Contribution of Gibberellin Deactivation by AtGA2ox2 to the Suppression of Germination of Dark-Imbibed Arabidopsis thaliana Seeds

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Gibberellin levels in imbibed Arabidopsis thaliana seeds are regulated by light via phytochrome, presumably through regulation of gibberellin biosynthesis genes, AtGA3ox1 and AtGA3ox2, and a deactivation gene, AtGA2ox2. Here, we show that a loss-of-function ga2ox2 mutation causes an increase in GA4 levels and partly suppresses the germination inability during dark imbibition after inactivation of phytochrome. Experiments using 2,2-dimethylGA4, a GA4 analog resistant to gibberellin 2-oxidase, in combination with ga2ox2 mutant seeds suggest that the efficiency of deactivation of exogenous GA4 by AtGA2ox2 is dependent on light conditions, which partly explains phytochrome-mediated changes in gibberellin effectiveness (sensitivity) found in previous studies.

Keywords: Arabidopsis thaliana — AtGA2ox2 — Gibberellin — Gibberellin 2-oxidase — Phytochrome — Seed germination.

Abbreviations: FR light, far-red light; 2ODD, 2-oxoglutarate-dependent dioxygenase; R light, red light.

Gibberellins are tetracyclic diterpenoids, some of which act as hormones in various aspects of plant growth and development. In many plant species, bioactive gibberellins act as stimulators of seed germination. In Arabidopsis, severe gibberellin-deficient mutants, such as ga1-3 and ga2-1, are defective in seed germination, and de novo gibberellin biosynthesis after the onset of imbibition is thought to be necessary for seed germination based on the inhibitory effect of gibberellin biosynthesis inhibitors on germination (Koornneef and van der Veen 1980, Nambara et al. 1991, Mitchum et al. 2006). Classes of 2-oxoglutarate-dependent dioxygenases (2ODDs) are involved in the final stage of gibberellin biosynthesis, including gibberellin 3-oxidase that is responsible for the formation of bioactive gibberellins (Fig. 1; Hedden and Kamiya 1997, Olszewski et al. 2002, Yamaguchi 2006). In Arabidopsis, GA4 has been thought to be the major active form, although GA1 (13-hydroxylated GA4) also exists in most Arabidopsis tissues at lower concentrations (Yamaguchi 2006). Deactivation mechanisms are crucial for effective regulation of hormone levels. The best characterized gibberellin deactivation reaction is gibberellin 2-oxidation, which is catalyzed by another class of 2ODDs. Recently, the occurrence of additional deactivation mechanisms by a cytochrome P450 monooxygenase and methyltransferases in plants has been demonstrated (Zhu et al. 2006, Varbanova et al. 2007), which indicates that gibberellin deactivation is catalyzed by a number of enzymes that belong to multiple categories.

In Arabidopsis, seed germination is critically dependent on light conditions, and phytochromes play a role in the perception of light by the seed (Shinomura 1997). Among five phytochrome species in Arabidopsis, phyB is present in dry seeds in part as the active form (Pfr) (Shinomura et al. 1994). Once phyB is inactivated by irradiation with a far-red (FR) light pulse shortly after the start of imbibition, germination is suppressed during the following imbibition in darkness. In the dark-imbibed seeds, phyB mediates the low fluence response and stimulates germination in response to red (R) light in an FR light-reversible manner, while phyA accumulates during dark imbibition and promotes seed germination by sensing extremely low fluence light in a wide range of wavelengths (very low fluence response) (Shinomura et al. 1994, Shinomura et al. 1996). Although multiple phytochrome species are involved in the induction of germination in response to varying wavelengths and intensities of light, the effect of light on gibberellin metabolism in the seed has been best studied in the phyB-dependent germination condition in Arabidopsis, as well as in similar light conditions in lettuce (Lactuca sativa L. cv. Grand Rapids). In Arabidopsis, R light is thought to promote
seed germination in part through increasing gibberellin levels, presumably as a result of up-regulation of *AtGA3ox1* and *AtGA3ox2* and down-regulation of *AtGA2ox2* (Fig. 1; Yamaguchi et al. 1998, Oh et al. 2006, Seo et al. 2006). Up- and down-regulation of gibberellin 3-oxidase and gibberellin 2-oxidase genes, respectively, by R light has also been found in dark-imbibed lettuce seeds (Toyomasu et al. 1998, Nakaminami et al. 2003). Gibberellin 3-oxidase and gibberellin 2-oxidase are each encoded by a multigene family, but only a specific member(s) in each family is likely to be responsible for light regulation of gibberellin levels in both *Arabidopsis* and lettuce seeds. Among seven genes encoding gibberellin 2-oxidase in *Arabidopsis*, only *AtGA2ox2* is expressed at relatively high levels in dark-imbibed seeds, while expression of other *AtGA2ox* genes stays at very low levels in imbibed seeds irrespective of light conditions (Ogawa et al. 2003, Yamauchi et al. 2004, Oh et al. 2006, Seo et al. 2006). However, these previous studies are solely based on transcript analyses, and it remains to be investigated whether up-regulation of gibberellin deactivation by *AtGA2ox2*, in addition to down-regulation of gibberellin synthesis, plays any significant role in lowering gibberellin levels and suppressing germination in dark-imbibed seeds.

