Nitrate does not compete with abscisic acid as a substrate of AtNPF4.6/NRT1.2/AIT1 in *Arabidopsis*

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Abbreviations: ABA, abscisic acid; GA, gibberellin; IAA, indole-3-acetic acid

We identified a member of the *Arabidopsis* NRT1/PTR FAMILY (NPF), AtNPF4.6, as an abscisic acid (ABA) transporter, AIT1. AtNPF4.6 was originally characterized as a low-affinity nitrate transporter NRT1.2. We hypothesized that the competition between nitrate and ABA as substrates for AtNPF4.6 might be involved in the interactions between nitrate and ABA signaling. However, the ABA transport activity of AtNPF4.6 was not inhibited by an excess amount of nitrate. In addition, the *npf4.6* mutant was less sensitive to ABA than the wild type during germination irrespective of nitrate concentrations in the media. Furthermore, nitrate promoted germination of both wild type and *npf4.6* in the presence of ABA. These results do not support the idea of a physiological linkage between nitrate and ABA signals through AtNPF4.6.

Abscisic acid (ABA) is a plant hormone that plays crucial roles in many aspects of plant life cycles, including the regulation of seed dormancy and germination, as well as plant responses to abiotic and biotic stress.[1,2] So far, a number of genes involved in the biosynthesis, catabolism, perception, and signal transduction of ABA have been identified.[3-7] These studies demonstrated that endogenous ABA levels as well as sensitivities (or responsiveness) of plants to ABA are important for the physiological responses. Recently, 2 ATP-binding cassette (ABC)-type ABA transporters, AtABCG25 and AtABCG40, were identified in *Arabidopsis*.[8,9] AtABCG25 mediates ABA export from cells whereas AtABCG40 mediates ABA import into cells. Mutants defective in the transporters showed altered responses to ABA in terms of seed germination and/or stomatal aperture, suggesting that ABA transport is an important element in the regulation of ABA-mediated physiological processes.

By using a modified yeast two-hybrid screening system with the ABA receptor PYR/PYL/RCAR and PP2C protein phosphatases, we identified the third ABA transporter [ABA-IMPORTING TRANSPORTER (AIT) 1], a member of the NRT1/PTR FAMILY (NPF), NPF4.6.[10,11] Our study suggests that NPF4.6 functions as an ABA importer,[11] although we cannot exclude the possibility that it is a bidirectional transporter as reported for some NPF proteins.[12,13] Interestingly, AIT1 is identical to the previously characterized low-affinity nitrate transporter NRT1.2.[14] Km values of AtNPF4.6 for nitrate and ABA were approximately 5.9 mM and 5 μM, respectively, suggesting that ABA is the more efficient substrate of the transporter. However, nitrate concentrations in soils and plants are much higher than

endogenous ABA levels.[15,16] Thus it is still possible that AtNPF4.6 functions as a transporter for both nitrate and ABA.

