The Arabidopsis NRT1/PTR FAMILY protein NPF7.3/NRT1.5 is an indole-3-butyric acid transporter involved in root gravitropism

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Active membrane transport of plant hormones and their related compounds is an essential process that determines the distribution of the compounds within plant tissues and, hence, regulates various physiological events. Here, we report that the Arabidopsis NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY 7.3 (NPF7.3) protein functions as a transporter of indole-3-butyric acid (IBA), a precursor of the major endogenous auxin indole-3-acetic acid (IAA). When expressed in yeast, NPF7.3 mediated cellular IBA uptake. Loss-of-function npf7.3 mutants showed defective root gravitropism with reduced IBA levels and auxin responses. Nevertheless, the phenotype was restored by exogenous application of IAA but not by IBA treatment. NPF7.3 was expressed in pericycle cells and the root tip region including root cap cells of primary roots where the IBA-to-IAA conversion occurs. Our findings indicate that NPF7.3-mediated IBA uptake into specific cells is required for the generation of appropriate auxin gradients within root tissues.

Significance

Plant roots grow toward gravity. The phenomenon is known as “root gravitropism” as early described by Charles Darwin, and it has been well established that the plant hormone auxin plays a central role in this response. The major endogenous auxin, indole-3-acetic acid (IAA), is mainly synthesized from tryptophan via indole-3-pyruvic acid, whereas indole-3-butyric acid (IBA) is a minor precursor of IAA. Previous studies have shown that IAA derived from IBA plays a specific role in regulating lateral root development. However, in the present study we demonstrate through the identification and characterization of IBA transporter NPF7.3/NRT1.5 that IAA synthesized from IBA regulates root gravitropism.
membrane (17). Here, we report that NPF7.3, originally identified as a low-affinity nitrate transporter (NRT1.5) and later shown to be a proton/kg potassium antiporter in Arabidopsis (23, 24), functions as an IBA transporter. We found that npf7.3 mutant roots were not able to respond properly to gravity. Our transport assays in yeast demonstrated that NPF7.3 efficiently mediated IBA uptake into the cells. Asymmetric distribution of auxin activities in root tips following the reorientation of roots was not observed in the npf7.3 mutants, and the defect was restored by IAA but not by IBA treatment. These results suggest that cellular IBA uptake mediated by NPF7.3 contributes to the production of IAA required to fully induce Arabidopsis root gravitropism.

Results

Mutants Defective in NPF7.3 Exhibit Altered Root Gravitropism. Some of the 53 members of the Arabidopsis NPF proteins transport plant hormones such as IAA, abscisic acid (ABA), gibberellin (GA), and jasmonates (jasmonoyl isoleucine; JA-Ile) (19). Thus, we hypothesized that there are still unidentified plant hormone transporters in this protein family. We examined the physiological functions of several members of the NPF proteins more closely based on their transport activities in yeast and/or their expression patterns available from public databases. NPF7.3 was one of the candidates since its gene expression was affected by biotic and abiotic stresses as well as IAA treatment [efp Browser. bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (25)]. NP7.3 was originally characterized as a low-affinity bidirectional nitrate transporter NRT1.5 that facilitates xylem loading of nitrate from root pericycle cells (23). NPF7.3 has also been reported to function as a proton/kg potassium antiporter (also referred to as LKS2) that mediates xylem loading of potassium (24). Previous studies showed that mutants defective in NPF7.3 exhibit phenotypes related to root growth especially when nitrate or potassium concentrations in the growth media were low (23, 24); however, we found that two allelic mutants of NPF7.3 showed a wavy root growth phenotype when grown on vertical plates containing half-strength Murashige-Skoog (MS) medium, which is rich in nitrate and potassium (more than 10 mM ammonium nitrate and 9 mM potassium nitrate) and 1% sucrose (Fig. 1A).

