Phased small RNA–mediated systemic signaling in plants

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Systemic acquired resistance (SAR) involves the generation of systemically transported signal that arms distal plant parts against secondary infections. We show that two phased 21–nucleotide (nt) trans-acting small interfering RNA3a RNAs (tasi-RNA) derived from TAS3a and synthesized within 3 hours of pathogen infection are the early mobile signal in SAR. TAS3a undergoes alternate polyadenylation, resulting in the generation of 555- and 367-nt transcripts. The 555-nt transcripts likely serves as the sole precursor for tasi-RNAs D7 and D8, which cleave Auxin response factors (ARF) 2, 3, and 4 to induce SAR. Conversely, increased expression of ARF3 represses SAR. Knockout mutations in TAS3a or RNA silencing components required for tasi-RNA biogenesis compromise SAR without altering levels of known SAR-inducing chemicals. Both tasi-ARFs and the 367-nt transcripts are mobile and transported via plasmodesmata. Together, we show that tasi-ARFs are the early mobile signal in SAR.

INTRODUCTION

Systemic acquired resistance (SAR) is a form of systemic immunity that protects distal uninfected parts of the plant against secondary infections. SAR involves the generation of mobile signals in the primary infected leaves, which, when translocated to distal tissue, activate defense responses resulting in disease resistance (1–3). The signal is first transmissible and moves throughout the plant (4). The production of the mobile signal is thought to occur within 3 hours of primary infection (5), and the infected leaf must remain attached for at least 4 and 6 hours after inoculation for SAR to be induced in cucumber (6) and Arabidopsis (7), respectively. This suggests that the signal is translocated within 4 to 6 hours of infection. However, the degree of protection against secondary infection is substantially higher if primary infected leaf is not excised (4). This suggests that the infected leaf continues to generate and transmit the SAR signal over time.

Although the identity of this early mobile signal remains elusive, many SAR-inducing factors, including some chemicals that move systemically, have been discovered. These include, salicylic acid (SA) (8, 9) and its methylated derivative MeSA (10), azelaic acid (AzA) (11), glycerol-3-phosphate (G3P) (7, 12), the free radicals nitric oxide (NO) and reactive oxygen species (ROS) (13), pimelic acid (Pip) (14, 15) and its derivative N-hydroxy Pip (NHP) (16, 17), pinene volatiles (18, 19), and extracellular nicotinamide adenine nucleotide [(e)NAD(P)] (20). These chemicals confer systemic resistance when applied exogenously and are required for pathogen-induced SAR. Extensive genetic, molecular, and biochemical analysis has shown that many of these SAR inducers operate in a bifurcate pathway, with additional nonlinear interactions among them (7, 18, 21–24).

Transport of chemicals associated with SAR can occur either via the apoplastic or symplastic compartments (1). For instance, pathogen infection increases SA and G3P/AzA levels in the apoplastic and symplastic compartments, respectively (22). The transport route for other SAR-associated chemicals remains unknown. Symplastic transport is regulated by plasmodesmata (PD), and defects in PD permeability do not affect apoplastic transport of SA. A portion of the total SA in leaves is partitioned into cuticular waxes (25). Mutants with defective cuticles, which exhibit increased transpiration, show increased partitioning of SA in their cuticle wax (25), suggesting an important role for transpirational pull in transport of SA. Further research has shown that distal transport of SA is essential for SAR (25, 26). Notably, mutants defective in the transport of SA or G3P accumulate reduced levels of Pip in the distal tissues (14, 15). Together, these observations suggest that coordinated transport and feedback regulation among various chemical signals is an important aspect of SAR activation. While several SAR-inducing chemical signals have been identified, the identity of an early mobile signal that is translocated within 4 to 6 hours of primary infection and conditions SAR in the distal leaves remains unresolved.

RESULTS

Overexpression of DRB2 and a mutation in AGO7 compromises SAR

Among the SAR-associated proteins, the double-stranded (ds-) RNA binding (DRB) proteins 1, 2, and 4 (27, 28) were of particular interest to us due to their involvement in RNA silencing and the proposed antagonistic functions of DRB2 and DRB4 (29). To resolve the precise roles of these DRB proteins, we assayed the effects of their overexpression on SAR. We generated transgenic Col-0 plants expressing DRB1, DRB2, DRB3, DRB4, and DRB5 via the 35S promoter and assayed the expression levels of the respective transgene (fig. S1, A and B). At least two independent lines per transgene were analyzed (fig. S1B). Transgene overexpression corresponded to increased accumulation of DRB1 and DRB4 proteins in the respective transgenic lines (fig. S1, C and D). Protein levels for DRB2, DRB3, and DRB5 could not be assayed because antibodies against these proteins showed nonspecific cross-reactivity to multiple bands on Western

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blots. Nevertheless, the presence of the zippy phenotype (characterized by downward curled leaf margins) in the 35S-DRB2 plants confirmed DRB2 overexpression as reported previously (fig. S1A) (29). Notably, the zippy phenotype of 35S-DRB2 plants was similar to the morphological phenotype of the dbr4 mutant (27, 28, 30). The 35S-DRB2 plants contained wild-type levels of DRB4 (fig. S1E), suggesting that zippy phenotype in 35S-DRB2 plants is not associated with changes in DRB4 levels. The dbr4 mutant is compromised in SAR (28), therefore, we assayed SAR in 35S-DRB2 and other DRB overexpressing lines. Only 35S-DRB2 plants were compromised in SAR (Fig. 1A), although these plants showed a normal hypersensitive reaction (HR) (quantified by assaying electrolyte leakage) and PR-1 induction (fig. S1, F and G) as well as wild-type–like levels of SA and Pip (fig. S1, H and I) in their infected leaves. Together, these results suggested that a factor other than SA or Pip was responsible for the compromised SAR phenotype of 35S-DRB2 plants.

**TAS3a regulates SAR**

Overexpression of DRB2 has previously been shown to antagonize the DRB4-mediated synthesis of trans-acting small interfering RNA (tasi-RNA) from TAS3a (29). TAS3 is one of four known *Arabidopsis* precursors of tasi-RNA [plant-specific class of small RNAs (sRNAs)] derived from microRNA (miRNA)–mediated cleavage of RNA precursors (fig. S2A). The TAS3 family is itself regulated by three members (3a to 3c) of which some expression is detectable in leaves and TAS3b is expressed at very low levels (fig. S2, B and C). The miRNA-generated cleavage products of TAS precursors are converted to dsRNA by RNA-dependent RNA polymerase 6 (DRDR6) followed by secondary cleavage by DCL4 (dicer-like 4) (fig. S2A). The tasi-RNA biogenesis from TAS3 specifically requires the miR390/Argonaute 7 (AGO7) complex (31, 32). TAS3-derived tasi-RNA represses ARF2, ARF3, and ARF4 to regulate leaf patterning, developmental timing, and lateral root growth (31–34). We considered the possibility that the compromised SAR phenotype in TAS3a plants may be associated with reduced levels of TAS3a-derived tasi-RNAs (fig. S2D). This was supported by the fact that mutations in miR390a, which is required for TAS3a-derived tasi-RNA biogenesis (34, 35), inhibited SAR (Fig. 1B). A nominal SAR seen in miR390a plants suggests a possible role for miR390b gene in TAS3a-derived tasi-RNA biogenesis (34).

