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ARTICLE

Arabidopsis NPF5.1 regulates ABA homeostasis and seed germination by mediating ABA uptake into the seed coat

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ABSTRACT

Abscisic acid (ABA) is a plant hormone that induces seed dormancy during seed development and inhibits seed germination after imbibition. Although ABA is synthesized in the seed coat (testa), endosperm, and embryo, the physiological roles of the hormone derived from each tissue are not fully understood. We found that the gene encoding an Arabidopsis ABA importer, *NPF5.1*, was expressed in the seed coat during seed development. Dry seeds of loss-of-function *npf5.1* mutants contained significantly higher levels of dihydrophaseic acid (DPA), an inactive ABA metabolite, than the wild type. The *npf5.1* mutant also had a slight increase in ABA content. An increase in DPA was prominent in the fraction containing the seed coat and endosperm. Seed germination of the *npf5.1* mutant was similar to the wild type in the presence of ABA or the gibberellin biosynthesis inhibitor paclobutrazol. However, a mutation in *NPF5.1* suppressed the paclobutrazol-resistant germination of *npf4.6*, a mutant impaired in an ABA importer expressed in the embryo. These results suggest that ABA uptake into the seed coat mediated by NPF5.1 is important for ABA homeostasis during seed development and for regulating seed germination.

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Introduction

The plant hormone abscisic acid (ABA) regulates several physiological processes throughout the plant life cycle.[1–3] During vegetative stages, ABA promotes stomatal closure to limit water loss. In addition, ABA regulates the expression of genes that are involved in plant responses to abiotic and biotic stresses. ABA is also required for the induction and maintenance of seed dormancy. These physiological responses are often associated with the changes in endogenous ABA levels. Therefore, it is important to understand how the hormone concentration is regulated through biosynthesis, catabolism (inactivation), and transport.

Recent studies have demonstrated that ABA movement within plants is actively regulated by transmembrane transporters.[4–6] Three types of transporter families, namely, the ATP-binding cassette (ABC), NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY (NPF), and Detoxification Efflux Carriers (DTX)/Multidrug and Toxic Compound Extrusion (MATE) proteins, are reported to be involved in ABA transport. In Arabidopsis, ABA synthesized in vascular tissues is released into the apoplastic space by the exporter protein ABCG25 and ultimately reaches guard cells, where the hormone is taken up by importer proteins ABCG40 and NPF4.6.[7–9] The amount of ABA transported from vascular tissues to guard cells is regulated by several transporters, such as ABCG17, ABCG18, DTX50, and NPF5.1.[8,10,11] In Arabidopsis seeds, ABCG25 and ABCG31 export ABA from the endosperm, whereas ABCG30 and ABCG40 import ABA into embryos.[12] These findings indicate that the movement of ABA within plants is regulated in a very complex manner.

In this study, we found that Arabidopsis *NPF5.1*, which encodes an ABA importer, was expressed specifically in the seed coat. Interestingly, mutants defective in *NPF5.1* (*npf5.1*) accumulated significantly higher levels of dihydrophaseic acid (DPA), an inactive ABA metabolite, in the seed coat and/or endosperm compared to the wild type. Possible functions of NPF5.1 in ABA homeostasis and the regulation of seed germination are discussed.

Materials and methods

Plant materials and growth conditions

Arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] accession Columbia-0 was used as the wild type. Mutants *npf4.6–1* (*ait1-1*) (SALK_146143), *npf5.1–1* (SALK_085919), and *npf5.1–2* (SALK_000464) were isolated in previous studies.[8,13] Transgenic plants expressing the *GUS* gene under the control of the *NPF5.1* promoter (*pNPF5.1:GUS*) were generated as described previously.[8] Seeds were sterilized with 70% (v/v) ethanol and then with 5% (v/v) NaClO (0.25% active chlorine) containing 1% (w/v) Tween 20. After washing with sterilized water, seeds were sown on plates containing half-strength MS medium and 0.8% (w/v) agar and incubated at 4°C for 3 days in the dark. Plates were then incubated at 22°C under continuous

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light (50 μ moles/m^2/s) for 7 days. Seedlings were transplanted to soil containing vermiculite and Metro-mix 350 (Sun Gro Horticulture) at a 3:1 ratio for seed propagation.