To clarify the contribution of gibberellin deactivation by *AtGA2ox2* to phytochrome regulation of seed germination, we obtained two independent insertion alleles of this gene: we designated the one in the Col-0 background as *ga2ox2-1* and the other in the No-0 background as *ga2ox2-2*. Fig. 2A shows that *ga2ox2-1* mutant seeds

![Fig. 1](https://academic.oup.com/pcp/article-abstract/48/3/555/2329832/fig1) Gibberellin biosynthesis and deactivation pathways in *Arabidopsis*. Only the major routes in the late stage of the pathway are shown. *3ox1*, *AtGA3ox1* (At1g15550); *3ox2*, *AtGA3ox2* (At1g80340); *2ox2*, *AtGA2ox2* (At1g30040). The arrow in a circle indicates up- or down-regulation by active phytochrome (Pfr) in dark-imbibed Arabidopsis seeds.

![Fig. 2](https://academic.oup.com/pcp/article-abstract/48/3/555/2329832/fig2) Light-dependent germination phenotypes of *ga2ox2* mutant seeds. Diagrams on the top of each panel depict light conditions. (A) Germination profiles of wild-type and *ga2ox2* mutant seeds under constant white light. (B) Germination percentage in darkness after irradiation with an FR light pulse. Germination was scored 4 d after the FR light pulse.
Germinated normally like wild-type seeds under constant white light. However, ga2ox2-1 mutant seeds germinated at significantly higher frequencies than did wild-type seeds when they were imbibed in darkness after irradiation with an FR light pulse that inactivates phyB in this condition (Shinomura et al. 1994, Shinomura et al. 1996) (Fig. 2B). A similar germination phenotype, although less prominent, was also observed for the ga2ox2-2 allele in the No-0 background (Fig. 2B).

To assess whether the increased germination frequency of ga2ox2 mutant seeds resulted from altered gibberellin 2-oxidase activity, we compared endogenous gibberellin levels between the wild type and the ga2ox2-1 mutant in dry and dark-imbibed seeds. As shown in Fig. 3, there was no significant difference in the levels of bioactive GA4 between these two genotypes in dry seeds. However, ga2ox2-1 seeds contained higher levels of GA3 than wild-type seeds during imbibition in darkness after FR light treatment. These results indicate that AtGA2ox2 plays a role in lowering GA4 levels in dark-imbibed wild-type seeds in the absence of active phytochrome. Thomas et al. (1999) reported that recombinant AtGA2ox2 protein was able to catalyze 2β-hydroxylation of both GA9 and GA3 to produce GA51 and GA34, respectively (Fig. 1), and that GA9 appeared to be a preferred substrate. Consistent with the predicted reduction in gibberellin 2-oxidase activity in the ga2ox2-1 mutant, the level of GA51 increased during dark imbibition in wild-type seeds, but not in ga2ox2-1 mutant seeds (Fig. 3). The presence of GA51 in ga2ox2-1 dry seeds is likely to be due to other AtGA2ox gene(s) that are expressed during seed maturation (Wang et al. 2004). The predicted reduction in the conversion of GA9 to GA51 in the ga2ox2 mutant would cause accumulation of GA9. However, the level of GA9 in dark-imbibed ga2ox2 mutant seeds was not significantly higher than that in wild-type seeds (Fig. 3). In addition, unlike the case with GA51, the level of GA34, another product of AtGA2ox2, was higher in ga2ox2 mutant seeds than that in wild-type seeds. This observation might be explained by the possible occurrence of other gibberellin 2-oxidase(s) in the presence of an elevated level of the substrate GA4, although this hypothesis needs to be verified using ga2ox2 seeds with additional loss-of-function mutations of other AtGA2ox genes. Taken together, our gibberellin analysis revealed an increase in the level of bioactive GA4 in the ga2ox2 mutant, but further experiments will be necessary to understand exactly how the metabolic flux is altered in ga2ox2 mutant seeds, e.g. by comparisons of metabolism of GA9 and GA4 between wild-type and mutant seeds.