We assayed SAR in a previously characterized T-DNA knockout (KO) line of TAS3a (fig. S2B) (33). The tas3a plants were compromised in SAR, whereas KO mutations in TAS3a or TAS3b did not inhibit SAR (Fig. 1D and fig. S2, B and E). Similar to 35S-DRB2 plants, the tas3a mutant showed zippy phenotype (Fig. 1C) and wild-type–like local resistance (fig. S2F) and HR (fig. S2G), suggesting that TAS3a specifically contributed to SAR. The tas3a plants showed wild-type–like PR-1 induction (fig. S2H) and accumulation of SA/SAs glucoside (SAG) (fig. S2I), Pip (fig. S2J), and G3P (fig. S2K). These plants also showed normal induction of SA (fig. S2L) and Pip (fig. S2M) in distal tissues and normal induction of AzA, G3P, and SA in petiole exudates (PEX) from pathogen-infected plants (fig. S2, N to P). Together, these results suggested that the SAR defect of tas3a mutant was not associated with defects in SA or Pip–AzA–G3P branches of the SAR signaling pathway. Consistent with these results, localized application of SA, Pip, AzA, or G3P did not restore SAR in tas3a plants (Fig. 1E). These results suggested that TAS3a likely functioned downstream or independent of SA, Pip, AzA, and G3P.

To test whether the compromised SAR phenotype due to the TAS3a mutation was associated with a defect in a RNA-derived signal, we assayed SAR in AGO mutants 1 and 7 as they play an important role in processing of TAS3a (fig. S2A). While the ago2, ago3, ago4, or ago5 mutants showed normal SAR, both ago1 and ago7 were compromised in SAR (fig. 1F), although they showed wild-type–like local resistance (fig. S3A). Pathogen-infected ago1 and ago7 mutants also accumulated wild-type–like levels of the SA marker PR-1 (fig. S3B) as well as SA and SA glucoside (SAG) (fig. S3C), Pip (fig. S3D), AzA (fig. S3E), and G3P (fig. S3F). The ago1 and ago7 plants also showed normal induction of SA (fig. S3G) and Pip (fig. S3H) in distal tissue. Consistent with these results, localized application of SA, Pip, or G3P could not restore SAR in ago1 or ago7 (Fig. 1G), suggesting that the SAR defects in these mutants was not associated with defects in SA, Pip, or G3P. Besides ago1 and ago7, mutations in DCL4, SGS3 (suppressor of gene silencing 3), and RDR6, all of which are required for processing of TAS3a (fig. S2A), also compromised SAR (Fig. 1H). Together, these results suggested that RNA biogenesis and thereby possibly an RNA species were essential for SAR.

**TAS3a and tasi-RNAs D7 and D8 confer robust SAR**

To test whether TAS3a RNA itself could serve as the SAR inducer, we tested the in vitro–transcribed mature 555–nucleotide (nt) TAS3a transcript (T-555) (fig. S4, A and B) in SAR assays. Exogenous application of single-stranded (ss) or ds short and long RNAs by spray and mechanical inoculation is an effective way to deliver RNA into cells [reviewed in (36)]. Although the process underlying uptake of nucleic acids by plant cells remain unknown, it is likely to overlap with the natural mechanisms that facilitate internalization of extracellular nucleic acids from microbial pathogens across plasma membrane (36). Exogenous application of the ss–T–555 effectively suppressed expression of the TAS3a target ARF3 (fig. S4C), indicating that infiltrated RNA was able to enter and induce a response in leaves. In contrast, exogenous application of ss–green fluorescent protein (GFP) transcript did not suppress ARF3 expression in wild-type plants, and ss–GFP or ss–T–555 did not suppress ARF3 expression in ago7 plants (fig. S4C). SAR assays carried out using ss–T–555 induced a robust SAR in wild-type plants, which was comparable to pathogen [Pseudomonas syringae expressing avrRpt2 (Pst-avrRpt2)]–induced SAR (Fig. 2A and fig. S4D). Localized application of 8.3 μM ss–T–555 RNA was sufficient to induce significant SAR in wild-type plants (fig. S4, D and E). Unlike T-555, exogenous application of GFP transcript did not induce SAR on Col-0 plants (fig. S4F). As expected, exogenously applied ss–T–555 was able to restore the SAR defect in the tas3a but not in the ago7 mutant (Fig. 2B). These results further indicate the effective uptake of ss–T–555 in the SAR bioassays and are consistent with the important role of AGO7 in the processing of TAS3a transcript (fig. S2A).