GUS staining

For GUS staining, developing seeds (15 days after flowering) were isolated from the silique envelopes. Seeds were incubated overnight in a solution composed of 50 mM sodium phosphate buffer (pH 7.2), 10 mM EDTA, 0.05% (v/v) Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide and 1 mM X-Gluc and then observed using a stereo microscope (Leica M205, Leica).

Germination assays

Surface sterilized seeds were sown on 0.8% (w/v) agar plates containing half-strength MS medium. Stock solutions of (±)-ABA (Sigma-Aldrich) and paclobutrazol (Wako) to be added to the media were prepared in DMSO at 1000× concentrations; DMSO was added to the control medium. Plates were incubated at 4°C in the dark for 3 days and then at 22°C under continuous light. Germination, defined as cotyledon greening, was scored after 4 days (for control and paclobutrazol treatments) or 7 days (for ABA treatment).

Measurements of ABA and ABA metabolites

ABA and ABA metabolites were extracted and purified as described previously.[14] ABA and ABA metabolites were quantified using a quadrupole/time-of-flight tandem mass spectrometer (Triple TOF 5600, SCIEX) and a Nexera HPLC system (SHIMADZU) equipped with a ZORBAX Eclipse XDB-C18 column (2.1 × 50 mm, Agilent). Compounds were separated by LC with a binary solvent system of water containing 0.01% (v/v) acetic acid (A) and acetonitrile containing 0.05% (v/v) acetic acid (B) at a flow rate of 0.4 ml/min. The experimental protocol was as follows: constant 3% solvent B from 0 to 0.5 min followed by a linear gradient of solvent B from 3 to 40% over 8.5 min. Retention times of the compounds were 3.24 min for dihydrophaseic acid (DPA) and d_3-DPA, 4.52 min for phaseic acid (PA) and d_3-PA, 5.93 min for ABA and d_6-ABA, and 4.53 min for ABA-glucose ester (ABA-GE) and d_5-ABA-GE. MS/MS conditions were as follows: ion spray voltage (kV) = −3.5, desolvation temperature (°C) = 600, declustering potential (V) = −100 (DPA and d_3-DPA), −90 (PA and d_3-PA, ABA and d_6-ABA), −120 (ABA-GE and d_5-ABA-GE), collision energy (V) = −20 (DPA and d_3-DPA), −16 (PA and d_3-PA), −15 (ABA and d_6-ABA), −16 (ABA-GE and d_5-ABA-GE), scan range (*m*/*z*) = 100–500, MS/MS transition (*m*/*z*) = 281/171 (DPA), 284/174 (d_3-DPA), 279/139 (PA), 282/142 (d_3-PA), 263–153 (ABA), 269–159 (d_6-ABA), 425–263 (ABA-GE), 430/268 (d_5-ABA-GE). Data were analyzed using SCIEX OS-Q 2.0 software (SCIEX).

Results and discussions

We previously reported that the transporter encoded by Arabidopsis *NPF5.1* (At2g40460) mediates cellular ABA uptake.[8] *NPF5.1* is expressed in vascular tissues and leaf mesophyll and epidermal cells during vegetative stages. Mutants defective in *NPF5.1* (*npf5.1*) exhibited higher leaf surface temperatures than in the wild type, suggesting that the protein negatively regulates stomatal closure by limiting the amount of ABA transported from vascular tissues, where the hormone is synthesized, to guard cells. The Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)[15,16] indicated that *NPF5.1* is also expressed in seeds, more specifically in the seed coat, during later stages of seed development. We confirmed the seed-coat specific expression of *NPF5.1* at 15 days after flowering by GUS staining of *pNPF5.1:GUS* seeds (Figure 1). We hypothesized that NPF5.1 might regulate some important aspects of seed physiology as an ABA transporter.

