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Research Area: Signaling and Response
Inter-tissue signal transfer of abscisic acid from vascular cells to guard cells

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One-sentence summary:
ABA biosynthetic enzymes and an ABA transporter expressed in vascular tissues induce stomatal closure, indicating ABA signal transfer from the vascular bundle is likely to be mediated by specific transporters.
Footnotes

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Abstract

Abscisic acid (ABA) is a phytohormone that responds to environmental stresses such as water deficiency. Recent studies have shown that ABA biosynthetic enzymes are expressed in the vascular area under both non-stressed and water-stressed growth conditions. However, specific cells in the vasculature involved in ABA biosynthesis have not been identified. Here, we detected the expression of two genes encoding ABA biosynthetic enzymes, ABA2 and AAO3, in phloem companion cells in vascular tissues. Furthermore, we identified an ATP-binding cassette transporter, AtABCG25, expressed in the same cells. Additionally, AtABCG25-expressing Sf9 culture cells showed an ABA efflux function. Finally, we observed that enhancement of ABA biosynthesis in phloem companion cells induced guard-cell responses, even under normal growth conditions. These results demonstrate that ABA is synthesized in specific cells and can be transported to target cells in different tissues.
Introduction

Hormones are chemical substances that exert a biochemical action on target cells at low concentrations. All multicellular organisms, including animals and plants, produce hormones to control their physiological status. In animals, many ordinary hormones are secreted from specific cells (such as endocrine cells) and transported to their target sites in other areas of the body. However, it remains unclear whether the concept of hormones as defined in animals is applicable to plants because plant hormones are not generally synthesized in specific cells but are broadly produced (Weyers and Paterson, 2001; Gaspar et al., 2003; Forestan and Vartotto, 2012).

Abscisic acid (ABA) is a key phytohormone that prevents water loss from the plant body by acting on guard cells, of which stomata (epidermal pores) compose the aerial organs in plants (Hetherington, 2001; Schroeder et al., 2001; Fan et al., 2004; Joshi-Saha et al., 2011). Gene and protein expression analyses using anti-sense RNA or antibodies specific for ABA biosynthetic enzymes in Arabidopsis have demonstrated that parenchyma cells in vascular bundles are the abundantly expression site of ABA biosynthesis under drought stress and well-watered growth conditions (Cheng et al., 2002; Koiwai et al., 2004; Endo et al., 2008). Because the vasculature is separated from guard cells, it has been suggested that ABA is transported from the site of synthesis to the site of action (Seo and Koshiba, 2011).

We previously found that an ATP-binding cassette (ABC) transporter family, AtABCG25, is expressed mainly in vascular tissues, and it is expected to function as an ABA exporter that transports ABA from inside to outside cells (Kuromori et al., 2010). According to this observation, we have proposed a working model: ABA is exported from ABA-synthesizing cells in vascular tissues by AtABCG25 to reach distant guard cells and induce stomatal closure (Kuromori and Shinozaki, 2010; Umezawa et al., 2010; Umezawa et al., 2011). However, which
parenchyma cells in vascular tissues express ABA biosynthetic enzymes or AtABCG25 transporting factor has not been identified.

Here, we explored whether specific cells express ABA biosynthetic enzymes or an ABA transporter, and found that their genes were expressed in phloem companion cells of vascular tissues. ABA synthesis in these cells enhances trans-signaling to distant guard cells of the epidermis. These results demonstrate that ABA is synthesized in specific cells and transported to target cells in another tissue. This is similar to endocrine hormones in animals and suggests that the ABA transport pathway between tissues in plants may be associated with specific transporters.

**Results and Discussion**

In *Arabidopsis*, the final two steps of ABA biosynthesis are enzymatic processes catalyzed by xanthoxin dehydrogenase, encoded by *ABA DEFICIENT 2* (*ABA2*), and abscisic aldehyde oxidase, encoded by *ABSCISIC ALDEHYDE OXIDASE 3* (*AAO3*) (Finkelstein and Rock, 2002; Schwartz et al., 2003; Nambara and Marion-Poll, 2005). To determine the location of ABA-biosynthesizing cells, approximately 0.9 kb of the *ABA2* promoter region and 1.2 kb of the *AAO3* promoter region, which covers the majority of intergenic regions adjacent to the coding genes, were cloned and used to drive expression of the nuclear-localized signal attached to Green Fluorescent Protein (nGFP) as a promoter-dependent reporter. Each of the recombinant vectors was transformed into *Arabidopsis*, and the green fluorescence signals were observed in transgenic plants.

