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Preferential Spreading of RNA Silencing Into the 3′ Downstream Region of the Transgene in Tobacco

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The amplification mechanism of short interfering RNAs (siRNAs) along the transgene sequence exists in RNA interference (RNAi). The RNA-dependent RNA polymerase synthesizes complementary RNAs by using the transgene mRNA as a template, and the secondary siRNAs are generated from the outside of primary RNAi target. Four independent RNAi vectors which produced primary siRNAs against distinct regions of the tobacco endoplasmic reticulum ω-3 fatty acid desaturase gene (*NtFAD3*) were transiently expressed in leaves of the *NtFAD3*-overexpressed transgenic plants. Regardless of the RNAi vector used, the secondary *NtFAD3* siRNAs were generated preferentially from the 3′ downstream region of the transgene. Secondary siRNAs from the 5′ upstream region adjacent to the annealing site of primary siRNAs accumulated under the detection level. Our results suggest that different regulatory mechanisms are involved in the spreading of RNA silencing into 5′ upstream and 3′ downstream regions of the target sequence, respectively.

Keywords: RdRP, secondary siRNA, transitive RNAi, ω-3 fatty acid desaturase

RNA interference (RNAi) is induced by double-stranded RNAs (dsRNAs) which are cleaved into 20- to 25-nucleotide (nt)-long small interfering RNAs (siRNAs). Then, siRNAs guide cleavage of homologous transcripts (Hannon, 2002). Amplification of siRNAs in RNAi is inferred from spreading of target regions of RNA silencing outside the inducer sequence. This phenomenon has been designated transitive RNAi (Sijen et al., 2001; Vaistij et al., 2002). Transitive RNAi involves the production of secondary siRNAs for which RNA-dependent RNA polymerase (RdRP) has implicated in nematodes, fungi, and plants (Wasseneger and Krczal, 2006).

Most plant RNAi vectors transcribe an inverted repeat sequence which is processed into siRNAs (primary siRNAs) (Horiguchi, 2004; Hirai et al., 2007). Annealing of primary siRNAs to the homologous transgene's transcripts can trigger spreading of silencing region along the transgene (Vaistij et al., 2002; Himber et al., 2003; Kościańska et al., 2005; Miki et al., 2005; Petersen and Albrechtsen, 2005). The synthesis of secondary siRNAs has been observed in both 5′ upstream and 3′ downstream region relative to the primary silencing inducer sequences (Kościańska et al., 2005; Miki et al., 2005; Bleys et al., 2006). In contrast, preferential synthesis of secondary siRNAs against the 3′ downstream region but not against the 5′ upstream region was also observed (Petersen and Albrechtsen, 2005; Haque et al., 2007). In the latter case, lack of spreading into the 5′ upstream region may be explained by limited synthesis of complementary RNAs (cRNAs) (Petersen and Albrechtsen, 2005). The RdRPs are recruited during the amplification process of siRNAs and synthesize cRNAs from the 3′ end of target mRNAs (Tang et al., 2003). If processibility of RdRPs is limited, cRNA molecules corresponding to the 5′ upstream region are barely synthesized on the template which has a long gap between the silencing inducer sequence and the 3′ end of templates. Another possible explanation for the lack of transitivity of RNA silencing into the 5′ upstream region is provided by a recent analysis of RdRP-deficient mutants. RDR6, a plant RdRP, is involved in the amplification process of siRNAs (Wasseneger and Krczal, 2006). Several endogenous transcripts can serve as a template for RdRP-dependent siRNA amplification after microRNA (miRNA)-mediated cleavage, and endogenous secondary small RNAs are produced (termed *trans*-acting siRNAs) (Allen et al., 2005; Yoshikawa et al., 2005; Bonnet et al., 2006). The target transcript of the miRNA is cleaved into 5′ upstream and 3′ downstream fragments followed by production of *trans*-acting siRNAs (Allen et al., 2005; Yoshikawa et al., 2005). In the *rdr6* mutants, the *trans*-acting siRNAs corresponding to the 5′ upstream region are produced, but those from the 3′ downstream region are not (Ronemus et al., 2006). This result suggests that RDR6 is responsible for the production of *trans*-acting siRNAs corresponding to the 3′ downstream region and that other unidentified RdRPs are required for generation of *trans*-acting siRNAs from the 5′ upstream region. If the transgene transcripts are recognized preferentially by RDR6 during the spread of RNAi silencing, the 5′ upstream region of transgenes relative to the inducer sequence would be ignored in the amplification process of siRNAs.

We have investigated RNA silencing of a tobacco endoplasmic reticulum ω-3 fatty acid desaturase gene (*NtFAD3*) (Hamada and Kodama, 2006). The NtFAD3 protein catalyzes the conversion of linoleic acid (18:2) to α-linolenic acid (18:3). Transitivity can be observed in the crossbred plants between the *NtFAD3*-overexpressed transgenic plants

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Abbreviation: CaMV, Cauliflower mosaic virus; cRNA, complementary RNA; dsRNA, double-stranded RNA; GFP, green fluorescent protein; GUS, β-glucuronidase; IMPDH, inosine-5′-monophosphate dehydrogenase; MES, 2-morpholinoethanesulfonic acid; miRNA, microRNA; Nos, nopaline synthase; RdRP, RNA-dependent RNA polymerase; RNAi, RNA interference; siRNA, small interfering RNA

(named S24 line) and the RNAi transgenic plants against the *NtFAD3* gene (designated R11 line) (Tomita et al., 2004; Shimamura et al., 2007). The RNAi vector used in the production of R11 plants harbored *NtFAD3* cDNA fragments in an inverted repeat structure. In the crossbred plants (S24× R11), the secondary *NtFAD3* siRNAs were generated from the 3′ downstream region relative to inducer sequences (Shimamura et al., 2007). In this paper, we investigated the generation of secondary siRNAs in the S24 plants by transient expression of several RNAi vectors that harbored target regions spanning the entire *NtFAD3* transcripts. The results showed that transitivity into the 5′ upstream region was strictly limited irrespective of the location of the inducer sequences in the *NtFAD3* gene.

