The Arabidopsis Cupin Domain Protein AtPirin1 Interacts with the G Protein α-Subunit GPA1 and Regulates Seed Germination and Early Seedling Development

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Heterotrimeric G proteins are implicated in diverse signaling processes in plants, but the molecular mechanisms of their function are largely unknown. Finding G protein effectors and regulatory proteins can help in understanding the roles of these signal transduction proteins in plants. A yeast two-hybrid screen was performed to search for proteins that interact with Arabidopsis G protein α-subunit (GPA1). One of the identified GPA1-interacting proteins is the cupin-domain protein AtPirin1. Pirin is a recently defined protein found because of its ability to interact with the CCAAT box binding transcription factor. The GPA1–AtPirin1 interaction was confirmed in an in vitro binding assay. We characterized two atpirin1 T-DNA insertional mutants and established that they display a set of phenotypes similar to those of gpa1 mutants, including reduced germination levels in the absence of stratification and an abscisic acid–imposed delay in germination and early seedling development. These data indicate that AtPirin1 likely functions immediately downstream of GPA1 in regulating seed germination and early seedling development.

INTRODUCTION

Heterotrimeric G protein–mediated signal transduction is one of the most conserved eukaryotic signaling mechanisms and is responsible for the transmission of extracellular signals to intracellular effectors. Heterotrimeric guanine nucleotide binding proteins (G proteins) belong to the superfamily of regulatory GTP hydrolases (Kaziro et al., 1991). G proteins consist of three subunits: α (Gα), β (Gβ), and γ (Gγ). In its inactive state, the Gα-subunit is bound to a GDP molecule and is associated with a Gβγ-subunit. Upon activation by a G protein–coupled receptor, the Gα-subunit exchanges GDP for GTP and dissociates from the Gβγ-subunit. As a result, the Gα- and Gβγ-subunits are free to transduce the signal to intracellular effectors (reviewed by Sprang, 1997; Hamm, 1998). In animal systems, effector molecules include adenyllyl and guanylyl cyclases, phosphodiesterases, phospholipases, and phosphoinositide 3-kinases (reviewed by Spiegel et al., 1994; Marinissen and Gutkind, 2001). Little is known about plant G protein effectors. Recently, a small GTPase Rac1 was implicated in the downstream function of the Gα-subunit (Weiss et al., 1994), and two genes that encode Gγ-subunits were identified (Mason and Botella, 2000, 2001). Nevertheless, a number of largely pharmacological studies have suggested plant G protein involvement in a variety of processes, including blue light–mediated responses (Warpeha et al., 1991), red light–mediated responses (Romero and Lam, 1993), abscisic acid (ABA) and gibberellin signaling, regulation of ion channels, and pathogen resistance (reviewed by Assmann, 2002).

Recently, two independent T-DNA insertions that are likely to be null mutations were found in the Arabidopsis Gα-subunit–encoding gene GPA1 (Ullah et al., 2001). Analysis of these gpa1 mutants revealed that they have reduced cell division during hypocotyl and leaf formation (Ullah et al., 2001). Additionally, gpa1 mutants are impaired in the inhibition of stomatal opening by ABA (Wang et al., 2001). The gpa1 mutant seeds appear to be hypersensitive to sugars, hyposensitive to gibberellic acid, and insensitive to brassinosteroids. Furthermore, gpa1 mutant seeds exhibit less than wild-type germination levels in the presence of exogenous ABA (Ullah et al., 2002). Multiple phenotypes of gpa1 mutants suggest that GPA1 is an integration point of several signaling pathways. It is likely that GPA1 executes its diverse functions by interacting with a variety of regulatory and effector proteins; however, no GPA1-interacting proteins have been reported to date.

To further investigate the mechanism of Gα-subunit function in Arabidopsis, we performed a yeast two-hybrid screen to identify its interacting partners. One of the GPA1-interacting proteins found in the screen was AtPirin1, an ortholog of the human pirin protein. Pirin is a recently defined protein found as a result of its ability to interact with the CCAAT box binding transcription factor NFI/CTF1 (Wendler et al., 1997). We have established that AtPirin1 interacts specifically with GPA1 in

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yeast as well as in vitro. \textit{AtPirin1} transcript levels were found to be upregulated by ABA and low-fluence red light but not by low-fluence blue light. Two Arabidopsis mutants harboring T-DNA insertions in the \textit{AtPirin1} gene were characterized. It was observed that insertions in the \textit{AtPirin1} gene cause reduced levels of seed germination in the absence of stratification and that \textit{atpirin1} mutant seed germination is hypersensitive to exogenous ABA in a manner similar to that of \textit{gpa1} mutants. Additionally, we observed that \textit{atpirin1} mutant plants initiate primary shoots and flower earlier than wild-type plants.

