Developmental Defects Mediated by the P1/HC-Pro Potyviral Silencing Suppressor Are Not Due to Misregulation of AUXIN RESPONSE FACTOR 8[1][OPEN]

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Plant viral suppressors of RNA silencing induce developmental defects similar to those caused by mutations in genes involved in the microRNA pathway. A recent report has attributed viral suppressor-mediated developmental defects to up-regulation of AUXIN RESPONSE FACTOR 8 (ARF8), a target of miR167. The key piece of evidence was that the developmental defects in transgenic Arabidopsis (Arabidopsis thaliana) expressing viral suppressors were greatly alleviated in the F1 progeny of a cross with plants carrying the arf8-6 mutation. Arf8-6 is a SALK line T-DNA insertion mutant, a class of mutations prone to inducing transcriptional silencing of transgenes expressed from the 35S promoter. We have reinvestigated the role of ARF8 in viral suppressor-mediated developmental defects, using two independent arf8 mutations and the P1/HC-Pro potyviral suppressor of silencing. Progeny of a cross between P1/HC-Pro transgenic Arabidopsis and the arf8-6 T-DNA insertion mutant showed little effect on the P1/HC-Pro phenotype in the F1 generation, but almost all arf8-6/P1/HC-Pro progeny had lost the phenotype in the F2 generation. However, the loss of phenotype in the F2 generation was not correlated with the number of functional copies of the ARF8 gene. Instead, it reflected transcriptional silencing of the P1/HC-Pro transgene, as evidenced by a pronounced decrease in P1/HC-Pro mRNA and the appearance of 35S promoter small interfering RNAs. Furthermore, an independent loss-of-function point mutation, Arf8-8, had no detectable effects on P1/HC-Pro phenotype in either the F1 or F2 generations. Together, these data argue against the previously reported role of increased ARF8 expression in developmental defects caused by P1/HC-Pro.

Eukaryotes have evolved an elaborate network of RNA silencing pathways mediated by small regulatory RNAs (for review, see Axtell et al. [2011] and Borges and Martienssen [2015]). These pathways play two major roles in plants. One is to regulate expression of endogenous genes, and the other is to defend against invading nucleic acids, such as viruses, transposons, and—more recently—transgenes. MicroRNAs (miR-NAs) are the small RNAs involved in regulation of endogenous genes and play an important role in development, while small interfering RNAs (siRNAs) function in defense against invading nucleic acids. Both of these classes of small RNA are produced by RNase III-like ribonucleases called DICER-LIKE (DCL) in plants; however, miRNAs and siRNAs differ not only in function but also in biogenesis. In Arabidopsis, DCL1 processes miRNAs from endogenous, imperfectly double-stranded transcripts to produce a duplex containing the miRNA and the opposite strand, the miRNA*. In contrast, siRNAs are processed by DCL2, DCL3, or DCL4 from the usually perfectly dsRNA that triggers the defensive arm of RNA silencing. MiRNA and siRNA duplexes are methylated by HUA ENHANCER1 (HEN1), and one strand incorporates into an RNA-induced silencing complex to guide the Argonaute silencing effector proteins to complementary target sequences. Thus, partially overlapping biosynthetic pathways lead to two classes of small RNAs with distinct functions.

To counteract antiviral silencing, many plant viruses have evolved proteins that suppress RNA silencing. It was noted early on that transgenic plants expressing P1/HC-Pro, the first discovered plant viral suppressor of silencing (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998), exhibited pronounced developmental abnormalities (Anandalakshmi et al., 2000) in addition to being suppressed for silencing. These developmental defects were first observed in tobacco (Anandalakshmi et al., 2000; Mallory et al., 2002a) but were subsequently characterized in greater detail in Arabidopsis (Arabidopsis thaliana; Kasschau et al., 2003; Chapman et al., 2004; Mlotshwa et al., 2005). In Arabidopsis, P1/HC-Pro-mediated

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developmental defects include reduced size, serrated leaves, late flowering, and abnormalities in floral morphology resulting in reduced fertility. Some of these developmental abnormalities resemble ones caused by mutations in genes involved in plant miRNA biogenesis and function. In addition, *P1/HC-Pro* transgenic lines display clear defects in both the biogenesis and function of miRNAs. MiRNAs are present at higher levels in the *P1/HC-Pro* lines, but the usually labile miRNA* also accumulates (Mallory et al., 2002b; Chapman et al., 2004). In addition, although miRNA levels are generally increased, so are their mRNA targets, suggesting a reduction in miRNA function (Kasschau et al., 2003; Chapman et al., 2004). This correlation of developmental defects with defects in the miRNA pathway led to the logical idea that the abnormalities in morphology are caused by interference with miRNA control of developmental pathways (Kasschau et al., 2003; Chapman et al., 2004).

Following up on this idea, a subsequent study investigated the possibility that ectopic overexpression of *DCL1*, which encodes the Dicer that produces miRNAs in plants, might rescue the defects in the miRNA pathway and thereby alleviate the developmental abnormalities in *P1/HC-Pro* transgenic Arabidopsis. Surprisingly, although overexpression of *DCL1* did largely alleviate the developmental defects, it did not correct the P1/HC-Pro-associated defects in the miRNA pathway: Levels of miRNAs and their targets were unchanged from those seen in the parental *P1/HC-Pro* line (Mlotshwa et al., 2005). These data indicate that P1/HC-Pro-mediated defects in development do not result from general impairments in miRNA biogenesis or function. However, because only a few miRNAs and their targets were examined, the study could not rule out the possibility that the developmental defects were due to interference with one or a small set of miRNA-controlled genes. Consistent with that idea, it was later reported that the developmental defects in *P1/HC-Pro* transgenic plants are due to up-regulation of *AUXIN RESPONSE FACTOR 8* (*ARF8*), a target of miR167 that plays a key role in auxin signaling and developmental patterning (Jay et al., 2011).

