Plant Signaling & Behavior 5:3, 264-266; March 2010; © 2010 Landes Bioscience

Reactive oxygen species derived from impaired quality control of photosystem II are irrelevant to plasma-membrane NADPH oxidases

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Key words: reactive oxygen species (ROS), photosystem II repair cycle, chloroplast, FtsH, NADPH oxidase, D1 protein, protein turnover

Abbreviations: cpROS, chloroplastic reactive oxygen species; DAB, 3,3'-deaminobenzidine; NBT, nitroblue tetrazolium; PSII, photosystem II

Submitted: 11/11/09

Accepted: 11/11/09

Previously published online: www.landesbioscience.com/journals/psb/article/10604

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Addendum to: Kato Y, Miura E, Ido K, Ifuku K, Sakamoto W. The variegated mutants lacking chloroplastic FtsHs are defective in D1 degradation and accumulated reactive oxygen species. Plant Physiol 2009; 151:1790-801; PMID: 19767385; DOI: 10.1104/pp.109.146589.

Protein quality control plays an important role in the photosynthetic apparatus because its components receive excess light energy and are susceptible to photooxidative damage. In chloroplasts, photodamage is targeted to the D1 protein of Photosystem II (PSII). The coordinated PSII repair cycle (PSII disassembly, D1 degradation and synthesis, and PSII reassembly) is necessary to mitigate photoinhibition. A thylakoid protease FtsH, which is formed predominantly as a heteromeric complex with two isoforms of FtsH2 and FtsH5 in Arabidopsis, is the major protease involved in PSII repair. A mutant lacking FtsH2 (termed *var2*) shows compromised D1 degradation. Furthermore, *var2* accumulates high levels of chloroplastic reactive oxygen species (cpROS), reflecting photooxidative stress without functional PSII repair. To examine if the cpROS produced in *var2* are connected to a ROS signaling pathway mediated by plasma membrane NADPH oxidase (encoded by *AtRbohD* or *AtRbohF*), we generated mutants in which either *Rboh* gene was inactivated under *var2* background. Lack of NADPH oxidases had little or no impact on cpROS accumulation. It seems unlikely that cpROS in *var2* activate plasma membrane NADPH oxidases to enhance ROS production and the signaling pathway. Mutants that are defective in PSII repair might be valuable for investigating cpROS and their physiological roles.

Photosynthetic apparatus components receive excess light energy that can ultimately engender photoinhibition.[1,2] Chloroplasts are therefore equipped with molecular systems to minimize accumulation of photodamaged proteins.[3] Photosystem II, a large pigment-protein complex located in the thylakoid membrane, transfers electrons to plastoquinone and drives oxidation of water molecules using light energy.[4,5] Because PSII is an initial and rate-limiting step of electron flow, photosynthetic organisms have evolved a unique mechanism of protein quality control (PSII repair cycle), in which the damage is centralized into the reaction center D1 protein, and in which PSII is recycled efficiently.[6,7] Several lines of evidence from genetic and biochemical studies indicate that a prokaryotic ATP-dependent metalloprotease FtsH plays a critical role in D1 turnover of the PSII repair.[8-12]

In chloroplasts, FtsH forms a heteromeric complex with two major isoforms.[13,14] Mutants lacking either major isoform (*var2* lacking FtsH2 or *var1* lacking FtsH5) show leaf variegation forming white sectors that contain cells with aberrant plastids.[8,9,11,15] The variegated phenotype implies that FtsH is involved not only in D1 degradation but also in thylakoid development.[16,17] We conducted in vivo D1 degradation assays using "non-variegated" *var1* and *var2* mutants (owing to a trans-acting suppressor mutation *fug1*).[18,19] Results showed that both D1 degradation and PSII electron transport rates were impaired in these non-variegated lines.[19] Collectively, our results corroborate the important role of chloroplast FtsH in the PSII repair cycle. We also infer that the variegation phenotype in *var* mutants is separable from the defect in the PSII repair.