\section*{RESULTS}

\subsection*{The Arabidopsis G\textsubscript{\alpha}-Subunit Interacts with \textit{AtPirin1} in the Yeast Two-Hybrid Screen}

To identify potential components of G protein–mediated signaling pathways in plants, we performed a yeast two-hybrid screen of the Arabidopsis seedling library using a full-length Arabidopsis G\textsubscript{\alpha}-subunit (GPA1) as “bait.” Screening of \textasciitilde1.9 \times 10^6 transformants yielded 16 positive clones that specifically activate \textit{His3} and \textit{LacZ} reporter genes. Restriction pattern comparison indicated that four of these positive clones contained partial cDNAs of the same gene. Sequence analysis revealed a protein homologous with human pirin (Wendler et al., 1997). Therefore, we named this GPA1-interacting protein \textit{AtPirin1}. The remaining GPA1-interacting clones will be described elsewhere. \textit{AtPirin1} clones caused specific activation of \textit{His3} and \textit{LacZ} reporter genes and did not interact with the lamin protein used as a control bait (Figure 1A). The interaction between the Arabidopsis proteins ETR1 and CTR1 (Clark et al., 1998) served as a positive control for the assay (Figure 1A). The two shortest GPA1-interacting \textit{AtPirin1} cDNA clones corresponded to the 170 C-terminal amino acids of the \textit{AtPirin1} protein, indicating that the first 117 N-terminal amino acid residues are not critical for the interaction with GPA1 (Figure 2A). The two longest interacting \textit{AtPirin1} cDNA clones lack the 64 N-terminal amino acid residues (Figure 2A).

\textit{AtPirin1} Interacts with GPA1 in Vitro

To confirm that \textit{AtPirin1} binds directly to GPA1, we studied their interactions using an in vitro binding assay. For these experiments, the GPA1 coding region was overexpressed as a glutathione S-transferase (GST) fusion in \textit{Escherichia coli} and immobilized on glutathione-Sepharose beads. Beads coated with GST alone served as a control (Figure 1B). In vitro binding assays were performed with \textit{AtPirin1} obtained by coupled in vitro transcription/translation. After multiple washes, bound proteins were separated on SDS-containing polyacrylamide gels. Autoradiographic detection revealed that \textit{AtPirin1} interacts with GPA1 and not with GST alone (Figure 1C).

Additionally, we tested whether \textit{AtPirin1} interaction with GPA1 depends on the GPA1 activation state. For this analysis, we examined the in vitro activation of GPA1 by incubation with GTP\gammaS (a nonhydrolyzable GTP analog) before the binding assay. In this experiment, \textit{AtPirin1} displayed no significant preference for either the active or the inactive form of GPA1 (data not shown).
AtPirin1 is a Conserved Protein Belonging to the Cupin Superfamily

Conceptual translation of AtPirin1 cDNA revealed that it encodes a 287-amino acid protein. Database searches identified several more pirin genes in the Arabidopsis genome. AtPirin1 is closely related to two of its family members, AtPirin2 and AtPirin3, with 66% identity and 75% amino acid similarity (Figure 2A). Sequence alignments revealed that pirin is present in mammals, plants, and a variety of prokaryotic species, whereas there are no putative pirin orthologs in three model

Figure 2. AtPirin1 Amino Acid Sequence Analysis, AtPirin1 Gene Structure, and Locations of the T-DNA Insertions.

(A) Alignment of the deduced amino acid sequences of AtPirin1, AtPirin2, AtPirin3, AtPirin4, tomato pirin (TOMpirin), and human pirin (HUMpirin). The alignment was generated using the CLUSTAL X program with default parameters. Identical amino acids are highlighted with a black background, and similar residues are highlighted with a gray background. Regions corresponding to motifs 1 and 2 of the cupin domain are indicated. The single and double asterisks mark residues corresponding to the beginning of the longest and shortest GPA1-interacting AtPirin1 clones, respectively, identified in the two-hybrid screen. Arrows indicate the positions of T-DNA insertions in the atpirin1-1 and atpirin1-2 mutants.

(B) Scheme of the AtPirin1 gene. Relative positions of potential cis elements mentioned in the text are shown. The positions of the atpirin1-1 and atpirin1-2 T-DNA insertions are indicated. Boxes represent exons, and motifs 1 and 2 of the cupin domain are marked by vertical and horizontal hatching, respectively.
eukaryotic species with fully sequenced genomes: Saccharomyces cerevisiae, Caenorhabditis elegans, and Drosophila melanogaster.

Pirin does not appear to have any known functional domains; however, it contains a two-motif sequence characteristic of the cupin domain (Figure 2A). Based on the three-dimensional structure of several cupin proteins, it was established that the two-motif cupin domain contains residues implicated in forming a β-barrel fold and in metal binding (Gane et al., 1998; Dunwell et al., 2000). The β-barrel fold also is associated with the high levels of thermal stability of cupin superfamily members (Dunwell et al., 2001). The superfamily of cupin proteins is the most functionally diverse of any family described to date and includes enzymes, transcription factors, seed storage proteins, auxin binding proteins, stress-related proteins (spherulins and germin-like proteins), and other proteins (Dunwell et al., 2000, 2001).

The fact that the two shortest GPA1-interacting AtPirin1 cDNA clones found in the two-hybrid screen do not contain the cupin domain (Figure 2A) indicates that the cupin domain is not required for interaction with the Gα-subunit.

**AtPirin1 mRNA Levels Are Regulated by Abscisic Acid and Red Light**

Sequence analysis of the 5’ regulatory region of AtPirin1 revealed the presence of several potential cis-acting regulatory elements, including two ACGT elements and one TT motif at 308, 430, and 512 bp upstream of the AtPirin1 ATG codon (Figure 2B). ACGT elements usually are defined as 8- to 10-bp sequences with the core sequence ACGT (Busk and Pages, 1998; Leung and Giraudat, 1998); they include the ABRE-like elements (abscisic acid–responsive elements) and the G-box elements. ABREs are found in numerous promoters of ABA- and stress-inducible genes (Leung and Giraudat, 1998). The strong ABRE (CAGGTG), found 430 bp upstream of the AtPirin1 gene ATG codon, is identical to the core sequence of the G-box element. G boxes are found in promoters of several light-regulated genes, such as CAB, RBCS, and CHS (Busk and Pages, 1998).