We were interested in further investigating the role of *ARF8* in P1/HC-Pro-mediated developmental defects because the conclusion that up-regulation of *ARF8* is responsible for the defects is in conflict with the earlier work on the effect of overexpression of *DCL1* on P1/HC-Pro phenotype (Mlotshwa et al., 2005). Importantly, one of the miRNA and target pairs looked at in Mlotshwa et al. (2005) was miR167 and its target, *ARF8*. Increased accumulation of *ARF8* mRNA was unaffected by overexpression of *DCL1* in *P1/HC-Pro* plants, even though the P1/HC-Pro developmental phenotype was largely eliminated. Moreover, the developmental phenotypes of *P1/HC-Pro*-expressing plants differ from those caused by mutation of the *miR167* target site in *ARF8* or the redundantly acting *ARF6*. P1/HC-Pro-expressing plants have narrow serrated leaves, narrow sepals and petals, and short stamen filaments (Kasschau et al., 2003; Chapman et al., 2004; Mlotshwa et al., 2005). In

contrast, *mARF8* or *mARF6* plants with mutated *miR167* target sites do not have serrated leaves or narrow flower organ but do have elongated stamen filaments and swollen (indehiscent) anthers and are also female sterile due to arrested ovule integument growth (Wu et al., 2006). Based on those results, up-regulation of *ARF8* would not appear to be correlated with or responsible for P1/HC-Pro-mediated developmental defects. To resolve these apparent discrepancies, therefore, we further characterized the effects of *arf8* mutations on the P1/HC-Pro phenotype using the same mutation as in the published work (Jay et al., 2011) as well as a second, independent mutation. Our results argue against the previously reported role of increased *ARF8* expression in developmental defects caused by *P1/HC-Pro*.

RESULTS

The P1/HC-Pro Developmental Phenotype Is Largely Retained in the F1 Generation of a Cross with the *arf8-6* T-DNA Insertion Mutant

Developmental defects in a *P1/HC-Pro* transgenic Arabidopsis line were reported to be greatly alleviated in the F1 progeny of a cross with plants carrying the *arf8-6* mutation (Jay et al., 2011). To try to reproduce that result, we used the same *arf8-6* SALK line as in that study and crossed it to our established *P1/HC-Pro* transgenic Arabidopsis line (Mlotshwa et al., 2005, 2008; Endres et al., 2010). The *P1/HC-Pro* line is hemizygous for the turnip mosaic virus coding region, expresses high levels of the *P1/HC-Pro* mRNA, and has a severe developmental phenotype (Mlotshwa et al., 2005; Fig. 1). In the F1 generation, about half (124/238) of the progeny of the cross had the P1/HC-Pro phenotype, while about half (114/238) did not. Genotyping 69 of the nonphenotypic plants revealed that none had the *P1/HC-Pro* transgene, and all but three had the *arf8-6* mutation, indicating that absence of P1/HC-Pro phenotype in the F1 progeny population did not reflect any effect of the *arf8-6* mutation but simply reflected absence of the *P1/HC-Pro* transgene. Among 71 F1 plants confirmed to be both hemizygous for *P1/HC-Pro* and heterozygous for *arf8-6*, all had the P1/HC-Pro phenotype. Thus, in our system, hemizygous *arf8-6* does not cause widespread, strong reduction in P1/HC-Pro phenotype in the F1 generation. A minority of our P1/HC-Pro phenotypic F1 progeny, however, showed a subtle reduction in developmental abnormalities (Fig. 1, F1), suggesting that effects of the *arf8-6* mutation might be better detected in the F2 generation of the cross.

The P1/HC-Pro Phenotype Is Largely Lost in the F2 Generation of the *arf8-6* Cross, But the Loss Is Independent of Whether the *arf8-6* Mutation Is Hemizygous or Homozygous

Because an effect of the *arf8-6* mutation on the phenotype of our *P1/HC-Pro* line might be more evident
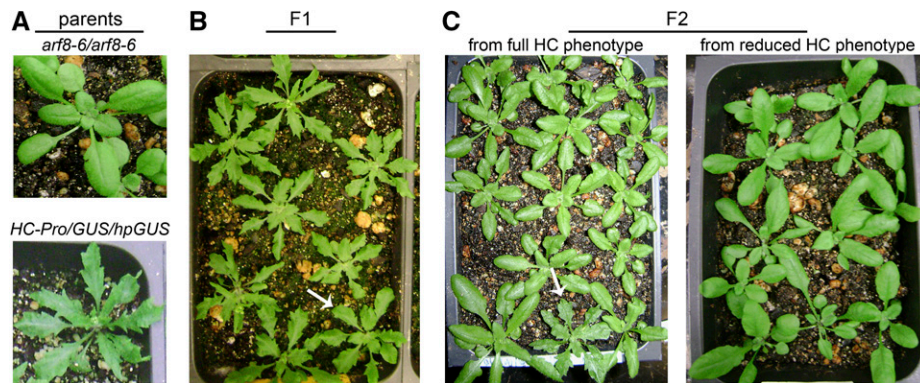
Figure 1. The P1/HC-Pro phenotype is largely retained in the F1 but lost in the F2 generation of a cross with the *arf8-6* mutant. Representative parental plants plus F1 and F2 progeny of the cross between *arf8-6* and *P1/HC-Pro* lines are shown. A, The *arf8-6/arf8-6* parental line has a wild-type phenotype, whereas the *P1/HC-Pro/GUS/hpGUS* parent exhibits the characteristic phenotype of our *P1/HC-Pro* lines. B, P1/HC-Pro phenotypic F1 progeny of the cross, showing that most have the full P1/HC-Pro phenotype and that only a minority have a reduced phenotype (arrow). C, Full and reduced P1/HC-Pro phenotype F1 plants from which RNA was isolated were allowed to self. Seeds from each of the F1 phenotypic groups were pooled separately before planting. No P1/HC-Pro phenotypic F2 progeny carrying *arf8-6* were obtained from the reduced phenotype F1 plants (right pot), while a minority of the F2 progeny from the full phenotype F1 plants exhibited the P1/HC-Pro phenotype (left pot, arrow).

