PALACKÝ UNIVERSITY IN OLOMOUC

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CENTRE OF REGION HANÁ FOR BIOTECHNOLOGICAL AND AGRICULTURAL RESEARCH

DEPARTMENT OF BIOPHYSICS

DOCTORAL THESIS

The study of physical and chemical signaling pathways related to light, abiotic and biotic stresses

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Contents

Declaration I

I hereby declare that this thesis has been written by me using the resources listed in the section “References“.

In Olomouc, Jan Hlavinka

List of papers

The thesis is based on the following papers. These papers are referred in the text by the roman numerals below and are enclosed in the Supplement.

I. **Hlavinka J,** Nauš J, Špundová M (2013) Anthocyanin contribution to chlorophyll meter readings and its correction. *Photosynthetic Research* **118**: 277–295.

II. **Hlavinka J**, Nauš J, Fellner M (2013) Spontaneous mutation *7B-1* in tomato impairs blue light-induced stomatal opening. *Plant Science* **209**: 75–80.

III. **Hlavinka J**, Nožková-Hlaváčková V, Floková K, Novák O, Nauš J (2012) Jasmonic acid accumulation and systemic photosynthetic and electrical changes in locally burned wild type tomato, ABA-deficient *sitiens* mutants and *sitiens* pretreated by ABA. *Plant Physiology and Biochemistry* **54**: 89–96.

IV. Novák J, Pavlů J, Novák O, Nožková V, Špundová M, **Hlavinka J**, Koukalová Š, Skalák J, Černý M, Brzobohatý B (2013) High cytokinin levels induce a hypersensitive-like response in tobacco. *Annals of Botany* **112**: 41–55.

V. Prokopová J, Mieslerová B, Hlaváčková V, **Hlavinka J,** Lebeda A, Nauš J, Špundová M (2010) Changes in photosynthesis of *Lycopersicon* spp. plants induced by tomato powdery mildew infection in combination with heat shock pre-treatment. *Physiological and Molecular Plant Pathology* **74**: 205–213.

Declaration II

I declare that my role in the preparation of mentioned papers was as following:

- **I.** Chief author – part of measurements, data processing and interpretation, preparation of manuscript
- **II.** Chief author – design of experiments, all measurements, data processing and interpretation, preparation of manuscript
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- **IV.** Co-author – collaboration on measurements
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In Olomouc, Jan Hlavinka

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List of publications

1. Prokopová J, Mieslerová B, Hlaváčková V, **Hlavinka J**, Lebeda A, Nauš J, Špundová M (2010) Changes in photosynthesis of *Lycopersicon* spp. plants induced by tomato powdery mildew infection in combination with heat shock pre-treatment. *Physiological and Molecular Plant Pathology* **74**: 205–213. (Cited 3 times on Web of Science)
2. **Hlavinka J**, Nožková-Hlaváčková V, Floková K, Novák O, Nauš J (2012) Jasmonic acid accumulation and systemic photosynthetic and electrical changes in locally burned wild type tomato, ABA-deficient *sitiens* mutants and *sitiens* pretreated by ABA. *Plant Physiology and Biochemistry* **54**: 89–96.
3. Novák J, Pavlů J, Novák O, Nožková V, Špundová M, **Hlavinka J**, Koukalová Š, Skalák J, Černý M, Brzobohatý B (2013) High cytokinin levels induce a hypersensitive-like response in tobacco. *Annals of Botany* **112**: 41–55. (Cited once on Web of Science)
4. **Hlavinka J**, Nauš J, Fellner M (2013) Spontaneous mutation *7B-1* in tomato impairs blue light-induced stomatal opening. *Plant Science* **209**: 75–80.
5. **Hlavinka J**, Nauš J, Špundová M (2013) Anthocyanin contribution to chlorophyll meter readings and its correction. *Photosynthesis Research* **118**: 277–295.

Participations on conferences

Hlavinka J, Leonhardt N, Vavasseur A, Nauš J, Fellner M (2012) Spontaneous mutation *7B-1* in tomato impairs blue light-induced stomatal opening (Plant Biology 2012, Austin, TX, USA)

Hlavinka J, Hlaváčková V, Prokopová J, Mieslerová B, Špundová M, Piterková J, Lenka Luhová L, Novák O, Lebeda A, Nauš J (2010) Effect of heat shock pre-treatment of tomato plants (*Lycopersicon chmielewskii*) on their interaction with biotrophic pathogen (*Oidium neolycopersici*) (The 15th International Congress of Photosynthesis, Beijing, China)

Hlavinka J (2009) Photosynthetic responses of susceptible and moderately resistant tomato (*Lycopersicon esculentum* cv. Amateur a *Lycopersicon chmielewskii*) plants to tomato powdery mildew (*Oidium neolycopersici*) infection and heat shock pre-treatment (7th International Conference of Ph.D. Students on Experimental plant Biology, Brno, Czech republic)

Prokopová J, Mieslerová B, Piterková J, Hlaváčková V, **Hlavinka J**, Špundová M (2008) Effect of heat shock pre-treatment of tomato plants (*Lycopersicon esculentum* cv. Amateur) on their resistance to biotrophic pathogen (*Oidium neolycopersici*) infection (International Conference on Biotic Plant Interactions, Brisbane, Australia)

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Abbreviations

9-AC – anthracene-9-carboxylic acid
A – assimilation rate
AAO3 – *Arabidopsis* aldehyde oxidase 3
ABA – abscisic acid
ABF – ABA-response element binding factor
ADP – adenosine diphosphate
AHK2-4 – *Arabidopsis* histidine kinase 2 to 4
AHP1-5 – *Arabidopsis* histidine phosphotransferase protein 1 to 5
Ala – alanine
AOC – allene oxide cyclase
AP – action potentials
ARR – *Arabidopsis* response regulator
Asp – aspartic acid (D)
A. thaliana – *Arabidopsis thaliana* L.
ATP – adenosine triphosphate
BL – blue light
CAB – chlorophyll a/b-binding protein
Chl(a+b) – chlorophyll content
CHLH – Mg-chelatase H subunit
C_i – intercellular CO_2 concentration
COI1 – coronatine insensitive 1
cp-actin – chloroplast-actin
cry1-2 – cryptochromes 1 and 2
Cys – cysteine
DAI – days after inoculation
DAS – DQXVP, acidic, STAES
DAT – day after DEX treatment
DEX – dexamethasone
E – transpiration rate
FAD – flavinadeninedinucleotide
FC – fusicoccin
FRL – far red light
FKF1 – flavin-binding Kelch repeat F-box protein
FMN – flavinmononucleotide
FNR1 – ferredoxin: NADP oxidoreductase
GCR2 – G-protein coupled receptor 2
GE – gas exchange
g_s – stomatal conductance
His – histidine (H)
HR – hypersensitive response
HS – heatshock
I_{Ca} channels – hyperpolarization-activated Ca^{2+}-permeable channels
Ile – isoleucine
iP – isopentenyladenine
IPT – ATP/ADP isopentenyltransferase
JA – jasmonic acid
JAZ1 – jasmonate ZIM-domain
L1 – first leaf counted from the apex of the plant

$L1^{st}$ – first leaf counted from the plant base
LA – linolenic acid
Leu – leucine
LKP2 – LOV Kelch protein
LOV1 – Light-Oxygen-Voltage 1
MeJA – methyl esters JA
MTHF – methenyltetrahydrofolate
NAD^+ – nicotinamide adenine dinucleotide
$NADP^+$ – nicotinamide adenine dinucleotide phosphate
NADPH – reduced form of nicotinamide adenine dinucleotide phosphate
NIF – niflumic acid
NO – nitric oxide
NPR1 – nonexpresser of PR genes 1
O. neolycopersici – Oidium neolycopersici
OPDA – 12-oxo-phytodienoic acid
OST1 – open stomata 1
PA – phosphatidic acid
PAR – photosynthetically active radiation
PEP – phosphoenolpyruvate
Phe – phenylalanine
phot1-2 – phototropins 1and 2
phyA-E – phytochromes A to E
PHR – photolyase-homologous region
PIN – proteinase inhibitors
PP1, PP2C – protein phosphatase 1, protein phosphatase 2C
PR – pathogenesis-related
PSI, PSII – photosystem I, II
PYR/PYL/RCAR – pyrabactin resistance/PYR1 like/regulatory component of ABA receptor
RL – red light
ROS – reactive oxygen species
RuBisCO – ribulose-1,5-bisphosphate carboxylase/oxygenase
SA – salicylic acid
SD – standart devaiation
SE – standard error
SEP – surface electric potentials
Ser – serine
SIT – *sitiens*
SITA – ABA pre-treated SIT plants
SLAC1 – slow anion channel-associated 1
T-DNA – transfer DNA
T_C – partly collimated leaf transmittance
T_{Cf} – terminal TC
T_{Cmax} – maximal TC
T_{C0} – initial TC
Thr – threonine
Val – valine
VDE – violaxanthin de-epoxidase
VP – variation potential
WT – wild type
ZTL – Zeitlupe

Abstract

Plants are sensitive organisms that interact with their environment. They are exposed to many external stimuli (conditions) that affect their life. Some stimuli are non-stressful for plants (e.g. light of low intensity) whereas other (abiotic or biotic stresses) can damage plants organs. Since plants can not escape from the place, they should develop the way how to deal with external stimuli. External stimuli can activate specific signaling pathways leading to plant responses. The progress in discovering signaling pathways of plants is very fast, however, many questions are still unanswered. The full understanding of plant signaling pathway could improve for example the yield of agriculture production.

Presented work is divided into three parts that focus on the study of the signaling pathway of plants related to different stimuli (light, abiotic stress and biotic stress). In first part, the reaction of male sterile *7B-1* tomato mutant (reported to show specific behavior under blue light) to light (chloroplasts and stomatal movements) has been explored and related signaling pathways have been investigated. The results showed that both avoidance chloroplast movement and stomatal opening induced by blue light were impaired in *7B-1* mutant. The following investigation points out that the blue light signaling pathways are affected (possibly in the blue light receptor part) in *7B-1* mutant. Besides, both chloroplast movement and stomatal opening are also most likely affected by increased abscisic acid (ABA) level in *7B-1*. The obtained results contribute to the contemporary state of understanding the properties of *7B-1* mutant.

The second part of this thesis focuses on investigation of the signaling pathways leading to systemic response (in gas exchange) in reaction to abiotic stress (local burning of the leaf) for tomato plants. Surface electric potential (SEP) and endogenous phytohormones (ABA and jasmonic acid (JA)) content has been measured to reveal the possibility that they can act as physical or chemical signals participating on triggering the systemic response. Using ABA deficient mutant *sitiens* and *sitiens* pre-treated by ABA, the role of endogenous ABA in long-distance signaling has been evaluated. The obtained results indicate that the initial endogenous ABA content defines the steady-state of gas exchange parameters and affects the shape of the gas exchange parameters and SEP changes appearing after local burning. Coordinated action of ABA, JA and SEP is needed for triggering the systemic response in gas exchange parameters to local burning. In response to local burning, the shortage of ABA (which is a signaling molecule triggering the gas exchange reaction) in the *sitiens* mutant is partly compensated for by a pronounced accumulation of JA. The capability to compensate for the ABA shortage by higher JA accumulation in reaction to local burning in *sitiens* plants is maintained even in *sitiens* plants endogenously supplied with ABA. The results also suggest that the level of accumulated JA itself directly affects neither SEP nor gas exchange parameters.

The third part of presented thesis touches the problematic of biotic stress signaling. One of the particular problems solved herein is the investigation of the role of cytokinins in plant response to pathogen infection. For this purpose, the transgenic tobacco plants with dexametazone-inducible *ipt* gene (coding for the enzyme responsible for cytokynin biosynthesis and inserted to genome by certain pathogens) were selected. Activation of the *ipt* gene leads to dramatic increase of endogenous cytokinin level. Increased cytokinin levels

cause a hypersensitive-like response in tobacco leaves. Obtained results lead to suggestion that increases in chloroplastic hydrogen peroxide levels orchestrate the molecular processes underpinning the hypersensitive-like response. An inhibition of photosynthesis, stomatal closure as well as increases in stress hormone levels and oxidative damage of membranes are included in the hypersensitive–like response. Altogether, the data indicate that plant defense against pathogen attack can be mediated by cytokinins.

Further, within the third part of the thesis, the role of plant resistance/susceptibility and effect of heat shock (HS) pre-treatment of plant in reaction to pathogen infection (*Oidium neolycopersici*) has been investigated in tomato. Leaves of both susceptible and moderately resistant tomato genotypes infected by *O. neolycopersici* showed only negligible decrease in the photosynthetic functions during 9 days after inoculation. This result indicates that the rate and the extent of the photosynthetic response to the pathogen are not markedly changed by different degree of resistance/susceptibility of plants to pathogen. Exposing of moderately resistant tomato genotype to HS pre-treatment did not change significantly its resistance and photosynthetic response. However, increased susceptibility to *O. neolycopersici* has been observed in HS pre-treated susceptible genotype. The hypothesis that this increase of susceptibility could be caused by the earlier activation of the cell-wall invertase that results from a higher demand for carbohydrates in HS-induced defense reactions has been pronounced. The consequent accumulation of hexoses would lead to the earlier feed-back inhibition of photosynthesis. It has been speculated that HS-induced changes in resistance/susceptibility to pathogen could be associated with heat shock influence on host photosynthesis.

Altogether, the results presented in this work contribute to contemporary understanding of the signaling pathways related to three kinds of stimuli (light, abiotic and biotic stress) in different levels of plant body (cell, leaf, whole plant). They show the complexity of plant signaling in reaction to external stimuli.

Souhrn

Rostliny jsou citlivé organismy interagující s okolním prostředím. Jsou vystavovány mnoha externím stimulům (podmínkám), jež ovlivňují jejich život. Některé stimuly jsou pro rostliny nestresující (např. světlo nízké intenzity), zatímco jiné (abiotické nebo biotické stresy) mohou poškodit rostlinné orgány. Jelikož rostliny nemohou uniknout ze svého stanoviště, musely si vyvinout způsob, jak se vypořádat s vnějšími stimuly. Vnější stimuly mohou aktivovat specifické signální cesty vedoucí k reakcím rostliny. Pokrok ve výzkumu signálních cest u rostlin je velice rychlý, avšak některé otázky stále zůstávají nezodpovězené. Plné pochopení signálních cest u rostlin by mohlo například vylepšit výtěžek zemědělské produkce.

Předkládaná práce je rozdělena do tří částí, jež jsou zaměřeny na studium signálních cest rostlin související s různými stimuly (světlo, abiotický stres a biotický stres). V první části práce byla studována reakce mutanta *7B-1* (se sterilními samčími částmi květů, vykazujícího specifické chování na modrém světle) na světlo (pohyb chloroplastů a průduchů) spolu se souvisejícími signálními cestami. Výsledky ukazují, že úhybný pohyb chloroplastů a otevírání průduchů způsobené modrým světlem jsou u *7B-1* ovlivněny mutací. Následné experimenty ukázaly, že signální cesty modrého světla jsou u mutanta *7B-1* poškozeny (nejspíše v části obsahující receptory modrého světla). Kromě toho jsou pohyby chloroplastů a průduchů u mutanta *7B-1* také pravděpodobně ovlivněny zvýšeným obsahem kyseliny abscisové (ABA). Získané výsledky přispívají k současnému stavu porozumění vlastností mutanta *7B-1*.

Druhá část této práce se zaměřuje na studium signálních cest u rostlin rajčete vedoucích k systémové odpovědi (v gazometrických parametrech) v reakci na abiotický stres (lokální popálení listu). Povrchové elektrické signály (SEP) a obsah endogenních fytohormonů (ABA a jasmonová kyselina (JA)) byly měřeny, aby bylo otestováno, zdali nemohou působit jako fyzikální a chemické signály účastnící se spuštění systémové odpovědi. Role endogenní ABA při přenosu signálu na dlouhou vzdálenost byla studována s použitím mutanta s nedostatkem ABA (*sitiens*) a *sitiens* s endogenně dodanou ABA. Získané výsledky naznačují, že počáteční endogenní obsah ABA definuje hodnoty ustáleného stavu gazometrických parametrů před popálením a ovlivňují podobu změn v gazometrických parametrech a v SEP, které se objevují po lokálním popálení. Pro spuštění systémové reakce v gazometrických parametrech (odpovědi na lokální popálení) je zapotřebí koordinovaná akce ABA, JA a SEP. Při odpovědi na lokální popálení je nedostatek ABA (která je signální molekulou spouštějící reakci v gazometrických parametrech) v mutantech *sitiens* částečně kompenzován výraznou akumulací JA. Schopnost kompenzovat nedostatek ABA vyšší akumulací JA při reakci na lokální popálení v rostlinách *sitiens* je zachována i v *sitiens* s endogenně dodanou ABA. Výsledky také ukazují, že samotná hladina akumulované JA neovlivňuje ani SEP ani gazometrické parametry.

Třetí část předkládané práce se dotýká problematiky signalizace související s biotickým stresem. Jedním z problémů řešených v této části je zjištění role cytokininů při reakcích rostlin na patogenní infekci. Pro tento účel byly vybrány transgenní rostliny tabáku obsahující gen *ipt* (kódující enzym odpovědný za biosyntézu cytokininu a vložený do genomu rostliny určitým druhem patogena), jehož expresi je možno vyvolat pomocí dexametazonu (aktivace genu). Aktivace genu *ipt* vede k dramatickému nárůstu endogenního obsahu cytokininů.

Zvýšená hladina cytokininů způsobuje v rostlinách tabáku reakci, která je podobná hypersensitivní reakci. Obdržené výsledky vedou k domněnce, že zvýšení hladiny peroxidu vodíku v chloroplastech řídí molekulární procesy, které jsou podstatou reakce podobné reakci hypersenzitivní. Reakce podobná reakci hypersenzitivní zahrnuje inhibici fotosyntézy, zavření průduchů a dále zvýšení obsahu stresových hormonů a oxidativní poškození membrán. Získané výsledky ukazují, že obrana rostliny proti patogennímu útoku může být zprostředkována cytokininy.

Ve třetí části této práce je dále popsána studie role resistence/náchylnosti rostlin a vliv tepelného ošetření rostlin na reakci vyvolanou patogenní (*Oidium neolycopersici*) infekcí v rostlinách rajčete. Listy náchylného a středně rezistentního genotypu rajčete infikované patogenem *O. neolycopersici* vykazovaly pouze zanedbatelné omezení fotosyntetických funkcí během devíti dní po inokulaci patogenem. Výsledky naznačují, že rychlost a rozsah odpovědi fotosyntézy na potogenní infekci nejsou významněji ovlivněny různým stupněm rezistence/náchylnosti rostlin k patogenu. Vystavení středně rezistentního genotypu rajčete tepelnému ošetření nezměnilo významně jeho resistenci ani odpovědi fotosyntézy. Avšak u tepelně ošetřených náchylných genotypů byla pozorována zvýšená náchylnost k *O. neolycopersici*. Předpokládáme, že tento nárůst náchylnosti by mohl být způsoben dřívější aktivací invertázy buněčné stěny, která je dána vyšší potřebou uhlovodíků během obranných reakcí způsobených tepelným ošetřením. Následné hromadění hexóz by vedlo k dřívější zpětné inhibici fotosyntézy. Domníváme se, že změny v rezistenci/náchylnosti k patogenu vyvolané tepelným ošetřením by mohly souviset s vlivem tepelného ošetření na fotosyntézu hostitele.

Výsledky prezentované v této práci přispívají k současnému pochopení signálních cest souvisejících s třemi druhy podnětů (světlo, abiotický a biotický stres) na různých úrovních rostliny (buňka, list, celá rostlina). Tyto výsledky ukazují složitost signalizace u rostlin v reakci na vnější podněty.

1. Introduction

Higher plants occur in the most places of the Earth. Some of them can survive even in extreme conditions prevailing in deserts or tundra. Their spreading is one of the factors contributing to expansion and development of human civilization. Many of plant species have been cultivated from everlasting for their important nutrition value, some of them have got interesting medicinal use, some have been used for constructions, etc. Although the plants were exploited by humans for all known history, they have been considered not to actively react to external conditions changes. However, in several last decades, the attitude to the plants has remarkably changed and the reactions of the plants to various conditions have been broadly investigated.

During their lifetime, the plants are exposed to the common (non stressful) changes of environmental condition, mainly light (e.g. diurnal changes of light intensity and direction). In this case, the light is not only a source of energy for photosynthesis, but it is, as well, one of the factors (signal) most important for the plant development and life. The light serves as a signal that regulates photomorphogenesis, phototropism, photoperiodism and stomatal aperture.

However, plants are as well exposed to many stressful conditions. The stressful conditions are often divided according to the origin of the stress to biotic (caused by another living organism or biological structure, e.g. herbivore, fungal pathogen or viral infection) and abiotic (the stress is of physical or chemical origin, e.g. changing of seasons, wind, excessive or low irradiation of plant organs, excessive or low nutrient or water content of soil, air pollution etc.).

Since the plants can not escape from the place, they should develop a way how to deal with external stimuli. Both non stressful and stressful conditions can activate specific signaling pathways leading to responses by which plants can optimize e.g. the utilization of light radiation, water or nutrition resources and which minimize the detrimental consequences of the stressing conditions for their survival, development and growth.

In the following, the term “signal” is understood as every process between the stimulus (change of conditions) and the reaction of a plant structure (level higher than a molecule, i.e.: an organelle, cell, tissue, organ, whole plant). This thesis consist of three parts which aims to cover three important cases of plant signaling: the first one is focused to the light signaling within plant cells, the second one to the long distance signaling in reaction to abiotic stress and third one to the signaling in reaction to biotic stress.

2. Present state of knowledge

2.1 Light signaling

Light (especially of lower intensities that do not cause photosynthesis photoinhibition) is widely utilized by plants among other as a signaling factor which can regulate developmental processes (e.g. germination, de-etiolation, transition to flowering) and function of plant organs, cells and organelles (e.g. leaf movement, stomata and chloroplasts movement). The primary step in plant light signaling is an interaction of photons of incoming light with specific chromoproteins – photoreceptors. The plants developed several groups of photoreceptors sensitive to different qualities of light such as color, intensity or direction.

2.1.1 Photoreceptors of blue light

Up to now, several groups of blue-light photoreceptor have been identified in higher plants, primarily, cryptochromes, phototropins, and the Zeitlupe family (ZTL/FKF1/LKP2). All of them contain flavins as the chromophore (Banerjee and Batschauer 2005).

2.1.1.1 Cryptochromes

Cryptochromes are photoreceptors of blue light (BL) that regulate growth, development (e.g. de-etiolation, phototropism and flowering time) and the circadian clock in plants (Banerjee and Batschauer 2005; Liu *et al.* 2011). Three cryptochromes (cry1, cry2, cry3) were identified in *Arabidopsis thaliana* L. (*A. thaliana*). cry1 and cry2 are primarily presented in the nucleus, however, cry 3 probably functions in the chloroplasts and mitochondria (Liscum *et al.* 2003; Liu *et al.* 2011). Major part of the cryptochromes is formed by the 500 amino acids long sequence, which is similar to the DNA-photolyase. This photolyase-homologous region (PHR) part binds non-covalently the chromophore flavinadeninedinucleotide (FAD) (Banerjee and Betschauer 2005; Liscum *et al.* 2003). cry1 and cry2 possibly bound also the second chromophore MTHF (methenyltetrahydrofolate) to PHR, whereas no chromophore other than FAD has been identified in cry3. Additional domain (containing DAS – i.e. three motifs: DQXVP, acidic, STAES; Lin and Shalitin 2003) with different length and sequence within cry1 and cry2 is presented at the C-terminus of the proteins. The C-terminal domain of cry2 containing a signal for nuclear localization is required for a nuclear import. cry3 lacks a C-terminal extension, however, it carries the extension at the N-terminal end (amino acids 1 – 40) which is required for the import of cry3 into the chloroplasts and mitochondria (Banerjee and Betschauer 2005; Liu *et al.* 2011). The scheme of cry1, cry2 and cry3 structures is depicted in Fig. 1.

Absorption of BL probably induces photoreduction of FAD and causes a cyclic electron transfer through a redox form of flavin. Probably, cry is also autophosphorylated in reaction to BL absorption. These changes lead to conformation changes of cry (Liu *et al.* 2011). The C-terminus of cry1 and cry2 than physically interacts with several proteins including COP1 (constitutive photomorphogenic 1), a repressor of photomorphogenesis in darkness (Banerjee and Batschauer 2005; Lin and Shalitin 2003; Liscum *et al.* 2003; Liu *et al.* 2011).

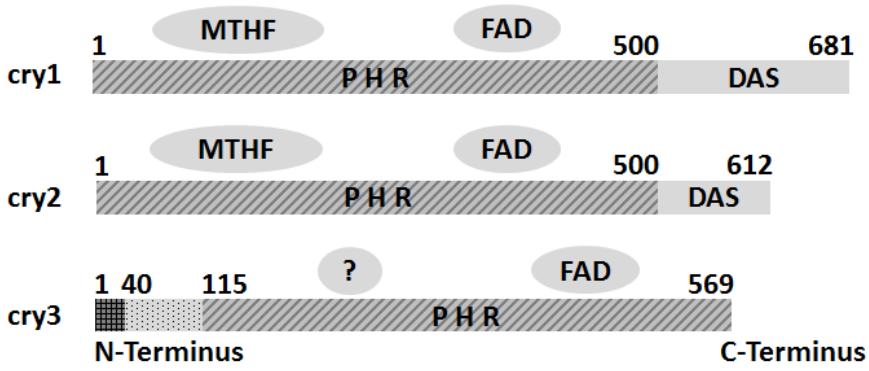

Fig. 1. The scheme of cry1, cry2 and cry3 structures. Chromophores are marked by an elliptic shape (MTFH – methenyltetrahydrofolate, FAD – flavin adenine dinucleotide, ? – possible, yet non-identified chromophore). Proteins are marked by the rectangular shapes of different color connected together (one color represents one function part of protein, PHR – photolyase-homologous region, DAS – three motifs: DQXVP, acidic, STAES). For additional information see text. Aminoacids are numbered above the protein from N-terminus to C-terminus. Adopted from Banerjee and Batschauer (2005).

2.1.1.2 Phototropins

Phototropins phot1 and phot2 (Briggs *et al.* 2001) are BL and UV-A (320-500 nm) photoreceptors, that mediate or regulate phototropism, chloroplast movement and stomatal opening (Brigs and Christie 2002; Kagawa 2003; Kinoshita *et al.* 2001; Sakai *et al.* 2001). In addition, the rapid inhibition of dark-grown stem growth by BL (de-etiolation) is probably mediated by phot1 (Brigs and Christie 2002; Folta and Spalding 2001). Receptor phot1 also plays a role in BL-mediated calcium uptake (Baum *et al.* 1999) and might play a role in BL-induced membrane depolarization (Folta and Spalding 2001). Phototropins are located mainly in the cell membranes (Kong *et al.* 2013; Sakamoto and Briggs 2002). Both phot1 and phot2 contain more than 900 amino acids and have the same domain structure with LOV1 (Light-Oxygen-Voltage 1) and LOV2 domains of about 100 amino acids long and the Ser/Thr kinase domain in the C-terminal half (Banerjee and Batschauer 2005; Briggs and Christie 2002) The LOV domains bind the FMN (flavinmononucleotide) chromophore (Briggs and Christie 2002). The scheme of phot1 and phot2 structures is depicted in Fig. 2.

In the dark or ground state, the phototropin is unphosphorylated and inactive (FMN is in the ground state) (Christie 2007). It is believed, that an exposition of phototropin FMN chromophore to BL results in a formation of an excited singlet state, which subsequently decays into a flavin triplet state. The triplet state in turn decays to form of a covalent bond between the C(4a) of FMN and a cysteine residue within the conserved NCRFL motif of the LOV2 domain what leads to an activation of the kinase domain (reviewed in: Briggs and Christie 2002; Celaya and Liscum 2005; Christie 2007). Consequently, it leads to the autophosphorylation of the photoreceptor and possibly phosphorylation of a yet-unidentified protein substrate. In darkness, this process is reversed and forms a photocycle (Christie 2007). Some proteins (e.g. 14-3-3 protein) are associated to the phototropins and are involved in the phototropin signaling (e.g. Banerjee and Batschauer 2005; Kinoshita *et al.* 2003).

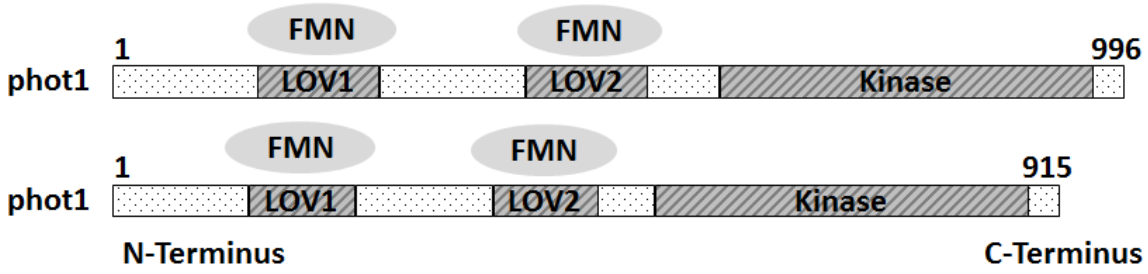

Fig. 2. The scheme of phot1 and phot2 structures. Chromophores are marked by an elliptic shape (FMN – flavinmononucleotide). Proteins are marked by the rectangular shapes of different color connected together (one color represents one function part of protein, LOV1 – Light-Oxygen-Voltage 1, LOV2 – Light-Oxygen-Voltage 2). For additional information see text. Aminoacids are numbered above the protein from N-terminus to C-terminus. Adopted from Banerjee and Batschauer (2005).

2.1.1.3 The Zeitlupe family (ZTL/FKF1/LKP2)

Three flavoproteins Zeitlupe (ZTL), flavin-binding Kelch repeat F-box protein (FKF1) and LOV Kelch protein (LKP2) are members of the Zeitlupe photoreceptor family in *A. thaliana*. These photoreceptors take part in controlling inhibition of hypocotyl growth and other de-etiolation responses, clock function and flowering time (Banarjee and Batschauer 2005; Fankhauser and Staiger 2002; Takase *et al.* 2011). These three proteins structure consist of the N-terminal LOV domain followed by the F-box part, and the C-terminal six Kelch repeats. As in phototropins, the chromophore FMN is bind to the LOV domain and goes through a photocycle very similar to that of the LOV domain of the phototropins but without the dark recovery (Banerjee and Batschauer 2005). The scheme of ZTL, FKF1 and LKP2 structures is depicted in Fig. 3.

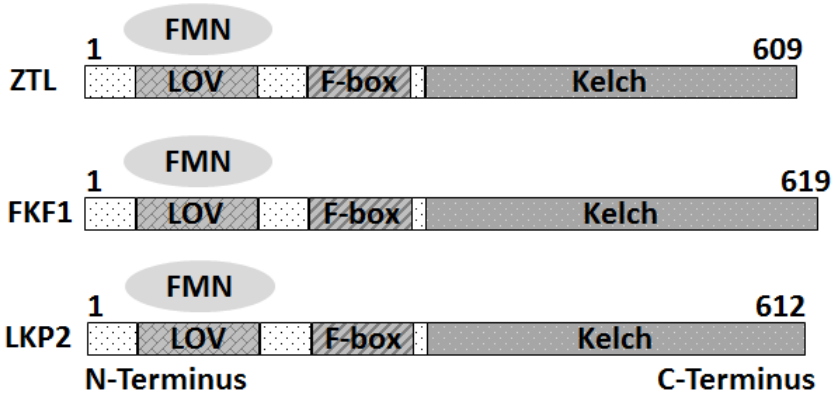

Fig. 3. The scheme of ZTL, FKF1 and LKP2 structures. Chromophores are marked by an elliptic shape (FMN – flavinmononucleotide). Proteins are marked by the rectangular shapes of different color connected together (one color represents one function part of protein, LOV – Light-Oxygen-Voltage, F-box – F-box part, Kelch – six Kelch repeats). For additional information see text. Aminoacids are numbered above the protein from N-terminus to C-terminus. Adopted from Banerjee and Batschauer (2005).