In contrast to the ACGT elements, little is known about the TT motif. The TT motif, also called the TCGT motif (TTCGTGT), was found originally in the distal promoter region of the desiccation- and ABA-inducible Dc3 gene of carrot and appears to be involved in ABA-induced gene expression in vegetative tissues (Busk and Pages, 1998).

The finding of potential cis-regulatory elements in the promoter of the AtPirin1 gene prompted us to test the effects of ABA and different light conditions on AtPirin1 transcript levels. RNA gel blot analysis of poly(A)+ RNA did not reveal any transcript; therefore, we performed relative quantitative reverse transcription (RT)-PCR.

RT-PCR revealed that AtPirin1 is present at similar levels in Arabidopsis seedlings and mature plants (data not shown). The effect of ABA on AtPirin1 transcript levels was tested by spraying 9-day-old plants with 100 μM ABA and harvesting tissue 4 h later. This ABA treatment increased AtPirin1 transcript levels (Figure 3A). The increase was approximately fivefold, as determined by RT-PCR product densitometric analysis of AtPirin1 and 18S relative quantitative amplification standards. This result suggests that ABA-inducible cis-acting regulatory elements in the AtPirin1 promoter region are functional.

When we tested AtPirin1 transcript levels under different light conditions, we found that a single pulse of low-fluence red light (10^4 μmol·m^{-2}·s^{-1}) causes an increase in AtPirin1 transcript levels compared with those in dark-grown seedlings (Figure 3B). A single pulse of low-fluence blue light did not elicit a noticeable response (Figure 3B). Densitometric analysis of AtPirin1 and 18S amplification levels indicated that the pulse of low-fluence red light leads to an approximately threefold increase in AtPirin1 transcript levels in dark-grown Arabidopsis seedlings.
Identification of atpirin1 T-DNA Insertional Mutants

To examine the role of AtPirin1 in plants, we obtained two different mutant lines, atpirin1-1 and atpirin1-2, that carry a T-DNA insertion in the AtPirin1 gene. The atpirin1-1 mutant harboring a T-DNA insertion in intron 5 of the AtPirin1 gene (Figure 2B) was found in a PCR screen of a collection of 60,480 T-DNA–inserted Arabidopsis lines using an AtPirin1-specific primer and a T-DNA left border–specific primer (Krysan et al., 1999). The genetic background for this mutant line is ecotype Wassilewskija (Ws).

The atpirin1-2 mutant harboring a T-DNA insertion in exon 4 of the AtPirin1 gene (Figure 2B) was obtained from the Salk Institute Genomic Analysis Laboratory. The genetic background for this mutant line is ecotype Columbia (Col). DNA gel blot and PCR analyses of atpirin1 mutants and their progeny revealed that there is only one T-DNA insertion in each of these mutant lines (data not shown).

No homozygous atpirin1-1 insertional mutants were found because of the developmental arrest of embryos at the globular stage of development, whereas the development of heterozygous atpirin1-1 mutant seeds was indistinguishable from that of the wild type (data not shown). Introduction of the AtPirin1 transgene under a moderate protochlorophyllide oxidoreductase A (PorA) promoter or a constitutive 35S promoter of Cauliflower mosaic virus did not lead to complementation of the embryo-lethal phenotype in atpirin1-1 mutants. atpirin1-2 homozygous mutants do not display the embryo-lethal phenotype. Together, these data suggest that the atpirin1-1 homozygous lethality probably is caused by a mutation in a linked gene.

For phenotypic analysis, comparisons were made between heterozygous atpirin1-1 and heterozygous and homozygous atpirin1-2 mutant plants as well as between two null alleles of GPA1, gpa1-1 and gpa1-2. Note that atpirin1-1 and atpirin1-2 seeds, referred to herein as “heterozygous,” are in fact the progeny of heterozygous plants and, as determined by PCR analysis, represent an ~2:1 mixture of heterozygous and wild-type seeds in the case of atpirin1-1 and an ~1:2:1 mixture of homozygous, heterozygous, and wild-type seeds in the case of atpirin1-2.

Germination of atpirin1 Mutant Seeds Is Delayed in the Absence of Stratification

In the absence of stratification treatment (4°C for 48 h), germination of atpirin1-2 homozygous mutant seeds was delayed compared with that of wild-type seeds (Figure 4A). There was a slight delay in the germination of atpirin1-1 (Figure 4B) and atpirin1-2 (Figure 4A) heterozygous seeds as well. Stratification brought atpirin1-1 and atpirin1-2 germination to wild-type levels (Figure 4). Importantly, germination of the GPA1 homozygous mutant lines gpa1-1 and gpa1-2 also was delayed somewhat under the conditions tested (Figure 4B). As with atpirin1, stratification increased gpa1-1 and gpa1-2 germination to wild-type levels (Figure 4B). Germination profiles of the atpirin1 and gpa1 mutants suggest an increased state of dormancy that is alleviated by stratification, which is known to be a dormancy-breaking factor.

