the light shows that in the *ahk2 ahk4* mutant, light was able to eliminate the increase in lipid peroxidation connected to non-functional AHK2 and AHK4. Similarly, the promoted decrease in photosynthetic parameters during dark senescence in *ahk2 ahk3*, caused by non-functional AHK2 and AHK3, was not only reversed by light, but the photosynthetic performance of this mutant was even better than in WT. Even though our results do not allow us to identify the exact intersection of the CK and light signalling pathways, we have clearly shown that light is able to substitute the disrupted CK signalling during senescence of detached leaves. The observed effect of light on the senescence of leaves with given CK status is summarized in scheme in Figure 6.

4.6 | Is AHK3 a mediator of the effect of *c*Z on senescence?

The CKs of *c*Z type constitute a distinct group of CKs, as these CKs are, unlike others, synthesized via a pathway known as mevalonate (MVA) biosynthetic pathway (Kasahara et al., 2004). *c*Z CKs occur ubiquitously in the plant kingdom (Gajdošová et al., 2011), but their function is largely unknown. An increase in relative content of *c*Z-type CKs has been repeatedly observed under growth-limiting conditions induced by various external or internal factors (for a review, see Schäfer et al., 2015). Senescence appears to be one such factor (Gajdošová et al., 2011; Šmehilová et al., 2016). Our results show the most marked increases in *c*Z level in detached leaves kept for 6 days in darkness (Table 3). During correlation analysis, we have found a strong negative correlation between *c*Z content and Chl content and PSII function in WT as well as in mutants except of *ahk2 ahk4* (Table 4). This finding indicates that the content of *c*Z increases when the photosynthetic performance substantially decreases. It supports a hypothesis by Gajdošová et al. (2011), who suggest that *c*Z-type CKs might ensure a basal CK activity necessary for the maintenance of essential physiological processes under conditions characterized by the lack of energy sources that lead to down-regulation of the main active CKs.

The increase in the *c*Z content might be caused either by the depletion of *t*Z by the action of *cis-trans* isomerase (Spíchal, 2012), or by the activation of MVA biosynthetic pathway. The reason for the preference of MVA pathway over the methylerythritol phosphate (MEP) pathway, necessary for the synthesis of other CKs except *c*Z, can be the availability of its precursors. Although the MEP pathway requires ATP/ADP/AMP precursors, MEV pathway does not. Therefore, the preference of the MVA pathway over the MEP pathway could be a possible survival strategy of leaves facing the ATP/ADP/AMP exhaustion, which can be expected to happen for example after 6 days in darkness. Under such conditions, the precursors of the MVA pathway might be more accessible and/or dispensable than the ATP/ADP/AMP precursors of the MEP pathway.

Interestingly, unlike in WT and other mutants, a positive correlation was found between *c*Z content and the photosynthetic parameters in the *ahk2 ahk4* mutant. This finding indicates that the AHK3 receptor may have an important role in mediating *c*Z action, which is supported by genuine *c*Z affinity to AHK3 (Romanov, Lomin, & Schmülling, 2006) and by the finding that AHK3 was significantly activated by *c*Z (Spíchal et al., 2004). Gajdošová et al. (2011) further tested the activity of exogenously applied CKs of the *c*Z type and compared it to their *trans*-counterparts in various CK bioassays. In the senescence assay, using darkened detached oat (as well as wheat and maize) leaves, the *c*Z had lower activity than *t*Z; however, its effect on Chl retention in leaves was significant. Among dark-kept leaves of all tested double mutants, the *ahk2 ahk4* exhibited the lowest increase in *c*Z level, but this relatively small increase was—in connection with the solely functional AHK3 receptor—sufficient for considerable Chl retention (Figures 1 and 2e). Interestingly, in WT, which also has functional AHK3 and relatively high *c*Z content, we have found a negative correlation between the *c*Z content and the photosynthetic parameters. A question arises why there is an opposite trend than in *ahk2 ahk4*. Possible explanation could be offered by increased abundance of AHK3, which could be expected in the mutant with dysfunction of the other two CK receptors. Therefore, the relative importance of AHK3-mediated *c*Z signalling in the *ahk2 ahk4* double mutant is much higher than in the WT, where all three CK

receptors are functional. This assumption, however, needs to be proved by further investigation.

5 | CONCLUSION

We have shown that the content of endogenous CKs and individual CK forms differs significantly in detached *Arabidopsis* leaves kept under various light conditions. The content of *t*Z forms decreased pronouncedly after 6-day-long incubation of detached leaves, no matter whether the leaves were kept in the dark or under GL or HL. However, the content of iP + iPR decreased only in darkness, and in leaves kept under light, it was significantly elevated. This increase in iP + iPR content was accompanied by highly maintained Chl content and PSII function. The elevated content of *c*Z in detached leaves kept in darkness supports the hypothesis that *c*Z plays a major role in the maintenance of basal leaf viability under growth-limiting conditions, when the synthesis of the main active CKs is suppressed. On the basis of correlation analysis performed on CK receptor double mutants, we propose that AHK3 is the main mediator of this *c*Z action.

Although all of the studied CK receptor double mutants had increased constitutive content of CKs, the dark senescence-induced increase in lipid peroxidation was retarded only in mutant with functional AHK4 receptor and partially also in mutant with functional AHK2. AHK4 thus seems to have the main responsibility for CK-induced inhibition of lipid peroxidation, followed by AHK2.

The CK receptor double mutants also enabled us to investigate in more detail the mechanism of the effect of light on senescence. Senescence-induced changes in the double mutants kept in darkness and under both light intensities indicate that light can compensate the disrupted CK signalling caused by loss-of-function mutation in the AHK receptors. On the basis of this hypothesis, we have suggested a scheme (Figure 6) that summarizes contributions of particular CK receptors, their main endogenous CK ligands, and light in the maintenance of photosynthesis and low lipid peroxidation in detached leaves of *Arabidopsis*.

ACKNOWLEDGMENTS

This work was supported by Grant LO1204 (Sustainable Development of Research in the Centre of the Region Haná) from the National Programme of Sustainability I, Ministry of Education, Youth and Sports, Czech Republic. We thank to Lukáš Spíchal from Laboratory of Growth Regulators, who kindly provided us with seeds of receptor double mutants of *Arabidopsis thaliana*. Special thanks belong to Miroslav Strnad for critical reading of the manuscript and Iva Ilíková for language editing.

ORCID

Alexandra Husičková http://orcid.org/0000-0002-9753-8119

REFERENCES

Abbasi, A. R., Hajirezaei, M., Hofius, D., Sonnewald, U., & Voll, L. M. (2007). Specific roles of α- and γ-tocopherol in abiotic stress responses of transgenic tobacco. *Plant Physiology*, *143*, 1720–1738.

Ananieva, K., Ananiev, E. D., Doncheva, S., Georgieva, K., Tzvetkova, N., Kamínek, M., … Malbeck, J. (2008). Senescence progression in a single darkened cotyledon depends on the light status of the other cotyledon in *Cucurbita pepo* (zucchini) seedlings: Potential involvement of cytokinins and cytokinin oxidase/dehydrogenase activity. *Physiologia Plantarum*, *134*, 609–623.

Argueso, C. T., Raines, T., & Kieber, J. J. (2010). Cytokinin signaling and transcriptional networks. *Current Opinion in Plant Biology*, *13*, 533–539.

Barber, J., & Andersson, B. (1992). Too much of a good thing: light can be bad for photosynthesis. *Trends in Biochemical Sciences*, *17*, 61–66.

Biswal, U. C., & Biswal, B. (1984). Photocontrol of leaf senescence. *Photochemistry and Photobiology*, *39*, 875–879.

Boonman, A., Prinsen, E., Voesenek, L., & Pons, T. L. (2009). Redundant roles of photoreceptors and cytokinins in regulating photosynthetic acclimation to canopy density. *Journal of Experimental Botany*, *60*, 1179–1190.

Brouwer, B., Gardeström, P., & Keech, O. (2014). In response to partial plant shading, the lack of phytochrome A does not directly induce leaf senescence but alters the fine-tuning of chlorophyll biosynthesis. *Journal of Experimental Botany*, *65*, 4037–4049.

Buchanan-Wollaston, V. (1997). The molecular biology of leaf senescence. *Journal of Experimental Botany*, *48*, 181–199.

Causin, H. F., Roberts, I. N., Criado, M. V., Gallego, S. M., Pena, L. B., Ríos, M. D., & Barneix, A. J. (2009). Changes in hydrogen peroxide homeostasis and cytokinin levels contribute to the regulation of shade-induced senescence in wheat leaves. *Plant Science*, *177*, 698–704.

Chory, J., Reinecke, D., Sim, S., Washburn, T., & Brenner, M. (1994). A role for cytokinins in de-etiolation in *Arabidopsis* (det mutants have an altered response to cytokinins). *Plant Physiology*, *104*, 339–347.

Cortleven, A., Marg, I., Yamburenko, M. V., Schlicke, H., Hill, K., Grimm, B., … Schmülling, T. (2016). Cytokinin regulates the etioplast-chloroplast transition through the two-component signaling system and activation of chloroplast-related genes. *Plant Physiology*, *172*, 464–478.

Cortleven, A., Nitschke, S., Klaumunzer, M., AbdElgawad, H., Asard, H., Grimm, B., … Schmülling, T. (2014). A novel protective function for cytokinin in the light stress response is mediated by the ARABIDOPSIS HISTIDINE KINASE2 and ARABIDOPSIS HISTIDINE KINASE3 receptors. *Plant Physiology*, *164*, 1470–1483.

Cortleven, A., & Schmülling, T. (2015). Regulation of chloroplast development and function by cytokinin. *Journal of Experimental Botany*, *66*, 4999–5013.

Cortleven, A., & Valcke, R. (2012). Evaluation of the photosynthetic activity in transgenic tobacco plants with altered endogenous cytokinin content: Lessons from cytokinin. *Physiologia Plantarum*, *144*, 394–408.

Danilova, M. N., Kudryakova, N. V., Voronin, P. Y., Oelmüller, R., Kusnetsov, V. V., & Kulaeva, O. N. (2014). Membrane receptors of cytokinin and their regulatory role in *Arabidopsis thaliana* plant response to photooxidative stress under conditions of water deficit. *Russian Journal of Plant Physiology*, *61*, 434–442.

Dobrev, P. I., & Kamínek, M. (2002). Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *Journal of Chromatography A*, *950*, 21–29.

Frébort, I., Kowalska, M., Hluska, T., Frébortová, J., & Galuszka, P. (2011). Evolution of cytokinin biosynthesis and degradation. *Journal of Experimental Botany*, *62*, 2431–2452.

Gajdošová, S., Spíchal, L., Kamínek, M., Hoyerová, K., Novák, O., Dobrev, P. I., … Motyka, V. (2011). Distribution, biological activities, metabolism, and the conceivable function of *cis*-zeatin-type cytokinins in plants. *Journal of Experimental Botany*, *62*, 2827–2840.

Gan, S. S., & Amasino, R. M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. *Science*, *270*, 1986–1988.

Gan, S. S., & Amasino, R. M. (1996). Cytokinins in plant senescence: From spray and pray to clone and play. *BioEssays*, *18*, 557–565.

Gan, S. S., & Amasino, R. M. (1997). Making sense of senescence: Molecular genetic regulation and manipulation of leaf senescence. *Plant Physiology*, *113*, 313–319.