Although localized application of T–555 did not induce SA, Pip, or ROS accumulation (fig. S5, A to C), it did nominally increase G3P levels (fig. S5D), suggesting a link between G3P and TAS3a in SAR signaling. To test this association further, we assayed TAS3a levels in gly1 gly1 and sid2 plants, which are defective in G3P and SA biosynthesis, respectively (7, 37). Notably, both gly1 gly1 and sid2 showed reduced basal levels of TAS3a, with gly1 gly1 showing significantly lower levels than sid2 (Fig. 2C). To determine whether reduced levels of TAS3a transcript could account for the SAR defect of gly1 gly1 and sid2 plants, we assayed their SAR in response to gly1 gly1 and sid2 plants, we assayed their SAR in response to gly1 gly1 and sid2 plants, we assayed their SAR in response to...
Fig. 1. A mutation in \textit{ago}1, \textit{ago}7, and \textit{tas}3\textalpha, or overexpression of \textit{DRB}2 compromises SAR. (A and B) SAR response in distal leaves of 35S-\textit{DRB} \textit{miR390}a (B) plants treated locally with MgCl\textsubscript{2} or \textit{P}. \textit{syringae} expressing \textit{avrRpt2} (\textit{Pst-avrRpt2}). CFU indicates colony forming units. dpi, days post infection. (C) Morphological phenotypes of 4-week-old Col-0, \textit{tas}2, \textit{tas}3\textalpha, and \textit{tas}3\textbeta plants. Only \textit{tas}3\textalpha plants showed a characteristic of zippy phenotype. (D) SAR response in distal leaves of \textit{tas} plants treated locally with MgCl\textsubscript{2} or \textit{Pst-avrRpt2}. (E) SAR response in distal leaves of Col-0 and \textit{tas}3\textalpha plants treated locally with mock (MgCl\textsubscript{2}), \textit{Pst-avrRpt2}, SA (500 \textmu M), Pip (1000 \textmu M), AzA (1000 \textmu M), or G3P (100 \textmu M). (F) SAR response in distal leaves of \textit{ago} mutants treated locally with MgCl\textsubscript{2} or \textit{Pst-avrRpt2}. The \textit{ago1-27} mutant showed nominal SAR in two of six repeats. (G) SAR response in distal leaves of Col-0, \textit{ago1-27}, or \textit{ago7-1} plants treated locally with water, \textit{Pst-avrRpt2}, SA (500 \textmu M), Pip (1000 \textmu M), or G3P (100 \textmu M) + \textit{Pst-avrRpt2}. (H) SAR response in distal leaves of Col-0, \textit{dcI}4-1, \textit{sgs}3-13, and \textit{rd}6-11 plants treated locally with MgCl\textsubscript{2} or \textit{Pst-avrRpt2}. Asterisks in (A), (B), (D) to (G), and (H) denote a significant difference with respective mock-inoculated samples (t test, \textit{P} < 0.0005). These experiments were repeated three (A, B, E, G, and H), four (D), or six (F) times with similar results.
Fig. 2. TAS3a and tasi-RNAs D7 and D8 confer robust SAR. (A) SAR response in distal leaves of Col-0 plants treated locally with MgCl₂, Pst-avrRpt2, or T-555 (8.3 µM). The virulent pathogen (DC3000) was inoculated 48 hours after local treatments. (B) SAR response in distal leaves of Col-0 and tas3a and ago7 plants treated locally with MgCl₂, Pst-avrRpt2, or T-555. (C) Real-time qRT-PCR showing relative expression levels of TAS3a in Col-0, gly1 gli1, and sid2 plants. The error bars indicate SD (n = 4). (D) SAR response in distal leaves of Col-0, gly1 gli1, and sid2 plants treated locally with MgCl₂, Pst-avrRpt2, or T-555. (E and F). Real-time quantitative stem-loop RT-PCR showing relative expression levels of tasi-RNAs D7 (E) and D8 (F) in Col-0 plants treated with T-555. The error bars indicate SD (n = 3). hpi, hours post infection. (G to I) Real-time quantitative stem-loop RT-PCR showing relative expression levels of tasi-RNAs D7 (left panel) and D8 (right panel) in Col-0, ago7 (H), gly1 gli1 (I), or sid2 and ald1 (J) plants after mock (MgCl₂) or Pst-avrRpt2 inoculations. The error bars indicate SD (n = 3). (K) SAR response in distal leaves of Col-0 plants treated locally with MgCl₂, Pst-avrRpt2, and single-stranded (ss) or double-stranded (ds) D7 (each at 5 µM concentration). (L to N) SAR response in distal leaves of indicated genotypes treated locally with MgCl₂, tasi-RNAs D7 or D8 (each at 5 µM concentration), or T-555 (8.36 µM concentration). Asterisks denote a significant difference [t test; P < 0.05 (G to I); P < 0.005 (E and F); P < 0.0001 (A, B, C, D, K, L, and N)]. These experiments were repeated two (E and F), three (D, K, L, and N), four (B, C, and G to I), or five (A) times with similar results.
localized application (infiltrated into specific leaves) of T-555. T-555 application restored robust SAR in gly1 gli1 but not sid2 plants (Fig. 2D). These results suggest that TAS3a operates in association with SA and downstream of G3P to confer SAR. TAS3a has been reported to be responsible for the biogenesis of multiple 21-nt tasi-RNAs, of which two (D7 and D8) target ARFs and are designated tasi-ARFs (31). To test whether T-555–induced SAR was associated with increased levels of TAS3a-derived tasi-ARFs (31), we assayed the levels of D7 and D8 in pathogen (Pst-avrRpt2)–infected or T-555–treated plants (table S1). Time-course analysis showed that both D7 and D8 were induced in wild-type plants within 3 hours of T-555 and Pst-avrRpt2 inoculation, with declining levels at later time points (Fig. 2, E to G). Consistent with their defect in tasi-ARF biogenesis (31), ago7 plants accumulated reduced levels of D7/D8 (Fig. 2H). Although this early pathogen-responsive induction of tasi-ARF was also observed in gly1 gli1 and sid2 plants, these levels were significantly lower than in wild-type plants (Fig. 2, I and J). This correlated with the reduced TAS3a accumulation in these mutants (Fig. 2C). The reduced basal levels of D7 tasi-ARF in gly1 gli1 plants further correlated with their zippy and early flowering phenotypes (fig. S6, A and B). In contrast, ald1 plants accumulated wild-type–like levels of D7 tasi-ARF (Fig. 2), suggesting that Pip or NHP was not required for the biogenesis of TAS3a or tasi-ARFs.

To determine whether D7 and D8 served as SAR signals, we assayed their ability to induce SAR. Exogenous application of either ss- or ds-D7 conferred equally effective SAR in wild-type plants (Fig. 2K and fig. S7A). In contrast, two other TAS3a-derived tasi-RNA, ss-D1 and ss-D5 RNAs, which do not target ARFs, did not confer SAR on Col-0 plants (fig. S7B). Similar to T-555, both D7 and D8 conferred SAR in gly1 gli1 and tas3a plants (Fig. 2L). Unlike T-555, D7/D8 was able to restore SAR in ago7, rdr6, and dcl4 plants (Fig. 2M), consistent with the proposed function of these proteins in the tasi-RNA biosynthesis (fig. S2A). However, neither D7 nor D8 induced SAR in ago1 plants (Fig. 2N), suggesting that AGO1 was required for a downstream step involving tasi-ARFs D7 and D8. Together, these results indicate that TAS3a–induced SAR is associated with tasi-ARF D7 and D8 accumulation, which function as SAR inducers in an AGO1-dependent manner.

Considering the efficacy of SAR against a broad spectrum of pathogens, we assayed D7–conferred SAR against the hemibiotrophic fungal pathogen Colletotrichum higginsianum. The primary leaves of Col-0 and tas3a plants were infiltrated with MgCl2, Pst avrRpt2, or D7, and 48 hours later, the distal leaves were spot inoculated with C. higginsianum. Fungal infection was monitored by assessing increasing in lesion size over time. Preinfection with Pst avrRpt2 resulted in significantly reduced C. higginsianum infection in Col-0 but not in ago7 or tas3a plants (fig. S7, C to E). Localized treatment with D7 restored SAR to C. higginsianum in both ago7 and tas3a (fig. S7, C to E). Similarly, localized application of D7 also conferred increased resistance against the oomycete pathogen Pythium irregulare (fig. S7F), confirming the broad spectrum efficacy of TAS3a-mediated systemic signaling. Notably, in comparison to Col-0 and tas3a plants, ago7 plants showed enhanced susceptibility to both C. higginsianum and Pythium, and D7 treatment was able to confer wild-type–like resistance against both these pathogens (fig. S7, C to F).

