

NPY Genes Play an Essential Role in Root Gravitropic Responses in *Arabidopsis*

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ABSTRACT Plants can sense the direction of gravity and orient their growth to ensure that roots are anchored in soil and that shoots grow upward. Gravitropism has been studied extensively using *Arabidopsis* genetics, but the exact mechanisms for gravitropism are not fully understood. Here, we demonstrate that five *NPY* genes play a key role in *Arabidopsis* root gravitropism. *NPY* genes were previously identified as regulators of auxin-mediated organogenesis in a genetic pathway with the AGC kinases *PID*, *PID2*, *WAG1*, and *WAG2*. We show that all five *NPY* genes are highly expressed in primary root tips. The single *npv* mutants do not display obvious gravitropism defects, but the *npv1 npv2 npv3 npv4 npv5* quintuple mutants show dramatic gravitropic phenotypes. Systematic analysis of all the *npv* double, triple, and quadruple combinations demonstrates that the five *NPY* genes all contribute to gravitropism. Our work indicates that gravitropism, phototropism, and organogenesis use analogous mechanisms in which at least one AGC kinase, one *NPH3/NPY* gene, and one ARF are required.

Key words: Light signaling; signal transduction; development; root biology; auxin.

INTRODUCTION

Plants reorient their growth in response to changes in directional signals such as light and gravity. Both phototropic and gravitropic responses allow plants to orient their roots and shoots in the right directions to ensure their survival. *Arabidopsis* shoots grow toward a light source whereas roots grow away from the light source. Both shoots and roots can also sense the direction of gravity and adjust their growth directions accordingly. Roots grow toward the direction of gravity while shoots grow against the direction of gravity. Gravitropism allows plants to anchor their roots in soil for nutrient uptake and to grow their stems straight in the air. Phototropism ensures that plants receive sufficient light for photosynthesis and plant growth.

A common factor that affects both gravitropism and phototropism is the plant hormone auxin (Morita, 2010; Zourelidou et al., 2009). Molecular genetic studies in *Arabidopsis* have demonstrated that auxin biosynthesis, polar transport, and signaling are essential for plants to properly respond to gravity stimuli. The auxin biosynthetic gene *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/SHADE AVOIDANCE3/TRANSPORT INHIBITOR RESPONSE2 (TAA1/SAV3/TIR2)* is expressed asymmetrically in response to gravistimuli and *TIR2* contributes to a positive regulatory loop required for root gravitropism (Yamada et al., 2009). Mutations in the auxin influx carrier

AUX1 completely abolish *Arabidopsis* root gravitropic curvature (Bennett et al., 1996). Disruption of the auxin efflux carrier *PINFORMED 2/WAVY GROWTH 6/ETHYLENE INSENSITIVE ROOT 1/AGRAVITROPIC 1 (PIN2/WAV6/EIR1/AGR1)* also affects root gravitropism (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998). *Arabidopsis* plants treated with the auxin transport inhibitor N-1-Naphthylphthalamic Acid (NPA) display a complete agravitropic root growth (Marchant et al., 1999). Auxin signaling components *INDOLE-3-ACETIC ACID 3/SHORT HYPYCYOTYL 3 (IAA3/SHY2)* (Tian and Reed, 1999), *AUXIN RESPONSE FACTOR 7 (ARF7)*, and ARF 19 also play a pivotal role in root gravitropism (Li et al., 2006; Okushima et al., 2005; Wilmoth et al., 2005). Auxin is also essential for shoot gravitropic responses because NPA treatments or dominant mutations in *IAA19* and *IAA7/AUXIN RESISTANT 2 (AXR2)* lead to agravitropic shoot growth (Nagpal et al., 2000; Tatematsu et al., 2004). The critical role of auxin in phototropism was demonstrated by many early

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physiological studies (Holland et al., 2009). Inactivation of the auxin influx carrier *AUX1* leads to defects in phototropism in *Arabidopsis* hypocotyls (Stone et al., 2008). *Arabidopsis* genetics has also uncovered important auxin signaling components required for proper phototropic responses (Harper et al., 2000; Tatematsu et al., 2004; Yang et al., 2004). *Arabidopsis non-phototropic hypocotyl 4 (nph4)/arf7* mutants cannot curve toward directional blue light (Harper et al., 2000). Dominant mutations in *IAA19* also abolish phototropism in hypocotyls (Tatematsu et al., 2004). Interestingly, *ARF7* is a shared component in gravitropism and phototropism, suggesting that auxin plays a very similar role in both types of tropism-mediated directional growth.

