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Original Paper

Isolation of *Arabidopsis* mutants that have altered sensitivity to staurosporine and ABA

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Abstract We have developed a sensitive system to investigate the possible roles of protein phosphorylation in ABA signaling in *Arabidopsis*. We used staurosporine, a protein kinase inhibitor, to suppress the inhibitory effects of ABA on seed germination and the opening/greening of cotyledons. Using staurosporine, we performed a large genetic screen for *asa* (*altered sensitivity to staurosporine and ABA*) mutants and identified novel weak alleles of *abi4* and *abi5* mutants as *asa1* and *asa2* mutants. This method will be useful in isolating weak alleles of mutants and in selecting mutants with altered sensitivity to chemical compounds.

Key words: ABA, liquid culture, phosphorylation, protein kinase, staurosporine.

The plant hormone abscisic acid (ABA) plays important roles in seed development and stress tolerance (Assmann 2003; Finkelstein et al. 2002; Yamaguchi-Shinozaki and Shinozaki 2005). Mutations that decrease sensitivity to ABA have been chiefly identified by screening seedlings on solid medium supplemented with ABA (Finkelstein 1994; Koornneef et al. 1984). This system usually requires a relatively high ABA concentration. For example, the ABA-insensitive (*abi*) mutants *abi1-1*, *abi2-1* and *abi3-1* were screened on solid medium supplemented with 10 μM ABA (Koornneef et al. 1984). The relatively weaker *abi4-1*, *abi5-1* and *abi5-2* mutants were identified using 3 μM ABA (Finkelstein 1994). Protein phosphorylation has been shown to be involved in ABA signaling (Assmann 2003). Two dominant mutations, *abi1-1* and *abi2-1*, result in the ABA-insensitive phenotypes. *ABI1* and *ABI2* encode PP2C-type protein phosphatases (Leung et al. 1994, 1997; Meyer et al. 1994). Some ABA-controlled processes are affected by the application of protein kinase and phosphatase inhibitors (Armstrong et al. 1995; Schmidt et al. 1995). OPEN STOMATA1 (OST1), a serine/threonine protein kinase, is involved in controlling the opening and closing of guard cells (Mustilli et al. 2002). The gene was identified as *SRK2E* by a combination of biochemical analysis and reverse genetics (Yoshida et al. 2002). The protein kinase activity of OST1/SRK2E is activated by ABA and low humidity (Yoshida et al. 2002).

However, the substrates for these ABA signaling protein kinases and phosphatases are not yet known. To grasp the molecular mechanisms, a sensitive method for screening *Arabidopsis* mutants with altered sensitivity to ABA is needed. We tested whether protein kinase inhibitors affect ABA signaling in the early developmental stages of *Arabidopsis*. We developed a novel selection method based on a liquid culture system. Our results show that staurosporine, a protein kinase inhibitor, may affect the ABA signaling cascade of *Arabidopsis*. Our system will be useful in isolating weak alleles of mutations in this pathway.

Materials and methods

Plant materials and growth conditions

The L*er*, Col and Ws ecotypes of *Arabidopsis* were used. *abi1-1*, *abi2-1*, *abi3-1*, *abi4-1* and *abi5-1* were described previously (Finkelstein 1994; Koornneef et al. 1984). *Arabidopsis* seeds were surface-sterilized and grown hydroponically in 60-mm suspension culture dishes (430589; Corning, NY, USA) with 5 ml MS liquid medium supplemented with or without ABA (A1049; Sigma, MI, USA) and staurosporine (569397; Calbiochem, CA, USA). K252a (420298; Calbiochem)

Abbreviations: ABA, abscisic acid; *abi*, *abscisic acid insensitive*; *asa*, *altered sensitivity to staurosporine and ABA*.

was also used in place of staurosporine. The culture dishes were gently shaken with a rotary shaker (NR-20; Taitec, Saitama, Japan) under LD (16 h light/8 h dark) conditions for 7 days. For screening of the *asa* mutants, T-DNA insertional lines (CS5452, CS5601, CS5626; ABRC), fast-neutron- and EMS-mutated M2 seeds (Lehle Seeds, Round Rock, USA) were selected in MS liquid medium supplemented with 500 nM ABA and 10 nM staurosporine. CS5390 in Ws-4 background was used as a control for the *asa1-asa5* mutants obtained from the T-DNA insertional lines.

Quantitative RT-PCR (Q-PCR) analysis of ABI4 *expression*

After DNase treatment, cDNA was produced from oligo (dT_{15}) primer using the SuperScript First-Strand Synthesis System (Invirtogen). The cDNA was used as a template for PCR amplification with each specific primer set.