It is difficult to observe fluorescent signals in the leaf vascular site of intact plants because of the strong auto-fluorescence by chloroplasts. Therefore, we examined various aerial tissues to detect clear signals. We observed an array of nuclear fluorescence of nGFP along the
vascular veins in the center of anther filaments in both transgenic plants expressing \textit{ABA2} promoter-driven nGFP and those expressing \textit{AAO3} promoter-driven nGFP using confocal laser scanning microscopy (Figure 1A and B). nGFP fluorescence was also observed close to (but not in) xylem vessels in sepals or petals in both transgenic plants (Figure 1E and F), although occasionally, it was detected in the sepal epidermis of the \textit{AAO3} promoter-driven transgenic plants. These results demonstrated that the expression patterns of the two promoters overlap in the vascular area. Furthermore, the expression of \textit{ABA2} and \textit{AAO3} is limited to particular cells and is not expressed broadly through every parenchyma cell in the vascular tissue. When observing belowground components, a similar pattern of fluorescence was detected in the root vasculature of \textit{ABA2} promoter-driven transformants (Figure 1I), although there were only faint signals in \textit{AAO3} promoter-driven transformants.

To identify cells expressing \textit{ABA2} and \textit{AAO3} in the vascular area, we first examined a publicly available database of a high-resolution microarray map in \textit{Arabidopsis} (Brady et al., 2007). Compared with vascular cell types, both \textit{ABA2} and \textit{AAO3} have expression patterns similar to \textit{SUCROSE-PROTON SYMPORTER 2 (SUC2)}, which is commonly used as a gene specifically expressed in phloem companion cells (Stadler and Sauer, 1996; Imlau et al., 1999). Based on this information, it is possible that the specific cells expressing \textit{ABA2} and \textit{AAO3} are phloem companion cells. Phloem companion cells are located adjacent to phloem sieve tubes, which form a line in the vascular tissues (Oparka and Turgeon, 1999). We generated transgenic plants to observe \textit{SUC2} promoter-driven nGFP and determined that the fluorescence in the transformants formed a clear array along the vasculature (Figure 1D, H, and K).

To confirm whether \textit{ABA2} or \textit{AAO3} promoter-driven fluorescent cells co-localize with \textit{SUC2} promoter-driven fluorescent cells, we generated transgenic plants expressing \textit{SUC2} promoter-driven Cyan Fluorescent Protein (CFP) and crossed them with the plants expressing
ABA2 promoter-driven nGFP or AAO3 promoter-driven nGFP. By visualizing two types of fluorescent proteins, we found that both ABA2 promoter-driven nGFP and AAO3 promoter-driven nGFP co-localized with SUC2 promoter-driven CFP (Figure 2A, B and D; Supplemental Figure S1). These results indicated that ABA2 and AAO3 are expressed in phloem companion cells.

Next, we investigated cells expressing AtABCG25. Approximately 2.0 kb of the AtABCG25 promoter region was used to detect promoter-dependent nGFP expression. A similar pattern of fluorescence for ABA2 and AAO3 was detected in aerial components of AtABCG25 promoter-driven transformants (Figure 1C and G). No clear signals were observed in roots, but ABA-treated plants showed similar signals (Figure 1J). We previously found that AtABCG25 expression was induced by ABA treatment (Kuromori et al., 2010). We then crossed transgenic plants expressing AtABCG25 promoter-driven nGFP with transgenic plants expressing SUC2 promoter-driven CFP. By visualizing the two fluorescent proteins, we found that AtABCG25 promoter-driven nGFP and SUC2 promoter-driven CFP co-localize (Figure 2C and E). These results indicated that (in addition to ABA2 and AAO3) AtABCG25 is predominantly expressed in phloem companion cells.

The co-localization of cells expressing ABA2, AAO3, and AtABCG25 supports our working model that AtABCG25 plays the role of an ABA exporter in ABA-biosynthesizing cells (Kuromori and Shinozaki, 2010; Umezawa et al., 2010; Umezawa et al., 2011). To assess ABA efflux activity of AtABCG25, we expressed AtABCG25 cDNA in cultured Sf9 insect cells. After adding ABA isotope to cell cultures, Sf9 insect cells expressing AtABCG25 contained lower radioactive counts of ABA isotopes remaining in cells than did control cells containing the empty vector (Figure 3A). This indicated that ABA excretion was more efficient in cells expressing AtABCG25 than in control cells. This count reduction was not detected when other
isotope-labeled phytophormones, indole-3-acetic acid (auxin), or jasmonic acid were added to cell cultures (Figure 3B and C), indicative of the specificity of the target molecule. These results support the conclusion that AtABCG25 is an ABA exporter with efflux activity from inside to outside cells.