when plants are homozygous rather than hemizygous for the mutation, we examined the phenotypes of the F2 progeny of the cross (after self-pollination of the F1 plants). Progeny of the slightly reduced P1/HC-Pro phenotypic F1 plants had completely lost the phenotype in the F2 generation (Fig. 1, F2), whether plants were hemizygous or homozygous for *arf8-6*. P1/HC-Pro phenotypic progeny obtained from the slightly reduced P1/HC-Pro phenotype F1 plants all lacked the *arf8-6* T-DNA insertion. Thus, although complete loss of P1/HC-Pro phenotype occurred in this population of the F2 progeny of the *arf8-6* cross, the loss did not require that *arf8-6* be homozygous. The P1/HC-Pro phenotype was also lost in the F2 progeny of the fully P1/HC-Pro phenotypic F1 plants but was not total in this case (Fig. 1, F2). Only about 25% (67/271) of this F2 population had a P1/HC-Pro phenotype, and most (50/67) had a reduced phenotype. All of these reduced P1/HC-Pro phenotype plants had *arf8-6*, but only 11 out of the 50 were homozygous for the mutation. Thus, progressive loss of P1/HC-Pro phenotype occurred in successive generations of the *arf8-6* cross but was independent of whether the *arf8-6* mutation was hemizygous or homozygous. Of the 17 fully P1/HC-Pro phenotypic plants obtained in this F2 population, 10 had the *arf8-6* mutation. Two of the 10 were homozygous for *arf8-6*, showing that the full P1/HC-Pro phenotype can occur even in the complete absence of *ARF8* expression.

Altogether, our results suggest that some mechanism other than reduced *ARF8* expression is responsible for alleviation and loss of P1/HC-Pro phenotype in plants carrying the *arf8-6* mutation. Because the *arf8-6* mutation is a SALK line T-DNA insertion mutation, one likely candidate is transcriptional silencing induced by the T-DNA. The T-DNAs used to generate SALK lines and several other T-DNA insertion lines carry an extraneous cauliflower mosaic virus 35S promoter that is known to transcriptionally silence 35S promoter-driven transgenes elsewhere in the plant genome (Daxinger et al., 2008).

Such unexpected silencing effects can generate misleading results, especially in studies involving viral suppressors of silencing, because such suppressors are not effective against transcriptional silencing. The *P1/HC-Pro* transgene in our lines and the one in the work of Jay et al. (2011) are both expressed from the 35S promoter. Therefore, one likely possibility is that transcriptional silencing is the mechanism responsible for alleviation and loss of P1/HC-Pro phenotype in plants also carrying the *arf8-6* mutation.

Indications of 35S Promoter-Induced Transcriptional Silencing Are Evident in the Reduced P1/HC-Pro Phenotype F1 Progeny of the *arf8-6* Cross

To see whether transcriptional silencing of 35S promoter-driven transgenes was occurring in our *arf8-6* mutant plants, we performed RNA gel blot analysis of high and low M_r RNA isolated from F1 and F2 progeny of the *arf8-6* cross. For the purpose of the molecular analysis, the *P1/HC-Pro* line we used for the cross contained two additional transgenes that provide an assay for silencing suppression (Mlotshwa et al., 2008). These additional loci are a sense *uidA* transgene (*GUS*) encoding β-glucuronidase and a hairpin transgene (*hpGUS*) that posttranscriptionally silences the *GUS* locus (Béclin et al., 2002). Expression of each of the three transgenes in this *P1/HC-Pro* line is under the control of the cauliflower mosaic virus 35S promoter. The *hpGUS* and *GUS* transgenes function not only as reporters for suppression of posttranscriptional silencing by P1/HC-Pro, but also as additional indicators of transcriptional silencing because

each is expressed from a 35S promoter. On their own, however, these two transgenes do not induce transcriptional silencing (Béclin et al., 2002; Mlotshwa et al., 2008).

For the RNA gel blot analysis, we examined RNA from plants that had P1/HC-Pro, the arf8-6 mutation, as well as both the hpGUS and GUS transgenes. In the F1 progeny of the cross, 50 plants were heterozygous for the arf8-6 mutation and contained all three of the other transgenes. Of these, 38 plants retained the full P1/HC-Pro phenotype, while 12 showed a slight reduction in the phenotype. RNA gel blot analysis shows that fully P1/HC-Pro phenotypic arf8-6 plants have the same levels as the P1/HC-Pro parent for all high and low M_r RNA species examined (Fig. 2, lanes 1–6): P1/HC-Pro is expressed at about the same level and suppresses posttranscriptional silencing equally well, as indicated by the highly increased accumulation of GUS and hpGUS mRNA compared to the non-P1/HC-Pro controls (Fig. 2, compare lanes 1–6 with lanes 15–19). The GUS locus gives rise to a transcript that is slightly longer than the full-length hpGUS transcript; however, a prominent high M_r hpGUS RNA species that also accumulates corresponds to the loop of the hairpin (Mlotshwa et al., 2008). With respect to low M_r RNA species, the fully P1/HC-Pro phenotypic arf8-6 plants and the P1/HC-Pro parent show the same increased accumulation of miRNAs and of primary siRNAs, which are the siRNAs from the stem of the hpGUS transcript (Fig. 2, compare lanes 1–6 with lanes 15–19).

Although the difference in phenotype between F1 progeny having the full P1/HC-Pro phenotype and those having a slightly reduced phenotype is quite subtle, the difference in the RNA data of these two groups is very striking. The RNA data for the slightly reduced phenotype plants present a very complicated picture because transcriptional silencing and P1/HC-Pro suppression of posttranscriptional silencing both appear to be occurring to some degree. 35S promoter siRNAs are associated with transcriptional silencing induced by T-DNA insertion mutants (Mlotshwa et al., 2010), providing a good indicator for 35S promoter homology-dependent transcriptional silencing. 35S siRNAs were not evident in any of the fully P1/HC-Pro phenotypic arf8-6 F1 plants (Fig. 2, lanes 3–6) or in plants that lacked the arf8-6 mutation (Fig. 2, lanes 1 and 2 with lanes 18 and 19). In contrast, we detected 35S promoter siRNAs in RNA from all of the slightly reduced P1/HC-Pro phenotype arf8-6 F1 plants assayed (Fig. 2, lanes 7–14), indicating that transcriptional silencing had begun in this population. Consistent with the very subtle reduction in phenotype, however, accumulation of P1/HC-Pro mRNA appears unaffected (Fig. 2, compare lanes 7–14 with lanes 1–6). In contrast, accumulation of GUS and hpGUS mRNA as well as primary siRNAs from hpGUS is much lower than in the fully P1/HC-Pro phenotypic plants (Fig. 2, compare lanes 7–14 to lanes 3–6), although still higher in some cases than in plants lacking P1/HC-Pro (Fig. 2, compare lanes 7–14 to lanes 15–19). Thus, despite the onset of transcriptional silencing, P1/HC-Pro suppression of posttranscriptional silencing appears to be occurring in at least some of the mildly reduced phenotype F1