Hensel, L. L., Grbić, V., Baumgarten, D. A., & Bleecker, A. B. (1993). Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in Arabidopsis. *Plant Cell*, *5*, 553–564.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., & Sakakibara, H. (2008). Regulation of cytokinin biosynthesis, compartmentalization and translocation. *Journal of Experimental Botany*, *59*, 75–83.

Huang, F. Y., Philosoph-Hadas, S., Meir, S., Callaham, D. A., Sabato, R., Zelcer, A., & Hepler, P. K. (1997). Increases in cytosolic Ca^{2+} in parsley mesophyll cells correlate with leaf senescence. *Plant Physiology*, *115*, 51–60.

Hwang, I., Sheen, J., & Müller, B. (2012). Cytokinin signaling networks. *Annual Review of Plant Biology*, *63*, 353–380.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., … Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. *Nature*, *409*, 1060–1063.

Kasahara, H., Takei, K., Ueda, N., Hishiyama, S., Yamaya, T., Kamiya, Y., … Sakakibara, H. (2004). Distinct isoprenoid origins of *cis*- and *trans*-zeatin biosyntheses in Arabidopsis. *Journal of Biological Chemistry*, *279*, 14049–14054.

Khanna-Chopra, R. (2012). Leaf senescence and abiotic stresses share reactive oxygen species-mediated chloroplast degradation. *Protoplasma*, *249*, 469–481.

Kim, H. J., Ryu, H., Hong, S. H., Woo, H. R., Lim, P. O., Lee, I. C., … Hwang, I. (2006). Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 814–819.

Klerk, H., Rebers, M., & van Loon, L. C. (1993). Effects of light and regulators on senescence-related changes in soluble-proteins in detached oat (*Avena sativa* L.) leaves. *Plant Growth Regulation*, *13*, 137–145.

Kurepin, L. V., & Pharis, R. P. (2014). Light signaling and the phytohormonal regulation of shoot growth. *Plant Science*, *229*, 280–289.

Lepistö, A., & Rintamäki, E. (2012). Coordination of plastid and light signaling pathways upon development of *Arabidopsis* leaves under various photoperiods. *Molecular Plant*, *5*, 799–816.

Lichtenthaler, H. K. (1987). Chlorophylls and carotenoids—Pigments of photosynthetic biomembranes. *Methods in Enzymology*, *148*, 350–382.

Lim, P. O., Kim, H. J., & Nam, H. G. (2007). Leaf senescence. *Annual Review of Plant Biology*, *58*, 115–136.

Liu, L., Li, H. X., Zeng, H. L., Cai, Q. S., Zhou, X., & Yin, C. X. (2016). Exogenous jasmonic acid and cytokinin antagonistically regulate rice flag leaf senescence by mediating chlorophyll degradation, membrane deterioration, and senescence-associated genes expression. *Journal of Plant Growth Regulation*, *35*, 366–376.

Liu, X. H., & Huang, B. R. (2002). Cytokinin effects on creeping bentgrass response to heat stress: II. Leaf senescence and antioxidant metabolism. *Crop Science*, *42*, 466–472.

Lomin, S. N., Krivosheev, D. M., Steklov, M. Y., Arkhipov, D. V., Osolodkin, D. I., Schmülling, T., & Romanov, G. A. (2015). Plant membrane assays with cytokinin receptors underpin the unique role of free cytokinin bases as biologically active ligands. *Journal of Experimental Botany*, *66*, 1851–1863.

Maeda, H., Sakuragi, Y., Bryant, D. A., & DellaPenna, D. (2005). Tocopherols protect *Synechocystis* sp strain PCC 6803 from lipid peroxidation. *Plant Physiology*, *138*, 1422–1435.

Mähönen, A. P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P. N., & Helariutta, Y. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. *Genes & Development*, *14*, 2938–2943.

Mira-Rodado, V., Sweere, U., Grefen, C., Kunkel, T., Fejes, E., Nagy, F., … Harter, K. (2007). Functional cross-talk between two-component and phytochrome B signal transduction in Arabidopsis. *Journal of Experimental Botany*, *58*, 2595–2607.

Mok, D. W. S., & Mok, M. C. (2001). Cytokinin metabolism and action. *Annual Review of Plant Physiology and Plant Molecular Biology*, *52*, 89–118.

Nauš, J., Prokopová, J., Řebíček, J., & Špundová, M. (2010). SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. *Photosynthesis Research*, *105*, 265–271.

Noodén, L. D., Guiamét, J. J., & John, I. (1997). Senescence mechanisms. *Physiologia Plantarum*, *101*, 746–753.

Noodén, L. D., Hillsberg, J. W., & Schneider, M. J. (1996). Induction of leaf senescence in *Arabidopsis thaliana* by long days through a light-dosage effect. *Physiologia Plantarum*, *96*, 491–495.

Novák, O., Hauserová, E., Amakorová, P., Doležal, K., & Strnad, M. (2008). Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. *Phytochemistry*, *69*, 2214–2224.

Novák, O., Tarkowski, P., Tarkowská, D., Doležal, K., Lenobel, R., & Strnad, M. (2003). Quantitative analysis of cytokinins in plants by liquid chromatography-single-quadrupole mass spectrometry. *Analytica Chimica Acta*, *480*, 207–218.

Oh, E., Kang, H., Yamaguchi, S., Park, J., Lee, D., Kamiya, Y., & Choi, G. (2009). Genome-wide analysis of genes targeted by PHYTOCHROME INTERACTING FACTOR 3-LIKE5 during seed germination in *Arabidopsis*. *Plant Cell*, *21*, 403–419.

Okada, K., Inoue, Y., Satoh, K., & Katoh, S. (1992). Effects of light on degradation of chlorophyll and proteins during senescence of detached rice leaves. *Plant and Cell Physiology*, *33*, 1183–1191.

Perilli, S., Moubayidin, L., & Sabatini, S. (2010). The molecular basis of cytokinin function. *Current Opinion in Plant Biology*, *13*, 21–26.

Pilz, J., Meineke, I., & Gleiter, C. H. (2000). Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. *Journal of Chromatography B*, *742*, 315–325.

Riefler, M., Novák, O., Strnad, M., & Schmülling, T. (2006). *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell*, *18*, 40–54.

Roberts, I. N., Caputo, C., Kade, M., Criado, M. V., & Barneix, A. J. (2011). Subtilisin-like serine proteases involved in N remobilization during grain filling in wheat. *Acta Physiologiae Plantarum*, *33*, 1997–2001.

Romanov, G. A., Lomin, S. N., & Schmülling, T. (2006). Biochemical characteristics and ligand-binding properties of *Arabidopsis* cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. *Journal of Experimental Botany*, *57*, 4051–4058.

Sakakibara, H. (2006). Cytokinins: Activity, biosynthesis, and translocation. *Annual Review of Plant Biology*, *57*, 431–449.

Schäfer, M., Brütting, C., Meza-Canales, I. D., Grosskinsky, D. K., Vankova, R., Baldwin, I. T., & Meldau, S. (2015). The role of *cis*-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. *Journal of Experimental Botany*, *66*, 4873–4884.

Singh, S., Letham, D. S., & Palni, L. M. S. (1992). Cytokinin biochemistry in relation to leaf senescence. VIII. Translocation, metabolism and biosynthesis of cytokinins in relation to sequential leaf senescence of tobacco. *Physiologia Plantarum*, *86*, 398–406.

Smart, C. M. (1994). Gene expression during leaf senescence. *New Phytologist*, *126*, 419–448.

Smart, C. M., Scofield, S. R., Bevan, M. W., & Dyer, T. A. (1991). Delayed leaf senescence in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium*. *Plant Cell*, *3*, 647–656.

Šmehilová, M., Dobrůšková, J., Novák, O., Takáč, T., & Galuszka, P. (2016). Cytokinin-specific glycosyltransferases possess different roles in cytokinin homeostasis maintenance. *Frontiers in Plant Science*, *7*, 1264.

Spíchal, L. (2012). Cytokinins—Recent news and views of evolutionally old molecules. *Functional Plant Biology*, *39*, 267–284.

Spíchal, L., Rakova, N. Y., Riefler, M., Mizuno, T., Romanov, G. A., Strnad, M., & Schmülling, T. (2004). Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. *Plant and Cell Physiology*, *45*, 1299–1305.

Špundová, M., Popelková, H., Ilík, P., Skotnica, J., Novotný, R., & Nauš, J. (2003). Ultra-structural and functional changes in the chloroplasts of detached barley leaves senescing under dark and light conditions. *Journal of Plant Physiology*, *160*, 1051–1058.

Špundová, M., Slouková, K., Hunková, M., & Nauš, J. (2005). Plant shading increases lipid peroxidation and intensifies senescence-induced changes in photosynthesis and activities of ascorbate peroxidase and glutathione reductase in wheat. *Photosynthetica*, *43*, 403–409.

Van Staden, J., Cook, E., & Noodén, L. D. (1988). Cytokinins and senescence. In L. D. Noodén, & A. Leopold (Eds.), *Senescence and aging in plants* (pp. 281–328). London: Academic Press.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H., & Mizuno, T. (2001). The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. *Plant and Cell Physiology*, *42*, 107–113.

Svačinová, J., Novák, O., Plačková, L., Lenobel, R., Holík, J., Strnad, M., & Doležal, K. (2012). A new approach for cytokinin isolation from Arabidopsis tissues using miniaturized purification: Pipette tip solid-phase extraction. *Plant Methods*, *8*, 17.

Sweere, U., Eichenberg, K., Lohrmann, J., Mira-Rodado, V., Bäurle, I., Kudla, J., … Harter, K. (2001). Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. *Science*, *294*, 1108–1111.

Takáč, T., Šamajová, O., Vadovič, P., Pechan, T., Košútová, P., Ovečka, M. …., & Šamaj, J. (2014). Proteomic and biochemical analyses show a functional network of proteins involved in antioxidant defense of the *Arabidopsis anp2anp3* double mutant. *Journal of Proteome Research*, *13*, 5347–5361.

Tao, G.-Q., Letham, D. S., Palni, L. M. S., & Summons, R. E. (1983). Cytokinin biochemistry in relation to leaf senescence I. The metabolism of 6-benzylaminopurine and zeatin in oat leaf segments. *Journal of Plant Growth Regulation*, *2*, 89–102.

Thimann, K. V. (1985). The senescence of detached leaves of *Tropaeolum*. *Plant Physiology*, *79*, 1107–1110.

Thomas, H. (1978). Enzymes of nitrogen mobilization in detached leaves of *Lolium temulentum* during senescence. *Planta*, *142*, 161–169.

Todorov, D., Alexieva, V., & Karanov, E. (1998). Effect of putrescine, 4-PU-30, and abscisic acid on maize plants grown under normal, drought, and rewatering conditions. *Journal of Plant Growth Regulation*, *17*, 197–203.

Ueguchi, C., Sato, S., Kato, T., & Tabata, S. (2001). The AHK4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in *Arabidopsis thaliana*. *Plant and Cell Physiology*, *42*, 751–755.

Vandenbussche, F., Habricot, Y., Condiff, A. S., Maldiney, R., Van der Straeten, D., & Ahmad, M. (2007). HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*. *Plant Journal*, *49*, 428–441.

Vescovi, M., Riefler, M., Gessuti, M., Novák, O., Schmülling, T., & Lo Schiavo, F. (2012). Programmed cell death induced by high levels of cytokinin in Arabidopsis cultured cells is mediated by the cytokinin receptor CRE1/AHK4. *Journal of Experimental Botany*, *63*, 2825–2832.

