High Light-Dependent Phosphorylation of Photosystem II Inner Antenna CP29 in Monocots Is STN7 Independent and Enhances Nonphotochemical Quenching

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Phosphorylation of the photosystem II antenna protein CP29 has been reported to be induced by excess light and further enhanced by low temperature, increasing resistance to these stress factors. Moreover, high light-induced CP29 phosphorylation was specifically found in monocots, both C3 and C4, which include the large majority of food crops. Recently, knockout collections have become available in rice (*Oryza sativa*), a model organism for monocots. In this work, we have used reverse genetics coupled to biochemical and physiological analysis to elucidate the molecular basis of high light-induced phosphorylation of CP29 and the mechanisms by which it exerts a photoprotective effect. We found that kinases and phosphatases involved in CP29 phosphorylation are distinct from those reported to act in State 1-State 2 transitions. In addition, we elucidated the photoprotective role of CP29 phosphorylation in reducing singlet oxygen production and enhancing excess energy dissipation. We thus established, in monocots, a mechanistic connection between phosphorylation of CP29 and nonphotochemical quenching, two processes so far considered independent from one another.

In eukaryotic photosynthesis, light-dependent reactions are performed by two supramolecular complexes, PSI and PSII, which catalyze light harvesting and electron transport from water to NADP⁺. To this aim, water is oxidized by PSII, which, in turn, is oxidized by PSI, which becomes a reductant for ferredoxin-NADP⁺(+) oxidoreductase and NADP⁺ (Nelson and Ben-Shem, 2004). The two photosystems are functionally connected by the plastoquinone (PQ) and cytochrome (cyt) b₅/f, which catalyze the building of the transthylakoid proton gradient, which is dissipated by ATP synthase (ATPase) activity for ATP synthesis from ADP and inorganic phosphate (Pᵢ). PSII and PSI have clearly distinct absorption spectra, with PSI-light-harvesting complex I (LHCI) complexes being enriched in red-shifted absorption forms (Gobets and van Grondelle, 2001). Within canopies, this leads to differential excitation depending on available light quality. This effect needs to be compensated to avoid imbalance of electron transport rates, yielding either photoinhibition or decrease of photon use efficiency. Two major regulatory mechanisms counteract these effects. (1) State 1-State 2 transitions are active in limiting light conditions (Rintamäki et al., 2000) and inhibited by reduction of a disulfide bridge in high light (HL; Lemeille et al., 2009). This mechanism is activated by overreduction of PQ to plastocynin (PQH₂) through activation of a thylakoid bound kinase, STN7, acting on LHCII (Depège et al., 2003; Bellaﬁore et al., 2005). This causes a fraction of PSII antenna system, mainly Lhcb2 (Leoni et al., 2013), to be transferred to PSI in stroma-exposed membranes. The consequent increase in PSI antenna size (Galka et al., 2012) bursts the electron transfer rate and reequilibrates PQ/PQH₂ redox poise, thus causing feedback inactivation of kinase activity. A phosphatase, PPF1-TAP38 (Pribil et al., 2010; Shapiguzov et al., 2010), dephosphorylates LHClII, allowing for its return to PSI in grana partitions. (2) The process of nonphotochemical quenching (NPQ) is rather aimed to photoprotection from excess light (Horton and Ruban, 2005; de Bianchi et al., 2010). In this condition, saturation of downstream metabolic reactions causes depletion of ADP and Pᵢ and inhibition of ATPase activity, which normally brings back protons from lumen to the stromal compartment. This causes accumulation of protons in the lumen, triggering dissipation into heat of the energy absorbed in excess by PSII (de Bianchi et al., 2010; Niyogi and Truong, 2013). Thus, the synergic, although independent, activities of State-1-State 2 transitions and NPQ cover the needs for regulation of photosynthesis over the wide dynamic range of light intensity experienced by plants when shaded or by daily light changes. However, a number of experimental results do not fit within this scheme: phosphorylation of PSII antenna proteins, namely CP29 (LHCB4) has been reported to be induced in very HL and further enhanced by low temperature (Bergantino et al., 1995), implying inhibition of LHCl protein phosphorylation by HL is not a general feature of plant photosynthesis. Moreover, CP29 phosphorylation has been shown to be protective in...
condition of HL combined with low temperature (Mauro et al., 1997), a major stressing factor limiting crop productivity. HL-induced CP29 phosphorylation has been specifically found in monocots, either C3 or C4 (Bergantino et al., 1998), which include the large majority of food crops, thus making the study of this process of interest for both basic and applied research. In dicots, CP29 phosphorylation has been detected at very low level (Fristedt and Vener, 2011) and targeted to different sites within the N-terminal domain with respect to monocots (Testi et al., 1996). Although early research focused on biochemical and physiological characterization of CP29 phosphorylation (Croce et al., 1996; Mauro et al., 1997; Hwang et al., 2003), genetic dissection of this regulation process has been hampered by lack of genetic resources. More recently, rice (Oryza sativa) has become a model organism for monocots, and knockout collections have become available. In this study, we have used reverse genetics coupled to biochemical and physiological analysis to elucidate the molecular basis of HL-induced phosphorylation of CP29 and the mechanisms by which it exerts a photoprotective effect. We found that a different set of kinases and phosphatases is involved in CP29 reversible phosphorylation with respect to that reported to act in State 1-State 2 transitions and that the photoprotection effect is mediated by an enhancement of excess energy dissipation. These results establish, for the first time, a mechanistic connection between thylakoid protein phosphorylation and NPQ, so far believed to be independent processes.

RESULTS

Kinetics of CP29 Phosphorylation and Recovery

Rice leaves from plants grown in a greenhouse for 8 weeks and incubated in the dark for 6 h were exposed to white light of 1,000 μmol photons m⁻² s⁻¹ for different periods. After 15- or 30-min of illumination, leaves were further incubated in the dark. Samples were harvested at different times and cooled in ice water slurry, and chloroplasts were isolated. SDS-PAGE analysis of thylakoid proteins, followed by immunoblots with anti-CP29 antibody, detected two bands with apparent molecular mass of 30 and 34 kD (Fig. 1A). At t = 0, only the faster migrating band was detected. Upon light exposure, a slow migrating band appeared and accumulated with time, while the intensity of the fast-migrating band decreased. Densitometric analysis showed that the 34-kD band was accumulated to 30% and 40% upon 15 and 30 min of light exposure, respectively.