Q-PCR amplification was performed using the Lightcycler FastStart DNA Master SYBR Green I (Roche Diagnostics) with each gene specific primer set. The reaction was performed as followed: an initial denaturation step (95°C for 10 min), followed by 40 cycles of denaturation (94°C for 10 s), annealing (55°C for 10 s), and extension (72°C for 10–22 s). Fluorescence data was acquired at the end of each extension cycle. A melting curve was carried out as follows: 95°C for 0 s, 65°C for 15 s, followed by a temperature increase of 0.1°C/s to 95°C for 0 s. Fluorescence was measured continually during this melting curve cycle. The temperature transition rate was 20°C/s in all cases, except for extension (2°C/s). Fluorescence signal of each sample was normalized with that of *Ubiqutin* (*UBQ*). Expression analysis of *ABI4* was preformed with Q-PCR from imbibed seeds after 20hr. Primers used were as follows: Ubi-F 5′-GTACTTTGGCGGATTACAACATC, Ubi-R 5′-GAATACCTCCTTGTCCTGGATCT, ABI4-F 5′-ACCGACCTTAGGGATGCTCATCGTA and ABI4-R 5′-GGAACATCAGTGAGCTCGATGTC.

Results

Antagonistic effects of staurosporine and ABA on seed germination and seedling growth of Arabidopsis

To examine whether phosphorylation/dephosphorylation processes play important roles in *Arabidopsis* ABA signaling, we tested the germination of L*er* and Col wild-type seeds in MS medium supplemented with ABA and a variety of protein kinase inhibitors. The greening and expansion of cotyledons and the initiation of true leaf development were normal when wild-type plants were grown on standard culture medium with MS salts (Figure 1A). However, these processes were greatly

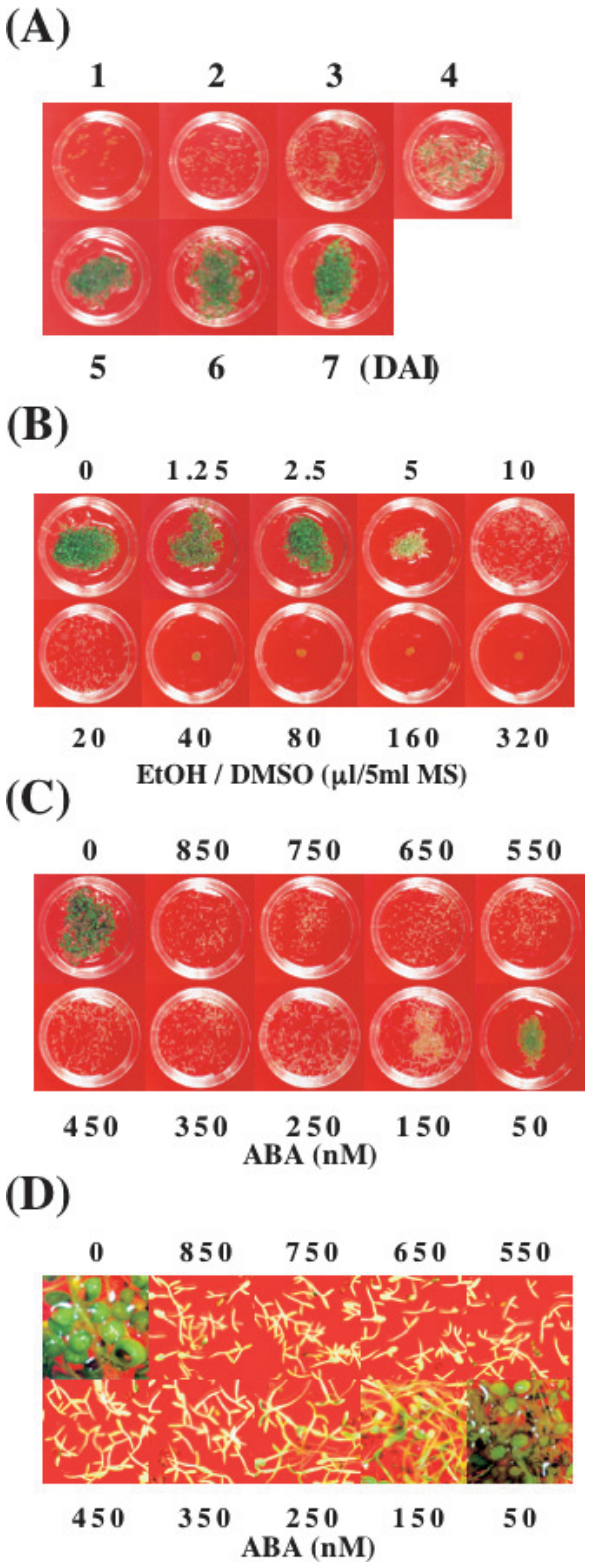

Figure 1. ABA inhibits seed germination and seedling growth of *Arabidopsis* in a liquid culture system. (A) Seed germination and seedling growth of *Arabidopsis* in a liquid culture system. Numbers indicate days after imbibition (DAI). (B) Low concentrations of ethanol (EtOH) and DMSO did not affect seed germination or seedling growth in the liquid culture system. Numbers indicate volumes of ethanol and DMSO in 5 ml MS medium. (C, D) ABA affected greening/opening of cotyledons and root growth. Numbers indicate the concentrations of ABA (nM).