Vlčková, A., Špundová, M., Kotabová, E., Novotný, R., Doležal, K., & Nauš, J. (2006). Protective cytokinin action switches to damaging during senescence of detached wheat leaves in continuous light. *Physiologia Plantarum*, *126*, 257–267.

Vylíčilová, H., Husičková, A., Spíchal, L., Srovnal, J., Doležal, K., Plíhal, O., & Plíhalová, L. (2016). C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus. *Phytochemistry*, *122*, 22–33.

Weaver, L. M., & Amasino, R. M. (2001). Senescence is induced in individually darkened Arabidopsis leaves but inhibited in whole darkened plants. *Plant Physiology*, *127*, 876–886.

Werner, T., Köllmer, I., Bartrina, I., Holst, K., & Schmülling, T. (2006). New insights into the biology of cytokinin degradation. *Plant Biology*, *8*, 371–381.

Wingler, A., von Schaewen, A., Leegood, R. C., Lea, P. J., & Quick, W. P. (1998). Regulation of leaf senescence by cytokinin, sugars, and light. Effects on NADH-dependent hydroxypyruvate reductase. *Plant Physiology*, *116*, 329–335.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., … Mizuno, T. (2001). The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant and Cell Physiology*, *42*, 1017–1023.

Zacarias, L., & Reid, M. S. (1990). Role of growth-regulators in the senescence of *Arabidopsis thaliana* leaves. *Physiologia Plantarum*, *80*, 549–554.

Zdarska, M., Dobisová, T., Gelová, Z., Pernisová, M., Dabravolski, S., & Hejátko, J. (2015). Illuminating light, cytokinin, and ethylene signalling crosstalk in plant development. *Journal of Experimental Botany*, *66*, 4913–4931.

Zhang, H. S., & Zhou, C. J. (2013). Signal transduction in leaf senescence. *Plant Molecular Biology*, *82*, 539–545.

Zubo, Y. O., Yamburenko, M. V., Selivankina, S. Y., Shakirova, F. M., Avalbaev, A. M., Kudryakova, N. V., … Börner, T. (2008). Cytokinin stimulates chloroplast transcription in detached barley leaves. *Plant Physiology*, *148*, 1082–1093.

Zwack, P. J., & Rashotte, A. M. (2013). Cytokinin inhibition of leaf senescence. *Plant Signaling and Behavior*, *8*, e24737.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Content of DHZ forms (pmol/g of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL, 120 µmol photons m^{-2} s^{-1}) or higher light (HL, 400 µmol photons m^{-2} s^{-1}). Means and SD (n = 3) are presented; n.d., under detection limit. Statistically significant differences (compared to WT, $P < 0.05$) are indicated in bold.

Table S2. Content of *m*T forms (pmol/g of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL, 120 µmol photons m^{-2} s^{-1}) or higher light (HL, 400 µmol photons m^{-2} s^{-1}). Means and SD (n = 3) are presented; n.d., under detection limit.

How to cite this article: Janečková H, Husičková A, Ferretti U, et al. The interplay between cytokinins and light during senescence in detached *Arabidopsis* leaves. *Plant Cell Environ*. 2018;41:1870–1885. https://doi.org/10.1111/pce.13329

Plant Physiology and Biochemistry 136 (2019) 43–51

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Exogenous application of cytokinin during dark senescence eliminates the acceleration of photosystem II impairment caused by chlorophyll *b* deficiency in barley

Helena Janečková, Alexandra Husičková, Dušan Lazár, Ursula Ferretti, Pavel Pospíšil, Martina Špundová*

Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Biophysics, Faculty of Science, Palacký University, Šlechtitelů 241/27, Olomouc, 783 71, Czech Republic

ARTICLE INFO

Keywords:
6-Benzylaminopurine
Chlorina
Chlorophyll fluorescence
Leaf senescence
OJIP
Photosystem II photochemistry
Xanthophylls

ABSTRACT

Recent studies have shown that chlorophyll (Chl) *b* has an important role in the regulation of leaf senescence. However, there is only limited information about senescence of plants lacking Chl *b* and senescence-induced decrease in photosystem II (PSII) and photosystem I (PSI) function has not even been investigated in such plants. We have studied senescence-induced changes in photosynthetic pigment content and PSII and PSI activities in detached leaves of Chl *b*-deficient barley mutant, *chlorina f2*^{f2} (*clo*). After 4 days in the dark, the senescence-induced decrease in PSI activity was smaller in *clo* compared to WT leaves. On the contrary, the senescence-induced impairment in PSII function (estimated from Chl fluorescence parameters) was much more pronounced in *clo* leaves, even though the relative decrease in Chl content was similar to wild type (WT) leaves (*Hordeum vulgare* L., cv. Bonus). The stronger impairment of PSII function seems to be related to more pronounced damage of reaction centers of PSII. Interestingly, exogenously applied plant hormone cytokinin 6-benzylaminopurine (BA) was able to maintain PSII function in the dark senescing *clo* leaves to a similar extent as in WT. Thus, considering the fact that without BA the senescence-induced decrease in PSII photochemistry in *clo* was more pronounced than in WT, the relative protective effect of BA was higher in Chl *b*-deficient mutant than in WT.

1. Introduction

Leaf senescence, a final stage of leaf life preceding its death, is important for plant with respect to nutrient remobilization. Leaf senescence is accompanied by a massive degradation of chlorophyll (Chl) and by inhibition of photosynthetic processes including photosystem II (PSII) photochemistry (Oh et al., 1996; Špundová et al., 2003, 2005; Vlčková et al., 2006; Kusaba et al., 2007; Talla et al., 2016; Janečková et al., 2018) and photosystem I (PSI) activity (Nath et al., 2013; Krieger-Liszkay et al., 2015). In the literature, there is no consensus whether the decrease of photosynthetic activity of PSII precedes the inhibition of PSI or *vice versa* (e.g., Nath et al., 2013; Krieger-Liszkay et al., 2015).

Leaf senescence is regulated by many factors, including plant hormones cytokinins. Cytokinins are known to slow down senescence, decelerate senescence-associated degradation of photosynthetic pigments and deterioration of photosynthetic function (Oh et al., 2005; Vlčková et al., 2006; Talla et al., 2016; Vylíčilová et al., 2016). Recent investigations have shown that Chl *b* also plays an important role in the regulation of leaf senescence. Mutants with higher Chl *b* content appear to have slower senescence-related degradation of Chl, light-harvesting

Abbreviations: ABS/RC, apparent antenna size of active reaction center of photosystem II; BA, 6-benzylaminopurine; CAO, chlorophyllide *a* oxygenase; car, carotenoids (sum of carotenes and xanthophylls); Chl, chlorophyll; *clo*, *chlorina f2*^{f2} mutant; DEPS, the de-epoxidation state of xanthophylls; $(dV/dt)_0$, the initial slope of the O-J chlorophyll fluorescence rise; F_v/F_m, maximal quantum yield of photosystem II photochemistry in the dark-adapted state; F_v'/F_m', the maximal quantum yield of photosystem II photochemistry in the light-adapted state; LHC(s), light-harvesting complex(es); OJIP, chlorophyll fluorescence induction transient; PSI, photosystem I; PSII, photosystem II; P700, primary electron donor of photosystem I; RCI, reaction center(s) of photosystem I; RCII, reaction center(s) of photosystem II; RE_0/ABS, quantum yield of electron transport from reduced Q_A to final acceptors of photosystem I; VAZ, content of xanthophylls (violaxanthin, antheraxanthin, and zeaxanthin); V_J, the relative variable fluorescence at the J step of OJIP curve; δR_0, the efficiency of electron transport from reduced plastoquinone to final acceptors of photosystem I; $\Phi_{f,D}$, quantum yield of constitutive non-regulatory dissipation processes in the light-adapted state; Φ_{NPQ}, quantum yield of regulatory non-photochemical quenching in the light-adapted state; Φ_P, the effective quantum yield of PSII photochemistry in the light-adapted state

* Corresponding author.
E-mail address: martina.spundova@upol.cz (M. Špundová).

https://doi.org/10.1016/j.plaphy.2019.01.005
Received 21 September 2018; Received in revised form 19 December 2018; Accepted 3 January 2019
Available online 04 January 2019
0981-9428/

complexes (LHCs) and thylakoid membranes (Kusaba et al., 2007; Sakuraba et al., 2012; Voitsekhovskaja and Tyutereva, 2015). At the same time, a recent study with *pgl* rice mutant has shown that Chl *b* deficiency was associated with increased Chl degradation, accumulation of reactive oxygen species, and electrolyte leakage during both natural senescence of flag leaves and dark-induced senescence of detached leaves (Yang et al., 2016). Kusaba et al. (2007) has also mentioned faster Chl degradation in dark-incubated detached leaves of *cao-2* rice mutant deficient in Chl *b*. Although these studies suggest that senescence-related changes are accelerated in plants lacking Chl *b*, the question whether and how Chl *b* deficiency affects senescence-induced inhibition of PSII and PSI function has not been addressed yet.

In order to broaden knowledge about the effect of missing Chl *b* on senescence, we have studied the changes in Chl and carotenoid (car) content and changes in PSII and PSI activity in dark-senescing detached leaves of *chlorina f2*^{f2} (*clo*) barley mutant. The *clo* mutant is deficient in Chl *b* due to the mutation in chlorophyllide *a* oxygenase (CAO), the enzyme responsible for the conversion of chlorophyllide *a* to chlorophyllide *b* and thus crucial for biosynthesis of Chl *b* (Mueller et al., 2012). The *clo* mutant has also lower contents of Chl *a* and car compared to WT (Štroch et al., 2004, 2008). The mutant is deficient in light-harvesting complexes Lhcb1, Lhcb6 and Lhca4, and has reduced amount of Lhcb2, Lhcb3 and Lhcb4 (Bossmann et al., 1997). The amount of LHCs of PSII and PSI is reduced by about 80 % and 20 %, respectively (Ghirardi et al., 1986). The more reduced amount of LHCs of PSII (LHCII) is in *chlorina* mutants compensated by an increased amount of reaction centers of PSII (RCII) and a greater ratio of RCII/RCI (Ghirardi et al., 1986).

The *chlorina* mutants generally have similar or only slightly lower efficiency of PSII photochemistry (Leverenz et al., 1992; Štroch et al., 2004, 2008) and oxygen evolution (Havaux and Tardy, 1997) than WT plants. However, under stress conditions such as high light or high temperature, the PSII efficiency is more reduced in the mutants (Leverenz et al., 1992; Havaux and Tardy, 1997; Peng et al., 2002; Štroch et al., 2008; Tyutereva et al., 2017) than in WT. The increased stress-sensitivity of the PSII photochemistry in the *chlorina* mutant has been attributed to its reduced amount of LHCs, resulting from missing Chl *b* (Havaux and Tardy, 1997).

In this work, we have studied how the Chl *b* deficiency in *clo* mutant changes the progress of dark senescence of detached leaves, with special focus given on the description of senescence-induced changes in the function of PSII. As cytokinins are known decelerators of senescence, we also wanted to find out whether and to what extent is exogenously applied cytokinin 6-benzylaminopurine able to suppress the supposedly pronounced senescence in Chl *b*-deficient *clo* mutant.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of wild-type barley (*Hordeum vulgare* L. cv. Bonus; WT) and *chlorina f2*^{f2} (*clo*) mutant were soaked in deionized water for 24 h before sowing and then transferred into pots containing perlite with Hoagland solution. Pots were placed in a growth chamber under controlled conditions of 16 h light (150 µmol of photons m^{-2} s^{-1})/8 h dark, 22/20 °C and 60% relative air humidity.

