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LEAF SENESCENCE AS A LIGHT-DEPENDENT PROCESS

HABILITATION THESIS

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Abstract

The habilitation thesis deals with leaf senescence, a fundamental process of plant's life that is essential for seed production and plant viability in the next generation or season, as allowing plants to recycle leaf nutrients. The understanding of senescence mechanisms is important not only with respect to scientific questions, but also in context of maintaining or increasing plant productivity and minimizing postharvest quality loss during transportation and storage. As a part of leaf development, senescence is primarily induced and controlled by endogenous factors, but it is pronouncedly modulated by environmental factors including light. During the past two decades there has been a significant progress in the understanding the senescence mechanisms and regulation, but they are still far from being elucidated.

In the first part of the thesis, recent knowledge of some aspects of leaf senescence (such as chloroplast and chlorophyll degradation, impairment of photosynthesis, and cytokinin action) is briefly summarized. The attention is also paid to a role of light in senescence processes. In the second part of the thesis, main results of our publications (chronologically ordered) dealing with leaf senescence are described. The relevant papers are included in the Appendix.

It has been shown that light pronouncedly affects rate of chlorophyll degradation and inhibition of photosynthetic processes, activity of xanthophyll cycle and antioxidative enzymes, and level of oxidative damage. Light also modifies the antisenescence effect of exogenous cytokinin and endogenous level of main cytokinin types and forms in detached leaves. It has been suggested that the delaying effect of light on senescence of detached *Arabidopsis* leaves may be related to the persisted biosynthesis of isopentenyladenine. It has been revealed that light can eliminate an acceleration of senescence-associated processes caused by the loss-of-function mutation in cytokinin receptors. Finally, it has been observed that chlorophyll *b* deficiency accelerates decrease in photosynthetic activity during dark-induced senescence of detached barley leaves and that exogenous cytokinin is able to eliminate this effect, probably through the stabilization of photosynthetic reaction centres.

Abstrakt

Habilitační práce se zabývá senescencí listu, základním procesem života rostliny. V rámci tohoto procesu dochází k recyklaci látek obsažených v listu, což významně přispívá k tvorbě semen a podporuje životaschopnost rostliny v příští generaci či sezóně. Pochopení mechanismů senescentních procesů je důležité nejen z hlediska základního výzkumu, ale i v souvislosti se zachováním nebo zvýšením produktivity zemědělských rostlin a minimalizací posklizňových ztrát během transportu a uskladnění. I když je senescence jako jedna z fází vývoje listu primárně spouštěna a řízena endogenními signály, významně ji ovlivňují faktory prostředí včetně světla. Během posledních dvaceti let došlo k významnému pokroku v pochopení mechanismů a regulace senescence, mnoho otázek však zůstává stále nezodpovězených.

V první části habilitační práce jsou stručně shrnuty nejnovější poznatky o některých aspektech senescence listů, zejména o degradaci chloroplastů a chlorofylu, inhibici fotosyntézy a působení cytokininů. Pozornost je věnována také roli světla v senescentních procesech. V druhé části práce jsou popsány hlavní výsledky našich publikací (chronologicky řazených) zabývajících se senescencí listů. Příslušné publikace jsou uvedeny v příloze.

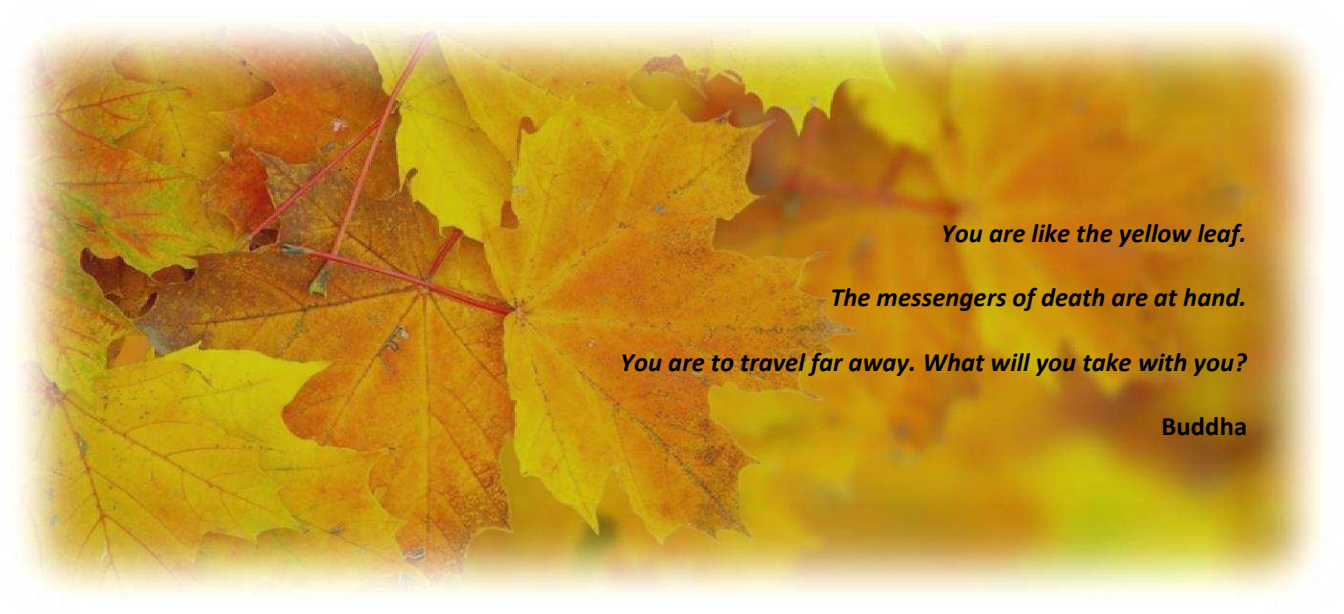
Bylo prokázáno, že světlo výrazně ovlivňuje rychlost degradace chlorofylu a inhibici fotosyntetických procesů, aktivitu xantofylového cyklu a antioxidačních enzymů a míru oxidačního poškození. Dále bylo ukázáno, že světlo modifikuje antisenescenční účinek exogenního cytokininu a také endogenní hladiny hlavních typů a forem cytokininů v oddělených listech. Předpokládáme, že zpomalující účinek světla na senescenci oddělených listů *Arabidopsis* může souviset s přetrvávající biosyntézou isopentenyl-adeninu. Bylo zjištěno, že světlo může eliminovat zrychlení senescentních procesů v mutantních rostlinách s nefunkčními receptory cytokininů. V poslední naší práci bylo ukázáno, že nedostatek chlorofylu *b* urychluje pokles fotosyntetické aktivity během senescence oddělených listů ječmene ve tmě a že exogenní aplikace cytokininu toto urychlení eliminuje, pravděpodobně prostřednictvím stabilizace fotosyntetických reakčních center.

Abbreviations

A	antheraxanthin
AHK	Arabidopsis histidine kinase
APX	ascorbate peroxidase
ARR	Arabidopsis response regulator
ATG(s)	autophagy gene(s)
B (light)	blue (light)
BAP	N^6-benzylaminopurine
CCV(s)	CHLOROPLAST VESICULATION-containing vesicle(s)
Chl	chlorophyll
CK(s)	cytokinin(s)
CKX	cytokinin oxidase/dehydrogenase
CLH	chlorophyllase
CRY	cryptochrome
CWINV	cell wall-invertase
*c*Z	*cis*-zeatin
DEPS	de-epoxidation state of xanthophyll cycle pigments
DMSO	dimethylsulfoxide
FR (light)	far-red (light)
GR	glutathione reductase
HPt	histidine phosphotransfer proteins
iP	isopentenyladenine
iPR	isopentenyladenine riboside
IPT	isopentenyl transferase
LHC(s)	light-harvesting complex(es)
LHCII	light-harvesting complex of photosystem II
LOOH(s)	lipid hydroperoxide(s)
LOX	lipoxygenase
LP	lipid peroxidation
MDA	malondialdehyde
*m*T	*meta*-topolin
NPQ	non-photochemical chlorophyll fluorescence quenching
PAO	pheophorbide *a* oxygenase
PG(s)	plastoglobule(s)
PHY(s)	phytochrome(s)
PL(s)	phospholipase(s)
PSI	photosystem I
PSII	photosystem II
PUFA(s)	polyunsaturated fatty acid(s)
qP	photochemical quenching of chlorophyll fluorescence
qN	non-photochemical quenching of chlorophyll fluorescence
R (light)	red (light)
RC(s)	reaction center(s)
RCB(s)	RUBISCO-containing bodies
RCI	reaction centre of photosystem I
RCII	reaction centre of photosystem II
ROS	reactive oxygen species
RUBISCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SAG(s)	senescence-associated gene(s)

SAV(s)	senescence-associated vacuole(s)
TBARs	thiobarbituric acid-reactive substances
TF(s)	transcription factor(s)
*t*Z	*trans*-zeatin
*t*ZR	*trans*-zeatin riboside
V	violaxanthin
Z	zeaxanthin
$\Phi_{f,D}$	quantum yield for constitutive non-regulatory dissipation processes
Φ_{NPQ}	quantum yield for regulatory non-photochemical quenching
Φ_{P}	effective quantum yield of PSII photochemistry in light-adapted state
Φ_{PSII}	actual quantum yield of photosystem II photochemistry

Content



1 INTRODUCTION

The habilitation thesis deals with leaf senescence, a fundamental process in plant kingdom. Efficient senescence is important for seed production and plant viability in the next generation or season. On the other hand, premature senescence can reduce yield and quality of crop plants. In addition, senescence pronouncedly contributes to postharvest loss in vegetable and ornamental plants. The understanding of senescence mechanisms is therefore important not only with respect to scientific questions, but also in context of maintaining or increasing plant productivity and minimizing the postharvest quality loss during transportation and storage. This issue has grown in importance in the context of global climate change and ensuring sufficient food for the population worldwide.

Senescence includes unique physiological, molecular and genetic mechanisms. As a part of plant or leaf development, it is primarily induced and controlled by endogenous factors, but it is also pronouncedly modulated by environmental factors including light. During the past two decades there has been a significant progress in the understanding the senescence mechanisms and regulation, but they are still far from being elucidated.

In the first part of the thesis, recent knowledge of some aspects of leaf senescence such as chloroplast deterioration, chlorophyll degradation, impairment of photosynthesis, and cytokinin action is summarized. The attention has also been paid to a role of light in senescence processes. In the second part of the thesis, main results of our publications (chronologically ordered) dealing with leaf senescence are briefly described. The relevant papers are included in the Appendix. Citations of these publications are written in bold blue in the text of the first part, citations of other publications I co-authored are written in blue.

1.1 Leaf senescence

1.1.1 Definition of leaf senescence

The word 'senescence' derives from Latin word *senescere* which means 'to grow old'. In Merriam-Webster online dictionary, senescence is defined as 'the state of being old or the process of becoming old', or as 'the growth phase in a plant or plant part (such as a leaf) from full maturity to death'. The term 'senescence' is used by both animal and plant physiologists but its exact meaning can differ. In animals (and humans), senescence is usually synonymous with aging. The meaning of senescence in plants is described below.

In plants we can distinguish two basic types of senescence: mitotic and post-mitotic. Mitotic (or replicative or proliferative) senescence, demonstrated in the arrest of cell division, occurs in shoot apical meristem, in fruits and in leaves at very early stages of development (for a review, see *e.g.* Gan 2007). Post-mitotic senescence is typical for mature leaves and floral petals. It can be divided into three phases: initiation, re-organization, and termination (Fig. 1). The thesis deals with the post-mitotic leaf senescence.

Senescence is often an annual event. Due to its importance for agriculture, leaf senescence of annual crops (*e.g.* wheat, barley, and rice) has been most intensively studied. Additionally, as in other areas of plant research, *Arabidopsis* is used as an important (annual) model plant. These plants show monocarpic senescence which is associated with formation and maturation of seeds. In some cases, the removal of reproductive structures delays leaf and whole-plant senescence (*e.g.*, Srivalli and Khanna-Chopra 2004). Whole-plant senescence in *Arabidopsis* is controlled by reproduction as well, but the onset of leaf senescence is determined mainly by age-related processes, not by the appearance of flowers and seeds (Hensel et al. 1993).

In contrast to the annuals (more precisely, to monocarpic plants), in perennial plants leaf senescence may not be directly associated with seed production. However, a re-translocation of nutrients (*e.g.*, nitrogen, phosphorus, sulphur, metal ions, and carbon skeletons) from senescing plant parts to surviving ones occurs in both plant types. In the annual plants, nutrients are transported to the seeds, while in the perennial plants nutrients are stored in surviving organs such as roots, bulbs, or bark. Leaf senescence can be therefore primarily imagined as a process during which leaf cell metabolism switches from anabolism to catabolism and in which cellular components are actively degraded and remobilized. The final stage of leaf senescence is the leaf death, but it is actively hindered until all nutrients have been removed. This is clearly demonstrated by the reversibility of leaf senescence: a completely yellow leaf (when majority of its nutrients is mobilized) can be induced to re-green by various treatments (*e.g.*, Zavaleta-Mancera et al. 1999). Such reversibility indicates that leaf senescence is controlled from the start to the finish.

Plant senescence is considered to be an evolutionary acquired 'strategy' being critical for plant fitness, survival and reproduction. Integration of multiple internal and external signals allows the plant to regulate timing, rate and nature of senescence processes. Leaf senescence as a part of normal leaf development (referred as developmental, natural or age-dependent senescence) is primarily induced and controlled by endogenous factors (such as age,

reproductive development, and phytohormone levels) (Fig. 1) and is considered to be a type of programmed cell death (PCD) controlled by a genetic program (Nam 1997). Cell death during natural senescence comes slowly than during other types of PCD to ensure the efficient remobilization of nutrients.

As senescence is obviously linked to previous development of plant, it should be seen from perspective of plant's life history. Leaf senescence is influenced not only by the contemporary internal and external conditions, but also by all previous events that co-determine properties and fitness of the plant. Thus, progress of senescence can vary pronouncedly between plants or leaves whose properties or fitness differ before start of senescence, even though the conditions during senescence are the same.

Senescence proceeds heterogeneously through the plant (usually starting from the plant base) and also through area and cross-section of an individual leaf. Within the senescing leaf, individual cells are usually at many different phases of senescence. The veinal tissue stays green and alive until the final stage of senescence whereas interveinal tissues undergo senescence-associated changes including de-greening (Gan and Amasino 1997, Niewiadomska et al. 2009). Obviously, this spatial heterogeneity of leaf senescence allows maximizing the transport of nutrients from the leaf. An unequal rate of senescence was found also in epidermal, guard, and mesophyll cells (Keech et al. 2007).

1.1.2 Senescence under stress conditions

The timing and progress of developmental senescence is pronouncedly modulated by environmental factors (Fig. 1). When plants suffer from abiotic or biotic stresses, premature leaf senescence is often induced (*e.g.*, Kyseláková et al. 2011). Under such conditions, it can be beneficial for the plant to start premature senescence of the leaves that are not photosynthetically productive. However, the leaves formed represent a significant investment for the plant therefore the start of senescence has to be strictly controlled to avoid its induction under only temporarily undesirable conditions.

The environmental stress factors affect plant productivity and yield by altering plant cell homeostasis and modifying source-sink relations (for a review, see *e.g.* Albacete et al. 2014). Drought, excess or low light intensity, high and low temperatures, soil salinity and nutrient deficiency are major abiotic stress factors impairing plant productivity and food quality. The global climate change will probably generate further deterioration of environmental conditions resulting in more severe decrease in plant fitness and yield.

A decrease in both, source and sink, can occur during stress conditions. Premature and/or accelerated leaf senescence accompanied by a decrease in chlorophyll (Chl) content and by impairment of photosynthetic apparatus leads to a decrease in the source strength due to inhibition of leaf photosynthesis. On the other hand, the decreased sink strength caused by reduced growth of sink tissues under stress conditions leads to accumulation of assimilates in the source leaves, which can cause a feedback inhibition of photosynthesis. In addition, stress conditions can impair also transport processes from source to sink which further decreases the sink strength and may contribute to the limitation of growth and productivity.

The annual and perennial plants developed different 'strategies' to deal with stress conditions (Munné-Bosch 2008, Sade et al. 2018). The annual plants usually accelerate their transition to a reproductive stage and increase allocation of nutrients into seeds (Albacette et al. 2014). The stress conditions induce leaf senescence at whole-plant level, starting in older leaves and continuing to younger ones, which is accompanied by the gradual remobilization of nutrients to seeds. Thus, in case of the annual plants, the stress-induced senescence can contribute to successful plant survival or reproduction.

Unlike the annual plants, the perennial ones allocate biomass and nutrients preferentially to vegetative tissues (for example, to roots), which allows surviving the stress conditions and subsequent plant recovering (*e.g.*, Zwicke et al. 2015). Usually, senescence is induced only in the oldest leaves of perennial plants (Munné-Bosch 2008, Sade et al. 2018). A positive (Pérez-Ramos et al. 2013) as well as negative correlation (Zwicke et al. 2015) between leaf senescence and plant survival have been found in stressed perennial grasses. Thus, in some cases the maintenance of photosynthesis and source strength under stress conditions could be for survival of the perennial plants more important than leaf senescence.

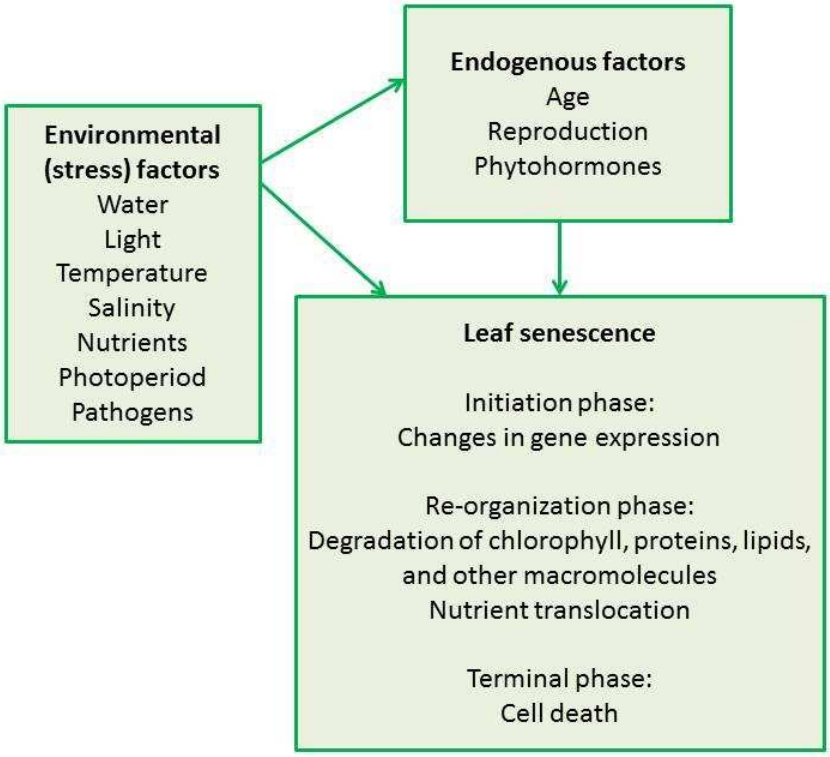


Fig. 1 A scheme of regulation of leaf senescence by major environmental and endogenous factors

1.1.3 Systems used in senescence studies

Different 'senescence model systems' are used in studies of leaf senescence (Fig. 2), having specific advantages and disadvantages. Developmental senescence of plants growing under natural field conditions represents the most natural system, but too many factors (known and unknown) can affect the senescence process and results can be unrepeatable and difficult to interpret. Results obtained on plants growing and undergoing developmental senescence under controlled conditions are better reproducible. In some senescence studies cotyledons are used, but their senescence may not be the same as in true leaves (*e.g.*, Weaver and Amasino 2001) as some specific organ-dependent differences (including gene expression) can be expected (*e.g.*, Brown and Hudson 2015).

When detached leaves are used instead of the intact ones, the situation is simplified due to abolishing an impact of the plant on the leaf and interruption of the nutrient transport from the leaf. However, as these effects can pronouncedly change the progress of senescence, results obtained with the detached leaves may not be representative for senescence of the intact ones. Finally, dark-induced senescence of whole plants or leaves (detached or intact ones, individually darkened) is often used (Fig. 2). The dark-treatment allows synchronizing and accelerating the senescence process (Weaver and Amasino 2001) and results are well reproducible. However, due to the absence of light and photosynthesis, processes induced by sugar starvation are prevailing, which may not fully correspond to natural senescence. Indeed, a comparative transcriptome analysis in *Arabidopsis* revealed significant differences in both gene expression and signalling pathways between developmental senescence and senescence induced by darkening of whole plants (Buchanan-Wollaston et al. 2005). Van der Graaff et al. (2006) showed pronounced differences in gene expression between individually darkened intact leaves and leaves during developmental senescence. A different response of the gene response has been found also between individually darkened intact leaves and leaves of whole darkened plants (Law et al. 2018). These findings indicate that senescence processes are not the same in the particular 'model systems' therefore results obtained with one system should not be generalized for the others. Interactions between the leaf and the rest of the plant as well as light conditions significantly influence the way leaf senescence will run.

1.1.4 Leaf senescence and gene expression

As a highly complex process, leaf senescence is strictly regulated at multiple levels, including chromatin-mediated regulation and regulation at transcriptional, post- transcriptional, translational and post-translational level (for a review, see *e.g.*, Woo et al. 2013). The chromatin-mediated regulation of leaf senescence involves histone modification and changes in chromatin architecture (Ay et al. 2009, Chen et al. 2016). The transcriptional regulation consists in a pronounced alteration of gene expression (see below). The post-transcriptional regulation is mediated mainly by microRNAs and tasiRNAs (trans-acting small-interfering RNAs). Finally, phosphorylation and ubiquitylation are the main post-translational modifications regulating leaf senescence (Woo et al. 2013).

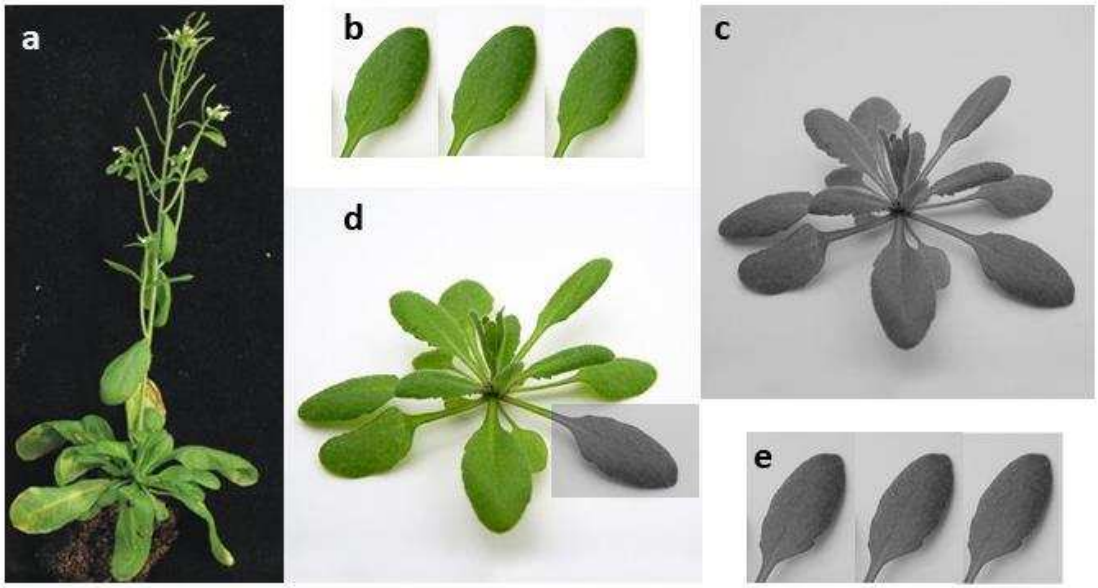


Fig. 2 Systems used in leaf senescence studies. **a** – developmental senescence under normal (light) conditions; **b** – detached leaves senescing under light conditions; **c** – whole plants darkened; **d** – individually darkened intact leaves (the rest of the plant is under normal light conditions); **e** – detached leaves senescing in dark.

Dramatic re-programming of cellular metabolism and degradation of cellular structures during leaf senescence are connected with extensive changes of gene expression. Thousands of 'senescence-associated genes' (*SAGs*) have been identified by transcriptomic analysis (*e.g.*, Lin and Wu 2004, Breeze et al. 2011). Generally, genes involved in degradation of cellular components, transport of nutrients, and detoxification of oxidative metabolites are induced or up-regulated during leaf senescence, while genes related to photosynthesis are down-regulated (Gan and Amasino 1997, Lin and Wu 2004, Wojciechowska et al. 2018). *SAG*-encoded proteins include proteases, nucleases, lipid-, carbohydrate-, and nitrogen-metabolizing enzymes, stress-responsive proteins, and transcription regulators (Buchanan-Wollaston et al. 2003). Most of known autophagy genes (*ATGs*) are co-ordinately up-regulated at an early stage of developmental senescence (van der Graaff et al. 2006). Further, genes whose products are components of signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) cascades are induced (Guo et al. 2004). It appears that senescence-promoting genes as well as anti-senescence regulators are up-regulated during leaf senescence in order to control precisely its initiation and progression.

A 'leaf senescence database' (LSD) of genes potentially involved in the senescence regulation has been developed as a useful tool for the further study of molecular aspects of leaf senescence (Liu et al. 2011). Using LSD, Li et al. (2012) created gene networks and identified common regulators of leaf senescence in *Arabidopsis*.

Although many *SAGs* are induced during both developmental and dark-induced senescence, the transcriptome analysis revealed that the senescence program differs significantly during these two types of senescence (Park et al. 1998, Buchanan-Wollaston et al.

2005, van der Graaff et al. 2006, Breeze et al. 2011). Interestingly, gene expression profiles differed also between senescing flag and second leaves of rice (Lee et al. 2017) indicating the different senescence progress in the particular leaves coordinated on the plant level.

The expression of *SAGs* is controlled by an activation of transcription factors (TFs). In microarray expression analysis, more than 200 transcription factors have been found to regulate leaf senescence (Buchanan-Wollaston et al. 2003, Miao et al. 2004, Buchanan-Wollaston et al. 2005). Two TF families, NAC and WRKY, have been identified as the major TFs regulating senescence. More than 30 NAC genes were found to be significantly up-regulated during leaf senescence of *Arabidopsis* (for a review, see Woo et al. 2013), including *ORE1* (positive regulator of senescence) and *JUB1* (negative regulator of senescence). It seems that NAC TFs integrate different internal and external stimuli with the developmental age (Woo et al. 2013). Regarding WRKY TFs, WRKY53 (positive regulator of dark-induced senescence), WRKY54, and WRKY70 (both negative regulators of senescence) were found to regulate leaf senescence (Besseau et al. 2012). The interactions of WRKY TFs apparently contribute to the fine-tuning senescence regulation at the transcriptional level.

Recently, a time-course gene-expression profiling of *Arabidopsis* leaves has revealed that senescence is regulated by time-evolving networks based on the temporal transition of interactions among senescence regulators including TFs (Breeze et al. 2011, Woo et al. 2016, Kim et al. 2018). For example, the effect of a 'NAC troika' (consisting of ANAC017, ANAC082, and ANAC090 TFs) has shifted from positive to negative at a pre-senescent stage (Kim et al. 2018).

Woo et al. (2016) reported that during leaf senescence of *Arabidopsis*, a transcriptional coordination of chloroplast and nuclear genes encoding photosynthetic proteins increases. The authors assume that the enhanced coordination of chloroplast and nuclear transcriptomes during senescence allows the optimal transition of chloroplasts from source of energy and assimilates to source of nutrients for recycling. Existence of a system specialized for transcriptional coordination between the nucleus and chloroplasts has been proposed (Woo et al. 2016).

The findings suggest that the regulation of senescence is extremely complex, variable in time and dependent on the conditions of senescence. It can be assumed that the senescence regulation is different in various types of plants (for example, in annuals and perennials) and can differ also among species or cultivars. Importantly, properties and fitness of the plant, co-determined by the environmental conditions in which it evolves, can significantly influence the progress of senescence. However, these questions are not yet taken into account in senescence studies.

1.2 Effect of light on leaf senescence

Light plays an essential role in plant life. It is indispensable for photosynthesis and acts as a signal for plant and leaf development. Photoreceptors (namely phytochromes, phototropins and cryptochromes) as well as chloroplasts serve as sensors in light sensing-mediated pathways regulating gene expression. It is known that leaf senescence is influenced by light, but this knowledge is based mainly on the finding that in darkened or shaded leaves senescence

is accelerated. The role of light in regulation of senescence under natural conditions is not yet elucidated. The effect of light on senescence processes has been viewed mainly in terms of providing the energy for photosynthesis (thereby life sustaining), but now it is clear that light influences senescence by several other routes such as generation of reactive oxygen species (ROS) (*e.g.*, Causin and Barneix 2007), signalling *via* light receptors and interactions with phytohormones (*e.g.*, Zdarska et al. 2015). Both light quantity (intensity and photoperiod) and quality (*i.e.*, spectral composition) are important (Noodén and Schneider 2004, Marchetti et al. 2018).

The question remains, what is the main cause of accelerated senescence in the absence or deficiency of light. Is it the lack of assimilates and energy due to not working or inhibited photosynthesis or the absence of light signals suppressing senescence? According to Liebsch and Keech (2016), the starvation is a 'driving force' for the senescence induction and acceleration in darkened or shaded leaves, but the phytochrome-mediated light signalling could be superior to the starvation in the inhibition of senescence. It is not excluded that both the starvation and light-signalling absence are required for the senescence induction and that also other factors can play a role (Liebsch and Keech 2016).

The light regulation of leaf senescence is largely determined by a 'light history' of plant or leaf and can differ, for instance, between sun- and shade-acclimated plants. Responses to the light treatment can differ also between intact and detached leaves because of diverse source-sink relations and interactions with the rest of plant. Both negative effect (senescence-delaying) and positive effect (senescence-accelerating) of light have been shown. The senescence promoting effect is usually observed when the light intensity and/or dose are increased (*e.g.*, Procházková and Wilhelmová 2004, Velez-Ramirez et al. 2017). However, as mentioned, senescence can be induced and/or accelerated also by darkening, shading or shortening of photoperiod.

1.2.1 Leaf senescence in dark

Dark is known to accelerate pronouncedly the rate of leaf senescence compared to the 'normal' (growth) light conditions. This acceleration has been found in both individually darkened intact leaves and detached leaves (*e.g.*, Weaver and Amasino 2001, Causin et al. 2006, Keech et al. 2007, **Janečková et al. 2018**). However, darkening of whole *Arabidopsis* plants for several days does not induce the senescence process (Weaver and Amasino 2001, Keech et al. 2007, Law et al. 2018). In the leaves of whole darkened plants, both chloroplasts and mitochondria were largely preserved, Chl content and photosynthetic capacity remained high, while rate of mitochondrial respiration decreased (Keech et al. 2007).

On the contrary, in the individually darkened intact leaves the photosynthetic function decreased rapidly as well as the Chl content and chloroplast number and size, but the rate of mitochondrial respiration was maintained. The faster decline in photosynthetic function was accompanied by a more pronounced down-regulation of genes encoding photosynthetic proteins (Law et al. 2018). The authors suggest that in the leaves of whole darkened plants, metabolism switches to 'stand-by mode' to preserve the photosynthetic apparatus as long as possible. In contrast, in the individually darkened leaves, the senescence process is rapidly

induced, cellular components (including the photosynthetic apparatus) are degraded, all in order to translocate nutrients efficiently from the darkened leaves to the other parts of the plant (Keech et al. 2007, Law et al. 2018).

1.2.2 Leaf senescence under light

Compared to darkened 'senescence system', the light treatment usually slows down the rate of senescence-associated changes (*e.g.*, Hidema et al. 1992, Kar et al. 1993, Chang and Kao 1998, **Špundová et al. 2005a**, **Janečková et al. 2018**). In the last years, the slowing down effect of light (continuous light of low intensity or light pulses) on senescence is practically used to delay post-harvest senescence of green vegetables, for example fresh basil (Costa et al. 2013) or broccoli (Favre et al. 2018).

It is assumed that the delay of senescence by light is not only due to functional photosynthesis providing assimilates and energy, but also due to light signals inhibiting the onset of senescence (see part 1.2.3). In some cases, light treatment does not delay senescence of detached leaves compared with dark (*e.g.*, **Špundová et al. 2003**, **Vlčková et al. 2006**), and when high light doses are used, senescence may be even accelerated. These different effects of light can be related to a balance between supply and demand of assimilates in the detached leaf as their transport from the leaf is eliminated. When the supply and demand of assimilates are roughly balanced, which can be expected in case of relatively low light intensity, the detached leaf can remain alive for a relatively long time. However, when the supply of assimilates outweighs their demand in the leaf (in case of higher light intensity or dose), a feed-back inhibition of photosynthesis may lead to over-excitation of photosynthetic apparatus, ROS accumulation and oxidative damage, thereby senescence will be accelerated.

In case of whole plants and intact leaves, a decrease in light intensity or length of photoperiod is known to start or promote senescence (*e.g.*, Weaver and Amasino 2001, **Špundová et al. 2005b**, Fracheboud et al. 2009, Brouwer et al. 2012). It is assumed that the decrease in light intensity below the compensation point (resulting in a negative carbon balance) induces changes in gene expression related to senescence (Brouwer et al. 2012). Law et al. (2018) reported that transcriptional response to shading is similar to a response in darkened leaves (at least during the first days) indicating similar signalling mechanisms and metabolic strategies. A reduced antioxidative protection (**Špundová et al. 2005b,** Causin et al. 2015) and decreased cytokinin content (Marchetti et al. 2018) can be also involved in the accelerated progress of senescence in the shaded leaves.

On the other hand, the application of supplemental light has begun to be used in practice to reduce senescence of shaded leaves in commercial canopies of leafy vegetables. For instance, supplemental lighting has been shown to retard senescence of outer leaves in lettuce grown in so-called 'plant factory with artificial lighting' and proposed to be the efficient way to improve yield and profitability of lettuce (Zhang et al. 2015).

In perennial deciduous trees, the shortening of photoperiod is thought to be one of the main factors starting leaf senescence (Lee et al. 2003, Fracheboud et al. 2009). However, the strict photoperiodic control of the onset of autumn senescence is not a rule as other factors might have interactive effects on senescence timing such as low temperatures, shortage

of water or nutrients, and pathogen infection (Fracheboud et al. 2009). In *Arabidopsis*, the length of photoperiod also affects leaf senescence but by other way than in the deciduous trees. A delay of leaf senescence under the short photoperiod and earlier senescence under the long photoperiod are typical for *Arabidopsis*. However, it has been proposed that in this case the light dose is the determining factor, not the length of photoperiod (Noodén et al. 1996).

1.2.3 Effect of red and far-red light

The light environment of individual leaves on the plant is different. Usually, there is a pronounced light gradient in plant canopies that increases with increasing canopy density. Within the plant canopy, not only light intensity is significantly reduced, but also light spectral composition is changed. Due to the spectral properties of Chl and other leaf pigments, red (R, wavelengths around 650 nm) and blue (B, 400-450 nm) light is strongly attenuated. A decreased ratio of R and far-red light (FR, wavelengths around 730 nm) is thought to contribute to triggering and/or accelerating senescence of bottom leaves. It has long been known that an application of R light delays leaf senescence compared to dark (reviewed by Biswal and Biswal 1984, Lers 2007). The deceleration of senescence by R light has been reported also in case of intact plants grown in a greenhouse (Wang et al. 2016). The effect of R light can be reversed by a subsequent illumination by FR which is a typical attribute of phytochrome-mediated response. There are two forms of phytochrome (PHY), the inactive (Pr) and active (Pfr) form. After absorption of R, Pr is converted to Pfr and Pfr is reverted back to Pr by absorption of FR. The acceleration of leaf senescence by the lower R/FR ratio can be associated with a higher accumulation of the inactive Pr.

The inhibition of leaf senescence by R light is thought to be mediated by PHYB (Brouwer et al. 2014, Sakuraba et al. 2014). In addition, the acceleration of leaf senescence by FR supplementation was found, and this effect is thought to be mediated by PHYA (Rousseaux et al. 1997, Brouwer et al. 2014). Recently, Lim et al. (2018) have specified that PHYA and PHYB antagonistically regulate FR-induced leaf senescence. This antagonism may be involved in fine-tuning leaf senescence under varying FR conditions (Lim et al. 2018). The role of PHYA has been proposed also in delaying senescence by low light intensities (up to 37 μmol photons m^{-2} s^{-1}) (Brouwer et al. 2012). Apart from PHYs, a role of other photoreceptors in the regulation of leaf senescence remains unknown.

Generally, PHYs regulate light responses by promoting degradation of phytochrome-interacting factors (PIFs), a family of basic helix-loop-helix (bHLH) TFs (Lorrain et al. 2008). Recently, an important role of PIFs in regulation of both dark-induced and natural senescence has been found (reviewed by Liebsch and Keech 2016). PIFs are induced during prolonged dark in a PHYB-dependent manner and promote dark-induced senescence in *Arabidopsis* (Sakuraba et al. 2014, Song et al. 2014). In light, PIFs are degraded *via* activation of PHYB, which suppresses PIF-dependent senescence induction. In dark, PHYB is inactive therefore PIFs are not degraded and can induce an expression of specific senescence TFs, which results in the induction of senescence.

1.2.4 Effect of blue light

There is limited information about the role of B light in senescence regulation. It has been shown that the B light-treatment cancels the induction and/or acceleration of senescence of both detached and intact wheat leaves caused by darkening or lowering R/FR ratio (Causin et al. 2006, Causin and Barneix 2007, Marchetti et al. 2018). A deceleration of leaf senescence by supplemental B light has been reported for grape plants (Wang et al. 2016). Photoreceptors mediating the effects of B light include cryptochromes, phototropins and members of a zeitlupe family. Almost nothing is known about their action during senescence. A role of cryptochrome 2 in the B light-mediated delaying senescence in soybean has been proposed (Meng et al. 2013). In *Arabidopsis*, B light enhanced an expression of two chlorophyllase genes, *CLH1* and *CLH2* (Banaś et al. 2011). The effect of B light was mediated mainly by cryptochromes and modulated by phototropins (Banaś et al. 2011). However, according to the latest findings, the chlorophyllase plays probably only a minor role in senescence-associated Chl degradation (see part 1.4.1).

1.2.5 Effect of excessive light

Not only the light insufficiency, but also its excess can induce or accelerate leaf senescence, although reasons may be different. In the case of high light intensity or unnatural long photoperiods (including continuous light), photoinhibition and photodamage of photosynthetic apparatus typically occur (*e.g.*, Procházková and Wilhelmová 2004, Velez-Ramirez et al. 2017), which can be considered as a main cause of the senescence acceleration. Metabolomic and transcriptomic analysis of tomato plants exposed to continuous light revealed a strong negative correlation between the inhibition of photosynthesis and accumulation of sucrose and starch (Velez-Ramirez et al. 2017). The authors suggested that the sugar accumulation down-regulated genes encoding enzymes of Calvin-Benson cycle.

The similar situation may occur also under 'normal' light conditions when the supply of assimilates overweighs their demand (*i.e.*, under sink limitation). Such a case may arise in detached leaves senescing under light and also in intact leaves when sink strength decreases, for example due to growth inhibition under low temperatures.

1.2.6 Effect of UV-radiation

In case of ultraviolet (UV) radiation, a senescence-inducing effect has been described (for a review, see Lers 2007). This effect is probably associated with an increased ROS generation and oxidative damage usually occurring in UV-treated plants (*e.g.*, John et al. 2001). The effect of UV-A (320-400 nm) on leaf senescence is unclear as both negative and positive regulation of senescence by UV-A was found (Lers 2007).

UV-B (280 – 320 nm) was found to accelerate Chl degradation, ROS accumulation, and inhibition of photosynthesis. A UV-B treatment of mature *Arabidopsis* leaves markedly up-regulates the expression of *SAGs* (John et al. 2001). However, it has been also shown that a pre-treatment by high-doses of UV-B slowed down Chl and protein degradation and decrease

in photosystem II photochemistry in detached *Arabidopsis* leaves during dark-induced senescence (Sztatelman et al. 2015). The authors suggest that UV-B (in the absence of visible light) activated signals interfering with a degradation pathway of light-harvesting complexes of photosystem II. In case of senescing flag leaf of rice, supplemental UV-B radiation caused a decrease in the rate of photosynthesis and an increase in oxidative damage, although the activity of antioxidative enzymes was elevated (Wang et al. 2015).

1.3 Leaf senescence and chloroplasts

1.3.1 Chloroplast degradation

Chloroplasts are primary energy suppliers for plants. They convert the energy from visible light (wavelengths of 400 – 700 nm) into chemical energy *via* the photosynthetic electron transport chain and proton accumulation in thylakoid lumen. In addition to photosynthesis, a number of other key metabolic processes of plant cell occur in chloroplasts, such as biosynthesis of amino acids, fatty acids, pigments and phytohormones. During the vegetative growth, the majority of plant nitrogen and other nutrients are localized in leaves and chloroplasts. It has been estimated that around 75 % of the total cellular nitrogen in leaves is localized in chloroplasts (Hörtensteiner and Feller 2002). For example, in pea leaves 75 – 80 % of total leaf nitrogen content was found in chloroplasts and about 70 % of chloroplast nitrogen was present in stroma (Makino and Osmond 1991).

During both developmental and stress-induced leaf senescence, chloroplasts are the first organelles undergoing changes. During transformation of chloroplasts into gerontoplasts, their role is switched from the source of carbon to source of nitrogen for recycling. RUBISCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) and other chloroplast proteins are gradually degraded and photosynthesis declines (see part 1.7). The volume and/or number of chloroplasts gradually decrease, thylakoid membranes are disintegrated and plastoglobules accumulate. Both plastidic and extraplastidic degradative pathways participate in chloroplast degradation. Currently, several chloroplast degradation pathways are supposed: piecemeal degradation of chloroplasts *via* senescence-associated vacuoles (SAVs), CHLOROPLAST VESICULATION-containing vesicles (CCVs) and RUBISCO-containing bodies (RCBs), autophagy of whole chloroplasts (chlorophagy), and selective chloroplast destruction *via* 13-lipoxygenase (13-LOX) (for reviews, see Ishida et al. 2014, Xie et al. 2015, Masclaux-Daubresse et al. 2017, Nakamura and Izumi 2018).

As a RUBISCO content usually decreases from early stages of senescence (earlier than the chloroplast number) and most or all proteolytic activity against RUBISCO is found in the vacuolar fraction (Wittenbach et al. 1982), it can be expected that RUBISCO is released from chloroplasts and subsequently degraded in other compartment(s). Chiba et al. (2003) revealed that during developmental senescence of wheat leaves, RUBISCO is localized in small spherical bodies (RCBs) occurring in cytoplasm and occasionally in the central vacuole. RCBs have a similar electron-staining density as chloroplast stroma and do not contain proteins from thylakoid membranes (for example, light-harvesting complexes, LHCs). RCBs are found in early stage of leaf senescence when the RUBISCO content starts to decline whereas Chl content is still

unchanged. It has been found that the RCB pathway is dependent on *ATG4* and *ATG5*, therefore it has been included among autophagy processes (Izumi and Nakamura 2018).

