Endogenous siRNAs Derived from a Pair of Natural *cis*-Antisense Transcripts Regulate Salt Tolerance in *Arabidopsis*

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SUMMARY

In higher eukaryotes, miRNAs and siRNAs guide translational inhibition, mRNA cleavage, or chromatin regulation. We found that the antisense overlapping gene pair of Δ^1-pyrroline-5-carboxylate dehydrogenase (*P5CDH*), a stress-related gene, and *SRO5*, a gene of unknown function, generates two types of siRNAs. When both transcripts are present, a 24-nt siRNA is formed by a biogenesis pathway dependent on DCL2, RDR6, SGS3, and NRPD1A. Initial cleavage of the *P5CDH* transcript guided by the 24-nt siRNA establishes a phase for the subsequent generation of 21-nt siRNAs by DCL1 and further cleavage of *P5CDH* transcripts. The expression of *SRO5* is induced by salt, and this induction is required to initiate siRNA formation. Our data suggest that the P5CDH and SRO5 proteins are also functionally related, and that the *P5CDH-SRO5* gene pair defines a mode of siRNA function and biogenesis that may be applied to other natural *cis*-antisense gene pairs in eukaryotic genomes.

INTRODUCTION

Despite the preponderance of large intergenic spaces, eukaryotic genomes contain many overlapping genes. For example, 4%–9% of human genes, up to 22% of *Drosophila melanogaster* genes, and approximately 10% of *Arabidopsis thaliana* genes are in convergent overlapping gene pairs, also known as natural *cis*-antisense gene pairs (Boi et al., 2004; Wang et al., 2005; Jen et al., 2005). Although their functional significance is unclear, one intriguing possibility is that overlapping transcripts in an antisense orientation form double-stranded RNAs that may be processed into small RNAs.

Small 21- to 25-nt, noncoding RNAs are important regulators of gene expression in both plants and animals (Carrington and Ambros, 2003; Bartel, 2004; He and Hannon, 2004). These small RNAs can be divided into two classes: microRNAs (miRNAs) and short interfering RNAs (siRNAs). miRNAs are found in a number of multicellular eukaryotes (Ambros, 2003; Carrington and Ambros, 2003; Bartel, 2004) and are generated from longer hairpin precursors by the Ribonuclease III-like enzyme Dicer. After incorporation into an Argonaute-containing *R*NA *I*nduced *S*ilencing *C*omplex (RISC) (Hannon, 2002; Meister and Tuschl, 2004), base pairing between miRNAs and complementary target mRNAs guides sequence-specific translational inhibition or transcript cleavage (Bartel, 2004). miRNAs have a well-documented role in allowing developmental regulation of multigene families (Palatnik et al., 2003; Carrington and Ambros, 2003; Bartel, 2004).

siRNAs differ from miRNAs in that they are generated from long double-stranded RNAs. siRNAs were first identified in plants undergoing posttranscriptional gene silencing (PTGS; Hamilton and Baulcombe, 1999), and subsequently diverse sets of endogenous siRNAs have been found in plants and animals (Llave et al., 2002; Sunkar and Zhu, 2004; Ambros, 2003; Aravin et al., 2003; Tang et al., 2003; Reinhart et al., 2002). The biogenesis of small RNAs in plants is especially complex. In *Arabidopsis,* there are four Dicer-like (DCL) proteins (Schauer et al., 2002), six predicted RDRs (Mourrain et al., 2000), and ten predicted Argonautes (Morel et al., 2000). DCL1 is required for the production of ~21-nt miRNAs and *trans*-acting siRNAs (tasiRNAs) (Park et al., 2002; Peragine et al., 2004; Vazquez et al., 2004b). Allen et al. (2005) demonstrated that an initial DCL1-dependent, miRNA-guided cleavage of tasiRNA primary transcripts sets the 21-nt phase for accurate tasiRNA formation. DCL4 is responsible for the processing of 21-nt tasiRNAs (Gasciolli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005). Generation of tasiRNAs also involves RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and SUPPRESSOR OF GENE SILENCING 3 (SGS3) (Peragine et al., 2004, Vazquez et al., 2004b).

DCL2 is involved in the production of some viral siRNAs (Xie et al., 2004) and may substitute for DCL3 or DCL4 when they are absent (Gasciolli et al., 2005). DCL3 and RDR2, as well as the RNA polymerase IV encoded by *NRPD1A/SILENCING DEFECTIVE 4,* cooperate in generating heterochromatin-associated 24-nt siRNAs from various

retroelements and transposons, 5S ribosomal RNA genes, endogenous direct and inverted repeats, and transgenes containing direct repeats (Chan et al., 2004; Xie et al., 2004; Zilberman et al., 2004; Herr et al., 2005; Onodera et al., 2005). The appearance of these siRNAs has been correlated with DNA and histone methylation at the homologous chromatin (Hamilton et al., 2002; Volpe et al., 2002; Zilberman et al. 2004; Xie et al., 2004).

Proline metabolism and particularly Δ^1-pyroline-5-carboxylate (P5C), an intermediate in proline synthesis and catabolism, have a key role in stress response and accumulation of reactive oxygen species (ROS). P5C can also promote apoptosis, and this may be mediated, at least partly, through ROS accumulation. In yeast, disruption of $PUT2$, which encodes Δ^1-pyroline-5-carboxylate dehydrogenase (P5CDH), an enzyme that catabolizes P5C, leads to decreased cell growth and ROS accumulation (Nomura and Takagi, 2004; Deuschle et al., 2001). In plants as well, exogenously applied P5C increases ROS production, reduces growth, and induces a number of stress-responsive genes (Deuschle et al., 2004; Hellmann et al., 2000; Iyer and Caplan, 1998). Inactivation of P5CDH causes hypersensitivity to proline and P5C (Deuschle et al., 2004). Also, P5CDH is induced at pathogen infection sites where programmed cell death and ROS accumulation occur (Ayliffe et al., 2002). These lines of evidence, along with the long-studied role of proline accumulation in plant and microbial stress tolerance (Zhu, 2002) indicate that proline and P5C metabolism are likely to be highly regulated processes impacting ROS accumulation, stress response, and cell death across a range of eukaryotes.

We report here that $Arabidopsis\ P5CDH$ and $SRO5,$ an overlapping gene of unknown function in the antisense orientation, generate both 24-nt and 21-nt siRNAs. We have termed these siRNAs nat-siRNAs because they are derived from natural antisense transcripts. Upon induction of $SRO5$ by salt stress, a 24-nt $SRO5-P5CDH$ nat-siRNA is produced by a biogenesis pathway requiring DCL2, RDR6, SGS3, and NRPD1A. Cleavage of the $P5CDH$ transcript guided by the 24-nt nat-siRNA sets the phase for accurate production of further 21-nt $P5CDH$ nat-siRNAs by DCL1. The nat-siRNAs downregulate the expression of $P5CDH$ by causing mRNA cleavage. Downregulation of $P5CDH$ leads to proline accumulation that is important for salt tolerance but also causes increased ROS production, which is normally counteracted by the SRO5 protein. Thus, the $SRO5-P5CDH$ nat-siRNAs together with the P5CDH and SRO5 proteins are key components of a regulatory loop controlling ROS production and stress response. The nat-siRNA-mediated crossregulation of $P5CDH$ and $SRO5$ mRNAs and the functional relationship of these two proteins suggest a regulatory model that may be applied to other cis-antisense gene pairs.

RESULTS

Salt Stress Induces an Endogenous siRNA from a Pair of Antisense Genes

Previously, we cloned miRNAs and putative siRNAs from $Arabidopsis$ plants treated with various abiotic stresses (Sunkar and Zhu, 2004). One putative siRNA (clone # P96-F02) matched the overlapping region between the 3′ end of the $P5CDH$ ORF and the 3′UTR of an unknown gene on the opposite strand (At5g62520), which has recently been designated $SRO5$ (Figure 1A). These two genes generate convergent transcripts that overlap by 760-nt, and the cloned siRNA sequence matched a 21-nt region (1873–1893) of the $SRO5$ 3′UTR and was complementary to the corresponding region of the $P5CDH$ ORF.

