Protein Tyrosine Kinases and Protein Tyrosine Phosphatases Are Involved in Abscisic Acid-Dependent Processes in Arabidopsis Seeds and Suspension Cells[1]

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Protein tyrosine (Tyr) phosphorylation plays a central role in many signaling pathways leading to cell growth and differentiation in animals. Tyr phosphorylated proteins have been detected in higher plants, and the roles of protein Tyr phosphatases and protein Tyr kinases in some physiological responses have been shown. We investigated the involvement of Tyr phosphorylation events in abscisic acid (ABA) signaling using a pharmacological approach. Phenylarsine oxide, a specific inhibitor of protein Tyr phosphatase activity, abolished the ABA-dependent accumulation of *RAB18* (responsive to ABA 18) transcripts. Protein Tyr kinase inhibitors like genistein, tyrphostin A23, and erbstatin blocked the *RAB18* expression induced by ABA in Arabidopsis (*Arabidopsis thaliana*). Stomatal closure induced by ABA was also inhibited by phenylarsine oxide and genistein. We studied the changes in the Tyr phosphorylation levels of proteins in Arabidopsis seeds after ABA treatment. Proteins were separated by two-dimensional gel electrophoresis, and those phosphorylated on Tyr residues were detected using an anti-phosphotyrosine antibody by western blot. Changes were detected in the Tyr phosphorylation levels of 19 proteins after ABA treatment. Genistein inhibited the ABA-dependent Tyr phosphorylation of proteins. The 19 proteins were analyzed by matrix-assisted laser-desorption ionization time-of-flight/time-of-flight mass spectrometry. Among the proteins identified were storage proteins like cruciferins, enzymes involved in the mobilization of lipid reserves like aconitase, enolase, aldolase, and a lipoprotein, and enzymes necessary for seedling development like the large subunit of Rubisco. Additionally, the identification of three putative signaling proteins, a peptidyl-prolyl isomerase, an RNA-binding protein, and a small ubiquitin-like modifier-conjugating enzyme, enlightens how Tyr phosphorylation might regulate ABA transduction pathways in plants.

The phytohormone abscisic acid (ABA) regulates several aspects of plant development and adaptation to stress (Finkelstein and Rock, 2002). In seeds, ABA is involved in the acquisition of storage nutrients, desiccation tolerance especially by the activation of late-embryogenesis abundant (LEA) protein genes, maturation, and dormancy (Bewley, 1997). During vegetative growth, ABA is a central signal that triggers plant responses to various adverse environmental conditions, including drought, high salinity, and cold (Leung and Giraudat, 1998). Dehydration triggers the accumulation of ABA, which prevents excessive water loss by reducing stomatal aperture (Schroeder et al., 2001). Drought resistance is also achieved by changes in gene expression. In plants exposed to hydric stress, ABA activates the expression of LEA protein genes, which are thought to exert protective mechanisms against drought because of their high hydrophilicity (Ramanjulu and Bartels, 2002).

There has been substantial progress in the characterization of ABA signaling pathways. Recently, three different ABA receptors have been identified: the RNA-processing protein FCA, the H subunit of Mg-chelatase, and the G protein-coupled receptor GCR2 (Hirayama and Shinozaki, 2007). Elements acting early in ABA signal transduction include reactive oxygen species, calcium ions, G proteins, phospholipases C and D, and ion channels (Finkelstein et al., 2002). Many genetic studies have shown that ABA signaling is modulated by the phosphorylation of proteins on Ser/Thr residues (Hirayama and Shinozaki, 2007). Protein Tyr phosphatases (PTPs) have been shown to be involved in ABA signaling pathways. Specifically, a potent PTP inhibitor, phenylarsine oxide (PAO), modifies several ABA-dependent responses. PAO impairs the induction of *RAB16* (responsive to ABA 16) expression in barley (*Hordeum vulgare*) aleurone protoplasts, prevents stomatal closure in *Commelina communis*, and enhances the inhibition of germination in

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Arabidopsis (*Arabidopsis thaliana*) seeds (Knetsch et al., 1996; MacRobbie, 2002; Reyes et al., 2006). The Arabidopsis mutant *phs1-3*, which is affected in a PTP, is hypersensitive to ABA during germination (Quettier et al., 2006). Although the role of protein Tyr phosphorylation in plant physiology is less documented than for animals (Hunter, 1998), there is evidence for their involvement. Tyr phosphorylated proteins have been detected in carrot (*Daucus carota*) cells, *Mimosa pudica* leaves, Arabidopsis hypocotyls, and Arabidopsis suspension cells (Barizza et al., 1999; Kameyama et al., 2000; Huang et al., 2003; Sugiyama et al., 2008). In addition, Tyr phosphorylation modulates embryogenesis in coconut (*Cocos nucifera*), and copper modulates Tyr phosphorylation of proteins in rice (*Oryza sativa*) roots (Islas-Flores et al., 1998; Hung et al., 2007). PTPs can be divided into two groups based on their phosphoamino acid specificity: Tyr-specific PTPs and dual-specificity PTPs (DsPTPs). Tyr-specific PTPs dephosphorylate phosphotyrosine but not phosphoserine/phosphothreonine, whereas the DsPTPs dephosphorylate all three. Protein Tyr kinases (PTKs) are classified in the same way: specific PTKs phosphorylate only Tyrs, while dual-specificity PTKs (DsPTKs) phosphorylate Ser/Thr and Tyrs. Several PTPs from Arabidopsis and other species have been characterized (Gupta et al., 1998; Xu et al., 1998; Fordham-Skelton et al., 1999). A diverse group of about 20 genes encoding putative PTPs have been identified in the Arabidopsis genome (Kerk et al., 2002). No plant Tyr-specific kinases have been identified based on homologies with animal PTKs. It has been suggested that in plants, as in yeast, DsPTKs mediate the Tyr phosphorylation of proteins (Rudrabhatla et al., 2006).

Here, we used a pharmacological approach to investigate the involvement of PTKs and PTPs in the ABA signal transduction pathways leading to *RAB18* expression and to stomatal closure in Arabidopsis. To estimate how PTK and PTP activities are regulated by ABA, we analyzed the phosphoproteome of Arabidopsis seeds treated or untreated with ABA. We specifically looked for proteins whose Tyr phosphorylation status was modulated by ABA and thereby identified 11 proteins. We discuss the possible functions of these proteins in seeds during germination.

RESULTS

PTPs and PTKs Are Involved in ABA-Induced *RAB18* Expression

To investigate involvement of Tyr phosphorylation in ABA signaling, we took a pharmacological approach using specific PTK and PTP inhibitors. In Arabidopsis suspension cells, 10 μM ABA elicited expression of the *RAB18* gene, whereas dimethyl sulfoxide (DMSO) alone did not (Fig. 1). When PAO (1–10 μM), a highly specific PTP inhibitor, was added with ABA, a dose-dependent inhibition of *RAB18* induction was observed (Fig. 1A). By contrast, addition of dephostatin (100 μM) or α-naphthyl phosphate (NAP; 200 μM) did not modify ABA-induced *RAB18* expression (Fig. 1, B and C). This suggests that at least one PAO-sensitive PTP is involved in ABA signaling that leads to *RAB18* expression.

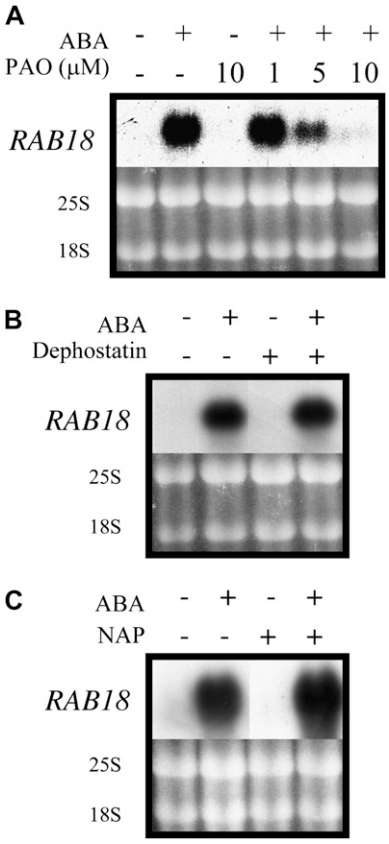

Figure 1. ABA induction of *RAB18* gene expression is mediated by PTP activities in Arabidopsis suspension cells. Northern-blot analysis of total RNA (10 μg) from cells incubated for 3 h with ABA (10 μM) and various PTP inhibitors is shown. A, Phenylarsine oxide (1 to 10 μM). B, Dephostatin (100 μM). C, NAP (200 μM). Control cells were treated with DMSO alone. Ethidium bromide staining of *25S* and *18S* rRNAs is shown as the control.

