Cytokinin delays dark-induced senescence in rice by maintaining the chlorophyll cycle and photosynthetic complexes

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Abstract

The phytohormone cytokinin (CK) is known to delay senescence in plants. We studied the effect of a CK analog, 6-benzyl adenine (BA), on rice leaves to understand the possible mechanism by which CK delays senescence in a drought- and heat-tolerant rice cultivar Nagina22 (N22) using dark-induced senescence (DIS) as a surrogate for natural senescence of leaves. Leaves of N22-H-dgl162, a stay-green mutant of N22, and BA-treated N22 showed retention of chlorophyll (Chl) pigments, maintenance of the Chl a/b ratio, and delay in reduction of both photochemical efficiency and rate of oxygen evolution during DIS. HPLC analysis showed accumulation of 7-hydroxymethyl chlorophyll (HmChl) during DIS, and the kinetics of its accumulation correlated with progression of senescence. Transcriptome analysis revealed that several plastid-localized genes, specifically those associated with photosystem II (PSII), showed higher transcript levels in BA-treated N22 and the stay-green mutant leaves compared with naturally senescing N22 leaves. Real-time PCR analyses showed that genes coding for enzymes associated with Chl a/b interconversion and proteins associated with light-harvesting complexes maintained higher transcript levels up to 72 h of DIS following BA treatment. The pigment–protein complexes analyzed by green gel remained intact in both N22-H-dgl162 and BA-treated N22 leaves even after 96 h of DIS. Thus, CK delays senescence by accumulation of HmChl and up-regulating genes in the Chl cycle, thereby maintaining the Chl a/b ratio. Also, CK treatment retains higher transcript levels of PSII-related genes, resulting in the stability of photosynthetic pigment complexes and functional stay-greenness in rice.

Key words: Chlorophyll cycle, cytokinin, dark-induced senescence, hydroxymethyl chlorophyll, pigment–protein complex, rice, stay green.

Introduction

In higher plants, photosynthesis is the process by which light energy from the sun is converted to chemical energy. Chlorophylls (Chls), associated with two pigment–protein complexes, namely photosystem I and II (PSI and PSII), located in the thylakoid membranes of the chloroplast, play a central role in this process. Chl a and Chl b, the main...
constituents of the photosynthetic apparatus, are identical molecules for the side chain at C7, which is a methyl group in the former and a formyl group in the latter (Rüdiger, 2002). Chl \(_a\) is synthesized from glutamate by several steps and is converted to Chl \(_b\) via an intermediate, 7-hydroxymethyl chlorophyll (HmChl), in a cyclic reaction called the Chl cycle, which is well conserved across all land plant species and is important for photosynthesis by green leaves (Tanaka and Tanaka, 2007). The primary energy conversion reactions of photosynthesis are executed in the reaction center (RC), a complex of several proteins, pigments, and cofactors, which, along with the light-harvesting complexes (LHCs), form PSI and PSII. Chl \(_a\) is a component of the RC and LHC complexes of both PSI and PSII. The LHC comprises Chl \(_a\), Chl \(_b\), xanthophylls/carotenoid pigments, and proteins (Lhca1–Lhca4 in PSI, Lhcb1–Lhcb6 in PSII) which collectively form the antenna complex. Conversion of Chl \(_a\) to Chl \(_b\) is by two-step oxidation catalyzed by the enzyme chlorophyll \(_a\) oxygenase (Cao) in rice (Morita et al., 2005). Chl \(_b\) is then converted back to Chl \(_a\) by two-step reduction, thus constituting a cycle. The first reduction is by Chl \(_b\) reductase which requires heterodimerization of non-yellow coloring 1 (Nyc1) and Nyc1-like (Nol) gene products in rice (Kusaba et al., 2007; Sato et al., 2009) to yield HmChl, and a second reduction is by HmChl reductase (Hcar), resulting in Chl \(_a\) (Sakuraba et al., 2013). The Chl cycle plays a crucial role in the greening process, light acclimatization, and leaf senescence.

Leaf senescence is the endogenously regulated degradation process resulting in irreversible yellowing and finally death of the organ. Several genes that were found to be highly differentially expressed during senescence have been referred to as senescence-associated genes (SAGs), and many of these have been used as molecular markers indicative of the process. Genetic variants of plants showing retention of leaf greenness and delay of senescence exhibit the stay-green phenotype (Hörtensteiner, 2006). Degradation of the photosynthetic pigment Chl \(_a\) is one of the major events during leaf senescence. Stay-green mutants are ideal subject for studying Chl degradation (Cha et al., 2002; Armstead, 2007; Jiang et al., 2007; Morita et al., 2009; Sato et al., 2009; Schelbert et al., 2009). Five categories of stay-green traits have been described (Thomas and Howarth, 2000), of which two categories, Type A and B, are of agronomic importance because the delay of senescence in Type A plants is due to delay in initiation whereas in Type B plants it is due to the slower progression of Chl degradation and loss of photosynthetic efficiency.