Using an oligonucleotide probe complementary to the 21-nt siRNA, we could not detect any siRNAs in $Arabidopsis$ plants grown under normal conditions (Figure 1B); however, a 24-nt siRNA (referred to hereafter as 24-nt $SRO5-P5CDH$ nat-siRNA) was detected in NaCl-treated adult plants and seedlings. Occasionally, a very weak signal at 21-nt could also be seen in the salt-stressed plants (not shown); this signal likely corresponds to the cloned 21-nt siRNA. No 24-nt $SRO5-P5CDH$ nat-siRNA signal was found in plants exposed to several other stress or homone treatments (Figure 1B), demonstrating that the induction of 24-nt $SRO5-P5CDH$ nat-siRNA is highly specific to NaCl stress.

To define the sequence of the 24-nt $SRO5-P5CDH$ nat-siRNA, we hybridized the small RNA blots with oligonucleotide probe A (5′-GGG**GACCCGAGAGGGGCCGGGATA**-3′) and oligonucleotide probe B (5′-**GACCCGAGAGGGGCCG GGATA**GGG-3′) (the sequences in bold letters are complementary to the 21-nt cloned siRNA). Probe A but not B detected the 24-nt $SRO5-P5CDH$ nat-siRNA from salt-stressed plants (data not shown). This suggests that the 24-nt $SRO5-P5CDH$ nat-siRNA sequence is likely 5′-UAUCCCGGCCC CUCUCGGGUCCCC-3′ (Figure 1C). This sequence was confirmed by a modified small RNA cloning approach (Table S1).

Although siRNAs are generated in the double-stranded form, often only one of the strands is incorporated into RISC and can be detected (Schwarz et al., 2003; Khvorova et al., 2003). Twenty-four nucleotide $SRO5-P5CDH$ nat-siRNA was detected with an antisense (relative to $SRO5$) probe but not with a sense probe (Figure 1C), suggesting that the sense strand as shown in Figure 1A is stable and the other strand is rapidly degraded. Furthermore, examination of the 24-nt $SRO5-P5CDH$ nat-siRNA sequence indicates that the sense strand sequence conforms to the asymmetry rule for siRNA stability (Schwarz et al., 2003).

24-nt $SRO5-P5CDH$ nat-siRNA Is Produced by a Unique Biogenesis Pathway

To define the components required for the formation of 24-nt $SRO5-P5CDH$ nat-siRNA, it was examined in salt stressed plants of small RNA biogenesis mutants. The 24-nt $SRO5-P5CDH$ nat-siRNA was detected in salt-treated wild-type plants of all ecotypes tested such as Landsberg (Ler), Columbia, C24, and Nossen (Figure 2). However, it was not detected in the $hen1$ mutant. Instead, a larger-sized signal was found in $hen1$ plants (Figure 2A). The 24-nt $SRO5-P5CDH$ nat-siRNA was present in $dcl1$, $dcl3$, and $dcl4$ but not in $dcl2$. The $sgs3$, $nrpd1a$, and $rdr6$ mutations also blocked the accumulation of 24-nt $SRO5-P5CDH$ nat-siRNA (Figure 2B). In contrast, the $rdr2$ mutation did not have any
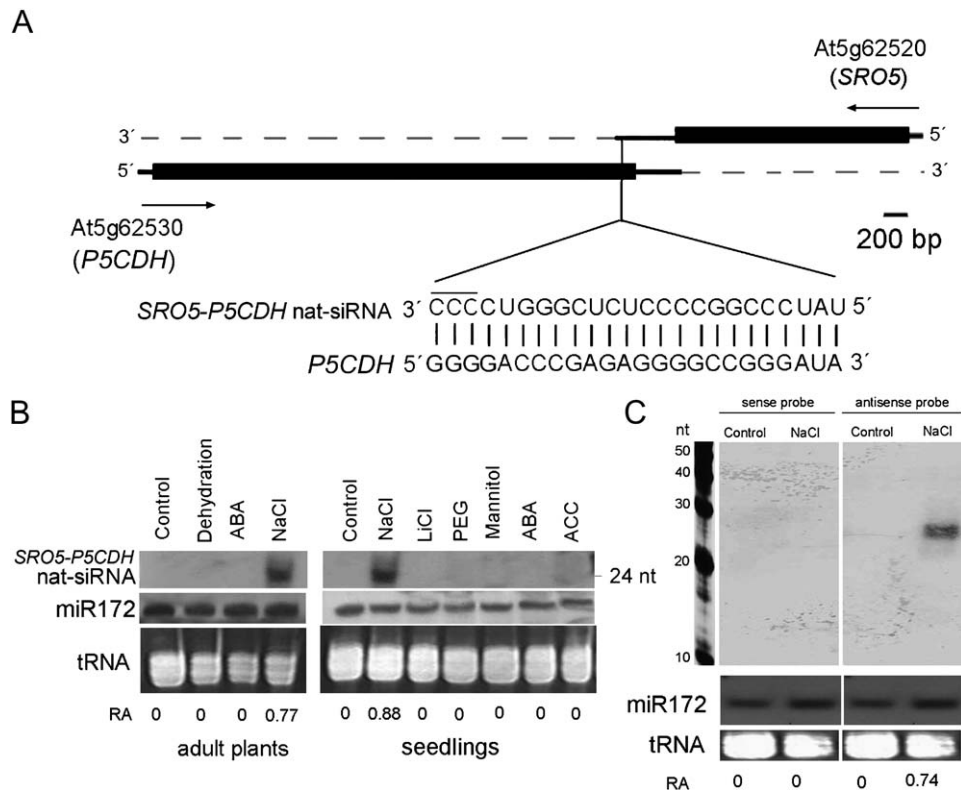
Figure 1. Salt Stress Induces a 24-nt nat-siRNA from the *SRO5-P5CDH cis*-Antisense Overlapping Genes

(A) Genomic structure of *SRO5* (At5g62520) and *P5CDH* (At5g62530) genes. Arrows indicate the direction of transcription. Thick and thin solid lines represent ORF and UTR regions, respectively. Sequence of the 24-nt *SRO5-P5CDH* nat-siRNA is aligned with P5CDH mRNA. The three nucleotides marked by a line on top were deduced from RNA blot analysis and confirmed by primer extension assays (Table S1).

(B) Accumulation of the *SRO5-P5CDH* nat-siRNA is specifically induced by salt stress. (Left panel) Twenty-day-old soil grown plants were exposed to the following stress or hormone treatments: dehydration (withholding watering for 10 days); ABA (spraying with 100 µM ABA with samples collected 24 hr later); and NaCl (watering with 300 mM NaCl for 2 days). (Right panel) Ten-day-old seedlings exposed to the following treatments in liquid media for 6 hr: Control (nutrient medium), 150 mM NaCl, 15 mM LiCl, 30% (w/v) PEG-8000, 250 mM Mannitol, 100 µM ABA, or 10 µM ACC.

(C) Northern blot of small RNA extracted from control or salt-treated (150 mM, 6 hr) seedlings. The sense and antisense probes are described in the text.

effect on 24-nt *SRO5-P5CDH* nat-siRNA, although it abolished the accumulation of siRNA 1003. The *HYL1* gene has been shown to be required for the accumulation of several miRNAs (Han et al., 2004; Vazquez et al., 2004a), including miR172, and the level of 24-nt *SRO5-P5CDH* nat-siRNA was reduced in the *hyl1* mutant plants (Figure 2B). These results suggest that accumulation of the 24-nt *SRO5-P5CDH* nat-siRNA is dependent on DCL2, RDR6, SGS3, and NRPD1A. This pathway is different from those producing heterochromatin-related siRNAs or tasiRNAs, suggesting that the *SRO5-P5CDH* nat-siRNA defines a new class of siRNAs.

Salt Stress Regulates *SRO5* and *P5CDH* Transcript Levels and *P5CDH* mRNA Cleavage

To understand the mechanism of salt stress induction of the *SRO5-P5CDH* nat-siRNA, we investigated whether the expression of *SRO5* or *P5CDH* is regulated by salt stress. *SRO5* was not expressed in plants grown under normal conditions, but its expression was induced by NaCl treatment

(Figures 2C and 2D). In contrast, *P5CDH* expression was reduced by NaCl treatment (Figures 2C and 2D). These results suggest that, upon salt treatment, the *SRO5* and P5CDH mRNAs can form a dsRNA that is then processed by DCL2 to generate the 24-nt *SRO5-P5CDH* nat-siRNA.