Incubation of Arabidopsis suspension cells with ABA (10 μM) and genistein (100 μM), a specific PTK inhibitor, blocked *RAB18* expression (Fig. 2A). This effect was specific to genistein, as addition of daidzein (100 μM), a biologically inactive analog, did not alter *RAB18* expression (Fig. 2A). *RAB18* expression induced by ABA was also inhibited when cells were treated with erbstatin (100 μM) and tyrphostin A23 (100 μM), which are both specific epidermal growth factor receptor-type PTK inhibitors (Fig. 2, B and C). Conversely, when lavendustin A (20 μM), tyrphostin A25 (100 μM), or tyrphostin AG490 (100 μM) was added with ABA, the accumulation of *RAB18* transcripts was not modified (Fig. 2, C and D). These

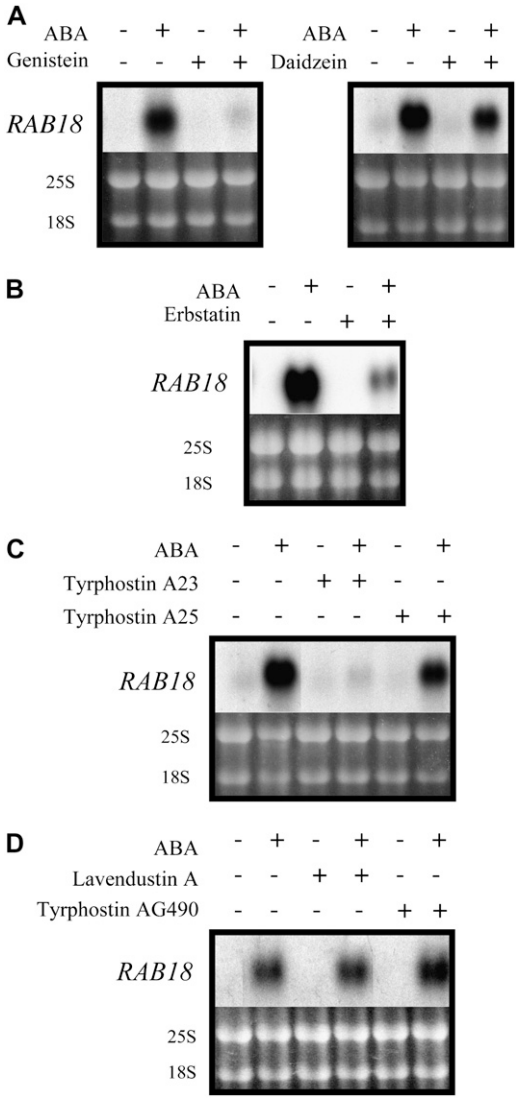

Figure 2. PTKs are involved in the signal transduction pathway leading to *RAB18* gene expression in Arabidopsis suspension cells. Northern-blot analysis of total RNA (10 μg) from cells incubated for 3 h with ABA (10 μM) and various PTK inhibitors is shown. A, Genistein (100 μM) and daidzein (100 μM). B, Erbstatin (100 μM). C, Tyrphostin A23 (100 μM) and tyrphostin A25 (100 μM). D, Lavendustin A (20 μM) and tyrphostin AG490 (100 μM). Ethidium bromide staining of *25S* and *18S* rRNAs is shown as the control.

observations suggest that PTKs specifically sensitive to genistein, tyrphostin A23, and erbstatin are required for ABA signaling.

PTPs and PTKs Regulate Stomatal Closure Induced by ABA

As ABA plays a crucial role in the control of stomatal movements, we studied the influence of Tyr phosphorylation on ABA-induced stomatal closure using PAO and genistein. Figure 3 shows that ABA induces 50% stomatal closure (stomatal aperture, 1.5 μm) compared with the control (stomatal aperture, 3.5 μm) in Arabidopsis leaf epidermis. ABA-induced stomatal closure was impaired when leaf epidermal strips were incubated with ABA and PAO or with ABA and genistein (Fig. 3). These results revealed that both PTK and PTP are also elements of the ABA signaling pathway that leads to stomatal closure.

ABA Modulates Protein Tyr Phosphorylation in Arabidopsis Seeds

We investigated whether the level of protein Tyr phosphorylation changes when germination is inhibited by ABA. Using PAO, Reyes et al. (2006) have already demonstrated that at least one PTP is involved in ABA signaling that leads to *RAB18* expression during seed germination. The up-regulation of ABA-induced *RAB18* expression was enhanced when seeds of *phs1-3*, a mutant affected in a DsPTP activity, were imbibed with ABA for 2 d (Quettier et al., 2006). This result also suggested that during this duration of imbibition, an ABA-regulated DsPTP is involved in the control of germination. We analyzed the two-dimensional (2D) electrophoresis protein patterns in the same experimental conditions that allowed the detection of the ABA-regulated DsPTP PHS1. Only about 20% of Arabidopsis seeds germinate after 2 d on ABA-supplemented medium. This incubation time corresponds strictly to the stage at which the radicle emerges in control seeds, also called the phase of germination sensu stricto. Consequently, the differences observed could not simply result from different developmental stages achieved by control seeds and ABA-treated seeds.

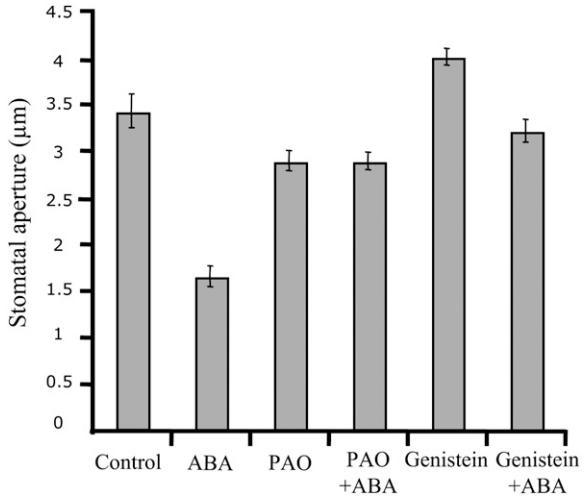

Figure 3. Effects of PAO and genistein on stomatal closure induced by ABA in Arabidopsis. Preopened stomata were exposed to ABA (10 μM), PAO (10 μM), ABA (10 μM) and PAO (10 μM), genistein (100 μM), and ABA (10 μM) and genistein (100 μM) for 3 h. The stomata were preincubated with PAO or genistein for 30 min before adding ABA. Values represent means of 70 measurements. Each experiment was repeated three times. Error bars indicate SE.

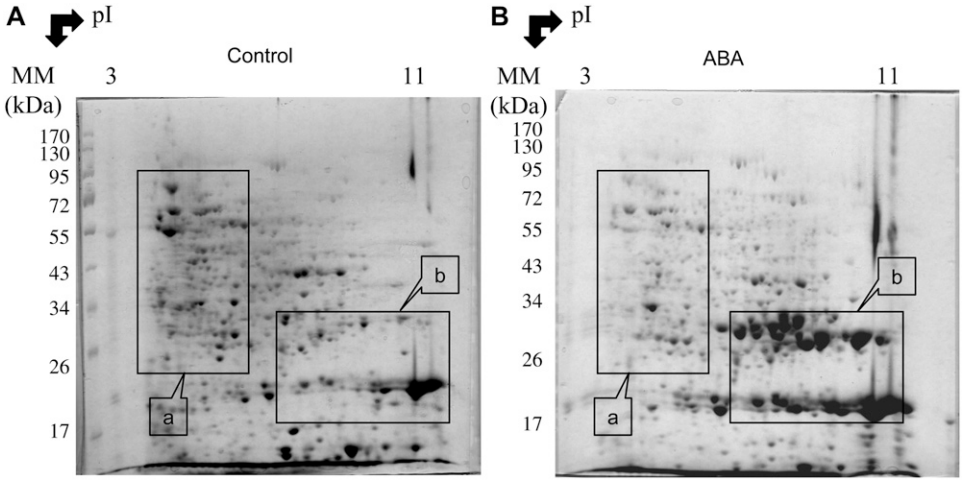

Figure 4. ABA modulates protein abundance in Arabidopsis seeds. Seeds were incubated for 2 d in water or in ABA (10 μM). An equal amount of each total protein extract (1.25 mg) was loaded onto each gel. The results shown are representative of at least three repetitions. 2D gels of total proteins were stained with Coomassie Brilliant Blue. MM, Molecular mass markers. A, Coomassie Brilliant Blue-stained 2D gel of total proteins from seeds imbibed for 2 d in water. B, Coomassie Brilliant Blue-stained 2D gel of total proteins from seeds imbibed for 2 d in the presence of ABA. Windows a and b locate the areas of the gel where the protein abundance is modulated by ABA.