Figure 1. CP29 phosphorylation kinetic in rice. A, Immunoblot of rice-isolated chloroplasts assayed with anti-CP29 polyclonal antibody. Before chloroplast isolation, leaves have been treated with HL (1,000 μmol photons m⁻² s⁻¹) for different time lengths (Tx indicates minutes of illumination after 6-h dark adaptation) and then dark incubated (Rx indicates minutes of dark incubation upon HL treatment). Tris-Gly SDS-PAGE 15% plus Urea 3M; 1 μg of total chlorophyll (Chl) per lane. B, Densitometrical analysis of immunoblot in A, determining the amount of P-CP29 with respect to the total amount of CP29 (totCP29). Average values have been obtained considering two independent biological replicates. C, Alkaline phosphatase (AlkPh) treatment on rice-isolated monomeric antenna complexes (obtained according to Betterle et al., 2009) from HL-treated samples. P-CP29 dephosphorylation has been evaluated as in A. One-quarter microgram of Chl per lane.
exposure, respectively (Fig. 1B). Upon dark recovery, the relative amplitude of the 34-kD band decreased by 7% to 8% in the first 15 min, after which the decay was slower. Alkaline phosphatase-treated thylakoids only showed the 30-kD band (Fig. 1C), implying that the change in mobility of CP29 was due to phosphorylation, as previously shown in corn (Zea mays) and barley (Hordeum vulgare; Bergantino et al., 1998). These results show that rice CP29 was rapidly phosphorylated in HL and slowly dephosphorylated in the dark.

Identification of the CP29 Phosphorylation Site

Previous work with the monocot corn (Testi et al., 1996) showed HL-dependent phosphorylation of CP29 occurring at Thr-83 of the mature protein, a conserved site in many plant species (Chen et al., 2013). Different phosphorylation sites were reported for Arabidopsis (Arabidopsis thaliana) CP29 (AtCP29; Fristedt and Vener, 2011). To verify whether rice CP29 (OsCP29) was phosphorylated in corn- or Arabidopsis-like site(s) upon HL treatment, we analyzed the two CP29-reactive polypeptides by mass spectrometry (MS) analysis for the detection of phosphopeptides. To this aim, thylakoids from dark-adapted and HL-treated wild-type rice leaves were solubilized by n-Dodecyl β-D-maltoside and fractionated by Suc gradient ultracentrifugation (Supplemental Fig. S1A). The Suc bands 2 (B2) containing monomeric LHC subunits, including CP29, was further fractionated by SDS-PAGE, and the presence of Phosphorylated CP29 (P-CP29) was verified by western blot (Supplemental Fig. S1B). Densitometry of the Coomassie Blue-stained gel yielded consistent results with western-blot analysis, implying that the antibody had similar reactivity for the phosphorylated and unphosphorylated forms of the polypeptide (Supplemental Fig. S2). The SDS-PAGE bands corresponding to CP29 (from both dark-adapted and HL-treated samples) and P-CP29 were excised and analyzed by liquid chromatography-MS analysis in triplicate. No phosphorylated fragments were detected from the 30-kD band (CP29), while a phosphorylated Thr residue was detected in a fragment from the 34-kD band (Supplemental Table S1). This phosphorylated site, Thr-82 in the sequence of the mature protein, was the same site previously identified on corn P-CP29 as Thr-83. Any other HL-related phosphorylated residues were not detected in rice P-CP29 from fractions isolated from the wild type. In low and HL, we found no evidence of phosphorylation at Thr-112 or Thr-114, which were previously identified as phosphorylation sites in the dicot Arabidopsis (Hansson and Vener, 2003), or the Thr-33 and Ser-103 residues identified as phosphorylation sites in Chlamydomonas reinhardtii (Lemeille et al., 2009). These results, together with the recovery of the 30-kD apparent molecular mass upon alkaline phosphatase treatment (Fig. 1C), imply that OsCP29 is phosphorylated into Thr-82 upon HL treatment only. This posttranslational modification was responsible for the change in mobility of CP29 in SDS-PAGE gels and was the same modification occurring in corn (Testi et al., 1996). Moreover, we show that quantitative assessment of phosphorylated versus unphosphorylated forms of a specific protein, often difficult, becomes accessible in the case of CP29 because a single phosphorylation event causes change of SDS-PAGE mobility, thus allowing quantitative detection of phosphorylation based on Coomassie Blue stain or immunoblotting.

Roles of STN7 Kinase and PPH1 Phosphatase in CP29 Phosphorylation and Dephosphorylation

Previous work with Arabidopsis and C. reinhardtii showed that the LHC antenna proteins of PSII are phosphorylated by the STN7 kinase and dephosphorylated by the PPH1 phosphatase during State 1-State 2 transitions and that these gene products control the physiological changes associated, including the far-red light-induced changes of room temperature fluorescence, changes in 77-K emission spectra, and binding of LHCII to the PSI-LHCl complex (Depège et al., 2003; Bellafiore et al., 2005; Pribil et al., 2010; Shapiguzov et al., 2010).

To investigate the possible involvement of STN7 kinase and PPH1 phosphatase in rice CP29 phosphorylation, we identified a mutant of rice lacking STN7 from Oryza Tag Line (Centre de Coopération Internationale en Recherche Agronomique pour le Développement [CIRAD]; rice ssp. japonica ‘Nipponbare’, no. AJTH05) by screening the seeds by western blot using anti-STN7 antibody. In Figure 2A, we compared selected stn7 mutant with the wild type by immunoblotting analysis at different dilution: The signal for STN7 protein was detectable in the wild type but not in the mutant lanes even upon 20-fold dilution, suggesting that the level of STN7 kinase in the stn7 mutant was at least 20 times lower than in the wild type. The insertion site in this stn7 mutant was within an intronic region (OryGenesDB rice mutant database, http://orygenesdb.cirad.fr/; LOC_Os05g47560; Supplemental Fig. S3). We then proceeded to assess whether there was any level of leakiness in this genotype by analyzing the transcription of STN7 gene (Fig. 2B). It is shown that no Stn7 mRNA could be amplified in the mutant, while it was evident in the wild type. The phenotype of the stn7 mutant (Fig. 2C) was also characterized by a stable fluorescence level upon removal of far-red light. When compared to the contrasting behavior of the wild type, undergoing a fast fluorescence decline in the same conditions, this result strongly supports the mutant phenotype corresponded to the original stn7 mutant (Bellafiore et al., 2005). In addition, stn7 mutant exhibited a higher stationary fluorescence, implying plastoquinone pool was overreduced with respect to the wild type, likely due to a decreased excitation of PSI compared with the wild type. A knockout PPH1 (TAP38) mutant was obtained from Rice Mutant Database (rice ssp. japonica ‘Zhonghua 15’, no. 04Z110K94). In the absence of an antibody able to recognize rice PPH1 protein, we screened lines by pulse amplitude-modulated fluorometry (Fig. 3A). Mutant plants were characterized by a faster fluorescence decrease upon exposure to blue light with respect to the wild type.
wild type (Fig. 3A) and by a stable level of fluorescence upon removal of far-red light, implying that State 1-State 2 transitions, once induced by blue light, did not relax within the time of the measurement. Even in the case of pph1 mutant, the insertion site was mapped in an intronic sequence (Supplemental Fig. S3; http://orygenesdb.cirad.fr/Loc_Os01g37130), and the analysis of the transcription of the gene, however, revealed the mutant level of the mRNA below detection (Fig. 3B).

