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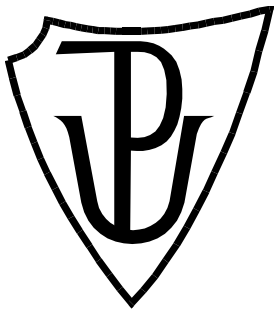

Regulatory aspects of shade-induced leaf senescence and cytokinin metabolism in *Triticum aestivum* L. (wheat)

DIPLOMA THESIS

Autor:	**Mitura Karel**
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V Olomouci dne .... *podpis diplomanta*

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Jméno a příjmení autora	Mitura Karel
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Abstract:

Listová senescence je biologický proces, který je závislý na působení mnoha endogenních a exogenních faktorů, mezi nimiž zejména viditelné záření hraje důležitou roli. Dlouhodobé stínění, úplné zastínění a také vysoká intenzita světla ovlivňují míru senescence listu. Většina rostlin ovlivňuje senescenci listů poměrem červeného a dalece červeného záření (R / FR). Studiem pšenice bylo zjištěno, že vyřazení modrého záření (MZ) vede k indukci signálu vyvolávajícího příznaky oxidativního stresu a senescence. Fytohormony představují jedny z hlavních endogenních faktorů, které svým účinkem stimulují nebo potlačují senescenci. Úloha cytokininů (CK) jako látek potlačujících stárnutí listů je již známá, avšak mechanismy regulace tohoto procesu v souvislosti se světelným zářením nebyly doposud zcela objasněny. Tato práce se zaměřuje na studium důsledků vyřazení určitých světelných vlnových délek v souvislosti s průběhem senescence a také metabolismem cytokininů u pšenice (*Triticum aestivum L.*) a ječmene (*Hordeum vulgare* cv. Golden Promise). Za tímto účelem byly podrobeny analýze 20 dnů staré listy pšenice, u kterých byla ustřihnutím indukovaná senescence. Inkubace těchto listů probíhala ve speciálních boxech s vodou, jejíž víčka byla potažena selektivními světelnými filtry značky Lee®. Cílem tohoto experimentu byla modulace toku světelného spektra, zejména v modré oblasti, ale také v poměru červené a dalece červené složky záření. Odběr vzorků listů byl v závislosti na průběhu senescence prováděn v různých časových intervalech. Posléze byly vzorky jednotlivých listů podrobeny analýze změn retence chlorofylu, aktivity cytokinin dehydrogenasy (CKX), exprese *CKX* genů a také vybraných genů souvisejících se senescencí.Vzhledem k vysoké komplexností pšeničného genomu, jehož hexaploidní uspořádání je dáno třemi (A, B, a D) subgenomy, představuje diploidní ječmen jednodušší model studia některých biologických procesů souvisejících se senescencí. Jsou diskutovány možnosti využití ječmene pro zvýšení a snížení genové exprese *HvCKX* v souvislosti se studiem listové senescence u pšenice.

Klíčová slova	Listová senescence, *Triticum aestivum* L., *Hordeum vulgare* cv. Golden Promise, Metabolismus Cytokininů, cytokinin dehydrogenasa, modré záření.
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Abstract:

Leaf senescence is a biological process which depends on many endogenous and exogenous factors, amongst which the light plays an important role. Prolonged exposure to darkness or leaf shading, as well as high light intensity can modify the senescence rate. While a decrease in the red to far red ratio (R/FR) was shown to trigger leaf senescence in several dicotyledoneous species, blue light (BL) suppression acts as a signal inducing oxidative stress symptoms and stimulating senescence in shaded leaves of wheat. Among the internal factors, it has been proved that plant phytohormones are able to accelerate or repress senescence. The role of cytokinins (CK) as agents delaying leaf senescence has been widely documented, nevertheless the mechanisms of regulation of this process together with light conditions remains unclear. The presented thesis is aimed on research the effect of specific light wavelengths deprivation on the progress of leaf senescence and CKs metabolism in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* cv. Golden Promise) plants. For that purpose, excised 20 day-old leaves of plants were incubated floating in boxes with water, whose lids were covered with Lee® light filters in order to modulate the flow of light in the blue spectral region. Leaf samples were collected at different time points and analyzed for changes in several parameters, for instance, chlorophyll and protein concentration, CKX activity, gene expression profiles of *CKX* and other several genes. Owing to the high complexity of wheat genome, a hexaploid plant with three (A, B, and D) subgenomes, diploid barley might represent a good genomic model for some biological processes as leaf senescence. Future possibilities of using barley plants with up or down-regulated expression of *HvCKX* genes for the study of leaf senescence in wheat are discussed.

Keywords	Leaf senescence, *Triticum aestivum* L., *Hordeum vulgare* cv. Golden Promise, cytokinin metabolism, cytokinin dehydrogenase, blue light.
Number of pages	67
Number of appendices	0
Language	English

Content

1. AIMS

Literature review on the topic of senescence process in plants, focused on the role of cytokinin (CKs) metabolism in the regulation of this mechanism.

Study of some of the processes involved in the signaling pathway of light, with the special emphasis on the role of CKs in regulating senescence in shaded leaves of wheat (*Triticum aestivum* L.).

Comparison of light responses in shaded leaves of wheat and barley plants (*Hordeum vulgare* cv. Golden Promise) to find out wheather these plants follow the same trends in the rate of senescence.

2. INTRODUCTION

2.1 The senescence process

Senescence is a process of aging and in the plant leaves represents the final stage of leaf development, being genetically controlled and depending on many endogenous as well as exogenous factors (Noodén *et al.* 1997). One of the most prominent endogenous factors which directly influence the onset of senescence are the phytohormones. It is known that ethylene and abscisic acid play a crucial role in causing programmed senescence, nevertheless, other plant hormones such as gibberellins, brassinosteroids, strigolactones and cytokinins can also modulate the rate of senescence. Among the exogenous factors that regulate leaf senescence, light plays an important role. Prolonged exposure to darkness, leaf shading and also high light intensity modify the rate of the senescence (Brouwer *et al.* 2012). Leaves beneath a dense canopy experience a marked reduction of the photon flux in the red (640-670 nm) and blue (400-450 nm) wavelengths due to the presence of foliar pigments (mainly chlorophyll). While a decrease in the red to far red ratio (R:FR) was shown to trigger leaf senescence in several dicotyledoneous species, blue light (BL) suppression acts as a signal inducing oxidative stress symptoms and triggers senescence in shaded leaves of wheat (Causin *et al.* 2006, Causin *et al.* 2009). Down-regulation of catalase (CAT) activity, increase in proteolytic activity, changes in the metabolism of cytokinins (CKs) and Ca^{2+} availability are involved in the regulation of this process. Whether wheat is unique in this response to BL or whether this is a characteristic response of monocotyledoneous plants, remains unclear.

The importance of senescence in cereals resides in the remobilization and recycling of nutrients that occurs along this process, from vegetative tissues (usually leaves), to the subsequent storage site, the grains.

2.1.1 Senescence in the cellular compartments

Stress, aging and other factors produce a loss of photosynthetic capacity and cause disruptions of membrane integrity (Quirino *et al*. 2000). Leaf senescence is usually accompanied by changes in cell structure, catabolism of chlorophyll and cessation of photosynthesis, degradation of important macromolecules such as proteins and membrane lipids (Buchanan-Wollaston 1997). The membrane-associated electron transports within photosystem I and II are rapidly reduced during senescence. These molecular changes result in a decline in photosynthetic activity, leading to diminished production of sacharides and ATP. As the progress of senescence is characterized by rapid changes in the entire expression pattern and it requires the injection of a vast amount of ATP, lipids are released from membranes, degraded and converted to AcetylCoA. Consequent imbalances in the ratio of unsaturated:saturated fatty acids reduce the membrane fluidity and increase its permeability. As the senescence progress, the level of protein and chlorophyll degradation products also rapidly increases and vacuoles become filled with toxic metabolites. At the terminal phase of senescence these accumulated metabolites are released into the cytosol. The nucleus of the cell operates the senescent gene expression profile and controls the degradation of all intracellular organelles and compartments, thus, its breakdown is the final step in the entire senescence process (Woo *et al.* 2013).

2.1.2 Chlorophyll degradation

The onset of senescence, at the cellular level, starts with chloroplast degradation and progresses to the destruction of chlorophyll. Chloroplast degradation begins with internal disruption of the grana stacks, which appears as swellings and ruptures of the thylakoids (Martinéz *et al.* 2008). Structural alterations give rise to new structures called gerontoplasts, which are characterized by a lack of stacked thylakoid membrane and by an elevated accumulation of electron dense lipid bodies called plastoglobuli that contain high levels of caroteinoids and anthocyanins (Keskitalo *et al.* 2005). Up to a certain point this transformation of the chloroplast is reversible (Thomas *et al*. 2003). The reversibility of this process serves to distinguish senescence from programmed cell death (PCD).

All plants produce chlorophyll *a* and chlorophyll *b*. The main difference between those structures is that chlorophyll *a* has a methyl group bound at position 7 in the tetrapyrrole macrocycle, while chlorophyll *b* has a formyl group in this position. Chlorophyll *a* is mainly present in the antenna complexes of PS1 and PS2 and also in light harvesting chlorophyll binding proteins (LHC) (Nelson and Yocum *at al.* 2006). Its main function is to harvest and transfer light energy. Chlorophyll *b* resides exclusively in the peripheral antenna complexes, and its main function involves participating in electron transfer reactions and the stabilization of the LHC (Tanaka and Tanaka 2011). The onset of senescence is launched by conversion of chlorophyll *b* to chlorophyll *a*. The first reaction involves reduction of the 7-formyl group in chlorophyll *b* to 7-hydroxymethyl. This step is catalysed by Chlorophyll *b* reductase, which is the member of the NADPH-dependent group of dehydrogenases. In the second step of chlorophyll *b* conversion, 7-hydroxymethyl is reduced to -methyl. The reaction is carried out by a Fe-S containing flavoprotein, 7-hydroxymethyl reductase (HCAR) (Fig. 1). After chlorophyll *b* conversion, all chlorophyll *a* molecules follow the same degradation pathway. Chlorophyll *a* breakdown is further continued by removal of Mg^{2+} ion and phytol group. The first reaction catalysed by Mg dechelatase leads to the formation of pheophytin. Following removal of phytol chain from the pheophytin is mediated by a serin protease, called pheophytinase (PPH). The outcome of this reaction is the formation of pheophorbide *a* (Morita *et al.* 2009). The chlorophyll macrocycle, which has lost a phytol and Mg^{2+} ion, is then subjected to an oxygenolytic cleavage reaction, carried out by a phaeophorbide *a* oxygenase (PaO), forming a red chlorophyll catabolite (Tanaka *et*

al. 2003). In the subsequent steps of degradation double bonds of this catabolite are hydrogenized to form primary fluorescent red chlorophyll catabolite (pFCC). This reaction is mediated by red chlorophyll reductase (RCCR) (Fig. 1) (Hörtensteiner *et al.* 2004), which belongs to the class of ferredoxin-dependent bilin reductases. All of the mentioned enzymatic reactions, beginning with the chlorophyll *b* conversion up to pFCC formation, occur in plastids, whereas the remaining products are transferred to the cytoplasmic space where they are further degraded. During its degradation, chlorophyll changes its colour. While chlorophyll *a* is a vivid green colour, pheophytin *a* and pheophorbide *a* are dull green, RCCR is red and pFCC is colourless.

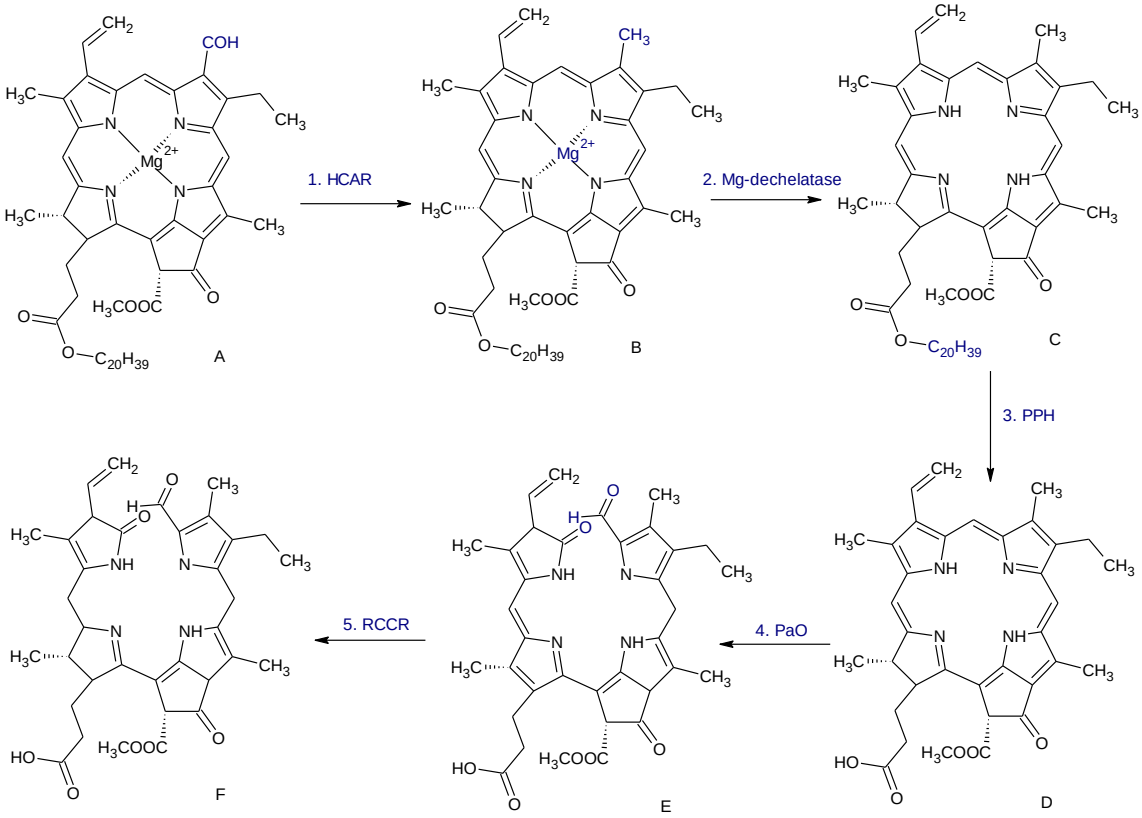

Figure 1- The chlorophyll breakdown reactions and conversions, A- Chlorophyll *b*, B- Chlorophyll *a*, C- Pheophytin *a*, D- Pheophorbide *a*, E- Red Chlorophyll Catabolite, F- Primary Fluorescent Red Chlorophyll Catabolite.

2.1.3 SAG genes

Senescence is also characterized by a new gene transcription profile, which is subject to post-transcriptional and post-translational regulation (Gan and Amasino 1997). The expression of many *SAG*s genes is typically enhanced during senescence (Buchanan-Wollaston 1997). The new expression profile includes the synthesis of several extracellular and intracellular oxidases, peroxidases, lipases, proteinases, kinases and RNases, as well as many transcription factors. This alterations in gene expression results in the degradation of various macromolecules, in nutrient translocation and an increased production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide ion (O_{2^-}) and nitric oxide (NO) (Joo *et al.* 2005). Depending on the reactions catalysed and their association with certain metabolic pathways, wheat *SAG*s were divided into different groups. For instance *TaSAG1*, *TaSAG3*, *TaSAG4*, *TaSAG5* and *TaSAG12* encode Aminotransferase, 2-oxoisovalerate dehydrogenase, Alanine-glyoxylate aminotransferase, Methylcrotonoyl-CoA carboxylase and cysteine protease, which are all enzymes associated with amino acid metabolism. *TaSAG7* and *TaSAG8* encode putative isocitrate lyase and 3-ketoacyl-CoA thiolase-like protein, which cohere with fatty acid metabolism. *TaSAG2* and *TaSAG6* encode seed proteins, whose role were not elucidated (Kajimura *et al.* 2010). According to the changes in expression patterns, *SAG*s can be classified as typically up-regulated and down-regulated. A commonly up-regulated gene is *TaSAG12*, which is widely used as a molecular marker for the progress of senescence. In contrast, a gene that is characteristically and significantly down-regulated during senescence is the *CAB* gene that encodes for the chlorophyll *a*/*b* binding protein (Gan and Amasino 1997). Significant changes of expression patterns during senescence emphasize the important role of transcription factors (Buchanan-Wollaston *et al.* 2005). NAC and WRKY represent two major groups of transcription factors, which participate on the regulation of senescence transcriptome (Guo *et al.* 2004). These two transcription factor families have been revealed to play a crucial role during *SAG*s activation in *Arabidopsis* and wheat (Miao *et al.* 2004). Within the *WRKY* family, *WRKY53* knock-out lines exhibited delayed senescence but the opposite effect was observed when *WRKY70* was silenced. Therefore, *WRKY53* was considered as a positive regulator, whereas *WRKY70* was shown to negatively affect senescence (Miao *et al.* 2004). Besides *WRKY*, over 30 *NAC* genes were reported to be significantly upregulated during *Arabidopsis* senescence (Kou *et al.* 2012).

2.1.4 Senescence and oxidative stress

2.1.4.1 Reactive oxygen species and antioxidant metabolism

ROS such as hydrogen peroxide, nitric oxide, superoxide ions, hydroxyl radicals and perhydroxyl radicals are inorganic molecules characterized by their high reactivity (Wojtaszek, 1997). These substances usually arise as side products of cell metabolism, mostly in energy-generating processes in chloroplasts and mitochondria. They can also be formed during biotic and abiotic stress exposure of the plants (Mittler, 2002), due to the disruption of cell homeostasis (Polle 2001). The largest amounts of ROS can be found in chloroplasts, but not negligible quantities can also be detected in the mitochondria, cell membrane systems, and in the cell wall (Mittler, 2002). These substances can also arise in the reactions catalyzed by amine oxidases peroxidases, especially wall peroxidase (Joo *et al.* 2005).

When ROS concentrations become excessive they exhibit toxic effects causing alterations in protein structure, DNA damage, purine oxidation, lipid peroxidation which eventually lead to cell death (Neill et al., 2002). Moreover, ROS were also reported to react with unsaturated acids in the membranes. This causes membrane peroxidation, resulting in permeability and transport disorders. Structural disruption to the membrane leads to peroxidative damage of the plasmalemma that can cause leakage of cell contents. Furthermore, ROS-induced membrane damage affects the respiratory activity of the mitochondria, and causes loss of ability to fix carbon chloroplast (Scandalios, 1990).

Nevertheless, the presence of ROS throughout plant development is essential. It has been shown that these substances participate in the regulation of various processes, serving as signalling molecules for gene expression and maintaining ion homeostasis. For instance, ROS have been implicated in SAG activation.

Hydrogen peroxide presents one of the most prominent effects of all the ROS due to its long half-life, diffusibility and stability. It is abundant and widely distributed in plant tissues. In contrast to animal cells, where even very low H_2O_2 concentrations have toxic effects, plant cells have evolved an extensive antioxidant system that helps to neutralize H_2O_2 toxicity. This system consists of catalase, ascorbate peroxidase and glutathione. Ascorbate peroxidase (APX, EC 1.11 1.11) utilizes ascorbate to reduce the H_2O_2 produced in chloroplasts to water. Ascorbate levels are maintained in a coupled

enzymatic reaction mediated by dihydroascorbate reductase (DHAR, EC 1.8.5.1), which oxidizes reduced glutathione (GSH) to regenerate ascorbate. Apart from chloroplasts, the ascorbate-glutathione cycle also occurs in mitochondria and peroxisomes (Jimenéz et al. 1998). During oxidative stress the H_2O_2 redox balance within cells is mainly maintained by the tetrameric, heme-containing catalase enzyme (CAT, EC 1.11 1.6) that mediates H_2O_2 conversion to water and oxygen. Catalase is the only enzyme that can catalyse the reaction without the need of reduction compounds (Scandalios *et al.* 1997). During photorespiration and β-oxidation of fatty acids, H_2O_2 is predominantly formed in peroxisomes, thus CAT enzymes are localized in this organelles (Mittler 2002). Several CAT isoenzymes have been described in plants and, according to their expression patterns and physiological properties, have been divided into three classes. The first class comprises catalases expressed in leaves that mainly catalyse the conversion of H_2O_2, which is formed during photorespiration. These enzymes display a light-dependent expression profile. The main feature of the second class is its high level of expression in vascular tissues. These enzymes mostly react in response to increased levels of stress-induced H_2O_2, but they have also been observed to participate on lignification (Dat *et al.* 2001). Contrary to the previous group, gene expression of these *CATs* is not influenced by light. The third class of CATs are mainly expressed in seeds and seedlings. These enzymes remove the H_2O_2 generated during fatty acid metabolism in glyoxysomes.

In senescing leaves of wheat, the spectral quality was shown to prominently influence CAT expression and CAT activity. When blue radiation was suppressed, CAT1 and CAT2 activity significantly decreased (Causin *et al.* 2015). The blue radiation has been shown to regulate the levels of cytosolic Ca^{2+} and Ca^{2+} binding activity of calmodulin (Yang and Poovaiah 2002). Blue light suppression increases the accumulation of cytosolic Ca^{2+}, which in turn stimulated ROS production.