A possible role of the 24-nt *SRO5-P5CDH* nat-siRNA is to cause cleavage of *P5CDH* mRNA. In addition to detecting a full-length *P5CDH* transcript, a *P5CDH* 3′UTR probe also hybridized with a smaller band corresponding to the size of the putative 3′ cleavage product. This putative 3′ cleavage product accumulated only in NaCl-treated plants (Figures 2C and 2D). Importantly, in *dcl2*, *sgs3*, *nrpd1a*, and *rdr6* mutants where the 24-nt *SRO5-P5CDH* nat-siRNA was not produced, there was less or no decrease in *P5CDH* full-length transcript level, and the 3′ cleavage product was not detected (Figures 2C and 2D). Interestingly, in *hen1* plants, salt stress still reduced the level of full-length *P5CDH* transcript and caused accumulation of the 3′ cleavage product. This suggests that the larger-sized siRNA in *hen1* mutant plants was still functional in causing *P5CDH* mRNA cleavage. Taken together, these
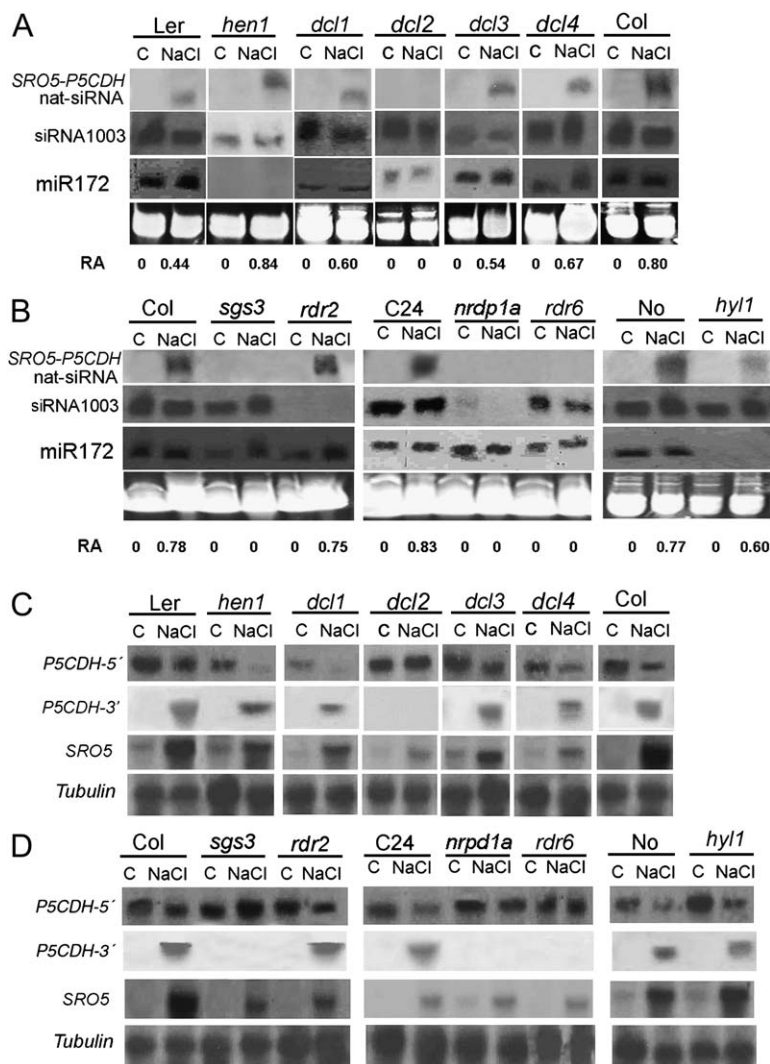
results suggest that salt stress triggers the expression of *SRO5*, leading to dsRNA formation and consequently generation of 24-nt *SRO5-P5CDH* nat-siRNA, which then downregulates *P5CDH* transcript levels through mRNA cleavage.
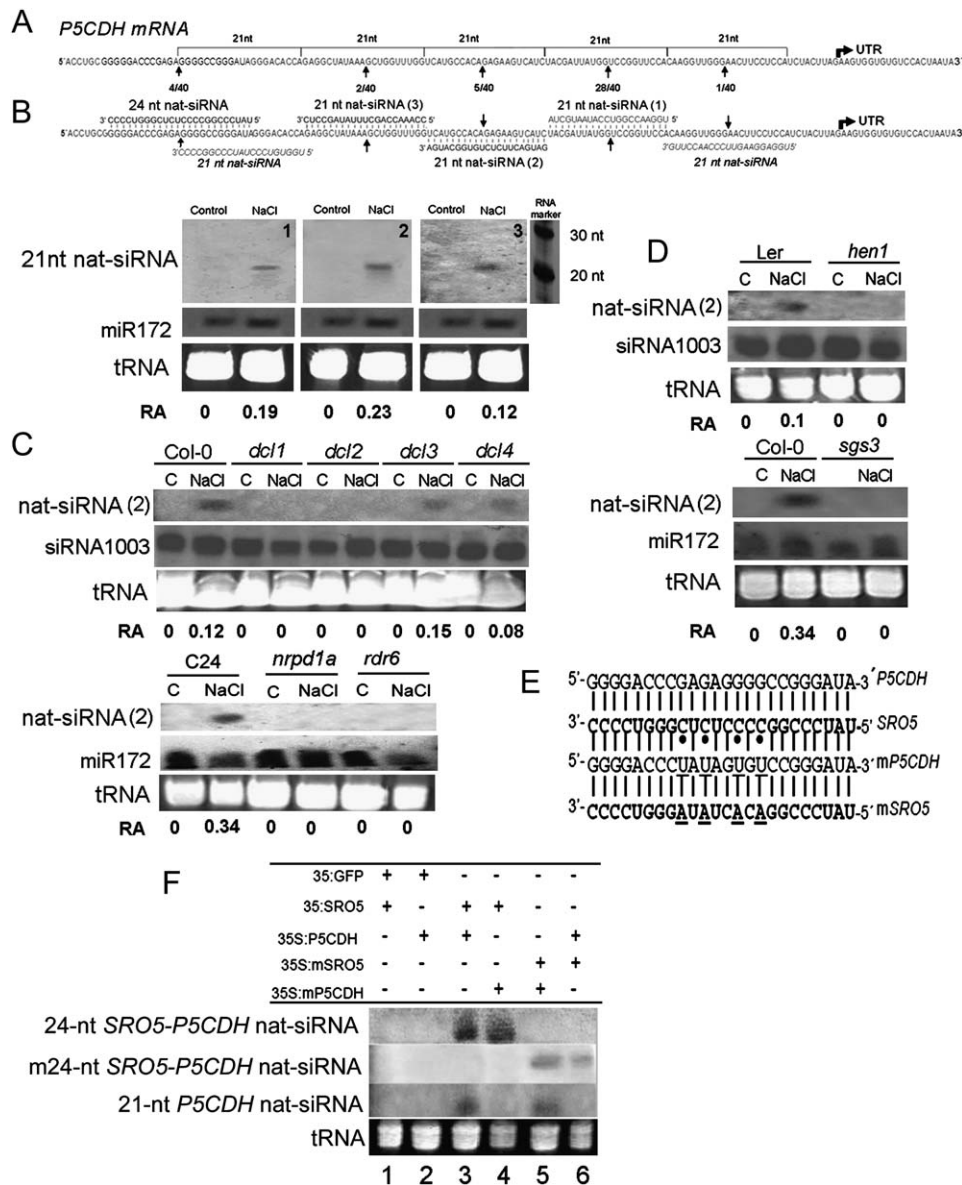
Cleavage Site Mapping of *P5CDH* mRNA Reveals Phased Processing of 21-nt *SRO5-P5CDH* nat-siRNAs

To verify that the 24-nt *SRO5-P5CDH* nat-siRNA indeed targets *P5CDH* mRNA for endonucleolytic cleavage, we carried out 5′ RACE assays using mRNA from salt-treated plants to map potential cleavage sites. Among several cleavage sites found in the *P5CDH* mRNA in wild-type plants, one site is between nucleotides 12 and 13 of the 24-nt *SRO5-P5CDH* nat-siRNA (Figure 3). This differs from other ARGONAUTE-catalyzed RNA cleavage sites which occur between nucleotides 10 and 11 (Allen et al., 2004). No cleavage products were found when the assay was performed with mRNA isolated from salt stress treated tissue of *rdr6* or *sgs3* plants or with mRNA from unstressed wild-type plants (data not shown).

The results support our hypothesis that the 24-nt *SRO5-P5CDH* nat-siRNA can direct *P5CDH* mRNA cleavage.