Figure 2. Transcriptional silencing is evident in F1 progeny of the arf8-6 cross that have a reduced P1/HC-Pro phenotype. Accumulation of high and low molecular weight RNA from parental and F1 progeny plants having the indicated genotypes and phenotypes was determined using RNA gel blot analysis. RNA from the GUS line was included as a control to show the position of the GUS transcript. Grouped lanes are all from the same gel. The high molecular weight blot was hybridized with a probe for GUS and hpGUS, then stripped and probed for P1/HC-Pro. Two separate low molecular weight (LMW) RNA gels were run. One LMW blot was probed for hpGUS primary siRNAs, while the other was probed for 35S siRNAs, then successively stripped and probed for the indicated miRNAs. Ethidium bromide (EtBr)-stained rRNA and the major RNA species in LMW RNA are shown as loading controls.
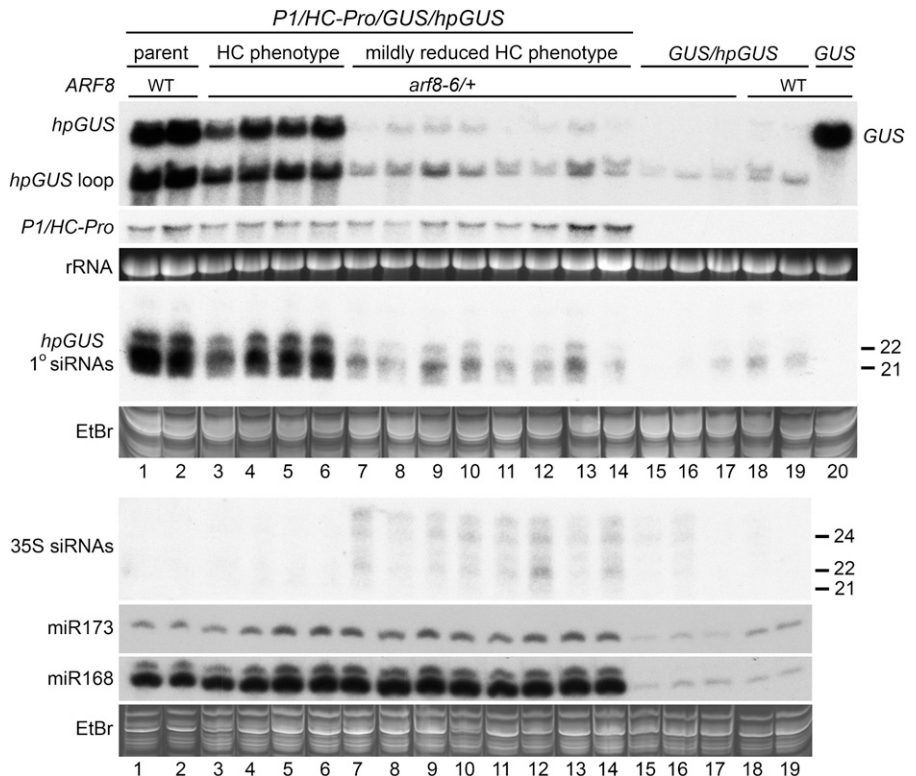
progeny. P1/HC-Pro activity in the mildly reduced phenotype F1 progeny is more clearly demonstrated by the increased miRNA accumulation in those plants compared to plants without P1/HC-Pro (Fig. 2, compare lanes 7–14 to lanes 15–19). Indeed, miRNA accumulation in the mildly reduced phenotype F1 progeny appears comparable to that in fully P1/HC-Pro phenotypic plants (Fig. 2, compare lanes 7–14 to lanes 1–6).

Thus, the *P1/HC-Pro/arf8-6* F1 plants are a mixed population. Most are completely normal for transgene expression and P1/HC-Pro activity, but some have started to transcriptionally silence 35S promoter-driven transgenes. It is interesting to note that 35S promoter-induced transcriptional silencing appears to have started even in *arf8-6* progeny that do not have the *P1/HC-Pro* transgene: 35S promoter siRNAs are very faintly detectable in RNA from two of the three *arf8-6/GUS/hpGUS* plants we analyzed (Fig. 2, compare lanes 15 and 16 with lanes 18 and 19).

35S Promoter-Induced Transcriptional Silencing Is Widespread and Well Developed in the F2 Progeny of the *arf8-6* Cross

RNA gel blot analysis of the F2 progeny of the *arf8-6* cross confirms that transcriptional silencing of 35S promoter-driven transgenes is occurring, even in the absence of the *P1/HC-Pro* transgene. All three of the *arf8-6*$^{-/-}$/*GUS*/*hpGUS* plants analyzed are transcriptionally silenced for the *GUS* and *hpGUS* transgenes. These plants show high levels of 35S siRNAs and have no detectable *GUS* and *hpGUS* mRNA or primary siRNAs from *hpGUS*, in contrast to *GUS/hpGUS* controls lacking *arf8-6* (Fig. 3, compare lanes 15–17 with lanes 18 and 19). Similarly, *P1/HC-Pro/arf8-6*$^{-/-}$/*GUS*/*hpGUS* F2 plants that have completely lost the P1/HC-Pro phenotype are clearly transcriptionally silenced for *P1/HC-Pro* as well as for the *GUS* and *hpGUS* transgenes. These plants have easily detectable levels of 35S siRNAs and accumulate little or no P1/HC-Pro mRNA and no *GUS* and *hpGUS* mRNA or primary siRNAs from *hpGUS*, compared to *P1/HC-Pro* controls lacking the *arf8-6* mutation (Fig. 3, compare lanes 7–14 with lanes 1 and 2). Surprisingly, however, miR168 accumulation is elevated compared to non*P1/HC-Pro* controls (Fig. 3, compare lanes 7–14 with lanes 15–19).