ABA induces stomatal closure through its action on guard cells, which are located in the epidermis and separated from vascular tissue (Hetherington, 2001; Schroeder et al., 2001; Fan et al., 2004; Joshi-Saha et al., 2011). To investigate the inter-tissue signal transfer of ABA, we induced ABA biosynthesis in phloem companion cells. We generated transgenic plants expressing SUC2 promoter-driven NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3) because it enhances ABA biosynthesis (Iuchi et al., 2001). Transgenic plants expressing SUC2 promoter-driven NCED3 did not show any visible phenotypes (Figure 4A). However, when plants were observed using a thermographic camera, we found that the leaf temperature of transgenic plants was significantly higher than that in wild-type plants under well-watered growth conditions (Figure 4A). Additionally, water loss from detached leaves of the transgenic plants was reduced compared with that from detached leaves of wild-type plants (Figure 4B). These results suggest that transgenic plants expressing SUC2 promoter-driven NCED3 contain limited stomata open on the leaves and that ABA biosynthesis induced in phloem companion cells influences guard cells in the absence of environmental stress.

Specific organs or cells that supply traditional phytohormones have not been identified. For example, auxin, the best-characterized phytohormone, is synthesized in many plant tissues through different pathways (Forestan and Varotto, 2012; Normanly, 2010; Zhao, 2010). In this study, we have indicated that phloem companion cells express ABA biosynthetic enzymes and an ABA transporter. Phloem companion cells are developmentally derived from the unequal division of phloem mother cells and are closely associated with phloem sieve-tube cells.
differentiated from phloem mother cells to develop the sieve-tube element (Oparka and Turgeon, 1999; Beck, 2010). Interestingly, the sieve tube–companion cell complexes are largely symplastically isolated from other parenchyma cells (Beck, 2010); thus, ABA exporters are required to secrete ABA from the site of biosynthesis.

In addition to vascular ABA synthesis, guard cell-autonomous ABA synthesis has been reported recently (Bauer et al., 2013). Guard cell-autonomous ABA synthesis could allow the plant to respond rapidly to changing environmental conditions to maintain water status homeostasis. According to this report, probably both of vascular ABA and guard cell-autonomous ABA may be related to stomatal regulation dependently or independently, however the initial induction and the spatiotemporal process of drought response at guard cells are not completely understood yet (Okamoto et al., 2009). Additionally, guard cells are arranging the physiological appearances also by diurnal regulation (Chen et al., 2012; Hills et al., 2012). The balance of the ABA synthesis in vasculature and in guard cells under stress or non-stress conditions is to be investigated on the basis to trigger and/or maintain guard cell responses.

ABA transport assays performed directly using insect cell culture were consistent with our previous vesicle transport experiments, in which the efflux activity was detectable as ABA uptake of the regenerated membrane vesicles, including inside–out transport (Kuromori et al., 2010). In both cases, we detected AtABCG25 export activity in a heterologous system of Sf9 insect culture cells. This suggested that ABA export activity of AtABCG25 could be activated under no or very little post-translational regulation. Based on the increased stomatal closure after enhancing ABA biosynthesis in phloem companion cells, we demonstrated a route of ABA signaling from phloem companion cells at the vasculature to epidermal guard cells, which is likely mediated by ABA exporters. In addition, two ABA importers, AtABCG40 and AIT1, have
been reported, indicating a complex system of inter-cellular ABA transport in plants (Kang et al., 2010; Kanno et al., 2012).

Floral stimulus proteins such as FT in Arabidopsis are known to be produced at phloem companion cells and to undergo long-distance movement into the shoot apex to initiate flowering (Corbesier et al., 2007; Mathieu et al., 2007). Thus, phloem companion cells may secrete different types of remote signals, likely corresponding to endocrine cells in animals. Because they supply multiple remote signals, phloem companion cells may play a role in integrating environmental recognition into the remote signaling output.