Interactions between nitrate and ABA signals have been suggested.[17-19] It has been suggested that the interactions occur at the level of gene expression.[20] However, as AtNPF4.6 transports both nitrate and ABA, it is also possible that the competition between nitrate and ABA as substrates for AtNPF4.6 is involved in the interactions of the 2 signals. An example for such an interaction is the auxin (indole-3-acetic acid; IAA) transport via AtNPF6.3/NRT1.1/CHL1 where competition between nitrate and IAA as substrates for the transporter affects lateral root development in *Arabidopsis*.[21] To evaluate the possibility, we first tested whether nitrate affects the ABA transport activity of AtNPF4.6 (**Fig. 1**). We used a modified yeast two-hybrid system with the ABI1 PP2C protein phosphatase fused to the GAL4 activation domain (AD; AD-ABI1) and the PYR1 ABA receptor fused to the GAL4 binding domain (BD; BD-PYR1) to detect ABA transport activities of AtNPF4.6.[11] If an ABA importer is expressed in the yeast cells, the interaction between AD-ABI1 and BD-PYR1 is induced at lower ABA concentrations in the medium compared with cells without transporters. ABA-dependent interactions between AD-ABI1 and BD-PYR1 were detected as the expression of the marker gene *lacZ* determined by a β-gal assay with *o*-nitrophenyl β-D-galactopyranoside as the substrate. Because AtNPF4.1 (AIT3) transports ABA and gibberellin [GA (GA$_3$)],[11] we performed a control experiment using ABA and GA$_3$ as competitive substrates for AtNPF4.1 (**Fig. 1A**). As expected, β-gal activities in the cells expressing AtNPF4.1 in the presence of 10 nM ABA
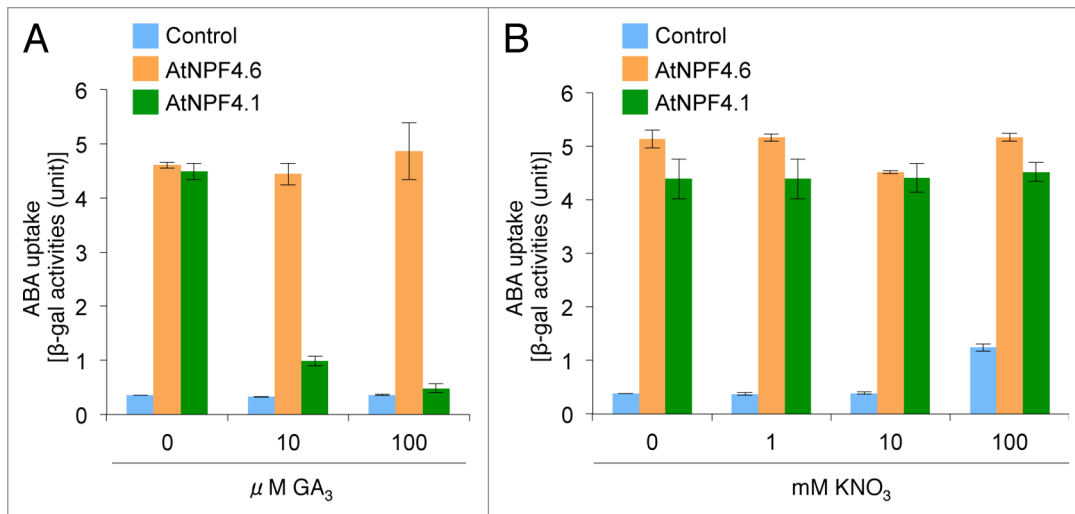
Figure 1. Effects of nitrate on ABA transport activities of AtNPF4.6. ABA transport activities of AtNPF4.6 and AtNPF4.1 were determined by a modified yeast two-hybrid system as described previously.[11] *AtNPF4.6*, *AtNPF4.1*, or the empty vector (Control) were introduced into yeast cells containing AD-ABI1 and BD-PYR1, and ABA-dependent interactions between AD-ABI1 and BD-PYR1 in the presence of 10 nM ABA were detected as the expression of lacZ as determined by a β-gal assay with o-nitrophenyl β-D-galactopyranoside as the substrate. (**A**) 0, 10, or 100 μM GA$_3$ was added as a competitor of ABA. (**B**) 0, 1, 10, or 100 mM nitrate (KNO$_3$) was added as a competitor of ABA. The assay was performed as described previously[10] except that yeast cells were cultured in synthetic dextrose (SD) medium without Leu, Trp, and Ura, instead of yeast extract-peptone-dextrose (YPD) medium. The β-gal activities detected under these conditions were relatively high. Differences in the ABA transport activities of AtNPF4.6 and AtNPF4.1 were small, probably due to saturated ABA uptake or β-gal reactions. Values are means ± SD of 3 biological replicates.

was inhibited by addition of 10 μM or higher concentrations of GA$_3$, indicating that AtNPF4.1-mediated ABA uptake into the cells was inhibited competitively by GA$_3$. On the other hand, consistent with our previous observation that AtNPF4.6 did not transport GA$_3$,[11] AtNPF4.6-mediated ABA uptake was not inhibited under the same conditions. These results demonstrated that this assay allowed the detection of the competition between ABA and other substrates. Next, we determined the effects of nitrate on ABA uptake through AtNPF4.6 (**Fig. 1B**). Because nitrate exists in soils and plant tissues at much higher concentrations than ABA,[15,16] we added up to 100 mM nitrate against 10 nM of ABA as a competitor. However, we could not detect any inhibitory effect of nitrate on ABA transport activities of AtNPF4.6 (and AtNPF4.1).

