Down-Regulation of the 26S Proteasome Subunit RPN9 Inhibits Viral Systemic Transport and Alters Plant Vascular Development

Hailing Jin*, Songtao Li, and Andy Villegas Jr.

Center for Plant Cell Biology, Department of Plant Pathology, University of California, Riverside, California 92521

Plant viruses utilize the vascular system for systemic movement. The plant vascular network also transports water, photosynthates, and signaling molecules and is essential for plant growth. However, the molecular mechanisms governing vascular development and patterning are still largely unknown. From viral transport suppressor screening using virus-induced gene silencing, we identified a 26S proteasome subunit, RPN9, which is required for broad-spectrum viral systemic transport. Silencing of *RPN9* in *Nicotiana benthamiana* inhibits systemic spread of two taxonomically distinct viruses, *Tobacco mosaic virus* and *Turnip mosaic virus*. The 26S proteasome is a highly conserved eukaryotic protease complex controlling many fundamental biochemical processes, but the functions of many 26S proteasome regulatory subunits, especially in plants, are still poorly understood. We demonstrate that the inhibition of viral systemic transport after *RPN9* silencing is largely due to alterations in the vascular tissue. *RPN9*-silenced plants display extra leaf vein formation with increased xylem and decreased phloem. We further illustrate that RPN9 functions at least in part through regulation of auxin transport and brassinosteroid signaling, two processes that are crucial for vascular formation. We propose that RPN9 regulates vascular formation by targeting a subset of regulatory proteins for degradation. The brassinosteroid-signaling protein BZR1 is one of the targets.

Virus movement within the plant occurs via local cell-to-cell movement through plasmodesmata and long-distance transport through the vasculature, mainly the phloem (Crawford and Zambryski, 1999; Scholthof, 2005). Compared with work on virus cell-to-cell movement (Zambryski and Crawford, 2000; Zambryski, 2004), there have been relatively few studies on virus long-distance movement in the vascular system (Santa Cruz, 1999). Using a modified *Tobacco mosaic virus* (TMV) expressing green fluorescent protein (GFP), Cheng and colleagues studied the systemic vascular invasion routes of TMV in *Nicotiana benthamiana* and determined that TMV could enter minor, major, or transport veins directly from nonvascular cells to produce a systemic infection (Cheng et al., 2000). Only a few host genes have been identified to be important for virus systemic spread (Scholthof, 2005). A vascular-specific Gly-rich protein inhibits the long-distance movement of *Turnip vein-clearing virus*, probably by inducing callose accumulation in the phloem cell walls (Ueki and Citovsky, 2002). RTM1 and RTM2, which function in phloem, were found to restrict long-distance movement of *Tobacco etch virus* (Chisholm et al., 2000, 2001; Whitham et al., 2000). However, the proteins identified so far only affect the systemic transport of specific viruses. Host components required for broad-spectrum virus systemic transport have yet to be identified.

An integrated vascular network is not only required for plant viral systemic transport, but is also essential for transporting water, nutrients, and signaling molecules during plant growth. The vascular system consists of phloem, xylem, and meristematic cells—procambium (Ye, 2002). The phloem forms a macromolecular trafficking network for transport of nutrients and signaling molecules that regulate physiological and developmental events at the whole-plant level (Ruiz-Medrano et al., 2001). The xylem consists of vessels and tracheary elements (TEs), which are essential for plant mechanical support and water transport. However, the molecular regulatory mechanisms of plant vascular development are still poorly understood.

A major research impediment in this field has been the small number of vascular development mutants because knockout mutations in central regulatory components of vascular development are likely to be lethal. Notably, most of the genetic mutants studied so far, including *monopteros* (*arf5*; Hardtke and Berleth, 1998), *axr6* (Hobbie et al., 2000), *bodenlos* (*iaa12*; Hamann et al., 1999), *vascular network defective 1* to *6* (*van1-6*; Koizumi et al., 2000, 2005), *scarface* (Deyholos et al., 2000; Sieburth and Deyholos, 2006), *lop1/tornado1* (Carland and McHale, 1996), *cotyledon vein pattern 1* and *2* (*cvp1*, *cvp2*; Carland et al., 2002; Carland and Nelson, 2004), *orc* (Willemsen et al., 2003), *fackel* (Jang et al., 2000), and *ifl1* (Zhong and Ye, 1999), show either reduced vascular formation or discontinuous vascular patterning. Furthermore, all these mutants also

* Corresponding author; e-mail hailingj@ucr.edu; fax 951–827–4294.

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display defects in other aspects of plant growth and development. Very few mutants exhibit increased vascular formation. Therefore, the in vitro zinnia (*Zinnia elegans*) TE induction system has long been used for vascular differentiation studies (Ye, 2002). Virus-induced gene silencing (VIGS) circumvents lethality by suppressing the expression of essential genes in developed plants and thus serves as an optimal technique for such research. Here we describe the identification of RPN9, which affects broad-spectrum viral systemic transport and regulates vascular development, from a viral transport suppressor screening using VIGS.

RPN9 encodes a subunit of the 26S proteasome, a highly conserved eukaryotic protease responsible for intracellular protein degradation. The 26S proteasome consists of a 20S catalytic core particle (CP) and a 19S regulatory particle (RP), and controls many fundamental biochemical processes by programmed degradation of regulatory protein targets besides its role in removing damaged or misfolded proteins. The ubiquitin/26S proteasome pathway is implicated in numerous diseases, including cancer and neurodegenerative diseases (Mani and Gelmann, 2005). In plants, the 26S proteasome degrades various regulators of diverse cellular processes, including cell division, stress responses, and hormone-signaling pathways (Chinnusamy et al., 2003; Moon et al., 2004; Smalle and Vierstra, 2004).

The capacity of protein selection for degradation by the proteasome is gained through the 19S RP, which recognizes, binds, and unfolds the target proteins, cleaves ubiquitin chains, and directs the target proteins into the lumen of the CP for degradation (Wolf and Hilt, 2004). However, our knowledge of these functions is still limited. The 19S RP is divided further into two subcomplexes, known as the base and the lid. The base contains six ATPase subunits, RPT1 to 6, and three non-ATPase subunits, RPN1, 2, and 10. The RPT subunits use ATP hydrolysis to facilitate channel opening, polyubiquitin chain binding, and protein unfolding (Lam et al., 2002; Hartmann-Petersen et al., 2003). RPN10 binds polyubiquitin chains and contributes to the turnover of multiple proteasome targets (Mayor et al., 2005). In plants, RPN10 regulates abscisic acid (ABA) signaling by targeting ABA-signaling proteins for degradation (Smalle et al., 2003). RPT2a is involved in the maintenance of Arabidopsis (*Arabidopsis thaliana*) meristems (Ueda et al., 2004). RPN1 is essential for Arabidopsis embryogenesis (Brukhin et al., 2005). The lid contains eight subunits, RPN3, RPN5 to 9, and RPN11 to 12. The functions of lid subunits are poorly understood. Some of them may function in a target-specific manner, presumably by recruiting specific E3 ligases or carrier proteins to deliver ubiquitinated targets to the 26S proteasome (Smalle and Vierstra, 2004). Arabidopsis RPN12 is required for normal cytokinin responses, likely by identifying specific proteins controlling these processes for degradation (Smalle et al., 2002; Smalle and Vierstra, 2004). However, the roles of RPN9 and other subunits of the 19S RP in higher plants, particularly during morphogenesis, are still unknown.