Germination of atpirin1 Mutant Seeds Is Hypersensitive to ABA

In the presence of 500 to 1000 nM ABA, germination of atpirin1 mutant seeds was delayed (Figures 5A to 5D). An ABA-imposed inhibition of gpa1 seed germination was observed recently, although under somewhat different growth conditions (Ullah et al., 2002). To fully compare the atpirin1 and gpa1 mutant phenotypes, all seeds were harvested, stored, and tested under identical conditions. Matched seed lots were planted on half-strength Murashige and Skoog (1962) medium lacking sucrose and Gamborg vitamins, stratified, and germinated at room temperature under continuous white light. On day 10, almost 80% of all wild-type Col seeds germinated, although only 31% of atpirin1-2 homozygous mutant seeds germinated, in
Matched seed lots on half-strength Murashige and Skoog (1962) agarose medium supplemented with ABA were stratified for 48 h at 4°C before being placed at 22°C under continuous white light for germination. Heterozygous and homozygous mutants are indicated by +/- and --/---, respectively, next to the corresponding mutant genotype.

(A) Germination of wild-type Col seeds and atpirin1-2 mutant seeds in the presence of the indicated concentrations of ABA at day 10 after stratification.

(B) Germination of wild-type Ws seeds and atpirin1-1, gpa1-1, and gpa1-2 mutant seeds in the presence of the indicated concentrations of ABA at day 10 after stratification.

(C) Germination of wild-type Col seeds and atpirin1-2 mutant seeds in the presence of 800 nM ABA over time (days after stratification).

(D) Germination of wild-type Ws seeds and atpirin1-1, gpa1-1, and gpa1-2 mutant seeds in the presence of 800 nM ABA over time (days after stratification).

Values shown in (A) to (D) are mean percentages of germination from three independent experimental replicates (each replicate included 70 to 150 seeds per line) with standard errors.

(E) Early seedling development of atpirin1-2 mutants is inhibited by ABA. No ABA (-) or 500 nM ABA (+) was added to the growth medium in phytatrays. Note that >50% of the mutant seeds shown are germinated as judged by radicle emergence (cf. with [A]).

(F) Early seedling development of atpirin1-1, gpa1-1, and gpa1-2 mutants is inhibited by ABA. No ABA (-) or 800 nM ABA (+) was added to the growth medium in phytatrays. Note that ~30 to 40% of the mutant seeds shown are germinated as judged by radicle emergence (cf. with [D]).

Photographs for (E) and (F) were taken on day 10.
the presence of 800 nM ABA (Figure 5C). Under identical conditions, >50% of the atpirin1-1 heterozygous seeds and >70% of gpa1-1 and gpa1-2 homozygous mutant seeds failed to germinate by day 10; however, only 31% of wild-type Ws seeds did not germinate under the same conditions (Figure 5D).

In addition to a reduced rate of seed germination, we found that ABA also inhibited early seedling development: It significantly delayed the rate of cotyledon expansion and greening in atpirin1 and gpa1 mutant seedlings. In the presence of exogenous ABA, even when the radicles of many atpirin1 and gpa1 mutant seedlings emerged, the majority of cotyledons failed to expand and to green (Figures 5E and 5F). Note that under the growth conditions tested, exogenous ABA inhibited the early seedling development of atpirin1 and gpa1 mutants to a much greater extent than it inhibited their germination rate (compare Figure 5C with 5E and Figure 5D with 5F); therefore, the seedling developmental delay cannot be explained simply by a delay in germination.

We performed a complementation test with atpirin1-1 heterozygous mutants to confirm that the observed ABA-sensitive phenotype is attributable to the genetic lesion in the AtPirin1 gene. Heterozygous atpirin1-1 plants were transformed with the AtPirin1 cDNA under the control of the PorA gene promoter. The PorA promoter is active predominantly in etiolated seedlings, and PorA mRNA disappears within the first 4 h of greening (Armstrong et al., 1995; Runge et al., 1996). Figures 6A and 6B show that the PorA-AtPirin1 transgene was able to complement the ABA-imposed reduction in germination levels of the atpirin1-1 mutant. Moreover, in one of the complementation lines, 1-1PorAp2, both ABA-related phenotypes of atpirin1-1 plants (reduced germination and inhibition of early seedling development) were complemented (Figure 6C). This complementation test confirms that the observed atpirin1 mutant phenotypes result from genetic lesions in the AtPirin1 gene.

In addition to being necessary for the regulation of several events during seed development, ABA is involved in responses to environmental stresses, including desiccation and high salt (Busk and Pages, 1998; Leung and Giraudat, 1998). No difference was noted in atpirin1 seed germination, seedling growth, or seedling development in the presence of 400 mM mannitol, 200 mM NaCl, or 10% polyethylene glycol. Similarly, mutant plants displayed no difference in wilting compared with wild-type plants (data not shown). Together, these data suggest that the role of AtPirin1 with regard to ABA-related processes is limited to seed germination and early seedling development.

Figure 6. Complementation of the atpirin1-1 ABA-Hypersensitive Phenotype in Plants Carrying the AtPirin1 Transgene.

Matched seed lots on half-strength Murashige and Skoog (1962) agarose medium supplemented with ABA were stratified for 48 h at 4°C before being placed at 22°C under continuous white light for germination. The atpirin1-1 heterozygous mutant genotype is indicated by +/-.

