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SHORT COMMUNICATION

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Arabidopsis BSD2 reveals a novel redox regulation of Rubisco physiology *in vivo*

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ABSTRACT

Plants need light energy to drive photosynthesis, but excess energy leads to the production of harmful reactive oxygen species (ROS), resulting in oxidative inactivation of target enzymes, including the photosynthetic CO_2-fixing enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). It has been demonstrated *in vitro* that oxidatively inactivated Rubisco can be reactivated by the addition of reducing agents. Busch et al. (in The Plant Journal, doi: 10.1111/tpj.14617, 2020) recently demonstrated that bundle-sheath defective 2 (BSD2), a stroma-targeted protein formerly known as a late-assembly chaperone for Rubisco biosynthesis, can be responsible for such reactivation *in vivo*. Here, we propose a working model of the novel redox regulation in Rubisco activity. Redox of Rubisco may be a new target for improving photosynthesis.

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Carboxylation reaction of Rubisco is renowned for its catalytic inefficiency due to a slow turnover rate, a low affinity for CO_2, and competing oxygenation reaction at the same active site.[1] Therefore, Rubisco is a major target to improve photosynthetic capacity or maximum rate of Rubisco carboxylation (V_{cmax}) through changing its catalytic properties or expression.[2] Attempt to improve Rubisco properties was, however, often hampered by the inability of reconstructing functional plant Rubisco (consists of eight large and eight small subunits: LS_8:SS_8). Therefore, recent success in producing functional plant Rubisco in *Escherichia coli* would facilitate improving Rubisco properties in coming years.[3] Another approach to improve photosynthetic capacity or accelerate V_{cmax} is to increase the Rubisco content. While overexpression of LS resulted in up to 30% increase in Rubisco content in rice, corresponding increase in CO_2 assimilation rate (A) was not realized.[4] In contrast, co-overexpression of LS, SS, and RAF1 (an assembly chaperone that binds and stabilizes LS_2)[3] in maize led to >30% increase in Rubisco content as well as 15% increase in maximum A, which correlated with increased *in vitro* V_{cmax} and plant fresh weight.[5] Likewise, our transgenic Arabidopsis with 80% greater concentration of BSD2 (a small Zn-finger protein that works in the final exchange of LS_8:SS_8 from LS_8:$BSD2_8$)[3] (BSD2ox) resulted in 20% increase in *in vivo* V_{cmax}, as well as 20% increase in shoot dry mass.[6] However, unlike the overexpression of RAF1,[5] both the number of total catalytic site and Rubisco content were unaffected. Instead, apparent catalytic efficiency for Rubisco carboxylation (*in vivo* V_{cmax} per total catalytic site) was found to be improved by 40%, suggesting increase in the proportion of active Rubisco.

To be catalytically active, mature Rubisco requires conformational repair by AAA+ chaperone Rubisco activase (Rca), which removes inhibitory sugar phosphates (Sugar-P) from the active site when the stromal ATP/ADP ratio increases with photophosphorylation and thereby facilitates the carbamylation (solid line box in Figure 1).[7] Notably, the Rca-mediated Rubisco activation is regulated in a redox-dependent manner, partly modulated by thioredoxins that activate Rca by reducing cysteine residues of the larger isoform.[8] Rca plays a significant role for the dynamic activation of Rubisco and is regarded as a potential target for photosynthetic improvement.[9–11] Intriguingly, our study demonstrated that the improvement of the apparent catalytic efficiency could be attained without mediating through the Rca, but by directly manipulating the redox state of Rubisco (dash line box in Figure 1). As was annotated by the DnaJ-like Zn-finger motifs (Figure 2a), recombinant BSD2 (rBSD2) had protein disulfide reductase (PDR) activity *in vitro*.[6] Moreover, the rBSD2 could reactivate Rubisco that has been oxidized by hydrogen peroxide (H_2O_2), leading to the hypothesis that BSD2 reactivates Rubisco oxidized by ROS and thereby maintains the photosynthetic capacity. Indeed, proteomic approaches have identified Rubisco cysteines as primary targets for oxidants like H_2O_2 *in vivo*[16] as well as *in vitro*.[17] Since cysteine thiol group can be easily oxidized by molecular oxygen in alkaline conditions,[18] the oxidation of Rubisco by O_2 may also occur when the stroma pH increases due to the proton gradient across the thylakoid membrane generated during photosynthesis. Our hypothesis is further supported by the fact that none of the freshly isolated Rubisco was oxidatively inactive in