Interplay of phytohormones can regulate senescence, and it is known that cytokinins (CKs), auxins, and gibberellins (GA\(_3\)) delay, while salicylic acid, jasmonic acid, abscisic acid (ABA), and ethylene accelerate leaf senescence. The auxin indole-3-acetic acid (IAA) is known to be involved in retarding senescence in detached senescing leaves of Arabidopsis (Noh and Amasino, 1999; Cohen et al., 2003). In plants, IAA is synthesized by two major pathways, namely a tryptophan-independent pathway where indole-3-glycerol phosphate (IGP) is the direct precursor, and a tryptophan-dependent pathway where tryptophan derived from IGP is the precursor (Mano and Nemoto, 2012). In contrast, ethylene and ABA promote senescence. Exogenous application of ethylene promotes visible yellowing of leaves, and several ethylene biosynthesis genes are up-regulated during senescence (van der Graaf et al., 2006). Ethylene is synthesized in a few highly regulated steps by conversion of \(\text{S}-\text{adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, which is then converted to ethylene by ACC oxidase (ACO). Transgenic broccoli containing an antisense ACC oxidase gene showed major reduction in ethylene production and improvement in head color changes after harvest (Henzi et al., 2000). ABA is converted to phasic acid as a result of hydroxylation by ABA-8’ hydroxylase (Ahs), a cytochrome P450 monooxygenase. The synthesis as well as the breakdown of ABA regulates its endogenous levels. A lesion-mimic rice mutant (spl3) is insensitive to ABA and shows delayed dark-induced and natural senescence (Wang et al., 2015).

The plant hormone CK retards senescence in detached leaves and promotes greening in plants (Richmond and Lang, 1957; Kakimoto, 2003). Heterologous expression of the Agrobacterium tumefaciens isopentyl transferase (IPT) gene coding for an enzyme which catalyzes the rate-limiting step of CK production results in increased levels of endogenous CK in plants (Akiyoshi et al., 1984). Transgenic plants of important crop species including rice, cassava, and cotton expressing the IPT gene under the SAG12 promoter convincingly show that CK delays leaf senescence (Lin et al., 2002; Zhang et al., 2010; Liu et al., 2012). The stay-green phenotype caused by overexpression of the IPT gene induced extreme drought tolerance (Rivero et al., 2007) and delayed stem and leaf senescence (Ma and Liu, 2009). CKs play a role in preserving the structure and function of the photosynthetic machinery under stress conditions (Cherniav, 2009). CK increases sink activities by stimulating assimilate accumulation in chloroplasts of older leaves (Criado et al., 2009). The role of CK in the biosynthesis of Chl, stimulation of tetrapyrrole biosynthesis, chloroplast transcription (Zubo et al., 2008), and enhancement of photosynthetic efficiency has also been demonstrated (Yaronskaya et al., 2006). CK has been reported to retard Chl degradation (Kao, 1980; Jordi et al., 2000). However, the actual step of CK involvement in the Chl metabolic pathway and the mechanisms by which it could retard senescence are not known. An ethylmethane sulfonate (EMS)-induced, stay-green mutant (N22-H-dgl162) of the Nagina22 (N22) rice cultivar showing delayed senescence was earlier identified in a field screen under prolonged drought and heat conditions (Panigrathy et al., 2011). The aim of this study was to investigate the mechanism of CK-mediated delay of dark-induced senescence (DIS) in rice, taking cues from the stay-green mutant. We studied the effect of 6-benzyl adenine (BA) on the photosynthetic apparatus in rice leaves during DIS by green gel analysis and HPLC profiling of the pigments and their identification by MS/MS analysis. We analyzed the transcriptome of BA-treated versus untreated N22 leaves to identify genes induced by CK during DIS, Real-time PCR analyses of genes associated with PSII, senescence, and the Chl cycle were carried out to understand the possible mechanism by which CK delays senescence in rice leaves.
Materials and methods

Plant growth conditions

For all experiments, rice seedlings were grown in similar size pots (five plants per pot) containing field soil, in a greenhouse with controlled temperature settings of 30 °C/25 °C during 16 h light (20 μmol m⁻² s⁻¹) of photosynthetic photon flux/8 h dark cycle, respectively, and 60% constant humidity. Fifteen plants at the four-leaf stage were covered with cardboard boxes for dark treatment in the greenhouse. The middle portion of the third leaf from the apex was taken in all experiments. Treatment of detached leaves with a 2 mg ml⁻¹ solution of BA was as described by Jiang et al. (2007), followed by exposure to DIS at different time intervals ranging from 0 h to 96 h with or without BA. Dark treatment was used as a means to induce senescence.

Photosynthesis measurement

Detached leaves were blotted dry after treatments at various time points and transferred into a leaf disc oxygen electrode chamber (LD-2; Hansatech Instruments Ltd, King’s Lynn, UK). The topmost capillary matting was moistened with 200 μl of 1 M bicarbonate buffer (pH 9.0), which results in a gaseous atmosphere of ~5% (v/v) CO₂ in the chamber. The leaf discs were arranged on this matting symmetrically in three successive rings of one, six, and 12. Oxygen in the chamber was calibrated for every sample as per the manufacturer’s instruction. Photosynthetic oxygen evolution was measured at 25 °C by a computer program, supplied by the manufacturer.

Chlorophyll fluorescence measurement

A portable pulse amplitude-modulated fluorescence meter (Walz, Effeltrich, Germany) was used to obtain measurements of leaf Chl fluorescence. The measurements were taken at 20 °C and in green light. Initial (Fₒ), maximal (Fm), and variable (Fm–Fᵣ) Chl fluorescence were determined directly after dark acclimation. To obtain Fm, a light pulse of 2000 μmol m⁻² s⁻¹ was applied. The quantum yield under illumination [(Fm–Fᵣ)/Fm] was determined during the measurement. The experiment was carried out three times.