We detected about 400 protein spots on 2D gels stained with Coomassie Brilliant Blue using Image Master 2D Platinum software (Fig. 4). Comparison of the 2D protein patterns of seeds imbibed for 2 d in water (control) with those of seeds imbibed for 2 d in ABA (10 μM) showed that ABA modifies the abundances of several proteins (Fig. 4). For example, some proteins of around 20 to 30 kD appear to up-accumulate in ABA-treated seeds (Fig. 4, window b). Conversely, other proteins, especially acidic proteins, are less abundant in ABA-treated seeds than in control seeds (Fig. 4, window a). An anti-phosphotyrosine antibody was used on western blots to detect modifications in the Tyr phosphorylation status of proteins from ABA-treated seeds. Coomassie Brilliant Blue-stained total protein patterns were largely different from Tyr phosphorylated protein patterns (Figs. 4 and 5). When the two sets of patterns were compared with Image Master 2D Platinum software, it was found that of the total of around 400 proteins, about 140 are Tyr phosphorylated. Interestingly, many heavily Coomassie Brilliant Blue-stained protein spots (i.e. abundant proteins) were not, or were only weakly, detected with the anti-phosphotyrosine antibody (Figs. 4 and 5). Conversely, many spots that were weakly stained with Coomassie Brilliant Blue (i.e. minority proteins) were readily detected with the anti-phosphotyrosine antibody (Figs. 4 and 5). Comparison of the Tyr phosphorylation profiles (control versus ABA-treated seeds) indicates that the level of Tyr phosphorylation of several proteins was unchanged after ABA treatment (Fig. 5, A and B, spots labeled with diamonds). After 2 d of imbibition with ABA, the Tyr phosphorylation level of 19 proteins appeared to be modified (Fig. 5B). Of these, eight proteins were Tyr dephosphorylated (Fig. 5C, spots in circles) and 11 were Tyr phosphorylated (Fig. 5C, spots in squares). Using Image Master 2D Platinum software, we estimated the relative abundance of proteins (amount of protein in ABA-treated seeds divided by amount of protein in control seeds) and the relative Tyr phosphorylation (Tyr phosphorylation level in ABA-treated seeds divided by Tyr phosphorylation level in control seeds; Table I). For each spot, the relative abundance appeared to be different from the relative Tyr phosphorylation, suggesting that these two mechanisms are regulated independently one from the other by ABA. In order to further study the activation of PTKs by ABA, the effect of genistein on the Tyr phosphorylation induced by ABA was analyzed. We established the protein Tyr phosphorylation profiles of seeds imbibed for 2 d in 100 μM genistein and those of seeds imbibed for 2 d in 10 μM ABA and 100 μM genistein (Fig. 6). Visual comparison of the Tyr phosphorylation profiles of seeds treated with ABA with those of seeds treated with both ABA and genistein indicates that genistein reduces the level of Tyr phosphorylation for the 11 proteins (Fig. 6A). Quantitative analysis using Image Master 2D Platinum software shows that Tyr phosphorylation status of these proteins was decreased in seeds treated with both ABA and genistein, compared with the seeds treated with ABA only (Fig. 6B). This result confirms the specificity of the detection of Tyr phosphorylation by the PY20 antibody. The amplitude of the decrease in Tyr phosphorylation varies from 0.2 arbitrary units (Fig. 6B, spot B) to 2.6 arbitrary units (Fig. 6B, spot S). This decrease is more important for proteins L, H, G, S, N, Q, O, and P

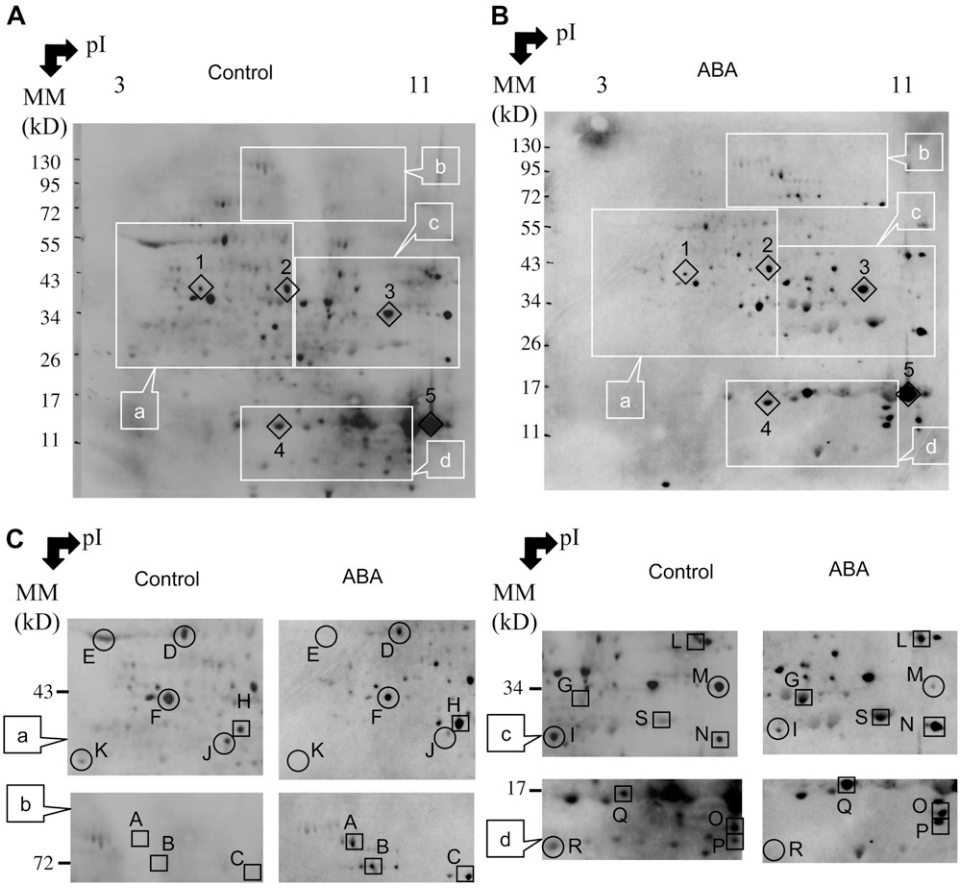

Figure 5. Tyr phosphorylation levels of Arabidopsis seed proteins are regulated by ABA. Total proteins were extracted from seeds and separated on 2D gels. Tyr phosphorylated proteins were detected by western blot with an anti-phosphotyrosine antibody. MM, Molecular mass markers. A, Immunoblot of 2D gel of total proteins from seeds imbibed for 2 d in water. B, Immunoblot of 2D gel of total proteins from seeds imbibed for 2 d in the presence of ABA (10 μM). The Tyr phosphorylation level of proteins labeled with diamonds was unaffected by ABA. The indicated portions of the immunoblots are reproduced in C. C, Enlarged windows (a–d) of immunoblots as shown in A and B for control (left) and ABA-treated (right) seeds. The proteins phosphorylated on Tyr residues in response to ABA are squared. Tyr residues of proteins shown in circles are dephosphorylated in the seeds after ABA treatment. These proteins (spots A–S) were analyzed by MALDI-TOF-TOF MS (Table I).

compared with proteins A, B, and C (Fig. 6B). This result strongly suggests that not only one but several ABA-induced PTKs phosphorylate these proteins on Tyr residues. The PTKs involved in ABA signaling are differentially blocked by genistein. The PTKs that act on proteins L, H, G, S, N, Q, O, and P are more sensitive to genistein than those that phosphorylate proteins A, B, and C.