Surprisingly, the 5′ RACE assay also revealed four additional cleavage sites in the *P5CDH* mRNA (Figure 3A). One of these was the most frequent cleavage site (28 out of 40). These additional cleavage sites are 21-nt apart and in-phase with each other. We hypothesized that cleavage guided by the 24-nt *SRO5-P5CDH* nat-siRNA may set the phase for processing of *P5CDH* transcript by RDR into dsRNA and then dicing by a different DCL, which generates new 21-nt *P5CDH* nat-siRNAs. We designed three oligonucleotide probes in an attempt to detect these predicted 21-nt *P5CDH* nat-siRNAs (1, 2, and 3; Figure 3B). Each of these probes detected a 21-nt *P5CDH* nat-siRNA in salt-stressed plants. Analysis of one of these siRNAs (probe 2) in the same mutants analyzed above showed that generation of 21-nt *P5CDH* nat-siRNAs was dependent on DCL1, DCL2, HEN1, RDR6, SGS3, and NRPD1A (Figures 3C and 3D).

We also looked for, but failed to find, siRNAs deriving from sequences upstream of and in-phase with the 24-nt

Figure 3. Cleavage Site Mapping of *P5CDH* mRNA and the Detection of 21-nt nat-siRNAs

(A) *P5CDH* mRNA cleavage sites detected by RLM-5′ RACE. Numbers indicate the frequency of cleavage at each site. The positions of predicted 21-nt nat-siRNAs are indicated.

(B) Diagram showing the sequences of 24-nt and 21-nt nat-siRNAs aligned with the *P5CDH* mRNA (upper panel) and detection of the 21-nt nat-siRNAs (lower panel). Sequences in italics are 21-nt nat-siRNA predicted but not confirmed by Northern blots. One hundred micrograms of low-molecular-weight RNA per lane was probed with oligo probes, 1, 2, and 3, complementary to 21-nt nat-siRNAs numbered. The number below the blot indicates the relative abundance (RA) of the nat-siRNA with respect to the loading control miR172.

(C and D) Accumulation of 21-nt nat-siRNA number 2 in different mutants. The number below the blot indicates relative abundance (RA) of the nat-siRNA with respect to the loading control miR172 or siRNA 1003.

(E) Alignment of the 24-nt nat-siRNA region in wild-type and mutated (m*SRO5* and m*P5CDH*) versions of *SRO5* and *P5CDH* used for transient expression in *N. benthamiana*. Two mutations were introduced on either side of the site of 24-nt nat-siRNA-directed *P5CDH* cleavage.

(F) Coexpression of various combinations of wild-type and mutant *SRO5* and *P5CDH* constructs in *N. benthamiana*. As a control, SRO5 and P5CDH were also coexpressed with an unrelated GFP transcript. Small RNA blots were prepared from leaves collected 3 days after infiltration and probed with oligoes complementary to wild-type 24-nt *SRO5-P5CDH* nat-siRNA, the mutated 24-nt *SRO5-P5CDH* nat-siRNA sequence (m24-nt nat-siRNA), and a mixture of three 21-nt *P5CDH* nat-siRNAs (probes 1, 2, and 3; Figure 3B).

SRO5-P5CDH nat-siRNA-directed cleavage site by using a mix of 21-nt oligonucleotide probes complementary to the upstream sequences. Additionally, we hybridized the small RNA blots with 24-nt oligonucleotide probes designed to detect other potential 24-nt siRNAs produced in-phase with (either downstream or upstream of) the 24-nt SRO5-P5CDH nat-siRNA (probe sequences used are described in Table S2). These probes failed to detect any siRNA signal (not shown), suggesting that only one 24-nt SRO5-P5CDH nat-siRNA accumulates. Since these 24-nt probes are completely out of phase with the 21-nt P5CDH nat-siRNAs, the result also suggests a lack of siRNAs not in-phase with the 24-nt SRO5-P5CDH nat-siRNA-directed cleavage site, although the experiment cannot rule out the existence of other out-of-phase siRNAs.

The combined data suggest that the 21-nt P5CDH nat-siRNAs were generated by DCL1, and their production was dependent on production of the 24-nt SRO5-P5CDH nat-siRNA by DCL2. To directly test whether formation of the 24-nt SRO5-P5CDH nat-siRNA is required for the formation of 21-nt nat-siRNAs, we transiently expressed combinations of wild-type and mutated P5CDH and SRO5 transcripts (mP5CDH and mSRO5) in Nicotiana benthamiana leaves. mP5CDH and mSRO5 were mutated to introduce four mismatches with the complementary wild-type sequence in the region corresponding to the 24-nt SRO5-P5CDH nat-siRNA (Figure 3E). However, mP5CDH and mSRO5 were perfectly complementary to each other at the 24-nt SRO5-P5CDH nat-siRNA site. Small RNA blots from tissue coexpressing these transcripts were probed with oligonucleotides complimentary to the wild-type and m24-nt SRO5-P5CDH nat-siRNA and with a mixture of 21-nt P5CDH nat-siRNAs (probes 1, 2, and 3; Figure 3B).

The results of this experiment support the hypothesis that 24-nt SRO5-P5CDH nat-siRNA is produced first and required for the formation of 21-nt P5CDH nat-siRNAs. When wild-type P5CDH and SRO5 transcripts were coexpressed in N. benthamiana leaves, both 24-nt and 21-nt SRO5-P5CDH nat-siRNAs were produced (Figure 3F, lane 3). Expression of either gene in combination with the unrelated GFP transcript did not produce any nat-siRNAs (Figure 3F, lanes 1 and 2), demonstrating that both the P5CDH and SRO5 transcripts must be present to generate nat-siRNAs. When wild-type SRO5 was coexpressed with mP5CDH, wild-type 24-nt SRO5-P5CDH nat-siRNA was still produced from the SRO5 transcript but no 21-nt P5CDH nat-siRNAs could be detected (Figure 3F, lane 4). This demonstrated that the initial 24-nt SRO5-P5CDH nat-siRNA could be produced from the long, double-stranded SRO5-P5CDH transcripts despite the mismatches. However, this wild-type 24-nt SRO5-P5CDH nat-siRNA was presumably unable to guide cleavage of mP5CDH due to the mismatches and thus no 21-nt nat-siRNAs could be formed. A similar result was observed from coexpression of P5CDH and mSRO5 (Figure 3F, lane 6). Coexpression of mP5CDH and mSRO5 led to the production of both m24-nt and 21-nt nat-siRNAs (Figure 3F, lane 5). Thus, while initial processing of the SRO5-P5CDH double-stranded RNA is tolerant of

mismatches, subsequent production of 21-nt nat-siRNAs is dependent on perfect complementarity between the 24-nt SRO5-P5CDH nat-siRNA and the P5CDH transcript.

Role of SRO5-P5CDH nat-siRNAs in Proline Metabolism and Salt-Stress Tolerance

To determine the physiological role of the salt stress-induced SRO5-P5CDH nat-siRNAs, we compared the levels of salt stress-induced proline accumulation in various mutant plants. In dcl2, sgs3, rdr6, and nrpd1a, which lacked SRO5-P5CDH nat-siRNAs and cleavage of the P5CDH transcript, proline accumulation was not significantly induced by salt stress or was induced to a lesser extent than in the corresponding wild-type (Figure 4A). This result is consistent with their inability to downregulate P5CDH under stress, thereby causing continued proline catabolism and reduced proline accumulation. In contrast, the dcl1 and rdr2 mutants, which were able to degrade P5CDH mRNA, had wild-type levels of proline accumulation under salt stress (Figure 4A). The wild-type level of proline accumulation in dcl1 indicates that although the 21-nt P5CDH nat-siRNAs were not produced, the 24-nt SRO5-P5CDH nat-siRNA alone was sufficient to cause downregulation of P5CDH (Figure 3C). Whether or not the 21-nt P5CDH nat-siRNAs are required to downregulate P5CDH under other stress conditions will be of interest for future experiments.

We also examined the proline and salt-stress sensitivity of nrpd1a and sgs3, two mutants that do not have strong pleiotropic phenotypes and grow well under nonstressed conditions. Consistent with previous observations (Hellmann et al., 2000), high levels of exogenous proline inhibited seedling growth of the C24 and Col wild-types (Figures 4B and 4C). The nrpd1a and sgs3 mutants were clearly more tolerant of exogenous proline (Figures 4B and 4C). This proline tolerance may be caused by a reduced cleavage of P5CDH mRNA leading to greater P5CDH activity and reduced P5C levels in the proline-treated seedlings. Conversely, these mutants were less tolerant of salt stress, which is consistent with their inability to control proline catabolism and accumulate protective amounts of proline.