Earlier studies have shown that phytochrome-dependent light signaling pathways affect the effectiveness of exogenous GA4 (or a mixture of GA4 and GA7) in inducing germination of gibberellin-deficient seeds in Arabidopsis and related species (Hilhorst et al. 1986, Hilhorst and Karssen 1988, Derkx and Karssen 1993, Yang et al. 1993). These results suggest that the gibberellin sensitivity of the seed is regulated by light in these species, although the molecular mechanisms have been unknown. Up-regulation of AtGA2ox2 expression in the absence of active phytochrome (Fig. 1) led us to speculate that the reduced effectiveness of exogenous GA4 in dark-imbibed seeds in previous reports might be (in part) explained by effective deactivation by AtGA2ox2. To address this question, we examined the effect of exogenous GA4 and 2,2-dimethylGA4 on germination of ga1-3 mutant seeds in the dark after FR light or FR/R light pulse treatment. 2,2-DimethylGA4 is a synthetic analog of GA4 that was previously designed to be resistant to 2β-hydroxylation (Fig. 4A; Beale and MacMillan 1981). The biological activity of 2,2-dimethylGA4 is equivalent to or sometimes higher than that of GA4 in various plant systems (Hoad et al. 1981). We prepared recombinant AtGA2ox2 protein in Escherichia coli and confirmed that 2,2-dimethylGA4 was not metabolized by AtGA2ox2, while the majority of GA4...
was converted to GA34 in the same assay conditions (data not shown). Fig. 4B shows that 2,2-dimethylGA4 was significantly more effective in inducing ga1-3 germination than GA4 when the seeds were imbibed in the dark after an FR light pulse. However, these two chemicals were nearly equally effective in inducing ga1-3 germination after an R light pulse, which activates phyB in this condition (Shinomura et al. 1994) and down-regulates AtGA2ox2 expression (Fig. 4C). These observations are consistent with the idea that the reduced effectiveness of GA4 in the absence of active phytochrome is partly attributable to the elevated GA4 metabolism by gibberellin 2-oxidase.

In order to determine whether AtGA2ox2 was truly responsible for deactivation of exogenous GA4, we compared the effectiveness of GA4 and 2,2-dimethylGA4 in wild-type and ga2ox2 mutant seeds. As shown in Fig. 5, 2,2-dimethylGA4 was more effective than GA4 in inducing germination of wild-type seeds that were imbibed in the...
dark after an FR light pulse. However, these two compounds were almost equally effective in inducing germination of qa2ox2 mutant seeds in the same conditions (Fig. 5). These results support our interpretation that AtGA2ox2 is responsible for deactivation of exogenous GA4 in dark-imbibed seeds in the absence of active phytochrome. Taken together, our results suggest that light-dependent alterations in gibberellin effectiveness observed in previous studies (Hilhorst and Karssen 1988, Derkx and Karssen 1993, Yang et al. 1993) can be partly explained by phytochrome-regulated AtGA2ox2 gene expression.

It has been unknown whether phytochrome-regulated gibberellin synthesis and deactivation occur in the same cell types in a seed. To address this question, we employed in situ hybridization to examine cellular localization of AtGA2ox2 mRNA. We found that the AtGA2ox2 transcript accumulated predominantly in the cortex of embryonic axis (mainly in the hypocotyl area) in dark-imbibed seeds after FR light irradiation, as observed for AtGA3ox1 and AtGA3ox2 transcripts in light-imbibed seeds in our previous study (Yamaguchi et al. 2001; also shown in Fig. 6 as a reference). These results indicate that phytochrome-dependent changes in gibberellin levels are achieved through altered gibberellin synthesis and deactivation that mainly take place in the same cell types.

Concerted up- and down-regulation of gibberellin biosynthesis and deactivation genes, respectively, or vice versa, has previously been observed in response to endogenous and/or environmental cues in several plant species (Toyomasu et al. 1998, O’Neill and Ross 2002, Nakaminami et al. 2003, Yamauchi et al. 2004, Oh et al. 2006, Seo et al. 2006). However, the contribution of gibberellin deactivation to the change in gibberellin content and the observed physiological responses by such signals remains unknown, due to the lack of studies using loss-of-function mutants defective in gibberellin deactivation in each context. In the present study, we have demonstrated that the lack of gibberellin deactivation by AtGA2ox2 causes an increase in GA4 levels and partly suppresses the non-germination of dark-imbibed wild-type seeds after an FR light pulse. However, the removal of AtGA2ox2 alone was not sufficient to allow germination at high frequencies in this unfavorable light condition (Fig. 2B), probably due in part to the repression of gibberellin biosynthesis genes, AtGA3ox1 and AtGA3ox2 (Fig. 1; Yamaguchi et al. 1998). Altogether, these results support the idea that light-regulated gibberellin biosynthesis in concert with

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Fig. 5 Effects of exogenous GA4 and 2,2-dimethylGA4 on germination of wild-type and qa2ox2 mutants. Seeds were imbibed in darkness after an FR light pulse, and the germination percentage was scored as described in Fig. 4A. Comparisons between wild-type and qa2ox2 mutant seeds in (A) Col-0 and (B) No-0 backgrounds.
deactivation contributes to effective control of bioactive gibberellin levels.