Materials and Methods

Vector construction

A 0.9-kb ABA2 promoter region, a 1.2-kb AAO3 promoter region, and a 1.2-kb SUC2 promoter region were amplified using KOD plus polymerase (Toyobo) with the primer sets ABA2pro-fw (5’-CACCTATCATCAATTCATCATGTAAACAATAA-3’) and ABA2pro-rev (5’-AATAGGCTTTAGCTCTTAGATCTTTCTTT-3’), AAO3pro-fw (5’-CACCTTGAAAGTGATAAACAACTTACATAGTG-3’) and AAO3pro-rev (5’-CAGAATTTTTCCAA TTATAAGGTTAGA T-3’), and SUC2pro-fw2 (5’-CACCTTCATATTAATTTCCACACACCAAGTTAC-3’) and SUC2pro-rev3 (5’-ATTTGACAAACCAAGAAAGTAAGA -3’), respectively. Each promoter region was cloned into the pENTR/D-TOPO vector (Invitrogen). The AtABCG25 promoter clone was described previously (Kuromori et al., 2010). Each clone was integrated into the nuclear-localized signal-attaching GFP vector pBGGN (Inplanta Inovations Inc.). Additionally, the SUC2 promoter clone was integrated into the CFP vector pHGC (Kubo et al., 2005). An ORF clone of
the NCED3 (At3g14440) gene was amplified from the Arabidopsis Columbia genome temperate with the primer set NCED3_TOPO_F2 (5’- CACCATGGCTTCTTTCACGGCAA -3’) and NCED3_TOPO_RS (5’- TCACACGACCTGCTTCGCCAAATCA T -3’) and cloned into the pENTR/D-TOPO vector (Invitrogen). To generate a plasmid of SUC2 promoter-driven NCED3, NCED3 ORF blunt fragments digested with NotI and AscI were inserted into the AscI site blunted just after the SUC2 promoter region in the SUC2 promoter-nGFP vector.

**Plant growth and observations**

Each constructed vector was electroporated into Agrobacterium GV3101 for introduction into Arabidopsis by floral dipping of an Agrobacterium-mediated transformation system. In the T2 transgenic plants, fluorescent observation was performed using a confocal laser scanning microscope (Carl Zeiss) in accordance with the manufacturer’s instructions. We observed similar fluorescent patterns in more than four independent transgenic lines of each vector. Furthermore, after cross-pollination between T2 plants, plants of the next generation were used for subsequent confocal observation of the dual fluorescent proteins. For ABA treatment, seedlings of 1-week-old transgenic plants were soaked in 10 μM ABA solution for 20 h.

Plants were germinated and grown on 0.5× MS medium containing 1% (w/v) sucrose and 0.8% (w/v) agar in a growth chamber or in soil under well-watered conditions at 22°C ± 2°C and 60–70% relative humidity under a 16-h light/8-h dark cycle. Thermal images were captured using an infrared thermography device (FLIR). After taking thermal images, each transgenic plant expressing SUC2 promoter-driven NCED3 was confirmed to contain vector insertion based on PCR genotyping with the primer set (5’- AAGTGTCTTGGAGAATCGAACG -3’) and (5’- TGGAGTCTACAGGACCCTATC -3’).
**Transport assay using Sf9 insect culture cells**

AtABCG25 was expressed in Sf9 cells using a baculovirus expression system (Invitrogen), as described previously (Kuromori et al., 2010). Sf9 cells (1 × 10^6 cells mL^-1) were infected with the P3 virus (1/100 v/v) and cultured in liquid culture medium, Sf-900 III SFM (Gibco), with 4% FBS (Gibco), 100 units mL^-1 penicillin, and 100 μg mL^-1 streptomycin in a shaking incubator at 100 rpm and 28°C for 48 h. Cells were collected by centrifugation at 1000 rpm for 5 min, washed with culture medium twice, and finally resuspended at a final concentration of 25 mg mL^-1. Isotope solution of [3H]abscisic acid (GE Healthcare), [3H]Indolylacetic acid (Perkin Elmer), or [3H]Jasmonic acid (American Radiolabeled Chemicals, Inc.) was added (1/1000 v/v), and the solution incubated at room temperature for 16 min. A total of 100 μl of each sample was passed through a 0.45-μm membrane filter (Millipore), and the filter was washed with 2 ml of liquid culture medium. The radioactivity retained on the filter was determined using a liquid scintillation counter (ALOKA).

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**Literature Cited**


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Figure legends

Figure 1. Localization of ABA2, AAO3, AtABCG25 or SUC2 promoter-driven nGFP in Arabidopsis vascular tissues.

A–D, Confocal images were taken in the filaments of transgenic plants expressing ABA2 (A,
ABA2pro::nGFP), AAO3 (B, AAO3pro::nGFP), AtABCG25 (C, AtABCG25pro::nGFP), and SUC2 (D, SUC2pro::nGFP) promoter-driven nGFP. Fluorescence images (top panel) and bright-field images (bottom panel) are shown. Scale bars: 40 µm.

E–H, Confocal images were taken in the sepals or petals of transgenic plants expressing ABA2 (E, ABA2pro::nGFP), AAO3 (F, AAO3pro::nGFP), AtABCG25 (G, AtABCG25pro::nGFP), and SUC2 (H, SUC2pro::nGFP) promoter-driven nGFP. nGFP localization is shown in green, and chlorophyll autofluorescence is shown in red. Triangles show xylem vessels. Scale bars: 4 µm.