The first step of root gravitropic response is to sense the direction of gravity—a process that is still poorly understood. Gravity sensing is believed to take place in the specialized cells called statocytes. Statocytes are located at the root cap and contain dense, starch-filled amyloplasts that may provide information of the direction of gravity when changing positions inside the statocytes in response to gravity changes (Morita, 2010). However, it is not clear how gravity signals are converted to chemical signals that eventually lead to the differential growth. In contrast, the molecular mechanism of phototropism is much better defined. *Arabidopsis* molecular genetic studies have defined several *non-phototropic hypocotyl* loci (*nph1*, *nph2*, *nph3*, and *nph4*) important for phototropism in hypocotyls (Liscum and Briggs, 1995). As discussed above, *NPH4* encodes the *ARF7* (Harper et al., 2000). *NPH1* and *NPH2* encode the photoreceptor PHOTOTROPIN1 (PHOT1) and PHOTOTROPIN2 (PHOT2), respectively (Sakai et al., 2001). PHOT1 and PHOT2 share 58% amino acid sequence identity and are responsible for the perception of directional blue light. Phototropins contain two light-sensing LOV (light, oxygen, or voltage) domains at the N-terminal region and a Ser/Thr kinase domain at the C-terminal region (Christie et al., 1998; Huala et al., 1997). Upon photo-excitation of the LOV domains in phototropins, the C-terminal kinase domain is activated and consequently the photoreceptor is autophosphorylated (Christie et al., 1998). Therefore, the phototropins define the starting point of a signaling pathway ultimately responsible for the phototropic curvature. PHOT1 is localized on plasma membrane, but upon blue light treatment, a small fraction of PHOT1 is released to the cytoplasm (Sakamoto and Briggs, 2002; Wan et al., 2008). *NPH3*, which encodes a plant-specific, plasma membrane-bound protein, has been shown to play an essential role in phototropic response (Motchoulski and Liscum, 1999). Inactivation of *NPH3* completely abolishes the curvature of hypocotyls to directional blue light (Motchoulski and Liscum, 1999). *NPH3* belongs to a large family with 32 members in the *Arabidopsis* genome. Interestingly, *RPT2* (Inada et al., 2004), an *NPH3* homolog, is required for root phototropic responses. Both *NPH3* and *RPT2* physically interact with PHOT1 (Inada et al., 2004; Motchoulski and Liscum, 1999), but the exact mechanisms for *NPH3* and *RPT2* in phototropism are not understood. However, it is known that *NPH3* is phosphorylated in the dark and dephos-

phorylated in light (Pedmale and Liscum, 2007). It has been suggested that an unidentified phosphatase is activated by phototropins directly or indirectly upon light perception. It is also suggested that *NPH3* is phosphorylated by an unknown kinase other than PHOT1 and PHOT2 (Pedmale and Liscum, 2007). Although the entire phototropism signaling pathway has not been solved, it is clear that PHOT1/PHOT2, *NPH3/RPT2*, and *ARF7* are important for the phototropic pathway.

We previously reported the isolation of an *Arabidopsis* mutant called *np1* (*naked pins in yucca (yuc) mutants 1*) that formed pin-like inflorescences in the *yuc1 yuc4* double-mutant background, but not in wild-type background (Cheng et al., 2007). Mutant *np1* is allelic to *enhancer of pinoid (pid) 1 (enp1)* (Trembl et al., 2005) and *macchi-bou 4 (mab4)* (Furutani et al., 2007), which were isolated as *pid* enhancers. The *yuc1 yuc4* double mutants are defective in auxin biosynthesis (Cheng et al., 2006; Zhao et al., 2001). *PID* is involved in regulating auxin transport/signaling (Benjamins et al., 2001; Christensen et al., 2000). Interestingly, *NPY1* is homologous to *NPH3*, which is known to participate in phototropism. Although *np1* alone does not have obvious developmental defects, simultaneous, inactivation of *NPY1* and two of its close homologs *NPY3* and *NPY5* leads to the formation of pin-like inflorescences (Cheng et al., 2008), a phenotype also observed in *pin1* (Galweiler et al., 1998), *pid* (Christensen et al., 2000), *yuc1 yuc4 np1* triple mutants (Cheng et al., 2007), and *monopteros (mp)* (Przemeck et al., 1996). *PID* is a Ser/Thr kinase that is homologous to PHOT1 and PHOT2 and belongs to the AGC kinase super-family. AGC kinase is the collective name for cAMP-dependent protein kinase A, cGMP-dependent protein kinase G, and phospholipids-dependent protein kinase C (Galvan-Ampudia and Offringa, 2007). *MP* encodes *ARF5* (Hardtke and Berleth, 1998), a close homolog of *NPH4/ARF7*. We proposed that auxin-regulated organogenesis and phototropism use analogous mechanisms for which at least one *NPH3-like* gene, one AGC kinase, and one ARF are required (Cheng et al., 2007, 2008).