2.2 Light as factor regulating senescence

The electromagnetic radiation provided by the sun supply energy for all plants to perform photosynthesis and incorporate saccharides, therefore light represents one of the main physiological factors that positively affects the growth and development of plants. Throughout the development, plants utilize different pigments to capture sun-light energy. Owing to the constitution of light receptors, plants perceive: ultraviolet (340-400 nm), blue (400-500 nm), red (600-700 nm) and far red (700-800 nm) radiation. The red and far-red light is sensed by photoreceptors called phytochromes (Chen *et al.* 2004). This bilin chromophore contains proteins that participate in the regulation of flowering, circadian rhythms, germination of the seeds and the synthesis of chlorophyll. Blue light spectrum is perceived by cryptochromes (CRY), phototropins (PHOT) and ZTL (Zeitlupe) family members (Cashmore *et al.* 1999). Whereas phototropins regulate phototropism, chloroplast movement, and stomatal opening, cryptochromes usually mediate photomorphogenesis and circadian rhythms (Lin, 2002). Structurally, cryptochromes are arranged in an orthogonal bundle that consists of alpha helices, several loops and a few β-sheets. These photoreceptors also contain a FAD cofactor and a light harvesting chromophore, usually pterin or 5, 10-methenyltetrahydrofolic acid (Brautigam *et al*. 2004). There are two cryptochromes encoded in *Arabidopsis* genome, cryptochrome 1 (CRY1) and CRY 2, whereas rice and wheat present four CRY subfamilies (Xu *et al*, 2009). Moreover, the response to BL is different between dicots and monocots, for instance, whereas in *Arabidopsis* CRY1 stimulates expansion of cotyledon and represses hypocotyl elongation, in rice OsCRY1 induces inhibition of coleoptile and seedling elongation (Zhang *et al*. 2006).

At the molecular level, cryptochromes regulate gene expression via two different processes: proteolysis and transcription (Liu *et al.* 2011). It has been shown in *Arabidopsis thaliana* that CRY2 interacts and activates the cryptochrome-interacting transcription factor (CIB1) regulating for instance the flowering time. CRY2-interacting CIB1 protein is strongly accumulated in plants exposed to blue light, but significantly decreases in the absence of blue light due to its degradation by the 26S proteasome. Meng *et al.* (2013) working on transgenic soybean, surprisingly showed opposite regulation in soybean, where CIB1 activates transcription of senescence-associated genes such as *WRKY53* and promotes senescence. Analyses of CRY2-CIB1 interactions revealed that, while CRY2 represses leaf senescence, CIB1 promotes it. Photo-excited

soybean CRY2 physically interacts with CIB1 and supresses its E-box binding and transcriptional activation of WRKY53, inhibiting leaf senescence. Little is known about the effects of environmental cues, especially light quality, on the expression of *TaCRY* and *TaCIB* genes and their interaction during leaf senescence.

2.3. Phytohormones in senescence

Phytohormones are low molecular weight organic substances, which control plant development, growth and physiology. Substances that possess plant hormone activity include; ethylene, auxins, cytokinins, gibberellic acid, abscisic acid, jasmonic acid and salicylic acid. In addition, many other signalling molecules such as stringolactons, have been identified (Gomez-Roldan *et al.* 2008). According to the effect and mode of action, phytohormones can be grouped according to their senescence-promoting and senescence-delaying activities. The best characterized senescence-inducing compound is ethylene. Ethylene activates hydrolytic enzymes and induces chlorophyll and protein degradation. Ethylene treated plants and fruits exhibit early senescence (Buchanan-Wollaston *et al.* 2003). As ethylene synthesis is carried out by aminocyclopropanecarboxylate oxidase (ACC), *ACC* knock-out lines display retarded senescence. Besides ethylene, jasmonic acid (JA) was reported to induce *SAG*s expression, therefore it was considered to be an important senescence activator (He *et al*. 2002). While salicylic acid was shown to trigger senescence via activation of WRKY53 transcription factor, the exact mechanism of brassinosteroid action is not yet fully understood. Abscisic acid (ABA) delays the onset of plant senescence by the regulation of stomatal closure and inhibition of ripening. On the other hand, exogenous treatment with ABA has been shown to induce *SAGs* expression, as it was confirmed in the work of Zhang (Zhang et al. 2012) where *SAG113* encoding phosphatase PP2C was shown to be up-regulated in response to ABA. In contrast to the positive regulators, cytokinins have been reported to act as typical senescence delaying agents. Several studies based on exogenous application of CK on senescing tissues revealed a significant delay of senescence. An interesting approach to the study of modulated cytokinin levels was proposed by Gan and Amasino (1997), who produced transgenic tobacco plants overexpressing the *IPT* gene, which encodes a key enzyme of CK biosynthesis. The *IPT* gene was under the control of the *SAG12* promoter, which expression is induced during senescence. As far as senescence proceeded, increased levels of cytokinins were produced and senescence was suppressed. Moreover,

phenotyping analysis revealed that this transgenic tobacco maintained increased photosynthetic activity and extended life cycle. Senescence delaying effects were also described for auxins and gibberellic acid. Although the final effect of phytohormones is achieved by their mutual interplay, the present thesis will be focus only on cytokinins because they were already proved to display significant senescence retarding effects in leaves of wheat but not further information is available of how the light quality affects this mechanism.

2.3.1Cytokinins: structure and function

Cytokinins are low molecular weight organic compounds derived from adenine substituted in position N^6. The N^6- bound lateral side chain can exhibit either isoprenoic or aromatic properties and differ from each other by the presence and distribution of hydroxyl groups, double bonds (Mok *et al.*, 2000). Isoprenoid cytokinins are ubiquitous substances that contain a laterally bound isopentenyl chain. According to the structure of the side chain, we can distinguish: isopentenyladenine (iP) (Fig. 2- A), a reduced form – dihydrozeatin -, and hydroxylated form - zeatin. Zeatin-type cytokinins usually occur as *cis* and *trans* configurational isomers (Sakakibara et al., 2006). *Trans*-zeatin (Fig. 2- B) is in comparison to its *cis* stereoisomer characterized by high activity in stimulating cell division. *Cis*-zeatin (Fig. 2- C) is regarded as a compound with low activity, but its physiological significance and function is not yet fully understood. Moreover, in *Poaceae*, *cis*-zeatin represents the most ubiquitous form of endogenous cytokinins, contributing the most to the whole CK content (Gajdošová *et al*. 2011). It is believed that the prevalence of cis-zeatine and its derivates in *Poaceae* family might be associated with the switching from vegetative to generative reproductive growth (Sykorová *et al.* 2008). Aromatic cytokinins include kinetin and benzyl adenine (BAP) (Fig. 2- D, E). These aromatic substances are present in plant tissues mostly in hydroxylated forms, called topolins. Topolins were first isolated from poplar leaves, and structurally differ from each other only by the position of the hydroxyl group (Fig. 2- E), thus *ortho*- and *meta*-topolins can be distinguished (Strnad et al., 1997).

CK regulation in the plant involves controls at the level of biosynthesis mediated by isopentenyl transferases (IPT), degradation by cytokinin dehydrogenases (CKX), reversible inactivation catalysed by *O*-glucosyltransferases (ZOG) and glucoside reactivation carried out by β-glucosidases (GLU). Cytokinins play many physiological

and regulatory functions in the plants. The most important of them are: stimulation of cell division, reduction of apical dominance, stimulation of the stem branching, root growth inhibition, regulation and deceleration leaf senescence (Gan and Amasino. 1995). They play a very important role during all phases of plant development, from seed germination to senescence. At the cellular level these phytohormones either induce or down-regulate gene expression. Cytokinin responding genes, which display up-regulation are mostly associated with the light regulation (*PHYA*, *PSK1*, *CIP8*, *PAT1*, *APRR*), nitrate metabolism (*NTR2*, *NIA*) sugar metabolism (*STP1*, *SUS1*) and also other hormones- ethylene (*ETR2*, *EIN3*) and gibberellins (*GAI*, *RGA*) (Brenner *et al.* 2005). In contrast, *WOX5*, *AUX1*, *LAX2* represent regulatory genes whose expression was shown to be down-regulated by cytokinin (Zhang et al. 2013). Cytokinins also induce expression of genes that are associated with mitosis and chloroplast development.

The role of cytokinins (CK) as agents delaying leaf senescence has been widely documented, and its endogenous decrease is one of the main events associated with the development of senescence. Although very little is known about their exact mechanism of action, there is evidence that retarded senescence is a consequence of CK stimulation of antioxidant metabolism (Zavaleta-Mancera *et al.*, 2007).

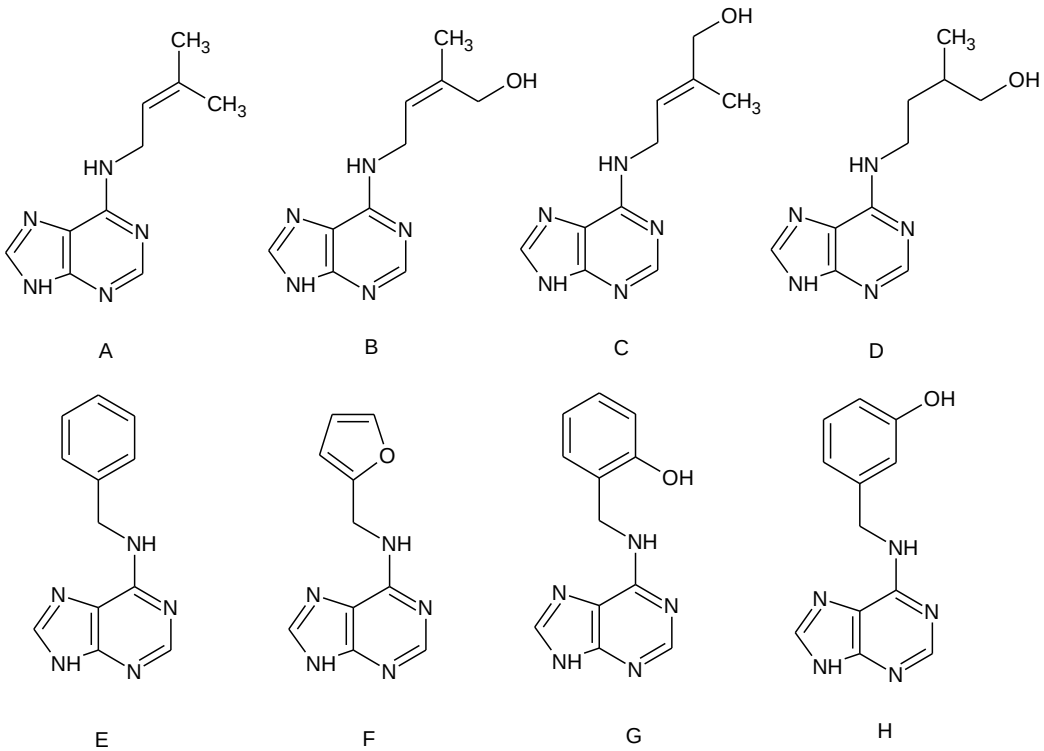

Figure 2- Cytokinins: Isoprenoic CK: A- N^6-isopentenyladenine (iP) B- *trans*-Zeatin, C- *cis*-Zeatin, D- Dihydrozeatin Aromatic CK, E- Benzyladenine, F- Furfuryladenine (Kinetin), E- *ortho*- Topolin, F- *meta*- Topolin.

2.3.2 Biosynthesis

Elevated concentrations of endogenous cytokinins are present predominantly in young dividing tissues, especially in young leaves and the tops of the stems and roots (Emery *et al.,* 2000). Cytokinin biosynthesis is catalysed by adenosine phosphate-isopentenyl transferase (Fig. 3) (IPT; EC 2.5.1.27) (Takei *et al*., 2001). This enzyme mediates the addition of either dimethylallyl pyrophosphate (DMAPP), or less commonly 1-hydroxy2-methyl-2-(E)-butenyl-4-diphosphate (HMBDP), to ATP, ADP or AMP (Fig. 3) (Sakakibara *et al*. 2005). Although synthesis of most abundant CK is mediated by adenylate isopentenyl transferases, cis-Zeatin biosynthesis is carried out by tRNA isopentenyl transferase enzymes (Miyawaki *et al*. 2006). Isoprenoid side chains of CK are formed in plants in two separate pathways. The mevalonate pathway begins with the condensation of three acetyl-CoA, followed by reduction to mevalonate, which is then subjected to double phosphorylation to form mevalonate-5-diphosphate. Further steps include phosphorylation, decarboxylation, isomerization and lead to final DMAPP formation (Lange *et al.,* 2000). Second pathway called methylerytritol phosphate involves pyruvate and glyceraldehyde-3-phosphate condensation. Next coupled reactions form methyleritriol phosphate, which is then associated with cytidylphosphate. Finally, HMBDP is obtained by further phosphorylation and cyclization. Products of both metabolic pathways serve not only as side chain donors, but are also utilized as precursors for monoterpenes, diterpenes, sesquiterpenes, sterols, carotenoids, chlorophyll plastoquinone and ubiquinone (Lange *et al.,* 2000). After N-prenylation carried out by IPT enzymes, CKs are stored as nucleotides. The last step in cytokinin biosynthesis involves hydrolytic activation, which is catalysed by phosphoribohydrolase enzymes (LOG). Water dependent LOGs cleave nucleotides to ribose-5-monophosphate and biologically active free bases (Fig. 3). Presence of LOGs was first identified in rice, then subsequently also in Arabidopsis. To obtain *trans*-Zeatin, isopentenyladenosine-5-phosphate is hydroxylated by specific cytochrome P450 monooxygenase (Kakimoto *et al.,* 2003). Trans-Zeatin reduction by specific zeatinreductase provides dihydrozeatin (Fig. 3).

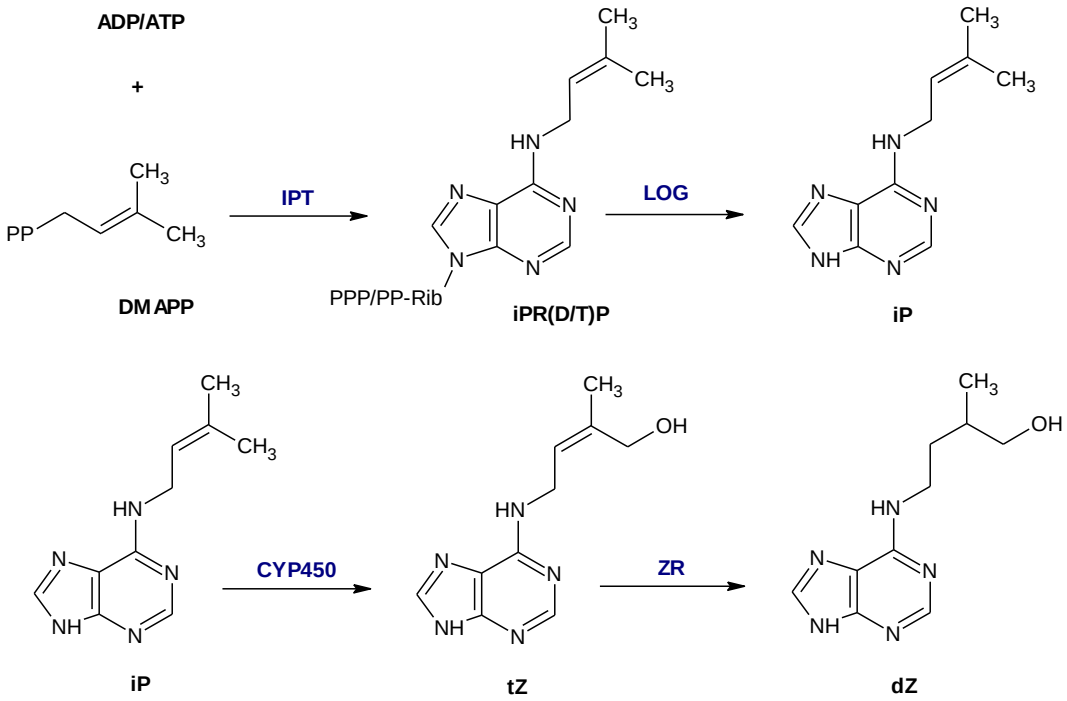

Figure 3- Scheme of CK biosynthesis: isopentenyltransferase (IPT)- N-prenylation of dimethylallylpyrophosphate (DMAPP) and ATP. Nucleotide activation by phosphoribohydrolase (LOG). Isopentenyladenine (iP) hydroxylation mediated by cytochrome P450 monooxygenase (CYP450) forms *trans*-Zeatin. Zeatin reductase (ZR)- reduction to dihydro- Zeatin.

2.3.3 Signal transduction

Cytokinins are signalling molecules that usually act at very low concentrations, (1-50 pmol.g^{-1} fresh weight) (Galuszka *et al.*, 2008). *Trans*-zeatin type distributed to the tissues mainly by xylem, whereas cytokinins derived from iP are transported by phloem. In order to ensure transportation to designated plant tissues, CK are at first transformed into purine nucleotides and nucleosides and as such, they can utilize specific nucleoside transporters (Hirose *et al.*, 2008). Cytokinin-mediated signals are perceived in the two-step histidyl-aspartyl (Asp-His) signalling system. At first CK molecule is captured by a histidine kinase receptor. This transmembrane receptor is composed of two receiver domains, CHASE domain and transmitter. CK binding to the CHASE domain causes changes in its structural conformation, which results in the phosphorylation of the transmitting and receiving domains (Mougel and Zhulin 2001). In *Arabidopsis* the CHASE domain is associated with AHK2, AKH3 and CRE1 / AHK4 histidine kinase receptors (Inoue *et al.*, 2001). In order to elucidate AHK function, several loss-of-function transgenic lines were described for *Arabidopsis*. As shown in publication Kim *et al.* 2006, AHK3 is one of the most important CK receptors involved in the senescence

delaying effect of CKs. *AHK3* overexpression resulted in extended leaf longevity and delayed senescence symptoms. *akh3* loss of function mutant showed faster chlorophyll degradation, reduced expression of *ARR* type A and decreased sensitivity to exogenous CK compared to WT. All symptoms mentioned contributed to early senescence in this mutant (Kim *et al.* 2006). In the next step of CK transduction, signal is transmitted to the regulatory proteins of cytokinin response (RR). This transfer is mediated by specific histidine-phosphotransferase proteins (HPS). RR can be divided into two classes: type A and B, which differ in their structural and functional properties (Mizuno *et al*., 2004). These response proteins have been characterized best in *Arabidopsis*, but their presence has also been confirmed in monocots, particularly in maize (*Zea mays*) and rice (*Oryza sativa*) (Asakura *et al.* 2003). Type B RRs contain a receiver domain fused to the GARP DNA-binding domain. Regarding the DNA binding mechanism, these regulatory proteins utilize helix-turn-helix motif, where specific DNA sequences are recognized by the GARP domain (Hosoda *et al.* 2002). This structural feature enables these proteins to function as important transcription factors. Transfer of phosphate to the B-type proteins induces the expression of RRs type A genes. This indicates that RRs type B act as transcription factors that activate expression of CK regulated genes. The presence of the DNA-binding domains and the ability to function as a transcriptional activator has been demonstrated in *Arabidopsis thaliana* for: ARR1, ARR2, ARR10 ARR11, ARR12, ARR13, ARR14, ARR18, ARR19, ARR20 and ARR21 (Imamura *et al.*, 2003). According to recent findings most of type B proteins contain C-terminal domain, which encompasses nuclear localization signals. GFP method confirmed the presence of most ARR in nucleus. In contrast to type A RRs, levels of type B RRs are not significantly changed with increased content of endogenous or exogenous CK. ARR type-A are considered to be primary transcriptional targets of cytokinin signalling. These type A response regulators have income domain, that comprises a region consisting of Asp-Asp-Lys (Stock *et al*., 2000). Recent findings suggest, that type A RRs can either act as a negative or positive regulators of CK induced gene expression. In *Arabidopsis*, group of type A proteins involves: ARR3, ARR4, ARR5, ARR6, ARR7, ARR8, ARR9, ARR15, ARR16, ARR17. Although the majority of RR type A proteins is localized in the nucleus, ARR16 and ARR17 were also found in the cytoplasmic space (Kiba *et al*. 2002). Interestingly, as was demonstrated in the work of D Agostino *et al*. (2000), CK treatment causes a rapid increment of ARR type A transcript. It was also demonstrated recently that each type A protein has its own characteristic expression pattern in

response to CK. Mutations in *ARR3*, *ARR4*, *ARR5* and *ARR6* resulting in increased sensitivity of *Arabidopsis* to red light perception has also been reported recently which emphasises the role of type A RR proteins as negative regulators of red light signal transmission (Jennifer *et al.* 2004). Whereas single *ARR* silencing in *Arabidopsis* does not cause evident phenotypic alterations such an *RR* manipulation in monocots exhibits severe morphological changes. Research of genetically engineered monocots revealed, that for instance *ZmRR3* is associated with geometric patterning of maize leafs, while *OsRR6* is regulator of the growth (Hirose *et al.* 2007).

