

Mobile gene silencing in *Arabidopsis* is regulated by hydrogen peroxide

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ABSTRACT

In plants and nematodes, RNAi can spread from cells from which it is initiated to other cells in the organism. The underlying mechanism controlling the mobility of RNAi signals is not known, especially in the case of plants. A genetic screen designed to recover plants impaired in the movement but not the production or effectiveness of the RNAi signal identified *RCI3*, which encodes a hydrogen peroxide (H₂O₂)-producing type III peroxidase, as a key regulator of silencing mobility in *Arabidopsis thaliana*. Silencing initiated in the roots of *rci3* plants failed to spread into leaf tissue or floral tissue. Application of exogenous H₂O₂ reinstated the spread in *rci3* plants and accelerated it in wild-type plants. The addition of catalase or MnO₂, which breaks down H₂O₂, slowed the spread of silencing in wild-type plants. We propose that endogenous H₂O₂, under the control of peroxidases, regulates the spread of gene silencing by altering plasmodesmata permeability through remodelling of local cell wall structure, and may play a role in regulating systemic viral defence.

Subjects Cell Biology, Developmental Biology, Molecular Biology, Plant Science

Keywords Mobile silencing, *Arabidopsis*, Type III peroxidase, Peroxide, Plasmodesmata, Catalase, Cell-to-cell transport, RNAi, Small RNA

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INTRODUCTION

Growth and development in multi-cellular organisms is co-ordinated by local and long-distance communication between cells and tissues. In plants, signaling molecules, including RNAs, are transported locally between cells, and long-distance in the phloem to provide developmental and defence information (Lough & Lucas, 2006; Sparks, Wachsman & Benfey, 2013). Small RNAs are critical regulators of plant development and defense, and can be mobile. For example, the endogenous miRNAs miR165/6, tasiRNA (tasiR-ARF), and miR394 serve as morphogen-like signals, forming gradients to determine cell fate during leaf and root development (Chitwood et al., 2009; Carlsbecker et al., 2010; Skopelitis, Husbands & Timmermans, 2012; Knauer et al., 2013). Plant virus-induced small interfering RNAs (siRNAs) can also move throughout the plant from the original site of infection to prime the defence mechanism against further virus invasion (Nelson & Citovsky, 2005; Voinnet, 2005).

Small interfering RNAs derived from transgenic hairpin RNAs also spread both locally and over long distances (Palauqui et al., 1997; Voinnet & Baulcombe, 1997; Himber et al., 2003; Brosnan et al., 2007; Liang, White & Waterhouse, 2012). Experiments in

Arabidopsis thaliana showed that these siRNAs will move from rootstocks expressing a hairpin RNA targeting a green fluorescent protein (GFP) sequence across graft junctions to silence GFP expression in grafted shoot tissue (Brosnan et al., 2007). Although the hairpin-derived silencing signals generated in roots have been shown to move gradually from cell to cell into the shoot, rather than moving through the phloem (Liang, White & Waterhouse, 2012), the factors controlling silencing mobility in plants are unknown.

In the nematode *Coenorhabditis elegans*, the movement of silencing signals is facilitated by the dsRNA-channels SID-1 and SID-2 (Winston, Molodowitch & Hunter, 2002; Feinberg & Hunter, 2003), but there is no clear homologue in *Arabidopsis*. Instead, the spread of silencing in plants is thought to be regulated, in part, by transport through plasmodesmata (PD), the channels that link the cytoplasm of adjacent plant cells, although direct genetic evidence is lacking (Melnyk, Molnar & Baulcombe, 2011). We sought to identify components that influence RNAi mobility in plants using a mutagenesis approach.

MATERIALS AND METHODS

EMS (ethyl methane sulfonate) mutagenesis and screening

As our starting material we used a transgenic *Arabidopsis thaliana* line, RtSS (Root to Shoot Silencing), in which 35S-GFP is expressed in all tissues. This line also expresses a root-specific promoter from tobacco, TobRb7, which controls the expression of LhG4-GR in the cell cytoplasm. When Dexamethasone (Dex) is present, the transcriptional activator, GR, enters the nucleus and binds to the 6xOp promoter, inducing bidirectional transcription of a GFP-silencing hairpin, hpGE, together with a GUS reporter, as described previously (Liang, White & Waterhouse, 2012). GFP silencing was then monitored using both fluorescence dissecting (Leica MZFLIII or Zeiss Stereo Lumar) and compound microscopes (Zeiss Axioimager) (Liang, White & Waterhouse, 2012), and all images were assembled and adjusted using Adobe Photoshop. EMS mutagenesis of RtSS was essentially performed as described by Kim, Schumaker & Zhu (2006). Initial trials showed that most of the potential mutants could not transmit the phenotype to the next generation. Hence, we used a higher concentration of EMS (2.5% ethanol and 0.6% EMS) to generate the M1 plants that formed the basis of this mutant screen. We pooled 200–300 M1 plants to provide M2 seeds. Around 100–150 M2 seeds were sown in each plate (150 cm²) onto 10 μM Dex-containing MS medium, grown for three weeks, then transferred to soil to screen for shoot silencing. In total, we screened 90,000 M2 seedlings derived from 110,000 M1 seeds, and identified 93 individual M2 plants showing root silencing but with no shoot silencing or very delayed shoot silencing. Potential mutants were allowed to self-fertilise for at least three generations to exclude environmental influences. Only one mutant, M397, showed stable genetic inheritance of the delayed silencing phenotype in 5 continuous self-fertilised generations.

