The DOF Protein DAG1 and the DELLA Protein GAI Cooperate in Negatively Regulating the *AtGA3ox1* Gene

Dear Editor,

Seed germination is controlled by multiple endogenous and environmental factors, which are integrated to trigger this developmental process at the right time. Gibberellins (GAs) are known to induce this process, and the levels of GAs are modulated by light—one of the most important environmental factors affecting seed germination. The bHLH transcription factor PIL5 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 5) is the master repressor of light-mediated seed germination in *Arabidopsis* (Oh et al., 2004). In seeds kept in the dark, PIL5 activates transcription of the *GAI* (*GA INSENSITIVE*) gene (Peng et al., 1997; Oh et al., 2007), a DELLA transcriptional regulator that represses GA-mediated processes (Sun, 2011). GAI plays a role in many growth processes with both unique and overlapping functions with another DELLA protein: RGA (REPRESSOR OF *ga1-3*) (Dill and Sun, 2001).

Also, the DOF transcription factor DAG1 (DOF AFFECTING GERMINATION1) acts in the light-mediated seed germination pathway downstream of PIL5: *DAG1* expression is reduced in seeds irradiated for 24 h with red (R) light, and this reduction is dependent on PIL5 as, in *pil5* mutant seeds, *DAG1* expression is reduced irrespective of light conditions (Gabriele et al., 2010).

Null mutant seeds *dag1* need a fluence rate six times lower than wild-type to germinate (Papi et al., 2000, 2002); similarly, *gai–t6rga28* double mutant seeds require less R light fluences than wild-type ones to germinate (Oh et al., 2007).

To further clarify the role of DAG1 in light-mediated seed germination, we focus here on the functional relationship between DAG1 and GAI in the control of this process.

We have recently demonstrated that DAG1 specifically represses *AtGA3ox1* expression. In *dag1* mutant seeds, only this GA biosynthetic gene was up-regulated, while the level of expression of *AtGA3ox2* and *AtGA2ox2* were unchanged compared to the wild-type (Gabriele et al., 2010). A very similar expression profile of *AtGA3ox1* was shown by Oh et al. (2007) in *gai–t6rga28* double mutant seeds. To verify whether GAI plays a role in the regulation of GA metabolic genes, and in particular of *AtGA3ox1*, we performed a quantitative RT–PCR (RT–qPCR) analysis on *gai-t6* mutant seeds. The level of the *AtGA3ox1* transcript was highly increased in the *gai-t6* null mutant compared to the wild-type, both in seeds imbibed in the dark and those exposed to R light (Figure 1A), while expression of *AtGA3ox2* and *AtGA2ox2* was not significantly altered. Since—similarly to *DAG1* inactivation—*GAI* inactivation specifically affected *AtGA3ox1* expression, we decided to verify whether the presence of GAI is necessary for DAG1-mediated repression of *AtGA3ox1*. In agreement with our hypothesis, promoter analysis of GAI-regulated genes revealed a significant enrichment of DOF-binding sites (Gallego-Bartolomé et al., 2011), suggesting that these transcription factors may mediate GAI activity.

We used the *dag1DAG1–HA* (Gabriele et al., 2010) and the *dag1gai-t6DAG1–HA* lines, which overexpress *DAG1* respectively in the *dag1* and *dag1gai-t6* mutant backgrounds. Both these lines expressed the DAG1–HA chimeric protein as revealed by immunoblot analysis (Supplemental Figure 1). As expected, the expression of *AtGA3ox1* in *dag1DAG1–HA* seeds was highly reduced compared to wild-type both in the dark and under R light, due to overexpression of *DAG1–HA*, whereas *AtGA3ox1* was strongly overexpressed in *dag1gai-t6DAG1–HA* seeds (Figure 1B and 1C), suggesting that both DAG1 and GAI are involved in the regulation of *AtGA3ox1*.