D7 and D8 tasi-RNA are the mobile RNA signals

We next tested the mobility of D7 and D8 tasi-RNA because systemic mobility is a key feature of SAR. Although grafting is one of the useful techniques to examine transport of mobile molecules, we were unable to use this to assay transport because successful grafting in Arabidopsis is limited to specific developmental stages and requires sterilized conditions, which do not coincide with the age window required to conduct SAR assays. Accordingly, as an alternative approach, we tested the presence of these tasi-RNAs in PEX as a measure of their mobile nature. Higher levels of D7 and D8 tasi-ARFs were detected in PEXavrRpt2 compared to PEXMgCl2 (Fig. 3A). Localized application of T-555 also resulted in an increase in D7 levels in the PEX of healthy plants (Fig. 3B). To further confirm the systemic mobility of D7, we assayed localization and systemic movement of 6-carboxyfluorescein (FAM)–tagged D7 RNA. The FAM-tagged RNAs have been used in earlier studies to induce RNA interference (38, 39) and thus are considered valid, biologically active, and proxies for D7. D7-FAM conferred SAR on Col-0 plants (fig. S8A), indicating that D7-FAM was biologically functional. Both D7-FAM and FAM localized to cytosol, but unlike FAM, a major percentage of D7-FAM was detected in the vasculature within 4 hours of infiltration (Fig. 3C). Furthermore, D7-FAM was also seen in the vasculature of distal leaves within 4 hours, thus supporting its mobile nature (Fig. 3C). The presence and systemic distribution of D7-FAM was further ascertained by high-performance liquid chromatography (HPLC) of the RNA extracted from local and distal tissue of D7-FAM–infiltrated Col-0 plants. The analysis confirmed the integrity of D7–FAM after infiltration (Fig. 3D) and showed that ~10% of intact D7-FAM were transported to distal leaves (Fig. 3E). These results, together with the rapid (3 hours) pathogen-induced increase in D7/D8, suggested that these tasi-ARFs may be the early mobile SAR signal that play an essential role in preparing the distal tissue against secondary infections. Consistent with this notion, Col-0 and tas3a plants showed ~75% overlap in the differentially expressed genes of infected tissue but only 7.1 and 27.9% overlap in induced and repressed genes in their distal tissue, respectively (fig. S8B and tables S2 to S7).

If D7 and D8 served as the key mobile signals in SAR, then PEX collected from any genotype unable to accumulate these tasi-RNAs should not be able to confer SAR on wild-type plants. Consistent with their reduced D7 and D8 levels (Fig. 2, H and I), PEXavrRpt2 from gly1 gli1 and ago7 was unable to confer SAR on Col-0 (Fig. 3, F and G). Likewise, PEXavrRpt2 from tas3a and sid2 mutant plants was also unable to confer normal SAR on Col-0 plants (Fig. 3, H and I).

In contrast, PEXavrRpt2 from ald1 plants conferred normal SAR on Col-0 plants (Fig. 3I). Furthermore, the PEXavrRpt2 from ago7 was able to confer SAR on Col-0 plants when mixed with the wild-type D7 RNA but not the mutant D7m RNA that was mutated in four conserved residues resulting in impaired binding to the target site (Fig. 3) and table S1 (40). Together, these results suggest a strong association between tasi-ARF levels and SAR-inducing ability of PEXavrRpt2.

Next, we assayed the systemic transport of D7 by monitoring the movement of D7–FAM in 35S-PDLP5, which show reduced symplastic transport via PD (22, 41). The 35S-PDLP5 plants showed significantly reduced transport of D7–FAM to the distal tissue (fig. S8C), and this, in turn, was consistent with inability of T555 or D7 to confer SAR on 35S-PDLP5 plants (Fig. 4, A and B). Likewise, PEXavrRpt2 from 35S-PDLP5 plants were unable to confer SAR on Col-0 plants (Fig. 4C). Together, these results suggested that D7 is systemically transported via the PD. As an additional test, we
Fig. 3. D7 and D8 are critical mobile tasi-RNAs generated from T-555. (A) Real-time quantitative stem-loop RT-PCR showing relative expression levels of tasi-RNAs D7 and D8 in PEX collected from Col-0 plants inoculated with buffer (MgCl₂) or Pst-avrRpt2. (B) Real-time quantitative stem-loop RT-PCR showing relative expression levels of tasi-RNA D7 in PEX collected from Col-0 plants infiltrated with T-555 (8.3 μM). (C) Confocal micrograph showing localization of D7-FAM and FAM in local and distal tissues of Col-0 plants 4 hours after treatments. The field of view corresponded to a magnification of ×10 or ×20. Merged images represent overlays of GFP and transmittance micrographs. (D) HPLC fluorescence–based detection of D7-FAM. Col-0 plants were locally infiltrated with 10 μM D7-FAM, and total RNA from local and distal leaves was extracted 4 hours after infiltration and analyzed by reverse-phase chromatography. A minor contaminating peak at 5.5 min in FAM elutes before the D7-FAM peak. LU represents luminescence units. (E) Relative levels of D7-FAM present in the total RNA samples extracted from ~100 mg of local and distal leaves. (F to I) SAR response in Col-0 plants infiltrated with MgCl₂ (PEXₘ₉₅) or Pst-avrRpt2 (PEXₐ₉₅) PEX collected from Col-0 or gly1 gli1 (F), ago7 (G), tasi3a (H), or sid2 and ald1 (I) plants. (J) SAR response in Col-0 plants infiltrated with MgCl₂ (PEXₘ₉₅) or Pst-avrRpt2 (PEXₐ₉₅) PEX collected from Col-0 or ago7 plants. One set of PEXₐ₉₅ from ago7 plants was mixed with wild-type or mutant (m) D7 tasi-RNA before infiltration into Col-0 leaves. The error bars in (A) and (B) represent SD (n = 3). Asterisks denote a significant difference [t test; P < 0.005 (A and B); P < 0.0001 (F to J)]. These experiments were repeated two (E) or three (A to D and F to J) times with similar results.
avrRpt2 (PEXavrRpt2). One set of PEX was treated with 10 μM RNase A for 1 hour before infiltration of primary leaves. At 48 hours after infiltration of primary leaves, the distal leaves were inoculated with virulent pathogen (DC3000) or D7 tasi-RNA (TAS3a). The virulent pathogen (DC3000) was inoculated 48 hours after local treatments. Asterisks denote a significant difference with respective mock-inoculated samples (t test, P < 0.0001). This experiment was repeated three times with similar results. (D) SAR response in Col-0 plants infiltrated with PEX collected from Col-0 or 35S:PDLP5 plants. The distal leaves were inoculated with virulent pathogen at 48 hours after infiltration of primary leaves. Asterisks denote a significant difference with respective PEXMGCl2 (t test, P < 0.0001). This experiment was repeated three times with similar results.

assayed the effect of ribonuclease (RNase) treatment on PEXMGCl2 and PEXavrRpt2. The RNase-treated PEXavrRpt2 was unable to induce SAR on wild-type plants (Fig. 4D). This correlated with the significant reduction in D7 in PEXavrRpt2 after RNase treatment (Fig. S8D) without altering the levels of SA or AzA in PEXavrRpt2 (fig. S8, E and F).