(A) Germination of two independent complementation lines, 1-1PorAp1 and 1-1PorAp2, carrying AtPirin1 cDNA under the control of the PorA promoter in the atpirin1-1 mutant background. Germination was tested in the presence of the indicated concentrations of ABA at day 10 after stratification. Wild-type Ws seeds and atpirin1-1 mutant seeds were included for comparison.

(B) Germination of two independent complementation lines, 1-1PorAp1 and 1-1PorAp2, wild-type Ws seeds, and atpirin1-1 mutant seeds in the presence of 800 nM ABA over time (days after stratification).

Values presented in (A) and (B) are mean percentages of germination from three independent experimental replicates with standard errors.

(C) Early seedling development of the 1-1PorAp1 and 1-1PorAp2 complementation lines. Wild-type (Ws) and atpirin1-1 mutant seeds were included for comparison. No ABA (-) or 800 nM ABA (+) was added to the growth medium in phytotrays.
Additionally, we tested atpirin1 sensitivity to gibberellic acid (GA), because GA is known to function as an antagonist of ABA during seed germination and gpa1 mutant seed germination appears to be less sensitive to GA (Ullah et al., 2002). However, under the conditions tested, we observed no statistically significant difference from the wild type in atpirin1 germination and early seedling development (data not shown).

The Transition from Vegetative to Reproductive Growth Is Accelerated in atpirin1 Mutants

atpirin1-1 mutant plants initiate primary shoots and start flowering earlier than wild-type plants, such that a few days before the onset of flowering, atpirin1 mutants produced primary shoots of significant length, whereas wild-type plants had very short shoots (Figure 7A). To analyze this phenotype further, we measured the time needed for the transition from vegetative to reproductive growth in atpirin1 mutants and wild-type plants. Time to flowering was determined by counting the number of days after stratification until the day the first flower opened. Under long-day growth conditions, which are known to induce flowering in Arabidopsis plants, 50% of atpirin1-1 plants were flowering in <23 days; however, only 10% of wild-type Ws plants were flowering at the same time (Figure 7B). atpirin1-2 homozygous plants also displayed a similar flowering phenotype (Figure 7C). No statistically significant difference in flowering time was noted when atpirin1 plants were grown under short-day conditions (data not shown).

These results support the hypothesis that AtPirin1 functions as a moderate negative regulator of flowering under long-day conditions but is not involved in primary shoot and flowering initiation. gpa1 mutants do not display any alterations in flowering time under the conditions tested (data not shown), suggesting that AtPirin1 is involved in regulating flowering time in a GPA1-independent manner.

DISCUSSION

It is likely that the Arabidopsis Gα-subunit, GPA1, integrates multiple signaling pathways (Assmann, 2002) and therefore interacts with numerous effector and regulatory proteins. To date, other than the G protein β-subunit, no GPA1-interacting proteins have been reported. We have identified the 31-kD AtPirin1 protein as a GPA1-interacting partner that, along with GPA1, plays a defined role in seed germination and early seedling development.

Pirin was isolated initially from mammals through its physical interaction with the CAAT-box binding transcription factor NFI/CTF1 (Wendler et al., 1997). Subsequently, pirin was found to interact with the ankyrin-repeat domain of the proto-oncoprotein Bcl-3, a nucleus-localized member of the IκB family of transcriptional regulators (Dechend et al., 1999). It is well established that G proteins regulate the activity of a number of transcription factors through various effectors and/or other intermediates (Neves et al., 2002). GPA1 interaction with AtPirin1 may represent an example of a direct connection between G proteins and transcriptional regulation.

A perfect CAAT-box sequence along with several of the sur-
rounding bases defines the core element of the low-fluence blue light response element in the pea Lhcβ1*4 promoter (Folta and Kaufman, 1999). Plant pirin’s potential to interact with a Gα-subunit and a CAAT-box factor, both of which are implicated in the low-fluence blue light system activation of Lhcβ1*4 (Warpeha et al., 1991; Folta and Kaufman, 1999), suggests a role in blue light signal transduction in addition to the ABA pathways identified here.

Two cis-acting elements, an ACGT core and a TT motif, implicated in ABA- and light-mediated transcription regulation are found in the 5′ regulatory region of the AtPirin1 gene. The ACGT core element is thought to be the target of basic domain/Leu zipper transcription factors (Menkens et al., 1995; Leung and Giraudat, 1998). It has been shown that a phytochrome-interacting factor, PIF3, a member of the basic helix-loop-helix family, also binds an ACGT element in a sequence-specific manner and regulates the expression of photoresponsive genes (Martinez-Garcia et al., 2000). AtPirin1 transcript levels increase in response to a single pulse of low-fluence red light and exogenous ABA, suggesting that at least some of the potential upstream cis elements are active. These data, along with the recent finding that overexpression of GPA1 enhances phytochrome-mediated responses during seedling development (Okamoto et al., 2001), suggest that both AtPirin1 and GPA1 may be involved in the light regulation of seedling development.

Two atpirin1 mutant lines, atpirin1-1 and atpirin1-2, that harbor distinct T-DNA insertions were used in this study. Both mutant lines display a set of very similar phenotypes with regard to seed germination, ABA sensitivity, and flowering time, indicating that these pleiotropic effects are the result of T-DNA insertions in the AtPirin1 gene. Also, the Pora-Pirin transgene complements the ABA-imposed delay in the germination and early seedling development of atpirin1-1.