Here we demonstrate that down-regulation of *RPN9*, but not two other 26S proteasome subunits, inhibits the systemic spread of two taxonomically distinct viruses. Inhibition of virus spread may be largely due to the alteration of vascular formation with reduced phloem and induced xylem. Our further analysis shows that RPN9 functions at least in part through regulation of auxin transport and brassinosteroid (BR) signaling. Given the established role of the 26S proteasome in programmed degradation of regulatory proteins, we propose that RPN9 regulates plant vascular development by targeting a subset of regulatory proteins for degradation. Our data suggest that the BR-signaling protein BZR1 is one such target.

RESULTS

RPN9-Silenced Plants Inhibit Systemic Transport of Two Taxonomically Distinct Viruses

The *RPN9* gene was identified in a functional high-throughput VIGS screen to identify genes necessary for viral infection and systemic spread in *N. benthamiana*, one of the best hosts for plant virus studies. A cDNA library was enriched for TMV-induced genes by suppression subtractive hybridization between cDNAs prepared from TMV-challenged and untreated *N. benthamiana* plants. The cDNA library was cloned into *Potato virus X* (PVX)-derived silencing vector pGr106 and introduced into Agrobacterium for high-throughput silencing in wild-type *N. benthamiana*. GFP-labeled TMV was inoculated on the silenced leaves (start from the third or fourth leaf above the VIGS agroinfiltration sites) 2 weeks after Agrobacterium inoculation for visualization of the viral infection and systemic spread in the silenced plants (Jin et al., 2002). One of the genes that attenuated viral systemic spread after silencing was *RPN9* (DQ226994), which encodes a subunit of the 26S proteasome regulatory complex.

The onset of viral infection foci was clearly visible on the inoculated leaves of both *RPN9*-silenced plants (*RPN9*$^-$) and pGr106 empty vector-treated controls at 4 days postinoculation (4 DPI) of TMV-GFP (Fig. 1A). The infection foci enlarged to a similar size in both *RPN9*$^-$ plants and control leaves, which indicates that cell-to-cell movement of TMV-GFP was not affected. However, the virus failed to achieve systemic spread in *RPN9*-silenced plants because no GFP fluorescence was detected on the upper uninoculated leaves at 10 DPI (Fig. 1A). In contrast, the virus invaded the vascular tissue of control plants and spread systemically to upper uninoculated leaves (Fig. 1A, 10 DPI). Real-time reverse transcription (RT)-PCR analysis showed that *RPN9* mRNA was reduced by 80% to 95% in the silenced leaves beginning at 8 days postsilencing induction (DPS) relative to the empty vector-treated control (Fig. 1B). VIGS induces RNA degradation

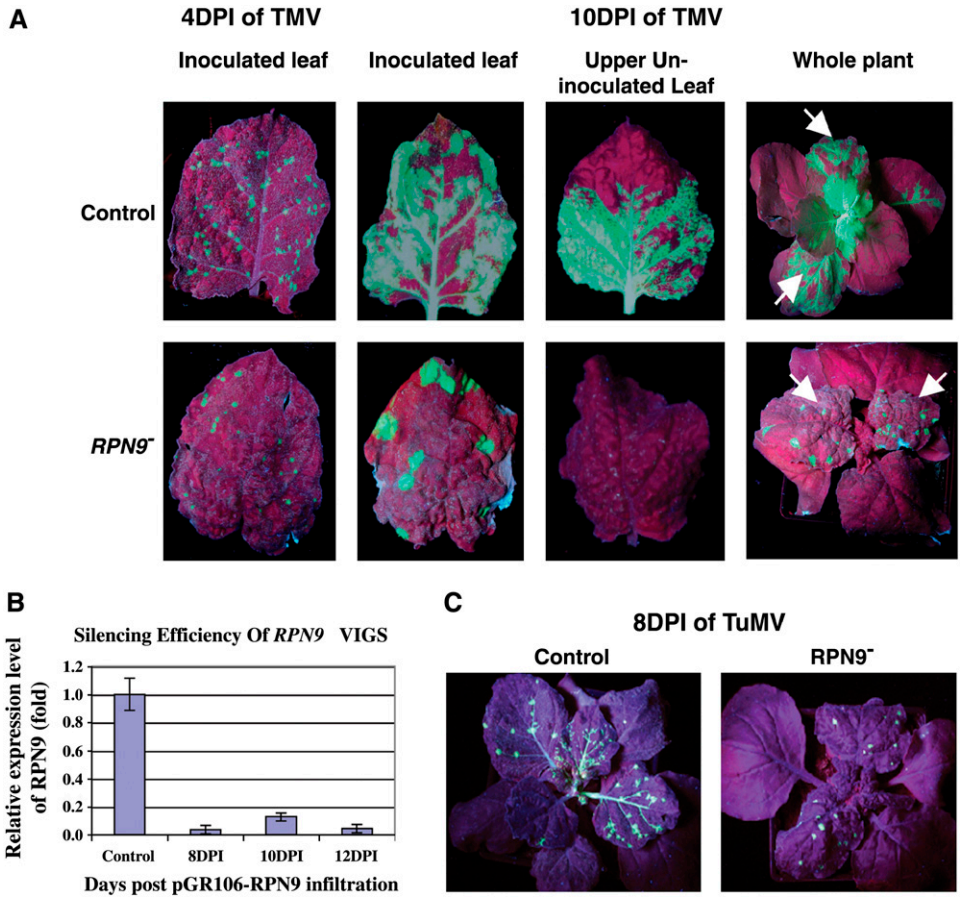

Figure 1. *RPN9* is required for TMV systemic spread. A, Systemic spread of TMV-GFP is inhibited in *RPN9*$^-$ plants. TMV-GFP infection foci were green under UV light and the noninfected area showed red due to chlorophyll. Arrows indicate inoculated leaves. B, VIGS of *RPN9* is efficient. Suppression of *RPN9* expression reached more than 10-fold after 8 DPS; similar results were obtained from two silencing experiments with a total of six individual plants for each time point. Primers were designed from sequence outside of the silencing region. C, Systemic spread of TuMV-GFP is attenuated in *RPN9*$^-$ plants. Pictures were taken at 8 DPI TuMV-GFP.

based on nucleotide sequence identity. Southern analysis under stringent conditions showed at most two copies of *RPN9*, which might share greater than 80% identity in the *N. benthamiana* genome (data not shown). Database searches revealed that two *RPN9* genes sharing more than 87% identity are present in Arabidopsis and tomato (*Lycopersicon esculentum*). VIGS of *RPN9* in *N. benthamiana* very likely silences both homologs because the silencing insertion is about 1 kb and covers the conserved region.