**TAS3a undergoes APA**

Because generation of D7 and D8 involves phased cleavage of TAS3a, we expected the pathogen-infected plants to show reduced accumulation of TAS3a transcript. Time-course analysis showed that TAS3a expression in the infected leaf declined within 3 hours after pathogen inoculation, with lowest levels by 12 to 24 hours (Fig. 5A). In contrast, TAS3a levels were significantly induced in PEXavrRpt2 within 3 hours, accumulating to highest levels by 12 hours (Fig. 5B). This suggested that TAS3a transcript was rapidly transported away from the site of pathogen infection. Consistent with this notion, the PEXavrRpt2 from 35S:PDLP5 plants showed significantly less transport of TAS3a transcript (Fig. 5C).

Considering that TAS3a undergoes phased cleavage, it was unlikely that the intact transcript was being transported systemically. Analysis of high throughput 3′-end tag reads that map to the TAS3a gene showed that TAS3a transcripts undergoes alternate polyadenylation (APA), resulting in the generation of T-555 and ~T-370 (Fig. 5D and fig. S9, A and B). The 5′-APA site corresponding to ~T-370 showed heterogeneity and generated variable length transcripts that only contained the 5′ miR390-AGO7 binding site (figs. S4A and S9A). 3′-RACE (rapid amplification of cDNA ends) analysis of RNA from pathogen-infected Col-0 leaves showed that both T-555 and T-367 were markedly reduced within 24 hours of infection (Fig. 5E). In contrast, 3′-RACE analysis of PEX RNA showed a significant increase in the levels of T-367 in PEXavrRpt2 (Fig. 5F). Together, these results suggest that rapid processing of T-555 and systemic transport of T-367 contribute to the decline in both transcripts at the site of pathogen infection. We next tested the SAR-inducing ability of the T-367. While exogenously applied T-367 induced SAR on wild-type plants, it failed to do so in the tas3a plants (Fig. 5G). This suggested that the presence of the full-length processable TAS3a transcript was required for T-367 activity in SAR.

**TAS3a confers SAR by negatively regulating ARF3**

Because tasi-ARFs target ARF expression, we tested the roles of the TAS3a-responsive ARF2, ARF3, and ARF4 in SAR using plants that were either defective for ARF expression (KO mutants) or expressed increased ARF3 (transgenic plants expressing ARF3-GUS via the ARF3 native promoter in wild-type background). The arf2/arf3/arf4 plants were SAR competent (Fig. 6A). To determine functional redundancy among the ARFs, we generated plants overexpressing artificial microRNAs (35S:miR-ARF) that targeted all three ARFs (ARF2, ARF3, and ARF4) (fig. S10A) (34, 42). As previously reported (34, 42), the 35S:miR-ARF plants showed developmental phenotype and did not yield viable seeds precluding us from assaying defense phenotypes. The T1 generation of 35S:miR-ARF plants showed basal expression of PR-1 (fig. S10B), suggesting that down-regulation of ARFs was not associated with constitutive activation of the SA pathway.

In contrast to arf mutants, transgenic ARF3-expressing plants (Fig. S10, C and D) exhibited the zippy phenotype (fig. S10E) and were compromised for SAR (Fig. 6B). Moreover, the zippy phenotype was more pronounced in plants expressing a mutant, tasi-ARF un-cleavable form, of ARF3 (ARF3m). As expected, ARF3m-expressing plants contained higher ARF3 transcript levels (fig. S10C) (31). Furthermore, these plants were compromised for SAR (Fig. 6B), although they accumulated wild-type levels of the SAR inducers SA, Pip, ROS, and G3P (fig. S11).

If TAS3a-derived tasi-ARFs regulated SAR by down-regulating ARF expression, then increased TAS3a would be expected to compensate the SAR defect of ARF3-overexpressing plants. Localization of T-555 restored SAR in ARF3-GUS but not ARF3m-GUS plants (Fig. 6C). Next, we assayed SAR using a mutant form of D7 (D7m) that does not target ARF3. Unlike D7, D7m was unable to confer SAR on Col-0 plants (Fig. 6D). These results suggest that a threshold level of TAS3a and thereby tasi-ARFs are required to down-regulate ARF3 expression, and this notion is consistent with increased expression of ARF2, ARF3, and ARF4 observed in tas3a plants (fig. S12). Pathogen infection of ARF3m-GUS plants did not deplete either 555- or 367-nt TAS3a transcripts (Fig. 6E). Consistent with these data, the ARFm-GUS plants showed significantly reduced levels of D7 tasi-RNA levels in the pathogen-infected leaves (Fig. 6F).
and the distal uninfected leaves (Fig. 6G). Furthermore, PEX\textsubscript{avrRpt2} from ARF3m-GUS plants was unable to confer SAR on wild-type plants (Fig. 6H). Together, these results suggest that increased ARF3 levels negatively regulate pathogen-induced tasi-RNA biosynthesis. ARF3 was recently shown to bind to the AGO7 promoter (43), suggesting that higher levels of ARF3 might regulate tasi-ARF levels by negatively regulating AGO7 transcription. However, localized application of D7/D8 restored SAR in ARF3-GUS but not ARF3m-GUS plants (Fig. 6I), although ARF3m-GUS plants showed wild-type–like transport of D7-FAM (fig. S13). Together, these results support the hypothesis that increased ARF3 expression suppresses SAR by down-regulating tasi-ARF and by targeting additional unknown factors.

**DISCUSSION**

In summary, we show that TAS3a is an important regulator of SAR, and TAS3a-derived tasi-ARFs D7 and D8 are the early mobile inducers of SAR (Fig. 7). We propose that ARF3 suppresses unknown positive regulator(s) of SAR. Pathogen infection resulting in tasi-ARF generation negatively regulates ARF2, ARF3, and ARF4 to activate SAR by relieving the suppression of the unknown positive SAR regulator(s). ARF3 also suppresses pathogen-responsive tasi-ARF biosynthesis, generating a feedback regulatory loop.