2.1.2 Photoreceptors of red light

The photoreceptors of Red/Far Red Light (RL/FRL) are phytochromes. In *A. thaliana*, there were find five types of phytochromes (phyA to phyE) (Li *et al.* 2011). phyA is the primary photoreceptor responsible for mediating photomorphogenic responses in FRL, phyB predominantly regulates de-etiolation responses in RL. Phytochomes regulate or mediate mainly seed germination, seedling de-etiolation and shade avoidance (Li *et al.* 2011). All phytochromes exist as dimmers that are composed of two 125 kDa polypeptides, each carrying a covalently linked open-chain tetrapyrrol chromophore (Gyula *et al.* 2003). Phytochromes are synthesized in the cytosol in the dark in their physiologically inactive RL-absorbing Pr form (Gyula *et al.* 2003; Li *et al.* 2011). Absorption of RL photoconvert (lead to the conformation changes) the Pr form to the physiologically active Pfr form, which absorbs FRL. The Pfr form can be transformed back into the Pr form by absorption of FRL. The Pfr form of phyA is light labile, whereas phyB-phyE are light stable (Gyula *et al.* 2003; Fankhauser and Staiger 2002; Li *et al.* 2011). Beside the conformational change of the phytochrome molecule, light causes the autophosphorylation of phyA and the phosphorylation of other proteins (Fankhauser 2000; Gyula *et al.* 2003). Light also induces phyA – phyE translocation into the nucleus of the cell which modulates their distribution in the nucleus and cytoplasm (Gyula *et al.* 2003).

2.1.3 Signaling pathways of light

There exist several reactions of plant cells or organ to light. However, complete signaling pathways for many of them are quite obscure. The following parts describe two signaling pathways of light: the chloroplast movements and stomatal opening.

2.1.3.1 Chloroplast movement

The chloroplast movement is induced by BL in most plant species (e.g. Wada *et al.* 2003). The RL-induced chloroplast movement has not been detected for the majority of higher plants, although, it has been observed for some ferns and lower plants (Augustynowicz and Gabryś 1999; Kadota and Wada 1999; Wada 2005). An accumulation response of chloroplasts under illumination of low intensity and an avoidance response under illumination of high intensity were observed. The chloroplast accumulation response is believed to maximize photosynthetic efficiency within the cell, whereas the chloroplast avoidance response to minimize its photodamage (Wada 2005).

Detailed investigation of the phenomenon has been performed by Kagawa and Wada (1999) who irradiated the fern *Adiantum capillus-veneris* L. by the micro beams. They found that both weak and strong illumination lead to the chloroplast movement toward the illumination, however, the movement caused by the strong light is stopped in front of the illuminated area. When the strong light is switched off, chloroplasts move into the area that was formerly irradiated. These results indicate that the signal for the accumulation movement can traverse the distance of the cell, but the signal for the avoidance movement stays inside the irradiated area. Moreover, the results indicate that the signal for the avoidance movement is dominant to that one for the accumulation movement during the irradiation and the signal

for the accumulation response remains long after irradiation, but that one for the avoidance response diminishes immediately. It suggests that the signals for both responses are different, however, the mechanism of the chloroplast movement seems to be the same for both the accumulation and the avoidance responses (Wada 2005).

By the examination of *A. thaliana* L. mutant *nph1* deficient in the chloroplast avoidance movement it was found that BL photoreceptor phot2 is related to the chloroplast avoidance movement (Kagawa *et al.* 2001). However, the chloroplast accumulation movement was normal in these mutants. The accumulation response is missing in the mutant *phot1phot2*. This reveals that both phot1 and phot2 are involved in the chloroplast accumulation response in *A. thaliana* (Sakai *et al.* 2001). Even RL is usually ineffective in triggering the chloroplast movement, RL photoreceptors phytochromes are involved in the modulation of the light-induced chloroplast movement in *A. thaliana* (DeBlasio *et al.* 2003; Luesse *et al.* 2010).

Even though the light receptors for the chloroplast movement have been found, the question how the signal is transmitted to the chloroplasts is not well known (e.g. Kong and Wada 2011). A candidate for the signal intermediate is cytosolic Ca^{2+} (Harada and Shimazaki 2007; Stoelzle *et al.* 2003; Tlałka and Fricker 1999). Phototropin activated by BL absorption induces an income of Ca^{2+} from the extracellular space and⁄or from the inner Ca^{2+} stores such as the endoplasmic reticulum and vacuoles (for review see Harada and Shimazaki 2007). It is, however, still uncertain how the direction of the chloroplast movement is regulated.

The chloroplast movement seems to be mediated by the cell cytoskeleton (composed of actin filament and microtubules). Both actin filaments (for review see Takagi 2003) as well as microtubules (Sato *et al.* 2001) can be involved in the light-induced chloroplast translocation. However, most land plant species use the actin filaments exclusively (rather than the microtubules) for the chloroplast movement. This was supported by application of anti-actin drugs which inhibited chloroplast movement in various green plant species, whilst application of anti-microtubule drugs did not show these actions (Suetsugu and Wada 2007). Kadota *et al.* (2009) have identified short actin filaments called cp-actin filaments (chloroplast-actin filaments) on the chloroplast envelope of *A. thaliana*. Cp-actin filaments are associated with the chloroplast photorelocation and anchoring to the plasma membrane. The presence of cp-actin filaments depends on the actin-binding protein CHUP1 (chloroplast unusual positioning1) localized on the chloroplast envelope. Mutant *chup1* lacked cp-actin filaments but showed normal cytoplasmic actin filaments. The filaments of cp-actin showed rapid BL-induced relocalization to the leading edge of chloroplasts before and during photorelocation and are regulated by phot1 and phot2 (Kadota *et al.* 2009).

2.1.3.2 Stomatal opening

It is well known fact that stomata open under light and close in darkness. Even both RL and BL can take part in the stomatal opening, the BL is more effective in the stomatal opening compared to RL (Sharkey and Raschke 1981). Blue light acts as a signal and RL as both a signal and an energy source (Shimazaki *et al.* 2007) for the stomatal opening.

Blue-light-induced stomatal opening

Several BL-absorbing pigments have been proposed to be BL receptors in the stomatal opening. According to Zeiger and Zhu (1998), the receptor of BL is zeaxanthin. Together with cryptochromes (Li and Yang 2007; Mao *et al.* 2005), zeaxanthin is involved in the BL-induced stomatal opening. However, phototropins phot1 and phot2 (Briggs and Christie 2002; Kinoshita *et al.* 2001) are nowadays considered as BL photoreceptors associated with the plasma membranes of the guard cells (e.g. Sakamoto and Briggs 2002) triggering the stomatal opening.

Following description of stomatal opening is mainly based on studies with *A. thaliana* model plants. Absorption of BL activates (autophosforylates) phototropins (phot1 and phot2) (Celaya and Liscum 2005). Reverse process – dephosphorylation of phot2 – is catalyzed by PP2A (protein phosphatase 2) (Inoue *et al.* 2010). This fact was confirmed by the *A. thaliana* mutant *rcn1* impaired in dephosphorylation of phot2 which enhances the stomatal opening (Tseng and Briggs 2010). In contrary, the phosphatase that catalyzes the phot1 dephosphorylation is unknown. In guard cells, the immediate downstream component or the substrate for the phototropin kinase has not been identified yet (Inoue *et al.* 2010). The signal might be transmitted to the regulatory subunit of PP1 (protein phosphatase 1) and modulates the catalytic subunit of PP1. The catalytic subunit of PP1 acts as a positive regulator for stomatal opening on BL in *Vicia faba* L. (Takemiya *et al.* 2006). However, the regulatory subunit and substrate of PP1 have not been determined (Inoue *et al.* 2010).

Subsequently, the signal activates the plasma membrane P-type H^+-ATPase (Assmann *et al.* 1985; Kinoshita and Shimazaki 1999) that transports H^+ outwards across the plasma membrane and increases the negative electrical potential inside the cell (leading to hyperpolarization of the plasmatic membrane of guard cell) (Schroeder *et al.* 2001a). The threonin residue in the C-terminus of the H^+-ATPase is fosforylated and subsequently 14-3-3 protein is bound to the H^+-ATPase (Shimazaki *et al.* 2007). The guard cell plasma membrane H^+-ATPase can be as well activated by some fungal toxins e.g. fusicoccin (FC; Johansson *et al.* 1993). In contrary, the function of the H^+-ATPase activated by BL may be reversibly blocked by an increase of NO (nitric oxide) or H_2O_2 concentration (e.g. caused by elevated level of abscisic acid (ABA) during water stress) (Zhang *et al.* 2007; Zhang *et al.* 2004). It has been proposed that the activity of PP1 is inhibited by phosphatidic acid (PA, second messenger of ABA signaling) (Inoue *et al.* 2010).

The hyperpolarization of membrane drives K^+ uptake through the voltage-gated inward-rectifying K^+_{in} channels in the guard cell membrane (Assmann and Shimazaki 1999; Inoue *et al.* 2010; Schroeder *et al.* 2001a). Possibly, there is also an interaction between actin cytoskeleton and the guard cell membrane K^+_{in} channels in broad bean (*Vicia faba* L.). The K^+_{in} channels can be activated by the actin filament-depolymerizing agent cytochalasin D. This effect enhances the light-induced stomatal opening. When the actin filament stabilizer phalloidin was tested, the K^+_{in} channels were inhibited (Hwang *et al.* 1997). As the H^+-ATPase, also the K^+_{in} channels can be inhibited by the increase of NO or H_2O_2 concentration caused by an interaction of the guard cell with ABA (Inoue *et al.* 2010).

An extrusion of H^+ from cytosol is supposed to participate on the Cl^- uptake via the H^+/Cl^- symport (Assmann and Shimazaki 1999) or the Cl^-/OH antiport (Schroeder *et al.*

2001a). The stomatal opening is also promoted by an inhibition of S-type (slow-activating sustained) anion (Cl^-, $malate^{2-}$) channels in the plasma membrane of the guard cells caused by the activation of phototropins by BL absorption (Marten *et al.* 2007). The inhibition of the S-type anion channels can be caused also by an action of anion channels inhibitors such as niflumic acid (NIF), anthracene-9-carboxylic acid (9-AC), and others. It has been shown that adding an anion channel inhibitor (NIF or 9-AC) to the stomata leads to the stomatal opening (e.g. Forestier *et al.* 1998; Schwartz *et al.* 1995).

Blue light also stimulates an increase in the intracellular concentrations of $malate^{2-}$. $Malate^{2-}$ is produced by fission of chloroplast starch and subsequent glycolysis of its products in the cytosol of the guard cell. Phosphoenolpyruvate (PEP) formed by glycolysis is carboxylated to oxalacetate by the PEP carboxylase. The PEP carboxylase activity is increased by BL. The oxalacetate is then reduced by the cytosolic nicotinamide adenine dinucleotide (NAD+)-malate dehydrogenase and/or the chloroplast nicotinamide adenine dinucleotide phosphate ($NADP^+$)-malate dehydrogenase to $malate^{2-}$. Blue light also stimulates breakdown of starch which produce sucrose. Sucrose accumulates in the vacuole of the guard cell and acts as an osmoticum (Assmann and Shimazaki 1999; Lawson 2009).

There are H^+-ATPases in tonoplast of vacuole, which pump protons from the cytosol into the vacuole. Concentration gradient is driving the protons back to cytosol, which can be used by H^+/K^+ antiport to transfer K^+ ions into the vacuole. The formed cation gradient drives the anion ($malate^{2-}$ and Cl^-) flow to the vacuole through the channels in tonoplast (Roelfsema *et al.* 2005). The concentration of K^+ increases in the morning and decreases in the afternoon, while the concentration of sucrose begins to increase with decreasing K^+ concentration. Thus, the mechanism involving K^+ plays a role during the fast stomatal opening in the morning, however, sucrose maintains the stomata opened until twilight (Tallbot and Zeiger 1998).

The ions and/or sucrose accumulated in the vacuole decrease the osmotic potential of the guard cells what facilitates water influx into the guard cells leading to an increase of turgor pressure in the guard cells and to the stomatal opening (Schroeder et al. 2001a). Aquaporins probably participate in water influx into the guard cells (Huang et al. 2002). The scheme of signaling pathways of the stomatal opening on BL is depicted in Fig. 4.

Red-light-induced stomatal opening

It was proposed that responses of stomata to RL are of photosynthetic origin. Red light is absorbed by chlorophyll in chloroplasts of guard cells. Electron transport in guard cell chloroplasts driven by RL produces ATP (adenosine triphosphate) that is used by H^+-ATPase to pump H^+ out what leads to the stomatal opening (Lawson 2009). Fixation of CO_2 in the guard cells chloroplasts produces osmotically active sucrose under RL (Lawson 2009; Talbott and Zeiger 1998). However, recent studies showed that the RL-induced stomatal opening is independent of the concurrent photosynthetic rate of the guard cells and that it involves phytochrome signaling (Baroli *et al.* 2008; Talbott *et al.* 2003; Talbott *et al.* 2002; Wang *et al.* 2010).

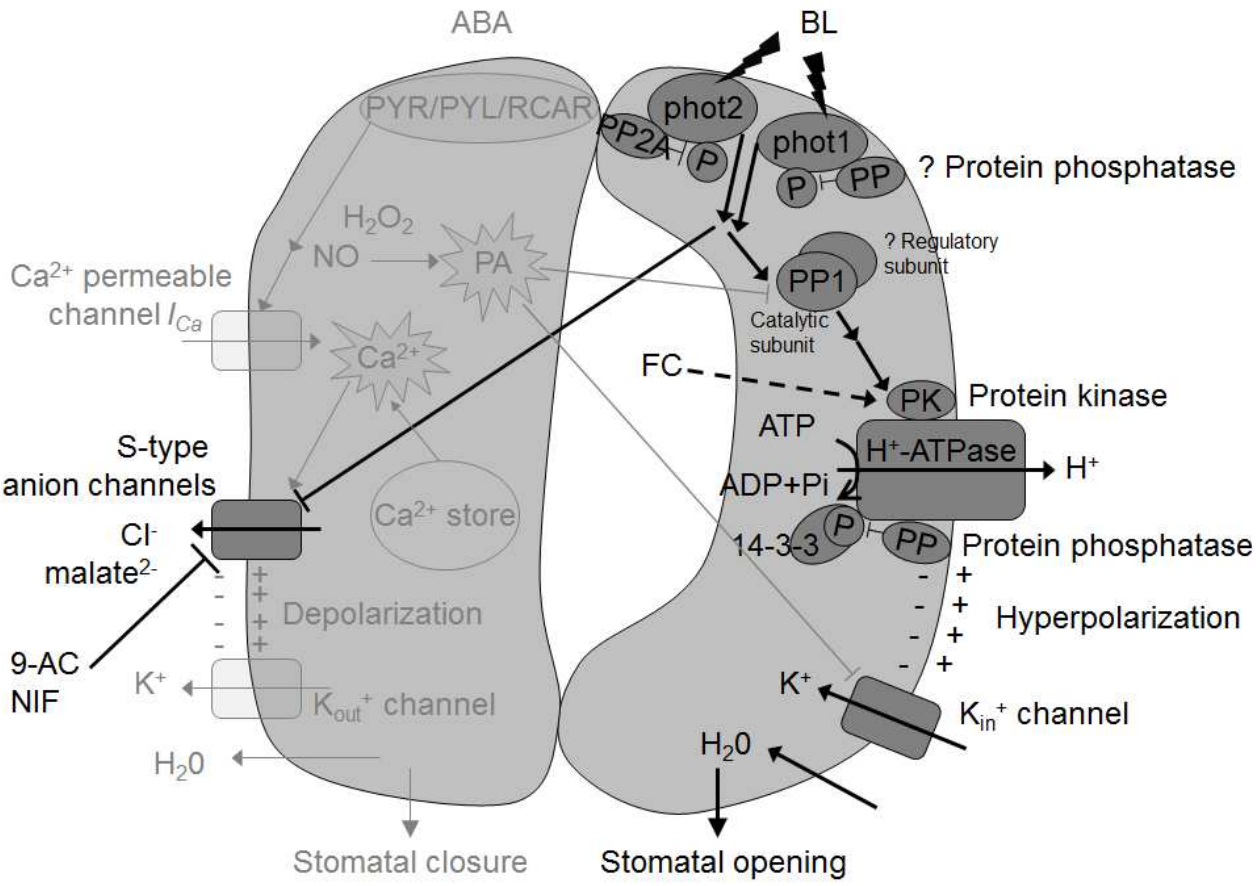

Fig. 4. Scheme of blue-light- (BL) induced stomatal opening. The processes involved are indicated by black coloration of lines (arrows indicate an activation and T-bar indicate an inhibition) and descriptive texts, components involved are of dark-grey coloration. BL percepted by phototropins phot1 and phot2 activates the H^+-ATPase and inhibits the S-type anion channels (PP2A – protein phosphatase 2A; P – phosphorylated protein; PP – protein phosphatase; PP1 – protein phosphatase 1; PK – protein kinase; 14-3-3 – 14-3-3 protein). The changes lead to water flux into the guard cell and to the stomatal opening. The processes involved in BL-induced stomatal opening are described in more detail in chapter 2.1.3.2. The action of anion channel inhibitors 9-AC and NIF and fusicoccin (FC) is also shown. The less visible components and lines (light-grey-colored) prevailing in the guard cell on the left side indicate crosstalk with processes triggered by ABA perception (ABA perception causes stomatal closure, see chapter 2.2.1.1 and Fig. 6). BL-induced stomatal opening can be inhibited or reversed by action of ABA. Adopted with slight modification from Inoue *et al.* (2010).

2.2 Stress signaling

Stress is an environmental factor that affects almost all aspects of plant life. The variety of possible plant responses to stress is very broad, however, some processes can be shared between biotic and abiotic stress responses. In reaction to stress, plants can change the production or degradation of certain chemical compounds (e.g. stress proteins, phytohormones, reactive oxygen species) and/or can change the physical properties of cell and tissues (e.g. permeability and charge of membranes). Both, chemical and physical changes act as a signal (chemical and physical) that leads to protective response of plants (e.g.

regulation of stomata aperture, specific gene expression). A local stimulation (stress action) of plant can cause responses detected locally (close to the site of stimulation), even systemically (far from the site of stimulation). Signals of chemical (e.g. Hlaváčková *et al.* 2006) origin, hydraulic (Mancuso 1999) and electric (e.g. Kaiser and Grams 2006) physical signals are supposed to mediate local and systemic reactions.

2.2.1 Chemical signals

Several group of chemical compound can act as a signaling factor in plants. The list of these compounds contains among others phytohormones (namely abscisic acid, jasmonates, cytokinins, salicylic acid and further hormones such as auxins, ethylene, gibberellins, brassinosteroids, etc.; e.g. Acharya and Assmann 2009), reactive oxygen species and stress proteins. One phytohormone can affect the action of another phytohormone, therefore the reaction of plant can be triggered or affected by one or several phytohormones. Little is known about their interaction in response to abiotic or biotic stress. Following parts briefly describe some properties of selected chemical compounds.

2.2.1.1 Abscisic acid

The phytohormone ABA serves as a regulator of growth and development processes (seed maturation, dormancy, inhibition of germination, photoregulation, inhibition of lateral root formation, senescence and flowering inhibition; Asselbergh *et al.* 2008). It serves as well as an endogenous messenger in response to biotic (e.g. Adie *et al.* 2007; Raghavendra *et al.* 2010; Ton *et al.* 2009) and abiotic stresses of plants (e.g. Raghavendra *et al.* 2010). Abiotic environmental stresses such as drought, high temperature, exposure to salty water or salinated soil and cold cause water loss and turgor decrease of plant body. ABA is synthesized in response to these stresses and is accumulated and redistributed within the plant body (Wilkinson and Davies 2002). ABA can stimulate root growth and increase root hydraulic conductivity that maximize water uptake by roots (Zhang *et al.* 1995). ABA also induces changes in stress-related gene expression patterns, which lead to various adaptive responses in the cell and at the whole plant levels (Leung and Giraudat 1998; Ramanjulu and Bartels 2002; Shinozaki and Yamaguchi-Shinozaki 2000). Nevertheless, ABA plays a crucial role in reducing water loss by stomatal closing or inhibiting stomatal opening (e.g. Acharya and Assmann 2009; Schroeder *et al.* 2001a). Stomatal closing induced by ABA reduces photosynthesis and also pathogen penetration (Melotto *et al.* 2006). Moreover, ABA functions as an essential signal in plant immunity to some necrotrophic pathogens (Adie *et al.* 2007). Since ABA-controlled processes are necessary for surviving of plant, ABA-deficient mutant (e.g. tomato mutant *sitiens* (SIT) impaired in the final step of ABA biosynthesis, Taylor *et al.* 1988) are of less viability due to susceptibility to water stress (e.g. Leung and Giraudat 1998).

ABA biosynthesis and transport

Abscisic acid biosynthesis begins with its precursor xanthoxin that is produced by carotenoide cleavage in chloroplasts. Cytosolic conversion of xanthoxin to ABA-aldehyde is followed by the final oxidation step leading to the ABA formation (Taylor *et al.* 2005). ABA biosynthesis

steps were reviewed in details elsewhere (e.g. Milborrow 2001; Nambara and Marion-Poll 2005; Taylor *et al.* 2005; Taylor *et al.* 2000).

The finding of the positioning of the enzyme involved in the last step of ABA biosynthesis – Arabidopsis aldehyde oxidase 3 (AAO3) – within plant (*A. thaliana*) helped to locate the place of ABA molecule origin. With the help of transgenic plants of *A. thaliana* (containing AAO3 with fluorescent mark), the place of ABA molecule origin was located in root tips, vascular bundles of roots, hypocotyls, inflorescence stems, and along the leaf veins, phloem companion cells and xylem parenchyma cells and in leaf guard cells (Koiwai *et al.* 2004). The obtained results indicate that ABA synthesized in vascular system is transported to various target tissues and cells. The possibility that guard cells are capable to synthesize ABA was also indicated in *Vicia faba* L. by Melhorn *et al.* (2008). However, it is possible as well that ABA synthesized in reaction to stress is transported to guard cells from the apoplast. ABA has been shown to move through the plant phloem or xylem (Wilkinson and Davies 2002). Therefore it can act as well as a long-distance signal. For instance, mechanical wounding, electric current application and burning caused local and systemic ABA accumulation in tomato and tobacco plants within 6 h (Herde *et al.* 1996). Hlaváčková *et al.* (2006) observed systemic ABA accumulation already 15 min after local burning.

ABA receptor and signaling

Absicic acid binds to the receptor in the plasma membrane or in the cytosol (Guo *et al.* 2011; Sirichandra *et al.* 2009). Protonated form of ABA penetrates through the plasma membrane (Pospíšilová 2003), although, ABA uptake by guard cell of *Commelina communis* L. could be driven also by carriers (Leung and Giraudat 1998). It was proposed that the complex of Mg-chelatase H subunit (CHLH) and ABA-binding protein ABAR (ABAR/CHLH) is an ABA receptor in *A. thaliana* (Shen *et al.* 2006) located in the chloroplast envelope (Guo *et al.* 2011). Another possible ABA receptor located in the plasma membrane, GCR2 (G-protein coupled receptor 2), was identified by Liu *et al.* (2007b) in *A. thaliana*. However, it is not clear if these two components are actually ABA receptors. Risk *et al.* (2009) showed that GCR2 does not bind ABA. Then, Tsuzuki *et al.* (2011) presented evidence that CHLH affects ABA signaling in the stomatal guard cells but is not itself an ABA receptor. Next possible plasma membrane ABA receptor was proposed to be GPCR (G-protein coupled receptor)-type G proteins GTG1 and GTG2 (Pandey *et al.* 2009). Another candidate to ABA receptor is cytosol/nucleus-localized PYR/PYL/RCAR (pyrabactin resistance/PYR1 like/regulatory component of ABA receptor) protein family (Guo *et al.* 2011; Hubbard *et al.* 2010; Kim *et al.* 2010).

ABA perception by its receptor PYR/PYL/RCAR proteins induces formation of ABA ligand-PYR/PYL/RCAR-PP2C (Protein Phosphatases 2C) complex (Ben-Ari 2012; Kim *et al.* 2010). Formation of this complex leads to subsequent inhibition of the PP2C phosphatase activity (for review see Guo *et al.* 2011). Without ABA, PP2C inhibits SnRK2 ((Sucrose-non-fermentation 1)-related kinases subfamily 2) kinases activity (Ben-Ari 2012; Guo *et al.* 2011; Hubbard *et al.* 2010; Kim *et al.* 2010). Active SnRK2 members phosphorylate downstream target proteins (Hubbard *et al.* 2010) and generate ABA responses (Fig. 5). Several SnRK2 targets have been identified both at the plasma membrane and in the nucleus, resulting in the

control of ion channels (the SLAC1 (slow anion channel-associated 1) anion channel), secondary messenger production (NADPH oxidases, where NADPH is reduced form of $NADP^+$), and gene expression (ABF (ABA-response element binding factor) family proteins) (Hubbard *et al.* 2010; Kim *et al.* 2010).

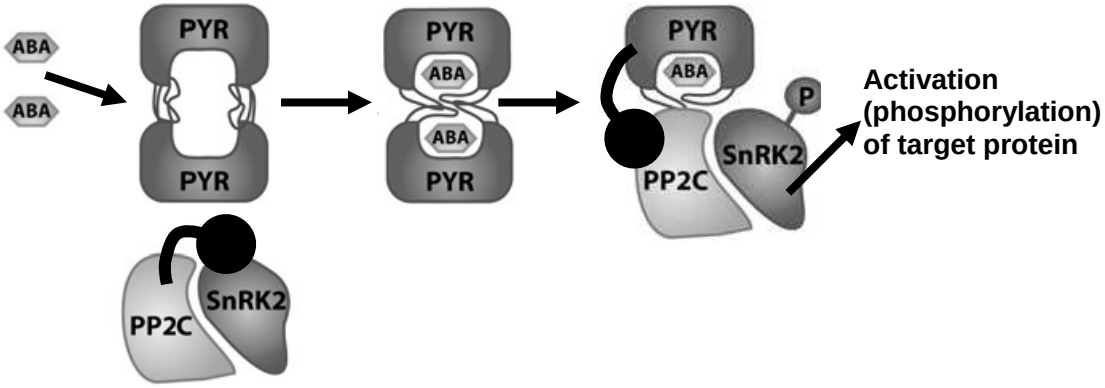

Fig. 5. Scheme of early ABA signal transduction. In the absence of ABA, PP2Cs inhibit protein kinase (SnRK2) activity (bottom left part of the figure). ABA is bound by intracellular PYR/PYL dimers (part of ABA receptor PYR/PYL/RCAR), which dissociate to form ABA ligand-PYR/PYL/RCAR-PP2C complexes. Complex formation inhibits the activity of the PP2C that allow activation of SnRK2s (right part of the figure). Activated SnRK2s phosphorylate target proteins (see text). Black curves terminated by black point indicate an inhibition. Adopted with slight modifications from Hubbard *et al.* (2010).

ABA-induced stomatal closing

Abscisic acid percepted by its receptor PYR/PYL/RCAR activates SnRK2 family members. Active SnRK2 members (e.g. OST1, open stomata 1) phosphorylate downstream target proteins (Hubbard *et al.* 2010), including NADPH oxidases, the SLAC1 anion channel and the ABF family proteins, and generate ABA responses (Kim *et al.* 2010).

Binding ABA to its receptor and subsequently activated OST1 directly interacts with and phosforylates the NADPH oxidases AtRBOHD and AtRBOHF that leads to an elevation of reactive oxygen species (ROS) in guard cell. ROS have been proposed to function as second messengers in ABA signaling in guard cells (Kwak *et al.* 2003). ROS (H_2O_2) activate I_{Ca} channels (hyperpolarization-activated Ca^{2+}-permeable channels) which transport Ca^{2+} from the extracellular space to the cytosol (Pei *et al.* 2000). Furthermore, $[Ca^{2+}]_{cyt}$ is elevated by release from the intracellular stores (Schroeder *et al.* 2001b). It is proposed that the Ca^{2+} channels in the membranes of intracellular compartments involved in ABA signaling can be activated by number of second messengers including ROS, NO, PA (phosphatidic acid), PIP3 (phosphatidyl-inositol-3-phosphate), IP3 (inositol-3-phosphate), IP6 (inositol-6-phosphate), and sphingolipids. However, the exact mechanism of early ABA signaling needs further research (Hetherington and Woodward 2003; Kim *et al.* 2010; Pospíšilová 2003). $[Ca^{2+}]_{cyt}$ elevations may be amplified by CICR (Ca^{2+}-induced Ca^{2+} release) from the vacuole. $[Ca^{2+}]_{cyt}$ increase activates the Ca^{2+}-dependent Slow Vacuolar (SV) channel (Schroeder *et al.* 2001b;

Ward *et al.* 1995). Oscillation of $[Ca^{2+}]_{cyt}$ is necessary for the guard cell closing (Schroeder *et al.* 2001a; Schroeder *et al.* 2001b).

$[Ca^{2+}]_{cyt}$ elevation activates Vacuolar K^+ (VK) channels, which are thought to mediate Ca^{2+} induced K^+ release from the vacuole. At resting $[Ca^{2+}]_{cyt}$, K^+ and anion efflux from the vacuole is mediated through Fast Vacuolar (FV) ion channel (Ward *et al.* 1995). $[Ca^{2+}]_{cyt}$ elevations also inhibit a phototropin mediated phosphorylation of the plasma membrane H^+-ATPase (Assmann and Shimazaki 1999; Zhang *et al.* 2007; Zhang *et al.* 2004) that transports H^+ out of the guard cell and inhibits activity of channel for K^+ uptake (K^+_{in}) (Schroeder *et al.* 2001b). By inhibiting these channels ABA suppresses BL-induced stomatal opening.

The increase of cytosolic $[Ca^{2+}]$ leads to activation of S-type (slow-activating sustained) and/or R-type (rapid transient) anion channels (Assmann 1993; Kim *et al.* 2010; Li *et al.* 2006; Schroeder *et al.* 2001a; Schroeder *et al.* 2001b) in the plasma membrane. Anion ($malate^{2-}$, Cl^-) efflux via anion channels causes membrane depolarization, which then activates K^+_{out} channel and leads to K^+ efflux (Kim *et al.* 2010; Schroeder *et al.* 2001a; Schroeder *et al.* 2001b). Anions must be released from the vacuole during the stomatal closure in response to ABA (Assmann and Shimazaki 1999). Furthermore, perception of ABA causes an alkalization of the guard cell cytosol. This alkalization enhances K^+_{out} channel activity (Schroeder *et al.* 2001a; Schroeder *et al.* 2001b). The long-term ion leakage linked with water efflux from the guard cells contributes to loss of guard cells turgor. This change leads to the stomatal closing (Assmann 1993; Li *et al.* 2006; Schroeder *et al.* 2001a). The scheme of ABA-induced stomatal closure is depicted in Fig. 6.

ABA may also cause cytoskeleton disruption in the guard cell by inactivation of the small GTPase protein AtRAC1. The function of AtRAC1 is to block actin cytoskeleton disruption (Lemichez *et al.* 2001). Thus, ABA contributes to guard cell actin cytoskeleton reorganization that is mediated by increased cytosolic Ca^{2+} levels (Hwang and Lee 2001). Besides, it is known that ABA inhibits PEP carboxylase and malate synthesis and induces malate breakdown in *Vicia faba* L. (Li *et al.* 2006). Finally, ABA can regulate the stomatal opening by influencing the expression of genes (e.g. Hetherington 2001).

ABA regulates photosynthesis

Since ABA regulates the stomatal aperture, CO_2 uptake into the leaf mesophyll by the stomata is regulated as well by ABA. For instance, ABA-induced decrease of transpiration rate (*E*) (caused by the stomatal closure) followed by decrease of assimilation rate (*A*) was observed after direct adding of ABA to the petioles of tomato leaves within 6 h (Herde *et al.* 1997). Slower (after 1 d) decrease of *E* and *A* was observed after the replacing the root medium of tobacco plants by ABA solution (Pospíšilová *et al.* 2009).