As discussed above, some *P1/HC-Pro/arf8-6*$^{-/-}$ F2 plants had a P1/HC-Pro phenotype (Fig. 1, F2). Most of these had a reduced phenotype, but a few had the full P1/HC-Pro phenotype. RNA gel blot analysis shows that 35S promoter-induced transcriptional silencing had started in the reduced phenotype plants, as evidenced by the presence of 35S siRNAs as well as reduced levels of *P1/HC-Pro*, *GUS*, and *hpGUS* mRNA and *hpGUS* primary siRNAs (Fig. 3, compare lanes 3 and 4 with lanes 1 and 2). In contrast, 35S promoter-induced
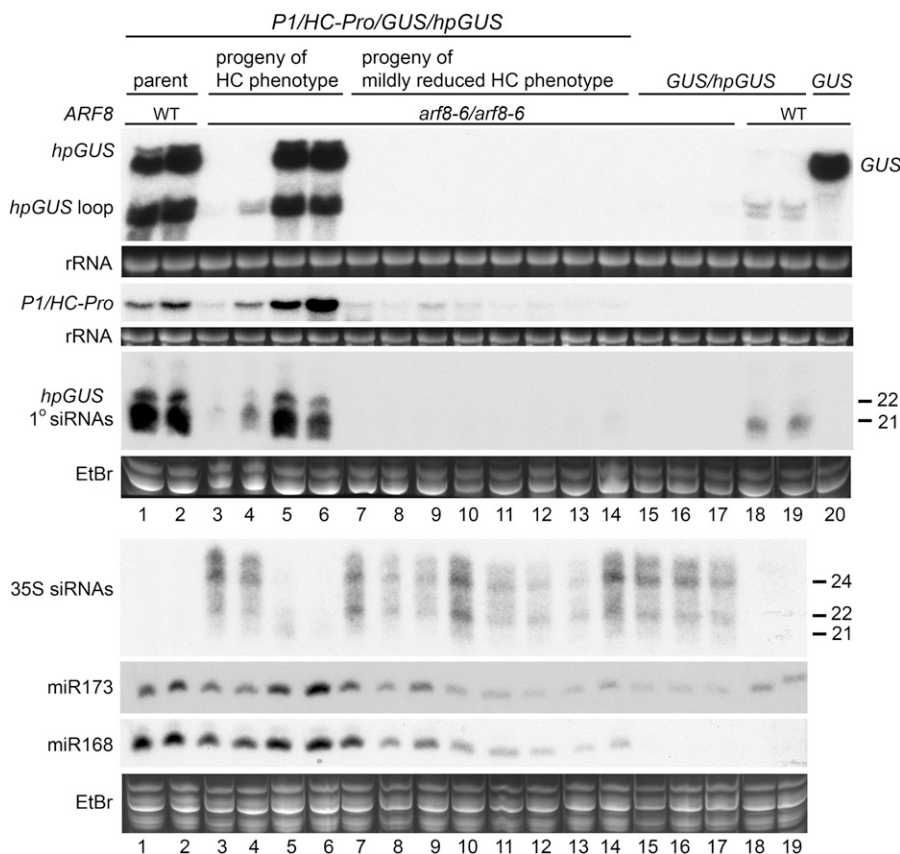
Figure 3. Transcriptional silencing is widespread in the F2 progeny of the *arf8-6* cross. Accumulation of high and low molecular weight RNA from the original parental lines and from F2 progeny having the indicated genotypes and F1 parental phenotypes was determined using RNA gel blot analysis. RNA from the *GUS* line was included as a control to show the position of the *GUS* transcript. Grouped lanes are all from the same gel. Two high molecular weight (HMW) RNA gels were run. One HMW blot was hybridized with a probe for *GUS* and *hpGUS*, while the other was probed for *P1/HC-Pro*. Two LMW RNA gels were run. One LMW blot was probed for *hpGUS* primary siRNAs, while the other was probed for 35S siRNAs, then successively stripped and probed for the indicated miRNAs. Ethidium bromide (EtBr)-stained rRNA and the major RNA species in LMW RNA are shown as loading controls.

Figure 4. Comparison of *arf8-3* and *arf8-8* developmental phenotypes. A to C, 5-week-old wild-type, *arf6-2 arf8-3* and *arf6-2 arf8-8* plants. D to H, Inflorescence apices of 5-week-old plants of indicated genotypes. D, E, and F were each assembled from multiple overlapping photographs. I, Measurements of floral organ lengths and petal widths of mature flowers of each genotype. Data are means ± SD (*n* = 13–18 flowers). In I, letters above each bar indicate values that were not statistically distinguishable by ANOVA at *P* < 0.05 based on Tukey's HSD statistic, calculated within each measurement class. As ARF6 and ARF8 act redundantly, the *arf6-2* background exposes the full phenotypic effects of the *arf8* mutations.
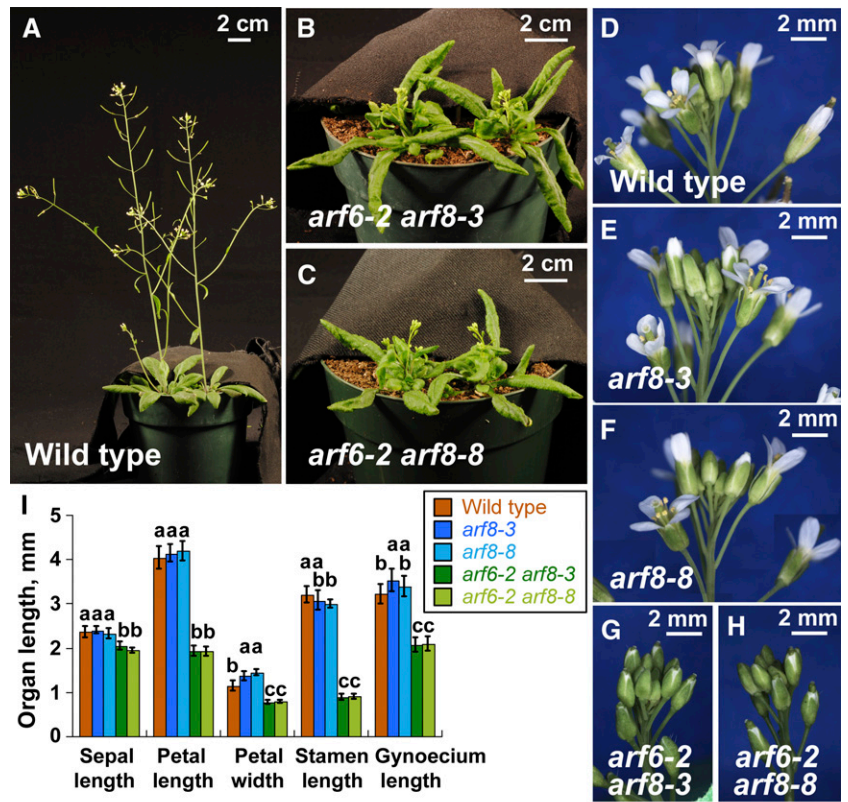