In our study, we used a previously characterized mutant allele (nrt1.5-4) (26) and a newly isolated allele (SI Appendix, Fig. S1) and identified here as npf7.3 mutants (npf7.3-5, npf7.3-6, npf7.3-8, and npf7.3-9). Four out of five alleles have been reported so far), respectively, for simplification. As compared with wild type, npf7.3 mutant roots had significantly increased wave frequencies with reduced wave intervals, whereas primary root lengths were almost comparable in the wild type and npf7.3 (Fig. 1 B–D). Consequently, npf7.3 mutants had lower root straightness indices (27) compared to wild type (Fig. 1E). Since several Arabidopsis mutants with wavy root growth display altered gravitropism (28, 29), we investigated the phenotype in npf7.3 primary roots. Indeed, gravitropic indices (27) in the npf7.3 mutants were significantly lower than those in wild type (Fig. 1F). To further validate the defects of npf7.3 in root gravitropic responses, vertical plates containing wild-type and npf7.3 seedlings were rotated to shift the gravity direction 90° (first rotation), and then the plates were rotated again back to the initial state (second rotation) after 24 h of incubation. This experiment was conducted in the absence of sucrose in the media, since the wavy root phenotype interferes with the measurement of root angles. Although most wild-type roots properly change their growth direction toward gravity (nearly 90°), npf7.3 mutant roots responded poorly to gravity (Fig. 1G). As a result, root angles were significantly larger and variable in npf7.3 compared with wild type; however, root elongation rates after reorientation of plants were comparable between wild type and the mutant (Fig. 1 H and J). We sometimes observed that root tips of npf7.3 were not attached to the surface of agar plates after reorientation. These plants were not used for measuring root angles and root growth rates. Finally, we confirmed that the disruption of root gravitropism observed in npf7.3 was restored by the expression of wild-type NPF7.3 coding sequence (CDS) under the control of its own promoter (2.1 kb) (Fig. 1J).

NPF7.3 Has IBA Uptake Activity. The plant hormone auxin plays a crucial role in root gravitropism (9). Therefore, we hypothesized that NPF7.3 somehow regulates auxin responses. Given that NPFs transport a variety of compounds including IAA, it is possible that NPF7.3 transports IAA and/or its related compounds. To assess this possibility, we conducted direct transport assays using yeast cells. Significantly larger amounts of IAA were accumulated in NPF7.3-expressing cells compared to the cells containing a control vector when the chemical concentration was 10 μM and the reaction buffer was acidic (pH 5.8) (Fig. 2A). We also examined whether IBA could be a substrate for NPF7.3 in the same conditions because the compound is considered to be an IAA precursor (8, 13). Interestingly, IBA also accumulated at a much higher level in the cells expressing NPF7.3 compared to the control cells (Fig. 2B). Moreover, IBA was more efficiently taken into the cells expressing NPF7.3 than IAA.

Kinetic analyses indicated that the Michaelis constant (Km) values of NPF7.3 for IAA and IBA were comparable (14 μM and 11 μM for IAA and IBA, respectively); however, NPF7.3 had a much higher maximum velocity value for IBA compared to IAA (9 pmol·1·107 cells·min·1 for IBA and 1.2 pmol·1·107 cells·min·1 for IAA) (Fig. 2C). When both IAA and IBA were present at the same concentration (10 μM) as substrates of NPF7.3, IBA was more preferentially transported into the yeast cells (Fig. 2D). Furthermore, IBA uptake mediated by NPF7.3 was only slightly inhibited when 10 times higher concentration of IAA relative to IBA was present as a competitor (100 μM IAA versus 10 μM IBA) (Fig. 2D). These results indicate that IBA is a better substrate for NPF7.3 than IAA. On the other hand, no significant effects on IBA uptake were observed even when the concentration of potassium nitrate (KNO3) was 10,000 times higher than that of IBA (100 mM KNO3 versus 10 μM IBA) in the reaction buffer.

Mutations in NPF7.3 Reduced Endogenous IBA Levels. To determine the possible involvement of NPF7.3 in IBA transport in vivo, we first measured the endogenous IBA and IAA content in plant tissues. We found that IBA levels in npf7.3 roots were reduced to approximately one-half of the wild-type levels (Fig. 3), whereas IBA in the shoots of both wild type and npf7.3 was undetectable. In contrast, IAA levels in both roots and shoots were unchanged in npf7.3 compared to wild type. We also determined endogenous levels of IAA metabolites in roots; however, these compounds accumulated to similar levels both in wild type and npf7.3 (SI Appendix, Fig. S2). In addition, no major difference was observed for endogenous levels of other plant hormones between wild type and npf7.3 (SI Appendix, Fig. S3).