Identification by Mass Spectrometry of the Proteins Whose Tyr Phosphorylation Level Is Modulated by ABA

For each of the 19 Tyr phosphorylated proteins, the spot was excised, digested with trypsin in gel, and analyzed by matrix-assisted laser-desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF-TOF MS). Table I lists the 11 Tyr phosphorylated proteins that were identified out of the 19 proteins detected. Proteins corresponding to spots L, M, and N (Fig. 5C) were not abundant enough to be analyzed by MS, and five other proteins were not identified (spots B, H, I, O, and P in Fig. 5C). Five proteins whose Tyr phosphorylation increased and six proteins whose Tyr phosphorylation decreased in response to ABA were identified with a high degree of confidence (Table I). Details of the analysis are given in Table I. The MALDI-TOF- and MALDI-TOF-TOF-generated peak lists were submitted to the database search program MASCOT. We report the MOWSE scores, the peptides matched, the sequence coverage, and the precursor peptides that were analyzed in MS/MS. The amino acid sequence is given for each precursor peptide (Table I). The five proteins that show an increase in Tyr phosphorylation after ABA treatment are three seed storage proteins, an aconitase, and a protein of unknown function in Arabidopsis (Table I; Fig. 5C, spots Q, C, S, A, and G, respectively). A BLASTP search of plant genome databases with the sequence At1g05510 (spot G) showed that it has a strong similarity to the embryo-specific protein Ose731 from rice (sequence 51038130). The protein spot C corresponds to a mixture of two cruciferin precursors, the 12S seed storage proteins CRA1 and CRC (Fig. 5C). The protein spots Q and S correspond to the CBR and the CRA1 cruciferin subunits, respectively (Fig. 5C). The six proteins that are Tyr dephosphorylated in response to ABA (Fig. 5) were identified as the Rubisco large subunit (spot D), an enolase (spot E), a Fru-bisP aldolase (spot F), an RNA-binding protein (spot K), the peptidyl-prolyl isomerase (PPIase) ROC4 (spot R), and a nucleoside-diphosphate sugar epimerase that is a small ubiquitin-like modifier (SUMO)-conjugating enzyme (spot J).

Table I. *Arabidopsis proteins whose Tyr phosphorylation varied significantly in seeds imbibed for 2 d in ABA*

Spot ID, Spot identity; AGI No., Arabidopsis Genome Initiative accession number; Theo, theoretical; MM, molecular mass in kD; Pep Mat, peptides matching; Seq Cov, sequence coverage in percentage; Prec Peak, precursor peak.

Spot ID	AGI No.	Protein Name and Family	Score MS/MS-MS	Theo MM	Theo pI	Pep Mat	Seq Cov	MS-MS Prec Peak	Amino Acid Sequences	Relative Phosphorylation[a]	Relative Abundance[b]
Storage proteins											
Q	At1g03880	12S seed storage protein CRB precursor	76/128	50.5	6.5	11	18	1,387.6 2,214.1	TNENAQVNTLAGR GLPLEVITNGYQISPEEAKR	7.72	9.93
C	At5g44120	Mixture: 12S seed storage protein CRA1	85/136	41	6.6	13	28	1,853.9 2,067.9	GLPLEVITNGFQISPEEAR VIPGCAETFQDSSEFQPR	2.39	NC[c]
	At4g28520	12S seed storage protein CRC		58.2	6.5	13	29				
S	At5g44120	Cruciferin precursor (CRA1)	101/156	52.5	7.6	12	21	1,611.8 2,067.9	FRDMHQKVEHIR VIPGCAETFQDSSEFQPR	3.98	6.91
Metabolism and energy											
A	At2g05710	Cytoplasmic aconitate hydratase	123/136	98	5.7	20	23	1,184.7 1,500	QVEIPFKPAR GVDRKDFNSYGSR	6.3	0.86
E	At2g36530	Enolase (2-phospho-D-glycerate hydrolyase)	122/152	47.6	5.5	14	57	2,004.1 2,252.2	IVLPVPAFNVINGGSHAGN GETEDTFIADLAVGLSTGQI	≤0.01	0.07
F	At4g38970	Fru-bisP aldolase	72/146	41.3	6.7	8	15	1,291.6 1,128.5	ATPEQVAAYTLK TAAYYQQGAR	0.86	0.34
Signaling elements											
J	At5g02240	Nucleoside-diphosphate sugar epimerase	95/176	27	6.1	9	38	1,155.6 1,926	ALFSQVTSRF KAEQYLADSGTPYTIIR	0.87	≤0.01
K	At2g37220	RNA-binding protein	140/152	30.7	5	11	30	1,080.5 1,558.7	VIYDRDSGR VSEAEARPPRRQY	≤0.01	≤0.01
R	At3g62030	Peptidyl-propyl isomerase ROC4	141/241	28.1	8.8	13	46	1,620.8 1,953	TLESQETRAFDVPK VTNKVYFDVEIGGEVAGR	≤0.01	0.07
Other processes											
D	AtCg00490	Rubisco large chain	221/296	52.9	5.8	23	34	1,465.8 1,684.9	TFQGPPHGIQVER NEGRDLAVEGNEIIR	0.86	0.72
G	At1g05510	Putative lipoprotein	85/166	27.2	6.2	12	40	1,878.9 2,177.1	LELREVDIKPVESVPR GLKLELREVDIKPVESVPR	4.72	≤0.01

[a]Normalized spot volumes in the seeds imbibed for 2 d in the presence of ABA (10 μM) divided by the normalized spot volume in the seeds imbibed for 2 d in water (ratio of protein Tyr phosphorylation in ABA-treated seeds over protein Tyr phosphorylation in control seeds) from three different gels and independent extractions; ≤0.01 means that the accumulation level of the corresponding protein in the seeds imbibed for 2 d in the presence of ABA (10 μM) was close to background. [b]Normalized spot volumes in the seeds imbibed for 2 d in the presence of ABA (10 μM) divided by the normalized spot volume in the seeds imbibed for 2 d in water (ratio of protein abundance in ABA-treated seeds over protein abundance in control seeds) from three different gels and independent extractions; ≤0.01 means that the accumulation level of the corresponding protein in the seeds imbibed for 2 d in the presence of ABA (10 μM) was close to background. [c]NC, Not calculated, as the levels of the corresponding protein in ABA-treated seeds and in control seeds were close to background.

DISCUSSION

PTKs and PTPs Are Involved in ABA Transduction Pathways Leading to *RAB18* Expression and Stomatal Closure

In animal cells, protein Tyr phosphorylation acts as an on-off switch in numerous pathways that regulate growth, differentiation, and oncogenesis (Hunter, 1998). The level of Tyr phosphorylation is regulated by the antagonistic action of PTKs and PTPs, which have been characterized according to their sensitivities to various inhibitors. Here, we used a pharmacological approach to assess the involvement of PTKs and PTPs in ABA signaling.

In Arabidopsis suspension cells, the inhibition of PTPs by PAO but not by NAP or dephostatin impaired the ABA induction of *RAB18* expression (Fig. 1). RAB18 belongs to the group 2 LEA proteins, also called dehydrins, and is phosphorylated in Arabidopsis seeds (Ramanjulu and Bartels, 2002; Irar et al., 2006). The accumulation of *RAB18* transcripts is induced by ABA in mature seeds (Parcy et al., 1994), in vegetative tissues (Lang and Palva, 1992), and in suspension cells (Jeannette et al., 1999). These results suggest that at least one PTP, which is PAO sensitive, is implicated in ABA signaling in Arabidopsis. Indeed, PAO is a highly specific PTP inhibitor that reacts with vicinal Cys residues of the PTPs active site (Garcia-Morales et al., 1990; Liao et al., 1991). The activity of plant DsPTP LePTPKIS1 is inhibited by PAO (Fordham-Skelton et al., 2002). In *M. pudica*, the Tyr dephosphorylation of actin induced by stimulation of the pulvinus is abolished by PAO (Kameyama et al., 2000). PAO also inhibits PTP activities in *Vicia faba* (Shi et al., 2005). NAP and dephostatin block PTPs by inhibiting competitively the fixation of the substrate in the active site (Davidson and Haslam, 1994; Umezawa et al., 2003; Partanen, 2008). The effects of NAP have not yet been studied on plant PTPs but on recombinant animal PTPs (Salve et al., 2000). Soybean (*Glycine max*) PTP has an