transcriptional silencing does not appear to have started in the few *P1/HC-Pro/arf8-6*$^{-/-}$ F2 plants that still have the full P1/HC-Pro phenotype. These plants look identical to the *P1/HC-Pro* parent for all high and low M_r RNA species examined (Fig. 3, compare lanes 5 and 6 with lanes 1 and 2). Thus, 35S promoter-induced transcriptional silencing appears well established in the vast majority of F2 progeny of the *arf8-6* cross, supporting our hypothesis that transcriptional silencing is the mechanism responsible for loss of P1/HC-Pro phenotype in *P1/HC-Pro/arf8-6* plants. The few *P1/HC-Pro/*

arf8-6 plants that retain some degree of P1/HC-Pro phenotype are ones in which transcriptional silencing has not yet been completely established.

The *arf8-8* Loss-of-Function Point Mutation Does Not Alleviate P1/HC-Pro-Mediated Developmental Defects

To test further whether loss of *ARF8* function can alleviate the P1/HC-Pro phenotype, we used the *arf8-8* mutant line, which has a point mutation at the junction of the fifth intron and sixth exon of *ARF8* (Reeves et al.,

Figure 5. The *arf8-8* mutation has no effect on the P1/HC-Pro phenotype in either the F1 or F2 generation of a cross to our *P1/HC-Pro* line. Representative parental plants plus F1 and F2 progeny of the cross between *arf8-8* and *P1/HC-Pro* lines are shown. A, The *arf8-8/arf8-8* parental line has a wild-type phenotype, whereas the *P1/HC-Pro/GUS/hpGUS* parent exhibits the characteristic phenotype of our *P1/HC-Pro* lines. B, P1/HC-Pro phenotypic F1 progeny of the cross, showing that all have the full P1/HC-Pro phenotype. C, P1/HC-Pro phenotypic F1 plants from which RNA was isolated were allowed to self, and seeds were pooled before planting. All *P1/HC-Pro* F2 progeny carrying arf8-8 exhibited the full P1/HC-Pro phenotype.
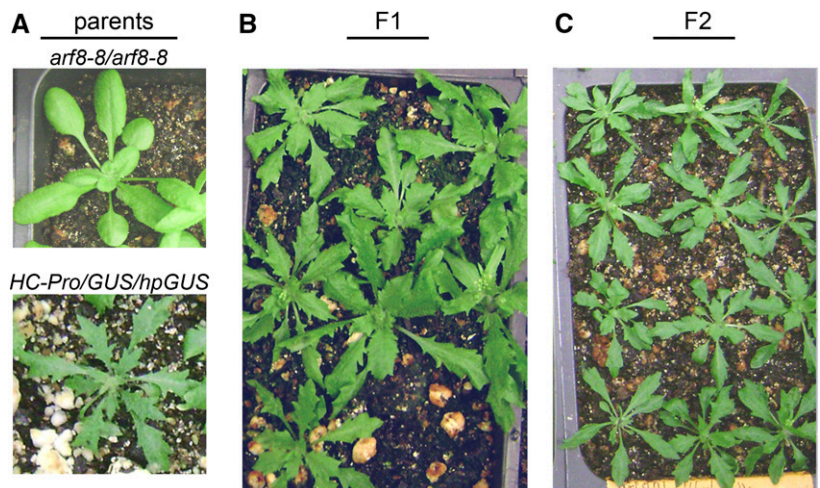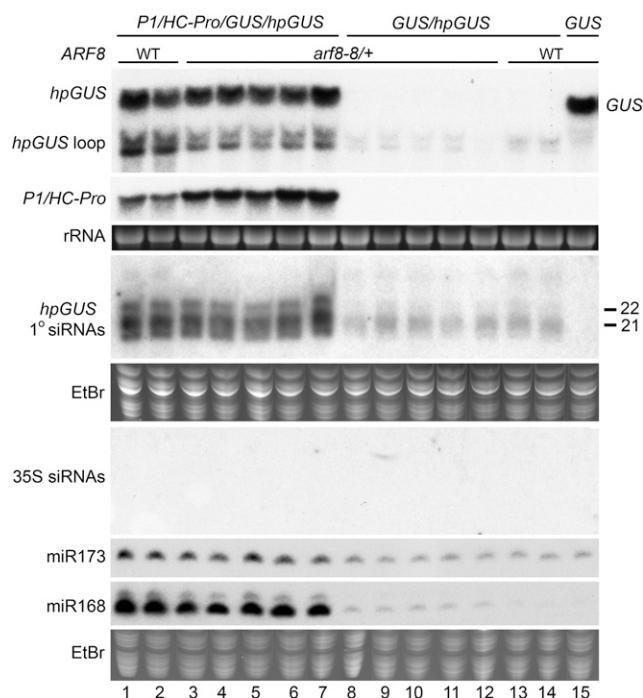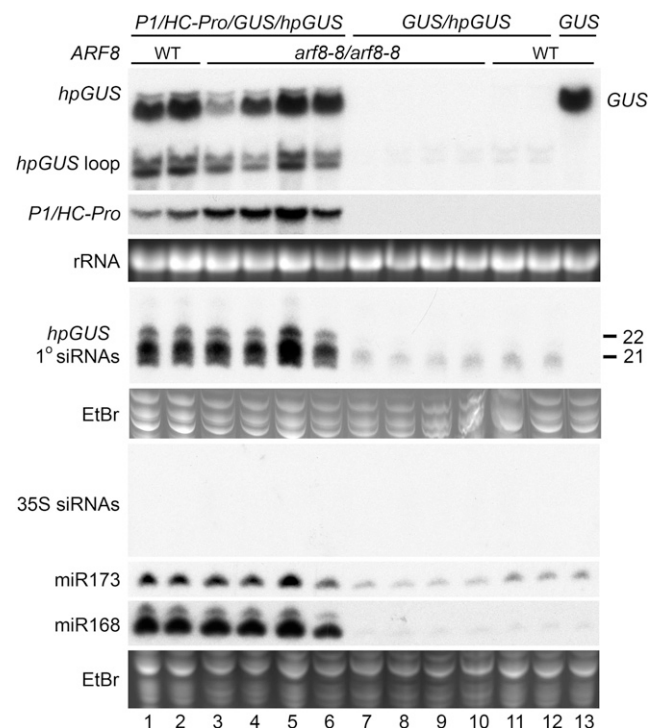
Figure 6. The *arf8-8* mutation has no effect on molecular measures of P1/HC-Pro function in the F1 generation of a cross to our *P1/HC-Pro* line. Accumulation of high and low molecular weight RNA from parental and F1 progeny plants having the indicated genotypes was determined using RNA gel blot analysis. RNA from the *GUS* line was included as a control to show the position of the *GUS* transcript. Grouped lanes are all from the same gel. The HMW blot was hybridized with a probe for *GUS* and *hpGUS*, then stripped and probed for *P1/HC-Pro*. Two separate LMW RNA gels were run. One LMW blot was probed for *hpGUS* primary siRNAs, while the other was probed for 35S siRNAs, then successively stripped and probed for the indicated miRNAs. Ethidium bromide (EtBr)-stained rRNA and the major RNA species in LMW RNA are shown as loading controls.