Eight days after the sowing, 4-cm segments were cut off from the primary leaves. Leaf segments were placed either into a 0.2% solution of dimethylsulfoxide (DMSO) or into a 10^{-5} mol l^{-1} solution of 6-benzylaminopurine (BA) in 0.2% DMSO (BA treated leaves). The leaf segments were then kept in the dark (other conditions were same as during plant growth). Measurements were performed immediately after the leaf detachment and on the 4th day after detachment.

2.2. Pigment analysis

For the determination of the content of pigments, the area of leaf samples was estimated and then the leaves were homogenized in liquid nitrogen, with $MgCO_3$ and 80% acetone. The homogenates were centrifuged at 4,000*g* and 4 °C for 10 min. The supernatant was used for the spectrophotometric estimation of Chl and total car contents (a sum of carotenes and xanthophylls) according to Lichtenthaler (1987) by a spectrophotometer Unicam UV550 (ThermoSpectronic, United Kingdom) and also for the quantification of individual xanthophylls (violaxanthin, V; antheraxanthin, A; zeaxanthin, Z) by high performance liquid chromatography (HPLC).

For the estimation of xanthophyll content (VAZ) by an HPLC system (Alliance e 2695 HPLC System, Waters, USA), the supernatant was filtered through 0.45µm PTFE membrane (Acrodisc, Waters, USA) into dark vials. The amount of 100 µl was injected into the HPLC system. A LiChroCART RP-18 (5 µm; 4.6 × 250 mm) column (Merck & Co., USA) was used. The analysis was performed by a gradient reverse-phase analysis (1.5 ml min^{-1} at 25 °C). The analysis started with isocratic elution using the mobile phase composed of acetonitrile, methanol and 0.1 mol l^{-1} Tris (pH 8) in the ratio 87:10:3 (v:v:v) for 10 min and was followed by a 2-min linear gradient using mobile phase composed of a mixture of methanol and n-hexane in the ratio 4:1 (v:v). Absorbance was detected at 440 nm using UV/VIS detector. The amount of pigments in samples was determined using their conversion factors (Färber and Jahns, 1998). The de-epoxidation state of xanthophylls (DEPS) was calculated according to Gilmore and Björkman (1994) as $(A + Z)/(V + A + Z) \times 100$ (%).

2.3. Chlorophyll fluorescence measurements

The Chl fluorescence induction transient (OJIP curves) and the quenching analysis were measured at room temperature on adaxial side of leaf samples. Freshly detached leaves (i.e., leaves before senescence induction) were dark-adapted for 25 min before the measurement. The OJIP curves were measured in the middle of leaf segments by Plant Efficiency Analyser (Hansatech Instruments, United Kingdom) for 2 s with excitation light intensity of 1100 µmol of photons m^{-2} s^{-1}. The initial slope of the O-J Chl fluorescence rise $(dV/dt)_O$, the relative variable fluorescence at the J step (V_J), and the specific energy flux *ABS/RC* were evaluated as follows (see Stirbet et al., 2018). The $(dV/dt)_O = 4(F_{300\mu s} - F_{50\mu s})/F_v$, where $F_{300\mu s}$ and $F_{50\mu s}$ are fluorescence intensities at the indicated times and F_v is variable fluorescence ($F_v = F_p - F_O$; F_0 is a minimal fluorescence and F_p is fluorescence at the P step). The $(dV/dt)_O$ parameter, defined as the maximal rate of the accumulation of the fraction of closed reaction centers of PSII (RCII) (Strasser et al., 2000), reflects the rate of excitation supply into the RCII and subsequently the rate of Q_A reduction. Parameter V_J, reflecting the fraction of reduced Q_A, was calculated as $(F_J - F_0)/F_v$, where F_J is fluorescence intensity at 2 ms. *ABS/RC* was calculated as $(dV/dt)_O/V_J \times F_p/F_v$ and reflects apparent antenna size of active RCII (Strasser et al., 2000). Further, the quantum yield of electron transport from reduced Q_A to final acceptors of PSI (RE_O/ABS) and the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI (δR_0) were estimated as follows: $RE_O/ABS = F_v/F_p \times (1 - V_I)$ and $\delta R_O = (1 - V_I)/(1 - V_J)$ (Stirbet et al., 2018). The measured OJIP curves as well as curves normalized to F_v are presented.

The quenching analysis was performed using PlantScreen (Photon Systems Instruments, Czech Republic) phenotyping platform (Humplík et al., 2015) according to the following protocol. At the beginning, the minimal fluorescence F_0 was determined using measuring flashes (duration of 10 µs) of red light (650 nm), which did not cause any closure of RCII. Then a saturating pulse (white light, 1900 µmol of photons m^{-2} s^{-1}, duration of 800 ms) was applied to measure maximal fluorescence F_m. After 90 s of dark-relaxation, when the measured fluorescence signal reached F_0, the leaf samples were exposed to actinic

light for 25 min (red light, 150 μmol of photons m^{-2} s^{-1}, the same intensity as used for plant growth). To determine the maximal fluorescence during the actinic light exposition (F_m'), a set of the saturating pulses was applied. The first pulse was applied 10 s after the actinic light was switched on and was followed by 9 pulses in 20 s intervals and then by 22 pulses in 59 s intervals.

The maximal quantum yield of PSII photochemistry in the dark-adapted state was estimated as $F_v/F_m = (F_m - F_0)/F_m$. The maximal quantum yield of PSII photochemistry in the light-adapted state was calculated as $F_v'/F_m' = (F_m' - F_0')/F_m'$, where F_0' is minimal fluorescence for the light-adapted state, which was calculated as $F_0/(F_v/F_m + F_0/F_m')$. The effective quantum yield of PSII photochemistry in the light-adapted state was calculated as $\Phi_P = (F_m' - F_t)/F_m'$, where F_t is fluorescence at time t measured immediately prior to the application of the saturating pulse. The quantum yield for regulatory non-photochemical quenching was calculated as $\Phi_{NPQ} = (F_t/F_m') - (F_t/F_m)$ and the quantum yield for constitutive non-regulatory dissipation processes was calculated as $\Phi_{f,D} = F_t/F_m$. The sum of Φ_P, Φ_{NPQ} and $\Phi_{f,D}$ equals unity (for a review, see Lazár, 2015). In the case of F_v'/F_m', Φ_P, Φ_{NPQ}, and $\Phi_{f,D}$, values obtained at the end of the actinic light exposition are presented.

2.4. Measurement of P700 oxidation

For estimation of light-induced oxidation of P700 (the primary electron donor of PSI), the I830 signal as a difference of transmittance at 875 nm and 830 nm was determined using Dual PAM 100 (Walz, Germany), see, e.g. Lazár (2013). The methodology assumes that P700 is fully reduced in the dark-adapted leaf and thus the I830 signal is zero. During illumination of the leaf, the I830 signal rises to a peak level reflecting an equilibrated maximal $P700^+$ level as a result of P700 oxidation by the charge separation and $P700^+$ reduction by plastocyanin. In both WT and *clo* leaves before senescence induction, the I830 signal reached the peak level at 17 ms. In senescing leaves, the level of $P700^+$ at 17 ms of illumination was expressed in % of the peak level observed in the leaves before senescence induction.

2.5. Statistical analysis

In all statistical testing, related data sets were first tested for normality (Kolmogorov-Smirnov test with Lilliefors' correction) and equality of variances (Levene Median test). If fulfilled, the Student's *t*-test or ANOVA test (with all pairwise multiple comparison by Holm-Sidak *post hoc* test) were used and if not fulfilled, the Mann-Whitney Rank Sum test or Kruskal-Wallis ANOVA on Ranks test (with all pairwise multiple comparison by Dunn's *post hoc* test) were used. The critical level of 0.05 was chosen for all tests (the P-value of the test is marked by *). If the P-value of a test was even lower than 0.01 or even lower than 0.001, the results are marked by ** or ***, respectively. All testing was performed using SigmaPlot version 11 (Systat Software, USA).

3. Results

3.1. Characterization of clo leaves before senescence induction

Leaves of the *clo* mutant had approximately half the Chl content compared to WT (Table 1). The content of Chl *a* was lower by about 30 %, while Chl *b* was not detected (Table 1). The content of carotenoids (car; sum of carotenes and xanthophylls) was also lower in *clo* (by about 30 % compared to WT). As a result of relatively more lowered content of Chl than car, *clo* had significantly lower *Chl/car* ratio than the WT (Table 1). Leaves of *clo* had also lower content of xanthophylls (VAZ) (by about 25 %; Table 1). However, the *VAZ/Chl* ratio and de-epoxidation state of the xanthophyll cycle pigment pool (DEPS) were higher in *clo* (Table 1), which indicates better photoprotection of photosynthetic apparatus in *clo* compared to WT.

Table 1
The content of pigments (mg per m^2 of leaf area), their ratios and maximal efficiency of PSII photochemistry in dark- (F_v/F_m) and light-adapted (F_v'/F_m') state in leaves of WT and *clo* mutant before senescence induction.

	WT	*clo*
Chl *a*	176 ± 24	**121 ± 4**
Chl *b*	51 ± 7	n. d.
Chl *a*+*b*	227 ± 31	**121 ± 4**
Chl *a*/*b*	3.4 ± 0.1	n. d.
car	43 ± 5	**30 ± 1**
Chl/car	5.3 ± 0.4	**4.0 ± 0.1**
VAZ	16.6 ± 1.5	**12.3 ± 1.0**
VAZ/Chl	0.074 ± 0.004	**0.102 ± 0.006**
DEPS (%)	2.4 ± 1.0	**4.3 ± 0.6**
F_v/F_m	0.802 ± 0.011	**0.792 ± 0.004**
F_v'/F_m'	0.776 ± 0.003	**0.720 ± 0.010**

Means and SD (n = 3–10 for pigments and n = 6 for fluorescence parameters) are presented; n. d., not determined. Statistically significant differences (compared to WT, $P < 0.05$, *t*-test, except of DEPS where Mann-Whitney Rank Sum test was used) are indicated in bold.

Besides the generally reduced content of photosynthetic pigments, the maximal quantum yield of PSII photochemistry in both dark-adapted state (F_v/F_m) and light-adapted state (F_v'/F_m') was slightly lowered in *clo* (Table 1, Fig. 1C). To determine whether *clo* had altered partitioning of absorbed light energy for photochemical and non-photochemical processes, the following parameters were evaluated in the light-adapted state: the effective quantum yield of PSII photochemistry (Φ_P), quantum yield of constitutive non-regulatory dissipation processes ($\Phi_{f,D}$) and quantum yield of regulatory non-photochemical quenching (Φ_{NPQ}). Together the sum of these quantum yields equals unity (Lazár, 2015). In *clo,* a slightly but significantly lower Φ_P and higher $\Phi_{f,D}$ and Φ_{NPQ} were observed (Fig. 1D), which indicates that lower fraction of absorbed light energy was used by PSII photochemistry and that more absorbed energy was dissipated *via* non-photochemical quenching processes.