Tasi-ARFs are generated and transported within 4 to 6 hours of primary infection, induce systemic resistance when applied in a localized manner, and are essential for SAR. PEX\textsubscript{avrRpt2} from plants unable to generate tasi-ARFs lacks SAR-inducing ability. Although many of the other SAR inducers exhibit physical mobility, this alone does not qualify them as the mobile signal. For instance, a SAR inducer may be mobile, but its mobility may not be functionally essential for SAR induction in the systemic tissue. As an example, the SAR inducers Pip and NHP are considered to be mobile (16, 17) and were proposed to function upstream of an unknown mobile signal that conditions SAR by promoting de novo synthesis of Pip and NHP in the systemic tissue (44). However, PEX\textsubscript{avrRpt2} from the ald1 mutant, which cannot synthesize Pip or NHP, can activate SAR in wild-type plants (Fig. 3I) (15). This indicates that mobile signal generation and transport from the infected tissue does not require Pip or NHP. Exogenous application of TAS3a or tasi-ARFs does not result in Pip accumulation. Conversely, ald1 plants accumulate normal levels of D7 tasi-ARF. This suggests that Pip is not required for the biogenesis of or signaling mediated by tasi-ARFs and vice versa.

In contrast, SA and G3P appear to positively regulate TAS3a and thereby tasi-ARFs for the following reasons: (i) Exogenous application of either SA or G3P resulted in nominal induction of TAS3a and tasi-ARF D7 (fig. S14). (ii) Both SA and G3P are required for

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**Fig. 5.** TAS3a undergoes APA. (A and B) Real-time qRT-PCR showing relative expression levels of TAS3a in inoculated leaves (A) or PEX (B). Schematics above each graph represents TAS3a (see figs. S2A and S4A). (C) Real-time qRT-PCR showing relative expression levels of 5′ TAS3a in PEX collected from mock (MgCl\textsubscript{2})– and Pst-avrRpt2–inoculated Col-0 and 35S-PDL5 plants. (D) Browser tracks showing 3′ end tag read coverage for the TAS3a gene. Mappings of reads from wild-type plants are displayed below a representation of the TAS3a RNA coding region. Detailed sequences for the proximal and distal poly(A) sites shown here are provided in fig. S9A. (E and F) PCR of the 3′-RACE reaction of TAS3a transcript variants in the total RNA from mock- and Pst-avrRpt2–inoculated leaf (E) or PEX (F) samples. (G) SAR response in distal leaves of Col-0 or tas3a plants treated locally with MgCl\textsubscript{2}, T-367, or T-555. The virulent pathogen (DC3000) was inoculated 48 hours after local treatments. The error bars in (A) to (C) represent SD (n = 4). Asterisks denote a significant difference from samples harvested at 0 time [t test; P < 0.001 (A and B)], between mock- and Pst-avrRpt2/TAS3a–inoculated samples [t test, P < 0.001 (C); P < 0.0001 (G)], or between the two Pst-avrRpt2–inoculated samples [t test, P < 0.0001 (B)]. These experiments were repeated three times with similar results.
Fig. 6. TAS3a confers SAR by negatively regulating ARF3. (A) SAR response in Col-0 and arf plants infiltrated with mock (MgCl₂) or Pst-avrRpt2. (B) SAR response in Col-0 and transgenic plants expressing ARF3-GUS or a mutant form of ARF3-GUS (ARF3m-GUS) lacking the TAS3a cleavage site. (C) SAR response in Col-0, ARF3-GUS, and ARF3m-GUS after localized inoculation with Pst-avrRpt2 or 8.3 μM T-555. (D) SAR response in distal leaves of Col-0 plants treated locally with MgCl₂, tasi-RNAs D7, or a D7 mutant that was altered in four conserved bases (each at 5 μM concentration; table S1). (E) PCR of the 3′-RACE reaction of TAS3a transcript variants in total RNA from mock- and Pst-avrRpt2-inoculated Col-0 and ARFm-GUS plants. Thirty-five cycles of PCR were performed for each PCR reaction. (F and G) Real-time quantitative stem-loop RT-PCR showing relative expression levels of tasi-RNAs D7 in local (F) and distal (G) leaves of Col-0 and ARFm-GUS plants. Plants were inoculated with Pst-avrRpt2 and sampled at indicated time points. (H) SAR response in Col-0 plants infiltrated with MgCl₂ (PEXmGUS) or Pst-avrRpt2 (PEXavrRpt2) PEX collected from Col-0 or ARFm-GUS plants. (I) SAR response in distal leaves of indicated genotypes treated locally with MgCl₂ or tasi-RNAs D7 + D8 (each at 5 μM concentration). For ALL SAR experiments, the distal leaves were inoculated with virulent pathogen at 48 hours after infiltration of primary leaves. Asterisks denote a significant difference with mock [t test; P < 0.0001 (A to C, D, H, and I) or P < 0.05 (F and G)]. These experiments were repeated twice (A, D, E, F, G, and I) or three times (B, C, and H) with similar results.
maintaining normal levels of \( \text{TAS3a} \) transcript and deficiencies in \( \text{SA} \) or \( \text{G3P} \) biosynthesis markedly reduce pathogen-induced \( \text{tasi-ARFs} \) levels. (iii) \( \text{PEX}_{avrRpt2} \) from \( \text{G3P} \)- or \( \text{SA} \)-deficient mutants lack \( \text{SAR} \)-inducing activity, as does \( \text{PEX}_{avrRpt2} \) from \( \text{tas3a} \) or \( \text{ago7} \) mutants (defective in \( \text{tasi-ARF} \) biogenesis). The slightly higher levels of \( \text{tasi-ARFs} \) in \( \text{sid2} \) plants versus \( \text{gly1 gli1} \) or \( \text{ago7} \) plants may account for the nominal \( \text{SAR} \)-inducing ability of \( \text{sid2} \) \( \text{PEX}_{avrRpt2} \).

\( \text{TAS3a} \) appears to operate downstream of \( \text{G3P} \) but requires \( \text{SA} \)-mediated signaling to confer \( \text{SAR} \) in distal tissue because exogenous application of \( \text{TAS3a} \) can restore \( \text{SAR} \) in \( \text{gly1 gli1} \) but not \( \text{sid2} \) plants. Previously, we showed that the distal transport of \( \text{SA} \) regulated by transpirational pull and water potential (25) is essential for \( \text{SAR} \) activation. The cuticle impaired mutants \( \text{mod1} \) and \( \text{acp4} \), which cannot maintain their water potential due to increased transpiration, exhibit significantly reduced distal transport of \( \text{SA} \), and this, in turn, is associated with their compromised \( \text{SAR} \) (25). However, \( \text{PEX}_{avrRpt2} \) from \( \text{mod1} \) and \( \text{acp4} \) is \( \text{SAR} \) competent (25), suggesting that \( \text{SA} \) transport is not essential for systemic mobility of the \( \text{SAR} \) signal (tasi-\( \text{ARFs} \)).