Exogenous Supplementation with IAA but Not IBA Restored Defective Gravitropic Responses of npf7.3. IBA is proposed to be converted to IAA through a process similar to fatty acid β-oxidation in peroxisomes, at least when applied exogenously to Arabidopsis (30). Therefore, we hypothesized that IBA taken inside the cells mediated by NPF7.3 has to be converted to IAA to induce physiological responses. To test this hypothesis, we investigated the effects of exogenous IBA and IAA treatments on root gravitropism of npf7.3. In control media, the angles of wild-type roots were mostly 90°, whereas npf7.3 roots responded less to gravity and had larger root angles compared to wild type (~120°). Addition of 10 or 50 nM IAA to the media did not affect the wild-type responses to gravity; however, the defective gravitropic responses observed in npf7.3 were restored at least partially when 50 nM IAA was present (Fig. 4). IBA supplementation (10 to 200 nM) slightly increased the population of wild-type roots.
indices (length. The gravitropic index is defined as the ratio of the root tip ordinate.

**Fig. 1.** Wavy root growth and altered root gravitropism observed in npf7.3. (A) Representative images of 7-d-old wild-type (WT) and npf7.3 (npf7.3-4 and npf7.3-6) seedlings grown on half-strength MS media containing 1.5% (wt/vol) agar and 1% (wt/vol) sucrose. (Scale bar, 1 cm.) (B-F) Lengths (B), wave numbers (C), wave lengths (D), straightness indices (E), and gravitropic indices (F) of primary roots measured in 7-d-old WT and npf7.3 (npf7.3-4 and npf7.3-6) seedlings. The straightness index is defined as the ratio of the direct distance from the origin of the root to the root tip and the primary root length. The gravitropic index is defined as the ratio of the root tip ordinate and the primary root length. Dots represent individual measurements, that responded less to gravity, but the majority of roots were still bent at a 90° angle. In these conditions, in contrast to the case with IAA treatment, IBA did not improve the impaired gravitropism of npf7.3.

Although the bulk IAA content in root tissues was comparable between wild type and npf7.3, local auxin activities might be affected in npf7.3 in the absence of cellular IBA uptake and, hence, subsequent IBA-to-IAA conversion. We first investigated the activities of an artificial auxin-inducible promoter DR5 (31) using a green fluorescent protein (GFP) or a β-glucuronidase (GUS) reporter (DR5rev:GFP and DR5:GUS, respectively). As reported previously, GFP fluorescence and GUS staining were observed in primary root tips and vascular tissues in the wild-type background grown on vertical plates (Fig. 5A and B). Similar expression patterns were observed in the npf7.3 background; however, the signal intensities were significantly reduced in the mutant. We then examined how wild type and npf7.3 respond to exogenous auxin in terms of DR5 promoter activities (Fig. 5C).

In the wild-type background, DR5:GUS expression was induced by both IBA and IAA in whole root tissues with higher expression levels in the root tip regions. DR5:GUS expression was induced by both IBA and IAA in the npf7.3 background as well. However, the responses to IBA were much reduced in the mutant compared to wild type, especially in root tips and elongation zones, whereas IAA treatment induced marker gene expression similarly in the wild-type and mutant backgrounds. We further analyzed the effects of root reorientation on the expression of DR5rev:GFP. When plants were grown on vertical plates, GFP signals were distributed symmetrically in the root tip regions of wild type and npf7.3, although lower signal intensities were observed for npf7.3 (Fig. 5A). The distribution of GFP signals was changed in the wild-type background within 4 h after rotating the plates 90° with higher intensities in the undersides, whereas the asymmetric distribution of GFP signals was less prominent in the npf7.3 mutant background (Fig. 5D). We also tested the effects of IBA and IAA on DR5rev:GFP expression (Fig. 5E). In the wild-type background, both IBA and IAA treatments enhanced auxin-responsive GFP signals and their asymmetric distribution in the root tip regions. IBA treatment, however, was not effective in inducing an asymmetric DR5rev:GFP expression, whereas IAA treatment restored asymmetric auxin responses in the npf7.3 background to the wild-type background level. These results are consistent with the observation that IAA but not IBA treatment restored the disrupted root gravitropism observed in npf7.3.

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Watanabe et al.
Complementation of Root Gravitropic Responses in npf7.3 by NPF7.2 That Has an IBA Transport Activity. NPF7.2, known as the low-affinity nitrate transporter NRT1.8, is the closest homolog of NPF7.3 (26). In many cases, homologous proteins have similar functions. Thus, we examined whether NPF7.2 could also transport IBA and found this to be the case (Fig. 6). NPF7.2 and NPF7.3 might play independent physiological roles in plants since their expression patterns are different (SI Appendix, Fig. S4) (25, 32). We reasoned that if IBA is the in vivo substrate of NPF7.3, NPF7.2 should be able to replace the function of NPF7.3 when expressed in the npf7.3 mutant background under the control of the NPF7.3 native promoter. Transgenic npf7.3 plants expressing NPF7.2 CDS under the control of a 2.1-kb promoter region of NPF7.3 (NPF7.3prom:NPF7.2npf7.3) showed gravitropic responses similar to wild type. In contrast, NPF4.6/NRT1.2/AIT1, an ABA transporter and also known as a low-affinity nitrate transporter, did not restore the impaired gravitropic responses observed in npf7.3.