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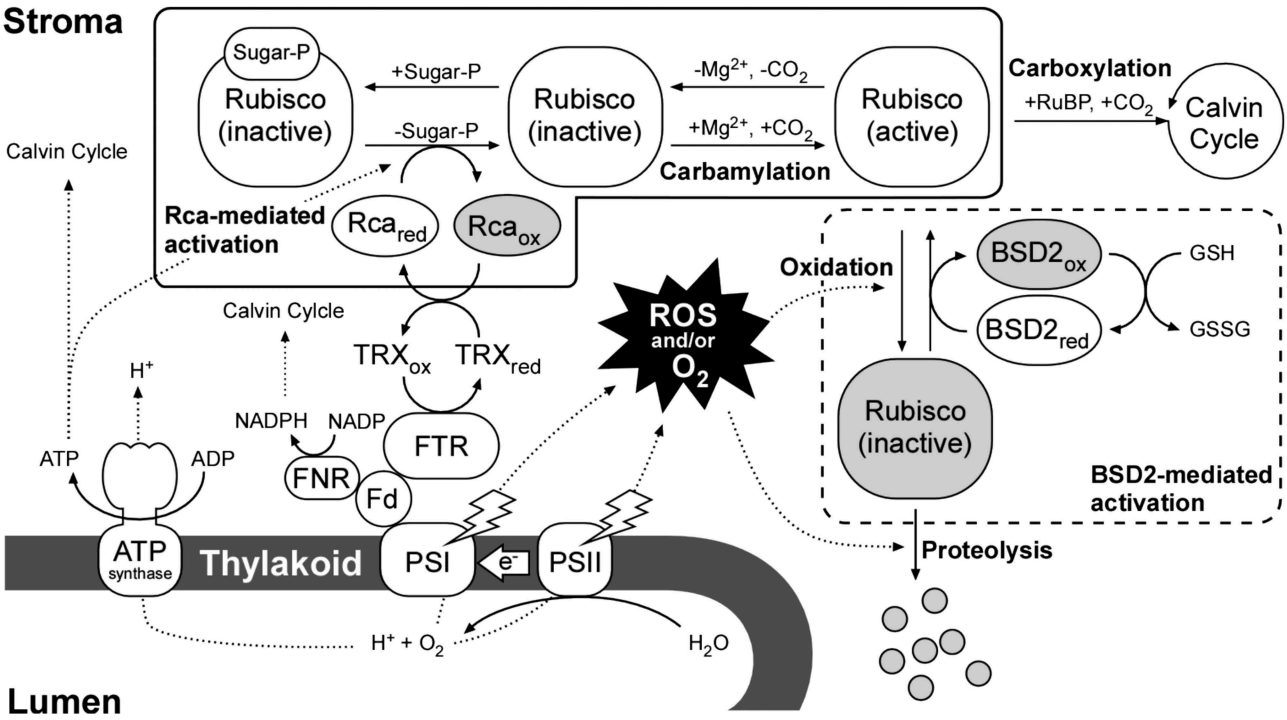

Figure 1. Redox regulation of Rubisco activity in chloroplast. Light energy absorbed by photosystem (PS) I and PSII on the thylakoid induce electron transport that generates primary reductants NADPH, while splitting of water evolves oxygen in the lumen. Thioredoxins (TRX) also accept reducing power from the electron transport chain, which in the reduced form are used for activating Calvin-cycle enzymes as well as Rubisco activase (Rca) in the stroma. The reduced Rca (Rca_{red}) then facilitates Rubisco to become active through carbamylation (solid line box). In the presence of oxygen, excess light energy can also lead to the production of harmful reactive oxygen species (ROS) which would oxidize Rubisco, causing from the reversible inactivation to proteolysis. Reduced BSD2 ($BSD2_{red}$) could reactivate the oxidized Rubisco (dashed line box). GSH: reduced glutathione, GSSG: oxidized glutathione, Fd: ferredoxin, FNR: ferredoxin-NADP reductase, FTR: ferredoxin-TRX reductase, RuBP: Ribulose-1,5-bisphosphate.

BSD2ox plants while 15% was so in control plants[6] (which could be attributable to about half of the gain in the apparent carboxylation efficiency). While we cannot exclude the possibility that the BSD2 affects the redox state of Rca, those *in vitro* assays were independent from the Rca.

Therefore, we examined the redox status of Rubisco LS in wild type *Arabidopsis* with non-reducing SDS-PAGE. The LS in the leaf extract was fragmented mostly to a reduced form of 50 kDa monomers, and much less to an oxidized form of 100 kDa dimers (lane 2 in Figure 2b). Incubation of the same leaf extracts with DTT reduced the dimers to the monomers (lane 1 in Figure 2b) whereas incubation with $CuCl_2$ oxidized the dimers to the monomers (lane 3–5 in Figure 2b), indicating the reversible response to the redox change. Progressive oxidation of Rubisco cysteines using disulfide/thiol mixtures at different ratios has shown that the carboxylation activity decreases as the redox potential decreases.[19,20] Based on their redox-dependent Rubisco inactivation profie,[19,20] the 15%-loss in Rubisco activity by oxidation[6] corresponds to much milder oxidative conditions than proteolytic sensitization, suggesting that the inactivation would not have been involved in significant conformational changes. Similarity in the redox-activity relationships among eukaryotic green-like Rubisco suggests that the critical cysteines are universally conserved.[20] Importantly, none of single substitutions of these conserved cysteines with serine in *Chlamydomonas* Rubisco eliminates the sensitivity of catalytic activity to disulfide exchange, strongly suggesting the highly redundant contribution of cysteine residues in oxidative inactivation.[20]