Root gravitropic responses require both *ARF7* and *ARF19* because *arf7 arf19* double mutants lack gravitropic responses in roots (Li et al., 2006; Okushima et al., 2005). It is also known that *PID* and its two close AGC kinase homologs *WAG1* and *WAG2* play important roles in root gravitropic responses (Santner and Watson, 2006; Sukumar et al., 2009). The AGC kinases *D6PKs* are also important for root gravitropism (Zourelidou et al., 2009). Therefore, it is likely that gravitropism also employs a mechanism analogous to those used in phototropism and auxin-mediated organogenesis. We hypothesized that *NPH3-like* genes are also required for gravitropic responses because both AGC kinases and ARFs have been demonstrated as regulators of gravitropism. In this paper, we show that five *NPY* genes (*NPY1-5*) contribute to *Arabidopsis* root gravitropic responses. We show that *NPY1*, *NPY2*, *NPY3*, *NPY4*, and *NPY5* are highly expressed in *Arabidopsis* roots with overlapping patterns. We systematically analyzed root gravitropic responses of all the single, double, triple, quadruple, and quintuple combinations of

the *npy* mutants. It is clear that all of the *NPY* genes analyzed in this paper play a role in root gravitropism. The single *npy* mutants did not show obvious defects in gravitropism, but many *npy* mutant combinations displayed significant defects in root gravitropic responses. The strongest gravitropic defects were observed in the *npy1 npy2 npy3 npy4 npy5* quintuple mutants. We conclude that plants use an AGC kinase-NPH3-like protein-ARF module analogous to those in auxin-regulated organogenesis and phototropism to regulate plant root gravitropic responses.

RESULTS

NPY Genes Are Highly Expressed at *Arabidopsis* Primary Root Tip

The AGC kinases PID, WAG1, and WAG2 were shown to regulate *Arabidopsis* root gravitropic response (Santner and Watson, 2006; Sukumar et al., 2009). We previously demonstrated that PID, WAG1, and WAG2 also play a critical role in auxin-mediated cotyledon formation (Cheng et al., 2007, 2008). We showed that the PID/WAG kinases work with *NPY* genes in the same genetic pathway to control organogenesis (Cheng et al., 2007, 2008). Therefore, we hypothesized that *NPY* genes are also important for gravitropism.

NPY1 and its four closest homologs share similar domain structures with a BTB domain at the N-terminal region and an NPH3 domain in the middle (Figure 1A). *NPY1*, *NPY3*, and *NPY5* are essential for the formation of flowers whereas the functions of *NPY2* and *NPY4* are not defined (Cheng et al., 2008). We first investigated whether the *NPY* genes are expressed in *Arabidopsis* primary roots. We used transgenic *Arabidopsis* plants that express the β -D-glucuronidase (GUS) gene under the control of a *NPY* gene promoter to determine the expression patterns of *NPY* genes in roots (Figure 1B). All five *NPY* genes (*NPY1* = At4g31820, *NPY2* = At2g14820, *NPY3* = At5g67440, *NPY4* = At2g23050, *NPY5* = At4g37590) were expressed in tips of *Arabidopsis* primary roots, but they displayed unique and overlapping patterns. *NPY1::GUS* was clearly observed in stele, root cap, and the meristem region (Figure 1B). *NPY2::GUS* displayed the highest expression in the quiescent center, but less expression in the columella root cap. *NPY2::GUS* was also expressed in the proximal meristem region (Figure 1B). Among the five *NPY::GUS* lines, *NPY3::GUS* expression was the only one whose expression was restricted in the vascular tissue (Figure 1B). We did not observe *NPY3::GUS* expression in the epidermis and columella root cap. *NPY4::GUS* had the highest expression levels compared to the other *NPY::GUS* lines (Figure 1B). *NPY4::GUS* was expressed in the root cap and in the meristem region, with the highest expression in the central domain (Figure 1B). The expression pattern of *NPY5::GUS* was very similar to that of *NPY2::GUS*, with the highest expression in the quiescent center (Figure 1B), but *NPY5::GUS* was also expressed throughout other tissues at the root tip (Figure 1B). The expression patterns of the *NPY*

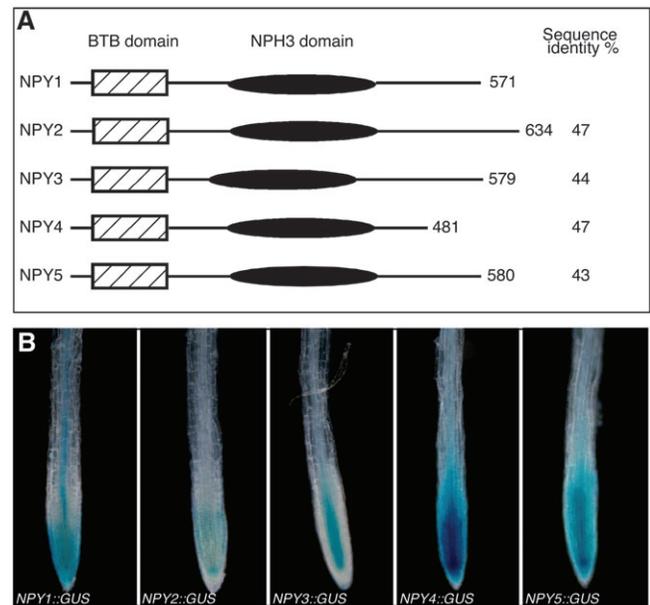


Figure 1. Expression Patterns of *NPY::GUS* in *Arabidopsis* Primary Root Tip.