Besides RCBs, there is an alternative pathway for extra-chloroplastic degradation of chloroplast protein *via* SAVs (Otegui et al. 2005). SAVs are found only in senescing leaves in chloroplast-containing cells (*i.e.*, mesophyll and guard cells) and only during developmental senescence. SAVs contain a senescence specific cysteine-protease, SAG12 (senescence-associated gene 12), while the formation of SAVs occur concomitantly with the *SAG12* expression (Otegui et al. 2005). Similarly to RCBs, only stromal proteins were detected in SAVs, not thylakoid proteins. However, the electron-staining density of SAVs is similar to that of the central vacuoles (*i.e.*, much lower than that of chloroplast stroma). As the formation of SAVs seems to be *ATG*-independent (Otegui et al. 2005), its mechanism probably differs from the mechanism of RCBs formation.

In addition to SAVs, another *ATG*-independent way of the piecemeal Chl degradation was proposed through CCVs (Wang and Blumwald 2014). A nucleus-encoded gene, *CV* (for chloroplast vesiculation) was identified to target and destabilize chloroplasts for protein degradation and to induce the formation of CCVs. Unlike RCBs and SAVs, CCVs contain thylakoid proteins. CCVs were found to be released from chloroplasts and translocated to vacuole. In contrast to SAVs, the *CV*-mediated chloroplast degradation occurs during stress-induced senescence (Wang and Blumwald 2014).

Under certain conditions, especially in the late stages of senescence or during dark-induced senescence, not only the size but also a number of chloroplasts decreases (*e.g.*, Keech et al. 2007). It is supposed that in such cases chloroplasts (reduced in size due to the RCBs and/or SAVs formation) are degraded *in toto* by chlorophagy (Wada et al. 2009). Chlorophagy is supposed to be activated also under accumulation of ROS caused by high-light conditions during senescence (Izumi and Nakamura 2018, Nakamura and Izumi 2018).

Recently, a key role of 13-LOX in the senescence-induced chloroplast destruction was revealed by Springer et al. (2016) in barley leaves. A link between 13-LOX and senescence was for the first time identified by Sharma et al. (2006). This enzyme attacks unsaturated fatty acids of the chloroplast envelope and thereby introduces 'holes' for mass export of stromal constituents into cytosol. The expression of 13-LOX is confined to senescent cells. Unique structural features of its NH_2 terminus appear to ensure the proper targeting to the chloroplasts but not to other cell organelles (Springer et al. 2016).

It can be assumed that the particular pathways of chloroplast degradation may be more or less active during different stages, types and conditions of senescence. The combination of these pathways may facilitate complete degradation of these large organelles and effective nutrient remobilization. For example, the RCB pathway is particularly active during sugar starvation due to darkening or inhibition of photosynthesis, while autophagy of entire chloroplasts may prevail under high-light conditions (Fig. 3, Izumi and Nakamura 2018).

The chloroplast degradation 'strategies' can vary also among plant species. For example, in wheat and barley, which are known to have very efficient nutrient remobilization from older to younger leaves and especially to the grains (Gregersen et al. 2008), both size and number of chloroplasts decrease gradually during senescence (Camp et al. 1982, Ono et al. 1995, Springer et al. 2015) and RCBs as well as chlorophagy are observed (Hörtensteiner and Feller

2002, Gregersen et al. 2008). The concomitant decrease in the chloroplast size and number has been found also in *Arabidopsis* (Wada et al. 2009). On the contrary, in the dicot common bean (*Phaseolus vulgaris* L.) the number of chloroplasts *per* mesophyll cell remains unaltered until the final phase of leaf senescence (Jenkins and Woolhouse 1981).

In addition to the decrease in the size and number of chloroplasts, their ultrastructure alters during senescence (Šesták 1985, Kutík et al. 1998, **Špundová et al. 2003**, Springer et al. 2015, Sobieszczuk-Nowicka et al. 2018). The senescence-associated changes of chloroplast ultrastructure include disappearance of thylakoid membranes, grana unstacking and loosening, swelling of intrathylakoid space, and chloroplast shrinkage. In parallel, a number and size of plastoglobules (PGs) increase (Lichtenthaler and Sprey 1966, Tuquet and Newmann 1980, Ghosh et al. 2001, Biswal et al. 2003, **Špundová et al. 2003**, Springer et al. 2015, Tominaga et al. 2018).

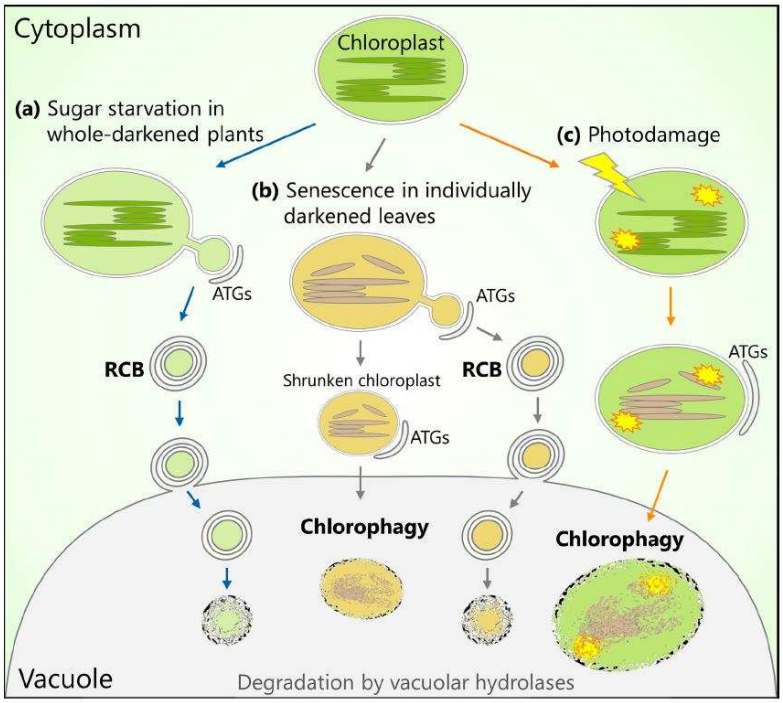


Fig. 3 A scheme of autophagy-related forms of chloroplast degradation under different conditions. (**a**) Darkening of whole plants (sugar starvation) – the RCB-pathway; (**b**) Darkening of individual leaves – combination of the RCB-pathway and chlorophagy of shrunken chloroplasts; (**c**) Excess light intensity (chloroplast photodamage) – chlorophagy of damaged chloroplasts prior activation of RCBs.
(Izumi and Nakamura 2018)

PGs are connected to the thylakoid membrane by the outer lipid half-bilayer and arise at the margin of the stromal thylakoid. The PG core contains neutral lipids including prenylquinones, triacylglycerols, fatty acid phytyl esters, carotenoids and others but not glycolipids (for reviews, see *e.g.* Besagni and Kessler 2013, van Wijk and Kessler 2017). Plastoquinol and tocopherol are the major constituents of the prenylquinones in PGs. For many years, PGs were supposed to be only a passive storage site for thylakoid degradation products

(Lichtenthaler 1969, Burke et al. 1984). However, now it is known that PGs contain also small proteome and metabolome (Lundquist et al. 2012, van Wijk and Kessler 2017) and are involved in metabolism of tocopherols, quinones, and carotenoids (van Wijk and Kessler 2017, Ksas et al. 2018). Recently has been shown that PGs play also a role in Chl degradation and phytol recycling (Vom Dorp et al. 2015) and in chloroplast photo-protection (Ksas et al. 2018).

1.3.2 Chloroplast movement

Chloroplast movement is one of the mechanisms by which plants respond to changing light environment (for a review, see *e.g.* Kong and Wada 2014). In higher plants, the light-induced chloroplast movement is induced by B light *via* phototropins. There are two types of light-induced chloroplast movement: accumulation and avoidance response. Under low light conditions, chloroplasts accumulate along the cell walls perpendicular to the incident light ('face' position). When exposed to high light, chloroplasts migrate to the cell walls parallel to the incoming light into 'side' position (avoidance response). As we have shown in our previous works, the chloroplast movement can be easily detected by measurement of a leaf optical transmittance (Nauš et al. 2010, 2016). During the avoidance response induced by strong light, the transmittance increases as chloroplasts relocate to the 'side' position. On the contrary, when the leaf is exposed to weak light, chloroplasts move back to the 'face' position and transmittance decreases (see Fig. 4).

It is thought that avoidance response protects chloroplasts from photo-oxidative damage under conditions of excess excitations (Brugnoli and Björkman 1992, Davis and Hangarter 2012, Cazzaniga et al. 2013). As such conditions commonly occur during senescence, avoidance movement might be important for the controlled and efficient chloroplast degradation. However, almost nothing is known about functioning of the light-induced chloroplast movement in senescing leaves, although its impairment could be expected. In our previous works on tobacco leaves, we have obtained indications that the chloroplast avoidance movement is inhibited during senescence as an extent of the strong B light-induced increase of leaf transmittance decreased with decreasing Chl content (Rolencová 2008, Nauš et al. 2010) and was lower in older barley leaves compared with younger ones (Nauš et al. 2016).

Preliminary results with senescing leaves of *Arabidopsis* (Fig. 4) showing a correlation between extent of the avoidance response and Chl content support this assumption. The inhibition of chloroplast movement in senescing leaves might be related to senescence-associated impairment of cytoskeleton found by Keech et al. (2010). In addition, an abnormal arrangement of chloroplasts has been found in cells of senescing leaves (*e.g.*, Wittenbach et al. 1982, **Vlčková et al. 2006**) indicating impaired chloroplast anchoring to plasma membrane, which might influence negatively the light-induced chloroplast movement. It cannot be excluded that the observed reduction of light-induced changes in transmittance was associated also with a decrease in the chloroplasts number. Interestingly, our results indicate that chloroplast movement may change after the plant transition from vegetative to generative phase (Nauš et al. 2010). Further investigations are needed to elucidate the role of chloroplast movement in leaf senescence.

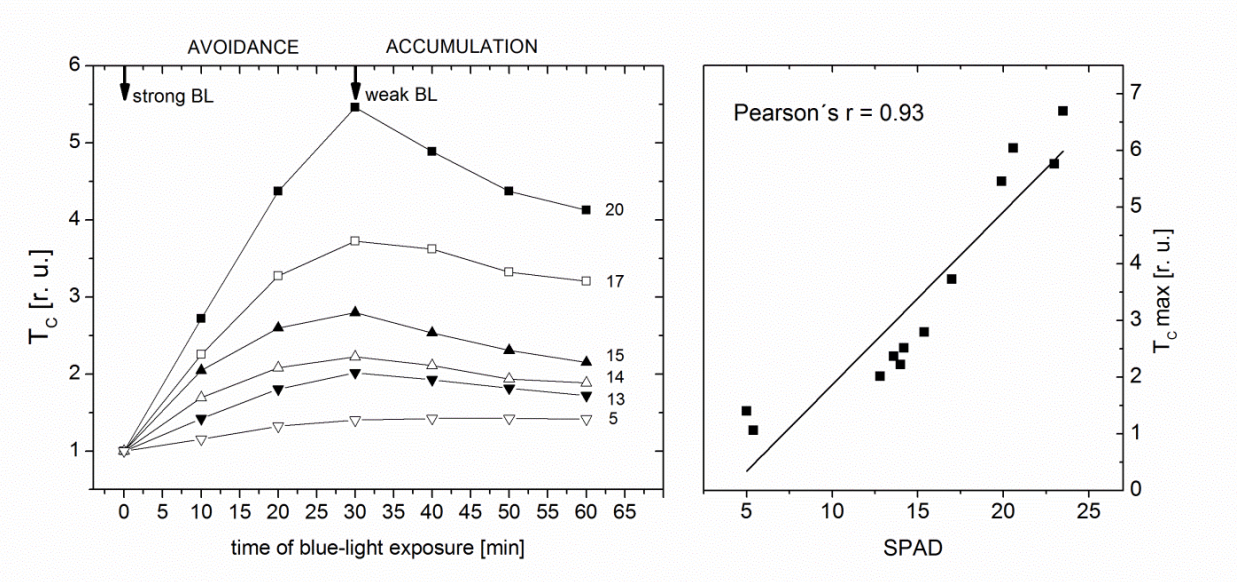


Fig. 4 Relative changes in collimated transmittance (T_C, measured at λ = 436 nm) of *Arabidopsis thaliana* L. (Col-0) leaves during exposition to strong blue-light (100 µmol photons m^{-2} s^{-1}; avoidance response) and weak blue-light (10 µmol photons m^{-2} s^{-1}; accumulation response) and correlation between the maximal T_C value (at time = 30 min) and chlorophyll content measured by SPAD-chlorophyllmeter.

1.4 Senescence-associated pigment changes

1.4.1 Chlorophyll degradation

Loss of green colour, the most apparent sign of leaf senescence, is caused by massive Chl degradation. Due to its light-absorbing ability, Chl has a phototoxic potential and can be dangerous for plant cells (especially when it is not integrated in photosynthetic pigment-protein complexes), therefore its metabolism (including degradation) is highly regulated (for a recent review, see *e.g.* Zhu et al. 2017).

Breakdown of Chl, a long lasting 'biological enigma', has been solved during the last three decades. Chl catabolites are now collectively termed phyllobilins and the biochemical pathway of Chl degradation 'PAO/phyllobilin pathway', as a key enzymatic breakdown step is catalysed by pheophorbide *a* oxygenase (PAO) (for an up-to-date review, see Kuai et al. 2018).

The PAO/phyllobilin pathway involves two distinct phases (Fig. 5). In the first phase, phototoxic free Chl molecules and their intermediates are degraded to colourless, primary fluorescent Chl catabolites (pFCC) inside chloroplasts, at thylakoid membranes. All reactions involved in the gradual conversion of Chl *b* to pFCC are common to all plant species in which Chl degradation has been investigated so far (Kuai et al. 2018). Based on the recent identification of $C3^2$-hydrolase as a chloroplast inner envelope-located oxygenase (named translocon at the inner chloroplast envelope 55, TIC55) (Hauenstein et al. 2016), and on the common occurrence of formyloxobilin- and dioxobilin-type non-fluorescent Chl catabolites (NCCs and DNCCs, respectively) deriving from $C3^2$-hydroxylated pFCC (hydroxyl-pFCC), the hydroxylation of pFCC has been included in the first phase of Chl breakdown (Kuai et al. 2018; Fig. 5).

Prior to its degradation by the PAO/phyllobilin pathway, Chl *b* has to be converted to Chl *a* by a reductive half of the 'chlorophyll cycle' (Tanaka and Tanaka 2011). The reduction of Chl *b* to Chl *a* is a two-step reaction catalysed by NYC1 (non-yellow coloring1) and NOL (NYC1-LIKE), and by hydroxymethyl Chl *a* reductase (HCAR).

Since its identification more than a century ago, chlorophyllase (CLH) hydrolysing Chl to chlorophyllide and phytol was assumed to be involved in senescence-associated Chl degradation, despite the fact that CLH proteins were found almost exclusively outside chloroplasts (especially in endoplasmatic reticulum and tonoplast). This was originally explained by extra-chloroplastic Chl dephytylation. Recently, CLH and chlorophyllide were proposed to play a role in plant defence against herbivores: in leaves damaged by herbivore, CLH has access to chloroplast-localized Chl (Hu et al. 2015). Currently, the Chl dechelatation is considered to be the first step of Chl *a* degradation during leaf senescence (Kuai et al. 2018; Fig. 5).

NYE (non-yellowing) protein (also called stay-green, SGR) has been recently identified as the enzyme degrading Chl *a* to pheophytin *a* as their Mg-dechelatase activity was revealed (Shimoda et al. 2016). The next step (dephytylation) is catalysed by pheophytinase (PPH). The next two steps are decisive in the loss of green colour: the ring-opening of pheophorbide *a* by PAO (the membrane-localized, nonheme Fe-containing Rieske-type mono-oxygenase) resulting in red Chl catabolite (RCC), and the reduction of RCC by RCC-reductase (RCCR) to pFCC.

The second phase of PAO/phyllobilin pathway involves further side-chain-modifying reactions, which all take place outside chloroplast, and include non-enzymatic isomeration of the fluorescent phyllobilins (pFCC and hydroxyl-pFCC) to non-fluorescent ones (NCCs and DNCCs) in acidic pH (Fig. 5). The occurrence of NCCs and DNCCs varies among plant species (Kuai et al. 2018). In *Arabidopsis*, CYP89A9 (localized to endoplasmatic reticulum and catalysing oxidative deformylation of FCC to respective DFCC) was identified to be responsible for DNCC formation. Demethylation of FCC was found to be catalysed by methylesterase 16 (MES16) localized in cytosol. Final Chl catabolites are transported and stored in vacuole of senescing cells.

The PAO/phyllobilin pathway might not to be the only way of Chl degradation. For instance, peroxidases were reported to be involved, however no respective degradation products have been identified (Kuai et al. 2018).

1.4.2 Carotenoids

Chloroplasts of higher plants usually contain the following carotenoids: α-carotene, β-carotene (located mainly in reaction centres), and xanthophylls – violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), neoxanthin and lutein (mainly associated with light-harvesting complexes). A content of overall carotenoids decreases during leaf senescence, however, a rate of their degradation is lower compared with Chl (Biswal 1995) thereby the ratio of carotenoid and Chl content usually increases during senescence (*e.g.*, **Janečková et al. 2019**). In senescing leaves, xanthophyll acyl esters are typically accumulated. It is thought that carotenoids released from thylakoid pigment-protein complexes are stored in PGs.

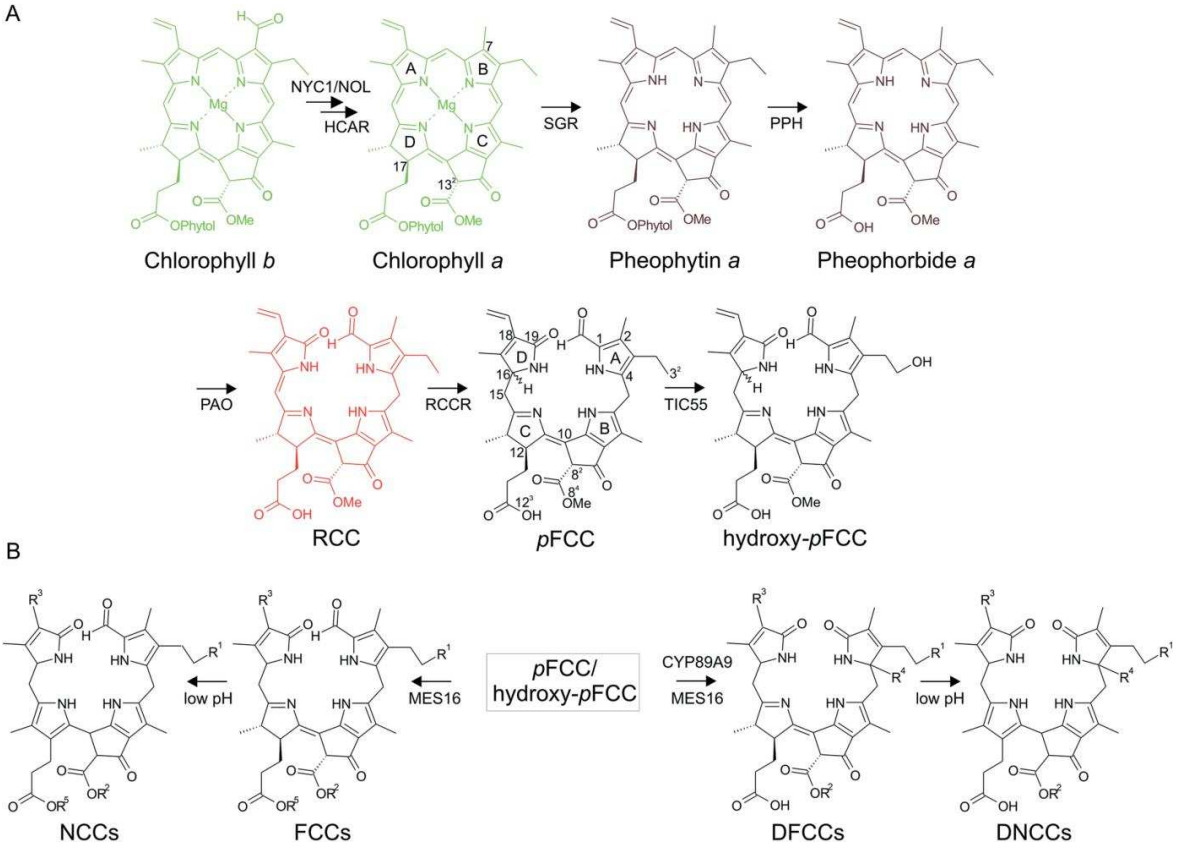


Fig. 5 The PAO/phyllobilin pathway of chlorophyll breakdown in leaves. (A) Reactions of the first part of the pathway from chlorophyll to hydroxy-pFCC that take place inside the chloroplast. (B) pFCC/hydroxy–pFCC-modifying reactions of the second part of the pathway that take place outside the chloroplast and lead to the diversity of phyllobilins. Note that MES16 and CYP89A9 are specific *Arabidopsis* enzymes, while all other enzymes are known from different plant species. Products: DFCCs, dioxobilin-type fluorescent chlorophyll catabolites; DNCCs, dioxobilin-type non-fluorescent chlorophyll catabolites; FCCs, formyloxobilin-type fluorescent chlorophyll catabolites; NCCs, formyloxobilin-type non-fluorescent chlorophyll catabolites; pFCC, primary fluorescent chlorophyll catabolite; RCC, red chlorophyll catabolite. Enzymes: CYP89A9, specific cytochrome P450 monooxygenase; HCAR, hydroxymethyl Chl *a* reductase; MES16, methylesterase 16; NOL, NYC1-LIKE; NYC1, non-yellow coloring1; PAO, pheophorbide *a* oxygenase; PPH, pheophytinase; RCCR, RCC-reductase; SGR, stay-green; TIC55, translocon at the inner chloroplast envelope 55. (Kuai et al. 2018, with permission).

The slower degradation of carotenoids during senescence may be associated with their photo-protective function. One of the important photo-protective mechanisms in senescing leaves is the xanthophyll cycle dissipating excess light energy as heat (Demmig-Adams and Adams 1992). Although the amount of xanthophyll cycle pigments (V, A, Z) decreases during senescence, their de-epoxidation state (DEPS = (A + Z)/(A + Z + V)) usually increases during senescence under field conditions (*e.g.*, García-Plazaola and Becerill 2001, Lu et al. 2001, 2003) as well as in detached leaves senescing under light (**Špundová et al. 2005a**, **Vlčková et al. 2006**). The increase in DEPS is higher when senescing leaves are exposed to higher light intensity (García-Plazaola and Becerill 2001, Lu et al. 2001). The protective role of xanthophylls during senescence is indicated also by the increase of (A + Z + V) content relative to content of Chl, which is generally observed in senescing leaves (*e.g.*, Lu et al. 2003, **Špundová et al. 2005a**).

1.4.3 Anthocyanins

Unlike Chl and carotenoids, anthocyanins are newly produced during leaf senescence. They are synthesized in cytosol and then transported to vacuoles (Tanaka et al. 2008). The anthocyanins distribution in leaves depends on plant species, they can accumulate in vacuoles of both upper and lower epidermis and also in mesophyll cells (Merzlyak et al. 2008). The anthocyanin synthesis and sequestration represent a considerable metabolic investment for cells. Although the role of anthocyanins has been thoroughly investigated, their function in senescing leaves is still unclear.

Generally, anthocyanins are thought to protect the photosynthetic apparatus in mesophyll cells from excess light intensity and consequent oxidative damage. *In vivo*, anthocyanins commonly absorb light of wavelengths between 500 – 600 nm, but their absorption above 600 nm was also reported (Merzlyak et al. 2008, Hlavinka et al. 2013). It has been suggested that synthesis and accumulation of anthocyanins during leaf senescence, when the risk of over-excitation and oxidative damage usually increases, is connected with their photo-protective effect (Hoch et al. 2001). The protection from oxidative damage should allow the efficient nutrients recycling during the re-organization phase of senescence. Consistently with this hypothesis, the anthocyanin content in falling tree leaves negatively correlated with a content of residual nitrogen (Lee et al. 2003).

However, in a number of studies, the photo-protective role of anthocyanins was not confirmed (Gould et al. 2018). There is increasing evidence that the massive biosynthesis of anthocyanins may serve as an alternative way of the decreasing sugar accumulation under conditions of reduced sink strength (Lo Piccolo et al. 2018). The sugar accumulation causes the feed-back inhibition of photosynthesis, down-regulates the expression of photosynthetic genes and it is thought to induce or accelerate leaf senescence (see also parts 1.2.5 and 1.7.3). The accumulation of anthocyanins might therefore delay a sugar-promoted senescence, maintain functional photosynthetic apparatus for a longer time and allow the more efficient nutrient translocation (Lo Piccolo et al. 2018).

1.5 Role of ROS in leaf senescence

It is generally thought that aging of an organism or cell is associated with oxidative stress caused by an accumulation of free radicals. Although the 'free radical theory of aging' (Harman 1956) has proved to be inaccurate (see *e.g.*, Pomatto and Davies 2018, Vina et al. 2018), the fact is that a level of oxidative damage increases with age (*e.g.*, Munné-Bosch and Alegre 2002). Oxidative stress and/or oxidative damage are caused by an accumulation of ROS (singlet oxygen, $^1O_2$; superoxide anion radical, $O_2^{\bullet -}$; hydroxyl radical, $OH^{\bullet}$; hydrogen peroxide, H_2O_2) due to an imbalance between their generation and elimination. Originally ROS were considered to have only the damaging effect, but now it is clear that ROS have an essential role in the regulation of key cellular processes including senescence.

ROS play multiple roles in senescing leaves. In the initiation phase, ROS participate in induction of re-programming of gene transcription (*e.g.*, Niewiadomska et al. 2009, Bieker et al. 2012, Bresson et al. 2018). At the beginning of re-organization phase, an increase in ROS content (*e.g.*, Smart 1994) leads to pigment-, protein-, and lipid-oxidation that is necessary for the nutrients remobilization (Hörtensteiner and Feller 2002). Finally, during the terminal phase, oxidative processes participate pronouncedly in the final cell and leaf destruction (Zimmermann and Zentgraf 2005). It is obvious that excessive accumulation of ROS should be avoided over the entire senescence process to allow the efficient nutrient recycling and to prevent from premature cell death. To save a low ROS content, an effective and fine-tuned antioxidant protection is needed, involving enzymes as well as low-molecular-weight antioxidant substances.

Several enzyme cycles participate in the antioxidative defence of plants. The enzymes ascorbate-peroxidase (APX) and glutathione reductase (GR) are the main enzymes of the ascorbate-glutathione cycle, a part of the water-water cycle in chloroplasts scavenging $O_2^{\bullet -}$ and H_2O_2 (Asada 1999). It is generally assumed that the capacity of antioxidant protection decreases during leaf senescence. However, the decrease in antioxidant activity occurs mainly in the latest stages of senescence and it is considered to be not the cause but a consequence of senescence (Dertinger et al. 2003). Different changes in activities of antioxidant enzymes and content of non-enzymatic antioxidants were reported in senescing leaves (for a review, see Procházková and Wilhelmová 2007) reflecting a complexity, time-spatial dependence and specifity of the antioxidant protection during senescence of different plant species under different conditions.

The initiation of senescence was found to coincide with an increase in H_2O_2 content in leaves of various species (*e.g.*, Niewiadomska et al. 2009, Bieker et al. 2012, Bresson et al. 2018), which was associated with a temporal decrease in activity of catalase and APX (Zimmermann et al. 2006, Niewiadomska et al. 2009, Bieker et al. 2012). In this case, the decrease in activity of antioxidant enzymes is probably controlled by cell to trigger senescence, as the increase in H_2O_2 content induces expression of TFs (Miao et al. 2007) and *SAGs* (Sabater and Martín 2013). For example, the expression of senescence-associated WRKY and NAC factors is controlled by H_2O_2 (Miao et al. 2007).

In some cases, especially during stress-induced senescence, the excessive ROS accumulation can cause earlier onset of the terminal phase thus the principal function

of senescence, the remobilization of nutrients from the leaf, is not fulfilled. However, it has been shown that the ROS accumulation needs not always to cause oxidative damage in senescing leaves (Vanacker et al. 2006, Niewiadomska et al. 2009, Pilarska et al. 2017). It is possible that in such cases the increased ROS content could activate some defence processes protecting cells from oxidative damage.

Nevertheless, leaf senescence is typically associated with an increased oxidative damage including lipid peroxidation (see part 1.6). The increase in oxidative damage has been found during developmental senescence (*e.g.*, Berger et al. 2001), premature senescence induced by plant shading (**Špundová et al. 2005b**) and dark-induced senescence of detached leaves (**Špundová et al. 2003**, Causin et al. 2006, **Vlčková et al. 2006**, **Janečková et al. 2018;** Fig. 6). Generally, the senescence-associated ROS accumulation is strengthened and level of oxidative damage increases under stress conditions. An origin of ROS can differ in dependence on light conditions during senescence. Under light conditions, chloroplasts can be considered as the main source of ROS (for a review of ROS generation in chloroplasts, see *e.g.* Pospíšil 2012). Besides the chloroplasts, other cellular compartments such as mitochondria and peroxisomes also contribute to the ROS generation.

1.6 Leaf senescence and cell membranes

During senescence, cell membranes undergo changes in composition and integrity. These senescence-associated changes occur asynchronously in different types of cellular membranes. The first membranes undergoing degradative changes are thylakoids (*e.g.*, Kolodziejek et al. 2003). The integrity of plasmatic membrane is usually maintained until late stages of leaf senescence as documented by relatively low ion leakage from senescing leaf tissue (*e.g.*, Oda-Yamamizo et al. 2016).

Both main membrane components, lipids and proteins, are degraded during senescence. Phospholipids are extensively catabolised. A content of fatty acid decreases, which leads to enhanced relative concentrations of sterols (Thompson et al. 1998, Hopkins et al. 2007). Senescence-induced changes in lipid compositions result in changed biophysical organization of membrane bilayer (*e.g.*, membrane rigidification, lipid phase separation) and consequent membrane leakiness (Thompson et al. 1998). In contrast to the cytoplasmic membranes, thylakoids do not undergo phase changes during senescence. The enzymatic lipid peroxidation is considered to be a main pathway of senescence-associated degradation of membrane lipids, in which four types of enzymes are involved: phospholipases (PLs), phosphatidic acid phosphatases, lipolytic acyl hydrolases and LOXs (Thompson et al. 1998).

The following scenario is supposed: PLs (activated *via* Ca^{2+}) release polyunsaturated fatty acids (PUFAs) from membrane phospholipids, the increase of PUFA content activates LOXs and LOXs transform PUFAs to lipid hydroperoxides (LOOHs). Then LOOHs are converted to various secondary products (Spiteller 2003). Galactolipids of thylakoid membranes (mono- and di-galactosyldiacylglycerol) are thought to be degraded by galactolipases, galactosidases and lipolytic acyl hydrolases during leaf senescence (Hopkins et al. 2007). Galactolipids are thought to serve as a source of energy for metabolism of senescent cells (Matile 2001). The released

fatty acids and their metabolites can be used for synthesis of acetyl-CoA. Subsequently, acetyl-CoA can be used directly for respiration or, *via* a glyoxylate pathway and gluconeogenesis, for production of carbohydrates (for a review, see Troncoso-Ponce et al. 2013). The senescence-enhanced expression of genes encoding enzymes for the β-oxidation and glyoxylate pathway (Buchanan-Wollaston et al. 2003) and increased β-oxidation activity (Law et al. 2018) indicate the importance of these processes in lipid degradation and remobilization during senescence.

An activity of PLs has been found to increase during senescence (*e.g.*, Antonacci et al. 2011). An increased LOX activity in senescing plant tissues has been repeatedly reported but in some case a decrease was found (for a review, see Siedow 1991). The LOX activity could be higher due to wounding of analysed tissues (Siedow 1991) or due to the senescence-associated increase in content of free PUFAs rather than due to the higher enzyme activity *per se* (Thompson et al. 1998). Berger et al. (2001) has assumed only a minor role of LOXs in senescence-associated lipid peroxidation. On the other hand, a temporal increase in LOX activity has been reported by Ye at al. (2000) during transition of *Arabidopsis* plants from vegetative to reproductive phase. According to Ye at al. (2000), the LOX activation and consequent increase in lipid peroxidation could induce *SAGs* and onset of leaf senescence. The role of LOXs and LOX-mediated lipid peroxidation in leaf senescence remains to be elucidated.

For estimation of level of lipid peroxidation, the quantification of malondialdehyde (MDA) (as a final product of lipid peroxidation) is most widely used. A low-specific MDA estimation based on spectrophotometric quantification of thiobarbituric acid-reactive substances (TBARs) (*e.g.*, **Špundová et al. 2003**, Causin et al. 2006, **Vlčková et al. 2006**) is gradually being replaced by more specific methods, for example, HPLC (Fig. 6, **Janečková et al. 2018**) or GC-MS (Pilarska et al. 2017) because the TBARs method can pronouncedly overestimate the MDA content (Liu et al. 1997). In our experiments, we have started to use *in vivo* detection of LOOH, primary products of lipid peroxidation, by a SPY-LHP probe (Soh et al. 2007). Using a confocal microscope, we have visualized the accumulation of LOOH in chloroplasts of dark-senescing leaves of wheat and *Arabidopsis* (Fig. 6B). The increased level of lipid peroxidation in senescing leaves was confirmed by a higher MDA content, as determined by HPLC according to **Janečková et al. (2018)** (Fig. 6A).

1.7 Leaf senescence and photosynthesis

1.7.1 Senescence-associated inhibition of photosynthesis

Senescence is typically accompanied by an inhibition of photosynthesis. A decrease in rate of CO_2 assimilation (*e.g.*, Lu and Zhang 1998, **Špundová et al. 2005b,** Tang et al. 2005) as well oxygen evolution is commonly observed (*e.g.*, Humbeck et al. 1996). The senescence-associated decrease in photosynthesis is caused by inhibition of both Calvin-Benson cycle reactions and primary photosynthetic reactions on thylakoid membranes. The inhibition of Calvin-Benson cycle, related mainly to a decrease in activity and content of RUBISCO (*e.g.*, Sobieszczuk-Nowicka et al. 2018), usually precedes the inhibition of primary photosynthetic reactions including PSII

photochemistry (*e.g.*, Camp et al. 1982, Grover 1993, **Špundová et al. 2005b**, **Vlčková et al. 2006**). The preferential inhibition of stromal reactions is thought to cause the ROS accumulation and consequent oxidative damage due to an imbalance between generation and consumption of electrons in the photosynthetic electron transport chain (*e.g.*, **Vlčková et al. 2006**).

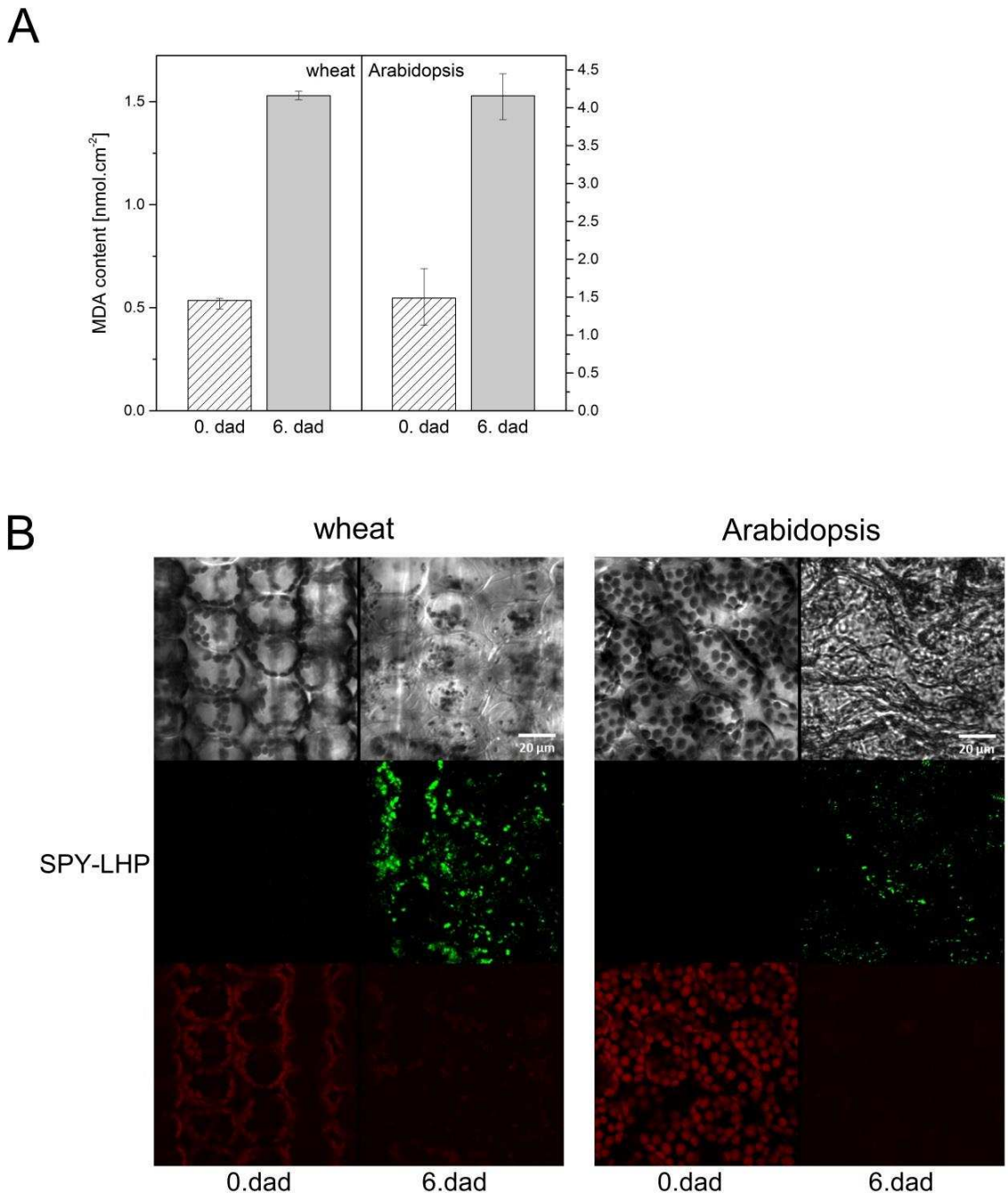


Fig. 6 Oxidative damage in wheat and *Arabidopsis* control (freshly detached) leaves (0. dad) and dark-senescing leaves on the 6th day after detachment (6. dad). A – MDA content *per* leaf area (determined by HPLC). Medians and quartiles are presented (n = 4). B – *in vivo* imaging of lipid hydroperoxides by confocal microscopy using a fluorescent probe SPY-LHP (Dojindo Molecular Technologies, USA).

Although PSII photochemistry is relatively stable during developmental senescence (*e.g.*, Miersch et al. 2000, Krupinska and Humbeck 2004, Tang et al. 2005), a rapid and pronounced decrease in PSII function has been reported in dark-senescing detached leaves (*e.g.*, **Špundová et al. 2003**, **Vlčková et al. 2006**, Keech et al. 2007, **Janečková et al. 2018**, Sobieszczuk-Nowicka et al. 2018). Steady-state parameters of Chl fluorescence induction reflecting photochemical quenching (qP) decreased during senescence, while parameters reflecting non-photochemical quenching processes (qN) usually increased (*e.g.*, Lu and Zhang 1998, **Janečková et al. 2018**). The decreased qP indicates that the portion of absorbed light energy used for photochemistry is lowered, thus the risk of ROS accumulation and photodamage increases in senescing leaves. The increased qN can serve as one of the protective mechanisms against photodamage of photosynthetic apparatus (Lu and Zhang 1998).

The energy partitioning in the leaf and its changes during senescence can be evaluated using parameters Φ_P (an effective quantum yield of PSII photochemistry in light-adapted state), Φ_{NPQ} (a quantum yield for regulatory non-photochemical quenching), and $\Phi_{f,D}$ (a quantum yield for constitutive non-regulatory dissipation processes) (for a review, see Lazár 2015). The advantage of using these parameters is that all are quantum yields, the sum of which is equal to unity, and thus they can be compared absolutely (Lazár 2015). Φ_P reflects the energy fraction used in PSII photochemistry, while the portion of energy allocated into non-photochemical quenching processes is reflected by both Φ_{NPQ} and $\Phi_{f,D}$. Φ_{NPQ} represents the quantum yield of regulatory quenching induced by illumination to protect photosynthetic apparatus against excess excitations and consequent ROS accumulation and oxidative damage, while $\Phi_{f,D}$ reflects the quantum yield of non-regulatory constitutive processes. In our work we have shown that in dark-senescing leaves of barley Φ_P decreased and $\Phi_{f,D}$ increased (as expected). Φ_{NPQ} was higher in the leaves with a partially functional PSII photochemistry and lower in the leaves, in which PSII photochemistry was almost completely inhibited (**Janečková et al. 2019**). This finding is consistent with the assumed protective role of regulatory quenching mechanisms in senescing leaves.

1.7.2 Degradation of photosynthetic proteins

As mentioned above, RUBISCO is thought to be one of the key factors responsible for the senescence-associated inhibition of photosynthesis (Grover 1993, Krupinska and Humbeck 2004, Tang et al. 2005). As RUBISCO is one of the major sources of cellular nitrogen, it is degraded (particularly its large subunit RbcL) in early stages of senescence. The RUBISCO degradation occurs mainly in vacuole (see part 1.3.1), but it can be degraded also in intact chloroplasts (for a review, see Feller et al. 2008). Other stromal enzymes (which are much less abundant than RUBISCO) are degraded with similar kinetics as RUBISCO (Krupinska and Humbeck 2004).

In next stages of senescence, before the Chl content starts to decline, a rate of photosynthetic linear electron transport decreases. It is attributed to a senescence-associated degradation of components of electron transport chain in thylakoid membranes, mainly Cyt b_6f complex and plastocyanin (*e.g.*, Grover 1993, Nath et al. 2013, Schöttler et al. 2017). Krieger-Liszkay et al. (2019) have suggested that during senescence alternative electron pathways (such as cyclic transport around PSII or PSI, electron transport *via* plastidial terminal oxidase,

and chlororespiration) become more important, as they may provide a sufficient generation of ATP needed for the controlled remobilization of thylakoid components.

The order of degradation of the individual pigment-protein complexes in thylakoid membranes depends on the conditions of senescence (*e.g.*, Mae et al. 1993) and seems to be also species-dependent. In different senescence studies, a different relative rate of photosystem II (PSII) and photosystem I (PSI) degradation has been found. The differences were found not only among various plant species, but also between different cultivars of one species (*e.g.*, Krieger-Liszkay et al. 2015). For example, a preferential decrease in PSI was found in rice (Tang et al. 2005) and barley (Miersch et al. 2000), while the higher stability of PSI was found during senescence of *Arabidopsis* (Nath et al. 2013) and tobacco (Schöttler et al. 2017).

The relative degradation rate differs also between reaction centres (RCs) and light-harvestings complexes (LHCs). For a comparison of these rates, a Chl *a*/Chl *b* ratio can be used, as Chl *a* is involved mainly in RCs and Chl *b* mainly in LHCs. In many senescence studies, a decrease in the Chl *a*/Chl *b* ratio was found indicating preferential degradation of RCs (*e.g.*, Tang et al. 2005, Nath et al. 2013, Schöttler et al. 2017, **Janečková et al. 2019**; Fig. 7 – wheat).