The relation between the fluorescence phenotype and the phosphorylation pattern in the two mutants was assessed by using antiphospho-Thr antibodies to detect changes in polypeptide phosphorylation pattern upon treatments with light conditions either favoring PQ reduction or oxidation and HL (stn7, Fig. 2D; pph1, Fig. 3C).

In dark-adapted wild-type leaves, P-LHCII could not be detected, while a clear signal appeared upon treatment with PSII light. An excess light treatment (1,000 μmol photons m⁻² s⁻¹) made P-LHCII signal disappear again (Fig. 2D). In addition to P-LHCII signal, two more P-Thr reactive bands were detected in all samples with mobility corresponding to CP43 and D1/D2 proteins. While the level of P-CP43 was high in all conditions, the P-D1/D2 signal was lower in the dark-adapted samples and highest in those treated with PSII light and excess light. Consistent with the role of STN7 and PPH1 gene products assessed in Arabidopsis, the stn7 mutant lacked P-LHCII signal in all conditions (Fig. 2D), while the pph1 mutant had constitutive levels of P-LHCII (Fig. 3C).

The above results show that stn7 mutation was effective in preventing the onset of State 1-State 2 transitions of LHCII phosphorylation, while pph1 mutation prevented the State 2-State 1 reversion, implying no redundancy of their respective enzymatic activities occurred in rice as previously reported in Arabidopsis. On this basis, we proceeded to assess the effect of these

Figure 2. Isolation and characterization of rice stn7 mutant. A, Immunoblot analysis using anti-STN7 antibody (αSTN7). Thylakoids have been isolated from wild-type (WT) and stn7 rice plants and then loaded on Tris-Tricine SDS-PAGE 10%. Wild-type 100% and stn7 100% correspond to 2 μg of Chl. An immunoblot analysis using anti-LHCII antibody (αLHCII) has been performed as an internal control. B, Reverse transcription (RT)-PCR measurement of gene-specific transcripts. Sequences of the oligonucleotides used are reported in “Materials and Methods.” The expected sizes of the PCR products are as follows: Stn7, 866 bp; and β-actin, 707 bp. M indicates molecular mass marker (1-kb Plus Ladder, Thermo Scientific). C, Analysis of state transition in stn7 mutant. Chlorophyll fluorescence emission was measured upon treatment with blue light and blue light supplemented with far-red (FR) light, which induce transition to State 2 and State 1, respectively. Data are expressed as arbitrary units (a.u.). D, Analysis of thylakoid phosphoproteins using anti P-Thr (Cell Signaling) antibody. Rice wild-type and stn7 mutant leaves were either dark adapted (D) or treated with HL (1,500 μmol photons m⁻² s⁻¹, 30 min) or PSII-specific light (PSII; 100 μmol photons m⁻² s⁻¹, 1 h, orange filter), and then thylakoids have been collected (Suorsa et al., 2004). Tris-Gly SDS-PAGE 15% plus Urea 3M; 0.75 μg of Chl per lane. E, Evaluation of rice stn7 mutant capacity to phosphorylate CP29 upon HL induction (1,500 μmol photons m⁻² s⁻¹, 30 min). Wild-type and stn7 rice leaves were illuminated, and then thylakoids have been collected and loaded on SDS-PAGE. Immunoblot analysis as in Figure 1A. One microgram of Chl per lane.
two mutations in CP29 phosphorylation. Figures 2E and 3D show the results of anti-CP29 immunoblotting of thylakoids from wild-type, stn7, and pph1 rice plants incubated in the dark, treated with excess light, or further recovered in the dark for 30 min. The three genotypes showed the same behavior as for the CP29 immunoreactive band pattern, implying that STN7 and PPH1 were not involved in CP29 phosphorylation in HL and recovery in the dark. Because the effect of stn7 and pph1 mutations had a complete control over LHCII phosphorylation and de-phosphorylation (Figs. 2D and 3C), this implies that these two phosphorylation/dephosphorylation events are controlled by distinct kinase/phosphatase systems.

An In Vitro Assay for LHCB4 Phosphorylation

To gain additional information of the mechanisms controlling CP29 phosphorylation, we verified the possibility of reproducing phosphorylation in vitro. To this aim, we isolated fully active chloroplasts from dark-adapted wild-type rice leaves that were incubated in the dark or exposed to HL (1,000 μmol m\(^{-2}\) s\(^{-1}\)) for 30 min with or without the addition of ATP. Aliquots were then submitted to SDS-PAGE and immunoblotting for detection of CP29 versus P-CP29 (Fig. 4A). Dark and HL-ATP samples only showed the unphosphorylated CP29 band, while HL plus ATP conditions led to the appearance of the 34-kD band corresponding to P-CP29. Similar results could be obtained in HL in presence of ADP plus Pi rather than ATP (Fig. 4B). The level of P-CP29 obtained was similar among functional chloroplasts and thylakoids, suggesting the kinase responsible for this reaction was associated with the thylakoid membranes.

We then utilized the in vitro assay for performing a pharmacological analysis of CP29 phosphorylation on isolated chloroplasts. We first proceeded to verify the effect of controlling the redox state of the PQ pool and cyt b\(_{6}/f\) complex. To this aim, chloroplasts isolated from dark-adapted rice leaves were illuminated (1,000 μmol m\(^{-2}\) s\(^{-1}\), 30 min) in the presence of 50 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; Fig. 5A), leading to a strong reduction of P-CP29 accumulation. Also, the addition of 50 μM 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB) yielded a strong reduction of P-CP29 level, which became undetectable.
when increasing the inhibitor concentration to 200 or 500 μM. Because DCMU and DBMIB respectively decrease and increase the redox state of PQH₂, these results indicate that the CP29 kinase is activated by the reduction PQH₂ and/or cyt b₆/f.