Pigment extraction and analysis

Leaf samples (~200 mg) were ground to a fine powder with liquid nitrogen, and pigments were extracted with 80% acetone. The samples were centrifuged at 15 000 g for 10 min and the supernatant was used for Chl estimation and HPLC analysis (Roca et al., 2004). Chls were determined according to Arnon (1949) using a Shimadzu UV-VIS spectrophotometer (model UV-2600, Japan). HPLC analysis of Chl pigments was done according to Jiang et al. (2007) by using an Agilent HPLC 1100 series equipped with a C-18 column (Waters Nova-Pak, 3.9 × 150 mm) and a dual absorbance detector. Separation was carried out on an elution gradient with the mobile phases (A) ion pair reagent (1 M ammonium acetate in water)/methanol (1:4, v/v) and (B) acetone/methanol (1:4, v/v), at a flow rate of 1.2 ml min⁻¹. The gradient was isocratic A for 4 min, isocratic B for 20 min, and a return to A for 6 min, and detection was at 660 nm. The absorption spectrum of each peak was obtained from their respective chromatograms in the HPLC profile. The eluted Chl pigment samples were collected in special eppendorf tubes. The HPLC-purified samples were vacuum centrifuged for 2 h to a powdered form for further analysis.

MS analysis

The dried pellet of HPLC-purified Chl pigments was dissolved in 50% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), and 1 μl of matrix (5 mg ml⁻¹ 50% ACN containing 0.1% TFA) was spotted on the MALDI (matrix-assisted laser desorption ionization) target plate. The sample was allowed to air dry. The mass spectra were acquired using a 4800 MALDI TOF-TOF analyzer obtained from Applied Biosystems (Foster City, CA, USA). The mass spectrometer was fitted with an Nd:YAG laser (355 nm) to ionize samples at 200 Hz. The ion path lengths of linear, reflector, and MS/MS modes were 1.5, 3, and 2.4 m, respectively. The instrument consists of a high-energy collision-induced (CID) cell, and spectra were obtained using air as the CID gas with 1 kV and 2 kV energy in the positive ion mode.

Microarray analysis

Total RNA was extracted with TRIzol reagent (Sigma Aldrich) from BA-treated and untreated detached N22 leaves after 72 h of DIS and used for hybridization of a Rice Affymetrix gene-chip (51K arrays) containing probe sets designed from 48 564 japonica and 10 260 indica gene sequences according to the Affymetrix GeneChip expression analysis technical manual. Three biological replicates were used for the experiment. Annotation of the differentially expressed probes was done using NetAffx software of Affymetrix and further validated using BLASTX search through NCBI. The microarray data have been submitted to the GEO repository; they were assigned the GEO accession number GSE55902, and can be viewed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55902

Quantitative PCR (qPCR) analysis

Total RNA from rice leaves was treated with RNase-free DNase I (Invitrogen) to remove DNA and used for reverse transcription with a superscript-III cDNA synthesis kit (Invitrogen). Real-time PCR was performed as described previously (Jisha et al., 2015). Rice OsActin1 was used as internal control, and relative gene expression levels were calculated using the 2⁻ΔΔCT method [ΔCₜ indicates –(Cₜ of target – Cₜ of OsActin)] and Cₜ is the threshold cycle number of the amplified gene.

Native gel electrophoresis of chlorophyll–protein complexes

The native Chl–protein complexes were separated as described by Allen and Staehelin (1991) with some modifications. To isolate thylakoid membranes, leaves were ground using a mortar and pestle with liquid nitrogen in an ice-cold grinding buffer (50 mM HEPES, pH 7.6, 0.3 M sorbitol, 10 mM NaCl, 5 mM MgCl₂), filtered through two layers of Miracloth, and centrifuged at 3000 g for 5 min at 4 °C. The supernatant was centrifuged at 20 000 g for 7 min and the pellet was then washed twice and resuspended in buffer containing 50 mM HEPES, pH 7.6, 0.1 M sorbitol, 10 mM NaCl, 5 mM MgCl₂. For electrophoresis, the resolving gel (8.0%) contained 25 mM TRIS-HCl (pH 8.8) and 10% glycerol, while the stacking gel (4.0%) contained 25 mM TRIS-HCl (pH 6.8) and 10% glycerol. The electrode buffer contained 25 mM TRIS, 192 mM glycine (pH 8.3), and 0.1% SDS. Before electrophoresis, the isolated thylakoid membrane suspension was centrifuged and the pellet was washed twice in 2 mM TRIS-maleate (pH 7.0) (Oh et al., 2003) and resuspended in solubilization buffer (4% digitonin, 1% glycerol). The samples were kept on ice for 30 min, and insoluble materials were removed by centrifugation at 15 000 g for 10 min. The samples were equally loaded onto each well and then normalized to the fresh weight; following electrophoresis, the green gel was photographed under visible light.

Western blot analysis

Rice leaves were ground into fine powder in liquid nitrogen and protein was extracted with 5 ml of ice-cold grinding buffer (50 mM HEPES, pH 7.6, 0.3 M sorbitol, 10 mM NaCl, 5 mM MgCl₂) per 150 mg FW of leaves. The homogenate was filtered through two layers of Miracloth and centrifuged at 3000 g for 5 min at 4 °C to remove the debris. The protein content was determined by Bradford assay (Biorad) and 50 μg of protein for each sample were diluted to 1× concentration using 5× SDS loading buffer (Takara) and subjected to SDS-PAGE after boiling. The proteins were separated by 10% SDS-PAGE, and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Amersham) according to standard procedures. Blots were probed with rabbit anti-PsbP [a 23kDa protein from the oxygen-evolving complex (OEC) of PSII] antibodies (1:2000) and detection was with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:5000) followed by the ECL-Plus Western blotting reagent.
Detection system (Roche) according to the manufacturer’s instructions. Antibodies were from Agrisera.

Statistics
The data presented are the mean values (±SE) of results from 3-4 experiments conducted on different days. The data were subjected to further statistical significance by one-way ANOVA using SigmaPlot Version 11.0.