Expression Patterns of NPF7.3. Previous studies have shown by in situ hybridization and histochemical analyses of promoter-GUS reporter lines that NPF7.3 was expressed in the pericycle cells adjacent to xylem (xylem pole pericycle cells) in mature root tissues (23, 33). This observation is supported by high-resolution and genome-wide Arabidopsis gene expression profiling of root tissues (SI Appendix, Fig. S4) (25, 32). The transcriptome analysis also suggested that NPF7.3 is expressed in the stelar of the root tip region as well. To investigate spatial expression patterns of NPF7.3 in detail, we generated transgenic Arabidopsis expressing GUS or GFP fused to a nuclear localization signal (NLS) under the control of the NPF7.3 promoter that was used for mutant complementation (Figs. 1F and 6). In accordance with previous reports, strong GUS activities were detected in the xylem pole pericycle cells in mature root zones; however, we detected GUS staining also in the pericycle cells close to phloem tissues to a lesser extent (Fig. 7 A and B). We further confirmed that GFP fluorescence was detected widely in pericycle cells (Fig. 7 E and F). In addition, we detected GFP fluorescence and GUS activities in the stelar of the root tip region and in columella root cap cells (Fig. 7 C, D, G, and H).

Effects of npf7.3 Mutations on IBA-Induced Lateral Root Formation. That exogenous IBA treatment induces lateral root formation is well documented (34). Thus, we determined whether this response is affected in npf7.3 (SI Appendix, Fig. S5). Although the number of lateral roots was comparable between wild type and npf7.3 when grown on hormone-free media, npf7.3 produced more lateral roots than wild type in response to IBA treatment. Furthermore, primary root elongation was less severely inhibited in npf7.3 compared to wild type. Since the npf7.3 response to IAA was similar to that of wild type, npf7.3 is likely to be hypersensitive to IBA in lateral root formation, as reported for pen3 ((15) and Fig. 5), but less sensitive to IBA in its inhibition of primary root elongation.

Discussion

Although NPF proteins were originally identified as nitrate or di/tripeptide transporters (18), it is now evident that the protein family transports many different compounds (19). The presence of a relatively large number of family members in a given plant species (e.g., 53 homologs in Arabidopsis) indicates that their biochemical and physiological functions are diverse (19). Upon cultivating several Arabidopsis mutants defective in NPFs, we first noticed a wavy root growth phenotype in npf7.3 unlike that of wild type (Fig. 1 A–F). Wavy patterns of root growth are influenced by several environmental factors such as gravity (35). We found that npf7.3 roots had a reduced ability to change their growth direction toward gravity compared to wild type (Fig. 1 G and H). This observation is somewhat contradictory to previous
studies since straight root growth with fewer wave numbers was reported to be associated with defective gravitropism, and vice versa (28, 29). Although the reason is unknown, it is possible that differences in growth conditions affected the phenotypes. In previous studies, plants were grown on inclined plates whereas we grew plants on vertical plates in this study. Also, we determined root gravitropic responses in the absence of sucrose, a media component normally present in previous studies. In any case, our observations indicate that NPF7.3 plays an important role in root gravitropism. NPF7.3 has been characterized previously as a nitrate and potassium transporter (23, 24). However, it was not tested in the present study since high amounts of IBA would rescue the phenotype by passive diffusion. Thus, IBA is now considered a precursor of IAA, although the Kmax values for the two substrates were comparable (Fig. 2C). Therefore, it is possible that IBA is the preferred substrate of NPF7.3 in vivo, even though endogenous IBA levels are significantly lower than IAA levels (Fig. 3). Interestingly, it was shown recently that Xenopus oocytes expressing NPF7.3 took up significantly lower amounts of plant hormones including ABA, GA, and JA-Ile compared to control oocytes injected with water (46). This is possibly because NPF7.3 affected proton uptake and hence pH inside the oocytes, which in turn influenced membrane permeabilities of the chemicals indirectly. We speculate that NPF7.3 is a multifunctional transporter.