Genome sequencing and genetic mapping

Both the M4 generation of M397 and the parent RtSS plants were subjected to Next Generation Sequencing using a protocol provided by the Australian Genome Research

Facility (AGRF NGS Submission Guide). A total of 2.56 Gb and 5.60 Gb Paired-End 100-bp sequence was generated using the Illumina Hiseq-2000 platform for M397 and RtSS, respectively. Primers used are listed in [Table S1](#). Bioinformatic analyses identified 4,522 and 7,470 potential SNPs from each genome, respectively, compared to the reference wild-type Columbia genome. Genetic tests showed M397 to be a single recessive mutant, therefore we reduced the total unique homozygous SNPs between M397 and the parent RtSS to 1,551 ([File S1](#)). We next used these SNPs to map the mutation in F3 families by Sanger sequencing, which showed that the mutation was strongly associated with Chr. 1 according to Wright's fixation index with $F_{st} > 0.1$ ([Wright, 1965](#)) ([Fig. S1](#)). We then selected 20 SNPs across Chr.1 to genotype all 142 F3 families. A linkage group was constructed on Chr.1 by calculating the allele frequency ([Fig. S1](#)). Only one SNP (AT1G05260, Chr. 1 position: 1530461; G to A) showed 100% frequency. Further genetic complementation confirmed that this SNP was the causal mutation.

H₂O₂ detection and breakdown

The H₂O₂-specific fluorescent probes, Peroxy Orange 1 (PO1, SML0688-5MG; Sigma) ([Dickinson, Huynh & Chang, 2010](#)) and 2,7-dichlorodihydrofluorescein diacetate (H₂ DCFDA, D6883-250MG; Sigma) ([Kristiansen et al., 2009](#)) were used to detect H₂O₂ in RtSS and wild-type Columbia and in *rci3-1* and *rci3-2* mutants. Roots of 10 to 13-day old plants were placed into 1.5 cm diameter wells, made with a core-borer, in MS agar medium. Each well was then filled with 100 µl of 50 µM PO1 (diluted from 5 mM stock solution in DMSO into pH 6.4 perfusion solution [Brauer et al., 1996](#)), to submerge the roots. Plates were then sealed with parafilm and placed into growth rooms (16 h light/8 h dark at 22 °C) and stained overnight. After 16–18 h of staining, plants were briefly rinsed with water before beginning the longitudinal sectioning and observation using a Zeiss Axioimager fluorescence microscope with a DsRed filter to detect the PO1 signal, and an AF488 filter to detect GFP and chlorophyll autofluorescence. Identical camera and software settings were used for all images. Fluorescence intensity over each hypocotyl was measured using ImageJ (<http://rsb.info.nih.gov/ij/>), and statistical significance was assessed using a Student's *t* test, as in [Dickinson, Huynh & Chang \(2010\)](#).

Treatments to modify PD or peroxide levels

Treatment media were prepared by adding chemicals directly to warm liquid MS agar medium. For H₂O₂ treatment, aliquots of 35% stock solution (Sigma, 349887-500ML) were added to Dex-containing MS agar medium just prior to setting. Catalase was filter-sterilized and added to near room temperature MS medium from 100 mg/ml stock in 50 mM potassium phosphate to give a final concentration of 1 mg/ml. For MnO₂ treatment, approximately 1 g autoclaved MnO₂ (529664-5G) powder was sprinkled onto the surface of Dex-containing MS medium before seeds were sown. For n-ethyl maleimide (NEM) treatment, plants germinated on medium with or without Dex for 4 days were transferred to 50 µM or 100 µM NEM-containing MS medium, with corresponding Dex treatment. Control plants were transferred to normal MS medium, and other controls were never treated with Dex. The number of plants showing silencing were recorded,

providing binary data (presence or absence of silencing) which were subjected to logistic regression analysis. Odds ratios greater than 1.0 indicate that a treatment is more likely to induce silencing than in controls, while odds ratios less than 1.0 indicate that a treatment is less likely to induce silencing, at calculated levels of significance (p value). In cases where percentage data were used to compare two treatments, the Wilcoxon signed rank test was applied to assess the significance of observed differences.