Previous studies have identified host components that only affect the systemic transport of specific viruses. To determine whether *RPN9*-silenced plants inhibit systemic transport of other viruses, we tested *Turnip mosaic virus* (TuMV), a potyvirus that is taxonomically very different from TMV, a tobamovirus. The systemic transport of GFP-tagged TuMV was also attenuated after *RPN9* silencing (Fig. 1C); no virus was visualized in the upper uninoculated leaves. Therefore, RPN9 is required for systemic transport of both TMV and TuMV.

RPN9$^-$ Plants Exhibit Greatly Increased Leaf Vein Formation

The inhibition of systemic spread of two taxonomically distinct viruses suggested that a common structure required for viral systemic transport, such as the vascular system, might be altered in *RPN9*$^-$ plants. Leaf-clearing experiments did not reveal obvious differences in vein patterning between *RPN9*$^-$ and control leaves. However, veins were slightly denser and clearly thicker in *RPN9*$^-$ leaves than the same order of veins in control leaves (Fig. 2A). Scanning electron microscopy (SEM) revealed that, at 14 DPS, double- and even triple-twisted veins were present on the abaxial surface of *RPN9*$^-$ leaves (Fig. 2B). Interestingly, the additional veins emerged adjacent to the existing veins (Fig. 2B, arrows), including both midveins and high-order veins. In wild-type plants, vascular bundles with normal auxin transport suppress the formation of new vascular strands in their immediate vicinity (Sachs, 1991). This inhibition is suppressed in *RPN9*$^-$ plants.

Increased Xylem and Reduced Phloem Were Observed in *RPN9*$^-$ Plants

Increased vein formation might be expected to aid viral systemic spread. However, long-distance movement of viruses was inhibited in *RPN9*$^-$ plants. To further explore this unexpected finding, we characterized the fine structures of the veins and stems of *RPN9*$^-$ plants. Compared to control plants that formed veins with a single vascular bundle, two or three vascular bundles were observed in *RPN9*$^-$ plants as

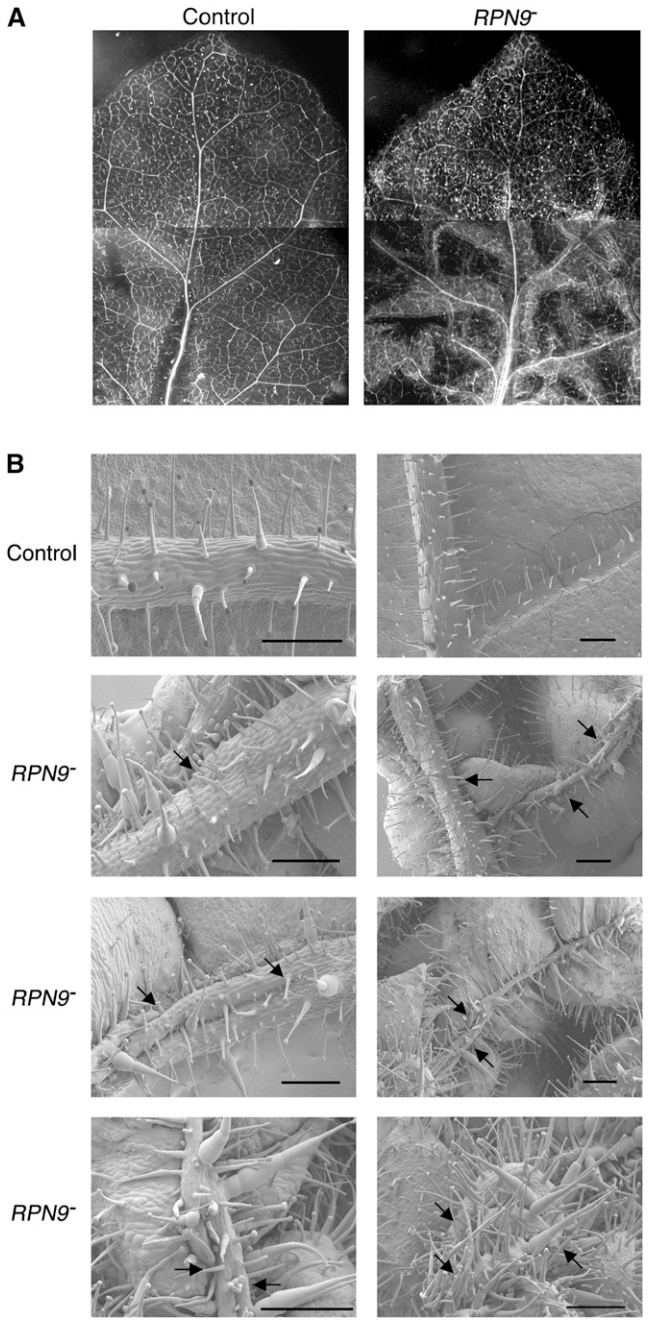

Figure 2. Increased vein formation on *RPN9*$^-$ leaves observed by leaf clearing (A) and SEM (B). Leaf tissue was collected at 14 DPS. Leaf clearing was done as described in Sieburth (1999). The abaxial side of leaves was examined under SEM. Arrows indicate the initiation sites of extra vein formation. Scale bars = 500 μm. More than 20 *RPN9*$^-$ leaves from three sets of independently silenced plants were examined and over 80% of the leaves displayed the extra vein formation phenotype.

early as 12 DPS (Fig. 3A, black circles). These vascular bundles contained increased xylem and decreased phloem in both veins (Fig. 3A) and stems (Fig. 3B). The phenotype became more severe at later stages of silencing (16 DPS or later) and the highly organized bicollateral vascular bundle patterning was disrupted (Fig. 3, A and B). Lignin deposition was greatly increased in *RPN9*$^-$ plants, which confirms that *RPN9* silencing promotes xylem formation (Fig. 3C). These data indicate that *RPN9* silencing suppresses phloem and promotes xylem, which may be the major cause of viral transport inhibition.