We previously showed that the *npf4.6* (*ait1*) mutant was less sensitive to exogenous ABA than the wild type during germination.[11] On the other hand, AtNPF4.6 did not transport the ABA agonist pyrabactin, and the sensitivity of *npf4.6* to pyrabactin was comparable to that of the wild type.[11] These findings indicate that *npf4.6* is defective in ABA transport rather than ABA signaling. However, it is unclear from these experiments whether nitrate competes with ABA as a substrate of AtNPF4.6. Other NPF members, together with AtNPF4.6, may be involved in the interactions between nitrate and ABA signals. We have shown that at least for members of NPF (AtNPF4.6/NRT1.2/AIT1, AtNPF4.5/AIT2, AtNPF4.1/AIT3, and AtNPF4.2/AIT4) exhibit ABA transport activity.[11] However, among mutants defective in AtNPF4.6, AtNPF4.5, and AtNPF4.1, only *npf4.6* showed an ABA-insensitive phenotype during germination. We were unable to obtain mutants

defective in AtNPF4.2. However, based on the relatively low ABA transport activity of AtNPF4.2,[11] its contribution to the physiological processes would be minor at best. Therefore, we investigated the effect of exogenously applied nitrate and ABA on seed germination of wild type and *npf4.6* (**Fig. 2**). As expected from previous observations of nitrate-induced germination of dormant *Arabidopsis* seeds,[22] nitrate promoted the germination and/or post-germination growth of non-dormant (after-ripened and stratified) wild-type seeds in the presence of 0.8 μM ABA, suggesting that nitrate negatively regulates the effect of ABA. If this phenomenon was caused by the inhibition of ABA transport through AtNPF4.6 by nitrate, *npf4.6* should not respond to nitrate because AtNPF4.6 is not functional in the mutant irrespective of nitrate availability. However, germination of *npf4.6* in the presence of ABA