2012). Based on phenotype, the *arf8-8* mutation appears indistinguishable from the loss-of-function T-DNA insertion mutation *arf8-3*. However, because *ARF8* acts partially redundantly with *ARF6*, the loss of function of *ARF8* alone has only a subtle effect, whereas the *arf8 arf6* double mutant is profoundly affected (Nagpal et al., 2005). Figure 4 shows that the *arf8-8* and *arf8-3* mutations have indistinguishable mild effects on vegetative and flower growth phenotypes (Fig. 4I; and compare Fig. 4, E and F, with D). In addition, the *arf8-8* mutation in combination with the *arf6-2* loss-of-function mutation causes the same strong phenotype as the *arf8-3 arf6-2* double mutant (Fig. 4I; and compare Fig. 4, B and C, with A and Fig. 4, G and H, with D). Thus, *arf8-8* appears phenotypically as a null allele. We crossed our *P1/HC-Pro/GUS/hpGUS* line to the *arf8-8* mutant line and examined the phenotypes of the F1 progeny. There were two classes of phenotype: P1/HC-Pro and wild type. All of the F1 progeny we obtained that had *P1/HC-Pro* in the heterozygous *arf8-8* background retained the distinctive P1/HC-Pro phenotype (Fig. 5, F1). None of the

nonphenotypic plants had the *P1/HC-Pro* transgene, and none of the *P1/HC-Pro* heterozygous *arf8-8* plants showed any reduction in P1/HC-Pro phenotype. The P1/HC-Pro phenotype was also retained in the F2 generation of the cross, whether plants were homozygous or heterozygous for the *arf8-8* mutation (Fig. 5, F2).

The *arf8-8* Loss-of-Function Point Mutation Does Not Affect P1/HC-Pro Function

Because P1/HC-Pro phenotype was unaffected by the *arf8-8* mutation (Fig. 5), we expected that molecular measures of P1/HC-Pro function would also be unaffected. RNA gel blot analysis of high and low M_r RNA isolated from F1 and F2 progeny of the *arf8-8* cross support that expectation. *P1/HC-Pro* plants hemizygous (Fig. 6) or homozygous (Fig. 7) for *arf8-8* look nearly identical to the *P1/HC-Pro* parent for all high and low M_r RNA species examined (Fig. 6, compare lanes 3–7 with lanes 1 and 2; Fig. 7, compare lanes 3–6 with

Figure 7. The arf8-8 mutation has no effect on molecular measures of P1/HC-Pro function in the F2 generation of a cross to our *P1/HC-Pro* line. Accumulation of HMW and LMW RNA from parental and F2 progeny plants having the indicated genotypes was determined using RNA gel blot analysis. RNA from the *GUS* line was included as a control to show the position of the *GUS* transcript. Grouped lanes are all from the same gel. Two HMW RNA gels were run. One HMW blot was hybridized with a probe for *GUS* and *hpGUS*, while the other was probed for *P1/HC-Pro*. Two LMW RNA gels were run. One LMW blot was probed for *hpGUS* primary siRNAs, while the other was probed for 35S siRNAs then successively stripped and probed for the indicated miRNAs. Ethidium bromide (EtBr)-stained rRNA and the major RNA species in LMW RNA are shown as loading controls.

lanes 1 and 2). P1/HC-Pro suppresses the *hpGUS* transgene-induced silencing in these plants, as indicated by the highly increased accumulation of *GUS* and hpGUS mRNA and mediates increased accumulation of miRNAs and hpGUS primary siRNAs compared to the non-*P1/HC-Pro* controls (Fig. 6, compare lanes 3–7 and 1 and 2 with lanes 8–14; Fig. 7, compare lanes 3–6 and 1 and 2 with lanes 7–12). Thus, reduced levels of functional *ARF8* in the absence of 35S promoter-induced transcriptional silencing affect neither P1/HC-Pro phenotype nor molecular measures of P1/HC-Pro function.

DISCUSSION

Our results clearly show that *ARF8* is not the key factor responsible for developmental defects in Arabidopsis expressing a turnip mosaic virus *P1/HC-Pro* transgene. Moderate up-regulation of *ARF8* was originally reported to underlie developmental defects in Arabidopsis induced by P1/HC-Pro and two other viral suppressors of silencing (Jay et al., 2011). The identification of *ARF8* as a candidate for this role came from determining what miRNA targets are upregulated in common in Arabidopsis expressing any one of the viral suppressors, P1/HC-Pro, P19, and P15, as well as in Arabidopsis *hen1-1* and *dcl1-9* mutants. The up-regulation in all cases was about 1.5- to 2-fold, suggesting that loss of just one copy of *ARF8* would greatly reduce or eliminate the developmental defects caused by the viral suppressors. Alleviation of the developmental defects by one copy of the *arf8-6* SALK insertion mutation was then taken as evidence that misregulation of *ARF8* was the key factor underlying viral suppressor-mediated developmental anomalies.