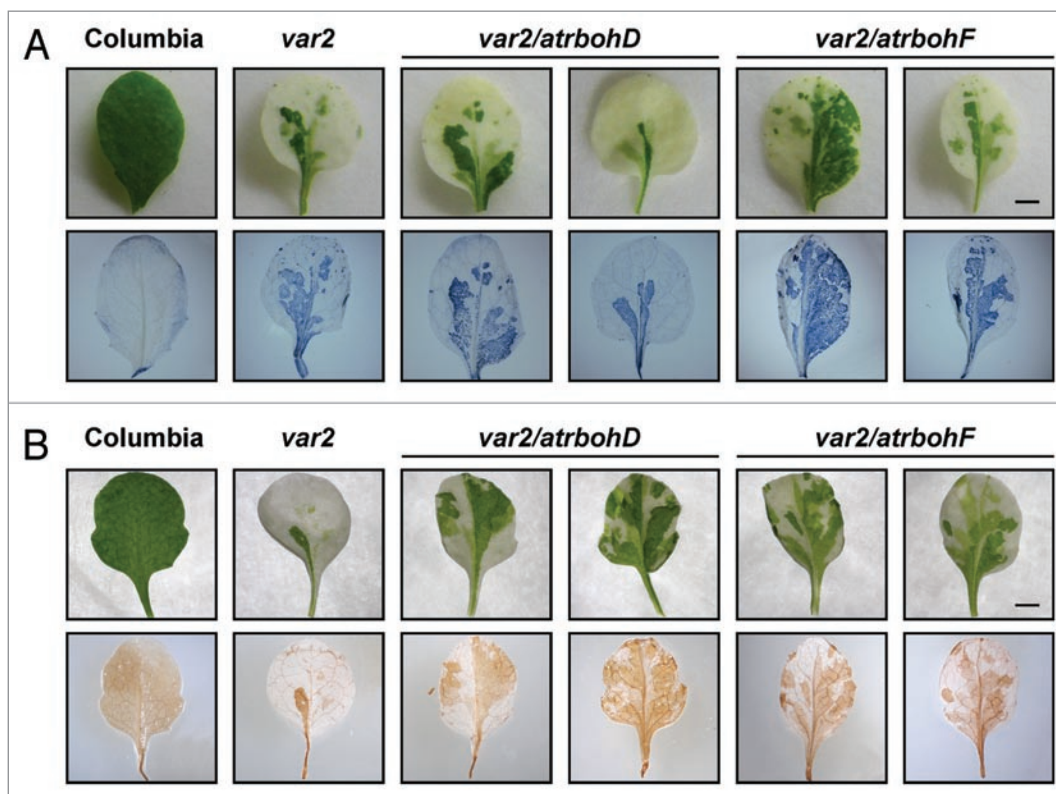

Figure 1. ROS accumulation in *var2* and *var2/atrboh* mutants. (A) In situ detection of superoxide by staining with NBT (blue, bottom panels) in four-week-old wild type (Columbia), *var2*, *var2/atrbohD*, *var2/atrbohF* leaves. Bar = 1 mm. (B) In situ detection of hydrogen peroxide by DAB staining (dark brown, bottom panels) in four-week-old wild type (Columbia), *var2*, *var2/rbohD*, *var2/rbohF* leaves. Bar = 1 mm.

Two important observations concomitant with impaired D1 degradation were made in our recent study.[19] One is the accumulation of PSII partial complexes in *var2*. Two-dimensional blue-native SDS-PAGE analysis demonstrated that thylakoid-membrane fractions from *var2* chloroplasts contained fewer PSII supercomplexes (representing functional PSII) and more partial PSII complexes (representing disassembled intermediates in the PSII repair cycle). These results indicate, although indirectly, that the impaired D1 degradation affects the disassembly/reassembly step of the PSII repair cycle. The other important observation is the accumulation of reactive oxygen species (ROS), such as superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) in *var2*. Results of NBT staining indicated that O_2^- is specially localized in chloroplasts of *var2* green sectors in a light-dependent manner. No NBT staining was detected in wild type under identical conditions. Similarly, DAB staining indicated that H_2O_2 is detectable in *var2* green sectors. High ROS in *var2* therefore demonstrates that chloroplasts suffer from photooxidative stress without a functional PSII repair system. Where these ROS are generated within chloroplasts remains unclear. We raise one possibility: that PSII partial complexes in *var2* contribute to ROS production because they potentially accumulate excitation energy that might not be used for water oxidation.

A considerable amount of chloroplastic O_2^- in *var2* might be converted rapidly to H_2O_2, which can then be exported to cytosol or to other organelles for detoxification. Simultaneously, H_2O_2 in cytosol might act as a signaling molecule and consequently affect responses to environmental stress.[20] We raised one possibility: cpROS in *var2* are influenced by an apoplastic oxidative burst that is mediated by plasma membrane-bound NADPH oxidases and which further activates downstream signaling cascades. For example, cpROS produced in guard cells of ozone-treated Arabidopsis were shown to activate certain NAPDH oxidases through the action of heterotrimeric G protein signaling.[21] Although Gα subunit activated by cpROS is primarily involved in oxidative bursts, Gβγ complexes appear to act on further production of cpROS.[21] These observations led us to examine whether high cpROS in *var2* are regulated by NADPH oxidases.

Ten genes for NADPH oxidases (*AtRbohA to AtRbohJ*) are reported in Arabidopsis.[22] Among these, *AtRbohD* and *AtRbohF* are expressed in mesophylls and are involved in cpROS signaling in guard cells.[22-24] To investigate the effect of these NADPH oxidases on cpROS accumulation in *var2*, we generated double mutants (*var2/atrbohD* and *var2/atrbohF*). The degrees of leaf variegation were similar in *var2* and the double mutants. Single *atrbohD* and *atrbohF* mutants did not accumulate detectable ROS (not shown). We observed strong signals in *var2/atrbohD* and *var2/atrbohF* double mutants both in NBT and DAB stains (**Fig. 1**). Overall, results showed no significant difference in the accumulation of cpROS

between *var2* and the double mutants. Furthermore, results of our microarray analyses demonstrated that expression levels of *AtRbohD* and *AtrbohF* are similar between *var2* green sectors and wild type (unpublished data). Taken together, these results suggest that no apparent NADPH oxidase activities in plasma membranes contribute to cpROS detected in *var2*.

Actually, ROS transiently generated by apoplastic NADPH oxidases are known to regulate a cell-death signaling pathway such as a hypersensitive response against pathogen infection.[25] Based on results of our current genetic and microarray analyses, we reason that, in *var2*, constitutive cpROS do not activate the ROS-mediated signaling pathway. Nevertheless, involvement of cpROS in signaling cascades has been suggested in other experimental systems. The mutants described in this report might be valuable for use in future studies.

Acknowledgements

We thank Dr. Jeffery L. Dangl for providing *atrboh* mutants. This work was supported by a Grant-in-Aid for Scientific Research from MEXT (16085207), the Asahi Glass Foundation, and the Oohara Foundation. E.M. is supported by a postdoctoral fellowship from JSPS.

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