altered with the addition of more than 500 nM ABA to the medium (Figure 1C, D). Under this condition, we screened for kinase inhibitors that could overcome the developmental arrest normally induced by ABA. Staurosporine partially suppressed the growth inhibition of Col (Figure 2A, C) and L*er* (Figure 2B) wild-type plants. By contrast, K-252a, a structural analog of staurosporine, enhanced the growth inhibition of ABA (Figure 3A). Other kinase inhibitors, such as Apigenin, PD98059, SB202190, SB203580, PD169316 (MAPK and MAPKK inhibitors; Calbiochem), did not affect growth inhibition (data not shown). ABA and staurosporine were usually dissolved in ethanol and DMSO, respectively. Less than 2.5 μl of ethanol or DMSO or both in 5 ml culture medium (v/v) did not affect the growth of the wild-type plants (Figure 1B). Therefore, we used 2 μl of ethanol or DMSO or both in all our experiments.

To further test the involvement of the staurosporine-sensitive step in ABA signaling, we analyzed the staurosporine sensitivity of three classes of ABA-insensitive mutants: *abi1-1*, *abi2-1* and *abi3-1* (Koornneef et al. 1984). As reported previously, all were less sensitive to ABA than were the wild-type plants (Figures 2B, 3B–D). Higher concentrations of staurosporine affected plant growth (Figure 2A, B, 3B–D). *abi3-1* was more sensitive to staurosporine than was L*er* (Figures 2B, 3D). The addition of 1000 nM staurosporine greatly affected the growth of *abi3* seedlings (Figure 3D). The sensitivity of other mutants was similar to that of L*er* (Figures 2B, 3B, C). These results suggest that staurosporine-sensitive molecule(s) may play pivotal roles in ABA signaling (Figure 4A).

Isolation of Arabidopsis *mutants that have altered sensitivity to staurosporine and ABA*

Taking advantage of the striking suppression by staurosporine of ABA-induced growth inhibition, we screened for *Arabidopsis* mutants displaying staurosporine hypersensitivity (Figure 4A). Altogether, 81 plants from ethyl methanesulfonate- (EMS; e.g., E2912, E3803 and E3908 in Figure 4B) or fast-neutron-mutagenized M2 seeds or from T-DNA insertion lines (e.g., T2, T10 and T19 in Figure 4B) were screened for growth in MS medium supplemented with 500 nM ABA and 10 nM staurosporine using the liquid culture system (Table 1). The greening and expansion of cotyledons and the initiation of true leaf development were blocked in Col, L*er* and Ws wild-type seedlings. Initially, we expected to isolate mutants with higher sensitivity to staurosporine (Figures 4A, 6, 7; gray bars).

Genetic analysis of asa *mutants*

Thirty mutants from four independent parental groups of the T-DNA insertion lines were further analyzed. T2 and