As the *clo* mutant is deficient in Chl *b* (Table 1) and consequently in LHCII (Ghirardi et al., 1986; Bossmann et al., 1997), a lower supply of excitations from LHCII to RCII can be expected. It should affect transient of Chl fluorescence induction (OJIP curve), as this curve reflects closure of RCII (Lazár, 2006) that depends on the rate of excitation supply. The typical OJIP curve was observed in the *clo* leaves, although the overall fluorescence signal was lower compared to WT (Fig. 2A). From normalized curves it is obvious that the J- and I-steps are both reached later in the *clo* leaves (Fig. 2B) than in WT, which reflects a slower reduction of Q_A as well as Q_B. This slower reduction consequently results in a lower transient accumulation of reduced Q_A, which is in turn reflected in a lower J-step. The lower relative height of J-step is quantitatively expressed by a lower V_J parameter (by about 14 %) (Fig. 2B and C). The slower Q_A reduction in the *clo* leaves is further indicated by $(dV/dt)_o$, which was lower by about 40 % (Fig. 2C) than in WT. Finally, a lower *ABS/RC* ratio (by about 25 % compared to WT; Fig. 2C) confirmed deficiency of LHCII in the *clo* leaves, as this ratio reflects an apparent antenna size of active RCII (Stirbet et al., 2018).

On the other hand, parameters of the OJIP curve reflecting electron transport to PSI, RE_o/ABS (the quantum yield of electron transport from reduced Q_A to final acceptors of PSI) and δ_{R0} (the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI) were higher in *clo* by 46 and 44 %, respectively (Fig. 2D). Finally, a relative amount of $P700^+$ was lower in *clo* (Fig. 2D).

3.2. Comparison of dark senescence-induced changes in WT and clo detached leaves

To induce senescence, leaves of WT and *clo* were detached and

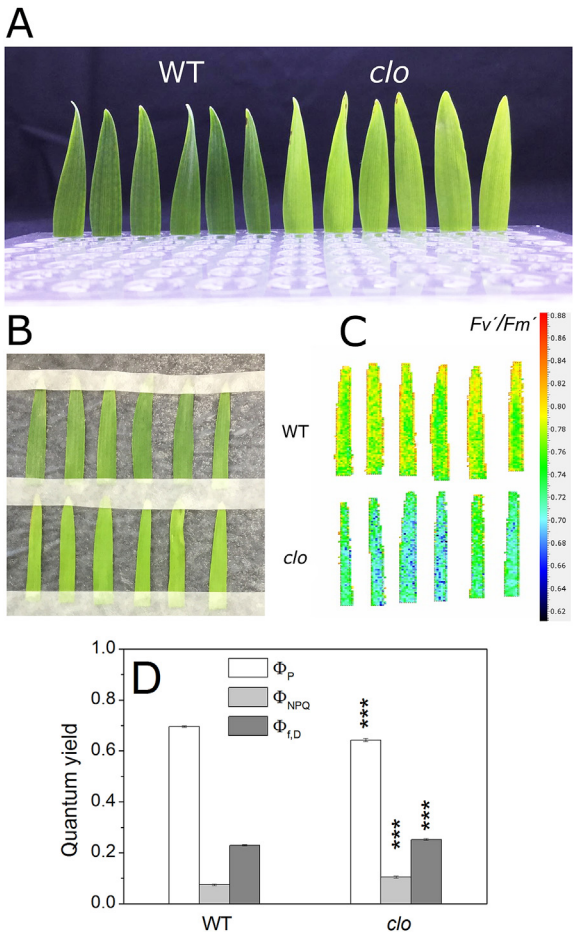

Fig. 1. Characterization of detached WT and *clo* leaves before senescence induction. A, phenotype; B, leaf segments used for the measurement of F_v'/F_m'; C, F_v'/F_m' in the area of the leaf segments; D, quantum yield of PSII photochemistry (Φ_P), regulatory non-photochemical quenching (Φ_{NPQ}), and constitutive non-regulatory dissipation processes ($\Phi_{f,D}$). Means and SD are presented, n = 6–10. Asterisks indicate statistically significant difference (Student's *t*-test) between WT and *clo* ($P < 0.001$).

subsequently incubated in control solution (0.2% DMSO) in the dark for 4 days. This incubation resulted in a significant decrease in Chl, car and VAZ content in all detached leaves (Fig. 3). The Chl content decreased by 82 % in WT and 87 % in *clo* (Fig. 3) and although the relative decrease in Chl content was similar in WT and *clo*, the absolute Chl content was pronouncedly lower in the *clo* senescing leaves (about 16 mg Chl m^{-2} compared to about 40 mg Chl m^{-2} in WT). As indicated by a decrease in Chl *a/b* ratio, the content of Chl *a* decreased in WT slightly more than Chl *b* (Fig. 3).

The content of car decreased in WT by about 60 %, whereas in *clo* this content was reduced only by about 40 % (Fig. 3). Similarly, the VAZ content decreased more in WT (by about 70 %) than in *clo* (by about 55 %; Fig. 3). The faster breakdown of Chl compared to car caused a significant decrease in the *Chl/car* ratio in both genotypes, more pronounced in *clo* (Fig. 3). In summary, the relative decrease in Chl content was similar in both *clo* and WT, but the relative decrease in the content of car and VAZ in *clo* was lower than in WT.

The loss of photosynthetic pigments during dark-induced senescence was associated with a decline in the maximal quantum yield of PSII photochemistry in the dark-adapted state in both *clo* and WT. Parameter F_v/F_m dropped by about 45 % in WT leaves and by about 70 % in *clo* (Fig. 4A), which indicates more pronounced impairment of PSII function in *clo* compared to WT. In fact, the real impairment of PSII function was much more pronounced in *clo*, because a considerable part of the area of measured leaves was already not photosynthetically functional enough for Chl fluorescence detection (i.e., the Chl fluorescence signal from *clo* leaves was so small that it was not distinguishable from a background signal, Fig. 4B) and these leaf parts were not included into the average F_v/F_m value (Fig. 4A). Thus the average F_v/F_m value is representative only for the (minimally) functional parts of leaves.

The decrease in F_v/F_m was accompanied by a decrease in Φ_P in both *clo* and WT, indicating decreased energy utilization by PSII photochemistry in the light-adapted state. The Φ_P value in *clo* was significantly lower than in WT (Fig. 5). On the other hand, Φ_{NPQ} and $\Phi_{f,D}$ increased in senescing leaves, indicating enhanced energy dissipation by means of non-photochemical processes. Unlike the leaves before senescence induction, the partitioning of absorbed light energy into regulatory or non-regulatory dissipation processes differed pronouncedly in WT and *clo*. While Φ_{NPQ} and $\Phi_{f,D}$ were comparable in WT, in *clo* $\Phi_{f,D}$ prevailed (Fig. 5). It indicates that in WT, energy non-utilized by PSII photochemistry was dissipated in both regulatory and non-regulatory processes to a similar extent, while in *clo*, the majority of this energy was dissipated *via* non-regulatory processes. This corresponds to the extreme impairment of PSII function in *clo* (Fig. 4B).

After 4 days of incubation in the dark, the shape of OJIP curve and the height of its individual steps changed in WT as well as in *clo* (compare Figs. 2A and 6A). In senescing WT leaves, the OJIP curve was more flat than in non-senescing ones due to the pronounced increase in the height of the O-step and decrease in the height of the P-step (Fig. 6A). Additionally, the normalized curve showed a relative increase in the J-step (compare Figs. 2B and 6B), reflected also in the increased parameter V_J (1.5-times when compared to leaves before senescence induction; Fig. 6C). The $(dV/dt)_o$ parameter also increased, but more (2.5-times) than V_J, thus *ABS/RC* proportional to their ratio increased more pronouncedly (4-times). The increase of *ABS/RC* suggests increase in apparent antenna size of active RCII, which in turn indicates preferential impairment of RCII compared to LHCII. This results in increased supply of excitations to remaining active RCII and thus a pronounced Q_A reduction can be observed in these RCII. We propose that the preferential RCII impairment was caused by their degradation, as the Chl *a/b* ratio decreased in the WT senescing leaves (Fig. 3). Since Chl *b* occurs mainly in LHCII, the decrease in the Chl *a/b* ratio reflects a relative decrease in RCII abundance (Leong and Anderson, 1984).

RE_o/ABS as well as δR_0 decreased in the senescing WT leaves by about 80 % and 45 %, respectively (Fig. 6D). The greater decrease in RE_o/ABS in comparison to δR_0 indicates that the electron transport efficiency decreased more within PSII than behind PSII and that RCI degradation was lower than degradation of RCII. This assumption is supported by a lower relative amount of $P700^+$ (Fig. 6D).

In senescing leaves of *clo*, changes in the OJIP curve were much more profound than in WT. The typical OJIP shape was missing and the curve became almost flat (compare Figs. 2A and 6A). The relative height of the J-step increased (V_J increased twice compared to the leaves before senescence), $(dV/dt)_o$ increased 4-times and *ABS/RC* increased 15-times (Fig. 6B and C). The extreme increase in *ABS/RC* was related also to very pronounced decrease in F_v/F_m. It means that the impairment of RCII during dark-senescence was much more pronounced in *clo* than in WT, which corresponds to more severe inhibition of PSII photochemistry described above.

Similarly to WT, parameters of the OJIP curve reflecting electron transport to PSI, RE_o/ABS and δR_0, decreased in the senescing leaves of *clo* (Fig. 6D). The decrease was again more pronounced in the case of RE_o/ABS (by 98 %) than in δR_0 parameter (by about 70 %), which indicates more pronounced impairment of electron transport within PSII than behind this complex and the preferential decrease in RCII compared to RCI. The relative amount of $P700^+$ was higher than in the senescing leaves of WT (Fig. 6D).

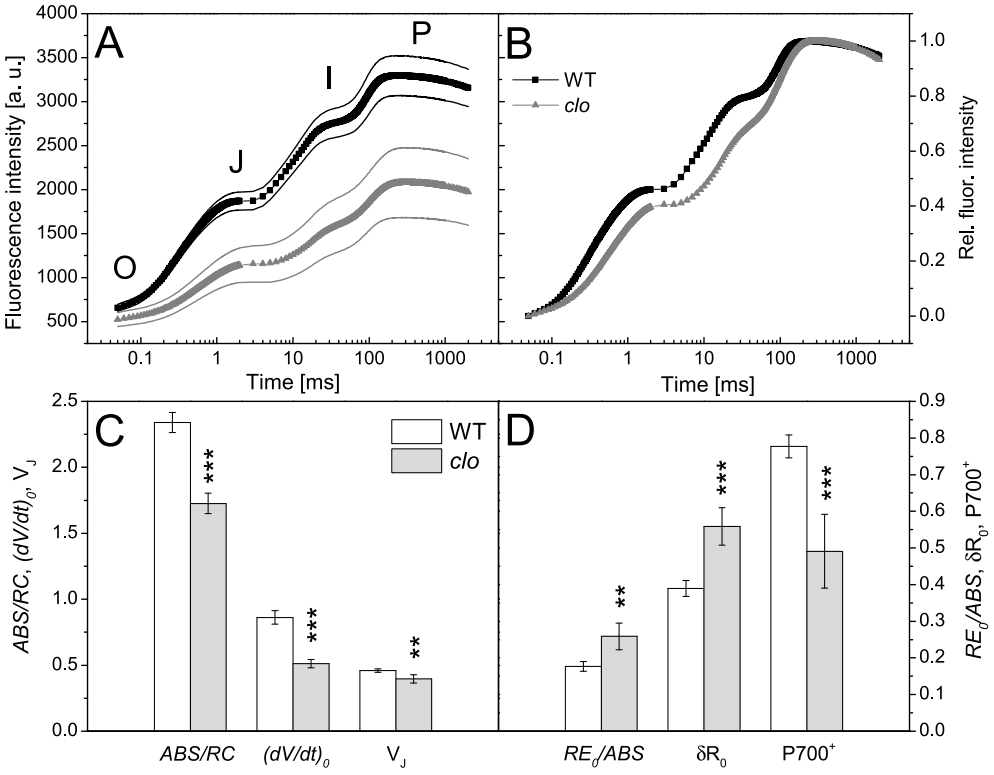

Fig. 2. Chl fluorescence induction transient (OJIP curves), related fluorescence parameters and changes in the PSI activity of detached WT and *clo* leaves before senescence induction. A, OJIP curves; B, the normalized OJIP curves; C, the apparent antenna size of active RCII (*ABS/RC*), the initial slope of the O-J fluorescence raise $(dV/dt)_O$, and the relative variable fluorescence at the J-step (V_J); D, quantum yield of electron transport from reduced Q_A to final acceptors of photosystem I (RE_O/ABS); the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI (δR_0) and the relative amount of oxidized primary electron donor of PSI, $P700^+$. Means and SD are presented, n = 6–7. Asterisks indicate statistically significant difference (Student's *t*-test) between WT and *clo* (**, P < 0.01; ***, P < 0.001).