The atpirin1 and gpa1 mutants display a significant reduction in germination rates in the absence of stratification. When germination rates were tested in the presence of exogenous ABA, both atpirin1 and gpa1 mutants displayed less than wild-type germination rates. Furthermore, exogenous ABA inhibited the expansion and greening of cotyledons in atpirin1 and gpa1 mutant seedlings. A subset of atpirin1 mutant phenotypes correspond to gpa1 mutant phenotypes, confirming that these interacting proteins function in the same pathway.

The reduced germination rates of the atpirin1 and gpa1 mutants likely are the result of the enhanced dormancy of these mutant seeds, which is further facilitated by the application of exogenous ABA, a plant hormone known to establish seed dormancy during embryo maturation (Bonetta and McCourt, 1998; Koornneef et al., 2002). The recent observation that overexpression of the Arabidopsis G protein–coupled receptor GCR1 abolishes seed dormancy (Colucci et al., 2002) further implicates the plant G protein pathway in the regulation of seed dormancy.

The two alleles have similar genetic characteristics with respect to flowering and germination in the absence of cold or ABA treatment. By contrast, atpirin1-1 and atpirin1-2 display apparent dominant and recessive natures, respectively, with regard to ABA effects on seed germination and early seedling development. The differences between atpirin1-1 and atpirin1-2 may reflect varietal differences with respect to ABA sensitivity and/or the maternally based ABA-mediated expression of the Atpirin1 gene.

atpirin1 and gpa1 are similar to a small group of mutants, including era1, fiery1, abh1, and sad1, that display increased seed dormancy and ABA hypersensitivity (Cutler et al., 1996; Hugouvieux et al., 2001; Xiong et al., 2001a, 2001b). These mutations are recessive, indicating that the proteins involved might be negative regulators of ABA action. Like atpirin1 and gpa1, these mutations have multiple phenotypes, some without evident connection to ABA signaling, suggesting that these proteins may be involved in the regulation of a number of different processes in plants.

It was suggested recently that gpa1 mutant seeds display lower germination rates in the presence of ABA as a result of the reduced sensitivity of these mutants to GA (Ullah et al., 2002). Under the germination conditions used in the present study (half-strength Murashige and Skoog [1962] medium lacking sucrose and Gamborg vitamins, cold pretreatment, and germination in continuous white light), mutants of the potential GPA1 effector, AtPirin1, had increased ABA sensitivity but did not display a reduction in GA sensitivity, suggesting that ABA hypersensitivity and GA hyposensitivity during gpa1 seed germination may result from lesions in two separate pathways.

Besides delaying the germination rates of atpirin1 and gpa1 mutants, ABA delays their overall seedling development: the majority of cotyledons failed to green and expand. Therefore, even though a significant fraction of mutant seeds germinated in the presence of low ABA concentrations, as judged by the full penetration of the seed coat by the root tip (Figures 5A to 5D), the seedlings still were growth arrested (Figures 5E to 5F). A similar phenotype has been reported for two other ABA-hypersensitive mutants, fiery1 and sad1 (Xiong et al., 2001a, 2001b). Previously, it was suggested that ABA inhibits cotyledon greening and cell expansion more strongly than the breakdown of the seed coat (Steber et al., 1998).

In addition to germination-related phenotypes, atpirin1 mutants exhibit a moderate early-flowering phenotype when grown under long-day conditions. Early-flowering mutant phenotypes are thought to result from the inactivation of genes required to repress flowering (Coulillard, 1995). Overexpression of the putative Arabidopsis G protein–coupled receptor GCR1 accelerates flowering as well as the expression of the flowering-inducing gene LFY (Colucci et al., 2002). However, we observed no difference in flowering time in gpa1 mutants, suggesting that AtPirin1-mediated flowering regulation is a G protein–independent process. A model illustrating AtPirin1’s involvement in flowering time regulation is presented in Figure 8 (at right).

Based on the AtPirin1 gene characterization and atpirin1 mutant phenotypes, there are two conceivable models for AtPirin1’s mode of action. The first model postulates that, with regard to germination and early seedling development, AtPirin1 functions in a negative feedback regulatory loop of ABA signaling and is involved in resetting the ABA signal transduction pathway (Figure 8). According to the second model, AtPirin1 also relieves the ABA-imposed inhibition of germination, although indirectly, through the activation of germination-pro-
Flowering, Early Seedling Development

Figure 8. Schemes of AtPirin1 Modes of Action.

Together with GPA1, AtPirin1 positively regulates seed germination and early seedling development by overcoming the negative effects of ABA and/or activating germination-promoting pathway(s). AtPirin1 mRNA levels are upregulated by ABA, suggesting the existence of a negative feedback regulatory loop of the ABA signaling pathway. Independent of GPA1, AtPirin1 is involved in the regulation of flowering time. Thick arrows indicate either known pathways or pathways established in this study.

motivating processes (Figure 8). These models are not mutually exclusive and, importantly, GPA1 can be envisioned to function together with AtPirin1 in both of them.

The isolation and characterization of the AtPirin1 protein and the characterization of its mutant phenotypes provide biochemical and genetic evidence that AtPirin1 is a multifunctional regulatory protein that likely operates immediately downstream of its interacting partner, GPA1, in regulating seed germination and early seedling development.

