The Arabidopsis Rho of Plants GTPase AtROP6 Functions in Developmental and Pathogen Response Pathways

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How plants coordinate developmental processes and environmental stress responses is a pressing question. Here, we show that Arabidopsis (Arabidopsis thaliana) Rho of Plants6 (AtROP6) integrates developmental and pathogen response signaling. AtROP6 expression is induced by auxin and detected in the root meristem, lateral root initials, and leaf hydathodes. Plants expressing a dominant negative AtROP6 (rop6DN) under the regulation of its endogenous promoter are small and have multiple inflorescence stems, twisted leaves, deformed leaf epidermis pavement cells, and differentially organized cytoskeleton. Microarray analyses of rop6DN plants revealed that major changes in gene expression are associated with constitutive salicylic acid (SA)-mediated defense responses. In agreement, their free and total SA levels resembled those of wild-type plants inoculated with a virulent powdery mildew pathogen. The constitutive SA-associated response in rop6DN was suppressed in mutant backgrounds defective in SA signaling (nonexpressor of PR genes [npr1]) or biosynthesis (salicylic acid induction deficient [sid2]). However, the rop6DN npr1 and rop6DN sid2 double mutants retained the aberrant developmental phenotypes, indicating that the constitutive SA response can be uncoupled from ROP function(s) in development. rop6DN plants exhibited enhanced preinvasive defense responses to a host-adapted virulent powdery mildew fungus but were impaired in preinvasive defenses upon inoculation with a nonadapted powdery mildew. The host-adapted powdery mildew had a reduced reproductive fitness on rop6DN plants, which was retained in mutant backgrounds defective in SA biosynthesis or signaling. Our findings indicate that both the morphological aberrations and altered sensitivity to powdery mildews of rop6DN plants result from perturbations that are independent from the SA-associated response. These perturbations uncouple SA-dependent defense signaling from disease resistance execution.

Rho of Plants (ROPs), also known as RACs (for clarity, the ROP nomenclature will be used throughout this article), comprise a plant-specific group of Rho family small G proteins. Like other members of the Ras superfamily of small G proteins, ROPs function as molecular switches, existing in a GTP-bound “on” state and a GDP-bound “off” state. In the GTP-bound state, ROPs interact with specific effectors that transduce downstream signaling or function as scaffolds for interaction with additional effector molecules (Berken and Wittinghofer, 2008). Conserved point mutations in the G1 (P loop) Gly-15 or the G3 (switch II) Gln-64, which abolish GTP hydrolysis, or the G1 Thr-20 or G4 Asp-121 that compromise GDP/GTP exchange, can form either constitutively active or dominant negative mutants, respectively (Feig, 1999; Berken et al., 2005; Berken and Wittinghofer, 2008; Sorek et al., 2010). Primarily based on studies with neomorphic mutants, ROPs have been implicated in the regulation of cytoskeleton organization and dynamics, vesicle trafficking, auxin transport and response, abscisic acid (ABA) response, and response to pathogens (Nibau et al., 2006; Yalovsky et al., 2008; Yang, 2008; Lorek et al., 2010; Wu et al., 2011; and refs. therein).

In Arabidopsis (Arabidopsis thaliana), there are 11 ROP proteins (Winge et al., 1997). Assigning specific functions to individual members of this family is difficult, however, because ROPs are functionally...
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RESULTS

ROP6 Expression Pattern and Subcellular Localization

Previously, we analyzed lipid modifications, membrane interaction dynamics, and the function of ROP6 in the regulation of cell polarity using the constitutively active rop6CA mutant expressed under the regulation of the cauliflower mosaic virus 35S promoter. Because of the functional redundancy of ROPs, the rop6 mutant phenotype is inseparable from that of wild-type plants. To overcome this difficulty and elucidate the specific function of ROP6, its promoter was isolated (Supplemental Fig. S1) and subcloned into the LhG4/pOp transcription transactivation system (Moore et al., 1998) to drive the expression of GUS, DsRed, GFP-ROP6, GFP-rop6CA, and GFP-rop6DN. To verify the expression pattern and phenotypes, three independent transgenic lines were analyzed for each expression construct.

In rosette leaves, ROP6 expression was detected at the tip and in the hydathodes at the leaf margin (Fig. 1, A–C). In cauline leaves, however, expression was not confined to the leaf margins, and leaf epidermis pavement cells expressing a constitutively active GFP-rop6CA (rop6CA) were rectangular with few and shallow lobes (Fig. 1F). No expression could be detected in the inflorescence meristem and in young flowers (Fig. 1D). In mature flowers, strong expression was detected in stamen filaments, pollen grains, and stigma and weak expression was detected in ovules (Fig. 1E). In the primary root, expression was detected in the root tip, was absent from the cell division zone, and increased in the stеле of the transition zone. In the root differentiation zone, expression increased in lateral root initials (LRI) and was already detected in lateral root founder cells in the pericycle (Fig. 1, G–I). The
The expression pattern of ROP6 in the root was further confirmed by whole-mount RNA in situ hybridization. Similar to expression detected with reporter genes fused to the ROP6 promoter, following hybridization with an antisense probe, ROP6 RNA was detected in the stele, LRI, and the tip of the emerging lateral root (Fig. 2).

Figure 1. Expression patterns of AtROP6 in the shoot and the root. A, B, D, E, G, and H, pROP6::GUS. C, F, J, and L, pROP6::GFP-rop6CA. I, pROP6::DsRed. A to C and F, In rosette leaves, ROP6 expression is primarily restricted to hydathodes, whereas in cauline leaves, it is detected throughout the epidermis. D, ROP6 expression could not be detected in the inflorescence meristem and young flowers. E, In mature flowers, expression is detected in stamen filaments and pollen grains, stigma, style, and weakly in ovules. G, In the root, expression is detected in the root cap, in and around the quiescent center; it is absent from the cell division and transition zone, and it reappears in the stele in the differentiation zone. H and I, Expression then drops again and reappears in LRI and founder cells. The arrow denotes a protoxylem file. J, Expression of ROP6 is induced by auxin and can be detected in pericycle cells as early as 3 h after induction. K, After 6 h, expression is spread to more cells in the pericycle. L, Twenty-four hours after induction, expression is spread throughout the pericycle and extensive cell divisions are detected. Bars = 100 μm (A), 50 μm (B, D, E, and G–L), and 20 μm (C and F). For more information, see Supplemental Figure S2.