RESULTS

Implementation and spread of RNAi can be genetically uncoupled

To investigate genetic factors involved in regulating silencing mobility, we generated a Root-to-Shoot mobile Silencing System (RtSS) in which a green fluorescent protein (GFP) reporter transgene is expressed throughout the plant but is specifically silenced in root tissue following the application of Dex. The system, triggered by localized hairpin RNA expression in the roots, generates a signal that moves cell-to-cell from the root, through the hypocotyl and into the shoot, visibly silencing the green fluorescence (Liang, White & Waterhouse, 2012). Treating seed from our RtSS line (T5 generation) with a chemical mutagen (EMS) and screening the Dex-induced progeny yielded plant lines with accelerated, retarded or abolished spread of silencing into the shoot tissue. From 110,000 seeds, 93 lines displayed root silencing that failed to spread into the shoots. The silencing in the root tissue demonstrated that components of the RNAi generation mechanism had not been compromised in these mutants and that they were likely to be defective in either signal mobility or the ability to respond to the signal. Of these lines, one showed stable inheritance of the trait for 5 generations. Longitudinal sections (Fig. 1A) revealed that the induction and initial shootward spread of silencing at the base of the hypocotyl in the mutant and wild-type RtSS plants were almost indistinguishable up to 5 days post induction (dpi). However, at 11 dpi there was a marked difference in spread, and by 21 dpi all of the wild-type RtSS plants but none of the mutant plants displayed silencing in the rosette leaves (Fig. 1 and Figs. S2E, S2F). In the wild-type, the silencing front migrated from the root-hypocotyl junction to the hypocotyl-epicotyl junction at a rate of 373 ± 65 μm per day ($N = 9$, similar to the rate of 377 ± 96 , $N = 12$, observed in Liang, White & Waterhouse (2012)), whereas in the mutant this was reduced to 110 ± 20 μm ($N = 9$) per day. Once the silencing front reached the hypocotyl-epicotyl junction, it migrated slowly towards the meristem at a rate of 56 ± 22 μm per day ($N = 14$) in the wild-type (Liang, White & Waterhouse, 2012), which was reduced to 20 ± 7 μm per day ($N = 9$) in the mutant. The rate of spread in the epicotyl of the mutant was so slow that the silencing front never reached the shoot apical meristem, and as a result neither the rosette leaves nor the floral bolt were silenced.

In *Arabidopsis* grafts or RtSS constructs, the silencing hairpin construct in the roots, which is against the first 400-bp fragment (the GF fragment) of the *GFP* gene, shows transitivity, such that silencing siRNAs in the graft scion or RtSS shoot are mostly against the 3' region of the gene (the P fragment) (Brosnan et al., 2007; Liang, White & Waterhouse, 2012). The roots contain both GF- and P-derived siRNAs (Liang, White & Waterhouse,