Since inactivation of *GAI* makes DAG1 unable to repress *AtGA3ox1* expression, we set to assess whether these two factors directly collaborate in regulating this GA biosynthetic gene. We performed chromatin immunoprecipitation (ChIP) assays using the *GAI–MYC* transgenic line constructed by Oh et al. (2007), and the *dag1DAG1–HA* line (Gabriele et al., 2010) as a positive control. Cross-linked and sonicated protein–DNA complexes were precipitated with anti-MYC and anti-HA antibodies, respectively. We amplified by real-time PCR (qPCR) three regions of the *AtGA3ox1* promoter containing different numbers of copies of DOF-binding sites (0, 2, and 15) (Figure 1D). As a negative control, we performed the same assays without adding the antibody, or with both antibodies on wild-type seeds (Supplemental Figure 2). The relative amounts of precipitated promoter fragments of *AtGA3ox1* by DAG1–HA are higher than the negative control, and the enrichment of the target fragment is proportional to the number of DOF sites present in the region. By contrast, the enrichment of precipitated promoter fragments of *AtGA3ox1* was very

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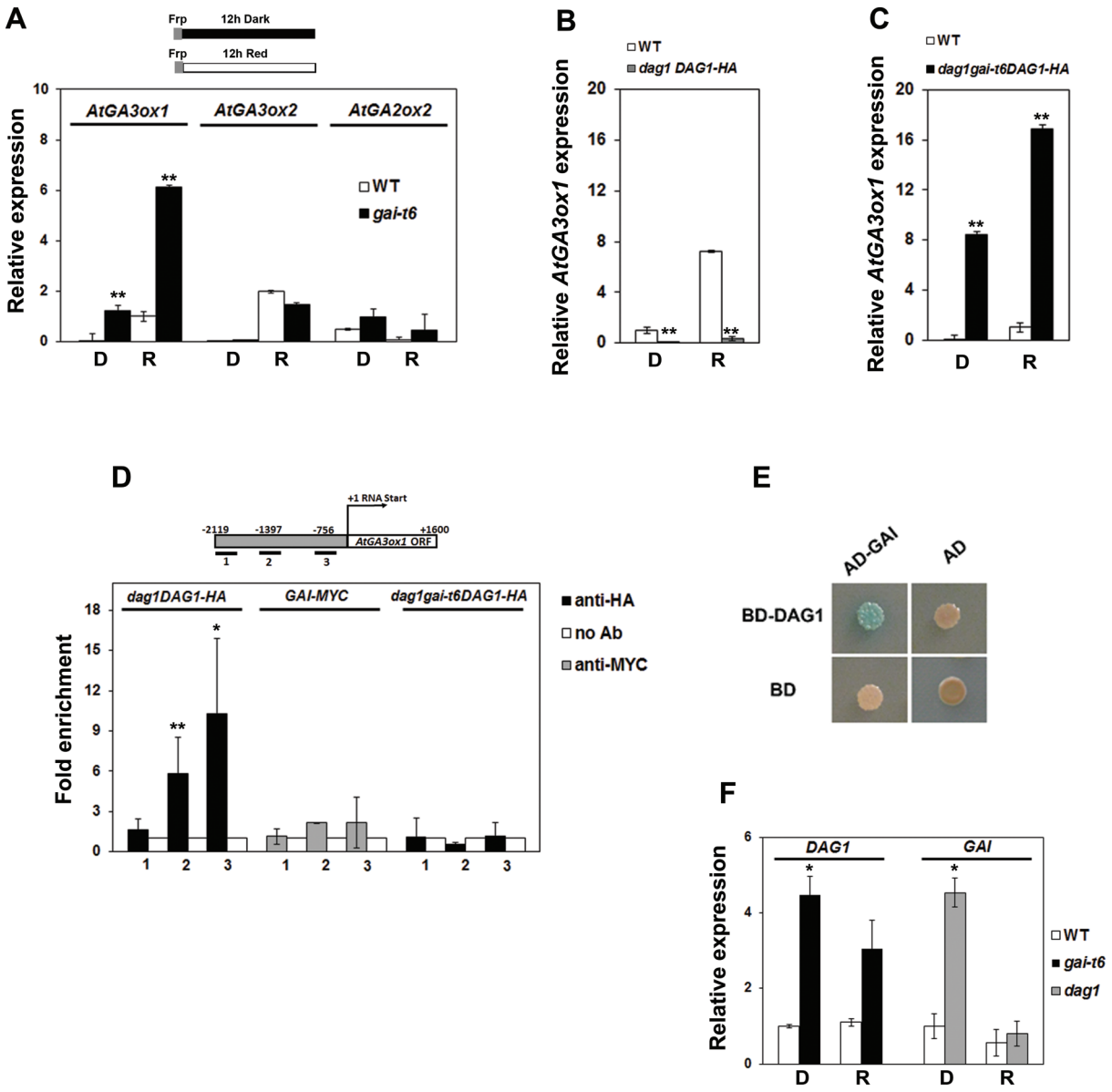

Figure 1 GAI Cooperates with DAG1 in Regulating *AtGA3ox1*.