The Function of RPN9 in Vascular Development Is Specific

RPN9 is a subunit of the 19S RP lid, which is a part of the 26S proteasome. To determine whether the phenotype observed is specific to *RPN9* silencing or is a common phenomenon of silencing any of the 26S proteasome subunits, genes encoding two other subunits, the α_4-subunit of the 20S CP and *RPN8* of the 19S lid complex, were silenced. Silencing the α_4-subunit resulted in whole-plant death at 10 DPS (Fig. 4A), which indicates that the α_4-subunit is absolutely required for plant growth. Silencing of *RPN8* induced early senescence and plant death at 14 to 16 DPS, but did not alter leaf morphology (Fig. 4A). No difference in vasculature was observed between control and *RPN8*$^-$ plants by either leaf clearing or SEM (data not shown). In contrast, *RPN9*$^-$ plants survived for 30 to 40 DPS, developing distinctive curled and crinkled leaf morphology. *RPN9*$^-$ leaves were slightly darker green and wider than the control (Fig. 4B). Interestingly, leaf senescence of *RPN9*$^-$ plants initiated from the veins after 28 DPS, whereas the control leaves were still green and healthy (Fig. 4B). Because TE differentiation is tightly coupled with secondary wall formation and leads to autonomous programmed cell death (Jones, 2001; Obara and Fukuda, 2003), the increased formation of xylem in the veins after *RPN9* silencing could explain the early senescence that initiated from veins. Real-time RT-PCR showed that the silencing efficiency was similar for all three subunits (Fig. 4C), indicating that the different VIGS-induced phenotypes were due to specific effects of each subunit rather than to a different degree of silencing. Thus, the phenotype of *RPN9* silencing in vascular development is rather specific.

Auxin Transport Is Reduced in *RPN9*$^-$ Plants

Various phytohormones and steroids have been implicated in vascular development and vein patterning (Fukuda, 2004). Polar auxin flow is essential for continuous vascular pattern formation (Scarpella et al., 2006). Auxin transport inhibitors induce the formation of a strong continuous vasculature along leaf margins (Mattsson et al., 1999; Sieburth, 1999). The asymmetrically localized auxin-efflux carrier, AtPIN1, is responsible for polarized auxin flow, which may lead to the formation of continuous columns of procambial cells (Galweiler et al., 1998; Fukuda, 2004). The Ser-Thr kinase PINOID is preferentially expressed in young vascular tissues and acts as a binary switch controlling the polar localization of PIN proteins (Christensen

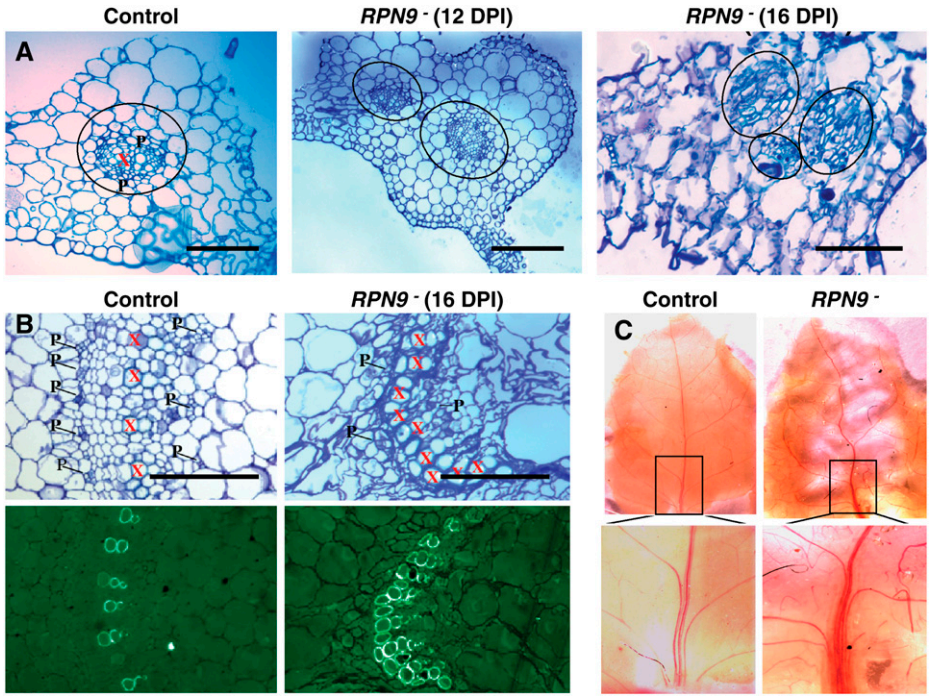

Figure 3. *RPN9*$^{-}$ plants exhibit multiple vascular bundles in the veins and increased xylem and reduced phloem in both veins and stems. X, Xylem (red); P, phloem (black). A, Cross section of leaf veins stained with toluidine blue. Vascular bundles are circled. The bicollateral pattern of the vascular bundle is indicated in the control. B, Cross section of stems stained with toluidine blue (top) or Auromine O (bottom). Auromine O staining enhances the autofluorescence of xylems (bright green). C, Increased lignin formation in *RPN9*$^{-}$ xylems visualized by phloroglucinol (saturated in 37% HCl) staining (Tamagnone et al., 1998), which specifically stains lignin red orange. Scale bars = 250 μm.

et al., 2000; Friml et al., 2004). Vascular bundles with high rates of auxin transport were found to inhibit the development of new vascular strands in their immediate vicinity (Sachs, 1991). *RPN9*$^{-}$ plants show double- and even triple-twisted veins (Fig. 2) and the extra veins emerged adjacent to the existing veins (Fig. 2, arrows). These phenotypes suggested that auxin transport might be altered in *RPN9*$^{-}$ plants, although the multiple veins were not concentrated along the leaf margins.

To test this hypothesis, we first analyzed the gene expression level of *PIN1* and *PINOID*, two major players in auxin transport. *PIN1* mRNA levels were similar in control and *RPN9*$^{-}$ plants (data not shown). In contrast, *PINOID* mRNA levels were reduced in *RPN9*$^{-}$ plants compared to the control (Fig. 5A). *PINOID* is preferentially expressed in young vascular tissues and controls the polar localization of PIN proteins for auxin transport (Christensen et al., 2000; Friml et al., 2004). The down-regulation of *PINOID* provides another indication that auxin transport might be affected in *RPN9*$^{-}$ plants. Therefore, we directly measured auxin polar transport activity in midveins using ^{14}C-labeled radioactive indole-3-acetic acid (IAA), a major form of endogenous auxin. IAA polar transport in *RPN9*$^{-}$ veins was reduced to approximately 57% of the control beginning at 10 DPS (Fig. 5, B and C). We detected IAA transport only in a basipetal direction, which was inhibited in the presence of 1-*N*-naphthylphtalamic acid (NPA), an auxin transport inhibitor (Fig. 5, B and C). The double and triple veins were not typically observed in *RPN9*$^{-}$ leaves until after 12 DPS and IAA transport did not show substantially more severe inhibition at later time points, which suggests that reduced IAA transport is likely the cause of the extra vein formation instead of a secondary effect of reduced phloem.

To confirm that the inhibition of auxin transport really induces vein formation in *N. benthamiana*, we germinated seeds in Murashisge and Skoog medium containing the auxin transport inhibitor NPA. The presence of 25 μM NPA induced thick vein formation (Fig. 5D), mimicking the phenotype of extra vein formation in *RPN9*$^{-}$ plants (Fig. 3C). Plants germinated on NPA arrested and could not be used for the viral infection assay. To examine the effect of extra vein formation on viral systemic transport, we sprayed 3-week-old *N. benthamiana* with 150 μM of NPA as described in Nemhauser et al. (2000). Leaves became crinkly and extra veins were observed in these leaves 1 week after NPA treatment, especially along the leaf margins (Fig. 5E). Systemic transport of TMV-GFP was clearly inhibited in plants that had been sprayed with NPA, but was not completely blocked (Fig. 5E). Virus could enter the vascular system and could occasionally be transported to the upper uninfected leaves (data not shown). These results suggest that inhibition of auxin transport indeed induces extra vein formation, which may partially inhibit viral systemic transport.