Primers
Primers used in this study are listed in Supplementary Table S1 at JXB online.

Results
Cytokinin-mediated changes in physiological parameters during DIS in rice

Detached leaves of an EMS-induced, stay-green mutant (N22-H-dgl162) of the drought- and heat-tolerant rice cultivar N22 remained green, whereas detached leaves of N22 turned yellow when subjected to DIS for 5 d (Fig. 1A). Exogenous application of CK to detached leaves has been shown to prevent senescence (Richmond and Lang, 1957). Treatment of

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**Fig. 1.** Effect of cytokinin treatment on rice leaves. (A) Phenotypic appearance of 4-week-old detached leaves of N22 with (+BA) or without BA (–BA) and N22-H-dgl162 (162) incubated in the dark for 5 d. (B) Total chlorophyll (mg g⁻¹ FW leaves). (C) Chl a/b ratio. (D) Photosynthetic oxygen evolution (µmol O₂ m⁻² s⁻¹). (E) Chlorophyll fluorescence parameters expressed in terms of the $F_v/F_m$ ratio in detached N22 rice leaves with and without BA treatment and N22-H-dgl162 leaves at different time intervals of dark incubation. Data represents mean values (± SE) from at least four independent experiments. Asterisks indicate statistically significant differences ($P<0.05$) between the control and BA-treated N22/N22-H-dgl162 cut leaves. (This figure is available in colour at JXB online.)
detached N22 leaves with BA, a synthetic CK, retarded yellowing, and the leaves remained green even after 120 h of DIS (Fig. 1A). Thus, treatment of N22 leaves with BA maintained greenness and retarded yellowing during DIS.

During the ontogenic progress of senescence, there is a sequential loss of Chl content and photosynthetic functions in barley leaves (Wiedemuth et al., 2005). The total Chl level was higher at all time points in N22-H-dgl162 and BA-treated N22 leaves when compared with untreated N22 leaves (Fig. 1B). The Chl alb ratio increased from 3.0 to 4.0 in BA-treated leaves after 24 h of DIS and remained constant till 72 h, followed by a slight decrease at 96 h. In the untreated N22 leaves, the Chl alb ratio decreased from 3.0 to 2.2 after 96 h of DIS. In N22-H-dgl162 leaves, the ratio remained constant at ~3.0 even after 96 h of DIS (Fig. 1C). Thus, maintaining both a higher Chl level and a higher Chl alb ratio distinguishes the mutant and BA-treated N22 leaves from untreated senescing N22 leaves.

The functionality of stay-greenness in mutant and BA-treated N22 leaves was evaluated during DIS by measuring the rates of photosynthetic oxygen evolution and characteristics of Chl fluorescence (Fv/Fm). Photosynthetic oxygen evolution measurements showed that the rate decreased from 5.4 μmol O₂ m⁻² s⁻¹ at 0 h to 3.6 μmol O₂ m⁻² s⁻¹ in BA-treated N22 and from 7.0 μmol O₂ m⁻² s⁻¹ to 4.5 μmol O₂ m⁻² s⁻¹ in N22-H-dgl162 leaves after 96 h of DIS. In the untreated N22 leaves, the rate of O₂ evolution decreased steadily to 2 μmol O₂ m⁻² s⁻¹ after 96 h of DIS (Fig. 1D). The Fv/Fm ratio is indicative of maximum efficiency of the PSII photochemical process. The Fv/Fm ratio remained constant at ~0.7 in N22-H-dgl162 and BA-treated N22 leaves but decreased steadily to 0.5 in untreated N22 leaves after 96 h of DIS (Fig. 1E). This result indicates that after 96 h of DIS the PSII apparatus functioned more efficiently in N22-H-dgl162 and BA-treated N22 leaves than in untreated N22 leaves.

Enhanced greenness under dark conditions in CK-treated leaves of rice is accompanied by accumulation of chlorophyll intermediates

The photosynthetic pigment profiles were analyzed by HPLC in order to study the stability of Chl pigments. Pigments extracted from fresh N22 leaves (0 h control) showed two peaks at 660 nm in the HPLC chromatogram, which correspond to Chl b and Chl a based on the absorption spectra, with retention time (tret min⁻¹) values of 10.3 and 10.9, respectively (Fig. 2A). In addition to the two peaks obtained in the 0 h control, the leaves without BA treatment showed a peak at 11.1 whereas BA-treated leaves and N22-H-dgl162 leaves showed two peaks at 10.5 and 11.1 after 72 h of DIS (Fig. 2A). To study the kinetics of the 10.5 peak accumulation, peak area was calculated from HPLC runs of BA-treated N22 samples at different time intervals of DIS. The accumulation kinetics showed a linear increase in the 10.5 peak area from 24 h to 96 h, with a decline only at 120 h of dark incubation (Fig. 2B). It is interesting to note that it is during this 96–120 h period that leaves begin to turn yellow.

MS was used to identify the Chl derivatives obtained by HPLC fractionation. One or more ionic species in solution can be detected and characterized based on the m/z values obtained by electron spray ionization (ESI)-MS. The pigment fractions separated by HPLC for individual samples were pooled and analyzed by applying positive ion mode. N22 leaves at 0 h showed two ionic species, Chl b (908.5) and Chl a (983.5), corresponding to the two HPLC peaks at 10.3 and 10.9 tret min⁻¹, respectively (Fig. 3). N22 leaves without BA treatment after 72 h of DIS showed three ionic species with m/z values of 893.5, 908.5, and 915 corresponding to Chl b, Chl a, and the sodium adduct of Chl a, respectively. On the other hand, BA-treated N22 leaves after 72 h of DIS showed several additional peaks, the most consistent being ionic species with m/z values of 910 and 925, which correspond to 7-hydroxymethyl Chl a (910) and hydroperoxyl Chl a (925).