2.3.4 Degradation

Cytokinin dehydrogenase (CKX, EC 1.5.99.12) is the enzyme that reversibly degrades CK to adenine and its corresponding side chain aldehyde (Schmülling *et al*., 2003). Regarding to the protein structure, CKX are characterized by the presence of two conserved domains: CK-binding and FAD^+-binding. Both of these domains are essential for correct course of enzymatic reaction. The enzyme oxidizes substrate by the reduction reaction that transfers two H^+ protons to an electron acceptor molecule, such as flavin adenine dinucleotide (FAD^+). This FAD^+ cofactor is covalently bound in the active site (Fig. 4) (Galuszka *et al*., 2001). Preferred substrates of this enzyme are isoprenoid cytokinins, aromatic ones undergo the cleavage reaction very reluctantly. It was demonstrated, that the exogenous application of aromatic cytokinins, (for instance 6-BAP), leads to their preferential inactivation via glycosylation (Frébort *et al*., 2011). While *Arabidopsis* genome encompasses seven genes encoding respective CKX isoenzymes, in rice are present eleven (Galuszka *et al.* 2004), in pea two (Vaseva-Gemisheva *et al.* 2005), and at least 13 putative members are present in barley and wheat (Galuszka *et al.* 2004). CKX occurs in relatively low amount in tissues of non-senescent plants, and both its activity and its expression increases under different stress conditions (Vaseva-Gemisheva *et al.* 2005; Perilli *et al*. 2010). The comparison of amino acid sequences of CKX revealed, that the individual recombinant proteins contain common regions of high sequence homology, which are mainly coincident in the structure of FAD^+ -binding domain. On the other hand outside these areas, particularly in the N-terminal sequences are these isoenzymes different (Schmülling *et al.*, 2003). Systematic analysis of cytokinin-degrading enzymes has proven that each isoform differs in certain biochemical and physiological properties. Functional diversity

of these enzymes is mainly displayed in pH optimum and substrate specificity (Galuszka *et al.*, 2007). It was shown that secretional CKX prefer as a substrate free base, while vacuolar CKX predominantly cleave N-glucosides (Galuszka *et al.*, 2007). Whereas CKX2, CKX4 and CKX6 exhibit the highest activity during degradation of isopentenyl adenine and *trans*-zeatin, other CKX isoenzymes predominantly cleave glucosides and nucleotides (Werner and Schmülling, 2009). Although are CKX enzymes predominantly localized in the apoplast and vacuoles, one isoform was also reported to be located in the cytosol (Šmehilová et al., 2009). CKX enzymes were best described in *Arabidopsis*, where the presence of CKX1 and CKX3 was indicated in vacuoles, CKX2, CKX4, CKX5 CKX6 were found in the apoplast and the remaining CKX7 was detected in cytosol. The function of the individual isoenzymes is likely to be influenced by different cellular localization. Studies on *Arabidopsis* demonstrated that particular isoforms significantly differ in substrate specificity. Cellular compartmentation of CKX enzymes was also described in maize (*Zea mays*), which serves as a reliable predicting model for all cereals including wheat and barley. Up to now was known that ZmCKX1 is located in apoplast, while ZmCKX10 was detected in cytosol and nucleus (Šmehilová *et al.* 2009). Latter ZmCKX2, ZmCKX4, ZmCKX5, ZmCKX8 and ZmCKX12 isoenzymes were found to be distributed in vacuoles (Zalabák *et al.* 2016 Data in press). Studies performed on different plant species suggest, that CKXs response is different to various stressful conditions and that the expression of this enzymes is tissue specific (Vasheva-Gemisheva *et al.* 2004). The same conclusion was drawn by Galuszka *et al*. (2004), who conducted work on the tobacco HvCKX2 gain-of-function mutant, namely that CKX expression is organ specific. To better characterize and clarify the roles of particular CKX isoenzymes in the maintenance of CK homeostasis, several gain-of-function and loss-of-function mutants have been researched. It has been demonstrated that CKX overexpression causes a significant decline in active cytokinins in *Arabidopsis* and tobacco. An increase in the rate of CK degradation disrupted CK homeostasis, which caused reduced chlorophyll retention, decreased soluble sugar concentrations in sink tissues, anomalies of cell cycle and several morphological alterations.

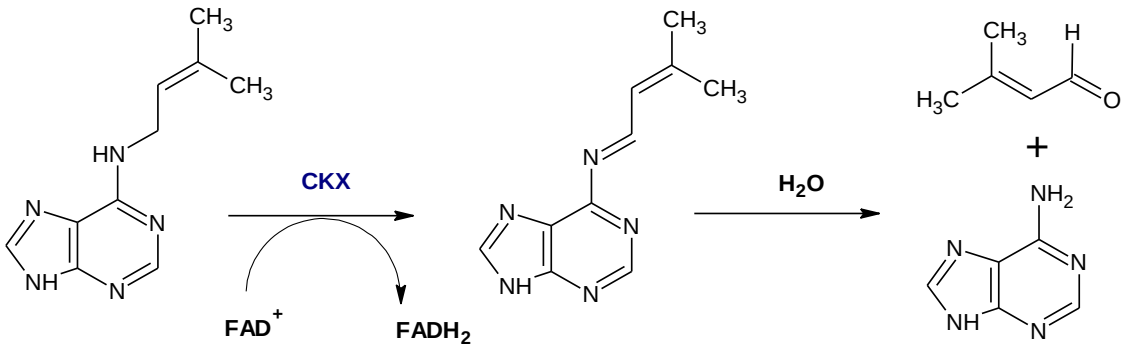

Figure 4. - Mechanism of reaction catalyzed by CKX enzyme. CKX enzyme has FAD^+ cofactor in active site, which catalyzes dehydrogenase reaction, Schiff base is being formed, in the next reaction with molecule of water is CK degraded on adenine and its side chain aldehyde.

2.3.5 Inactivation

The most common modifications of the CK adenine involve N-glycosylation. Glucose can be attached at position N^3, N^7 and N^9 (Fig. 5). Usually, after conjugation, the CK molecule loses its original biological activity. This deactivation is catalysed by UDP-glucosyltransferase enzymes (UGT, EC 2.4.1.X). UDP-glucose serves during this reaction as a donor of sugar moieties (Bowles *et al*., 2006). N^7- and N^9-glucosides are regarded as the final products of irreversible inactivation that can participate in detoxification pathways of plant metabolism (Sakakibara *et al*., 2006). Although N-glucosides are believed to be resistant to CKX cleavage, it was demonstrated, that some recombinant CKX utilize N^9-glucosides as a substrate (Galuszka *et al.* 2007).The other mechanism of CK inactivation involves attaching glucose to hydroxyl group of isopentenyl side chain. This reaction is mediated by UDP-glucosyltransferases. CK *O*-glucosides are resistant to CKX fission, and mostly participate in the maintenance of cell homeostasis. *O*-glucosides serve as a storage form of CK, in case of lack of free CK, are this substances converted back to its free forms in the reaction catalysed by β-glucosidase (Brzobohatý et al., 1993). Although the enzymatic activity of β-glucosidase is generally very low in mature plant tissues, it was shown that this enzyme plays a very important role during seedling development and releases free CK into the embryo by hydrolysis of *trans*-zeatine-*O*-glucoside and dihydrozeatine-*O*-glucoside. The presence of the highest amount of *O*-glucosides was detected in the vacuoles, thus it is believed that *O*-UGT specific enzymes have their greatest expression in these cellular compartments (Bajguz and Piotrowska, 2009). *Cis*-Zeatin (cZ) is generally regarded as a cytokinin with little or no activity, compared with the highly active trans-zeatin (tZ).

Although recent studies have suggested possible roles for cZ, its physiological significance remains unclear. Recently three putative cZ-*O*-glucosyltransferases (cZOG1, cZOG2, and cZOG3) were identified in rice. In the work of Kudo *et al.* (2012), it was shown that cZOGTs preferentially catalyse *O*-glucosylation of cZ and cZ-riboside rather than tZ and tZ-riboside. The same study revealed that some transgenic plants overexpressing the *cZOG1* and *cZOG2* genes exhibit delay of leaf senescence.

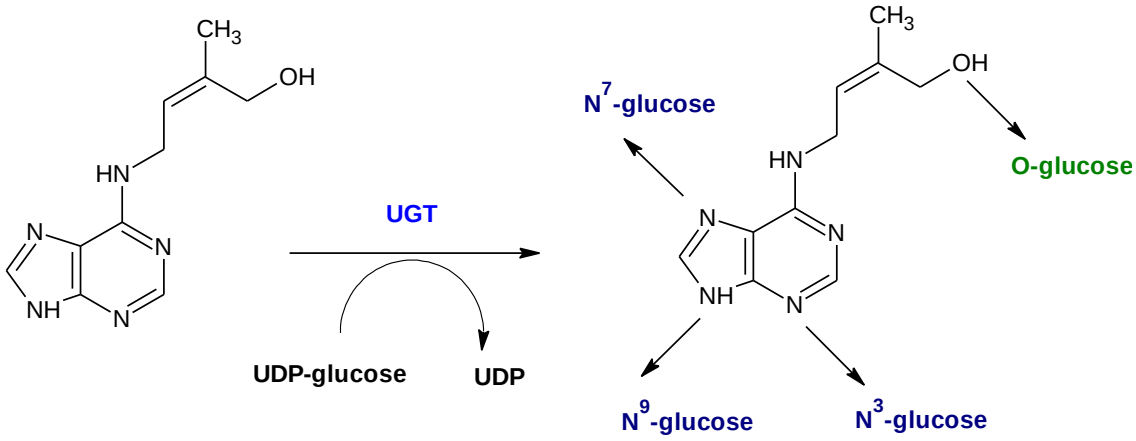

Figure 5- Mechanism of CK inactivation catalysed by glucosyltransferase enzyme. The glucose units are supplied from uridindiphosphateglucose. As shown, glucose can be attached to adenin ring either at position N^3, N^7, N^9, or on isopentenyl side chain, to hydroxyl.

2.4 Plants studied: Monocotyledoneus plants and *Poaceae* family

The flowering plants have been divided to dicotyledons and monocotyledons. In contrast to dicotyledon plants, which contain two embryonic leaves, monocotyledon plants contain only one embryonic leave (cotyledon). Monocotyledon plants comprise around 60 000 species, from which over 40 000 represent true grasses (*Poaceae*), and remaining 20 000 form orchids (*Orchidaceae*).

Poaceae is the family of grasses, which contains over 10 000 species. Their reproductive mechanism, anatomy and genetic variability have resulted in great ability of adaptation to the wide range of natural conditions. These features have contributed to their widespread distribution from the equator to the Arctic Circle (Feuillet *et al.* 2007). Since the beginning of agriculture (more than 10 000 years ago), cereals represent one of the most important cultivated crops on the earth. The main benefit of *Poaceae* agricultural crops is relatively high yield, nutritional value, long-term storage and easy transportation (Zohary and Hopf, 2000).

The largest group of domesticated plant species is represented by the genus *Triticeae*, whose members are estimated to form over a third of global cereal production (Feuillet *et al.*, 2007). *Triticeae* include the genera of *Hordeum*, *Triticum* and *Secale*. The most prominet role concerning world cereal production is played by a genus *Triticum* (wheat). Particular species and subspecies of *Triticeae* mostly differ in the size of the genome and its arrangements. According to the number of chromosomes, diploid (2n = 2x = 14) *Triticum monococcum L.*, *T. urartu T. boeoticum Boiss*, tetraploid (2n = 4x = 28), *T. turgidum L.*, *T. turgidum durum*, *T. turgidum dicoccum*, and hexaploid (2n = 6x = 42),: *T. spelta L. and T. aestivum L* can be distinguished. Regarding the hardness of the seeds, milling properties and quality of the flour, most important is *Triticum aestivum L.*, which represents over 90% of world wheat production. From the perspective of evolution very significant role was played by *Triticum monococcum* (Devos and Gale, 1997; Feuillet et al., 2007).

2.4.1 Wheat (*Triticum aestivum*)

As one of the most widely cultivated crops, wheat is a major source of food in the world and therefore an important plant to study with the prospect of improving crop yield. Bread wheat, *Triticum aestivum L*. is hexaploid cereal (2n = 6x = 42 AABBDD) whose genome is composed of three homologous subgenomes (A, B, D), which are derived from different species. The size of nowadays wheat genome is enormous it contains around 17 Gb (Bennett and Smith, 1991). The first step of wheat evolution was launched around 3,000,000 years ago by the hybridization of diploid wheat, *Triticum urartu* ; which served as a donor of genome A (Dvorak *et al.*, 1993) and unknown species *Sitopsis*, closely related to *Aegilops speltoides*, which provided genome B (Dvorak and Zhang, 1990). This breeding resulted in tetraploid *Triticum turgidum* formation (2n = 4x = 28, AABB).This *Triticum turgidum* was in comparison with its diploid ancestors, more vital, more adaptable to the changing environmental condition and had increased yield. *Triticum turgidum* was firstly domesticated and utilized as a cereal crop more than 10 000 years ago. In the past, different varieties of this tetraploid species were bred by farmers. However in the present days these breeds have, except for *Triticum turgidum ssp. Durum*, which is widely used for pasta production, little economic and agricultural significance. The second phenomenon, which has led to the wheat evolution, took place around 10 000 years ago. It was launched by the crossing of

Triticum turgidum durum with wild-type diploid species *Aegilops tauschii* (2n = 2x = 14, DD, Fig.). The genomic hybridization gave rise to new hexaploid species known as common wheat, *Triticum aestivum* (2n = 6x = 42, AABBDD). Genome D confers genes for better adaptation to the continental climate, and also encodes proteins, that improve properties of leavened wheat products. The combination of these traits and factors directly contributed to the global expansion of bread wheat (Feuillet et al., 2007). As particular genomes are derived from common ancestor, nucleotide diversity within these genomes is reduced. Very large and complex wheat genome constituted barriers for obtaining full wheats sequence data. In recent work, data for whole genome shotgun sequencing were compiled (Branchley *et al*. 2012). Gen analysis implied two strategies: 454 pyrosequencing and sequence comparison with ancestral genomes. As a result around 96000 genes were assigned, information of two thirds wheats genome was compiled. Comparison with ancestral sequences revealed loss of some gene families, what has probably occurred during its polyploidization and domestication (Branchley *et al*. 2012).

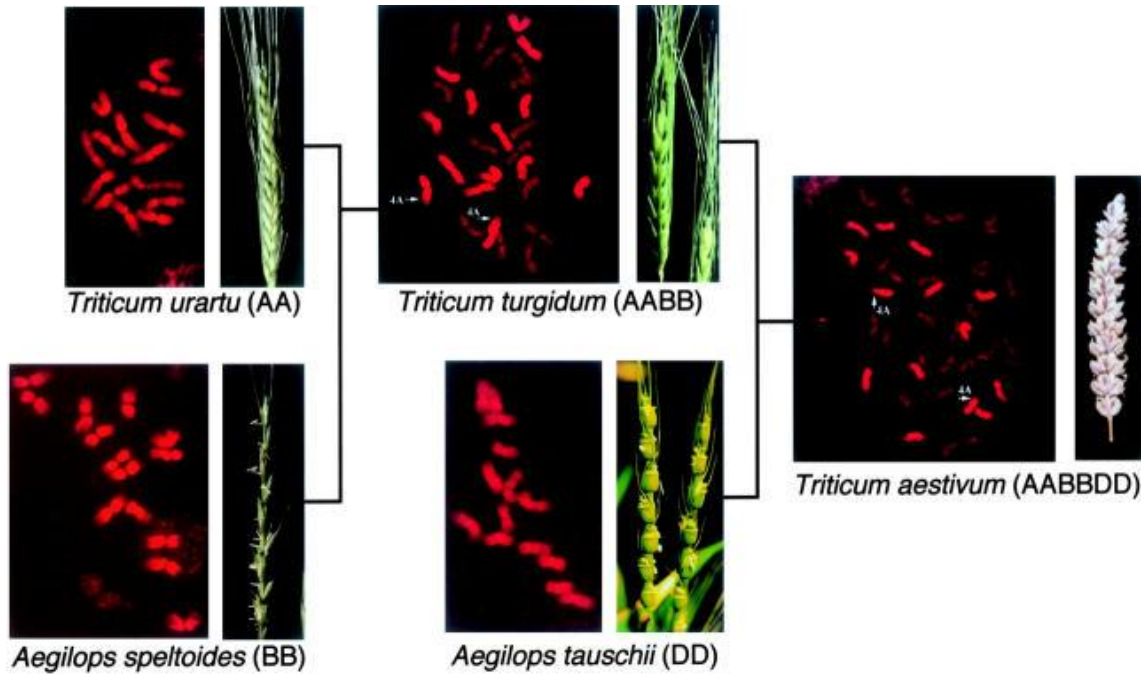

Figure 6- Gradual development of the wheat genome by crossing of its ancestors genomes. Around 3 millions years ago crossing of *Triticum Aegilops speltoides* and *Triticum Uartum* gave rise to tetraploid wheat, *Triticum turgidum* (AABB). Around ten thousand years ago, *Triticum turgidum* intersected with the grass *Aegilops tauschii*, which served as a donor of genome D. This phenomenon led to the evolution of hexaploid wheat, *Triticum aestivum* (AABBDD). Each letter represents the single copy of the genome, which encompasses seven chromosomes. Image was extracted from (Gill *et al.*, 2004).

2.4.2 Barley (*Hordeum vulgare*)

Barley represents the fourth most cultivated cereal in the world. This crop is mostly used for animal feed, malting and brewing. Concerning the genetic constitution, barley is a diploid organism, whose genome encompasses over 5 Gbp (Bennet and Smith 1976). Owing to its simpler diploid genome and direct close relationship, barley has been utilized as a reliable genomic model for bread wheat (*Triticum aestivum*). In the present work the effect of spectral quality was researched on genetically engineered barley cultivar, called Golden Promise.

Golden Promise is the variety of spring barley cultivar, which exhibits salt tolerance. This salt resistant cultivar was obtained by gamma-ray induced mutation of cultivar Maythorpe (Forster *et al.* 1994). Owing to its improved agricultural traits, such as short stature and earliness, it became desired commodity for malting. In response to salt stress conditions, this variety accumulates in comparison to Maythorpe variety lower levels of Na^+ in shoot tissue. When compared with Maythorpe, Golden Promise produces higher number of seeds of seeds and fewer sterile. The phenotype alterations have been inflicted by single recessive mutation called *GPert*.

3. METHODS

3.1.1Chemicals

4-Aminophenol (Sigma-Aldrich, USA),

Acrylamide (Sigma-Aldrich, USA),

Agarose (Amresco, USA),

Amidoblack (Merck, Germany),

Ammonium nitrate (Lachema, ČR),

Ammonium persulfate (Fluka, USA),

Bisacrylamide (Sigma-Aldrich, USA)

Boric acid (NeoLab, Germany),

Calcium nitrate tetrahydrate (Penta, ČR),

Citric acid (NeoLab, Germany),

Coomassie briliant blue R250 (Penta, ČR)

Copper sulfate pentahydrate (Lachner, ČR)

Dichlorophenolindophenol (Fluka USA)

Dimethylallyl-aminopurine (Sigma-Aldrich, USA)

Dimethylsulfoxide (Duchefa, Netherlands)

Dithiothreitol (Fluka, USA)

Dried low- fat milk (Laktino, ČR)

Ethanol 96% (Lachner, ČR)

Ethylendiamintetraacetic acid disodium salt (Penta ČR)

Glycerol (Lachner, ČR)

Glycin (Lachner, ČR)

Hydrogen peroxide (Lachner, ČR)

Leupeptin (Sigma-Aldrich, USA)

Lithium Chloride (Ambion, Lithuania)

Magnesium sulfate heptahydrate (Penta, ČR)

Manganese chloride tetrahydrate (Sigma-Aldrich, USA)

Mercaptoethanol (Sigma-Aldrich, USA)

N,N- dimethylformamid (Lach-Ner, ČR)

Nuclease-free water (Qiagen, Germany)

Osmocote (Substral, Germany)

Phenylmethylsulfonyl fluoride (Sigma-Aldrich USA)

Polyvinylpolypyrrolidone (Fluka USA)

Potassium dihydrogen phosphate (Lachema, ČR)

Potassium nitrate (Penta, ČR)

Bradford Protein assay (Bio-rad, USA)

Sodium dodecyl sulfate (Penta, ČR)

Sodium hydrogen phosphate (Lachema, ČR)

Sodium chloride (Lachner, ČR)

Sodium molybdate dihydrate (Penta, ČR)

Soil (Sondermischung, Germany)

Tetramethylethylenediamine (Sigma-Aldrich, USA)

Trichloracetic acid (Penta, ČR)

Tris (Duchefa, Netherlands)

Triton-X-100 (Sigma-Aldrich, USA)

Tween 20 (NeoLab, Germany)

Zink sulfate tetrahydrate (NeoLab, Germany

3.1.2 Enzymes and antibodies

Small Scale Phenol-Free Total RNA Isolation (RNAqueous®) Kit, TRI Reagent® Kit and Turbo DNase and 5x concentrated Turbo buffer (Ambion, Lithuania). RevertAid H Minus M-MULV reverse transcriptase (200000 $U.ml^{-1}$) and 5x concentrated buffer (Thermo Scientific, USA). Oligo(dT) primer (Sigma, USA). 10 mM dNTP (Fermentas, Canada). gb SG PCR Master Mix and ROX dye (Generi biotech, ČR) Primary antibody was polyclonal (obtained from Kristine Bilyeu), as a secondary antibody was used Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology). Luminol reagent for WB was Clarity Western ECL Substrate (Bio-rad, USA). Precision Plus Protein™ Standard and Bradford reagent for protein assay were from Bio-Rad, Lowry and Phenol Reagent were from Pierce (USA).