We were led to question the conclusions of the Voinnet group (Jay et al., 2011) for three reasons. The first was our prior work, which showed no correlation between P1/HC-Pro phenotype and *ARF8* expression (Mlotshwa et al., 2005). The second reason was a paradoxical result presented in the gel blot analysis of RNA isolated from *P1/HC-Pro* plants that carried the *arf8-6* mutation and a hairpin transgene that targets the gene encoding chalcone synthase (CHS; Jay et al., 2011; Fig. 5E). The paradoxical result was that the gel blot analysis in Figure 5E showed a total absence of *CHS* siRNAs in those plants, although Figure 5E also presented evidence that P1/HC-Pro was suppressing the hairpin transgene-induced posttranscriptional silencing. It has been well established, however, that primary siRNAs, such as those that would derive from the stem of a hairpin transgene, are not eliminated in P1/HC-Pro suppression of silencing (Johansen and Carrington, 2001; Mlotshwa et al., 2008; Zhang et al., 2008; Endres et al., 2010). Therefore, *CHS* siRNAs should have been present if P1/HC-Pro suppression of silencing was truly occurring, and their absence strongly suggested that some confounding factor had affected the Jay et al. (2011) P1/HC-Pro studies. Lastly, the P1/HC-Pro phenotypes differ in several aspects from those

caused by loss of miR167 regulation of *ARF6* or *ARF8* (Wu et al., 2006).

Our work shows that all the effects of the *arf8-6* T-DNA insertion mutation on P1/HC-Pro phenotype and on molecular indicators of P1/HC-Pro function are most likely due to 35S promoter homology-dependent transcriptional silencing induced by the *arf8-6* T-DNA insertion. In addition, the *arf8-8* loss-of-function point mutation has no effect on P1/HC-Pro phenotypes or on molecular measures of P1/HC-Pro function, further arguing against *ARF8* as the key factor responsible for developmental defects caused by *P1/HC-Pro* expression. Thus, the exact mechanism responsible for P1/HC-Pro-mediated developmental defects remains an open question. Interference with miRNA-controlled developmental pathways is a very attractive hypothesis, but ectopic overexpression of *DCL1* has been shown to separate P1/HC-Pro phenotype from effects on general miRNA biogenesis and function (Mlotshwa et al., 2005). Therefore, one or a few key miRNA-controlled factors might, in fact, underlie the developmental defects caused by P1/HC-Pro; however, our work shows that *ARF8* is not one of these key factors.

MATERIALS AND METHODS

Arabidopsis Lines

The mutant and transgenic lines used are all in the Columbia (Col-0) ecotype and have been described in previous studies. The *P1/HC-Pro/GUS/hpGUS* line is hemizygous for the *P1/HC-Pro* transgene and was previously published as *P1/HC-Pro*/6b4/306 (Mlotshwa et al., 2008). The *arf8-6* line is the University of Wisconsin T-DNA line WiscDsLox324F09 (Goetz et al., 2006). The *arf8-8* line has an ethyl methane sulfonate-generated G to A mutation in a splice acceptor site in *ARF8* (Reeves et al., 2012). The *arf8-3* null mutation line has a T-DNA insertion in the transcribed region of *ARF8* (Nagpal et al., 2005).

Genotyping

Hemizygous and homozygous *arf8-8* plants were genotyped using the PCR primers and *Eco*NI digestion of the PCR product as described previously (Reeves et al., 2012). For hemizygous and homozygous *arf8-6* genotyping, the T-DNA primer LB (5′-AACGTCCGCAATGTGTTATTAAGTTGTC-3′) was used with primers ARF8-6LP (5′-CGAGGAAAGGTGAAACCTAC-3′) and ARF8-6RP (5′-AGCTGTCAACATCTGGATTGG-3′). Primers ARF8-6LP and ARF8-6RP amplify a 1014 bp product from the wild-type locus, and primers ARF8-6RP and LB amplify a 500 bp product from the *arf8-6* locus. Genotyping for the *hpGUS* (= 306) and *GUS* (= 6b4) transgenes was performed as described previously (Mlotshwa et al., 2008). *P1/HC-Pro* primers HC-F (GTGCCCA-GAAGTTCAAGAGC) and HC-R (GTCAACGACTATGCCACTCCAACC) were used to confirm the presence of the *P1/HC-Pro* transgene in combination with phenotypic selection. P1/HC-Pro phenotype was evaluated based on the degree of dwarfing and leaf serration compared to wild-type Arabidopsis (*Arabidopsis thaliana*).

RNA Isolation and Gel Blot Analysis

High and low M_r total RNA was isolated from aerial tissues of individual flowering plants and gel blot analysis performed as described previously (Mlotshwa et al., 2005, 2008) [α-^{32}P]UTP-labeled antisense RNA probes to detect *GUS*, *hpGUS*, and P1/HCPro mRNAs or the sense RNA probes to detect *hpGUS* primary siRNAs and 35S-promoter siRNAs were prepared using an Ambion MAXIscript in vitro transcription kit (http://www.ambion.com) as described previously (Mlotshwa et al., 2008, 2010). The probes were hybridized to mRNA blots at 68°C in Ambion ULTRAhyb buffer or to siRNA blots at 42°C in Ambion ULTRAhyb-oligo buffer. Antisense oligonucleotide probes for detection of

miRNAs were prepared by end-labeling with ^{32}P using T4 polynucleotide kinase (New England Biolabs) and hybridized to miRNA blots at 42°C in Ambion ULTRAhyb-oligo buffer.

arf Mutant Phenotypic Analyses

Flower organs were dissected from mature open flowers of wild-type and *arf8* single mutants (2 or 3 flowers per apex) or from arrested flower buds at the equivalent position on the inflorescence of *arf6 arf8* double mutants (5–7 flowers per apex). Organs were placed flat on agar plates, and measured using a camera lucida attached to a dissecting microscope (Reeves et al., 2012). Mean lengths or widths measured from at least two sepals, petals, and long stamens for each flower were used in the summary graph in Figure 4.

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