RPN9 Silencing Alters BR Signaling

RPN9^{-} plants had curled crinkly leaves with increased xylem and reduced phloem in the vascular bundles. The silenced leaves were dark green and underwent early senescence initiated from the veins (Fig. 4B). These phenotypes were not observed in NPA-treated plants and cannot all be explained simply

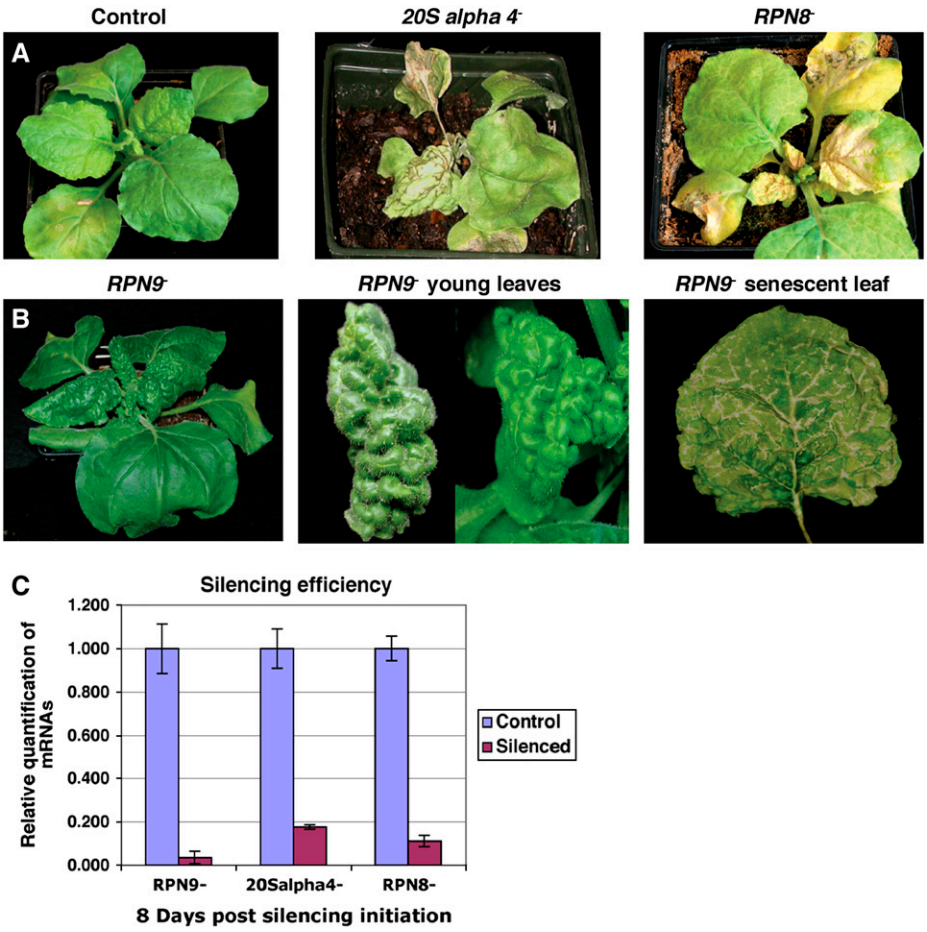

Figure 4. The phenotype of *RPN9* silencing is specific. A, 26S proteasome α_4-subunits (DQ226996) and *RPN8* (DQ226995)-silenced plants. B, *RPN9*$^-$ plants. All pictures were taken at 14 DPS, except for that of the *RPN9*$^-$ senescent leaf, which was taken at 28 DPS. C, Silencing efficiency was measured by real-time RT-PCR at 8 DPS. Two biological samples were tested and gave similar results. Error bars represent the SD of the mean.

by reduced auxin transport, but point to the likelihood of the involvement of the BR-signaling pathway in *RPN9*-mediated regulation. BRs are involved in xylem cell differentiation by regulating secondary wall formation and the programmed cell death that occurs during TE differentiation (Yamamoto et al., 1997). Increased endogenous BRs or increased BR perception promotes xylem and suppresses phloem formation (Szekeres et al., 1996; Yamamoto et al., 1997; Choe et al., 1999; Nagata et al., 2001; Cano-Delgado et al., 2004) and induces secondary wall formation and programmed cell death (Yamamoto et al., 1997). To test the effect of BR on plant growth, we grew *N. benthamiana* on Murashige and Skoog medium containing 100 nM brassinolide (BL; the most biologically active BR). The cotyledons and leaves of the BL-treated plants curled down at the sides (Fig. 6A), which is similar to the leaves of *RPN9*$^-$ plants (Fig. 4B). Exogenous IAA did not alter cotyledon and leaf morphology (Fig. 6A).

BRs are perceived by the receptor-like kinases BRI1, BRL1, and BRL3, which then inactivate the negative regulator BIN2 and stabilize its downstream transcription factors BZR1 and BES1 to promote BR-regulated gene expression (Cano-Delgado et al., 2004; Nemhauser and Chory, 2004; Vert and Chory, 2006). *BRL1* and *BRL3* are specifically expressed in vascular cells and a loss-of-function mutation in BRL1 causes reduced xylem and increased phloem differentiation (Cano-Delgado et al., 2004). Conversely, overexpression of BRI1 results in increased procambial activity leading to increased xylem differentiation. These results suggest the existence of a vascular cell-specific BR perception system. The *N. benthamiana* ortholog of the vascular-localized *BRL1* was clearly induced in *RPN9*$^-$ leaves (Fig. 6B), whereas the mRNA levels of *NbBRI1* and *NbBIN2* remained unchanged in *RPN9*$^-$ plants (data not shown). Gene expression analyses were carried out on samples collected 8 DPS, a time when the expression of *RPN9* was clearly suppressed (Fig. 1B), but the plants appeared morphologically normal. Thus, the observed changes in gene expression are likely to reflect direct effects of *RPN9* silencing rather than secondary effects from morphological alterations. Our results indicate a possible increase of BR perception in vascular tissues in *RPN9*$^-$ plants. Loss of function in *BRL1* causes increased phloem and reduced xylem differentiation (Cano-Delgado et al., 2004), which correlates perfectly with our data that *RPN9*$^-$ plants have elevated *BRL1* expression and display increased secondary xylem and reduced phloem.