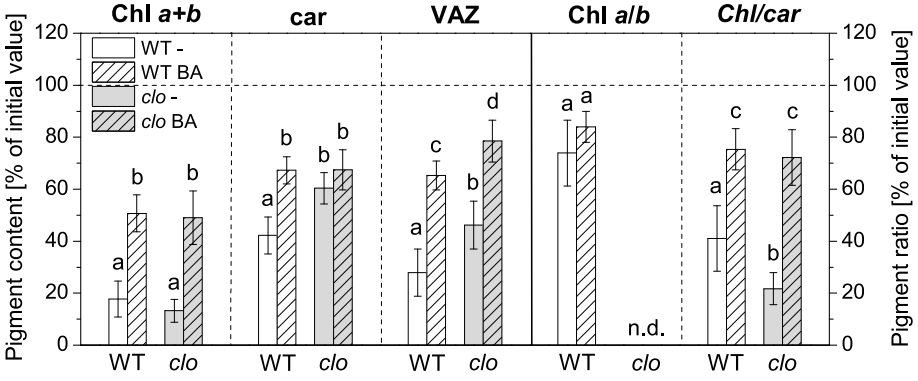

Fig. 3. Chlorophyll (Chl *a*+*b*), carotenoid (car) and xanthophyll (VAZ) content related to leaf area and Chl *a*/*b* and *Chl/car* ratios in detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (−) or with 6-benzylaminopurine (BA). Relative values (% of the initial values before senescence induction) are presented. Means and SD are shown, n = 6. Except of the Chl *a*/*b* ratio (analyzed by the *t*-test, P = 0.139), all other data were analyzed by ANOVA test (P < 0.001 in all cases) and statistically significant differences in following *post hoc* statistical testing (Holm-Sidak test) at P < 0.05 are indicated by different letters.

3.3. Effect of BA on senescence-induced changes in WT and clo leaves

To evaluate the effect of cytokinin on dark-senescing WT and *clo* leaves, detached leaves were incubated in BA (10^{-5} mol l^{-1}) solution and kept in the dark for 4 days. BA significantly reduced the degradation of photosynthetic pigments in both genotypes, the content of Chl and car decreased by about 50 % and 35 %, respectively (Fig. 3), and the *Chl/car* ratio by about 25 % (Fig. 3). The VAZ content decreased by about 35 % and 20 % in WT and *clo*, respectively (Fig. 3), and the Chl *a*/*b* ratio in WT leaves decreased by about 15 %.

BA also suppressed the senescence-induced decrease in PSII photochemistry in both WT and *clo* leaves (Figs. 4–6). In the presence of BA, F_v/F_m dropped only by 20 % during the senescence, which indicated that PSII photochemistry is relatively well maintained (Fig. 4A). This was also evidenced by a smaller decrease in Φ_P (i.e., utilization of absorbed light energy by PSII photochemistry) in both WT and *clo* (Fig. 5). In *clo* BA significantly suppressed the senescence-induced increase in $\Phi_{f,D}$ (Fig. 5).

The protective effect of BA on PSII function in senescing leaves was also reflected in less pronounced changes in the shape of OJIP curve (Fig. 6A) and smaller changes in corresponding parameters. In leaves undergoing senescence in the presence of BA, we have observed a smaller increase in relative height of the J-step (i.e., V_J) (Fig. 6B) and also the increase in *ABS/RC* and $(dV/dt)_O$ parameters was considerably smaller compared to leaves senescing in the absence of BA (Fig. 6C).

In both genotypes, BA suppressed the senescence-induced decrease in RE_O/ABS and δR_0; in *clo* the BA application even increased δR_0 by about 20 % (Fig. 6D). On the contrary, BA had no significant effect on the relative amount of $P700^+$ in either WT or *clo* (Fig. 6D).

The changes in parameters described above indicate that BA suppressed the senescence-induced impairment of PSII photochemistry in both WT and *clo*. Interestingly, in the presence of BA, the progress of senescence in *clo* became more similar to WT (Figs. 4–6), although in the absence of exogenous cytokinin the senescence-induced impairment of PSII function was much more pronounced in *clo*. The stronger effect of BA in the case of *clo* is further apparent from the significantly lower increase in *ABS/RC* and V_J (Fig. 6C). The more marked effect of BA on *clo* in comparison to WT was even more visible when an increased actinic light intensity (600 µmol of photons m^{-2} s^{-1}) was applied. In the untreated leaves of WT and *clo*, Φ_P was 0.13 and 0.14, respectively. In WT, BA improved Φ_P only non-significantly (to 0.25), while in *clo*, the Φ_P improvement (to 0.36) was statistically significant.

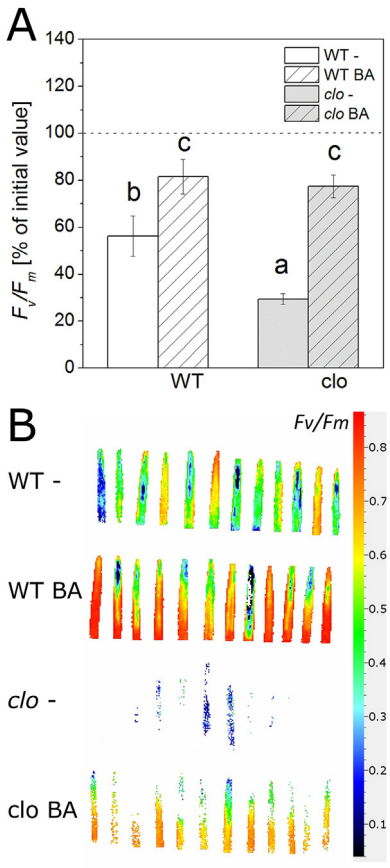

Fig. 4. The maximal efficiency of PSII photochemistry in dark-adapted state (F_v/F_m) in detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (−) or with 6-benzylaminopurine (BA). A, the relative F_v/F_m values (% of the initial values before senescence induction), means and SD estimated from measurable leaves are shown. Data were analyzed by ANOVA test ($P < 0.001$) and statistically significant difference in following *post hoc* statistical testing (Holm-Sidak test) at $P < 0.05$ are indicated by different letters. B, F_v/F_m in the area of detached WT and *clo* leaves.

4. Discussion

It has been reported that the Chl *b* deficiency accelerates senescence-related changes in rice (Kusaba et al., 2007; Yang et al., 2016). Faster Chl degradation was observed in detached leaves of Chl *b*-deficient rice mutant *cao-2* (Kusaba et al., 2007). Based on faster Chl degradation, increased accumulation of reactive oxygen species, and increased electrolyte leakage Yang et al. (2016) suggested faster senescence in *pgl* rice mutant with reduced Chl *b* content in case of naturally senescing flag leaves as well as in case of detached leaves kept in the dark. Nevertheless, deeper knowledge of senescence-associated impairment of photosynthetic apparatus including PSII and PSI function under Chl *b* deficiency is missing.

To find out whether the deficiency of Chl *b* accelerates senescence-induced impairment of PSII and PSI activities, we have investigated their changes (together with changes in photosynthetic pigment content) in detached leaves of the Chl *b*-deficient barley mutant senescing in the dark for 4 days. As cytokinins are known to partially protect photosynthetic activity during senescence (Oh et al., 2005; Vlčková et al., 2006; Talla et al., 2016), we have also studied the effect of exogenously applied BA and analyzed whether it is able to suppress the senescence-associated changes also in *clo*.

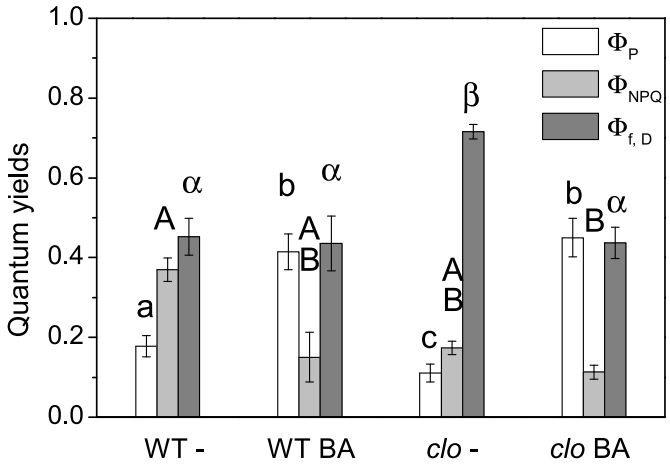

Fig. 5. Quantum yields of PSII photochemistry (Φ_P), regulatory non-photochemical quenching (Φ_{NPQ}), and constitutive non-regulatory dissipation processes ($\Phi_{f,D}$) of the detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (−) or with 6-benzylaminopurine (BA). Means and SD are shown, n = 6. Φ_P and $\Phi_{f,D}$ were analyzed by ANOVA test ($P < 0.001$ in both cases), followed by *post hoc* statistical testing (Holm-Sidak test) and Φ_{NPQ} was analyzed by Kruskal-Wallis ANOVA on Ranks test ($P < 0.001$), followed by *post hoc* statistical testing (Dunn's test). Statistically significant differences in the *post hoc* statistical testing at $P < 0.05$ are indicated by different letters.

4.1. Lower efficiency of PSII photochemistry in clo mutant before senescence induction

Leaves of *clo* mutant have lower content of photosynthetic pigments (Table 1). As expected due to the mutation in CAO (Mueller et al., 2012) and in agreement with literature (Štroch et al., 2004, 2008), Chl *b* was not detectable in *clo* (Table 1). Due to the lack of Chl *b*, the antenna size of PSII is substantially reduced in *clo*, as has been shown by lower abundance of LHCII proteins (Król et al., 1995; Bossmann et al., 1997) and by changes in emission and excitation Chl fluorescence spectra measured at 77 K (Štroch et al., 2004). We have confirmed the reduced functional size of LHCII in *clo* by lower *ABS/RC* ratio (Fig. 2C), reflecting lower amount of absorbed excitations per active (Q_A-reducing) RCII. The presence of smaller PSII antennae resulted in slower supply of excitations to the RCII, in slower Q_A reduction and smaller amount of reduced Q_A, which was evidenced by the lower $(dV/dt)_0$ and V_J parameters (Fig. 2C).