3.1.3 Buffers

Hoagland solutions: **1.** 1 M KH_2PO_4, **2.** 2 M $MgSO_4.7H_2O$, **3.** Micronutrients: 46 mM H_3BO_3, 0,48 mM $MnCl_2.4H_2O$, 0,765 mM $ZnSO_4.7H_2O$, 0,2 mM $CuSO_4.5H_2O$, 0,5 mM $Na_2MoO_4.2H_2O$, **4.** 1 M Fe-EDTA, **5.** 2 M KNO_3, 2 M $Ca(NO_3)_2.4H_2O$, 1 M NH_4NO_3.

Buffers for CKX activity:

CKX extraction (Wheat): 0,2 M Tris/ acetate buffer, 0,3% Triton-X-100, 1 mM PMSF pH=8.

CKX extraction (Barley): 0,2 M Tris/HCl, 150 mM NaCl, 0,1% Triton-X-100, 1 mM , 10 μM Leupeptin, pH=8.

McIlvaine: 200 mM citric acid, 200 mM Na_2HPO_4, pH= 6,5.

Buffers for Western blot:

Protein Extraction: 50 mM Tris, 150 mM NaCl, 20 mM $MgSO_4.7H_2O$, 2 mM Na_2EDTA, 1 mM PMSF, 1 mM DTT and 1% Triton-X-100, pH=7,7.

Cracking buffer: 6,25 mM Tris/HCl, 25% glycerol, 20% SDS, 200 mM DTT. 50 % (v/v) glycerol, 0.05 M EDTA, 0.125 % (w/v) bromphenol blue, 0.125 % (w/v) xylene violet, pH 8.0, 2,5% β-mercaptoethanol (25 μl added to 1 ml).

Acrylamide-Bisacrylamide: 30 % (w/v) acrylamide, 0.8 % (w/v) bisacrylamide.

10% Running gel: 5,9 ml H_2O, 5 ml 30% acrylamide, 3,8 ml 1,5 M Tris (pH=8,8), 150 µl 10% SDS, 150 µl 10% APS, 6 µl TEMED for final volume 15 ml.

4% Stacking gel: 4,1 ml H_2O, 1 ml 30% acrylamide, 0,75 ml 1M Tris (pH=6,8), 60 µl 10% SDS, 60 µl 10% APS, 6 µl TEMED for final volume 6 ml.

SDS – stock solution: 10 % (w/v) SDS.

Ammonium persulphate $(NH_4)_2S_2O_8$ (APS): 10 % (w/v) ammonium persulphate.

Running buffer (10 x concentrated): 25 mM Tris, 192 mM glycine, 35 mM SDS.

Running buffer (SDS-PAGE): 2,5 mM Tris, 19,2 mM glycine, 3,5 mM SDS.

Transference buffer (10 x concentrated): 192 mM Glycine, 25 mM Tris

Transference buffer (transfer): 19,2 mM Glycine, 2,5 mM Tris, 20 % methanol

TBS-T: 0,02 mM Tris, 0,5 M NaCl, 0.1 % (v/v) Tween 20, pH=7.5.

Blocking buffer: 0.02 M Tris, 0.5 M NaCl, 5 % (w/v) milk, pH 7.5

Amidoblack solution: 0,1 g of Amidoblack was dissolved in 80% Acetic acid, 4 ml of methanol, volume was comleted to obtain 0,162 mM Amidoblack.

Buffers for Coomassie Brilliant Blue R-250 staining:

Fixing buffer: 40 % (v/v) methanol, 10 % (v/v) acetic acid.

Coomassie Brilliant Blue R-250 solution: 0.1 % (w/v) Coomassie Brilliant Blue R-250, 15 % (v/v) acetic acid, 45 % (v/v) methanol.

Destaining buffer: 40 % (v/v) methanol, 10 % (v/v) acetic acid.

Buffers for incubation of membranes with antibodies:

Primary antibody buffer: 0.02 M Tris, 0.5 M NaCl, 1 % (w/v) powdered milk, pH 7.5, primary antibody (dilution from 1 / 1000)

Secondary antibody buffer: 0.02 M Tris, 0.5 M NaCl, 1 % (w/v) powdered milk, pH 7.5, secondary antibody (dilution 1 / 25000).

3.1.4 Instruments

Plant samples were homogenized by Mixer mill MM400. Weight of chemicals and samples were taken by analytical balance (Nahita, Japan). Buffer´s pH were adjusted with pH50 Bench pH-meter (XS instruments, Italy). Chlorophyll content was measured with CCM-300 chlorophyll content Meter (Opti Sciences, USA), Light intensity was measured with Light Sensor and Display Meter System (Skye Instuments Limited, UK), Concentration of proteins, CHL in DMF and CKX activity were measured by UV/VIS spectrophotometer 8453 (Agilent, USA). Samples were centrifuged either with centrifuge SCANSPEED 1730R (LaboGene, Denmark) or centrifuge Eppendorf 5417R (eppendorf, Germany) or Mikro 120 (Hettich, Germany) and vortexed with VSM-3 (Shelton Scientific, USA) and with FVL-2400N Combi-Spin (Biosan, Letonia). Nucleic acids concentration was measured with NanoDrop™ Lite Spectrophotometer (Thermo Scientific, USA). Thermocycler T-personal (Biometra, Germany). Pipeting Station (Agilent Technologies, USA.The plates were centrifuged in centrifuge NF400 (Nüve, Turkey), The transcript level was quantified by real-time PCR with ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA) and StepOnePlus™ System (Applied Biosystems, USA).). Shaker orbit 1000 (Labnet, USA). For chemioluminiscent detection of western Chemi Doc^{TM} MP imaging system (Biorad, USA) was used. SDS-PAGE separation of the proteins was performed in MINIPROTEOM tetrasystem (Biorad, USA) using ENDURO^{TM} power station (Labnet, Germany).

3.1.5 Used software

ACD Chemsketch (2010) was used for painting chemical structures.

StepOne Software v2.1 (Applied Biosystems)- for qRT-PCR analysis.

ViiA 7 RUO Software (Applied Biosystems)- for qRT-PCR analysis.

DataAssist (Applied Biosystems, USA) was used for the analysis of gene expression.

BioEdit (Hall, 1999) was used for comparison of CKX sequences.

Mega7.2 (Takamura, 2014) was used for phylogenic analysis.

3.2 Biological material

Wheat *Titicum aestivum* L. (cv. Inta oasis), barley *Hordeum vulgare* (cv. Golden promise).

3.2.1 Preparation of the seeds

Seeds were cleaned for 12 min in 10% v/v H_2O_2, and then washed several times in distilled water. Sterilised seeds were put to germinate in Petri dishes with wet paper and left in room temperature for about 48 hrs. When their roots started to emerge, they were transferred to soil. Barley seeds were disinfected for 12 min in 2% sodium hypochlorite, and then washed several times in distilled water. They were transferred to Petri dishes with wet paper. In order to stimulate germination, they were put for 48 hrs. in the fridge and then they were 1 day left at room temperature. When they started to emerge their roots, they were transferred into the soil.

3.2.2 Cultivation

Plants were grown under standard light conditions in a greenhouse, in pots containing a mixture of sand and perlite in a 2:1 ratio. After 4/5 days fertilization was started. Plants were cultivated for about 3-4 weeks, and meanwhile fertilized always twice a week with a modified Hoagland solution (Hoagland and Aron 1950). Plants were supplied with 40 mM NO_3^-, 10 mM NH_4^+, 10 mM Ca^{2+}, 18 mM K^+, 8 mM PO_4^{3-}, 4 mM Mg^{2+}, 4 mM SO_4^{2-}, 0,02315 mM BO_3^{3-}, 0,00024 mM Mn^{2+}, 0,00048 mM Cl^-, 0,00038525 mM Zn^{2+}, 0,000102 mM Cu^{2+}, 0,000291 MoO_4^{2-}, concentration is relativized on 1L of HS. Experiments were also conducted in phytotron (temperature 15 ˚C, humidity 74,5 %, photoperiod of 18 hours) in this case cultivated in soil containing 2.5g.L-1 of Osmocote fertilizer. In order to induce senescence, every 4^{th} totally expanded and mature leaf was excised (always the same leaves to assure homogenous material),and incubated in plastic boxes (12,5 cm × 12,5 cm, depth 1 cm) with water, whose lids were covered with special Lee filters (Fig. 8) (red#026, green #089 or blue #075), or with a transparent or white cover for controls (non filter, NF; white, W) and lined with aluminum foil on the sides and base to increase light reflection inside the box. Two types or controls were used, without shading (NF) or shading just to decrease radiation in the same proportions as filters but without affecting the light quality. For each treatment three independent biological replicates were performed. The use of cutted leaves not only facilitates the development of senescence in a relatively short time, but allows processing a larger amount of biomass without affecting the pattern of senescence observed in intact leaves (Causin et al. 2007) with similar light filters.

These filters used were chosen because although they differ in the percentage of transmission of blue light, photosynthetically active radiation (PPFD) does not differ significantly between the shading treatments and transmit different proportions of red (R=660 nm) to far red radiation (FR=730 nm) (Causin et al. 2006)**.** As shown in (Fig, 8), the transmission in the blue region for B is between 40 and 80%, R entirely deprive plants of BL meanwhile G transmits less than 1% (fig. 5).

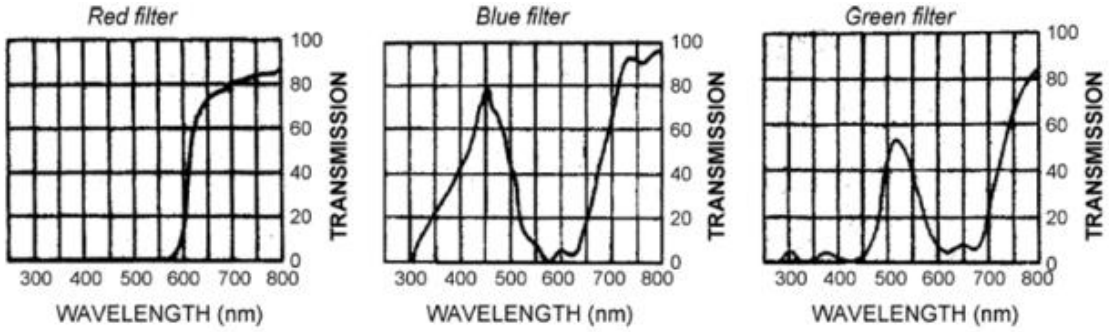

Figure 8- Transmission spectrum of Lee filters red (R), blue (B) and green (G). R does not transmit BL, G is characteristic with very low BL transmission.

The average values of PPFD (μmol m^{-2} s^{-1}) were 2,8 ± 0,1 for treatment W; 2,8 ± 0,15 for treatment B; 3,0 ± 0,1 for treatment G and 2,4 ± 0,1 for treatment R and 15,5 ± 0,21 for treatment NF.

3.2.3 Sampling procedure

Sampling was performed at 0, 24, 50, 80, 100 and 120 hs after scission. Samples were homogenized with liquid nitrogen in the mixer mill (frequency 27 Hz, 2 min) and determined weight fractions were separated and stored at -70ºC for further analysis. All experiments were repeated three times. Data shown are from one representative experiment.

3.3 Chemical analysis

3.3.1 Chlorophyll content evaluation

The use of chlorophyll degradation measurement as a marker of senescence is due to the close link between this degenerative process and the breakdown of the chlorophyll. Chlorophyll content was measured every time point (times could be modified according the rate of chlorophyll degradation and maturity of the leaves).

In some cases chlorophyll concentration was estimated using a nondestructive method, using a chlorophyll meter. The reading was carried out in the central part of the sheet (five measurements per sheet) and an average per biological replicate (total of three leaves) was calculated. Three biological replicates (each consisting of three leaves) were studied.

Depending on the experiment, chlorophyll content was also measured using the method of Porra (2002). 0.02 g of homogenized leaf biomass was extracted in 5 ml of N,N-dimethylformamide (DMF). Mixture was covered with aluminium and incubated for 72 hours at 4˚C. Absorbance was screened at 647 nm and 665 nm. The total chlorophyll concentration was quantified from the following equation (Porra 2002): $\mathrm{Chl\ a+b} = 17{,}67\mathrm{A}^{647} + 7{,}12\mathrm{A}^{665}$

3.3.2 Soluble protein concentration

Proteins were colorimetrically measured according to Bradford *et al*. (1976), using bovine serum albumin (BSA) as standard.

3.3.3 CKX activity measurement

The measurement of CKX activity is based on the conversion of iP to adenine. The side chain of CK molecule is being converted into aldehyde. The quantity of side chain aldehyde is proportional to the CK content, and can be directly determined in coupled reaction with paraaminophenol (Figure 9). CKX activity is indirectly proportional to the amount of aldehyde (Frébort *et al.* 2001). This means, that the lowest values of aldehyde concentration indicate the highest CKX activity. Total amount of extracted proteins was determined using the Bradford method. This method is based on a colorimetric reaction that shifts the absorbance of the Coomassie Brilliant blue dye. Under acidic conditions red form of this nonspecific dye is converted into its protein binding blue form. Coomassie dye donates its free electron to the ionisable groups of the protein. This results in hydrophobic and electrostatic interactions (positive amine groups in proximity with the negative charge of the dye). The absorbance is measured at a wavelength of 595 nm (Bradford 1976).

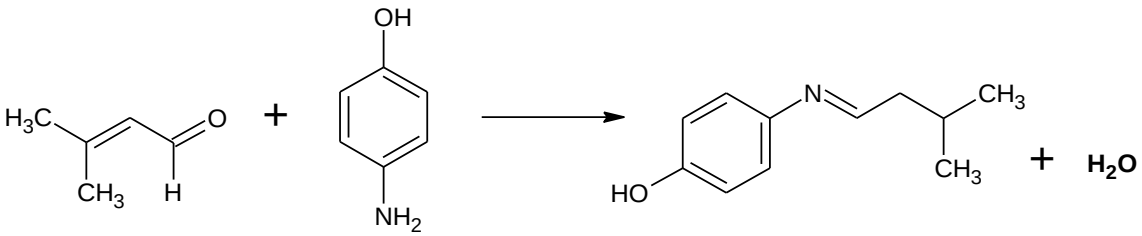

Figure 9- Reaction of side chain aldehyde (arising from side cytokinin molecule) with paraaminophenol. The cytokinin activity measurement utilizes schiff base chromofor. Absorbances are screened for 352 and 450 nm (Frébort *et al*. 2001).

Samples were extracted in the buffer containing 0,2 M Tris/ acetate buffer, 0,3% Triton-X-100, 1 mM PMSF pH=8. The buffer was added in ratio 1:3 (sample:buffer).The mixture was then incubated in ice for 1-2 hours, meanwhile samples were vortexed every 15 minutes. Cell debris was removed by maximun speed centrifugation for 10 minutes at 4˚C. Supernatant was then transferred into new tubes. For barley CKX extraction was used the buffer composed of 0,2 M Tris/HCl, 150 mM NaCl, 0,1% Triton-X-100, 1 mM , 10 μM Leupeptin, pH=8. For the set up of the reaccion 30 μl of sample was incubated over night at 37 ˚C with 200 μl of McIlvaine buffer, 40 μl of DCPIP redox dye. The samples differed from blank in addition of 10 mM iP, which serves as CKX substrate. After elapsing of time, proteins were precipitated with addition of 300 μl of 40% Trichloracetic acid (TCA) and centrifuged at maximun speed. In order to detect cleaved aldehyde, 200 μl of paraminophenol (PAF) was added (2% PAF in 6% TCA). Samples were vortexed and then centrifuged at maximun speed. The absorbance was screened at wavelengths of 352 and 450 nm.

3.4Analysis of gene expression

3.4.1 RNA isolation

Two kits were used for isolation of RNA: Small Scale Phenol-Free Total RNA Isolation (RNAqueous®) Kit and TRI Reagent® Kit.

3.4.1.1 RNAqueous kit

This technique is based on glass fiber filter separation. After cell lysis with chaotropic guanidinium solution, is extracted RNA electrostatically bound to the glass fiber housed filter. The given procedure enables to obtain high quality RNA for RT-PCR analysis (Zhan *et al.* 2012). 100 mg of homogenized leaf material was extracted with 1 mL

Lysis/Binding Solution, and samples were vortexed for 5 minutes and incubated for 5 minutes at room temperature. Centrifugation was performed (5 min, 4 ˚C, 19500 g) to remove cell debris and the supernatant was transferred into fresh tubes, mixed with equal volume of 64% ethanol and mixed about 5 times by inversion. The lysate/ethanol mixturewas transferred onto the columns provided on the kit (a filter cartridge assembled in a collection tube) and centrifuged 1 min at 10 000 g). Filter was kept and the flow-through was discarded. Later on, 700 µl of wash solution 1 was applied to the filter, centrifugated (1 min, 10 000 g) and again the flow through was discarded. 500 µl of wash solution 2/3 was applied twice, this step was also accompanied by centrifugations (1 min, 10 000 g) and the flow through was discarded.The filter cartridge was transferred to a fresh collection tube and the RNA was eluted with the addition of 100 µl of elution buffer (preheated to 75 ˚C) and centrifugation (15 000 g, 40 s, 20 ˚C).

3.4.1.2 Trizol

Trizol solution consists of phenol, guanidine isothiocyanate, and other components, which disrupts cells and dissolves its cell components. After treatment with trizol, chloroform is added. This enables to separate RNA, which is present in the clear upper aqueous layer from DNA and proteins in sediment (Chadderton *et al*. 1997). RNA can be further precipitated from the aqueous layer with isopropanol. 1 ml of TRI Reagent solution was added per each 100 mg of homogenized biomass and the mixture was then incubated for 5 min at room temperature. Samples were centrifuged at 12000 g, 10 min at 4˚C and the supernatant was transferred into new tubes, where 200 µl of chloroform was added. The homogenate was mixed by inversion, incubated at room temp. for 15 min and then centrifugated at 12000 g, 15 min at 4˚C. The aqueous upper phase was transferred to a fresh tube and then 500 µl of isopropanol was added. After mixing and incubating at room temp. for 10 min, centrifugation at 12000 g, 8 min at 4˚C was done The supernatant was removed and to clear the pellet containing the RNA, 1 ml of 75% ethanol was added, mixed and then centrifuged at 7500 g, 5 min at 4˚C. All residual ethanol was removed, pellet was air dried and dissolved in elution buffer.

3.4.2 DNAase treatment

In order to remove genomic DNA, samples were treated with DNAase enzyme. 5 µl of the buffer (10 times concentrated TURBO DNAase) and 3 µl of DNAse enzyme (600

U, 9,996 μkat), was added to 50 μl of the sample and mixed gently. The mixture was incubated for 45 min at 37 ˚C. Subsequently, 2 μl of DNAse enzyme (400 U, 6,664 μkat) was added and samples were incubated the same way as mention previously. Reaction was stopped by precipitation with LiCl. 30 μl of LiCl was added to 60 μl of the sample and mixed by vortexing. This mixture was left overnight at -20 ˚C. Later on, samples were centrifuged (15 min, 19 000 g, 4 ˚C) and the water phase was discarded and the pellet was washed with 1 ml of 75% ethanol. Centrifugation was performed (5 min, 7500 g, 4 ˚C). The pellet was air dried and dissolved in nuclease free water.

3.4.3 Reverse transcription

2 μg of isolated RNA was transcribed to cDNA with RevertAid H Minus M-MULV reverse transcriptase. 1 μl of 100 μM oligo (dT) was added to 12 μl of sample, mixed and incubated at 70 °C for 5 min in a thermocycler. After, per each sample 1 μl of the RT enzyme (200 U/ μl, 3,332 μkat), 4 μl of 5 times concentrated reverse transcriptase buffer and 2 μl of 10 mM deoxynucleotides (dNTPs) were added. RT programme was continued for 90 minutes at 42 ˚C, and the reaction was stopped by 10 min heat deactivation at 72 ˚C.

3.4.4 qPCR analysis

Real time PCR is molecular technique, which is based on the polymerase chain reaction (PCR) (Mullis *et al.* 1986). The amplification of targeted DNA is monitored after each cycle, by the intercalation of non-specific dye called Sybr green. RT-PCR method can either be used for quantitative detection of certain amount of DNA (Zipper *et al.*, 2004) or qualitative analysis, which involves comparison of relative changes in gene expression.