As a subunit of the 26S proteasome, RPN9 is likely to function through protein degradation. To determine whether RPN9 regulates degradation of many proteins or only a small number of targets, we analyzed total

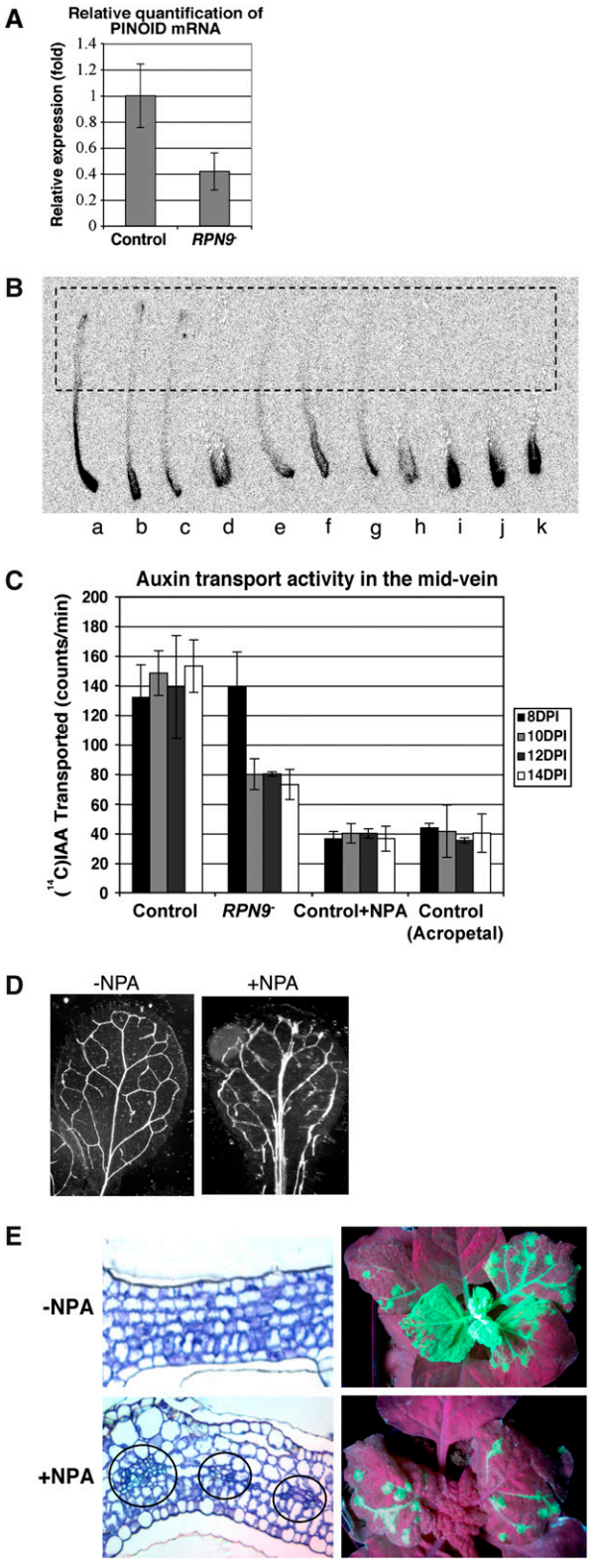

Figure 5. Auxin transport is reduced in *RPN9*$^{-}$ plants. A, *PINOID* mRNA is reduced at 8 DPS in *RPN9*$^{-}$ plants. Three biological samples were tested and gave similar results. Error bars represent the SD of the mean. B, Distribution of [^{14}C]IAA in the midveins at 10 DPS after 18-h incubation with labeled IAA. a to c, Control (basipetal). d, Control (acropetal). e to g, *RPN9*$^{-}$ (basipetal). h, *RPN9*$^{-}$ (acropetal). i to k, Control + 60 μM NPA (basipetal). The box indicates the upper 1-cm parts used for quantification. C, Amounts of [^{14}C]IAA transported to the upper 1 cm of vein sections after 18-h incubation. Error bars are the SD from three replicates for each treatment. The experiment was repeated once with a different set of silenced plants and gave similar results. D, NPA treatment induces vascular formation in wild-type *N. benthamiana* plants. Plants were grown on Murashige and Skoog medium without or with 25 μM of NPA for 3 weeks; leaves were cleared as described (Sieburth, 1999). E, NPA treatment induces vascular formation and partially inhibits viral transport. Cross sections of leaf margins are shown on the left, vascular bundles are circled. TMV-GFP was inoculated 1 week after NPA treatment and pictures were taken at 7 DPI of TMV-GFP infection.

polyubiquinated proteins. If RPN9 affects global protein degradation, we would expect to see an accumulation of polyubiquinated proteins in *RPN9*$^{-}$ plants, as was observed in the *rpn10* mutant in Arabidopsis (Smalle et al., 2003). We did not detect obvious differences in the level of total polyubiquinated protein between control and *RPN9*$^{-}$ plants (Fig. 6C). Therefore, we hypothesize that RPN9 regulates vascular development by targeted degradation of specific proteins.

Previous studies have shown that a downstream transcription factor of the BR-signaling pathway, BZR1, is regulated by 26S proteasome-mediated degradation (Wang et al., 2002). To determine whether BZR1 is a possible target of RPN9, we transiently expressed Arabidopsis BZR1-CFP in the *RPN9*$^{-}$ and control leaves and detected increased BZR1-CFP accumulation in *RPN9*$^{-}$ leaves (Fig. 6D). In contrast, we did not detect differences in BRI1-GFP accumulation between control and *RPN9*$^{-}$ plants (Fig. 6D). Thus, BZR1 is likely a target of RPN9-mediated protein degradation.

To determine whether *RPN9* silencing affects other proteins regulated by 26S proteasome degradation, we examined the protein level of ICE1 (Chinnusamy et al., 2003), a MYC transcription factor regulated by ubiquitin/26S proteasome-mediated proteolysis (Dong et al., 2006). We transiently expressed the AtICE1-GFP fusion and detected the degradation of ICE1-GFP after 16 h at 0°C in both *RPN9*$^{-}$ and control leaves (Fig. 6E), which indicates that ubiquitin/26S proteasome-mediated ICE1 degradation is not affected by *RPN9* silencing.