As mentioned above, IBA is converted to IAA (14, 37, 38). We therefore hypothesized that IBA taken into cells mediated by NPF7.3 is converted to IAA to induce root gravitropic responses. If this hypothesis is correct, exogenous application of IAA would complement the phenotype observed in npf7.3. In contrast, IBA treatment would be less effective in rescuing the npf7.3 phenotype since the mutant is impaired in IBA uptake into cells and thus not able to convert IBA to IAA efficiently. As expected, IBA, but not IAA, was able to restore the altered gravitropic responses in the npf7.3 mutant roots in our experimental conditions (Fig. 4). One may expect that application of an excess amount of IBA would rescue the phenotype by passive diffusion. However, it was not tested in the present study since high

**Fig. 3.** Endogenous levels of IBA and IAA in npf7.3. IBA (A) and IAA (B) levels in shoots and roots of 14-d-old wild-type (WT) and npf7.3 (npf7.3-4 and npf7.3-6) seedlings were quantified. Bars indicate SDs of the means. Dots represent individual measurements. Asterisks indicate significant differences compared with WT (*P < 0.05; **P < 0.01 by Tukey’s multiple comparison test).

was acidic (Fig. 2A and B), although we cannot exclude the possibility that NPF7.3 recognizes protonated IAA and IBA as substrates. The pH values for the apoplasm and cytosol are estimated to be 5.0 to 6.0 and 7.2 to 7.4, respectively (43, 44). Therefore, it is reasonable to propose again that NPF7.3 is an IAA/IBA importer. IAA and IBA are weak acids (negative logarithm of the acid dissociation constant = 4.75 and 4.83, respectively) with a carboxylic group and exist as protonated or ionized forms depending on pH (45). Their protonated forms can passively move through biological membranes relatively easily because of their hydrophobic natures. Thus, one may imagine that specific transporters are not required especially when IAA and IBA are taken into cells; however, at least for IAA uptake, the existence of specific transporters is evident (9).

Fig. 2 shows that IBA is less permeable to biological membranes compared to IAA. This observation suggests that cellular IBA uptake is also mediated by specific transporters. In our yeast transport assays, NPF7.3 transported IBA more efficiently than IAA, although the Kmax for the two substrates were comparable (Fig. 2C). Thus, it is possible that IBA is the preferred substrate of NPF7.3 in vivo, even though endogenous IBA levels are significantly lower than IAA levels (Fig. 3). Interestingly, it was shown recently that Xenopus oocytes expressing NPF7.3 took up significantly lower amounts of plant hormones including ABA, GA, and JA-Ile compared to control oocytes injected with water (46). This is possibly because NPF7.3 affected proton uptake and hence pH inside the oocytes, which in turn influenced membrane permeabilities of the chemicals indirectly. We speculate that NPF7.3 is a multifunctional transporter.

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concentrations of chemicals often induce side effects. These data suggest that auxin responses are reduced in npf7.3. Although bulk IAA content in whole root tissues was comparable between wild type and npf7.3 (Fig. 3), it is still possible that IAA levels are affected in very limited areas. We observed that auxin responses determined by DR5rev:GFP and DR5:GUS reporters were significantly lower in the root tip region of npf7.3 compared to wild type (Fig. 5 A and B). Although DR5:GUS expression in root tissues was similarly induced by exogenous IAA in wild-type and npf7.3 backgrounds, the reporter had a lower response to IBA in npf7.3 than the wild type (Fig. 5C). In addition, we found that the asymmetric distribution of auxin activities observed after reorientation of the roots was not clearly observed in the npf7.3 mutant background (Fig. 5D). Furthermore, npf7.3 roots treated with IAA, but not with IBA, showed asymmetric expression patterns of DR5rev:GFP upon reorientation (Fig. 5E). Finally, we confirmed that the expression of an NPF protein having an IBA transport activity (NP7.2) in the npf7.3 background under the control of the NP7.3 promoter complemented the npf7.3 phenotype (Fig. 6). Collectively, these data consistently support the idea that NP7.3 mediates cellular IBA uptake in root tissues, and a defect in this process results in a reduced ability to synthesize IAA from IBA and to establish proper auxin (IAA) gradients within roots in response to gravity.