To further examine the biogenesis of SRO5-P5CDH nat-siRNAs and their role in proline and salt-stress tolerance, we identified two homozygous Arabidopsis mutant plants with a T-DNA insertion in the ORF of SRO5 or P5CDH (Figure 5A). Northern blot analysis showed that expression of P5CDH and SRO5 was abolished in the respective mutants (Figure 5B). Neither mutant could produce the 24-nt SRO5-P5CDH nat-siRNA (Figure 5C) or 21-nt P5CDH nat-siRNAs (data not shown) under salt stress. In the sro5 knockout mutant, P5CDH full-length transcript accumulated to a higher level and was not reduced by salt stress (Figure 5B). These results further demonstrate that production of SRO5-P5CDH nat-siRNAs is dependent on the presence of the P5CDH and SRO5 antisense transcripts, and that SRO5-P5CDH nat-siRNAs are responsible for the downregulation of the P5CDH mRNA. Interestingly, SRO5 mRNA was detected in the p5cdh knockout mutant even without salt stress (Figure 5B). This indicates that either the p5cdh
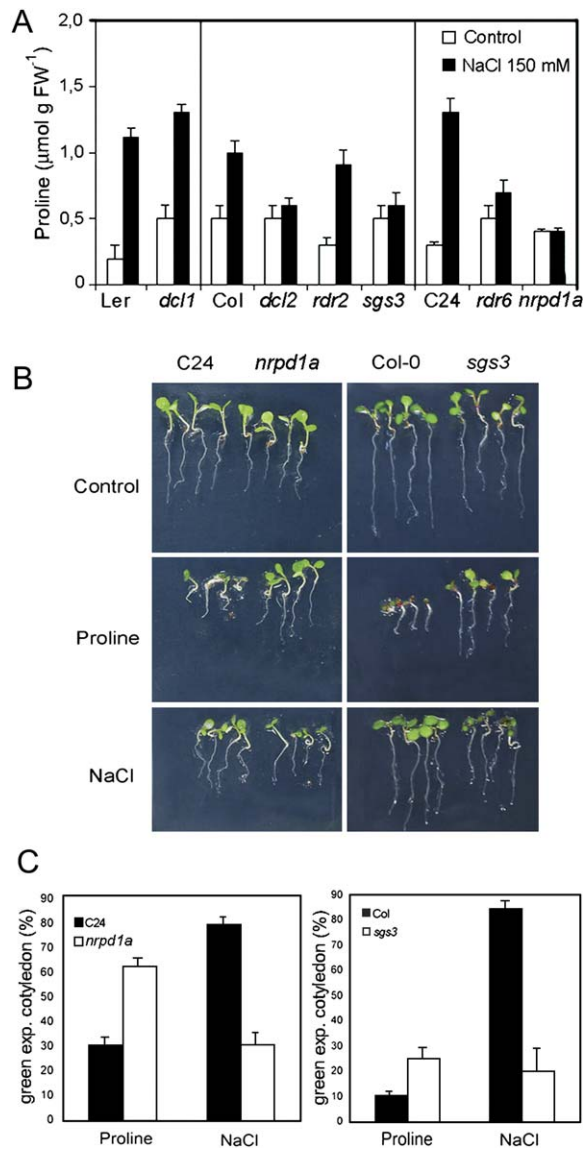
Figure 4. Proline Accumulation and Salt and Proline Sensitivity in Mutants Affected in *SRO5-P5CDH* nat-siRNA Accumulation

(A) Proline content in 10-day-old seedlings with or without 6 hr of exposure to 150 mM NaCl. Values represent means ± SD from three independent experiments.

(B) Response of *sgs3* and *nrpd1a* to NaCl and exogenous proline. Seedlings were germinated and grown for 7 days in 75 mM NaCl or 5 mM proline for Col-O and *sgs3*, and 75 mM NaCl or 2 mM proline for C24 and *nrpd1a*. Photos show representative seedlings.

(C) Tolerance to NaCl or exogenous proline quantified as the percentage of 7-day-old seedlings with green expanded cotyledons. Values represent means ± SD from three independent experiments.

knockout causes certain physiological stress (e.g., oxidative stress) that can induce *SRO5* expression, or that *SRO5* transcription or transcript stability is normally suppressed by the *P5CDH* gene through an unknown mechanism.

The *p5cdh* mutant accumulated more proline than wild-type under control conditions and slightly more under salt stress (Figure 5D). The mutant was slightly more tolerant to NaCl stress under our conditions, which is possibly related to its higher proline accumulation (Figures 5E and 5F). As reported previously (Deuschle et al., 2004), the *p5cdh* mutant was more sensitive than wild-type to exogenous proline (Figures 5E and 5F). In contrast, the *sro5* mutant accumulated less proline under salt stress (Figure 5D) and was more sensitive to NaCl stress (Figures 5E and 5G) and more tolerant of exogenous proline (Figures 5E and 5F). These phenotypes are all consistent with the increased expression of *P5CDH* in *sro5*.

Altered ROS Sensitivity and Accumulation in *sro5* Indicates a Functional Link between the SRO5 and P5CDH Proteins

Our results suggest that nat-siRNA regulation of *P5CDH* is dependent on the *SRO5* mRNA. However, *SRO5* is a coding gene, and we hypothesized that the SRO5 protein may also be functionally related to P5CDH. It has been shown that *P5CDH* downregulation leads to the accumulation of its substrate, P5C (Deuschle et al., 2004). P5C itself or glutamate semialdheyde, which is in spontaneous equilibrium with P5C, causes the accumulation of high levels of ROS (Deuschle et al., 2001; Nomura and Takagi, 2004). Therefore, the SRO5 protein may function in counteracting this ROS accumulation in a manner that balances the effects of reduced P5CDH activity.

We found that *sro5* plants were more sensitive to H_2O_2-mediated oxidative stress (Figure 6A). Also, there was substantially more accumulation of ROS, particularly H_2O_2, in salt-stressed *sro5* and *p5cdh* seedlings (Figures 6B and 6C). The increased ROS level in *p5cdh* under salt stress was expected based on previous observations that P5C can cause ROS accumulation (Deuschle et al., 2001; Nomura and Takagi, 2004). The observation that *sro5* had even greater ROS accumulation and sensitivity than *p5cdh* suggests a role for SRO5 protein in counteracting the increased ROS production caused by decreased P5CDH activity, either by blocking ROS production or increasing ROS detoxification.