Despite the smaller LHCII in *clo*, the efficiency of electron transport behind PSII to PSI was higher compared to WT which corresponds to the higher ratio RCII/RCI in *chlorina f2* mutant reported by Ghirardi et al. (1986). The higher electron flow behind PSII probably led to the lower relative amount of $P700^+$ (Fig. 2D). The reduced size of PSI antennae might also contribute to the decreased relative amount of $P700^+$ as *clo* is known to be deficient in the light-harvesting complex Lhca4 (Bossmann et al., 1997). Consistent with this assumption, it has been shown that kinetics of P700 oxidation was much slower in a rice mutant *dye1-1* with a severely reduced amount of Lhca4 (Yamatani et al., 2018).

The *clo* leaves had slightly less effective PSII photochemistry as indicated by lower values of the maximal quantum yield of PSII photochemistry in the dark-adapted state (F_v/F_m; Table 1) and of the maximal and effective quantum yield of PSII photochemistry in the light-adapted state (as F_v'/F_m' and Φ_P; Table 1, Fig. 1D). The slightly lower quantum yield of PSII photochemistry of *chlorina* mutants has been reported previously (Leverenz et al., 1992; Štroch et al., 2004, 2008).

The light energy that is not utilized by PSII photochemistry is dissipated *via* non-regulatory ($\Phi_{f,D}$) and/or regulatory (Φ_{NPQ}) non-photochemical quenching processes. $\Phi_{f,D}$ represents quantum yield of

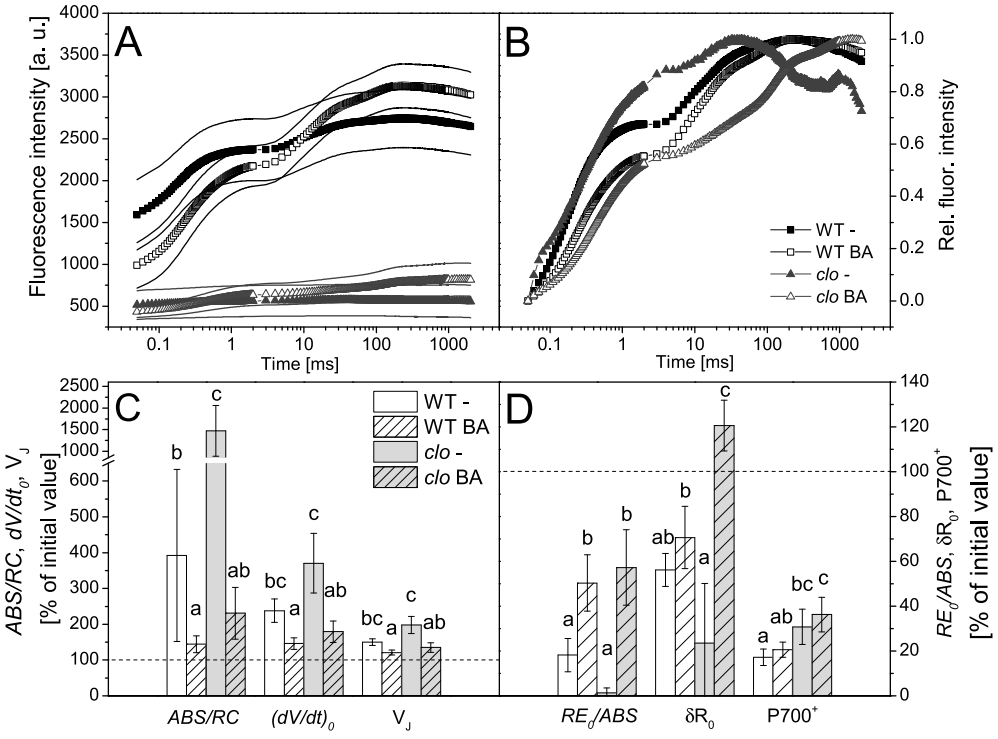

Fig. 6. Chl fluorescence induction transient (OJIP curves), related fluorescence parameters and changes in the PSI activity of detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (−) or with 6-benzylaminopurine (BA). A, OJIP curves; B, the normalized OJIP curves; C, the relative values of the apparent antenna size of active RCII (*ABS/RC*), the initial slope of the O-J fluorescence raise ($dV/dt)_0$, and the relative variable fluorescence at the J-step (V_J); D, quantum yield of electron transport from reduced Q_A to final acceptors of PSI (RE_0/ABS); the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI (δR_0) and the relative amount of oxidized primary electron donor of PSI, $P700^+$, expressed as % of the initial values before senescence induction. Means and SD are shown, n = 8–12. Data were analyzed by Kruskal-Wallis ANOVA on Ranks test ($P < 0.001$) and statistically significant differences in following *post hoc* statistical testing (Dunn's test) at $P < 0.05$ are indicated by different letters.

constitutive (basal) energy dissipation (for a review see Lazár, 2015), whereas Φ_{NPQ} is quantum yield of regulatory quenching, which is induced by illumination to protect the photosynthetic apparatus against excess light and consequent accumulation of reactive oxygen species and oxidative damage (Demmig-Adams et al., 2014). As mentioned above, *clo* had lower Φ_P (Fig. 1D), which indicates lower utilization of absorbed light energy by PSII photochemistry. The proportion of absorbed light energy allocated into non-photochemical quenching processes was higher compared to WT, as both non-regulatory ($\Phi_{f,D}$) and regulatory component (Φ_{NPQ}) were increased (Fig. 1D).

The regulatory non-photochemical quenching processes are related to activation of the xanthophyll cycle where zeaxanthin (Z) is formed by de-epoxidation of violaxanthin (V) through antheraxanthin (A). The extent of the de-epoxidation is expressed as DEPS. Compared to WT, the *clo* leaves were characterized by higher DEPS, by about 80 % (Table 1). Together with the higher *VAZ/Chl* ratio and higher relative content of car (indicated by the lower *Chl/car* ratio) (Table 1), it implies that the *clo* plants had an enhanced photoprotection of photosynthetic apparatus when they were grown under relatively low light intensity (150 µmol of photons m^{-2} s^{-1}). The higher protection against photoinactivation of RCII has been reported by Štroch et al. (2004) in *clo* plants grown under similar light intensity (100 µmol of photons m^{-2} s^{-1}). The higher photoprotection of *clo* could be associated with the existence of free (not bound to LHCs) zeaxanthin (Havaux et al., 2007; Štroch et al., 2008; Nezval et al., 2017).

4.2. Clo had much more impaired PSII function in dark senescing leaves than WT

It is generally known that leaf senescence is accompanied by the loss of photosynthetic pigments and impairment of photosynthetic function. In the dark senescing leaves, the photochemical activity of PSII is markedly reduced during a few days (Oh et al., 1996; Špundová et al., 2003; Vlčková et al., 2006; Janečková et al., 2018). In the detached leaves of WT, the content of photosynthetic pigments and PSII photochemistry decreased significantly after 4 days in the dark (Figs. 3 and 4). The increase in the *ABS/RC* ratio as well as decrease in the Chl *a/b* ratio indicated that RCII were damaged to a greater extent than LHCII. This is in agreement with higher $(dV/dt)_0$ and V_J parameters (Fig. 6C), indicating increase in the excitation supply into the active RCII, acceleration of Q_A reduction and thus the increased amount of reduced Q_A (Strasser et al., 2000; Stirbet et al., 2018). The PSII photochemistry was impaired as F_v/F_m and Φ_P decreased (Figs. 4 and 5), whereas the dissipation *via* both regulatory (Φ_{NPQ}) and non-regulatory non-photochemical quenching processes ($\Phi_{f,D}$) increased (Fig. 5). This indicates that the senescing WT leaves were still able to partially regulate the dissipation of excess light energy. The changes in RE_0/ABS, δR_0 and $P700^+$ parameters in the senescing WT leaves indicate that the PSII activity was more impaired during dark senescence than the activity of PSI.

As mentioned above, plants with enhanced Chl *b* content were reported to have slower leaf senescence (Kusaba et al., 2007; Sakuraba et al., 2012), while senescence of Chl *b*-deficient rice mutants was accelerated (Kusaba et al., 2007; Yang et al., 2016). Thus, in the case of *clo* mutant, we expected faster dark-induced senescence. Although the relative decrease in Chl content was similar in WT and *clo* (Fig. 3), the absolute Chl content was pronouncedly lower in the *clo* senescing leaves due to the lower Chl content in the leaves before senescence induction (Table 1). The pronounced decrease in Chl content in *clo* corresponded with more pronounced impairment of PSII function (Figs. 4–6). In fact, the senescing *clo* leaves had only minimal PSII activity after 4 days (Fig. 4B). The preferential senescence-induced impairment of RCII found in the WT leaves was even more pronounced in *clo,* as documented by extremely increased *ABS/RC* (and also by increased $(dV/dt)_0$ and V_J, Fig. 6C). Unlike WT, regulatory quenching processes were almost inactive and dissipation *via* non-regulatory processes prevailed, as indicated by pronouncedly increased $\Phi_{f,D}$ (Fig. 5).

Interestingly, despite the more pronounced impairment of PSII photochemistry, the activity of PSI was higher in *clo* than in WT (Fig. 6D). It seems that in the *clo* mutant the missing Lhca4 did not decrease the stability of PSI during senescence.

The substantially impaired PSII function in the dark-senescing leaves of *clo* is in agreement with the previous studies, reporting higher sensitivity of PSII photochemistry of *chlorina* barley mutants to stress-conditions (Leverenz et al., 1992; Peng et al., 2002; Štroch et al., 2008; Tyutereva et al., 2017). This higher sensitivity is probably related to Chl

b/LHC deficiency, as proper assembly of LHCII seems to stabilize the structure of PSII complexes and their function (Havaux and Tardy, 1997).

We can summarize that in the *clo* mutant, Chl *b* deficiency caused faster impairment of RCII and consequently faster loss of photochemical activity of PSII during dark senescence. On the contrary, the senescence-induced decrease in PSI activity was smaller in *clo* compared to WT leaves.

4.3. Protective effect of exogenous BA on PSII function in dark-senescing leaves was higher in clo

Application of exogenous cytokinins on senescing leaves slows down the degradation of photosynthetic pigments and preserves photosynthetic function, including PSII photochemistry (Oh et al., 2005; Vlčková et al., 2006; Talla et al., 2016; Vylíčilová et al., 2016). In the case of WT leaves, exogenously applied BA significantly reduced the senescence-induced decrease in Chl, car and xanthophyll contents and decrease in the *Chl/car* ratio (Fig. 3), as well as impairment of PSII function (Figs. 4–6). The protective effect of BA was observed also in *clo* and the senescence in the presence of BA was basically similar in both WT and *clo* (Figs. 4–6). Thus, considering the fact that in the absence of BA the PSII function in *clo* leaves was almost completely lost, the protective effect of BA was relatively more pronounced in *clo*. It seems that exogenous BA application suppressed the destabilizing effect of Chl *b*/LHC deficiency on PSII function in the dark-senescing *clo* leaves.

The exact mechanism by which cytokinins maintain PSII function during senescence is not known. It has been proposed that cytokinins could stabilize both LHCII (Oh et al., 2005; Talla et al., 2016; Vylíčilová et al., 2016) and RCII (Oh et al., 2005) in dark-senescing leaves, RCII stabilization being the key process for the maintenance of PSII photochemical activity (Oh et al., 2005). Based on our results we suppose that the protective effect of BA on PSII function in WT as well as in *clo* is based mainly on a pronounced suppression of the impairment of RCII.

