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 f2f2* (clo). After 4 days in the dark, the senescence-induced decrease in PSII activity was smaller in clo compared to WT leaves. On the contrary, the senescence-induced impairment in PSI 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 PSI function seems to be related to more pronounced damage of reaction centers of PSI. 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 PSI 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

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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 f2f2* mutant; DEPS, the de-epoxidation state of xanthophylls; (dV/dt)a, the initial slope of the O-J chlorophyll fluorescence rise; Fv/Fm, maximal quantum yield of photosystem II photochemistry in the dark-adapted state; Fv′/Fm′, the maximal quantum yield of photosystem II photochemistry in the light-adapted state; LHCs, 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; REd/ABS, quantum yield of electron transport from reduced QA to final acceptors of photosystem I; VAZ, content of xanthophylls (violaxanthin, antheraxanthin, and zeaxanthin); V0, the relative variable fluorescence at the J step of OJIP curve; SE, the efficiency of electron transport from reduced plastocyanine to final acceptors of photosystem I; Fd, quantum yield of constitutive non-regulatory dissipation processes in the light-adapted state; ΦNPQ, quantum yield of regulatory non-photochemical quenching in the light-adapted state; Fv, 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-senescent detached leaves of chlorina f24 (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 (Stroch et al., 2004, 2008). The mutant is deficient in light-harvesting complexes Lhcbl, Lhcb6 and Lhca4, and has reduced amount of Lhcb2, Lhcb3 and Lhcb4 (Bossmann et al., 1997). The amount of LHCs of PSI and PSII is reduced by about 80 % and 20 %, respectively (Ghirardi et al., 1986). The more reduced amount of LHCs of PSII (LHCCI) is in chlorina mutants compensated by an increased amount of reaction centers of PSI (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; Stroch 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 PSI 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 PSI 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 deacelorators of senescence, we also wanted to find out whether and to what extent is exogenously applied cytikinin 6-benzyaminopurine 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 f24 (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 10⁻³ mol l⁻¹ solution of 6-benzyaminopurine (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 MgCO₃ 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 carotenones 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 (VAY) by an HPLC system (Alliance c 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 an 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) × 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/dt₀), 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/ dt₀) = 4(F₃₀₀₅₃₀/F₅₀₀₅₃₀)F₅₀₀, where F₃₀₀₅₃₀ and F₅₀₀₅₃₀ are fluorescence intensities at the indicated times and F₅₀₀ is variable fluorescence (F₅₀₀ = F₇₀₀ – F₅₀₀, F₅₀₀ is a minimal fluorescence and F₇₀₀ is fluorescence at the P step). The (dV/ dt₀) parameter, defined as the maximal rate of the accumulation of the fraction of closed reaction centers of PSI (RCII) (Strasser et al., 2000), reflects the rate of excitation supply into the RCII and subsequently the rate of Qₐ reduction. Parameter Vₐ, reflecting the fraction of reduced Qₐ, was calculated as (F₉₁/F₅₀₀/F₅₀₀) and F₉₁ is fluorescence intensity at 2 ms. ABS/RC was calculated as (dV/ dt₀) /V J × F₉₁/F₅₀₀ and reflects apparent antenna size of active RCII (Strasser et al., 2000). Further, the quantum yield of electron transport from reduced Qₐ to final acceptors of PSI (RE/ABS) and the efficiency of electron transport from reduced plastoquinone to final acceptors of PSI (5R₀) were estimated as follows: RE/ABS = F₉₁/F₅₀₀ × (1 – V J) and 5R₀ = (1 – V J)/(1 – V J) (Stirbet et al., 2018). The measured OJIP curves as well as curves normalized to F₅₀₀ 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 F₀ 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 Fm. After 90 s of dark-relaxation, when the measured fluorescence signal reached F₀, 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 $F_{p}/F_{m} = (F_{m} - F_{0})/F_{m}$. The maximal quantum yield of PSII photochemistry in the light-adapted state was calculated as $F_{p}'/F_{m}' = (F_{m}' - F_{0})/F_{m}'$, where $F_{0}$ is minimal fluorescence for the light-adapted state, which was calculated as $F_{0}/(F_{p}/F_{m} + F_{p}/F_{m}')$. The effective quantum yield of PSII photochemistry in the light-adapted state was calculated as $\Phi_{p} = (F_{m}' - F_{0})/F_{m}'$, where $F_{1}$ 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 $\Phi_{NPQ} = (F_{m}' - F_{p}')/F_{m}'$ and the quantum yield for constitutive non-regulatory dissipation processes was calculated as $\Phi_{CD} = F_{0}/F_{m}'$. The sum of $\Phi_{p}$, $\Phi_{NPQ}$ and $\Phi_{CD}$ equals unity (for a review, see Lazár, 2015). In the case of $F_{p}'/F_{m}'$, $\Phi_{p}$, $\Phi_{NPQ}$, and $\Phi_{CD}$, 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 indicating an equilibrated maximal P700$^+$ level as a result of P700 oxidation by the charge separation and P700$^+$ reduction by plastoquinone for the light-adapted state, which was calculated as $F_{0}/(F_{p}/F_{m} + F_{p}/F_{m}')$. The effective quantum yield of PSII photochemistry in the light-adapted state was estimated as $\Phi_{f,D} = F_{0}/F_{m}$, where $F_{1}$ 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 $\Phi_{NPQ} = (F_{m}' - F_{p}')/F_{m}'$, where $F_{1}$ is fluorescence at time $t$ measured immediately prior to the application of the saturating pulse. The quantum yield for constitutive non-photochemical quenching was calculated as $\Phi_{CD} = F_{0}/F_{m}'$. The sum of $\Phi_{p}$, $\Phi_{NPQ}$ and $\Phi_{CD}$ equals unity (for a review, see Lazár, 2015). In the case of $F_{p}'/F_{m}'$, $\Phi_{p}$, $\Phi_{NPQ}$, and $\Phi_{CD}$, values obtained at the end of the actinic light exposition are presented.