A role for the SRO5 protein in ROS regulation is supported by its intracellular localization. SRO5 is predicted to be a mitochondrial protein, and we confirmed this by observing that transiently expressed SRO5-YFP fusion protein is targeted to the mitochondria (Figure S1). Proline catabolism occurs in the mitochondria, and P5CDH is localized on the matrix side of the inner mitochondrial membrane (Deuschle et al., 2001; Nomura and Takagi, 2004). Also, SRO5 shares partial sequence homology to RADICLE INDUCED CELL DEATH 1 (RCD1). RCD1 is involved in controlling ROS-induced cell death, and *rcd1* plants are hypersensitive to ozone, which causes oxidative stress (Ahlfors et al., 2004). Further support for a role of SRO5 protein will require demonstration that an untranslatable *SRO5* mRNA can cause *P5CDH* cleavage but cannot suppress ROS accumulation.
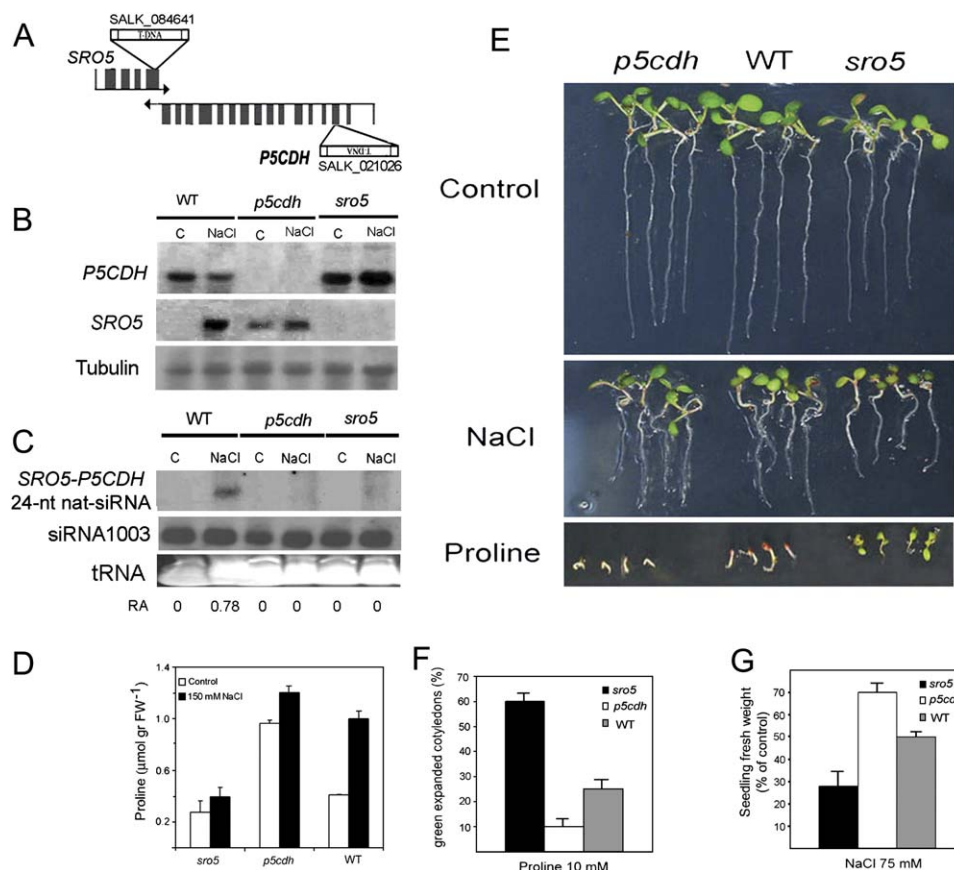
Figure 5. *SRO5-P5CDH* nat-siRNA Accumulation and Salt and Proline Responses in *sro5* and *p5cdh* Knockout Mutants

(A) Diagram of T-DNA insertion mutants isolated.

(B) Northern blot of *P5CDH* and *SRO5* mRNAs in wild-type, *p5cdh*, and *sro5* T-DNA lines. RNA (20 μg per lane) from 10-day-old seedling with or without 6 hr exposure to 150 mM NaCl were probed with *P5CDH* 5′ probe or *SRO5* probe.

(C) Northern blot of small RNAs. Eighty micrograms of low-molecular-weight RNA extracted from the same samples as in (B) was blotted and probed with an oligoprobe complementary to the 24-nt *SRO5-P5CDH* nat-siRNA. The number below the blot indicates relative abundance (RA) of the nat-siRNA with respect to the loading control siRNA 1003.

(D) Proline accumulation in wild-type, *p5cdh*, and *sro5* T-DNA lines after 6 hr exposure to 150 mM NaCl. Data are means ± SD (n = 10).

(E) Phenotypes of seedlings grown on 75 mM NaCl or 5 mM proline for 7 days.

(F) Proline response quantified as the percentage of seedlings with green expanded cotyledons. Data are means ± SD (n = 10).

(G) NaCl response quantified as seedling fresh weight relative to control not treated with NaCl. Data are means ± SD (n = 10).

Salt treatment causes oxidative stress, and we also found that application of H_2O_2 induced the expression of *SRO5*, induced the 24-nt *SRO5-P5CDH* nat-siRNA, and decreased P5CDH transcript levels (Figures 6D and 6E). The result suggests that the salt-stress induction of *SRO5* and *SRO5-P5CDH* nat-siRNA formation might be mediated by increased ROS under salt stress.

DISCUSSION

A Fine-Tuned Biological Regulatory Circuitry Involving nat-siRNAs

We have discovered a class of siRNAs, which we call nat-siRNAs, that are central components of a stress regulatory circuit (Figure 7). In this circuit, salt stress induces the expression of *SRO5* mRNA, and together with the *P5CDH* mRNA

already present, the *SRO5* mRNA forms dsRNA that is processed by DCL2 into 24-nt nat-siRNAs. The accumulation of detectable levels of the 24-nt *SRO5-P5CDH* nat-siRNAs also requires the presumably amplifying activity of the type IV RNA polymerase NRPD1A (Onodera et al., 2005; Herr et al., 2005), the RNA-dependent RNA polymerase RDR6, and SGS3, which is functionally coupled with RDR6 (Peragine et al., 2004). One of the 24-nt *SRO5-P5CDH* nat-siRNAs is stable and targets the *P5CDH* mRNA for cleavage. Initial cleavage of *P5CDH* mRNA guided by this 24-nt *SRO5-P5CDH* nat-siRNA establishes a phase for subsequent production of 21-nt nat-siRNAs by DCL1 and further degradation of *P5CDH* mRNA. The downregulation of *P5CDH* reduces proline degradation, thereby enhancing proline accumulation, which is beneficial to salt-stress tolerance. However, reduced P5CDH activity also leads to the accumulation
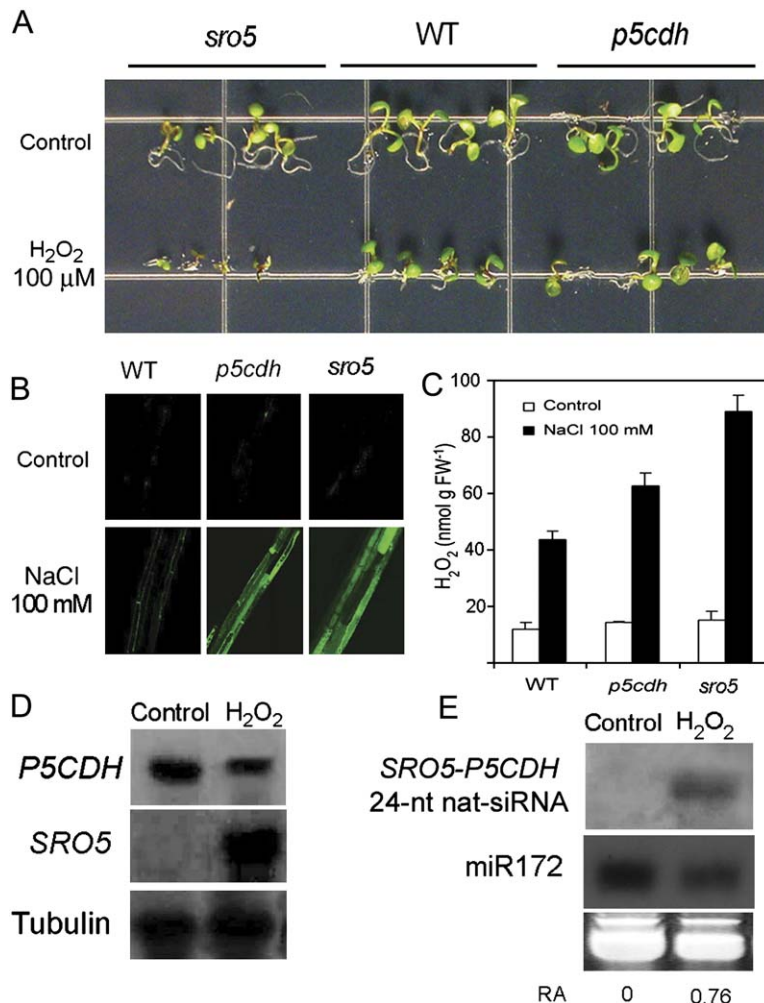
Figure 6. *SRO5* Is Required for Oxidative Stress Response

(A) Hypersensitivity of *sro5* mutant to oxidative stress induced by 100 µM exogenous H_2O_2.

(B) ROS staining in seedling roots after exposure to 100 mM NaCl for 12 hr.

(C) H_2O_2 accumulation quantified in seedlings exposed to 100 mM NaCl for 12 hr. Data are means ± SD (n = 10).

(D) Regulation of *P5CDH* and *SRO5* mRNAs by oxidative stress. Seedlings were treated or not treated (control) with 6 hr exposure to 10 mM H_2O_2.