Interestingly, glutathione (GSH) was more effective reductant for the PDR activity of rBSD2 than NADPH and NADH (i.e., reductants present *in vivo*) and its efficiency is comparable to DTT.[6] Glutathione and ascorbate are very abundant in the chloroplast stroma ranging between 0.5–3.5 and 20–300 mM, respectively, thereby constituting a pool of redox buffers against ROS.[21,22] While direct regulation of Rubisco activity by the chloroplastic glutathione pool is hindered by kinetic barriers impeding access to the critical residues, GSH would drive the reactivation with the aid of smaller intermediary thiol/disulfide exchangers.[23] In contrast to the light-dependent ferredoxin- or NADP-thioredoxin systems, the glutathione pool would be a stable source of reducing power when photosynthetic electron transport is diminished under stressful conditions.[21] It has been frequently noted that oxidation of Rubisco is physiologically relevant to senescence or stress scenarios, which are known to trigger a fast catabolism of Rubisco.[19,20] The reactivation of Rubisco by BSD2 might be therefore important for alleviating senescence or stress scenarios. Now, the regulation of Rubisco activation is extended to a new dimension – redox, which would also open new avenue for improving photosynthesis.

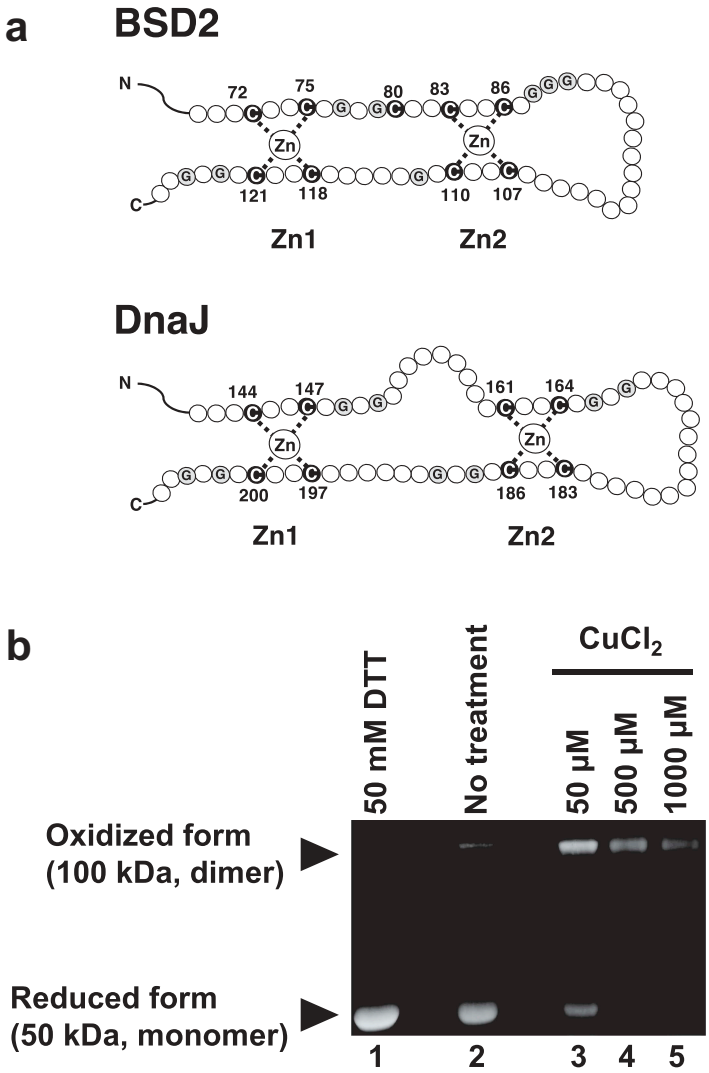

Figure 2. (a) Pictorial topologies of two Cys4-type Zn-finger motifs in BSD2 from Arabidopsis and DnaJ from *E. coli*. The location of cysteine (C) and glycine (G) residues in each protein are shown. BSD2 orthologues in green plants universally contain these domains,[12–14] which in *E. coli* DnaJ show either chaperone activity or thiol-disulfide oxidoreductase activity.[15] (b) Redox state of Rubisco large subunit (LS). Non-reducing SDS-PAGE was performed in leaf extracts from 3-week-old wild type plants. Leaf extracts were untreated (lane 2) or incubated with varying concentrations of $CuCl_2$ oxidant (lane 3 to 5) or 50 mM DTT reductant (lane 1). Note that oxidized and reduced LS were separated as 100 kDa LS dimers and 50 kDa monomers.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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