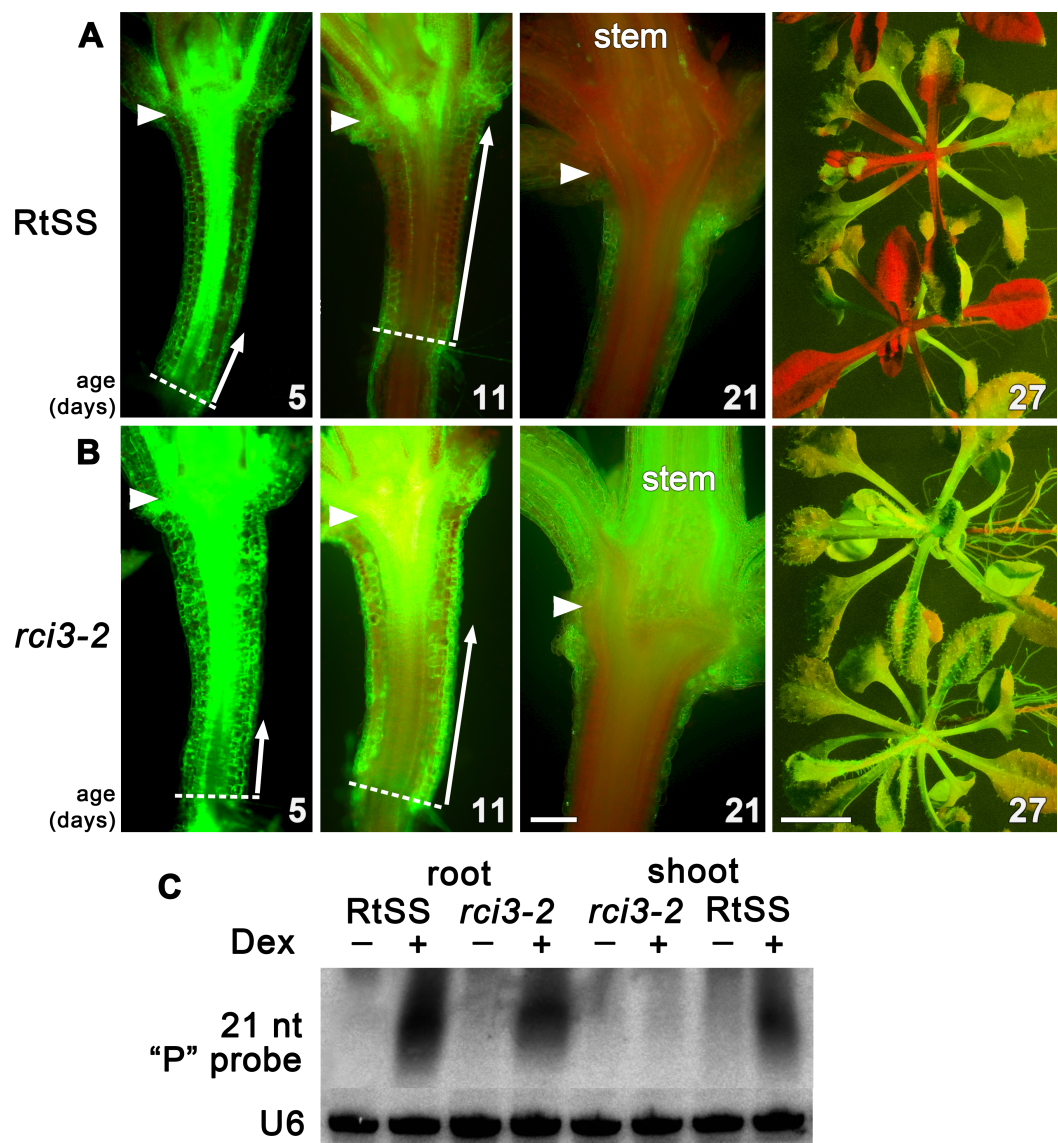


Figure 1 Silencing progress in mutant and wild-type RtSS *Arabidopsis* lines. (A) Median longitudinal sections of 5-, 11- and 21-day-old and intact 27-day-old RtSS germinated and grown on Dex-containing medium. (B) Median longitudinal sections of 5-, 11-, and 21-day-old and intact 27-day-old *rci3-2* germinated and grown on Dex. Arrowheads, hypocotyl-epicotyl junction; dashed line, root-hypocotyl junction; long arrows indicate the extent of silencing; stem, floral bolt stem. Bar = 200 μ m for 5–21-day-old plants; 1 cm for 27-day-old plants. (C) Small RNA northern blot of root and shoot tissue from WT-RtSS and *rci3-2* plants showing hairpin-derived secondary small RNA targeting the “P” region of GFP mRNA in all Dex-induced tissues except *rci3-2* shoots. U6, loading control.

2012). The abundant P region-derived siRNAs in both wild-type RtSS and mutant roots, and their presence in RtSS but not in the mutant shoots, confirmed that the mutants could generate but not mobilise these silencing signals (Fig. 1B). Apart from the reduced spread of Dex-induced silencing, mutant plants were phenotypically almost indistinguishable from wild-type plants, and showed normal induction of GUS expression by Dex (Fig. S3).

RCI3 (Rare Cold Inducible 3), a type III peroxidase, is required for mobile RNAi

In order to identify the mutated gene responsible for this loss of silencing spread into aerial tissues, we backcrossed our homozygous mutant line with its wild-type RtSS parent, deep sequenced the parental lines, and deep sequenced a pool of 44 F2 plants expressing the mutant phenotype. We identified 1,551 single nucleotide polymorphisms (SNPs) between the parental wild-type RtSS and the mutant (Table S1, Fig. S1), and mapped the mutation to the top arm of chromosome 1 between nucleotide position 863625 and 17192682. Further Sanger sequencing analysis using 20 SNPs and 12 F3 families refined this to a zone between SNP markers 1g001 and 1g012 (Table S1, Fig. S1) and ultimately to one mutation, at 1530461, in the RCI3 gene (At1g05260), which encodes a type III peroxidase. The mutation causes an Arg¹⁴⁵ to Lys substitution in a motif of the protein that is highly conserved from bryophytes to eudicots (Attwood *et al.*, 1994) (Fig. 2A) and substitution of this amino acid within the invariant GRRDG sequence seemed highly likely to compromise the function of the enzyme (Welinder *et al.*, 2002). To confirm that this was the cause of the significantly slowed silencing spread, we re-introduced a functional copy of RCI3 into the mutant background. A 4.5 kb genomic fragment containing the wild-type promoter, coding region and terminator sequence was used and restored root-to-shoot mobile silencing in 14 out of 20 transformants (Fig. S4). This demonstrates that RCI3 is required for silencing mobility in *Arabidopsis*.

H₂O₂ is an endogenous signal that regulates mobile RNAi

RCI3 is involved in the production of reactive oxygen species (ROS) (Llorente *et al.*, 2002; Kim, Ciani & Schachtman, 2010), so we analyzed our mutant (now termed *rci3-2*) and *rci3-1* (Kim, Ciani & Schachtman, 2010), a T-DNA insertion mutant, with H₂O₂-detecting dyes (Dickinson, Huynh & Chang, 2010). This revealed that H₂O₂ is endogenously produced and readily detectable in the wild-type RtSS parental line, whereas it is barely detectable in *rci3-1* and almost undetectable in *rci3-2* plants (Fig. 2B and Fig. S5). Overexpression of RCI3 increases ROS production (Kim, Ciani & Schachtman, 2010); therefore, we wondered whether treatment with H₂O₂ could restore silencing spread in *rci3-2* plants. Although slower to reach the shoots than in wild-type RtSS plants, 42 of 46 Dex-induced *rci3-2* plants treated with 1.5 mM H₂O₂ displayed shoot silencing by 36 dpi (Figs. 3A, 3B and 3E). Furthermore, H₂O₂ treatment accelerated the rate of silencing spread in wild-type RtSS plants (Figs. 3A, 3B and 3E). A logistic regression analysis of the data in Fig. 3E showed that increasing H₂O₂ concentration was significantly associated with shoot silencing (odds ratio = 1.78, 95% confidence interval = 1.51–2.10; *p* < 0.001), although the highest concentrations were sub-optimal. Peroxide treatment also increased the distance of spread from a vascularly-expressed silencing signal targeting phytoene desaturase (*AtSuc2:PDS*) (Smith *et al.*, 2007), which results in leaf bleaching (Figs. 3C and 3D). Indeed, all 66 Dex-induced RtSS plants growing on H₂O₂-containing medium showed accelerated silencing into the shoots 13 days later (Fig. 3F). However, if catalase was added to this medium to eliminate H₂O₂, none of 74 plants displayed any shoot

