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PHOR1: A U-Box GA Signaling Component With a Role in Proteasome Degradation?

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Abstract

Recent identification of U-box proteins as E3 ubiquitin ligases suggests that the U-box arm-repeat protein PHOR1, for which we have demonstrated a role in GA signal transduction, may play a role in GA signaling by ubiquitinating one or more components of the GA response pathway to target them for proteasome degradation. Here we show that PHOR1 function in GA signaling is not exclusive of potato plants, but it is also conserved in *Arabidopsis*. Three PHOR1-homologs have been identified in this plant species, which would correspond to PHOR1-orthologs. Experimental evidence has recently been obtained for the involvement of proteasome-dependent protein degradation in GA-mediated destabilization of the SLN1 DELLA protein, thus pointing to this repressor as a likely substrate for ubiquitination by the PHOR1 ubiquitin ligase activity.

Key words: U-box motif; RING finger protein; ubiquitin ligase; gibberellin signalling; GAI repressor

Introduction

Bioactive gibberellins (GAs) are tetracyclic diterpenoid hormones with a role in multiple plant growth and development processes. They control many aspects of plant growth such as stem elongation, seed germination and flower and fruit development, mediating also between certain environmental signals like photoperiod and the control of flowering time or tuber induction in potato (Hooley 1994; Swain and Olszewski 1996). Although substantial progress has been made during the last years concerning GA metabolism, with the isolation of all of the genes involved in GA biosynthesis, with the exception of GA 13-hydroxylase, and the understanding of many of the mechanisms by which endogenous GA content is regulated (Hedden and Phillips 2000), less is known about the mode of GA action.

Components of the GA signal transduction pathway, such as the GAI repressor, were isolated in a screening of mutants resembling GA-deficient mutants but that did not respond to the addition of GA. Unlike the GA biosynthetic mutations, the *gai* plants contain higher levels of bioactive GAs than do wild-type plants, and the levels of 20-oxidase and 3 beta-hydroxylase transcripts are increased in

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these plants, thus indicating that feedback regulation is perturbed in this mutant (Koornneef and others 1985; Peng and others 1997). The homolog of *GAI*, *RGA* was isolated in a screening for mutant alleles able to partially rescue the phenotype of the GA biosynthetic mutant *ga1-3* (Silverstone and others 1997). GAI and RGA belong to the plant-specific GRAS family of regulatory proteins (Pysh and others 1999). Unlike other members of the GRAS family they include an N-terminal conserved domain called the DELLA domain after a set of conserved amino acids in this region (Silverstone and others 1998). The semidominant *gai* allele shows a 51-bp in frame deletion that results in the loss of 17 amino acids within the DELLA domain (Peng and others 1997). Intragenic suppressor mutants of the *gai-1* mutation have been isolated, showing base pair substitutions that introduce premature stop codons in the coding region for the protein (Peng and Harberd 1993; Wilson and Somerville 1995). These loss-of-function null alleles restored the plant phenotype and were resistant to the GA biosynthesis inhibitor paclobutrazol, thus showing a constitutive GA response as the *rga* alleles (Silverstone and others 1997). Based on these observations it was hypothesized that the GAI and RGA proteins would act as negative regulators of the GA response, with the DELLA domain being required for de-repression of these inhibitors by GAs (Harberd and others 1998). According to this hypothesis, deletion of 17 amino acids within the DELLA domain in the gai-1 protein renders this repressor insensitive to GA regulation and as a consequence, the gai protein constitutively represses plant growth, having a dominant effect over the wild-type GAI allele that still recognizes the GA signal (Richards and others 2001).

Mutants with a similar dwarf GA-insensitive phenotype to the *Arabidopsis gai* mutant have also been isolated from other plant species. These include the *reduced height (Rht)* mutants of wheat and the *D8* and *D9* mutants of maize (Silverstone and Sun 2000). The wheat *Rht* mutants have been referred to as the green-revolution wheat because of their high yields that brought dramatic increases in worldwide wheat production since the 1950s. All these mutants were found to affect GAI and RGA ortholog genes, and to carry similar genetic lesions such as the *gai* mutation (Peng and others 1999). The *D8-1* and *D8-2023* mutants, for example, contain small in frame deletions in the DELLA domain, whereas the *D8-Mpl* allele has a 330 bp deletion at the N-terminus, encoding a truncated protein that lacks the first N-terminal 105 residues. *Rht-B1b* and *Rht-D1b* both contain a base substitution that introduces a stop codon in the DELLA domain. It has been claimed that in these mutants a protein is still produced through translational reinitiation, thus resulting in a truncated protein that lacks, as *D8-Mpl*, the original N-terminus (Peng and others 1999). These results strongly support the hypothesis that the DELLA domain in the N-terminus of the GAI/RGA family of GA-signaling components is responsible for modulating activity of these repressor proteins in response to GA. Consistent with this model, introduction of a 51-bp in frame mutation in the DELLA domain of the RGA protein was found to result in a semidominant dwarf phenotype that does not respond to GA treatment (Silverstone and others 2001). The green fluorescent protein fusion (GFP-RGA) has been shown to localize in the nucleus of transiently transformed onion cells or stably transformed *Arabidopsis* plants (Silverstone and others 1998). GA treatment induces a rapid disappearance of the GFP-RGA fluorescence from the nucleus, thereby causing a release from suppression and allowing the plants to activate GA downstream signals (Silverstone and others 2001). This disappearance is not observed in GFP-Δ^{DELLA}RGA transformed cells, in which a constitutive nuclear localization of GFP fluorescence is observed regardless of GA treatment (Dill and others 2001).