On the contrary, the Chl *a*/Chl *b* ratio increased in barley flag leaves during a late phase of developmental senescence, concomitantly with a decrease in Lhcb1, one of the outer LHCII apoproteins (Krupinska et al. 2012). According to the authors, the degradation of LHCII reduced a risk of photodamage of photosynthetic apparatus, which allowed a longer maintenance of photosynthesis and increased efficiency of nutrients remobilization. This assumption is supported by results of Mae et al. (1993) who found a delayed LHCII degradation in shaded plants of *Lolium temulentum*. The increase of Chl *a*/Chl *b* ratio has been also found during dark-induced senescence of detached leaves of *Arabidopsis* (Fig. 7; Pružinská et al. 2005, Sztatelman et al. 2015) although in this case the reason for preferential degradation of LHCs is not clear. Finally, Kolodziejek et al. (2003) did not found any changes in the Chl *a*/Chl *b* ratio in barley leaves (as well as in leaves of maize) developmental senescence. Similarly, Keech et al. (2007) reported the unchanged Chl *a*/Chl *b* ratio during dark-induced senescence of detached *Arabidopsis* leaves, which contradicts the above mentioned results by Pružinská et al. (2005) and Sztatelman et al. (2015). Thus, it seems that there is no universal scenario for the order of dismantling of individual photosynthetic components in thylakoid membranes during senescence.

An important role of the LHC degradation in the progress of senescence has been proposed (Sakuraba et al. 2012a). There is little information about mechanism of this degradation. A proteolytic activity against LHCII was found in thylakoid fraction of *Arabidopsis* leaves senescing in dark (Oh et al. 2004). Further, the proteolytic activity associated with thylakoid membranes and mediating degradation of Lhcb3 (one of the outer LHCII apoproteins) during dark-induced senescence was found in barley (Żelisko and Jackovski 2004) and *Arabidopsis* (Żelisko et al. 2005). In both latter cases, the proteases were characterized as Zn^{2+}-dependent metalloproteases. The authors assumed that these proteases could degrade various LHCII apoproteins, not only Lhcb3. Based on *in vitro* experiments, the protease FtsH6 (Fts, filamentation temperature sensitive) was thought to be responsible for LHCII degradation (Żelisko et al. 2005), however, according to later study of Wagner et al. (2011), FtsH6 seems to be unimportant for the LHCII degradation *in vivo*, including degradation in senescing leaves.

A critical factor for the LHC II degradation is the pigment binding. Before degradation by proteolysis, Chl molecules have to be dissociated from the apoproteins (Hörtensteiner and Feller 2002). The senescence-induced degradation of LHCII is initiated by the conversion of Chl *b* to Chl *a* and by Mg^{2+} dechelatation of Chl *a* (Fig. 5; Horie et al 2009, Shimoda et al. 2016). Impairment or inhibition of these steps of Chl degradation (for example, in some types of stay-green mutants) is usually associated with maintenance of both LHCII and thylakoid organisation (*e.g.*, Horie et al 2009, Tominaga et al. 2018).

Recent investigations have shown that Chl *b* plays an important role in the regulation of leaf senescence. In mutants with higher Chl *b* content, a slower senescence-associated degradation of Chl, LHCs and thylakoid membranes was found (Kusaba et al. 2007, Sakuraba et al. 2012b, Voitsekhovskaja and Tyutereva 2015). On the other hand, a recent study with *pgl* rice mutant has shown that Chl *b* deficiency was associated with increased Chl degradation, accumulation of ROS, and electrolyte leakage during both natural senescence of flag leaves and dark-induced senescence of detached leaves (Yang et al., 2016a). Kusaba et al. (2007) has also mentioned faster Chl degradation in dark-incubated detached leaves of *cao-2* rice mutant deficient in Chl *b*. In our work (**Janečková et al. 2019**) we have shown that the Chl *b* deficiency in the *chlorina* barley mutant accelerated pronouncedly the inhibition of PSII photochemistry during dark-induced senescence of detached leaves. We suppose that Chl *b* deficiency resulting in a substantial decrease in amount of LHCs destabilized RCII and accelerated their impairment during dark-induced senescence (**Janečková et al. 2019**).

It can be summarized that the senescence-associated inhibition of the primary photosynthetic reactions may have different reasons as the individual photosynthetic components in thylakoid membranes are degraded in different order being dependent on the 'senescence system', conditions (especially light conditions) during senescence and on plant species. This might be related to different ROS production and species-dependent strategy of nutrient remobilization, which can result in different way of chloroplast degradation (see part 1.3.1).

1.7.3 Chloroplast retrograde signalling during senescence

It has been proposed some time ago that the expression of nuclear *SAGs* is regulated by signals from chloroplasts (Hensel et al. 1993) but a role of the chloroplast retrograde signalling in regulation of leaf senescence has not yet been thoroughly studied. Zapata et al. (2005) have suggested that chloroplasts could induce leaf senescence by increasing a reduction level of electron carriers and by generating ROS due to increased activity of a thylakoid NADH dehydrogenase complex. There are indications that salicylic acid (produced by chloroplasts) could play a role in this regulation. Salicylic acid (SA) accumulates during natural senescence and is involved in the control of many nuclear *SAGs* (Morris et al. 2000, Buchanan-Wollaston et al. 2005). Further, SA has been identified to be responsible for high-light acclimation consisting in altered expression of nuclear genes (Szechynska-Hebda and Karpinski 2013) and 'high-light conditions' can be expected in senescing chloroplasts in which capture of excitation energy is higher than its utilization. As recently proposed, a WHIRLY1 protein could be involved in the SA retrograde signalling during senescence under high light conditions (Kucharewicz et al. 2017).

It is considered that sugars play a significant role in chloroplast retrograde signalling. However, their role in the induction and regulation of senescence is not yet clear as summarized by van Doorn (2008), and recently by Biswal and Pandey (2018) and Wojciechowska et al. (2018). It is difficult to determine mechanisms of senescence regulation by sugars because of its complexity. The sugar level in senescing leaves depends not only on the rate of their (photo)synthesis, but also on their demand, accumulation *via* degradation processes and also on their transportation from the leaf. All these processes may change during senescence and may also differ among plants or leaves senescing under different conditions and even among individual leaf parts and cells. There is a pronounced spatial distribution of sugars between cells and even inside the cell, but sugar contents are usually measured from the whole tissue, so the results may be misinterpreted. In addition, environmental factors influence the sugar accumulation and signalling during senescence (for a review, see Wingler et al. 2006). Zwack and Rashotte (2013) have proposed that the onset of senescence might be triggered by changes in sugar transport rather than changes in their content.

It is thought that sugar starvation as well as sugar accumulation can induce leaf senescence but it cannot be excluded that the changes in sugar content are the result of senescence, not the reason. After leaf maturation, a rate of photosynthesis gradually declines, which suggests that low sugar content is the cause of leaf senescence. This conception is supported by findings that exogenous sugars can eliminate the accelerating effect of dark on senescence of detached leaves. However, changes in gene expression in response to glucose treatment are close to changes occurred during developmental leaf senescence (Wingler et al. 2009). Further, gene expression during dark-induced senescence did not correspond to developmental senescence but resembled response to starvation in *Arabidopsis* cell cultures. It implies that high sugar level rather than starvation (and/or dark induce changes in gene expression) is characteristic of developmental leaf senescence. Indeed, high sugar levels are found at the beginning of senescence (*e.g.*, Wingler et al. 2006). However, it is unclear how sugars accumulate when the photosynthetic rate decreases.

Uncertainties are also regarding sugar-sensing mechanisms in senescing leaves. A model by Biswal and Pandey (2018) proposes the participation of two main sugar sensors, HKX1 (hexokinase 1; high-sugar sensor) and SnRK1 (sucrose nonfermenting-1-related protein kinase 1; low-sugar sensor) in the sugar-signalling during developmental senescence. According to this model, higher sugar level is sensed by HKX1, which causes the down-regulation of photosynthesis-associated genes. This down-regulation leads to the decrease in photosynthesis and the sugar starvation, which induces expression of *SAGs*. SnRK1 senses the low sugar content and activates degradation of cellular components to provide energy for execution and completion of the senescence program, including nutrient recycling. The final step involves the degradation of cell wall polysaccharides to provide energy during the terminal phase of senescence (Biswal and Pandey 2018).

Longevity of source leaves and duration of their photosynthetic activity may influence yield of crop plants. Both increased and reduced yield have been found in plants with delaying leaf senescence (Jameson and Song 2016, and references therein). The different results might be related to different nutrient (mainly N) conditions or to different sink-source limitations (*e.g.*, Borrill et al. 2015, Jameson and Song 2016). On the other hand, under stress conditions,

premature senescence can be induced as the source-limitation prevails, which may decrease the yield.

1.8 Cytokinin regulation of leaf senescence

1.8.1 Cytokinins

Cytokinins (CKs) are key plant hormones that regulate many processes of plant growth and development including cell division and differentiation, growth of shoot and root, apical dominance, leaf senescence, seed development, and also response to stress factors. There are two groups of naturally occurring CKs – isoprenoid and aromatic CKs that differ in a substituent at the N^6 position of adenine. Isoprenoid CKs, that represent the most abundant CKs, include isopentenyl (iP)-type and zeatin (Z)-type of CKs, which occur in either *cis* (*c*Z) or *trans* (*t*Z) form. Aromatic CKs include for instance N^6-benzylaminopurine (BAP) or *meta*-topolin (*m*T). CKs are present in plants in various chemical forms of different role and activity. Free CK bases have the highest activity in bioassays and are thought to be the most active forms of CKs (Spíchal et al. 2004, Sakakibara 2006). CK ribosides, as a transport form, were reported to be less active (Spíchal et al. 2004). CK homeostasis is maintained by their biosynthesis (by isopentenyl transferase, IPT), activation, reversible and irreversible inactivation, and degradation (by cytokinin oxidase/dehydrogenase, CKX).

The CK signalling pathway involves hybrid histidine protein kinases (HKs), histidine phosphotransfer proteins (HPts), and response regulators (RR) (for a review, see Keshishian and Rashotte 2015). After CK binding in CHASE (cyclase/HK-associated sensory extracellular) domain of HK, HK is autophosphorylated and then the phosphoryl group is transferred by phosphorylation of HPt to phosphorylate B-type RR in nucleus. In *Arabidopsis,* three CK receptors have been identified: AHK2, AHK3 and AHK4 (also known as CRE1 or WOL). The CK-dependent regulation of senescence is known to be mediated mainly by the AHK3 receptor, coupled with the phosphorylation/activation of the B-type response regulator ARR2 (ARABIDOPSIS RESPONSE REGULATOR2) (Kim et al. 2006, Riefler et al. 2006). The AHK2 receptor is considered to have a redundant function in the senescence regulation, while the AHK4 receptor is thought to have only minor role (Riefler et al. 2006).

An inverse relationship between endogenous content of active CKs and stage of senescence has been found in many studies (Gan and Amasino 1997). The onset of leaf senescence is usually associated with a decrease in the level of active CK forms (Buchanan-Wollaston 1997, Gan and Amasino 1997, Noodén et al. 1997), which can be caused by inhibition of CK biosynthesis and/or by faster CK degradation or inactivation. The main degradation pathway involves CKX (Werner et al. 2006), while CK inactivation occurs *via* N- and O-glucosylation.

1.8.2 Cytokinin effects on photosynthesis in senescing leaves

It is well known that both exogenous application of CKs and increase in their endogenous content can delay senescence (*e.g.*, Gan and Amasino 1997, Zavaleta-Mancera et al. 1999, **Vlčková et al. 2006**, Zwack and Rashotte 2013, **Janečková et al. 2019**). CKs have been widely reported to preserve Chl content and photosynthetic activity including PSII function in senescing leaves (Fig. 7; **Vlčková et al. 2006**, Zubo et al. 2008, Vylíčilová et al. 2016, **Janečková et al. 2019**) and also to mitigate lipid peroxidation (Huang et al. 1997, **Vlčková et al. 2006**, Liu et al. 2016, **Janečková et al. 2018**). Nevertheless, the exact mechanisms of these CK effects are not known. The CK-mediated maintenance of Chl content seems to be related to suppression of senescence-associated up-regulation of genes encoding enzymes of Chl degradation (*e.g.* Liu et al. 2016, Zhang et al. 2016). Regarding the preservation of PSII function, CKs were found to stabilize LHCII (Oh et al. 2005, Talla et al. 2016, Vylíčilová et al. 2016) as well as RCII (Oh et al. 2005, **Janečková et al. 2019**) in dark-senescing leaves, RCII stabilization being the key process for the maintenance of PSII photochemical activity (Oh et al. 2005). CKs have also been found to maintain both activity and content of RUBISCO in senescing leaves (Weidhase et al. 1987), which may pronouncedly contribute to the CK-promoting effect on photosynthesis. The suppression of lipid peroxidation may be associated with CK-mediated stimulation of antioxidative protection including activity of antioxidative enzymes (*e.g.*, Dertinger et al. 2003, Zavaleta-Mancera et al. 2007, Ren et al. 2018), activity of xanthophyll cycle (*e.g.*, **Vlčková et al. 2006**) and maintenance of carotenoid content (*e.g.*, **Janečková et al. 2019**).

The antisenescence effects of CKs are also related to down-regulation of *SAGs* (*e.g.*, Gepstein et al. 2003), up-regulation of genes encoding LHCII (Vylíčilová et al. 2016) and up-regulation and activation of apoplastic cell wall invertase (CWINV), a key enzyme of the sink-source regulation (Ehneß and Roitsch 1997, Lara et al. 2004). Both exogenous and endogenous CKs were found to increase the CWINV activity, which resulted in delayed senescence. CWINV is considered to be a key element of the CK-mediated delay of senescence (Lara et al. 2004).

It is assumed that the delaying senescence effects of CKs could be used to increase the yield. In this respect, an increase of endogenous CK levels *via* up-regulation of *IPT* (Guo and Gan 2014) or down-regulation of *CKX* (Jameson and Song 2016) as well as exogenous CK application (*e.g.*, Luo et al. 2018) are considered. However, as mentioned above, the suppression of senescence may not lead to the increased yield, and this also applies to the case of delayed senescence in plants with increased endogenous CK level (*e.g.*, Sýkorová et al. 2008). The positive CK effect on the yield is more convincing in the case of premature senescence under stress conditions (Guo and Gan 2014), *i.e.*, in the case of prevailing source limitation. For instance, the exogenous application of BAP has been reported to increase the yield in wheat grown under heat stress (Yang et al. 2016b) and in maize exposed to waterlogging (Ren et al. 2018).

1.8.3 Cytokinin derivatives

A number of CK derivatives with different biological activities have been prepared (for a recent review, see Hönig et al. 2018). Some of them have a higher antisenescent activity than BAP that

is the most widely CK tested for use in agriculture (for a review, see Koprna et al. 2016) and already used in biotechnological applications because of its activity, availability and affordability. Recently, two derivatives of 9-(β-D-arabinofuranosyl)-6-benzylaminopurine with methoxy- and hydroxy- group introduced to a *meta*-position of a benzyl ring (3MeOBAPA and 3OHBAPA, respectively) were synthesized (Doležal et al. 2018). Both compounds have significant senescence-delaying effects that are (quantitatively) species-specific. While 3OHBAPA was highly efficient in maintenance of Chl content and PSII function in wheat leaves, 3MeOBAPA has better protective function in *Arabidopsis* (Fig. 7). The higher antisenescence effect might be related to a pronouncedly reduced stimulation of ethylene production found in 3OHBAPA- and 3MeOBAPA-treated leaves (Fig. 8) as ethylene is known to promote leaf senescence (for a review, see Koyama 2014).

1.8.4 Cytokinin-mediated acceleration of leaf senescence

In some cases, CKs can accelerate leaf senescence (*e.g.*, Carimi et al. 2004, Rulcová and Pospíšilová 2001, Prokopová et al. 2010). The CK-mediated acceleration of leaf senescence may occur when high exogenous CK concentrations are used or when CK-treated leaves senesce under light (especially under light of higher intensity or dosage) (Zacarias and Reid 1990, **Vlčková et al. 2006**, Prokopová et al. 2010). The mechanism of this deleterious CK effect is not clear. It is possible that under light conditions the CK-mediated maintenance of photosynthetic apparatus (including LHCs) in combination with a feed-back inhibition of photosynthesis due to sugar accumulation can eventually lead to over-excitation of photosynthetic apparatus and consequent oxidative damage (**Vlčková et al. 2006**). The sugar accumulation might be caused by the above mentioned CK-mediated stimulation of CWINV. This hypothesis is supported by the finding that an extremely high level of endogenous CKs causes a pronounced increase in production of chloroplastic H_2O_2 and oxidative damage (Novák et al. 2013). On the other hand, an application of exogenous CK has diminished an injury of tomato plants caused by continuous light treatment and consequent feed-back inhibition of photosynthesis due to the sugar accumulation (Velez-Ramirez et al. 2017). The mechanism of the negative cytokinin action remains unresolved.

1.8.5 Interaction of cytokinins and light in detached leaves

It has been well documented that light influences levels of endogenous CKs as it participates in the regulation of CK biosynthesis, degradation, and transport (Boonman et al. 2009, Kurepin and Pharis 2014, Zdarska et al. 2015). As reviewed by Zdarska et al. (2015) there are only few studies dealing with the role of light in CK metabolism and their results are unambiguous. For instance, both up-regulation and down-regulation of *CKX* expression or stimulation and inhibition of CKX activity have been found in leaves under light/dark cycle compared to dark (Zdarska et al. 2015 and references therein).

It can be expected that the light regulation of senescence is related to the changes in CK levels. However, a detailed investigation of their interaction during senescence is missing.

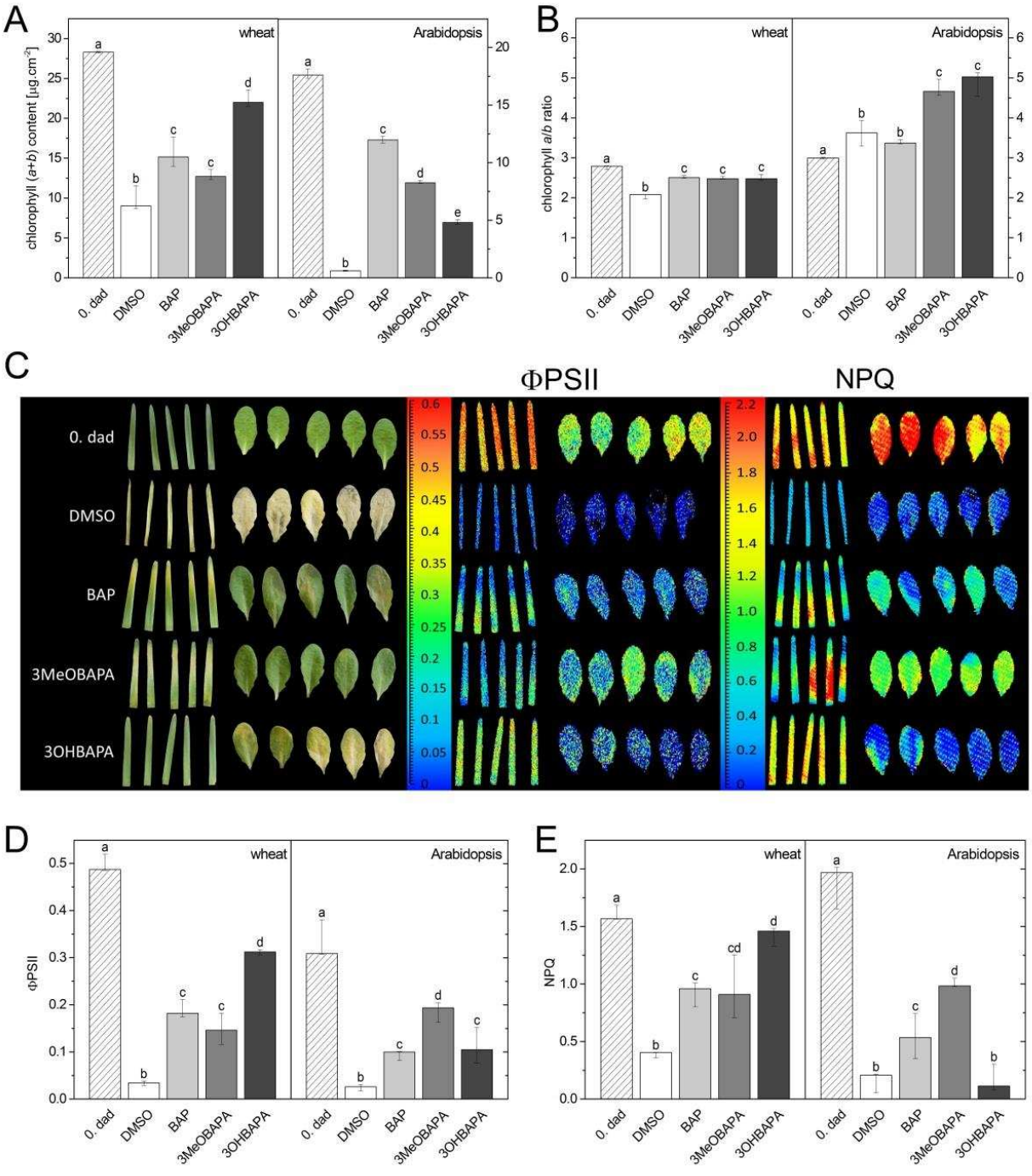


Fig. 7 Senescence-induced changes in detached leaves of wheat and *Arabidopsis* after 6 days in dark as affected by cytokinin BAP and arabinosides (3MeOBAPA, 3OHBAPA). A – chlorophyll content; B – chlorophyll *a*/chlorophyll *b* ratio; C – images of actual quantum yield of photosystem II photochemistry (Φ_{PSII}) and non-photochemical fluorescence quenching (NPQ) in the leaves; D, E – medians and quartiles of Φ_{PSII} and NPQ, respectively (n = 5). 0. dad (day after detachment) – control (mature) leaves. Different letters above the bars indicate statistically significant differences ($P < 0.05$).

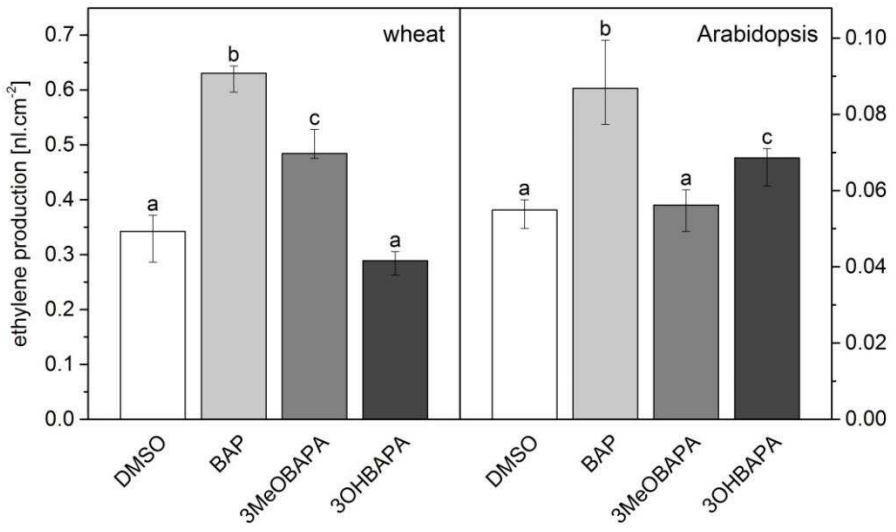


Fig. 8 Ethylene production in detached leaves of wheat and *Arabidopsis* incubated in 0.1% DMSO or in 10^{-5} mol l^{-1} solutions of BAP, 3MeOBAPA and 3OHBAPA and kept sealed in 8 ml vials in darkness for 6 days. Medians and quartiles are presented (n = 5-9). Different letters indicate a statistically significant difference (*P < 0.05*).

As described in part 1.2, light usually delays senescence of detached leaves, but it is not clear if and how endogenous CK level in leaves is modified after their detachment and how it is affected by light conditions. There are only a few studies dealing with the changes in the endogenous CK content in detached leaves kept under different light conditions and their results are contradictory. Zubo et al. (2008) have shown that in detached barley leaves, the content of zeatin derivatives increased two-fold under continuous light, whereas in leaves kept in darkness the change was much smaller. Two other studies provided conflicting results about the changes in the iP content: Roberts et al. (2011) have reported a decrease in iP + iPR content in detached leaves of wheat kept in dark, whereas Causin et al. (2009) have found a reduced iPR content in detached leaves of wheat exposed to light or shading treatments.

Recently, Marchetti et al. (2018) have reported a decrease in *t*Z content and almost unchanged iP content in detached shaded leaves of wheat. Interestingly, the authors have revealed that the level of CK metabolites and CKX activity in detached leaves depend on light spectral quality and that BL is important for the maintenance of active CKs *via* suppression of an increase in CKX activity caused by shading. However, Schlüter et al. (2011) have found that the increase in CKX activity was much higher in detached barley leaves kept in light/dark cycle than in darkened leaves. As senescence of the leaves under light/dark cycle was slower than in dark, the authors have suggested that light not only increased CK degradation, but also promoted the formation of active CKs in detached leaves (Schlüter et al. 2011).

In our recent work we have found that the content of endogenous CKs and their individual forms differ significantly in detached *Arabidopsis* leaves kept under various light conditions (**Janečková et al. 2018**). On the sixth day after detachment, the content of *t*Z + *t*ZR decreased pronouncedly, no matter whether the leaves were kept in dark or light. However, the content of iP + iPR decreased only in dark-senescing leaves, while in leaves kept under light

it was significantly elevated, which was accompanied by highly maintained Chl content and PSII function. A comparison of the senescence-induced changes in leaves of double CK receptor mutants kept in dark and under light indicates that light can compensate for the disrupted CK signalling caused by loss-of-function mutation in the AHK receptors.

Based on our results, we have suggested a scheme (Fig. 9) that summarizes contributions of particular CK receptors, content of iP + iPR and *t*Z + *t*ZR as main CK forms (CK), and light in the maintenance of photosynthesis (PS) and low lipid peroxidation (LP) in detached leaves of *Arabidopsis*. In dark, the (initial) CK content in WT leaves was not sufficient to maintain the high PS and low LP even though all three CK receptors were active. In the CK receptor double-mutants, the (initial) summary CK content was higher, the highest being in *ahk2 ahk3*. In this mutant, the pronouncedly increased CK level maintained the lowest LP because of the presence of AHK4 that is proposed to be the main receptor mediating CK effect on LP. The smallest increase in the CK content was found in *ahk2 ahk4*, but it was accompanied by the highly maintained PS due to the presence of the AHK3 receptor, the supposed main receptor mediating CK effect on PS during senescence, and by the highest LP.

In the *ahk3 ahk4* mutant, the CK content as well as the maintenance of PS and LP, were between *ahk2 ahk3* and *ahk2 ahk4*, indicating that AHK2 can partially take over the role of both these receptors in the regulation of PS as well as LP.

The pronounced promoting effect of light on the maintenance of high PS was associated with the increased level of iP + iPR. The highest content of iP + iPR in *ahk2 ahk3*, together with the functional AHK4, maintained the lowest LP. The elevation of the content of active CK forms, however, was not the only way how light inhibited senescence-associated changes, as the PS decrease in this mutant was small despite the poor effect of AHK4 on PS. The presence of light compensated also for the insufficient effect of receptors in *ahk2 ahk4* and *ahk3 ahk4* mutants, resulting in similar maintenance of LP and PS in comparison to WT. The effect of light was dependent on light dose. The effect of higher light (HL, 400 µmol photons m^{-2} s^{-1}) contrasted to growth light (GL, 120 µmol photons m^{-2} s^{-1}) in *ahk2 ahk3* and *ahk3 ahk4* mutants, which – due to the HL – maintained higher PS in spite of the inactive AHK3 receptor. The maintained PS, together with the elevated supply of excitations due to the increased light dose, resulted in the promotion of LP. In *ahk2 ahk3*, the LP increase was minimized by the AHK4 action, but in *ahk3 ahk4* the protection mediated by AHK2 was poor (**Janečková et al. 2018**).

In addition to the light influence on the CK levels, there is an extensive crosstalk between light- and CK-dependent signalling pathways at multiple levels that is involved in regulation of many processes of plant growth and development (Zdarska et al. 2015). It has been identified a number of components shared by both the types of signalling pathways, for example PHY and ARR4 (ARR of type A) (Sweere et al. 2001) and CRY and HY5 (transcription factor LONG HYPOCOTYL 5) (Vandenbussche et al. 2007). However, many questions concerning the downstream molecular mechanisms of light and CK interactions remain open. As summarized by Zdarska et al. (2015): 'Further knowledge is required of: the signalling intermediates acting downstream of phytochromes in the regulation of hormonal metabolism; mechanisms that mediate effects of light at tissue and cell levels; and the specificity of these phenomena in developmental contexts.'

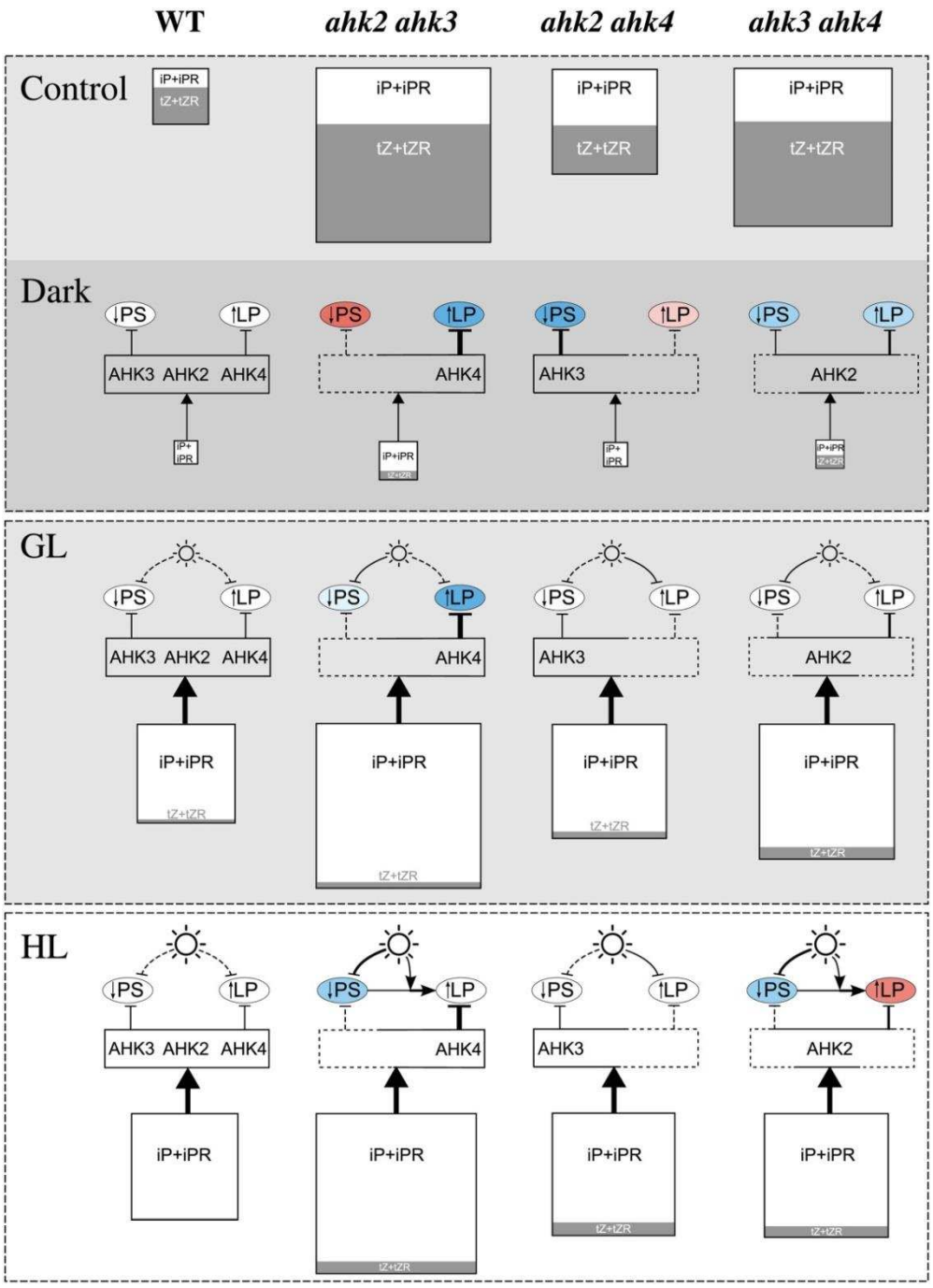


Fig. 9 The proposed scheme of the interplay between CKs and light during senescence of detached *Arabidopsis* leaves. Different genotypes are presented in columns, while horizontal sections represent situation after 6 days in darkness (Dark), under growth light (GL; 120 µmol photons m^{-2} s^{-1}) or higher light (HL; 400 µmol photons m^{-2} s^{-1}); the uppermost section shows the initial content of CKs in leaves immediately after the detachment (Control). The areas of the squares shown for particular variants are proportional to the summary content of iP, *t*Z, iPR and *t*ZR. The area of the square for WT controls represents 1.61 pmol g^{-1} of fresh weight. The white parts of the squares represent the sum of iP and iPR, the dark grey parts of squares represent the sum of *t*Z and *t*ZR. CK receptors that are active in each genotype are indicated in the rectangles. A "strength" of the CK signalling output on the senescence-associated decrease in photosynthetic parameters (↓PS; chlorophyll content and photosystem II function) and increase in lipid peroxidation (↑LP; evaluated from the MDA content) is represented by a dashed or full line of various thickness. Background colour of ↓PS and ↑LP represents marked difference in comparison to the respective WT; red shades indicate promotion; blue shades indicate inhibition of the senescence-associated changes. For explanation, see the text (**Janečková et al. 2018**, with permission).

2 AIMS OF THE THESIS

In the first phase of my research on senescence, the aim was to compare the structural and functional changes in chloroplasts of leaves senescing under different light conditions and evaluate the effect of light on these changes. Detached leaves as well as intact plants were used for study. Relationships among maintenance of chloroplast ultrastructure, Chl and carotenoid content, photosynthetic function, xanthophyll cycle and antioxidative enzyme activities, and oxidative damage were examined.

In the next phase, the research was extended to effect of CKs on the progress of leaf senescence. It was investigated how the effect of exogenous CK (*m*T) differed in dark- and light-senescing wheat leaves. Further, we investigated to what extent the slowing-down effect of light on senescence of detached *Arabidopsis* leaves was related to endogenous content of main forms and types of CKs. Using CK receptor mutants, a role of light in the senescence regulation in plants with impaired CK signalling was evaluated. Finally, it was examined whether the Chl *b* deficiency in *chlorina* barley mutant would accelerate leaf senescence and whether exogenous CK (BAP) would be able to slow down senescence-associated decrease in Chl content and photosynthetic function also in that case.

3 RESULTS

3.1 Ultra-structural and functional changes in the chloroplasts of detached barley leaves senescing under dark and light conditions

Špundová Martina, Popelková Hana, Ilík Petr, Skotnica Jiří, Novotný Radko, Nauš Jan

Journal of Plant Physiology 160, 1051-1058 (2003)

35% contribution (design of experiments, performance and evaluation of chlorophyll fluorescence and pigment measurements, writing a manuscript)

23 citations (without autocitations)

In this paper, we compared changes in chloroplast ultra-structure and photosystem II (PSII) photochemistry in detached primary leaves of barley (*Hordeum vulgare* L.) kept in dark or under continuous light (90 μmol photons m^{-2} s^{-1}) for 5 days. Typical senescence-induced changes in the chloroplast ultrastructure were observed in both dark- and light-senescing leaves: a shape of chloroplasts became more spherical, thylakoid membranes were gradually disintegrated and plastoglobules accumulated. In the chloroplasts of light-senescing leaves, starch grains occurred. A rate of overall Chl degradation was very similar in both the variants, thus no positive effect of light on Chl maintenance was observed. Different changes were found in the Chl *a*/Chl *b* ratio: it was almost unchanged in dark-senescing leaves, while under light it increased pronouncedly, which indicated a preferential degradation of light-harvesting complexes (LHCs) compared to reaction centres.

We supposed that the decrease in relative content of LHCs was induced by an over-excitation of photosynthetic apparatus due to the combined effect of leaf detachment and increased light dose (as compared with growth light conditions – 16 h light/8 h dark). The feed-back inhibition of photosynthesis could be expected as the starch accumulated in the chloroplasts. The decreased LHC content should reduce an amount of absorbed light and thereby protect the photosynthetic apparatus from over-excitation and oxidative damage. Indeed, the function of PSII photochemistry estimated from Chl fluorescence parameters (F_V/F_M and $\Delta F/F_M'$, maximal and actual quantum yield of PSII photochemistry, respectively, and M1/F(T30) – the ratio evaluated from a dependence of Chl fluorescence on temperature during linear heating of leaf and reflecting the function of PSII photochemistry as well) was highly maintained during 5 days after detachment. However, the content of TBARPs (thiobarbituric acid reaction products), which was thought to reflect the MDA (malondialdehyde) content and level of membrane lipid peroxidation, was largely increased as compared with control (mature) leaves and dark-senescing ones. Thus, the estimated level of (thylakoid) membrane damage did not correspond to the maintained PSII photochemistry. This is probably the case, when the level of lipid peroxidation was over-estimated due to non-specifity of the TBARPs method (see part 1.6).

3.2 Xanthophyll cycle activity in detached barley leaves senescing under dark and light

Špundová Martina, Strzałka Kazimierz, Nauš Jan

Photosynthetica 43, 117-124 (2005)

80% contribution (design, performance and evaluation of experiments, writing a manuscript)

2 citations (without autocitations)

In this work, we continued in the study of differences between the dark- and light-senescing detached barley leaves and evaluated senescence-associated changes in xanthophyll cycle activity. Similarly to the previous work, the light-senescing leaves were kept under continuous light of 90 µmol photons m^{-2} s^{-1}.

During 6 days after leaf detachment, we compared an actual de-epoxidation state (DEPS) of xanthophyll cycle pigments and DEPS after 4, 9, and 14 min of high-light exposure (500 µmol photons m^{-2} s^{-1}). In the dark-senescing leaves, the actual DEPS was zero (as expected) and the high-light induced increase in DEPS was reduced, but still functional during whole 6 days after detachment. The gradual inhibition of the violaxanthin de-epoxidation might be related to impairment of electron transport in thylakoid membranes and inhibition of thylakoid lumen acidification. In addition, dark-induced changes in thylakoid lipid composition could have contribute to this inhibition.

Under continuous light, senescence was delayed pronouncedly compared with dark as the Chl content, the PSII photochemistry and electron transport processes were highly maintained. Simultaneously, the actual DEPS increased from the 3rd day after detachment indicating a relative increase in amount of excitations in the leaves kept under continuous light. We supposed that the delay of senescence-associated changes was (at least partially) related to the efficient protection of chloroplasts from photo-oxidative damage. This photo-protection was probably more efficient than in the previous study where the Chl degradation was not slowed down compared with dark.

In the leaves kept under light, a faster and higher increase in DEPS was observed during the high-light treatment indicating the stimulation of xanthophyll cycle activity.

3.3 Plant shading increases lipid peroxidation and intensifies senescence-induced changes in photosynthesis and activities of ascorbate peroxidase and glutathione reductase in wheat

Špundová Martina, Slouková Kateřina, Hunková Miroslava, Nauš Jan

Photosynthetica 43, 403-409 (2005)

25% contribution (design of experiments, evaluation of photosynthetic parameters, writing a manuscript)

11 citations (without autocitations)

This paper deals with senescence of intact leaves of wheat (*Triticum aestivum* L.) grown during autumn (October – November) in a green-house under different light intensities (100 %, 70 %, and 40 %). The plant shading by a non-woven fabric started when plants were in a growth phase of three leaves. Measurements were performed in three terms (0, 6 and 12 days of shading) with the first, second and third leaves of the plant (counted from the plant base).

In the un-shaded plants, the Chl and total protein content as well as the rate of CO_2 assimilation and quantum yield of PSII photochemistry gradually decreased. On the contrary, a level of lipid peroxidation (estimated from the TBARs content) increased in all measured leaves, which correlated with a decrease in activity (related to fresh weight) of the main enzymes of ascorbate-glutathione cycle, ASCORBATE PEROXIDASE (APX) and GLUTATHIONE REDUCTASE (GR). These changes indicated that the plants were undergoing senescence during the experiment despite their relatively low age (37 – 49 days). We supposed that premature senescence was induced by relatively low-light conditions due to short days and low light intensities during the autumn. As the rate of CO_2 assimilation decreased faster than quantum yield of PSII photochemistry, the imbalance between supply and demand of excitations probably led to ROS accumulation and lipid peroxidation. It is not clear whether the decreased activity of APX and GR was a reason or consequence of the premature senescence.

The plant shading intensified the senescence-induced changes found in the un-shaded plants, especially in the oldest leaves. We expected lower ROS photo-generation and oxidative damage due to the decreased supply of excitations in the shaded plants. However, the shading increased pronouncedly the level of lipid peroxidation in the oldest leaves, which might be related to a more reduced activity of APX and GR. It seems that the shaded plants accelerated the senescence of the oldest leaves in order to support the growth and function of the younger leaves.

3.4 Protective cytokinin action switches to damaging during senescence of detached wheat leaves in continuous light

Vlčková Alexandra, Špundová Martina, Kotabová Eva, Novotný Radko, Doležal Karel, Nauš Jan

Physiologia Plantarum 126, 257-267 (2006)

30% contribution (contribution on design of experiments, partial measurements and data evaluation, supervision of writing a manuscript)

13 citations (without autocitations)

In this paper, we extended our senescence study to evaluation of effect of exogenously applied *meta*-topolin (*m*T, N^6-(*meta*-hydroxybenzyl)adenine; 10^{-4} mol l^{-1}), an aromatic naturally occurring cytokinin with a high antisenescence activity. As usual, we used detached leaves, in this case the primary leaves of wheat (*Triticum aestivum* L.) that were kept in dark or under continuous light (100 µmol photons m^{-2} s^{-1}) for 6 days after detachment. We estimated changes in Chl content, de-epoxidation state of xanthophylls (DEPS), chloroplast ultrastructure, rate of CO_2 assimilation, PSII photochemistry and level of lipid peroxidation.

The Chl content and rate of CO_2 assimilation decreased simultaneously in dark- and light-senescing leaves. A stomatal conductance decreased too, but a concomitant increase in intercellular CO_2 concentration indicated that the closing of stomata was not the main cause of the inhibited CO_2 assimilation in senescing leaves. Thus, light did not slow down the senescence-induced Chl degradation and decrease in CO_2 assimilation. However, light reduced the senescence-associated changes in chloroplast shape, arrangement, and ultrastructure, and also in efficiency of PSII photochemistry and electron transport in thylakoid membranes. On the contrary, the level of lipid peroxidation was slightly higher in the light-senescing leaves, which together with the increased DEPS indicated the imbalance between supply and demand of excitations, probably due to more inhibited stromal reaction in comparison with primary reactions of photosynthesis. We supposed a mild feed-back inhibition of photosynthesis in the light-senescing leaves.