However, in some cases, stomatal closing (decrease of stomatal conductance g_s) is not a single reason for the decrease of *A* since intercellular CO_2 concentration (C_i) is unchanged (Hlaváčková *et al.* 2006). ABA causes e.g. changes in photosynthetic pigments content (Agarwal *et al.* 2005; Pospíšilová *et al.* 2009). The exogenous treatment of barley seedlings with ABA reduced the rate of photosynthetic CO_2 fixation, the carboxylase activity of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (also for the bean plants, Seemann and Sharkey 1987) and the carbonic anhydrase, increased the rate of

photorespiration and the CO_2 compensation point (Popova *et al.* 1996; Popova *et al.* 1987). Abscisic acid can also affect the expression of genes encoding small and large subunits of RuBisCO (*rbsS* and r*bsL*) or *psbA* gene encoding the D1 protein and *cab* genes encoding proteins of light harvesting pigment protein complexes (Bray 2002; Pospíšilová *et al.* 2009). Pea seedlings treated by ABA showed an inhibited PSII (photosystem II) activity. Abscisic acid treatment had direct effect on donor side of PSII, on oxygen evolving complex. Abscisic acid treatment led to increase of the number of $PSII_\beta$ centers (Maslenkova *et al.* 1995).

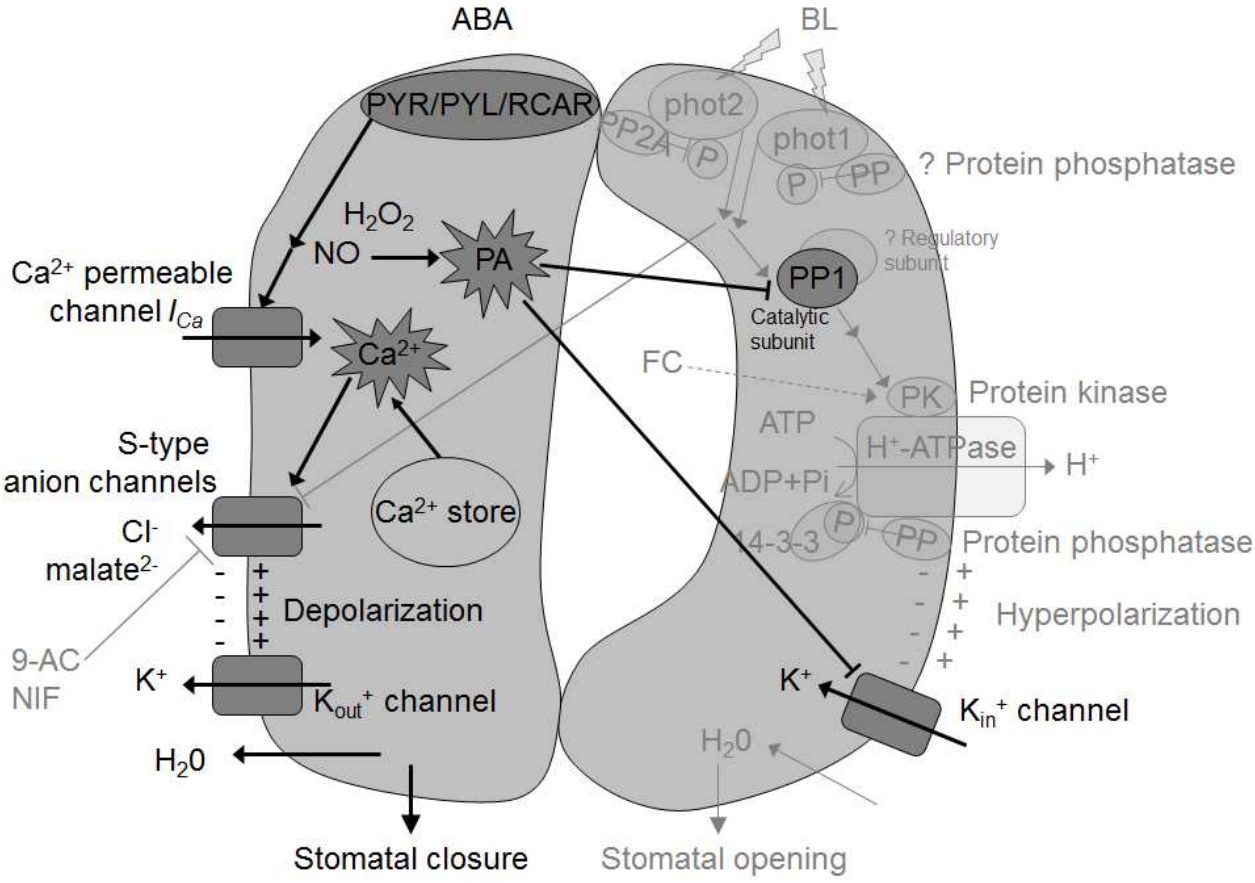

Fig. 6. Scheme of abscisic acid (ABA)-induced stomatal closure. The involved processes are indicated by black coloration of lines (arrows mean an activation and T-bars mean an inhibition) and descriptive text, involved components are of dark-grey coloration. ABA percepted by receptor PYR/PYL/RCAR activates Ca^{2+} permeable channel I_{Ca} that causes an accumulation of Ca^{2+} ions (star-shaped object) in cytosol. The Ca^{2+} activates S-type anion channels that cause anion efflux. Moreover, ABA perception leads to accumulation phosphatidic acid (PA) that causes an inhibition of catalytic subunit of PP1 and K_{in}^+channels both involved in BL-induced stomatal opening. The changes triggered by ABA perception lead to turgor loss of guard cells and guard cells are closing. The processes involved in the ABA-induced stomatal closure are described in more detail in chapter 2.2.1.1. The less visible components and lines (light-grey-colored) prevailing in the guard cell on the right side indicate crosstalk with processes triggered by BL perception (BL perception causes stomatal opening, see chapter 2.1.3.2 and Fig. 4). BL-induced stomatal opening can be inhibited or reversed by action of ABA. Adopted with slight modification from Inoue *et al.* (2010).

2.2.1.2 Jasmonates

Jasmonic acid (JA) and its derivates (Methyl esters JA (MeJA), glycosyl esters and amidelinked amino acid conjugates (JA-Ile, JA-Leu, JA-Val and JA-Phe)) known as jasmonates are lipid-derived phytohormones. Apart from their effect in plant growth and development (inhibition of growth and seed germination, promoting senescence, abscission, tuber formation and ripening; Pospíšilová *et al.* 2003), jasmonates are involved also in defense responses of plants to abiotic and biotic stresses. Jasmonates were, for instance, reported to accumulate in response to water stress (Creelman and Mullet 1995), UV-radiation (Mackerness et al 1999), ozone treatment and other abiotic stresses (Wasternack 2007).

JA is one of the main signaling molecules participating in response to herbivore attack (or other wounding) and to elicitors of wound responses such as oligogalacturonides and systemin. JA mediates wound signal transduction pathways locally and systemically (Wasternack 2007). For instance, the results of Herde *et al.* (1999) and Herde *et al.* (1996) showed that tomato plants accumulate JA locally and systemically in response to mechanical wounding, electrical current application and to local burning. Also Hlaváčková *et al.* 2006 observed fast JA accumulation (within an hour) after local burning in tobacco plants. In this case, JA (MeJA) induces formation of defense compounds such as proteinase inhibitors (PINs), toxic compounds such as nicotine and emission of volatiles which attract insect predators (de Bruxelles and Roberts 2001; Wasternack *et al.* 2006).

JA has been shown to be a specific signal in defense particularly to necrotrophic (kill host tissue and feed on the remains) pathogens in *A. thaliana* (Glazebrook 2005).

JA biosynthesis and transport

Jasmonic acid metabolic precursor OPDA (12-oxo-phytodienoic acid) is derived from linolenic acid (LA) released from chloroplast membranes via the octadecanoid pathway. It is believed that the initial steps of JA biosynthesis (i.e. from LA to OPDA) occur in stroma of the chloroplast. OPDA is transported from the chloroplast to peroxisome, where the enzymes for beta-oxidation producing JA are known to be located (Wasternack 2007; Wasternack *et al.* 2006). Subsequent metabolism of JA (described e.g. in Wasternack *et al.* 2006) can produce JA derivates. For additional information concerning JA biosynthesis see e.g. Wasternack (2007). Certain enzymes which participate on JA biosynthesis (AOC (allene oxide cyclase), AOS (allene oxide synthase), LOX (lipoxygenase)) could be localized in companion cells as well as in sieve elements. These compounds and also JA can move through plasmodesmata (Hause *et al.* 2003) and phloem (Wasternack *et al.* 2006). Volatile derivates of JA (e.g. methyl jasmonate) may be released and spread to distant sites also through air (Farmer and Ryan 1990).

It was reported that the accumulation of JA can be induced by a treatment of tomato leaves with polypeptide systemin (Peña-Cortés *et al.* 1995). Systemin is produced after local wounding from its precursor prosystemin and can be transported by phloem (de Bruxelles and Roberts 2001; Wasternack 2007; Wasternack *et al.* 2006). The following mechanism was proposed: systemin perception by its cell membrane located receptor SR160 (Scheer and Ryan 2002) activates JA biosynthetic enzymes such as AOC and leads to local rise in JA (Ryan 2000; Wasternack 2007). Since JA-dependent prosystemin expression and systemin-

dependent AOC expression are both located in vascular bundles, it has been suggested, that JA formation may be amplified (Narváez-Vásquez and Ryan 2004; Stenzel *et al.* 2003).

In addition to systemin, other compounds positively affect the JA biosynthesis and JA-induced gene expression in tomato (e.g. ABA (Herde *et al.* 1996), ethylene (O'Donnell *et al.* 1996), oligogalacturonides (Doares *et al.* 1995), H_2O_2 and fatty acid conjugates (Wasternack *et al.* 2006)). These compounds together with electric current play role in transmitting the wound signal to jasmonates (Seo *et al.* 1997).

JA receptor and signaling

Even JA-Val, JA-Leu and JA-Ala are bioactive JA-derivatives, JA-Ile is the most active derivative in causing the response (Acharya and Assmann 2009). Binding JA-Ile to the receptor COI1 (coronatine insensitive 1) (Yan *et al.* 2009) leads to an interaction of COI1 with multiple proteins which form the SCF^{COI1}E3 ubiquitin ligase complex. This complex provides JAZ1 (jasmonate ZIM-domain) proteins for degradation by the 26S proteasome. The degradation of JAZ1 (transcriptional repressors) promotes JA signaling (Acharya and Assmann 2009; Yan *et al.* 2009). Since MeJA does not promote COI1-JAZ1 interaction, MeJA may not be an active JA derivate. However, it is possible that MeJA can be metabolically converted to active form or it may interact with another member of JAZ family (Acharya and Assmann 2009).

JA induced stomatal closing

It was shown that JA/MeJA accumulates in plants under drought conditions (Creelman and Mullet 1995) and promotes stomatal closing (e.g. Munemasa *et al.* 2007). The capability of JA to close the stomata has been shown in several experiments. For instance, JA exogenously applied to growth medium of seedlings induced the stomatal closure in barley (Popova *et al.* 1988; Tsonev *et al.* 1998). Liu *et al.* (2002) applied JA exogenously to growth medium of seedlings of broad bean (*Vicia faba* L.) and observed the stomatal closure. A decline in *E* interpreted as the stomatal closure was observed after an adding JA to the petiole of tomato leaf (Herde *et al.* 1997). As in ABA dependent signaling pathway, production of ROS (via AtrbohD/F) and NO was observed in response to MeJA. Similarly to ABA, S-type anion channels, K^+_{out} channels, I_{Ca} channels activation and cytoplasmic alkalization were observed in *A. thaliana* guard cells in reaction to MeJA reception (Munemasa *et al.* 2007). Thus, the MeJA signaling pathway leading to the stomatal closure overlaps with ABA pathways, however, they differ in receptor parts (Acharya and Assmann 2009; Munemasa *et al.* 2007; Suhita *et al.* 2004).

Experiments performed with ABA-deficient tomato plants showed that physiological levels of ABA are required for the proper JA-mediated stomatal closure (Herde *et al.* 1997). It was reported that only very high (possibly toxic) MeJA concentrations in the transpiration stream cause the stomatal closure in barley (Horton 1991). These results indicate that JA is not the only compound which causes stomatal closure and the effect of jasmonates to stomata is limited.

JA regulates photosynthesis

Stomatal closure is not the only possibility how JA can regulate photosynthesis. It was reported that Me-JA inhibits PSII electron transport and O_2 evolving reactions in isolated thylakoid membranes of pea seedling (Maslenkova *et al.* 1995). Application of JA to barley seedling increases the rate of photorespiration and CO_2 compensation point (Popova *et al.* 1988). Similarly to ABA, JA inhibits biosynthesis and activity of enzymes involved in carbon fixation (RubisCO; Popova and Valkinova 1988; Rakwal and Komatsu 2001). JA action also leads to a change in thylakoid polypeptide patterns (Maslenkova *et al.* 1992). It was also reported that Me-JA reduced the chlorophyll content, chloroplast transcriptional activity and photosynthetic rate in *Cucurbita pepo* L. (Ananieva *et al.* 2007). Also the contents of chlorophyll a and b, as well as the photosynthetic rate and the carboxylase activity of RuBisCO in rice were decreased after JA methyl ester treatment (Wu and Pan 1998).

2.2.1.3 Cytokinins

Cytokinins are a group of phytohormones that are N^6 derivatives of the adenine with either isoprenoid or aromatic side chains (Frébort *et al.* 2011; Ha *et al.* 2012). Cytokinins with isoprenoid chain (e.g. isopentenyladenine (iP)-, trans-zeatin (tZ)-, cis-zeatin (cZ)- or dihydrozeatin-type derivatives) are widespread in higher plants. However, cytokinins with aromatic side chain, such as N^6-(meta-hydroxybenzyl) adenine, are found in plants at a lower abundance (Ha *et al.* 2012).

Cytokinins have been reported to promote cell division and root and shoot development. Cytokinins also delay leaf senescence, play a role in stress and pathogen responses. Moreover, they can serve as important signals for coordinating growth rates throughout the plant (Ha *et al.* 2012; Werner and Schmülling 2009).

Cytokinin biosynthesis and transport

Cytokinin precursor (iP nucleotide) is synthesized from adenosine diphosphate (ADP) or ATP and izoprenoid side chain donors by enzymes encoded by ATP/ADP isopentenyltransferase (IPT) genes *ipt*. Cytochrome P450 monooxygenases (CYP735A1 and CYP735A2 for *A. thaliana*) then catalyze hydroxylation of isopentenyladenine-type cytokinins. Cytokinin conversion from an inactive to an active (free basis) form is catalyzed by enzymes of LOG (LOnely Guy) family (El-Showk *et al.* 2013; Frébort *et al.* 2011). Cytokinin degradation is mediated by CKXs (cytokinin oxidases) (El-Showk *et al.* 2013; Frébort *et al.* 2011; Werner and Schmülling 2009). Genes for cytokinins biosynthesis and degradation are expressed and active in the shoot and the root tissues (El-Showk *et al.* 2013).

Members of PUP (purine permease) family of transporters (AtPUP1 and AtPUP2) were identified to have an affinity to cytokinins. AtPUP1 and AtPUP2 may also play a role in the loading and unloading of cytokinins for long-distance transport (Bürkle *et al.* 2003; Gillissen *et al.* 2000). Moreover, nucleoside-type cytokinins in *A. thaliana* can be transported by the members of ENT (equilibrative nucleoside transporter) family like SOI33/AtENT8 and AtENT3 (Sun *et al.* 2005). In *Oryza sativa* L., there was found that OsENT2 may play a role in long-distance transport of nucleosides in growing plants (Hirose *et al.* 2005). Hirose *et al.* (2008) hypothesizes that AtENT6 could also participate in the cytokinin nucleoside transport

of nucleoside-type (iP riboside) cytokinins in vascular tissues of *A. thaliana* (Hirose *et al.* 2008).

Cytokinins can be transported for longer distances via xylem and phloem, however, the way and the direction is dependent on cytokinin type (El-Showk et al 2013; Pospíšilová 2003).

Cytokinin receptor and signalling

Cytokinin signalling is mediated by phosphorelay systems (also called two component signaling systems). Histidine kinases (in case of *A. thaliana* named AHK2, AHK3, and AHK4/CRE1/WOL) have been identified as cytokinin receptors (El-Showk *et al.* 2013; Ha *et al.* 2012; Hwang *et al.* 2012). The function of all three receptors overlaps in shoot apical meristems and root cap columella. However, only two of them are specifically localized to the leaf parenchyma (AHK2 and AHK3), whereas the third one (AHK4) is localized to the root vasculature (Ha *et al.* 2012). Cited cytokinis receptor proteins are bounded to the plasmatic membrane as well as to the membrane of endoplasmatic reticulum (El-Showk *et al.* 2013).

When cytokinin is bound to the transmembrane CHASE (Cyclases/Histidine kinases Associated Sensory Extracellular) domain of histidine kinase, it induces conformational changes that trigger a phosphorelay (Ha *et al.* 2012, Hwang *et al.* 2012, Werner and Schmülling 2009). This comprises the transfer of phosphoryl group from a conserved His (H) to an Asp (D) within the receptor. The phosphoryl group is then transferred to five Arabidopsis histidine phosphotransferase proteins (AHP1-AHP5). Next member of this family AHP6 (pseudo-AHP) inhibits cytokinin signaling by competing with AHP1-AHP5 for phosphotransfer (El-Showk *et al.* 2013). The AHPs (AHP1-AHP5) continuously translocate between the cytosol and the nucleus. In the nucleus, AHPs phosphorylate aspartame of proteins named Arabidopsis response regulators (ARRs). Phosphorylation of the type A ARRs stabilizes them. The type A ARR is considered to act as inhibitors of cytokinin signaling, however, it was shown that ARR4 has upregulated phyB. The phosphorylation of type B ARR to nucleus leads to their bound to DNA which initiates transcription of cytokinin-responsive genes (including the type A ARRs) (El-Showk *et al.* 2013). The scheme of cytokinin signaling is depicted in Fig. 7. Recently, the cytokinis signaling is largely reviewed by Hwang *et al.* (2012).

Cytokins affect stomatal aperture and photosynthesis

Stomatal reaction to cytokinins can differ between plant species and depends on the concentration and cytokinin species. For instance, the stomatal opening of grass *Anthephora* was enhanced by both natural and synthetic cytokinins (Jewer and Incoll 1980). In *A. thaliana*, cytokinin 6-benzyladenine caused thr opening of stomata closed by ABA. Moreover, ABA-induced stomatal closure was inhibited in the cytokinin overexpressing *A. thaliana* mutant *amp1-1* (Tanaka *et al.* 2006). ABA-stimulated closure of maize stomata was reversed by zeatin and kinetin. The reversal of the ABA effect increased with the increasing cytokinin concentration. Both zeatin and kinetin applied to *Commelina communis* L. epidermis or leaf pieces at high concentration restricted the stomatal opening (Blackman and Davies 1983).

Mechanism of cytokinins induction of the guard cells opening is not fully clear yet, however, it has been shown that it can be related to decrease of H_2O_2 levels and NO levels within guard cells (She and Song 2006; Song *et al.* 2006).

Also the photosynthesis can be affected by cytokinin application. For example, it was showed that cytokinin application inhibits the photosynthesis in lettuce. This change can be similar to the changes observed during hypersensitive reaction caused by downy mildew infection (Prokopová *et al.* 2010). It is possible that photosynthetic genes are regulated by cytokinins as described e.g. by Zubo *et al.* (2009) for barley.

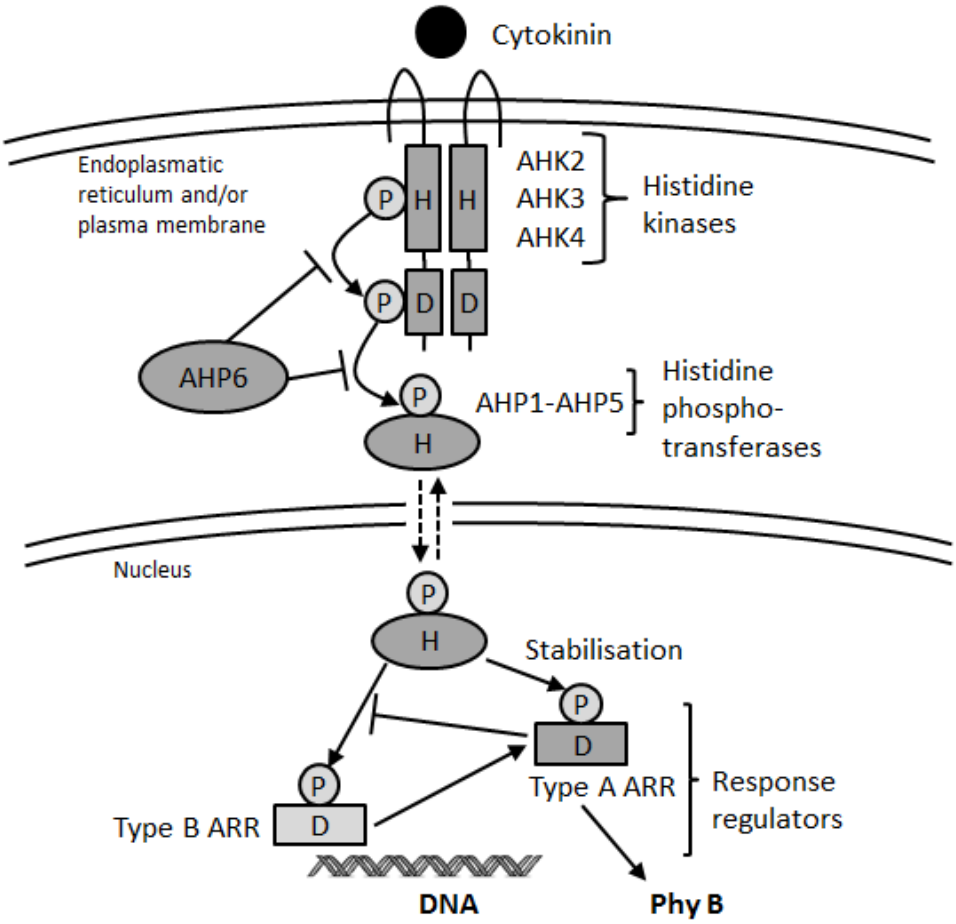

Fig. 7. Scheme of cytokinin signaling pathway. Cytokinin binds to the receptor *Arabidopsis* histidine kinases (AHKs). Interaction of cytokinin with AHK proteins induces conformational changes that trigger a phosphorelay (a phosphoryl group (P) is first transferred from conserved His (H) to Asp (D) residue within the receptor and is then relayed to *Arabidopsis* histidine phosphotransferase proteins (AHP1-AHP5)). AHP6 inhibits cytokinin signaling by competing with AHP1-5 for phosphotransfer. AHPs translocate to nucleus, where type A and B *Arabidopsis* response regulators (ARRs) are phosphorylated. The phosphorylated type B ARRs can bind DNA and initiate transcription of cytokinin-responsive genes. Type A ARR inhibits cytokinin signaling, although ARR4 can upregulate phy B. Adopted from El-Showk *et al.* (2013).

2.2.1.4 Salicylic acid

Salicylic acid (SA) is a phenolic compound that acts as a hormone as well as a stress signaling molecule in plants (Chen *et al.* 2009). As a plant hormone, it regulates e.g. seed germination,

plant growth, flower formation, seed production (Rivas-San Vicente and Plasencia 2011). Salicylic acid is also involved in defense responses of plants to abiotic and biotic stresses (Hao *et al.* 2011). SA mediates plant response to abiotic stresses such as drought, chilling, heavy metal tolerance, heat or osmotic stress (Drazic and Mihailovic 2005; Hao *et al.* 2011; Rivas-San Vicente and Plasencia 2011).

Endogenous SA accumulation upon pathogen attack can induce an expression of various defense-related genes or lead to the hypersensitive cell death response. In addition to the local reactions, SA activates systemic defense responses (activation of pathogenesis-related (PR) genes) in uninfected parts of the plant what enhance the resistance to subsequent infections. This effect known as the systemic acquired resistance is long-lasting and active against a broad range of pathogens, including viruses, bacteria, fungi, and oomycetes (Halim *et al.* 2006). However, wounding does not induce the accumulation of SA and SA-inducible PR proteins (Hlaváčková *et al.* 2006; Seo *et al.* 1997).

The possible SA biosynthetic pathways from its precursors cinnamate (produced from phenylalanine by phenylalanine ammonia lyase) and isochorismate are reviewed in Chen *et al.* (2009). The receptors of SA have been found recently by Fu *et al.* (2012). They showed that NPR1 paralogues NPR3 and NPR4 are SA receptors that bind SA with different affinities. NPR1 is transcription cofactor (nonexpresser of PR genes 1) required by the systemic acquired resistance in *A. thaliana*. The degradation of NPR1 acts as a molecular switch (Fu *et al.* 2012).

Salicylic acid was found to induce stomatal closing in *Vicia faba* L., lily or *Cucumis sativus* L. (Hao *et al.* 2011; Lu a Chen 2005; Mori *et al.* 2001) The results of Mori *et al.* (2001) suggest that ROS (Superoxide aniont) are generated in epidermal peels in response to SA. Hao *et al.* (2011) presented a hypothesis that stomatal closing is caused by a change in the balance of endogenous hormones (SA significantly increased endogenous ABA). Salicylic acid may regulate some other physiological processes, e.g. membrane permeability and photosynthesis (Arfan *et al.* 2007).

2.2.1.5 Reactive oxygen species

Reactive oxygen species is a group of molecules that are often produced by cells even under normal growth conditions. Intracellular ROS content is controlled by antioxidant system (by enzyme superoxide dismutase, catalase or peroxidase, and then by ascorbic acid, glutathione, tocopherols, carotenoids, anthocyanins etc.) (Kreslavski *et al.* 2012; Minibaeva and Gordon 2003). In the cases when ROS production is too rapid and strong to be eliminated by antioxidant system, an oxidative burst (a rapid and transient ROS production) occurs. The oxidative burst is one of the plant responses to both abiotic and biotic stress. It was reported that, for instance, stress conditions such as drought, wounding, salinity, extreme temperatures, excess light, heavy metals, xenobiotics, ultra-violet radiation, ozone, hypoxia, nutrient deficiency and pathogen attack can induce oxidative burst (Jaspers and Kangasjärvi 2010; Minibaeva and Gordon 2003; Torres *et al.* 2010). The rapid accumulation of ROS also precedes hypersensitive response (in which cells immediately surrounding the site infected by pathogen die rapidly what deprives the pathogen of nutrients and prevents its spread)

During stress, ROS can be produced in chloroplasts (photosynthetic electron transport chain), mitochondria (respiratory electron transport chain) or peroxisomes (reviewed e.g. in Kreslavski *et al.* 2012). Moreover, plasma membrane enzymes NADPH oxidase (Rboh), amino oxidases or cell wall peroxidases (POX) can contribute to ROS formation (Torres *et al.* 2010).

Reactive oxygen species are highly reactive molecules able to damage cellular components. ROS action is responsible for induction of processes such as lipid peroxidation, protein structural changes and inactivation, and DNA modifications (Kreaslavski *et al.* 2012; Torres *et al.* 2010). ROS also act as important cell signaling elements. ROS are involved in the regulation of gene expression (e.g. Gadjev *et al.* 2006, Miller *et al.* 2008). ROS can also activate MAPK (mitogen-activated protein kinase cascade). Crucial component in ROS signaling is Ca^{2+}. Ca^{2+} has been shown to activate both RbohC and RbohD which produce ROS. These ROS can activate I_{Ca} channels in plasma membrane (transport Ca^{2+} into cell) what increases cytosolic Ca^{2+} content (Jaspers and Kangasjärvi 2010). This Ca^{2+} accumulation is common step of several signaling pathways (e.g. ABA signaling) and was reported to regulate stomatal opening (among others).

2.2.2 Physical signals

2.2.2.1 Hydraulic signals

Hydraulic signals can be defined as self-propagating changes in water (fluid) pressure. These pressure changes quickly spread mainly through the fluid continuum in plant xylem (Malone 1993) and can participate on local or systemic response. Spreading of hydraulic signal observed after local wounding by heat or mechanical stimulation is manifested by changes of turgor pressure of cells, changes of a leaf thickness or of a diameter of stem (Malone 1992; Malone and Stanković 1991; Mancuso 1999). Malone (1993) pronounced the hypothesis that a localized wounding initiates hydraulic signals by destroying the cell membranes which released water (bounded in cell membranes) into the apoplast where it becomes available to the nearest xylem. The negative pressure in the xylem caused by transpiration draws the released sap what locally increases the xylem pressure. The xylem pressure change propagates basipetally and acropetally throughout the shoot as a hydraulic (pressure) signal (surge) (Malone 1993). The change in xylem pressure can be sensed by membrane-located mechano-sensitive channels or pumps of surrounding living cells and triggers electric potential response (e.g. action or variation potential, Malone and Stanković 1991; Mancuso 1999; Stanković *et al.* 1997). In reaction to incoming hydraulic signal, the change in turgor of epidermal cells can occur and this may results in hydropassive stomatal movement (Kaiser and Grams 2006).

Beside the hydraulic pressure surge, a mass flow ("hydraulic dispersal") from the wounded site is another component of the hydraulic signal. In plants, all cells of the shoot are initially in hydraulic equilibrium with their nearest xylem. Localized wound can lead to a sudden systemic increase of xylem pressure. All shoot cells will draw water from their local xylem to reach the new equilibrium. The water uptake by shoot cells will lead to the reduction of xylem pressure throughout the healthy tissue, which will further promote the entry of water from the wounded tissue into the xylem. This continues until all water released at the wound site is exhausted (Malone 1993). Shortly after wounding, all leaves still transpire. However,

the released fluid at the wounded site is more accessible source of water for the transpiration than the root medium. The transpiration from the healthy leaves will transiently tend to draw water also basipetally down the petiole of the wounded leaf, and possibly also down the stem towards the nodes of more basal leaves. Therefore, the flow can be transiently directed in both acropetal and basipetal direction in the stem and can carry fluid and solutes previously released to xylem after the wounding (Hlaváčková 2009; Malone 1994; Malone 1993). According to Malone (1993), the mass flow rate is 10mm s^{-1} and may last for several minutes. Elicitors carried by the mass flow can be sensed by ligand-modulated ion channels or receptors in the cell membranes and can participate in the systemic signaling after wounding (Malone 1994; Malone 1993).

2.2.2.2 Electric signals

Changing of environmental conditions or wounding leads to changes in the membrane potential of plant cells (e.g. Lautner *et al.* 2005; Malone and Stanković 1991; Mancuso 1999). In plants, authors distinguish several kinds of electrical signals: action potentials (APs), variation potentials (VPs), wound potential and systemic potential (Brenner *et al.* 2006; Mancuso 1999; Stahlberg *et al.* 2006; Zimmermann *et al.* 2009). While the wound potential is only local and stops a few millimeters from the wounded region (dying cells), APs, VPs or systemic potentials are often observed in long distances from wounded site (Brenner *et al.* 2006; Fromm and Lautner 2007; Trębacz *et al.* 2006; Zimmermann *et al.* 2009).

Action potentials

Plant APs are self-propagated signals mediated through the voltage-gated channels (Stanković *et al.* 1998) and evince similar properties to AP observed in animals. Along the plant body, spreading APs have a constant amplitude, a constant propagation velocity and a regular shape and fulfill an all-or-none law (Dziubińska *et al.* 2001; Stanković *et al.* 1998; Trębacz *et al.* 2006). APs are evoked by non-damaging stimuli as weak electrical or mechanical stimuli, light/dark transitions, pollination, re-irrigation or temperature shock (Fromm and Fei 1998; Stanković and Davies 1996; Stahlberg *et al.* 2006; Wildon *et al.* 1992).