Similar results were obtained with the rice *slender rice 1* (*slr1*) gene, whose mutation causes a constitutive GA-response with a slender phenotype similar to that of rice plants treated with GAs (Ikeda and others 2001). In contrast to *Arabidopsis*, rice has only one gene encoding the GAI/RGA-ortholog SLR1 and consequently, plants with a loss-of-function allele of *SLR1* show a strong constitutive GA-responsive phenotype (Ikeda and others 2001). This lack of redundancy is a significant advantage of rice with respect to other plant species in studying the mechanism of action of the members of the SLR1/GAI/RGA/RHT family of repressors. Using transgenic plants overproducing the GFP-SLR1 fusion protein has demonstrated that SLR1 acts in the nucleus to repress GA action, and that GA signal transduction is regulated by the appearance or disappearance of the SLR1 protein from the nucleus (Itoh and others 2002). Domain analysis revealed four domains with distinct functions in the SLR1 protein: a GA signal perception domain located at the N-terminus, a regulatory domain required for repression activity, a dimer formation domain essential for signal perception and repression activity, and a repression domain at the C terminus (Itoh and others 2002).

Another constitutive GA response mutant in *Arabidopsis* is *spindly* (*spy*) (Jacobsen and others

1996). The SPY protein is significantly similar to O-linked GlcNAc transferase (OGT) from animals. OGTs modify target proteins by adding a single GlcNAc group to Ser and/or Thr residues. There is a large body of evidence indicating that this modification has a regulatory role by interfering or competing with kinases for phosphorylation sites. Epistatic analysis has suggested that *SPY* and GAI act in the same pathway to suppress GA signaling (Jacobsen and others 1996). In consequence, it has been suggested that SPY possibly modifies GAI and RGA at the Ser/Thr-rich domain in the N-terminus, thus regulating activity of these repressor proteins (Thornton and others 1999; Sun 2000).

Other potential GA-signaling proteins uncovered by characterization of GA response mutants include the semidominant GA-insensitive mutant *short internodes* (*shi*) in *Arabidopsis* (Fridborg and others 1999) or the recessive GA-insensitive mutants *dwarf 1* (*d1*) in rice (Mitsunaga and others 1994), *pickle* (*pkl*) in *Arabidopsis* (Ogas and others 1999), *GA sensitivity* (*gse*) in barley (Chandler and Robertson 1999), and *sleepy 1* (*sly 1*) in *Arabidopsis* (Steber and others 1998), which likely correspond to loss-of-function mutants of positive regulators of the GA response. The *shi* mutation is caused by overexpression of the SHI gene that encodes a putative zinc finger protein proposed to be a negative regulator of GA signaling (Fridborg and others 1999). *D1* has been shown to encode a putative heterotrimeric G protein α-subunit, supporting earlier pharmacological studies which suggested that heterotrimeric G proteins are involved in GA signaling (Ashikari and others 1999; Ueguchi-Tanaka and others 2000). Positional cloning of *PKL*, in its turn, revealed that this gene encodes a CHD3 chromatin remodeling factor that regulates gene expression by transcriptional repression (Ogas and others 1999).

The U-Box/Arm-Repeat Protein PHOR1 is a Positive Regulator of GA Signaling

By RT-PCR screening of genes that were up-regulated in potato leaves of plants grown under tuber-inductive (SD) conditions, we have isolated clone PHOR1 (photoperiod regulated 1) encoding a novel component of the GA signaling pathway. Antisense inhibition of *PHOR1* produces a semi-dwarf phenotype that resembles that of GA-deficient plants. The antisense *PHOR1* lines show a reduced response to GA application and higher levels of GAs than wild-type controls, indicative of an impaired response to GAs. Like in the *Arabidopsis gai* mutant, levels of the 20-oxidase transcript were increased, and levels of the 2-oxidase transcript were reduced in the antisense *PHOR1* lines, indicating an altered feedback regulation of GA biosynthesis in these plants. PHOR1 encodes an arm-repeat-containing protein similar to the segment polarity gene *armadillo* from *Drosophila* (Amador and others 2001) and contains a U-box (UFD2 homology) domain in its N-terminal end. The highly conserved U-box domain was first identified at the C-terminus of the yeast UFD2 protein, a novel ubiquitination factor, designated as E4, that binds to the ubiquitin moieties of specific ubiquitinated proteins catalizing efficient multi-ubiquitination of these substrate proteins (Koegl and others 1999). Multi-ubiquitinated substrates are the preferred substrates of the 26S proteasome, thus implicating the UFD2 protein in selective protein degradation by the ubiquitin proteasome pathway (Ciechanover 1998).