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 carotenoids 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.

Besides the generally reduced content of photosynthetic pigments, the maximal quantum yield of PSI photochemistry in both dark-adapted state ($F_{p}/F_{m}$) and light-adapted state ($F_{p}'/F_{m}'$) 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 PSI photochemistry ($\Phi_{p}$), quantum yield of constitutive non-regulatory dissipation processes ($\Phi_{CD}$) and quantum yield of regulatory non-photochemical quenching ($\Phi_{NPQ}$). Together the sum of these quantum yields equals unity (Lazár, 2015). In clo, a slightly but significantly lower $\Phi_{p}$ and higher $\Phi_{CD}$ and $\Phi_{NPQ}$ were observed (Fig. 1D), which indicates that lower fraction of absorbed light energy was used by PSI 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 LHCCI (Ghirardi et al., 1986; Bossmann et al., 1997), a lower supply of excitations from LHCCI 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 QA as well as QB. This slower reduction consequently results in a lower transient accumulation of reduced QA, which is in turn reflected in a lower J-step. The lower relative height of J-step is quantitatively expressed by a lower VJ parameter (by about 14 %) (Fig. 2B and C). The slower QA reduction in the clo leaves is further indicated by $(dV/dt)_{0}$, 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 LHCCI 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/ABS (the quantum yield of electron transport from reduced QA to final acceptors of PSI) and $\delta_{0}$ (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

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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.
The loss of photosynthetic pigments during dark-induced senescence was pronouncedly lower in the clo genotype (by 98%) than in WT (Fig. 5). The decrease in Fv/Fm was accompanied by a decrease in Φps in both clo and WT, indicating decreased energy utilization by PSII photochemistry in the light-adapted state. The Φps value in clo was significantly lower than in WT (Fig. 5). On the other hand, Φnq and ΦD 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 Φnq and ΦD were comparable in WT, in clo ΦD 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-senescent 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 Vj (1.5-times when compared to leaves before senescence induction; Fig. 6C). The (dV/dθ) parameter also increased, but more (2.5-times) than Vj, 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 QA 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).

RE0/ABS as well as δR increased in the senescing WT leaves by about 80% and 45%, respectively (Fig. 6D). The greater decrease in RE0/ABS in comparison to δR indicates that the electron transport efficiency decreased more within PSII than behind PSII and that RCII 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 pronounced 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 (Vj increased twice compared to the leaves before senescence), (dV/dθ) 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 Fv/Fm. 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, RE0/ABS and δR were increased in the senescing leaves of clo (Fig. 6D). The decrease was again more pronounced in the case of RE0/ABS (by 98%) than in δR 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).
3.3. Effect of BA on senescence-induced changes in WT and clo leaves

To evaluate the effect of cytokinin on dark-senescent 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 decreased by about 15% (Fig. 3). The VAZ content decreased by about 35% and 20% in WT and clo, respectively (Fig. 3), and the Chl/car ratio in WT leaves decreased by about 15%.