To further explore possible function(s) of ROP6 in lateral roots, we examined its subcellular localization to an auxin-containing medium (10 μM naphthalene-acetic acid [NAA]; Fig. 1J). Six hours after transfer to auxin-containing medium, GFP-rop6CA was detected in many pericycle cells, whereas on control plates, it was detected only in a few cells (Fig. 1K). GFP-rop6CA was visible in many dividing pericycle cells 24 h after transfer to auxin (Fig. 1L). The results of the auxin induction experiments confirm that ROP6 expression is quickly induced by auxin during lateral root development. Furthermore, the identical expression pattern, which was detected when using GUS, DsRed, GFP-ROP6, GFP-rop6CA, or GFP-rop6DN (Figs. 1 and 3), and the fast (3-h) response to auxin treatments make it unlikely that the activation status of the transgenic ROP protein affected the AtROP6 expression pattern and the preliminary response to auxin.

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Figure 2. Expression of ROP6 in the roots determined by whole-mount in situ hybridization. A, Hybridization with a ROP6 gene-specific sense probe, negative control. B to D, An antisense ROP6 gene-specific probe. Expression was detected in the stele (B, arrow, and D), in the LRI (C), and in the meristematic region of the mature lateral root (D). B, Lateral root founder cells prior to the first anticlinal division. C, Lateral root primordia emerging from the primary root. D, Young lateral root. Bars = 20 μm. [See online article for color version of this figure.]

in the developing LRI. In lateral root founder cells in the pericycle, either GFP-ROP6 or GFP-rop6CA was localized in the cell at the side that is closer to the xylem pole (Fig. 3, A, B, I, and J). At later stages of lateral root development, GFP-ROP6 and GFP-rop6CA were localized such that they aligned the cell division planes (Fig. 3, C–P) and were absent from the side of the cell pointing toward the lateral root tip (Fig. 3, E–H, M, and N). This polar localization suggests that ROP6 relays a special signal in LRI cells. Similarly, it has been found that during stomata development in maize (Zea mays), the type I ROPs ROP2 and ROP9 enhance the polarization of asymmetric cell divisions (Humphries et al., 2011). Together, these data suggest that ROP-dependent polarization could be critical for asymmetric cell divisions. In contrast to GFP-ROP6 and GFP-rop6CA, the dominant negative GFP-rop6DN accumulated in the cytoplasm and nuclei in root and leaves (Fig. 3, Q–S), resembling the subcellular distribution of the nonprenylated GFP-rop6C195S mutant (Fig. 3T). Hence, nucleotide exchange is required for the recruitment of ROPs to the plasma membrane.

The Phenotype of rop6DN Plants

Next, we examined the phenotype of the pROP6>>GFP-rop6DN (rop6DN) plants (Fig. 4). The rop6DN plants were small and developed multiple inflorescence stems (Figs. 4, A and B, and 7, D and E). The rosette leaves of the rop6DN plants were curled, and their cauline leaves were twisted (Fig. 4, A–F). The stronger effect of rop6DN on the structure of cauline leaves compared with rosette leaves was closely correlated with its expression levels in the two leaf types (Fig. 1, A and F). Analysis of the cell structures in rosettes (Figs. 4, G and H, and 7, H and I) and cauline leaves (Fig. 4, I and J) revealed that pavement cells in the rop6DN leaves had fewer lobes and indentations. Quantitative analysis of cell structure, using a value designated the average polarity score, which is based on the ImageJ tools “circularity” and “Skeleton end points” (Sorek et al., 2010, 2011), shows that the rop6DN cells are significantly less polar compared with wild-type cells ($P \leq 0.001$, ANOVA, Tukey-Kramer test; Fig. 7, L–N).

Organization of the Cytoskeleton in Wild-Type and rop6DN Plants

ROPs have been implicated in cytoskeleton organization and dynamics, suggesting that the phenotype of rop6DN plants could be associated with impaired actin and MT functions. Therefore, we examined the organization of MT and actin in wild-type and rop6DN plants by immunostaining the cytoskeleton in rosette leaves (Fig. 5). In wild-type pavement cells, F-actin was organized in thin filaments and a few thicker bundles that were oriented at different angles. In addition, diffuse fluorescence likely corresponding to fine F-actin or G-actin could be seen (Fig. 5A; Supplemental Fig. S3, A–C). In comparison, in the rop6DN pavement cells, F-actin was primarily organized in thick bundles, many of which were arranged parallel to the long cell axis, and little diffuse fluorescence could be seen (Fig. 5B; Supplemental Fig. S3, E–H). The MTs were arranged at different angles in wild-type pavement cells (Fig. 5C; Supplemental Fig. S3, I–L), while in rop6DN pavement cells, they were arranged mainly in parallel arrays, perpendicular to the long axis of cells (Fig. 5D; Supplemental Fig. S3, M–P). Taken together, the immunostains demonstrate that both actin and MT were differentially organized in the rop6DN plants.