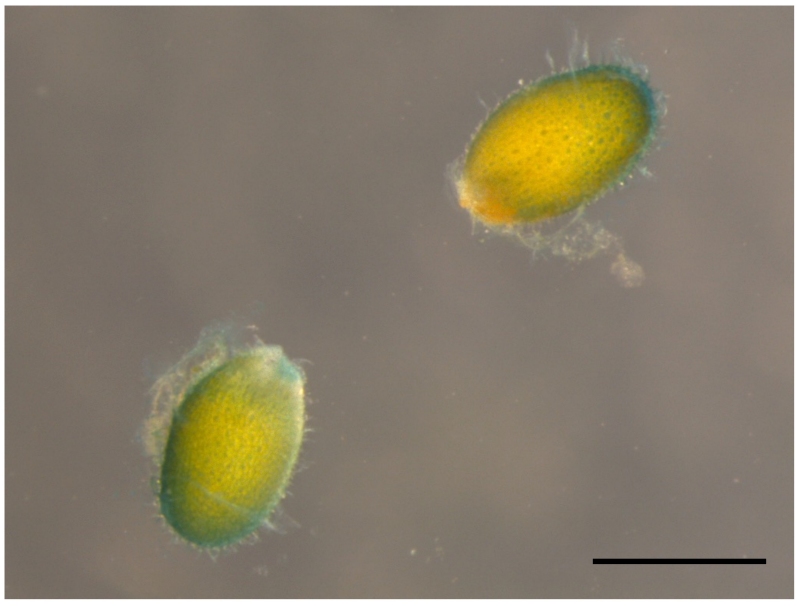

Figure 1. Expression of *NPF5.1* in seeds. Seeds of *pNPF5.1:GUS* plants at 15 days after flowering were stained for GUS activity. Bar = 0.5 mm.

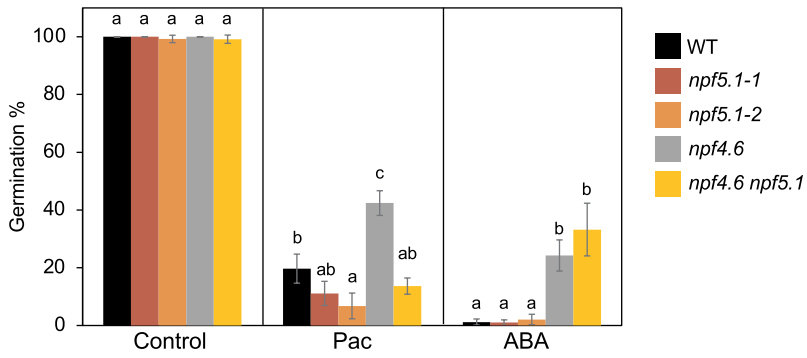

Figure 2. Germination of wild type (WT), *npf5.1–1, npf5.1–2, npf4.6–1* (*npf4.6*), and *npf4.6–1 npf5.1–1* (*npf4.6 npf5.1*) on the control medium (Control) or on media containing 50 μM paclobutrazol (Pac) or 0.5 μM ABA. Different letters indicate significant differences ($P < .05$) by Tukey's multiple comparison test determined for each group.

We next examined whether the loss of *NPF5.1* functions affects seed germination (Figure 2). Germination levels of seeds from both *npf5.1–1* and *npf5.1–2* alleles and the wild type were almost 100% within 4 days after stratification. Although several ABA-deficient and -insensitive mutants have been reported to germinate better than the wild type in the presence of gibberellin (GA) biosynthesis inhibitors such as paclobutrazol,[17,18] germination of *npf5.1* was inhibited to levels similar to the wild type. The sensitivity of *npf5.1* to exogenously applied ABA was also comparable to that of the wild type. In contrast, *npf4.6* germinated better than the wild type in the presence of ABA, as we reported previously.[13] In addition, *npf4.6* was less sensitive to paclobutrazol compared to the wild type. As *NPF4.6* is expressed in the embryo,[13] ABA uptake into the embryo may contribute significantly to seed sensitivity to exogenously applied ABA and paclobutrazol. Interestingly, a mutation in *NPF5.1* suppressed the paclobutrazol-resistant germination phenotype in the *npf4.6* mutant background. This observation may occur because ABA uptake into the seed coat mediated by NPF5.1 somehow affected ABA accumulation within the seeds.

Endogenous levels of ABA and its metabolites in whole dry seeds were analyzed to determine the impact of the *npf5.1* mutations on the homeostasis of the hormone (Figure 3). *npf5.1* contained slightly (about 1.3 times) but significantly greater amounts of ABA and ABA-GE than the wild type. In *npf4.6*, the two compounds were detected at the levels comparable to *npf5.1*. Additive effects of the *npf5.1* and *npf4.6* mutations on ABA and ABA-GE content were not observed for unknown reasons. To our surprise, DPA levels were markedly (3–4 times) increased in *npf5.1* compared to the wild type. The difference in the DPA content between the two *npf5.1* alleles was possibly due to different severities of the mutations: *npf5.1–1* contains T-DNA in the second intron, whereas T-DNA is inserted in the 3' UTR in *npf5.1–2*. DPA was detected at similar levels in the wild type and *npf4.6*, indicating that different mechanisms may have caused the increase in ABA and ABA-GE levels observed in *npf5.1* and *npf4.6*.