3.4.4.1 Step one plus analysis

All of the samples were firstly screened for Ct values for some housekeeping gene, an endogenous control with constant expression in all of the plant tissues. In order to obtain samples with similar CT values prior to analysis of all genes of interest, dilution of some samples was performed according to one housekeeping gene. All primers were used in a final concentration of 300 nM, except GADPH which was 500 nM. For RT-PCR analysis was used Master mix containing DNA Taq polymerase, dNTP, MgI_2. Master mix contained ROX dye (ratio 5:1). Each well contained 2.5 μl of cDNA, 2.5 μl

of diluted primers and 5 µl of the Master mix solution. The programe included: Initial heating (120 s, 95 ˚C), Denaturation 1 (600 s 95 ˚C), Denaturation 2 (15 s 95 ˚C) and Annealing (60 s, 60 ˚C). The number of repeated cycles was 40.

3.4.4.2 Via VII analysis

To research expression patterns of genes associated with CK metabolism, series of RT-PCR experiments were carried out. 3.75 µl of prepared premix containing mixture of Master mix and primers was transferred into the wells. Master mix contained ROX-dye in ratio (1:50). 1.25 µl of cDNA was dropped on the wall. 384 well plate was then centrifuged, The programe included initial heating (120 s, 95 ˚C), Denaturation 1 (600 s 95 ˚C), Denaturation 2 (15 s 95 ˚C) and Annealing (60 s, 60 ˚C). The number of repeated cycles was 40.

Reference samples were To and the endogenous controls used: *HvEF2* and *HvACT* for barley, and *HvEF2* and *TaGADPH* for wheat.

.**Table 1**- Primer sequences used for RT-PCR analysis.

PRIMERS	**Forward**	**Reverse**
Houskeeping	**5'-3' direction**	**5'-3' direction**
HvEF2	CCGCACTGTCATGAGCAAGT	GGGCGAGCTTCCATGTAAAG
TaGADPH	TTAGACTTGCGAAGCCAGCA	AAATGCCCTTGAGGGTTTCCC
HvACT	TGTTGACCTCCAAAGGAAGCTATT	GGTGCAAGACCTGCTGTTGA
CK metabolism	**5'-3' direction**	**5'-3' direction**
HvCKX1	TGTGGACAGTAACACAGCAGTTTAAC	CCGTGCCACCTACTATCAAATTT
TaCKX3	CGTGGCTCAACCTCTTCGTC	GTTCGGGTCCCACTTGCTC
TaCKX4	TGCTGTCTCTGGCTGAGATACATACAG	TGACGTCCTGTTGTCCCACTTTG
TaCKX6	CGACGAGATCTTACGGTTCT	GACCGATGGATCAGCCA
TaCKX8	GCCAAATATGATCCCCACGC	GTGTGCCAGCGGCAAGCACGGCA
TaCKX10	GGTAAGGTGGATAAGAGTTCTCTACTT	ATCTGAGTTGAGATAGTAGTGCATGGA
TaGLU2	GCTCTTCAACATCCCGCTCT	AGTCGGCGAGTTCCACAAAT
TacZOG1	TCATCTGGGTATTGCGCGAG	CTCAGAGAGCAGCTTGTCGT
TacZOG2	CCAGGTGATCGACAAAATGCC	CGGCATCAAAAGGCAAATCAG
TaHK3	TTAAAAACCTGGAGATCGATGGA	GGTGGGATGGTGTACCAAGAA
TaRR1	GGCTACGACCTCCTCAAAGCCA	GCGGCACATCCTTGCTCTGA
TaRR4	AGGAGGTGGGCGTGAATTTGA	TGCGACTTGAGCTTCTTCATGTCA
TaRR9	CGTGGATGACAGCGTCCTTGA	CTTCCTCCAGGCATCTGTTGATTC
TaCRY2	TCCGATATCAAGGAAAAGATCCA	GAGTGCACTTCGACTGAAGATGA
TaCIB1	GACGCCGCCTCTCAGGTT	TGCCCCATGTCCATCTGAA

3.5.1 Western- Blot

Protein extracts were obtained by homogenizing plant material in liquid nitrogen in mortar and pestle and extracting in ratio 1:3 with the extraction buffer containing 50 mM Tris, 150 mM NaCl, 20 mM $MgSO_4.7H_2O$, 2 mM Na_2EDTA, 1 mM PMSF, 1 mM DTT and 1% Triton-X-100, pH=7,7. For removal of phenolic compounds, one spoon of PVPP was added. Cell debris was removed by centrifugation at 20,000 g for 25 min at 4°C. The supernatant was transferred into fresh tubes and centrifuged at 20,000 g for 25 min at 4°C. Supernatant was collected and protein content was measured using Bradford method. 300 ug of the extracted proteins were applied into each well, as a marker of molar weights Pre-Stained Protein Ladder was used and, extracted proteins were subjected to the electrophoretic separation in a 10% denaturing SDS-polyacrylamide gel during 15 min at 100V and 60 min at 180 V, according to Schägger 1987 procedure. Proteins were transfered onto 0.45 μm polyvinylidene fluoride membrane (PVDF, Millipore), previously activated in methanol, during 1h at 100V using buffer composed of 192 mM Glycine, 25 mM Tris, 20 % methanol. Later on, the membrane was blocked with 5% powdered milk in blocking buffer containing: 0.02 M Tris, 0.5 M NaCl, 5 % (w/v) milk, pH 7.5 over night. The membrane was washed with TBS buffer containing 0.1% Tween-20 and incubated for 2 hours in TBS buffer supplemented with 1% powdered milk and a rabbit polyclonal antibody (ratio 1:1000) raised against ZmCKX1 (Bilyeu *et al*., 2001). The membrane was rinsed four times with TBS buffer supplemented with 0.1% Tween-20 then incubated in TBS buffer with 1% powdered milk and anti-rabbit IgG horseradish peroxidase conjugate (Sigma) (ratio 1:25000) for 1 hour. The secondary antibody was washed out in the same way as the primary antibody. Chemioluminiscent signal was detected using ECL substrate. The membrane was covered with 1 ml of substrate for 2 min, foil-wrapped, and visualized using ChemiDoc™ MP Imaging System with Image Lab™ Software (BioRad, USA) In order to ensure same amount of loaded proteins in all the wells, Coomassie (R-250) staining was performed in gels developed in parallel to the ones were used for blotting.

3.6.1 HPLC analysis of CK

Concentration of free cytokinins and conjugated forms was evaluated, in 5 mg of lyophilized material samples, by technical staff of the laboratory of Dr. Ondřej Novák, using a high performance liquid chromatograph (UPLC), coupled to a mass spectrometer electrospray ionization. The cytokinins and their derivates were separated on the basis of their retention time stability and detected according to the weight of the quasi-molecular ions $[M + H]^+$ (Novák *et al.* 2008). The analysed compounds were represented by different cytokinin groups (free base, riboside and glucoside).

The leaf samples analysed in this part correspond to an experiment experiment developed in Argentina in the same experimental conditions as in this thesis. This results belong to MSc. Cintia Marchetti and PhD Fabio Causin and will be soon published, altough they were provided for the development of this thesis in order to better understand the fenomenon of study.

3.7.1 Statistical analysis

In order to see whether obtained results were statistically significant, T-test was performed using Microsoft Excel Software. A significant effect was assumed given for $p < 0.05$.

3.8.1 Phylogeny analysis

HvCKX gene sequences were used as a query in a local BLAST analysis performed in BioEdit software with wheat sequences found in Triticum aestivum cDNA library, which is available to download from http://plants.ensembl.org/index.html. Local BLAST which enabled the assignment of wheat CKX sequences to appropriate barley CKX sequences on the basis of homology. Sequence alignment was done, and then the file containing all sequences was opened in MEGA 7.2 software for preparation of phylogenetical trees. The evolutionary was constructed using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-3067.2694) was chosen.

4. RESULTS

4.1 Wheat

4.1.1 Chlorophyll degradation

In all the treatments where the blue region of the spectra was altered (R and G) the decrease in chlorophyll content was significantly accelerated in comparison to wheat leaves under W or B filter, which displayed prolonged chlorophyll retention (Fig. 10). The lowest chlorophyll content was detected under R treatment, being significantly different from the rest of the treatments in 96 and 168 hours. The fast chlorophyll degradation in leaves under NF could be due to stress caused by high light intensity.

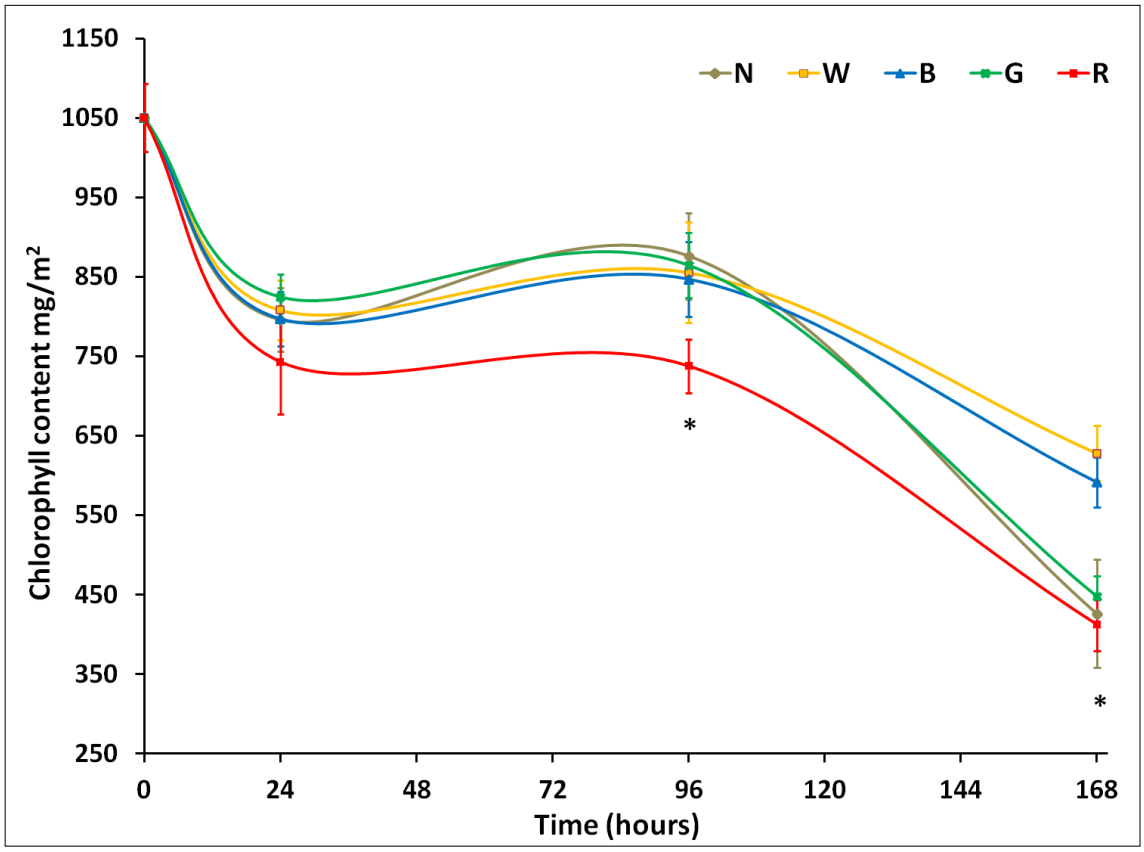

Figure 10- Chlorophyll content (mg/m^2) in excised wheat leaves (*Triticum aestivum* L.) exposed to white radiation (NF) or in the presence of white (W) or blue (B) or green (G) or red (R) filters. Mean values ± SD (n=3) are showed. Significative differences between treatments are marked with the asterisk.

4.1.2 Cytokinin content

To evaluate the effect of the light treatments on the endogenous content of cytokinins, the endogenous levels of these compounds and their derivatives were determined by UPLC analysis. To simplify the analysis due to the big number of samples, NF samples were not analyzed because its response was shown not to be connected to light quality but to light intensity. Moreover, R and G treatments were shown to behave in a similar way, thereforejust G treatment was analyzed.

Although all treatments displayed decreasing trends of free cytokinins in time, the lowest concentrations were detected in the samples deprived of BL (G treatment) (Fig. 11. A). Differences in the content of active cytokinin within all treatments were observable since the beginning of experiment and proportionally increased with the time. *Cis* isomer of Zeatin was shown to be the most prevalent form occurring in monocots, thus the changing endogenous levels of *cis*-Zeatin in response to senescence were analysed (Fig. 11. C). The levels of *Cis*-Zeatin decreased with the time in all treatments, but it drop in blue light suppressed leaves. Since *trans*-Zeatin is referred as the cytokinin with the highest activity, the endogenous levels of this compound were monitored. The lowest *trans*-Zeatin concentrations were measured in blue light deprived wheat leaves (Fig. 11. B), while the highest levels were found in control leaves. The endogenous levels of isopentenyladenine (iP) displayed ambiguous and fluctuating trends, however the lowest concentrations of iP were measured in the final time point in treatments, where blue light was suppressed (Fig. 11. D).

Interesting trends were observed in retention of CK *O*- glucosides, whose endogenous concentrations rise rapidly within treatments deprived of blue light (Fig. 12. A). Whereas blue light-deprived plants exhibited in comparison with the starting points nearly double levels of CK *O*-glucosides, the remaining B and W treatments were only slightly increased. N-glucosides did not significantly changed (Fig. 12. B). Although these inactive CK forms displayed fluctuations, their endogenous levels remained comparable with those measured in To. As cZ was in the highest rate converted into its riboside, it was important to monitor the levels of these compounds. Moreover *cis*-Zeatin riboside displays higher biological activity than cZ. Although the concentration of all CK ribosides is shown, the trend is given by cZ to cZR conversion. In the beginning of senescence endogenous CK ribosides rapidly increased especially in the blue light suppressed treatments, while in the final stage of senescence have the levels

of these compounds fallen abruptly. In comparison, control treatment displayed steadily soaring levels of CK ribosides and B treatments retained unchanged level of these compounds (Fig. 12 D). Interesting trends were observed for CK-ribotides, which maintained in the beginning very low levels, but then suddenly went up. The highest levels of cytokinin ribotides were measured in the blue light deprived leaves (Fig. 12 C), while the lowest in control. The formation of all *O*-glucosides, ribosides and ribotides, was prevalently caused due to conversion of *cis*-Zeatin.

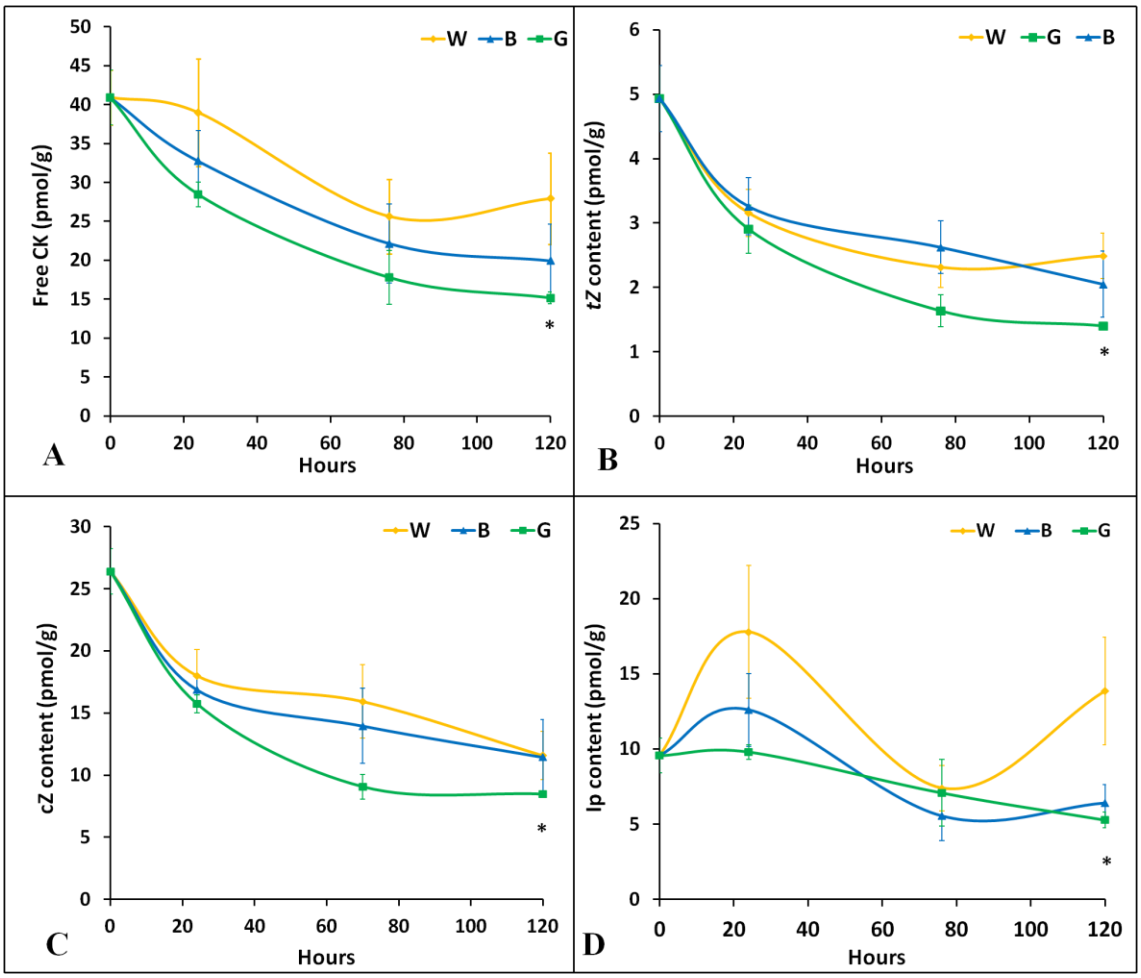

Figure 11- Concentrations of free cytokinins, depending on time **A**- Concentrations of free cytokinins: *trans*-Zeatin (tZ), *cis*-Zeatin (cZ) and isopentenyl adenine (iP) **B**- Endogenous concentrations of tZ. **C**- Concentration of cZ. **D**- Endogenous levels of iP. Data are presented in pmol per 1 g of leaf tissue. Vertical bars represent relative standard deviation. Significative differences between treatments are marked with the asterisk. These results were obtained from M Sc. Cintia Marchetti experiment, article in press.

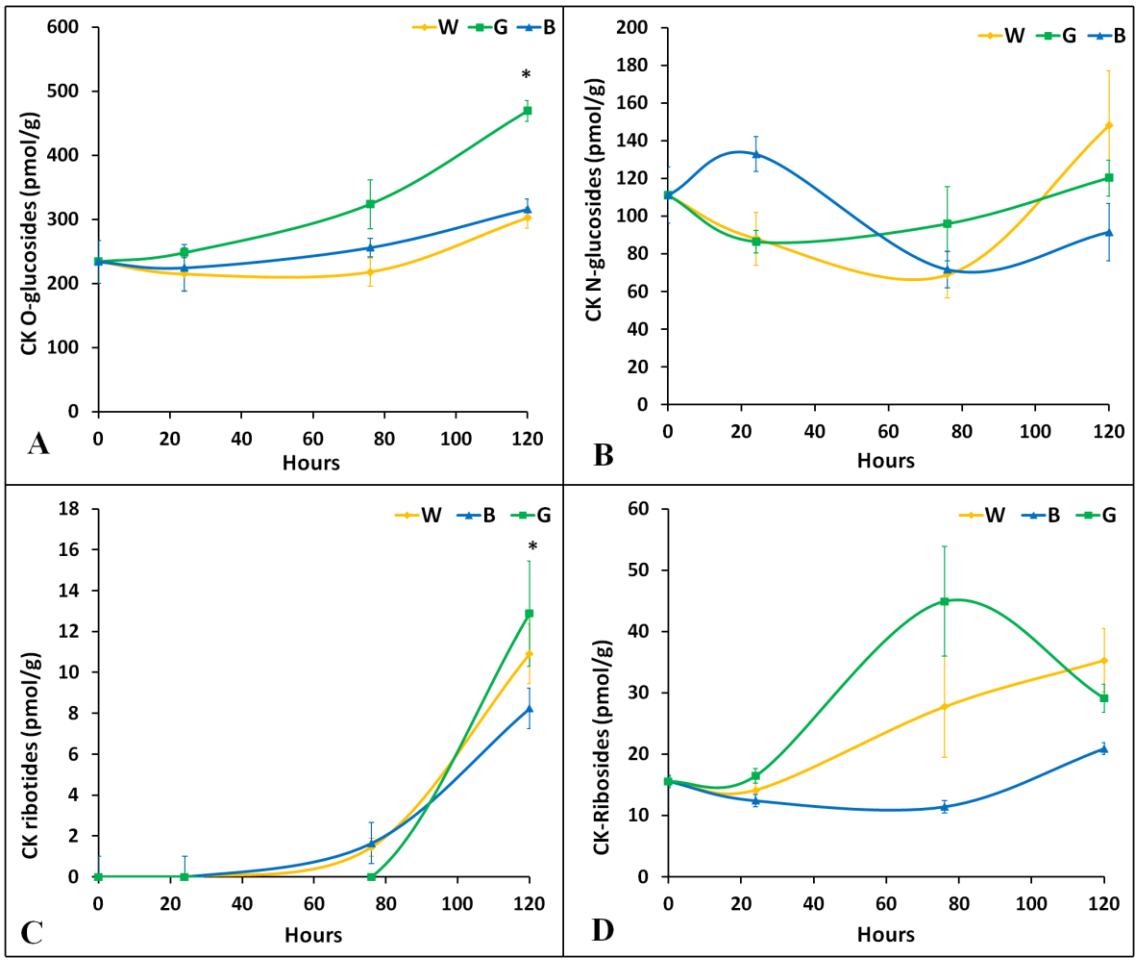

Figure 12- Concentrations of cytokinin derivates, depending on time **A**- Concentrations of cytokinin *O*-glucosides. **B**- Endogenous concentrations of cytokinin N-glucosides. **C**- Concentration of cytokinin ribotides. **D**- Endogenous levels of CK ribosides. Data are presented in pmol per 1 g of leaf tissue. Vertical bars represent relative standard deviation. Significative differences between treatments are marked with the asterisk. These results were obtained from M Sc. Cintia Marchetti experiment, article in press.