rop6DN-Associated Changes in Gene Expression

The obvious developmental perturbations of rop6DN plants prompted us to examine whether the phenotype of the rop6DN plants is associated with changes in gene expression. Gene expression in rop6DN, wild-type, pROP6>>GFP-ROP6 (ROP6), and pROP6>>GFP-rop6CA (rop6CA) plants (Supplemental Fig. S2) was analyzed using microarrays. Entire seedlings were used in the microarrays. To identify differentially expressed genes, we used the “rank product” method. The rank product analysis gives an estimate of false discovery rate while providing flexible means of
Figure 3. Subcellular localization of GFP-ROP6, GFP-rop6CA, and GFP-rop6DN during lateral root development. Subcellular localizations of GFP-ROP6 (A–H) and GFP-rop6CA (I–P) were observed in developing lateral roots in pROP6::GFP-ROP6 and pROP6::GFP-rop6CA seedlings. A, B, I, and J, Lateral root founder cells. GFP-ROP6 and GFP-rop6CA were localized along the side of the cell adjacent to the xylem pole (J, arrow). C, D, K, and L, Stage I/II LRI. The majorities of the GFP-ROP6 and GFP-rop6CA proteins moved and were localized along anticlinal cell borders (arrows). E, F, M, and N, Stage II/III LRI. The majority of GFP-ROP6 and GFP-rop6CA were localized along anticlinal and periclinal cell borders (arrows) separating the newly formed outer and inner layers. G, H, O, and P, Emerged and mature lateral roots with organized cell files. The majority of the GFP-ROP6 and GFP-rop6CA proteins were concentrated on proximal anticlinal cell borders (arrows). In the meristematic zone, the protein was distributed more or less evenly around the cells. Q to S, GFP-rop6DN was observed in pROP6::GFP-rop6DN seedlings in lateral root founder cells (Q), stage II/III LRI cells (R), and leaf epidermis pavement cells (S). The recruitment of GFP-rop6DN to the plasma membrane is compromised, and it accumulates in the cytoplasm and nuclei in roots (arrows). T, Non-prenylated GFP-rop6CA mutant accumulates in cytoplasm and nuclei (arrows) but, unlike GFP-rop6DN, does not affect cell polarity. Bars = 20 μm.
to assign a significance level to each gene (Breitling et al., 2004). Differentially expressed genes are listed in Supplemental Tables S1 to S5. Pairwise plots showing relative gene expression levels revealed that the expression of many genes was changed in rop6DN seedlings relative to wild-type, ROP6, and rop6CA plants (Fig. 6A). Gene expression data were analyzed using hierarchical (Fig. 6B) and K-means (Fig. 6, C–H) clustering algorithms to identify genes with similar expression patterns. The clustering analyses revealed a group of 14 genes that were strongly induced in rop6DN plants (Fig. 6, C and F), a group of 24 genes that were mildly up-regulated (Fig. 6, D and G), and a group of 30 genes that were suppressed in rop6DN plants relative to wild-type, ROP6, or rop6CA transgenic plants (Fig. 6, E and H). Functional categorization of the differentially expressed genes by Gene Ontology (Martin et al., 2004) revealed that many of the up-regulated genes in the rop6DN plants were reminiscent of SA-dependent defense responses (Supplemental Tables S1–S5; Supplemental Fig. S4). SA-mediated disease resistance is a mechanism of induced defense that confers protection against a broad spectrum of microorganisms. SA-mediated pathogen defense requires the accumulation of SA, which serves as a signaling molecule leading to the induction of pathogenesis-related proteins that contribute to pathogen resistance (Durrant and Dong, 2004; Conrath, 2006). The frequency of SA-associated genes among the up-regulated genes is statistically significant ($P \leq 0.05$) compared with their random up-regulation (Supplemental Table S4). Furthermore, expression analysis of the SA-associated genes in
Genevestigator (http://www.genevestigator.ethz.ch; Hruz et al., 2008) showed that their expression is primarily associated with biotic defense responses and not with other stress responses. Examination of the gene expression patterns of all the genes that were differentially expressed in rop6<sup>DN</sup> plants revealed that most of them are subject to regulation by biotic stress. Hence, the microarray study suggested that expression of the dominant negative rop6<sup>DN</sup> induces a constitutive SA-mediated defense response and that most if not all of the observed changes in gene expression can be explained by activated SA signaling.

The microarray data required validation of the gene expression pattern by an independent method and raised questions that required additional experimental work. The questions that we asked were as follows. (1) Are the levels of SA higher in the rop6<sup>DN</sup> plants compared with the controls, as would be expected from the gene expression analysis? (2) Is the developmental phenotype of the dominant negative rop6<sup>DN</sup> plants associated with a constitutive systemic acquired resistance response? (3) Are the rop6<sup>DN</sup> plants more resistant to pathogens as a result of the constitutive systemic acquired resistance response?

**rop6<sup>DN</sup> and the SA Response**

Validation of the microarray data was carried out by real-time quantitative reverse transcription PCR (qPCR) analysis of three genes that displayed strong differential expression in the rop6<sup>DN</sup> plants and are often used as markers for SA-dependent defense responses: (1) NIMIN1 (for nonexpresser of PR genes1/noninducible immunity1 interacting protein1; AT1G02450), (2) Pathogenesis-Related Protein5 (PR-5; AT1G75040), and (3) PR-1 (AT2G14610). The qPCR results show that NIMIN1, PR-5, and PR-1 expression levels are 4-, 12-, and 2,000-fold higher, respectively, in rop6<sup>DN</sup> relative to wild-type plants (Fig. 7, A–C), confirming the microarray results. Importantly, the expression levels were the same when RNA was extracted from 14-d-old seedlings (Supplemental Fig. S2), like the plants analyzed by microarrays or from mature flowering plants (Figs. 4 and 7).

To examine whether the changes in gene expression in the rop6<sup>DN</sup> plants are indeed associated with SA, we measured the levels of total and free SA in wild-type, ROP6, rop6<sup>CA</sup>, and two groups of rop6<sup>DN</sup> plants. One group is characterized by strong expression of the rop6<sup>DN</sup> protein, similar to the plants shown in Figures 3, 4, and 7, while the second group is characterized by very low expression levels of rop6<sup>DN</sup> and has a mild developmental phenotype (these plants are resistant to both kanamycin and basta, confirming that both transfer DNA (T-DNA) inserts of the transactivation expression system are present). In each of the lines, SA levels were compared with those in plants that were inoculated with spores of Golovinomyces orontii.

![Figure 5. Immunostaining showing actin and MT organization in Col-0 wild-type and GFP-rop6<sup>DN</sup> plants. Actin (red) and MTs (green) are shown in wild-type Col-0 (A and C) and GFP-rop6<sup>DN</sup> (B and D) leaf epidermal cells. Bars = 10 μm.](https://academic.oup.com/plphys/article/161/3/1172/6110578)
In contrast, in wild-type, ROP6, and rop6<sup>CA</sup> plants, SA levels only increase following inoculation with G. orontii. SA levels are also increased in the rop6<sup>DN</sup> plants with low transgene expression levels but at lower levels compared with rop6<sup>DN</sup> plants with high expression levels of the transgene (Fig. 6, I and J). The higher SA levels confirmed that the changes in gene expression detected in rop6<sup>DN</sup> plants are indeed associated with the SA-induced defense response.