![Graph showing enhanced greenness under dark conditions in CK-treated leaves of rice.](Image)
It is known that treatment of Chl b with sodium borohydride (NaBH₄) results in the formation of HmChl (Ito et al., 1996). In order to confirm the identity of the accumulated intermediate, authentic HmChl was prepared by reduction of Chl b with NaBH₄ and 8-hydroxyquinoline (8-HQ) in methanol. N22 leaves treated with 8-HQ and methanol alone showed a minor peak at 10.5 \( t_{\text{ret}} \) min\(^{-1}\) (Fig. 4A), which can be explained by the fact that addition of 8-HQ stabilizes HmChl. Addition of NaBH₄ resulted in a major peak at retention time 10.5 min and two other peaks at 10.2 min and 10.8 min (Fig. 4B). The absorption spectrum of the 10.5 min peak in BA-treated samples matched that of HmChl (Ito et al., 1996; Fig. 4C). These

![Mass spectra of HPLC-fractionated samples from N22 leaves at 0 h and 72 h of DIS without and with BA treatment. Three independent experiments were performed and only the consistently occurring peaks are labeled.](image-url)
results confirmed that HmChl accumulated in the dark-induced Chl extracts of BA-treated samples of N22 at 72 h of DIS.

**Cytokinin-mediated molecular events in delay of DIS**

To gain insight into the molecular events in the delaying of DIS by CK, microarray analysis of BA-treated versus untreated N22 control samples was carried out using the Affymetrix 3’UTR (untranslated region) rice genome array consisting of 57,381 probe sets. Genes showing a >1.5-fold difference with a P-value <0.05 from three biological replicates were selected. Up-regulated pathways included CK regulation, auxin biosynthesis, nitrogen metabolism, and response to oxidative stress, whereas cellulose biosynthetic, carbohydrate metabolic, ethylene-responsive, and GABA (γ-aminobutyric acid) shunt pathways were down-regulated. The details of differentially regulated genes are listed in Table 1.

Two genes of the A-type two-component CK signal transduction system (OsRR1 and OsRR4) and CK-responsive genes such as cytokinin dehydrogenase/oxidase (Cdg), iso-flavanol reductase, and dihydroflavanol reductase were up-regulated in the BA-treated N22 leaves, suggesting a negative feedback regulation commonly observed in many cell signaling systems (Hirose et al., 2007; Tsai et al., 2012) and homeostatic control of CK activity (Wang et al., 2011).

Several genes related to the biosynthesis of the auxin IAA via different pathways were up-regulated in BA-treated leaves during DIS (Table 1). The levels of ABA-8’ hydroxylase and a ubiquitin-ligase gene negatively regulating ABA biosynthesis were increased 7-fold and 2.5-fold, respectively, suggesting increased ABA catabolism upon BA treatment. The ethylene biosynthetic genes, ACC synthase and ACC oxidase, were down-regulated in BA-treated rice leaves under DIS. This indicates that the auxin pathway is up-regulated while ABA and ethylene pathways are down-regulated by CK during DIS.

Microarray analysis showed higher expression of several genes associated with PSII of the photosynthesis light reaction in BA-treated leaves when compared with untreated N22 leaves during DIS. These included Lhcb4 and Lhcbb6, genes encoding Chl alb-binding proteins (Cbp) from the LHC, and the oxygen-evolving enhancer genes PsbO and PsbP from the OEC of PSII. In addition, the cytochrome b$_{5}$f complex subunit, a component of the light reaction of photosynthesis, was up-regulated. Thus, CK regulates the light-harvesting and oxygen-evolving functions in the chloroplast. However, protoporphyrinogen oxidase (Ppo) involved in Chl biosynthesis showed lower expression. We also found that expression of Rubisco genes, glutamate synthase, and glutamate dehydrogenase genes involved in nitrogen metabolism were increased upon treatment of N22 leaves with BA during senescence induced by dark treatment. The apparent increased expression of photosynthesis-related genes in BA-treated leaves may well be due to a decrease in the transcripts in the untreated control. It has been shown previously that a high yield of the rice cultivar Akenohoshi was due to maintenance of rbcL and rbcS transcript levels during senescence, whereas the lesser yielding Nipponbare showed a decline in these transcripts and CK could account for the difference in reduction of Rubisco during senescence between cultivars (Ookawa et al., 2004).

SAGs which have been reported to be up-regulated during senescence in earlier studies were found to be down-regulated in BA-treated leaves under DIS. These include glutamate decarboxylase (Gad) and aminotransferase (Amt) involved in the GABA shunt pathway (Ansari and Chen, 2009), SAG 29, the SWEET gene (Mtn), hexokinase (Hks), and β-glucosidase (Bgs) involved in sugar metabolism, and a group of genes encoding nutrient transporters such as a sugar transporter gene, ABC transporter, and potassium transporter. Our results showed that among the SAGs known to be down-regulated during senescence but found to be up-regulated upon BA treatment were two invertases (Inv) involved in sugar metabolism. Increased expression of invertase has been reported to be required for delay of senescence mediated by CK in tobacco (Lara et al., 2004).