4.1.3 CKX activity

In order to investigate if a decrement in the endogenous CK content due to irreversible degradation by CKX is the cause of the increase in the senescence rate, CKX activity was measured.

As shown in Figure 13, CKX activity increased steadily with the time in all treatments, particularly in wheat leaves deprived of blue light (R and G treatments) and in the final stages of senescence. Probably due to relatively high light intensity, high CKX activity was measured also in (NF). This indicates in comparison with the second control (W), that light intensity can also significantly affect rate of CK degradation.

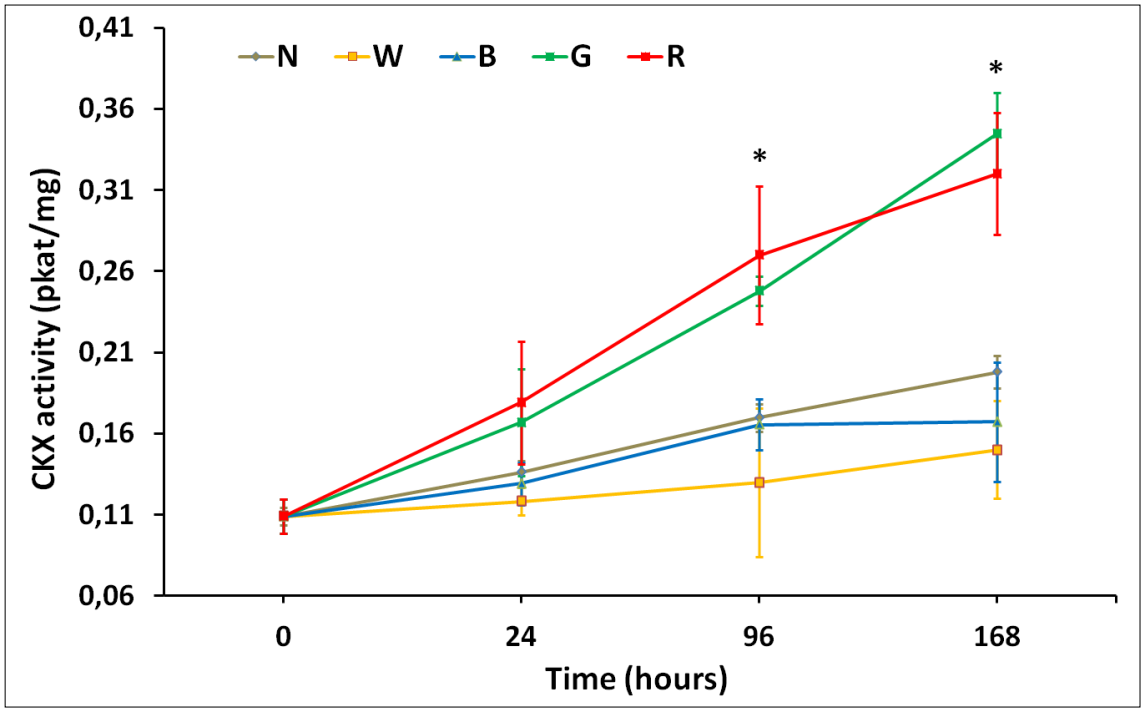

Figure 13- Cytokinin dehydrogenase enzymatic activity in excised wheat leaves (*Triticum aestivum* L.) exposed to white radiation (NF) or in the presence of white (W) or blue (B) or green (G) or red (R) filters. Mean values ± SD (n=3) are showed. Significative differences between treatments are marked with the asterisk.

4.1.4 Detection of TaCKX1 by Western Blot

In order to monitor levels of CKX1 protein, Western Blot analysis was performed. A band of approximately 70 kDa that would correspond to TaCKX1 wheat protein was detected. Increase in CKX1 protein level was found in the last time point in BL suppressed treatments. Interestingly, as shown in figure 14- A, the highest levels of CKX1 protein were present in green filter treated leaves. The levels of detected CKX1 protein are in agreement with the *CKX1* expression patterns, which tended to rapidly increase in the final stages of senescence (Fig. 16A).

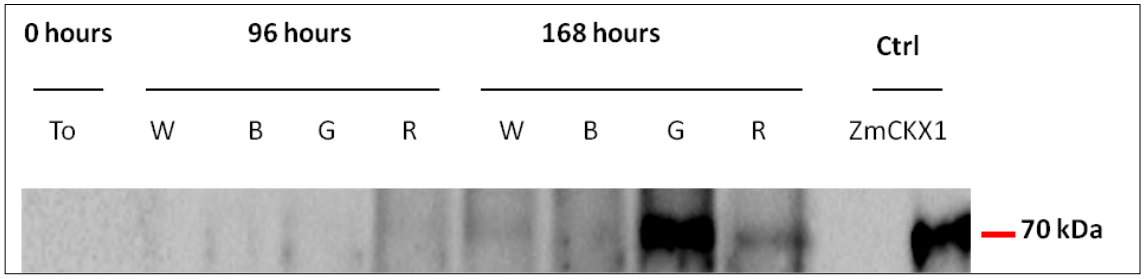

Figure 14- Western Blot analysis of CKX1 protein in wheat leaves. Samples were probed with primary antibody (Bilyeu *et al*., 2001). As a secondary antibody anti-rabbit IgG horseradish peroxidase conjugate was applied. Right side line and value represent molecular weight given by Pre-Stained Protein Ladder. The figure represents intensity of the signal in defined time points, which differed in irradiated spectral quality.

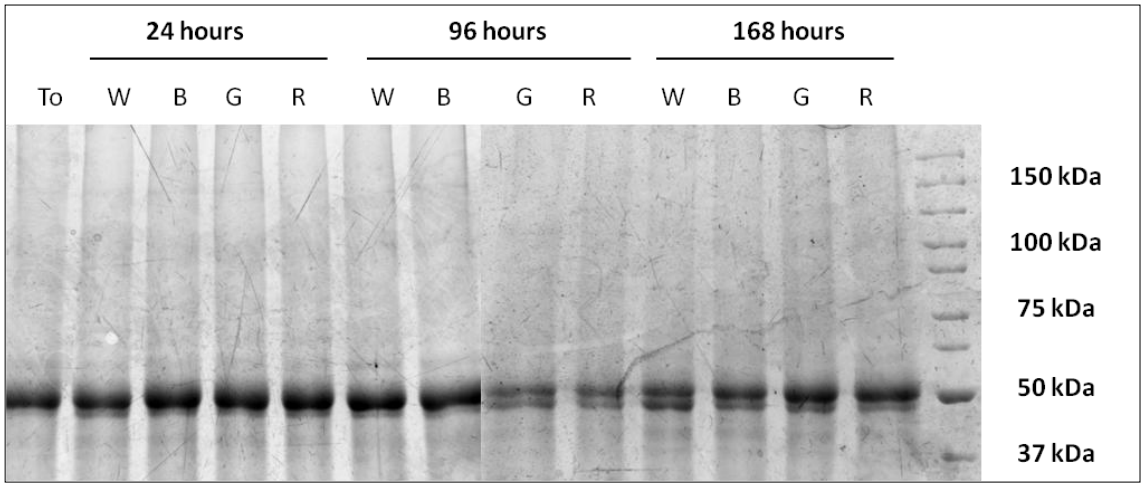

Figure 15 Coomassie staining of proteins separated by SDS-PAGE, which served as a control of the comparable amount of applied proteins. Lines and values at the right side of the figure represent molecular weights given by Pre-Stained Protein Ladder.

Analysis of gene expression

It is of utmost importance to clarify that as R and G treatments presented similar trends in chlorophyll degradation and cytokinin dehydrogenase activity, from both treatments only G was subjected to gene expression analysis in order to reduce the elevated amount of samples.

4.1.5 *TaCKX* expression

As cytokinins delay senescence, to research whether their inactivation and degradation enzymes change the level of their transcript with the progress of senescence, relative abundances of expression of the genes encoding cytokinin dehydrogenases, glucosyltransferases and *β*-glucosidases were investigated.

Whereas *TaCKX10* and *TaCKX4*, genes encoding cytokinin degrading enzymes exhibited down-regulation, *TaCKX1* and *TaCKX3* genes were significantly up regulated (Fig. 16). Interesting trends were observed for *TaCKX1*, whose relative abundances increased rapidly between 104 hours and 120 hours. Whereas, the highest *CKX1* expression was in 120 hours in leaves exposed to G and NF (Fig. 16 A), the remaining B and W filter exposed leaves displayed minor up-regulation of *TaCKX1* expression. The changes in *TaCKX1* expression within light treatments augmented in the final stages of senescence. Along with the time, blue light suppression was shown to act as a signal stimulating *TaCKX1* transcription. In contrary to *TaCKX1*, *TaCKX3* up-regulation did not seem to be induced by the blue radiation suppression. *TaCKX3* has gradually increased its expression up to the 104 hours in all treatments (Fig. 16 C). Paradoxically, *TaCKX3* displayed the reduced expression in blue light deprived leaves. From the rest of CKX genes, *TaCKX4* and *TaCKX10* exhibited in response to the senescence down-regulated expression patterns (Fig. 16 B, D). The rest of *TaCKX* genes presented constant or null expression (data not shown).

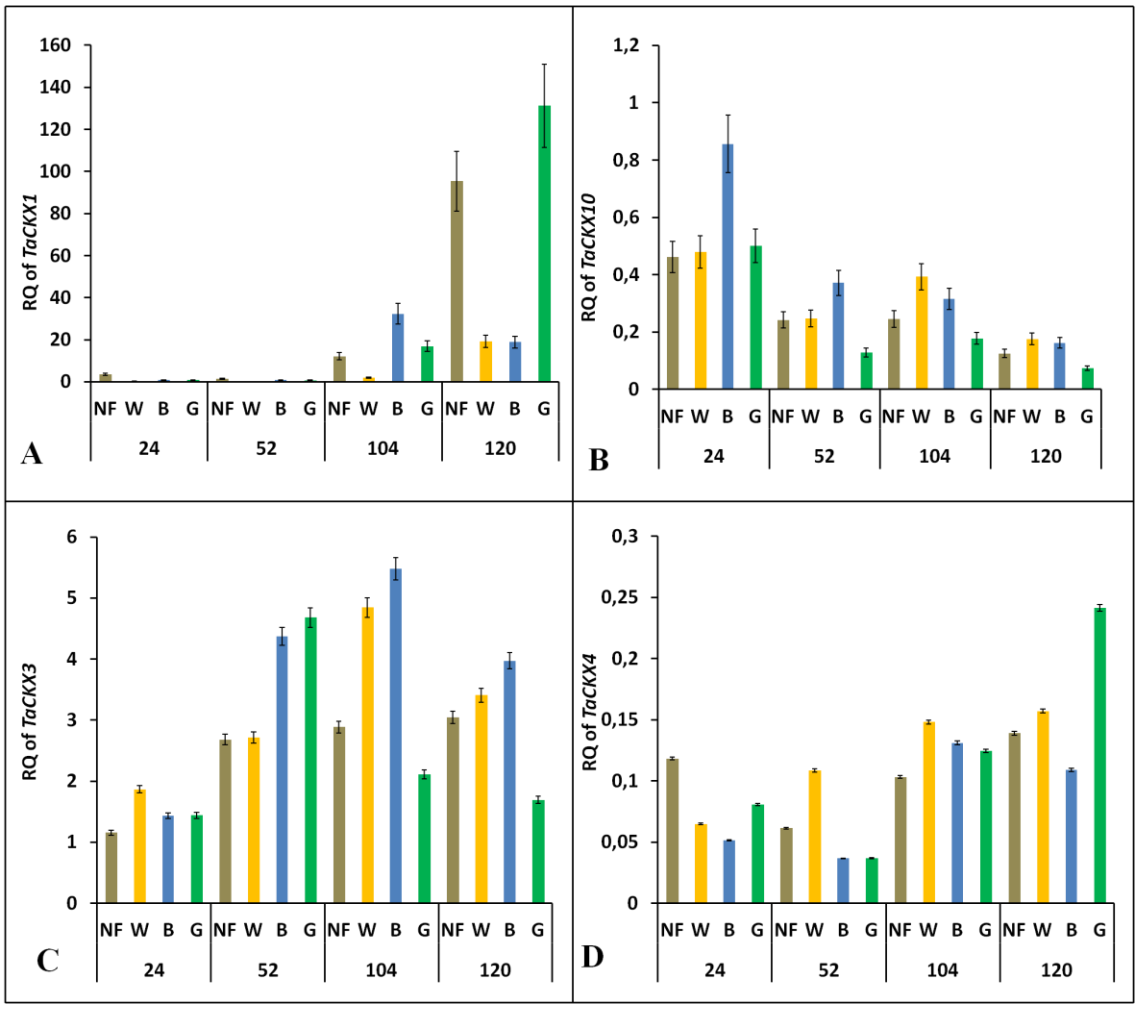

Figure 16- Relative expression of genes encoding proteins associated with cytokinin inactivation and degradation enzymes depending on time of particular light treatment (expressed in hours) **A**- Relative expression of *TaCKX1* (cytokinin dehydrogenase 1) **B**- relative expression of *TaCKX10* (cytokinin dehydrogenase 10), **C**- Relative expression of *TaCKX3* (cytokinin dehydrogenase 3), **D**- Relative gene expression of *TaCKX4* (cytokinin dehydrogenase 4).

***TacZOG* and *TaGLU* expression**

The other metabolic pathway responsible for the regulation of cytokinin metabolism involves inactivation mediated by cZOG1 and cZOG2 enzymes. It was shown that with the progress of senescence, concentrations of endogenous cytokinin *O*-glucosides rapidly increase (Fig. 12. A). In order to see correlation between arising *O*-glucosides and cZOGs, amount *cZOG1* and *cZOG2* transcript was investigated (Fig. 17). Whereas *cZOG1* remained down-regulated in comparison to To, *cZOG2* expression gradually increased with the time and in the last time point the higher expression was observed in treatment G. In order to find out whether the rate of cytokinin reactivation is changed during senescence, relative amount of *TaGLU1* encoding β-glucosidase was measured.

Along with the senescence *TaGLU1*displayed increased expression in all treatments (Fig. 17, C). Blue light light deprived leaves gradually increased *TaGLU1* expression.

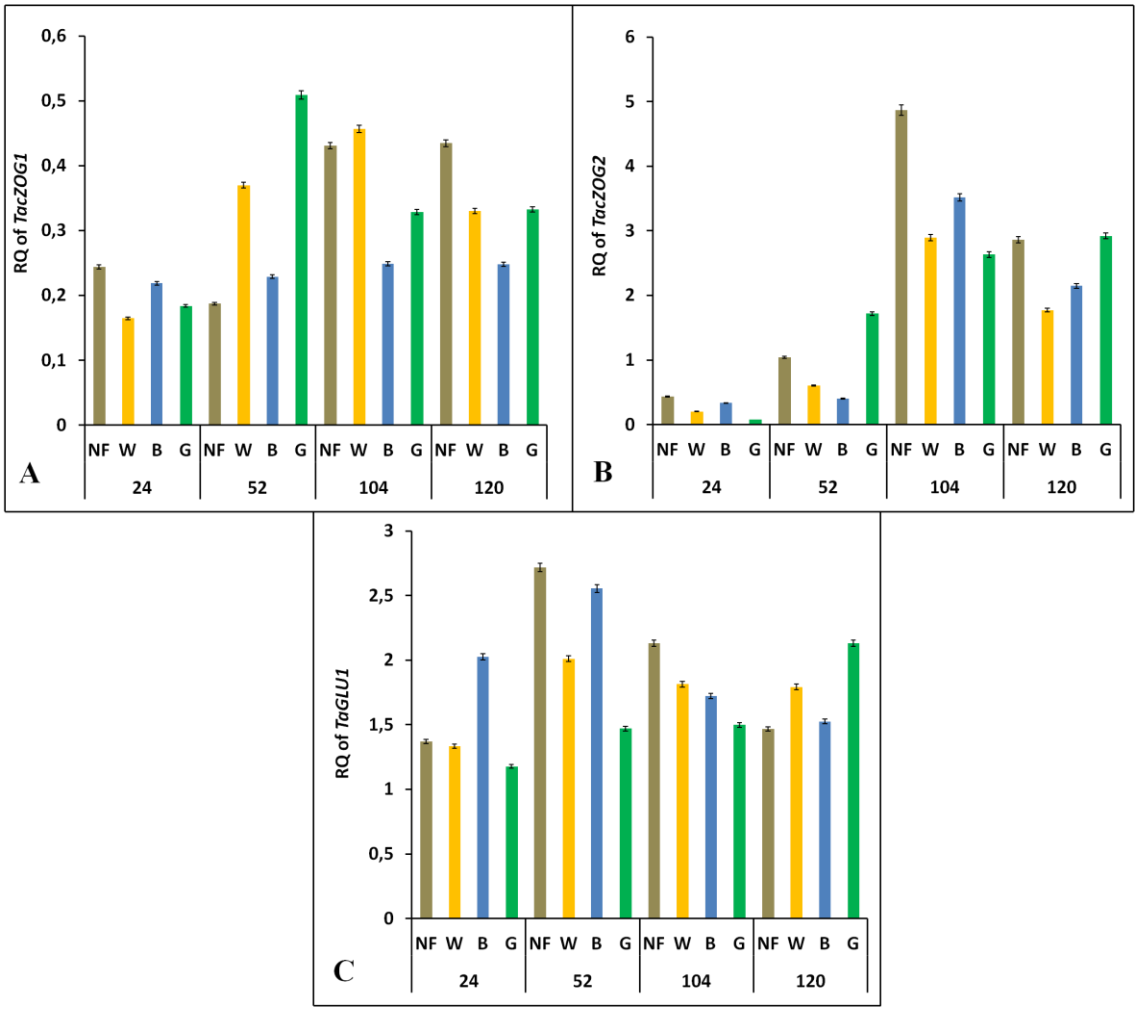

Figure 17- Relative abundances of genes associated with cytokinin inactivation and reactivation. **A**- Relative gene expression of *TacZOG1* encoding *cis*-Zeatin *O*-glucosyltransferase 1. **B**- Relative gene expression of *TacZOG2* encoding *cis*-Zeatin *O*-glucosyltransferase 2. **C**- Relative gene expression of *TaGLU1* encoding β-glucosidase.

4.1.6 *TaRR* and *TaHK* expression

In order to research whether the changes in CK metabolism correlate with variations in CK signal transduction pathways, expression profiles of genes that are responsible for CK signal relay were analysed. In general all genes associated with CK signal transmission exhibited down-regulation. The expression patterns of histidinkinase *TaHK3* were in comparison to To slightly down-regulated, except of G treatments, which displayed since 52 hours moderate up-regulation (Fig. 18, A). Concerning the

cytokinin response regulators, the amount of transcript was significantly reduced in all treatments in comparison with To. *TaRR1*, which encodes a type B response regulator, displayed in comparison to control, reduced expression (Fig. 18, B), especially in G treatment. Opposite trends were observed in the expression of type A response regulators. With the progress of senescence *TaRR4* augmented its expression, particularly in the treatments when BL was supressed (treatment G). Increased expression of type A regulatory genes was also found in NF treatments. It is important to notice that TaHK3 and TaRR4 followed similar trends. When compared to control, *TaRR9* exhibited moderately reduced level of transcript.