(A) The domain structures of *NPY* proteins. *NPY1* and its closest homologs have a BTB domain at the N-terminal region and an NPH3 domain in the middle.

(B) *NPY* gene expression patterns in root tip as defined by *NPY* promoter::GUS transgenic plants. The β -glucuronidase gene was under the control of an *NPY* promoter. The GUS lines of *NPY2*, *NPY3*, *NPY4*, and *NPY5* were stained for 15 min. The *NPY1::GUS* line was stained for 2 h. From left to right: *NPY1::GUS*, *NPY2::GUS*, *NPY3::GUS*, *NPY4::GUS*, and *NPY5::GUS*.

genes indicate that they are expressed in root tips and they probably play a role in root development. The expression analysis also suggests that the *NPY* genes probably have overlapping functions in root development.

NPY Genes Are Required for Root Gravitropic Response

We investigated whether inactivation of *NPY* genes had any effects on root gravitropic response. Disruption of a single *NPY* gene did not cause obvious defects in root development (data not shown), probably due to overlapping functions of the *NPY* genes. We then analyzed whether simultaneous inactivation of the five *NPY* genes led to any root phenotypes. We germinated seeds from a single plant that was *npy1^{+/-} npy2 npy3 npy4 npy5* and grew the seedlings on a vertical plate for 7 d at 23°C with 16 h light/8 h dark cycle. The seedlings were photographed, numbered, and genotyped. The *npy1 npy2 npy3 npy4 npy5* quintuple mutants lacked proper gravitropic responses (Figure 2A, arrow). For wild-type, the majority of the plants grew straight down toward the direction of gravity (90°), with a small percentage of seedlings deviating from 85° to 112° (Figure 2B). The roots of the *npy* quintuple mutants were in random directions within a range from 21° to 150° (Figure 2B).

We systematically analyzed all of the double, triple, and quadruple *npy* mutant combinations by growing them on