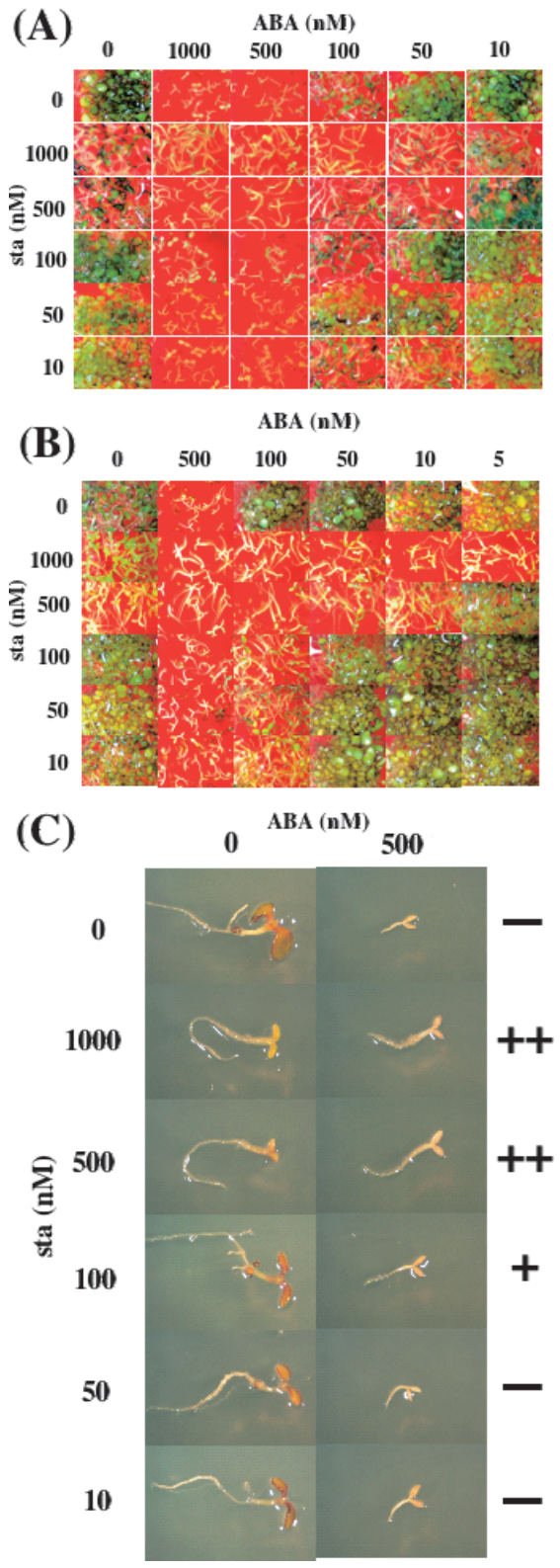

Figure 2. ABA inhibits germination, greening/opening of cotyledons and root growth. These inhibitions are partially suppressed by staurosporine, a protein kinase inhibitor. Staurosporine blocked ABA-induced growth inhibitions of *Arabidopsis* seedlings. Col (A, C) and L*er* (B) wild-types were grown hydroponically for 7 days with or without ABA and staurosporine. Numbers indicate the concentrations of ABA and staurosporine (nM). Strong, weak and no suppression of the ABA-dependent inhibition of root growth by staurosporine are represented by ++, + and −, respectively in (C).

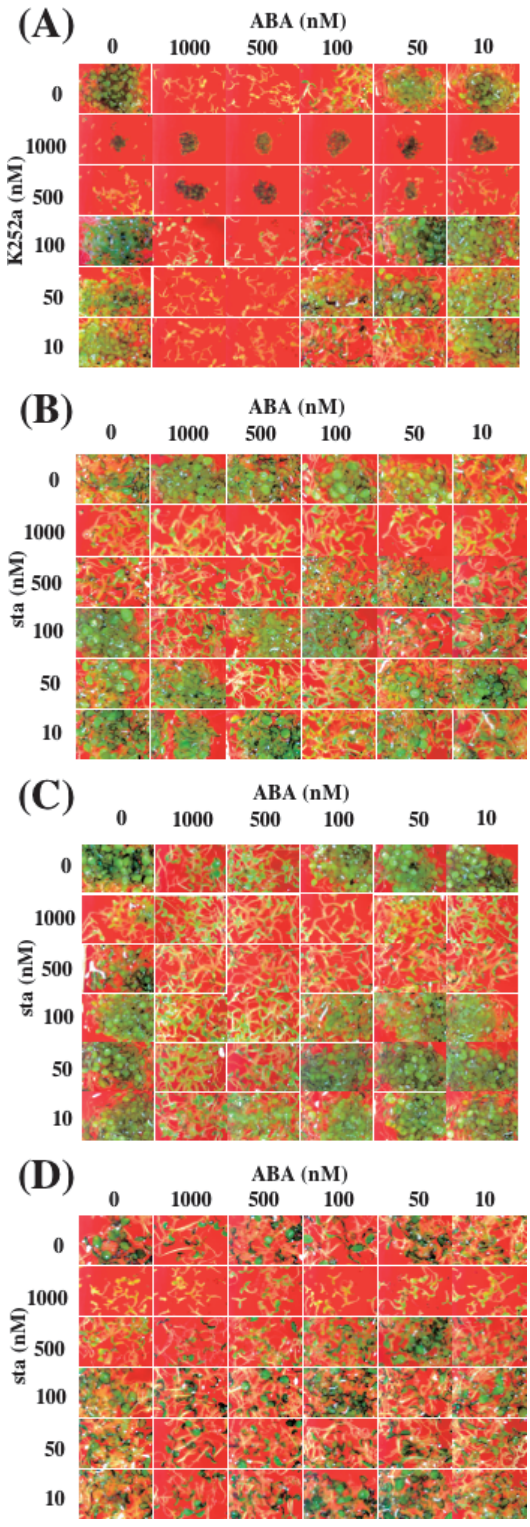

Figure 3. *abi* mutants are ABA-insensitive and *abi3-1* is more sensitive to staurosporine. Staurosporine blocked ABA-induced growth inhibitions of *Arabidopsis* seedlings (Figure 2), but K252a enhanced the inhibition of seed germination by ABA (A). *abi1-1* (L*er*; B), *abi2-1* (L*er*; C), *abi3-1* (L*er*; D) were grown hydroponically for 7 days with or without ABA, staurosporine and K252a. Numbers indicate the concentrations of ABA, staurosporine and K252a (nM).