Generation of AP in plants resemble to its generation in animals. The plasma membrane surface exposed to the cytosol of cells of higher plants is negatively electrically charged compared to the plasma membrane surface exposed to the extracellular medium. This forms the electric potential difference (the resting membrane potential, usually with values between -80 mV and -200 mV (Fromm and Lautner 2007).

A stimulus initiates passive Ca^{2+} ions influx into the cell. The Ca^{2+} ions accumulated in cytosol cause an opening of the Ca^{2+}-activated anion channels which allow the efflux of Cl^- ions. Cl^- ions leave the cell down their electrochemical gradient. This event reduces the membrane potential difference (a local depolarization of the membrane). When the stimulus is sufficiently strong to depolarize the membrane to a certain threshold, rapid and large membrane depolarization (AP) is triggered that propagates through the whole cell (Hlaváčková 2009; Fromm and Lautner 2007; Trębacz *et al.* 2006; Zimmermann and Felle 2009).

The results of Vodeneev *et al.* (2006) provide evidence that the depolarization phase of AP is also associated with Ca^{2+}-induced temporary suppression of plasma membrane H^+-ATPase. The duration of refractory period (the time during which the membrane can not be excited) after the generation of AP limits the generation of subsequent AP (Dziubińska *et al.* 2001; Fromm and Lautner 2007; Trębacz *et al.* 2006). When Cl^- channels begin to close and voltage-gated K^+ channels (allowing passive K^+ efflux from the cytosol) begin to open, the repolarization of the membrane occurs (Trębacz *et al.* 2006). Simultaneously, Ca^{2+} ions in cytoplasm evoke an opening of anion channels located in the tonoplast and Cl^- is transported from the vacuole into the cytoplasm. Finally, Ca^{2+} pumps in the plasmatic membrane translocate the cytosolic Ca^{2+} ions back to the apoplast. Thus, the positive charge in the cytosol is decreased and the membrane potential difference is restored to its initial value (Hlaváčková 2009).

At the local level (short distances), the fast transmition of APs is mediated by plasmodesmata which ensure the connection between cells (Fromm and Lautner 2007, Trębacz *et al.* 2006). This constitutes a network able to transmit APs in different directions (Trębacz *et al.* 2006). On the long distances, APs spread via the phloem sieve tubes (Fromm and Lautner 2007; Mancuso 1999), phloem parenchyma or protoxylem (Lautner *et al.* 2005; Mancuso 1999; Rhodes *et al.* 1996; Trębacz *et al.* 2006) i.e. through the living cells (Mancuso 1999). Electrical signals can leave the phloem pathway at any site via plasmodesmata to induce particular physiological responses in the neighboring tissue (Hlaváčková 2009). The speed of APs propagation depends on the species of plant. For example in *Vitis vinifera*, the speed was reported to be about 10 $cm \cdot s^{-1}$ (Mancuso 1999).

Variation potentials (VPs)

Variation (or slow wave) potentials appear in higher plants in response to strong and damaging stimuli such as wounding (e.g. organ excision (Fromm and Lautner 2007; Stahlberg and Cosgrove 1992) or crushing (Malone and Stanković 1991)), localized increase in xylem pressure (e.g. Malone and Stanković 1991; Stahlberg and Cosgrove 1997a). The stimulus widely used to generate VP is a local burning of plant tissue with flame (e.g. Dziubińska *et al.* 2001; Hlaváčková *et al.* 2006; Malone and Stanković 1991; Stankovic *et al.*1998; Wildon *et al.* 1992). In contrary to AP, VP varies with the intensity of the stimulus (From and Lautner 2007). The amplitude and propagation velocities of VPs decrease with increasing distance from the wounded site (Dziubińska *et al.* 2001; Davies *et al.* 1997; Stanković *et al.* 1998). VPs do not follow an all-or-nothing law. VPs share with APs a refractory period (much longer compared to AP, Brenner *et al.* 2006; Stahlberg and Cosgrove 1996).

Two hypotheses were proposed for the mechanism of VP propagation. Hydraulic hypothesis says that the local injury causes an increase in the xylem hydraulic pressure (spreading of hydraulic wave). The chemical hypothesis takes into account the diffusion of some wound-released substance (Ricca factor) from the injured site along the xylem. The hydraulic wave or Ricca factor spreads along the xylem and locally activates mechanosensitive or ligand-operated Ca^{2+} channels. Subsequent Ca^{2+} influx leads to Ca^{2+}-dependent activation of Cl^- channels and inactivation of the H^+-ATPase (Vodeneev *et al.* 2011). These changes lead to membrane depolarization. Therefore, VPs are local

consequences of a hydraulic or chemical signal spreading. The propagation rate of VP ranges from 0.1 to 10 $mm \cdot s^{-1}$ (Stanković *et al.* 1997).

In some cases, APs were registered on a background of VP (e.g. Hlaváčková 2009; Stahlberg and Cosgrove 1997b; Stanković *et al.* 1998). This AP has been probably caused by depolarization during VP. In contrary, only depolarization has never been reported to cause a VP, which suggesting that APs are unable to trigger VPs.

System potential

Along AP and VP, a new type of systemic electric activity has been recently introduced by Zimmermann *et al.* 2009. Addition of a variety of cations to leaves of *Vicia faba* L. and *Hordeum vulgare* that had been before injured by cutting caused voltage transients at the systemic leaf. Electric changes have been detected by an electrode inserted to substomatal cavities of open stomata. These voltage transients are characterized by initial hyperpolarization, they do not follow the all-or-none rule and depend on the concentration and type of added substance. Their propagation velocity is 5 to 10 cm min^{-1}. Therefore they differ from AP and VP. Systemic potentials are most likely generated and transmitted by H^+ pump activation.

Reactions to electric signal

In reactions to electric signal, gene expression, photosynthetic changes and hormones content change have been observed. For instance, Wildon *et al.* (1992) observed systemic pin2 gene expression after mechanical wounding and localized burning that generate electric signals. Moreover, electric-current application and localized burning (inducing VP) led to the accumulation of pin2 mRNA in potato and tomato plants (Herde *et al.* 1996).

The suggestion that a signal of electric origin could affect photosynthesis is supported by an observation that a local heat stimulation of *Mimosa pudica* L. plants that caused changes in membrane potential is followed by a transient decline in PSII quantum yield, CO_2 uptake rate and g_s (Koziolek *et al.* 2004; Kaiser and Grams 2006). Then, a systemic decline of gas exchange (GE) parameters was observed in tobacco plants after local burning that caused surface potential changes (Hlaváčková *et al.* 2006). Furthermore, electric changes generated by changing the root medium led to various responses in the parameters *A* and *E* in willow leaves (Fromm and Eschrich 1993). Also direct electric stimulation caused transient local and systemic responses in GE in tomato plants (Herde *et al.* 1995).

In addition, it has been described that hormone content can change in reaction to electric signal. For example, Peña-Cortés *et al.* (1995) pointed out the possibility that an electric signal spreading in tomato plants increased the systemic ABA content. Indirect indication that JA and ABA can accumulate in reaction to electric signals was showed by Herde *et al.* (1999) and Herde *et al.* (1996). They measured ABA and JA content in tomato plants 6 h after electrical (mechanical) stimulation (producing action potential) and local burning (producing variation potential), and they detected an accumulation of these hormones. A faster systemic accumulation of endogenous ABA and JA (detected during 1 h after local burning) in tobacco plants preceded by changes in SEP was observed by Hlaváčková *et al.* (2006) after the local burning.

3. Materials and methods

3.1 Light signaling

3.1.1 Chloroplast movement

3.1.1.1 Growth condition and plant material

Tomato spontaneous mutant *7B-1* and corresponding wild type (WT, *Solanum lycopersici* L. cv. Rutgers; Sawhney 1997; Fellner *et al.* 2001) were used for the chloroplast movement measurement. Plants were grown in a greenhouse in pots (80 × 80 × 70 mm, one seed per pot, 10 mm deep) filled by a soil (Potgrond H, Klasmann Deilmann GmbH, Geeste, Germany) and watered daily. To maintain a 16 h photoperiod, high-pressure sodium lamps PlantaStar E40/ES 400 W (Osram GmbH, Germany) were used as a light source additionally to sunlight. A temperature was maintained between 15 °C and 27 °C. Three-week-old plants were transferred to a growth chamber (SGC.170.PFX.J, Weiss–Gallenkamp Ltd., Loughborough, England). The regime in growth chamber consisted of 8 h dark (temperature 18 °C, relative air humidity 55 %), 14 h light (temperature 25 °C, relative air humidity 50 %, 100 μmol photons of photosynthetically active radiation (PAR) m^{-2} s^{-1}) with 1 h of linear light rise (sun rise) and 1 h light decrease (sun set). The plants were watered daily. The measurements were performed on 8 weeks old plants (4^{th} and 9^{th} leaf computed from the plant base; used notation is $L4^{th}$ and $L9^{th}$) and 10 weeks old plants ($L10^{th}$, the plants were before or in the flowering phase, but it has been supposed that the flowering phase does not affect the chloroplasts movement). The plants were taken out of growth chamber 30 min before measurement and transferred to a dark place in the laboratory to achieve the face (diastrophe) initial position of chloroplasts in cells (along cell walls perpendicular to the incident light, Frolec *et al.* 2010; Nauš *et al.* 2010).

3.1.1.2 Chlorophyll content (paper I)

The chlorophyll content (Chl(a+b)) was estimated by a SPAD-502 (nondestructive chlorophyll meter, Konica Minolta Sensing, Osaka, Japan), on the leaves selected for the measurement, see above. The measurement of SPAD values was performed after 30 min of dark adaptation of plants before the beginning of the chloroplast movement measurement (when chloroplasts were in diastrophe position). Five readings were registered on different places of given leaf and average value was computed.

The obtained SPAD values were used to read the values of Chl(a+b) from a calibration curve determined by analytical measurement. The calibration curve for SPAD values has been measured for tomato leaves of cultivar other than was used in our experiments, however, it is supposed that the calibration curve for cv. Rutgers is very similar. The following procedure has been executed: discs (14 mm in diameter) have been cut off from the leaf blade at the site used for the SPAD values measurement. The discs were frozen in liquid nitrogen, homogenized in 80 % acetone with a small amount of $MgCO_3$ and centrifuged (3600g for 5 min). The Chl(a+b) in the supernatant was determined (by Dr. Špundová) spectrophotometrically (Unicam UV550 Thermo Spectronic, Cambridge, UK) with spectral slit width of 1 nm according to Lichtenthaler (1987). SPAD reading linearly correlated

(r^2=0.955, p<0.0001) with leaf Chl(a+b). It holds: Chl(a+b) [μg cm^{-2}] = 5.48 + 1.02 × SPAD value.

The determination of Chl(a+b) by SPAD-502 can be precise enough when measuring green leaves (this was the case in above mentioned description of Chl(a+b) measuring). However, in some cases, especially when the measured leaves are of blue-violet-green color, the reading of SPAD-502 can be significantly affected by the presence of blue-violet pigments – anthocyanins. This can be caused by overlapping absorption spectra of chlorophyll and anthocyanins *in vivo.* The theoretical evaluation of the problem as well as the way how to correct the SPAD values for anthocyanins contribution has been described in detail in the paper I. The proposed correction for anthocyanins contribution can be used for chlorophyllmeters based on measurement of leaf transmittance on wavelength around 650 nm. Furthermore, the paper I describes how to correct the values of chlorophyllmeters based on measurement the leaf reflectance around 650 nm (e.g. these ones computing NDVI value) for anthocyanins contribution.

3.1.1.3 Chloroplast movement

The sample (first right leaflet (counted from terminal leaflet) of attached leaves) was fixed in a soft clip with an aperture (Nauš *et al.* 2008). The movement of chloroplasts from the face to the side (parastrophe) position (along cell walls parallel to the incident light, Frolec *et al.* 2010; Nauš *et al.* 2010) was induced by BL. The combination of the source of cold WL Schott KL 2500 (Schott Glas, Mainz, Germany) with the light piping (15 mm diameter) and the glass blue filter (Schott BG 12, thickness 1.5 mm) was used as a source of BL. The leaf area (4.5 mm in diameter) was illuminated from adaxial side for 30 min with BL of high intensity (340 ± 20 μmol photons m^{-2} s^{-1}) for the induction of avoidance movement and for following 45 min by BL of low intensity (8 ± 2 μmol photons m^{-2} s^{-1}) for the accumulation movement induction. The irradiances (PAR) were measured by a quantum radiometer LI-189 (LI-COR, Lincoln, NE, USA). The chloroplast movement was detected as changes of leaf transmittance (e.g. Gabryś *et al.* 1981).

3.1.1.4 Leaf transmittance

The partly collimated leaf transmittance (T_C) was measured according Frolec *et al.* (2010) and Nauš *et al.* (2008). The light transmitted by the leaf was conducted by a light pipe (diameter 3.5 mm) to the spectroradiometer LI-1800 (LI-COR, Lincoln, NE, USA). The distance between leaf and light pipe was 8 mm. The intensity of transmitted light was detected at 436 nm (absorption maximum of chlorophyll a) every 30 s. Finally, to obtain the value of T_C, the intensity of light transmitted through the leaf was divided by the intensity of light detected in the same arrangement but without the leaf sample.

Besides T_C at single wavelength (436 nm), the spectra of T_C were detected in the same arrangement. The spectra were measured before the beginning of chloroplast movement (chloroplasts were in face position, designation A), then 30 min later (chloroplasts in side position, designation B) and in the end of measurement (after 75 min, chloroplasts in face position, designation C). The WL (500 μmol photons m^{-2} s^{-1}, the source of cold WL, see above) illumination of sample was used for measuring the spectra of T_C. A typical curve of

chloroplast movement with marked meaning of used parameters, times of spectra measurement and light intensities is depicted on Fig. 8. The interval for spectra of T_C detection was restricted to 400-800 nm, because the signal in shorter and longer wavelengths was distorted by significant noise. To obtain the spectra T_C, the signal measured with inserted leaf was divided by the signal detected in the same arrangement without leaf.

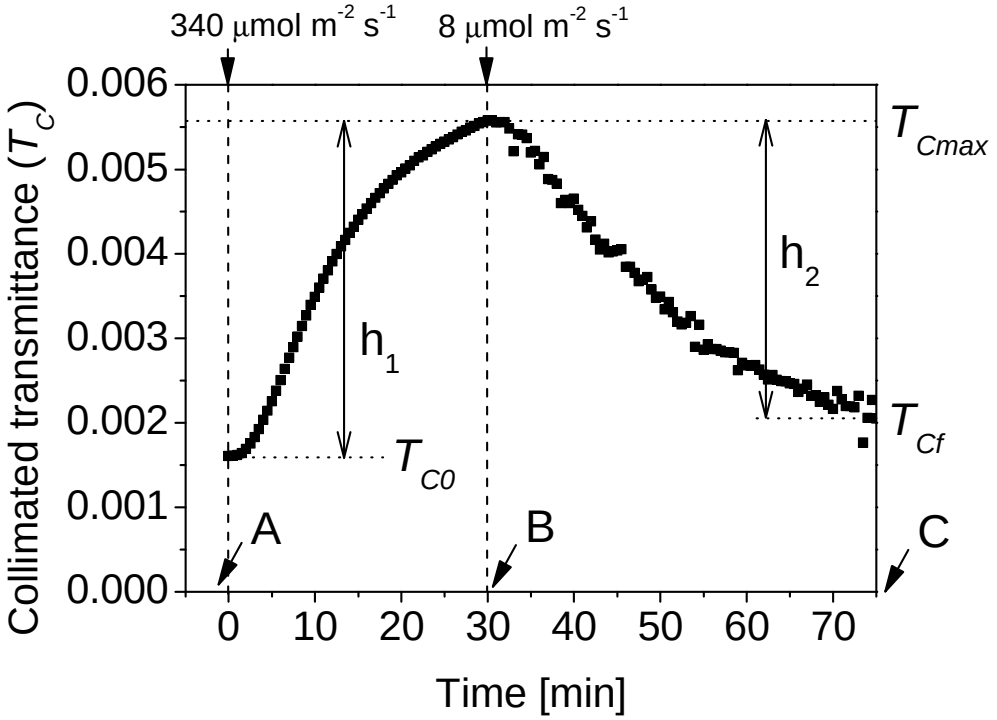

Fig. 8. Partly collimated transmittance (T_C) changes corresponding to avoidance movement (on blue light of high intensity (340 ± 20 μmol photons m^{-2} s^{-1}); 0 - 30 min) and accumulation movement (on blue light of low intensity (8 ± 2 μmol photons m^{-2} s^{-1}); 30 – 75 min) of chloroplasts in leaves. The spectra of partly collimated transmittance were measured before avoidance movement (A), after avoidance movement (B) and after accumulation movement (C). The initial (maximal and terminal, respectively) value of partly collimated transmittance T_{C0} (T_{Cmax} and T_{Cf}, respectively) and extent of avoidance (accumulation, respectively) movement h_1 (h_2, respectively) are indicated.

3.1.1.5 Statistical analysis

The statistical differences were tested using *t*-test or Mann–Whitney rank sum test depending on the statistical properties of the data. Mann–Whitney rank sum test was used when the data did not have normal distributions or did not have the same variances. *P*-value of the applied test was compared with the critical value, which was chosen as 0.05. There was statistically significant difference between data if $P < 0.05$. Statistical software SigmaStat (Systat, Chicago, USA) version 3.0 was used for the testing.

3.1.2 Stomatal movements

3.1.2.1 Plant material

To cultivate a plant material for measurement of the stomatal movement, the seeds of spontaneous mutant *7B-1* in tomato (*Solanum lycopersicum* L.) and its corresponding WT (cv. Rutgers) (Sawhney 1997; Fellner *et al.* 2001) were sown into soil 10 mm deep. The soil (pH (H_20) 5.5-6.5) was composed of weakly spread bright peat (H_2-H_5) and of deeply chilled dark peat (H_6-H_8). Enriched with fertilizer NPK 14:16:18, the soil was then mixed with rough sand. The dimensions of used pots were 0.1 m in height and 0.06 m in width. The pots with the seeds were placed in a home-made growth chamber and were watered once with a nutrient solution containing 0.8 mM $Ca(NO_3)_2$, 2 mM KNO_3, 60 μM K_2HPO_4, 695 μM KH_2PO_4, 1.1 μM $MgSO_4$, 20 μM $FeSO_4$, 20 μM Na_2EDTA, 74 nM $(NH_4)_6Mo_7O_{24}$, 3.6 μM $MnSO_4$, 3 μM $ZnSO_4$, 9.25 μM H_3BO_3, 785 nM $CuSO_4$ (Herbette *et al.* 2006). Only water was used for subsequent watering of growing seedlings. The controlled conditions in the growth chamber for plant cultivation include relative air humidity 70 %, temperature 22 °C/20 °C during day/night (8/16 h). Plants in growth chamber were illuminated by WL (200 μmol photons of PAR m^{-2} s^{-1}) coming from incandescent light source. The light irradiance was measured with quantum radiometer Li-Cor 185A (LI-COR, Lincoln, NE, USA). At the end of the night period, leaflets of first fully developed leaves of 3 to 5 weeks-old plants were harvested and were used for experiments.

3.1.2.2 Epidermal strip experiments

Samples for epidermal strip experiments were prepared as follows: the major veins were separated from a harvested leaflet and the rest of the leaflet was cut to small pieces (about 5 x 5 mm). Two randomly selected pieces were glued to a microscopic cover glass coated with a layer of low viscosity glue (Telesis 5, Pacoima, California). The pieces were facing the cover glass by the abaxial side. The upper cell layers were peeled off with an edge of a microscopic glass, so the abaxial epidermal cells only with viable stomata remained on the cover glass. On such prepared samples, the stomata were still able to move due to low viscosity of the glue. The cover glass with epidermal strips were floated by “adaxial” side up in Petri dishes containing 5 ml of the incubation solution (50 mM KCl with 10 mM MES, pH 6.0 (TRIS)). The samples in Petri dishes were incubated in darkness at 24 °C for 30 min to standardize the initial state.

In order to study stomatal opening induced by light, the Petri dishes with samples were placed to a box (ca. 0.125 m^3) illuminated by WL (300 μmol photons m^{-2} s^{-1}, incandescent light) and incubated at 24 °C. Placing the Petri dishes with samples on the surface of flowing water reduces a thermal effect of illumination on samples (warming). Covering the Petri dishes with color Supergel filters (Rosco Laboratories, Stamford, Connecticut, USA) was the way how the samples were illuminated by BL and RL. Blue filter no. 65 was used to provide BL of 60 μmol photons m^{-2} s^{-1}, and red filter no. 26 was selected to provide RL of 50 μmol photons m^{-2} s^{-1}. To investigate responses of dark-adapted stomata to FC, anion channels blockers 9-AC and NIF, the effectors were added to the Petri dishes with the samples before the illumination and the samples were incubated for 3 h. To investigate the effects on light-adapted stomata, the effectors (9-AC, NIF or ABA [(±)-cis, trans-ABA]) were added to the

samples 3 h after beginning of the illumination. The samples were then placed under the same light for subsequent 2.5 h. The concentration of the anion channels blockers and FC were 100 μM and 10 μM, respectively. ABA concentration sufficient to induce full stomatal closure was 1 μM. The dark-adapted samples were kept in the dark during all experimental time and stomatal aperture was measured at the end of experiment.

The microscopic cover glass with samples was pulled out from the Petri dish and placed on a microscopic slide resulting into a sample together with a drop of the incubating solution between cover glass and the slide. An optical microscope (Nikon, Tokyo, Japan) for stomatal aperture measurement was fitted with a camera lucida and a digitizing table Calcomp Drawing slate II (Houston Instrument, Austin, TX, USA) connected with a personal computer as described in (Leonhardt *et al.* 1997). In microscope, the sample was illuminated by very weak WL. During about 5 min, sixty stomatal apertures were measured on each sample and each condition. The figures show the mean and error bars (corresponding to a standard error (SE) calculated from several independent measurements). Number of independent measurement is stated in the figure legends. The statistical differences were tested according the same procedure as described above (see part 3.1.1.5).

Stomatal density (defined as number of stomata per mm^2) and stomatal index [defined as number of stomata / (number of stomata + number of epidermal cells)] was computed from microscopic pictures taken with the same microscopic objective magnification, the dimensions of pictures were estimated using the microscopic measure.

3.2 Abiotic stress signaling

3.2.1 Plant material, growth conditions

To explore systemic response to local burning, tomato WT plants (*Solanum lycopersicum* L. cv. Moneymaker) and ABA-deficient SIT mutant (Tal *et al.* 1966; Taylor *et al.* 1988) were cultivated. The seeds were inserted into a pot filled with seed soil substrate. Peat with dolomitic limestone (pH = 5.5-6.5, 80-100 μg N/l, 60-100 μg P_2O_5 /l, 100-150 μg K_2O/l; AGRO CS, Česká Skalice, Czech Republic) has been used as the soil substrate. The pot with sown seed has been covered with transparent plastic film and was placed in a growth chamber (Microclima 1750, Snijders Scientific, Tilburg, Netherlands). The condition in growth chamber includes temperature 25 °C, relative air humidity 50 % and 16 h light (130 μmol photons of PAR m^{-2} s^{-1})/8 h dark cycles. The soil in pot was watered once per two days with about 250 ml of water per the pot. The seeds grew in the pot for 14 d and after the seedlings were repotted to the pot filled with common soil substrate (pH = 5.5-7.0, Florcom SZ, BB Com, Letohrad, Czech Republic). The tomato plants were cultivated in the growth chamber in conditions mentioned above. The plants were fertilized once a week with about 250 ml of 1 g/liter Kristalon START fertilizer (NPK (19-6-20)+3 % MgO+7.5 % SO_3+ME; AGRO CS, Česká Skalice, Czech Republic) per pot. WT plants old 36-39 d and SIT plants old 43-46 d (the growth of SIT plants was slower compared to WT) were used for measurement of GE, detection of surface electric potentials (SEP) and for hormones quantification. All used plants were in the period before branching of a main stem. The measurements were performed on

26–35 cm tall WT or SIT plants with five to six fully developed leaves. Every leaf consisted of five main leaflets and of zero to four smaller leaflets.

Abscisic acid pre-treated SIT plants (SITA) were prepared as follows: isolated SIT mutants were watered with the 70 ml of 10 μM ABA solution (ABA dissolved in ethanol and then added to water in 1 volume percent concentration of ethanol) 24 h before the beginning of measurement. The beginning of measurement was defined as switching on the data logging of GE parameters, see below. The cited concentration of the ABA solution was expected to be sufficient to induce partial stomata closure (Herde *et al.* 1997). Actually, three different concentrations (10, 50 and 80 μM) of the ABA solution were tested. The result of the test was that 10 μM ABA was sufficient to stimulate a decrease of GE parameters of SIT mutants to levels similar to those of WT plants. To include the effect of ethanol on tomato plants, one day (24 h) before the beginning of measurement, isolated WT or SIT plants were watered with 70 ml of 1 % water solution of ethanol. One hour before the beginning of measurement, 250 ml of the tap water was added to the plate under the pot to prevent a desiccation during experiment.

3.2.2 Gas exchange and heat stimulation

A dark room with temperature about 25 °C served as a place with stable light conditions for measurement of GE parameters and simultaneously for SEP measurement. The parameters g_s, E, A and C_i were measured with a LI-6400 open gas exchange system (LI-COR, Lincoln, NE, USA). The measurement was performed on the attached untreated leaves (terminal leaflet of the second leaf counted from apex of the plant; used notation is L2; Fig. 9).

Before the beginning of measurement, the plants were dark-adapted for 15 min. Leaflets to be measured were inserted into leaf chamber of LI-6400 and equilibrated (about 5 min before beginning of measurement) there under standard conditions including the constant leaf temperature 25 °C, CO_2 concentration 380 μmol mol^{-1}, and constant relative air humidity 50 %. Data logging of GE parameters with 30 s interval begun 5 min before the measuring light (LED light source 6400-02B (LI-COR, Lincoln, NE, USA) providing RL (665 nm) and BL (470 nm); 320 μmol photons of PAR m^{-2} s^{-1}) was switched on. Since the measuring light and leaf chamber form a compact unit, only measured leaves (terminal leaflets of L2) were illuminated by the measuring light. When all measured parameters reached steady-state values (65 min after the beginning of the measurement and 60 min after the measuring light was switched on), the tip of the terminal leaflet of the youngest fully developed leaf (L1; Fig. 9A) of the plant (except of the control ones) was burned by a flame. The flame with temperature of about 430 °C (measured with a precision thermometer TM-917, Lutron electronic, Taipei, Taiwan) has been provided by a burning wooden skewer of 30.5 cm length and Ø 3 mm. The wooden skewer was moved back and forth below the tip of leaflet for 12 s. The GE measurement then continued for additional 65 min after the local burning. The light was switched off 60 min after burning.

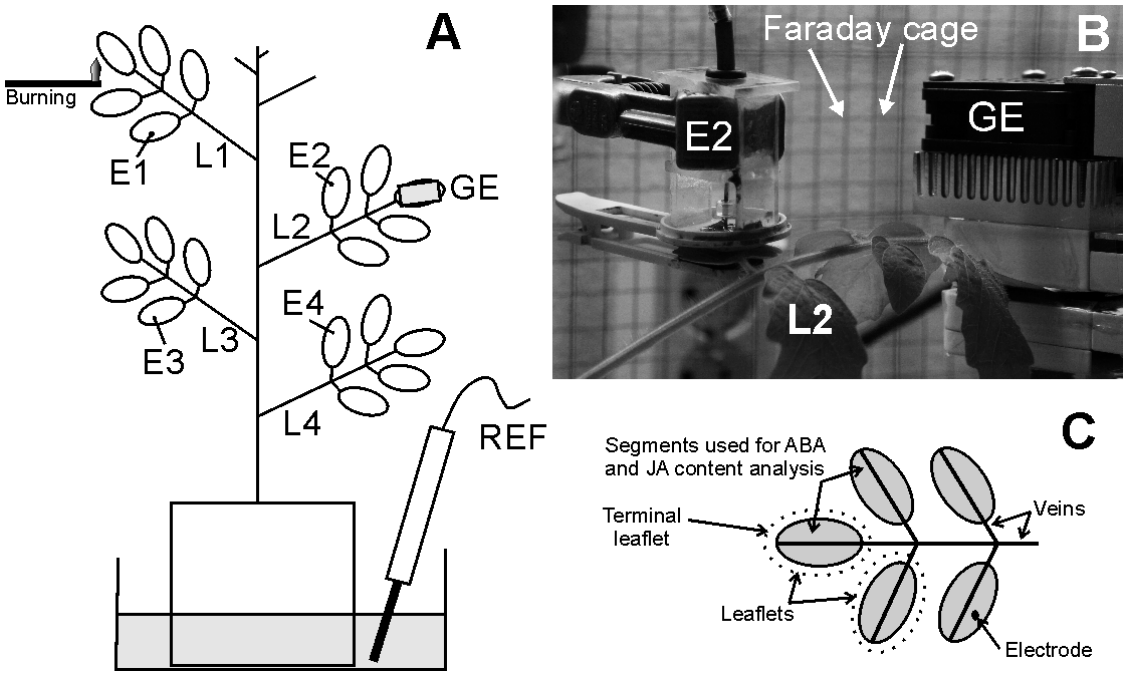

Fig. 9. Scheme of the experimental setup (part A). A tip of the terminal leaflet of the leaf L1 was burned. The electrodes (E1-E4) for the detection of surface electric potential were fixed on the left side of the second left leaflet of L1-L4. The reference electrode (REF) was placed in the root medium (water). Gas exchange (GE) parameters were measured on the terminal leaflet of L2. A picture of L2 with fixed electrode E2 and gas exchange leaf chamber (part B). Scheme of one leaf (part C) with marked placing of the electrode (black spot) and segments (grey color) used for analysis of endogenous ABA and JA content.

3.2.3 Surface electric potential measurements

Four-channel home-made device described in details in (Hlaváčková *et al.* 2006; Ilík *et al.* 2010) was used to record the SEP. The detection of the SEP has been ensured by four Ag/AgCl surface electrodes (Scanlab Systems, Prague, Czech Republic) placed to leaves L1-L4 (Fig. 9A). The electrodes were placed on left adaxial side of the second leaflet (from the terminal one) on the left side of the main veins (viewed from the adaxial leaf side; Fig. 9C). Fixation of electrodes to leaflet was ensured by a plastic clip. Drop of a high-conductive gel (SONO gel VITA, HELLADA, Prague, Czech Republic) improve the connection between the electrode and leaf surface.

Next (fifth) Ag/AgCl electrode inserted into a glass tube tipped by a glass frit and filled with 0.3 M KCl (the tip of electrode was dipped in the KCl solution) serves as reference electrode (Ilík *et al.* 2010). The tip of glass tube of reference electrode was immersed into water in the basin below the pot with the measured plant (Fig. 9A). The reference electrode was grounded, thus the single-ended setup was used. The contact between electrodes and leaflet has been left to reach steady-state levels for an hour before burning. Signal detected by electrodes was converted to digital form by A/D convertor and displayed on computer screen. Two minutes before burning a new measuring procedure was initiated and a signal was shifted to zero. The SEP measurements continued for following 63 min after the burning. All measurements (including GE measurement) were performed inside a Faraday cage.

3.2.4 Quantification of endogenous ABA and JA in leaves

The leaves previously used for GE and SEP measurements were cut down. Since vascular bundles might be potential site of ABA synthesis (Koiwai *et al.* 2004) (what could distort the results of ABA content determination), the veins and petioles of the leaves were removed (Fig. 9C). Resulting leaf segments were weighted, then packed to an aluminum foil and finally sunk into liquid nitrogen (77.3 K) for fast freezing. All samples were kept in refrigerator at -84 °C till the next processing (including purification of samples and quantification of endogenous ABA, JA content on ultra performance liquid chromatography - mass spectrometry; the process of hormones quantification was performed by co-author(s) of the paper III). The whole process has been described previously (Bergougnoux *et al.* 2009; Hlaváčková *et al.* 2006). Results reflect hormone content 85 min after burning (control plants were unburned).