We analyzed the expression of genes representative of various functions by qPCR. The expression of Cdg, OsRR1,
and OsRR4, genes involved in CK metabolism and signaling, and Ahs involved in ABA degradation was higher and PSII-related genes such as Cbp (Lhcb4) and Oep also showed higher expression in BA-treated samples during 72h DIS (Fig. 5A). On the other hand, Amt, Gad, Mtn, Hks, and Bgs genes involved in sucrose metabolism and Ppo involved in Chl

| Broad functional category | Gene locus ID | Gene name | Fold change |
|---------------------------|--------------|-----------|-------------|
| Cytokinin metabolism      | LOC_Os01g10110 | CK dehydrogenase/oxidase | 19.6 |
|                           | LOC_Os01g72330 | OsRR4 type-A response regulator | 3.0 |
|                           | LOC_Os04g36070 | OsRR1 | 3.1 |
|                           | LOC_Os03g06824 | Dihydroflavonol-4-reductase | 2.4 |
|                           | LOC_Os01g13610 | Isoflavone reductase | 2.9 |
| ABA metabolism            | LOC_Os08g36860 | ABA hydroxylase | 7.0 |
|                           | LOC_Os12g17880 | Ubiquitin protein ligase | 2.58 |
| Ethylene metabolism       | LOC_Os11g37520 | Ethylene overproducer 1-like | –1.9 |
|                           | LOC_Os02g53180 | ACC oxidase | –4.0 |
|                           | LOC_Os06g03990 | ACC synthase | –1.7 |
| Auxin metabolism          | LOC_Os03g03450 | Anthranilate phosphoribosyltransferase | 2.2 |
|                           | LOC_Os09g08130 | Indole-3-glycerol phosphate synthase | 8.1 |
|                           | LOC_Os07g08430 | Tryptophan synthase alpha-subunit | 2.4 |
|                           | LOC_Os06g42560 | Tryptophan synthase beta chain 2 | 1.8 |
|                           | LOC_Os02g42350 | Indole-3-acetonitrile nitrilase | 1.7 |
|                           | LOC_Os01g06660 | Indole-3-pyruvic acid decarboxylase | 3.5 |
|                           | LOC_Os12g32750 | Tryptamine monoxygenase | –2.0 |
| PSII complex              | LOC_Os07g04840 | PSBP-1, 23kDa oxygen evolving complex | 2.1 |
|                           | LOC_Os07g37030 | Cytochrome b6-f complex iron-sulphur subunit | 2.2 |
|                           | LOC_Os07g37240 | LHCB4.2 chlorophyll A-B binding protein | 2.6 |
|                           | LOC_Os11g3690 | PSBO1, oxygen-evolving enhancer protein 1 | 2.3 |
|                           | LOC_Os04g38410 | Lhcb6 chlorophyll A-B binding protein | 3.2 |
|                           | LOC_Os12g19470 | Ribulose bisphosphate carboxylase small chain | 2.9 |
|                           | LOC_Os12g19381 | Ribulose bisphosphate carboxylase small chain | 2.5 |
| GABA metabolism           | LOC_Os08g36320 | Glutamate decarboxylase | –2.6 |
|                           | LOC_Os03g44150 | Aminotransferase | –1.6 |
| Carbohydrate metabolism   | LOC_Os02g34560 | Plant neutral invertase | 2.6 |
|                           | LOC_Os11g07440 | Plant neutral invertase | 3.0 |
|                           | LOC_Os01g53930 | Hexokinase | –1.6 |
|                           | LOC_Os11g31190 | Nodulin MfN3 family protein | –3.6 |
|                           | LOC_Os12g29220 | SAC29 | –14.5 |
|                           | LOC_Os03g11420 | beta-Glucosidase (SAG) | –3.9 |
| Chlorophyll metabolism    | LOC_Os04g41260 | Protoporphyrinogen oxidase | –2.9 |
| Nitrogen metabolism       | LOC_Os04g45970 | Glutamate dehydrogenase (NAD+) | 2.8 |
|                           | LOC_Os07g46460 | Glutamate synthase | 2.2 |
| Oxidative stress          | LOC_Os10g38340 | Glutathione S-transferase | 4.2 |
|                           | LOC_Os01g72140 | Glutathione S-transferase | 10.5 |
|                           | LOC_Os01g27390 | Glutathione S-transferase | 9.9 |
|                           | LOC_Os10g38600 | Glutathione S-transferase | 5.7 |
|                           | LOC_Os10g38360 | Glutathione transferase | 5.1 |
|                           | LOC_Os10g38640 | Glutathione S-transferase 4 | 4.2 |
|                           | LOC_Os10g38340 | Glutathione transferase | 4.2 |
|                           | LOC_Os10g38780 | Glutathione S-transferase | 2.8 |
|                           | LOC_Os10g27210 | Glutathione S-transferase | 2.1 |
|                           | LOC_Os04g17050 | Glutaredoxin/GST | 2.1 |
|                           | LOC_Os09g23370 | Glutathione-dependent-formaldehyde | 1.9 |
|                           | LOC_Os01g72160 | activating enzyme | 1.9 |
|                           | LOC_Os12g26250 | Glutathione transporter | 1.8 |
| Triacylglycerol degradation| LOC_Os01g46370 | Triacylglycerol lipase | –1.9 |
|                           | LOC_Os05g11910 | GDSL-like lipase/acylhydrolase | –8.4 |
|                           | LOC_Os06g06290 | GDSL-like lipase/acylhydrolase | –9.5 |
| Transport genes           | LOC_Os02g17500 | Sugar transporter | –1.9 |
|                           | LOC_Os05g04610 | ABC transporter | –2.6 |
|                           | LOC_Os01g70490 | Potassium transporter | –1.6 |
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biosynthesis showed reduced expression, while the \textit{Inv} gene which is normally down-regulated during senescence showed increased expression in BA-treated leaves following 72 h of DIS (Fig. 5B). We also compared the expression of these genes during natural leaf senescence. Similar results were obtained when the expression of genes from naturally senescing third youngest N22 leaves from 48-day-old plants were compared with still green N22-\textit{H-dgl162} leaves of the same age and position in the plant (Fig. 5C, D), suggesting similarity of events during natural senescence and DIS. The only exception was expression of the \textit{Gad} gene in N22-\textit{H-dgl162} leaves, indicating that glutamate decarboxylase which converts glutamate to GABA is up-regulated only during natural senescence (Fig. 5D). It has been reported that GABA may have a role as a signal molecule in co-ordinating carbon:nitrogen balance during developmental and not dark- or starvation-induced senescence (Buchanan-Wollaston \textit{et al.}, 2005).