DISCUSSION

Many plant viruses rely on vascular structure, primarily the phloem, for their systemic transport. The vascular system is also essential for plant development. However, the molecular regulatory mechanisms of plant vascular development are still poorly understood. From a viral transport suppressor screen using VIGS in *N. benthamiana*, we have identified RPN9, a regulatory subunit of the 26S proteasome. Silencing *RPN9* inhibits the systemic transport of two taxonomically distinct viruses, which leads to the finding that

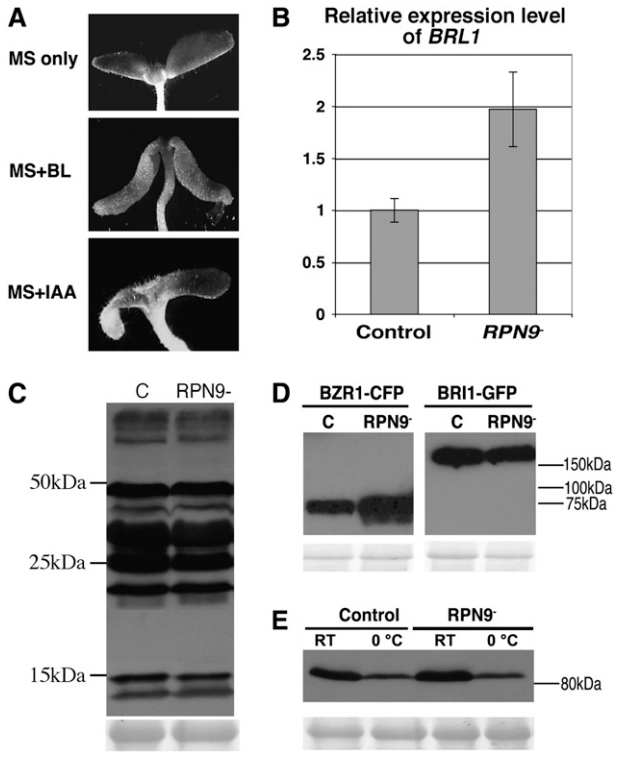

Figure 6. BR signaling is involved in *RPN9*-mediated regulation. A, *N. benthamiana* cotyledon curled down under BL treatment. Seedlings were grown on Murashige and Skoog medium with 100 nM BL or 10 μM IAA. Pictures were taken at 10 d postgermination. B, *BRL1* is induced in *RPN9*$^-$ plants. Three biological samples were tested and gave similar results. Error bars represent the SD of the mean. C, Western analysis using plant ubiquitin antibody reveals no obvious difference in poly-ubiquitin-conjugated proteins between control and *RPN9*$^-$ plants. D, Increased accumulation of BZR1-CFP in *RPN9*$^-$ leaves. BZR1-CFP and BRI1-GFP were transiently expressed in *RPN9*$^-$ and control leaves. Samples were collected at 66 h postagroinoculation and subjected to western analysis with GFP antibody. Large subunit of Rubisco (bottom) of same samples runs in parallel and is stained by Coomassie Blue for loading control. Similar results were obtained from three independent experiments. E, Degradation of GFP-ICE1was detected by western analysis with GFP antibody in both *RPN9*$^-$ and control plants. Total protein extracts before immunoprecipitation were checked on SDS-PAGE gel after Bradford protein quantification to ensure the same quantity of total proteins for immunoprecipitation; bands of the large subunit of Rubisco were presented here.

RPN9 regulates plant vascular development. Silencing of *RPN9* induced extra vein formation with increased xylem and reduced phloem, which may largely contribute to the inhibition of broad-spectrum viral systemic transport. This genetic evidence indicates that plant vascular development is regulated at the proteolysis level via a specific 26S proteasome subunit. RPN9 is a subunit of the 19S RP lid. Limited studies suggest that different subunits of 19S RP have well-differentiated functions (Glickman et al., 1999). On the basis of recent studies on RPN12a (Smalle et al., 2002), RPN10 (Smalle et al., 2003), and RPN1 (Brukhin et al., 2005) in plants that function specifically in cytokinin-, ABA-, and embryogenesis-signaling pathways, respectively, we hypothesize that RPN9 regulates vascular development via selective degradation of specific target proteins.

The extra vein formation in the immediate vicinity of the existing veins led us to the finding that auxin transport is reduced in *RPN9*$^-$ plants. The down-regulation of *PINOID* may contribute at least in part to the reduction of auxin transport in *RPN9*$^-$ plants. The roles of EMB30/GN and VAN3 in auxin transport and auxin signaling strongly indicate the involvement of vesicle transport in leaf vein formation (Geldner et al., 2003; Koizumi et al., 2005). Their roles in RPN9-mediated regulation have yet to be determined. Thus, we propose that RPN9 is likely to regulate auxin transport through the selective degradation of a negative regulator of *PINOID* that regulates polar localization of PIN proteins or components that regulate the vesicle transport of PIN proteins. Future protein accumulation analysis via a proteomic approach will help identify such proteins.

BRs have been shown to regulate plant vascular development. Up-regulation of the vascular-specific BR receptor BRL1 and the distinctive phenotype resulting from *RPN9* silencing strongly indicate the involvement of BR signaling in RPN9 regulation. *BRL1* is specifically expressed in vascular tissues and the *brl1* knockout mutant displays increased phloem and reduced xylem (Cano-Delgado et al., 2004). The elevated expression of *BRL1* observed in *RPN9*$^-$ plants is likely to increase BR perception and activate the BR-signaling pathway in the vascular system, which results in increased xylem and reduced phloem and early senescence initiated from the veins. BRL1 is likely to mediate a vascular-specific BR perception system. The phosphorylation and proteolytic degradation of BR-signaling transcription factors BZR1 and BES1 are critical steps of BR signal transduction pathways. Our results show that BZR1 is selectively stabilized in *RPN9*$^-$ plants, whereas degradation of ICE1 is not affected. Immunoblot analysis of total proteins with antiubiquitin antibody revealed no obvious difference between *RPN9*$^-$ plants and controls in accumulation of ubiquitin-conjugated proteins. These data suggest that *RPN9* silencing does not perturb the function of the 26S proteasome globally, but rather affects the *RPN9*-related functions specifically by accumulating a specific subset of protein targets, such as BZR1. Thus, we provided evidence that RPN9 specifically regulates vascular development at least in part by the mechanism of regulating auxin transport and BR signaling.

Several recent studies address cross talk and overlap between BR and auxin responses and indicate a complex interdependency between the two phytohormones (Mussig et al., 2002; Goda et al., 2004; Nemhauser et al., 2004). BRs and auxin were shown to act synergistically in the control of hypocotyl elongation and lateral root growth (Mandava, 1988; Bao et al., 2004; Nemhauser et al., 2004). Our results suggest there may be an interaction between auxin transport and BR signaling in plant vascular development.

Cytokinin also plays an important role in vascular development by regulating the formation of procambial cells (Mahonen et al., 2000; Fukuda, 2004). Real-time RT-PCR analysis on cytokinin receptor and signaling genes, *HK3*, *ARR15*, and *ARR5*, revealed essentially no difference between *RPN9*$^-$ and control samples. Thus, cytokinin might not play a direct role in *RPN9*-mediated regulation. Several transcription factors, such as MP (ARF5; Hardtke and Berleth, 1998), homeobox protein ATHB-8 (Baima et al., 2001), and APL, a Myb-like transcription factor promoting phloem differentiation and repressing xylem differentiation (Bonke et al., 2003), are involved in vascular patterning and development. We did not detect obvious alterations in expression of these genes in *RPN9*$^-$ plants by real-time RT-PCR (data not shown). However, RPN9 might still regulate the expression of these genes at the protein level.