5. Conclusion

We can conclude that the Chl *b* deficiency in the *clo* barley mutant leads to a substantial acceleration of the inhibition of PSII photochemistry during dark-induced senescence of detached leaves. We assume that this acceleration was due to the more pronounced impairment of RCII. It is in agreement with previous reports, describing higher sensitivity of RCII in *chlorina* mutants to unfavorable conditions (Havaux and Tardy, 1997). The application of exogenous BA was able to suppress the extreme impairment of PSII function in *clo* and the relative extent of the observed protective effect was even more pronounced in *clo* than in WT. It seems that the presence of Chl *b* is not decisive for the protective cytokinin effect on PSII photochemistry in dark-senescing leaves.

Further investigations are needed to clarify the specifics of senescence process in Chl *b*-deficient mutants as well as the mechanism of the cytokinin-mediated protection of photosynthetic apparatus and function in senescing leaves.

Author contributions

Helena Janečková designed and performed the experiments, analyzed the data, interpreted results and wrote the manuscript; Alexandra Husičková contributed on design and performance of the experiments, helped to interpret the results and revised the manuscript. Dušan Lazár designed the measuring protocol of quenching analysis and measurement of P700 oxidation, evaluated the data, and did statistical analysis; Ursula Ferretti performed the HPLC measurement and analyzed the data; Pavel Pospíšil supervised the HPLC measurement; Martina Špundová supervised the research, contributed on design of the experiments, helped to interpret the results, revised the manuscript and complemented the final writing. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by Grant No. LO1204 (Sustainable Development of Research in the Centre of the Region Haná) from the National Program of Sustainability I, Ministry of Education, Youth and Sports, Czech Republic, and by ERDF project "Plants as a tool for sustainable global development" (No. CZ.02.1.01/0.0/0.0/16_019/0000827). We thank to Michal Štroch from Department of Physics, University of Ostrava who kindly provided us with seeds of *clo* barley mutants. We also thank to Karel Doležal from Department of Chemical Biology and Genetics (Centre of the Region Haná for Biotechnological and Agricultural Research) who provided us with BA.

References

Bossmann, B., Knoetzel, J., Jansson, S., 1997. Screening of chlorina mutants of barley (*Hordeum vulgare* L.) with antibodies against light-harvesting proteins of PS I and PS II: absence of specific antenna proteins. Photosynth. Res. 52, 127–136.

Demmig-Adams, B., Garab, G., Adams III., W.W., Govindjee, 2014. Non-photochemical quenching and energy dissipation in plants, algae and cyanobacteria. In: In: Govindjee, Sharkey, T. (Ed.), Advances in Photosynthesis and Respiration, vol. 40 Springer, Dordrecht.

Färber, A., Jahns, P., 1998. The xanthophyll cycle of higher plants: influence of antenna size and membrane organization. Biochim. Biophys. Acta 1363, 47–58.

Gilmore, A.M., Björkman, O., 1994. Adenine nucleotides and the xanthophyll cycle in leaves. I. Effects of CO_2- and temperature-limited photosynthesis on adenylate energy charge and violaxanthin de-epoxidation. Planta 192, 526–536.

Ghirardi, M.L., McCauley, S.W., Melis, A., 1986. Photochemical apparatus organization in the thylakoid membrane of *Hordeum vulgare* wild type and chlorophyll *b*-less chlorina f2 mutant. Biochim. Biophys. Acta 851, 331–339.

Havaux, M., Dall'Osto, L., Bassi, R., 2007. Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in Arabidopsis leaves and functions independent of binding to PSII antennae. Plant Physiol. 145, 1506–1520.

Havaux, M., Tardy, F., 1997. Thermostability and photostability of photosystem II in leaves of the *chlorina-f2* barley mutant deficient in light-harvesting chlorophyll *a*/*b* protein complexes. Plant Physiol. 113, 913–923.

Humplík, J.H., Lazár, D., Fürst, T., Husičková, A., Hýbl, M., Spíchal, L., 2015. Automated integrative high-throughput phenotyping of plant shoots: a case study of the cold tolerance of pea (*Pisum sativum* L.). Plant Methods 11, 20.

Janečková, H., Husičková, A., Ferretti, U., Pržina, M., Pilařová, E., Plačková, L., Pospíšil, P., Doležal, K., Špundová, M., 2018. The interplay between cytokinins and light during senescence in detached Arabidopsis leaves. Plant Cell Environ. 41, 1870–1885.

Krieger-Liszkay, A., Trösch, M., Krupinska, K., 2015. Generation of reactive oxygen species in thylakoids from senescing flag leaves of the barley varieties Lomerit and Carina. Planta 241, 1497–1508.

Król, M., Spangfort, M.D., Huner, N.P.A., Öquist, G., Gustafsson, P., Jansson, S., 1995. Chlorophyll *a*/*b*-binding proteins, pigment conversions, and early light-induced proteins in a chlorophyll *b*-less barley mutant. Plant Physiol. 107, 873–883.

Kusaba, M., Ito, H., Morita, R., Iida, S., Sato, Y., Fujimoto, M., Kawasaki, S., Tanaka, R., Hirochika, H., Nishimura, M., Tanaka, A., 2007. Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. Plant Cell 19, 1362–1375.

Lazár, D., 2006. The polyphasic chlorophyll *a* fluorescence rise measured under high intensity of excitation light. Funct. Plant Biol. 33, 9–30.

Lazár, D., 2013. Simulations show that a small part of variable chlorophyll *a* fluorescence originates in photosystem I and contributes to overall fluorescence rise. J. Theor. Biol. 335, 249–264.

Lazár, D., 2015. Parameters of photosynthetic partitioning. J. Plant Physiol. 175, 131–147.

Leong, T.M., Anderson, J.M., 1984. Adaptation of the thylakoid membranes of pea chloroplasts to light intensities. II. Regulation of electron transport capacities, electron carriers, coupling factor (CF1) activity and rates of photosynthesis. Photosynth. Res. 5, 117–128.

Leverenz, J.W., Öquist, G., Wingsle, G., 1992. Photosynthesis and photoinhibition in leaves of chlorophyll *b*-less barley in relation to absorbed light. Physiol. Plantarum 85, 495–502.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.

Mueller, A.H., Dockter, C., Gough, S.P., Lundqvist, U., von Wettstein, D., Hansson, M., 2012. Characterization of mutations in barley *fch2* encoding chlorophyllide *a* oxygenase. Plant Cell Physiol. 53, 1232–1246.

Nath, K., Phee, B.-K., Jeong, S., Lee, S.Y., Tateno, Y., Allakhverdiev, S.I., Lee, C.-H., Nam, H.G., 2013. Age-dependent changes in the functions and compositions of photosynthetic complexes in the thylakoid membranes of *Arabidopsis thaliana*. Photosynth. Res. 117, 547–556.

Nezval, J., Štroch, M., Materová, Z., Špunda, V., Kalina, J., 2017. Phenolic compounds

and carotenoids during acclimation of spring barley and its mutant *Chlorina f2* from high to low irradiance. Biol. Plant. 61, 73–84.
Oh, M.H., Kim, J.H., Zulfugarov, I.S., Moon, Y.-H., Rhew, T.-H., Lee, C.-H., 2005. Effects of benzyladenine and abscisic acid on the disassembly process of photosystems in an *Arabidopsis* delayed-senescence mutant, *ore9*. J. Plant Biol. 48, 170–177.
Oh, S., Lee, S., Chung, I., Lee, C., Nam, H., 1996. A senescence-associated gene *of Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. Plant Mol. Biol. 30, 739–754.
Peng, C., Duan, J., Lin, G., Gilmore, A.M., 2002. Correlation between photoinhibition sensitivity and the rates and relative extents of xanthophyll cycle de-epoxidation in *chlorina* mutants of barley (*Hordeum vulgare* L.). Photosynthetica 40, 503–508.
Sakuraba, Y., Balazadeh, S., Tanaka, R., Mueller-Roeber, B., Tanaka, A., 2012. Overproduction of Chl *b* retards senescence through transcriptional reprogramming in Arabidopsis. Plant Cell Physiol. 53, 505–517.
Špundová, M., Popelková, H., Ilík, P., Skotnica, J., Novotný, R., Nauš, J., 2003. Ultra-structural and functional changes in the chloroplasts of detached barley leaves senescing under dark and light conditions. J. Plant Physiol. 160, 1051–1058.
Špundová, M., Strzałka, K., Nauš, J., 2005. Xanthophyll cycle activity in detached barley leaves senescing under dark and light. Photosynthetica 43, 117–124.
Stirbet, A., Lazár, D., Kromdijk, J., Govindjee, 2018. Chlorophyll *a* fluorescence induction: can just a one-second measurement be used to quantify abiotic stress responses? Photosynthetica 56, 86–104.
Strasser, R.J., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Mohanty, Yunusa, Pathre (Eds.), Probing Photosynthesis: Mechanism, Regulation & Adaptation. Taylor & Francis, London, pp. 443–480.
Štroch, M., Čajánek, M., Kalina, J., Špunda, V., 2004. Regulation of the excitation energy utilization in the photosynthetic apparatus of *chlorina f2* barley mutant grown under different irradiances. J. Photochem. Photobiol. B Biol. 75, 41–50.
Štroch, M., Lenk, S., Navrátil, M., Špunda, V., Buschmann, C., 2008. Epidermal UV-shielding and photosystem II adjustment in wild type and *chlorina f2* mutant of barley during exposure to increased PAR and UV radiation. Environ. Exp. Bot. 64, 271–278.
Talla, S.K., Panigrahy, M., Kappara, S., Nirosha, P., Neelamraju, S., Ramanan, R., 2016. Cytokinin delays dark-induced senescence in rice by maintaining the chlorophyll cycle and photosynthetic complexes. J. Exp. Bot. 67, 1839–1851.
Tyutereva, E.V., Evkaikina, A.I., Ivanova, A.N., Voitsekhovskaja, O.V., 2017. The absence of chlorophyll *b* affects lateral mobility of photosynthetic complexes and lipids in grana membranes of Arabidopsis and barley *chlorina* mutants. Photosynth. Res. 133, 357–370.
Vlčková, A., Špundová, M., Kotabová, E., Novotný, R., Doležal, K., Nauš, J., 2006. Protective cytokinin action switches to damaging during senescence of detached wheat leaves in continuous light. Physiol. Plantarum 126, 257–267.
Voitsekhovskaja, O.V., Tyutereva, E.V., 2015. Chlorophyll *b* in angiosperms: functions in photosynthesis, signaling and ontogenetic regulation. J. Plant Physiol. 189, 51–64.
Vylíčilová, H., Husičková, A., Spíchal, L., Srovnal, J., Doležal, K., Plíhal, O., Plíhalová, L., 2016. C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus. Phytochemistry 122, 22–33.
Yamatani, H., Kohzuma, K., Nakano, M., Takami, T., Kato, Y., Hayashi, Y., Monden, Y., Okumoto, Y., Abe, T., Kumamaru, T., Tanaka, A., Sakamoto, W., Kusaba, M., 2018. Impairment of Lhca4, a subunit of LHCI, causes high accumulation of chlorophyll and the stay-green phenotype in rice. J. Exp. Bot. 69, 1027–1035.
Yang, Y., Xu, J., Huang, L., Leng, Y., Dai, L., Rao, Y., Chen, L., Wang, Y., Tu, Z., Hu, J., Ren, D., Zhang, G., Zhu, L., Guo, L., Qian, Q., Zeng, D., 2016. *PGL*, encoding chlorophyllide *a* oxygenase 1, impacts leaf senescence and indirectly affects grain yield and quality in rice. J. Exp. Bot. 67, 1297–1310.