Interestingly, an effect of *m*T differed significantly in the dark- and light-senescing leaves. In dark, *m*T effectively reduced the senescence-induced changes in all parameters. Chloroplasts in the *m*T-treated darkened leaves remained ellipsoidal, accumulation of plastoglobules was reduced, and relative grana content was maintained, which indicated that *m*T suppressed chloroplast and thylakoid degradation. Further, the *m*T application slowed down Chl degradation, decrease in CO_2 assimilation, stomatal conductance, and PSII photochemistry, and increase in the level of lipid peroxidation.

In the light-senescing leaves, *m*T reduced the Chl degradation and accumulation of plastoglobules (indicating a lesser extent of deterioration of chloroplast components), but at the same time it did not prevent the decrease in rate of CO_2 assimilation. As a pronounced starch accumulation was found in chloroplasts of the *m*T-treated light-senescing leaves, we supposed that *m*T strengthened the feed-back inhibition of photosynthesis, which together with the suppressed Chl degradation (and probably also lesser deterioration of light-harvesting

complexes) led to strong over-excitation of photosynthetic apparatus. The activation of photo-protective xanthophyll cycle found in the light-senescing leaves treated by *m*T did not prevent photo-oxidative damage of membranes and PSII. We summarized that under continuous light, paradoxically, the *m*T-mediated suppression of degradation of photosynthetic apparatus eventually intensified oxidative damage, probably due to the combination of feed-back inhibition of stromal reactions and maintenance of light-harvesting complexes and excitation delivery.

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

Janečková Helena, Husičková Alexandra, Ferretti Ursula, Prčina Maroš, Pilařová Eva, Plačková Lenka, Pospíšil Pavel, Doležal Karel, Špundová Martina

Plant, Cell & Environment 41, 1870-1885 (2018)

20% contribution (contribution on design of experiments, interpretation of results, and writing a manuscript)

1 citation (without autocitations)

In this paper, the effect of light on senescence of detached *Arabidopsis* leaves was studied in the context of changes in endogenous cytokinin (CK) content and impaired CK reception. We estimated the changes in the content of 29 main CK forms and compared them with senescence-induced changes in Chl content, efficiency of PSII photochemistry and lipid peroxidation. To clarify the role of individual CK receptors (AHK2, AHK3, and AHK4) and to evaluate the role of light in the regulation of senescence in plants with impaired CK reception, three *Arabidopsis* AHK double mutants were used in our study, each of them with only one functional CK receptor.

The mature leaves of mutants without AHK3 receptor (*i.e.*, *ahk2 ahk3* and *ahk3 ahk4*) had constitutively lower Chl content (due to lacking AHK3 receptor that is thought to play the main role in CK-mediated stimulation of Chl biosynthesis), while their PSII photochemistry was not lower compared with wild type (WT) plants. Both the mutants had approximately two-fold overall content of CKs, and the sum of isopentenyl adenine (iP) and *trans*-zeatin (*t*Z) was increased even nine-fold and five-fold, respectively. Interestingly, a content of *cis*-zeatin (*c*Z) was decreased in these mutants. Qualitatively same (but smaller) changes of the CK contents were found in the *ahk2 ahk4* mutant.

In dark-senescing leaves, both the Chl content and PSII function decreased, more pronouncedly in WT and *ahk2 ahk3* and less in *ahk2 ahk4* and *ahk3 ahk4*. The different rate of the senescence-induced changes in individual genotypes was probably related to their different constitutive CK contents in combination with the functional CK receptor. In the *ahk2 ahk4* mutant, the slightly increased CK content was sufficient to delay senescence compared with WT due to the functional AHK3. On the contrary, the higher CK content did not maintain Chl content and PSII photochemistry in *ahk2 ahk3* as neither AHK3 nor AHK2 (main and ancillary receptor, respectively, mediating CK effect on Chl retention and PSII maintenance during leaf senescence) was functional in this mutant.

A senescence-induced increase in lipid peroxidation was higher in WT and *ahk2 ahk4*, and lower in *ahk2 ahk3* and *ahk3 ahk4*, which indicated a main role of the AHK4 receptor (and ancillary role of AHK2 receptor) in the CK-mediating protection against lipid peroxidation during senescence in dark. The total CK content decreased markedly in the dark-senescing leaves of all genotypes. No iP and only negligible residues of *t*Z were detected after 6 days. Proportional changes in the individual CK forms indicated a dominance of CK inactivation and degradation over CK biosynthesis with the only exception: the biosynthesis of *c*Z seemed to be stimulated

as the *c*Z content was increased in dark-senescing leaves of all genotypes compared with the mature leaves. This finding supports a hypothesis that *c*Z plays a major role in maintenance of basal leaf viability under energy-limiting conditions, when the biosynthesis of the main active CKs is suppressed. We proposed that AHK3 is the main CK receptor mediating this *c*Z action.

Compared with the dark-senescing leaves, light (two intensities were used, 120 and 400 µmol photons m^{-2} s^{-1}, with photoperiod 8 h light/16 h dark) suppressed the decrease in Chl content and PSII function as well as the increase in lipid peroxidation in all genotypes. The suppression of senescence-induced changes was accompanied by the increased content of iP indicating persistent (or even stimulated) iP biosynthesis in the detached leaves. On the contrary, the *t*Z content was reduced by a similar extent as in the dark-senescing leaves. Thus, the delaying effect of light on senescence of detached leaves might be related to the persisted iP biosynthesis. The biosynthesis of *t*Z seemed not to be influenced by light so the main factor influencing the *t*Z content would be the detachment of leaf, which corresponds to the assumed predominant synthesis of *t*Z in roots.

Interestingly, light eliminated the acceleration of senescence-induced changes found in the dark-senescing leaves of *ahk2 ahk3*. Thus, the disrupted CK signalling caused by the loss-of-function mutation in the AHK receptors can be compensated by light. This compensation might be related mainly to the stimulation of iP biosynthesis.

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

Janečková Helena, Husičková Alexandra, Lazár Dušan, Ferretti Ursula, Pospíšil Pavel, Špundová Martina

Plant Physiology and Biochemistry 136, 43-51 (2019)

25% contribution (contribution on design of experiments, interpretation of results, and writing a manuscript)

no citation

Changes in photosynthetic pigment content and PSII and PSI activities associated with dark-induced senescence were studied in a Chl *b*-deficient barley mutant, *chlorina f2*^{f2} (*clo*). This mutant is deficient in Chl *b* due to mutation in chlorophyll(id) *a* oxygenase, a key enzyme of Chl *b* biosynthesis. The *clo* mutant has also lower content of Chl *a* and carotenoids and pronouncedly reduced content of light-harvesting complexes (LHCs) compared with wild type (WT) plants.

After 4 days in dark, Chl and carotenoid content decreased in detached leaves of both WT and *clo*. The relative decrease in Chl content was similar in WT and *clo*, but due to the lower initial value, the absolute Chl content was pronouncedly lower in the senescing *clo* leaves. In both genotypes, the senescence-induced decrease in PSII function (attributed mainly to impairment of reaction centres) was higher compared to PSI. The electron transport within PSII was thought to be more inhibited than the transport behind PSII.

The inhibition of PSII function including the assumed impairment of RCII was much more pronounced in *clo* than in WT. In fact, the senescing *clo* leaves had only minimal PSII activity after 4 days in dark. We assume that the acceleration of PSII impairment in *clo* was caused by the Chl *b* deficiency associated with the lover LHCII content and consequent destabilization and damage of RCII.

Exogenously applied cytokinin N^6-benzylaminopurine (BAP, 10^{-5} mol l^{-1}) suppressed the senescence-induced degradation of pigment content and decrease in PSII function in the leaves of both WT and *clo*. The PSII function in the WT and *clo* senescing leaves treated by BAP was similar, thus BAP eliminated the acceleration of PSII impairment related to Chl *b* deficiency.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Leaf senescence is a key process in plant's life as it is important for nutrient recycling and consequently for seed production or plant survival. Leaf senescence is also very important from the agriculture point of view. In last years, a pronounced progress has been made in understanding the mechanism and regulation of senescence, but many questions remain still unanswered.

One of the unresolved problems is the role of light in the regulation of senescence. It has been shown that light influences senescence at multiple levels including photosynthesis and sugar supply, light signalling pathways, phytohormones, and ROS signalling and damage. Light can accelerate as well slow down leaf senescence, depending on plant species, leaf attachment/detachment, and other environmental conditions. It is known that there is close interaction between light and CKs, but the mechanism of this interaction remains unclear. Similarly, the mechanism of the CK regulation of senescence is far from clarification.

In our works, it has been shown that light pronouncedly affects the rate of Chl degradation and inhibition of photosynthetic processes, activity of xanthophyll cycle and antioxidative enzymes, and level of oxidative damage. Light also modifies the antisenescence effect of exogenous CK and endogenous level of main CK types and forms in detached leaves. It has been suggested that the delaying effect of light on senescence of detached *Arabidopsis* leaves may be related to the persisted biosynthesis of iP. It has been revealed that light can eliminate the acceleration of senescence-associated processes caused by the loss-of-function mutation in CK receptors. Finally, it has been observed that chlorophyll *b* deficiency accelerates decrease in photosynthetic activity during dark-induced senescence of detached barley leaves and that exogenous CK is able to eliminate this effect, probably through the stabilization of RCII.

In future research of leaf senescence, life history of studied plants should be taken into account as not only contemporary internal and external conditions, but also all previous events co-determine properties and fitness of the plant, which can subsequently influence the progress of senescence. For example, senescence of *Arabidopsis* plants grown under long photoperiod (or even under continuous light) may significantly differ from senescence of plants growing under short photoperiod. Furthermore, a more detailed time-course study of leaf senescence (including senescence-associated changes in gene expression) is needed to understand exact mechanisms of the senescence regulation. It could help resolve the question of the role of sugars in the regulation of senescence. Finally, tissues at different stages of senescence should be analysed separately as senescence proceeds heterogeneously through the plant as well as through the individual leaf.

I would like to conclude that even though we are the yellow leaves, we can live a fruitful life and contribute to the lives of our followers.

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6 APPENDIX (PUBLICATIONS)

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

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Ultra-structural and functional changes in the chloroplasts of detached barley leaves senescing under dark and light conditions

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Summary

Changes in the chloroplast ultra-structure and photochemical function were studied in detached barley (*Hordeum vulgare* L. cv. Akcent) leaf segments senescing in darkness or in continuous white light of moderate intensity (90 μmol m^{-2} s^{-1}) for 5 days. A rate of senescence-induced chlorophyll degradation was similar in the dark- and light-senescing segments. The Chl *a/b* ratio was almost unchanged in the dark-senescing segments, whereas in the light-senescing segments an increase in this ratio was observed indicating a preferential degradation of light-harvesting complexes of photosystem II. A higher level of thylakoid disorganisation (especially of granal membranes) and a very high lipid peroxidation were observed in the light-senescing segments. In spite of these findings, both the maximal and actual photochemical quantum yields of the photosystem II were highly maintained in comparison with the dark-senescing segments.

Key words: chlorophyll fluorescence – chloroplast ultra-structure – leaf senescence – photosystem II

Abbreviations: Chl = chlorophyll. – Chl (*a* + *b*) and Chl *a/b* = sum and ratio of Chl *a* and *b*, respectively. – F_0 = minimal Chl fluorescence intensity of the dark-adapted leaf. – F_M and F_M' = maximal Chl fluorescence intensity of the dark- and light-adapted leaf, respectively. – F(T30) = Chl fluorescence intensity at a temperature 30 °C in FTC. – FTC = fluorescence temperature curve (a dependence of the Chl fluorescence intensity on linearly increasing temperature). – F_V/F_M = $(F_M-F_0)/F_M$, maximal quantum yield of photosystem II photochemistry. – $\Delta F/F_M'$ = $(F_M'-F_t)/F_M'$, actual quantum yield of photosystem II photochemistry. – LHC = light harvesting complex. – M1 = the first fluorescence maximum (around 50 °C) in FTC. – M2 = the second fluorescence maximum (around 60 °C) in FTC. – PAR = photosynthetically active radiation. – PPCs = pigment-protein complexes. – PSI = photosystem I. – PSII = photosystem II. – Q_A = primary stable quinone acceptor of PSII. – RCII = reaction centres of PSII. – TBARPs = thiobarbituric acid reaction products

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Introduction

Plant senescence includes many processes proceeding at various levels of a plant organism. Within the plant cell the most conspicuous response to senescence occurs at the chloroplast level (Stoddart and Thomas 1982, Šesták 1985, Smart 1994, Noodén et al. 1997). Changes in chloroplast size, shape and ultra-structure have been found during progressive senescence (Šesták 1985, Synková et al. 1997, Kutík et al. 1998). Many stromal and membrane components in the chloroplast (including chlorophylls) gradually degrade during senescence (Grumbach and Lichtenthaler 1973, Grover and Mohanty 1993, Chang and Kao 1998, Rosiak-Figielek and Jackowski 2000). A typical feature of chloroplasts in senescing leaves is the increase in the number and/or size of osmiophilic plastoglobuli (Lichtenthaler and Sprey 1966, Lichtenthaler 1969, Tuquet and Newman 1980, Meier and Lichtenthaler 1982, Ghosh et al. 2001).

The senescence-induced changes in the chloroplast ultrastructure and composition are accompanied by significant functional changes, such as a decrease in the rate of CO_2 assimilation (Thomas and Stoddart 1980, Lu and Zhang 1998 a) and oxygen evolution (Lichtenthaler and Grumbach 1974, Humbeck et al. 1996). The inhibition of chloroplast functions is thought to be a consequence of decreasing activities of the photosystems and the Calvin cycle (Thomas and Stoddart 1980). The inhibition of the Calvin cycle is attributed mainly to a decline in the content and activity of Rubisco (Friedrich and Huffaker 1980, Camp et al. 1982, Okada and Katoh 1998).

Detached leaves or leaf segments are often used in senescence studies even if the senescence-induced changes in detached leaves may differ from the changes occurring in leaves attached to a plant (Grover and Mohanty 1993). The detached leaves, however, represent a simpler system in which the effect of the rest of the plant (including relocation of assimilates, water, and nutrients) is eliminated, and they are easy to handle and incubate under controlled conditions.

Light of moderate intensity is known to delay the senescence process in detached leaves as compared to the effects of darkness. Degradation of the chloroplast components (chlorophylls, proteins) and a decline in the photosynthetic function are usually slower under light conditions (Thimann et al. 1977, Kar et al. 1993, Chang and Kao 1998). The mechanism of the protective role of light during senescence of detached leaves remains unclear.

In this study we have compared senescence-induced processes in detached barley leaf segments kept in darkness and in continuous white light of moderate intensity ($90\,\mu mol\,m^{-2}\,s^{-1}$ of PAR). In order to contribute to a better understanding of the protective effect of light during senescence, we have focused on a comparison of the changes in chloroplast ultra-structure and PSII function.

Materials and Methods

Plant material and senescence conditions

Plants of spring barley (*Hordeum vulgare* L. cv. Akcent) were grown in a growth chamber at 21 ± 1 °C on an artificial medium composed of perlite and Knop's solution. The light regime was 8 h dark/16 h light (fluorescent light, Tesla Z 25W) with a PAR intensity of $90\,\mu mol\,m^{-2}\,s^{-1}$. The primary leaves of the plants in the growth phase 1.2 (two leaves) according to Feekes (1941) were used for measurements.

Leaf segments were cut off from the primary leaf blades (1.5 cm from the tip of the leaf, length of 5 cm) and placed into dishes with distilled water. The leaf segments floated on the water surface. The segments were kept under dark or light conditions (continuous PAR, intensity of $90\,\mu mol\,m^{-2}\,s^{-1}$) at an air temperature of 21 ± 1 °C.

Chlorophyll content determination

The content of chlorophyll *a* and *b* in the barley leaf segments was determined in 80 % acetone according to Lichtenthaler (1987) using a DU-8-UV-VISIBLE Spectrophotometer (Beckman, Fullerton, USA).

Determination of lipid peroxidation

The level of lipid peroxidation in the barley leaf segments was determined from the content of the thiobarbituric acid reaction products according to a modified procedure of Dan et al. (1996). One g of the leaf segments was homogenised in 10 mL of 0.1 % trichloroacetic acid (TCA) and a filtrated homogenate was centrifuged at 10,000 g_n for 10 min at 4 °C. A 2 mL aliquot of supernatant was mixed with 8 mL of 20 % TCA containing 0.5 % thiobarbituric acid (TBA). The mixture was heated in boiling water for 30 min and then quickly cooled in an ice-bath. After centrifugation at 10,000 g_n for 10 min at 4 °C, the absorbance of the supernatant at 532 nm (A_{532}) and 600 nm (A_{600}) was measured against 0.5 % TBA in 20 % TCA as a blank. The TBARP content was determined from a difference of absorbances at 532 nm and 600 nm. A DU-8-UV-VISIBLE Spectrophotometer (Beckman, Fullerton, USA) was used for the absorbance measurement.

Transmission electron microscopy

The barley leaf segments were cut into pieces of an approximate area of $1\,mm^2$. The leaf pieces were immersed in a mixture of 2 % glutaraldehyde and 1 % formaldehyde in a $0.1\,mol\,L^{-1}$ phosphate buffer (pH 7.4) for 2 hours (primary fixation) and then in 2 % osmic acid in the same buffer for 2 hours (second fixation). After dehydration in acetone and embedding in Durcupan ACM (Fluka), ultra-thin sections were cut, stained with uranium acetate and lead citrate, and examined under a transmission electron microscope Opton EM 109 (Carl Zeiss, Göttingen, Germany) at an accelerating voltage of 80 kV.

Chlorophyll fluorescence measurements

Chl *a* fluorescence was measured at room temperature with a modulation fluorometer PAM 2000 (Walz, Effeltrich, Germany). The F_V/F_M ratio [= $(F_M-F_0)/F_M$] was determined from the minimal Chl fluorescence intensity (F_0) and maximal fluorescence intensity (F_M) measured on the adaxial side of the barley leaf segments after 30 min of dark-adapta-

tion. F_0 was obtained upon excitation of the leaf segment with a weak measuring beam of 0.2 μmol $m^{-2} s^{-1}$, F_M was measured by means of a 0.8 s saturating pulse. The correct intensity and the length of the pulse were checked via the kinetics of the Chl fluorescence response within the pulse excitation.

The induction kinetics of the actual photochemical quantum yield of PSII ($\Delta F/F_M' = (F_M'-F_t)/F_M'$; Paillotin 1976, Genty et al. 1989) were examined in the leaf segments for approximately 14 min after switching on the actinic PAR of an intensity of approximately 90 μmol $m^{-2} s^{-1}$. F_M' was the maximal Chl fluorescence intensity during the actinic illumination and F_t was the Chl fluorescence intensity at the time *t* of the actinic illumination. F_M' was determined using additional saturation pulses. The first pulse was applied 2 s after switching on the actinic PAR, following 9 pulses in 20 s intervals, following 5 pulses in 40 s intervals, and the last 6 pulses in 80 s intervals.

The Chl fluorescence temperature curve (FTC) was measured as the dependence of Chl fluorescence intensity on linearly increasing temperature (Schreiber and Armond 1978, Nauš et al. 1992; see Fig. 1). The FTC of the barley leaf segments were measured with a fluorometer PAM 2000 (Walz, Effeltrich, Germany) by means of a weak measuring beam of 0.2 μmol $m^{-2} s^{-1}$ (F_0-conditions). Linear heating at a rate of 4 °C min^{-1} was used. The fluorescence intensities at a temperature of 30 °C [F(T30)], at a temperature of the first fluorescence maximum M1 (around 50 °C) and at the second fluorescence maximum M2 (around 60 °C) were determined (Fig. 1) and the ratios M1/F(T30) and M2/M1 were calculated. The M1/F(T30) ratio has been shown to correlate with the maximal photochemical yield of PSII under profound stress effect (Nauš et al. 1992). The relative height of the M2 maximum (which can be expressed by the M2/M1 ratio) has been shown to be inversely proportional to the relative amount of appressed thylakoids (Downton and Berry 1982, Ilík et al. 1995). We, therefore, used the M2/M1 ratio as a measure of changes in the granal thylakoid abundance. A general statistical description (medians and quartiles) was used in the case of the Chl fluorescence parameters (Lazár and Nauš 1998).

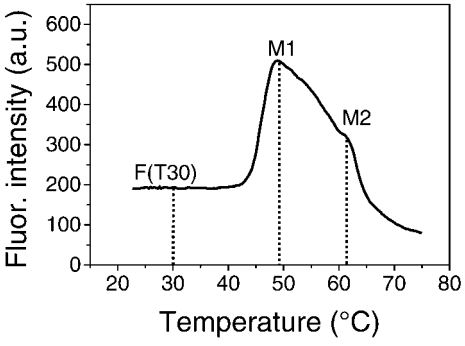

Figure 1. Typical chlorophyll fluorescence temperature curve measured with the control primary leaf of barley. The rate of linear heating was 4 °C min^{-1}. The fluorescence intensities at a temperature of 30 °C [F(T30)], at a temperature of the first fluorescence maximum M1 (about 50 °C) and at the second fluorescence maximum M2 (about 60 °C) were evaluated (dotted lines) and used for calculation of the ratios M1/F(T30) and M2/M1.

Results

Changes in leaf segments senescing in darkness

The chlorophyll (Chl) content in barley leaf segments gradually decreased after detachment (Table 1). In darkness the Chl *a* and Chl *b* contents decreased at a very similar rate as few pronounced changes in the Chl *a/b* ratio were found (Table 1). The only statistically significant change in the Chl *a/b* ratio (related to that of the control segments before detachment) was a mild decrease (by approximately 17 %) found for the segments on the 5th day after detachment.

The thylakoid organisation in chloroplasts, including the granal arrangement, was relatively conserved until the 4th day after detachment (Fig. 2 b, c). On the 5th day, disintegrated grana were found in about one quarter of the chloroplasts. At the same time, many translucent plastoglobuli occurred in chloroplasts (Fig. 2 d). In order to estimate the extent of lipid peroxidation in the leaf segments, we determined the content

Table 1. Changes in chlorophyll content (Chl *a* + *b* in μg per cm^2 of leaf area), ratio Chl *a/b* and ratio of fluorescence temperature maxima M2/M1 after detachment of the barley leaf segments senescing in continuous light (white light of intensity of 90 μmol $m^{-2} s^{-1}$) or dark conditions. Means and SD are shown (n = 5–8). In the case of the M2/M1 ratio, the medians and quartiles (in parentheses) are shown (n = 5–7). Statistically significant differences from control values are indicated by the letter «a», and between the samples senescing under light and dark conditions for corresponding time after detachment by the letter «b».

	Days after detachment	Chl (*a* + *b*) [μg cm^{-2}]	Chl *a/b*	M2/M1
Control	0	30.31 ± 1.60	2.92 ± 0.09	0.66 (0.65; 0.71)
Dark	3	10.22 ± 1.98 a***	3.37 ± 0.60	0.70 (0.64; 0.72)
	4	5.43 ± 1.05 a***	2.76 ± 0.42	0.74 (0.70; 0.81)
	5	3.30 ± 1.35 a***	2.43 ± 0.43 a*	0.78 (0.73; 1.03)
Light	3	12.38 ± 1.68 a***	3.95 ± 0.17 a***	0.79 (0.78; 0.83) (a, b)**
	4	5.65 ± 0.94 a***	3.70 ± 0.59(a, b)**	1.21 (1.16; 1.30) (a, b)***
	5	4.46 ± 1.23 a***	4.30 ± 0.41 (a, b)***	1.45 (1.42; 1.49) (a, b)***

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

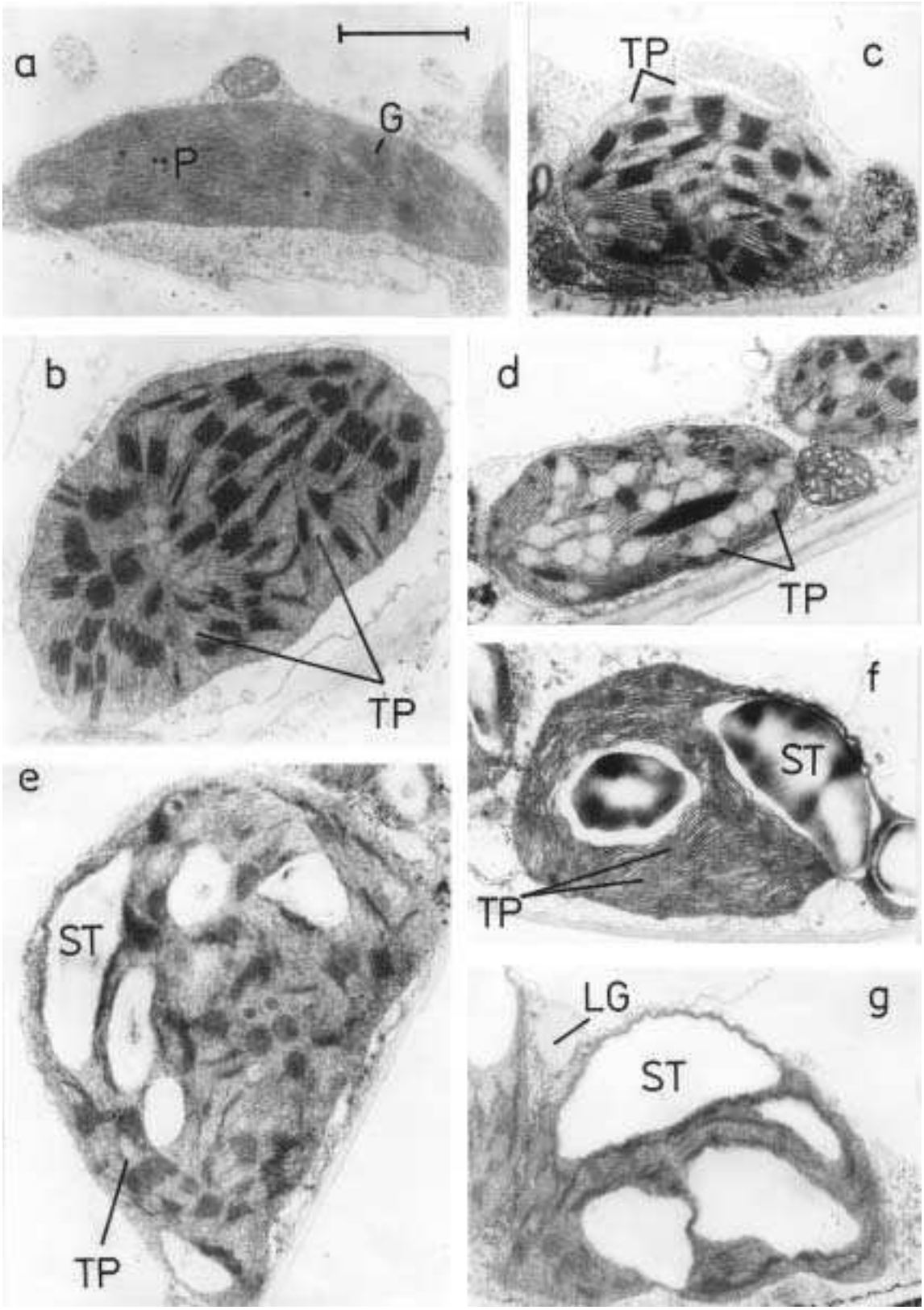

Figure 2. TEM micrographs of the chloroplasts from the control barley leaf (a) and from the leaf segments senescing in darkness (b, c, d – the 3rd, 4th and 5th day) or in continuous white light (e, f, g – the 3rd, 4th and 5th day). G – granum, LG – cytoplasmic lipid globule, P – plastoglobuli, ST – starch inclusion, TP – translucent plastoglobuli. The bar represents 1 μm, all the micrographs have the same magnification.

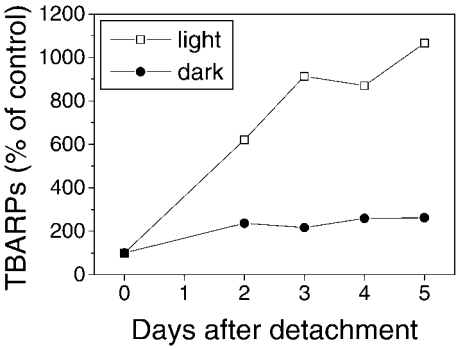

Figure 3. Changes in the relative content of TBARPs during the period after detachment in the barley leaf segments senescing in continuous white light (open squares) and in darkness (closed circles) expressed as percentage of the TBARPs content in the control leaves.

of thiobarbituric acid reaction products (TBARPs). From the 2nd day after detachment the content of TBARPs was approximately twofold compared to the control leaves (Fig. 3).

A measurement of Chl fluorescence induction was used for the estimation of changes in the PSII function during senescence of the leaf segments. The F_V/F_M ratio (interpreted as the maximal photochemical quantum yield of PSII – Kitajima and Butler 1975, Krause and Weis 1991, Govindjee 1995) decreased from 0.8 to 0.55 within the 5 days in the dark-senescing segments (Fig. 4 a). This decrease was mainly caused by a pronounced decline of the maximal fluorescence F_M (data not shown). The decrease in the photochemical function of PSII was confirmed by a decrease in the M1/F(T30) ratio (by about 40 %, Fig. 4 b) calculated from fluorescence temperature curves (FTCs, see Materials and Methods). As was shown in our previous work, a decrease in the M1/F(T30) ratio reflects a decrease in the PSII photochemistry (Nauš et al. 1992). The actual photochemical quantum yield of PSII

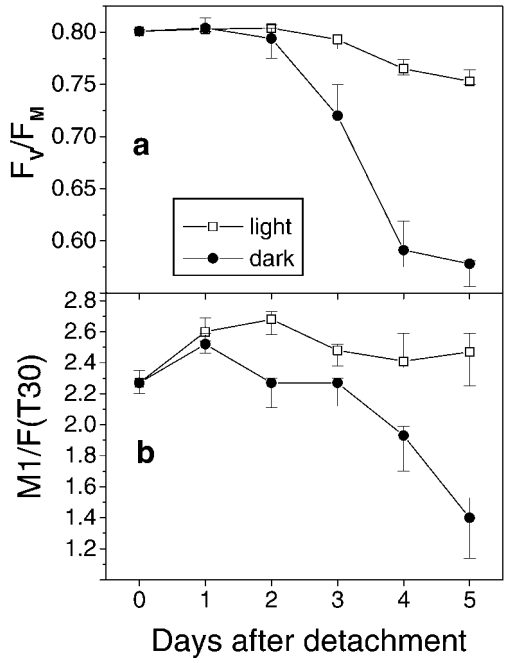

Figure 4. Changes in the Chl fluorescence parameters during the period after detachment in the barley leaf segments senescing in continuous white light (open squares) and in darkness (closed circles). a – F_V/F_M (measured with PAM 2000 fluorometer by the application of a 0.8 s saturation pulse on the dark-adapted leaf segments (n = 7); b – M1/F(T30), the ratio of fluorescence intensity in the M1 peak of the Chl fluorescence temperature curve and intensity at 30 °C (n = 5–7). Medians and quartiles are shown.

($\Delta F/F_M'$, Genty et al. 1989) during the transition of the photosynthetic apparatus from a dark- to a light-adapted state was also markedly inhibited during the period after detachment (Fig. 5).

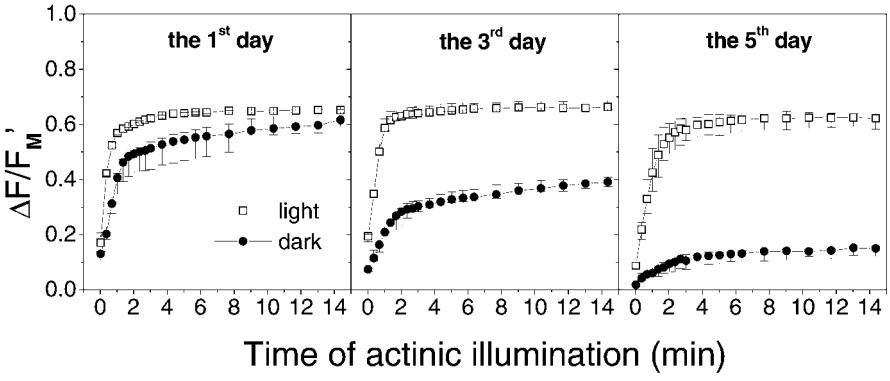

Figure 5. Dependence of the actual photochemical quantum yield of PSII ($\Delta F/F_M'$) on the time of actinic illumination (intensity of 90 μmol m^{-2} s^{-1}) in the barley leaf segments senescing in continuous white light or in darkness for 1 (the 1st day), 3 (the 3rd day) and 5 (the 5th day) days. The dependence observed in the control barley leaves (data not shown) was the same as that measured with the «light» segments on the 1st day after detachment. Measured with a PAM 2000 fluorometer using saturation pulses. Medians and quartiles are presented (n = 5–7).

Changes in leaf segments senescing in continuous white light

A decrease in the chlorophyll content during the period after detachment was similar to that found in the segments senescing in darkness (Table 1). The Chl *b* content, however, decreased faster in comparison with Chl *a* as is indicated by a significant increase in the Chl *a/b* ratio during the 5 days after detachment (Table 1). The pronounced increase in the Chl *a/b* ratio indicates a preferential decline in light-harvesting complexes of PSII (LHCII) (e.g., Anderson et al. 1988).

A typical attribute of the chloroplasts in the segments senescing in continuous white light was an occurrence of starch inclusions (Fig. 2 e–g) indicating persisting photosynthetic processes. The starch accumulation was probably caused by both a detachment of the leaf segments preventing the transfer of assimilates to other plant parts and by continuous light exposure without a dark period. The shape of the chloroplasts changed from elliptic to rather spherical and thylakoid arrangement (especially granal stacking) was gradually disturbed (Fig. 2 e–g). In order to roughly estimate what type of thylakoid membranes (granal or stromal) were impaired, we evaluated the ratio of Chl fluorescence intensities in M2 and M1 maxima from the measured FTCs (see Fig. 1 and Materials and Methods). The relative height of the high-temperature peak (M2) has been shown to be inversely proportional to the relative amount of appressed thylakoids (Downton and Berry 1982, Ilík et al. 1995). In our case the M2/M1 ratio gradually increased during the period after detachment (Table 1) indicating that the relative amount of granal thylakoids decreased in the light-senescing segments. This finding corresponds with the progressive disintegration of granal structure shown through electron microscopy (Fig. 2 e–g).

The disturbed thylakoid arrangement could be connected with peroxidation of thylakoid membrane components including lipids. A drastic increase in the content of TBARPs reflecting a very pronounced increase in the extent of lipid peroxidation was observed in the segments senescing in continuous light; on the 5th day after detachment, the content of TBARPs was 10 times higher than in the control segments prior to detachment (Fig. 3).

In spite of this pronounced lipid peroxidation, the PSII function was inhibited only slightly in the leaf segments senescing in continuous light as the F_V/F_M ratio decreased by merely 6 % during the 5-day senescence (Fig. 4 a). The higher M1/F(T30) values calculated from FTCs also indicated a better PSII function in comparison with the dark-senescing segments (Fig. 4 b). The induction kinetics of the $\Delta F/F_M'$ ratio within the transition of the photosynthetic apparatus from the dark- to light-adapted state was almost unchanged (Fig. 5). Thus we can conclude that in the leaf segments senescing in continuous white light both maximal and actual photochemical quantum yields of PSII were maintained to a great degree.

Discussion

A pronounced degradation of chlorophyll content was found in the barley leaf segments senescing in darkness and continuous white light (Table 1). Chl breakdown is the most typical symptom of leaf senescence (for a review see, for example, Grover and Mohanty 1993, Smart 1994) including the dark-induced senescence (Lichtenthaler and Grumbach 1974). Chl *a* and Chl *b* do not generally degrade at the same rate, various tendencies in the Chl *a/b* ratio have been observed with various plant species under various senescence conditions (e.g., Lichtenthaler and Grumbach 1974, Hidema et al. 1992, Lu and Zhang 1998 a, b). The various changes in the Chl *a/b* ratio indicating different relative rates in the degradation of individual PPCs could be connected mainly with different light conditions during senescence (Hidema et al. 1992). In our barley leaf segments senescing in darkness, the Chl *a/b* ratio was almost unchanged (Table 1). A slight decrease in Chl *a/b* found on the 5th day after detachment (Table 1) might indicate a lower amount of the PSI complexes containing preferentially Chl *a*. This interpretation is in agreement with the decreased gene expression and transcript accumulation of *psaA* and *psaB* genes coding for PSI apoproteins, which were reported for barley plants during dark-induced senescence (Krause et al. 1998).

A significant disturbance of thylakoid organisation, including distortion of granal arrangement observed from the 4th day of dark senescence, was accompanied by an increase in the number and size of translucent plastoglobuli (Fig. 2 c, d), which is a typical feature of chloroplast aging (Meier and Lichtenthaler 1982, Ghosh et al. 2001). The plastoglobuli in senescing chloroplasts are thought to contain components from disintegrated thylakoid membranes (Lichtenthaler 1969, Burke et al. 1984). The different electron transparency of the majority of plastoglobuli in the chloroplasts of control and senescing leaves (Fig. 2) indicates the changing lipid composition of the plastoglobuli during senescence.

Leaf senescence is usually accompanied by inhibition of the photosynthetic processes (Lichtenthaler and Grumbach 1974, Thomas and Stoddart 1980, Babani et al. 1998) including a decrease in PSII efficiency (Humbeck et al. 1996, Lu and Zhang 1998 a). In our case, both the maximal (F_V/F_M – Fig. 4 a) and actual ($\Delta F/F_M'$ – Fig. 5) quantum yields of the PSII photochemistry significantly decreased in the dark-senescing segments. A similar decrease in the F_V/F_M ratio has also been observed during the natural senescence of barley flag leaves under field conditions (Humbeck et al. 1996).

In our leaf segments senescing in continuous white light (90 $\mu mol\, m^{-2}\, s^{-1}$ of PAR), the rate of decrease in the total chlorophyll content was similar to that observed for the dark-senescing segments (Table 1), although light of moderate intensity (up to approximately 100 $\mu mol\, m^{-2}\, s^{-1}$) is known to retard the senescence-induced decrease in Chl content in detached leaves (e.g., Thimann et al. 1977, Kar et al. 1993, Chang and Kao 1998). The discrepancy between our results

and the results of other authors could be caused by different light conditions during senescence in relation to the light conditions during plant cultivation. In our case, plants were grown under a periodic light regime and the leaf segments were exposed for several days to continuous light of the same intensity. This change could represent a relative increase in the light dose absorbed by the leaf tissue causing an increased risk of photo-oxidative damage including photodestruction of pigment molecules.

In our light-senescing leaf segments Chl *b* degraded faster than Chl *a* (increasing Chl *a/b* ratio – Table 1), indicating a preferential decrease in LHCII, the Chl-protein complex with the lowest Chl *a/b* ratio (approximately 1.2; e.g., Melis 1991). The preferential decrease in LHCII should lessen the risk of photo-oxidative damage due to a relative decrease in the absorption cross-section of photosystems. However, in spite of the decreased LHCII in the light-senescing leaf segments, the level of oxidative damage of lipid molecules drastically increased during senescence (Fig. 3). Interestingly, the PSII function was highly maintained in these segments (Figs. 4, 5). Thus it seems that the level of lipid peroxidation need not generally be in direct relationship to the thylakoid membrane functions as has been suggested by Chang and Kao (1998). These authors demonstrated that an increased level of lipid peroxidation in detached leaves of rice senescing in white light (40 $\mu mol\, m^{-2} s^{-1}$) was not accompanied by a decrease in membrane integrity.

We can conclude that in our barley leaf segments continuous white light had a negative effect on the senescence-induced ultrastructural changes in the chloroplasts, probably due to the pronounced photo-oxidative damage. This light had a very strong positive effect, however, on the maintenance of the PSII function. How the PSII function is preserved in the case of strong lipid peroxidation within chloroplasts remains unclear.

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Xanthophyll cycle activity in detached barley leaves senescing under dark and light

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Abstract

Senescence-induced changes in the xanthophyll cycle activity and chlorophyll (Chl) fluorescence parameters were compared in detached barley (*Hordeum vulgare* L.) leaf segments kept for 6 d in darkness or under continuous "white light" (90 μmol m^{-2} s^{-1}). Before detachment of the leaf segments, the plants were grown at periodic regime [12 h light (90 μmol m^{-2} s^{-1})/12 h dark]. The de-epoxidation state of the xanthophyll cycle pigments (DEPS) in the leaf samples was determined immediately (the actual DEPS), after 1 h of dark-adaptation (the residual DEPS), and during 14 min of a high-irradiance (HI) exposure (500 μmol m^{-2} s^{-1}) (HI-induced DEPS). In the light-senescing segments, senescence was delayed pronouncedly compared to dark-senescing ones as the Chl content, the photosystem 2 photochemistry, and electron transport processes were highly maintained. Further, the actual DEPS increased, probably due to the increased mean photon dose. The HI-induced increase in the DEPS was stimulated in the light-senescing segments, whereas it was slowed down in the dark-senescing ones. However, after the 14 min HI-exposure of the dark-senescing segments the HI-induced DEPS was not markedly lower than in the mature leaves, which indicated the maintenance of the xanthophyll cycle operation.

Additional key words: ageing; carotenoids; chlorophyll fluorescence induction; de-epoxidation state; *Hordeum vulgare*; photosystem 2.

Introduction

Senescence of plant leaves is their final ontogenetic phase (Šesták 1985), comprising many physiological, biochemical, and molecular events that occur in a highly controlled manner (for reviews, see *e.g.* Grover and Mohanty 1993, Smart 1994, Matile 2001). Leaf senescence is characterized by a decrease in the rate of photosynthesis. This decrease is caused mainly by a progressive loss of chlorophyll (Chl). However, it is also associated with a decline in activities of photosystems and dark reactions of the Calvin cycle. The senescence-induced inhibition of the dark reactions (caused mainly by a decline in amounts and activities of stromal enzymes, especially ribulose-1,5-bisphosphate carboxylase/oxygenase) usually precedes a decrease in function of pigment-protein complexes in thylakoid membranes (Camp *et al.* 1982, Grover 1993). Especially, the photosystem 2 (PS2) photochemistry seems to be diminished rather slightly during senescence of intact leaves (Lu and Zhang 1998a,b, Špundová *et al.* 2003).

Senescence of plants grown under natural conditions is significantly affected by many environmental factors, especially by irradiance, temperature, nutrition, and water supply, and by the periodic circadian irradiation. Compared to the progressive senescence proceeding in darkness, the moderate irradiance usually retards the degradation of photosynthetic components (Thimann *et al.* 1977, Kar *et al.* 1993, Klerk *et al.* 1993, Chang and Kao 1998) and supports conservation of the photosynthetic activity (Kar *et al.* 1993, Špundová *et al.* 2003). However, when irradiance exceeds some critical level, the senescence-induced changes are accelerated (Hidema *et al*. 1992, Kar *et al*. 1993, Biswal 1995), most probably due to an

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Abbreviations: A, antheraxanthin; Car, sum of carotenoids; Chl, chlorophyll; DEPS, de-epoxidation state of the xanthophyll cycle pigments; HI, high irradiance; MGDG, monogalactosyldiacylglycerol; NPQ, non-photochemical chlorophyll fluorescence quenching; PAR, photosynthetically active radiation; PS2, photosystem 2; V, violaxanthin; Z, zeaxanthin.
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increasing photo-oxidative damage to the photosynthetic apparatus caused by over-excitation (Munné-Bosch and Allegre 2002). Under field conditions, the protective and deleterious effects of irradiation may appear successively in a periodic manner.