Within plant, fungal, and animal kingdoms, RPN9 is the most divergent subunit of the 26S proteasome (Yang et al., 2004). It contains a PINT/PCI domain that is present in several subunits of the 19S RP lid, COP9/signalosome complex, and translation initiation factor eIF3 (Wei and Deng, 2003), reflecting a possible common evolutionary ancestry. RPN9 is not essential in yeast (*Saccharomyces cerevisiae*) and *Caenorhabditis elegans* (Takeuchi et al., 1999, 2002), but its deletion results in temperature-sensitive growth arrest, reduced 26S proteasome accumulation, and incomplete 26S proteasome assembly in yeast (Takeuchi et al., 1999; Takeuchi and Toh-e, 2001). Recently, it was demonstrated that silencing of *RPN9*, *RPN3*, and the 20S core α_6-subunit activates programmed cell death after silencing (Kim et al., 2003), which implies an essential role for *RPN9* in plants. This result agrees with our data that *RPN9* silencing promotes xylem formation, a process of programmed cell death in plants, and induces early senescence initiating from leaf veins. Our data also suggest that *RPN9* is essential in plants and specifically regulates plant vascular development and leaf vein formation. This evidence indicates that the 26S proteasome regulates plant vascular development via a specific regulatory subunit RPN9, possibly by selective degradation of regulatory components in auxin transport and BR signaling. Further identification of the regulatory protein targets of RPN9 will help elucidate the complex molecular mechanism of plant vascular development.

MATERIALS AND METHODS

Plant Growth Conditions

Nicotiana benthamiana plants and Arabidopsis (*Arabidopsis thaliana*) plants were grown in a plant growth room at 22°C under a 16-h light/8-h dark cycle. For BL and auxin treatments, seedlings were grown on Murashige and Skoog medium with 100 nM BL or 10 μM IAA.

VIGS Screen

A subtraction cDNA library was made by suppression subtractive hybridization between cDNAs prepared from TMV-challenged *N. benthamiana* (mixture of 1-, 2-, 4-, 8-, 24-, and 48-h postinoculation) and unchallenged plants with a PCR-select cDNA subtraction kit (BD Biosciences). The cDNA library was cloned into the PVX-derived silencing vector pGr106 (Takken et al., 2000) and introduced into Agrobacterium *GV2260.* The VIGS screen was conducted by toothpick inoculation on the abaxial side of the leaves of 4-week-old plants with Agrobacterium colonies carrying silencing constructs. Agrobacterium carrying a TMV binary construct expressing GFP with a coat protein promoter was infiltrated into wild-type *N. benthamiana* plants for virus propagation. TMV-GFP leaf sap was extracted and inoculated on silenced leaves 14 d after toothpick inoculation for monitoring viral transport as described in Jin et al. (2002).

Histological Analysis

Leaf and stem tissues were fixed and dehydrated for SEM analysis as described (Dong et al., 2005). Dehydrated samples were critical-point dried, sputter coated, and observed under a SEM (XL30-FEG; Philips).

For light microscopy, leaf and stem tissues were fixed, dehydrated, embedded in Technovit 7100 resin (Kulzer), and sectioned at 4-μm thickness as described (Dong et al., 2005). Sections were stained with 0.5% toluidine blue or 0.01% Auromine O (fluorescence dye) for visualization under a Nikon Microphot FXA microscope (Nikon).

Real-Time RT-PCR

Samples were collected at 8 DPI and subjected to RNA extraction and cDNA synthesis as described (Jin et al., 2002). Real-time PCR was performed on an iCycler (Bio-Rad) with SYBR Green (Bio-Rad). Each sample was measured in triplicate and calculated by the standard curve method (iCycler guide). The Actin gene was used as an internal reference. Real-time primers used for *N. benthamiana* genes are as follows: Actin forward, 5′-CCCAAAGGCTAATCGTGAAA-3′, reverse, 5′-ACCATCACCAGAGTCCAACA-3′; PINOID forward, 5′-GCAGCGTAGCTTCGTTTGAT-3′, reverse, 5′-TTGTTGGGACCCACGAGTAT-3′; BRL1 forward, 5′-TATACGAATACATGAAATGGGGAAG-3′, reverse, 5′-AGCATTTACCAGCCTTGCCATTCC-3′; *RPN9* forward, 5′-ATGGCCGCTTTAGAGTACTT-3′, reverse, 5′-TGGTACAGATCTGATAGCGTA-3′; RPN8 forward, 5′-TTTAGATCGACTGACAGAGAAC-3′, reverse, 5′-ATTCATCTATTTTAGGTACCAAG-3′, and 20S α_4-subunit forward, 5′-AAGTCTACTCCCAAGCTTCAG-3′, reverse, 5′-CAGTAACAGGATCCTCAACAG-3′. Primers for quantifying RPN9, RPN8, and the 20S α_4-subunit were designed from outside the silencing region. All PCR products were sequenced and confirmed to be the correct clones. All gene expression analyses were repeated at least three times on different sets of silenced plants with comparable results, and the data presented are means of triplicates for each condition in one representative experiment. Error bars are the SD of the mean.

Auxin Transport

IAA transport activity was measured as described (Okada et al., 1991). Two-centimeter lengths of leaf midveins were cut from *RPN9*$^-$ and control leaves of similar size and placed into inorganic Murashige and Skoog medium containing [^{14}C]IAA at 1.45 μM (0.08 μCi/mL) with or without 60 μM NPA in upright (acropetal) and inverted (basipetal) orientations. Distribution of labeled IAA was visualized by exposing the veins to a phosphor-imaging plate after 18 h of incubation. The amount of incorporated radioactivity within the upper 1-cm pieces was measured by a liquid scintillation counter.

Polar Auxin Transport Inhibitor Treatments

For seedling assays, seeds were germinated on Murashige and Skoog medium with 25 μM NPA (Uniroyal Chemical). Leaves were taken after 10 to 14 d for leaf-clearing analysis. For the viral infection assay, 3-week-old plants were sprayed with a heavy mist of 100 or 200 μM NPA with 0.01% Silwet L-77 (a surfactant; Lehle Seeds) as described in Nemhauser et al. (2000). Mock treatments were performed with distilled water containing 0.01% Silwet L-77.

Transient Expression Assay

Agrobacterium GV2260 (OD_{600} = 1.0) carrying various GFP fusion constructs (BRI-GFP, BZR1-CFP, or GFP-ICE1) was infiltrated into *RPN9*$^-$ and control leaves at 9 DPI. Leaf discs were collected at 66 h postinoculation and subjected to western analysis with GFP antibody sc-8334 (Santa Cruz), which

recognizes both GFP and cyan fluorescent protein fusions. For detecting GFP-ICE1 fusion, the total protein extract was immunoprecipitated with GFP antibody prior to western-blot analysis.

Sequences of the mRNA fragments used for silencing RPN9 (DQ226994), RPN8 (DQ226995), and the 20S α_4-subunit (DQ226996) have been deposited in GenBank.

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