**Expression levels of genes involved in Chl a/b conversion**

Expression levels of the four genes involved in the Chl \textit{a/b} cycle were analyzed by qPCR to follow the kinetics of regulation. BA-treated N22 leaves were compared with untreated N22 leaves at 0, 24, 48, 72, and 96 h of DIS. Expression of the \textit{Actin} gene was used as an internal control to normalize the expression levels in all the samples. There was an increase in expression of \textit{Cao} within 24 h of BA treatment, with the level remaining high till 72 h of DIS, whereas the level decreased in untreated samples after 72 h of DIS when compared with untreated non-DIS control, suggesting that \textit{CaO} is repressed under senescence. \textit{Nol} and \textit{Hcar} showed a similar pattern of expression to \textit{CaO}, being strongly expressed 72 h after DIS in response to BA treatment, and showed a decrease in expression in DIS leaves compared with untreated, non-DIS control (Fig. 6). \textit{Nyc1} showed reduced expression in both untreated and BA-treated samples, indicating that it is strongly repressed during dark treatment.

**Cytokinin stabilizes chlorophyll–pigment complexes**

Microarray analysis indicated that PSII-related genes were differentially expressed, and Chl analysis revealed that Chl \textit{a/b} ratios and \textit{Fv}/\textit{Fm} values were maintained during DIS in N22-\textit{H-dgl162} and BA-treated N22 leaves, suggesting intactness of pigment–protein complexes. To study the effect of BA treatment on the stability of Chl–protein complexes, we performed non-denaturing green gel analysis. In the untreated leaves at 0 h, three distinct major bands representing the RC–LHC complex, the LHCs, and free pigments were observed (Fig. 7A). In the untreated N22 leaves, the complexes were drastically reduced by

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**Fig. 5.** Transcript levels of genes differentially expressed in microarray analysis. Total RNA was extracted from N22 leaves subjected to 72 h of DIS, with and without BA treatment (A, B) or the third youngest leaf of 48-day-old N22 and N22-\textit{H-dgl162} rice plants (C, D). Real-time PCR was performed with primers for genes which include (A, C) phytohormone- and PSII-related: ABA hydroxylase (Abs), CK dehydrogenase (Cdg), OsRR1 (Rr1), OsRR4 (Rr4), Lhcb4 chlorophyll A-B binding protein (Cbp), and PSBP-1, 23 kDa oxygen-evolving complex (Oep); (B, D) senescence-associated genes (SAGs): aminotransferase (Amt), glutamate decarboxylase (Gad), SAG29 (Mtn), protoporphyrinogen oxidase (Ppo), hexokinase (Hks), \(\beta\)-glucosidase (Bgs), and plant neutral invertase (Inv). \textit{Actin} was used as internal standard. Data represent the mean ± SE from three independent biological replicates, and an asterisk indicates a \(P\)-value of 0.05.
Fig. 6. Expression pattern of genes encoding enzymes involved in the Chl a/b cycle. Total RNA was extracted from N22 leaves without and with BA treatment for 0, 24, 48, and 72 h of dark incubation. Real-time PCR was performed with primers for (A) Cao (chlorophyllase a oxygenase); (B) Nol (Nyc1-like); (C) Nyc1 (non-yellow coloring 1); and (D) Hcar (hydroxymethyl Chl a reductase). Primer details are given in Supplementary Table S1. OsActin was used as control.

Fig. 7. Electrophoretic analysis of pigment–protein complexes. (A) Green gel analysis of thylakoid membranes isolated from cut leaves of N22 and N22-H-dgl162 incubated in the dark with or without BA treatment at different time intervals and solubilized with a detergent. Samples loaded into each well of the non-denaturing native gel were normalized to the fresh weight of leaves. Labels indicate: RC-LHC, reaction center–light harvesting complex; LHC, light-harvesting complex; FP, free pigment. (B) Western blot analysis of PsbP protein from untreated and BA-treated leaves of N22 and N22-H-dgl162 leaves at 0 h and different times of dark incubation. Extracts were prepared in 5 ml of extraction buffer for 150 µg FW of leaf for each sample and 50 µg of protein was loaded on each lane after estimation using the Bradford method. Anti-Psb antiserum was used to detect protein, and a single band of ~23 kDa was obtained. (This figure is available in colour at JXB online.)
72 h of DIS and degraded by 96 h of DIS, whereas all the bands remained intact in N22-H-dgl162 and BA-treated N22 leaves after 96 h of DIS (Fig. 7A). However, BA treatment of N22-H-dgl162 affected the complexes, and degradation was observed at 96 h of DIS. Hence, Chl–pigment complexes were stable in N22-H-dgl162 and BA-treated N22 leaves during DIS in rice leaves.