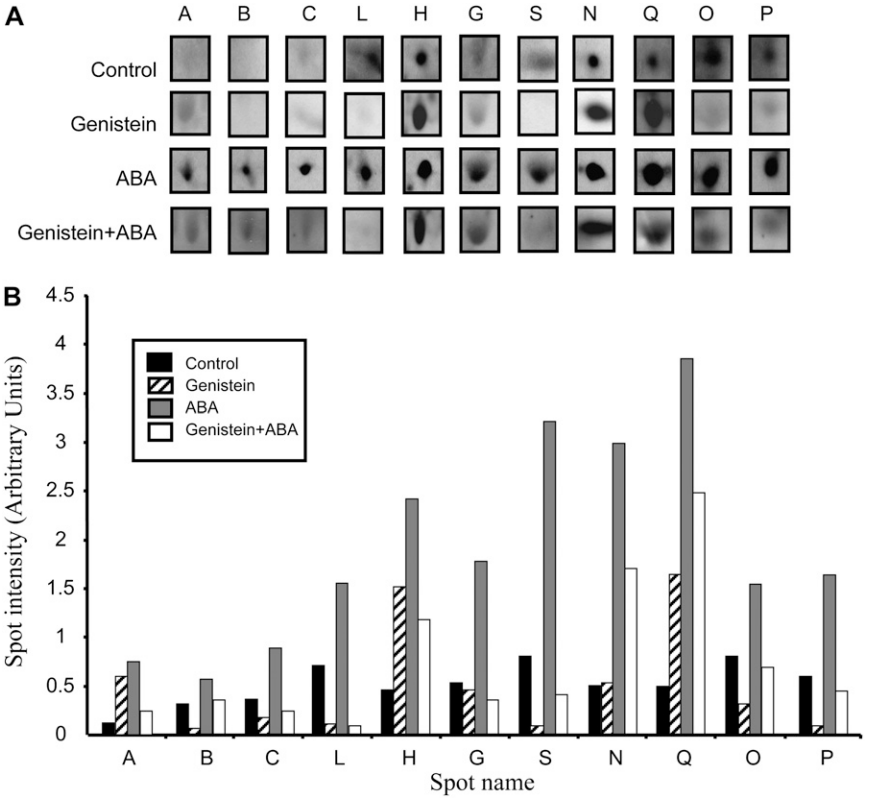

Figure 6. Effect of genistein on the Tyr phosphorylation induced by ABA. Seeds were imbibed with 0.1% DMSO (Control), 100 μM genistein (Genistein), 10 μM ABA (ABA), or both genistein and ABA (Genistein+ABA) for 2 d. Total proteins were extracted from seeds and separated on 2D gels. Tyr phosphorylated proteins were detected by western blot with PY20 anti-phosphotyrosine antibody, and spots showing variations in ABA-dependent Tyr phosphorylation were quantified. A, Close-up view of the 11 spots corresponding to proteins whose Tyr phosphorylation is induced by ABA. B, Quantitation of the spot intensities. Images were quantitated as described in "Materials and Methods." The results are means of three experiments.

activity inhibited by dephostatin (Fordham-Skelton et al., 1999). The lack of effect of NAP and dephostatin on ABA-induced *RAB18* expression could be explained by the mechanism of inhibition of these inhibitors, which is different from that of PAO. PTP involved in ABA signaling is selectively inhibited by PAO. In accordance with our results, PAO inhibits the ABA-induced expression of *RAB16*, the ortholog of *RAB18*, in barley aleurone cells (Knetsch et al., 1996). We also observed that ABA-induced stomatal closure is blocked by PAO (Fig. 3). Likewise, PAO inhibits the stomatal closure induced by ABA in *C. communis* (MacRobbie, 2002). Nevertheless, PAO enhances ABA-induced *RAB18* expression in Arabidopsis seeds (Reyes et al., 2006). This discrepancy could be explained by cross talk between ABA transduction pathways and other signaling pathways. Despite the fact that the role of PTP in ABA signaling is established, the mechanisms controlling these complex pathways remain unknown. Genetic studies have confirmed that DsPTPs are involved in ABA signaling. The Arabidopsis mutant *ibr5*, defective in a DsPTP, is not sensitive to the inhibition of root elongation induced by ABA (Monroe-Augustus et al., 2003). The DsPTP mutant *phs1-3* is hypersensitive to ABA during germination and stomatal responses (Quettier et al., 2006).

As the level of Tyr phosphorylation of a protein is determined by the balanced activity of a PTP and a PTK, we investigated the role of PTKs in ABA signaling using specific PTK inhibitors. We showed that genistein, but neither its inactive analog daidzein nor lavendustin A, impaired the ABA-induced *RAB18* expression (Fig. 2) and that genistein also blocked ABA-induced stomatal closure (Fig. 3). These results suggest an activation by ABA of a PTK specifically sensitive to genistein. Genistein and lavendustin A are broad-range PTK inhibitors and act by competitively binding to the ATP binding site of the kinase (Akiyama et al., 1987; Onoda et al., 1989; Levitzki and Gazit, 1995). A Tyr kinase activity purified from *Catharanthus roseus* is blocked by genistein (Rodriguez-Zapata and Hernandez-Sotomayor, 1998). Genistein inhibited a DsPTK from pea (*Pisum sativum*; Rudrabhatla and Rajasekharan, 2004). In maize (*Zea mays*), lavendustin A inhibited a DsPTK activity (Trojanek et al., 2004). In lemon (*Citrus limon*) seedlings, production of inositol-1,4,5-trisphosphate induced by *Alternaria alternata* is blocked by lavendustin A (Ortega et al., 2005). More selective inhibitors have been used to characterize the PTK activities involved in ABA signaling. In Arabidopsis suspension cells, tyrphostin A23 impaired the regulation of *RAB18* expression more efficiently than erbsatin, and tyrphostin A25 did not affect *RAB18* expression (Fig. 2). Erbstatin and tyrphostins have similar molecular structures and block PTKs by competitively binding to both the substrate-binding site and the ATP-binding site (Levitzki and Gazit, 1995; Levitzki and Mishani, 2006). Tyrphostins inhibit the activity of a DsPTK in pea (Rudrabhatla and Rajasekharan, 2004). An erbstatin analog, methyl-2,5-dihydroxycinnamate, blocks a PTK involved in the transduction pathway leading to inositol-1,4,5-

trisphosphate production in lemon seedlings (Ortega et al., 2005). The differences in the inhibition of *RAB18* could be due to the selectivity of each inhibitor toward the PTK involved in the transduction pathway. Tyrphostin A23 and erbstatin might block PTK activity by two different mechanisms. Alternatively, the differential behaviors observed between the two inhibitors may also be explained by the involvement of more than one PTK in the ABA signaling pathway. In that case, one PTK would be specifically sensitive to tyrphostin A23 and the other one would have a sensitivity toward erbstatin. Finally, tyrphostin AG490, a highly selective PTK inhibitor that blocks an intracellular PTK in animals (Levitzki and Mishani, 2006), did not modify the accumulation of *RAB18* transcripts (Fig. 2). This suggests that PTKs might be preferentially associated with membranes. DsPTK activities have already been detected in plants (Hirayama and Oka, 1992; Ali et al., 1994; Rudrabhatla and Rajasekharan, 2004), but their involvement in ABA signal transduction is less well documented. To our knowledge, these results provide the first evidence for the involvement of PTKs in ABA signaling in Arabidopsis.

Tyr Phosphorylation of Several Proteins Is Modified during the Inhibition of Germination in Arabidopsis Seeds by ABA

We used a proteomic approach coupled with immunodetection to measure the Tyr phosphorylation levels of proteins modulated by ABA. These proteins are potential targets of PTKs and PTPs involved in ABA signaling. Tyr phosphorylated proteins from Arabidopsis suspension cells were first detected by western blot. However, after ABA treatment for 10 to 30 min, no significant change in the level of Tyr phosphorylation of the proteins was detected (data not shown). Previous studies of an Arabidopsis suspension cell phosphoproteome with the fluorescent phosphoprotein dye Pro-Q Diamond indicated that about 10 proteins are phosphorylated on either Ser, Thr, or Tyr residues after ABA treatment (El-Khatib et al., 2007). Therefore, we chose to investigate the effect on protein Tyr phosphorylation when ABA inhibits seed germination. In Arabidopsis seeds, PTP activities have been implicated in ABA signaling (Quettier et al., 2006; Reyes et al., 2006).