One of the protective mechanisms of plants against the over-excitation is the xanthophyll cycle, the reversible conversion of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) (Demmig-Adams and Adams 1992, Horton *et al.* 1996, Choudhury and Behera 2001). The extent of this conversion is usually expressed as the de-epoxidation state (DEPS) of the xanthophyll cycle pigments. Numerous studies have shown a close positive correlation between the DEPS and the non-radiative dissipation of absorbed photon energy reflected in the non-photochemical quenching (NPQ) of Chl fluorescence (*e.g.* Demmig-Adams 1990, Demmig-Adams and Adams 1996).

Senescence-induced changes in the xanthophyll cycle have not been yet fully elucidated. Biswal (1995) has implied a senescence-induced inhibition of the xanthophyll cycle. But recent studies have shown an increase in the DEPS during senescence under field conditions, especially in the leaves exposed to high irradiance (HI) (Murchie *et al.* 1999, García-Plazaola and Becerill 2001, Lu *et al.* 2001a,b, 2003, Yang *et al.* 2001, Jiao *et al.* 2003). Their results indicate that the xanthophyll cycle plays a role in photoprotection of the photosynthetic apparatus in senescing leaves through the thermal dissipation of excess photon energy (García-Plazaola and Becerill 2001, Lu *et al.* 2001a,b). All mentioned studies investigated only the "actual" DEPS in the leaves exposed to particular irradiance. Changes in the DEPS during HI-exposure of dark-adapted leaves reflecting the activation of the xanthophyll cycle have not been yet studied during senescence.

Further, the leaves attached to plants were used in all the mentioned studies. The connection of the leaves with the plant enables the source-sink relations, the transport of nutrients from the senescing leaves to the rest of plant, and the signal regulation of some processes in these leaves (Matile 2001). In the present study we used barley leaves detached from plants, *i.e.* a system without a possibility of translocation of degradation products and without any influence of the rest of plant. The natural light/dark circadian changes were eliminated by keeping the barley leaf segments in darkness or under continuous irradiation (90 μmol m^{-2} s^{-1}) for 6 d at constant temperature and water supply. We have followed senescence-induced changes in the actual DEPS as well as changes in the DEPS during HI-exposure (500 μmol m^{-2} s^{-1}, 14 min) of dark-adapted leaf samples in order to determine changes in the activity of the xanthophyll cycle during senescence. The kinetics of the HI-induced V de-epoxidation has been compared with the induction kinetics of non-photochemical Chl fluorescence quenching (NPQ) and $1 - q_P$ parameter.

Materials and methods

Plants and senescence conditions: Plants of spring barley (*Hordeum vulgare* L.) were grown in a growth chamber at 23 °C on an artificial medium composed of perlite and Knop's solution. The irradiation regime was 12 h dark/12 h light (fluorescent radiation) with a PAR irradiance of 90 μmol m^{-2} s^{-1}. The mature primary leaves of the plants in the growth phase of two leaves (the growth phase 1.2 according to Feekes 1941) were used for measurements. Leaf segments were cut off from the primary leaf blades (1.5 cm from the tip of the leaf, length of 5.0 cm) and placed into dishes with distilled water during the first two hours of the dark period. The leaf segments floated on the water surface. The segments were kept under dark or light (continuous PAR, irradiance 90 μmol m^{-2} s^{-1}) at an air temperature of 23 °C.

Pigment analyses: Leaf segments were frozen in liquid nitrogen. Pigments were extracted from the segments with 80 % acetone and a small amount of $MgCO_3$. The extract was centrifuged (3 600×*g*, 5 min) and the filtered supernatant was used for spectrophotometric (*Specord M40*, *Carl Zeiss*, Jena, Germany) estimation of Chl and total carotenoid (Car) contents (Lichtenthaler 1987), and for HPLC determination of relative changes in individual pigments of the xanthophyll cycle. The isocratic reverse-phase HPLC was performed according to a slightly modified procedure of Färber and Jahns (1998). The HPLC system consisted of a *ProStar 230* Delivery Module, *ProStar 32*0 UV-VIS detector *(Varian Analytical Instruments*, Walnut Creek, USA), and a reverse phase column (5 μm particle size, *250/4 RP18*, *Lichrocart*, Germany). The pigments were eluted isocratically for 13 min with acetonitrile : methanol : 0.1 M Tris (87 : 10 : 3, v/v) followed by a 12-min isocratic elution with methanol : ethyl acetate (34 : 16, v/v) at a flow rate of 33.3 mm^3 s^{-1}. Absorbance was measured at 440 nm. The de-epoxidation state of the xanthophyll cycle pigments (DEPS) was calculated as DEPS = (Z + A)/(V + A + Z) (Gilmore and Björkman 1994), where Z is zeaxanthin, A is antheraxanthin, and V is violaxanthin. The actual DEPS, residual DEPS after 1 h of dark-adaptation (for control leaves and light-senescing segments), and "HI-induced DEPS" in three time points (4, 9, and 14 min) of a HI-exposure ("white light", 500 μmol m^{-2} s^{-1}) were evaluated. A difference between the HI-induced DEPS and residual DEPS was estimated and presented as ΔDEPS.

Chl fluorescence was measured at room temperature (23 °C) with a modulation fluorometer *PAM 2000* (*Walz*,

Effeltrich, Germany). The $F_V/F_M = (F_M - F_0)/F_M$ was determined from the minimal Chl fluorescence intensity (F_0) and maximal fluorescence intensity (F_M) measured on the adaxial side of the barley leaves or leaf segments. The control (attached) leaves were measured during the fifth and sixth hours of the dark period, the light-senescing segments were measured after 1 h of dark-adaptation. F_0 was obtained upon excitation of the leaf segment with a weak measuring beam of 0.2 μmol m^{-2} s^{-1}, F_M was measured by means of a 0.8-s saturating pulse (≈6 000 μmol m^{-2} s^{-1}).

After the F_V/F_M measurement, the induction kinetics of Chl fluorescence parameters was recorded during 14 min of HI-exposition of the leaf samples ("white light", 500 μmol m^{-2} s^{-1}). During fluorescence induction the saturating pulses (0.8 s, 6 000 μmol m^{-2} s^{-1}) were applied in order to determine F_M' (= maximal Chl fluorescence intensity during the actinic irradiation). The first two pulses were applied 2 and 22 s after switching on the HI, following 9 pulses in 40 s intervals, and the last 6 pulses in 80 s intervals. After each pulse, HI was switched off and far-red radiation was applied for 3 s in order to measure F_0' (= minimal Chl fluorescence intensity during the actinic irradiation). The induction kinetics was measured each day within 6 d after detachment (except the first day after detachment when no significant changes in the studied parameters were expected). For better readability only the kinetics measured in the control leaves and 2, 4, and 6 d after detachment is presented (Fig. 4).

The excitation pressure on PS2 was calculated from a coefficient of photochemical fluorescence quenching q_P (van Kooten and Snell 1990) as $1 - q_P = 1 - (F_M' - F_t)/(F_M' - F_0')$, where F_t was the fluorescence intensity at time t. Non-photochemical fluorescence quenching (NPQ) was calculated as $F_M/F_M' - 1$ according to Bilger and Björkman (1990). A general statistical description (medians and quartiles) was used in the case of Chl fluorescence parameters (Lazár and Nauš 1998).

Results

The content of Chls and total Cars did not change significantly in barley leaf segments kept under continuous irradiation (Table 1). The ratios of contents of Chl/Car and Chl/xanthophyll cycle pigments (V+A+Z) slightly decreased after the detachment (Table 1).

In the dark-senescing segments of barley leaves a gradual decrease in Chl and Car contents was found together with a decrease in Chl/Car, indicating a relatively more rapid decrease in the Chl content in comparison with Cars (Table 1). The Chl/(V + A + Z) ratio decreased, too. On the 6th d after detachment it was reduced by about 50 % compared with the control leaves and segments senescing under continuous irradiation. The pronounced decrease in the relative amount of Chls was caused by the decrease in their content (Table 1) rather than by an increase in the xanthophyll cycle pigment pool.

Changes in the F_V/F_M ratio in the light-senescing leaf segments were almost negligible; the values above 0.80 within 6 d after detachment indicated a nearly unchanged maximal efficiency of PS2 photochemistry (Fig. 1). In the dark-senescing segments, F_V/F_M decreased to about 0.65.

The actual de-epoxidation state of the xanthophyll cycle pigments (DEPS, estimated as a ratio (A + Z)/(A + Z + V) in the control (attached) leaves was about 0.06

Table 1. Changes in contents of chlorophyll (Chl) and carotenoids (Car) and ratios Chl/Car, Chl *a*/*b*, and Chl/xanthophyll cycle pool pigments (V+A+Z) (V, violaxanthin; A, antheraxanthin; Z, zeaxanthin) in senescing segments of barley leaves. The last ratio was evaluated from HPLC results ($n = 3$), the other parameters from spectrophotometric measurements ($n = 5$). The segments were kept under continuous irradiation (90 μmol m^{-2} s^{-1}) or in the dark. Means and SD are shown. Statistically significant differences from control values are indicated by the letter a, and between the segments kept under light and dark conditions for corresponding day after detachment by the letter b. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$.

	Time after detachment [d]	Chl *a*+*b* [g m^{-2}]	Car *x*+*c* [g m^{-2}]	Chl/Car	Chl *a*/*b*	Chl/(V+A+Z)
Control	0	23 ± 3	4.4 ± 0.5	5.3 ± 0.1	3.6 ± 0.2	7.9 ± 0.3
Light	2	20 ± 4	4.4 ± 0.8	4.7 ± 0.2 a***	3.5 ± 0.2	6.7 ± 0.6 a**
	3	24 ± 3	4.6 ± 0.7	5.2 ± 0.2	3.1 ± 0.2 a**	7.0 ± 0.2 a***
	4	27 ± 4	5.4 ± 0.8	4.9 ± 0.1 a**	3.3 ± 0.1 a*	6.9 ± 0.2 a***
	5	20 ± 5	4.2 ± 1.0	4.7 ± 0.2 a***	3.2 ± 0.1 a*	7.4 ± 0.6
	6	18 ± 6	4.3 ± 1.3	4.2 ± 0.2 a***	3.5 ± 0.1	7.2 ± 0.2 a**
Dark	2	18 ± 5	4.0 ± 1.1	4.5 ± 0.2 a***	3.5 ± 0.1	6.4 ± 0.4 a***
	3	14 ± 3 a***, b***	3.5 ± 0.6 a**, b**	4.0 ± 0.3 a***, b***	3.3 ± 0.1 a*	5.4 ± 0.6 a***, b*
	4	10 ± 3 a***, b***	3.3 ± 0.3 a**, b***	2.9 ± 0.6 a***, b***	3.2 ± 0.1 a*	5.8 ± 1.2 a**
	5	7 ± 2 a***, b***	2.7 ± 0.4 a***, b***	2.6 ± 0.7 a***, b***	3.1 ± 0.1 a**	5.5 ± 0.2 a***, b**
	6	6 ± 2 a***, b**	2.6 ± 0.4 a***, b***	2.2 ± 0.7 a***, b***	3.2 ± 0.3	4.0 ± 0.8 a***, b**

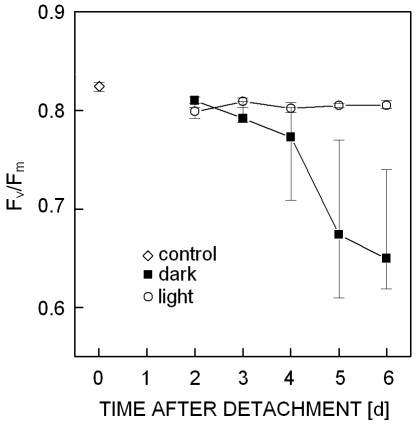

Fig. 1. The maximal efficiency of photosystem 2 photochemistry (F_V/F_M) in the control (attached) barley leaves (◇) and in the leaf segments senescing in darkness (■) and under continuous light (○). Medians and quartiles are shown; n = 10–12.

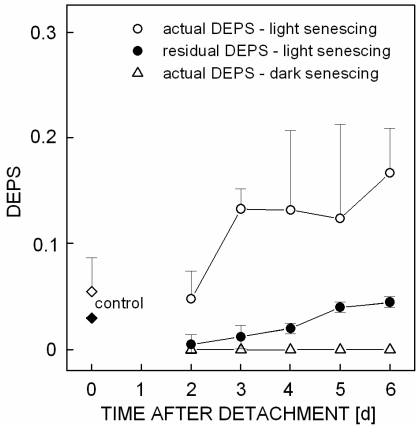

Fig. 2. The actual (*open symbols*) and residual (*solid symbols*) de-epoxidation state of the xanthophyll cycle pigments (estimated as the amount of A and Z related to amounts of A, Z, and V) in the control (attached) barley leaves (*diamonds*) and in the light senescing (*circles*) and dark senescing (*triangles*) barley leaf segments. The residual DEPS was determined after 1 h of dark adaptation. Means and SD, n = 3.

at the end of the light period (Fig. 2). The residual DEPS (*i.e.* the DEPS after 1 h of dark-adaptation) was about 0.03 as a part of Z was converted back to V. There was a pronounced increase in the actual DEPS in the light-senescing segments from the 3rd day after detachment. The residual DEPS slightly decreased on the 2nd d after detachment in comparison with the control leaves, and then a slight increase was found. The actual DEPS in the dark-senescing segments was zero as neither Z nor A were detectable from the 2nd d after detachment (Fig. 2).

The HI-exposure of the control leaves and senescing segments was aimed to reveal the rate and extent of conversion of V into A and Z during HI treatment. This exposure caused an increase in the DEPS designated as ΔDEPS (ΔDEPS = "HI-induced DEPS" – residual DEPS, Fig. 3). In the control leaves the ΔDEPS reached a relatively low plateau after 4 min of HI. In the light-senescing segments the HI-exposure caused a more pronounced increase in the ΔDEPS, especially after 9 and 14 min. The de-epoxidation of V was strongly delayed in the dark-senescing segments as an increase in the ΔDEPS was gradually slowed down. However, the ΔDEPS after 14 min of the HI-exposure was even slightly higher on the 2nd and 4th d or non-significantly lower on the 6th d after detachment in comparison with the control leaves (Fig. 3).

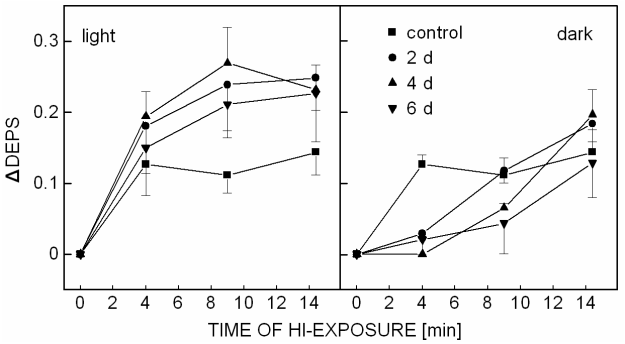

Fig. 3. The high-irradiance (HI) induced de-epoxidation state of the xanthophyll cycle pigments (estimated as ΔDEPS = actual DEPS – residual DEPS) during the HI (500 μmol m^{-2} s^{-1}) exposure of the control (attached) barley leaves and leaf segments senescing in darkness (dark) and under continuous light (light) for 2, 4, and 6 d after detachment. Means and SD, n = 3.

The induction kinetics of Chl fluorescence parameters (NPQ and 1 – q_P) was measured during 14 min of the HI-exposure in the same leaf samples for which the ΔDEPS was followed. In the case of control leaves and light-senescing segments, NPQ should be also understood as an increment (*i.e.* ΔNPQ) as in the case of the ΔDEPS, because the F_M value estimated after 1 h of dark-adaptation of these samples might have been lowered by the residual DEPS (Fig. 2). In the control leaves, the induction kinetics of NPQ was characterized by a gradual increase to a steady-state value (of about 2.3) (Fig. 4*A*). The increase in NPQ in the light-senescing segments during the first 4 min of HI was almost the same as in the control leaves (except a slight acceleration observed in the segments on the 2nd d after detachment). However, there was a suppression of the subsequent NPQ increase and steady-state values. The steady-state value of NPQ decreased on the 2nd d after detachment to about 1.3 and remained near this value (Fig. 4*A*). Interesting changes were found in the NPQ induction kinetics of the dark-senescing segments. The increase of NPQ induced by HI became bi-phasic: after an initial increase within the first 4 min there was a plateau and subsequent more rapid increase even without reaching saturation. The NPQ increase was gradually suppressed in the dark-senescing segments and the NPQ value after 14 min of HI decreased significantly on the 6th d after detachment (Fig. 4*A*).

The senescence-induced changes in the 1 – q_P

induction kinetics were analogous to those in the NPQ kinetics. The decrease in value of $1 - q_P$ during the HI-exposure was accelerated in the segments senescing under continuous irradiation in comparison with control leaves (Fig. 4*B*). In the dark-senescing segments this decrease was gradually inhibited within 6 d after detachment.

Discussion

The continuous exposure of the senescing barley leaf segments to the cultivation irradiance (90 μmol m^{-2} s^{-1}) markedly retarded the Chl degradation in comparison with the situation in darkness (Table 1) and maintained the maximal efficiency of PS2 photochemistry (F_V/F_M; Fig. 1). Induction kinetics of NPQ and $1 - q_P$ was even accelerated compared to the control leaves (Fig. 4). However, in the control leaves the kinetics was measured within the fifth and sixth hours of the dark period so that the activation of the photosynthetic apparatus could be slowed down in comparison with the light-senescing segments that were dark-adapted for 1 h.

Moderate irradiance retards the senescence-induced symptoms such as a decrease in contents of photosynthetic pigments (*e.g.* Thimann *et al.* 1977, Kar *et al.* 1993, Meir and Philosoph-Hadas 1995) and proteins (*e.g.* Klerk *et al.* 1993, Chang and Kao 1998), in PS2 photochemistry (*e.g.* Kar *et al.* 1993, Špundová *et al.* 2003), and in some other functions. However, there is an increasing risk of photo-oxidative damage to the photosynthetic apparatus when senescing leaves are irradiated (Munné-Bosch and Allegre 2002). A supply of $NADP^+$ as a final acceptor of electrons in the photosynthetic transport chain decreases due to an inhibition of stromal reactions so that probability of the electron flow to molecular oxygen, accumulation of the reactive oxygen species, and photodamage to the chloroplast components increase. One of the protective mechanisms against photodamage is the pH-dependent thermal dissipation of excited states occurring in the PS2 antenna pigment bed. This process involves the de-epoxidation of V through A to Z which is triggered by the acidification of thylakoid lumen (Demmig-Adams and Adams 1992, Horton *et al.* 1996). A higher de-epoxidation has been recently found in the leaves of the plants senescing under natural conditions, which indicated that the xanthophyll cycle played a role in the photoprotection of the photosynthetic apparatus in senescing leaves. The increase in the actual DEPS during natural senescence was observed in the attached leaves of field-grown wheat (Lu *et al.* 2001a,b, 2003) and rice (Murchie *et al.* 1999, Yang *et al.* 2001), and in leaves of beech (García-Plazaola and Becerill 2001). The senescence-induced increase in the actual DEPS was more pronounced in the leaves exposed to higher irradiance (at midday in comparison with the morning: Murchie *et al.* 1999, Lu *et al.* 2001a,b, 2003, Yang *et al.* 2001, Jiao *et al.* 2003, or in the sun leaves compared to the shade ones: García-Plazaola and Becerill 2001). These results indicate that a long lasting exposition of senescing leaves to HI leads to a higher DEPS.

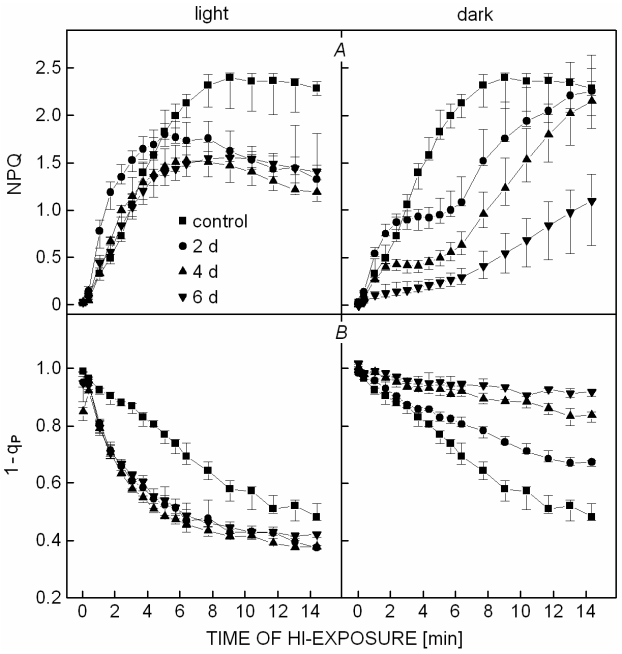

Fig. 4. Induction kinetics of NPQ (non-photochemical quenching of chlorophyll fluorescence) (*A*) and $1 - q_P$ (*B*) in the control (attached) leaves of barley and leaf segments senescing in darkness (dark) and under continuous irradiation (light) for 2, 4, and 6 d after detachment during their high-irradiance exposure (500 μmol m^{-2} s^{-1}). Medians and quartiles are shown; $n = 6$.

In our case, the actual DEPS was also increased in the light-senescing leaf segments (Fig. 2) even if they were exposed to a relatively low irradiance (90 μmol m^{-2} s^{-1}). However, a long-time photon dose was doubled compared to that before the detachment, because the plants were growing under a periodic irradiation (12 h light/12 h dark) whereas the segments were kept under continuous irradiation. Afitlhile *et al.* (1993) have reported a decrease in the actual DEPS in detached barley leaves senescing under continuous irradiation. However, they used lower irradiance (46 μmol m^{-2} s^{-1}) and the photon dose was not increased after detachment as in our case.

The increased actual DEPS might contribute to the maintenance of Chl content and PS2 photochemistry in the light-senescing segments (Fig. 2) because the protective effects of Z and A in thylakoid membranes include a regulation of the thylakoid membrane fluidity (Gruszecki and Strzalka 1991, Strzalka and Gruszecki 1997, Tardy and Havaux 1997) and inhibition of membrane lipid peroxidation (Havaux *et al.* 2000). The slightly increased residual DEPS in the light-senescing segments (Fig. 2) indicated the slowed-down epoxidation of Z and A back to V.

In HI-treated (500 μmol m^{-2} s^{-1}) dark-adapted leaf

samples, the xanthophyll cycle was activated, and the level of de-epoxidation (ΔDEPS) increased. The rate and extent of conversion of V into A and Z was stimulated in the light-senescing segments, which was indicated by the more rapid increase in the ΔDEPS and by the higher ΔDEPS values after 14 min of HI-exposure in comparison with the control leaves (Fig. 3). An increase in the DEPS should be accompanied by a corresponding increase in NPQ (Demmig-Adams 1990, Demmig-Adams and Adams 1996). Such changes were found during senescence in the field (Murchie *et al.* 1999, Lu *et al.* 2001a,b, 2003, Jiao *et al*. 2003). In our case such correlation might be valid in the initial phases of the induction kinetics (compare Figs. 3 and 4*A*). However, the steady-state values of NPQ in the light-senescing segments were lower (despite of the higher ΔDEPS) in comparison with the control leaves. Similar results were reported by Kurasová *et al.* (2002) for barley plants acclimated to HI: the lowest NPQ was observed in the leaves with the highest DEPS. Kurasová *et al.* (2002) suggested a specific HI-acclimation of barley without a pronounced increase in non-radiative dissipation (see also Čajánek *et al.* 1999). In our case, the decrease was observed also in steady-state values of SV_0 in the light-senescing segments (data not shown). SV_0 is the quenching of minimal Chl fluorescence and it might be related to the part of non-radiative dissipation localized within light-harvesting complexes (Pospíšil 1997, Špunda *et al.* 1997). Therefore this quenching should better correspond to the DEPS. However it was not our case. A reason for the lower non-radiative dissipation in the light-senescing segments with the higher DEPS remains misty.

During senescence in darkness, similar changes in the induction kinetics of both ΔDEPS and NPQ were observed (Figs. 3 and 4*A*). The increase in the ΔDEPS and NPQ was gradually slowed down and the steady state could not be reached within the 14 min of HI-exposure. The gradual retardation of V de-epoxidation and slowing down of the NPQ increase might be connected with a partial inhibition of lumen acidification and impairment of the electron transport processes in thylakoid membranes, which was indicated by the gradual inhibition of $1 - q_P$ kinetics (Fig. 4*B*). The slowing down of the $1 - q_P$ decrease during the HI-exposure can be interpreted as the decrease in the electron withdrawing from PS2 reaction centres (Björkman and Demmig-Adams 1995). The inhibition of the linear electron transport was reported for the senescing leaves (Grover and Mohanty 1993 and references therein).

Other explanation of the delayed conversion of V into A and Z might be connected with changes in the lipid composition of thylakoid membranes during senescence in darkness. According to a model of Latowski *et al.* (2002) the activity of violaxanthin de-epoxidase is associated with monogalactosyldiacylglycerol (MGDG) domains in the thylakoid membranes. The authors suggest that V has to diffuse into such MGDG-enriched domains to be converted into A and Z. During dark-induced senescence a degradation of galactolipids (preferentially MGDG) precedes the degradation of other membrane lipids (Gut and Matile 1989, Meir and Philosoph-Hadas 1995). Wanner *et al.* (1991) have shown for dark-senescing barley leaves that the carbon of acyl residues originating from galactolipids is utilized for gluconeogenesis. So we can speculate that the inhibited kinetics of the V de-epoxidation could be associated with an extension of a diffusion path of the V to the MGDG-enriched domains caused by a decrease in a relative content of MGDG in the thylakoid membranes of the dark-senescing segments.

Summing up, the increased actual DEPS in the barley leaf segments senescing under continuous cultivation irradiance indicated that the xanthophyll cycle participated in the protection of the photosynthetic apparatus from over-excitation similarly as in case of the attached leaves senescing under field conditions and exposed to the increased irradiance. The increased level of V de-epoxidation was induced by the increase in the mean photon dose. The conversion of V into A and Z induced by the HI-exposure was stimulated in the light-senescing segments. This process was slowed down in the dark-senescing segments (probably due to the slowing down of the thylakoid lumen acidification), but it was still functional for 6 d after detachment.

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Plant shading increases lipid peroxidation and intensifies senescence-induced changes in photosynthesis and activities of ascorbate peroxidase and glutathione reductase in wheat

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Abstract

Plants of spring wheat (*Triticum aestivum* L. cv. Saxana) were grown during the autumn. Over the growth phase of three leaves (37 d after sowing), some of the plants were shaded and the plants were grown at 100 (control without shading), 70, and 40 % photosynthetically active radiation. Over 12 d, chlorophyll (Chl) and total protein (TP) contents, rate of CO_2 assimilation (P_N), maximal efficiency of photosystem 2 photochemistry (F_V/F_P), level of lipid peroxidation, and activities of antioxidative enzymes ascorbate peroxidase (APX) and glutathione reductase (GR) were followed in the 1st, 2nd, and 3rd leaves (counted according to their emergence). In un-shaded plants, the Chl and TP contents, P_N, and F_V/F_P decreased during plant ageing. Further, lipid peroxidation increased, while the APX and GR activities related to the fresh mass (FM) decreased. The APX activity related to the TP content increased in the 3rd leaves. The plant shading accelerated senescence including the increase in lipid peroxidation especially in the 1st leaves and intensified the changes in APX and GR activities. We suggest that in the 2nd and 3rd leaves a degradation of APX was slowed down, which could reflect a tendency to maintain the antioxidant protection in chloroplasts of these leaves.

Additional key words: ageing; chlorophyll fluorescence; CO_2 assimilation; plant topography; *Triticum aestivum*.

Introduction

Plant senescence is a systemically organized heterogeneous process within the whole plant organism. Both plant signalling and gene expression are among the regulating factors in whole plant senescence, however, it is still discussed to what degree plant organ senescence is a matter of pure genetic, hormone, or combined control (Thomas *et al.* 2003).

There is an insertion gradient of leaf characteristics within a plant determined by different phases of ontogeny of subsequently formed leaves and by various micro-environmental conditions, especially irradiance (Šesták 1985). A typical phenomenon of whole plant senescence is a remobilization of nutrients from the lower (older) leaves to the upper (younger) ones. For this reason, the topography of a plant should be followed when studying whole plant senescence. The topographic measurement of leaf characteristics (*i.e.* measurement on several leaves of different insertion) makes possible to determine structural and functional heterogeneity within the plant that can differ under different conditions. The concept of topography gradients of measured parameters [*e.g.* the chlorophyll (Chl) fluorescence ones] might be of relevance (Matoušková *et al.* 1999). There is also an age gradient of structural and functional parameters within one leaf (*e.g.* Buschmann 1981, Nauš *et al.* 1985, Šesták and Šiffel 1997).

Leaf senescence is characterized by a decrease in the rate of photosynthesis. This decrease is associated with a progressive loss of Chls and with a decline in activities of the photosystems and reactions of the Calvin cycle. The senescence-induced inhibition of the Calvin cycle reactions (caused mainly by a decline in amounts and activities of stromal enzymes, especially ribulose-1,5-bisphosphate carboxylase/oxygenase) usually precedes

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Abbreviations: APX, ascorbate peroxidase; Chl, chlorophyll; DM, dry mass; FM, fresh mass; GR, glutathione reductase; LA, leaf area; PAR, photosynthetically active radiation; PS2, photosystem 2; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TP, total proteins.
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a decrease in function of the pigment-protein complexes in thylakoid membranes (Camp *et al.* 1982, Grover 1993). In particular, photochemistry of photosystem 2 (PS2) is diminished very little during senescence of leaves (Špundová *et al.* 2003).

A substantial decrease in net photosynthetic rate (P_N) accompanied by only a slight decrease in PS2 photochemistry can lead to photoinhibition and an increased generation of reactive oxygen species (ROS) in senescent leaves. Upon weakening of the anti-oxidative defence mechanisms oxidative degradation of pigments, proteins, and lipids is initiated (Munné-Bosch and Allegre 2002, Jing *et al.* 2003). From this point of view, a decrease in irradiance should lead to a lower photo-generation of ROS and a lower oxidative damage. The leaves of low insertion usually grow under low irradiance so a low oxidative damage could be expected. On the other hand, a lower P_N due to limitation of excitation energy may cause a shortage of cell energy and a failure of anti-oxidative defence mechanisms so that oxidative damage could increase in these leaves.

Several enzyme cycles participate in the anti-oxidative defence of plants. The antioxidant enzymes ascorbate peroxidase (APX) and glutathione reductase (GR) are the main enzymes of the ascorbate-glutathione cycle that is a part of the water-water cycle in chloroplasts (Asada 1999, 2000) but also appears in other organelles (Jiménez *et al.* 1997). The water-water cycle scavenges ROS (superoxide and hydrogen peroxide) photo-generated mostly by thylakoid membranes. Senescence of detached leaves (Feng *et al.* 2003), seeds (Goel and Sheoran 2003), or stressed plants (Jiang and Huang 2001) is usually accompanied by a decrease in APX and GR activities in parallel with an increase of lipid peroxidation. In naturally senescing cucumber cotyledons, the GR activity decreased whereas APX activity increased (Kanazawa *et al.* 2000). Irradiance can influence significantly the senescence-induced changes in anti-oxidative enzyme activities (Kar *et al.* 1993, Kanazawa *et al.* 2000, Yamazaki and Kamimura 2002). This phenomenon is not usually taken into account in senescence studies.

In this project we investigated the effect of reduced irradiance on senescence of wheat leaves in different insertion. We focused on changes in lipid peroxidation and in the APX and GR activities in order to determine how the decrease in irradiance affects these changes in the senescing leaves.

Materials and methods

Plants and their shading: Plants of spring wheat (*Triticum aestivum* L. cv. Saxana) were grown in pots with an artificial medium composed of perlite and Knop's solution at autumn season (October, November) in a greenouse in Olomouc, Czech Republic. Irradiance changed according to weather conditions; the maximal irradiance at midday was around 500 μmol(photon) m^{-2} s^{-1} of photosynthetically active radiation (PAR). The day temperature varied between 16 and 24 °C and the relative air humidity was 33–52 %. 37 d after sowing the pots with plant seedlings were divided into three groups. Two of them were shaded with a non-woven fabric (permeable to water) so that three irradiances were set: 100 % (un-shaded plants), 70 %, and 40 % of PAR. The first, second, and third leaves of the plants (counted from the shoot base) were used for measurements that were carried out on the day of shading (0 d) and on the 6^{th} and 12^{th} d of shading. In order to eliminate (at least partly) an effect of age gradient within the leaf, only middle part of leaf blades was used for measurements. The shading slowed down the growth rate of plants. The growth phases according to Feekes (1941) are shown in Table 1.

Chl and total protein (TP) contents: Chl content was determined spectrophotometrically (*Unicam UV550, Thermospectronic*, Cambridge, UK) in 80 % acetone extract prepared from the middle part of leaf blades (centrifugation: 5 min, 3 600×*g*) according to Lichtenthaler (1987). The content of TP was estimated by a modified method of Lowry *et al.* (1951) using ovalbumin as a protein standard.

P_N was measured using an open gasometric system (*LCA–4, ADC*, Hoddesdon, UK). Middle parts of blades (625 mm^2) of attached wheat leaves were placed into a leaf chamber (CO_2 concentration 350 μmol mol^{-1}, temperature 24 °C) and dark-adapted for 7 min, then the "white" actinic irradiation [290 μmol(photon) m^{-2} s^{-1}] was switched on. P_N was measured within the 19^{th} and 22^{nd} min of the actinic irradiation every 30 s and the mean value was estimated.

Maximal efficiency of PS2 photochemistry [$F_V/F_P = (F_P - F_0)/F_P$] was measured at room temperature with a fluorometer *PEA* (*Hansatech*, King's Lynn, UK) from the adaxial side of pre-darkened (15 min) leaves. F_0 was the minimal Chl fluorescence intensity and F_P was the fluorescence intensity at a P-level of the O-J-I-P transient (Strasser and Govindjee 1992). The excitation irradiance was 4 800 μmol(photon) m^{-2} s^{-1}, time of detection was 2 s. General statistical description (medians and quartiles) was used for the F_V/F_P ratio (Lazár and Nauš 1998).

Fresh mass (FM) and dry mass (DM) were determined from a set of 15 leaf blade segments cut 1 cm from apex and 1 cm from the leaf base. FM was determined immediately after detachment of the segments, DM was determined after oven-drying of the segments for 1 h at 105 °C. Both quantities were related to the leaf area (LA).

Lipid peroxidation in wheat leaves was determined from the content of thiobarbituric acid (TBA) reaction products according to a modified procedure of Dan *et al.* (1996): 0.5 g of segments from the middle part of leaf blades was homogenized in 5 cm^3 of 0.1 % trichloroacetic acid (TCA). A filtrated homogenate was centrifuged at 10 000×*g* for 10 min at 4 °C. A 1 cm^3 of supernatant was mixed with 4 cm^3 of 20 % TCA containing 0.5 % TBA. The mixture was heated in boiling water for 30 min and then quickly cooled in an ice-bath. After centrifugation at 10 000×*g* for 10 min at 4 °C, the absorbances of the supernatant at 532 nm (A_{532}) and 600 nm (A_{600}) were measured (*Unicam UV550, ThermoSpectronic*, Cambridge, UK) using 0.5 % TBA in 20 % TCA as a blank. A 1 cm cuvette was used. A difference of absorbances ($\Delta A = A_{532} - A_{600}$) reflecting the content of TBA reaction products was used as an indicator of lipid peroxidation (Dan *et al.* 1996).

Leaf homogenate was prepared from the leaf blade segments cut 1 cm from apex and 1 cm from the leaf base. Two grams of segments were placed in aluminium foil, immersed in liquid nitrogen (see also Jiang and Huang 2001) for 2 min, and then ground. The leaf powder was overlaid by 10 cm^3 of the extraction buffer (5 °C). The composition of extraction buffer was 0.1 M Tris (pH 7.8), 1 mM dithiothreitol, 1 mM $EDTANa_2$, 1 % (m/v) *Triton X-100*, 4 % (m/v) polyvinylpolypyrrolidone, and 5 mM ascorbic acid. The mixture was homogenized in an interrupted regime (*Ultra-Turrax T 25B, IKA–Labortechnik*, Germany) for 1 min (13 500 rotations per min). Then the homogenate was exposed for 1 min to ultrasound (*Transsonic T 460 H, Elma*, Germany). After 30 min incubation on ice the homogenate was centrifuged (21 000×*g* for 10 min at 4 °C). The supernatant was filtered through 4 layers of gauze and placed to Eppendorf cuvettes. All the steps were done at about 4 °C. The homogenate was then stored at –80 °C for subsequent enzymatic assays and TP content determination.

APX and GR activities: The APX activity was assayed according to Nakano and Asada (1981) by measuring the decrease in absorbance at 290 nm that reflects the ascorbate oxidation. The reaction mixture for measuring of the APX activity contained 95 mM HEPES-EDTA (pH 7.0), 0.5 mM Na-ascorbate, 0.88 mM H_2O_2, and 50 mm^3 of the leaf homogenate in 3 cm^3 of the mixture. The GR activity was assayed using a modified method of Foyer and Halliwell (1976) by measuring the decrease in absorbance at 340 nm that reflects the consumption of NADPH. The reaction mixture for measuring of the GR activity contained 91 mM Tris-EDTA (pH 7.8), 0.1 mM NADPH, 1.0 mM glutathione (oxidized form, GSSG), and 100 mm^3 of the leaf homogenate in 3 cm^3 of the mixture. The absorbance changes were measured by a spectrophotometer (*Unicam UV550, Thermospectronic*, Cambridge, UK) during the 2 min of the reaction. A 1 cm cuvette was used; the exact temperature 25 °C and sample stirring were insured by a special cuvette holder. The spectral slit-width was 1 nm.

Results

In order to describe the situation of the whole plant during senescence, a method of plant topography (see Šesták 1985, Šesták and Šiffel 1997, Matoušková *et al.* 1999) was used. The first three fully developed leaves of wheat plants were followed. The youngest developing leaves (4th and 5th) were not measured (see Table 1).

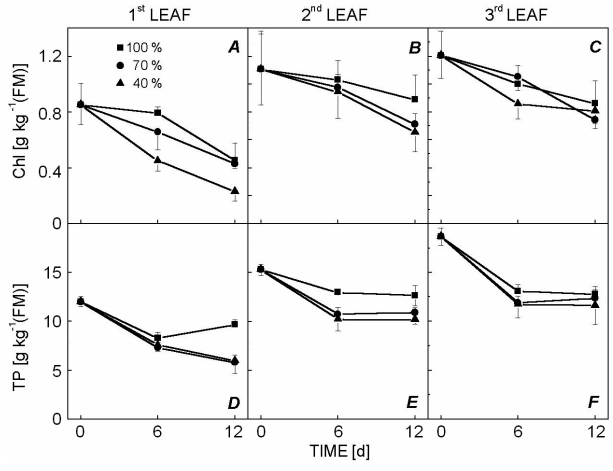

Fig. 1. Chlorophyll (Chl) (*A–C*) and total protein (TP) (*D–F*) contents related to the fresh mass (FM) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. Means and SD, $n = 6$.

Table 1. The growth phase, *i.e.* number of developed leaves (Feekes 1941) of wheat plants (*Triticum aestivum* L. cv. Saxana) grown under different irradiances. In brackets, the number of developed leaves is shown.

Time of experiment [d]	Growth phase 100 % PAR	70 % PAR	40 % PAR
0	1.3 (3)	1.3 (3)	1.3 (3)
6	1.4 (4)	1.4 (4)	1.3 (3)
12	1.5 (5)	1.4 (4)	1.4 (4)

A decrease in Chl and TP contents was observed in all the measured leaves during the experiment (Fig. 1). The lowest values were found in the 1st leaf. The plant shading stimulated the decrease in Chl and TP contents, especially in the 1st leaves (Fig. 1*A,D*). Similar trends were found in P_N (Fig. 2*A-C*) and maximal efficiency of PS2 photochemistry (F_V/F_P) (Fig. 2*D–F*). In all the measured leaves, a relative decrease in P_N with plant ageing was

higher than a relative decrease in F_V/F_P, which indicated a more pronounced senescence-induced inhibition of stromal reactions in comparison with PS2 photochemistry.

The FM (related to the LA) was mostly slightly higher in the 1st leaves in comparison with the 2nd and 3rd ones (Fig. 3*A–C*). This observation indicates that the plants were sufficiently supplied with water and did not relocate it from the oldest leaves to the younger ones. The DM (related to the LA) was usually slightly higher in the 3rd leaves compared with the older leaves (Fig. 3*D–F*). No pronounced changes (up to about 15 %) were found in the FM and DM during plant ageing or after plant shading.

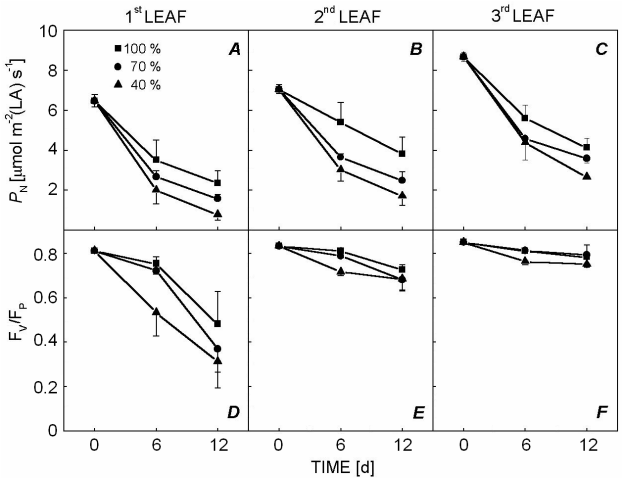

Fig. 2. The net photosynthetic rate (P_N) related to the leaf area (LA) (*A–C*) and the maximal efficiency of photosystem 2 photochemistry (F_V/F_P; *D–F*) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. For P_N, means and SD (n = 3), and for F_V/F_P, medians and quartiles (n = 7) are shown, respectively.

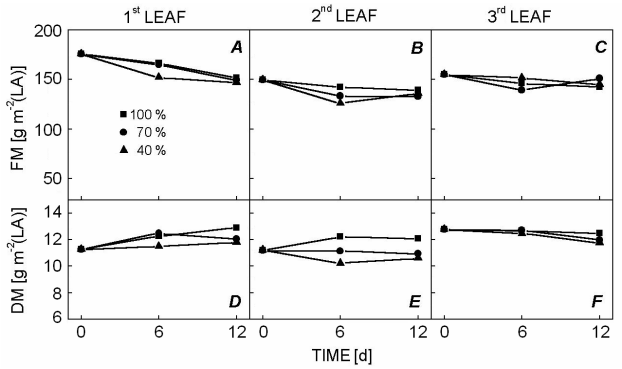

Fig. 3. The fresh mass (FM; *A–C*) and dry mass (DM; *D–F*) related to the leaf area (LA) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances.

The lipid peroxidation increased during ageing in all the measured leaves (Fig. 4). In the un-shaded plants, the highest relative increase (of about 160 %) was found in the 3rd leaves (Fig. 4*C*) whereas a relative increase found in the 1st leaves was smaller (of about 40 %) because of a relatively high lipid peroxidation at the beginning of experiment (Fig. 4*A*). Plant shading stimulated the increase in lipid peroxidation slightly in the 3rd leaves and considerably in the 1st leaves (Fig. 4*A*,*C*).