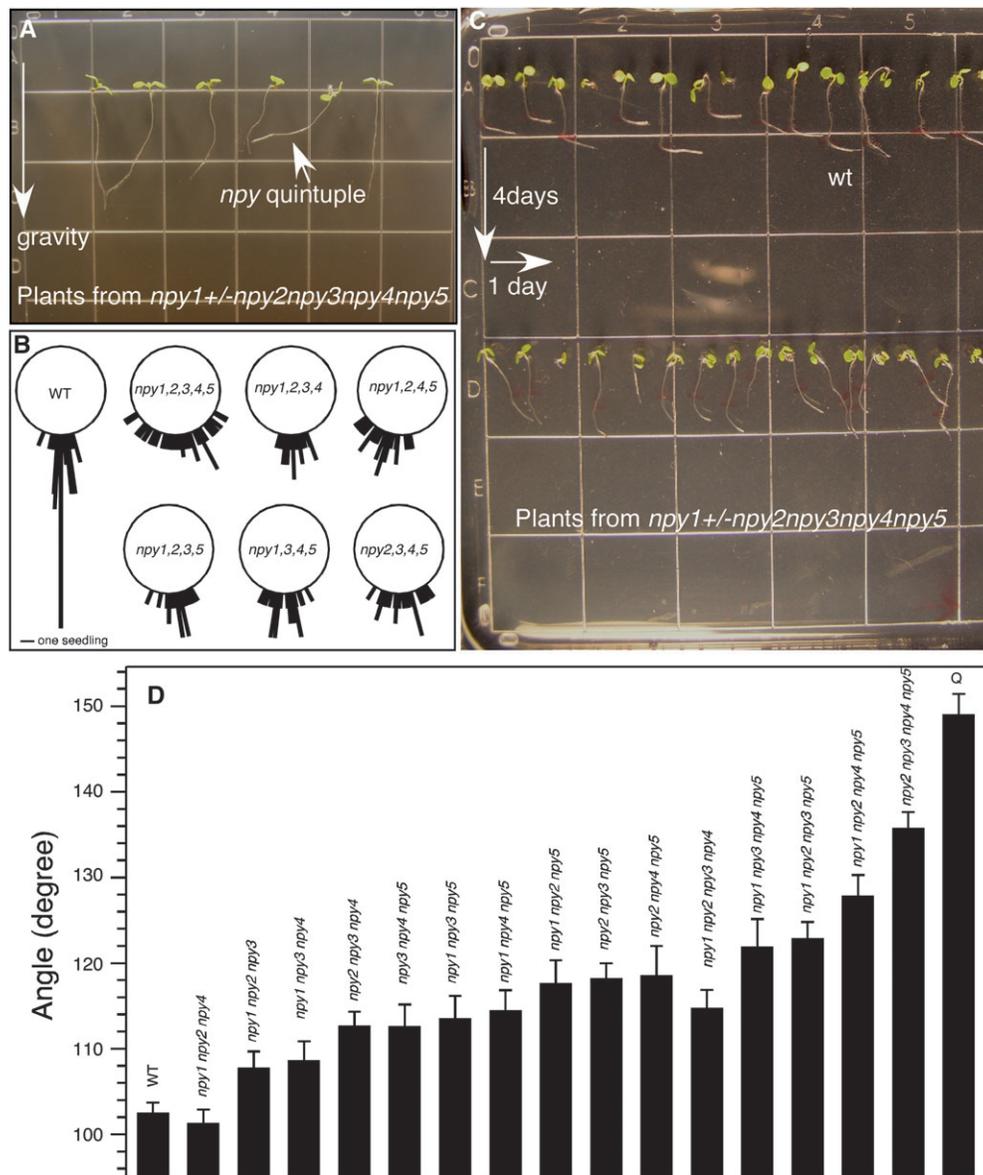


Figure 2. NPY Genes Contribute to Root Gravitropism.

(A) Seeds from a plant of *npv1^{+/-} npv2 npv3 npv4 npv5* were germinated and grown vertically for 7 d. The *npv* quintuple mutants (arrow head) showed defects in gravitropism. The direction of gravity is also marked with an arrow.

(B) Directions of root growth of wild-type and *npv* mutants. Plants were grown vertically for 4 d. The angle between the gravity vector and root were measured and are shown here. The bar refers to one seedling. If two seedlings have the same angle, the line at that angle is twice of the length of the bar. The total number of seedlings analyzed for each genotype were: 49 for WT, 45 for *npv1, 2, 3, 4, 5* quintuple, 24 for *npv1, 2, 3, 4* quadruple, 27 for *npv1, 2, 4, 5* quadruple, 27 for *npv1, 2, 3, 5* quadruple, 32 for *npv1, 3, 4, 5* quadruple, and 28 for *npv2, 3, 4, 5*.

(C) Responses to a directional change of gravity. Plants were grown vertically for 4 d and then the plates were turned 90° for 1 d. The top row is wt and bottom row is plants from *npv1^{+/-} npv2 npv3 npv4 npv5*. Note: wild-type roots bend about 90° while the *npv* mutants do not bend much.

(D) Quantitative analysis of gravitropism in *npv* mutant combinations. Plants were grown vertically for 4 d and the plates were turned 90° for 1 d. The angle of root bending was measured. The angle for perfect gravitropism is 90° and the angle for non-gravitropic growth is 180°. Q refers to the *npv1 npv2 npv3 npv4 npv5* quintuple mutants. At least 20 seedlings were analyzed for each genotype shown in Figure 2D.

vertical plates. The double mutants and triple mutants did not display obvious gravitropic defects in this assay, but the quadruple mutants displayed various degrees of gravitropism defects (Figure 2B). Among the quadruple mutants, *npv1*

npv2 npv4 npv5 and *npv2 npv3 npv4 npv5* had the strongest phenotypes (Figure 2B).

We then used a more sensitive and quantitative assay to determine gravitropism defects among the *npv* mutant

combinations. Seedlings were grown vertically for 4 d and then the plates were turned 90° to change the gravity vector. The angle of root bending was then measured (Figure 2C). Perfect gravitropism leads to the formation of a near 90° angle whereas non-gravitropism will have an angle of 180° (Figure 2C). Wild-type roots bent about 90° to follow the direction of gravity (Figure 2C), but *npv* mutant combinations displayed defects in reorienting their root growth (Figure 2C). In this more sensitive assay, we observed that all of the *npv* triple mutants except *npv1 npv2 npv4* triple mutants displayed significant gravitropism defects (Figure 2D). All of the *npv* quadruple mutants except *npv1 npv2 npv3 npv4* had stronger gravitropism defects than the *npv* triple mutants (Figure 2D). We conclude that all of the *NPY* genes contribute to root gravitropic responses.

Effects of *npv* Mutants on Root Elongation and DR5–GUS Expression

Auxin is known to regulate gravitropism. NPY1, NPY3, and NPY5 are known to participate in auxin-mediated organogenesis (Cheng et al., 2008). We used the auxin reporter DR5–GUS (Sabatini et al., 1999) to analyze auxin responses in the *npv* mutants. As shown in Figure 3A, DR5–GUS is highly expressed in the root tip in wild-type *Arabidopsis*. DR5–GUS is expressed in the root cap and quiescent center. The DR5–GUS expression patterns in *npv2 npv3 npv4 npv5* are very similar to those in wild-type (Figure 3A).

We also measured root elongation with and without IAA in the media. The *npv2 npv3 npv4 npv5* quadruple mutants had slightly shorter root than wild-type (Figure 3B). The *npv* quadruple mutants were not resistant to exogenous auxin in the root elongation assay (Figure 3B).