3.2.5 Computed parameters and statistical analysis

Initial steady-state values of GE parameters were computed as the arithmetic mean of values from last 5 min before the burning. The start of systemic reaction of GE parameters after burning was defined as 5 % decrease from the initial steady-state level. The start of local and systemic reaction of SEP after burning was defined as 10 % increase from the resting potential level. The differences between measured data were tested according the description above (see part 3.1.1.5).

3.3 Biotic stress signaling

3.3.1 The role of cytokinins in plant-pathogen interaction

3.3.1.1 Plant material and treatments

Experiments were performed on transgenic plants with DEX-inducible *ipt* gene expression. Transgenic plants *CaMV35S>GR>ipt* (pOp6-ipt/LhGR-N, lines 303 and 307; Šámalová *et al.* 2005) and the corresponding wild-type *Nicotiana tabacum* 'SR1 Petit Havana' was grown in a growth chamber AR-36L (Percival Scientific, Perry, IA, USA). The conditions in growth chamber were set up to 16 h light at 24 °C / 8 h dark at 21 °C. The plants were illuminated by the cool white fluorescent lamps (100 μmol photons of PAR m^{-2} s^{-1}). For all experiments, 5-weeks-old plants were used. A dexamethasone (DEX) solution with a final concentration of 20 mM was prepared by diluting a 20 mM stock solution in 96 % ethanol with tap water. A 50 ml aliquot of the diluted solution was applied to the soil by watering 3 h before the start of the dark period. Control plants were watered with 0.096 % ethanol in tap water (mock).

The samples were collected from non-damaged tissue in the middle of the light period at the times after DEX application indicated in figures legend (from 0 to 4 day after DEX treatment - DAT). Collected samples were frozen in liquid nitrogen and stored at –80 °C.

3.3.1.2 Leaf gas exchange

The third or fourth leaves (counted from the plant base) of plants adapted to darkness for 30 min were used to measure the leaf GE. Measurement of the leaf GE was performed on a LI-6400 open gas exchange system (LI-COR, Lincoln, NE, USA). To measure light-induced changes of A and g_s, the leaf were inserted into chamber and acclimated in darkness to chamber conditions (mass flow of air 200 µmol s^{-1}, relative air humidity 50 %, block temperature 24.5 °C, CO_2 concentration 390 µmol mol^{-1}). After 5 min, the measuring light (LED light source 6400-02B (LI-COR, Lincoln, NE, USA) providing RL (665 nm) and BL (470 nm); 330 µmol photons of PAR m^{-2} s^{-1}) was switched on.

Furthermore, the CO_2 response of photosynthesis (A/C_i curve) was measured according to the method described in detail in paper IV.

3.3.1.3 Chlorophyll fluorescence

Parameters of slow induction kinetic were measured on the adaxial sides of the third or fourth attached leaves. The measurement was performed with an instrument which can be used to monitor chlorophyll fluorescence and P700 absorbance simultaneously (Dual-PAM-100 Chlorophyll Fluorescence & P700 Photosynthesis Analyzer, Heinz Walz GmbH, Effeltrich, Germany). The measuring protocol is described in paper IV. The estimated parameters were: the non-photochemical chlorophyll fluorescence quenching (NPQ), the relative rates of electron transport via photosystem I (PSI) and PSII – ETR(I) and ETR(II), respectively – and the quantum yield of non-photochemical energy dissipation due to donor side limitations of PSI [Y(ND)] at steady-state. The measurement of cited parameters was performed by a co-author of paper IV.

In addition, images of steady-state NPQ were recorded on the adaxial side of the third or fourth attached leaf with a fluorescence imaging system FluorCam 700MF (Photon Systems Instruments, Czech Republic). The leaf sample was irradiated for 10 min with actinic light (100 µmol photons of PAR m^{-2} s^{-1}) and the measurement protocol described in Prokopová et al. (2010) was applied.

3.3.1.4 Other methods performed

Endogenous levels of phytohormones (cytokinins, ABA, JA and SA) quantification, determination of leaf dry matter content, spectroscopic determination of chlorophyll content in the samples, quantification of hydrogen peroxide, visualization of hydrogen peroxide and the extent of lipid peroxidation within samples are methods that have been performed by co-authors of paper IV and are described in detail ibidem.

Furthermore, quantification of steady-state transcript levels of genes coding for bacterial isopentenyl transferase (IPT), chlorophyll a/b-binding protein (CAB), ferredoxin:NADP oxidoreductase (FNR1), violaxanthin de-epoxidase (VDE), pathogenesis-related protein 1b (PR-1b) and acidic phosphatase (PR-Q) were performed by co-author(s) on reverse transcription followed by quantitative PCR (RT–qPCR) using the fluorescent dye SYBR Green I. For details see paper IV.

3.3.2 The role of plant resistance/susceptibility in reaction to patogen infection and the effect of heat shock pre-treatment

3.3.2.1 Plant material and pathogen isolate

Two tomato genotypes were used for measuring chlorophyll fluorescence and GE parameters: susceptible genotype (*Solanum lycopersicum* L. cv. Amateur) and the moderately resistant one (*Solanum chmielewskii* (Rick, Kesickii, Forbesand Holle) Spooner, Anderson and Jansen). Tomato seeds were sown in plastic pots (7 cm in diameter) filled by Perlite (PERLIT, Šenov u Nového Jičína, Czech Republic). Seedlings with fully developed cotyledons were transferred to plastic pots (7 cm diameter) filled with a garden soil/peat (2/1, v/v). The seedlings were watered twice a day. Once a week, seedlings were fertilized by 50 ml of the fertilizer solution (5 g/l Cristalon fertilizer (NPK (19-6-20) + 3 % Mg; Hydro-Czech Republic, Prague, Czech Republic)) per pot. Plants were grown in pest-and pathogen-free glasshouse tempered to 22 °C at day/18 °C at night. Photoperiod in the glasshouse copied the ambient outside conditions and the illumination was supported 12 h a day by the artificial light (100-120 µmol photons m^{-2} s^{-1}). Measurements were performed from February to May (during 4 months).

Isolate of *Oidium neolycopersici* (C-2) was cultivated according description in paper V.

3.3.2.2 Heatshock pre-treatment

Selected 8-10 weeks-old plants were inserted into a cultivation box (SANYO E&E Europe BV, Etten-Leur, Netherlands) where they were exposed to 40.5 °C for 2 h and illuminated with WL (45 µmol photons m^{-2} s^{-1}). In the case of infected plants the heatshock (HS) pre-treatment was applied immediately before the inoculation. The rest of plants (that was not exposed to HS pre-treatment and was kept in the glasshouse under conditions described above) serve as controls.

3.3.2.3 *Oidium neolycopersici* inoculation

Leaves of tomato cv. Amateur covered (>80 %) by fresh sporulating mycelium of *Oidium neolycopersici* (*O. neolycopersici*, 8-10 days old) were used for the inoculation. The third and fourth leaves (counted from the plant base up) of selected plants (8-10 weeks old) were inoculated on the adaxial side by surface contact (dusting/tapping) with infected leaf. The group of plants was treated in the same way as inoculated plats (tapping), however, with uninfected leaf (mock control of inoculation).

After the inoculation, all plants (inoculated and non-inoculated with or without HS pre-treatment) were kept in a growth chamber. The temperature in growth chamber was set up to 22 °C a day /18 °C night with the light condition 12 h light (100 µmol photons m^{-2} s^{-1})/12 h dark. Measurement was performed at 2, 3, 4, 7 and 9 days after inoculation (DAI). However, an infection caused defoliation of *L. chmielewskii* leaves and therefore the parameters could be measured only up to 4 DAI in plants without HS pre-treatment. The leaves of HS pre-treated L. *chmielewskii* plants were measurable up to 7 DAI (but only two leaves were left on the plants and could be used for the measurements).

3.3.2.4 Chlorophyll fluorescence measurement

Chlorophyll fluorescence was monitored by a co-author of paper V on the adaxial side of the tip leaflet of the attached third or fourth leaf at room temperature using a FluorCam700MF imaging system (Photon Systems Instruments, Brno, Czech Republic). Images of the maximal quantum yield of photosystem II (PSII) photochemistry (F_V/F_M) and the non-photochemical chlorophyll fluorescence quenching (NPQ) as well as average values of F_V/F_M, the maximal fluorescence of the dark-adapted sample (F_M) and steady-state NPQ are presented in paper V. The measuring protocol and calculation of parameters are described in details ibidem.

3.3.2.5 Leaf gas exchange

Leaf GE was measured on the tip leaflets of the attached third or fourth leaves. An open gas exchange system LCA-4 (ADC BioScientific, Hoddesdon, UK) was used for measurement. After finishing the chlorophyll fluorescence measurement, the plant was light-adapted in a growth chamber for 30 min in following conditions: 100 µmol photons of PAR m^{-2} s^{-1}, 22 °C and relative air humidity 45 %. The light-adapted plant was removed from the chamber and the tip leaflet (chosen regions without any necrotic lesions) was placed into a gasometric leaf chamber, actinic light (320 µmol photons m^{-2} s^{-1}, WL) was switched on and these conditions were kept: leaf temperature 24 °C, mass flow of air 100 µmol s^{-1}, CO_2 concentration 350 µmol mol^{-1}, relative air humidity 40 %. The leaflet was equilibrated in the chamber for 1 h and GE parameters (A, g_s, C_i) were measured. The parameter A was related to Chl(a+b) in the measured leaflet area (A_{chl}; pmol CO_2 μg^{-1} s^{-1}).

3.3.2.6 Content of photosynthetic pigments

The area of the tomato leaflet delimited by the gasometric leaf chamber served as a sample for spectroscopic determination of the content of chlorophyll a, chlorophyll b and total carotenoids. More detailed description of the measurement can be found in paper V (measurement has been performed by a co-author).

3.3.2.7 Statistic alanalysis

Samples from four different plants were used for each treatment. The measured GE parameters are presented relatively to their values at 0 DAI (= 100 %). The values of the parameters at 0 DAI are mentioned in figures legends (in some cases the values at 0 DAI were not same in different variants). Statistical evaluation of data is described in paper V.

4. Aims of thesis

The experimental part of this thesis has been designed to cover three fields related to plant signaling: light signaling, abiotic and biotic stress signaling.

Within the light signaling, the aim was to explore the reaction of *7B-1* mutant to light (chloroplasts and stomata movements) and investigate the related signaling pathways.

Within the abiotic stress signaling, the aims was to investigate the signaling pathways leading to systemic response to abiotic stress and the role of phytohormone ABA in these responses using the ABA-deficient mutant *sitiens*

Within the biotic stress signaling, the aims were:

- to investigate the role of cytokinins in plant response to pathogen infection

- to explore the role of plant resistance/susceptibility and effect of heat shock pre-treatment of plant in reaction to pathogen infection

5. Results and discusions

5.1 Light signaling

Plants are very often exposed to non-stressful conditions including the light illumination of lower intensities (not causing the photosynthesis photoinhibition). In this case, the light can serve as a signaling factor important for triggering or regulation of some important plant processes. The proper function of signaling pathways for the light is essential for plant survival. An important system for the study of the reaction to light is a mutant affected in light signaling. If the mutant is, besides, the male sterile, it can be a choice material for plant breeders for several reasons, including its use in backcrossing, interspecific hybridization, and in F1 hybrid seed production. This is one of the reasons, why the male-sterile mutant was selected to be investigated for reactions to the light. However, if the male sterile mutant is sensitive to abiotic or biotic stresses, its use in breedings programs is limited (Rao *et al.* 1990). In contrast, the resistance of the male sterile mutants to various stresses will certainly improve practical applications, e.g. in the hybrid seed industry.

In tomato (*Solanum lycopersicum* L.), a spontaneous recessive single gene mutant *7B-1* (Sawhney 1997) is characterized by reduced de-etiolation of hypocotyl growth, tall stature of adult plant, elevated levels of endogenous ABA and chlorophyll, and reduced levels of gibberellins, auxin, ethylene and cytokinins (Bergougnoux *et al.* 2012; Fellner *et al.* 2005; Fellner *et al.* 2001). The mutant shows reduced responsiveness to various abiotic stresses specifically in BL conditions (Fellner and Sahwney 2001, 2002). It was further showed that the *7B-1* mutation confers a BL-specific lower sensitivity to coronatine produced by *Pseudomonas syringae* pv. *tomato* (Bergougnoux *et al.* 2009). Current results revealed that the mutant has defects in phototropic responses (Bergougnoux *et al.*, unpublished data), and shows changes in g_s, A and intrinsic water-use efficiency under BL (Ježilová *et al.* 2012). The pleiotropic nature of the *7B-1* mutation suggests that a basic element involved in a BL signaling pathway(s) is affected.

To determine the effect of the *7B-1* mutation on BL signaling pathways, both chloroplast and stomatal movement (known to be caused by BL) were explored on *7B-1* mutant. The WT plant has been used as a reference system. The basic elements of light signaling for these two movements are well described for many plant species.

5.1.1 Chloroplast movement

Selected leaves ($L4^{th}$ and $L9^{th}$, and $L10^{th}$ of older plants - see part 3.1.1.1) of both WT and *7B-1* plants adapted to darkness conditions were used to measure the chloroplast movement. At first, the Chl(a+b) of desired leaves was determined by measuring the SPAD values. Resulting chlorophyll contents (re-calculated to units [$\mu g\ cm^{-2}$], see part 3.1.1.2) are shown on Fig. 10. The results show that there is not statistically significant difference between Chl(a+b) of WT and *7B-1* mutant for $L4^{th}$ and $L9^{th}$. However, there is statistical significant difference between Chl(a+b) of WT and *7B-1* detected on $L10^{th}$.

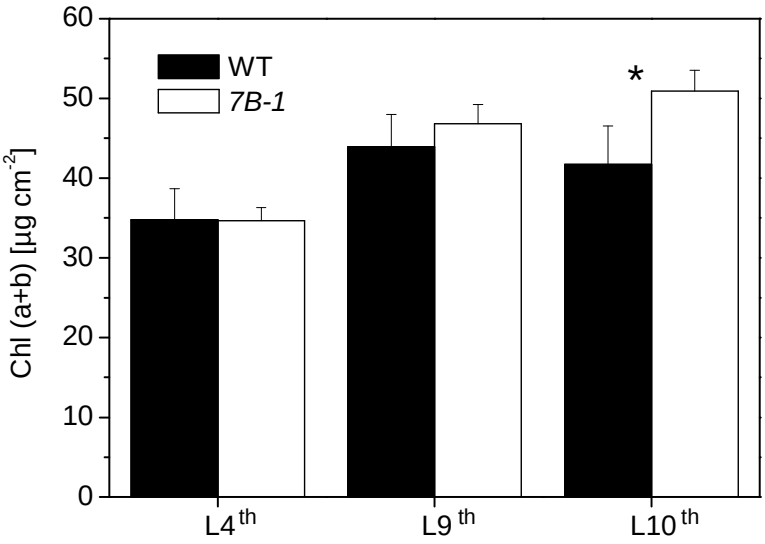

Fig. 10. Chlorophyll content in leaves (L4th and L9th, and L10th of older plants, counted from the plant base up) of WT (black columns) and *7B-1* (white columns). Significant difference between WT and *7B-1* is marked by an asterisk, arithmetic mean ± SD are shown, n = 5 - 7.

Subsequently, a selected dark-adapted leaf was inserted into the leaf clip and the measurement of T_C was used for monitoring of the chloroplast movement. The value of initial T_C (T_{C0}) has been recorded immediately after switching on the BL (T_{C0} is arithmetic mean of the first three recorded T_C values, see Fig. 8). Since chloroplasts previously dark - adapted are in face position with regard to the incident light while absorbing as much light as possible (maximum), the value of T_{C0} is minimal. Absolute values of T_{C0} seem to be correlated with Chl(a+b) in leaves. The higher Chl(a+b) the lower value of T_{C0} (compare Chl(a+b) in Fig. 10 and T_{C0} in Fig. 11).

Rapidly after switching on the BL of high intensity (340 ± 20 µmol photons m^{-2} s^{-1}), the illuminated chloroplasts begin to move towards the side cell walls as much as possible to get the chloroplasts out from the high intensity of light. At the same time, the leaf transmittance for the light increases and T_C increased too. This response is governed by phot2 and is designed as the avoidance movement. Changes in T_C caused by chloroplasts movements are shown in Fig. 11.

The chloroplasts were illuminated by BL of high intensity for 30 min to reach the side position. After this time, the T_C was about the highest (maximal T_C; designed as T_{Cmax}). Even the T_{C0} of *7B-1* is lower for L9th (and L10th) compared with WT, the results show that the chloroplasts of *7B-1* leaves move with lower extent compared to those of WT (Fig. 11).

Since the values of T_{C0} and T_{Cmax} differ between WT and *7B-1*, parameter h_1 (defined as: $h_1 = T_{Cmax} - T_{C0}$) was evaluated. When it is assumed that the cells of WT and *7B-1* leaves are of similar size, this parameter reflects the extent of the avoidance chloroplast movement. The values of parameter h_1 are shown in Fig. 12.

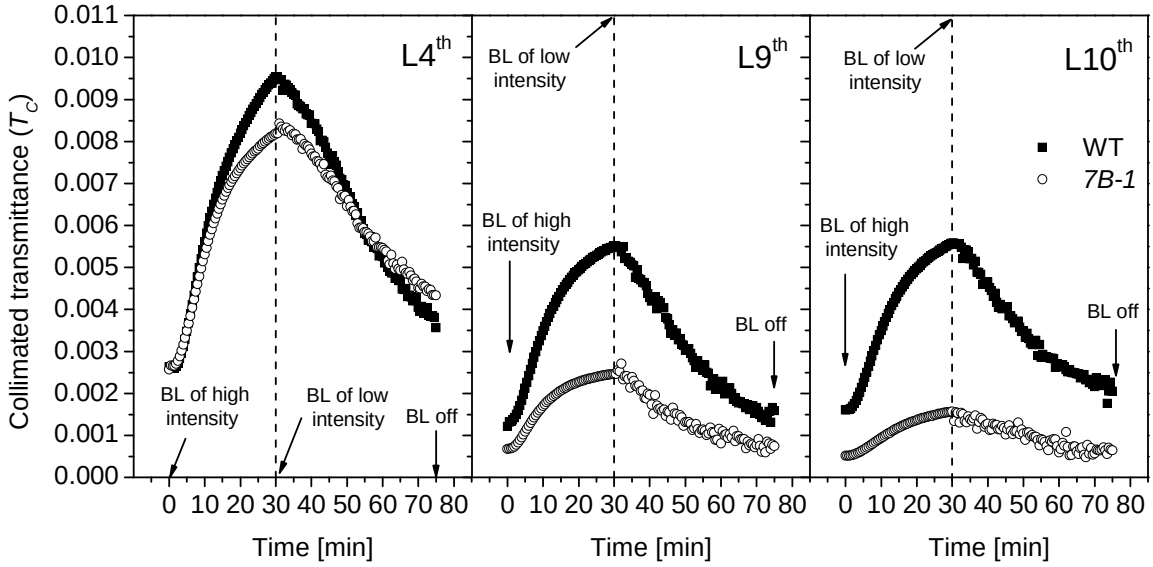

Fig. 11. Changes in collimated transmittance (T_C) of L4th, L9th and L10th of WT and *7B-1* plants induced by illuminating the sample by the blue light of high intensity (340 ± 20 μmol photons m^{-2} s^{-1}; 0 min - 30 min, the avoidance movement of chloroplasts) and by illuminating the sample by the blue light of low intensity (8 ± 2 μmol photons m^{-2} s^{-1}; 30 min – 75 min, the accumulation movement of chloroplasts). The average curves are shown, n = 5 - 7.

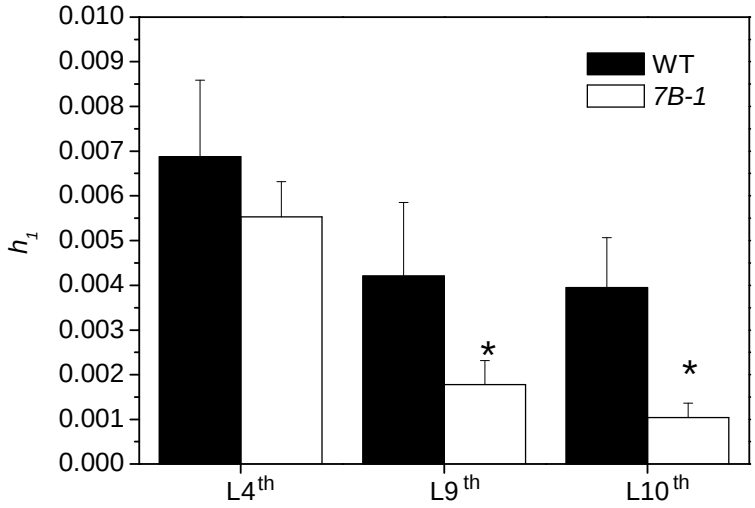

Fig. 12. Values of parameter h_1 (defined as: $h_1 = T_{Cmax} - T_{C0}$) calculated for L4th, L9th and L10th of WT (black columns) and *7B-1* (white columns) for the avoidance chloroplasts movement. Significant differences between WT and *7B-1* are marked by asterisk, arithmetic means ± SD are shown, n = 5 - 7.

The value of h_1 measured on L4th of *7B-1* is slightly lower compared to WT. However, significantly lower value of h_1 in *7B-1* compared to WT was observed on L9th. Since the Chl(a+b) of both WT and *7B-1* is not significantly different for L4th and L9th, the significantly lower value of h_1 in *7B-1* compared to WT probably do not reflect differences in Chl(a+b) of

both kinds of plants. It rather reflects the differences in BL-induced chloroplasts movement between *7B-1* and WT. The measurement of chloroplasts movements was repeated on L10th of older plants. The extent of the chloroplast movement remained in this case similar to L9th measured before although the Chl(a+b) of L10th of *7B-1* was significantly higher than chlorophyll content of WT. The obtained results suggest that in the case of avoidance movement, the chloroplasts of *7B-1* move less than those of WT.

After 30 min, chloroplasts that reached the side position were exposed to BL of lower intensity (8 ± 2 μmol photons m^{-2} s^{-1}) and began to move back to the initial face position. This movement is governed by both phot1 and phot2 and is designed as accumulation movement. The accumulation movement is also shown in Fig. 11.

Similarly to parameter h_1, the parameter h_2 ($h_2 = T_{Cmax} - T_{Cf}$, where T_{Cf} is terminal T_C computed as arithmetic mean of the last three values of T_C measurement, Fig. 8). The values of parameter h_2 are shown in Fig. 13.

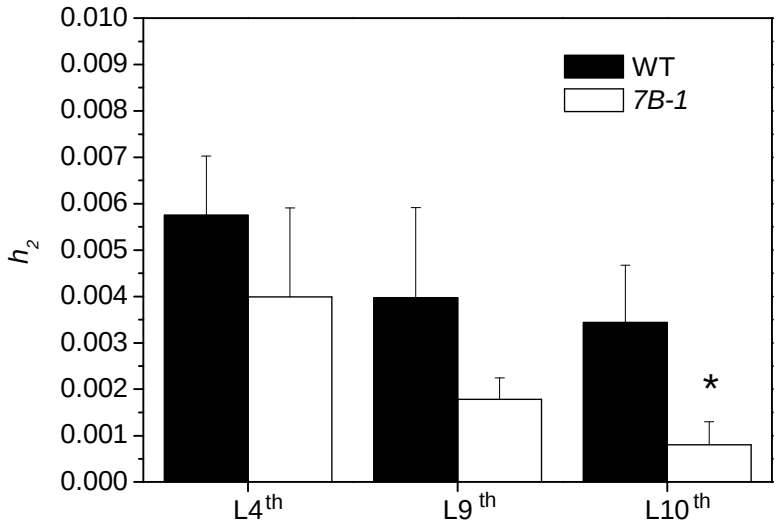

Fig. 13. Values of parameter h_2 (defined as $h_2 = T_{Cmax} - T_{Cf}$) calculated for L4th, L9th and L10th of WT (black columns) and *7B-1* (white columns) for the accumulation chloroplast movement. Significant differences between WT and *7B-1* are marked by asterisk, arithmetic mean ± SD are shown, n = 5 - 7.

Similarly to parameter h_1, the values of parameter h_2 of *7B-1* were lower compared to WT. However, the significant difference between WT and *7B-1* was detected only for L10th. Obtained results seem to show that in the case of the accumulation movement, the chloroplasts of *7B-1* move less than that of WT. However, it is possible that the lower extent of the accumulation movement h_2 in *7B-1* is caused only by the lower extent of the avoidance movement (parameter h_1) in *7B-1*. To reflect the difference between the extents of the avoidance and accumulation movements, parameter h_2/h_1 has been calculated (Fig. 14). If the value h_2/h_1 is close to 1, the chloroplasts accumulation movement is of the same extent as the avoidance movement. If mutant *7B-1* is impaired in accumulation chloroplast movement, the h_2/h_1 of *7B-1* would be lower than 1 and significantly different (lower) from the h_2/h_1 value of WT at the same time. However, the ratio h_2/h_1 for *7B-1* is not significantly different from WT for L4th, L9th and L10th, even the values of h_2/h_1 are lower than 1 for L4th and L10th and about equal to 1 for L9th (Fig. 14).

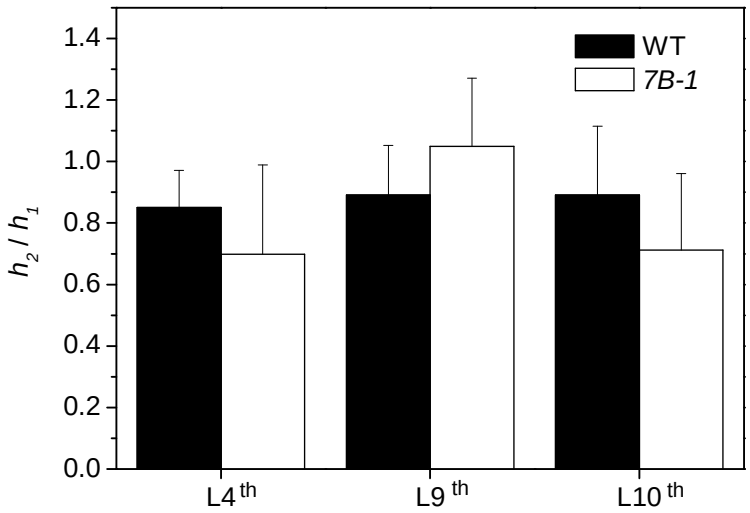

Fig. 14. Values of parameter h_2/h_1 calculated for L4th, L9th and L10th of WT (black columns) and *7B-1* (white columns). Arithmetic means ± SD are shown, n = 5 - 7.

In previous, it was supposed that BL-induced changes of T_C correspond to chloroplasts movement. To show that the effect was caused only by the chloroplast movement and not by the chloroplast decomposition or other process (leaf drying, leaf infiltration etc.), the T_C spectra of leaves were detected three times in the same arrangement as measurement of BL-induced changes of T_C. First T_C spectrum was read before the beginning of T_C measurement (A), second at the time when T_C reached maximal value (B) and third after the end of T_C measurement (C). An example of detected T_C spectra is shown in Fig. 15.

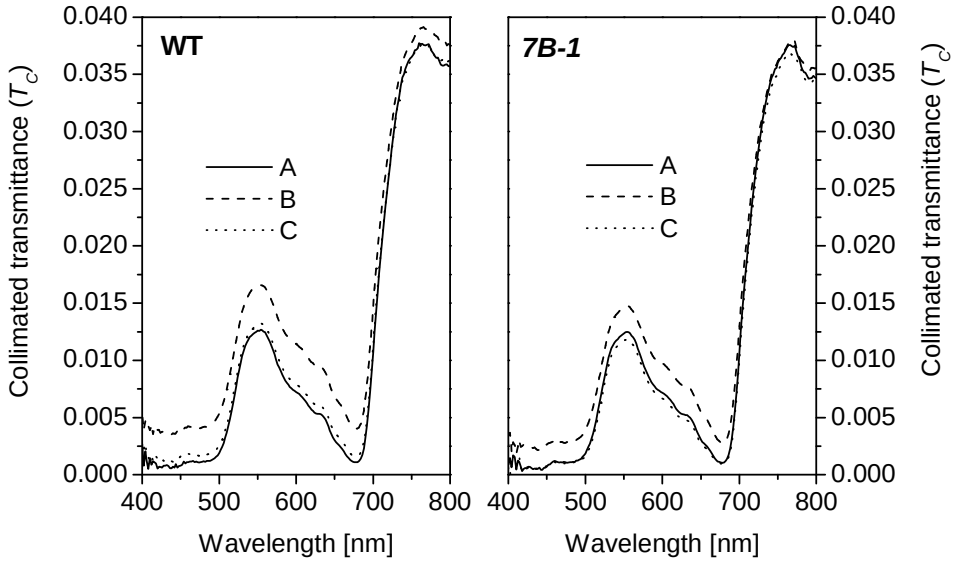

Fig. 15. Examples of spectra of collimated transmittance measured before the chloroplast movement (A), after the avoidance chloroplast movement (B) and after the accumulation movement (C) of WT (left part) and *7B-1* (right part) in L9th.

It is clear from the results that all values of T_C spectra A increased uniformly to values of T_C spectra B. Thus, the shape of T_C spectra did not change. Since T_C spectra did not change the shape, processes like chloroplast decomposition etc. can be excluded and an increase of values of T_C spectra A to B is assigned to the BL-induced avoidance chloroplasts movement. Illumination of chloroplasts in the side position by BL of low intensity decreased the T_C of spectra B to values of T_C spectra C, while the shape of the spectra remained approximately the same. The shape and values of T_C spectra A were nearly the same as values of T_C spectra C indicating that the chloroplasts illuminated by BL of low intensity move back to initial position and no other process but the accumulation movement of chloroplast can be seen during the observation.

Taking together, the obtained results show that the BL-induced avoidance chloroplast movement is reduced in *7B-1* plants. Interestingly, the impairment in the accumulation chloroplast movement in *7B-1* mutants have not been proved properly, however, it cannot be completely excluded. Chloroplast movement in tomato plants can be triggered only by BL, the RL is ineffective in triggering the chloroplast movement in tomato (measured, but results not shown). On the basis of the results, it can be suggested that signaling pathway of BL that leads to the avoidance but not to the accumulation chloroplast movement is impaired in *7B-1* plants. Since the avoidance movements of chloroplasts under BL are affected in *7B-1* in comparison to WT and since chloroplast movement is induced by interaction of BL with phot2, it can be speculated that phot2 (e.g. Kagawa *et al.* 2001) could be affected by the *7B-1* mutation. If the phot2 is affected in *7B-1*, the accumulation movement of chloroplasts in *7B-1* could be predominantly caused by the activation of phot1. However, the results do not show direct evidence that *7B-1* mutation affects phototropin.

Chloroplast movement can be sensitive to ABA. Königer *et al.* (2010) showed that chloroplasts in guard cells of *A. thaliana* have kept their position in the middle of guard cells irrespective of light treatment. Thus, the limitation of chloroplast movement caused by ABA can be also responsible for the reduced chloroplast movement in ABA-overproducing *7B-1* (Fellner *et al.* 2001).

At the date, it is accepted that, besides chloroplast movements, also stomatal responses to BL are dependent on functional BL photoreceptors phot1 and phot2. Taking into account the obtained results concerning the chloroplast movement under BL, the next question to be solved is whether *7B-1* mutant also exhibits different reactions to BL in stomatal responses.

5.1.2 Stomatal movement (paper II)

To explore the BL signaling pathways important for stomatal opening, the microscopic measurement of stomatal aperture was used for WT and *7B-1* leaves. Firstly, the physiological study of epidermal surface cells was performed. It showed that the stomatal density of WT was significantly higher than that of *7B-1* (Fig. 16A), however, the stomatal index (Fig. 16B; multiplied by 100 corresponding to percentage fraction of stomata among all cells) was similar in both genotypes. These characteristics are manifested in a different arrangement and size of epidermal cells within *7B-1* and WT. However, stomata of both genotypes were of the

similar size and surrounded by similar number of epidermal cells (Fig. 16C). Therefore, the results of stomatal apertures of WT and *7B-1* stated below should be comparable.