The study of P-CP29 function requires a system for selective inhibition of CP29 phosphorylation or the availability of a mutant lacking the CP29 kinase, so far not identified. Alternatively, selective prevention of P-CP29 accumulation could be obtained by the use of kinase inhibitors (Chen et al., 2009) that could be tested in the in vitro phosphorylation assay, thus avoiding the problems of permeability often encountered in vivo. As described by Chen et al. (2009), we tested different kinase inhibitors: U0126, an inhibitor of Mitogen-activated protein kinase (MAPK), W7, an inhibitor of Ca²⁺-dependent protein kinase, and k252a, an alkaloid kinase inhibitor derived from the fungus Nocardiopsis spp. (Ruegg et al., 1989). When rice chloroplasts from dark-adapted leaves were treated with HL in the presence of U0126 and W7 inhibitors, we obtained the same P-CP29 level as in the control sample, treated with HL but without inhibitor (Fig. 5B). Differently, in the presence of k252a, no P-CP29 was detected, irrespective from light conditions, implying this alkaloid is a strong inhibitor of the CP29 kinase in agreement with previous results (Chen et al., 2009). We used 25 μM for U0126, 100 μM W7, and 20 μM K252a (Chen et al., 2009). The inhibitory concentration value for CP29 phosphorylation in rice chloroplast (chlorophyll concentration of 40 μg mL⁻¹) by k252a was determined at 2 μM (Supplemental Fig. S4). It is interesting to note that in the presence of k252a, P-CP29 level is even lower than in dark-adapted thylakoids, implying that, upon blocking of the CP29 kinase, a phosphatase specific for CP29 can proceed. It is also interesting to note that k252a strongly reduces the intensity of the two high molecular mass bands in the immunoblotting with antiphospho-Thr (Supplemental Fig. S5), suggesting this is also an inhibitor of the kinase(s) involved in PSII core complex phosphorylation.

**PSII Photoprotection and P-CP29**

The availability of an inhibitor for P-CP29 accumulation allows for experimental verification of the physiologic effect of this phosphorylation event. Previously, a photoprotective effect was suggested based on the differential behavior of near-isogenic lines (Bergantino et al., 1995). To this aim, we exposed isolated chloroplasts at...
1,000 μmol m⁻² s⁻¹ in the presence of and without k252a assessing photoinhibition by measuring the maximum photochemical efficiency of PSII in the dark-adapted state (Fv/Fm). As reported in Figure 6A, the inhibitor caused a faster decrease of Fv/Fm during HL treatment, supporting the idea of a photoprotective activity for P-CP29. Photo-inhibition is induced by ROS accumulation (Havaux et al., 2007; Dall’Osto et al., 2010): we measured the production of singlet oxygen during illumination of isolated chloroplasts by using a fluorescent probe (Flors et al., 2006; Dall’Osto et al., 2007; Betterle et al., 2010) and observed that the presence of k252a led to an increased production of ¹O₂ (Fig. 6B). NPQ dissipates light energy absorbed in excess, thus decreasing the production of ¹O₂ while CP29 was shown to have a major role in NPQ (de Bianchi et al., 2011) by activating zeaxanthin radical cation (Holt et al., 2005; Ahn et al., 2008). To verify the hypothesis that accumulation of P-CP29 was correlated to an increased NPQ activity, we measured NPQ in isolated chloroplasts in the presence or absence (control [CTR]) of k252a inhibitor (Fig. 7A). The chloroplast preparation obtained from rice showed a PSII quantum yield of 0.795 ± 0.05. NPQ measured in CTR was reproducible, yielding values of 1.15 ± 0.04 with an energy quenching (qE) component of 0.65 ± 0.03, as measured after 10-min recovery in the dark. Maximum NPQ amplitude was only slightly lower in presence of k252a (1.06 ± 0.04), while a strong effect was observed on dark relaxation kinetics, twice as faster in k252a-treated samples compared with CTR. Thus, the presence of P-CP29 could be associated with an increased inhibitory quenching (qI). Because the difference in NPQ activity increased with time during light exposure, we repeated the 15-min actinic light treatment three times with 10-min dark intervals in between. Upon three cycles of exposure to actinic light, CTR chloroplasts reached an NPQ value of 2.0 compared with an NPQ value of 1.25 in k252a-treated samples (Fig. 7C), implying that CP29 phosphorylation led to a 60% increase in NPQ activity, due to both an increase of qI and qE. It should be noticed that the increase of qI cannot be ascribed to photoinhibition because Fv/Fm was higher in CTR with respect to the k252a sample (Fig. 7D). It is interesting to note that the comparison between the inhibition of CP29 phosphorylation and the reduction in NPQ and qI yielded k252a concentration-dependent curves with a similar behavior (Supplemental Fig. S4).

Figure 6. Effect of CP29 kinase inhibitor on photoprotection of intact chloroplasts. A, Fv/Fm in functional rice wild-type (WT) chloroplasts during illumination with white light of 1,000 μmol photons m⁻² s⁻¹. These chloroplasts have been treated in the presence or absence of Ser/Thr inhibitor k252a, and two independent batches of them have been analyzed. Crt, Control samples. B, Singlet oxygen production in functional chloroplasts in the presence or absence of Ser/Thr inhibitor k252a. Functional chloroplasts have been treated with red light of 1,500 μmol photons m⁻² s⁻¹, and singlet oxygen production has been measured at different time points using the fluorescent probe Singlet Oxygen Sensor Green (SOSG; Betterle et al., 2010). a.u., Arbitrary unit.

Is LHCII/PSII Core Phosphorylation or Zeaxanthin Accumulation Involved in Quenching Reactions?

In rice, thylakoid protein phosphorylation involves not only CP29 but also LHCII and PSII core. To dissect the effect of these different phosphorylation events, we first considered the case of LHCII. In principle, the light intensity used in our experiments was well above the inhibition level for STN7 kinase (Rintamäki et al., 2000; Lemelle et al., 2009), suggesting P-LHCII should not be involved. NPQ measurements with Os-stn7 mutant showed no difference with respect to the wild type, irrespective from whether the measurement was performed in isolated chloroplasts or in vivo (Fig. 7, A and B). This was consistent with Figure 3C showing no P-LHCII signal in HL-treated wild-type samples.