Many textbooks illustrate that IAA is actively synthesized in the shoot apex and transported toward the root tips via phloem tissues and/or cell-to-cell polar transport mechanisms (9). Recent studies, however, have demonstrated that IAA synthesized locally in roots also plays important roles in root growth and development (47, 48), even though it is largely unknown how IBA is transported within plants. Given that IBA levels are reduced in npf7.3 roots compared to wild type, NP7.3 might play a role in retaining IBA in root tissues and/or IBA transport from shoots to roots. In this study, we found that NP7.3 was expressed predominantly in pericycle cells in mature root tissues, whereas we were unable to detect its presence in above ground tissues (Fig. 7 A, B, E, and F). Perhaps IBA is normally transported from roots to shoots via xylem in wild type, and NP7.3 facilitates IBA uptake into pericycle cells from xylem. In fact, IBA was reported to be transported from roots to shoots at least when applied exogenously (49). In this case, the loss of NP7.3 function would increase the amount of IBA translocated from roots to shoots and, thus, bulk IBA levels in the roots would be reduced. This hypothesis is not fully proven yet since we were unable to detect IBA in shoot tissues possibly due to imperfections affecting liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis and/or rapid degradation of IBA in shoots. Interestingly, we also found that NP7.3 was expressed in the root tip region (stele cells and columella root cap cells) of primary roots (Fig. 7 C, D, G, and H). Columella root cap cells are considered to be important hubs to perceive and transmit gravity signals and to generate asymmetric IAA distribution (9), IBA-to-IAA conversion is active in the same region (50, 51), indicating that NP7.3 is required for efficient IAA production from IBA. IAA synthesized from IBA could be exported from columella root cap cells upon reorientation, thereby rapidly inducing an auxin response on the gravity side. Again, the site of IBA synthesis is not known. However, we propose that part of the IBA taken into pericycle cells from xylem is transported to the root tip regions through phloem tissues and/or other unknown transport mechanisms. It is also possible that IBA is converted to IAA in pericycle cells and then transported to the shoot apex.
The NPF7.3 promoter (NPF7.3pro:NPF7.2) showed altered responses to exogenous IBA treatment. 

We showed that NPF7.2 also has an IBA transport activity; however, the gene is not expressed in the root tip region according to a public database. Our study demonstrates the role of endogenous IBA in regulating root gravitropic responses. Why the IBA pathway is required to fully induce gravitropism in addition to the main IAA biosynthesis pathway remains an enigma. Furthermore, it will be important to establish how IBA is synthesized in plants. A public database indicates that the expression of NPF7.3 is regulated by biotic and abiotic stresses. Thus, it will also be informative to know whether the expression of NPF7.3 is related to IBA transport. If not, what is the substrate of NPF7.3, and what is the physiological role of the protein under stressed conditions? These questions will be answered in future studies.

**Methods**

**Plant Materials and Growth Conditions.** Arabidopsis [Arabidopsis thaliana (L.) Heynh.] accession Columbia-0 (Col-0) was used as the wild type for all experiments in this work. Mutant lines obtained from the Arabidopsis Biological Resource Center used in experiments were as follows: npf7.3-4

![Fig. 6.](image)

Complementation of npf7.3 with another NPF protein having IBA transport activity. Activities of NPF7.2 and NPF4.6 to transport IBA were determined in yeast when the substrate concentration was 10 μM (Left). Yeast cells containing the empty pYES2-DEST52 vector were analyzed as the control. Asterisks indicate significant differences compared with the controls (**P < 0.001 by Student’s t-test). Root angles of wild type (WT), npf7.3 (npf7.3-4 and npf7.3-6), and npf7.3 expressing NPF7.2 or NPF4.6 under the control of the NPF7.3 promoter (NPF7.3pro:NPF7.2 and NPF7.3pro:NPF4.6, respectively) were measured 24 h after the first rotation (Right). Horizontal bars represent the median, boxes represent the middle 50% of the distribution, and whiskers represent the entire spread of the data. Different letters indicate statistically significant differences by Dunn’s multiple comparison test (P < 0.05).