Effects of *npv* Mutations on the Formation of Starch Granules in Root Tips

Gravity sensing statocytes contain dense, starch-filled amyloplasts that can be visualized by Lugol staining. Lugol solution contains iodine that reacts with starch granules to generate a bright blue color. We studied whether the gravitropic defects of *npv* mutants were caused by disruption of starch granule formation. In wild-type root tips, differentiated columella cells displayed strong staining (Figure 4). We observed similar staining patterns in *npv2 npv3 npv4 npv5* quadruple mutants.

DISCUSSION

In this report, we provide evidence showing that the five *NPY* genes contribute to *Arabidopsis* root gravitropism. This work also demonstrates that gravitropism uses mechanisms analogous to those employed in phototropism and organogenesis. Gravitropism, phototropism, and organogenesis all require the plant hormone auxin, AGC kinases, NPH3-like proteins, and ARFs.

The *arf7 arf19* double mutants show agravitropic root growth (Li et al., 2006; Okushima et al., 2005). Both *ARF7*

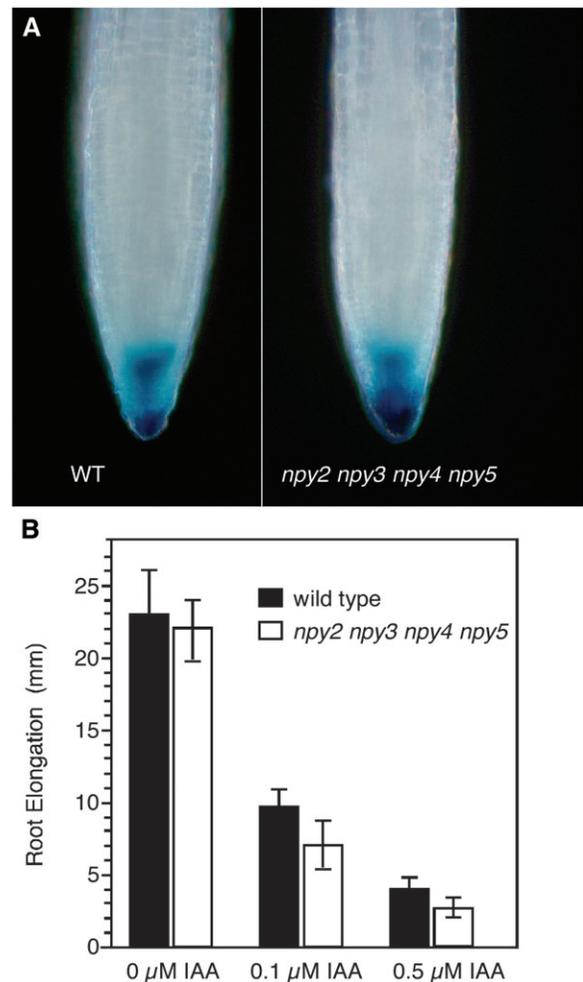


Figure 3. The Effects of *npv* Mutations on Auxin Responses.

(A) The auxin reporter DR5–GUS expression in wild-type (left) and *npv2 npv3 npv4 npv5* quadruple mutants (right) are shown. More than 10 seedlings for each genotype were analyzed. The typical staining patterns were shown.

(B) Auxin response in *npv2 npv3 npv4 npv5* quadruple mutants. Plants were grown vertically for 4 d and then transferred to media with and without IAA. The root elongation of wild-type and *npv2 npv3 npv4 npv5* quadruple mutants were measured after additional 4 d. At least 20 seedlings for each genotype and each auxin concentration were measured.

and *ARF19* are expressed in roots (Li et al., 2006; Okushima et al., 2005). *ARF7* is mainly expressed in vascular tissue in roots and *ARF19* is highly expressed in the root tip including root cap, root meristem, and elongation Zone (Li et al., 2006; Okushima et al., 2005). Two groups of AGC kinases, PID/WAG kinases and D6PK kinases, have been implicated in gravitropism (Santner and Watson, 2006; Sukumar et al., 2009; Zourelidou et al., 2009). The AGC kinases *WAG1* and *WAG2* are highly expressed at root tips, but their expression is excluded from the root cap (Santner and Watson, 2006). *PID::GUS* is mainly expressed in the vascular tissue proximal to the root meristem (Benjamins et al., 2001). *PID::GUS* is not expressed in

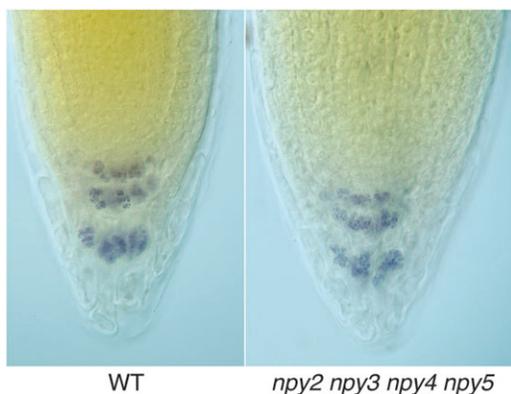


Figure 4. Formation of Starch Granules in Root Tips.