Our experiments using 2,2-dimethylGA₄, a synthetic analog of GA₄ resistant to gibberellin 2-oxidase, provided evidence that altered gibberellin 2-oxidase activity is partly responsible for phytochrome-mediated changes in gibberellin effectiveness of imbibed ga1-3 mutant seeds (Figs. 4, 5). Nevertheless, we speculate that additional factor(s) should also be involved in this phytochrome-dependent process, because R light treatment still slightly increased the effectiveness of 2,2-dimethylGA₄ on germination of ga1-3 mutant seeds (Fig. 4B, C). One possible factor contributing to this phenomenon may be phytochrome regulation of ABA levels, which was also observed in the ga1-3 mutant background (Seo et al. 2006). It is also important to note that gibberellin 2-oxidation is not the only deactivation mechanism. Therefore, we cannot exclude the possibility that 2,2-dimethylGA₄ applied to the seed is metabolized by other enzymes, such as cytochrome P450s (Zhu et al. 2006) and methyltransferases (Varbanova et al. 2007), and that any of these enzymes is also regulated by phytochrome. In addition, genetic analysis using mutants defective in individual member(s) of the DELLA protein family suggest that some of these negative regulators of the gibberellin response pathway may also play a role in light regulation of seed germination (Cao et al. 2005). Further investigations will be necessary to uncover the whole picture of how light signals are translated into hormonal signals to control seed germination.

Materials and Methods

Arabidopsis thaliana ecotypes Columbia-0 (Col-0) and Nossen-0 (No-0) were used as wild type in this study. ga2ox2-1 (Salk_051749) in the Col-0 background was obtained from the Arabidopsis Biological Resource Center (The Ohio State University, Columbus, OH, USA), and ga2ox2-2 (RATM15-4417-1H) in the No-0 background was obtained from RIKEN BioResource Center (Tsukuba, Japan). Both alleles are likely to be null, because the insertion disrupts an exon sequence. The ga1-3 allele (originally found in the Ler background; Koornneef and van der Veen 1980) backcrossed to Col-0 six times was used in the experiments (courtesy of Dr. Tai-ping Sun, Duke University, Durham, NC, USA).

For germination tests, the seeds were soaked with 0.02% Triton-X solution, rinsed with water, and then incubated at 22 °C on wet filter paper (3MM, Whatman, Maidstone, UK) under various light conditions. Germination tests were carried out using triplicate samples (each containing 50–100 seeds). Seeds were scored as germinated when radicle protrusion was visible. For dark incubation, seeds were handled under a dim green safety light. The FR light pulse treatment consisted of 1 min of FR light irradiation (91 μmol m⁻² s⁻¹) supplied from light-emitting diodes (MIL-IF18, Sanyo Biomedical Inc., Osaka, Japan) passed through an FR acrylic filter (D deroglass A900, 2 mm thick, Asahikasei, Japan). The R light pulse treatment consisted of 1 min of R light irradiation (120 μmol m⁻² s⁻¹) supplied from light-emitting diodes (MIL-R18, Sanyo Biomedical Inc.). To compare germination frequencies across different genotypes, we grew parental plants on soil at the same time under identical conditions (continuous light at 22 °C). Harvested mature seeds were stored at room temperature in a desiccator at 30% humidity for at least 2 months to allow after-ripening before germination tests. Germination phenotypes were confirmed using at least two independently prepared sets of seeds.

Gibberellin measurements were carried out by gas chromatography-selected ion monitoring using 2H-labeled gibberellins as internal standards, as described previously (Gawronska et al. 1995). Labeled gibberellins were purchased from Professor Lewis Mander (Australian National University, Canberra, ACT, Australia).

In situ hybridization experiments were carried out using digoxigenin-labeled antisense cRNA probes as described previously (Yamaguchi et al. 2001). To generate the AtGA2ox2 antisense probe, the full-length AtGA2ox2 cDNA (about 1.1 kb) was used as a template.
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References