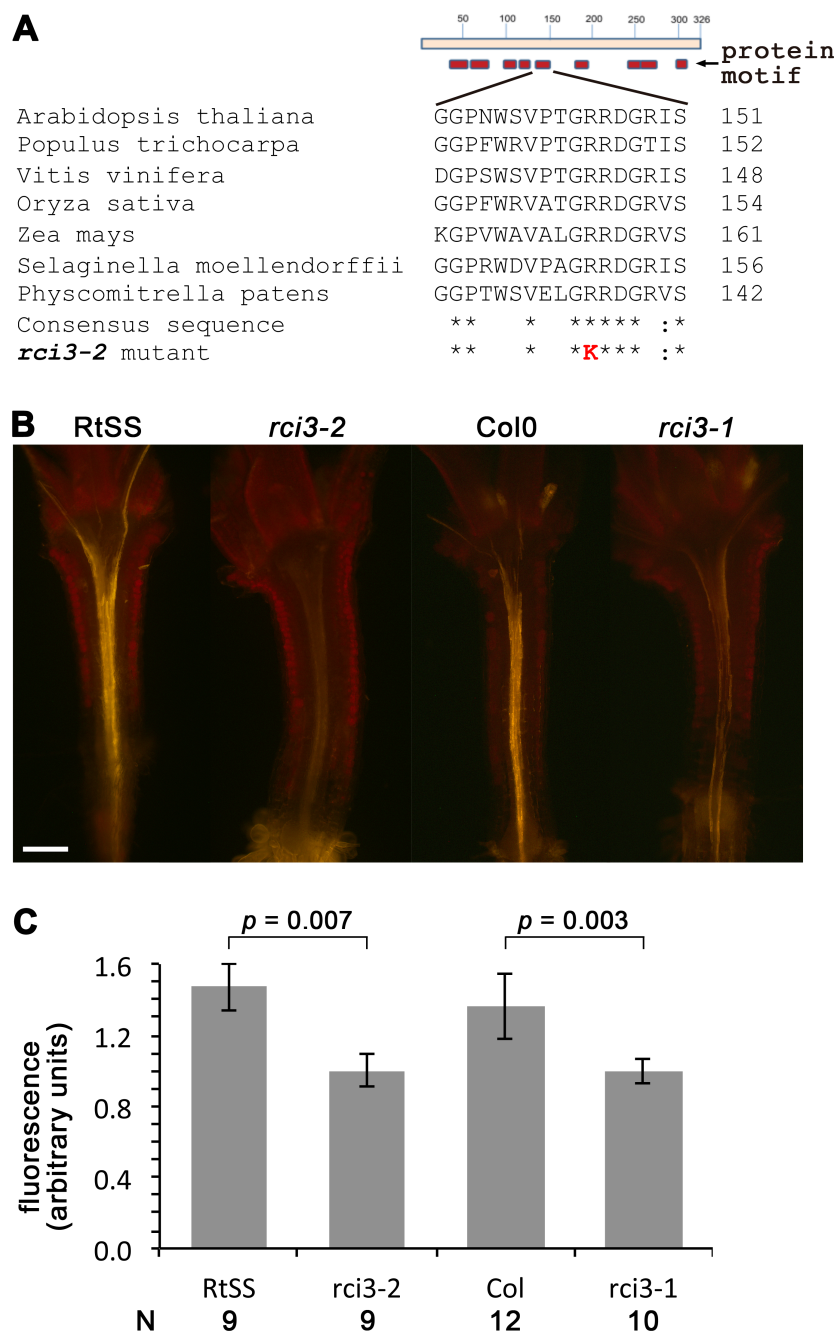


Figure 2 Mutation in RCI3/AtPrx03 reduces peroxide in *rci3-2*. (A) Protein structure and conserved motifs. Red boxes indicate the conserved motifs in plant type III peroxidases. *rci3-2* has an R-to-K mutation in the GRRDG sequence within the fifth motif (number of conserved terminal serine residue in this motif indicated at right), which is conserved in all land plants. Stars indicate conserved amino acid residues. (B) Longitudinal median sections of wild-type 12-day-old RtSS and Col show strong orange fluorescence after staining with H₂O₂-specific indicator PO1, whereas *rci3-2* and *rci3-1* show weak fluorescence. Bar = 200 μ M. (C) PO1 fluorescence was quantified; to account for any differences in background fluorescence from GFP, RtSS was normalised with respect to *rci3-2*, and Col0 was normalised with respect to *rci3-1*. Bars = 95% confidence intervals; P value from two-tailed unpaired Student's *t* test. N, number of plants.