Finally, we determined where the accumulation of ABA and DPA occurred in seeds. Mature seeds were dissected into the embryo and the remaining tissues containing the seed coat and endosperm within 1 h of imbibition, and ABA and DPA were quantified separately from the two fractions (Figure 4). In *npf5.1*, slight increases in the ABA levels compared to wild type were observed in the embryos and the testa/endosperm fraction. DPA was accumulated to much higher levels (about 4 times relative to the wild type) in the testa/endosperm fraction of *npf5.1*. In contrast, the compound present in embryos was only slightly (about 1.3 times) higher in *npf5.1* than the wild type.

ABA is synthesized in the seed coat, endosperm, and embryo during Arabidopsis seed development.[19,20] Crosses between wild-type and ABA-deficient mutants have shown that ABA synthesized in maternal tissues, including the seed coat, is transported to embryos.[14,21] Given that *NPF5.1* is expressed in the seed coat (Figure 1), we hypothesized that the NPF5.1 transporter protein plays a role in retaining ABA within seed coats. Loss of *NPF5.1* functions would, thus, first result in an increase in the amount of ABA transported from the seed coat to the neighboring tissue, namely the endosperm. One of the genes (*CYP707A2*) encoding ABA 8'-hydroxylases, the rate-limiting enzyme of ABA catabolism in Arabidopsis, is highly expressed in the endosperm.[22] Therefore, ABA transported from the seed coat to the endosperm may be easily converted successively to 8'-hydroxylated ABA, PA, and finally to DPA in *npf5.1*. Although the increase in ABA levels observed in *npf5.1* was not prominent, possibly due to a higher turnover rate, the mutation was sufficient to affect seed germination performances, at least in the *npf4.6* background (Figure 2). Different effects of *npf4.6* and *npf5.1* mutations on seed germinability and endogenous levels of ABA and its metabolites (Figures 2–4) support the idea that NPF4.6 and NPF5.1 function at distinct sites (the embryo and seed coat, respectively) and regulate different physiological processes. Quantification of ABA and DPA also suggests that an excess amount of either or both compounds accumulated in the endosperm of *npf5.1* is transported to the embryo (Figure 4). Several ABA transporters that function in seeds have been identified;[12] however, it is not fully understood how ABA and ABA metabolites move within seed tissues. Further identification of transporters and the visualization of ABA-related compounds at the tissue or cellular levels are required to answer this question.