To examine whether the developmental phenotype of the rop6<sup>DN</sup> plants is linked to the constitutive SA-mediated response, the rop6<sup>DN</sup> plants were crossed to mutants in NPR1, which is required for SA-mediated signaling (Cao et al., 1997), and Salicylic Acid Induction Deficient2 (SID2)/Enhanced Disease Susceptibility16 (EDS16), which is required for pathogen-inducible SA biosynthesis (Nawrath and Métraux, 1999; Supplemental Figs. S4 and S5). It has been shown that both npr1 and sid2 plants fail to induce the expression of PR genes and display enhanced disease susceptibility to pathogens (Cao et al., 1997; Nawrath and Métraux, 1999; Kinkema et al., 2000; Stein et al., 2006). The expression of NIMIN1, PR-5, and PR-1 is suppressed in the rop6<sup>DN</sup> npr1 and rop6<sup>DN</sup> sid2 double mutant plants, resembling their expression in naive wild-type plants (Fig. 7, A–C). The mutant analysis confirmed that the expression of PR genes in the rop6<sup>DN</sup> mutant plants requires SA biosynthesis and NPR1 function, as is known to occur in authentic pathogen-induced SA-mediated defense responses (Durrant and Dong, 2004).

The macroscopic phenotype of both rop6<sup>DN</sup> npr1 and rop6<sup>DN</sup> sid2 double mutants resembles the rop6<sup>DN</sup> single mutants. The plants were small, the rosette and cauline leaves were uneven and twisted, and the plants lost their apical dominance and developed numerous adventitious inflorescence stems (Fig. 7, D–G). Qualitative (Fig. 7, H–K) and quantitative (Fig. 7, L–N) analyses of leaf epidermis pavement cell structure showed that cells of rop6<sup>DN</sup> npr1 and rop6<sup>DN</sup> sid2 double mutants resembled the rop6<sup>DN</sup> single mutant and are significantly ($P \leq 0.001$, ANOVA, Tukey-Kramer test) different from wild-type plants. The phenotypic analysis of the rop6<sup>DN</sup> npr1 and rop6<sup>DN</sup> sid2 double mutant plants indicates that the developmental phenotype of the rop6<sup>DN</sup> plants can be uncoupled from the constitutive SA-dependent defense response. Hence, the overall small plant size, loss of apical dominance, twisted leaves, and deformed cells observed in the rop6<sup>DN</sup> plants are not associated with the constitutive SA-dependent defense response.

Pathogen Response of rop6<sup>DN</sup> Plants

Mutants in SA biosynthesis or signaling enhance disease susceptibility to pathogens, while the induction of SA-dependent defense responses reduces pathogen growth, especially against biotrophic pathogens such as powdery mildews (Durrant and Dong, 2004).
Figure 7. PR gene expression and phenotypes of rop6DN npr1 and rop6DN sid2 double mutants. A to C, Expression analysis of NIMIN1 (A), PR-5 (B), and PR-1 (C) by qPCR in wild-type (WT), rop6DN, rop6DN npr1, and rop6DN sid2 plants. For each gene, its relative expression level in the wild type was taken as 1. Note the suppression of expression of each of the three genes to wild-type levels in the rop6DN npr1 and rop6DN sid2 double mutants. D to G, Plants of the wild type (D), rop6DN (E), rop6DN npr1 (F), and rop6DN sid2 (G). H to K, Abaxial epidermis pavement cells. Bars = 20 μm. L to N, Quantitative parameters of epidermal cell structure. L, Number of lobes. M, Circularity. N, Average polarity score. Note that the rop6DN single mutant and the rop6DN npr1 and rop6DN sid2 double mutants have the same plant and cell structures. [See online article for color version of this figure.]
Hence, it was expected that the rop6<sup>DN</sup> plants would display increased resistance to G. orontii due to the high levels of SA and PR gene expression that greatly exceed the SA levels seen in wild-type plants after G. orontii infection (Fig. 6, I and J). To test this, wild-type, ROP6, rop6<sup>CA</sup>, rop6<sup>DN</sup>, rop6<sup>DN</sup> WT (plants that phenotypically resembled wild-type plants and in which no visible GFP-rop6<sup>DN</sup> expression could be detected, although they were resistant to both the

**Figure 8.** Susceptibility to the powdery mildew fungus. A, Wild-type (WT), GFP-rop6<sup>DN</sup> (ROP6<sup>DN</sup>), GFP-rop6<sup>DN</sup> with low GFP-rop6<sup>DN</sup> expression and weak phenotype (ROP6<sup>DN</sup> WT), SA biosynthesis mutants eds1-2, sid2-1, and sid2-2, and double mutants of GFP-rop6<sup>DN</sup> with sid2-1 (ROP6<sup>DN</sup> sid2-1) and with the SA signaling mutant npr1-2 (ROP6<sup>DN</sup> npr1-2) plants are susceptible to the powdery mildew fungal pathogen G. orontii. B, Fungal entry rates of G. orontii on GFP-rop6<sup>DN</sup> (6DN) and on GFP-rop6<sup>DN</sup> plants with low expression levels and weak phenotype (6DN WT) are lower compared with the entry rate on wild-type, GFP-ROP6 (R6), and GFP-rop6<sup>CA</sup> (6CA) plants. C, Fungal entry rates of Bgh on GFP-rop6<sup>DN</sup> (6DN) and on GFP-rop6<sup>CA</sup> (6CA) plants. The entry rates of Bgh on the GFP-rop6<sup>CA</sup> plants are higher than on wild-type and GFP-ROP6 plants. D, G. orontii conidiospore formation was significantly lower on GFP-rop6<sup>DN</sup> (6DN), GFP-rop6<sup>DN</sup> sid2-1 (6DN sid2-1), and GFP-rop6<sup>DN</sup> npr1-2 (6DN npr1-2) compared with wild-type and GFP-rop6<sup>DN</sup> WT (6DN WT) plants. Conidiospore formation was strongly enhanced compared with the wild type on eds1-2 and slightly enhanced on sid2-1 and sid2-2 SA biosynthesis mutants. E, Treatments with the SA analog BTH (black bars) reduced G. orontii conidiospore formation and further enhanced resistance of GFP-rop6<sup>DN</sup> plants. Gray bars show mock-treated (water) plants.
low-expressing rop6DN plants at early stages of pathogenesis (48 h post inoculation; Supplemental Fig. S7). As expected, enhanced disease susceptibility was detected in the eds1-2 mutant that lacks a key regulatory node of plant immune responses known to limit the growth of host-adapted virulent pathogens (Poraty et al., 2005).