![Fig. 7.](image)

Spatial expression patterns of NPF7.3 in roots. (A–D) GUS-stained primary roots of NPF7.3pro:GUS transgenic plants. Seven-day-old seedlings grown on vertical plates were subjected to GUS staining at 37 °C for 24 h. (A) GUS activities in the mature root zone of a primary root. (B) Transverse sections of the mature root zone of a primary root. (C and D) GUS activity in the root tip of a primary root. (E–H) Representative images of GFP fluorescence in transgenic plants expressing GFP fused to an NLS under the control of NPF7.3 promoter (NPF7.3pro:GFP-NLS). (E and F) Longitudinal sections (Upper) and transverse sections (Lower) of mature root tissues of the primary roots. Dashed lines in the upper panels indicate the positions of the transverse sections. (G and H) Longitudinal sections of a primary root tip (G) and a magnified image of the root cap (H). T1 plants were selected on half-strength MS plates (placed horizontally) containing 25 mg/L hygromycin and 0.8% (wt/vol) agar for 5 d and transferred onto vertical plates without antibiotics. GFP fluorescence was observed after subsequent growth for 7 d. GFP fluorescence and propidium iodide staining of cell walls are shown in green and magenta, respectively. (Scale bars, 50 μm.) Arrowheads indicate the positions of xylem pole pericycle cells.
were vertically placed in growth chambers at 22 °C under continuous light. Cross was not added to the media unless otherwise indicated. To break dormancy, seeds were incubated at 4 °C for 3 d in the dark, and the plates were vertically placed in growth chambers at 22 °C under continuous light.

The extracts were dissolved with 50 mM potassium phosphate buffer (KPB) (pH 5.8 or 7.0) and centrifuged and then resuspended with SG media containing 2% (wt/vol) galactose and 1% (wt/vol) raffinose without uracil (SG, –Ura) to induce the production of NPF proteins. After centrifugation, yeast cells were resuspended in 1% (vol/vol) acetic acid by vortexing and sonication and then completely dried under N2 flow at 40 °C. The samples were resuspended in 1% (vol/vol) acetic acid by vortexing and sonication and then completely dried under N2 flow at 40 °C. The extracts were dissolved in 50 mM potassium phosphate buffer (KPB) (pH 5.8 or 7.0). The extracts were dissolved with 50 mM potassium phosphate buffer (KPB) (pH 5.8 or 7.0) and incubated with the buffer containing IBA or IAA at 25 °C for 10 min. For competition assays, the cells were incubated with IBA and either potassium nitrate or IAA. Concentrations of the substrates are indicated in the figure legends. Yeast cells were then collected by centrifugation and followed by washing three times with 50 mM KPB (pH 7.0). We defined the end of the reactions as the first wash with KPB (pH 7.0). The cells were stored at –80 °C until extraction. Quantification of IBA and IAA by LC-MS/MS was performed as described above.

Hormone Measurements. To extract IBA and IAA from plant tissues, lyophilized samples were homogenized by vortexing with zirconia beads in 80% (vol/vol) acetonitrile containing 1% (vol/vol) acetic acid and isotope-labeled IBA (13C8, 15N1)-IAA) and/or IAA ([methylene-2H2]-IAA) as internal standards. Homogenates were incubated in the dark for 16 h at 4 °C and then centrifuged at 3,000 × g for 5 min at 4 °C. The supernatants were dried under N2 flow at 40 °C. The samples were resuspended in 1% (vol/vol) acetic acid and sonicated for 10 min. The samples were then combined and then sonicated for 10 min. The extracts were dissolved with 50 μL 1% (vol/vol) acetic acid and subjected to LC-MS/MS analysis.

To extract IBA and IAA from yeast cells, frozen samples were incubated for 2 h at 4 °C in 80% (vol/vol) acetonitrile containing 1% (vol/vol) acetic acid and [methylene-2H2]-IAA) and/or [methylene-2H2]-IAA) as internal standards. Supernatants were collected by centrifugation at 20,000 × g for 5 min and then completely dried under N2 flow at 40 °C. The samples were resuspended in 1% (vol/vol) acetic acid by vortexing and sonication and then loaded onto Oasis WAX cartridges (Waters) preconditioned with 0.1 M potassium hydroxide, and 1% (vol/vol) acetic acid, followed by washing with 20% (vol/vol) acetonitrile containing 1% (vol/vol) acetic acid. IBA and IAA were eluted with 60% (vol/vol) acetonitrile containing 1% (vol/vol) acetic acid and then completely dried under N2 flow. The extracts were dissolved with 50 μL 1% (vol/vol) acetic acid and subjected to LC-MS/MS analysis.

In the experiments presented in SI Appendix, Fig. S3, IAA, IAA–α-amino acid conjugates (IAA–aspartate (IAA-Asp) and IAA–glutamate (IAA-Glu)), and 2-oxoindole-3-acetic acid (OIAA) were extracted and purified as described previously (57). In this analysis, [phenyl-13C6]-IAA was used as an internal standard instead of [methylene-2H2]-IAA.