\( \text{TAS3a} \) undergoes APA, which results in the generation of the \( \text{T-367} \) and \( \text{T-368} \). The \( \text{T-367} \) contains both 5′ and 3′ \( \text{miR390-AGO7} \) binding sites, whereas \( \text{T-367} \) contains only the 5′ \( \text{miR390-AGO7} \) binding site corresponding to 5′ of the 11 phased \( \text{tRNAs} \), including \( \text{D7} \) and \( \text{D8} \). Because \( \text{biogenesis} \) of tasi-\( \text{ARFs} \) is initiated upon cleavage at the 3′ \( \text{miR390-AGO7} \) target site, \( \text{T-367} \) is unlikely to generate properly phased \( \text{D7} \) and \( \text{D8} \) tasi-\( \text{ARFs} \). Thus, \( \text{T-555} \) is the likely precursor for SAR-bioactive \( \text{D7} \) and \( \text{D8} \) tasi-\( \text{RNAs} \). Yet, \( \text{T-367} \) is able to induce \( \text{SAR} \) in wild-type plants but not the \( \text{tas3a} \) mutant. One possibility is that \( \text{SAR} \) activation requires de novo synthesis of tasi-\( \text{ARFs} \) in the distal tissue, and this may be facilitated by \( \text{T-367} \). Tasi-\( \text{RNA} \) levels in distal tissue are ~10-fold higher than in \( \text{PEX} \). Understanding how \( \text{T-367} \) contributes to \( \text{SAR} \) is important for further dissecting SAR signaling. Identifying the SAR-regulating targets of \( \text{ARF3} \) is important as well. This is especially important for understanding how \( \text{plants} \) regulate the switch between growth and defense because \( \text{ARF3} \) is important for normal growth and development and is known to target hundreds of \( \text{genes} \) in healthy \( \text{plants} \) (25).

**MATERIALS AND METHODS**

**Plant growth conditions and genetic analysis**

Plants were grown in MTPS 144 Conviron (Winnipeg, MB, Canada) walk-in chambers at 22°C, 65% relative humidity, and 14-hour light and 10-hour dark photoperiod. These chambers were equipped with cool white fluorescent bulbs (Sylvania, FO96/841/XP/ECO). The photon flux density of the day period was 106.9 μmol m−2 s−1 (measured using a digital light meter; Phytotronic Inc., MO). Plants were grown on autoclaved PRO-MIX soil (Premier Horticululture Inc., PA, USA). Soil was fertilized once using Scotts Peter’s 20:10:20 peatlite special general fertilizer that contained 8.1% ammonical nitrogen and 11.9% nitrate nitrogen (www.scotts.com). Plants were irrigated using deionized or tap water. The \( \text{tas3a} \) (\( \text{GK-621G08} \)) and \( \text{tas3b} \) (\( \text{GK-649H12} \)) plants used in this study are described earlier (33, 45). The \( \text{tas2} \) homozygous plants were identified from SALK insertion line (014168) obtained from the Arabidopsis Biological Resource Center (ABRC) database. The \( \text{ago1-27} \) hypomorphic mutant is described earlier (46). The \( \text{ago7-1}, \text{sgs3-13} \), and \( \text{rdr6-11} \) seeds were obtained from the ABRC database. The gly1 gli1 double mutant plants were generated by crossing gly1-1 with gli1-1, and both these genotypes are described earlier (7, 47). All genotypes and primers used for genotyping are provided in the table S1.

**Generation of transgenic plants**

For transgenic overexpression of \( \text{DRBs} \), the cDNA spanning the coding region was cloned into pGW2B2 vector, which, after confirmation of the DNA sequence, was transformed into Col-0 plants. The \( \text{35S-miR-ARF} \) construct was provided by A. Maizel (34). The transgenic plants were selected on plates containing kanamycin (50 μg/ml) and hygromycin (17 μg/ml).

**RNA extraction, quantitative real-time PCR, 3′-RACE, and in vitro transcription**

Small-scale extraction of RNA from two or three leaves (per sample) was performed with the TRIzol reagent (Invitrogen) following the manufacturer’s instructions. RNA quality and concentration were determined by gel electrophoresis and determination of \( \text{A}_{260} \). Reverse transcription (RT) and first-strand cDNA synthesis were carried out using SuperScript II (Invitrogen) or EpiScript (Lucigen Corp.) reverse transcriptase. Quantitative RT (qRT)–polymerase chain reaction (PCR) was carried out as described before (48). Each biological replicate was run in triplicates, and \( \text{ACTINII} \) (At3g18780) or \( \text{UBC2} \) (leaf)/\( \text{UBC9} \) (PEX) expression levels were used as internal controls.
control for normalization. Cycle threshold values were calculated by SDS 2.3 software (Applied Biosystems).

The cDNA synthesis for 3′-RACE was carried out using the dt17-adapter primer using SuperScript II or EpiScript reverse transcriptase. PCR amplification of the target was performed using the TAS3a forward and a primer complementary to the adapter primer (table S1).

For sRNA expression analysis, total RNA extracted using TRIzol reagent was precipitated with isopropanol and dissolved in 300 μl of diethyl pyrocarbonate (DEPC) water. A final concentration of 5% polyethylene glycol and 0.5 M NaCl was added to this, and after a 30-min incubation on ice, the high–molecular weight RNA was recovered by centrifugation at 10,000g for 10 min. sRNA in the supernatant was precipitated with three volumes of ethanol for a 2-hour incubation at −20°C. The sRNA pellet was washed with 70% ethanol, air-dried, and suspended in DEPC-treated water. RNA was quantified, and ~1 μg was used for stem-loop qPCR as described earlier (49). Expression level of D7 and D8 tasi-ARFs was quantified by normalizing against a standard curve prepared using D7(+) and D8(+) oligonucleotides or U6 that was used as an internal control for normalizations. The primers used for qRT and stem-loop PCRs are listed in table S1. Synthesis of ds oligo was carried out as described before (50).

The synthesis of TAS3a or GFP RNA was carried out by in vitro transcription using T7 RNA polymerase. The TAS3a sequences were cloned in the pBlueScript-SK+ vector, which, after confirmation of transcription using T7 RNA polymerase. The sequences were TAS3a (49).

Protein extraction and immunoblot analysis
Proteins were extracted in buffer containing 50 mM tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 5 mM dithiothreitol, and 1× protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentration was measured by the Bio-Rad Protein Assay (Bio-Rad, CA). For Ponceau S staining, polyvinylidene difluoride membranes were incubated in Ponceau S solution [40% methanol (v/v), 15% acetic acid (v/v), and 0.25% Ponceau S (w/v)]. The membranes were destained using deionized water. Proteins (~150 μg) were fractionated on a 12 to 15% SDS–polyacrylamide gel electrophoresis gel and subjected to immunoblot analysis using α-DRB antibodies. The DRB1 and DRB4 antibodies have been described earlier (27). Immunooblots were developed using an enhanced chemiluminescence detection kit (Roche) or alkaline phosphatase–based color detection.