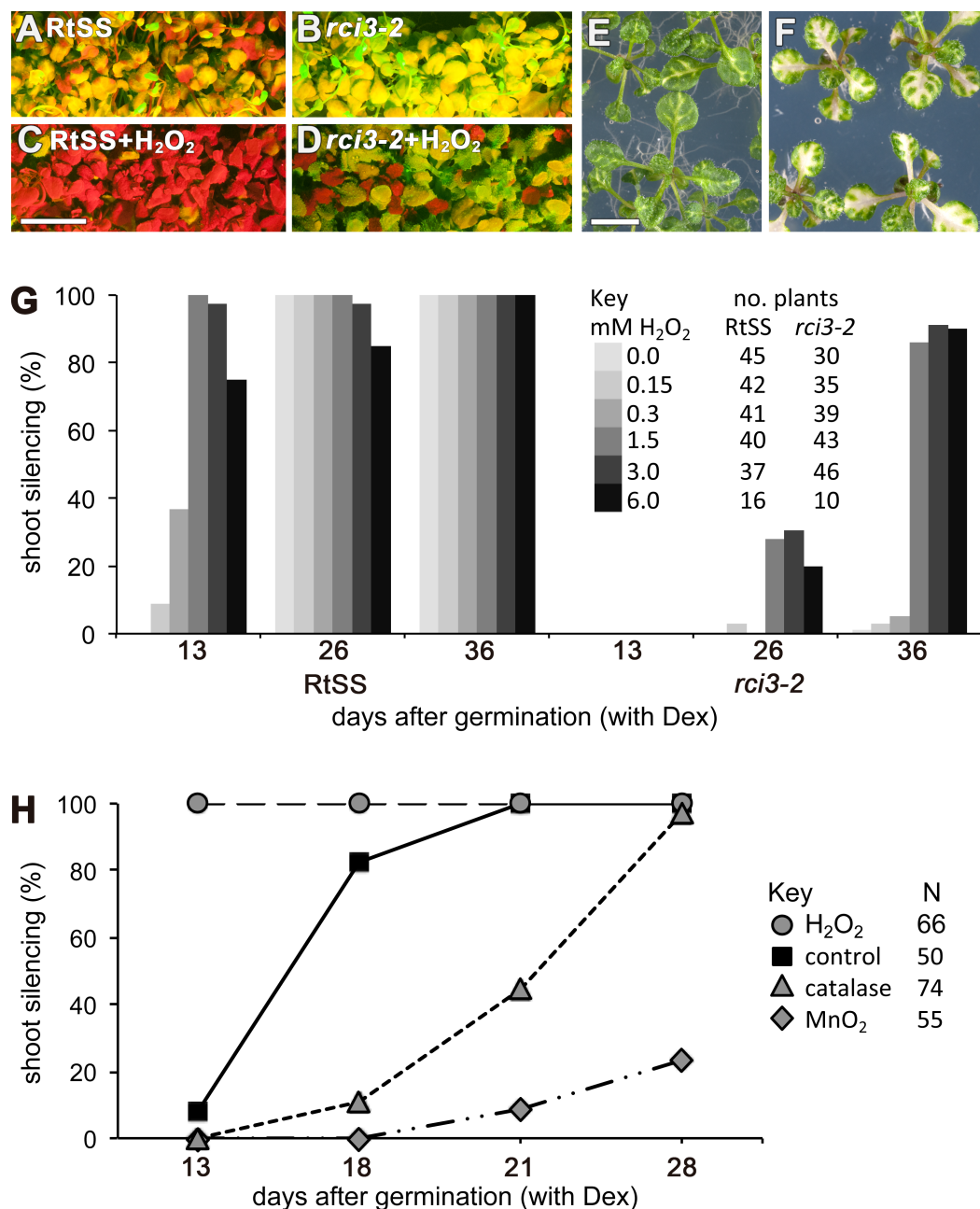


Figure 3 Effects of H_2O_2 or its breakdown catalysts on silencing in *RtSS* or *rci3-2*. (A) *RtSS* and (B) *rci3-2* plants grown for 26 d on Dex medium only. (C) *RtSS* and (D) *rci3-2* plants grown on medium containing H_2O_2 for 26 d after Dex-induction of silencing. (E) *AtSuc2:PDS* plants on MS medium. (F) *AtSuc2:PDS* plants grown on 1.5 mM H_2O_2 showed enhanced spread of silencing. (G) Effect of different concentrations of H_2O_2 on the percentage of shoots showing silencing in Dex-induced *RtSS* and *rci3-2* plants. (H) H_2O_2 accelerated, and blocking H_2O_2 with catalase or MnO_2 slowed, the percentage of shoots showing silencing in Dex-induced *RtSS* plants. N, number of plants.

silencing after the same time period (Fig. 3F). Furthermore, medium containing catalase also delayed silencing spread in Dex-induced wild-type-RtSS (Fig. 3F and Fig. S6). In this case, a logistic regression analysis of the data in Fig. 3F showed that the presence of catalase was significantly associated with reduced shoot silencing (odds ratio = 0.01, 95% confidence interval = 0.005–0.029; $p < 0.001$).