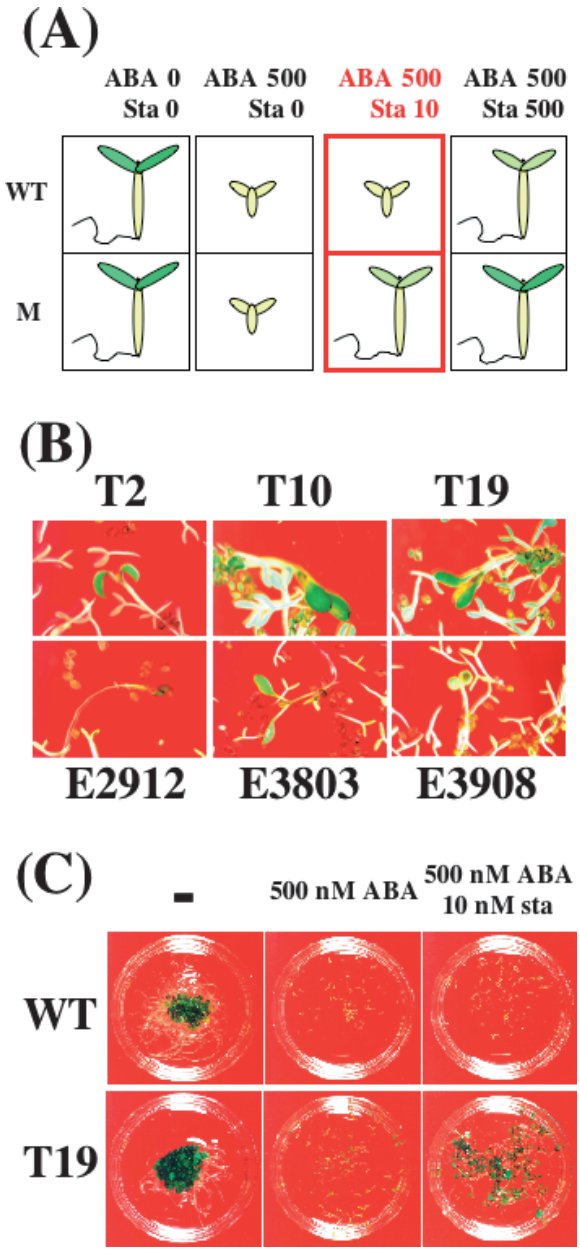

Figure 4. Screening of mutants with altered sensitivity to staurosporine and ABA. (A) Mutants with higher sensitivity to staurosporine (Sta). Root elongation and greening/opening of cotyledons of wild-type plants were inhibited by 500 nM ABA (Figure 1). High concentration of staurosporine (500 nM) partially suppressed the inhibition by ABA, but low concentration of staurosporine (10 nM) did not (Figure 2). In our screening, *Arabidopsis* mutants that grew in a liquid medium supplemented with 500 nM ABA and 10 nM staurosporine shown in red were selected. (B) The first screening of *asa* mutants from the seed collections of T-DNA tagged lines (T2, T10 and T19) and EMS-mutagenized Col seeds (E2912, E3803 and E3908). Plants were grown hydroponically in MS liquid medium supplemented with 500 nM ABA and 10 nM staurosporine. (C) The *asa* mutant phenotype was heritable. The next generation of the T19 line shown in (B) was grown in MS medium with or without 500 nM ABA and 10 nM staurosporine.

T8, T10 and T15, T18, T19, and T24 were obtained from the four parental groups, and these seven mutants were used for further analysis. The mutants were backcrossed to Ws wild-type plants. Genetic analysis of the F1 progeny showed that all the mutations except T24 were recessive (Table 2). The F2 progeny resulting from the backcrosses showed approximately a 3 : 1 segregation ratio (T2, T8, T10, T15, T18 and T19) and a 1 : 3 ratio (T24) of wild type to mutant (chi-square test, $P>0.05$; Table 2). Therefore, all the mutants were caused by single nuclear mutations. Allelism tests showed that the mutants fell into at least three complementation groups: group 1 (T2), group 2 (T10, T15 and T19) and group 3 (others) (Table 3). The *asa* phenotype of T24 behaved as dominant, and T24 was obtained from different pools than those of the three complementation groups. These results suggest that *asa* mutations occur at least in five distinct genes in *Arabidopsis*. Therefore T19, T15, T10, T2, T8, T24 and T18 were named *asa1-1*, *asa1-2*, *asa1-3*, *asa2-1*, *asa3-1*, *asa4-1* and *asa5-1*, respectively.

Because the *abi* mutants (*abi1-1*, *abi2-1*, *abi3-1*, *abi4-1*, and *abi5-1*) also grew under this condition (data not shown), we tested the effect of a low concentration of staurosporine on the growth of isolated mutant plants. The ABA-induced growth inhibition of the *asa1-1* (Figure 4C), *asa1-2*, *asa2-1*, *asa3-1* and *asa5-1* mutants (data not shown) was suppressed by 50 or 10 nM staurosporine. *asa4-1* grew slowly in medium supplemented with 500 nM ABA, but this growth inhibition was largely suppressed when 10 nM staurosporine was added (data not shown).