There are about 400 proteins in both Arabidopsis control and ABA-treated seeds, as estimated by Coomassie Brilliant Blue staining after 2D gel separation (Fig. 4). Similarly, Rajjou et al. (2007) detected 475 proteins in Arabidopsis seeds. Nevertheless, the 2D patterns of total proteins were different for control seeds compared with seeds imbibed for 2 d in ABA (Fig. 4). Treatment with ABA induced a loss of some acidic proteins and an accumulation of some proteins, in particular some 20- to 30-kD proteins (Fig. 4, windows a and b). Similarly, Chibani et al. (2006) estimated that the amounts of 57 proteins decreased and the amounts of seven proteins increased in nondormant Arabidopsis seeds in response to ABA. It was suggested, therefore, that protein abundance is modulated through proteolytic mechanisms (Chibani et al., 2006). We detected about 140 Tyr phosphorylated protein spots in extracts from Arabidopsis seeds. Therefore, this kind of posttranslational modification may play an important role in seeds. Similar approaches based on immunodetection have demonstrated the role of Tyr phosphorylation in pulvinus movement in *M. pudica* and hypocotyl growth in Arabidopsis (Kameyama et al., 2000; Huang et al., 2003). Tyr phosphorylation of at least 19 seed proteins was modulated after ABA treatment: eight were Tyr dephosphorylated and 11 were Tyr phosphorylated (Fig. 5). When seeds were imbibed with both ABA and genistein compared with seeds imbibed with ABA alone, the level of Tyr phosphorylation of the 11 proteins was decreased (Fig. 6). The differences observed in the genistein-induced decrease of Tyr phosphorylation suggest that not only one but several PTKs with differential sensitivities to genistein are involved in the ABA signaling pathway in Arabidopsis seeds. This result also supports the hypothesis that the spots detected by western blot are protein Tyr phosphorylated.

We identified 11 of these 19 proteins by MS and estimated their relative abundance and relative Tyr phosphorylation (Table I). The amounts of two proteins increased and the amounts of eight proteins decreased in response to ABA (Table I). The Tyr phosphorylation level of five proteins increased and that of six proteins decreased (Table I). The differences observed between their relative abundance and their relative Tyr phosphorylation level suggest that ABA regulates the accumulation and the Tyr phosphorylation of these proteins separately. We tested the possibility that the 2D gel spots analyzed by MS could correspond to protein mixtures. During database searches, the MASCOT search engine automatically detects significant protein mixtures of up to six components. Among all of the spots analyzed, only spot C appeared to be a mixture of the two seed storage proteins CRA1 and CRC (Table I). The unmatched peptides that were not selected for the identification of the proteins in the first attempt were used to achieve a second database search with MASCOT in order to identify a less abundant protein that could also be present in the spot. In all cases, these unmatched peptides did not allow the identification of a protein with a significant MOWSE score. Thus, there is a high probability that all of the spots analyzed, with the exception of spot C, correspond to the single protein that has been shown to be Tyr phosphorylated by western blot. Also, using Netphos 2.0, a sequence-based prediction database for phosphorylation sites, we could identify at least one Tyr residue for each protein identified as a putative phosphorylation site. Altogether, these points strongly suggest that the 11 spots that have been identified correspond to Tyr

phosphorylated proteins. However, analysis of the MS spectra of these proteins showed that phosphopeptides were in very low abundance compared with the normal peptides and could not be detected by MS (data not shown). The phosphopeptides have a poor ionization efficiency because of the phosphoryl group and so frequently correspond with low-intensity peaks (Ficarro et al., 2002). The realistic amount of starting material for the characterization of phosphorylated sites in proteins separated on a gel needs to be 10 to 100 times higher than the amount needed to identify a protein on the gel by MALDI peptide mass mapping (Larsen et al., 2001). The selective enrichment of the tryptic digest for phosphopeptides has been performed by TiO_2 chromatography, but the detection of the phosphopeptides by combined MS and MS/MS experiments was unsuccessful (data not shown). Hydroxy acid-modified metal oxide chromatography, recently developed (Sugiyama et al., 2008), or immobilized metal ion affinity chromatography (Collins et al., 2007) could lead to a more appropriate enrichment for these phosphopeptides of very low abundance and thus allow us to determine the Tyr phosphorylation sites of the proteins detected by MS.

Given that ABA regulates the Tyr phosphorylation of these 11 proteins when germination is inhibited, the proteins identified may have a role in the regulation of germination or be elements of the ABA signaling pathways. We classified the proteins according to these two main categories. The role of Tyr phosphorylation during germination is discussed below in relation to the function of the Tyr phosphorylated proteins identified.

Germination-Related Proteins Involved in the Mobilization of Stored Reserves and Preparation for Seedling Growth Are Tyr Phosphorylated

In higher plants, major seed storage proteins include the 12S globulins, also called cruciferins, and the 2S albumins, also called napin or arabin (Fujiwara et al., 2002). Cruciferins are the most abundant storage proteins in Arabidopsis seeds. There are three genes encoding 12S globulins in the genome of the Columbia ecotype, designated CRA1, CRB, and CRC (Pang et al., 1988). The cruciferins are synthesized in developing embryos in a precursor form consisting of a single 50-kD protein chain. At later stages, the precursor form is cleaved into an α-subunit (30 kD) and a β-subunit (20 kD) that remain linked together by a single interchain disulfide bond and interact noncovalently to form hexameric complexes (Fujiwara et al., 2002). Mature cruciferins are subsequently broken down during germination and used by the seedling as an initial nutrient source (Bewley, 1997). We detected the mature 12S storage protein CRA1 (Fig. 5, spot S) and its precursor form (Fig. 5, spot C) in Arabidopsis seeds. This suggests that the protein maturation process giving rise to the formation of α- and β-chains was not complete in dry seeds. In addition, we noted that cruciferins are more abundant in Arabidopsis seeds treated with ABA than in control seeds (Table I). This is probably because they are mobilized during germination in control seeds and, conversely, their degradation is inhibited in ABA-treated seeds. These proteins are also Tyr phosphorylated (Table I). We noted that in control seeds, cruciferins (Fig. 5, spots C, Q, and S) are dephosphorylated on Tyr residues. Phosphorylation of storage proteins in Arabidopsis seeds, and more specifically their Tyr phosphorylation, has been reported previously (Irar et al., 2006; Wan et al., 2007). In the PP2C-impaired mutant *abi1-1*, cruciferins have higher Tyr phosphorylation levels compared with the wild type, and this suggested that Tyr phosphorylation plays a role in ABA signaling (Wan et al., 2007). It may be suggested that Tyr phosphorylation induced by ABA prevents cruciferin proteolysis. Alternatively, the dephosphorylation of Tyrs could promote their degradation during seed imbibition.

In Arabidopsis seeds, triacylglycerols (TAGs) are the major storage lipids. During germination, TAGs are converted to sugar that provides the energy necessary for seedling development (Penfield et al., 2006). Accordingly, Arabidopsis seeds are well equipped with the enzymes necessary to degrade stored TAGs. Consistent with this, we identified three enzymes involved in TAG catabolism and subsequent energetic metabolism: an aconitase (Fig. 5C, spot A; Table I), a 1,6-Fru-bisP aldolase (Fig. 5C, spot F; Table I), and an enolase (Fig. 5C, spot E; Table I). Their relative abundance diminishes in response to ABA (Table I). In keeping with these results, aconitase and 1,6-Fru-bisP aldolase accumulate prior to radicle protrusion during seed germination, and aconitase levels decrease when germination is inhibited by exogenous ABA (Gallardo et al., 2002; Chibani et al., 2006). These enzymes are all Tyr phosphorylated, and there is a particularly marked increase in aconitase Tyr phosphorylation in response to ABA. Tyr phosphorylation of these three enzymes has not been reported before in plants but has been shown in animal cells (Buonocore et al., 1999; Kim et al., 2007). Phosphorylation or dephosphorylation of these enzymes on Tyr residues may regulate their abundance and/or their activity.

We detected a protein with an unknown function in Arabidopsis (Fig. 5C, spot G; Table I). The abundance of this protein decreases and its Tyr phosphorylation increases in ABA-treated seeds (Table I). By sequence homology (BLASTP analysis), we showed that it is related to the embryo-specific protein Ose731 from rice, which has a lipoprotein domain like oleosins. Oleosins are specific proteins associated with lipid reserves, where they stabilize oil bodies in seeds during desiccation and facilitate the hydrolysis of these reserves by lipases during germination (Keddie et al., 1992). Similarly, the levels of oleosins decrease after ABA treatment in *Brassica napus* and rice (Holbrook et al., 1991; Konishi et al., 2005). By labeling proteins with [^{32}P]orthophosphate, oleosins have been shown to be phosphorylated in *B. napus* (Holbrook et al., 1991). Tyr phosphorylation, therefore, may regulate oil body

biogenesis or oleosin targeting and thus modulate the hydrolysis of lipid reserves.