We interpreted this to be a consequence of the catalase accelerating the breakdown of endogenous pools of H_2O_2 in the plants. Applying a different H_2O_2 -breakdown catalyst (MnO_2) also caused slower silencing spread in both Dex-induced wild-type-RtSS (Fig. 3F; logistic regression; odds ratio <0.001 ; $p < 0.001$) and in plants with vascular-pattern PDS silencing, both catalase and MnO_2 reduced the distance of silencing spread from veins (Fig. S6). Collectively, the results demonstrate that H_2O_2 is an endogenous signal that regulates the rate of silencing mobility in plants.

Increased PD permeability requires RCI3

Previous work has shown that H_2O_2 enhances PD permeability to a symplastic dye (Rutschow, Baskin & Kramer, 2011). Therefore, the restoration of silencing in *rci3-2* by H_2O_2 treatment might be a result of enlarged PD. To test this idea, we applied *n*-ethyl maleimide (NEM), which can increase transport via PD (White & Barton, 2011; Liang, White & Waterhouse, 2012). As previously reported (Liang, White & Waterhouse, 2012), 100 μ M NEM enhanced the rate of spread of gene silencing in RtSS plants (Fig. S7), but did not restore silencing spread into the shoots of *rci3-2* plants (Fig. S7). This suggests that the rescue of silencing spread in *rci3-2* plants by H_2O_2 treatment is due to a specific effect of H_2O_2 and that NEM cannot cause PD enlargement without the enzymatic activity of RCI3.

DISCUSSION

We have demonstrated that H_2O_2 regulates the mobility of small RNA-mediated gene silencing, and extensive studies have shown that H_2O_2 and/or peroxidase-generated reactive oxygen species (ROS) can cause cell wall loosening (Fry, 1998; Schweikert, Liskay & Schopfer, 2000; Schopfer, 2001; Liskay, van der Zalm & Schopfer, 2004; Passardi, Penel & Dunand, 2004; Muller et al., 2009; Kunieda et al., 2013). We postulate that localised increase in H_2O_2 concentration relaxes the cell wall structure, enabling cell wall-embedded PD remodelling and opening and thus changing their transport capacity (Fig. 4). Evidence of localising peroxidases at the plasma membrane and PD in tomato cambial cell walls (Ehlers & van Bel, 2010) supports this suggestion, and mutants showing altered PD transport, such as *ise1* (Stonebloom et al., 2009), *ise2* (Stonebloom et al., 2012) and *gat1* (Benitez-Alfonso et al., 2009) are also associated with changes in ROS production. Moreover, since grafted rootstocks and scions show bursts of H_2O_2 , peroxidase and catalase activity a few days after grafting (Fernandez-Garcia, Carvajal & Olmos, 2004), their effects on PD may contribute to the variation in success rates of transmitting silencing signals across graft junctions (Voinnet et al., 1998; Crete et al., 2001; Liang, White & Waterhouse, 2012).

Peroxide signalling plays a significant role in many cell processes, and may influence the expression of certain miRNAs (Li et al., 2011). One possibility is that peroxide levels regulate components of the silencing pathway. However, in *Arabidopsis*, a whole-genome

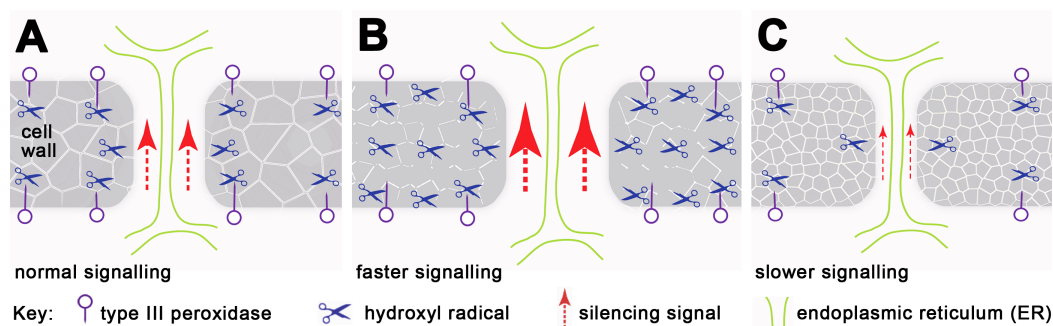


Figure 4 Working model for regulation of silencing spread by peroxidases around PD. Each panel represents a single PD traversing the cell wall. (A) Cell wall-localised Type III peroxidases maintain H_2O_2 through hydroxylic and peroxidative cycles (reviewed in [Passardi, Penel & Dunand, 2004](#)), in which H_2O_2 modifies cell wall components via cross-linking and depolymerization, thus maintaining a functional cell wall network and PD size. (B) With increased H_2O_2 , the cell wall undergoes depolymerization and loosening, allowing PD to enlarge, with increased transport capability through the cytoplasmic sleeve (data based on this work and [Benitez-Alfonso et al., 2009](#); [Stonebloom et al., 2009](#); [Stonebloom et al., 2012](#)). (C) Reduced H_2O_2 or mutation in peroxidase-dependent cross-linking or depolymerization causes cell wall stiffening, and presumably also reduces PD apertures (this work and [Fry, 1998](#); [Schweikert, Liskay & Schopfer, 2000](#); [Schopfer, 2001](#); [Liskay, van der Zalm & Schopfer, 2004](#); [Passardi, Penel & Dunand, 2004](#); [Muller et al., 2009](#)).