Western blot analysis was performed to examine the change in the amount of PsbP, a component of the OEC of PSII which showed a 5-fold increase in transcript levels in microarray analysis. In N22, the PsbP levels remained constant until 72 h following BA treatment during DIS, by which time degradation was observed in the untreated samples (Fig. 7B). The level of Psb protein was maintained in the N22-H-dgl162 mutant without BA treatment even at 96 h of DIS, whereas BA treatment caused degradation by 72 h of DIS. These results were in agreement with the degradation of pigment–protein complexes observed using green gel analysis. The changes in protein level support the possibility that the increase in transcript levels of photosynthetic genes in BA-treated N22 samples in comparison with untreated controls could be because of the degradation of transcripts in untreated controls rather than up-regulation of the genes.

Discussion

The plant hormone CK is known to delay senescence in plants. BA-treated detached N22 leaves and untreated detached leaves of the N22-H-dgl162 mutant showed a delay in senescence when subjected to DIS, a commonly used procedure for artificially inducing senescence by dark treatment of detached leaves to obtain uniformity and save time while performing experiments (Oh et al., 2003; Chrost et al., 2004). The physiological parameters indicated that delays in initiation of Chl degradation and loss of photosynthetic efficiency contribute to delayed senescence. This signifies the Type A category of stay-greeness in N22-H-dgl162, an agronomically important trait for crop productivity (Hörtensteiner, 2009). Further characterization of N22-H-dgl162 would help to identify the mutation.

Our microarray data showed a higher level of transcripts for several plastid-localized photosynthesis-related genes such as Rubisco activase, Rubisco small chain, cytochrome b_{6}f complex, PsbO, PsbP, and Cph in BA-treated leaves when compared with untreated leaves during DIS. Also the level of PsbP protein remained constant upon BA treatment during DIS. On the other hand, a Chl biosynthetic gene (Ppo) was down-regulated, suggesting that BA treatment maintains Chl levels by preventing degradation rather than inducing its synthesis, and protects the photosynthetic machinery during DIS, similar to the effect reported in wheat (Zavaleta-Mancera et al., 2007).

Porra et al. (1994) showed by MS and ^18O labeling that an oxygenase as opposed to a hydratase mechanism is operational in a two-step conversion of Chl a to Chl b. HPLC and MS/MS data revealed additional peaks in BA-treated leaves with m/z values of 910 and 925, suggesting accumulation of HmChl and aldehyde hydrate derivatives of Chl a during DIS. Cytokinin delays rice senescence | 1849

Porra et al. (1994) showed by MS and ^18O labeling that an oxygenase as opposed to a hydratase mechanism is operational in a two-step conversion of Chl a to Chl b. HPLC and MS/MS data revealed additional peaks in BA-treated leaves with m/z values of 910 and 925, suggesting accumulation of HmChl and aldehyde hydrate derivatives of Chl a during DIS. The enzyme which catalyzes the oxidation of Chl a to Chl b in the two-step process, is up-regulated following BA treatment within 24 h of DIS, suggesting that CK enhances the conversion of Chl a to Chl b. The appearance of an additional peak in HPLC and accumulation of HmChl and aldehyde hydrate species of Chl a in the leaf tissues also occurs at the same time. Also, we found that transcript levels of Hcar and Nol were reduced during DIS in untreated N22 samples, whereas BA-treated samples showed increased expression of both genes from 24 h, with maximum expression at 72 h, suggesting their role in Chl turnover. Hcar and Nol are reported to be drastically down-regulated during DIS and highly up-regulated during greening of etiolated seedlings (Sakuraba et al., 2013). Overexpression of Cao (Tanaka et al., 2001), as well as a decrease in Chl alb ratios under low light intensities (Tanaka and Tanaka, 2011), has been reported to affect enlargement of the antenna size of PSII. Although BA treatment resulted in higher levels of Cao, this did not affect the Chl alb ratios, suggesting maintenance of antenna size during DIS. The increased expression of Cao did not affect the Chl b levels during DIS, probably because of the feedback mechanism mediated by its N-terminal domain (Yamasato et al., 2005) and also due to increased levels of other enzymes in the Chl cycle. Green gel analysis showed intact pigment–protein complexes in BA-treated leaves in comparison with untreated leaves during DIS. All these results suggest that CK delays the DIS via accumulation of HmChl, probably by regulating the conversion of Chl a to Chl b and maintaining the Chl alb ratios and pigment–protein complexes.

In summary, we report that CK retards senescence in cut leaves of N22 following dark treatment and HmChl accumulates in response to CK treatment. Our results show that HmChl levels increase till 96 h following CK treatment, thereby maintaining the Chl alb ratio during the delay of dark-induced leaf senescence. The accumulation of HmChl appears to have an adaptive value since it is stable, has a similar molecular structure and absorption spectra to Chl a and Chl b, and is probably incorporated in the pigment–protein complexes functioning as a light-harvesting pigment as suggested by Nagane et al. (2010). The light energy absorbed by HmChl may be transferred to neighboring pigments without producing reactive oxygen species since hmc1 mutants of Arabidopsis thaliana which accumulate HmChl do not show necrosis and cell death (Nagane et al., 2010). It is known that under dark conditions, Chl a is irreversibly degraded to catabolites, resulting in a major decrease in levels of total Chl, breakdown of pigment–protein complexes, and yellowing of leaves (Hörtensteiner, 2006). HmChl may be serving as a stable substrate ensuring continued availability of Chl a which would otherwise be degraded and not be available for conversion to Chl b, thereby maintaining the Chl alb ratio. We conclude that CK affects the Chl alb interconversion cycle and maintains the stability of photosynthetic pigment complexes, resulting in prolonged greenness during senescence.

Supplementary data

Supplementary data are available at JXB online.

Table S1. List of primers used in this study.
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