Table 1. Summary of *asa* mutants.

	Independent parental groups	Isolated lines (2nd screening)
T-DNA	4	30
EMS	7	36
Fast N	8	15
total	19	81

For screening of the *asa* mutants, T-DNA insertional lines (T-DNA), fast-neutron (Fast N)- and EMS-mutated M2 seeds were selected in MS liquid medium supplemented with 500 nM ABA and 10 nM staurosporine.

Identification of ASA1 *and* ASA2 *genes*

Allelism tests showed that both *asa1-1* (T19) and *asa1-2* (T15) were allelic to *abi4-1* (Table 3). Therefore we compared sequences of the *ABI4* gene of two *asa* mutants with those of Ws wild-type plants: *asa1-1* had a T-DNA insertion in the *ABI4* promoter 2100 bp upstream of the initiation codon (ATG) (Figure 5A), while *asa1-2* had a complicated mutation (rearrangement) in the end of the exon and the 3′-non coding region (Figure 5A). Both mutations substantially decreased *ABI4* mRNA levels (Figure 5B). The mutations were found to be new alleles of *abi4*; therefore *asa1-1* and *asa1-2* were renamed *abi4-201* and *abi4-202* (Figure 5A).

Allelism tests showed that *asa2-1* was allelic to *abi5-*

Table 2. Segregation of *asa* mutations[a].

Female	Male	sta (nM)	S[b]	R[b]	X^{2c}
Ws	T2	10	64	16	1.07[d]
Ws	T8	10	45	13	0.21[d]
Ws	T10	10	74	18	1.45[d]
Ws	T15	10	65	20	0.10[d]
Ws	T18	10	62	7	8.12
Ws	T18	50	60	19	0.04[d]
Ws	T19	10	66	21	0.03[d]
Ws	T24	10	47	120	0.08[d]
Ws	Ws	10	73	0	ND[e]
Ws	Ws	50	60	0	ND[e]

[a] The *asa* mutants were backcrossed to Ws wild-type plants and F2 generation of the crosses was tested for their growth in MS liquid medium supplemented with 500 nM ABA and 10 or 50 nM staurosporine (sta).

[b] R (resistant) and S (sensitive) indicate the F1 seedlings grew or did not grow in MS liquid medium supplemented with 500 nM ABA and 10 nM staurosporine.

[c] The X^2 is given for the ratio of 3 : 1 or 1 : 3 (wild-type/mutant) for T2, T8, T10, T15, T18 and T19, or T24, respectively.

[d] Not significant at $P=0.05$.

[e] Not determined.

Table 3. Summary of complementation tests.

	T2	T8	T10	T15	T18	T19	T24	*abi3*	*abi4*	*abi5*	Col	Ws	L*er*
T2		S	S	S	S	S		S	S	R	S	S	S
T8	S					S							
T10	S					R							
T15	S					R							
T18	S					S							
T19	S	S	R	R	S	R		S	R	S	S	S	S
abi1	R					R							
abi2	R					R							
abi3	S					S							
abi4	S					R							
abi5	R					S						S	
Col	S					S							
Ws	S	S		S	S	S	R	S	S	S			S

R (resistant) and S (sensitive) indicate the F1 seedlings grew or did not in MS liquid medium supplemented with 500 nM ABA and 10 nM staurosporine.

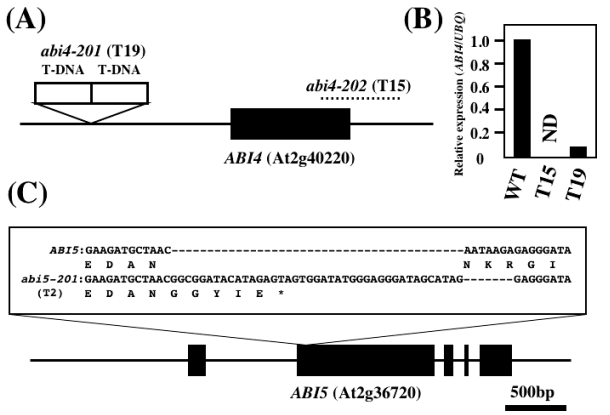

Figure 5. *asa1* and *asa2* are novel alleles of *abi4* and *abi5*, respectively. (A) *asa1-1* (T19) and *asa1-2* (T15) are novel alleles of *abi4* and were renamed *abi4-201* and *abi4-202*, respectively. Two T-DNAs are inserted in the *ABI4* promoter at −2100 in *abi4-201*/*asa1-1*. The end of the exon and the 3′- non coding region of the *ABI4* gene (At2g40220) are rearranged in *abi4-202*/*asa1-2*. (B) The *ABI4* mRNA levels were lower in T15 (*abi4-202*/*asa1-2*) and T19 (*abi4-201*/*asa1-1*). ND indicates "not detectable." (C) *asa2-1* (T2) is a novel allele of *abi5* and was renamed *abi5-201*. *abi5-201* has a 41-bp insertion and 7-bp deletion at the beginning of the second exon of the *ABI5* gene (At2g36720).