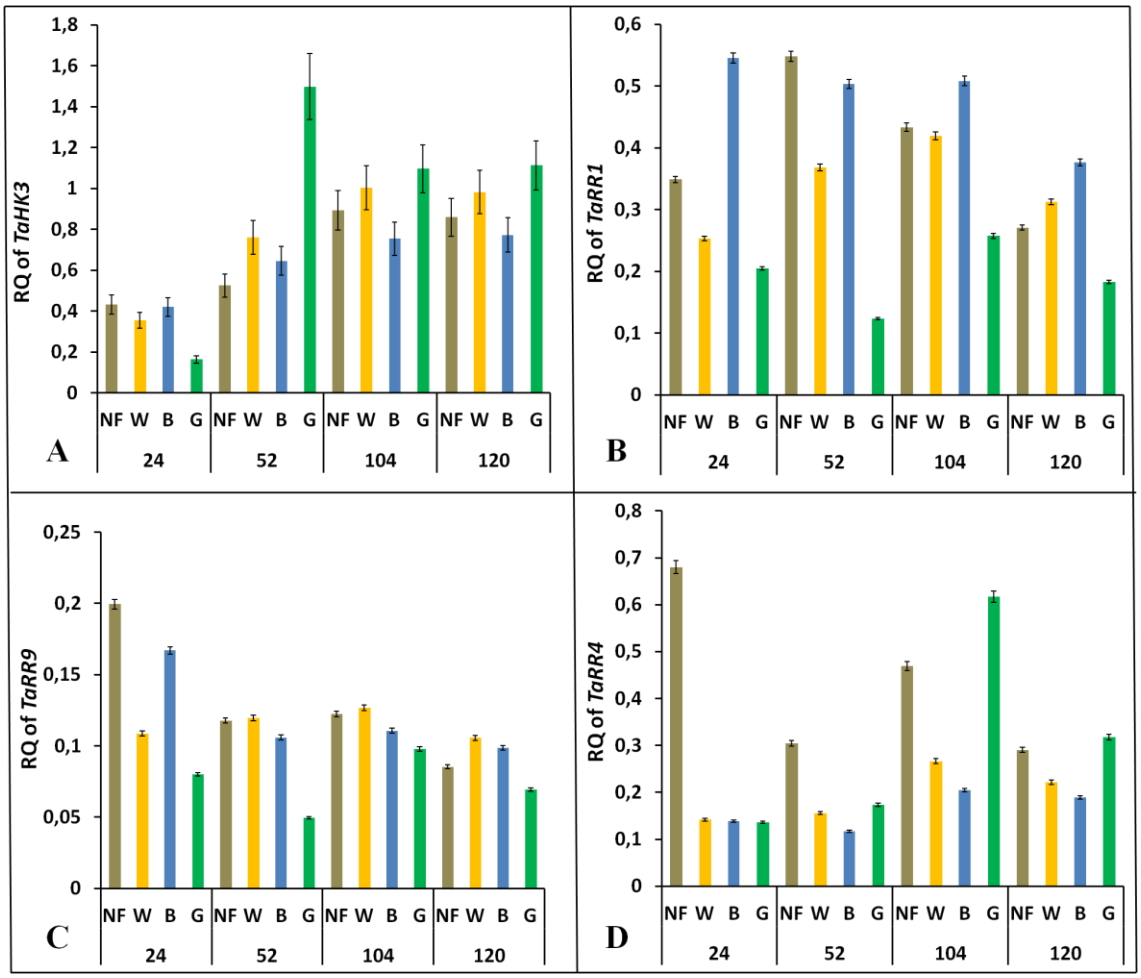

Figure 18- Relative gene expression of wheat genes encoding proteins associated with CK signal transduction depending on the time of particular light exposure (expressed in hours). **A**- Relative gene expression of *TaHK3* histidinkinase receptor. **B**- Relative gene expression of *TaRR1* encoding CK response regulator. **C**- Relative gene expression of *TaRR9* encoding CK response regulator. **D**- Relative gene expression of *TaRR4* encoding CK response regulator. Vertical bars represent standard deviation.

4.1.7 *TaCIB1* and *TaCRY2* expression

As light treatments supplied different proportions of blue light, it was important to monitor expression of blue light receptors and proteins involved in blue light signalling. With the proceeding senescence, all researched treatments displayed, except W and NF treatments in 104 hours, significant down-regulation in relative amount of *CIB1* transcript (Fig. 19 A). The consistent down-regulation was also observed in *CRY2* expression patterns (Fig. 19 B). The comparative expression analysis has not revealed in researched treatments significant alterations. The level of *CRY2* and *CIB1* transcript was not affected but the light quality.

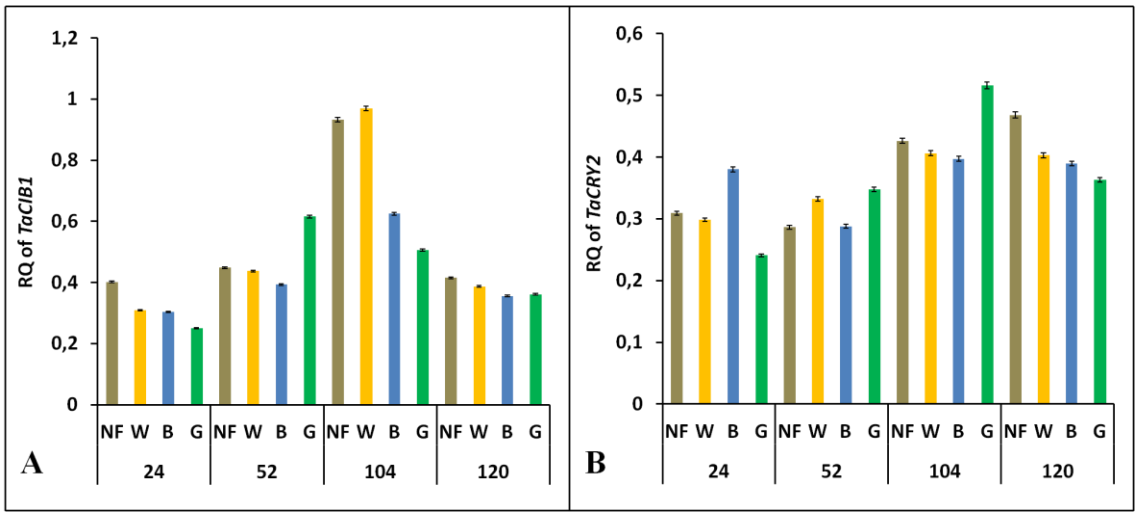

Figure 19- A- Relative expression of *TaCIB1* encoding cryptochrome interacting helix-loop-helix transcription factor, **B-** Relative expression of *TaCRY2* encoding cryptochrome receptor. Vertical bars represent standard deviation.

4.1.8 Phylogeny analysis of *TaCKX*s

In order to better characterize and elucidate evolution of *TaCKX*, a phylogeny tree was constructed. On the basis of homology, wheat's CKX sequences were compared and aligned with CKX sequences from other cereals: maize (*Zea mays*), rice (*Oryza sativum*) and barley (*Hordeum vulgare*). As shown in Fig. 20, 27 *CKX* sequences were found to be distributed within wheat´s genome. 8 *TaCKX* sequences were determined in genome A, 11 in genome B and the remaining 8 in genome D. As noticeable, wheat's TaCKXs share the closest homology with the barley HvCKX. Except for HvCKX10, each HvCKX has at least one homologous corresponding sequence. The most homologous sequence to HvCKX10 was wheat's Traes 6AS 4B78E3665.1, which was on the base of homology assigned to HvCKX7. Moreover, TaCKX10 was shown to share high sequence homology with HvCKX2 (Song *et al*. 2012), therefore one of Traes3B, which was assigned to HvCKX2.2 probably corresponds to wheat´s CKX10. TaCKX1 shares high sequence similarity with orthologues from other monocot species, including HvCKX1 in barley, ZmCKX1 in maize and OsCKX1 in rice as also shown in (Song *et al*. 2012). Phylogeny analysis confirmed 95 % sequence homology between TaCKX1 and HvCKX1, which coincided on 130 amino acid positions, including C-terminal sequences (Galuszka *et al.* 2004). Maximum likelihood of sequence homology was also identified for HvCKX4, TaCKX4 and OsCKX4 (Song *et al*. 2012). Regarding the protein sequence homology, close relation was revealed between TaCKX3, TaCKX8 and HvCKX3, HvCKX8, OsCKX3, OsCKX3 (Mameaux *et al.* 2012). In agreement with Mameux *et al.* 2012 HvCKX2.1, HvCKX2.2 and OsCKX2 shared close sequence homology with appropriate wheat's sequences. A separate branch formed for ZmCKX11, ZmCKX12 indicates different origin of these sequences.

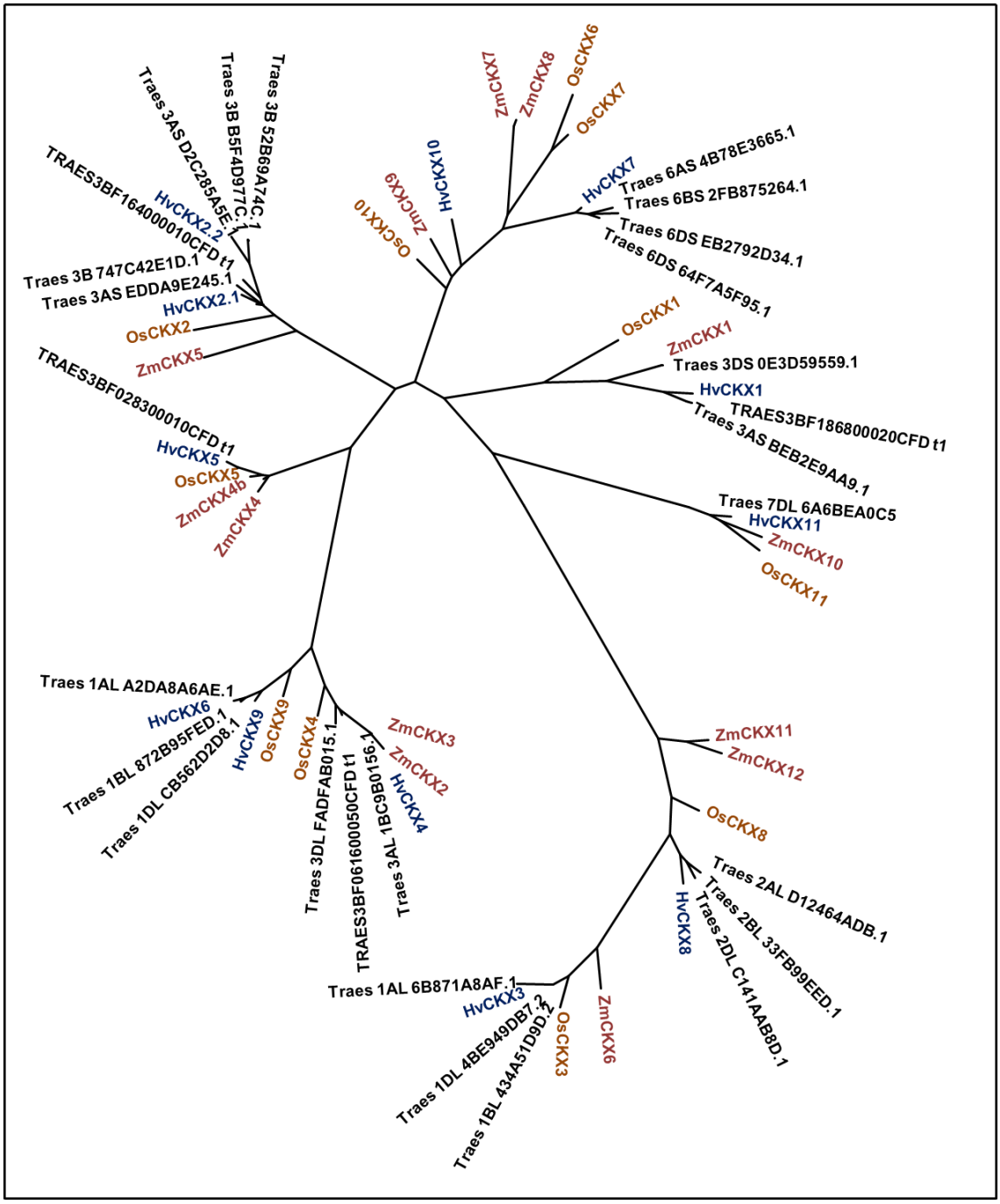

Figure 20- Evolutionary comparison of CKX protein sequences. ZmCKX from maize (*Zea mays*) are marked in orange, OsCKX from rice (*Oryza sativa*) are green, HvCKX from barley (*Hordeum vulgare*) are blue, and wheat CKX (Traes- *Triticum aestivum*) in black. The second capital letter in the wheat's sequence is related to genome, in which sequence was found, A corresponds to A subgenome, B corresponds to B subgenome, D corresponds to D subgenome. The number indicates, in which chromosome the sequence was found. Evolutionary analysis was performed in MEGA7 software.

4.2 Barley

4.2.1 Chlorophyll content

Barley senescing leaves followed similar trends in chlorophyll degradation as wheat. Decreased rate of chlorophyll degradation was measured in leaves were blue light was not suppressed (Fig. 21).

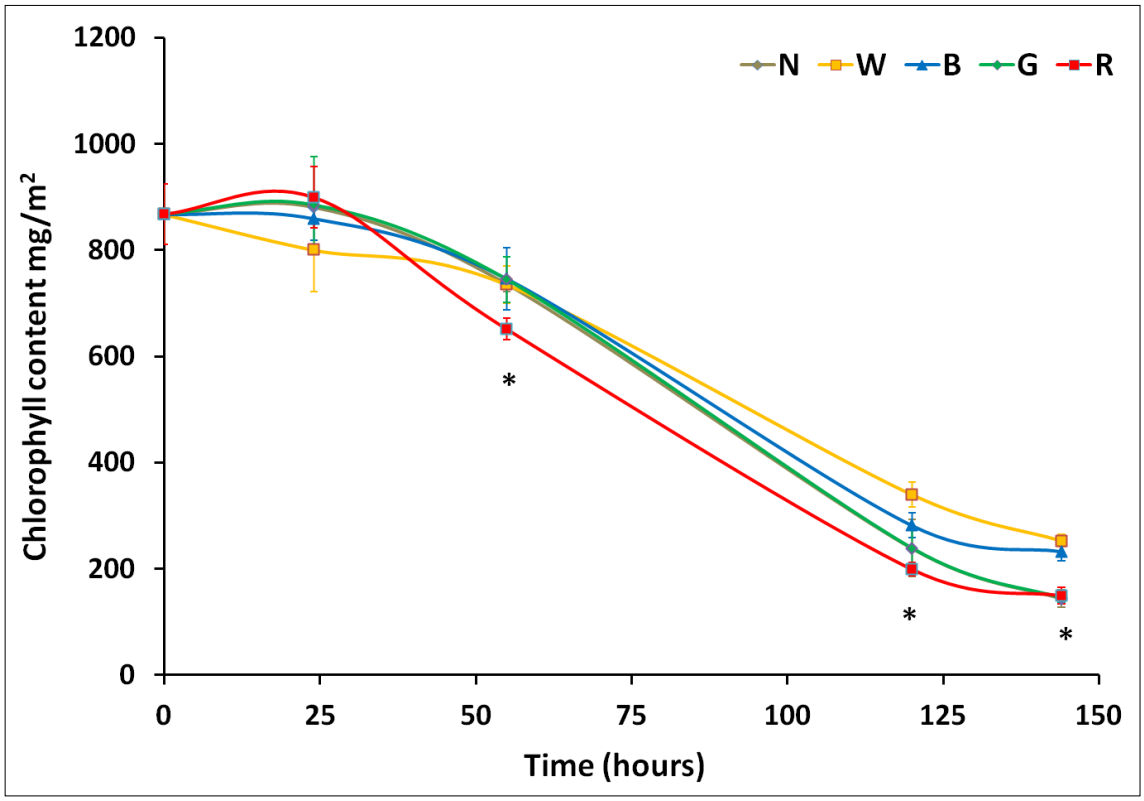

Figure 21- Graph of Chlorophyll content (mg/m^2) depending on time (hours) in Barley leaves (*Hordeum vulgare*). Each treatment is marked accordingly to the colour of the filter, except of white treatment (control) is marked yellow, and non filter treatment is marked grey. Vertical bars represent standard deviation. Significative differences between treatments are marked with the asterisk.

4.2.2 CKX activity

Up to the first 55 hours of exposure, only moderate increment of cytokinin dehydrogenase activity was measured (Fig. 22). Whereas in B and W activity was low, R, G and NF displayed increment of CKX activity. In contrast to wheat, barley leaves exhibited significantly higher activity in red filter treatments.

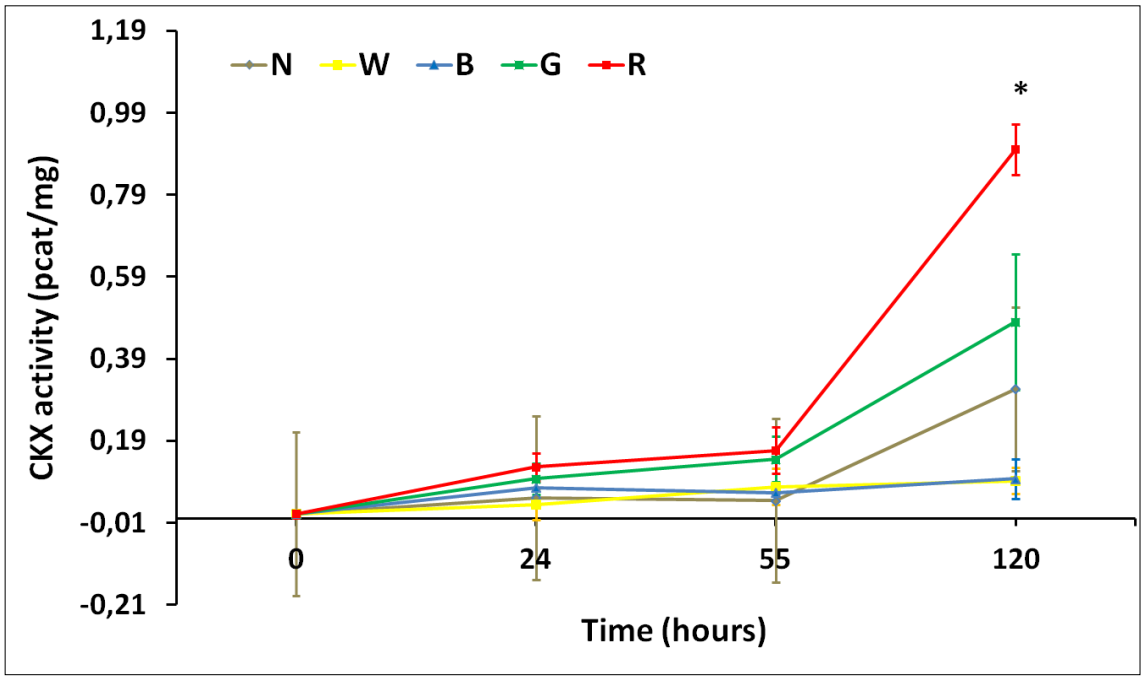

Figure 22- Cytokinin dehydrogenase enzymatic activity expressed in pkat per mg of isolated protein extract depending on time (hours), in Barley leafs (*Hordeum vulgare*), Each treatment is marked accordingly to the colour of the filter, except of white treatment (control) is marked yellow, and non filter treatment is marked grey. Vertical bars represent standard deviation. Significative differences between treatments are marked with the asterisk.

4.2.3 Detection of HvCKX1 by Western Blot

In order to see if the absence of the blue radiation stimulates the activity of cytokinin dehydrogenase, western blot detection of this protein was performed. Interestingly the highest intensity of the blotted protein was found in 120 hours in all blue light deprived treatments (Fig. 23). Significantly lower, but detectable amount of CKX1 protein was present in leaves treated with W and B filter. In previous time points the signal was almost not detected, similarly as in To.

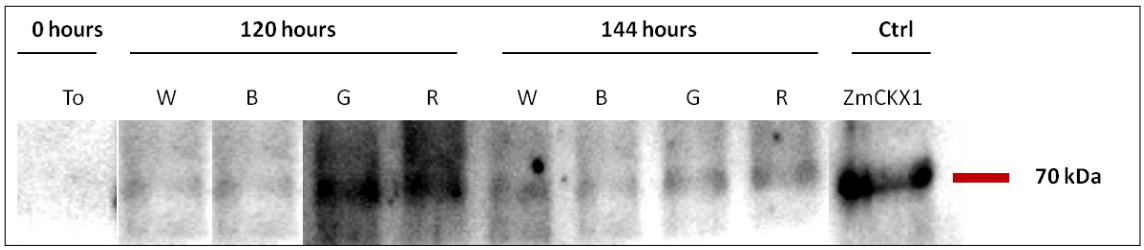

Figure 23- Western Blot analysis of CKX1 protein in barley leaves. Samples were probed with primary antibody (Bilyeu *et al*., 2001). As a secondary antibody anti-rabbit IgG horseradish peroxidase conjugate was applied. Right side line and value represent molecular weight given by Pre-Stained Protein Ladder. The figure represents intensity of the signal in defined time points of barley leaves, which differed in irradiated spectral quality.