To obtain a more accurate account of infection phenotypes on the different lines, fungal entry into plant cells from each of the different lines was determined (as percentage entry at single plant-powdery mildew interaction sites). The results of this quantitative analysis show that 48 h after inoculation, the fungal entry rates into the rop6DN plants were around 65% compared with 90% in wild-type, ROP6, and rop6CA plants. The fungal entry rates on rop6DN plants with low expression of the transgene were around 80%, slightly lower compared with wild-type, ROP6, and rop6CA plants and higher compared with rop6DN plants with high transgene expression levels (Fig. 8B). These data indicate that the constitutive SA-dependent defense response in the rop6DN plants results in a moderate increase in preinvasive defense responses to the powdery mildew fungus, despite even higher constitutive SA levels than in wild-type plants found after pathogen challenge (Fig. 6, I and J).

We also determined preinvasion infection phenotypes after inoculation with Bgh, a nonadapted powdery mildew that fails to colonize Arabidopsis due to effective extracellular (preinvasive) resistance responses at early stages during fungal pathogenesis (Collins et al., 2003). At 48 h post inoculation, the entry rates of Bgh sporelings into wild-type Columbia (Col-0) leaf epidermal cells were around 10% (Fig. 8C). In comparison, the entry rates of virulent G. orontii at 48 h post inoculation were approximately 90% (Fig. 8B). The entry rates of Bgh into epidermal cells expressing a constitutively active rop6CA were higher, averaging between 16% and 20% (Fig. 8C). Interestingly, in a compatible interaction in barley expressing constitutively active ROP mutants, entry rates of Bgh were also higher compared with the wild type (Schultheiss et al., 2002, 2003; Opalski et al., 2005; Hoeft et al., 2011). Significantly, Bgh entry rates of rop6DN epidermal cells were even higher, reaching approximately 30% (Fig. 8C). Taken together, these data suggest that the disruption in cytokinesis organization impaired extracellular disease resistance responses of the rop6DN plants toward Bgh, albeit the SA levels in these plants were constitutively elevated.

Next, we quantified the reproductive fitness of the host-adapted G. orontii powdery mildew by counting fungal conidiospores at a late stage after pathogen challenge (7 d after inoculation) in wild-type, rop6DN, rop6DN WT (low levels of rop6DN expression), sid2-1, eds1-2, sid2-2, rop6DN sid2-1, and rop6DN npr1-2 plants. As expected, G. orontii reproductive fitness on eds1-2 plants was greatly increased compared with the wild type and was moderately increased on sid2-1 and sid2-2 plants (Fig. 8D; Supplemental Fig. S8). In contrast, G. orontii reproductive fitness on rop6DN plants was significantly lower compared with the wild type (Fig. 8D; Supplemental Fig. S8). Importantly, reproductive fitness of G. orontii remained significantly lower than wild-type levels in rop6DN sid2-1 and rop6DN npr1-2 double mutants and was comparable to or only slightly higher than in rop6DN single mutants (Fig. 8D; Supplemental Fig. S8). To further substantiate these findings, we tested G. orontii reproductive fitness following pretreatments with the SA analog benzothiadiazole (BTH). The BTH treatments caused decreased G. orontii reproductive fitness in wild-type, ROP6, rop6CA, rop6DN, and rop6DN Wt, plants, demonstrating that SA can confer enhanced disease resistance to G. orontii in Arabidopsis. In rop6DN plants, BTH treatments had an additive effect, further reducing the already lower reproductive fitness of the fungus (Fig. 8E; Supplemental Fig. S9). Taken together, the extensive data set provides evidence that rop6DN mediates an enhanced disease resistance phenotype to host-adapted G. orontii independently of the SA-mediated defense pathway.

**DISCUSSION**

**Plant Development and Hormonal Response and Transport**

In the absence of other families of signaling small GTPases in plants, ROPs were suggested to function as regulators of diverse signaling pathways (Yang, 2002). The results in our work are consistent with earlier findings and implicate ROP6 in both developmental and pathogen response regulation. The expression pattern of ROP6 is highly specific and closely resembles that of auxin-regulated genes both in leaves and roots. Correspondingly, the small plant size, loss of apical dominance, and twisted leaf phenotypes of plants expressing a dominant negative rop6DN under the regulation of the ROP6 promoter resemble mutant plants that are affected in auxin signaling, transport, and biosynthesis. Because the phenotype of rop6 T-DNA knockout mutants is indistinguishable from wild-type plants, it is likely that the rop6DN mutant affected functions of other ROPs, which are expressed in the same tissue as ROP6. Hence, by expressing rop6DN under the regulation of its native promoter, we overcame functional redundancy between ROPs while maintaining specificity and analyzing ROP function in a developmental context.

It has recently been proposed that ROPs are activated by auxin through a mechanism that involves ABP1 (Xu et al., 2010). Activated ROPs undergo transient S-acylation and partition into lipid rafts, a process that is required for their function (Sorek et al., 2007, 2010) and that induces localized inhibition of endocytosis (Bloch et al., 2005). In lipid rafts, ROPs interact with their effector ICR1 (Sorek et al., 2010), which regulates the recruitment of PIN auxin efflux transporters to polar domains in the plasma
membrane and is required for directional auxin transport (Hazak et al., 2010). ICR1 transcription is induced by auxin, and similar to ROP6, it is also expressed in LRI (Hazak et al., 2010). Our results (Fig. 3, Q–S) indicate that the dominant negative rop6DN accumulates in the cytoplasm and nuclei, where it presumably interacts with ROPGEFs, inhibiting their function in ROP activation (Feig, 1999; Berken et al., 2005; Berken and Wittinghofer, 2008). In addition, the altered cytoskeleton organization in the rop6DN plants (Fig. 5) may interfere with auxin transport. It has been demonstrated that actin is required for PIN recycling (Geldner et al., 2001) and MTs are required for polar PIN localization (Boutté et al., 2006; Kleine-Vehn et al., 2008; Heisler et al., 2010). Hence, the expression of rop6DN could have inhibited ROP function in regulating auxin transport.

Epidermal Cell Structure and Growth

The changes in rosette leaf pavement cell morphology, which were detectable in independent transgenic lines, suggest that although ROP6 is primarily expressed at the leaf tip and hydathodes (Fig. 1, A–C), it may also be expressed in pavement cells at levels that are below detection with GUS and GFP reporter genes. Alternatively, the dominant negative rop6DN may affect pavement cell morphology indirectly. For example, it has recently been shown that perturbations in auxin biosynthesis or distribution affect pavement cell morphology (Xu et al., 2010). Because ROP6 expression in rosette leaves corresponds to sites of high auxin response, it is possible that suppression of ROP function at these sites affects auxin response and/or distribution. Furthermore, the overall small size of the rop6DN plants and the development of multiple inflorescence stems indicate that the dominant negative rop6DN has non-cell-autonomous effects.