Although UFD2 is the only U-box-containing protein in yeast, two UFD2 homologs have been identified in humans. In addition to these two UFD2 homologs, 19 other predicted U-box-containing proteins have been identified in the human genome (Koegl and others 1999). In these proteins, the U-box-conserved domain is flanked by a putative protein-protein interaction domain, which is thought to participate in recruiting substrate proteins to ubiquitination and proteasomal targeting. In a database search for plant U-box (PUB) proteins, about 63 U-box proteins, far more than in any metazoan species, have been identified in *Arabidopsis* (Patterson 2002). The U-box domain in these proteins is flanked by different subsets of conserved domains, defining five subclasses of PUB proteins that are likely to be involved in diverse biological functions (Azevedo and others 2001). It is intriguing why plants have evolved a much higher number of U-box proteins than other organisms. Increased complexity of plant U-box proteins suggests that they are involved in functions that are particularly vital for plants. The *Brassica ARC1* gene, required for self-incompatibility (Gu and others 1998), and the potato *PHOR1* gene, with a GA-signaling function (Amador and others 2001), are the only *PUB* genes functionally characterized to date. We need to unravel the function of other U-box proteins to understand why this family of proteins has been evolutionarily favored in plants.

GA-Dependent Migration of PHOR1 into the Nucleus

Subcellular localization studies using a translational fusion of the PHOR1 protein to GFP showed ambiguous results; cytosolic distribution of GFP fluorescence or a nuclear localization was observed,

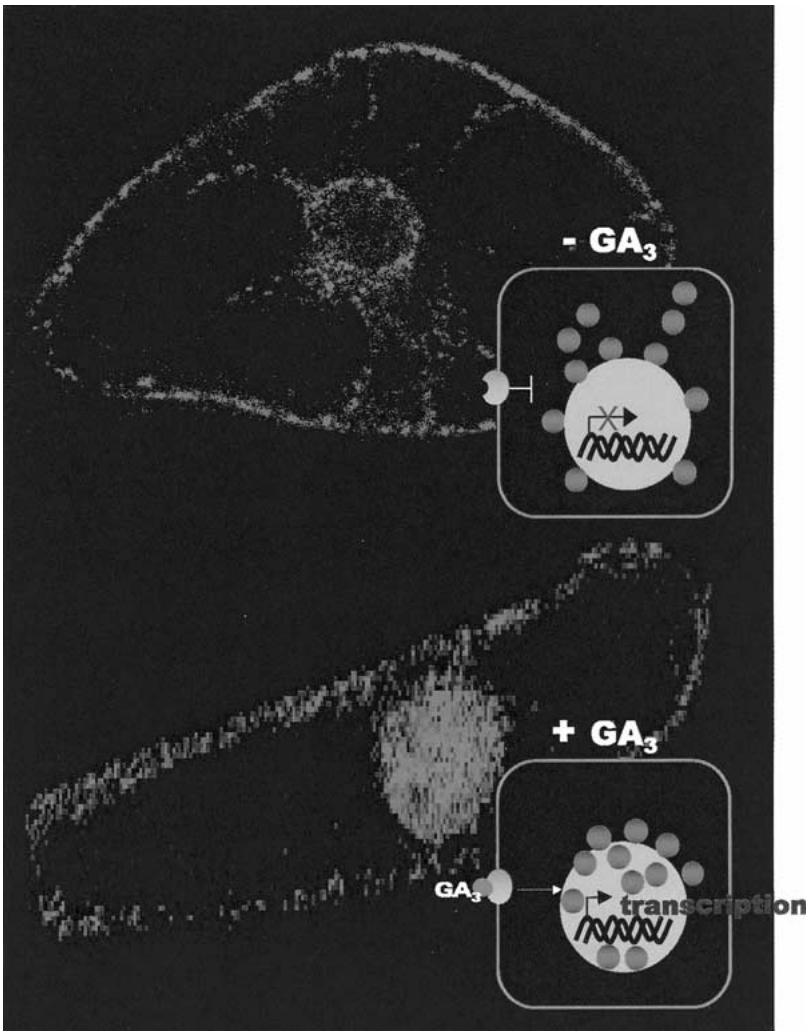

Figure 1. GA-dependant nuclear import of PHOR1. Confocal micrographs showing a cytosolic localization of the PHOR1-GFP fusion in ancymidol-treated cells and a nuclear localization of the protein in cells treated with GA_3.

depending on the cell analyzed. To assay whether this variable pattern of localization was due to a transient migration of the protein to the nucleus, cells were pretreated with the inhibitor of GA synthesis ancymidol or with an extra dose of the hormone. In these experiments, in the absence of the GA signal, as in ancymidol-treated cells, PHOR1-GFP was localized in the cytosol, while GA treatment led to a nuclear translocation of the fusion protein (Figure 1). GA application to ancymidol-treated cells induced accumulation of the PHOR1-GFP protein into the nucleus within 2–4 h of hormone treatment. In addition, migration of the protein into the nucleus was transitory, with a cytosolic distribution of fluorescence again observed after 12 h of treatment with the hormone. Confocal scanning microscopy was used to demonstrate that the fluorescent protein was localized inside the nucleus and not attached to the outer nuclear envelope membrane. Interestingly, cytosolic distribution of the fluorescence signal was found not to be homogeneous but to show a characteristic spotted pattern indicative of the association of PHOR1 in a multiprotein complex.