The case of PSII core phosphorylation was more difficult to tackle because, as reported in Supplemental Figure S5, k252a significantly decreased phosphorylation of PSII core subunits, in addition to preventing P-CP29, potentially leading to a (still unknown) quenching effect. To verify whether this was the case, we studied the k252a effect on Arabidopsis, a species where CP29 is not phosphorylated or to a very low level under the control of
STN7 kinase (Bergantino et al., 1998; Bellaﬁore et al., 2005; Tikkanen et al., 2006; Wunder et al., 2013) and for which knockout mutants are available for both Stn7 and Stn8 genes encoding kinases involved in LHCII and PSII core phosphorylation, respectively (Bellaﬁore et al., 2005; Bonardi et al., 2005). Supplemental Figure S6 shows the NPQ kinetics during three consecutive actinic light exposures (1,000 μmol photons m⁻² s⁻¹ at 23°C) separated by dark recovery, as for rice in Figure 7C. The kinetics of NPQ rise and recovery were identical for chloroplasts from wild-type, stn7, and stn8 plants, and the addition of k252a did not produce any change in quenching activity in any of these genotypes, none of which is active in CP29 phosphorylation. It was important to verify that k252a was effective in blocking STN7 and STN8 kinases in Arabidopsis (Supplemental Fig. S7), leading to a reduction of CP43, D1, D2, and LHCII phosphorylation level in HL-treated functional chloroplasts alike in rice samples shown in Supplemental Figure S5. The results in Supplemental Figure S7 show that HL treatment led to an increased level of CP43 and D1/D2 phosphorylations, which were instead prevented in the presence of k252a to a larger extent with time of treatment. Another PSII core subunit well known to be phosphorylated, PSII reaction center protein H (PsbH; Aro et al., 2004) was not resolved in our gel system due to its low Mr (approximately 10 kD). These results are consistent with the differences in quenching activities of CTR versus k252a-treated rice (Fig. 7) being caused by differences in CP29 phosphorylation only, without contribution by phosphorylation of PSII core subunits.

We finally considered the hypothesis that differences in accumulation of zeaxanthin could be involved in HL-dependent NPQ enhancement. An enhancing effect of...
zeaxanthin on quenching reactions has been reported (Niyogi et al., 1998; Dall’Osto et al., 2005; Nilkens et al., 2010; Ruban and Johnson, 2010), thus opening the possibility that k252a-dependent differences in zeaxanthin accumulation during HL might cause at least part of the differences in quenching reported in Figure 7A for rice. To verify this hypothesis, we measured both P-CP29 accumulation and zeaxanthin accumulation in control and k252a-treated rice chloroplasts during 15-min HL treatment and subsequent dark relaxation (Supplemental Figs. S5 and S8). We observed that a low level of P-CP29 was present in the dark (percentage P-CP29 per total CP29), which then increased in HL. The level of P-CP29 in k252a-treated sample, instead, decreased in the light and remained low over the subsequent dark treatment, suggesting that the CP29 kinase was active in the dark in the presence of added ATP, while its inhibition allowed for phosphatase activity to proceed. Zeaxanthin accumulation was the same in CTR and in the presence of k252a, implying the difference in zeaxanthin accumulation was not the reason for differential quenching in rice chloroplasts (Supplemental Fig. S8).

Localization of P-CP29 in Thylakoid Domains

Previous work in C. reinhardtii has shown that CP29 phosphorylation leads to its migration from grana membranes, where it participates to PSII supercomplexes and stroma membranes to become part of a PSI-LHCl-LHCII-CP29 supercomplex (Kargul et al., 2005; Drop et al., 2014). In rice, dissociation of PSII supercomplexes with migration of P-CP29 to stroma-exposed membranes was also reported (Liu et al., 2009; Chen et al., 2013). To verify whether phosphorylation changed the localization of CP29 in the rice photosynthetic apparatus, we isolated thylakoid membranes from rice plants either dark adapted or exposed to HL (1,000 μmol m⁻² s⁻¹, 30 min). These thylakoids were then fractionated into grana membranes and stroma lamellae by detergent treatment and differential centrifugation (Barbato et al., 2000; Sirpiö et al., 2007;
Morosinotto et al., 2010) and analyzed by SDS-PAGE and immunoblotting (Fig. 8A). Figure 8A shows the Coomassie Blue-stained SDS-PAGE gel loaded with the thylakoid, grana, and stroma lamellae preparations: a small portion of PSI contaminants is present in grana membranes, although a strong enrichment of PSII and PSI/ATPase in, respectively, grana membranes and stroma lamellae is evident. P-CP29 was detected in whole thylakoids and the grana fraction with P-CP29 per total CP29 ratio of 30% in both fractions. Instead, no P-CP29 could be detected in the stroma membrane fraction. Pigment analysis showed that the chlorophyll a/b ratio of thylakoids, grana membranes, and stroma lamellae was, respectively, 3.25 ± 0.03, 2.35 ± 0.05, and 6.70 ± 0.1, irrespective of whether the samples derived from dark-adapted or HL-treated plants. Also, the Chl amount recovered in the fraction from the two conditions was equal. With the aim of verifying whether amount recovered in the fraction from the two conditions was equal. With the aim of verifying whether the HL-induced reversible phosphorylation of CP29 in monocots is catalyzed by the same set of enzymes that were previously found to be active in the major PSII antenna LHCCI. Moreover, we assessed the nature of the photoprotective activity consequent to CP29 phosphorylation.

**CP29 Kinase and Phosphatase Activities**

Isolation of rice insertional mutants inactivating stn7 and pph1 genes was instrumental in assessing the independence of CP29 phosphorylation on the activity of LHCCI kinase and phosphatase involved in State 1-State 2 transitions. stn7 and pph1 mutations were effective in preventing LHCCI phosphorylation and dephosphorylation in rice (Figs. 2D and 3C) as well as fluorescence changes associated with this process (Figs. 2C and 3A), in agreement with previous reports in the dicot Arabidopsis (Bellaire et al., 2005; Pribil et al., 2010; Shapiguzov et al., 2010). However, these mutations were ineffective in preventing the accumulation of P-CP29 or its dephosphorylation in the dark (Figs. 2E and 3D).

We conclude that neither STN7 nor PPH1 are involved in reversible CP29 phosphorylation in rice. This is likely the case with corn and barley, as suggested by the similar behavior of P-CP29 in these species in being promoted by excess light conditions and enhanced by concomitant stress conditions rather than inhibited at relatively low irradiance (Rintamäki et al., 1997). These results imply that, in addition to regular State 1-State 2 transitions active in green algae (Allorent et al., 2013) and dicots (Bellaire et al., 2005), an additional phosphorylation-dependent regulation mechanism evolved in monocots for response to excess light conditions. Thus, reversible phosphorylation of the two subunits of PSI antenna system, LHCCI and CP29, are independent in rice, at variance with the case of Arabidopsis, where STN7 activity was responsible for both LHCCI phosphorylation and the low level of CP29 phosphorylation found (Tikkanen et al., 2006; Fristedt and Vener, 2011). In C. reinhardtii, CP29 was strongly phosphorylated (Kargul et al., 2005; Allorent et al., 2013) and yet was found to be STT7 dependent. Thus, the lack of STT7/STN7 involvement in monocot CP29 phosphorylation is consistent with the different conditions activating this process: STN7 activity is up-regulated by a reduced plastquinone pool (Zito et al., 1999; Tikkanen et al., 2011) induced by PSI light or unbalanced excitation of PSI and PSI. However, STN7 activity is also inhibited by reduced ferredoxin/thioredoxin (Vainonen et al., 2005; Lemeille et al., 2009), leading to the inhibition of LHCCI phosphorylation in HL (Fig. 2D). We show that CP29 kinase is associated with thylakoid membranes (Fig. 4B) and is activated upon reduction of cyt b6/f (Fig. 5A), similar to STN7 kinase in Arabidopsis (Wunder et al., 2013). Although sharing with STN7 the activation by reduced PQQ, CP29 kinase is not inactivated by HL, which is required for CP29 phosphorylation. This strongly suggests