METHODS

Plant Materials and Growth Conditions

Both Columbia (Col) and Wassilewskija (Ws) ecotypes of Arabidopsis thaliana were used as wild-type controls for phenotype comparison. For germination assays, seeds were placed in 15-cm Petri dishes on filter paper saturated with distilled water and, where specified, stratified (4°C for 48 h) before being placed at room temperature in continuous white light (20 μmol·m⁻²·s⁻¹) for germination. For hormonal assays, stress tolerance assays, and RNA isolation from abscisic acid (ABA)–treated plants, seeds were planted in phytatrays (Sigma) on half-strength Murashige and Skoog (1962) 0.8% (w/v) agarose medium lacking sucrose and Gamborg vitamins, stratified, and germinated as described above.

For flowering tests, plant transformation, and bulking up of seeds, plants were grown on Metro-Mix 200 growing soil medium (Scotts, Maysville, OH) under white light supplied by cool-white fluorescent bulbs (90 μmol·m⁻²·s⁻¹). The atpirin1-1 T-DNA insertion line was isolated by screening the α population of the Arabidopsis T-DNA insertion lines generated in the Ws ecotype at the Arabidopsis Knockout Facility at the University of Wisconsin (Madison). The atpirin1-2 T-DNA insertion line in the Col ecotype background was obtained from the Salk Institute Genomic Analysis Laboratory through the ABRC (Columbus, OH).

Yeast and Bacterial Strains

For the yeast two-hybrid experiments, the Saccharomyces cerevisiae L40 strain was used. The partial genotype of L40 is MATα his3-200 trp1-901 leu2-3,112 ade2 lys2::(LexAop)-HIS3 ura3::(LexAop)-LacZ GAL4 (Vojtek et al., 1993). Escherichia coli strain KCB (Clontech Laboratories, Palo Alto, CA), which is auxotrophic for Leu and Trp, was used for plasmid recovery from yeast. E. coli protease-deficient strain BL21 was used for glutathione S-transferase (GST) fusion protein expression.

Plasmid Construction

For the two-hybrid assay, protein fusions to the DNA binding domain of the bacterial repressor LexA were made using the plasmid pLexA-NLS, a modified version of the pBTM116 plasmid (Vojtek et al., 1993) containing nuclear localization signal. The GPA1 cDNA was obtained by PCR from pCIT1828 plasmid using primers designed to incorporate partial EcoRI and XhoI restriction sites. The GPA1 bait plasmid was created by cloning the GPA1 cDNA into the pLexA-NLS plasmid (EcoRI-SalI) after excoll (Promega) modification of PCR product ends (Kaluz et al., 1992). For in vitro protein association assays, the restriction fragment of GPA1 cDNA (EcoRI-XhoI) was subcloned into the GST fusion expression vector pGEX-4T-1 (Amersham Pharmacia Biotech). For the complementation test of the atpirin1-1 mutant, the AtPirin1 cDNA was obtained by reverse transcription (RT)–PCR using primers designed to incorporate partial Ncol restriction sites. After excoll modification of the PCR product ends, the AtPirin1 cDNA was cloned into the vector pBSK101.4PorA (Ncol), a modified version of pBSK101.3 (Folta and Kaufman, 1999) in which the β-glucuronidase (GUS; uidA) coding region was replaced with a multiple cloning site (EcoRI-HindIll-NcoI) and the PorA gene promoter was released from the pAG3/6 plasmid and inserted into HindIll-NcoI sites. The PorA–AtPirin1 fusion was moved into the plant transformation vector pLS9600, a derivative of pPZP100 (Hajdukiewicz et al., 1994). pLS9600 contains an additional cassette consisting of the minimal blue light promoter (the −95 to +2 fragment of the PsLhcb1’4 promoter), the GUS gene, and the nopaline synthase terminator (Folta and Kaufman, 1999).

Yeast Two-Hybrid Screening

The GPA1 bait construct was used to screen the Kim and Theologis λACT two-hybrid cDNA library (ABRC). Yeast transformations were performed using the lithium acetate/polyethylene glycol–based method (Gietz and Sughio, 1988). Standard yeast growth medium was prepared as described in the Yeast Protocol Handbook from Clontech. The S. cerevisiae reporter strain L40 was transformed first with the GPA1 bait vector and subsequently with the Arabidopsis two-hybrid cDNA library. Transformants were plated on 15-cm synthetic dextrose plates (Trp⁺, Leu⁺, Lys⁺, His⁺) to select for His prototrophy. The His⁺ colonies were re-streaked on 5-bromo-4-chloro-3-indolyl–β-D-galactoside–containing selective medium, and Lac-Z reporter gene activity was monitored visually. Library plasmids from His⁺ “LacZ” yeast colonies were rescued by direct transformation from LacZ⁺ cells into the E. coli strain KCB according to a standard protocol. E. coli colonies grown on medium lacking Leu were selected. Positive clones were sequenced at the Cancer Research Center DNA Sequencing Facility at the University of Chicago.