The APX activity related to FM was lower in the 1st leaves compared with the 2nd and 3rd ones during whole experiment (Fig. 5*A–C*). During plant ageing, the APX activity decreased slightly in all the measured leaves. In the 1st leaves, this decrease was stimulated by plant shading (Fig. 5*A*). The APX activity related to the TP content did not almost change in the 1st and 2nd leaves and

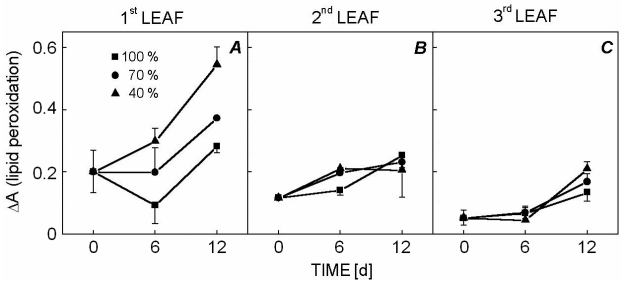

Fig. 4. Lipid peroxidation (expressed as the difference of absorbances $\Delta A = A_{532} - A_{600}$ reflecting the content of products of thiobarbituric acid reaction) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. Means and SD, n = 3.

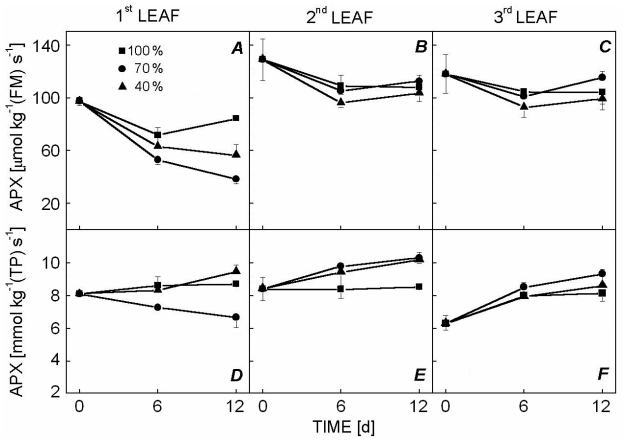

Fig. 5. The activity of ascorbate peroxidase (APX) related to the fresh mass (FM; *A–C*) and total protein content (TP; *D–F*) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. Means and SD, n = 3.

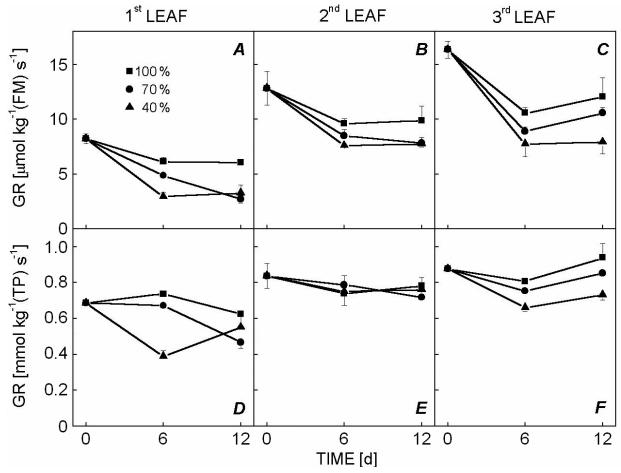

Fig. 6. The activity of glutathione reductase (GR) related to the fresh mass (FM; *A–C*) and total protein content (TP; *D–F*) in the first three leaves of wheat plants. Leaves are counted according to their emergence (from plant base to apex, 1st leaf = the primary leaf). The plants were grown in the greenhouse under normal (100 %) and reduced (70 and 40 %) irradiances. Means and SD, $n = 3$.

Discussion

The changes in parameters measured in the un-shaded wheat plants revealed that these plants were undergoing the senescence process during the experiment despite their relatively low age (37–49 d). Probably the plants suffered from the low irradiance and short day due to their growing in the autumn. During the 12 d of the experiment, the Chl and TP contents (Fig. 1), and P_N and maximal efficiency of PS2 photochemistry (Fig. 2) decreased while the rate of lipid peroxidation increased (Fig. 4) in all the measured leaves. The values of the mentioned parameters indicated that senescence of the 1st leaves had already started before the beginning of experiment and became more pronounced during it. The most pronounced senescence of the oldest leaves corresponds to the expected remobilization of nutrients from these leaves to the younger parts of the plant.

Senescence is accompanied by an increasing generation of ROS and consequent oxidative damage (Munné-Bosch and Allegre 2002, Jing *et al.* 2003). One of the reasons for this phenomenon is an increasing imbalance between generation and consumption of electrons in the photosynthetic electron transport chain caused by a preferential inhibition of stromal reactions compared with the PS2 photochemistry (Camp *et al.* 1982, Grover 1993, Špundová *et al.* 2003). The inhibition of stromal reactions leads to an increasing probability of the electron flow to molecular oxygen, consequently ROS accumulate and photo-damage to the chloroplast components increases. Most probably that was the situation in the un-shaded wheat plants—the relative decrease in P_N during plant ageing was much higher than the relative decrease in the PS2 photochemistry (F_V/F_P) (Fig. 2) and lipid peroxidation increased (Fig. 4).

Oxidative damage to the cellular components can be mitigated by operation of anti-oxidative enzymes. We investigated senescence-induced changes in the activities of APX and GR, the main enzymes of the ascorbate-glutathione cycle that is a part of the water-water cycle in chloroplasts (Asada 1999, 2000). In order to interpret changes in the APX and GR activities correctly, attention was paid to the choice of reference quantity because it can influence the nature of trends. The enzyme activities were related to FM, DM, and TP contents. The FM and DM changed minimally during senescence (Fig. 3) therefore they both could be taken as the proper reference quantities. The changes of APX and GR activities related to both the FM and DM were nearly the same so only the FM was chosen as the representative quantity (Figs. 5*A–C*, 6*A–C*). The APX and GR activities related to the TP content (Figs. 5*D,E*, 6*D,E*) should indicate their relative degradation in comparison with the rate of general degradation of proteins.

In the un-shaded plants, the activity of anti-oxidative system seemed not to be stimulated during senescence as the APX and GR activities related to the FM decreased with the increase in leaf age and lipid peroxidation. However, the APX activity related to the TP content barely changed with ageing in the 1st and 2nd leaves and even increased in the 3rd leaves (Fig. 5*D–F*). Hence the degradation of the APX might be slower than degradation of total proteins, especially in the 3rd leaves. The slower degradation of the APX could reflect a tendency to maintain the antioxidant protection of chloroplast components as about 90 % of the total leaf APX activity is in the increased in the 3rd leaves (by about 30 %) during ageing of the un-shaded plants. Shading of the plants stimulated this increase in both the 2nd and 3rd leaves (Fig. 5*E,F*). The smallest stimulation of increase or even decrease (in 40 % PAR) was found in the 1st leaves (Fig. 5*A*).

The GR activity related to the FM was again lowest in the 1st leaves (Fig. 6*A*) in comparison with the younger leaves and decreased with plant ageing in all the measured leaves (Fig. 6*A–C*). Shading of the plants stimulated this decrease. A relative decrease in activity related to the FM was more pronounced in the case of GR in comparison with APX, especially in the shaded plants (compare Figs. 5 and 6). The GR activity related to the TP content decreased only slightly in the 1st and 2nd leaves of un-shaded plants (Fig. 6*D,E*), in the 3rd ones even slightly increased (by about 6 %) (Fig. 6*F*). Plant shading stimulated the decrease in the 1st leaves and decreased the activity in the 3rd leaves (Fig. 6*D,F*).

chloroplasts (Gillham and Dodge 1986). The GR activity related to TP did not increase significantly with ageing, which indicates that the rate of GR degradation was probably similar to the rate of TP degradation but higher than the rate of APX degradation.

Shading of the plants intensified the senescence-induced decrease in Chl and TP contents, P_N, and maximal efficiency of PS2 photochemistry. This effect was most pronounced in the 1st leaves. A decrease in supply of excitations due to plant shading should slow down the photosynthetic electron generation and consequently suppress the photo-generation of ROS and oxidative damage. Contrary to this expectation, the rate of lipid peroxidation mostly increased in the shaded plants, especially in the 1st leaves (Fig. 4*A*). The high increase of lipid peroxidation in the 1st leaves might be caused by the more pronounced degradation of the APX and GR: their activities related to the FM (and to the TP content too in the case of GR) decreased faster (in most cases) than in the 1st leaves of unshaded plants. Similarly, the APX activity related to the FM decreased with decreasing irradiance in leaves of *Cucurbita pepo* and *Vinca major* (Logan *et al.* 1998) or pea (Gillham and Dodge 1987). Yamazaki and Kamimura (2002) also explained a decrease in the APX activity (related to the LA) within rice plants from the top to bottom leaves as a consequence of decreasing irradiance but the lower activity in the bottom leaves seemed to be caused simply by senescence as in our un-shaded plants.

On the other hand, the decrease in APX activity related to FM was not markedly stimulated in the 2nd and 3rd leaves of the shaded plants in comparison with the unshaded ones (Fig. 5*B*,*C*). Moreover, the increase of APX activity related to the TP content was intensified in these leaves (Fig. 5*E*,*F*). These results indicate a tendency in the younger leaves of shaded plants to slow down a senescence-induced decrease in the APX activity.

Summing up, the shaded plants accelerated senescence of the 1st leaves probably in order to maintain the functionality of the younger leaves. In the latter leaves we suppose a more pronounced tendency to maintain the antioxidant protection in chloroplasts. However, the "low-light" stress was probably too strong because the situation of the 2nd and 3rd leaves got worse too, as compared with the un-shaded plants. The main reason of the accelerated senescence of the oldest leaves was probably the pronouncedly increased oxidative damage due to weakening of anti-oxidative protection.

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Protective cytokinin action switches to damaging during senescence of detached wheat leaves in continuous light

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The effect of exogenous application of the cytokinin *meta*-topolin [mT; N^6-(*meta*-hydroxybenzyl)adenine] on artificial senescence of detached wheat leaves (*Triticum aestivum* L. cv. Hereward) was studied and compared in leaves senescing under continuous light (100 μmol photons m^{-2} s^{-1}) and darkness. Senescence-induced deterioration in structure and function of the photosynthetic apparatus was characterized by reduction in chlorophyll content, maximal efficiency of photosystem (PS) II photochemistry (F_v/F_m) and the rate of CO_2 assimilation, by increase in the excitation pressure on PSII ($1 - q_P$) and a level of lipid peroxidation and by modifications in chloroplast ultrastructure. While in darkened leaf segments mT effectively slowed senescence-induced changes in all measured parameters, in light-senescing segments the effect of mT changed into opposite a few days after detachment. We observed an overexcitation of photosynthetic apparatus, as indicated by pronounced increases in the excitation pressure on PSII and in a deepoxidation state of xanthophyll cycle pigments, marked starch grain accumulation in chloroplasts and stimulation of lipid peroxidation in light-senescing leaf segments in mT. Possible mechanisms of acceleration of senescence-accompanying decrease in photosynthetic function and increase in lipid peroxidation during mT influence are discussed. We propose that protective mT action in darkness becomes damaging during artificial senescence in continuous light due to overexcitation of photosynthetic apparatus resulting in oxidative damage.

Introduction

Plant senescence is an internally programmed degradation leading to death. Because chloroplasts appear to be the initial target of senescence and because chlorophyll (Chl) breakdown is so prominent, the loss of Chl and the associated yellowing of leaves are one of the markers that are commonly used as the indicators of plant senescence (Noodén et al. 1997). It is known that exogenous application of cytokinins usually slows senescence-induced changes, including the decrease in Chl content (e.g. Selivankina et al. 2001), plastoglobuli formation (Hudák et al. 1996, Paramonova et al. 2002, Zavaleta-Mancera et al. 1999), an increase in

Abbreviations – A, rate of CO_2 assimilation; Chl, chlorophyll; DEPS, deepoxidation state of the xanthophyll cycle pigment pool; mT, *meta*-topolin; PSII, photosystem II; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TBARPs, thiobarbituric acid reactive products; VAZ, overall content of xanthophylls.

membrane leakiness (Kraus et al. 1993, Liu and Huang 2002) and decrease in photosynthetic parameters such as the rate of CO_2 assimilation (A) (Čatský et al. 1996, Gan and Amasino 1995, Rulcová and Pospíšilová 2001), the photochemical Chl fluorescence quenching (q_P) or the maximal photochemical efficiency of PSII (F_v/F_m) (for review see Synková et al. 1997).

Cytokinins have also been reported to slow a senescence-accompanying increase in lipid peroxidation [(determined as the level of thiobarbituric acid reactive products (TBARP)] (Huang et al. 1997, Hung and Kao 1998, Liu and Huang 2002, Thomas et al. 2005, Todorov et al. 1998). The probable mechanism through which cytokinins retard lipid peroxidation is their influence on the activity of antioxidative system enzymes that were induced (or retarded its decrease) by cytokinins (e.g. Cag et al. 2003, Dertinger et al. 2003, Dhindsa et al. 1982, Durmuş and Kadioğlu 1998, Liu and Huang 2002). Cytokinins also increase the synthesis of carotenoids, which are known to protect the reaction centres from the detrimental effects of light and oxygen too (Chernyad'ev 2000).

Although the effects of cytokinins on senescence processes have been widely described, the molecular mechanisms of this action are poorly understood. Furthermore, contrary to the generally observed retardation of senescence, in some cases, the application of cytokinins causes an acceleration of senescence (Carimi et al. 2003, 2004, Zacarias and Reid 1990), with exogenous applications of high cytokinin concentrations having an opposite effect to the lower concentration (Genkov et al. 1997, Pospíšilová et al. 1993, 2001, Rulcová and Pospíšilová 2001). The effect of cytokinin treatment on senescence can also depend on light conditions. Zacarias and Reid (1990) observed different effects of exogenous cytokinins on senescent leaf disks excised from *Arabidopsis thaliana* leaves kept under continuous light or dark conditions. Benzyladenine (10 μ*M* and higher concentration) retarded the decrease in Chl content in dark but accelerated it in light. This opposite effect of high cytokinin concentrations in comparison with lower ones is always observed under continuous light or on day/night cycle, suggesting that light plays an important role in the cytokinin action. The unusual action of cytokinins in stimulating senescence is not yet understood.

There is also evidence of an additive effect of continuous light and cytokinin treatment retarding the decrease in Chl and soluble protein contents (10 μ*M* benzyladenine, Klerk et al. 1993; 45 μ*M* benzyladenine, Weidhase et al. 1987). Therefore, it would be interesting to know more about the action of cytokinins in this respect and to prove or disprove a common action of both, cytokinins and light, in senescence. In the literature, little attention has been given to the effect of light conditions on cytokinin action during senescence. As far as we know, cytokinin effects on plants or leaves senescing under different light conditions have not yet been studied in connection with changes in photosynthetic apparatus and processes.

As detached leaves of wheat are well-stated biological system often used for senescence bioassays of cytokinin activity (e.g. Holub et al. 1998, Kamínek et al. 1987, Strnad et al. 1994, Tarkowská et al. 2003), we have investigated an influence of a cytokinin – *meta*-topolin (mT) [N^6-(*meta*-hydroxybenzyl)adenine] – on the photosynthetic apparatus in detached wheat leaves senescing under continuous light or dark treatments. mT is a recently identified, naturally occurring, aromatic cytokinin (Strnad et al. 1997). It is a derivative of benzyladenine, whose cytokinin activity is relatively well documented. However, the biological activity of mT is higher than that of benzyladenine (even higher or comparable with the most active isoprenoid cytokinin, zeatin) in senescence bioassays (Cag et al., 2003, Holub et al. 1998). Also, our preliminary results proved its highest activity to slow down the senescence in detached wheat leaves; therefore, we choose mT for this study. The aim of our work was a more comprehensive study, which could contribute to understanding of mechanisms of cytokinin action.

Materials and methods

Plant material and induction of leaf senescence

Seeds of winter wheat (*Triticum aestivum* L. cv. Hereward) were sown in perlite supplied with Knop solution and placed into a growth chamber maintained at $22 \pm 2^\circ$ C under 16/8-h day/night cycle (100 μmol photons m^{-2} s^{-1}) and relative humidity 50–55%. After 8 days, in the growth phase of the second leaf [1.2 according to Feekes (1941)], the mature primary leaves were cut off 5 cm below the leaf tips to induce artificial senescence. The leaf segments were erected into vertical position with basal ends put into 100 μ*M* solution of cytokinin mT dissolved in 0.5% dimethylsulfoxide or into a pure 0.5% dimethylsulfoxide solution in distilled water. The latter leaf segments were used as control variants, because no significant differences were found between these segments and segments put into distilled water (in all measured parameters). mT [N^6-(*meta*-hydroxybenzyl)adenine] was prepared by a method described in Holub et al. (1998). Half of the leaf segments were kept in continuous light (100 μmol photons m^{-2} s^{-1}); the second half were

kept in darkness for next 6 days. The other conditions were the same as during plant growth. All measurements were performed in the middle region of the leaf segments after 1-h adaptation to darkness.

Pigment analyses

For pigment analyses, 3-cm-long middle parts of leaf segments were immediately frozen in liquid nitrogen. The frozen leaf segments were homogenized in 80% acetone with a small amount of $MgCO_3$, and the homogenate was centrifuged at 3600 *g* for 5 min. The supernatant was used for spectrophotometric estimation (Unicam UV 500, Thermo Spectronic, Cambridge, UK) of Chl *a* and *b* content per unit leaf area according to Lichtenthaler (1987). Filtered supernatant (filtration through a 45-μm filter) was also used for determination of the amount of xanthophyll cycle pigments. Their separation was performed by gradient reversed-phase HPLC (Alliance 2695 Separations Module, Waters, Milford, MA) using reversed-phase column 250/4 RP 18 (LiChroCART, Darmstadt, Germany) kept at 25° C and PDA detector (2996 Waters). Elution with a solvent system which consisted of acetonitrile, methanol and 0.1 *M* Tris (pH 8.0) in a ratio of volumes 87:10:3 (12 min at flow rate 1.2 ml min^{-1}) was followed by 3-min linear gradient (flow rate 1 ml min^{-1}) to second solvent which was a 34:16 mixture (v/v) of methanol and ethyl acetate (12 min, flow rate 1 ml min^{-1}). Absorbance of eluted pigments was detected at 440 nm. Factors converting the measured area below the absorption peak to the relative pigment content were used according to Färber et al. (1997). The deepoxidation state of the xanthophyll cycle pigment pool was calculated as DEPS = (0.5 A + Z)/(A + V + Z) and overall content of xanthophylls as overall content of xanthophylls = V + A + Z (where A is antheraxanthin, V is violaxanthin and Z is zeaxanthin content per unit leaf area) (Demmig-Adams and Adams 1996).

Chl fluorescence

Chl fluorescence was measured at a room temperature using a modulation fluorometer PAM 2000 (Walz, Effeltrich, Germany) from the adaxial side of the leaf segments. The minimal fluorescence intensity F_o in a dark-adapted state was measured by analytic modulated light (0.2 μmol photons m^{-2} s^{-1}); the maximal fluorescence intensities in the dark-adapted state (F_m) and after 14 min of the adaptation to white actinic light (F_m') were measured by 0.8-s saturating pulses (8000 μmol photons m^{-2} s^{-1}). After the F_m' measurement, the actinic light (400 μmol photons m^{-2} s^{-1}) was switched off, and the far-red light was applied for 3 s in order to measure the minimal fluorescence intensity in the light-adapted state (F_o'). The excitation pressure on PSII (Demmig-Adams 1990) was calculated from a coefficient of photochemical Chl fluorescence quenching q_P as $(1 - q_P) = (F_s - F_o')/(F_m' - F_o')$ (van Kooten and Snel 1990) where F_s was steady-state fluorescence intensity measured after 14-min adaptation to actinic light. The maximal photochemical efficiency of PSII photochemistry was evaluated as $F_v/F_m = (F_m - F_o)/F_m$ (Kitajima and Butler 1975). A general statistical description (medians and quartiles) was used in the case of the Chl fluorescence parameters (Lazár and Nauš 1998).

Gas exchange

Leaf gas exchange was measured using an open gasometric system LCA-4 (ADC, Hoddesdon, UK). 2.5-cm-long middle parts of four leaf segments were placed into a leaf chamber (CO_2 concentration 350 μmol mol^{-1}, temperature 23.1 ± 0.5° C and partial water vapour pressure 1.0 ± 0.2 kPa) for the last 15 min of the 1-h adaptation to darkness and then the white actinic light (435 μmol photons m^{-2} s^{-1}) was switched on. After about 15 min of the light adaptation, the rate of CO_2 assimilation (A) intercellular CO_2 concentration (C_i) and stomatal conductance to water vapour (g_s) were estimated according to von Caemmerer and Farquhar (1981).

Microscopy

Samples for light and electron microscopy were taken at the 4th hour of photoperiod in the case of attached leaves; the detached segments were taken directly from the senescing conditions (without dark adaptation). As in the other methods, all microscopy measurements were performed on the middle region of leaf segments. We cut down the edges of the leaves and cut pieces (approximately 1 mm^2) from leaf tissue 2.5 cm below the leaf tips. Six tissue pieces were immediately fixed in 2.5% glutaraldehyd in 0.1 *M* phosphate buffer (pH 7.2) for 12 h (6° C). After three washes, the tissue pieces were postfixed in 1% osmium tetroxide in phosphate buffer (0.1 *M*, pH 7.2), dehydrated through a graded series of acetone, transferred to propylene oxide and embedded in Durcupan ACM (Fluka, Buchs, Switzerland) resin. For light microscopy, 0.5–1-μm sections were stained with toluidine blue and photographed (immersion objective magnification of 100). For transmission electron microscopy, ultrathin sections were prepared from the same embedded material, stained with Reynold's lead citrate and examined and photographed under Zeiss Opton EM 109 electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 50 kV. Areas

of chloroplasts and their components were counted with computer analysis program ACC 5.0 (SOFO, Brno, Czech Republic).

Lipid peroxidation

The level of lipid peroxidation in the leaf segments was determined from the content of TBARPs according to a modified procedure of Dan et al. (1996). 0.5 g of the segments was frozen in liquid nitrogen, homogenized in 5 ml of 0.1% trichloracetic acid (TCA) and the homogenate was centrifuged at 10 000 *g* for 10 min at 4° C. A 1-ml aliquot of supernatant was mixed with 4 ml of 20% TCA containing 0.5% TBA (v/v). The mixture was heated in boiling water for 30 min and then quickly cooled in an ice bath. The absorbance of the mixture was measured against 0.5% TBA in 20% TCA as a blank using an UV-visible spectrophotometer (Unicam UV 500, Thermo Spectronic, Cambridge, UK). A difference of absorbancies at 532 and 600 nm reflecting the level of TBARPs was used as an indicator of the level of lipid peroxidation.

Results

Artificial senescence of detached primary wheat leaves kept under both the continuous light and dark conditions was characterized by rapid degradation of Chls (Fig. 1). mT slowed down the Chl degradation in both the dark- and light-senescing leaf segments; however, its effect was more pronounced under the dark treatment.

Four days after the leaf detachment, the influence of mT on changes in chloroplast shape, arrangement and ultrastructure were evaluated. In the control dark-senescing segments, the chloroplast shape changed from ellipsoidal to pronouncedly more spherical and plastids assembled into huddles (Fig. 2C). Marked disorganization of thylakoid membranes together with accumulation of plastoglobuli were observed too (Fig. 2D, Table 1). mT in darkness strongly retarded these senescence-induced alterations. Chloroplasts

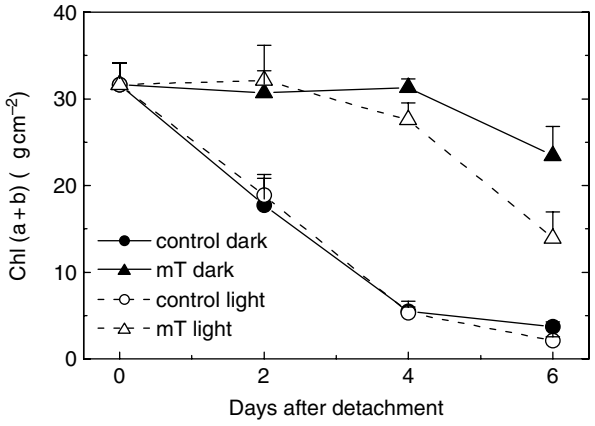

Fig. 1. Changes in chlorophyll (Chl) (*a* + *b*) content during artificial senescence of control (circles) and meta-topolin (mT)-affected (triangles) wheat leaf segments kept in darkness (closed symbols) or continuous light (open symbols). Data represent means + SD of five measurements.

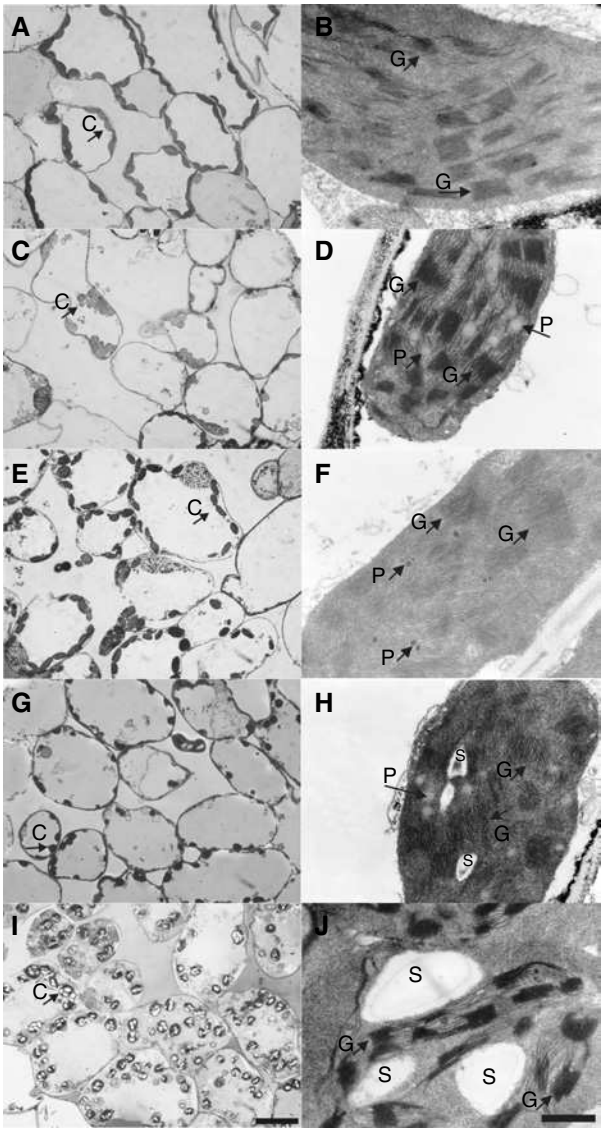

Fig. 2. Light microscopy (left column) and transmission electron microscopy (right column) images of mesophyll cells and chloroplasts in mature wheat leaves (A, B) and senescing leaf segments on the 4th day after detachment: control solution, darkness (C, D); meta-topolin (mT) solution, darkness (E, F); control solution, continuous light (G, H) and mT solutions, continuous light (I, J). C, chloroplast; G, granum; P, plastoglobule; S, starch grain. Scale bars shown in the lowest photographs are equivalent to 20 μm and 0.5 μm for all LM and TEM images, respectively.

Table 1. Structural characteristics of chloroplasts from primary wheat leaves before detachment (Mature) and from leaf segments senescing in continuous light (light) or darkness (dark) at the 4th day after detachment and treatment of meta-topolin (mT) or control (Control) solution. Grana, plastoglobuli and starch contents were calculated as percentage of total chloroplast area. The values represent medians (upper; lower quartiles) from 50 chloroplasts. n.d., not detected.

	Grana (%)	Plastoglobuli (%)	Starch grains (%)
Mature	18 (14; 22)	n.d.	n.d.
Control dark	13 (10; 21)	3.2 (1.7; 4.1)	n.d.
mT dark	21 (18; 25)	0.4 (0.3; 0.5)	n.d.
Control light	17 (14; 20)	1.4 (1.0; 2.0)	0.0 (0.0; 0.8)
mT light	12 (10; 14)	n.d.	15.0 (9.2; 20.0)

remained ellipsoidal and were still located at cell edges (Fig. 2E); the accumulation of plastoglobuli was suppressed and the relative grana content was higher in comparison with the control dark-senescing segments (Fig. 2F, Table 1).

In the light-senescing segments, the chloroplast shape and arrangement were not changed so much as in the darkness in the case of control segments (Fig. 2G,H). However, mT promoted modifications of the chloroplast shape to round and of the chloroplast layout to a more distant from cell edges (Fig. 2I,J). Hence, although these changes, which are usually observed during senescence (e.g. Schmidt 1988), were effectively slowed down by mT in darkness, in light there was observed a rather opposite effect. By contrast, mT retarded the plastoglobuli accumulation in both the light- and dark-senescing segments (Table 1). This observation indicates that mT markedly slowed down an overall degradation of chloroplast components, as the plastoglobuli are thought to contain a material from disintegrated thylakoid membranes (Burke et al. 1984, Lichtenthaler 1969).

During senescence of the leaf segments in light, starch inclusions occurred in chloroplasts (Table 1), which is a sign of assimilate accumulation. The starch accumulation might be associated with the detachment of leaf segment from the rest of plant preventing the transfer of assimilates to other plant parts and/or with the continuous light exposure without a dark period (Špundová et al. 2003). The starch accumulation in the light-senescing segments was very markedly stimulated by mT (Fig. 2I,J; Table 1).

A decrease in the rate of CO_2 *A* was observed in both the dark- and light-senescing control leaf segments (Fig. 3A). The *A* decrease was accompanied by a pronounced decline in the g_s (Fig. 3B). The g_s decline (and consequently a lower CO_2 exchange between intercellular spaces and external air) was probably not the primary cause of the decrease in *A*, however, because the intercellular concentration of CO_2 (C_i) was even

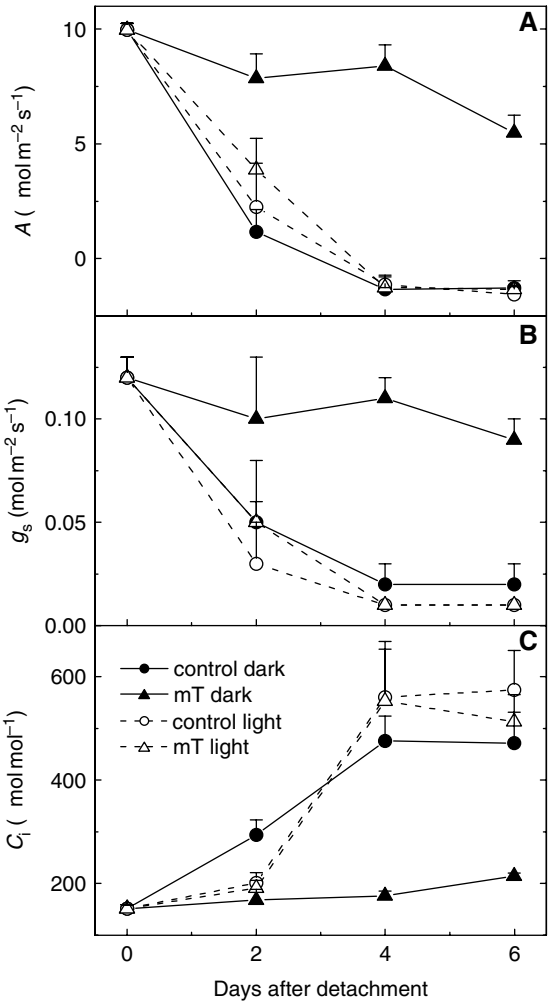

Fig. 3. Changes in the rate of CO_2 assimilation (*A*, A), stomatal conductance (g_s, B) and intercellular CO_2 concentration (C_i, C) during artificial senescence of control (circles) and meta-topolin (mT)-affected (triangles) wheat leaf segments kept in darkness (closed symbols) or continuous light (open symbols). Data represent means + SD of three measurements.

increased (Fig. 3C). This observation might indicate an inhibition of CO_2-consuming processes in mesophyll tissue. From the 4th day after detachment, CO_2-releasing processes prevailed as *A* was below the zero value. The inhibition of *A* in the control segments was caused not only by the decrease in Chl content (see Fig. 1), because a similar *A* decrease was observed when the *A* was estimated per Chl content (data not shown).

mT applied on the darkened leaf segments suppressed the senescence-induced changes in *A*, g_s and C_i effectively, but its application on the light-senescing segments had no significant effect on changes in any of these parameters (Fig. 3). The *A* decreased in the light-senescing segments in mT similarly as in the control segments, despite the more than five times higher Chl content on the 4th and 6th day after detachment (Fig. 1). In the case of light-senescing segments in mT, the *A* inhibition could be associated with the mentioned pronounced accumulation of starch in their chloroplasts (Fig. 2I,J; Table 1). It is known that the accumulation of assimilates in chloroplasts can lead to a feedback inhibition of photosynthesis (Friedrich and Huffaker 1980), which is connected with down-regulation of photosynthetic gene expression (Krapp et al. 1993, Krapp and Stitt 1995, Smeekens 1998).

The senescence-induced decrease in *A* was accompanied by an increase in the excitation pressure on PSII ($1 - q_P$) (Fig. 4A,B). The increase in $1 - q_P$ was also slowed down by mT only in the dark-senescing segments. Under light, the mT treatment even stimulated the $1 - q_P$ increase in comparison with the control segments, except the 2nd day after detachment (Fig. 4B). On the 6th day after detachment, the PSII function was inhibited so much that no fluorescence signal was detected in the light-senescing segments in mT. The accelerated senescence-induced changes in $1 - q_P$ in the latter segments could be explained by the above mentioned feedback inhibition of stromal reactions. Such inhibition can enhance the excitation pressure on PSII due to decreased amount of $NADP^+$, a final acceptor of electrons from photosynthetic electron transport chain.

An opposite effect of mT during the artificial senescence under the light and dark conditions was observed in the maximal photochemical efficiency of PSII (F_v/F_m) too (Fig. 4C,D). In the darkness, mT maintained the F_v/F_m values almost unchanged even on the 6th day after detachment in comparison with the mature leaves, whereas in the control, the F_v/F_m values decreased markedly. In the light, the maximal photochemical efficiency of PSII was slightly (but statistically significantly) higher in segments in mT (0.816 ± 0.003) than in the control ones (0.790 ± 0.003) on the 2nd day, only then it declined rapidly. The acceleration of the F_v/F_m decrease by mT in the light-senescing segments was less pronounced than the changes in $1 - q_P$ parameter (compare relative differences in F_v/F_m and $1 - q_P$ values measured on the 4th day after detachment, Fig. 4B,D), which reflects lesser sensitivity of PSII photochemistry to the assumed feedback inhibition of stromal reactions.

The maximal fluorescence intensity (F_m) decreased similarly after detachment in all measured variants except the dark-senescing segments in mT (Fig. 4E,F), although, causes for this decrease were probably different. In the control segments (both the light and dark senescing), the major cause for the F_m decrease was probably the fast decline in Chl content (see Fig. 1). This explanation could not be used for the F_m decrease in the light-senescing segments in mT, however, because of their relatively high Chl content. We suppose that F_m was decreased by a pronounced stimulation of zeaxanthin-mediated quenching processes in these segments, because from the 4th day after detachment, the xanthophyll cycle pigment pool was markedly deepoxidated even after 1-h dark adaptation (Fig. 5A) (note that F_m was measured after 1-h adaptation to darkness). The overall content of xanthophylls was also increased by mT (Fig. 5B). Interestingly, the very high DEPS found in the light-senescing segments in mT correlates with the high excitation pressure on PSII ($1 - q_P$) reflecting over-reduction of photosynthetic electron transport chain.

The xanthophyll cycle is one of mechanisms protecting plants against overexcitation (e.g. Demmig-Adams 1990, Young 1991) that can lead to an excessive accumulation of reactive oxygen species and consequently to photo-oxidative damage to the photosynthetic apparatus. Nevertheless, even the very high DEPS (about 0.7–0.8 after 1 h of dark adaptation) did not prevent lipid peroxidation in the light-senescing segments in mT, as a high concentration of TBARPs was indicated by the very high difference between absorbancies at 532 and 600 nm (Fig. 5C). In a case of this parameter, mT had again the opposite effect in the light- and dark-senescing leaf segments. mT significantly decreased the level of lipid peroxidation in the dark-senescing segments, whereas markedly stimulated its increase in the light-senescing ones.

Discussion

It is well known that exogenous application of cytokinins usually retards senescence-induced processes; in some cases, however, the opposite effect was observed. From our results, we assume that the cytokinin influence on artificial senescence strongly depends on light

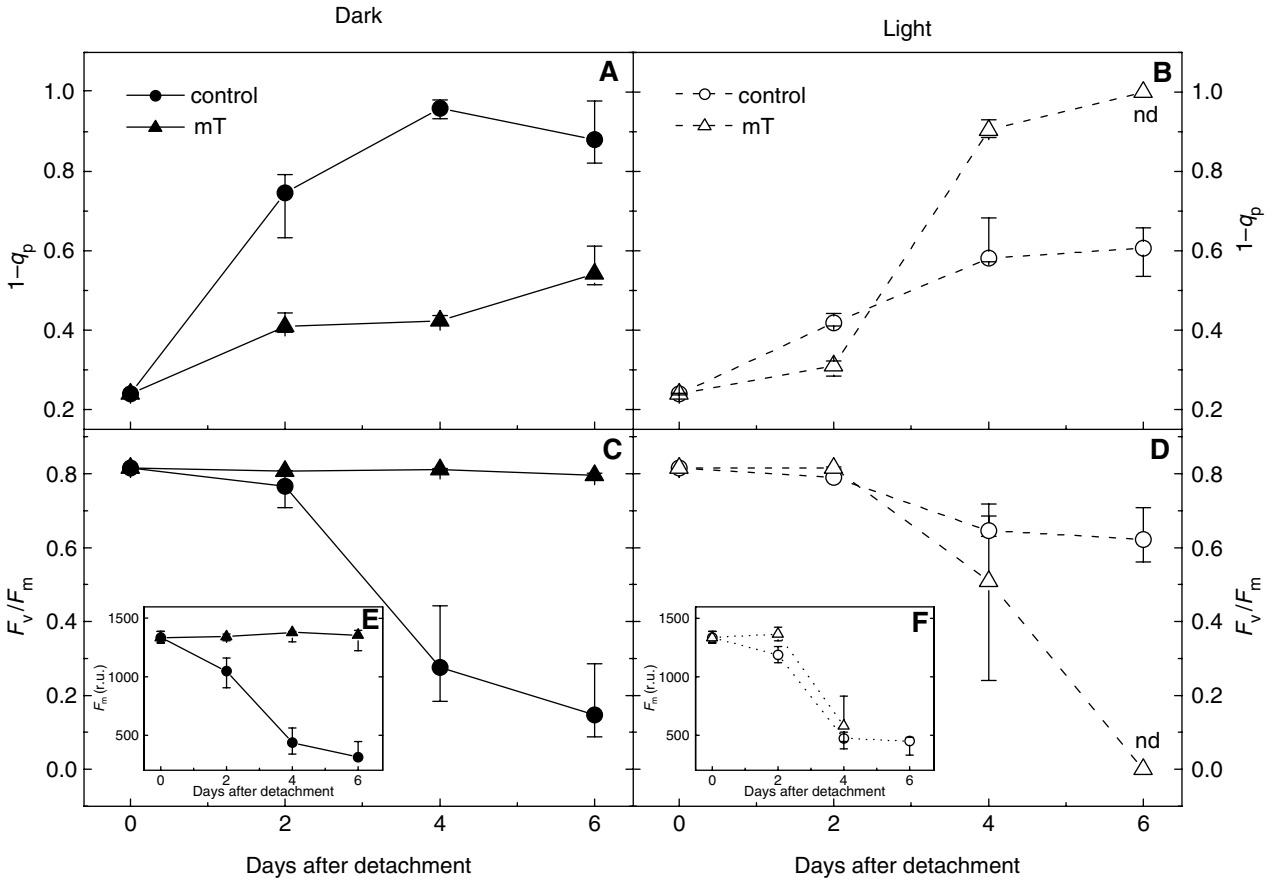

Fig. 4. Changes in parameters of chlorophyll (Chl) *a* fluorescence $1 - q_P$ (A, B), F_v/F_m *ratio* (C, D) and F_m (E, F) during artificial senescence of control (circles) and meta-topolin (mT)-affected (triangles) wheat leaf segments kept in darkness (closed symbols) or continuous light (open symbols). On the 6th day after detachment, Chl fluorescence was not detectable in the light-senescing segments in mT; therefore, only assumed extreme values are shown (points labelled as nd). Data represent medians and quartiles of six measurements.

conditions. Exogenous application of mT had a protective effect on detached wheat leaves senescing in darkness while under continuous light the protective action of mT switched to damaging in a few days after the detachment.

Exogenously applied mT markedly retarded progression of artificial senescence in darkened detached primary wheat leaves. It slowed down the senescence-accompanying changes of structural parameters (Chl degradation, plastoglobuli formation, chloroplast shape and position and lipid peroxidation) as well as parameters of photosynthetic function (A, $1 - q_P$, F_v/F_m). Accordingly, our results of mT application on leaf segments senescing in darkness are in agreement with cytokinin effects usually described in literature (e.g. Huang et al. 1997, Hung and Kao 1998, Kraus et al. 1993, Li et al. 2000, Weidhase et al. 1987).

Under light conditions, mT induced similar improvement of photosynthetic function in comparison with control segments in the first stage of artificial senescence (2 days after leaf detachment) only. On the 2nd day after leaf detachment, the lower DEPS, lower excitation pressure on PSII ($1 - q_P$) and slightly, but statistically significantly, higher maximal photochemical efficiency of PSII (F_v/F_m) were found in the light-senescing segments in mT in comparison with control, which indicated a better-maintained PSII photochemistry and linear electron transport. Also A was slightly higher in leaf segments in mT than in control leaf segments kept in the light (Fig. 3A).

One of mechanisms how exogenously applied cytokinins preserve photosynthetic activity is their effect on a key enzyme of carbon *meta*bolism – ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); it is known that cytokinins are able to slow down the

decrease in content and activity of Rubisco (e.g. Abdelghani et al. 1991, Chernyad'ev and Monakhova 2003, Weidhase et al. 1987) and stimulate de novo Rubisco synthesis (Kusnetsov et al. 1994, Lerbs et al. 1984). This might be also our case as we have observed higher *A* in mT-affected leaves compared with controls under both the light and dark conditions, however, under light conditions only on the second day after detachment.

The assumed higher Rubisco activity together with probable stimulation of invertase activity (which is also known to be promoted by cytokinin application; for review see Roitsch and Ehness 2000) could be the ways through which mT induced so strong starch grains formation in chloroplasts of the light-senescing segments (Fig. 2I,J; Table 1). Cytokinins have already been reported to promote the storage of assimilates in the form of starch within chloroplast by for example Paramonova et al. (2002), Wilhelmová and Kutík (1995) and Zavaleta-Mancera et al. (1999).