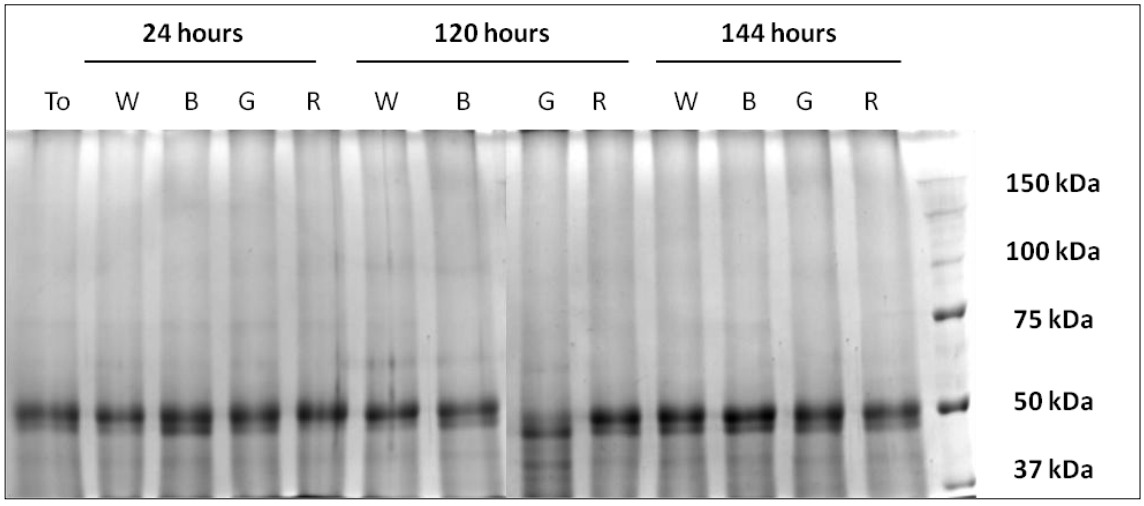

Figure 24- Coomassie staining of proteins separated by SDS-PAGE which served as a control of the comparable amount of applied proteins. Lines and values at the right side of the figure represent molecular weights given by Pre-Stained Protein Ladder.

4.2.4 *HvCKX1* gene expression

In order to monitor the effect of spectral quality on the *HvCKX* gene expression, which was proved to be crutial in wheat plants, analysis of this gene was performed. The particular stages of blue light deprived senescence were accompanied by soaring expression patterns of *HvCKX1* (R and G treatments) (Fig. 24). W and B treatments displayed with the time increased *HvCKX1* abundances, but the blue light contributed to the lower amount of *HvCKX1* transcript.

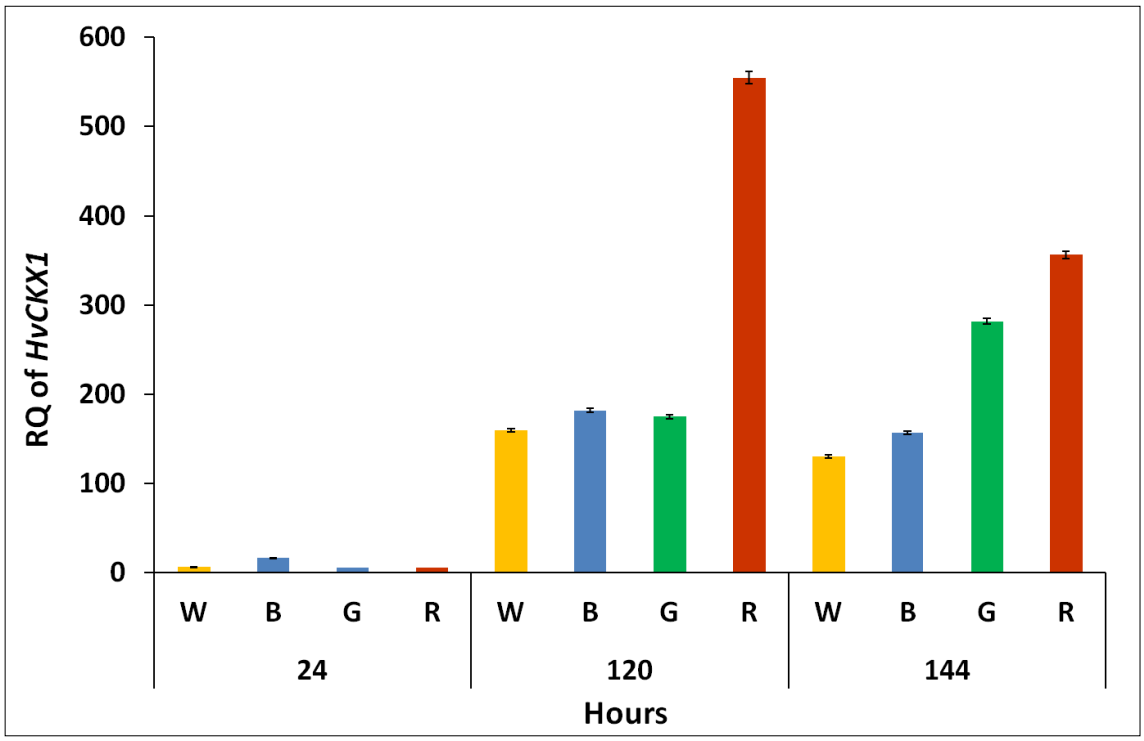

Figure 24- Expression of HvCKX1 gene in barley leaves exposed to different light treatments Each treatment is marked accordingly to the colour of the filter, except of white treatment (control) is marked yellow, and non filter treatment is marked grey. Vertical bars represent standard deviation.

5. DISCUSSION

Acquired data from the present experiment is in agreement with the previous results concerning the blue light radiation as a crucial factor modulating the rate of senescence in shaded wheat leaves (Causin *et al.* 2011). In the present work was experimentally confirmed that blue radiation delays chlorophyll breakdown. Observed differences between the treatments in chlorophyll retention and cytokinin dehydrogenase activity indicate a light-dependent response. The differences in chlorophyll retention could have been also caused by different levels of 5-aminolevulenic acid, which is the key component of chlorophyll synthesis. 5-aminolevulenic acid arises in the reaction catalysed by 5-aminolevulenic acid dehydratase (ALAD), an enzyme, whose activity was shown to be stimulated by cytokinins (Hukmani and Tripathy 1994). There is also evidence that cytokinins affects the activities of other enzymes like for instance Mg protoporphyrin IX chelatase and Mg protoporphyrin methyltransferase, which are implicated in chlorophyll synthesis (Yaronskaya *et al.* 2006). The changes in cytokinin metabolism, which were inflicted by spectral quality, might have either amplified or attenuated signal inducing chlorophyll synthesis. The consequence of this phenomenon was successive much longer chlorophyll degradation, which resulted in diminished senescence. The enhanced chlorophyll degradation in control NF leaves could correspond to increased light intensity and stressful conditions (Brouwer *et al.* 2012).

Since cytokinin has been implicated in the regulation of senescence, intriguing quest concerns the influence of spectral quality in relation to cytokinin homeostasis. As long as senescence has proceeded, exhibited blue light deprived leaves significantly increased activity of cytokinin dehydrogenase. In connection, the analysis of cytokinin content has revealed that blue light treated plants maintained much higher levels of endogenous cytokinins than blue light suppressed ones. Interesting was that, in the beginning of senescence, active cytokinins were inactivated mainly by *O*-glucosylation, while in the final stages these compounds were prevalently degraded by cytokinin dehydrogenase enzyme. The increment of cytokinin *O*-glucosides in the beginning phase of senescence might have been associated with their transport into vacuoles (Kato *et al.* 2002). Moreover, *O*-glucosylation assures temporary storage of active cytokinins, which are in this form more resistant to the CKX cleavage (Mok and Mok, 2001). In the case that active cytokinins are required, *O*-glucosides are reversely cleaved by β-glucosidase. The expression patterns of *cZOG2* genes augmented with the progress of

senescence, thus cZOG2 is considered as a key enzyme mediating *O*-glucosylation of cytokinins during leaf senescence. Although, *TacZOG2* displayed light quality dependent expression, it is not known whether this enzyme is responsible for rapid rise of *O*-glucosides in the beginning phase. According to Song *et al.* (2012), another isoform of TacZOG could also be involved in an increased formation of *O*-glucosides.

In the present work was shown also that blue radiation regulated cytokinin homeostasis by the inhibition of CKX1 transcription and translation. During senescence *CKX1* consistently increased expression in all light treatments, but highest abundances were measured in the blue light deprived treatments. The patterns of *CKX1* expression correspond to those found by Song *et al.* 2012, who has researched *CKX1* expression in defined points after wheat's anthesis. Western blot analysis of CKX1 has confirmed that the absence of blue light induced increase in CKX1 protein. The rapid decrement of active cytokinins in the final phase of senescence was correlated with the enhanced activity of CKX1 enzyme. All these data suggest that in the final phases of senescence CKX1 represents key enzyme, which is scavenging active cytokinins and their derivates. It would be also intriguing to investigate whether the increment of CKX1 activity is also associated with the rapid decrement of cZR content. The localization of TaCKX1 and HvCKX1 remains unknown as well as the effect of the environment on their enzymatic activity. Due to high sequence homology wheat's and barley CKX1 could be compared to maize ZmCKX1 (Fig. 20). Since ZmCKX1 was shown to be located in apoplast (Šmehilová *et al*. 2009) localization of TaCKX1 and HvCKX1 was predicted in the same cellular compartments. The pH in apoplast is during standard conditions around 6-5, in addition with the proceeding senescence pH decreases in these compartments to 5,5 (Mattsson *et al.* 2003). These molecular changes might create optimal conditions for CKX1 activity, as it was shown for *Arabidopsis* CKX1 degrading zeatin riboside (ZR) (Galuszka *et al.* 2007). The probable mechanism, which explains altered trends in CKX1 in particular light treatments was proposed by Causin *et al.* 2015, who has considered the blue radiation as a crucial exogenous factor regulating Ca^{2+} homeostasis. The blue light suppression increases levels of cytosolic Ca^{2+}, which results in the rapid decrement of catalase activity. Catalase is an enzyme, which is responsible for regulation of H_2O_2 homeostasis. The inhibition of catalase activity, has contributed to the rapid increment of H_2O_2, which in turn acted as an agent triggering

for instance SAGs expression (Balazadeh *et al.* 2011). In our work was shown that *TaCKX1* and *HvCKX1* behave as typical SAGs.

Since cytokinins are known to display pleiotropic effects, it is important to monitor genes associated with their signal relay. Therefore, the effect of light quality in relation to *HK* and *RRs* expression was investigated. Increased levels of endogenous CK elicit stronger signal transduction. As reported by Kim et al. 2006, augmented CK signal should be correlated with enhanced *HK3* response. According to these findings, the highest expression of *TaHK3* is expected in treatments which exhibited elevated levels of cytokinins (B, W). Our obtained data have not supported these findings. We have shown that blue light deprived leaves, the ones with lowest CK content, stimulated expression of *TaHK3*. Possible explanation of *TaHK3* up-regulation could be associated with more effective perception of decreased cytokinin signal. The analysis of *TaHK3* expression revealed that wheat responds to imbalanced cytokinin homeostasis using opposite mechanism than *Arabidopsis*. Although *TaHK3* is known to be primary responsible for cytokinin response in *Arabidopsis* (Kim et al. 2006), it would be interesting to monitor expression of *TaCRE1* and *TaHK4*, which also encode proteins responsible for the perception of cytokinin signal. Although level of *TaRR4* and *TaRR9* transcript is correlated with the endogenous cytokinin content of the tissue (Song 2012), spectral quality did not seem to significantly affect *TaRR* expression. Decreased levels of endogenous cytokinins inflicted moderate up-regulation of type-A *RRs* genes. It is interesting that changed *TaRR4* expression displayed time-dependent profile. In contrast to type A *RRs*, type B *TaRR1* displayed down-regulated pattern. As TaRR1 is transcription factor, which regulates many CK-stimulated genes, it would important to analyse these genes. However, *TaIPTs* are expressed at relatively low levels in the starting phase of wheat's leaf senescence (Song 2012), it has not affected the levels of active cytokinins, which have displayed decreasing trends. On the other hand, the increment in the endogenous levels of cytokinin ribotides (Fig. 12 C) suggests possible association with the activity of IPT enzymes, in which according to Song *et al.* 2012 TaIPT2 and TaIPT5 could have been implicated.

In respect of the blue light perception, *TaCRY2* and *TaCIB1* abundances did not seem to be affected by different light conditions. The unchanged level of *CRY2* transcript could be substantiated by light independent expression of this gene. The same conclusion was drawn by Xu *et al*. 2009, who has shown that meanwhile exposed to certain light

wavelengths, *CRY2* maintained comparable level of expression in all treatments. According to Xu *et al*. 2009, *TaCRY1* expression could be more affected by the spectral quality. In addition, cryptochromes were described to interact with the red/ far red light receptors and phototropins. This interaction mediates regulation of various physiological processes. In order to determine, whether CRY2 remained in response to changed light conditions intact, WB analysis would need to be conducted. However *TaCIB1* seemed to display light independent expression, it is not known whether these trends were correlated to the protein level. It would be interesting to monitor if the levels of TaCIB1 protein decrease in the absence of blue light, as shown in *Arabidopsis*. According to our results blue light might have mediated CRY2 dependent inactivation of CIB1 (Liua *et al.* 2013), which prevented CIB1 to activate SAGs.

The highest decrement of active cytokinins was due to cZ depletion. This points out a possible important role of this cZ derivates during senescence. Among all analysed cytokinins, decrement of cZ contributed in the highest rate to the conversion to respective *cis*-Zeatin-riboside (cZR) (Fig. 12 D). In contrast to cZ, is cZR characterised with relatively high biological activity (Doležal *et al.* 2007, Gajdošová *et al.* 2011). Interestingly, the highest activities of cZR were observed in the wheat senescence bioassay performed by the mentioned articles. The amplified physiological response to cZR is attributed to its more efficient transportation and higher resistance to CKX degradation. In addition, ambiguous expression patterns of genes associated with cytokinin signal transduction could be justified by very low sensitivity of cZR to both AHK3 and AHK4 receptors (Doležal *et al.* 2007). It would be interesting to investigate, whether blue light suppression contributes to the enhanced conversion of cZ to its respective riboside. We have shown that abrupt changes in the level of cZR, which were detected after 120 hours in blue light deprived treatment, could have significantly accelerated the final stage of senescence. The absence of blue light has triggered in the final stage of senescence faster degradation of active cytokinins. When active cytokinins were depleted, CKX1-mediated degradation of cytokinin ribosides was launched.

To compare and to better understand blue light suppression response on monocot senescence, two independent experiments were conducted. Although both plants seemed to follow similar trends in blue light inhibited senescence, slight differences were observed between these closely related crops. Since the beginning till 55 hours, blue light deprived barley leaves have displayed comparable CKX activity with control.

In 120 hours have the trends rapidly changed. CKX activity was in comparison to the previous time point increased more than 5 times in R and more than 3 times in G. The increment of activity was correlated to the soaring amount of *HvCKX1* transcript as well as to the increased amount of detected HvCKX1 protein. The changes in the activity were accompanied by the abrupt slump of chlorophyll content, in percentage the highest loss of was also in R and G. Wheat's senescence was characterized by slowed rate of chlorophyll degradation especially in the final stages wheat leaves retained higher levels of chlorophyll. Alterations in chlorophyll retention could be also explained by the involvement of different molecular mechanism. Whereas wheat's senescence is accompanied by rapid decrease in the number of chloroplasts (Ono *et al.* 1995), barley displays stable number of chloroplasts, but decreased protein and chlorophyll content (Krupinska *et al.* 2006). This work has shown that blue light has inhibiting effects on the expression, synthesis and activity of CKX1 in both barley and wheat. Interesting was that wheat leaves displayed the highest levels of CKX1 protein when blue light transmission was less than 1 %, while barley leaves maintained highest amount of CKX1 protein when blue light was entirely suppressed. These findings suggest that wheat's senescence is in contrast to the barley senescence influenced by the ratio of blue and red radiation. In our work was shown that blue light has significant effect on wheat's senescence, but the whole process is also orchestrated by certain proportions of red radiation.

The phylogenetic analysis showed high sequence similarity between the analysed species, which suggests that CKX genes were evolved in these crops from one common ancestor. The coincident N-terminal and C-terminal sequences enable to predict cellular localization of these proteins. The knowledge of sequence renders information about possible physiological and biochemical properties of these enzymes. The compiled information about entire wheat´s *TaCKX* sequences would raise the possibility of investigating the expression of particular homologous *TaCKX* independently. It could be determined whether wheat activates expression of only one TaCKX, or possibly all sequences, which encode respective isoform are expressed under defined conditions during wheat´s lifespan.

6. CONCLUSION

The present work has proved that spectral quality perturbs the homeostasis of cytokinins in monocots. It was possible to corroborate the hypothesis that blue light deprivation accelerates senescence in wheat leaves exposed to shading stress. In addition, it has been determined that the onset of senescence is associated with a decrease in endogenous active cytokinins content, particularly *trans* and *cis*-zeatin levels, and an increment of cytokinin-*O*-glucosides.

As senescence proceed, CKX activity was increased, nevertheless in the beginning phase this didn´t correlate with a decay on endogenous CKs. Hence, in the homeostasis of CKs in our conditions, inactivation by glycosylation of active forms could have played a crucial role rather than degradation by CKX, which had an important role but in the final stages of senescence. It would be important to analyse if the formation of cytokinin *O*-glucosides mediates transient storage of cytokinins, which are resistant to CKX or possibly these substances have function in senescent metabolism. In the final stage of senescence, blue light suppression proportionally correlated with an increment of cytokinin dehydrogenase activity, which mediated accelerated cytokinin degradation. During wheat´s lifespan many cytokinin-degrading enzymes mediate the regulation of cytokinin homeostasis. Only two of these cytokinin regulating isoenzymes have been shown to be up regulated during senescence. However, in the absence of blue light, only CKX1 exhibited significant up regulation. The obtained findings are in agreement with Song *et al.*, 2012, who has shown that *TaCKX1* is strongly up-regulated during leaf senescence but *TaCKX2* and *TaCKX6* are barely expressed during leaf senescence. All these data emphasize the importance of *TaCKX1*, which could be behaving in barley and wheat as a typical *SAG* gene. Interesting was that these monocots exhibited slightly different response to blue radiation. Whereas barley displayed the highest *HvCKX1* expression in the absence of blue light, *TaCKX1* expression in wheat was the highest when blue light transmission was less than 1%.

Our findings suggest that senescing wheat leaves display complex regulation of cytokinins. Wheat leaves that were deprived of blue light compensated diminished concentration of active cytokinins by an increased expression of histidin kinase receptors, which have amplified the sensitivity to the cytokinin. CKX enzymatic activity, relative gene expression and chlorophyll content results confirmed that blue light has contributed to the senescence-delaying effect of cytokinins in monocots. Blue light suppression was shown to have stimulatory effect on the formation of cytokinin *O*-

glucosides and cytokinin dehydrogenase activity. Whereas in the beginning of senescence free cytokinins participated in delaying senescence, in the later phases cZR assumed the crucial senescence-regulating role. In the initial phase was decrement of active cytokinins proportional to the increment of arising cytokinin *O*-glucosides, the final stages of senescence were in blue light deprived leaves accompanied by rapid decrement of cZR. Disrupted levels of cZR have corresponded to the rapid increment of CKX1 activity.

The importance of the study of senescence in crops lays on the possibility of increase grain biomass and crop yield through the manipulation of the length of photosynthesis (Thomas and Ougham, 2014). As it was shown in Gan and Amasino (1995) transgenic plants of tobacco with increased life span has risen the yield up to 40% under controlled condition. As TaCKX1 was shown to be a crucial enzyme, further studies need to be performed in plants with silenced CKX1 gene. Barley plants with down-regulated HvCKX1 expression through RNAi-based silencing have been obtained (Zalewski *et al.*, 2010) and these genetically engineered plants were characteristic with increased grain yield. As in this thesis the spectral quality was shown to regulate *CKX1* expression in both monocots similarly, and also that it would be interesting to research the consequences of *TaCKX1* silencing in wheat, but the complexity of its genome makes it difficult, barley could be used for this purpose as it is a simpler organism in respect of genome size.

7. LITERATURE

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8. ABREVIATIONS

At	*Arabidopsis thaliana*
BL	Blue light
CIB1	Cryptochrome interacting helix loop helix transcription factor
CK	cytokinins
CKX	Cytokinin dehydrogenase
CRY	Cryptochrome
cZ	*Cis*-Zeatin
cZOG	*Cis*- Zeatin *O*-glucosyltransferase
cZR	*Cis*-Zeatin riboside
DHZ	Dihydrozeatin
DMF	N,N-dimethylformamid
FAD	Flavin adenin dinucleotide
GLU	β-glucosidase
HK	Histidin kinase
Hv	*Hordeum vulgare*
CHL	Chlorophyll
iP	Isopentenyldadenine
Os	*Oryza sativa* (rice)
RR	Response regulators
Ta	*Triticum aestivum*
To	Time 0 sampling point
tZ	*Trans*-Zeatin
Zm	Zea mays (maize)