Levels of the large subunit of Rubisco decreased and its Tyr phosphorylation levels decreased in seeds treated with ABA (Fig. 5, spot D; Table I). This Rubisco subunit has already been detected in Arabidopsis seeds (Chibani et al., 2006). Mann and Turner (1988) have shown that the large subunit of Rubisco is phosphorylated on Tyr residues in *Rhodomicrobium vannielii*. The phosphorylation of this subunit may be involved in the assembly of Rubisco (Foyer, 1985). When ABA inhibits germination, the dephosphorylation on Tyr residues of the large subunit of Rubisco could impair the assembly of the enzyme. As the seedlings have not started their growth, this mechanism could allow the negative regulation of the activity of Rubisco.

Tyr Phosphorylation of Putative ABA Signaling Elements in Arabidopsis Seeds

We identified a PPIase, ROC4, that decreases and is dephosphorylated in ABA-treated seeds (Fig. 5C, spot R; Table I). Accordingly, Tyr phosphorylation of PPIases has been observed recently in Arabidopsis (Sugiyama et al., 2008). PPIases are ubiquitous enzymes involved in protein folding, translocation through biological membranes, and signal transduction (Shaw, 2002). The PPIase family consists of three subfamilies called FK506-binding proteins (FKBPs), cyclophilins, and parvulins (Dolinsky and Heitman, 1997). FKBP73 expression is induced by ABA in wheat (*Triticum aestivum*; Kurek et al., 2000), and cyclophilin expression is stimulated by abiotic stresses and ABA (Kong et al., 2001). Regulation of the PPIase activity by dephosphorylation has been suggested, as in two-hybrid experiments the Arabidopsis cyclophilin ROC7 interacts with RCN1, a PP2A-type protein phosphatase (Jackson and Soll, 1999). In plants, Pro cis-trans isomerization is emerging as a critical step in signaling cascades, as it is a basic molecular switch that may provide a mechanism for selecting distinct pools of binding partners and subsequently modulating signal transduction. An added level of regulation might be achieved by Tyr phosphorylation, which could regulate the PPIase activity of ROC4.

We also identified an RNA-binding protein predicted to be located in the chloroplast (Fig. 5C, spot K; Table I). According to the BLASTP analysis, this protein is related to the RNA-binding protein GRPA from maize encoded by a gene whose expression is induced by ABA and that can be phosphorylated (Gomez et al., 1988; Testi et al., 1996). Many genetic approaches have led to the identification of RNA-binding proteins that act in ABA signaling during germination and drought tolerance (Kuhn and Schroeder, 2003). In Arabidopsis, the expression of 10 genes encoding putative RNA-binding proteins is regulated by ABA (Raab et al., 2006). Several RNA-binding proteins have been shown to be Tyr phosphorylated in Arabidopsis (Sugiyama et al., 2008). Their phosphorylation status has been shown to impair RNA binding (Lisitsky and Schuster, 1995). Thus, in seeds imbibed with ABA, the Tyr dephosphorylation of the RNA-binding protein encoded by *At2g37220* might modify its RNA-binding activity and thereby regulate gene expression.

A nucleoside-diphosphate sugar epimerase, which markedly decreases and is slightly dephosphorylated on Tyr residues in response to ABA, has also been identified (Fig. 5C, spot J; Table I). It is related to a SUMO-conjugating enzyme that is a component of the sumoylation pathway in Arabidopsis (Kurepa et al., 2003). SUMO proteins are polypeptide tags that play a major role in various cellular processes by regulating protein-protein interactions and subcellular localization or by antagonizing ubiquitination (Vierstra and Callis, 1999). Two loci encoding related proteins, designated SUMO-Conjugating Enzymes (SCE) 1a and 1b, have been identified in Arabidopsis (Kurepa et al., 2003). Sumoylation has been implicated in ABA responses through the determinants AtSUMO1/2 and AtSCE1 (Lois et al., 2003). Overexpression of AtSUMO1 or AtSUMO2 reduced, and cosuppression of AtSCE1 enhanced, sensitivities to the ABA-mediated inhibition of root growth (Lois et al., 2003). Also, AtSUMO1/2 overexpression induced the expression of ABA-responsive genes *RD29A* and *AtPLC1* (Lois et al., 2003). Thus, sumoylation contributes to the regulation of ABA signaling. In animal cells, the SUMO E3 ligase Pc2 is phosphorylated by the protein kinase HIPK2 (Roscic et al., 2006). Regulation of the ABA signaling pathway in which sumoylation is implicated is possibly achieved by Tyr phosphorylation.

CONCLUSION

In conclusion, we have shown that Tyr phosphorylation events are involved in ABA signaling. Using specific inhibitors of PTPs and PTKs, we have demonstrated that these enzymes are elements of the ABA transduction pathways. We also have achieved a phosphoproteomic analysis that allowed us to identify proteins whose Tyr phosphorylation is modulated by ABA in seeds. Proteins that have previously been shown to play a role during germination, in particular during the initial mobilization of seed proteins and lipid reserves, have been identified. Some of these Tyr phosphorylated proteins are putative ABA signaling elements. The study of their putative signaling functions will be engaged using a reverse genetic approach. Finally, as all of these Tyr phosphorylated proteins are substrates of PTKs and PTPs that are regulated by ABA, an exciting challenge will be to identify these ABA-regulated PTKs and PTPs.

MATERIALS AND METHODS

Chemicals

(±)-cis-trans-ABA, NAP, genistein, daidzein, lavendustin A, PAO, and tyrphostins A23, A25, and AG490 were from Sigma-Aldrich. Erbstatin and

dephostatin were purchased from Calbiochem. The anti-phosphotyrosine antibody (clone PY20) was purchased from Sigma-Aldrich, and trypsin (Gold MS grade) was from Promega. ABA was added to Arabidopsis (*Arabidopsis thaliana*) suspension cells in DMSO to give a final concentration of 0.1%.

Arabidopsis Suspension Cells

Arabidopsis ecotype Columbia suspension cells were obtained as described by Axelos et al. (1992). Cells were cultured in 200 mL of culture medium (Jouanneau and Péaud-Lenoël, 1967), pH 6.8, in 500-mL Erlenmeyer flasks under continuous white light (40 μE m^{-2} s^{-1}) with 130 rpm orbital agitation at 24°C. The suspension was subcultured weekly, and all of the experiments were done with 4-d-old cells.

Stomatal Aperture Bioassays

Arabidopsis ecotype Columbia plants were grown on soil plus 1:3 vermiculite under short-day conditions (8 h of light at 22°C and 16 h of darkness at 20°C) for 6 weeks. Paradermal sections of abaxial epidermis were taken from leaves harvested at the end of the night. Epidermal peels were incubated at 20°C for 3 h in a stomata-opening medium (20 mM KCl, 10 mM iminodiacetic acid, and 10 mM MES-KOH, pH 6.25) in the light to allow stomata to open completely. Then, the epidermal peels were incubated at 20°C in the light for 3 h in the same medium with ABA (10 μM) or 0.1% DMSO (control). PAO and genistein were added 30 min before the addition of ABA. Stomatal apertures were measured with an optical microscope (Microphot-FXA; Nikon) fitted with a camera (Digital Sight DS-L1; Nikon).

RAB18-Responsive Test and Northern-Blot Analysis

Suspension cells (in 5-mL aliquots) were incubated for 3 h in normal culture conditions. PTK and PTP inhibitors dissolved in DMSO were added with ABA. The viability of the cells during the 3-h incubation with inhibitors was systematically checked with Trypan blue (data not shown). Northern blots were hybridized with a 700-bp *RAB18* cDNA probe labeled with [α-^{32}P] dCTP by random priming (Ready To Go kit; GE Healthcare) according to the protocol described previously (Jeannette et al., 1999). The *RAB18* cDNA probe (GenBank accession no. X68042) contained the coding sequence (minus the first 100 bp of 5′ sequence after the ATG codon) and the 3′ noncoding sequence ending with the polyadenylation site of the gene. Ethidium bromide staining was used to check that equal amounts of RNA were loaded in each well. All experiments were performed at least in triplicate.