**DISCUSSION AND CONCLUSION**

P-CP29 has been previously reported in several species from green algae to monocots. In the case of C. reinhardtii, CP29 phosphorylation is dependent on State Transition7 Kinase (STT7) kinase and has been reported to be involved in state transitions (Kargul et al., 2005; Allorent et al., 2013). In Arabidopsis, a dicot, CP29 is weakly detectable upon treatment with PSI light, and its phosphorylation is under the control of STN7 kinase, homologous to STT7 (Tikkanen et al., 2006; Fristedt and Vener, 2011). Within monocots, CP29 phosphorylation has been reported in rice (C3; Hwang et al., 2003), barley (Bergantino et al., 1998), and corn (C4; Mauro et al., 1997; Bergantino et al., 1998), and yet in these species, no association was established with State 1-State 2 transitions. Rather, a photoprotective effect was reported against cold stress (Bergantino et al., 1995) and, more recently, to other environmental stresses, such as water and salt stresses in monocots (Chen et al., 2009; Liu et al., 2009). Also, an effect in modulating the size of PSI-LHCCI supercomplexes was suggested (Chen et al., 2009; Fristedt and Vener, 2011).

In this study, we investigated whether the HL-induced reversible phosphorylation of CP29 in monocots is catalyzed by the same set of enzymes that were previously found to be active in the major PSII antenna LHCCI. Moreover, we assessed the nature of the photoprotective activity consequent to CP29 phosphorylation.
overreduction of electron transport chain due to a de-
accumulation level was reported in cold and HL
STN7 (Friso et al., 2010). Moreover, the highest P-CP29
with thylakoid in corn through MS analysis, in addition to
2005) and is the only Ser/Thr kinase enzyme associated
not inhibited by HL (Bonardi et al., 2005; Vainonen et al.,
Hypothesis is supported by the report that STN8 activity is
CP29 kinase is still unknown. One candidate is STN8. This
Arabidopsis depends on the kinase rather than to differ-
ration site in rice P-CP29 was at Thr-82 in the mature
protein: this residue is conserved in Arabidopsis LHCB4.1
and LHCB4.2 but not in LHCB4.3, suggesting that the
different phosphorylation behavior of CP29 in rice and
Arabidopsis depends on the kinase rather than to differ-
ences in the substrate sequence. At present, the identity of
CP29 kinase is still unknown. One candidate is STN8. This
hypothesis is supported by the report that STN8 activity is
not inhibited by HL (Bonardi et al., 2005; Vainonen et al.,
and is the only Ser/Thr kinase enzyme associated
with thylakoid in corn through MS analysis, in addition to
STN7 (Friso et al., 2010). Moreover, the highest P-CP29
accumulation level was reported in cold and HL
(Bergantino et al., 1995), a condition known to induce
overreduction of electron transport chain due to a de-
creased demand for reducing equivalent by the Calvin-
Benson cycle (Savitch et al., 2011). The alignment of
STN8 protein of Arabidopsis and rice (Supplemental Fig.
S9) shows that the rice homolog has a significant longer N
terminus compared with the Arabidopsis one, consistent
with a different substrate binding activity. Although we
could not have access to a monocot snl8 mutant, the STN8
hypothesis is supported by the results of the pharmaco-
logical analysis using intact isolated chloroplasts. This ap-
proach was attempted before (Bergantino et al., 1995; Chen
et al., 2009) using drug delivery by leaf infiltration, yield-
ing results of difficult interpretation likely due to problems
with diffusion of the chemicals in the leaf tissue to the
target site. We found the isolated chloroplast method
much more reliable, and among the three kinase inhibitors
assayed in this work, only one, namely k252a (an alkaloid
kinase inhibitor), was effective in preventing CP29 phos-
phorylation in HL (Fig. 5B), in agreement with previous
results (Chen et al., 2009). K252a also caused a decreased
CP43 phosphorylation level (Supplemental Fig. S5), sug-
gesting the two reactions were caused by the same kinase,
while phosphorylation of PSII core subunits has been
shown to be catalyzed by STN8 (Bonardi et al., 2005; Nath
et al., 2013). Interestingly, k252a treatment shows that the
effect of CP29 phosphatase was complete when CP29 ki-
nase has been inactivated. The level of P-CP29 decreased
upon direct (by k252a; Fig. 5B) or indirect (see DCMU and
DBMB; Fig. 5A) kinase inactivation during HL treatment
with respect to the starting level in dark samples.

Physiological Effect of CP29 Phosphorylation

Previous work with near-isogenic lines in corn reported
an increased resistance to photoinhibitory treatment in the
presence of P-CP29 (Mauro et al., 1997). Moreover, dis-
sociation of PSII supercomplexes and migration of CP29
from grana to stroma-exposed membranes has been
reported (Liu et al., 2009), which might potentially in-
crease PSII activity and alleviate PQ overreduction, similar
to previous reports with Chlamydomonas spp. (Kargul
et al., 2005; Drop et al., 2014). Our results with rice are in
contrast with these latter reports and strongly indicate
that CP29 does not migrate to stroma lamellae upon
phosphorylation but remains in the grana partitions.
Although we cannot offer a clear explanation for the dis-
crepancy of our results with respect to those reported (Liu
et al., 2009), we notice that properties of fractions puta-
tively deriving from grana partitions, grana margins, and
stroma lamellae in Liu et al. (2009) only exhibit small
differences in the content of D1 and LHCII, which is un-
expected in the light of the extreme lateral heterogeneity
of thylakoid membranes (Anderson et al., 2012), possibly
as a consequence of the drought stress applied in that
study. In our hands, extreme lateral separation was
obtained for PSII plus LHCII versus PSI plus ATPase,
respectively, in grana versus stroma lamellae (Fig. 8A).
CP29 signal in stromal fraction was very low and did
not increase upon HL treatment, irrespective from its
phosphorylation state (Fig. 8A). Further analysis of grana
membranes showed that PSII-LHCII supercomplexes
were maintained (Fig. 8B, bands B6–B9), even in pres-
ence of P-CP29 in HL-treated samples, with the excep-
tion of the larger complexes (Fig. 8B, bands B10/B11),
which are composed by C2S2M and C2S2M2 PSII dimers,
as previously reported (Caffarri et al., 2009). These
results imply that while P-CP29 caused dissociation of
largest supercomplexes into complexes with smaller size,
remained in grana membranes. This suggests that
whatever the physiological effect of P-CP29, it is exerted
within grana domains and within the inner layer of the
PSII antenna system close to the PSII core. It is worth
noting that upon HL treatment, a phosphorylation of
PSII core subunits was also observed, as reported in
Supplemental Figure S5, thus we cannot exclude that
CP43, D1/D2, and/or PsbH phosphorylation might also
have a role in PSII destabilization upon HL as, previ-
ously reported for Arabidopsis (Tikkkanen et al., 2008).