(E) Accumulation of 24-nt *SRO5-P5CDH* nat-siRNA is induced by H_2O_2. Small RNA (100 µg per lane) from the same tissues used in (D) were probed with an oligonucleotide complementary to the 24-nt *SRO5-P5CDH* nat-siRNA. The number below the blot indicates relative abundance (RA) of the nat-siRNA with respect to the loading control miR172.

of the toxic metabolic intermediate P5C and ROS accumulation, which is probably counteracted by the SRO5 protein either through a direct detoxification activity or regulatory mechanism. The initial induction of *SRO5* mRNA by salt stress may be mediated by oxidative stress that is an inevitable consequence of salt stress. We propose that the insights gained from this characterization of the *P5CDH-SRO5* cis-antisense gene pair will be useful in the study of the many other such gene pairs present in eukaryotic genomes.

Phased Processing of nat-siRNAs

siRNAs are extremely diverse, and their biogenesis is especially complex in plants (Xie et al., 2004; Baulcombe, 2004; Meister and Tuschl, 2004). siRNAs from transposons and other repeat sequences are important for RNA-dependent DNA methylation and heterochromatin formation (Zilberman et al., 2004; Xie et al., 2004). Biogenesis of these siRNAs requires NRPD1A, RDR2, and DCL3 (Xie et al., 2004). The tasiRNAs that target specific mRNAs for cleavage require RDR6, SGS3, DCL1, and DCL4 (Peragine et al., 2004, Vazquez et al., 2004b; Allen et al., 2005; Gasciolli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005). In contrast, our

data suggest that the nat-siRNAs described here are a unique class in which the 24- and 21-nt nat-siRNAs are generated by DCL2 and DCL1, respectively.

Unexpectedly, the production of the 24-nt *SRO5-P5CDH* nat-siRNA is also dependent on the atypical DNA-dependent RNA polymerase NRDP1A. The fact that formation of the nat-siRNAs but not *P5CDH* or *SRO5* transcript accumulation is dependent on NRPD1A suggests a DNA-independent function of this mysterious protein. The role of RDR6 is also intriguing since it is required not just for the formation of 21-nt nat-siRNAs, where the need to generate a new RNA strand is clear, but, also for the generation of the 24-nt *SRO5-P5CDH* nat-siRNA, where dsRNA from the *SRO5-P5CDH* cis-antisense transcripts should already be present. Perhaps RDR6 is required for subsequent amplification of the 24-nt *SRO5-P5CDH* nat-siRNA generated from the initial antisense transcript pair.

Our results show that initial cleavage of *P5CDH* mRNA guided by this 24-nt *SRO5-P5CDH* nat-siRNA sets the phase for accurate processing by DCL1 to yield the 21-nt *P5CDH* nat-siRNAs. Production of these 21-nt nat-siRNAs is also dependent on RDR6, SGS3, and NRPD1A. It is
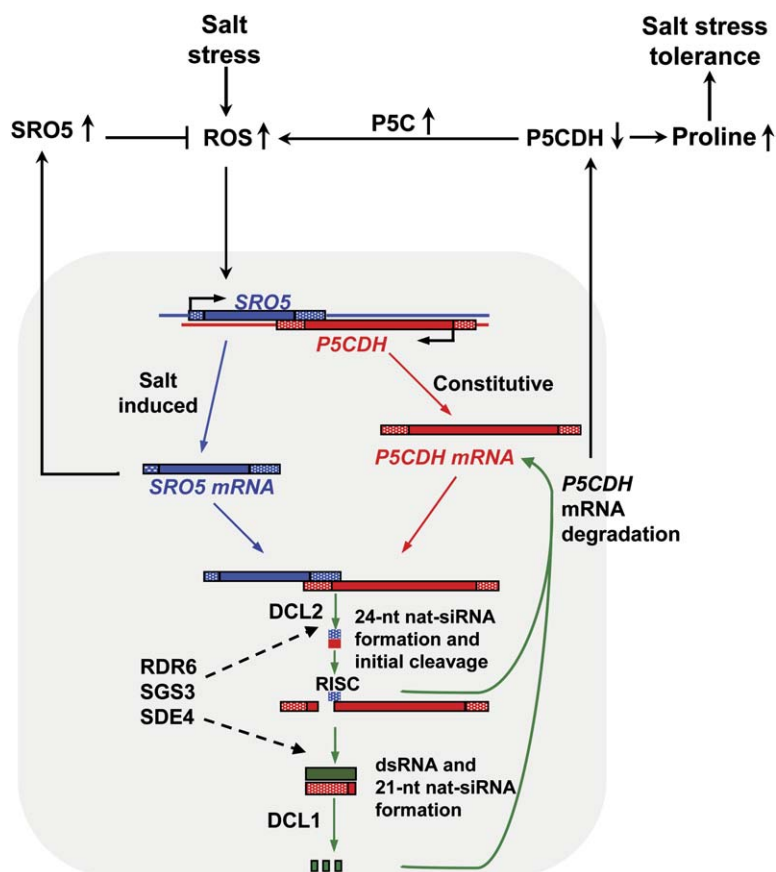
Figure 7. Diagram of Phased Processing of *SRO5-P5CDH* nat-siRNAs and Their Role in a Salt-Stress Regulatory Loop

Salt stress induces ROS accumulation which in turn induces the expression of *SRO5*. This initiates a series of nat-siRNA processing steps (shown in the shaded area of the model) that result in the downregulation of *P5CDH*. When *SRO5* is induced, dsRNA can form by annealing of the *SRO5* and *P5CDH* transcripts (note that poly-A tails of the mRNAs are omitted for clarity), and this initiates DCL2-dependent formation of 24-nt nat-siRNA. This initial cleavage of the *P5CDH* mRNA causes phased generation of 21-nt nat-siRNAs by a DCL1-dependent mechanism and additional cleavage of the *P5CDH* transcript. RDR6, SGS3, and NRPD1A are suggested to be involved in the formation of both 24- and 21-nt *SRO5-P5CDH* nat-siRNAs. The importance of *P5CDH* downregulation by nat-siRNAs is further illustrated by the functional relationship between the SRO5 and P5CDH proteins (portion of model outside the shaded area). Degradation of *P5CDH* mRNA decreases P5CDH activity and proline degradation. Decreased proline degradation contributes to salt-induced proline accumulation and salt tolerance but, at the same time, leads to the ROS accumulation caused by buildup of the proline catabolic intermediate P5C. This ROS accumulation is potentially harmful but is also a signal to initiate further stress responses. The SRO5 protein counteracts P5C-induced ROS accumulation, thus completing a regulatory loop that allows fine tuning of ROS accumulation and stress response.

possible that this dependency is due to the phasing requirement for the 24-nt nat-siRNA. Alternatively, these components may be needed to process the cleaved *P5CDH* mRNA fragment into dsRNA that is then competent for dicing by DCL1. In the absence of 21-nt nat-siRNA formation, as in *dcl1*, the 24-nt *SRO5-P5CDH* nat-siRNA is sufficient to downregulate the *P5CDH* transcript under the salt-stress conditions used in our experiments. It may be that 21-nt nat-siRNA-guided degradation of *P5CDH* mRNA serves to ensure *P5CDH* downregulation. Nonetheless, future investigations to determine whether the 21-nt nat-siRNAs might have additional functions are needed.

Another important question that arises from these experiments is that of how a specific 24-nt *SRO5-P5CDH* nat-siRNA is initially generated from the relatively long (760-nt) *P5CDH-SRO5* overlapping region. This situation may be analogous to the generation of a single, precise miRNA from a hairpin precursor with a long dsRNA-like stem. Alternatively, many different 24-nt siRNAs may be produced from the long overlap, but only one is stable and incorporated into the RISC to guide subsequent mRNA cleavage.

Implications for the Regulation and Function of Genes in Natural *cis*-Antisense Pairs

Our proposed regulatory model (Figure 7) has implications beyond the regulation of *P5CDH* and *SRO5*. In both animals and plants, genome analyses have found thousands of genes in convergent overlapping pairs that can generate complementary transcripts (Boi et al., 2004, Wang et al., 2005; Jen et al., 2005). Various expression profiling approaches, such as genome tiling arrays (Yamada et al., 2003; Bertone et al., 2004) and MPSS (*m*assively *p*arallel *s*ignature *s*equencing; Brenner et al., 2000), have detected widespread antisense transcription throughout genomes. As illustrated by the *SRO5-P5CDH* example here, these antisense transcript pairs can form dsRNAs that are processed by a Dicer into nat-siRNAs. In the *SRO5-P5CDH* example, one of the overlapping genes (*SRO5*) is inducible. This may be a widespread scenario as our preliminary analysis indicates that most of the nearly 2000 genes in convergent overlapping pairs in *Arabidopsis* are regulated by various environmental or hormonal stimuli (T. Girke and J.-K.Z., unpublished data). Our preliminary survey has detected several other nat-siRNAs from *cis*-antisense gene pairs, and all of these nat-siRNAs are inducible and detected only under specific abiotic or biotic stress-treatment conditions (our unpublished data). Consistent with their inducible nature, these nat-siRNAs including the nat-siRNAs from the *SRO5-P5CDH* pair were not identified by recent deep small RNA profiling by the MPSS method, which was carried out with non-stressed plants (Lu et al., 2005). Whether or not these induced nat-siRNAs also downregulate the corresponding noninducible overlapping gene by mRNA cleavage is an important topic for further investigation.