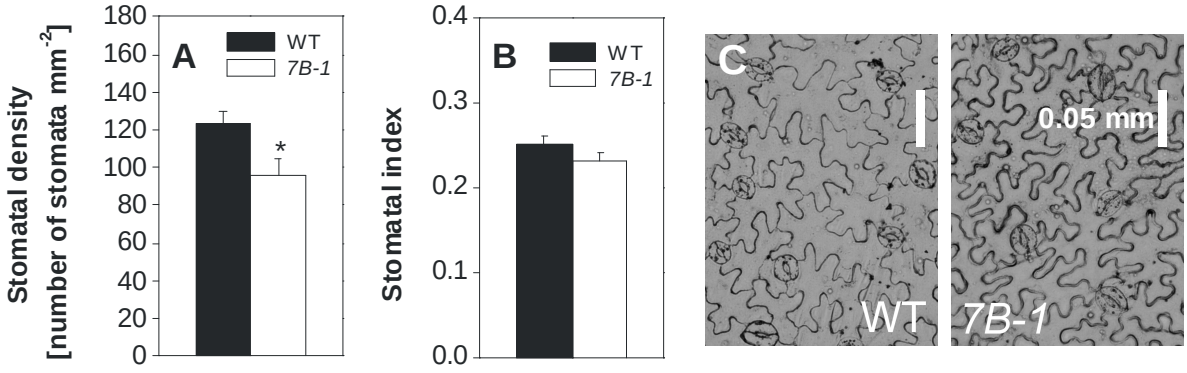

Fig. 16. Stomatal density (A), stomatal index (B) of WT (black column) and *7B-1* (white column) samples. Arithmetic means ± SE are shown, a number of evaluated samples was 21. Statistically significant difference between WT and *7B-1* is marked with an asterisk. Microscopic picture (C) of WT and *7B-1* leaves shows the dimensions (white segment is a measure) and arrangement of cells. Adopted with slight modifications from the paper II.

When observing the stomata in the dark, their aperture in *7B-1* mutant was similar to that in WT leaves. Illumination of sample with BL induced stomatal opening in WT leaves, whereas the *7B-1* stomata were insensitive to BL (Fig. 17A). In *7B-1* mutant, normal stomatal opening was induced only by the RL illumination and the aperture was comparable to that observed in WT plants (Fig. 17B). Illumination the samples by WL opened the mutant stomata less than the stomata of WT, but a significant difference was observed only after 5.5 h of WL exposure (Fig. 17C).

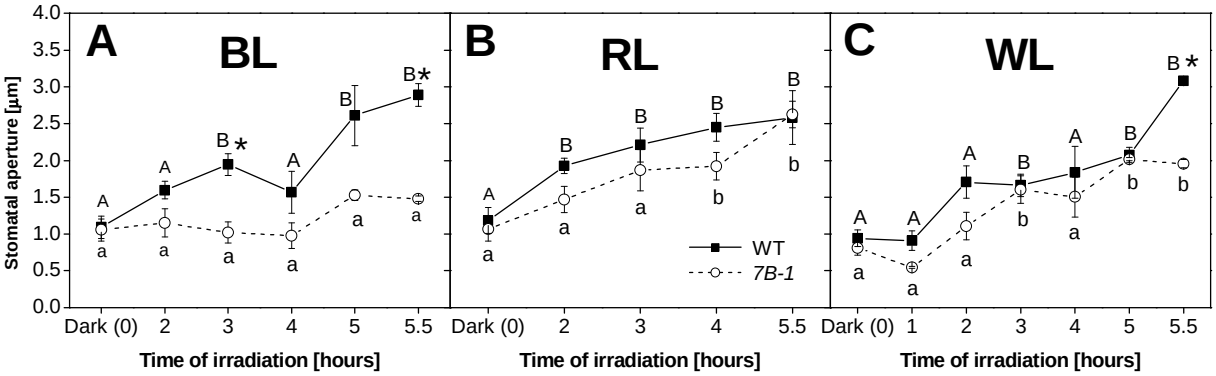

Fig. 17. Time course of stomatal aperture of WT (black squares and solid lines) and *7B-1* (white dots and dashed lines) mutant in reaction (A) to blue light (BL), (B) to red light (RL) and (C) to white light (WL). Arithmetic means ± SE of 3 independent experiments are shown (60 stomata per condition and per experiment). Letters A, B indicate significantly different groups for WT, letters a, b indicate significantly different groups for *7B-1*; asterisks indicate significant differences between WT and *7B-1* in one specific condition. Adopted with slight modifications from paper II.

The data are consistent with our previously published results. For instance, it was earlier reported that 20-day-old *7B-1* seedlings showed limited transpiration under fluorescent tube light (Fellner *et al.* 2001) corresponding to more closed stomata of *7B-1* than of WT. Recently, it was demonstrated in 5-week-old seedlings that parameter g_s measured under BL on *7B-1* is lower compared to WT (Ježilová *et al.* 2012). The results could suggest that *7B-1* mutation impairs an element of phototropin signaling pathways within the guard cells. To get more information about this defect, we further studied the *7B-1* stomatal responses to FC, two anion channel blockers 9-AC and NIF, and to ABA.

Fusicoccin has been found to activate H^+-ATPase in plasma membrane of guard cells (Johansson *et al.* 1993), one of the processes involved in the light-induced stomatal opening. In order to show whether the extent of activation of guard cell plasma membrane H^+-ATPases differ between WT and *7B-1*, the responses of dark-adapted stomata of both genotypes to FC were compared (Fig. 18). Stomata treated by FC and/or exposed to WL considerably opened similarly in the WT and *7B-1* leaves. The similar stomatal responses of *7B-1* and WT to FC (Fig. 18) point out that the extent of the activation of H^+-ATPase is probably not impaired by the *7B-1* mutation. This result also demonstrates that the function of H^+-ATPase itself is intact in *7B-1*.

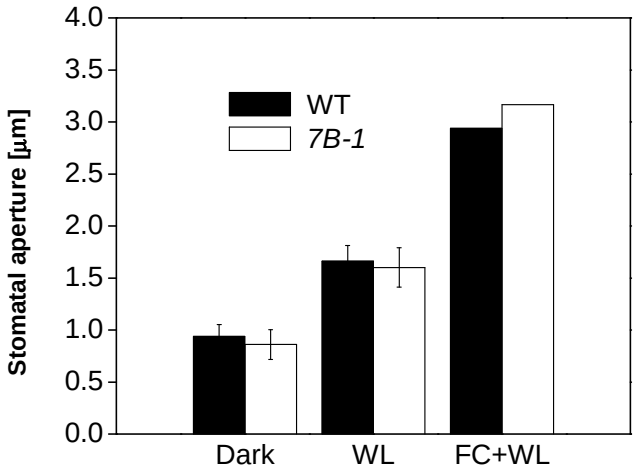

Fig. 18. Aperture of the dark-adapted stomata of WT (black column) and *7B-1* (white column) and aperture of stomata non-treated or treated by fusicoccin (FC) before 3 h lasting exposition to white light illumination (WL or FC + WL respectively). Arithmetic means ± SE of 3 independent experiments (1 in case of FC + WL) are shown (60 stomata per condition and per experiment). Adopted with slight modifications from paper II.

Next tool useful for investigating the stomatal signaling pathways are the ion channel blockers. For instance, the fact that the anion channel blockers regulate stomatal movements (Schroeder *et al.* 1993) has been used to support the mechanism of stomatal closure consisting in opening of the anion channels promoting membrane depolarization. It was shown that the anion channel blockers 9-AC and NIF block current through R-type and S-type anion channels of plasma membrane of *Vicia faba* L. guard cells (Marten *et al.* 1993; Marten *et al.* 1992; Schroeder *et al.* 1993; Schwartz *et al.* 1995). According to Marten *et al.* (1992), NIF inhibits reversibly the R-type anion channels in plasma membrane of guard cells more than 9-

AC, whereas the S-type of anion channel is more inhibited by 9-AC than by NIF (White and Broadley 2001).

In our case, adding the anion channel blocker 9-AC (100 μM) to dark-adapted epidermal strips induced stomatal opening in both genotypes (WT and *7B-1*) in a similar extent (Fig. 19). This result is in accordance with observation on *A. thaliana*, where the anion channel blocker 9-AC induces stomatal opening in the dark (Forestier *et al.* 1998).

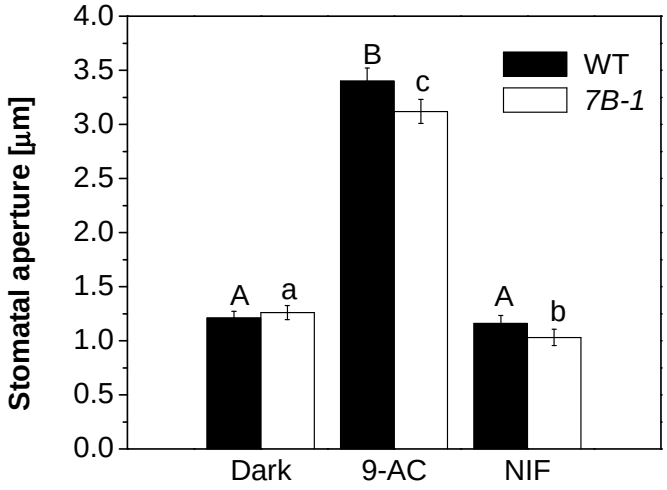

Fig. 19. Reaction of dark-adapted stomata of WT (black columns) and *7B-1* (white columns) tomato mutant to 3 h long action of ion channel blockers 9-AC (100 μM) and NIF (100 μM) in dark. Arithmetic means ± SE of 8 independent experiments are shown (60 stomata per condition and per experiment). Letters A, B indicate significantly different groups for WT, letters a, b indicate significantly different groups for *7B-1*. Adopted with slight modifications from paper II.

The effect of 9-AC on stomata was also observed with both genotypes pre-exposed (before 9-AC application) for 3 h to BL (RL or WL respectively) and afterwards exposed to BL (RL or WL respectively) for 2.5 h (Fig. 20A-C).

The obtained results indicate that the function of 9-AC-sensitive anion channels per se is not likely impaired in *7B-1* stomata. Since the 9-AC blocks the S-type anion channels involved in stomatal opening (Schwartz *et al.* 1995) and it was observed that in WT the BL did not further increases the stomatal aperture promoted by 9-AC (Fig. 20A), it seems that 9-AC and BL block the similar type of anion channels. This result is supported by observed slight 9-AC-induced increase of the stomatal aperture under RL (Fig. 20B). Interestingly, although containing blue and red part of spectra, WL in combination with 9-AC promoted stomatal opening less than BL (or RL) in combination with 9-AC in both genotypes (Fig. 20C). The explanation of this result is not clear. However, it differs from the result obtained on *Commelina communis* L., where stomatal opening induced by WL could be enhanced by 9-AC (Schwartz *et al.* 1995).

The second anion channel blocker NIF (100 μM) acted differently on stomatal movement as compared to 9-AC, and this was observed in both genotypes tested. The stomata did not respond to NIF in darkness (Fig. 19). This is in accordance with the results of Forestier *et al.* (1998) on *A thaliana* plants. It suggests that NIF inhibits anion channels in

plasma membrane that are different from those inhibited by light (S-type). It is also possible that NIF inhibits the Vcl anion channels in tonoplasts of vacuoles (Pei *et al.* 1996). When the stomata of WT pre-exposed by BL (RL or WL respectively) for 3 h were treated by NIF and subsequently exposed to BL (RL or WL respectively) for 2.5 h, the stomatal aperture was reduced (Fig. 20A-C). Since the stomatal aperture of *7B-1* under BL is low, the effect of NIF on stomata under BL was not considerable (the stomata remained almost closed, Fig. 20A). When the stomata of *7B-1* pre-exposed by RL (or WL respectively) for 3 h were treated by NIF and subsequently exposed to RL (or WL respectively) for 2.5 h, the stomatal aperture was reduced (Fig. 20B, C) as in the case of WT. Thus, NIF prevented stomatal opening in any light conditions tested in our experiments. This leads to a hypothesis that NIF inhibits those anion channels working opposite to the anion channels inhibited by light (S-type).

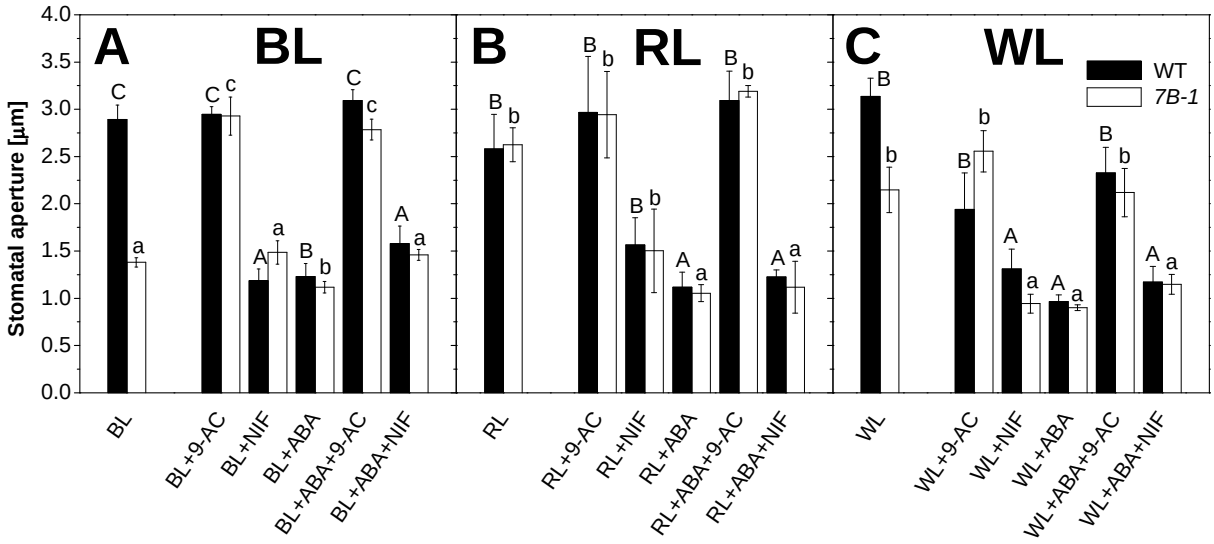

Fig. 20. Reaction of stomata of WT (black columns) and *7B-1* (white columns) tomato mutant illuminated for 3 h with (A) blue light (BL), (B) red light (RL) or (C) white light (WL) to anion channels blockers 9-AC and NIF, to ABA and to ABA together with the anion channels blockers 9-AC and NIF. After adding the effectors, the samples have been put back under (A) BL, (B) RL or (C) WL for subsequent 2.5 h. Arithmetic means ± SE of 3 independent experiments are shown (60 stomata per condition and per experiment). Letters A, B, C indicate significantly different groups for WT, letters a, b, c indicate significantly different groups for *7B-1*. Adopted with slight modifications from paper II.

Taken together, data presented above contribute to our hypothesis that *7B-1* is impaired in early BL signaling pathway for stomatal opening (i.e. in signaling component(s) preceding the inhibition of S-type of anion channel in plasma membrane of guard cell).

It is well known that stomata close in the presence of ABA and that ABA inhibits stomatal opening (e.g. Schroeder *et al.* 2001a). It was shown in several reports that *7B-1* is an ABA overproducer (Bergougnoux *et al.* 2009; Fellner *et al.* 2001; Piterková *et al.* 2012), while it was demonstrated that ABA amount increases especially in BL-grown plants and that BL-induced accumulation of ABA was significantly higher in *7B-1* than in WT (Bergougnoux *et al.* 2009; Piterková *et al.* 2012). Therefore, stomatal opening of *7B-1* in BL may be

inhibited by the increased endogenous ABA content. However, in several systems, it was also reported that *7B-1* is less sensitive to BL in various responses, e.g. seed germination and hypocotyl elongation (Bergougnoux *et al.* 2012; Bergougnoux *et al.* 2009; Fellner and Sawhney 2002; Piterková *et al.* 2012). Currently, we also revealed reduced responses of *7B-1* specific for phototropins, such as phototropism, early stage of BL-induced inhibition of hypocotyl elongation (unpublished results of Fellner, Bergougnoux, and Ježilová) and chloroplast movement (see the chapter 5.1.1). We therefore suggest that reduced stomatal aperture in *7B-1* in BL reflects, on the one hand, elevated level of ABA in *7B-1* mutant (i.e. increased stimulation of S-type anion channels), and the reduced sensitivity to BL mediated by phototropin signaling pathway on the other hand (i.e. reduced inhibition of S-type anion channels) (Fig. 21).

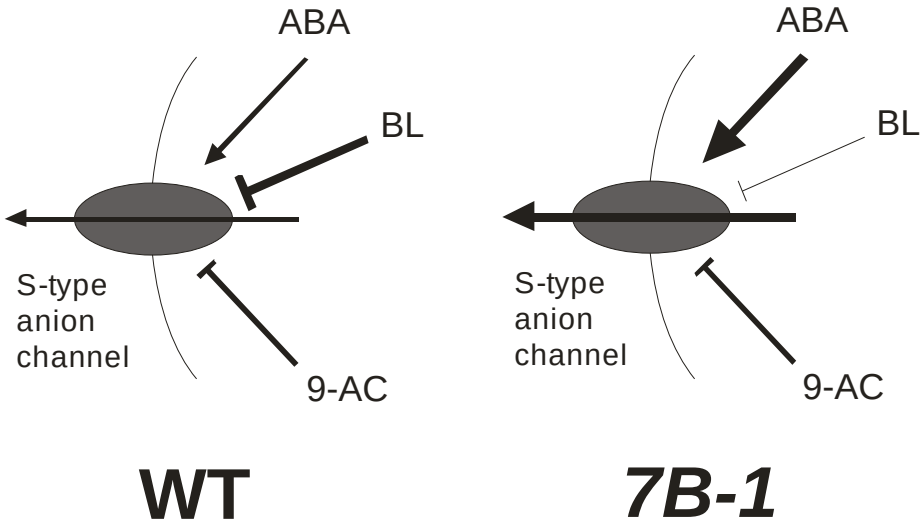

Fig. 21. Model explaining differential responses of WT and *7B-1* stomata to ABA and blue light (BL) and similar responses to anion channel blocker 9-AC. Our data suggest that reduced stomatal aperture in *7B-1* mutant is a consequence of elevated ABA content and reduced BL signaling in *7B-1* mutant. In addition, similar responses of WT and *7B-1* to 9-AC also indicate that ABA, BL and 9-AC take action likely in the similar type of anion channels. Arrows and T-bars represent positive and negative effects, respectively. Adopted from paper II.

We still do not have direct evidence that *7B-1* has a defect in receptor phot1 or phot2. But since phot1 and phot2 functionally cooperate in BL responses (phototropism, chloroplast movement, stomata opening) (Christie 2007), it is possible that the defect in one of the phototropin receptors can affect final responses regulated by the second phototropin receptor.

Further, ABA effect on stomata was investigated. Abscisic acid was used as a factor that in contrast to light promotes stomatal closure or inhibits stomatal opening. Different responses to ABA in WT and ABA-overproducing *7B-1* (Fellner *et al.* 2001) were expected. Stomatal opening in WT leaves induced by BL, RL or WL was completely inhibited by adding 1 μM ABA to the samples (Fig. 20A-C). Like in WT, ABA closed stomata in *7B-1* samples exposed to RL or WL (Fig. 20B, C). Because of the insensitivity to BL, the sensitivity of *7B-1* to ABA is not obvious in our experimental protocol (Fig. 20A). However, it was previously reported that the *7B-1* mutant shows BL-specific resistance to ABA (Fellner

and Sawhney 2002). Under WL, the stomata of WT were more sensitive to endogenously applied ABA than the stomata of *7B-1* (Fig. 20C).

The degree of reversibility of the ABA-induced stomatal closing by anion channel blockers (9-AC and NIF) was tested. It was showed that in WT and at all light conditions tested, 9-AC restored stomatal opening inhibited by ABA (Fig. 20A-C). These results correspond to the results of Schwartz *et al.* (1995) and Forestier *et al.* (1998) obtained with *Comellina communis* L. and *A. thaliana*. They confirm that in tomato there is a competition between a signaling component activated by ABA, which causes an activation of anion channel (Inoue *et al.* 2010), and by 9-AC (or light) which causes an inhibition of anion channel. The full restoration of stomatal opening by 9-AC was also observed in *7B-1*. The 9-AC-induced restoration of stomatal opening in BL in the presence of ABA was of similar extent in WT and *7B-1* samples.

It indicates that ABA, BL and 9-AC take action in the similar type of anion channels (Fig. 21). Whatever the light conditions were, the stomatal closure induced by ABA in WT and *7B-1* mutant could not be overcome by blocker NIF (Fig. 20A-C). In accordance to obtained results, minimal effect of NIF on ABA-induced stomatal closing was also observed by Forestier *et al.* (1998) in *A. thaliana* plants. It supports our presumption that NIF inhibits anion channels working opposite to those inhibited by 9-AC. Since in our experiments NIF always showed negative effect on stomatal opening, it is also possible that the anion channel blocker NIF in the concentrations used is toxic for tomato stomata.

The experiments performed with the stomata guard cells revealed that additionally to the previously reported BL-specific characters, *7B-1* is also insensitive to the BL-induced stomatal opening. Using FC, anion channel blockers and ABA suggests that the *7B-1* mutation affects the signaling functioning in the inhibition of anion channels in plasma membrane of guard cells that is involved in stomatal opening. Our data indicate that the previously reported increased endogenous ABA content in *7B-1* along with reduced mutant responses to BL is likely responsible for the inhibition of stomatal opening in BL.

The obtained results lead to a conclusion that both avoidance chloroplast movement and stomatal opening under BL are impaired in *7B-1*. This points out that the BL signaling pathways (possibly in BL-receptor part) are affected by *7B-1* gene. Besides, both chloroplast movement and stomatal opening are also most likely affected by the increased ABA in *7B-1*. The results contribute to the contemporary understanding of *7B-1* mutation. Although, more work has to be done in order to completely describe the behavior of *7B-1*.

The results described in the chapter 5.1.2 have been presented in paper II.

5.2 Abiotic stress signaling (paper III)

In natural conditions, the plants are as well exposed to many stressful stimuli which activate the signaling pathways different from the signaling pathways of light examined in chapter 5.1. The local injury of a plant can lead to effects that are detectable far from the site of the injury. This effect is known as a systemic response. Systemic response to a local injury can include among others the inhibition of photosynthetic processes, limitation of water loss by stomata closure (Hlaváčková *et al.* 2006; Kaiser and Grams 2006; Lautner *et al.* 2005; Peña-Cortés *et al.* 1995; Van Sambeek and Pickard 1976) or accumulation of chemical compounds (JA, ABA; e.g. Hlaváčková *et al.* 2006). It is supposed that the systemic reactions can be initiated by signals of electrical (e.g. Kaiser and Grams 2006), hydraulic (Mancuso 1999), or chemical (e.g. Hlaváčková *et al.* 2006) origin.

There is little information about the impact of endogenous ABA level (ABA can act as signaling molecule by itself) on systemic reaction to abiotic stress and related signaling pathways. Therefore, the short-term reaction in GE to local burning (chosen as a standard stimulus inducing both electrical and chemical changes) has been measured together with the electrical signal (SEP) and the endogenous content of phytohormones (ABA and JA; the measurement performed by co-authors of paper III) on WT tomato plants, SIT mutants (ABA-deficient) and SIT mutants pre-treated with ABA (SITA).

First step in exploration the effect of the endogenous ABA content on reaction of tomato plants (WT, SIT mutants and SITA) to local burning is to determine the influence of ABA content on initial state (steady-state) of GE parameters before burning. Coming out from the analysis of endogenous hormones content (The endogenous ABA content in WT and SITA plants was similar and in SIT mutants, the content of ABA was significantly lower (about 30% of ABA content in WT, Fig. 24). The global endogenous JA content in WT, SIT and SITA plants was similar (Fig. 25).), it is supposed that the differences described below were caused predominantly by the different endogenous ABA content in plants.

The measurement of GE showed that steady-state values of g_s, E, A and C_i in SIT are significantly higher compared to WT plants (Table 1).

Table 1
Initial steady-state levels of GE parameters (before local burning). The results represent the arithmetic means ± SD, n ≥ 4. Values followed by the same letter are not significantly different (only in columns).

	g_s [mol (H_2O) m^{-2} s^{-1}]	E [µmol (H_2O) m^{-2} s^{-1}]	A [µmol (CO_2) m^{-2} s^{-1}]	C_i [µmol (CO_2) mol^{-1}]
WT	0.22 ± 0.05^a	3.06 ± 0.47^a	7.92 ± 1.03^a	304.09 ± 6.49^a
SIT	0.85 ± 0.27^b	6.75 ± 1.41^b	10.52 ± 0.67^b	340.84 ± 8.48^b
SITA	0.20 ± 0.01^a	2.83 ± 0.16^a	6.27 ± 0.33^c	314.98 ± 3.60^c

Since ABA is one of the main components inhibiting the stomatal opening and inducing the stomatal closure in WT (e.g. Acharya and Assmann 2009; Schroeder *et al.* 2001a), the lower endogenous ABA content in SIT plants could be a reason for the higher number of opened stomata in SIT compared to WT plants (Nagel *et al.* 1994). In addition, higher number of stomata (one of the morphological features of SIT mutants (Tal 1966, Nagel *et al.* 1994)),

can also contribute to the observed differences between SIT and WT. Higher steady-state value of A and C_i in SIT plants (Table 1) is probably a result of higher g_s. The observed effects of lower ABA content on the steady state GE parameters were suppressed by the addition of external ABA to SIT plants (SITA). In SITA, the values of g_s, E and C_i were restored to the WT level, although, A was lower. On the basis of obtained results it seems that an increase in endogenous ABA content can lower A both directly in chloroplasts and indirectly by the stomata closure. It was reported before that in the long term, ABA is also able to regulate the photosynthesis by changing photosynthetic pigment content (Agarwal *et al.* 2005; Pospíšilová *et al.* 2009), RuBisCO gene expression and activity (Pospíšilová *et al.* 2009; Popova *et al.* 1987; Seemann and Sharkey 1987), or PSII gene expression and function (Bray 2002; Maslenkova *et al.* 1995).

Local burning of the tip of the terminal leaflet of the upper leaves (L1, Fig. 9) lead to fast (starting within 3 min after burning) systemic decline in GE parameters (firstly observed in g_s, Fig. 22) detected in the terminal leaflet of the nearest leaf underneath (L2, Fig. 9) in WT. This is in agreement with the data published elsewhere (e.g. Hlaváčková *et al.* 2006; Kaiser and Grams 2006). However, to our knowledge, the systemic GE reaction to local burning in SIT and SITA plants was detected for the first time.

There are differences in the shape of GE reactions depending on the endogenous ABA content. After reaching the minimum, the GE parameters in WT and SITA plants slightly rose to a new steady-state level, different from that observed before burning (Fig. 22). However, in SIT plants, the GE parameters rose only very slowly from the local minimum and no subsequent steady-state levels were reached within 1 h after burning (Fig. 22). These results showed the possible effect of overall endogenous ABA content (inhibition of stomata opening and/or photosynthesis) during later phases of GE reaction to local burning.

It seems from our results that the fast systemic decrease in A in WT, SIT and SITA plants after the burning is not only the consequence of stomatal closure (which begin to decline first). If stomatal conductance was the only limiting factor of A, the parameters A and C_i would follow the time course of g_s after local burning. However, C_i in SIT plant almost did not react systemically (Fig. 22H), while the systemic reaction of g_s and A was clearly visible. Therefore, it seems that A decline after local burning partly independently to g_s.

Since there was observed a fast systemic reaction in GE parameters, some signal (triggered by local abiotic stress – local burning) has to spread from the site of stimulation to the site of reaction and trigger the reaction in GE. A suggestion that a signal of electrical origin can affect photosynthesis has been presented by many authors. For instance, it has been shown that a local stimulation of *Mimosa pudica* L. plants by a flame causes changes in membrane potential, followed by a transient decline in PSII quantum yield, CO_2 uptake and g_s (Kaiser and Grams 2006). Besides, electrical signal generated by the change of the root medium has been reported to induce various responses in the A and E in willow leaves (Fromm and Eschrich 1993). Moreover, even direct electrical stimulation can lead to transient local and systemic responses in GE in tomato plants (Peña-Cortés *et al.* 1995). There are even some evidences that the electrical signal can be triggered or affected by ABA. For exemple, replacing the root medium (mannitol) by the solution of ABA has been shown to induce fast changes in root and leaf membrane potential of willow plants (Fromm *et al.*. 1997; Fromm and Eschrich 1993). Herde *et al.* (1998) have observed a different shape of electrical signals

in SIT mutants compared to WT plants after local burning. Also locally, on the cellular level, the reception of ABA by stomata guard cells triggers a signaling pathway leading to a change in the membrane potential of the guard cells (Konrad and Hedrich 2008; Schroeder *et al.* 2001a). To explore more deeply this phenomenon, the measurement of SEP aside the measurement of GE has been performed in reaction to local burning on all three kinds of tomato plants (WT, SIT and SITA).

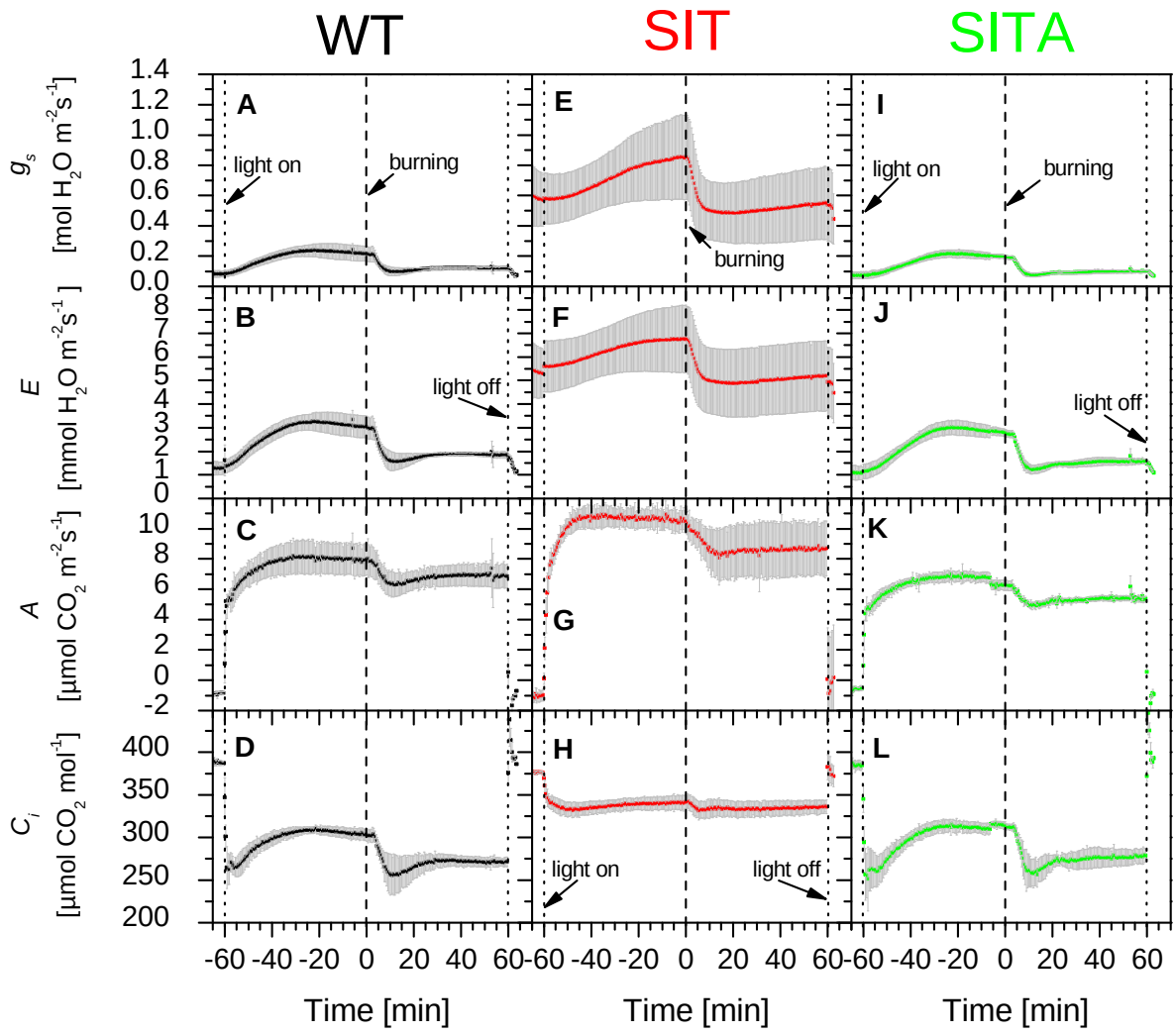

Fig. 22. Systemic response in gas exchange parameters (g_s, E, A and C_i) detected on leaf L2 (see Fig. 9) of WT, *sitiens* (SIT) and SIT pre-treated by endogenous ABA (SITA) tomato plants after local burning of the leaf L1 (0 min, central dashed lines). One hour before burning of the leaf L1, the light was switched on (left dotted lines) and 1 h after burning it was switched off (right dotted lines). Mean curves and SD are shown, n ≥ 4. Adopted with slight modifications from paper III.