Figure 1 shows that P-CP29 accumulation is a very
rapid process upon exposure to excess light. In these
conditions, NPQ is also activated, allowing for fast heat
dissipation of the excess energy absorbed (Li et al., 2000;
Kühlheim et al., 2002; Horton et al., 2008). A relation be-
tween NPQ and CP29 phosphorylation has been previ-
ously suggested (Mauro et al., 1997). Here, we exploited
the k252a inhibitor for studying the relation between
accumulation of P-CP29 and NPQ in fully functional
isolated chloroplasts exposed to excess light. A clear
effect was detected on NPQ rise kinetic and relaxation
kinetic in the dark (Fig. 7, A and B). Dark-adapted,
P-CP29-depleted chloroplast underwent a small NPQ
increase and a strong delay in its relaxation. Due to the
slow dephosphorylation kinetic (Fig. 1), repeated light
exposures led to progressively increasing NPQ ampli-
tude (Fig. 7C). Thus, these NPQ kinetic changes in both


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the rise and decay were abolished by k252a and were absent in the Arabidopsis wild type as well as in stn7 and stn8 mutants (Supplemental Fig. S6), where CP29 did not accumulate. Because k252a did not affect the synthesis of zeaxanthin (Supplemental Fig. S8), an NPQ enhancer (Niyogi et al., 1998), we conclude that P-CP29 had an enhanced quenching capacity versus CP29 and that this process is physiologically active in monocots, but not in the dicot Arabidopsis plants. The photoprotective effect of the additional NPQ induced in P-CP29 is evident from the slower rate of NPQ dark relaxation of both the wild type and stn7 rice mutants (Fig. 7A) and by the decreased rate of singlet oxygen produced (Fig. 6B). The use of the Arabidopsis wild type and stn7 and stn8 mutants as controls for the treatment with k252a inhibitor allowed us to correlate NPQ and photoprotection phenotypes with the phosphorylation of CP29 in rice. Exposure of Arabidopsis chloroplasts to k252a did not induce any significant NPQ variation. We cannot exclude that in monocots, phosphorylation of CP29 and PSII core sub-units cooperatively yields the increased photoprotection phenotype observed, and a deeper investigation of PSII core phosphorylation is required. However, the comparison of the Arabidopsis wild type and stn7/stn8 mutants and the rice wild type treated or untreated with k252a inhibitors strongly suggests a peculiar role of P-CP29 in photoprotection.

The Mechanism of Quenching in P-CP29

Enhanced quenching in the presence of P-CP29 is consistent with the decrease in amplitude and slower NPQ kinetic in plants lacking CP29 (de Bianchi et al., 2011) and with the rate-limiting activity of CP29 in transferring excitation energy from outer antenna to PSII core complex (Caffarri et al., 2011). It is interesting to consider the consequences of CP29 phosphorylation: MS analysis showed that mature rice CP29 is phosphorylated at the Thr-82 residue (Supplemental Table S1), located at the N terminus, exposed to the stromal surface of thylakoids. This position is homologous to Thr-83, previously reported for ZmCP29. No other phosphorylated residues were detected in OsCP29, implying that the changes in conformation previously detected (Testi et al., 1996) are specially associated with this phosphorylation event. CP29 spectral properties are also modulated by zeaxanthin binding (Croce et al., 1996; Crimi et al., 2001). Previous work on the isolated P-CP29 plant demonstrated that phosphorylation does not induce the protein to undergo a dissipative state in detergent solution (Crimi et al., 2001): on the basis of this result, we propose that phosphorylation-dependent conformational change of CP29 in monocots affects the overall PSII supercomplexes conformation, as evidenced by the partial disassembly of PSII-LHCII supercomplexes in smaller complexes (Fig. 8C), leading to formation of quenched states in a zeaxanthin-like long-living mechanism. In this context, we cannot exclude that protein-protein interaction in PSII-LHCII supercomplexes leads to formation of some specific quenching sites in P-CP29, which are inactivated when the protein is extracted by detergent treatment. For example, previous work in thylakoid membranes purified from Arabidopsis showed the formation of carotenoid radical cations responsible for energy dissipation upon NPQ induction in a high fraction (33%) of monomeric proteins (Holt et al., 2005): upon investigation of isolated proteins, the yield of carotenoid radical cation formation dramatically decreased to less than 1% (Avenson et al., 2008). A similar protein environment-dependent activation of quenching sites in P-CP29 cannot be excluded.

Among monomeric LHCB proteins, CP26 and CP24 showed the highest rates of zeaxanthin binding upon exposure to HL (Betterle et al., 2010), while CP29 has lower binding rates (Morosinotto et al., 2002). We propose that monocots developed an additional response to fast and repeated exposure to excess light by pretriggering the energy-dissipative activity through a change of conformation in CP29, caused by N-terminal phosphorylation. This mechanism adds to the zeaxanthin-dependent NPQ mechanisms, which have its preferential target in CP26 (Dall’Osto et al., 2005) and CP24 (Betterle et al., 2010) and is likely to be more efficient than violaxanthin-to-zeaxanthin exchange in conditions of low temperature when the rate of the lipid diffusion step required for violaxanthin deepoxidation and binding to the L2 site of Lhcb proteins is decreased. Also, zeaxanthin formation requires ascorbate, a reducing cofactor, which is also involved in a number of redox reactions and might become limiting due to its use in concomitant pathways such as the water-water cycle (Asada, 1999). In the cold, ATP level is strongly increased by reduced requirement by the Calvin-Benson cycle, a condition favoring kinase activity.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Rice (Oryza sativa) lacking STN7 was obtained from Oryza Tag Line (CIRAD; rice japonica ‘Nipponbare,’ no. AJTH05; Larmande et al., 2008), whereas rice lacking PP1H/TAP38 was obtained from the Rice Mutant Database (rice japonica ‘Zhonghua 15,’ no. 04Z10K94; Zhang et al., 2006). Plants were grown in a greenhouse at 28°C/35°C with natural light during warmer seasons or artificial light (400 μmol photons m−2 s−1) during late autumn and winter (14-h-day/10-h-night photoperiod).