The next question addressed was which of the two PHOR1 conserved domains mediate the nuclear import of the protein? For this purpose, fusion constructs of the U-box and arm-repeat domains to GFP were generated and transformed into tobacco BY2 cells. In these experiments, the ARM-GFP fusion directed a constitutive nuclear localization of the green fluorescence, whereas the Ubox-GFP fusion retained a GA-dependent migration of the protein into the nucleus. Nuclear accumulation of the Ubox-GFP fusion, however, appears to result from a free nuclear diffusion of the protein (the fusion is about 45 kDa in size, which is below the exclusion size of the nuclear pore) and not from an active nuclear localization signal mediated nuclear transport because, in a similar experiment, a Ubox-GFP-GUS fusion was not detected in the nucleus. Thereby, GA-dependent migration of the Ubox-GFP

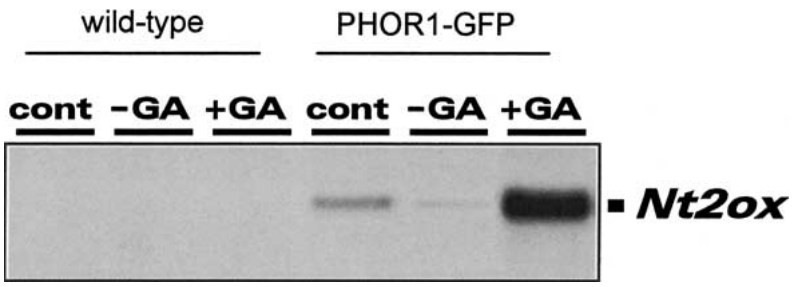

Figure 2. Expression of the tobacco *Nt2ox* gene, encoding GA 2-oxidase, in wild-type BY2 cells and cells expressing the PHOR1-GFP fusion. Cells were either non-treated (cont) or treated with 5 mg/L ancymidol (−GA) or 50 μM GA_3 (+GA) for 12 h. Total

fusion is not mediated by a nuclear localization signal (NLS) present in the U-box region of the protein but by a protein-protein interaction domain present in this region that would mediate interaction of PHOR1 with a cytosolic protein complex, thus retaining the protein in the cytosol. This interaction would be disrupted by GAs, with the PHOR1 protein then being targeted to the nucleus by a nuclear migration signal present in the arm-repeat domain.

BY2 Cells Over-Expressing the PHOR1-GFP Fusion Protein Show an Enhanced Response to GAs

To demonstrate that migration of PHOR1 into the nucleus correlates with an efficient activation of the GA response in the suspension cells, we analyzed the levels of expression of the tobacco *Nt2ox* gene, encoding GA 2-oxidase, in the cells treated with the inhibitor ancymidol or with GAs. GA 2-oxidase catalyzes inactivation of GA_1 into GA_8 and has been reported to be subjected to a positive mechanism of feed-back regulation, with higher levels of transcript detected in GA-treated plants (Thomas and others 1999). Interestingly, in these studies we did not observe an activation of the *Nt2ox* gene in control BY2 cells treated with the hormone, but a clear increase in *Nt2ox* mRNA levels upon GA-application was observed in the cells over-expressing the PHOR1-GFP fusion protein (Figure 2). Basal levels of expression of the *Nt2ox* transcript were slightly higher in the cells over-expressing the PHOR1-GFP fusion than in the controls (Figure 2). Ancymidol application to cells over-expressing the PHOR1-GFP fusion resulted in lower levels of the *Nt2ox* transcript, and application of GA_3 or a combination of GA_3 and the inhibitor ancymidol, was able to induce a strong increase in the levels of accumulation of the *Nt2ox* mRNA in these cells (Figure 2).

These observations demonstrate that the PHOR1-GFP fusion protein is functionally active in BY2 cells and that over-expression of this protein fusion results in a vast increase in the response of BY2 cells to GAs. This positive effect on GA signaling is exacerbated by the fact that BY2 suspension cells respond very poorly to GA application and that PHOR1 over-expression appears to recover the ability of these cells to respond to GAs. The reason why tobacco BY2 cells hardly respond to GA application is presently not well understood. Indeed, we have observed that stably transformed cells over-expressing the PHOR1-GFP fusion gradually lose their GA-response ability, with a parallel loss of their capacity to transport the fusion protein into the nucleus. However, further research should be conducted to verify this loss of nuclear uptake of PHOR1 and to define the mechanisms involved in repression of the GA response in these cells.

Arabidopsis Lines Over-Expressing the Potato PHOR1 Protein are Partly Resistant to Paclobutrazol

We have analyzed whether PHOR1 function as a signaling intermediate is conserved in other plant species by over-expressing the PHOR1 protein in *Arabidopsis* plants. *Arabidopsis* lines accumulating high levels of the potato protein were selected by protein blot analysis using an antibody raised against the purified PHOR1 protein. As shown in Figure 3, these lines were slightly larger than the untransformed controls and were partially resistant to the inhibitory effect of paclobutrazol (PAC) on seed germination.