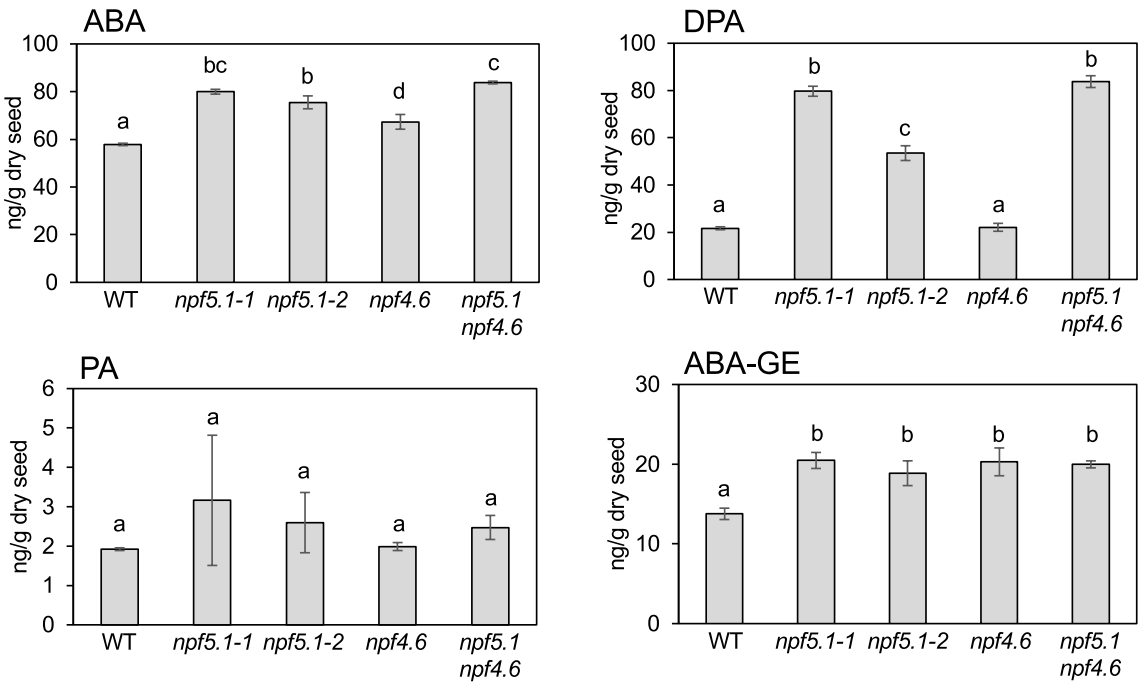

Figure 3. Endogenous levels of ABA, phaseic acid (PA), dihydrophaseic acid (DPA) and ABA-glucose ester (ABA-GE) in mature dry seeds of wild type (WT), *npf5.1–1*, *npf5.1–2*, *npf4.6–1* (*npf4.6*), and *npf4.6–1 npf5.1–1* (*npf4.6 npf5.1*). Different letters indicate significant differences ($P < .01$) determined by Tukey's multiple comparison test.

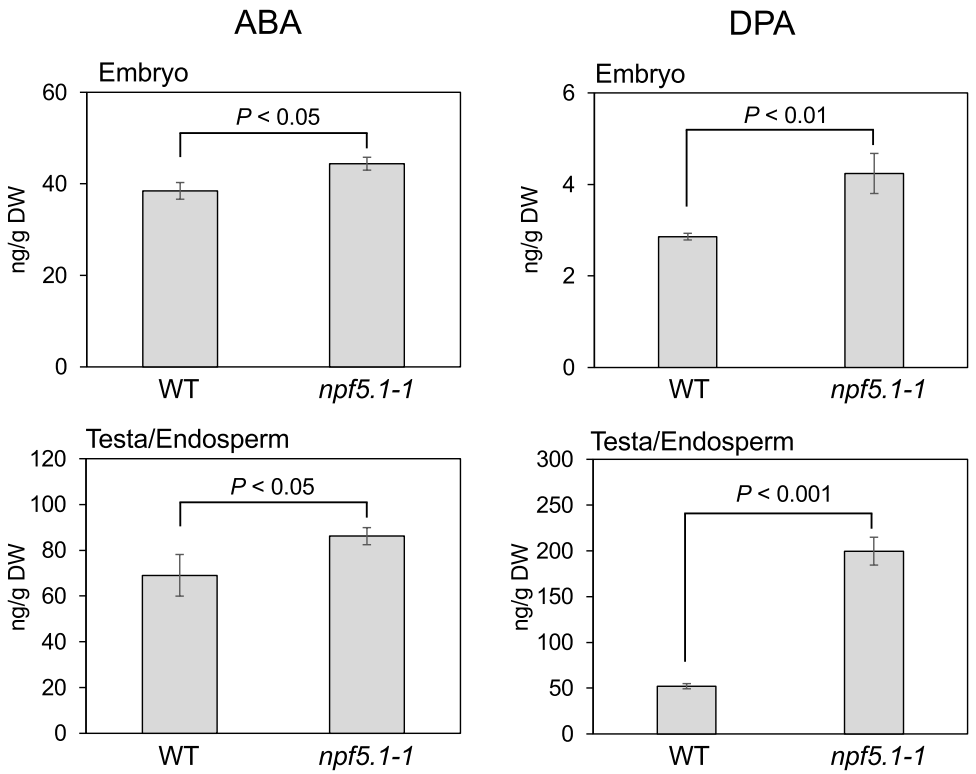

Figure 4. Comparison of ABA and dihydrophaseic acid (DPA) levels in embryos and the fraction containing seed coats and endosperms (testa/endosperm) between wild type (WT) and *npf5.1* (*npf5.1–1*). Significant differences were determined by Student's *t*-test.

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