was promoted by nitrate similarly to that of the wild type (**Fig. 2**). Also, if AtNPF4.6 was involved in the nitrate-mediated promotion of seed germination in the presence of ABA, the ABA insensitive germination phenotype of *npf4.6* should be masked at high nitrate concentrations. However, *npf4.6* remained ABA-insensitive even in the presence of 10 mM nitrate, a saturating nitrate concentration with respect to the promotion of germination in the presence of ABA (**Fig. 2**). Thus, it appears that the ABA-insensitive phenotype of *npf4.6* is independent of the function of AtNPF4.6 as a nitrate transporter. Taken together, our experiments do not support the idea that AtNPF4.6 mediates interactions between nitrate and ABA signals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
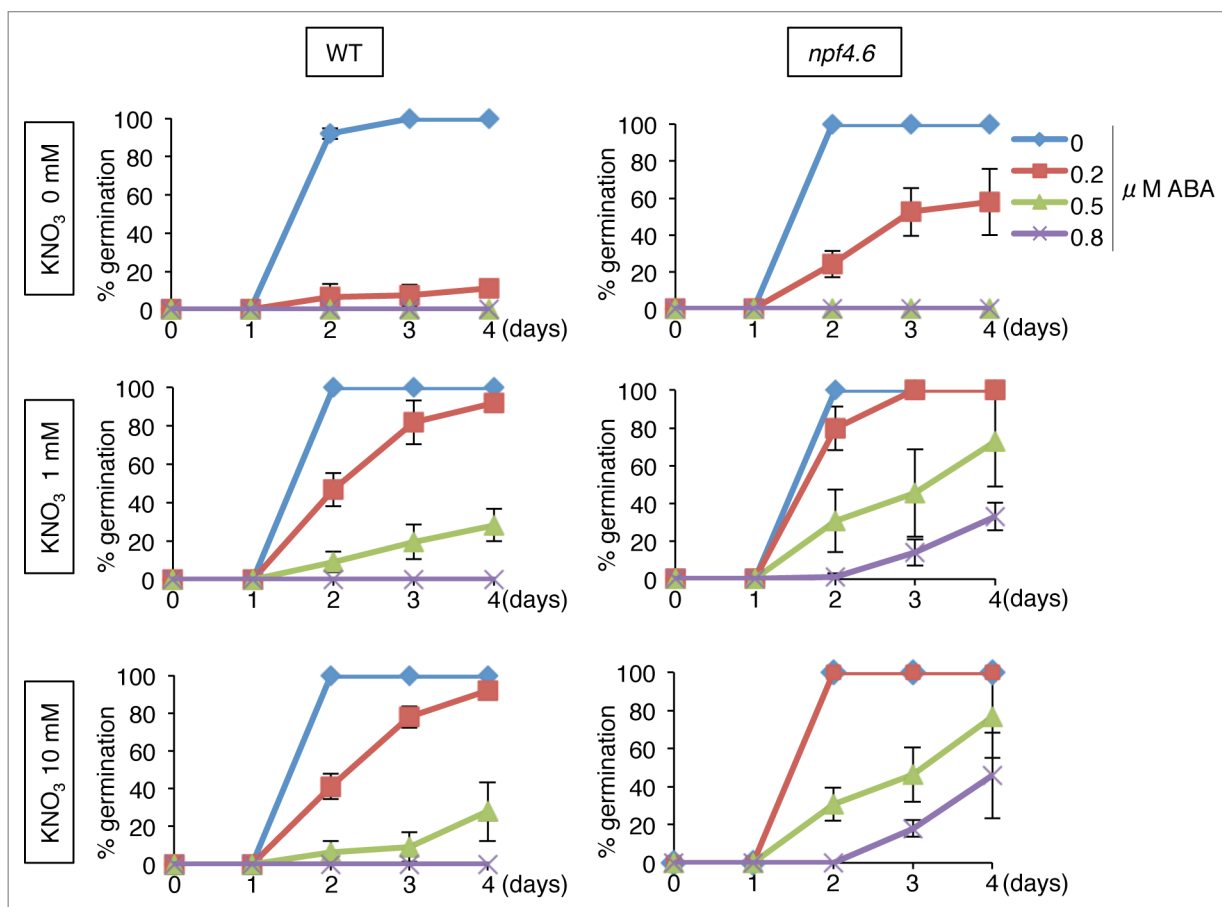
Figure 2. Germination of wild type and *npf4.6* in the presence of nitrate and ABA. Seeds of wild type (Col-0 accession) and *npf4.6* (*ait1–1*) were surface-sterilized in a solution containing 5% (vol/vol) NaClO and 0.05% (vol/vol) Tween 20, rinsed with water, and sown on 0.8% (w/vol) agar plate (pH 6.5) containing 0.5 × Murashige and Skoog salts, MES (0.5 g/l), ABA (0, 0.2, 0.5, or 0.8 μM), and nitrate (KNO₃; 0, 1, or 10 mM). Stock solutions of ABA (1,000 × concentration) were prepared in DMSO and added to the media after autoclaving. Ten and 9 mM KCl was added to the media containing 0 and 1 mM KNO₃, respectively, to maintain equal osmotic pressure among the treatments. After sowing, plates were incubated at 4 °C in the dark for 3 d. Germination rates were scored at 0, 1, 2, 3, and 4 d after transferring the plates to light conditions at 22 °C. Germination was determined as greening of cotyledons. Values are means ± SD of 3 biological replicates.

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