I–K, Confocal images were taken in roots of transgenic plants expressing ABA2 (I, ABA2pro::nGFP), AtABCG25 (J, AtABCG25pro::nGFP), and SUC2 (K, SUC2pro::nGFP) promoter-driven nGFP. Confocal images of J were taken after ABA treatment. Fluorescence images (top panel) and bright-field images (bottom panel) are shown. Scale bars: 50 µm.

**Figure 2. Colocalization of ABA2, AAO3, or AtABCG25 promoter-driven nGFP and SUC2 promoter-driven CFP in Arabidopsis vascular tissues.**

Confocal images of vascular tissues of filaments (A–C) and roots (D, E) were taken after crossing between the transgenic plants expressing ABA2 (A and D, ABA2pro::nGFP x SUC2pro::CFP), AAO3 (B, AAO3pro::nGFP x SUC2pro::CFP), and AtABCG25 (C and E, AtABCG25pro::nGFP x SUC2pro::CFP) promoter-driven nGFP and transgenic plants expressing SUC2 promoter-driven CFP. Confocal images of E were taken after ABA treatment. The two fluorophores CFP and nGFP were simultaneously visualized; CFP localization is shown in cyan (top left panel), nGFP localization is shown in green (bottom left panel), and both were merged (top right panel). Two fluorescence images and bright-field images were merged (bottom right panel). Triangles show xylem vessels.
Figure 3. Transport assay of isotope-labeled phytohormones by AtABCG25-expressing Sf9 culture cells.

Abscisic acid (A, $[^3]$HABA), indole-3-acetic acid (B, $[^3]$HIAA), or jasmonic acid (C, $[^3]$HJA) efflux activity was measured in Sf9 culture cells expressing AtABCG25 and empty vector (vec). Each bars represents the mean ± SD ($n$ = 4).

Figure 4. Transpiration phenotypes of transgenic plants expressing $SUC2$ promoter-driven $NCED3$.

A. Thermal images of the transgenic plants expressing $SUC2$ promoter-driven $NCED3$. Rosette leaves of 5-week-old wild-type plants (Col) and two independent transgenic plants expressing $SUC2$ promoter-driven $NCED3$ (SUC2pro-NCED3_1, SUC2pro-NCED3_2) were imaged by a visible-light camera (left panel) and an infrared thermography device (right panel).

B. Transpiration ratio of the transgenic plants expressing $SUC2$ promoter-driven $NCED3$. The water loss of the detached rosette leaves of 5-week-old plants was determined as a percentage of the initial fresh weight. Values are shown as means ± S.D. of three independent plants.
Figure 1
Figure 1. Localization of *ABA2*, *AAO3*, *AtABCG25* or *SUC2* promoter-driven nGFP in *Arabidopsis* vascular tissues.

A–D, Confocal images were taken in the filaments of transgenic plants expressing *ABA2* (A, *ABA2pro::nGFP*), *AAO3* (B, *AAO3pro::nGFP*), *AtABCG25* (C, *AtABCG25pro::nGFP*), and *SUC2* (D, *SUC2pro::nGFP*) promoter-driven nGFP. Fluorescence images (top panel) and bright-field images (bottom panel) are shown. Scale bars: 40 µm.

E–H, Confocal images were taken in the sepals or petals of transgenic plants expressing *ABA2* (E, *ABA2pro::nGFP*), *AAO3* (F, *AAO3pro::nGFP*), *AtABCG25* (G, *AtABCG25pro::nGFP*), and *SUC2* (H, *SUC2pro::nGFP*) promoter-driven nGFP. nGFP localization is shown in green, and chlorophyll autofluorescence is shown in red. Triangles show xylem vessels. Scale bars: 4 µm.

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Confocal images of vascular tissues of filaments (A–C) and roots (D, E) were taken after crossing between the transgenic plants expressing *ABA2* (A and D, *ABA2pro::nGFP* x *SUC2pro::CFP*), *AAO3* (B, *AAO3pro::nGFP* x *SUC2pro::CFP*), and *AtABCG25* (C and E, *AtABCG25pro::nGFP* x *SUC2pro::CFP*) promoter-driven nGFP and transgenic plants expressing *SUC2* promoter-driven CFP. Confocal images of E were taken after ABA treatment. The two fluorophores CFP and nGFP were simultaneously visualized; CFP localization is shown in cyan (top left panel), nGFP localization is shown in green (bottom left panel), and both were merged (top right panel). Two fluorescence images and bright-field images were merged (bottom right panel). Triangles show xylem vessels.
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