Local burning induced both local and systemic changes of SEP in all measured leaves (L1-L4) of WT, SIT and SITA plants (Fig. 23). The onset of SEP changes (begin of the increase to local maximum) was observed within 0.5 min (in local leaves, L1) or within 1.5 min (in systemic leaves, L2-L4) after burning. SEP changes in leaves L3 and L4 were analogous to L2. The local SEP maximum in systemic leaves was detected significantly later

after burning than in local leaves (data not shown). The maximal amplitudes of SEP decreased with increasing distance from the burned site (data not shown).

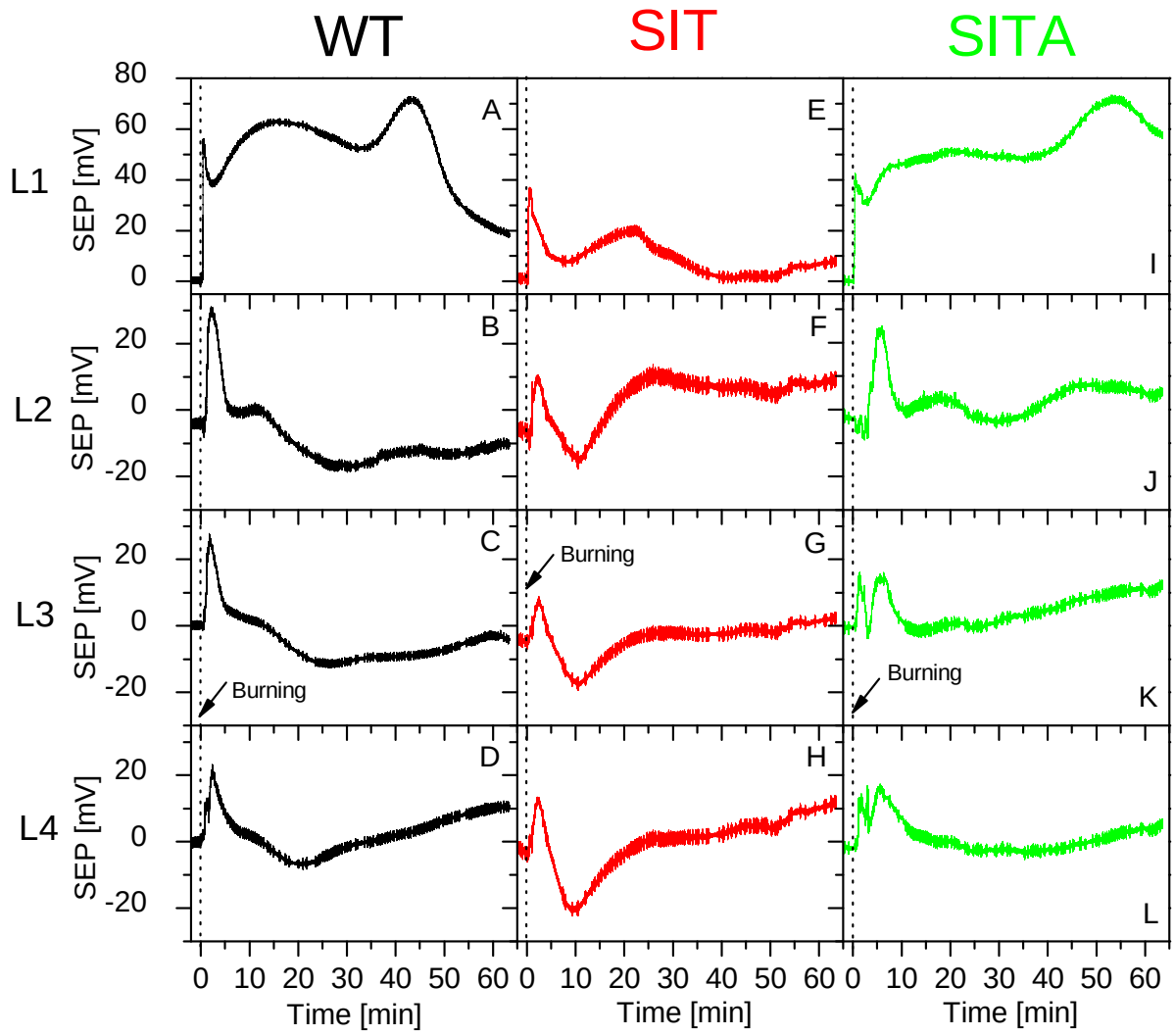

Fig. 23. The time course of the surface electric potentials (SEP) detected locally (leaf L1) and systemically (L2-L4) on WT, *sitiens* (SIT) and SIT pre-treated by endogenous ABA (SITA) tomato plants after local burning of the tip of the terminal leaflet of L1 (left dotted line). Representative curves are shown, $n \geq 4$.

The common feature of the time courses of SEP recorded systemically in WT plants was a fast increase to a local maximum (depolarization) and a subsequent decrease (repolarization) continuing through a shallow minimum to a new resting potential level (Fig. 23). Interestingly, different systemic SEP time courses were observed after local burning in SIT leaves (Fig. 23). In this case, the increase in SEP level to a local maximum was followed by a decrease to a sharp local minimum and then a significant increase combined with oscillation led to a new resting potential level (Fig. 23). With SITA plants, the observed features of SEP time course were remarkably similar to WT plants within about 1 h after burning.

Detected SEP changes correspond to changes in extracellular electrical potential, which reflects ion imbalances in the apoplast (Ilík *et al.* 2010). The wavelike form of the signal (Fig. 23) and the gradual decrease in the amplitude with increasing distance from the burned leaf are considered as typical features of variation potential (VP, e.g. Stanković *et al.* 1998).

The SEP time courses observed after local burning of tomato WT leaves correspond to the previously reported ones (Ilík *et al.* 2010; Vian and Davies 2006). Minor differences in SEP courses compared to those published by Vian and Davies (2006) can be caused by different detection site (petioles) or different length of the stimulus (1.5 s). It has been already shown that in sunflower, SEP signals can vary depending on the site of the measurement (leaves, petioles) (Dziubińska *et al.* 2001).

Since the time courses of SEP in SIT plants differed from those in WT and SITA plants (Fig. 23), our results indicate that the global features of SEP time courses can be affected by endogenous ABA content. This is supported by the fact that the SEP changes observed in WT and SITA plants were similar (Fig. 23). The effect of ABA on SEP has been described previously by Herde *et al.* (1998), who has observed different features in the time courses of electrical potential in veins of WT and SIT tomato plants. According to the explanation of Herde *et al.* (1998), ABA can inhibit the closure of Ca^{2+} membrane channels (possibly involved in the generation of membrane potential) during the reaction to the heat stimulation. ABA may also down-regulate H^{+}-ATPase and control other ionic channels involved in the generation of the membrane potential (Herde *et al.* 1998). It is also important to take into account that ABA (JA as well) can be indirectly involved in gene expression (e.g. *pinII* gene (Herde *et al.* 1999) or pin mRNA and proteins (Doares *et al.* 1995)) and that eventual changes in the concentration of some membrane transport proteins could also affect the detected electrical signals (Herde *et al.* 1998). Since VP has been suggested to reflect the local changes in membrane potential induced by a passage of a hydraulic signal (Malone and Stanković 1991; Mancuso 1999), ABA can influence SEP also by the modulation of plant hydraulic properties, i.e. via the regulation of aquaporin activity (Parent *et al.* 2009).

Our results indicate that, on the contrary to ABA, JA has relatively small (if any) effect on SEP. This is based namely on the fact that even though SITA plants accumulated substantially higher amount of JA after burning than WT (see below, Fig. 25), the SEP signals observed in SITA and WT were very similar (Fig. 23).

The effect of electrical signal on GE has been previously described in tomato, maize, poplar, mimosa or tobacco plants (Gramms *et al.* 2009; Hlaváčková *et al.* 2006; Kaiser and Grams 2006; Lautner *et al.* 2005; Peña-Cortés *et al.* 1995). The timing of SEP changes and changes in GE parameters in distant leaves after burning suggests that SEP might be able to somehow trigger stomata closure. Namely, the first systemic SEP changes (0.5 - 1.5 min) and SEP local maxima were followed by the beginning of systemic changes in GE parameters (after about 3 min). Since the stomatal guard cells lack plasmodesmata (which ensure an electrical connection between adjacent cells) stomata cannot react directly to SEP changes. However, Kaiser and Grams (2006) have proposed that stomata of mimosa can move hydropassively (by changing turgor of epidermal cells) in reaction to electrical signals generated after local burning. Stomatal closure could be affected not only by the electrical signal (VP) itself, but also by the preceding hydraulic signal (heat induced increase in

hydraulic pressure (Malone and Stanković 1991)). Also changes in photosynthesis can be triggered by the SEP changes as reported in (Gramms *et al.* 2009; Kaiser and Grams 2006).

It is possible that SEP might be able to affect the later (after about 15 min) phases of GE reaction after local burning. Both, the time course of GE reaction and SEP in SIT differed from those in WT and SITA plants. This suggests that SEP and/or ABA can somehow affect the later phases of GE reaction.

It has been suggested that ABA and JA are involved in signaling pathways leading to GE reaction to local burning (e.g. Herde *et al.* 1997; Hlaváčková *et al.* 2006; Hlaváčková and Nauš 2007). It was shown that a fast systemic ABA accumulation (during 15 min, followed by a decrease of ABA concentration) in tobacco plants occurs together with a decrease in g_s, *E* and *A* after local burning (Hlaváčková *et al.* 2006). Since in our case the ABA accumulation was observed only locally in the burned tomato leaf (leaf L1; 85 min after burning), ABA probably participated only in the local stomata closure and local decrease in *A*. The fact that the increased ABA content can lead to a decrease in GE parameters can be supported by the results of Herde *et al.* (1997). After the addition of ABA to petioles of detached tomato leaves they have observed a local decrease in *E* followed by a subsequent decrease in *A*. Our results showed that stomata in distant leaves began to close despite of low ABA systemic accumulation (see Fig. 22 for L2 and Fig. 24). This suggests that ABA is not the only one signal component in systemic signaling leading to GE reaction and some other components contribute (e.g. SEP, JA). However, the possibility that ABA could accumulate systemically in shorter time (before 85 min) as described for tobacco plant in (Hlaváčková *et al.* 2006) cannot be excluded and we still consider ABA as one of the signaling components which can trigger the GE reaction.

Another signaling component that spreads systemically after the local burning throughout the plant is JA, which can also be possibly involved in systemic changes of GE parameters. Local burning was described before to induce local and/or systemic accumulation of JA in tobacco plants (within 60 min (Hlaváčková *et al.* 2006)) and in WT and SIT tomato plants (after 6 h (Herde *et al.* 1999, Herde *et al.* 1996)). Here, we report fast local and systemic JA accumulation (within 85 min) in WT and SIT tomato plants (Fig. 25), which could be related to the observed decrease in GE parameters. The fact that the presence of JA in leaves can lead to the stomata closure was demonstrated by exogenous application of JA in barley (Popova *et al.* 1988). At the same time, the addition of JA to the petiole of WT tomato leaf led to a primary decrease in *E* (after 1 h) followed by a decrease in *A* (Herde *et al.* 1997). Moreover, JA was found to inhibit PSII electron transport and O_2 evolving reactions in isolated thylakoid membranes (Maslenkova *et al.* 1995) and to change the polypeptide content of isolated thylakoid (Maslenkova *et al.* 1992). JA can also inhibit biosynthesis and activity of enzymes involved in carbon fixation (RuBisCO (Popova and Valkinova 1988; Rakwal and Komatsu 2001)). Systemic accumulation of JA observed after burning can be considered as one of the factors participating in the GE response to the local burning.

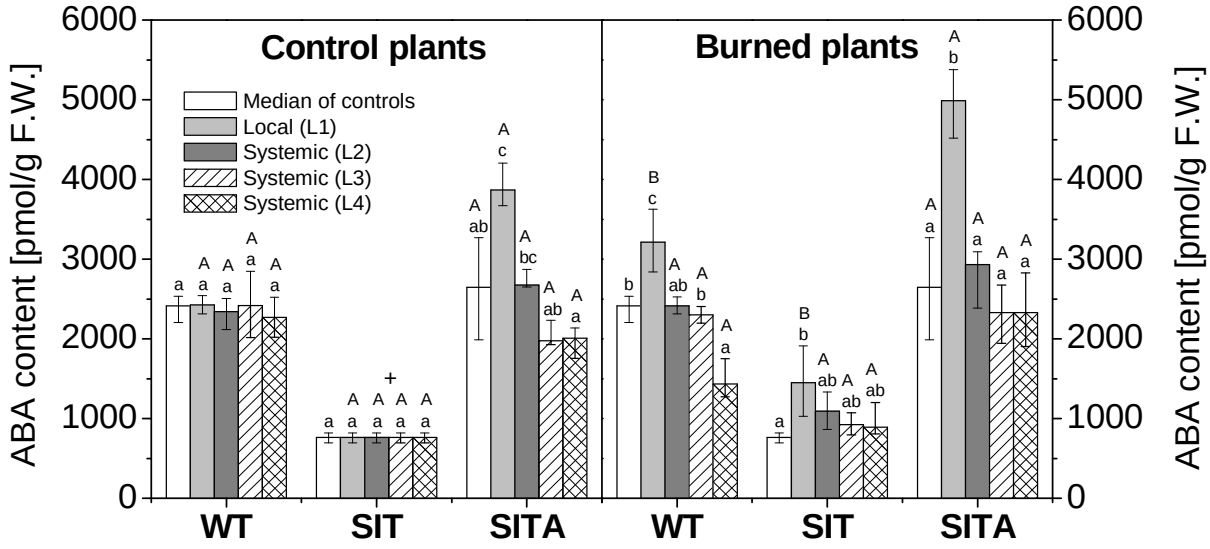

Fig. 24. Content of endogenous ABA in the local (L1) and in the systemic (L2, L3, L4) leaves of WT, *sitiens* (SIT) and SIT pre-treated by endogenous ABA (SITA) tomato control and locally burned plants at the time corresponding to 85 min after local burning. Medians are shown, error bars reflects lower and upper quartile, n ≥ 3. The medians of all leaves of control plants are represented by white-colored columns. Different uppercase letters denote significant differences between one column (L1- L4) of the burned plants and the same column of control plants within the same plant type (WT, SIT or SITA). Different lowercase letters indicate significant differences among columns of one plant type of control or burned plants. Mark + indicates that the median of ABA content of all SIT leaves was used instead of the ABA content of individual leaves (due to technical reasons, it was not possible to detect the ABA content in individual leaves). Adopted with slight modifications from paper III.

The JA-mediated GE response in SIT tomato plants has been studied by Herde *et al.* (1997). When the petioles of SIT leaves were incubated in JA, the *E* (reflecting g_s) decreased only a little. However, when SIT petioles were pre-treated with ABA, the stomata closed in response to JA. A decrease in *A* was observed in both cases (Herde *et al.* 1997). This suggests that the JA-mediated stomata response requires the presence of ABA in tomato plants, which is present also in SIT mutant although in a lower amount (Fig. 24). Hlaváčková *et al.* (2006) proposed that coordinated action of ABA and JA is involved in the regulation of short-term systemic GE responses of tobacco plants. The authors observed a correlation between the changes of ABA and JA content (ABA accumulation within 15 min followed by JA accumulation within 60 min) and GE parameters after local burning. Since the endogenous levels of JA in their case started to increase when endogenous levels of ABA reached the peak (after 10 min), it supports a view that ABA accumulation may stimulate JA accumulation (Peña-Cortés *et al.* 1995; Seo *et al.* 1997).

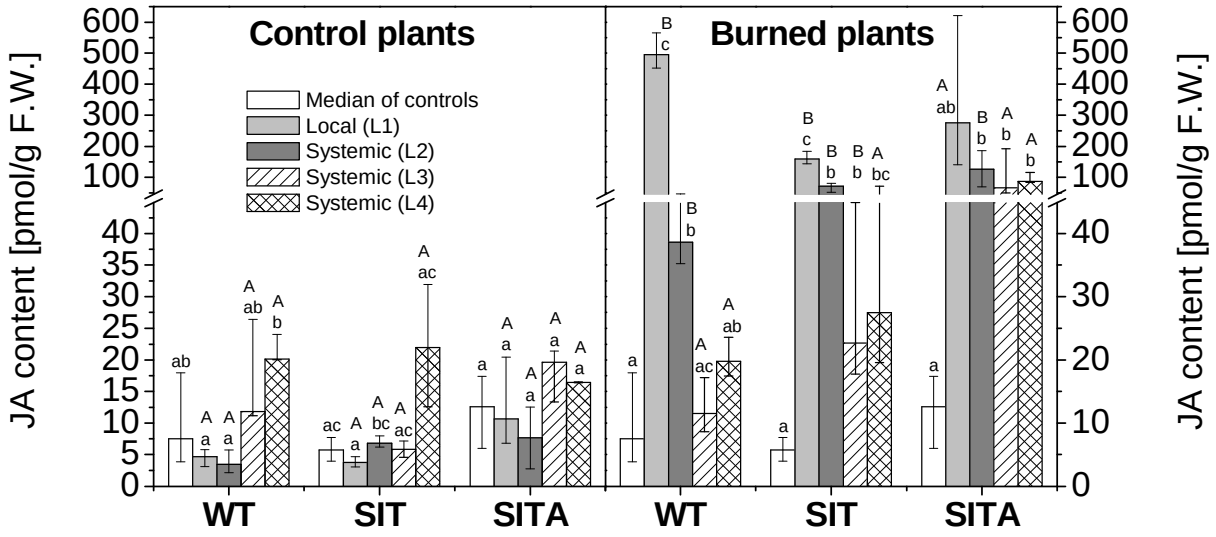

Fig. 25. Content of endogenous JA in the local (L1) and in the systemic (L2, L3, L4) leaves of WT, *sitiens* (SIT) and SIT pre-treated by endogenous ABA (SITA) tomato control and locally burned plants at the time corresponding to 85 min after local burning. For details and meaning of letters, see legend of Fig. 24. Adopted with slight modifications from paper III.

Our results obtained with the SIT mutant lead to a suggestion that JA can partly compensate for the shortage of ABA in the GE reaction to burning. This is evidenced by a higher systemic level of JA in SIT after burning (Fig. 25). The higher systemic accumulation of JA after burning in SIT compared to WT seems to be the property of SIT mutant (see Fig. 25 for SITA). If it is assumed that ABA is the most important hormone regulating dominantly the JA effect on GE (e.g. Herde *et al.* 1997), then this dominancy was strengthened in our case in SIT plants (i.e. lower ABA content in SIT leaves compared to WT leaves leads to an increased JA effect on GE). This hypothesis is supported by the finding that the content of ABA was similar in SITA and WT but the content of JA in SITA was several times higher than in WT due to the suspected effect of strengthened dominancy. It is interesting to note that the newly described systemic accumulation of JA in SITA did not importantly influence the GE parameters. It can be speculated that the amount of JA was well above the concentration that "saturates" its effect on GE parameters. At the same time, the proposed inhibitory effect of JA on photosynthesis (Maslenkova *et al.* 1995; Maslenkova *et al.* 1992; Popova and Valkinova 1988; Rakwal and Komatsu 2001) was not observed in our experiments.

In summary, our results obtained on WT, SIT and ABA-pre-treated SIT tomato plants indicate that the endogenous ABA content defines the steady-state of GE and affects the shape of the GE and SEP changes appearing after local burning. Coordinated action of ABA, JA and SEP is needed in triggering the GE response to local burning what has already been suggested (e.g. Hlaváčková *et al.* 2006). The shortage of ABA (which is a signaling molecule triggering the GE reaction) in the SIT mutant is partly compensated for by a pronounced accumulation of JA in response to burning. The capability to compensate for the ABA shortage by higher JA accumulation in reaction to local burning in SIT plants is maintained

even in SIT plants endogenously supplied with ABA. The results also suggest that the level of accumulated JA itself directly affects neither SEP nor GE parameters. A model of signaling pathways participating in the induction of the changes in GE parameters in the response to local burning is proposed (Fig. 26).

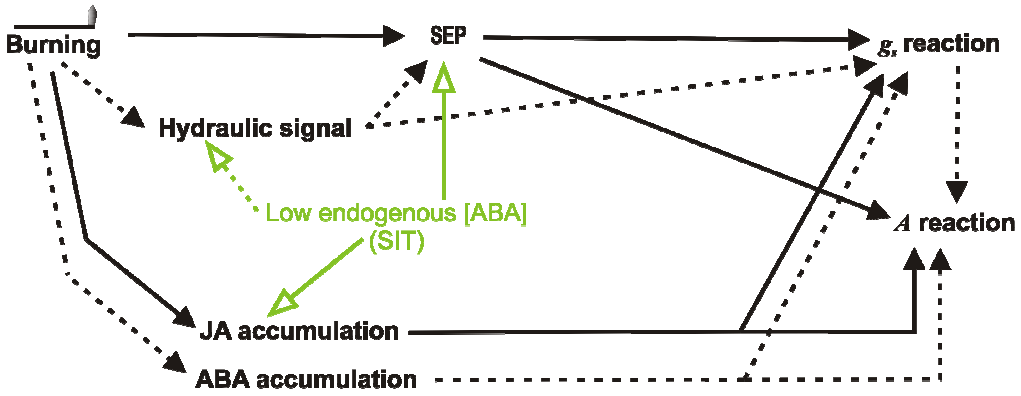

Fig. 26. Model of proposed signaling pathways (full-headed arrows) participating in our case in the reaction of gas exchange parameters (g_s and A) after local burning. The effect of low endogenous ABA concentration in SIT mutant on signal pathways is shown and marked by green white-headed arrows. Dashed lines describe the hypothetical signaling pathways (see discussion section). Adopted with slight modifications from paper III.

The results described in this section (chapter 5.2) have been presented in paper III.

5.3 Biotic stress signaling

5.3.1 The role of cytokinins in plant-pathogen interaction (paper IV)

Pathogen infection is one of the biggest challenges to explore in contemporary agriculture, due to the fact that various widespread pathogens destroy a big part of plant production. There is a big effort to clarify the signaling pathways leading to plant defense action against pathogens. One of many processes triggered by a pathogens action on plant is the hypersensitive response. During the hypersensitive response, plant cells near the infection site are rapidly killed by plant, depriving the pathogen of nutrients and preventing its spread. However, the signals participating on the triggering the hypersensitive response are still poorly understood.

Cytokinins ensure a wide range of functions in plant–pathogen interactions. Some pathogen infections have been reported to cause changes in cytokinin levels in the infected plants. The change (increase) in cytokinin level can then facilitate disease development. For example, *Agrobacterium tumefaciens*, the causal agent of crown gall disease in a number of dicot species (including some important crops), inserts T-DNA (transfer DNA) from a Ti (tumour-inducing) plasmid into the plant genome. This T-DNA carries a gene necessary for the key cytokinin biosynthetic enzyme IPT (isopentenyl transferase), and genes for auxin and opines biosynthesis. While high cytokinin and auxin levels convert the transformed cells into rapidly growing tumours, opines secreted from the transformed cells serve as a source of carbon and nitrogen for the *Agrobacterium* (Chilton *et al.* 1977). On the other hand, there are several lines of evidence linking cytokinins to the induction of cell death (e.g. Šámalová *et al.* 2005).

To explore the potential roles of cytokinins in the hypersensitive response to pathogen attacks, transgenic tobacco plants *CaMV35S* > GR > *ipt* (pOp6-ipt/LhGR-N, lines 303 and 307; Šámalová *et al.* 2005) containing gene *ipt* and the corresponding WT (SR1) have been selected. The *ipt* gene expression in transgenic plants can be induced by DEX. The *ipt* gene expression causes an increase of endogenous cytokinin level in transgenic plants. This has been concluded from the detected dramatic increases in the levels of both cytokinin bases and their ribosides and ribotides within first day after DEX (20 µM) treatment (1 DAT) in leaves (without necrotic lesion, see below) of 5-week-old transgenic tobacco plants. These increases of cytokinin amount leveled off over the following 24 h (2 DAT), reaching values about four orders of magnitude higher than those observed in non-activated samples. In general, following *ipt* activation, the cytokinin pool was consistently somewhat higher in line 303 than in line 307, which is consistent with the stronger phenotypic response (see below) observed in line 303. Lower cytokinin accumulation was observed after treatment with DEX of lower concentration (1 µM). The endogenous level of cytokinin was measured by co-authors of paper IV and their results are not shown in the graphical form.

Consequently, *ipt* activation by DEX (20 µM) triggers distinct phenotypes in leaves depending on their developmental stage. Slightly translucent zones appeared on expanded horizontal leaves around 50 h after exposure to DEX. Within the first 4 d after *ipt* activation, older leaves that had already achieved a horizontal orientation but were still growing developed necrotic lesions without any prior or accompanying chlorosis, wilted, and

eventually died in the more responsive line 303. This DEX effect was concentration dependent. Since the chlorophyll content (measured by co-authors of paper IV, data not shown) in leaves did not change much and no chlorosis have been visible, it seems that the leaves did not get senescent and therefore the responses observed in our work were more similar to the hypersensitive response – a type of cell death associated with pathogen infection. Young, upward-oriented leaves of activated transgenic plants developed chlorosis but only rarely formed lesions. This seems to be in contradiction to general consideration that cytokinins are positive regulators of chloroplast biogenesis and function (Brenner *et al.* 2005; Černý *et al.* 2011; Lochmanová *et al.* 2008). Nevertheless, chlorosis has been observed in some transgenic plants (e.g. maize) that overproduce cytokinins, however, just like an accompaniment of stress conditions (e.g. nutrient deprivation; Robson *et al.* 2004). Thus, the chlorosis in young leaves of *ipt*-expressing plants is unlikely to result directly from the effects of cytokinins. The chlorosis might result from the decreased assimilate availability due to extensive damage in older leaves and/or reductions in the uptake and transport of mineral nutrients from the soil. In addition to lesion formation, growth of the roots decreased by 25% in plants with an increased cytokinin level at 4 DAT (measured by co-authors of paper IV, data not shown). Cytokinins were before reported to negatively affect the uptake of mineral nutrients by roots (including nitrate, ammonium, phosphate and iron; reviewed in Rubio *et al.* 2009). This is consistent with the observation that the chlorosis only became apparent 4 DAT. Nevertheless, our data cannot exclude the possibility that increased cytokinin levels *per se* caused the chlorosis in young leaves directly.

The hypersensitive-like nature of reported cytokinin response is further demonstrated by the observed changes in various biochemical and molecular parameters prior to the appearance of the first islets of dead cells in affected leaves. One of the early consequences of the increased cytokinin levels in transgenic plants was an increase in the levels of chloroplast-associated ROS (hydrogen peroxide), which was accompanied by the oxidative membrane damage, increases of levels of stress hormones (ABA, JA, and SA) and PR transcripts (PR-1b (pathogenesis-related protein 1b) and PR-Q (acidic phosphatase)), and decreases in the abundance of transcripts related to photosynthesis (CAB (chlorophyll a/b-binding protein), FNR1 (ferredoxin: NADP oxidoreductase) VDE (violaxanthin de-epoxidase)). Cited parameters were measured by co-authors of paper IV, data not shown.

Coming out from our data (the amount of cytokinin and leaf necrosis appearance depended on used DEX concentration) and the results published elsewhere (high doses of exogenous cytokinin increase the levels of ROS in tobacco BY2 cells (Mlejnek *et al.* 2003) and milder increases in cytokinin levels stimulate enzymes involved in ROS detoxification (Barna *et al.* 2008; Zavaleta-Mancera *et al.* 2007), we concluded that cytokinins have two separate and concentration-dependent effects on the cellular ROS pool.

It has been shown that hydrogen peroxide plays a role in responses to biotic (either abiotic) stress. Analysis of transcripts reported by Vandenabeele *et al.* (2003) demonstrated that hydrogen peroxide production is closely linked to the levels of stress hormones such as SA, JA and ethylene. Cytokinins were reported to modulate SA signaling to enhance resistance against *Pseudomonas syringae* (Choi *et al.* 2010), and a system analysis revealed a synergism between cytokinin and SA in plant disease networks (Naseem *et al.* 2012). However, cytokinin has not been reported to directly stimulate either SA or JA accumulation.

On the contrary, transgenic tobacco explants that were propagated *in vitro* and overproduced cytokinins due to the *ipt* expression driven by a promoter of the RuBisCO small subunit showed decreased SA levels relative to those in an untransformed control (Schnablová *et al.* 2006). Treatment of poplars with exogenous cytokinins did not affect JA levels in undamaged leaves. However, cytokinin priming increased the wound-inducible accumulation of JA during insect attacks on the poplar leaves (Dervinis *et al.* 2010). It thus seems that increases in cytokinin amount in our plants after DEX activation cause a production of hydrogen peroxide which mediates the increase in the levels of SA and JA.

The key component of systemic defense responses of plants to pathogen infection is the induction of PR protein expression. SA-mediated signal transduction cascade (reviewed by van Loon 1997) may be responsible for the induction of PR protein expression. Furthermore, a number of PR proteins are encoded by late cytokinin response genes (Rashotte *et al.* 2003). Taking this into account, it is possible that our results showing the activation of the PR-1b and PR-Q could be direct consequence of the cytokinin signaling.

Hypersensitive response, ROS levels and photosynthesis are intertwined at several levels (reviewed Kangasjärvi *et al.* 2012). There are evidences that chloroplastic ROS production contributes to plant immunity (Karpinski *et al.* 1999; Vandenabeele *et al.* 2004). ROS can be produced within chloroplasts (photosynthetic electron transport reactions, Hideg *et al.* 2002; photoactivation of free chlorophyll). However, high level of ROS inhibits photosynthesis (Vandenabeele *et al.* 2003). Our results showed downregulation of CAB, FNR1 and VDE transcripts related to photosynthesis within 43 h of *ipt* activation, which might indicate a reduction in photosynthetic activity. Since downregulation of related genes was observed also following the activation of *ipt* gene in *A. thaliana* (Hoth *et al.* 2003) it can be related to cytokinin signaling. Together with the fact that chlorophyll levels remained unchanged, the downregulation of CAB transcript levels might indicate the presence of free chlorophyll in the chloroplasts. This could contribute to ROS generation in the tobacco leaves that developed lesions following *ipt* activation. Possible reduction of photosynthetic activity was supported by the analyses of chlorophyll fluorescence (measured by co-authors of paper IV, data not shown) and CO_2 assimilation (Fig. 27A) following *ipt* activation.