Jen et al. (2005) have argued against dsRNA- or siRNA-mediated RNA cleavage as a mode of regulation of antisense overlapping gene pairs. Their proposal is based largely on gene expression data from DNA microarrays that showed apparent coexpression of *cis*-antisense gene pairs. We believe that such microarray data offers only an incomplete picture of the regulation of overlapping genes because it may not distinguish full-length transcripts from stable 3′ cleavage products. Thus, we think that the proportion of *cis*-antisense gene pairs that are genuinely coexpressed remains an open question.

The *SRO5-P5CDH* example implies a functional link between proteins encoded by antisense overlapping genes such that regulating the two genes in a converse manner is beneficial. Whether or not such a functional link also exists between other pairs of antisense overlapping genes remains to be determined. In most cases, only one gene in the antisense overlapping pair has a defined function or contains known domains; thus, the possibility of a functional link between proteins encoded by overlapping genes offers an approach to identify the physiological function of a number of unknown genes.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

Arabidopsis thaliana mutants, *rdr2-1, dcl2-1, dcl3-1, and dcl4-1*, were a generous gift of Jim Carrington (Center for Gene Research and Biotechnology, Oregon State University). *dcl1-9* and *hen1-1* were provided by Xuemei Chen. *sde1* (*rdr6* in this study*)* and *sde4/nrpd1a* were kindly provided by David Baulcombe (John Innes Center for Plant Science Research, Sainsbury Laboratory, United Kingdom). *sgs3* was kindly provided by Herve Vaucheret (Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, Versailles, France). *hyl1* was a gift from Nina Federoff (The Huck Institute of Life Science, Pennsylvania State University).These mutants were in the Columbia (Col-0), Landsberg *erecta* (Ler), Nosssen-0 (No), or C24 genetic backgrounds as indicated in the text and figures. T-DNA insertion mutants of *P5CDH* (salk_021026) and *SRO5* (salk_084641) were identified using the SIGnAL website (http://signal.salk.edu) and obtained from ABRC.

Seedlings were grown in half-strength MS (3% w/v sucrose) agar plates under 16/8 hr light/dark cycle of fluorescent light (120 $\mu E/min/m^2$) at 22°C. After 10 days, the seedlings were treated by immersing the roots in half-strength MS liquid media (3% w/v sucrose) with or without the addition of salt, osmotic agents (PEG, mannitiol), or hormones, at concentrations indicated in the figure legends. Tissue was collected 6 hr after the treatment. In the experiments with adult plants, 20-day-old plants grown in pots and kept in a growth chamber with a 16/8 hr light/dark cycle at 22°C were used.

Tolerance of salt or exogenous proline was assessed by sowing seeds on half-strength MS agar plates (3% sucrose) containing the concentrations of proline indicated in the text or figure legends. After 7 days, the seedling response (survival or fresh weight) was quantified. For *p5cdh* and *sro5* mutants, the seeds were pre-soaked in MS solution containing 1 mM proline for 48 hr before they were subjected to the above tolerance assays.

RNA Analysis

Total RNA was extracted from 10-day-old seedlings with Trizol reagent (Invitrogen). Twenty micrograms of total RNA was separated on 1.2% formaldehyde-MOPS agarose gels and blotted onto Hybond-N+ membranes (Amersham Biosciences). Hybridization was carried out at 65°C using PerfectHyb Plus buffer (Sigma). Probes were labeled with ^{32}P-dCTP using a Ready-To-Go DNA Labeling Kit (Amersham Biosciences).

Blots were washed twice in 2× SSC and 0.1% SDS for 20 min at 65°C and once in 1× SSC 0.1% SDS.

For detection of low-molecular-weight RNA, total RNA was purified from at least 1 gram of seedlings using hot-phenol extraction followed by two extractions of phenol/chloroform/isoamyl alcohol (25/24/1). Total RNA were precipitated with ethanol and pellet was dissolved in 750 microliter DEPC-water. High molecular weight RNA were selectively precipitated from the total RNA by the addition of one volume of 20% PEG-1M NaCl (Llave et al., 2002). The resulting low-molecular-weight-enriched RNA (80–100 μg) was then separated by 17% denaturing polyacrylamide gels and electrically transferred to Hybond-N+ membranes. Low-molecular-weight RNA blots were probed with miR172, siRNA1003, and 24 and 21-nt nat-siRNA DNA oligonucleotides complementary to small RNA sequences. The probes were end-labeled with γ^{32}P-ATP using T4 kinase (NEB). Unincorporated nucleotides were removed using G-25 spin columns (Amersham) as per manufacturer's instructions. Blots were prehybridized for at least 1 hr and hybridized overnight at 38°C using PerfectHyb Plus buffer (Sigma). The blots were washed twice in 2× SSC and 0.2% SDS for 15 min at 38°C and once with 1× SSC 0.1% SDS for 15 min at 38°C. Membranes were exposed to X-ray films and to Typhoon phosphoimager and relative abundance (RA) was estimated using Image Quant software 5.2.

Transient Expression in *Nicotiana benthamiana*

Site-directed mutagenesis was performed to generate *P5CDH* and *SRO5* genes (mP5CDH and mSRO) mutated in the region complementary to the 24-nt *SRO5-P5CDH* nat-siRNA. Both mutant and wild-type *P5CDH* and *SRO5* were incorporated into the binary vector pMDC32 with expression under control of the 35S promoter. These constructs were transformed into *Agrobacterium tumefaciens* strain 3301. Overnight cultures grown in presence of 30 μM acetosyringone were harvested by centrifugation, and cells resuspended in 10 mM MgCl₂, 10 mM (pH 5.6), and 150 μM acetosyringone to an OD$_{600}$ of 0.5. After 2 hr incubation at room temperature, *Agrobacterium* suspension was infiltrated into expanding leaves of *N. benthamiana* using a needleless syringe (Llave et al., 2002). Leaves were harvested 3 days after infiltration and small RNA extraction and blotting performed as described above.

RLM 5′ RACE

A modified procedure for RNA ligase-mediated rapid amplification of cDNA ends (RLM 5′RACE) was performed using the GeneRacer Kit (Invitrogen). Total RNA was extracted from 10-day-old seedlings using the TRIZOL method described above. The GeneRacer RNA Oligo adaptor was directly ligated to total RNA (100 ng) without calf intestinal phosphatase treatment. The GeneRacer oligo dT primer was then used for cDNA synthesis. Initial PCR was carried out using the GeneRacer 5′ Primer and *P5CDH* RACE primer 1 (5′-CCCTCTACCCCTTGACTAAGTCAACG-3′), following the protocol of Llave et al. (2002). Nested PCR was carried out using 1 μl of the initial PCR reaction, the GeneRace 5′ nested primer, and *P5CDH* RACE primer 2 (5′-CTTATTCCCTCACACGGAAACTACTCG-3′). RACE fragments were cloned and sequenced after gel purification.

Proline and ROS Determination

Proline was quantified using the acid ninhydrin assay (Deuschle et al., 2004). For ROS detection, 7-day-old seedlings were transferred to media containing 100 mM NaCl for 12 hr. The salt-treated and untreated control seedlings were then incubated with 50 μM 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA, Molecular Probes) for 30 min following the methods of Shin and Schachtman (2004). Fluorescence images were obtained with a Leica SP2 confocal microscope.

For quantitative measurement of H₂O₂ production, 7-day-old seedlings were transferred to media with and without 100 mM NaCl and incubated for 12 hr. Salt-treated and control seedlings were extracted using 20 mM sodium phosphate buffer (pH 6.5) according to the protocol of Shin and Schachtman (2004) and H₂O₂ quantified using an Amplex red

Supplemental Data

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