Starch granules were visualized by Lugol staining. The left root tip is from wild-type and the right is from *npy2 npy3 npy4 npy5* quadruple mutants. At least 10 seedlings for each genotype were stained. The typical patterns were shown here.

the root cap and meristem (Benjamins et al., 2001). *D6PK::GUS* and *D6PKL2::GUS* show high expression in the elongation zone and *D6PKL1::GUS* and *D6PKL3::GUS* are expressed in columella cells and elongation zone. The five *NPY* genes discussed in this paper are expressed in root tips (Figure 1B). *NPY2* and *NPY5* have high expression in the quiescent center while *NPY3* is not expressed in the columella root cap (Figure 1B). The root cap, quiescent center, and meristem have at least one expressed *NPY* gene. The expression patterns of *ARF 7*, *ARF19*, AGC kinases, and the *NPY* genes overlap significantly in the root tip, which is consistent with the idea that these genes function in the same genetic pathway to control gravitropic responses.

The central columella cells in the root cap are believed to be the main sites for gravity sensing. The root curvature takes place at the elongation zone in response to a gravistimulus (Holland et al., 2009). It is proposed that the asymmetric auxin flow from the gravity-sensing cells to the lateral root cap and the elongation zone after gravity stimulation is responsible for the gravitropic curvature (Holland et al., 2009). The auxin efflux carrier PIN3 is expressed in the columella cells and PIN3 protein relocates laterally within columella cells in response to gravistimulation to mediate lateral auxin transport (Friml et al., 2002). Therefore, PIN3 was suggested as a key component to translate directional signal sensed in the statocyte to a directional flow of auxin. However, *PIN3*-null mutants only have subtle gravitropic defects (Friml et al., 2002). PID has been shown to phosphorylate PIN proteins and to regulate PIN localization (Michniewicz et al., 2007). It is unlikely that PID, WAG1, and WAG2 regulate PIN3 localization in columella cells because the three AGC kinases are not expressed in columella cells. Studies with *PID::PID-GFP* transgenic lines showed that PID is expressed in the epidermis and endodermis in the elongation zone (Michniewicz et al., 2007). However, *PID::GUS* showed a completely different expression pattern in roots (Benjamins et al., 2001). *PID::GUS* is exclusively expressed in

vascular tissue, suggesting that either PID proteins move from the site of synthesis or one of the transgenic lines does not reflex the actual *PID* expression pattern. The D6PK co-localizes with PIN1, PIN2, and PIN4 (Zourelidou et al., 2009). However, it is still an open question whether phosphorylation of PIN proteins by AGC kinases is part of the mechanism for converting gravity signals to auxin flow. The *NPY* genes are expressed in the right places for regulating gravitropism. It will be very interesting to investigate auxin transport and PIN protein localization in *npy* mutants.

Gravitropism, phototropism, and organogenesis are involved in differential growth. In phototropism and gravitropism, differential auxin distribution, which is triggered by directional stimuli, causes differential cell elongation and organ curvature. In organogenesis, developmental signals cause the formation of an auxin maximum that initiates the formation of an organ and that is required for organ outgrowth away from the meristem. The three processes all require at least one AGC kinase, one NPH3-like protein, and one ARF. Interestingly, some components are shared whereas others are unique to a particular process. For example, ARF7 is required for both gravitropism and phototropism whereas ARF5 is essential for organogenesis. The PID/WAG kinases are involved in both organogenesis and gravitropic responses, but gravitropism requires additional AGC kinases. The PID/WAG and D6PK kinases are believed to interact with PIN proteins directly to regulate auxin transport whereas the substrates for the AGC kinases PHOT1 and PHOT2 are not known. It is not clear whether AGC kinases PHOT1 and PHOT2 can also interact with and phosphorylate PIN proteins. PHOT1 and PHOT2 are known to physically interact with NPH3 and RPT2 whereas the direct partners of *NPY* proteins are not known. Interestingly, gravitropism appears to have more genetic redundancy than phototropism and organogenesis. It is known that two AGC kinases (PHOT1 and PHOT2), one NPH3 protein, and one ARF (ARF7) are required for hypocotyl phototropic responses. The formation of flowers needs one AGC kinase (PID), three NPH3-like proteins (NPY1, NPY3, and NPY5), and one ARF (ARF5). Gravitropism uses at least seven AGC kinases (PID, WAG1, WAG2, and four D6PKs), at least five NPH3-like genes, and two ARFs.