Endogenous ABA, jasmonic acid (JA), JA-ile, trans-zetatin (tZ), isopentenyl adenine (iP), and salicylic acid (SA) were extracted and purified as described previously (55).

IAA-Asp, IAA-Glu, and oxIAA were analyzed using an Agilent 6420 Triple Quadrupole Mass Spectrometer (Agilent) equipped with a ZORBAX Eclipse XDB-C18 column. Conditions for LC and MS/MS analyses were the same as described previously (57). For analyzing other chemicals, a Nexera high-performance liquid chromatography (HPLC) system (Shimadzu) coupled with a quadrupole/time-of-flight tandem mass spectrometer (Triple TOF 5600; AB Sciex) was used. Conditions for LC and for MS/MS analyses of IBA and IAA are summarized in SI Appendix, Tables S2 and S3. LC and MS/MS conditions for other chemicals were the same as previously described (55).

Chemicals. IBA, IAA, [methylene-2H2]-IAA, and ABA were purchased from Sigma-Aldrich. [13C8, 15N1]-IBA was synthesized according to the literature (58) from [13C8, 15N1]-indole (CNLM-4786-0.1; Cambridge Isotope Laboratories) and was purified by silica gel column chromatography (CHCl3-MeOH = 10:1). The purity of isolated [13C8, 15N1]-IBA was determined by HPLC in combination with authentic [methylene-2H2]-IBA. Isopentenyl 1-3H2-oxIAA, [Phenyl-13C6]oxIAA, [13C15N]-IAA-Asp, and [13C15N]-IAA-Glu were synthesized (12, 59). JA, JA-ile, and [13C8]tZ were gifts from Yusuke Jikumaru, RIKEN, Yokohama, Japan. [3H]ABA was purchased from Tokyo Chemical Industry Co. [3H]J2A was purchased from OIChemical. [3H]IAA was purchased from Isotec.
pENTR4-GFP-NLS by PCR using primers NRT7.3pro-GFP-NLS-F and NRT7.3pro-GFP-NLS-R. The PCR fragments were cloned using an In-Fusion Cloning Kit with the linear pENTR-NPF7.3pro vector amplified by inverse PCR using primers pENTR-NPF7.3pro-F and pENTR-NPF7.3pro-R. The construct was cloned into the pGBWB501 vector (61) by LR reactions. The final recombinant binary vector was introduced into GV3101 by electroporation and transformed into plants by the floral dip method.

Histochemical Gus Analysis. Gus activity was detected using a staining solution composed of 50 mM sodium phosphate buffer (pH 7.2), 10 mM ethylene glycol betadecanoate, 0.05% (vol/vol) Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 1 mM X-Gluc. Samples were mounted in chloral hydrate after stopping the reaction with 70% (vol/vol) ethanol and then observed using an Olympus BX53 microscope (Olympus).

For the preparation of root sections, Gus-stained samples were fixed with 0.05 M sodium cacodylate buffer (pH 7.4) containing 4% (wt/vol) paraformaldehyde and 2% (vol/vol) glutaraldehyde overnight at 4 °C. Fixed root samples were dehydrated through a graded ethanol series and embedded in Technovit 7100 resin (Kulzer). Thin sections (1 μm thick) were obtained using a glass knife on an ultramicrotome (EM UC7; Leica) and then observed using a fluorescence microscope (Olympus).

GUS activity was detected using a staining solution composed of 50 mM sodium phosphate buffer (pH 7.2), 10 mM ethylene glycol betadecanoate, 0.05% (vol/vol) Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 1 mM X-Gluc. Samples were mounted in chloral hydrate after stopping the reaction with 70% (vol/vol) ethanol and then observed using an Olympus BX53 microscope (Olympus).

Microscopic Observations of Gfp Signals. Gfp-fluorescent images were obtained using a confocal laser scanning microscope LSM700 (Carl Zeiss). Root tips were excised from whole seedlings and then stained with a 30 μM propidium iodide solution to visualize cell walls. Spatial distributions and intensities of Gfp signals were quantified using the ZEN software (Carl Zeiss).

Statistical Analysis. Significant differences between two groups were determined by Student’s t test. Significant differences among three or more groups were evaluated by Tukey’s or Dunn’s multiple comparison test. All statistical tests were performed using statistical software Prism 8 (GraphPad Software Inc.) or R ver. 3.6.1 (R Core Team) in RStudio ver. 1.2.5001 (RStudio Team).

Data Availability. All study data are included in the paper and in Appendix.
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