The rosette and cauline leaves of the rop6DN plants have a wavy or twisted appearance (Fig. 4), likely owing to uneven cell growth rates in different parts of the leaf. Analysis of pavement cell growth dynamics in developing Arabidopsis cotyledons showed that cell growth is coordinated between groups of neighboring cells, and it was suggested that this coordination is required for the maintenance of flattened leaf surface (Zhang et al., 2011). Close comparison of the scanning electron microscopy images of wild-type and rop6DN rosette and cauline leaf epidermal cells shows that the pavement cells are flatter in the wild type compared with rop6DN (Fig. 4, compare G with H and I with J). Furthermore, the cells of the rop6DN plants range in size and shape: some cells are rectangular or cubical, while other cells develop more lobes (Fig. 7). The bulging of the rop6DN epidermal cells probably occurred due to isotropic and uncoordinated cell growth that may have altered the mutual pressures that cells exert on their neighbors. Pavement cells of plants expressing constitutively active ROP mutants also display isotropic growth, but they are usually more rectangular and even in their size. The leaf margins of the ropCA mutants usually bend downward, but the leaves are not wavy or twisted (Li et al., 2001; Fu et al., 2002; Bloch et al., 2005; Sorek et al., 2010). This suggests that the isomorphic cell growth cannot fully explain the uncoordinated cell growth that leads to leaf waviness and twisting. It could be that the expression of rop6DN disrupted a non-cell-autonomous mechanism that is responsible for coordinating cell growth in leaves.

Cytoskeleton Organization

Thick GFP-mTalin-labeled F-actin bundles have previously been observed in plants expressing a dominant negative ROP2 (rop2DN) under the regulation of the 35S promoter. It was further shown that the abundance of diffuse F-actin was reduced following transient expression of rop2DN. Interestingly, the same study showed that the expression of rop2CA had the opposite effect, causing an increase in the abundance of diffuse F-actin (Fu et al., 2002). Using a different experimental system, our data similarly show that the abundance of diffuse F-actin was low and that F-actin was primarily organized in thick bundles in the rop6DN plants (Fig. 5; Supplemental Fig. S3). The effect of constitutively active rop6CA or rop11CA led to the formation of thick actin bundles and eliminated the zone of fine F-actin at the root hair tip (Molendijk et al., 2001; Bloch et al., 2005, 2011). This may indicate that activated ROPs possibly have different effects on actin organization in different cell types. An alternative and tempting explanation is that ROP cycling between active and inactive states is required for F-actin turnover and the formation of fine F-actin. The constitutively active mutants are locked in the GTP-bound state (Berken and Wittinghofer, 2008; Sorek et al., 2010). Hence, when signaling requires the binding and release of effectors, it should be impaired when the dissociation of the GTPase from target proteins is compromised. Thus, the bundling of actin filaments may reflect an inhibition of the ROP switch mechanism. How ROPs regulate actin nucleation and stability is yet an open question (Hussey et al., 2006; Deeks and Hussey, 2009).

The MT analysis in this work demonstrates that in many fully developed pavement cells, MTs were organized in transverse or oblique orientation relative to the long cell axis (Fig. 5; Supplemental Fig. S3). Earlier studies with plants expressing rop2DN showed that the cortical MTs have transverse orientation during early stages of pavement cell growth but are randomly organized in mature cells (Fu et al., 2002). The discrepancy between our data and the previous study could be explained by differences in the experimental system, such as the different ROPs used in each
experiment, different expression systems, the level of transgene expression, and plant growth conditions. The observed changes in MT organization could have resulted from changes in tissue biomechanics or from the direct influence by ROPs or both. In cells of the shoot apical meristem, the orientation of cortical MTs was shown to correspond to biomechanical stress from the surrounding tissue (Hamant et al., 2008). It was further shown that both cortical MT orientation and the localization of PIN1 auxin efflux carrier likely respond to the same biomechanical regulator (Heisler et al., 2010). In developing metaxylem cells, the ICR1 homolog MIDD1/RIP3/ICR5 is recruited to specific plasma membrane domains by ROP11, where it interacts with and induces the severing of cortical MTs, leading to secondary cell wall pit formation (Oda et al., 2010; Oda and Fukuda, 2012). Interestingly, adaxial pavement cells of icr1 mutant plants have a cubical shape lacking lobes and indentations, resembling ROP mutants (Lavy et al., 2007). During pavement cell growth, ROP6 is activated by auxin and stabilizes MTs via its effector RIC1 (Fu et al., 2005; Xu et al., 2010). Hence, ROPs may modulate MTs via ICR and RIC family proteins. Analysis of pavement cell growth dynamics showed little correlation between MT organization and orientation and cell structure (Zhang et al., 2011). Clearly, more work is required to understand the complex relation between ROP function, cell growth, MT organization, and cell wall formation and flexibility.

The SA Response

The microarray studies were carried out on young seedlings before the developmental abnormalities induced by the expression of rop6DN became apparent (Supplemental Fig. S2). The logic behind this experimental design was that by looking at changes in gene expression prior to the stage where the phenotype of the plant becomes apparent, it should be possible to define whether the dominant negative ROP induces changes in gene expression and possibly identify the key genes whose alteration leads to the developmental aberrations. However, the microarray analysis revealed that the most significant changes in gene expression induced by rop6DN are associated with SA-dependent defense responses. Down-regulation of lignin biosynthesis by silencing the expression of hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (HCT) in alfalfa (Medicago sativa) and Arabidopsis led to retarded plant growth, which has been associated with a constitutive SA response. Growth of HCT-silenced plants was regained in HCT-RNAi sid2 but not in HCT-RNAi npr1 double mutants (Gallego-Giraldo et al., 2011a, 2011b). In contrast to the HCT-silenced plants, the phenotype of the rop6DN npr1 and rop6DN sid2 double mutants, in which the SA-dependent defense response is blocked, resembled that of rop6DN single mutant plants, indicating that the developmental perturbations induced by rop6DN are unrelated to the constitutive SA-dependent response.