microarray screen of responses to exogenous H_2O_2 found no change in transcriptome level of *RDR1*, *RDR6*, *DCL4* or *AGO1* ([Cheng, Zhang & Guo, 2013](#)), which are key genes required for post-transcriptional gene silencing. Therefore, we conclude that the reduced silencing spread we observed in the *rci3-2* mutant was due to effects on cell-to-cell transport of the silencing signal, rather than to any effects on the silencing machinery itself.

One puzzle in this explanation is that *RCI3* is found predominantly in roots ([Llorente et al., 2002](#)), with some expression seen in hypocotyl and leaf tissue only after cold treatment. Yet we observed reduced peroxide levels ([Figs. 2B, 2C](#) and [Fig. S5](#)) and slower silencing spread through the hypocotyl in the absence of cold treatment. The latter did not appear to be a consequence of slower initiation of the signal in roots, since the length of silenced hypocotyl was similar up to 5 days after induction of the signal by Dex ([Fig. 1A](#)), and the roots appeared to become silenced at similar rates in both wildtype *RtSS* and mutant *rci3-2* plants ([Figs. S2B–S2D](#)). If there were any effect of lower peroxide levels on RNAi signal induction and amplification, it was too subtle to be detected in our experiments.

A major role of RNAi is to defend against virus infection ([Waterhouse, Wang & Lough, 2001](#)), and the ability of silencing signals to move and spread through the plant is a key component of this defence ([Dunoyer & Voinnet, 2005](#); [Molnar, Melnyk & Baulcombe, 2011](#)). As a counter-strategy, viruses encode silencing suppressor proteins (SSPs) that neutralise the degradative and/or signal amplification components of the mechanism ([Ding & Voinnet, 2007](#)). Recent studies have shown that the SSPs of some viruses can physically interact with H_2O_2 scavenging enzymes; for example, the 2b SSP of CMV ([Inaba et al., 2011](#)) and the p26 SSP of PepMV ([Mathioudakis et al., 2013](#)) both interact with a catalase, which enhances virus accumulation. We showed here that exogenous H_2O_2 also increased the rate of silencing spread, and that application of either catalase, or the H_2O_2

breakdown catalyst MnO_2 , greatly reduced silencing spread. Therefore, we suggest that the extracellular type III peroxidase, RCI3, as a regulator of peroxide levels and silencing spread, may be involved in defence responses to virus infection. However, direct evidence for its role in the plant defence network is currently lacking.

Regulation of symplastic cell-to-cell transport by type III peroxidases also has a broader evolutionary relevance, since these cell wall enzymes are not found in aquatic algae but exist in all land plants (Passardi, Penel & Dunand, 2004; Mathe et al., 2010). The ability to modify transport via PD provides a means of adjusting both short- and long-distance information flow through complex multicellular tissues; this ability appears to have been hijacked by plant viruses, and, in turn, can be modified by the plant in viral defense. The coincidence of type III peroxidases appearing only in the terrestrial plant line suggests to us that it is a key evolutionary adaptation to life on land.

CONCLUSIONS

Increasing evidence demonstrates that redox states are critical for the regulation of PD-mediated transport. Our findings have now unambiguously established a direct genetic link between a hydrogen peroxide-producing type III peroxidase and the regulation of small RNA-mediated silencing mobility. Manipulation of hydrogen peroxide levels in vivo and in vitro altered silencing movement in two independent systems: our root-to-shoot systemic silencing system; RtSS, and the cell-to-cell short distance silencing system; and AtSUC2-PDS. These results strongly support the conclusion that hydrogen peroxide plays a role in the control of silencing signal movement. Considering the role of ROS in regulating the movement of other signals, we can reasonably envisage that this mechanism may be more widespread than previously thought, extending beyond the role of H_2O_2 in stress signalling. Future experiments should examine the idea that ROS production provoked by different sorts of biotic or abiotic stimuli might be beneficial to cell survival, given its role in ramping up the movement of associated endogenous biological signals.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Commonwealth Scientific and Industrial Research Organization.

Competing Interests

At the time this research was carried out and written up, both Dacheng Liang and Rosemary G. White were employees of CSIRO Plant Industry.

Author Contributions

- Dacheng Liang and Rosemary G. White conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Peter M. Waterhouse conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The mutant sequence was mapped to reference the TAIR Columbia genome. Details were provided in [File S1](#).

Data Deposition

The following information was supplied regarding the deposition of related data:

Sequence data have been deposited in the Sequence Read Archive, Project ID PRJNA269088; <http://www.ncbi.nlm.nih.gov/sra/?term=PRJNA269088>.

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.701#supplemental-information>.

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