The AGC kinase-NPH3-like protein-ARF genetic module may not be limited to phototropism, gravitropism, and organogenesis. Disruption of *SETH6/At2g47860*, which encodes an NPH3-like protein, greatly affected pollen germination and pollen tube growth (Lalanne et al., 2004). Two *Arabidopsis* AGC kinases (AGC1.5 and AGC1.7) are critical for polarized growth of pollen tubes (Zhang et al., 2009), suggesting that the two AGC kinases and *SETH6* may also participate in the same pathway. The *defectively organized tributaries (dot) 3* mutant has defects in vein patterning (Petricka et al., 2008). *DOT3/AT5g10250* is also homologous to NPH3, sharing 36% sequence identity and 54% similarity at the amino acid level with NPH3 (Petricka et al., 2008). Interestingly, auxin is also a main regulator for vascular development. Therefore, the AGC

kinase–NPH3-like protein–ARF module appears to be widely used in different biological processes to regulate directional growth. This hypothesis is also consistent with the fact that AGC kinases, NPH3-like proteins, and ARFs all belong to large families.

METHODS

Plant Materials

The *npv* single mutants were all T-DNA insertion mutants (Alonso et al., 2003). The insertion sites of the *npv* mutants were described previously (Cheng et al., 2007, 2008). The genotyping primers for the *npv1*, *npv2*, *npv3*, *npv4*, and *npv5* mutants were also described previously (Cheng et al., 2007, 2008).

NPY::GUS transgenic lines were constructed as described below. *NPY* promoters were amplified by PCR and cloned into the vector pBI101.3 to drive the *GUS* gene expression. The PCR primers for each *NPY* promoter are:

NPY1P1: 5'-ATTTCTTCGCTTGTTTACCAAAGGAAGACAC-3';

NPY1P2: 5'-GGGAGTAAAGCTGCCGGCGTTGAG-3';

NPY2P1: 5'-GTCTCGAAGATTCAGAAACAGCC-3';

NPY2P2: 5'-CTTCTCCCCCTAGGATAACTC-3';

NPY3P1: 5'-TTCTTAACGTTGACTGAGTTTGAG-3';

NPY3P2: 5'-TTCTCACAATAAAACAAAGCCAG-3';

NPY4P1: 5'-GGAGTCTTGAGTCATCGTTTTACG-3';

NPY4P2 : 5'-TTTTTCTATCTCTTTAATAAGTT-3';

NPY5P1: 5'-GGTTCATCTCACAATGATCAAGC-3';

NPY5P2: 5'-GATGAAGAAGAACTGATAGAAGAAG-3'.

The *NPY::GUS* constructs were transformed into wild-type *Arabidopsis* Columbia background by the floral dipping method (Clough and Bent, 1998). Lines with the most consistent *GUS* expression patterns were used in this study.

Beta-Glucuronidase (GUS) Staining for *NPY::GUS* Lines

Five-day-old seedlings grown on 0.5 X MS media in light were used for *GUS* staining. *NPY2::GUS*, *NPY3::GUS*, *NPY4::GUS*, and *NPY5::GUS* were highly expressed in root tips and they were stained for only 15 min. The *NPY1::GUS* lines were relatively weaker and were stained for 2 h. All of the *NPY::GUS* lines would be over-stained if they were stained for more than 3 h. *GUS* staining procedure was conducted according to what was described previously (Jefferson et al., 1987). The samples were photographed in dark field using a Leica DM5000 microscope.

Measurements of Gravitropic Responses

Arabidopsis seeds were surface-sterilized with 75% ethanol for 10 min and 100% ethanol for another 10 min. The seeds were allowed to dry on a piece of 3M filter paper in a sterile tissue culture hood. Then the seeds were sown on plates containing 0.5 X MS medium and 0.65% agar. After 2 d at 4°C, the plates were then placed vertically in an incubator at 23°C. After 4 d, the plates were photographed and the angle between gravity and the primary root were determined using NIH Image J,

a free program that can be downloaded from <http://rsbweb.nih.gov/ij/>.

Gravitropic responses were also measured by growing seedlings on 0.5 X MS for 4 d on a vertical plate and then turning the plates 90° so that the seedlings were perpendicular to the gravity vector. The plates were photographed 24 h later. Angles of root curvature were analyzed using the NIH Image J program.

Root Elongation Assay and DR5–GUS Expression

The auxin reporter DR5–GUS was used to study the auxin responses of *npv* mutants. The DR5–GUS marker was crossed into *npv* mutants. Both wild-type and *npv* mutants were grown on 0.5 X MS media vertically for 5 d. Then, the seedlings were stained for *GUS* activities for 3 h.

Four-day-old light-grown seedlings were transfer from 0.5 X MS agar plates to 0.5 X MS agar plates containing various concentrations of indole-3-acetic acid (IAA). The positions of root tips were marked with a sharpie marker. The seedlings were allowed to grow for four more days vertically. The plates were then photographed. The root elongation was analyzed with the NIH Image J program.

Starch Granule Staining

Four-day-old seedlings were first fixed in FAA (3.7% formaldehyde, 5% acetic acid, and 50% ethanol) at 4°C overnight. The samples were washed once in 50% ethanol. Then, the samples were placed in lugol solution (0.37% iodine, 0.71% potassium iodide) for 1 min, followed with 2 min in chloral hydrate solution (80 g chloral hydrate, 20 ml glycerol, 20 ml water). Pictures were taken immediately.

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