1. Therefore we compared sequences of the *ABI5* gene of the *asa2-1* mutant with those of Ws wild-type plants. The *asa2-1* had a 7-bp deletion and a 41-bp addition in the beginning of the second exon of the *ABI5* gene (Figure 5C). This mutation, which causes a premature stop codon and a truncation of the protein, was a new allele of *abi5*; therefore *asa2-1* was renamed *abi5-201*.

Discussion

Staurosporine is a specific inhibitor of protein kinase C (PKC) in animal cells, and a loss-of-function mutant of *pkc* in yeast cells is hyper-sensitive to staurosporine (Levin and Bartlett-Heubusch 1992). Staurosporine inhibits the biosynthesis of cell walls in *S. cereviciae* and therefore the yeast cells do not grow on the solidified medium supplemented with staurosporine. A MAP kinase kinase kinase, BCK1, functions as a downstream factor for the PKC in *S. cereviciae*. Loss-of-function of *bck1* was also recovered as a mutant with higher sensitivity to staurosporine. These results suggest that not only the direct targets of the inhibitor but also downstream factors of them can be screened as mutants with increased sensitivity to the inhibitor. We expected that mutations in the genes encoding protein kinase(s) sensitive to staurosporine and downstream factors of the protein kinases in ABA signaling could be recovered in our screening (Figure 6). In our system, staurosporine appears to decrease the ABA sensitivity of *Arabidopsis* plants, suggesting that staurosporine might inhibit the possible kinase(s) directly or indirectly involved in ABA signaling. We did not identify protein kinase gene

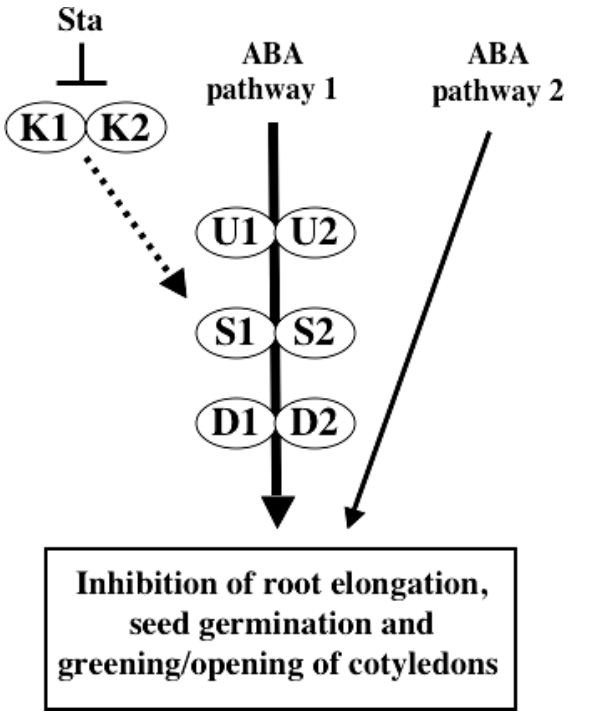

Figure 6. Hypothetical model on protein kinases as potential targets of staurosporine in the ABA signaling. More than two independent ABA pathways are thought to control the root elongation, seed germination and greening/opening of cotyledons. Staurosporine (Sta) is an inhibitor of protein kinases and partially suppressed the inhibition of root elongation and greening/opening of cotyledons by ABA. In this model, two protein kinases with redundant functions (K1 and K2) have positive roles in the ABA pathway 1 and inhibited by staurosporine. Loss-of-function of the kinases may result in decrease the activity of the ABA pathway 1 to inhibit the root elongation, seed germination and greening/opening of cotyledons. Two protein kinases (K1 and K2) phosphorylate and activate the two substrate proteins with redundant functions (S1 and S2). Upstream factors (U1 and U2) and downstream factors (D1 and D2) of the S1 and S2 are also involved in the ABA pathway. The screening shown in this work is expected to recover mutations in all of the factors (K, U, S and D) shown here.

mutations in the screening, perhaps because the screening was not saturated. Alternatively, if *Arabidopsis* has genes with redundant ABA signaling functions, a monogenic mutation might not display the phenotype at our staurosporine concentration. A larger-scale screening with our system would identify these protein kinase gene mutations.