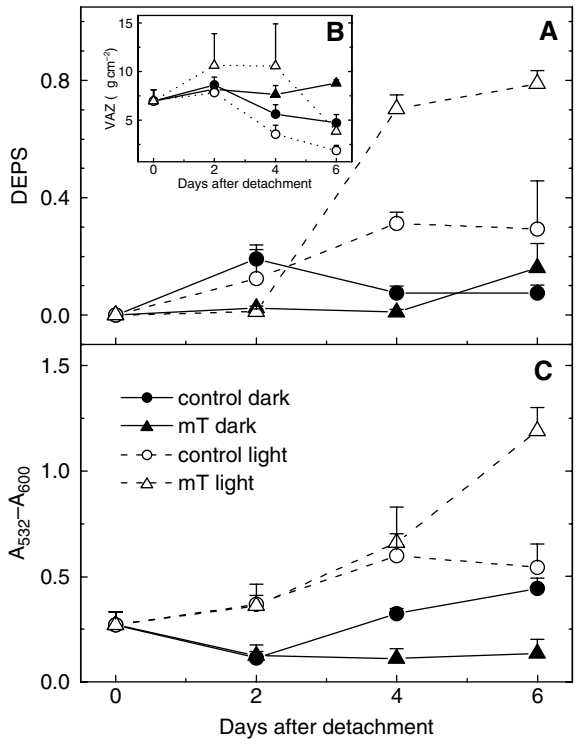

Fig. 5. Changes in the deepoxidation state of the xanthophylls expressed as deepoxidation state of the xanthophyll cycle pigment pool (DEPS) = (0.5 A + Z)/(A + V + Z) (A), overall content of xanthophylls overall content of xanthophylls (VAZ) = V + A + Z per unit leaf area (B) and content of thiobarbituric acid reactive products calculated as a difference of absorbancies at 532 and 600 nm (C) during artificial senescence of control (circles) and meta-topolin (mT)-affected (triangles) wheat leaf segments kept in darkness (closed symbols) or continuous light (measured after 1-h dark adaptation; open symbols). Data represent means + SD of three measurements.

It seems that in our case mT induced assimilate production and accumulation in the detached leaves initially; however, this accumulation led paradoxically to the deterioration of photosynthetic function via feedback inhibition in later stages of artificial senescence (the 4th and 6th days after detachment). Note that although *A* was inhibited in control and mT-treated light-senescing segments to a similar extent on the 4th and 6th days (Fig. 3A), in the control segments the Chl content was only about 20% in comparison with the mT-treated ones (Fig. 1). The feedback inhibition of stromal reactions probably caused limited availability of $NADP^+$, a final electron acceptor in the photosynthetic electron transport, as we assume from an increase in the excitation pressure on PSII ($1 - q_P$). By contrast, the PSII photochemistry was still effective on the 4th day after detachment (F_v/F_m was about 0.5, Fig. 4D). Hence, an imbalance between the generation and the demand for electrons might have occurred; this probably led to PSII overexcitation (as indicated by the increased $1 - q_P$ and DEPS) and supposedly to production of reactive oxygen species. Although on the 4th day after detachment there was still no observable enhancement of lipid peroxidation in comparison with the control light-senescing segments, it was clearly detectable 2 days later (Fig. 5C). This delay of the onset of oxidative damage was probably caused by the mentioned DEPS enhancement (Fig. 5A), supported by increased overall content of xanthophylls (Fig. 5B), which should suppress the overexcitation of photosynthetic apparatus and its consequences. Cytokinin activation of antioxidative system could also take part in the delay of oxidative damage onset as cytokinins have been reported to induce the activity of some antioxidative system enzymes (e.g. Cag et al. 2003, Dertinger et al. 2003, Dhindsa et al. 1982, Durmuş and Kadioğlu 1998, Liu and Huang 2002). The lesser degradation of chloroplast components on the 4th day was also reflected by inhibited formation of plastoglobuli (Fig. 2J, Table 1). However, on the 6th day after detachment, the photooxidative damage in light-senescing leaves in mT probably outweighed the protective mechanisms, and the lipid peroxidation increased much more than in control light-senescing segments.

Based on our results, we conclude that mT promoted function of photosynthetic apparatus during artificial senescence (in the dark; in continuous light until the 2nd day after leaf detachment); however, its simultaneous incidence with light treatment led to an inordinate accumulation of assimilates and consequently to oxidative damage. Even the enhanced deepoxidation of

xanthophyll cycle pigments was not able to avert photooxidative damage in the end.

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ORIGINAL ARTICLE

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

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Abstract

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

KEYWORDS

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

1 | INTRODUCTION

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

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

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

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

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

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

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

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

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

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

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

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

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

2.2 | Chl content assay

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

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

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

2.3 | Chl fluorescence measurement

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

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

2.4 | Determination of malondialdehyde content

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

2.5 | Identification and quantification of endogenous CKs

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

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

2.6 | Correlation and statistical analyses

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

3 | RESULTS

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

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

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

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

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

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

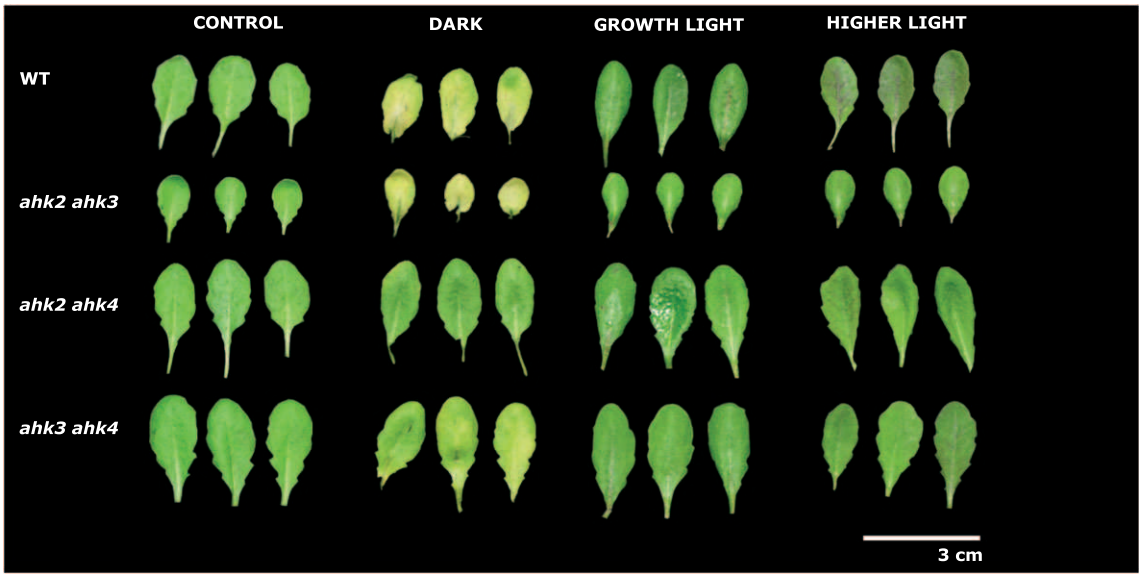

FIGURE 1 Phenotype of leaves of wild-type (WT) and receptor mutants immediately after the detachment (control) and after 6 days under dark, growth light (120 µmol photons m^{-2} s^{-1}), or higher light (400 µmol photons m^{-2} s^{-1}) conditions

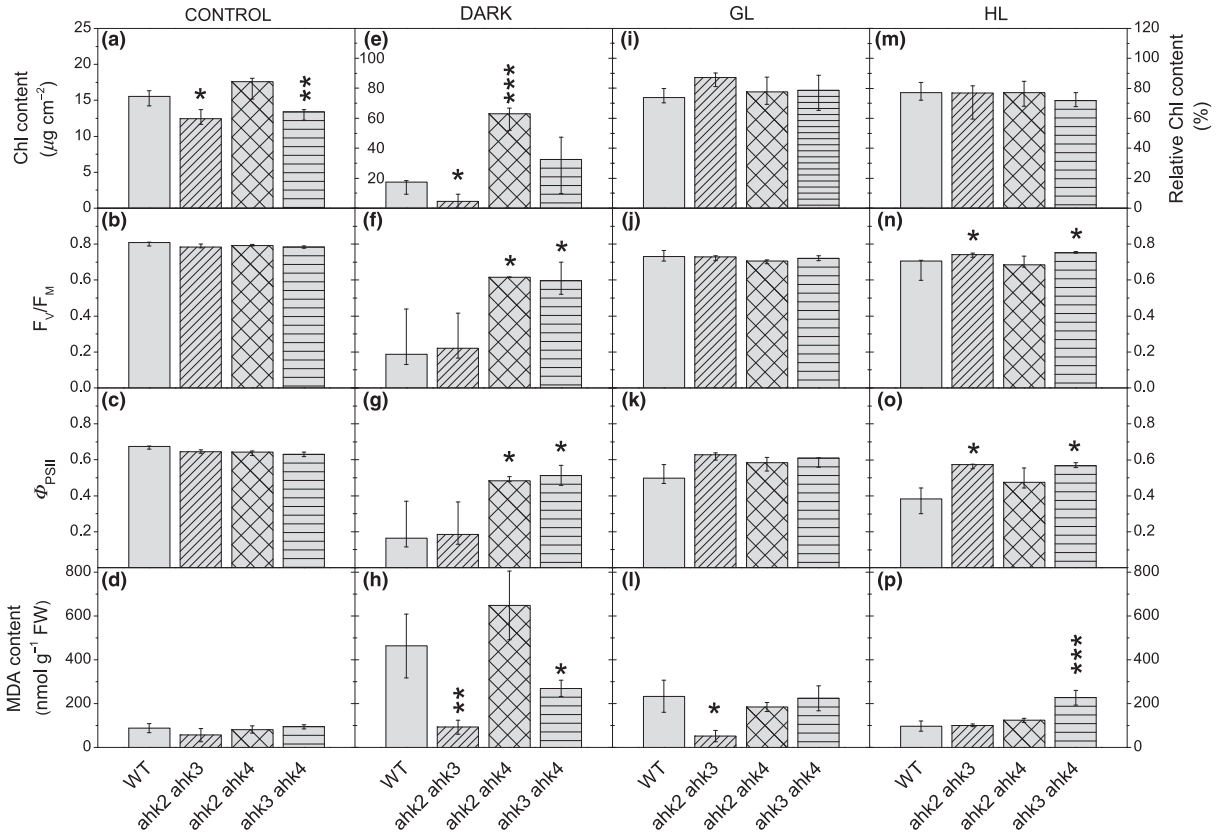

FIGURE 2 Chlorophyll (Chl) content, F_V/F_M (the maximal quantum yield of photosystem II photochemistry in dark-adapted state), Φ_{PSII} (the maximal quantum yield of photosystem II photochemistry in light-adapted state), and malondialdehyde (MDA–DNPH adduct) content estimated by high-performance liquid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

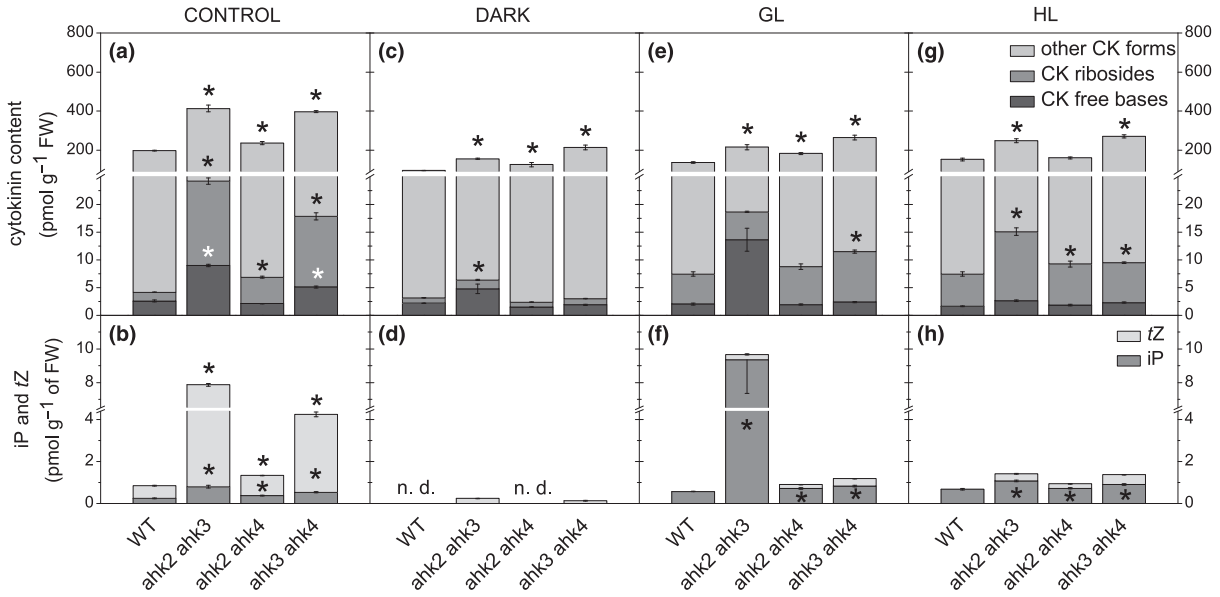

FIGURE 3 Endogenous content of total cytokinin (CK) free bases (iP, *t*Z, *c*Z, and *m*T; black), cytokinin ribosides (iPR, *t*ZR, *c*ZR, DHZR and *m*TR; dark grey), and other cytokinin forms (iP7G, iP9G, iPR5′MP, *t*ZOG, *t*ZROG, *t*Z7G, *t*Z9G, *t*ZR5′MP, *c*ZOG, *c*ZROG, *c*Z7G, *c*Z9G, *c*ZR5′MP,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

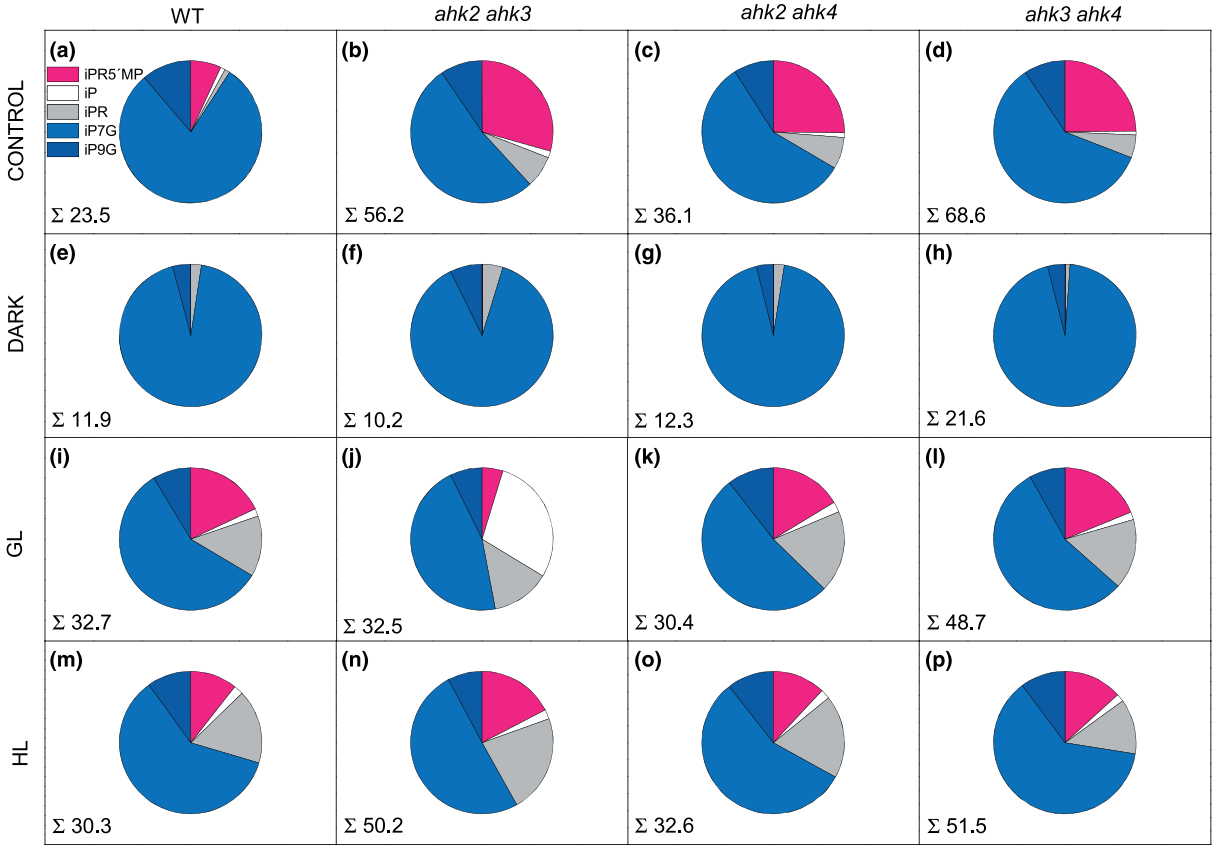

FIGURE 4 The overall content of iP forms (Σ; pmol/g of fresh weight) and relative amount of particular iP forms (iPR5′MP, N^6-(Δ^2-isopentenyl) adenosine 5′-monophosphate; iP, N^6-(Δ^2-isopentenyl)adenine; iPR, N^6-(Δ^2-isopentenyl)adenosine; iP7G, N^6-(Δ^2-isopentenyl)adenine 7-glucoside; iP9G, N^6-(Δ^2-isopentenyl)adenine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

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

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

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

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

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

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

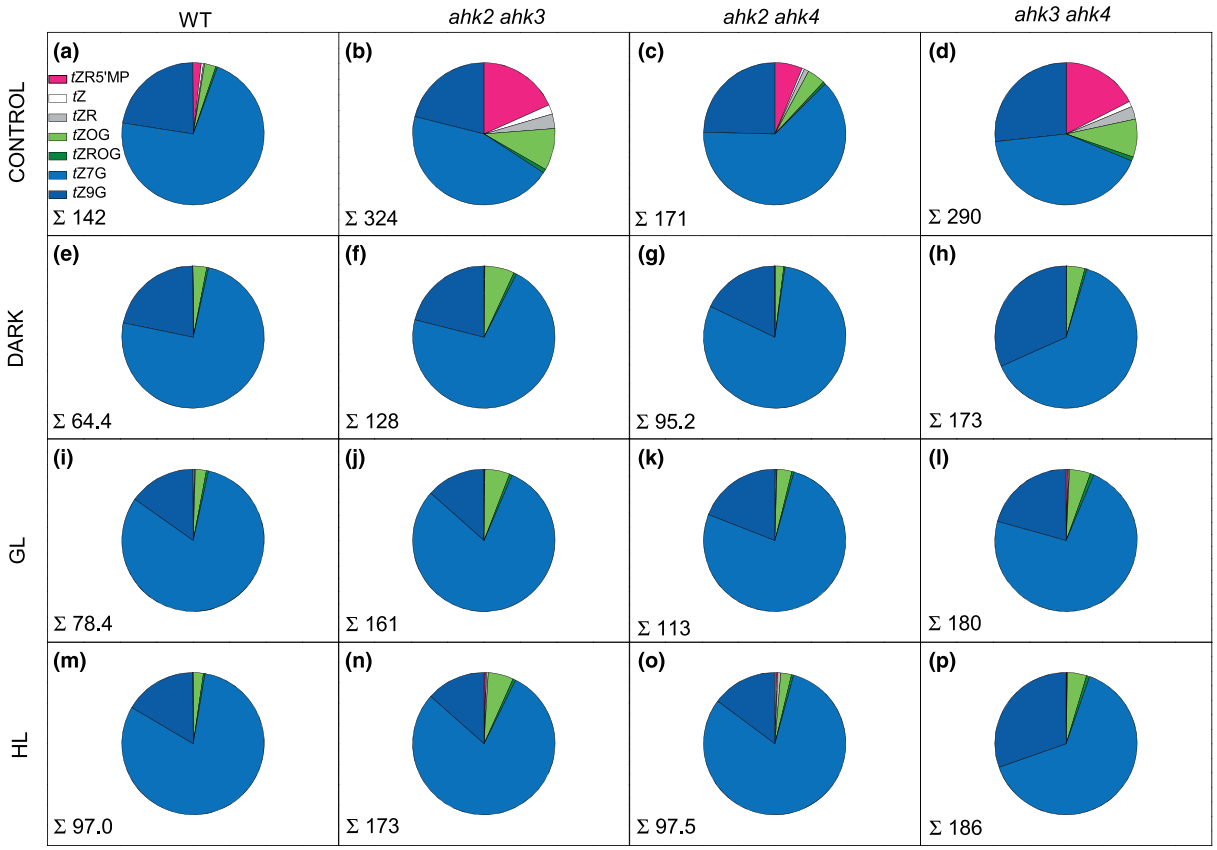

FIGURE 5 The overall content of *t*Z forms (Σ; pmol/g of fresh weight) and relative amount of particular *t*Z forms (*t*ZR5′MP, *trans*-zeatin riboside 5′-monophosphate; *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside; *t*ZOG, *trans*-zeatin *O*-glucoside; *t*ZROG, *trans*-zeatin riboside *O*-glucoside;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

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

3.5 | MDA content was influenced differently by GL and HL

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4 | DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

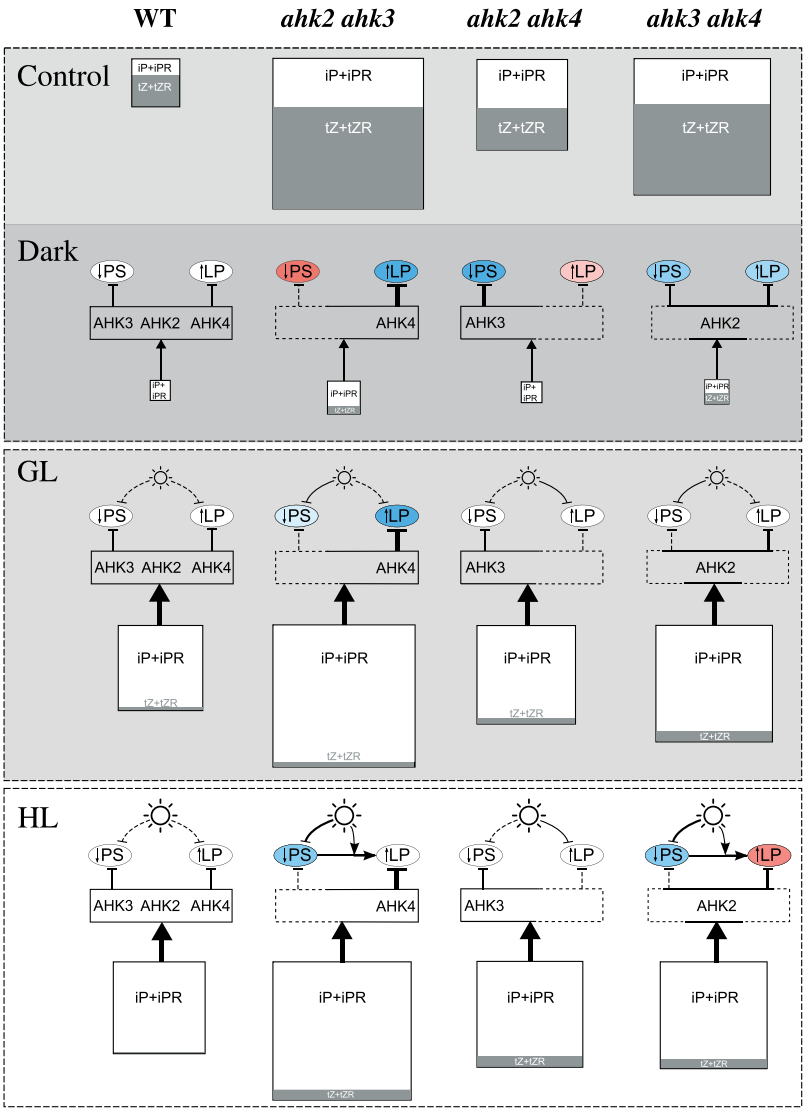

FIGURE 6 The proposed scheme of the interplay between cytokinins (CKs) and light during senescence of detached *Arabidopsis* leaves. Different genotypes are presented in columns, whereas horizontal sections represent situation after 6 days in darkness (dark), under growth light (GL; 120 µmol photons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

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

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

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

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

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

4.5 | Can light substitute CK signalling?

It has been well documented that there is an extensive crosstalk between light and CKs at multiple levels (Argueso et al., 2010; Oh et al., 2009; Sweere et al., 2001; Vandenbussche et al., 2007). Therefore, we have analysed how light can modify senescence in mutants where two out of three CK receptors are non-functional.

As expected, the dysfunction of AHK3 receptor led to constitutively lower Chl content (Danilova et al., 2014; Riefler et al., 2006) in both the *ahk2 ahk3* and *ahk3 ahk4* double mutants (Figure 2a). After 6-day-long incubation of detached leaves in darkness, the most pronounced decrease in Chl content was observed in mutant where both AHK3 and AHK2 were non-functional (Figures 1 and 2e). Surprisingly, this acceleration of Chl loss was not observed under GL treatment (Figures 1 and 2i). Even more surprisingly, under HL conditions, both mutants without functional AHK3 had markedly better PSII function in comparison with WT (Figure 2n,o). These results are even more interesting in the light of the detailed analysis of the CK content. Under HL conditions, the leaves of the *ahk2 ahk3* mutant contained 9 times lower amount of iP compared with GL conditions (Figure 3f, h); nevertheless, the photosynthesis was much better protected than in WT (Figure 2o). The disrupted CK signalling via AHK3 thus led to lower Chl content in darkness; nevertheless, under GL, it was compensated by light, and under HL, we could observe even improvement of the photosynthetic function in comparison with WT. Light thus seems to have the ability to substitute the insufficient CK signalling, and under HL conditions, it was able to even reverse the negative effect of AHK3 dysfunction. Possible explanation is offered by AHK-mediated activation of type A ARRs that negatively regulate response to CK as well as to light (Mira-Rodado et al., 2007; Sweere et al., 2001). The missing AHK3-mediated feedback inhibition of photosynthesis by CK and/or by light might explain the seemingly surprising finding that the mutants with non-functional AHK3 had more maintained photosynthesis during senescence in HL.

The ability of light to compensate the insufficient CK signalling would further explain the surprising drop in the level of iP under HL (but not GL) in the *ahk2 ahk3* mutants. The intensity of GL was supposedly not high enough to fully compensate the insufficient CK signalling in *ahk2 ahk3*, and the iP accumulation was thus markedly promoted (Figure 4j). However, in leaves exposed to HL intensity, the relative content of iP significantly dropped in favour of other, less biologically active

iP forms (Figure 4n). Under HL conditions, the light was probably strong enough to compensate the impaired CK signalling and as a result, iP could be converted to less active iP metabolites. Furthermore, this drop in iP content demonstrates that light is able to protect photosynthetic apparatus also by other ways than by increasing endogenous levels of active CKs. We have shown that HL was able to minimize the decrease in PSII efficiency in the *ahk2 ahk3* mutant leaves even though the content of CK active forms was lower than in leaves kept at GL (cf. Figure 3 f,h). In addition, we still have to keep in mind that this *ahk2 ahk3* mutant, in which we have observed the light-dependent maintenance of photosynthetic function, has inactive AHK3 and AHK2 receptors that are responsible for the CK-dependent modulation of photosynthetic activity.

Unlike WT and *ahk2 ahk4*, both the *ahk2 ahk3* and *ahk3 ahk4* mutants showed unexpected increase in lipid peroxidation under HL compared with GL (Figure 2l,p), which could be explained by the above-discussed strongly maintained photochemistry of PSII under HL (Figure 2n,o). Earlier, we have shown that in detached leaves with relatively well-preserved photosynthesis, the elevated supply of excitations due to HL dose might lead to an imbalance between the generation and demand of electrons in electron transport chain (Vlčková et al., 2006). This imbalance in turn increases excitation pressure on PSII (1-qP) and leads to higher production of reactive oxygen species and lipid peroxidation. Such situation probably occurred in detached leaves of both the *ahk2 ahk3* and *ahk3 ahk4* mutants exposed to HL, which were characterized by relatively high PSII efficiency (Figure 2n,o), and the measurement confirmed high excitation pressure under HL conditions (1-qP; data not shown). Although in the dark the MDA content in the leaves of these mutants was markedly lower than in WT (Figure 2h), under HL conditions, its content was similar (*ahk2 ahk3*) or even markedly higher (*ahk3 ahk4*) than in WT (Figure 2p). It seems that in *ahk2 ahk3*, the increase in lipid peroxidation was partially suppressed via the AHK4-mediated CK-dependent protection, making the MDA content comparable with WT. However, in *ahk3 ahk4*, the CK-dependent protection mediated by AHK2 was not sufficient for the suppression of lipid peroxidation and the MDA content rose pronouncedly.

Comparison of senescence in the dark and in the light shows that in the *ahk2 ahk4* mutant, light was able to eliminate the increase in lipid peroxidation connected to non-functional AHK2 and AHK4. Similarly, the promoted decrease in photosynthetic parameters during dark senescence in *ahk2 ahk3*, caused by non-functional AHK2 and AHK3, was not only reversed by light, but the photosynthetic performance of this mutant was even better than in WT. Even though our results do not allow us to identify the exact intersection of the CK and light signalling pathways, we have clearly shown that light is able to substitute the disrupted CK signalling during senescence of detached leaves. The observed effect of light on the senescence of leaves with given CK status is summarized in scheme in Figure 6.

4.6 | Is AHK3 a mediator of the effect of *c*Z on senescence?

The CKs of *c*Z type constitute a distinct group of CKs, as these CKs are, unlike others, synthesized via a pathway known as mevalonate (MVA) biosynthetic pathway (Kasahara et al., 2004). *c*Z CKs occur ubiquitously in the plant kingdom (Gajdošová et al., 2011), but their function is largely unknown. An increase in relative content of *c*Z-type CKs has been repeatedly observed under growth-limiting conditions induced by various external or internal factors (for a review, see Schäfer et al., 2015). Senescence appears to be one such factor (Gajdošová et al., 2011; Šmehilová et al., 2016). Our results show the most marked increases in *c*Z level in detached leaves kept for 6 days in darkness (Table 3). During correlation analysis, we have found a strong negative correlation between *c*Z content and Chl content and PSII function in WT as well as in mutants except of *ahk2 ahk4* (Table 4). This finding indicates that the content of *c*Z increases when the photosynthetic performance substantially decreases. It supports a hypothesis by Gajdošová et al. (2011), who suggest that *c*Z-type CKs might ensure a basal CK activity necessary for the maintenance of essential physiological processes under conditions characterized by the lack of energy sources that lead to down-regulation of the main active CKs.

The increase in the *c*Z content might be caused either by the depletion of *t*Z by the action of *cis-trans* isomerase (Spíchal, 2012), or by the activation of MVA biosynthetic pathway. The reason for the preference of MVA pathway over the methylerythritol phosphate (MEP) pathway, necessary for the synthesis of other CKs except *c*Z, can be the availability of its precursors. Although the MEP pathway requires ATP/ADP/AMP precursors, MEV pathway does not. Therefore, the preference of the MVA pathway over the MEP pathway could be a possible survival strategy of leaves facing the ATP/ADP/AMP exhaustion, which can be expected to happen for example after 6 days in darkness. Under such conditions, the precursors of the MVA pathway might be more accessible and/or dispensable than the ATP/ADP/AMP precursors of the MEP pathway.

Interestingly, unlike in WT and other mutants, a positive correlation was found between *c*Z content and the photosynthetic parameters in the *ahk2 ahk4* mutant. This finding indicates that the AHK3 receptor may have an important role in mediating *c*Z action, which is supported by genuine *c*Z affinity to AHK3 (Romanov, Lomin, & Schmülling, 2006) and by the finding that AHK3 was significantly activated by *c*Z (Spíchal et al., 2004). Gajdošová et al. (2011) further tested the activity of exogenously applied CKs of the *c*Z type and compared it to their *trans*-counterparts in various CK bioassays. In the senescence assay, using darkened detached oat (as well as wheat and maize) leaves, the *c*Z had lower activity than *t*Z; however, its effect on Chl retention in leaves was significant. Among dark-kept leaves of all tested double mutants, the *ahk2 ahk4* exhibited the lowest increase in *c*Z level, but this relatively small increase was—in connection with the solely functional AHK3 receptor—sufficient for considerable Chl retention (Figures 1 and 2e). Interestingly, in WT, which also has functional AHK3 and relatively high *c*Z content, we have found a negative correlation between the *c*Z content and the photosynthetic parameters. A question arises why there is an opposite trend than in *ahk2 ahk4*. Possible explanation could be offered by increased abundance of AHK3, which could be expected in the mutant with dysfunction of the other two CK receptors. Therefore, the relative importance of AHK3-mediated *c*Z signalling in the *ahk2 ahk4* double mutant is much higher than in the WT, where all three CK

receptors are functional. This assumption, however, needs to be proved by further investigation.

5 | CONCLUSION

We have shown that the content of endogenous CKs and individual CK forms differs significantly in detached *Arabidopsis* leaves kept under various light conditions. The content of *t*Z forms decreased pronouncedly after 6-day-long incubation of detached leaves, no matter whether the leaves were kept in the dark or under GL or HL. However, the content of iP + iPR decreased only in darkness, and in leaves kept under light, it was significantly elevated. This increase in iP + iPR content was accompanied by highly maintained Chl content and PSII function. The elevated content of *c*Z in detached leaves kept in darkness supports the hypothesis that *c*Z plays a major role in the maintenance of basal leaf viability under growth-limiting conditions, when the synthesis of the main active CKs is suppressed. On the basis of correlation analysis performed on CK receptor double mutants, we propose that AHK3 is the main mediator of this *c*Z action.

Although all of the studied CK receptor double mutants had increased constitutive content of CKs, the dark senescence-induced increase in lipid peroxidation was retarded only in mutant with functional AHK4 receptor and partially also in mutant with functional AHK2. AHK4 thus seems to have the main responsibility for CK-induced inhibition of lipid peroxidation, followed by AHK2.

The CK receptor double mutants also enabled us to investigate in more detail the mechanism of the effect of light on senescence. Senescence-induced changes in the double mutants kept in darkness and under both light intensities indicate that light can compensate the disrupted CK signalling caused by loss-of-function mutation in the AHK receptors. On the basis of this hypothesis, we have suggested a scheme (Figure 6) that summarizes contributions of particular CK receptors, their main endogenous CK ligands, and light in the maintenance of photosynthesis and low lipid peroxidation in detached leaves of *Arabidopsis*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Content of DHZ forms (pmol/g of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL, 120 µmol photons m^{-2} s^{-1}) or higher light (HL, 400 µmol photons m^{-2} s^{-1}). Means and SD (n = 3) are presented; n.d., under detection limit. Statistically significant differences (compared to WT, $P < 0.05$) are indicated in bold.

Table S2. Content of *m*T forms (pmol/g of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL, 120 µmol photons m^{-2} s^{-1}) or higher light (HL, 400 µmol photons m^{-2} s^{-1}). Means and SD (n = 3) are presented; n.d., under detection limit.

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SUPPORTING INFORMATION

Supplemental Table S1. Content of DHZ forms

Table S1. Content of DHZ forms (pmol g^{-1} of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL, 120 µmol photons m^{-2} s^{-1}) or higher light (HL, 400 µmol photons m^{-2} s^{-1}). Means and SD (n = 3) are presented; n.d., under detection limit. Statistically significant differences (compared to WT, $P < 0.05$) are indicated in bold.

Line	DHZ	DHZOG	DHZR	DHZROG	DHZ7G	DHZ9G	DHZR5´MP
Control							
WT	n.d.	n.d.	0.09 ± 0.00	n.d.	4.48 ± 0.33	0.25 ± 0.01	n.d.
ahk2 ahk3	n.d.	0.39 ± 0.01	**0.22 ± 0.01**	n.d.	**15.8 ± 0.3**	**0.80 ± 0.03**	0.23 ± 0.01
ahk2 ahk4	n.d.	0.05 ± 0.00	0.09 ± 0.01	n.d.	**5.35 ± 0.22**	**0.33 ± 0.00**	n.d.
ahk3 ahk4	n.d.	0.17 ± 0.01	**0.16 ± 0.00**	n.d.	**16.4 ± 1.0**	**0.87 ± 0.03**	0.17 ± 0.01
Dark							
WT	n.d.	n.d.	0.08 ± 0.01	n.d.	1.58 ± 0.15	0.07 ± 0.00	n.d.
ahk2 ahk3	n.d.	0.10 ± 0.01	0.10 ± 0.00	n.d.	**4.28 ± 0.37**	**0.20 ± 0.01**	n.d.
ahk2 ahk4	n.d.	n.d.	0.07 ± 0.00	n.d.	2.10 ± 0.08	0.10 ± 0.01	n.d.
ahk3 ahk4	n.d.	n.d.	0.08 ± 0.01	n.d.	**4.95 ± 0.43**	**0.25 ± 0.02**	n.d.
GL							
WT	n.d.	n.d.	0.11 ± 0.01	n.d.	1.82 ± 0.12	0.07 ± 0.01	n.d.
ahk2 ahk3	n.d.	0.11 ± 0.01	**0.08 ± 0.01**	n.d.	**4.30 ± 0.11**	**0.18 ± 0.01**	n.d.
ahk2 ahk4	n.d.	0.08 ± 0.00	0.12 ± 0.00	n.d.	**3.54 ± 0.24**	**0.13 ± 0.01**	n.d.
ahk3 ahk4	n.d.	0.13 ± 0.00	0.12 ± 0.00	n.d.	**7.21 ± 0.16**	**0.25 ± 0.01**	n.d.
HL							
WT	n.d.	n.d.	0.07 ± 0.00	n.d.	2.12 ± 0.04	0.08 ± 0.01	n.d.
ahk2 ahk3	n.d.	0.12 ± 0.00	**0.09 ± 0.00**	n.d.	**5.39 ± 0.13**	**0.19 ± 0.01**	n.d.
ahk2 ahk4	n.d.	n.d.	**0.09 ± 0.01**	n.d.	**2.54 ± 0.14**	0.10 ± 0.01	n.d.
ahk3 ahk4	n.d.	0.07 ± 0.00	0.07 ± 0.01	n.d.	**7.29 ± 0.43**	**0.24 ± 0.02**	n.d.

Supplemental Table S2. Content of mT forms

Table S2. Content of mT forms (pmol g^{-1} of fresh weight) in control leaves and detached leaves incubated for 6 days under dark, growth light (GL, 120 µmol photons m^{-2} s^{-1}) or higher light (HL, 400 µmol photons m^{-2} s^{-1}). Means and SD (n = 3) are presented; n.d., under detection limit.

Line	mT	mTR	mT9G
Control			
WT	1.53 ± 0.17	n.d.	n.d.
ahk2 ahk3	1.02 ± 0.17	n.d.	n.d.
ahk2 ahk4	0.70 ± 0.05	n.d.	n.d.
ahk3 ahk4	0.80 ± 0.13	n.d.	n.d.
Dark			
WT	1.81 ± 0.02	n.d.	n.d.
ahk2 ahk3	3.93 ± 0.84	n.d.	n.d.
ahk2 ahk4	1.31 ± 0.00	n.d.	n.d.
ahk3 ahk4	1.43 ± 0.08	n.d.	n.d.
GL			
WT	1.28 ± 0.16	n.d.	n.d.
ahk2 ahk3	3.73 ± 0.59	n.d.	n.d.
ahk2 ahk4	0.79 ± 0.08	n.d.	n.d.
ahk3 ahk4	0.96 ± 0.12	n.d.	n.d.
HL			
WT	0.78 ± 0.08	n.d.	n.d.
ahk2 ahk3	0.93 ± 0.13	n.d.	n.d.
ahk2 ahk4	0.67 ± 0.15	n.d.	n.d.
ahk3 ahk4	0.66 ± 0.07	n.d.	n.d.

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Research article

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

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ABSTRACT

Recent studies have shown that chlorophyll (Chl) *b* has an important role in the regulation of leaf senescence. However, there is only limited information about senescence of plants lacking Chl *b* and senescence-induced decrease in photosystem II (PSII) and photosystem I (PSI) function has not even been investigated in such plants. We have studied senescence-induced changes in photosynthetic pigment content and PSII and PSI activities in detached leaves of Chl *b*-deficient barley mutant, *chlorina f2*^{f2} (*clo*). After 4 days in the dark, the senescence-induced decrease in PSI activity was smaller in *clo* compared to WT leaves. On the contrary, the senescence-induced impairment in PSII function (estimated from Chl fluorescence parameters) was much more pronounced in *clo* leaves, even though the relative decrease in Chl content was similar to wild type (WT) leaves (*Hordeum vulgare* L., cv. Bonus). The stronger impairment of PSII function seems to be related to more pronounced damage of reaction centers of PSII. Interestingly, exogenously applied plant hormone cytokinin 6-benzylaminopurine (BA) was able to maintain PSII function in the dark senescing *clo* leaves to a similar extent as in WT. Thus, considering the fact that without BA the senescence-induced decrease in PSII photochemistry in *clo* was more pronounced than in WT, the relative protective effect of BA was higher in Chl *b*-deficient mutant than in WT.

1. Introduction

Leaf senescence, a final stage of leaf life preceding its death, is important for plant with respect to nutrient remobilization. Leaf senescence is accompanied by a massive degradation of chlorophyll (Chl) and by inhibition of photosynthetic processes including photosystem II (PSII) photochemistry (Oh et al., 1996; Špundová et al., 2003, 2005; Vlčková et al., 2006; Kusaba et al., 2007; Talla et al., 2016; Janečková et al., 2018) and photosystem I (PSI) activity (Nath et al., 2013; Krieger-Liszkay et al., 2015). In the literature, there is no consensus whether the decrease of photosynthetic activity of PSII precedes the inhibition of PSI or *vice versa* (e.g., Nath et al., 2013; Krieger-Liszkay et al., 2015).

Leaf senescence is regulated by many factors, including plant hormones cytokinins. Cytokinins are known to slow down senescence, decelerate senescence-associated degradation of photosynthetic pigments and deterioration of photosynthetic function (Oh et al., 2005; Vlčková et al., 2006; Talla et al., 2016; Vylíčilová et al., 2016). Recent investigations have shown that Chl *b* also plays an important role in the regulation of leaf senescence. Mutants with higher Chl *b* content appear to have slower senescence-related degradation of Chl, light-harvesting

Abbreviations: ABS/RC, apparent antenna size of active reaction center of photosystem II; BA, 6-benzylaminopurine; CAO, chlorophyllide *a* oxygenase; car, carotenoids (sum of carotenes and xanthophylls); Chl, chlorophyll; *clo*, *chlorina f2*^{f2} mutant; DEPS, the de-epoxidation state of xanthophylls; $(dV/dt)_0$, the initial slope of the O-J chlorophyll fluorescence rise; F_v/F_m, maximal quantum yield of photosystem II photochemistry in the dark-adapted state; F_v'/F_m', the maximal quantum yield of photosystem II photochemistry in the light-adapted state; LHC(s), light-harvesting complex(es); OJIP, chlorophyll fluorescence induction transient; PSI, photosystem I; PSII, photosystem II; P700, primary electron donor of photosystem I; RCI, reaction center(s) of photosystem I; RCII, reaction center(s) of photosystem II; RE_0/ABS, quantum yield of electron transport from reduced Q_A to final acceptors of photosystem I; VAZ, content of xanthophylls (violaxanthin, antheraxanthin, and zeaxanthin); V_J, the relative variable fluorescence at the J step of OJIP curve; δR_0, the efficiency of electron transport from reduced plastoquinone to final acceptors of photosystem I; $\Phi_{f,D}$, quantum yield of constitutive non-regulatory dissipation processes in the light-adapted state; Φ_{NPQ}, quantum yield of regulatory non-photochemical quenching in the light-adapted state; Φ_P, the effective quantum yield of PSII photochemistry in the light-adapted state

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complexes (LHCs) and thylakoid membranes (Kusaba et al., 2007; Sakuraba et al., 2012; Voitsekhovskaja and Tyutereva, 2015). At the same time, a recent study with *pgl* rice mutant has shown that Chl *b* deficiency was associated with increased Chl degradation, accumulation of reactive oxygen species, and electrolyte leakage during both natural senescence of flag leaves and dark-induced senescence of detached leaves (Yang et al., 2016). Kusaba et al. (2007) has also mentioned faster Chl degradation in dark-incubated detached leaves of *cao-2* rice mutant deficient in Chl *b*. Although these studies suggest that senescence-related changes are accelerated in plants lacking Chl *b*, the question whether and how Chl *b* deficiency affects senescence-induced inhibition of PSII and PSI function has not been addressed yet.