PAC inhibits GA biosynthesis at the kaurene oxidase reaction, producing a depletion of the endogenous levels of GAs. Germination of *Arabidopsis* seeds is blocked in the presence of PAC, this inhibitor causing dwarfism if applied after germination. When imbibed on medium containing PAC, the seeds of plants over-expressing PHOR1 (PHOR1-OE) showed a higher percentage of germination than the wild-type controls, over the whole range of PAC concentrations assayed. Twenty-five percent of the PHOR1-OE seeds germinated in 10^{-4} M PAC, a concentration that completely inhibited germination of wild-type seeds.

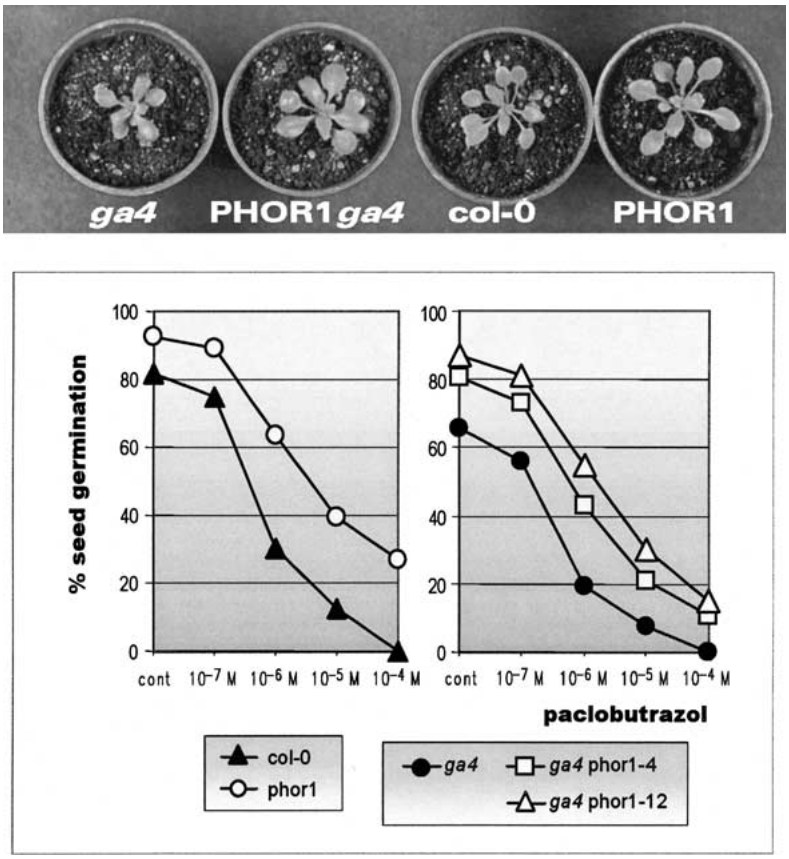

Figure 3. Phenotype of wild-type *Arabidopsis* and *ga4* mutant plants over-expressing the potato PHOR1 protein. (Top): PHOR1 over-expression partially overcomes the dwarf phenotype of the *ga4*-deficient mutant. Over-expressers are larger and leaves are less green than the mutant plants. (Bottom): Effects of increasing concentrations of PAC on seed germination of wild-type and *ga4* mutant lines over-expressing the potato PHOR1 protein. At the highest PAC concentration, only the PHOR1 seeds were able to germinate with 25% germination and 15% germination observed, respectively, in the wild-type and *ga4* allele backgrounds.

PHOR1 effect was more evident when these lines were crossed to a GA-deficient background such as the *ga4* mutant, affected in the 3beta-hydroxylase activity converting GA_{20}/GA_9 into bioactive GA_1/GA_4 (Chiang and others 1995). PHOR1 over-expression in the *ga4*-deficient background partially suppressed the GA-deficient phenotype of this mutant, with PHOR1-OE *ga4* plants being less dwarfed and with paler green color leaves than the *ga4* plants (Figure 3A). As shown for wild-type plants, homozygous seeds obtained from the cross PHOR1-OE × *ga4* were also partially resistant to paclobutrazol, with approximately 15% of the seeds achieving germination on medium containing 10^{-4} M PAC, whereas a complete inhibition of germination was observed in *ga4* seeds. These results indicate that PHOR1 would exert in *Arabidopsis* a similar function as in potato, with the PHOR1 over-expresser plants being more sensitive to GAs and exhibiting an increased resistance to paclobutrazol compared to the nontransformed controls. In addition, these findings show that PHOR1 function has been conserved in *Arabidopsis* and thereby proteins related to PHOR1 are likely to have a GA signaling function in this plant species.

Three Gene Copies Encode PHOR1 in *Arabidopsis*

Comparison of the PHOR1 amino acid sequence with the proteins deduced from the complete *Arabidopsis* genome sequence retrieved three ORFs coding for proteins that share substantial homology with PHOR1. An alignment of the *Arabidopsis* and potato proteins is shown in Figure 4. Phylogenetic tree analysis showed that genes *HIM1* (ABO20752, *AtPUB28*) and *HIM3* (ABO10076, *AtPUB27*) are more closely related to each other than gene *HIM2* (ABO26654, *AtPUB29*). By RNA blot analysis we could verify that whereas *HIM1* and *HIM3* mRNAs accumulate in all plant tissues, transcript *HIM2* is mainly detected in flowers (data not shown). These findings indicate that genes *HIM1* and *HIM3* are likely to have a redundant function in vegetative tissues, while *HIM2* would be specific in floral organs.