A light-induced increase in the *A* was inhibited (Fig. 27A) in DEX activated plants, which indicated downregulation of RuBisCO activation. Moreover, electron transport through PSII and PSI was inhibited, there was an increase in the amount of energy dissipated at the donor side of PSI due to reduced electron donation and there was an increase in the dissipation of excitation energy by heat emission, as demonstrated by an increase in NPQ (Fig. 28). The need to dissipate excess chlorophyll excitation energy via NPQ reflects a decrease in electron consumption by CO_2 assimilation resulting from the downregulation of RuBisCO activity, together with a decrease in CO_2 availability due to reduced stomatal conductance (Fig. 27B).

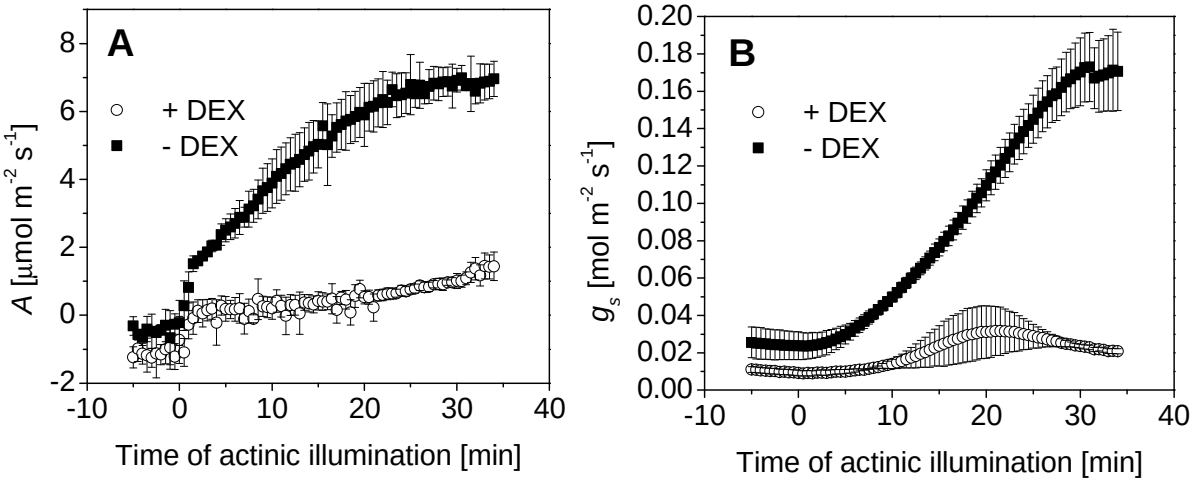

Fig. 27. Changes in A (A) and g_s (B) induced by light. Five-week-old plants of *CaMV35S* > *GR* > *ipt* line 303 were treated with 50 ml of 20 mM dexamethasone (+DEX) or 0.096% ethanol (–DEX), and both g_s and A were determined 67 h (3 DAT) after DEX application. Means and SD are shown (n = 3). The photosynthetic photon flux density of actinic light was 330 µmol photons of PAR m^{-2} s^{-1}.

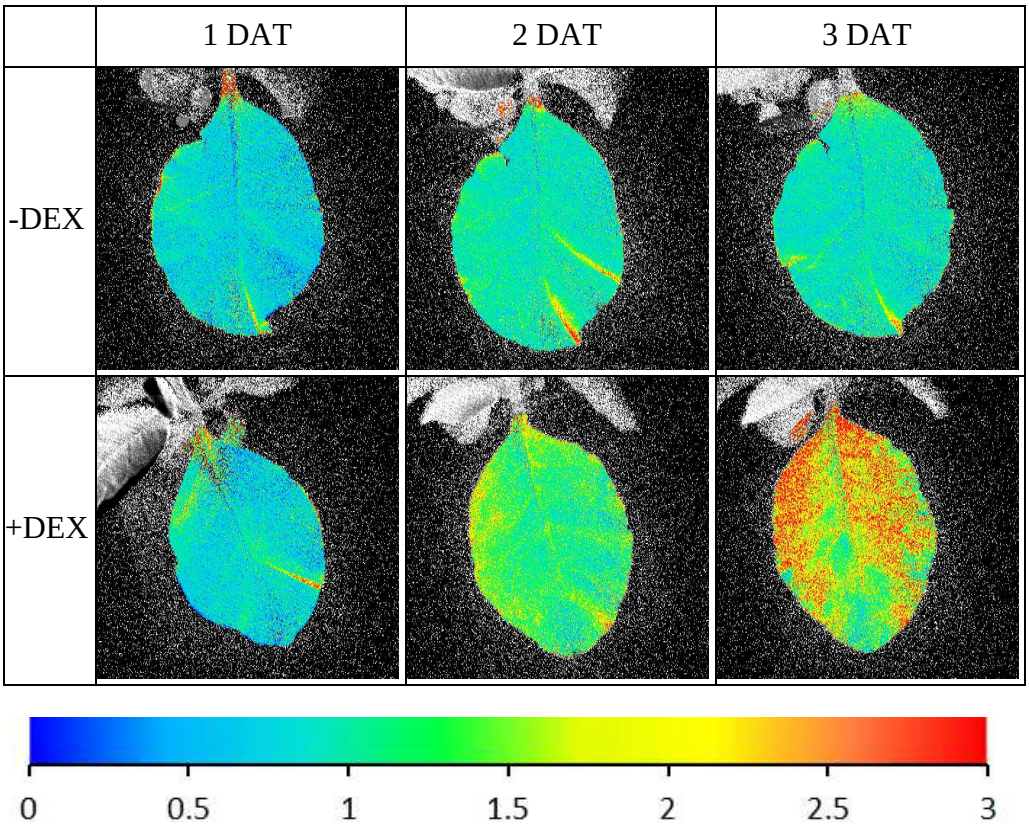

Fig. 28. Images of NPQ in false color in leaves of *CaMV35S* > GR > *ipt* plants following *ipt* activation. Plants of line 303 were treated with 50 ml of 20 mM dexamethasone (+DEX) or 0.096% ethanol (–DEX). NPQ was determined under steady-state conditions (10 min of actinic light, 100 µmol photons of PAR m^{-2} s^{-1}) on 1, 2 and 3 DAT.

Decreases in CO_2 assimilation reportedly lead to an excess of excitation energy, the formation of ROS and hypersensitive-like cell death (Liu et al. 2007a; Mateo et al. 2004). However, the hypersensitive-like reaction observed in our system induced by cytokinis did not seem to be mainly contributed by the decrease in CO_2 availability. This was tested by treatment of leaves with lanolin paste (as described in Mateo et al. 2004) which itself was not sufficient to trigger this response on a time scale of 4 d.

Microscopic observation of stoamatal closure corresponded with decrease of stomatal conductance (Fig. 27B). Cytokinins are reported to promote stomatal opening and decrease sensitivity to ABA, which can cause stomatal closure. However, the responses to both hormones are often dependent on their abundance (reviewed by Acharya and Assmann 2009). In our system, cytokynin level elevation is accompanied by the ABA level increase.

Similar result has been previously observed in tobacco plants overexpressing the *Sho* gene (which codes for a cytokinin biosynthetic enzyme from *Petunia hybrida*, Polanská *et al.* 2007). The elevated ABA levels in these plants correlate with stomatal closure. The occurrence of cross-talk between cytokinin and ABA on the metabolic level can be further supported by an analysis of transgenic tobacco overexpressing *Arabidopsis* cytokinin oxidase/dehydrogenase AtCKX3. In these plants, the decrease in cytokinin levels caused by AtCKX3 activity was accompanied by a decrease in ABA content (Polanská *et al.* 2007). Since our observation showed both ABA and hydrogen peroxide level increase, it seems that these two molecules together are probably responsible for observed stomatal closure following *ipt* activation in *CaMV35S* > GR > *ipt* tobacco plants. The stomatal closure probably represents an insufficient attempt to prevent the water losses that accompany elevated cytokinin levels. However, the stomatal closure also protects the plant against pathogen invasion (Melotto *et al.* 2006) and so would be an expected response to observed expression of pathogen-related hormones.

In conclusion, activating the *ipt* gene by DEX in transgenic tobacco plants leads to dramatic increase of endogenous cytokinin level. Increased cytokinin levels cause a hypersensitive-like response in tobacco leaves. Obtained results lead to a suggestion that increases in chloroplastic hydrogen peroxide levels orchestrate the molecular processes underpinning the hypersensitive-like response. The hypersensitive-like response includes inhibition of photosynthesis, increases in stress hormone levels, oxidative damage of membranes and stomatal closure. Altogether, the data indicate that plant defense against pathogen attack can be mediated by cytokinins. This conclusion is consistent with the results of recent dynamic modeling studies and systems analyses performed by Naseem *et al.* (2012). There were identified multiple cytokinin-mediated regulatory interactions in plant disease networks.

The results presented in the chapter 5.3.1 have been presented in paper IV.

5.3.2 The role of plant resistance/susceptibility in reaction to patogen infection and the effect of heat shock pre-treatment (paper V)

As described above, the endogenous cytokinin level increase can cause a hypersensitive-like response in tobacco plants. An infection of plant by a pathogen which would be able to modify *ipt* gene expression that is responsible for elevated endogenous cytokinin level can lead to photosynthesis inhibition. However, inhibition (reduced rate) of photosynthesis can occur also in cases when plant is infected by a different type of biotrophic pathogen (Akhkha *et al.* 2003; Chou *et al.* 2000; Huang 2001; Gordon and Duniway 1982; Lebeda *et al.* 2008; Moll *et al.* 1995; Roberts and Walters 1988; Sabri *et al.* 1997; Walters and McRoberts 2006; Wright *et al.* 1995a) (e.g. powdery mildew) that is not able to affect *ipt* gene expression.

Photosynthetic processes inhibition caused by powdery mildew infection can be of various origins. Powdery mildew can lower the supply of light energy due to covering of the leaf surface by mycelium (Yurina *et al.* 1996) and can inhibit CO_2 influx due to stomata closure (Gordon and Duniway 1982). It can also affect photosynthesis indirectly, via pathogen-induced changes in source-sink relations and nutrient remobilization towards infection sites (Fotopoulos *et al.* 2003; Kocal *et al.* 2008; Scholes *et al.* 1994; Wright *et al.* 1995b). This can result in the inhibition of the Calvin cycle (Gordon and Duniway 1982; Chou *et al.* 2000; Scholes *et al.* 1994; Wright *et al.* 1995a) and in the inhibition of photosynthetic light reactions in thylakoid membranes (Magyarosy *et al.* 1976; Moll *et al.* 1995). It has been suggested that the down-regulation of photosynthesis during powdery mildew infection is caused by pathogen-induced higher activity of cell-wall invertase, which leads to the accumulation of hexose sugars (Scholes *et al.* 1994; Swarbrick *et al.* 2006; Wright *et al.* 1995b) and subsequently to feed-back inhibition of the expression of some photosynthetic genes (especially *cab* and *rbcS* - RuBisCO small subunit) (Chou *et al.* 2000; Fotopoulos *et al.* 2003; Scholes *et al.* 1994; Swarbrick *et al.* 2006). The increase in both cell-wall invertase activity and hexose content is preferentially observed in directly invaded regions of the infected leaves (Chou *et al.* 2000; Roberts and Walters 1988; Swarbrick *et al.* 2006). In our work, we focus on investigation the effect of economically important powdery mildew fungus *O. neolycopersici* on photosynthesis in tomato leaves.

Mature leaves of two 8-10 weeks-old genotypes (susceptible - *Solanum lycopersicum* and moderately resistant - *Solanum chmielewskii*) were chosen for the study to reveal whether different degree of resistance/susceptibility could modify the rate and the extent of photosynthetic changes in infected leaves. Leaf GE, chlorophyll fluorescence and content of photosynthetic pigments were measured during 9 days after inoculation (DAI) by biotrophic powdery milldew fungus *O. neolycopersici* (the chlorophyll fluorescence and content of photosynthetic pigments were measured by co-authors of paper V and their results are not shown in graphical form).

A typical progress of the infection was observed in interaction of *O. neolycopersici* with susceptible tomato genotype. Conidia germination, appressorium and haustorium formation and superficial mycelium development proceeded up to 2 DAI on the adaxial leaf side of susceptible tomato genotype and almost the whole area of the infected leaves was covered with sporulating mycelium at 9 DAI. In susceptible genotype, the infection had only a minimal effect on parameter A_{Chl} (*A* expressed per a unit of Chl(a+b)) while the Chl(a+b) and

carotenoid content have not been affected by infection). Stomatal conductance g_s was decreased on 7 and 9 DAI. Gas exchange parameters are depicted in Fig. 29.

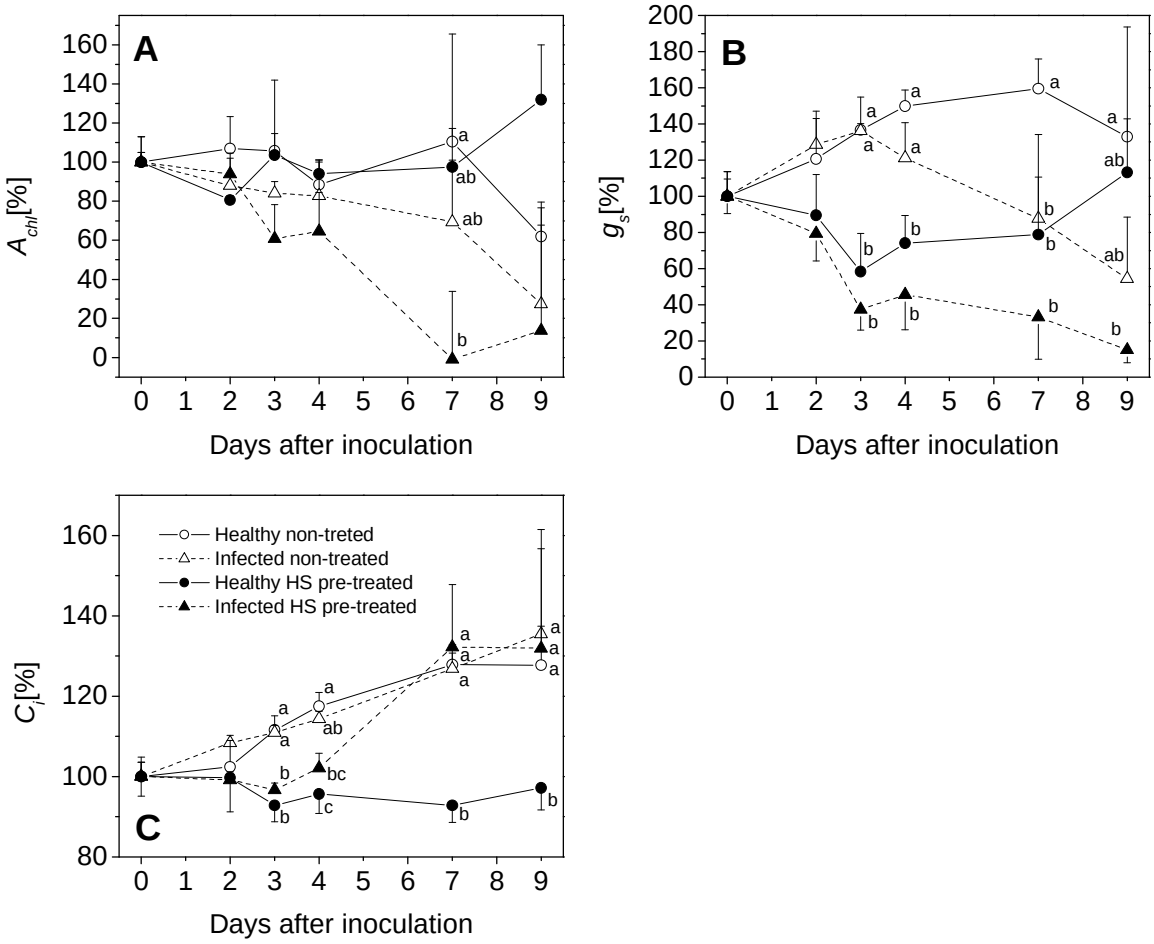

Fig. 29. The percentage changes in A_{chl} (A; 100 % = 3.9 pmol μg^{-1} s^{-1} for non-treated and 3.1 pmol μg^{-1} s^{-1} for HS pre-treated leaves), g_s (B; 100 % = 0.19 mol m^{-2} s^{-1} for both non-treated and HS pre-treated leaves) and C_i (C; 100 % = 217 μmol mol^{-1} for non-treated and 262 μmol mol^{-1} for HS pre-treated leaves) in healthy and powdery mildew (*O. neolycopersici*) infected leaves of the susceptible tomato genotype (*Solanum lycopersicum* L. cv. Amateur) during first 9 days after inoculation. Points at "0 dai" always represent leaves before inoculation and heat shock pre-treatment. Arithmetic means and SD are shown, n = 4; statistically significant differences are indicated by different letters.

The chlorophyll a/b ratio was decreased at 9 DAI in susceptible genotype. Parameters F_V/F_M and NPQ changed very slightly in the regions covered by a white coat of mycelium.

The results presented above show only negligible changes in photosynthetic parameters during 9 DAI despite the progressive development of the pathogen in infected leaves compared to non-infected leaves. This indicates that, despite the proposed activation of the cell-wall invertase by powdery mildew (Fotopoulos *et al.* 2003; Scholes *et al.* 1994; Swarbrick *et al.* 2006; Wright *et al.* 1995b), hexose sugars did not accumulate in the infected leaves during this period and thus no feed-back inhibition of photosynthetic processes was observed. This suggestion is supported by the results of Essmann *et al.* (2008), who showed that in tobacco plants with suppressed cell-wall invertase activity, carbohydrate accumulation

and inhibition of photosynthesis after pathogen attack were significantly reduced. Whether the cell-wall invertase was not activated in the susceptible tomato or whether it was activated but the pathogen demand for sugars was so high that they did not accumulate (as proposed e.g. in Walters *et al.* 2006) remains to be solved.

More pronounced inhibition of photosynthesis of susceptible genotype caused by *O. neolycopersici* could be expected in the later stages of the infection (about 14 DAI) when leaves covered by sporulating mycelium became progressively chlorotic and necrotic (premature senescence occurs most probably due to the activation of cell-wall invertase (Lara *et al.* 2004; Scholes *et al.* 1994; Swarbrick *et al.* 2006; Wright et al 1995b)). Most pronounced accumulation of hexoses (caused by the activation of cell-wall invertase) has been observed in these senescent regions of infected leaves where the consequent inhibition of photosynthetic processes takes place (Chou *et al.* 2000; Roberts and Walters 1988). In our case, the impairment of photosynthetic function in the leaf regions covered by the sporulating mycelium was indicated by a mild decrease in the F_V/F_M ratio and increase in NPQ, revealed by the fluorescence images at 9 DAI. The participation of the leaf shading by mycelium (Yurina *et al.* 1996) and pathogen-induced stomatal closure (Gordon and Duniway 1982) on assumed decrease of photosynthesis in the later stages of infection cannot be excluded. If mycelium which shades the leaf surface is responsible for decrease of photosynthesis, the photosynthetic apparatus should acclimate to lower intensity of PAR. This was, in our case, indicated by significantly lower chlorophyll a/b ratio at 9 DAI. Well documented effect of pathogen infection on stomatal movement (causes stomata closure; e.g., León *et al.* 1996; Prats *et al.* 2006) also observed in our experiments (as a decrease of g_s, Fig. 29B) should lead to lower C_i because the photosynthetic processes in chloroplasts were not inhibited, as suggested by the chlorophyll fluorescence data. However, high C_i was found in the infected leaves at the end of the experiment (Fig. 29C). This discrepancy could be explained by the infection-induced stimulation of respiration rate (e.g. Akhkha *et al.* 2003; Sabri *et al.* 1997; Scott and Smillie 1966) that could increase the CO_2 concentration in intercellular spaces.

It has been shown that the temperature can influence plant resistance (and/or susceptibility) in many host-pathogen interactions (Judelson and Michelmore 1992). High temperature (pre) treatment of plants has been reported to be followed by the induction of resistance (Judelson and Michelmore 1992; Schweizer *et al.* 1995; Vallélian-Bindschedler *et al.* 1998) as well as the increase in susceptibility to pathogen (Chamberlain and Gerdemann 1966; Chen *et al.* 2003). It has been proposed that high temperatures can influence plant resistance to powdery mildew by affecting the pathogen (e.g. inhibition of virulence gene expression); by affecting the host (e.g. induction of resistance genes); by affecting the interaction of pathogen/host (e.g. dysfunction of some virulence gene products) (Ge *et al.* 1998).

The existence of temperature-sensitive resistance genes has been confirmed by many authors (e.g. Judelson and Michelmore 1992). However, cultivars lacking the resistance genes showed the temperature-influenced resistance to powdery mildew (Ge *et al.* 1998). Therefore, probably other gene(s) or other factor(s) participate on the influence of the plant resistance to powdery mildew induced by higher temperature.

It was reported that host defense reactions (such as cell-wall reinforcement (Schweizer *et al.* 1995; Vallélian-Bindschedler *et al.* 1998) or changes in the relative rate and types of synthesized proteins (Chen *et al.* 2003; Schweizer *et al.* 1995)) can be modulated by heat shock (HS). Heat shock is also connected with the oxidative stress signaling and responses (for review see e.g. Wahid *et al.* 2007). Heat treatment of leaves leads to an induction of the oxidative burst that can be linked to the induction of plant resistance (Vallélian-Bindschedler *et al.* 1998).

The inhibition of photosynthetic processes induced by higher temperatures is well known (e.g. Camejo *et al.* 2005). Lower production of carbohydrates can be a consequence of this inhibition (Wahid *et al.* 2007). Additionally, an increase in the invertase activity has been recently reported to be induced by HS (Kaur *et al.* 2009).

The information mentioned above (the pathogen-induced changes in photosynthesis and in cell-wall invertase activity) can lead to a question whether the resistance (/susceptibility) of the host plants to pathogen can be affected by their heat stress pre-treatment via changing the supply of carbohydrates. To check the hypothesis that the changes in resistance/susceptibility to pathogen induced by HS could be associated with HS influence on host photosynthesis, the photosynthetic changes induced by pathogen were investigated in plants pre-treated with HS.

The control reaction to HS pre-treatment on healthy leaves has been observed firstly. Healthy tomato leaves exposed to HS pre-treatment (40.5 °C, 2 h) did not show any visual damage. Compare to HS non-treated plants, a temporary increase in carotenoid content (4 and 7 DAI), decrease in g_s (3, 4 and 7 DAI, Fig. 29B) and significantly lower C_i values from 3 DAI (Fig. 29C) were observed in HS pre-treated plants. The NPQ of HS pre-treated healthy leaves increased heterogeneously across the leaflets. However, the average values of NPQ of HS pre-treated leaves did not differ significantly from those of the non-treated leaves.

The progress of infection in HS pre-treated plants was similar as in the HS non-treated plants (the white mycelium started to form on the adaxial leaf surface) for first 2 DAI. However, chloroses appeared on leaf surface and developed progressively to necroses since 3 DAI. Since this effect was not observed in the mock control leaves (leaves inoculated without pathogen), both treatments (HS and infection) were needed to develop this reaction.

Despite the symptoms of pre-mature senescence (chloroses/necroses) development, an observed decrease in Chl(a+b) in HS pre-treated leaves was not significant compared to infected leaves (although, pigment content was determined in the leaf regions that were used for GE measurement – the regions without any necrotic lesions have been chosen). Significantly lower value of chlorophyll a/b ratio than in leaves of both non-infected variants was detected on HS pre-treated infected leaves at 9 DAI.

Besides, significantly lower value of rate of A_{Chl} has been observed at 7 DAI in leaves exposed to HS pre-treatment and infection (Fig. 29A) compared to HS non-treated healthy leaves. The more pronounced decrease of g_s value of HS pre-treated infected leaves was observed compared to g_s of HS nontreated infected plants from 3 DAI to 9 DAI (Fig. 29B). Value of C_i of HS pre-treated infected leaves was found to be lover 3 and 4 DAI compared to infected HS non-treated leaves (Fig. 29C).

Compared to only infected leaves, the decrease (even non-significant) of parameter F_V/F_M has been observed for HS pre-treated infected leaves. Since minimal fluorescence F_0 did not change, observed decrease in F_V/F_M ratio was mainly caused by a decrease in maximal

fluorescence value (F_M). The value of F_V/F_M was heterogeneous across the leaflet while the value of F_V/F_M was null in the necrotic parts (indicating total loss of photosynthetic functions). The average values of NPQ did not differ significantly from NPQ values in leaflets of all the others variants, however, the changes of NPQ were heterogeneous within infected leaflet (in the regions with a relatively high F_V/F_M the NPQ was increased whereas in the chlorotic and necrotic regions with low (or zero) F_V/F_M, NPQ was low (or zero) too.

The finding that the photosynthesis was inhibited (decrease of the A_{Chl}) seems to be connected with the occurrence of the symptoms of pre-mature senescence (chlorosis/necroses) induced by co-action of infection and HS pre-treatment (in the HS pre-treated plants without infection, neither chloroses/necroses nor significant inhibition of photosynthetic processes were found). Even though, the decrease of A_{Chl} had to be more pronounced due to the occurence of necrotic lesions. However, it seems that HS could represent a (mild) stress that activated some protective processes (van Loon 2000) in the tomato plants. In our results, this is indicated by the transient increase in carotenoid content and/or by higher NPQ (van Loon 2000) in HS pre-treated healthy leaves.

It is supposed that the induction of defense reactions to HS pre-treatment could lead to an increased demand for carbohydrates. The increased demand for carbohydrates together with the infection leads to the activation of the cell-wall invertase, hexose accumulation and consequent inhibition of photosynthesis (earlier than in the infected leaves without HS pre-treatment). Another possibility deals with the fact that the light intensity used during the HS pre-treatment was lower compared to the glasshouse conditions. A temporary decrease in the photosynthetic rate due to decreased PAR intensity leads to lower production of assimilates. This could deepen a disproportion between demand and supply of carbohydrates and could amplify the activation of the cell-wall invertase in the HS pre-treated and infected leaves.

Due to a great heterogeneity in the inhibition of photosynthesis (parameters F_V/F_M and NPQ) across the HS pre-treated infected leaf, we suppose that the extent of the assumed feed-back inhibition of photosynthesis (caused by the proposed hexose accumulation) was low in the green regions and higher in the chlorotic regions. In the green regions, Calvin cycle reactions were slowed down probably by a mild accumulation of hexoses (Chou *et al.* 2000, Scholes *et al.* 1994). Accumulated ATP led to the increase in ΔpH across thylakoid membranes and consequently to a NPQ increase. The increase in NPQ is believed to prevent the photosynthetic transport chain from over-reduction and damage (for review see Maxwell *et al.* 2000; Roháček 2002). The increase in NPQ has been reported also for the powdery mildewed leaves of wheat (Wright *et al.* 1995a).

On the contrary, we suppose more pronounced accumulation of hexoses (Chou *et al.* 2000; Roberts and Walters 1988; Swarbrick *et al.* 2006) in the chlorotic regions. This causes the impairment of Calvin cycle and also of the reactions in thylakoid membranes including PSII photochemistry. Calvin cycle inhibition leads to lower ΔpH across thylakoid membranes and consequently lower NPQ. In the chlorotic and necrotic regions of the leaves, the inhibition of PSII photochemistry could be caused by photoinhibition and consequent oxidative damage due to the over excitation of photosynthetic apparatus (Vlčková *et al.* 2006).

At the same time, an identical investigation of photosynthesis reaction to *O. neolycopersici* as described above for susceptible tomato plants was performed on moderately resistant tomato plants - *Solanum chmielewskii*. There has been observed a typical progress of powdery mildew interaction with moderately resistant tomato genotype. On the surface of infected leaves of moderately resistant genotype, a mild development of the mycelium was apparent 2 DAI. Since 2 DAI, the hypersensitive response (HR) was observed in form of small black spots (representing clusters of dead cells) in the infected leaves. The HR spots suppressed pronouncedly the pathogen development (Huang 2001; Huang *et al.* 1998). The reaction of leaves to the pathogen invasion continued by a consecutive nercotization, wilting and bending of leaves followed by a final abscission (since 6 DAI). All infected leaves were downfallen by 7 DAI. Powdery mildew infection did not cause any significant changes in the measured photosynthetic parameters in moderately resistant genotype.

The HS pre-treated plants of *Solanum chmielewskii* did not show any pronounced differences in the progress of the pathogen and HR. Moreover, any significant impairment of photosynthesis was observed in the HS pre-treated infected leaves compared to the infected leaves without HS. These results indicate that the HS pre-treatment did not change the level of plant resistance in the *Solanum chmielewskii* - *O. neolycopersici* interaction. On the basis of this result, it can be suggested that early hexose accumulation (that could support the plant resistance (Essmann *et al.* 2008; Kocal *et al.* 2008)) is not probably induced by HS. Except early hexose accumulation, also the expression of some race-specific resistance genes can be dependent on temperature (Lebeda *et al.* 2008) as has been shown for other host-pathogen interactions (e.g. *Lactuca* spp. - *Bremia lactucae*). The resistance based on race-specific genes (e.g. *Dm6*, *Dm7* and *Dm11*) became less effective or even ineffective at lower temperatures and vice versa (Judelson and Michelmore 1992). Host-pathogen interaction described in our case is race-specific (e.g. Lebeda and Mieslerová 2002) and monogenic resistance is involved (Li *et al.* 2007). Our observations reveal that HS pre-treated and non-treated plants show the similar level of resistance. This indicates that our HS pre-treatment did not affect the race-specific resistance genes. Nevertheless, temperature pre-treatment different from that used could affect the resistance of *Solanum chmielewskii* plants to *O. neolycopersici*.

It can be summarized that leaves of both susceptible and moderately resistant tomato genotypes infected by *O. neolycopersici* showed only negligible decrease in the photosynthetic functions during 9 DAI. Therefore, the rate and the extent of the photosynthetic response to the pathogen are not markedly changed by different degree of resistance/ susceptibility of plants to pathogen. The HS pre-treatment of moderately resistant tomato genotype did not change significantly its resistance and photosynthetic response. However, the HS pre-treatment of the susceptible genotype increased its susceptibility to *O. neolycopersici*. It was hypothesized that this increase of susceptibility could be caused by the earlier activation of the cell-wall invertase that results from a higher demand for carbohydrates in HS-induced defense reactions. Consequently, the accumulation of hexoses would lead to the earlier feed-back inhibition of photosynthesis. It can be speculated that HS-induced changes in resistance/susceptibility to pathogen could be associated with heat shock influence on host photosynthesis.

The results described in the chapter 5.3.2 have been presented in paper V.

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7. Supplement

Following papers that were published are attached:

Paper I. **Hlavinka J,** Nauš J, Špundová M (2013) Anthocyanin contribution to chlorophyll meter readings and its correction. *Photosynthetic Research* **118**: 277–295.

Paper II. **Hlavinka J**, Nauš J, Fellner M (2013) Spontaneous mutation *7B-1* in tomato impairs blue light-induced stomatal opening. *Plant Science* **209**: 75–80.

Paper III. **Hlavinka J**, Nožková-Hlaváčková V, Floková K, Novák O, Nauš J (2012) Jasmonic acid accumulation and systemic photosynthetic and electrical changes in locally burned wild type tomato, ABA-deficient *sitiens* mutants and *sitiens* pretreated by ABA. *Plant Physiology and Biochemistry* **54**: 89–96.

Paper IV. Novák J, Pavlů J, Novák O, Nožková V, Špundová M, **Hlavinka J**, Koukalová Š, Skalák J, Černý M, Brzobohatý B (2013) High cytokinin levels induce a hypersensitive-like response in tobacco. *Annals of Botany* **112**: 41–55.

Paper V. Prokopová J, Mieslerová B, Hlaváčková V, **Hlavinka J,** Lebeda A, Nauš J, Špundová M (2010) Changes in photosynthesis of *Lycopersicon* spp. plants induced by tomato powdery mildew infection in combination with heat shock pre-treatment. *Physiological and Molecular Plant Pathology* **74**: 205–213.