In order to broaden knowledge about the effect of missing Chl *b* on senescence, we have studied the changes in Chl and carotenoid (car) content and changes in PSII and PSI activity in dark-senescing detached leaves of *chlorina f2*^{f2} (*clo*) barley mutant. The *clo* mutant is deficient in Chl *b* due to the mutation in chlorophyllide *a* oxygenase (CAO), the enzyme responsible for the conversion of chlorophyllide *a* to chlorophyllide *b* and thus crucial for biosynthesis of Chl *b* (Mueller et al., 2012). The *clo* mutant has also lower contents of Chl *a* and car compared to WT (Štroch et al., 2004, 2008). The mutant is deficient in light-harvesting complexes Lhcb1, Lhcb6 and Lhca4, and has reduced amount of Lhcb2, Lhcb3 and Lhcb4 (Bossmann et al., 1997). The amount of LHCs of PSII and PSI is reduced by about 80 % and 20 %, respectively (Ghirardi et al., 1986). The more reduced amount of LHCs of PSII (LHCII) is in *chlorina* mutants compensated by an increased amount of reaction centers of PSII (RCII) and a greater ratio of RCII/RCI (Ghirardi et al., 1986).

The *chlorina* mutants generally have similar or only slightly lower efficiency of PSII photochemistry (Leverenz et al., 1992; Štroch et al., 2004, 2008) and oxygen evolution (Havaux and Tardy, 1997) than WT plants. However, under stress conditions such as high light or high temperature, the PSII efficiency is more reduced in the mutants (Leverenz et al., 1992; Havaux and Tardy, 1997; Peng et al., 2002; Štroch et al., 2008; Tyutereva et al., 2017) than in WT. The increased stress-sensitivity of the PSII photochemistry in the *chlorina* mutant has been attributed to its reduced amount of LHCs, resulting from missing Chl *b* (Havaux and Tardy, 1997).

In this work, we have studied how the Chl *b* deficiency in *clo* mutant changes the progress of dark senescence of detached leaves, with special focus given on the description of senescence-induced changes in the function of PSII. As cytokinins are known decelerators of senescence, we also wanted to find out whether and to what extent is exogenously applied cytokinin 6-benzylaminopurine able to suppress the supposedly pronounced senescence in Chl *b*-deficient *clo* mutant.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of wild-type barley (*Hordeum vulgare* L. cv. Bonus; WT) and *chlorina f2*^{f2} (*clo*) mutant were soaked in deionized water for 24 h before sowing and then transferred into pots containing perlite with Hoagland solution. Pots were placed in a growth chamber under controlled conditions of 16 h light (150 µmol of photons m^{-2} s^{-1})/8 h dark, 22/20 °C and 60% relative air humidity.

Eight days after the sowing, 4-cm segments were cut off from the primary leaves. Leaf segments were placed either into a 0.2% solution of dimethylsulfoxide (DMSO) or into a 10^{-5} mol l^{-1} solution of 6-benzylaminopurine (BA) in 0.2% DMSO (BA treated leaves). The leaf segments were then kept in the dark (other conditions were same as during plant growth). Measurements were performed immediately after the leaf detachment and on the 4th day after detachment.

2.2. Pigment analysis

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

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

2.3. Chlorophyll fluorescence measurements

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

The quenching analysis was performed using PlantScreen (Photon Systems Instruments, Czech Republic) phenotyping platform (Humplík et al., 2015) according to the following protocol. At the beginning, the minimal fluorescence F_0 was determined using measuring flashes (duration of 10 µs) of red light (650 nm), which did not cause any closure of RCII. Then a saturating pulse (white light, 1900 µmol of photons m^{-2} s^{-1}, duration of 800 ms) was applied to measure maximal fluorescence F_m. After 90 s of dark-relaxation, when the measured fluorescence signal reached F_0, the leaf samples were exposed to actinic

light for 25 min (red light, 150 µmol of photons m^{-2} s^{-1}, the same intensity as used for plant growth). To determine the maximal fluorescence during the actinic light exposition (F_m'), a set of the saturating pulses was applied. The first pulse was applied 10 s after the actinic light was switched on and was followed by 9 pulses in 20 s intervals and then by 22 pulses in 59 s intervals.

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

2.4. Measurement of P700 oxidation

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

2.5. Statistical analysis

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

3. Results

3.1. Characterization of clo leaves before senescence induction

Leaves of the *clo* mutant had approximately half the Chl content compared to WT (Table 1). The content of Chl *a* was lower by about 30 %, while Chl *b* was not detected (Table 1). The content of carotenoids (car; sum of carotenes and xanthophylls) was also lower in *clo* (by about 30 % compared to WT). As a result of relatively more lowered content of Chl than car, *clo* had significantly lower *Chl/car* ratio than the WT (Table 1). Leaves of *clo* had also lower content of xanthophylls (VAZ) (by about 25 %; Table 1). However, the *VAZ/Chl* ratio and de-epoxidation state of the xanthophyll cycle pigment pool (DEPS) were higher in *clo* (Table 1), which indicates better photoprotection of photosynthetic apparatus in *clo* compared to WT.

Table 1
The content of pigments (mg per m^2 of leaf area), their ratios and maximal efficiency of PSII photochemistry in dark- (F_v/F_m) and light-adapted (F_v'/F_m') state in leaves of WT and *clo* mutant before senescence induction.

	WT	*clo*
Chl *a*	176 ± 24	**121 ± 4**
Chl *b*	51 ± 7	n. d.
Chl *a*+*b*	227 ± 31	**121 ± 4**
Chl *a*/*b*	3.4 ± 0.1	n. d.
car	43 ± 5	**30 ± 1**
Chl/car	5.3 ± 0.4	**4.0 ± 0.1**
VAZ	16.6 ± 1.5	**12.3 ± 1.0**
VAZ/Chl	0.074 ± 0.004	**0.102 ± 0.006**
DEPS (%)	2.4 ± 1.0	**4.3 ± 0.6**
F_v/F_m	0.802 ± 0.011	**0.792 ± 0.004**
F_v'/F_m'	0.776 ± 0.003	**0.720 ± 0.010**

Means and SD (n = 3–10 for pigments and n = 6 for fluorescence parameters) are presented; n. d., not determined. Statistically significant differences (compared to WT, $P < 0.05$, *t*-test, except of DEPS where Mann-Whitney Rank Sum test was used) are indicated in bold.

Besides the generally reduced content of photosynthetic pigments, the maximal quantum yield of PSII photochemistry in both dark-adapted state (F_v/F_m) and light-adapted state (F_v'/F_m') was slightly lowered in *clo* (Table 1, Fig. 1C). To determine whether *clo* had altered partitioning of absorbed light energy for photochemical and non-photochemical processes, the following parameters were evaluated in the light-adapted state: the effective quantum yield of PSII photochemistry (Φ_P), quantum yield of constitutive non-regulatory dissipation processes ($\Phi_{f,D}$) and quantum yield of regulatory non-photochemical quenching (Φ_{NPQ}). Together the sum of these quantum yields equals unity (Lazár, 2015). In *clo,* a slightly but significantly lower Φ_P and higher $\Phi_{f,D}$ and Φ_{NPQ} were observed (Fig. 1D), which indicates that lower fraction of absorbed light energy was used by PSII photochemistry and that more absorbed energy was dissipated *via* non-photochemical quenching processes.

As the *clo* mutant is deficient in Chl *b* (Table 1) and consequently in LHCII (Ghirardi et al., 1986; Bossmann et al., 1997), a lower supply of excitations from LHCII to RCII can be expected. It should affect transient of Chl fluorescence induction (OJIP curve), as this curve reflects closure of RCII (Lazár, 2006) that depends on the rate of excitation supply. The typical OJIP curve was observed in the *clo* leaves, although the overall fluorescence signal was lower compared to WT (Fig. 2A). From normalized curves it is obvious that the J- and I-steps are both reached later in the *clo* leaves (Fig. 2B) than in WT, which reflects a slower reduction of Q_A as well as Q_B. This slower reduction consequently results in a lower transient accumulation of reduced Q_A, which is in turn reflected in a lower J-step. The lower relative height of J-step is quantitatively expressed by a lower V_J parameter (by about 14 %) (Fig. 2B and C). The slower Q_A reduction in the *clo* leaves is further indicated by $(dV/dt)_o$, which was lower by about 40 % (Fig. 2C) than in WT. Finally, a lower *ABS/RC* ratio (by about 25 % compared to WT; Fig. 2C) confirmed deficiency of LHCII in the *clo* leaves, as this ratio reflects an apparent antenna size of active RCII (Stirbet et al., 2018).

On the other hand, parameters of the OJIP curve reflecting electron transport to PSI, RE_o/ABS (the quantum yield of electron transport from reduced Q_A to final acceptors of PSI) and δ_{R0} (the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI) were higher in *clo* by 46 and 44 %, respectively (Fig. 2D). Finally, a relative amount of $P700^+$ was lower in *clo* (Fig. 2D).

3.2. Comparison of dark senescence-induced changes in WT and clo detached leaves

To induce senescence, leaves of WT and *clo* were detached and

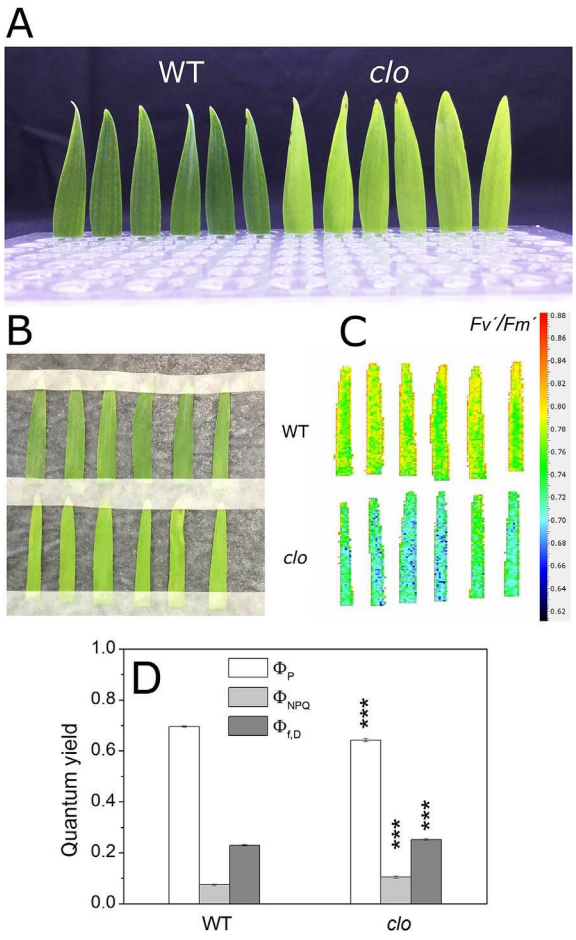

Fig. 1. Characterization of detached WT and *clo* leaves before senescence induction. A, phenotype; B, leaf segments used for the measurement of F_v'/F_m'; C, F_v'/F_m' in the area of the leaf segments; D, quantum yield of PSII photochemistry (Φ_P), regulatory non-photochemical quenching (Φ_{NPQ}), and constitutive non-regulatory dissipation processes ($\Phi_{f,D}$). Means and SD are presented, n = 6–10. Asterisks indicate statistically significant difference (Student's *t*-test) between WT and *clo* ($P < 0.001$).

subsequently incubated in control solution (0.2% DMSO) in the dark for 4 days. This incubation resulted in a significant decrease in Chl, car and VAZ content in all detached leaves (Fig. 3). The Chl content decreased by 82 % in WT and 87 % in *clo* (Fig. 3) and although the relative decrease in Chl content was similar in WT and *clo*, the absolute Chl content was pronouncedly lower in the *clo* senescing leaves (about 16 mg Chl m^{-2} compared to about 40 mg Chl m^{-2} in WT). As indicated by a decrease in Chl *a*/*b* ratio, the content of Chl *a* decreased in WT slightly more than Chl *b* (Fig. 3).

The content of car decreased in WT by about 60 %, whereas in *clo* this content was reduced only by about 40 % (Fig. 3). Similarly, the VAZ content decreased more in WT (by about 70 %) than in *clo* (by about 55 %; Fig. 3). The faster breakdown of Chl compared to car caused a significant decrease in the *Chl/car* ratio in both genotypes, more pronounced in *clo* (Fig. 3). In summary, the relative decrease in Chl content was similar in both *clo* and WT, but the relative decrease in the content of car and VAZ in *clo* was lower than in WT.

The loss of photosynthetic pigments during dark-induced senescence was associated with a decline in the maximal quantum yield of PSII photochemistry in the dark-adapted state in both *clo* and WT. Parameter F_v/F_m dropped by about 45 % in WT leaves and by about 70 % in *clo* (Fig. 4A), which indicates more pronounced impairment of PSII function in *clo* compared to WT. In fact, the real impairment of PSII function was much more pronounced in *clo*, because a considerable part of the area of measured leaves was already not photosynthetically functional enough for Chl fluorescence detection (i.e., the Chl fluorescence signal from *clo* leaves was so small that it was not distinguishable from a background signal, Fig. 4B) and these leaf parts were not included into the average F_v/F_m value (Fig. 4A). Thus the average F_v/F_m value is representative only for the (minimally) functional parts of leaves.

The decrease in F_v/F_m was accompanied by a decrease in Φ_P in both *clo* and WT, indicating decreased energy utilization by PSII photochemistry in the light-adapted state. The Φ_P value in *clo* was significantly lower than in WT (Fig. 5). On the other hand, Φ_{NPQ} and $\Phi_{f,D}$ increased in senescing leaves, indicating enhanced energy dissipation by means of non-photochemical processes. Unlike the leaves before senescence induction, the partitioning of absorbed light energy into regulatory or non-regulatory dissipation processes differed pronouncedly in WT and *clo*. While Φ_{NPQ} and $\Phi_{f,D}$ were comparable in WT, in *clo* $\Phi_{f,D}$ prevailed (Fig. 5). It indicates that in WT, energy non-utilized by PSII photochemistry was dissipated in both regulatory and non-regulatory processes to a similar extent, while in *clo*, the majority of this energy was dissipated *via* non-regulatory processes. This corresponds to the extreme impairment of PSII function in *clo* (Fig. 4B).

After 4 days of incubation in the dark, the shape of OJIP curve and the height of its individual steps changed in WT as well as in *clo* (compare Figs. 2A and 6A). In senescing WT leaves, the OJIP curve was more flat than in non-senescing ones due to the pronounced increase in the height of the O-step and decrease in the height of the P-step (Fig. 6A). Additionally, the normalized curve showed a relative increase in the J-step (compare Figs. 2B and 6B), reflected also in the increased parameter V_J (1.5-times when compared to leaves before senescence induction; Fig. 6C). The $(dV/dt)_o$ parameter also increased, but more (2.5-times) than V_J, thus *ABS/RC* proportional to their ratio increased more pronouncedly (4-times). The increase of *ABS/RC* suggests increase in apparent antenna size of active RCII, which in turn indicates preferential impairment of RCII compared to LHCII. This results in increased supply of excitations to remaining active RCII and thus a pronounced Q_A reduction can be observed in these RCII. We propose that the preferential RCII impairment was caused by their degradation, as the Chl *a*/*b* ratio decreased in the WT senescing leaves (Fig. 3). Since Chl *b* occurs mainly in LHCII, the decrease in the Chl *a*/*b* ratio reflects a relative decrease in RCII abundance (Leong and Anderson, 1984).

RE_o/ABS as well as δR_0 decreased in the senescing WT leaves by about 80 % and 45 %, respectively (Fig. 6D). The greater decrease in RE_o/ABS in comparison to δR_0 indicates that the electron transport efficiency decreased more within PSII than behind PSII and that RCI degradation was lower than degradation of RCII. This assumption is supported by a lower relative amount of $P700^+$ (Fig. 6D).

In senescing leaves of *clo*, changes in the OJIP curve were much more profound than in WT. The typical OJIP shape was missing and the curve became almost flat (compare Figs. 2A and 6A). The relative height of the J-step increased (V_J increased twice compared to the leaves before senescence), $(dV/dt)_o$ increased 4-times and *ABS/RC* increased 15-times (Fig. 6B and C). The extreme increase in *ABS/RC* was related also to very pronounced decrease in F_v/F_m. It means that the impairment of RCII during dark-senescence was much more pronounced in *clo* than in WT, which corresponds to more severe inhibition of PSII photochemistry described above.

Similarly to WT, parameters of the OJIP curve reflecting electron transport to PSI, RE_o/ABS and δR_0, decreased in the senescing leaves of *clo* (Fig. 6D). The decrease was again more pronounced in the case of RE_o/ABS (by 98 %) than in δR_0 parameter (by about 70 %), which indicates more pronounced impairment of electron transport within PSII than behind this complex and the preferential decrease in RCII compared to RCI. The relative amount of $P700^+$ was higher than in the senescing leaves of WT (Fig. 6D).

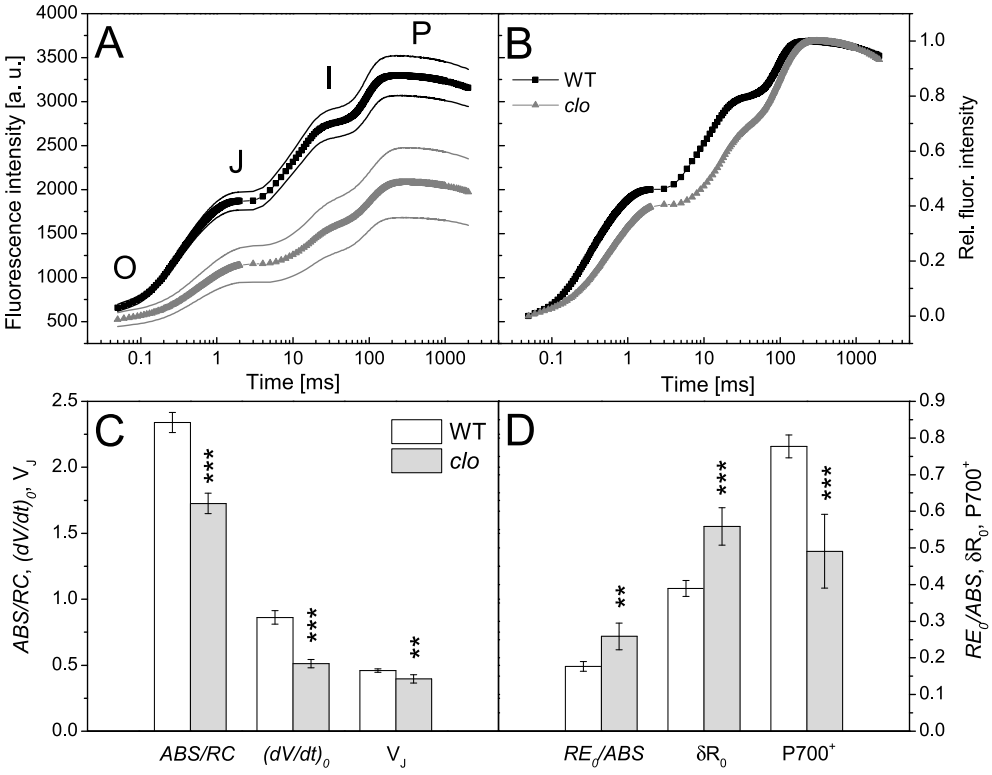

Fig. 2. Chl fluorescence induction transient (OJIP curves), related fluorescence parameters and changes in the PSI activity of detached WT and *clo* leaves before senescence induction. A, OJIP curves; B, the normalized OJIP curves; C, the apparent antenna size of active RCII (*ABS/RC*), the initial slope of the O-J fluorescence raise $(dV/dt)_O$, and the relative variable fluorescence at the J-step (V_J); D, quantum yield of electron transport from reduced Q_A to final acceptors of photosystem I (RE_O/ABS); the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI (δR_0) and the relative amount of oxidized primary electron donor of PSI, $P700^+$. Means and SD are presented, n = 6–7. Asterisks indicate statistically significant difference (Student's *t*-test) between WT and *clo* (**, $P < 0.01$; ***, $P < 0.001$).

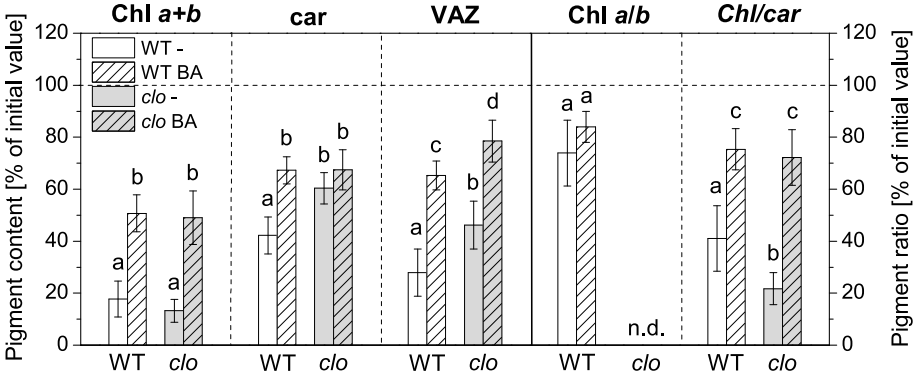

Fig. 3. Chlorophyll (Chl $a+b$), carotenoid (car) and xanthophyll (VAZ) content related to leaf area and Chl a/b and *Chl/car* ratios in detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (−) or with 6-benzylaminopurine (BA). Relative values (% of the initial values before senescence induction) are presented. Means and SD are shown, n = 6. Except of the Chl a/b ratio (analyzed by the *t*-test, $P = 0.139$), all other data were analyzed by ANOVA test ($P < 0.001$ in all cases) and statistically significant differences in following *post hoc* statistical testing (Holm-Sidak test) at $P < 0.05$ are indicated by different letters.

3.3. Effect of BA on senescence-induced changes in WT and clo leaves

To evaluate the effect of cytokinin on dark-senescing WT and *clo* leaves, detached leaves were incubated in BA (10^{-5} mol l^{-1}) solution and kept in the dark for 4 days. BA significantly reduced the degradation of photosynthetic pigments in both genotypes, the content of Chl and car decreased by about 50 % and 35 %, respectively (Fig. 3), and the *Chl/car* ratio by about 25 % (Fig. 3). The VAZ content decreased by about 35 % and 20 % in WT and *clo*, respectively (Fig. 3), and the Chl a/b ratio in WT leaves decreased by about 15 %.

BA also suppressed the senescence-induced decrease in PSII photochemistry in both WT and *clo* leaves (Figs. 4–6). In the presence of BA, F_v/F_m dropped only by 20 % during the senescence, which indicated that PSII photochemistry is relatively well maintained (Fig. 4A). This was also evidenced by a smaller decrease in Φ_P (i.e., utilization of absorbed light energy by PSII photochemistry) in both WT and *clo* (Fig. 5). In *clo* BA significantly suppressed the senescence-induced increase in $\Phi_{f,D}$ (Fig. 5).

The protective effect of BA on PSII function in senescing leaves was also reflected in less pronounced changes in the shape of OJIP curve (Fig. 6A) and smaller changes in corresponding parameters. In leaves undergoing senescence in the presence of BA, we have observed a smaller increase in relative height of the J-step (i.e., V_J) (Fig. 6B) and also the increase in *ABS/RC* and $(dV/dt)_O$ parameters was considerably smaller compared to leaves senescing in the absence of BA (Fig. 6C).

In both genotypes, BA suppressed the senescence-induced decrease in RE_O/ABS and δR_0; in *clo* the BA application even increased δR_0 by about 20 % (Fig. 6D). On the contrary, BA had no significant effect on the relative amount of $P700^+$ in either WT or *clo* (Fig. 6D).

The changes in parameters described above indicate that BA suppressed the senescence-induced impairment of PSII photochemistry in both WT and *clo*. Interestingly, in the presence of BA, the progress of senescence in *clo* became more similar to WT (Figs. 4–6), although in the absence of exogenous cytokinin the senescence-induced impairment of PSII function was much more pronounced in *clo*. The stronger effect of BA in the case of *clo* is further apparent from the significantly lower increase in *ABS/RC* and V_J (Fig. 6C). The more marked effect of BA on *clo* in comparison to WT was even more visible when an increased actinic light intensity (600 µmol of photons m^{-2} s^{-1}) was applied. In the untreated leaves of WT and *clo*, Φ_P was 0.13 and 0.14, respectively. In WT, BA improved Φ_P only non-significantly (to 0.25), while in *clo*, the Φ_P improvement (to 0.36) was statistically significant.

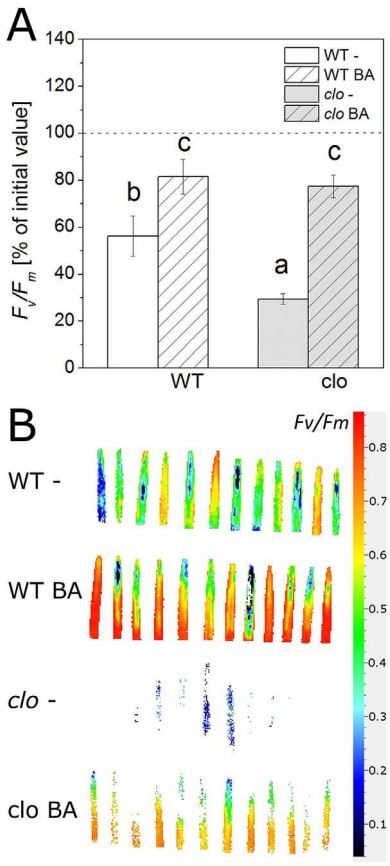

Fig. 4. The maximal efficiency of PSII photochemistry in dark-adapted state (F_v/F_m) in detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (−) or with 6-benzylaminopurine (BA). A, the relative F_v/F_m values (% of the initial values before senescence induction), means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

4. Discussion

It has been reported that the Chl *b* deficiency accelerates senescence-related changes in rice (Kusaba et al., 2007; Yang et al., 2016). Faster Chl degradation was observed in detached leaves of Chl *b*-deficient rice mutant *cao-2* (Kusaba et al., 2007). Based on faster Chl degradation, increased accumulation of reactive oxygen species, and increased electrolyte leakage Yang et al. (2016) suggested faster senescence in *pgl* rice mutant with reduced Chl *b* content in case of naturally senescing flag leaves as well as in case of detached leaves kept in the dark. Nevertheless, deeper knowledge of senescence-associated impairment of photosynthetic apparatus including PSII and PSI function under Chl *b* deficiency is missing.

To find out whether the deficiency of Chl *b* accelerates senescence-induced impairment of PSII and PSI activities, we have investigated their changes (together with changes in photosynthetic pigment content) in detached leaves of the Chl *b*-deficient barley mutant senescing in the dark for 4 days. As cytokinins are known to partially protect photosynthetic activity during senescence (Oh et al., 2005; Vlčková et al., 2006; Talla et al., 2016), we have also studied the effect of exogenously applied BA and analyzed whether it is able to suppress the senescence-associated changes also in *clo*.

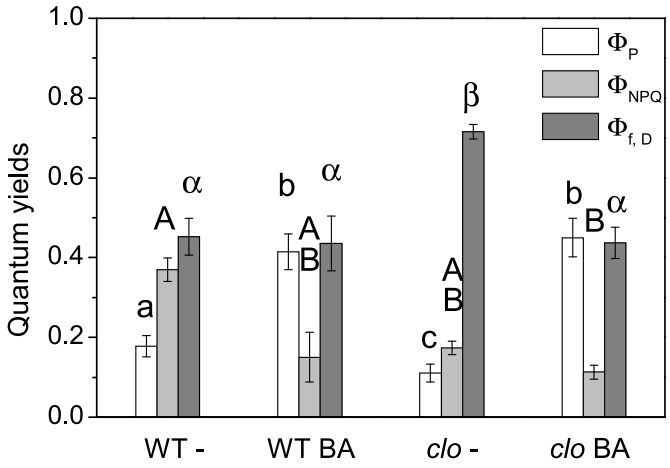

Fig. 5. Quantum yields of PSII photochemistry (Φ_P), regulatory non-photochemical quenching (Φ_{NPQ}), and constitutive non-regulatory dissipation processes ($\Phi_{f,D}$) of the detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (−) or with 6-benzylaminopurine (BA). Means and SD are shown, n = 6. Φ_P and $\Phi_{f,D}$ were analyzed by ANOVA test ($P < 0.001$ in both cases), followed by *post hoc* statistical testing (Holm-Sidak test) and Φ_{NPQ} was analyzed by Kruskal-Wallis ANOVA on Ranks test ($P < 0.001$), followed by *post hoc* statistical testing (Dunn's test). Statistically significant differences in the *post hoc* statistical testing at $P < 0.05$ are indicated by different letters.

4.1. Lower efficiency of PSII photochemistry in clo mutant before senescence induction

Leaves of *clo* mutant have lower content of photosynthetic pigments (Table 1). As expected due to the mutation in CAO (Mueller et al., 2012) and in agreement with literature (Štroch et al., 2004, 2008), Chl *b* was not detectable in *clo* (Table 1). Due to the lack of Chl *b*, the antenna size of PSII is substantially reduced in *clo*, as has been shown by lower abundance of LHCII proteins (Król et al., 1995; Bossmann et al., 1997) and by changes in emission and excitation Chl fluorescence spectra measured at 77 K (Štroch et al., 2004). We have confirmed the reduced functional size of LHCII in *clo* by lower *ABS/RC* ratio (Fig. 2C), reflecting lower amount of absorbed excitations per active (Q_A-reducing) RCII. The presence of smaller PSII antennae resulted in slower supply of excitations to the RCII, in slower Q_A reduction and smaller amount of reduced Q_A, which was evidenced by the lower $(dV/dt)_0$ and V_J parameters (Fig. 2C).

Despite the smaller LHCII in *clo*, the efficiency of electron transport behind PSII to PSI was higher compared to WT which corresponds to the higher ratio RCII/RCI in *chlorina f2* mutant reported by Ghirardi et al. (1986). The higher electron flow behind PSII probably led to the lower relative amount of $P700^+$ (Fig. 2D). The reduced size of PSI antennae might also contribute to the decreased relative amount of $P700^+$ as *clo* is known to be deficient in the light-harvesting complex Lhca4 (Bossmann et al., 1997). Consistent with this assumption, it has been shown that kinetics of P700 oxidation was much slower in a rice mutant *dye1-1* with a severely reduced amount of Lhca4 (Yamatani et al., 2018).

The *clo* leaves had slightly less effective PSII photochemistry as indicated by lower values of the maximal quantum yield of PSII photochemistry in the dark-adapted state (F_v/F_m; Table 1) and of the maximal and effective quantum yield of PSII photochemistry in the light-adapted state (as F_v'/F_m' and Φ_P; Table 1, Fig. 1D). The slightly lower quantum yield of PSII photochemistry of *chlorina* mutants has been reported previously (Leverenz et al., 1992; Štroch et al., 2004, 2008).

The light energy that is not utilized by PSII photochemistry is dissipated *via* non-regulatory ($\Phi_{f,D}$) and/or regulatory (Φ_{NPQ}) non-photochemical quenching processes. $\Phi_{f,D}$ represents quantum yield of

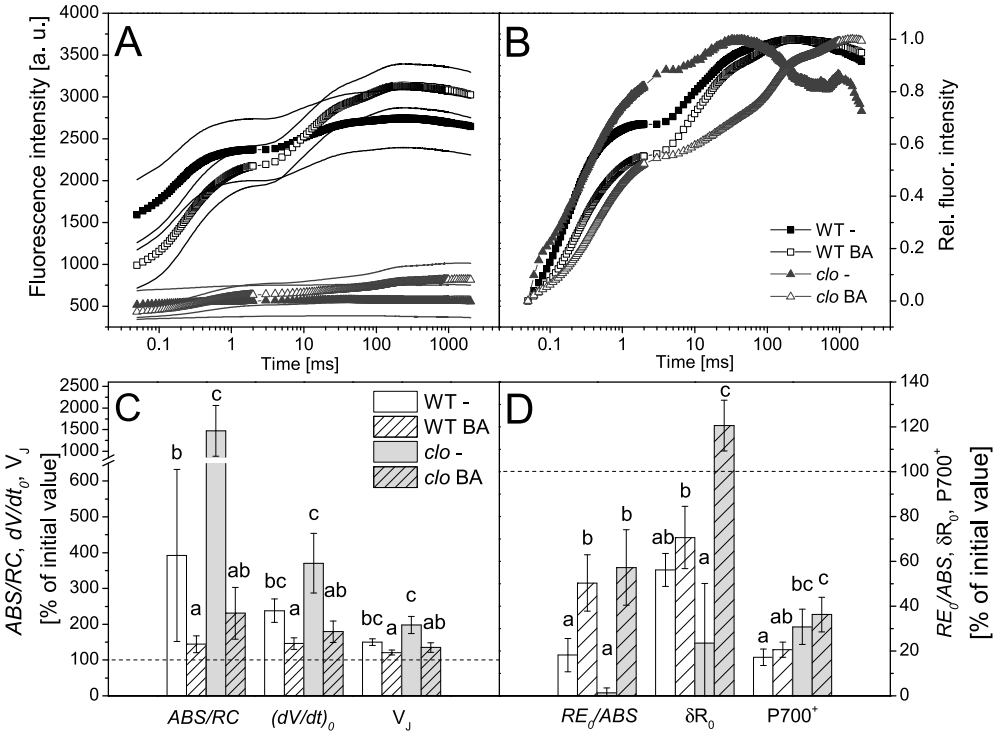

Fig. 6. Chl fluorescence induction transient (OJIP curves), related fluorescence parameters and changes in the PSI activity of detached WT and *clo* leaves kept for 4 days in the dark in 0.2% DMSO solution without (−) or with 6-benzylaminopurine (BA). A, OJIP curves; B, the normalized OJIP curves; C, the relative values of the apparent antenna size of active RCII (*ABS/RC*), the initial slope of the O-J fluorescence raise ($dV/dt)_0$, and the relative variable fluorescence at the J-step (V_J); D, quantum yield of electron transport from reduced Q_A to final acceptors of PSI (RE_0/ABS); the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI (δR_0) and the relative amount of oxidized primary electron donor of PSI, $P700^+$, expressed as % of the initial values before senescence induction. Means and SD are shown, n = 8–12. Data were analyzed by Kruskal-Wallis ANOVA on Ranks test ($P < 0.001$) and statistically significant differences in following *post hoc* statistical testing (Dunn's test) at $P < 0.05$ are indicated by different letters.

constitutive (basal) energy dissipation (for a review see Lazár, 2015), whereas Φ_{NPQ} is quantum yield of regulatory quenching, which is induced by illumination to protect the photosynthetic apparatus against excess light and consequent accumulation of reactive oxygen species and oxidative damage (Demmig-Adams et al., 2014). As mentioned above, *clo* had lower Φ_P (Fig. 1D), which indicates lower utilization of absorbed light energy by PSII photochemistry. The proportion of absorbed light energy allocated into non-photochemical quenching processes was higher compared to WT, as both non-regulatory ($\Phi_{f,D}$) and regulatory component (Φ_{NPQ}) were increased (Fig. 1D).

The regulatory non-photochemical quenching processes are related to activation of the xanthophyll cycle where zeaxanthin (Z) is formed by de-epoxidation of violaxanthin (V) through antheraxanthin (A). The extent of the de-epoxidation is expressed as DEPS. Compared to WT, the *clo* leaves were characterized by higher DEPS, by about 80 % (Table 1). Together with the higher *VAZ/Chl* ratio and higher relative content of car (indicated by the lower *Chl/car* ratio) (Table 1), it implies that the *clo* plants had an enhanced photoprotection of photosynthetic apparatus when they were grown under relatively low light intensity (150 µmol of photons m^{-2} s^{-1}). The higher protection against photoinactivation of RCII has been reported by Štroch et al. (2004) in *clo* plants grown under similar light intensity (100 µmol of photons m^{-2} s^{-1}). The higher photoprotection of *clo* could be associated with the existence of free (not bound to LHCs) zeaxanthin (Havaux et al., 2007; Štroch et al., 2008; Nezval et al., 2017).

4.2. *Clo had much more impaired PSII function in dark senescing leaves than WT*

It is generally known that leaf senescence is accompanied by the loss of photosynthetic pigments and impairment of photosynthetic function. In the dark senescing leaves, the photochemical activity of PSII is markedly reduced during a few days (Oh et al., 1996; Špundová et al., 2003; Vlčková et al., 2006; Janečková et al., 2018). In the detached leaves of WT, the content of photosynthetic pigments and PSII photochemistry decreased significantly after 4 days in the dark (Figs. 3 and 4). The increase in the *ABS/RC* ratio as well as decrease in the Chl *a/b* ratio indicated that RCII were damaged to a greater extent than LHCII. This is in agreement with higher $(dV/dt)_0$ and V_J parameters (Fig. 6C), indicating increase in the excitation supply into the active RCII, acceleration of Q_A reduction and thus the increased amount of reduced Q_A (Strasser et al., 2000; Stirbet et al., 2018). The PSII photochemistry was impaired as F_v/F_m and Φ_P decreased (Figs. 4 and 5), whereas the dissipation *via* both regulatory (Φ_{NPQ}) and non-regulatory non-photochemical quenching processes ($\Phi_{f,D}$) increased (Fig. 5). This indicates that the senescing WT leaves were still able to partially regulate the dissipation of excess light energy. The changes in RE_0/ABS, δR_0 and $P700^+$ parameters in the senescing WT leaves indicate that the PSII activity was more impaired during dark senescence than the activity of PSI.

As mentioned above, plants with enhanced Chl *b* content were reported to have slower leaf senescence (Kusaba et al., 2007; Sakuraba et al., 2012), while senescence of Chl *b*-deficient rice mutants was accelerated (Kusaba et al., 2007; Yang et al., 2016). Thus, in the case of *clo* mutant, we expected faster dark-induced senescence. Although the relative decrease in Chl content was similar in WT and *clo* (Fig. 3), the absolute Chl content was pronouncedly lower in the *clo* senescing leaves due to the lower Chl content in the leaves before senescence induction (Table 1). The pronounced decrease in Chl content in *clo* corresponded with more pronounced impairment of PSII function (Figs. 4–6). In fact, the senescing *clo* leaves had only minimal PSII activity after 4 days (Fig. 4B). The preferential senescence-induced impairment of RCII found in the WT leaves was even more pronounced in *clo,* as documented by extremely increased *ABS/RC* (and also by increased $(dV/dt)_0$ and V_J, Fig. 6C). Unlike WT, regulatory quenching processes were almost inactive and dissipation *via* non-regulatory processes prevailed, as indicated by pronouncedly increased $\Phi_{f,D}$ (Fig. 5).

Interestingly, despite the more pronounced impairment of PSII photochemistry, the activity of PSI was higher in *clo* than in WT (Fig. 6D). It seems that in the *clo* mutant the missing Lhca4 did not decrease the stability of PSI during senescence.

The substantially impaired PSII function in the dark-senescing leaves of *clo* is in agreement with the previous studies, reporting higher sensitivity of PSII photochemistry of *chlorina* barley mutants to stress-conditions (Leverenz et al., 1992; Peng et al., 2002; Štroch et al., 2008; Tyutereva et al., 2017). This higher sensitivity is probably related to Chl

b/LHC deficiency, as proper assembly of LHCII seems to stabilize the structure of PSII complexes and their function (Havaux and Tardy, 1997).

We can summarize that in the *clo* mutant, Chl *b* deficiency caused faster impairment of RCII and consequently faster loss of photochemical activity of PSII during dark senescence. On the contrary, the senescence-induced decrease in PSI activity was smaller in *clo* compared to WT leaves.

4.3. Protective effect of exogenous BA on PSII function in dark-senescing leaves was higher in clo

Application of exogenous cytokinins on senescing leaves slows down the degradation of photosynthetic pigments and preserves photosynthetic function, including PSII photochemistry (Oh et al., 2005; Vlčková et al., 2006; Talla et al., 2016; Vylíčilová et al., 2016). In the case of WT leaves, exogenously applied BA significantly reduced the senescence-induced decrease in Chl, car and xanthophyll contents and decrease in the *Chl/car* ratio (Fig. 3), as well as impairment of PSII function (Figs. 4–6). The protective effect of BA was observed also in *clo* and the senescence in the presence of BA was basically similar in both WT and *clo* (Figs. 4–6). Thus, considering the fact that in the absence of BA the PSII function in *clo* leaves was almost completely lost, the protective effect of BA was relatively more pronounced in *clo*. It seems that exogenous BA application suppressed the destabilizing effect of Chl *b*/LHC deficiency on PSII function in the dark-senescing *clo* leaves.

The exact mechanism by which cytokinins maintain PSII function during senescence is not known. It has been proposed that cytokinins could stabilize both LHCII (Oh et al., 2005; Talla et al., 2016; Vylíčilová et al., 2016) and RCII (Oh et al., 2005) in dark-senescing leaves, RCII stabilization being the key process for the maintenance of PSII photochemical activity (Oh et al., 2005). Based on our results we suppose that the protective effect of BA on PSII function in WT as well as in *clo* is based mainly on a pronounced suppression of the impairment of RCII.

5. Conclusion

We can conclude that the Chl *b* deficiency in the *clo* barley mutant leads to a substantial acceleration of the inhibition of PSII photochemistry during dark-induced senescence of detached leaves. We assume that this acceleration was due to the more pronounced impairment of RCII. It is in agreement with previous reports, describing higher sensitivity of RCII in *chlorina* mutants to unfavorable conditions (Havaux and Tardy, 1997). The application of exogenous BA was able to suppress the extreme impairment of PSII function in *clo* and the relative extent of the observed protective effect was even more pronounced in *clo* than in WT. It seems that the presence of Chl *b* is not decisive for the protective cytokinin effect on PSII photochemistry in dark-senescing leaves.

Further investigations are needed to clarify the specifics of senescence process in Chl *b*-deficient mutants as well as the mechanism of the cytokinin-mediated protection of photosynthetic apparatus and function in senescing leaves.

Author contributions

Helena Janečková designed and performed the experiments, analyzed the data, interpreted results and wrote the manuscript; Alexandra Husičková contributed on design and performance of the experiments, helped to interpret the results and revised the manuscript. Dušan Lazár designed the measuring protocol of quenching analysis and measurement of P700 oxidation, evaluated the data, and did statistical analysis; Ursula Ferretti performed the HPLC measurement and analyzed the data; Pavel Pospíšil supervised the HPLC measurement; Martina Špundová supervised the research, contributed on design of the experiments, helped to interpret the results, revised the manuscript and complemented the final writing. All authors read and approved the final manuscript.

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