Seed Protein Extraction

Arabidopsis ecotype Columbia dry seeds (at least 200 seeds) were sown on plates on moistened filter paper and kept for 2 d in the dark at 4°C. Then, seeds were placed on 10 μM ABA (or 0.1% DMSO for the control) in short-day conditions (8 h of light at 22°C and 16 h of darkness at 20°C) for 2 d. Thus, studies were restricted to the germination sensu stricto phase. For the pharmacological studies, the Arabidopsis dry seeds were sown on moistened filter paper imbibed with 100 μM genistein. After 2 d in the dark at 4°C, seeds were imbibed with both 10 μM ABA and 100 μM genistein (or 0.1% DMSO and 100 μM genistein for the control) in short-day conditions for 2 d.

Seeds collected from two independent plates were ground in liquid nitrogen in a mortar. The fine powder obtained was suspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 10 mM NaF, 1 mM dithiothreitol [DTT], 10 mM α-glycerophosphate, 0.2 mM Na_3VO_4, 0.1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 8,000*g* for 30 min at 4°C. Aliquots of the supernatant were frozen in liquid nitrogen and then stored at −30°C. Protein concentrations were estimated using the Bradford assay (Bradford, 1976).

2D Gel Electrophoresis and Protein Staining

For 2D gel electrophoresis, samples containing 1.25 mg of total proteins were precipitated with an equal volume of 10% TCA for 30 min at 4°C and washed with acetone and ethanol at −20°C. The pellet was resuspended in rehydration buffer (Destreak solution; GE Healthcare) with 0.5% ampholines (GE Healthcare). Passive rehydration was carried out on 18-cm pH 3 to 11 nonlinear immobilized pH gradient strips (GE Healthcare) at 25°C for 12 h. Isoelectric focusing using the Multiphor system (GE Healthcare) was performed at 18.5°C successively at 150 V for 1 h, 300 V for 1 h, 600 V for 1 h, 1,200 V for 1 h, 2,400 V for 1 h, and 3,500 V for 4 h. The strips were then equilibrated for 15 min in a buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 10 mg mL^{-1} DTT and then washed for 15 min with the same buffer without DTT but with 25 mg mL^{-1} iodoacetamide. For the second dimension, the strips were loaded onto 10% polyacrylamide gels and were run at 25 mA per gel for 4 h. Two gels were run in parallel (Protean II; Bio-Rad). The gels were stained with Coomassie Brilliant Blue (G-250; Bio-Rad) and scanned with an Epson Perfection 4990 photo scanner.

Western-Blot Analysis

After 2D gel electrophoresis, proteins were transferred to nitrocellulose membranes using a semidry transfer unit (SemiPhor; Hoefer). The transfer was carried out in 25 mM Tris-HCl, pH 9.2, 192 mM Gly, 0.1% SDS, and 10% methanol at 350 mA for 1 h. For the detection of Tyr phosphorylated proteins, the membranes were saturated in TBS buffer (10 mM Tris-HCl, pH 7.5, and 100 mM NaCl) supplemented with 0.1% Tween 20 (TBST) and 3% bovine serum albumin for 12 h. The membrane was incubated with an anti-phosphotyrosine antibody conjugated to peroxidase (clone PY20; Sigma) for 2 h and washed twice in TBST buffer for 30 min each and once in TBS buffer for 30 min. The blot was developed using the ECL Plus Western Blotting System (GE Healthcare). The films were scanned with an Epson Perfection 4990 photo scanner. Analysis of the scans with Image Master 2D Platinum software (GE Healthcare) included gel cropping, anchor spot selection, alignment, subtraction of the background, and spot volume normalization to allow for differences in staining intensities.

Peptide Mass Mapping and Sequencing

After Image Master 2D Platinum analysis, the protein spots of interest were excised from the two Coomassie Brilliant Blue gels (control or ABA) with a sterile pipette tip, and each gel plug was placed in a separate tube. Gel plugs were washed twice with 100 μL of 50% acetonitrile to remove the Coomassie Brilliant Blue stain and then dehydrated using a SpeedVac. The gel plugs were rehydrated in 100 mM NH_4HCO_3 containing 10 mM DTT and 0.2% EDTA (15 μL) for 30 min at 56°C. The gel plugs were then incubated in 100 mM NH_4HCO_3 containing 50 mM iodoacetamide (50 μL) for 30 min in the dark at 20°C. Plugs were washed twice in 100 mM NH_4HCO_3 (200 μL), treated with 200 μL of acetonitrile, and dehydrated using a SpeedVac. Finally, samples were rehydrated in 5 μL of porcine modified trypsin (Gold MS grade; Promega) solution at 12.5 ng μL^{-1} in 50 mM NH_4HCO_3 for 15 min at 37°C. For protein digestion and passive peptide extraction, 50 mM NH_4HCO_3 (15 μL) was added and the samples were incubated at 37°C for 16 h. Tryptic peptides were extracted twice in acetonitrile:5% aqueous trifluoroacetic acid (TFA; 1:1, v/v). The extract was dehydrated in the SpeedVac, and the peptides were solubilized in 0.1% aqueous TFA (10 μL). A small aliquot (1 μL) of each extract was eluted in 1 μL of α-cyano-4-hydroxycinnamic acid (10 mg mL^{-1}; Sigma-Aldrich) in acetonitrile:0.1% aqueous TFA (1:1, v/v), then spotted onto an Applied Biosystems 4700 target plate and allowed to dry.

Peptide mass maps and partial peptide sequences of tryptic peptides were generated by MALDI-TOF-TOF using an Applied Biosystems 4700 Analyzer. All MALDI-TOF spectra were obtained in positive reflection mode with a mass range of 700 to 4,000 mass-to-charge ratio. All samples were irradiated with UV light (355 nm) from an Nd:Y YAG AG laser with a repetition rate of 200 Hz; 5,000 and 10,000 laser shots were acquired and averaged into MS spectra and MS/MS spectra, respectively. The samples were analyzed at 20 kV of source acceleration voltage with two-stage reflection in MS mode. In the MS/MS experiment, collision energy was set to 1 kV (N_2 gas pressure, 5×10^{-7} T). MS spectra were externally calibrated with the 4700 standard peptide mixture of des-Arg-1-bradykinin, angiotensin I, Glu-1-fibrinopeptide B, adrenocorticotropic hormone (ACTH) clip 1 to 17, ACTH clip 18 to 39, and ACTH clip 7 to 38 (Applied Biosystems). For MS/MS spectra, the peaks were calibrated using the fragment of the protonated angiotensin I molecule. At least two peptides with the highest abundance in the MS spectrum were selected and studied by MALDI-TOF-TOF.

Database Searching and Protein Identification

MALDI-MS- and MALDI-MS/MS-generated peak lists and sequence data were submitted to the database search program MASCOT (Matrix Science)

and compared with known proteins in three different databases. We used the National Center for Biotechnology Information (NCBI) nonredundant Arabidopsis database, the MSDB database, and the Uniprot database. Parameters for protein identification included a mass tolerance of 75 ppm, a maximum of two missed trypsin cleavages per peptide, and the acceptance of carbamidomethylation of Cys residues and oxidation of Met residues. Positive identifications depended on a MOWSE score (M_r search algorithm) of around 60, the location of missed cleavages, and whether significant peaks in the MS spectra were those used for the analysis. Finally, both the M_r and pI of each database-matched protein were compared with those of the sample by referring to its position on the 2D gels. The putative sequences from the MS/MS analysis were subjected to BLAST searches of NCBI databases to identify protein function. A peptide charge state of +1, fragment mass tolerance of 75 ppm, and MS/MS tolerance of 0.15 D were used for the MS/MS ion search. For MASCOT MS/MS ion searches, the identified proteins had to be in the top hit with more than two peptide sequences matched in the NCBI BLAST search. A matched protein was accepted if it ranked as a hit with a single-peptide match.

We also used the Netphos Web server (http://cbs.dtu.dk/services/NetPhos) to predict potential Tyr phosphorylation sites for the proteins identified. We entered the identified protein sequence in FAST format for prediction of phosphorylation sites at Tyr, Ser, and Thr residues.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g03880, At5g44120, At4g28520, At2g05710, At2g36530, At4g38970, At5g02240, At2g37220, At3g62030, AtCg00490, and At1g05510.

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