Pathogen infection and collection of phloem exudate
Inoculations with P. syringae DC 3000 were conducted as described before (51). The bacterial cultures were grown overnight in King’s B medium containing rifampicin and/or kanamycin. For analysis of SAR, the primary leaves were inoculated with Mglycine or the Pst-avrRpt2 bacteria [106 colony-forming units (CFU) ml−1], and 48 hours later, the systemic leaves were inoculated with virulent bacteria (106 CFU ml−1). Unless noted otherwise, samples from the systemic leaves were harvested at 3 days post inoculation.

Inoculations with C. higginsianum and P. irregulare were carried out as described before (52–54). D7 (5 μM) was infiltrated into abaxial surface of Col-0 and ago7 plants 48 hours before inoculation of distal leaves with C. higginsianum or root inoculation with P. irregulare. Three leaves per plant were infiltrated with D7.

PEX was collected in DEPC-treated water as described earlier (7, 13). PEX was collected for 3 to 48 hours and assayed for bacterial growth to ensure that it did not contain any viable bacteria. PEX RNA was extracted using the TRIzol reagent and quantified using NanoDrop, and cDNA was synthesized from PEX. RNA was evaluated for contamination with leaf RNA by assaying for amplification of RubisCO transcript (55). Each sample was run in triplicates, and UBC9 expression levels were used as internal control for normalization. Cycle threshold values were calculated by SDS 2.3 software (Applied Biosystems).

Transport assays
FAM-tagged D7 RNA used in transport studies was custom synthesized (Integrated DNA Technologies or Eurofins Genomics). D7-FAM (5 to 10 μM) was infiltrated into abaxial surface of 3-week-old Arabidopsis leaves. Three leaves per plant were infiltrated with ~0.05 ml of D7-FAM solution. The plants were then kept in a growth chamber set at 14-hour light and 10-hour dark photoperiods, and the leaves were analyzed by confocal microscopy.

High-performance liquid chromatography
Reverse-phase LC of FAM and D7-FAM was carried out by running standards and RNA samples on the C18 column using a 0 to 50% gradient of 25 mM tris (pH 8.0) and acetonitrile. FAM and D7-FAM were detected using a fluorescence detector set at excitation at 492 nm and emission at 520 nm. The levels of D7-FAM were quantified using D7-FAM calibration curve.

Chemical and RNA treatments
SA, G3P, AzA, and Pip treatments were carried out by using 500-, 100-, 1000-, and 1000-μM solutions, respectively. TAS3a RNA was suspended at a concentration of 0.83 to 83 μM, and ~40 μl was infiltrated per leaf. AzA was prepared in methanol and diluted in water. SA, G3P, and Pip were prepared and diluted in water. All dilutions were freshly prepared before performing biological experiments.

Trypan blue staining
Microscopic proliferation of C. higginsianum was analyzed by trypan blue staining of inoculated leaves as described before (56).

G3P, SA, and Pip quantifications
G3P quantifications were carried out as described earlier (7) and reconfirmed using gas chromatography–mass spectrometry (GC-MS). SA and SAG were extracted and measured from ~0.1 g of fresh weight leaf tissue, as described before (56). Pip quantifications were carried out using GC-MS (57). For quantification of SA and AzA in PEX, the samples were dried under nitrogen, suspended in acetonitrile, and derivatized with N-methyl-N- (tert-butyldimethylsilyl) trifluoroacetamide containing 1% tert-butyldimethylchlorosilane and analyzed by GC-MS.

RNA sequencing
The RNA concentration and quality were determined using NanoPhotometer and Agilent 2100 Bioanalyzer. Sequencing libraries were constructed, and Illumina paired-end sequencing was performed using the HiSeq2500 platform at Novogene Corporation. Each genotype and treatment contained three biological replicates. All of the raw reads (~150 nts) were filtered to exclude reads that failed the built-in Failed Chastity Filter in the Illumina software according to
the relation “failed-chastity ≤ 1,” using a chastity threshold of 0.6, on the first 25 cycles. Likewise, reads with adaptor contamination were discarded, low-quality reads were masked with ambiguous sequences “N,” and reads with more than 10% Q < 20 were removed. All the filtered reads were de novo assembled using Trinity (RRID: SCR_013048, version trinityrnaseq_r2013_08_14) with paired-end method and default parameters as previous study on optimal assembly strategy (58). The clean data were aligned to the Arabidopsis genome using HISAT2. The filtered reads were assembled into transcripts using StringTie. Gene expression levels were quantified as count number and processed, as described earlier (60, 61). In brief, reads from the individual wild-type samples were trimmed to remove sequencing adapter sequences and the oligo-dT primer, the wild-type reads were pooled, and the two pools were mapped to the Arabidopsis TAS3a region. For these analyses, the CLC Genomics Workbench suite of tools was used. Results were displayed as browser tracks as shown in Fig. 4D and fig. S9A. These reads define the mRNA-poly(A) junctions such that the 3’-ends of mapped reads coincide with polyadenylation sites.

Confocal microscopy

For confocal imaging, samples were scanned on an Olympus FV1000 microscope (Olympus America, Melville, NY). GFP was excited using a 488-nm laser line, and water-mounted sections of leaf tissue were examined using water immersion PLAPo60XWLSM2 (numerical aperture: 1.0) objective. GFP images were acquired at a scan rate of 10 ms per pixel. Olympus Fluoview1.5 was used to control the microscope, image acquisition, and the export of Tag Image File Format files.

Conductivity assays

Electrolyte leakage was measured in 4-week-old plants. Leaves were infiltrated with MgCl2 or Pst-avrRpt2 (106 CFU/ml). After inoculation ~5 leaf discs per plant (7 mm) were removed with a cork borer, floated in distilled water for ~30 min, and subsequently transferred to tubes containing 5 ml of distilled water. Conductivity of the solution was determined with a National Institute of Standards and Technology–traceable digital conductivity meter (Thermo Fisher Scientific) at the indicated time points. SD was calculated from four replicate measurements per genotype per experiment.

Statistics and reproducibility

For pathogen assays, ~16 plants/genotypes/treatments were analyzed in a single experiment. At least four biological replicates/genotypes/treatments were plated in each experiment. For metabolite quantification, ~12 plants/genotypes/treatments were analyzed in each experiment. Each biological replicate used for pathogen assays and metabolite quantification contained leaves that were harvested from multiple plants. For RNA sequencing, three biological replicates were analyzed, and each contained leaves were harvested from multiple plants. For qRT-PCR, three biological replicates were analyzed in each experiment, and each biological replicate was run in triplicates. Experiments were repeated at least two to six times with a different set of leaves as indicated in the figure legends. Unless otherwise mentioned, error bars indicate SD.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abm8791

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