Overall identity between the *Arabidopsis* and potato homologs is 54–56%. These proteins share nearly identical U-box domains, with a somewhat lower percentage of homology observed within the arm-repeat region. This agrees with our previous observation that this region would correspond to a

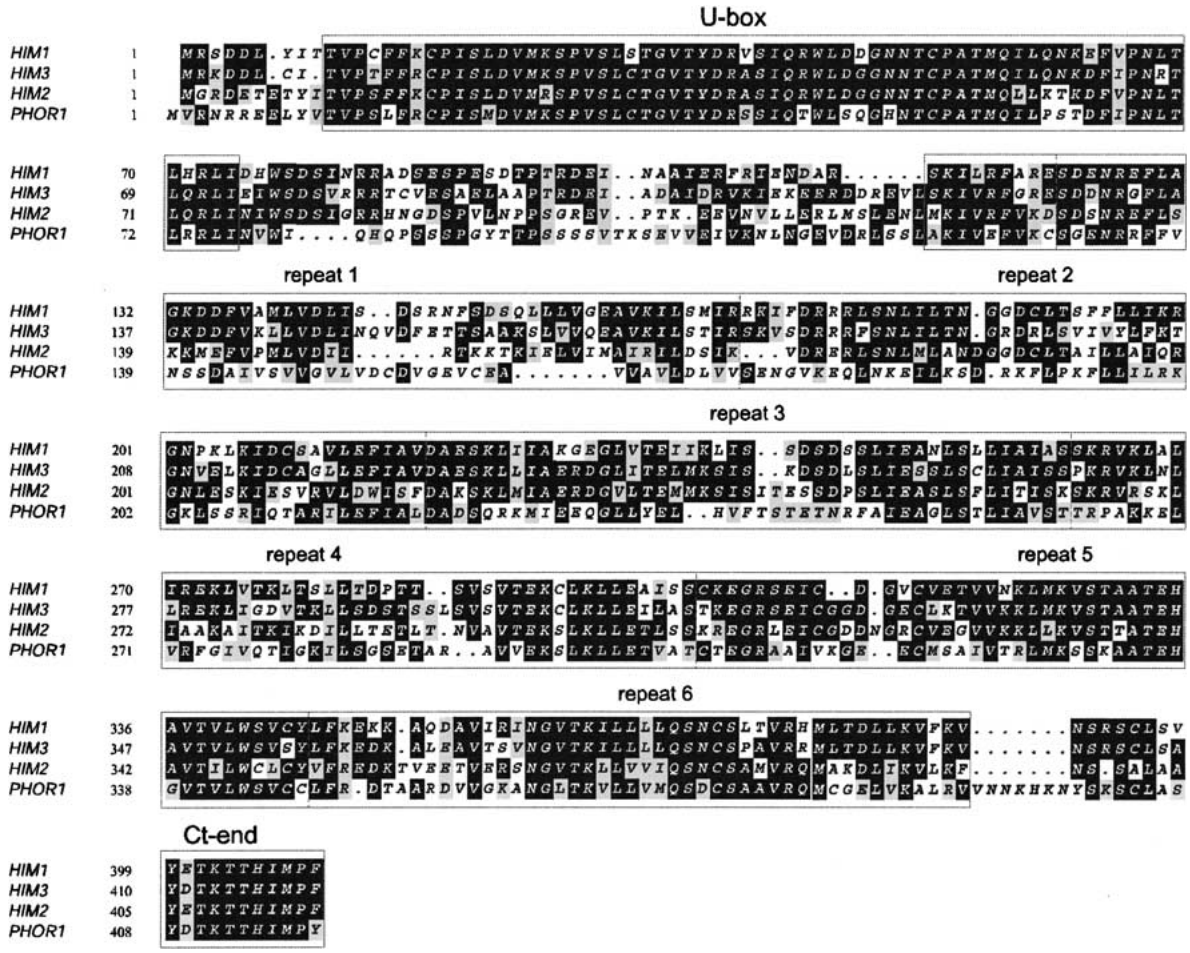

Figure 4. Sequence alignment of the *Arabidopsis* homologs and potato PHOR1 proteins. The U-box and arm/repeat domains are indicated. Similarity is very high within the U-box domain. The highly conserved residues in the extreme C-terminal region are also indicated.

structural domain, and that conservation at the level of secondary structure rather than preservation of the primary amino acid sequence is important for function of this domain (Amador and others 2001). According to this observation, the conserved hydrophobic residues in the potato repeats, reported to be involved in repeat-repeat interaction and therefore in domain folding, are also highly conserved in the *Arabidopsis* homologs, this being consistent with a conserved secondary structure of these proteins. Interestingly, 11 identical residues are observed at the C-terminal end of the potato and *Arabidopsis* proteins, except for a conservative substitution of the C-terminal tyrosine residue in PHOR1 which is substituted by a phenylalanine residue in all the *Arabidopsis* homologs (Figure 4). These residues are not conserved in any of the other *Arabidopsis* plant U-box (PUB) proteins, indicating that the conserved C-terminal end may play an important role in PHOR1 function.