RT-PCR Analysis

For RT-PCR, total RNA was isolated from 10-week-old plants with the Spectrum Plant Total RNA Kit (Sigma-Aldrich). RT was performed using M-MLV reverse transcriptase with oligo(dT) primer and 1 μg of total RNA and was followed by 37 cycles of PCR amplification. In parallel, amplification of the housekeeping gene flactin transcript with primers collected from the literature (Bt et al., 2011) has been performed from the same complementary DNAs as the loading control. The primers used for STN7 (Loc_Os05g47560) and PpH1 (Loc_Os03g57330) genes were as follows: STN7 (forward) AACGGACAGCAGCCTCATAC; STN7 (reverse) TCAGCCACGGTTTCTTTGTA; PpH1 (forward) CTTGTGTGTCGCCACATTGG; and PpH1 (reverse) AATATCTGCCCCAGCAGAG.

Pigment Analysis

Pigments were extracted and then separated and quantified by HPLC as previously described (Gilmore and Yamamoto, 1991).
Membrane Isolation

Functional chloroplasts/thylakoids were isolated as previously described (Casazza et al., 2001). Experiments with functional chloroplasts/thylakoids were performed at a chlorophyll concentration of 40 μg mL\(^{-1}\) in the presence of 10 μM methylviologen, 0.5 μM ATP, and 15 μM Na-ascorbate. To investigate the mechanism of CP29 phosphorylation, functional chloroplasts were treated with U0126 (MAPK inhibitor, Sigma-Aldrich), W7 (Calcium-Dependent Protein Kinases inhibitor, Sigma-Aldrich), and K252a (Ser/Thr protein kinases inhibitor, LC Laboratories), using the same concentrations previously indicated for in vivo experiments by Chen et al. (2009).

Grana membranes have been prepared according to Morosinotto et al. (2010), and stroma lamellae have been prepared according to Sirpiö et al., 2007 and Barbatò et al. (2000). PSII supercomplexes have been prepared from grana membranes according to Caffarri et al. (2009). Sodium fluoride (10 μM) was added to all the buffers used for preparation of grana membranes, stroma lamellae, and PSII supercomplexes.

Gel Electrophoresis and Immunoblotting

SDS-PAGE analysis was performed with the Tris-Gly buffer system (Laemmli, 1970) and 15% (w/v) acrylamide concentration, with the addition of 3 μl urea to the running gel to separate phosphorylated and unphosphorylated CP29 polypeptides. For western-blot analysis, chloroplast or thylakoid samples were loaded on SDS-PAGE and electrobotted on nitrocellulose membranes, and proteins were detected with homemade anti-Cp29 (Bergantino et al., 1995), anti-STN7 (Lemeille et al., 2009), or anti-Rubisco serum, using alkaline phosphatase-SDS-PAGE and electroblotted on nitrocellulose membranes, and proteins phosphorylated CP29 polypeptides. For measurements of the PSII photoinhibition, functional chloroplasts were illuminated with white light of 10,000 μmol photons m\(^{-2}\) s\(^{-1}\) and then \(F_{v}/F_{m}\) ratios were subsequently followed at irradiances of 20 μmol photons m\(^{-2}\) s\(^{-1}\) at 48°C (Aro et al., 1994), using Fluorcam FC-800 fluorescence imaging system.

In Vivo Fluorescence and NPQ Measurements

State transition experiments were performed on leaves according to established protocols (Jensen et al., 2000). Preferential PSII excitation was avoided by illumination with blue light (40 μmol photons m\(^{-2}\) s\(^{-1}\)), excitation of PSI was achieved using far-red light from a light-emitting diode light source (Heinz-Walz, 102-FR) applied for 15 min simultaneously with blue light. NPQ was measured through chlorophyll fluorescence on whole leaves or functional chloroplasts at room temperature with a PAM 101 fluorimeter (Heinz-Walz; Andersson et al., 2001), a saturating light pulse of 4,500 μmol photons m\(^{-2}\) s\(^{-1}\) for 0.8 s, and white actinic light of 1,000 μmol photons m\(^{-2}\) s\(^{-1}\) supplied by a KL1500 halogen lamp (Schen). For NPQ measurements, intact chloroplasts were dissolved immediately before analysis in an optimized hypotonic buffer containing 100 mM sorbitol, 5 mM MgCl\(_2\), 10 mM NaCl, 20 mM KCl, 30 μM HEPEs, and 0.03% (w/v) agarose (40 μg mL\(^{-1}\) chloroplasts final chlorophyll concentration, supplemented with cofactors as previously discussed below). NPQ was calculated according to the following equations (Baker, 2008): NPQ = \(F_{v}/F_{m}^{0}\)/\(F_{v}/F_{m}\), where \(F_{v}/F_{m}^{0}\) is the maximal fluorescence from dark-adapted/light-adapted leaves measured after the application of a saturating flash. For NPQ measurements on functional chloroplasts, final 0.05% (w/v) agarose was added to avoid chloroplasts sedimentation. NPQ kinetics during three consecutive periods of illumination were measured using Fluorcam FC-800 (PSI) fluorescence imaging system (PSII), allowing contemporaneous analysis of multiple samples and preventing chloroplasts deterioration during such long analyses.

Singlet Oxygen Production

Singlet oxygen production in isolated functional chloroplasts was measured using Singlet Oxygen Sensor Green dye (Flors et al., 2006), as described (Beterle et al., 2010), illuminating the samples with red light of 1,500 μmol photons m\(^{-2}\) s\(^{-1}\).

Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Identification of the HL-dependent phosphorylated residues of P-Cp29.

**Supplemental Figure S2.** Analysis of anti-Cp29 antibody sensitivity compared with Coomassie Blue gel staining.

**Supplemental Figure S3.** Transfer DNA insertion mutagenesis sites in stn7 and pph1 mutants.

**Supplemental Figure S4.** Inhibition of Cp29 phosphorylation and NPQ components upon treatment with different concentrations of kinase inhibitor k252a.

**Supplemental Figure S5.** Analysis of rice thylakoid phosphoproteins upon NPQ induction in HL.

**Supplemental Figure S6.** NPQ kinetics of wild-type, stn7, and stn8 Arabidopsis chloroplasts during three consecutive periods of illumination.

**Supplemental Figure S7.** Analysis of Arabidopsis thylakoid phosphoproteins upon NPQ induction.

**Supplemental Figure S8.** Time course of CP29 phosphorylation and zeaxanthin production in the presence or absence of inhibitor k252a.

**Supplemental Figure S9.** Sequence comparison of STN7, STN8, and CP29 (Lhcb4) of Arabidopsis and rice.

**Supplemental Table S1.** Identification of the phosphorylation site in LOC_Os06g372401.
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