ROPs and Pathogen Response

As described in the introduction, ROP function was linked to plant defense responses in several studies. Results in this work (Figs. 6–8) suggest that the possible suppression of SA-dependent defense response execution by activated ROPs or by loss of MT-associated ROPGAP1 function (Hoefle et al., 2011; Huesmann et al., 2011) may also contribute to the enhanced susceptibility of the respective mutants. Loss-of-function mlo mutant alleles in Arabidopsis and barley are resistant to host-adapted powdery mildews, and this preinvasive resistance depends on polarized exocytosis regulated by PEN1/ROR2 syntaxins (Collins et al., 2003; Consonni et al., 2006; Kwon et al., 2008). Infection by the Bgh powdery mildew was shown to induce a redistribution of MLO, ROR2, and other proteins involved in the defense response to plasma membrane microdomains, which could be lipid rafts, just below sites of attempted fungal ingress (Bhat et al., 2005). The actin and MT cytoskeleton are disorganized in dominant negative ROP mutants (Fu et al., 2002; this work), possibly affecting directed secretion and, in turn, plant defense responses to the powdery mildew fungus. Hence, impaired extracellular resistance responses of the rop6DN plants toward Bgh may have resulted from the disorganized cytoskeleton. Consistent with this interpretation, pharmacological inhibitors and genetic interference by ectopic expression of an actin-depolymerizing factor-encoding gene, HvADF, impaired extracellular resistance responses to Bgh entry in barley (Miklis et al., 2007), and silencing of the HvRAB- and HvRAC1-interacting protein kinase HvRBK1 disrupted MT organization and enhanced susceptibility to Bgh (Huesmann et al., 2012). Of note, however, preinvasive resistance responses to Bgh at early stages during pathogenesis are essentially independent of an intact SA defense pathway (Consonni et al., 2006; Stein et al., 2006). This could explain why extracellular resistance responses to Bgh in rop6DN plants are impaired despite constitutively elevated SA levels.

The BTH treatments (Fig. 8; Supplemental Fig. S9) demonstrated that SA-dependent defense responses can confer resistance to G. orontii in Arabidopsis. Hence, we cannot exclude a small contribution of the SA-dependent defense response to the reduced reproductive fitness of G. orontii seen on rop6DN plants. However, the double mutants of rop6DN with sid2 or npr1 show that the reduced reproductive fitness of G. orontii on rop6DN plants was largely a rop6DN-dependent and SA-independent defense response.

CONCLUSION

Our results imply that ROPs regulate highly complex defense responses to powdery mildews that can be detected at early and late stages of the infection process. ROPs either enhance or reduce early preinvasive
disease resistance responses to the tested host-adapted and nonadapted powdery mildews, presumably via their effects on the cytoskeleton and intracellular trafficking. At later stages of fungal pathogenesis, interference with ROP function reduces fungal reproductive success by mechanisms that remain to be elucidated. The mechanism underlying the constitutive activation of SA accumulation/signaling in rop6DN plants remains unclear. One possibility is that perturbation of the cytoskeleton or default secretion by rop6DN mimics a pathogen attack. Alternatively, similar to HCT-RNAi plants (Gallego-Giraldo et al., 2011b), release of cell wall materials or changes in cell wall integrity may activate SA responses in rop6DN plants. Changes in cell wall integrity may have also contributed to the susceptibility of the rop6DN plants to Bgh. Irrespective of this, our results implicate ROPs as important regulators of plant development as well as in SA-dependent and SA-independent defense execution.

MATERIALS AND METHODS

Molecular Cloning

All plasmids and primers are listed in Supplemental Tables S6 and S7, respectively. Plasmid construction was carried out using standard molecular cloning techniques. ARIP66 was cut from pSY700 with SacI and then was subcloned to pGFP-MRC to create pSY811. PCR-based site-directed mutagenesis (QuikChange kit; Stratagene) was used to create a constitutively active mutant (ArROP6CA) by substituting Gly-15 with Val and Thr-30 with Asn. pSY811 was used as template with the following primer sets: SYP189 + SYP190, PCR-based site-directed mutagenesis (QuikChange kit; Stratagene) was used to create a constitutively active mutant (AtROP6CA) by substituting Gly-15 with Val and Thr-30 with Asn. pSY811 was used as template with the following primer sets: SYP189 + SYP190, PCR-based site-directed mutagenesis (QuikChange kit; Stratagene) was used to create a constitutively active mutant (AtROP6CA) by substituting Gly-15 with Val and Thr-30 with Asn. pSY811 was used as template with the following primer sets: SYP189 + SYP190, PCR-based site-directed mutagenesis (QuikChange kit; Stratagene) was used to create a constitutively active mutant (AtROP6CA) by substituting Gly-15 with Val and Thr-30 with Asn. pSY811 was used as template with the following primer sets: SYP189 + SYP190, PCR-based site-directed mutagenesis (QuikChange kit; Stratagene) was used to create a constitutively active mutant (AtROP6CA) by substituting Gly-15 with Val and Thr-30 with Asn. pSY811 was used as template with the following primer sets: SYP189 + SYP190, PCR-based site-directed mutagenesis (QuikChange kit; Stratagene) was used to create a constitutively active mutant (AtROP6CA) by substituting Gly-15 with Val and Thr-30 with Asn.

GUS Staining

GUS staining was carried out as described previously (Weigel and Glazebrook, 2002).

Whole-Mount in Situ Hybridization

Tissue preparation and whole-mount in situ hybridization were performed essentially as described previously (Brewer et al., 2006; Hejatko et al., 2006). A 244-bp fragment from the 3′-untranslated region of ROP6 was amplified by PCR from Arabidopsis genomic DNA and was used as template to create gene-specific digoxigenin-labeled antisense/sense RNA probes. The digoxigenin-labeled probes were prepared by in vitro transcription according to the manufacturer’s instructions (Roche).