U-Box Proteins are a New Class of U3 Ubiquitin Ligases

The U-box domain has recently been implicated in ubiquitin-dependent protein degradation. This 70-amino acid motif, designated as U-box after the yeast ubiquitination factor UFD2, is related to the RING finger motif of E3 ubiquitin ligases but lacks the conserved cysteine residues involved in Zn^{+2} coordination. Modeling studies predicted the U-box domain to adopt a tertiary structure very similar to that of a RING finger without the association of Zn^{2+} ions (Aravind and Koonin 2000). The first U-box proteins identified, UFD2, CHIP and NOSA, were all initially described as proteins that function in the ubiquitin proteasome pathway. In addition, several recent reports indicate that U-box domains indeed function as ubiquitin ligases (Hatakeyama and others 2001; Jiang and others 2001) that selectively ubiquitinate cellular proteins to

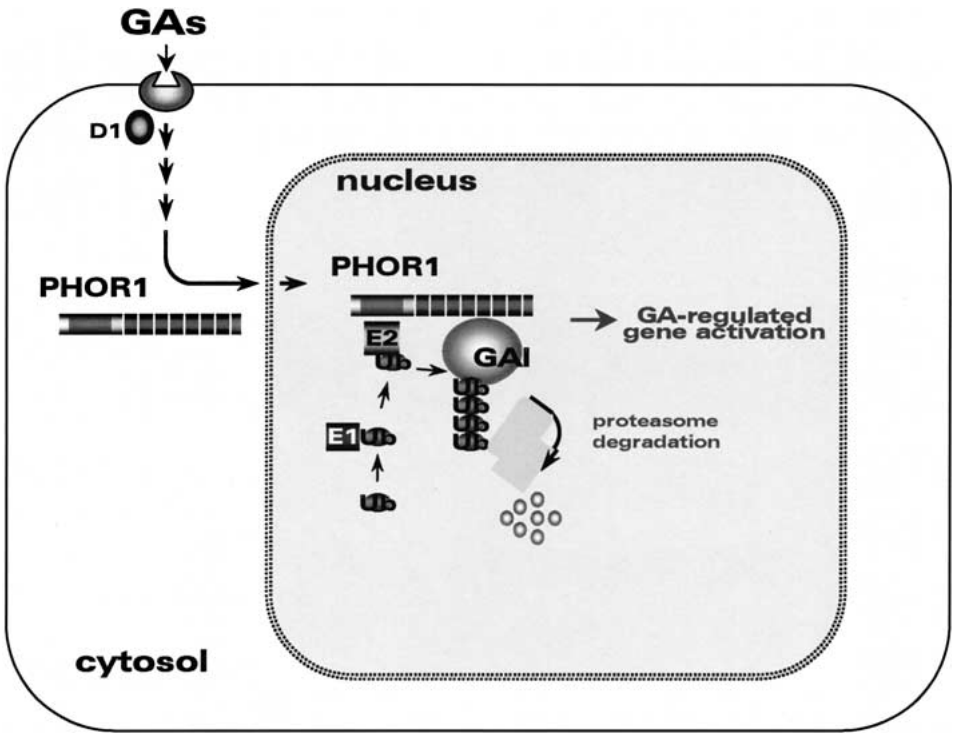

Figure 5. Hypothetical model of PHOR1 action. Bioactive GA binds to an as yet unidentified GA receptor, activates second messengers and G proteins (D1) and causes PHOR1 to be localized to the nucleus. In the nucleus PHOR1, as a single protein or as part of a multiprotein complex, ubiquitinates the repressor RGA/GAI and targets it for degradation by the proteasome system. In the absence of GA, PHOR1 is localized in the cytosol and the repressors RGA/GAI are stable, thus inhibiting the GA response.

target them for degradation by the proteasome pathway.

E3 ubiquitin ligases were classified into two types: the HECT type E3 ligases and the RING-finger type E3 ubiquitin ligases. The HECT domain contains a conserved active-site cysteine residue that binds ubiquitin residues charged to the HECT-E3 enzyme by a partner E2 conjugating enzyme. The RING-type E3 are ubiquitin ligases with a RING-finger motif(s). These ligases appear not to be covalently bound to Ub, but to recruit through the RING finger domain E2s to the vicinity of the proteins to be ubiquitinated, thus mediating ubiquitination by facilitating the direct transfer of Ub from E2-Ub to the Lys residue in the target protein. The U-box proteins constitute a third family of E3 enzymes which by analogy to the RING finger, are likely to recruit E2s facilitating the transfer of ubiquitin residues from E2-Ub to the target protein. Mutational analysis of the U-box protein UIP5 has indeed shown that the U-box domain of this protein mediates direct interaction with E2 enzymes (Pringa and others 2001), with potential protein-protein interaction domains being identified in several U-box proteins (Azevedo and others 2001). These domains are likely to be involved in binding specific substrate proteins to target them for degradation, with U-box proteins therefore being E3 ubiquitin ligases closely related to the RING-finger proteins (Patterson 2002).