(A) Relative expression level of *AtGA3ox1*, *AtGA3ox2*, and *AtGA2ox2* in wild-type (WT) and *gai-t6*.

(B, C) Relative expression level of *AtGA3ox1* in *dag1DAG1–HA* (B), and in *dag1gai-t6DAG1–HA* (C).

(D) Top: Graphic representation of the *AtGA3ox1* promoter. Underlying thick lines marked by numbers (1, 2, 3) refer different promoter fragments used for qPCR, containing 0, 2, and 15 DOF sites, respectively. Bottom: Chromatin from *dag1DAG1–HA*, *GAI–MYC*, *dag1gai-t6DAG1–HA* seeds was immunoprecipitated with anti-HA or anti-MYC antibodies, and the amount of DNA was measured by qPCR. The values of fold enrichment are the average of three independent experiments presented with SD values.

(E) Yeast two-hybrid assay with DB–DAG1 and AD–GAI. The single constructs were used as negative controls.

(F) Relative expression level of *DAG1* in WT (Col-0) and *gai-t6*, and of *GAI* in WT (Ws-4) and *dag1* seeds.

Relative mRNA levels of each gene are presented by the ratio of the corresponding mRNA level of WT in D (B, G) or in R (A, C), which was set to 1. 12-h imbibed seeds were exposed to dark (D) and red (R) light. Similar results were obtained from three independent biological replicates, and one representative experiment is presented with SD values. Significant differences were analyzed by *t*-test (* $P \leq 0.05$; ** $P \leq 0.01$). Light conditions are indicated by the diagrams on the top. FRp, far red pulse. All the primers used are listed in Supplemental Table 1.

low when GAI–MYC was precipitated with the anti-MYC antibody (Figure 1D). Furthermore, to verify whether the presence of GAI is required for the binding of DAG1 to the promoter of *AtGA3ox1*, we performed a ChIP assay using the *dag1gai-t6DAG1–HA* line. The enrichment of promoter fragments of *AtGA3ox1* precipitated by DAG1–HA was similar to that of the negative control, indicating that GAI is necessary for the binding of DAG1 to the DOF sites in the *AtGA3ox1* promoter (Figure 1D).

We carried out a yeast two-hybrid assay with full-length DAG1 fused to the GAL4 DNA-binding domain (DB–DAG1) and a prey consisting of full-length GAI fused to the GAL4 activation domain (AD–GAI) (Figure 1E and Supplemental Figure 3). Both protein constructs examined expressed the predicted fusion proteins in yeast, as determined by immunoblot analysis (Supplemental Figure 3).

Since our data show a direct interaction between DAG1 and GAI, we examined whether DAG1 and GAI would mutually affect their expression, by performing RT–qPCR in *dag1* and *gai-t6* mutant seeds imbibed for 12 h in the dark or under R light. In the absence of GAI, we observed a significant increase in *DAG1* transcript level irrespective of light conditions, suggesting that GAI impinges on *DAG1* expression (Figure 1F). Similarly, inactivation of *DAG1* clearly affects *GAI* expression, as the amount of *GAI* mRNA was increased up to 2.5-fold in *dag1* mutant seeds imbibed in the dark compared to wild-type seeds (Figure 1F).

Taken together, our results demonstrate that GAI indeed cooperates with DAG1 in repressing *AtGA3ox1*, and that it directly interacts with DAG1. In addition, DAG1 and GAI mutually affect their expression.

Attempts to demonstrate direct DNA-binding capability of DELLA proteins have been so far unsuccessful, so the main mode of action of these proteins in controlling transcription is thought to occur via sequestering of, or interacting with, transcription factors. In agreement with this notion, very recently, two modes of action for DELLA proteins have been proposed, namely the interfering and the targeting model (Park et al., 2013). According to the latter, GAI has been proposed to directly regulate target genes, possibly by interacting with other transcription factors (Gallego-Bartolomé et al., 2011). Moreover, promoter analysis of GAI-regulated genes showed a strong enrichment of DOF-binding sites, suggesting that these transcription factors may mediate GAI activity (Gallego-Bartolomé et al., 2011). Because all DOF proteins recognize very similar target sequences due to their highly conserved DNA-binding domain (Yanagisawa, 2002), these transcription factors are likely to interact with other proteins in order to ensure specificity. Indeed, our data show that GAI is necessary for DAG1 binding to the *AtGA3ox1* promoter, and that it functions by directly interacting with DAG1. Nevertheless, as some aspects of the GAI and DAG1 cooperation are still unclear, further experiments will be required to fully elucidate this novel molecular mechanism.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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