Expression of the GST–GPA1 Fusion Protein

The GST–GPA1 fusion protein was expressed in E. coli strain BL21. Protein expression was induced with 100 μM isopropyl-β-D-thiogalactopyranoside, and extracts were prepared by treating cells for 3 min at 0°C with lysozyme solution (1 mg/mL lysozyme and 2.5 mM Tris, pH 8.0) followed by a brief sonication. Recombinant proteins were affinity-purified.
Identification of the \textit{atpirin1-1} T-DNA Insertion Mutant

The \( \alpha \) population of \(~\)64,000 Arabidopsis T-DNA insertion lines at the University of Wisconsin Arabidopsis Knockout Facility was screened using \textit{pirinTdir} primer (5'-ATACATGCTACGGGAGGTATCCACTGACA-3') according to the facility’s protocol (www.biotech.wisc.edu/Arabidopsis/). The progeny of a plant heterozygous for a T-DNA insertion in the \textit{AtPirin1} gene were genotyped by PCR using \textit{pirinTdir} primer and JL202 T-DNA left border–specific primer (www.biotech.wisc.edu/Arabidopsis/). As a result of the embryonic lethality of plants homozygous for a T-DNA insertion in the \textit{AtPirin1} gene, heterozygous plants were distinguished by histochemical GUS staining of flowers and selected for propagation. Flowerers were chosen for GUS staining because the T-DNA insert vector contains the GUS reporter gene driven by the flower-specific \textit{apetala3} promoter. GUS histochemical staining was conducted essentially as described (Jefferson et al., 1987). In brief, freshly excised flowers were immersed in GUS staining solution containing 8 mM 5-bromo-4-chloro-\( \beta \)-d-glucuronide cyclohexylammonium salt (Gold BioTechnology, St. Louis, MO) and 2% Triton X-100 in 50 mM sodium phosphate buffer, pH 7.2, and then incubated at 37°C overnight.

Germination Assay

Germination rates were compared between seed lots that were produced, harvested, and stored under identical conditions. Seventy to 150 seedlings from wild-type (Col and Ws), \textit{atpirin1-1}, \textit{gpa1-1}, \textit{gpa1-2}, and \textit{atpirin1-2} plants were planted in triplicate. Seeds were considered germinated when the radicles completely penetrated the seed coat. Germination was scored daily for 10 days after seeds were placed at room temperature.

Hormonal and Stress Tolerance Assays

The effects of ABA on seed germination were studied by determining the germination rates of 70 to 150 seeds planted in triplicate on growth medium containing ABA (mixed isomers; Sigma). For direct comparisons of germination rates at a particular ABA concentration, each phytatray was subdivided and all seed lines were planted on the same phytagtray. The effects of GA on seed germination were determined as described for ABA except that 1 or 10 \( \mu \)M GA (Sigma) was added to the medium. The effects of mannitol, polyethylene glycol, and NaCl were studied in a similar manner.

Complementation Analysis

Complementation constructs carrying the \textit{AtPirin1} cDNA under the control of the \textit{PorA} promoter are described above. \textit{atpirin1-1} heterozygous Arabidopsis plants were transformed by vacuum infiltration (Bechtold et al., 1993). Transgenic plants were selected by resistance to the herbicide Finale (Roussel Uclaf, Montvale, NJ) and by histochemical GUS staining of leaves. \textit{atpirin1-1} heterozygous plants carrying the \textit{PorA-AtPirin1} transgene were selected by PCR.

Flowering Time Analysis

Wild-type and mutant plants for corresponding experimental replicates were grown in the same tray, which was rotated 180° every 24 h. For each experimental replicate, 25 to 40 plants were analyzed per line. Long-day conditions consisted of 16 h of light followed by 8 h of darkness. The onset of flowering was defined as the day when the first flower opened. \textit{atpirin1-1} heterozygous plants were selected by histochemical GUS staining of flowers as described above.

Expression Studies by Relative Quantitative RT-PCR

To study the effects of ABA, 9-day-old Arabidopsis seedlings were sprayed with 100 \( \mu \)M ABA or sterile distilled water and plant tissue was harvested 4 h later. To study the effects of light, plant was performed as described (Folta and Kaufman, 1999). Six-day-old dark-grown Arabidopsis seedlings were irradiated with a single pulse of low-fluence blue or red light (104 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)). Plant tissue was harvested 2 h later. Total RNA was isolated with TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RNA was used as a template for first-strand DNA synthesis using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA). PCR amplification was performed using Elongase Enzyme Mix (Invitrogen Life Technologies); the optimal concentration of Mg\(^{2+}\) for these amplification reactions was 1.3 mM. PCR amplifications were performed in a final volume of 23 \( \mu \)L with RtpirinD (5'-TGACGT-ATAGAAACAAATGTTGAC-3') and \textit{pirinTrev} (5'-GTAACCTCTAACACT-TGAGCTTTACAC-3'), \textit{AtPirin1}-specific primers that distinguish between the \textit{AtPirin1} transcript and genomic DNA contaminants. Conditions were chosen in the linear range of amplification for the products and were as follows: 30 cycles of 94°C for 20 s, 94°C for 15 s, 60°C for 30 s, and 72°C for 2 min, followed by 72°C for 2 min. QuantumRNA 18S Internal Standards (Ambion, Austin, TX) were used as a relative quantitative amplification control. To achieve levels of amplification comparable to those of the \textit{AtPirin1} PCR products, 18S-specific primers were equilibrated with 18S PCR Competimers in a ratio of 2:8, which corresponds to the abundance levels of rare transcripts. The PCR products were resolved on 1% agarose gel, and amplification levels were determined using the one-dimensional multidensitometry tool on an Alphalager 2000 (Alpha Innotech, San Leandro, CA).
Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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The Arabidopsis Cupin Domain Protein AtPirin1 Interacts with the G Protein α-Subunit GPA1 and Regulates Seed Germination and Early Seedling Development
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