BA also suppressed the senescence-induced decrease in PSI photochemistry in both WT and clo leaves (Figs. 4–6). In the presence of BA, F₀/Fₕ dropped only by 20% during the senescence, which indicated that PSI photochemistry is relatively well maintained (Fig. 4A). This was also evidenced by a smaller decrease in Φₚ (i.e., utilization of absorbed light energy by PSI photochemistry) in both WT and clo (Fig. 5). In clo BA significantly suppressed the senescence-induced increase in Φₚ,Δ (Fig. 5).

The protective effect of BA on PSII function in senescent 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., utilization of abscorbed light energy by PSII photochemistry) in both WT and clo (Fig. 6B). 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, Φₚ was 0.13 and 0.14, respectively. In WT, BA improved Φₚ only non-significantly (to 0.25), while in clo, the Φₚ 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 statistical testing (Holm-Sidak test) at $P < 0.05$ and statistically significant difference in following post hoc statistical testing (Holm-Sidak test) at $P < 0.001$ 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 pgi 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.

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 LHClII 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 LHClII in clo by lower ABS/RC ratio (Fig. 2C), reflecting lower amount of absorbed excitations per active (QA-reducing) RCII. The presence of smaller PSI antennae resulted in slower supply of excitations to the RCII, in slower QA reduction and smaller amount of reduced QA, which was evidenced by the lower ($dV/dt$) and $V_{I}$ parameters (Fig. 2C).

Despite the smaller LHClII in clo, the efficiency of electron transport behind PSII to PSII 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 $\Phi_{P}$; 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 ($\Phi_{NPQ}$) and/or regulatory ($\Phi_{D}$) non-photochemical quenching processes. $\Phi_{D}$ represents quantum yield of
constitutive (basal) energy dissipation (for a review see Lazár, 2015), whereas \( \Phi_{NPQ} \) 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 (Demming-Adams et al., 2014). As mentioned above, \( \text{clo} \) had lower \( \Phi_F \) (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 (\( \Phi_{NPQ}^{\text{NR}} \)) and regulatory component (\( \Phi_{NPQ}^{\text{R}} \)) 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 \( \text{clo} \) 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 \( \text{clo} \) plants had an enhanced photoprotection of photosynthetic apparatus when they were grown under relatively low light intensity (150 \( \mu \text{mol of photons m}^{-2} \text{s}^{-1} \)). The higher protection against photoinactivation of RCII has been reported by Štroch et al. (2004) in \( \text{clo} \) plants grown under similar light intensity (100 \( \mu \text{mol of photons m}^{-2} \text{s}^{-1} \)). The higher photoprotection of \( \text{clo} \) could be associated with the existence of free (not bound to LHGs) zeaxanthin (Havaux et al., 2007; Štroch et al., 2008; Nezval et al., 2017).

4.2. \( \text{clo} \) 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 LHCl.

This is in agreement with higher \( (dV/dt)_0 \) and \( V_f \) 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 \( \Phi_F \) decreased (Figs. 4 and 5), whereas the dissipation via both regulatory (\( \Phi_{NPQ}^{\text{R}} \)) and non-regulatory non-photochemical quenching processes (\( \Phi_{NPQ}^{\text{NR}} \)) 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 \( \text{RE}_{0}/\text{ABS}, \text{SR}_0 \), 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 \( \text{clo} \) mutant, we expected faster dark-induced senescence. Although the relative decrease in Chl content was similar in WT and \( \text{clo} \) (Fig. 3), the absolute Chl content was pronouncedly lower in the \( \text{clo} \) senescing leaves due to the lower Chl content in the leaves before senescence induction (Table 1). The pronounced decrease in Chl content in \( \text{clo} \) corresponded with more pronounced impairment of PSII function (Figs. 4–6). In fact, the senescing \( \text{clo} \) 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 \( \text{clo} \), as documented by extremely increased ABS/RC (and also by increased \( (dV/dt)_0 \) and \( V_f \) (Fig. 6C). Unlike WT, regulatory quenching processes were almost inactive and dissipation via non-regulatory processes prevailed, as indicated by pronouncedly increased \( \Phi_{NPQ}^{\text{NR}} \) (Fig. 5).

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

The substantially impaired PSII function in the dark-senescing leaves of \( \text{clo} \) is in agreement with the previous studies, reporting higher sensitivity of PSII photochemistry of \text{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, as shown in specific antenna proteins. Photosynth. Res. 52, 127–136.

Husiš and Vlčková et al., 2006; Talla et al., 2016; Vylíčková 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 LHCCI (Oh et al., 2005; Talla et al., 2016; Vylíčková 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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