Fixation and Immunostaining

Fixation and immunostaining were carried out essentially as described previously (Chaimovitch et al., 2012). To stabilize actin and MT, specimens were fixed for 5 min with 500 μm N-maleimidooxybenzoylhydroxyssucinimide ester in MT-stabilizing buffer (MTSB) containing 100 mM PIPES, pH 6.8, 5 mM EGTA, 5 mM MgSO4, 7H2O, and 1% Triton X-100 (v/v) buffer and, in turn, for 1 h in freshly prepared 8% parafomaldehyde in MTSB. Fixation was carried out at room temperature in the dark. Samples were then rinsed three times in MTSB and treated with the following enzyme mixture: 2% cellulase Onzuka R-10 and 1% (w/v) pectinase, protease inhibitor cocktail (Sigma), and 20 μM phenylmethylsulfonyl fluoride for 10 min. After washing in MTSB for 30 min, the samples were squashed on coverslips coated with poly-L-Lys (1 mg mL−1; Sigma-Aldrich). Then, the cells were retreated for 10 min with enzyme mixture (2% cellulase Onzuka R-10 and 1.5%/w/v pectinase) with protease inhibitors. This step was followed by a rinse in phosphate-buffered saline (PBS) with 1% Triton X-100 (v/v) for 30 min and then incubation in PBS containing 1% (w/v) bovine serum albumin for 20 min. To reduce alkaline-induced auto-fluorescence, samples were treated for 5 min with 10 mg mL−1 sodium borohydride in PBS and then washed in PBS for 20 min. For immunofluorescence detection of MTs, specimens were incubated overnight at room temperature with primary sheep anti-aβ-tubulin polyclonal antibodies (Cytoskeleton) and mouse anti-actin monoclonal (clone C4) antibodies (ICN Biomedical) at a dilution of 1:100. Samples were then washed in PBS and incubated with anti-sheep secondary antibodies conjugated to Alexa Fluor 488 and anti-mouse secondary antibodies conjugated to Alexa Fluor 594 (Molecular Probes) diluted 1:100 for 1 h at room temperature. Mounting was done in 50% glycerol/water (v/v).

Plant DNA and RNA Isolation and qPCR

Plant genomic DNA was isolated with the GenElute Plant Genomic DNA miniprep kit according to the manufacturer’s instructions (Sigma). Total RNA was isolated with the SV Total RNA isolation kit according to the manufacturer’s instructions (Promega). The RNA from the qPCR experiments was extracted from both 14-d-old seedlings and mature flowering plants. Complementary DNA first-strand synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (Promega). Quantifications with the primer sets (Supplemental Table S7) were carried out by qPCR using an ABI Prism 7700 StepOnePlus Instrument (Applied Biosystems). Study samples were run in triplicate on eight-well optical PCR strips (Applied Biosystems) in a final volume of 10 μL. Primers were designed using the Roche Universal Probe Library (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). The PCR cycles were run as follows: 10-min initial denaturation at 95°C, followed by 40 subsequent cycles of 15 s of denaturation at 95°C, and 1 min of annealing and elongation at 60°C. The specificity of the unique amplification product was determined by melting-curve analysis according to

Auxin Induction

For auxin induction, seeds were germinated in the presence of the auxin transport inhibitor N-1-naphthylphthalamic acid followed by transfer to growth medium containing 10 μM auxin (NAA), as described previously (Hilmanen et al., 2004). Alternatively, seeds were germinated on medium lacking N-1-naphthylphthalamic acid and were, in turn, treated with 10 μM NAA.

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Counts were carried out as described previously (Wessling and Panstruga, LCS, and Adobe Photoshop.

Image analysis was performed with Zeiss AxioVision, ImageJ, Leica splitter, and a spectral detector set between 530 and 560 nm. Scanning electron microscopy was performed with an argon laser set to 514 nm, a 456/514-nm double dichroic beam splitter, and a spectral detector set between 505 and 530 nm. DsRed was visualized by excitation with an argon laser at 488 nm, a 500-nm beam splitter, and a spectral detector set between 530 and 560 nm. Scanning electron microscopy was carried out using a JEOL JSM-840A scanning electron microscope. Image analysis was performed with Zeiss AxioVision, ImageJ, Leica LCS, and Adobe Photoshop.

SA Measurements, Fungal Entry Rates, Conidiospore Counts, and BTH Treatments

Measurements of free and conjugated SA levels were carried out by analytical HPLC essentially as described previously (Bartsch et al., 2006). Colovonycyes orontii and Blumeria graminis f. sp. hordei conidiospore inoculations were carried out as described previously (Consonni et al., 2006). Conidiospore counts were carried out as described previously (Wessling and Panstruga, 2012). BTH treatments were carried out essentially as described previously (Lawton et al., 1996). Briefly, 4-week-old plants were sprayed with 42.6 μg mL⁻¹ water-solubilized BTH 24 h prior to inoculation by G. orontii conidiospores. Control plants were sprayed with water (BTH mock control). For all infection assays, plants were grown in a phytochamber with the following settings: 65% humidity; temperature of 22°C from 9 AM to 7 PM and 20°C from 7 PM to 9 AM; and 10/14-h light/dark cycles. Light intensity was 100 μE m⁻² s⁻¹.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Nucleotide sequence of the AirROP6 promoter.

Supplemental Figure S2. Fourteen-day-old Col-0 (wild type), rop6CA (6CA), and rop6D (6DN) seedlings grown on vertical-orientated plates.

Supplemental Figure S3. Immunostaining of actin and microtubule in wild-type and GFP-rop6DN leaf epidermis cells.

Supplemental Figure S4. Immunostaining of actin and microtubule in wild-type and GFP-rop6DN leaf epidermis cells.

Supplemental Figure S5. Molecular identification of rop6DN npr1 and rop6DN sid2 double mutants.

Supplemental Figure S6. Susceptibility to the powdery mildew fungus.

Supplemental Figure S7. G. orontii fungal hyphae.

Supplemental Figure S8. G. orontii conidiospores formation.

Supplemental Figure S9. Susceptibility to G. orontii following SA analog BTH treatments.

Supplemental Table S1. Differentially expressed genes in GFP-rop6DN versus the wild type (Col-0).

Supplemental Table S2. Differentially expressed genes in GFP-rop6DN versus GFP-ROP6.

Supplemental Table S3. Differentially expressed genes in GFP-rop6DN versus GFP-rop6CA.

Supplemental Table S4. Gene Ontology of differentially expressed genes in the GFP-rop6DN lines.

Supplemental Table S5. Strong differential expression of SAR associated genes in the GFP-rop6DN lines.

Supplemental Table S6. Plasmins used in this study.

Supplemental Table S7. Oligonucleotide primers used in this study.

Supplemental Table S8. Transgenic Arabidopsis lines used in this study.

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