Genetic analysis indicates that *asa4-1* (T24) is dominant mutation (Table 2). Only two dominant alleles, *abi1-1* and *abi2-1*, have been reported for the ABA-insensitive mutants. *ABI1* and *ABI2* encode closely related PP2C proteins. Both of *abi1-1* and *abi2-1* have exactly same amino acid substitution at same position. This type of mutation appears quite rare. The *abi* phenotype of *asa4-1* is much weaker than those of *abi1-1* and *abi2-1* (data not shown), suggesting that *asa4* might be different from *abi1* and *abi2*.

Figure 7 shows a hypothetical model based on our results. At least two independent pathways have been

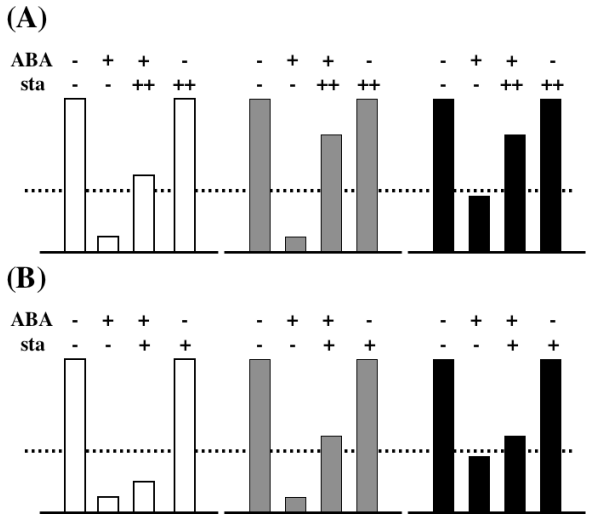

Figure 7. Hypothetical models to explain the *asa* phenotypes. White and black bars indicate wild-type plants and *asa* mutants, such as *asa1* and *asa2*. Gray bars indicate a putative mutant with hypersensitivity to staurosporine and normal sensitivity to ABA. + and − for ABA (A, B) indicate with or without 500 nM ABA, respectively. ++ (A), + (B) and − (A, B) for staurosporine (sta) indicate a high concentration (500 nM), low concentration (10 nM) or no staurosporine, respectively. Dashed lines indicate the threshold levels of activity for greening/opening of cotyledons and root growth. ABA inhibits greening/opening of cotyledons and root growth in wild-type seedlings (white bars). The high, but not the low, concentration of staurosporine suppressed the growth inhibitions. The *asa* mutants (black bars) have weak, but still significant, *abi* phenotypes. Therefore, the basal activity for greening/opening of cotyledons and root growth may be higher than that of wild-type seedlings. The low concentration of staurosporine may be sufficient for greening/opening of cotyledons and root growth even in the presence of ABA. Initially, we looked for mutants shown by the gray bars. Sensitivity to staurosporine and ABA may be highly related because *abi3* has an increased sensitivity to staurosporine (Figure 3D).

proposed for ABA signaling based on phenotypes of double mutants: the ABI1/ABI2 and ABI3/ABI4/ABI5 pathways (Finkelstein 1994). In our test, *abi3-1* showed increased sensitivity to staurosporine (Figure 3D), but *abi1-1* and *abi2-1* appeared similar to the wild-type control (Figures 2A, B, 3B, C). Two novel alleles of *abi4* and one of *abi5* were identified in our screening. These mutants grew better in 500-nM ABA than did the wild-type plants, even without 10 nM staurosporine (Figure 4C). Thus, *asa1* and *asa2* may have weak, but still significant, *abi* phenotypes at low concentrations of ABA, although it takes longer time to see clear phenotypes than those of strong alleles of the *abi* mutants. These results suggest that the method shown here will be useful in isolating weak alleles of mutants and in selecting mutants with altered sensitivity to chemical compounds.

Our results suggest that the staurosporine-sensitive step(s) may be in the ABI3/ABI4/ABI5 pathway. ABI5 has been shown to be phosphorylated by protein kinase (Lopez-Molina et al. 2001). ABI5 and closely related proteins in *Arabidopsis* and other plants may be direct substrates or downstream factors for the putative staurosporine-sensitive kinases involved in ABA signaling (Johnson et al. 2002; Uno et al. 2000).

The whole genome of *Arabidopsis* has been sequenced (*Arabidopsis* Genome Initiative 2000), and knockout mutants produced by T-DNA tagging are available for most *Arabidopsis* genes (Alonso et al. 2003; Pennisi 2004). A pronounced redundancy in the *Arabidopsis* genome is evident in segmental duplications and tandem arrays, and many other genes with high levels of sequence conservation are scattered over the genome (*Arabidopsis* Genome Initiative 2000). Thus, identifying monogenic mutations that cause severe effects on the plant's sensitivity to various conditions is difficult.

Various drugs can be powerful tools for identifying mutants with altered responses to the chemical compounds. Thus, the application of a drug targeting certain enzymes should decrease the sum of their activity. Under the proper conditions, it should be possible to identify even monogenic mutations whose phenotypes are visible only if enzymatic activity is very low.

Acknowledgments

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