Genetic analysis in potato and more recently in *Arabidopsis*, has demonstrated that the U-box protein PHOR1 would play a role as a component in GA signaling (Amador and others 2001). Recognition of U-box proteins as E3 enzymes identifies this signaling intermediate as a E3 ubiquitin ligase enzyme involved in ubiquitination of one or more components of the GA signal transduction pathway, to target them for degradation by the proteasome pathway. Experimental evidence for the involvement of protein degradation in GA signaling has been provided by the observation that activation of the GA response is linked with disappearance of the RGA/SLR1 repressor protein from the nucleus (Silverstone and others 2001; Itoh and others 2002). Therefore, the RGA/SLR1 repressor family would be an obvious candidate for ubiquitination and targeting to proteasome degradation by the PHOR1 ubiquitin ligase enzyme (Figure 5). In support of this hypothesis, proteasome function has recently

been shown to be required for GA-dependent degradation of the GAI/RGA barley homolog SLN1 (Fu and others 2002). Using specific inhibitors of proteasome function, these authors were able to show that proteasome-mediated protein degradation is necessary for GA-mediated destabilization of SLN1, with these inhibitors not only blocking destabilization of SLN1, but also affecting aleurone α-amylase gene expression and seedling leaf extension growth (Fu and others 2002).

Modification of proteins by the attachment of the polypeptide ubiquitin has been increasingly shown to be an important and common mechanism to control stability of key regulatory proteins in animal and plant cells (Weissman 2001). Proteasome-mediated protein degradation has been shown to be involved in a variety of plant cellular responses including photomorphogenesis (Osterlund and others 2000), auxin (Gray and others 2001), jasmonic acid (Xie and others 1998) and elicitor responses, floral development (Samach and others 1999), senescence (Woo and others 2001), cold response (Lee and others 2001) and disease resistance pathways (Austin and others 2002; Azevedo and others 2002). The specificity of ubiquitination is controlled by ubiquitin E3 ligases which recognize the protein substrate and facilitate ubiquitin transfer from the E2 ubiquitin conjugating enzyme to the target protein. RING-H2 finger domain E3 ligases represent a major type of ubiquitin E3 ligases which were first identified in plants. These enzymes can consist of a single protein containing the RING-H2 finger domain, like COP1, or a multiple subunit complex as in the case of SCF1 (Freemont 2000). The SCF complex is composed of Skp1, Cul1 (or CDC53), an F-box protein and the RING-finger protein Roc1/RBx1. Within this complex, the Cul1/Roc1 complex is the catalytic site and the RING finger domain is the binding site for the ubiquitin E2 conjugating enzyme (Jackson and others 2000). SCF E3s have a particularly important role in regulating response to the hormone auxin. Response to these hormones depends on the degradation of the Aux/IAA family of transcriptional repressors, which are recognized and ubiquitinated by the SCF^{TIR1} E3 ligase complex and targeted for degradation by the 26S proteasome (Gray and others 2001).

The *Arabidopsis* COP1 RING finger protein, in its turn, functions as a putative E3 ubiquitin ligase negatively regulating light-responsive gene expression and photomorphogenesis (Deng and others 1992). COP1 controls the degradation of HY5, a bZIP transcription factor that activates light-regulated gene expression (Osterlund and others 2000). Identification of the RING finger proteins ELS with a function in elicitor-induced gene expression (Takai and others 2002), SINAT5 with a function in auxin signal attenuation, or HOS1, with a negative regulatory function in cold signal transduction (Lee and others 2001) was also recently reported. The rice elicitor-responsive EL5 protein has been shown to be membrane-bound and to function as E3 ubiquitin ligase, negatively regulating the plant defense response as part of a feed-back regulation mechanism (Takai and others 2002). The SINAT5 *Arabidopsis* homolog of the *Drosophila* SINA RING-finger protein has been shown to have ubiquitin protein ligase activity and to ubiquitinate the transcription activator NAC1, thus targeting this protein for ubiquitin-mediated proteolysis to downregulate auxin signals in plant cells (Xie and others 2002). *Arabidopsis* HOS1, in its turn, has been shown to be a negative regulator of low temperature-responsive gene transcription. Mutations in *HOS1* lead to enhanced cold induction of genes such as *RD29A*, *COR15A*, *KIN1* and *ADH*, and the mutant plants have reduced capacity for freezing tolerance (Ishitani and others 1998). The HOS1 protein, like COP1 or the U-box protein PHOR1, exhibits nucleo-cytoplasmic partitioning in response to environmental stimuli. COP1 is localized in the nucleus in the dark and translocates to the cytoplasm in response to light signals (von Arnim and Deng 1994). HOS1 was found to reside in the cytoplasm at normal growth temperatures and to accumulate in the nucleus in response to low temperature treatments (Lee and others 2001). Similarly, we have shown that PHOR1 accumulates in the cytosol in the absence of GAs, but migrates to the nucleus after GA application. Therefore, these proteins appear to function as signaling intermediates between cold- and GA-generated cytosolic signals and the nuclear compartment, where they may play a role in regulating gene transcription.

Further studies are required to demonstrate whether PHOR1 does function as an E3 ubiquitin ligase and to define the targets of its ubiquitin ligase activity. Because of PHOR1 function in GA signaling, it is tempting to speculate that this U-box protein would control the turnover of RGA/SLN1. However, whether PHOR1 can directly or indirectly interact with the RGA/SLN1 repressor has not yet been established. Work using yeast two-hybrid analysis and *in vitro* interaction studies is in progress in our laboratory to prove this interaction.

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