MATERIALS AND METHODS

Plant material

pTF1SIIn harbored the *NtFAD3* cDNA fragment from 52 to 1366 (GenBank accession no. D26509) in a sense orientation relative to the promoter sequence (Hamada et al., 1998). pTF1AGS contained two 498-bp sequences (corresponding to the nucleotide positions 181-678) of the *NtFAD3* cDNA in an inverted repeat structure (Tomita et al., 2004). *Nicotiana tabacum* cv. SR1 was transformed with pTF1SIIn and pTF1AGS, and the S24 and R11 lines were used as a representative line showing successful overexpression and the RNAi of the *NtFAD3* gene, respectively (Hamada et al., 1998; Tomita et al., 2004).

Plasmid construction

A binary vector, pSMAH621 (constructed by Ichikawa et al., personal communication), was used for construction of the following *NtFAD3*-related plasmids (Fig. 1). In this plasmid, the Cauliflower mosaic virus (CaMV) *35S* promoter, GUS gene and the terminator of nopaline synthase *GUS* gene sequences were cloned into the *Hin*dIII/*Xba*I, *Xba*I/*Sac*I, and *Sac*I/*Eco*RI sites, respectively. We prepared an *NtFAD3*-sense construct, pSO-NtFAD3, by replacing the *Hin*dIII-*Sac*I fragment of pSMAH621 with the *Hin*dIII-*Sac*I fragment of pTF1SIIn. The RNAi plasmids against the *NtFAD3* gene were constructed by using pSH-GUS1007 according to the procedures described in Hirai et al. (2007). This plasmid harbors the same inverted repeat sequences as those of pTF1AGS. The *Xba*I and *Apa*I sites were added to the 5′ and 3′ ends of each antisense-oriented *NtFAD3* fragment by means of PCR with primers harboring these restriction enzyme sites. Similarly, *Xho*I and *Sac*I sites were created at the 5′ and 3′ ends of the corresponding *NtFAD3* sense fragments, respectively, by PCR. The *Xba*I-*Apa*I and *Xho*I-*Sac*I fragments of pSH-GUS1007 were replaced with each *Xba*I-*Apa*I *NtFAD3* antisense fragment and *Xho*I-*Sac*I *NtFAD3* sense fragments to produce the following plasmids pSO-hp-I to pSO-hp-IV.

A control plasmid, pIR-IMPDH, harbors the 500-bp-long tea IMPDH sequences (GenBank accession no. EU106658) in an inverted repeat manner. The details of this plasmid will be reported elsewhere. The inverted repeat sequence consisting of the 700-bp cDNA fragment encoding a tobacco RdRP (NtRDR6) (nucleotide positions 1890-2559, GenBank accession no. AB361628) was similarly prepared in according to the procedure described above, and then inserted into *Xba*I-*Sac*I region of the pSMAH621 to generate an RNAi vector, pSH-hp-RDR6 (Fig. 1).

Stable expression of the *NtRDR6* RNAi construct in the R11 plants

The homozygous R11 plants were transformed by the leaf-disc method using *A. tumefaciens* C58 containing pSH-hp-RDR6. Regenerated plants were selected on MS medium containing 750 mg/L Augmentin (GlaxoSmithKline) and 5 mg/L hygromycin B. To strictly select the transgenic plants with pSH-hp-RDR6, the concentration of hygromycin B increased by 5 mg/L increments every 7 d to a concentration of 20 mg/L at final. The hygromycin-resistant offspring were transferred to soil, and then, the insertion of an inverted repeat sequence against the *NtRDR6* gene was determined by PCR. The resulting tobacco plant containing both pTF1AGS and pSH-hp-RDR6 was designated R11-ΔRDR6, and was subjected to an analysis of transitivity.

Stable expression of the *NtFAD3* RNAi constructs in tobacco hairy roots

The S24 seedlings homozygous for the T-DNA insertion locus were transformed by the leaf-disc method using *Agrobacterium tumefaciens* R1000 carrying the *NtFAD3* RNAi vectors (pSO-hp-I to -IV) or pSMAH621 as a control vector. Hairy roots were produced on Murashige-Skoog (MS) medium containing 750 mg/L Augmentin and 5 mg/L hygromycin B (Kodama et al., 1997). The fatty acid composition of each hairy root was determined as previously described (Kodama et al., 1994).

Agroinfiltration-mediated transient expression of the *NtFAD3* RNAi constructs

The S24, R11 and R11-ΔRDR6 plants were grown under continuous illumination at 25°C. The young but fully expanded leaves of the about 2-month-old plants were used for agroinfiltration (Llave et al., 2000; Zhao et al., 2005). Individual *Agrobacterium* colonies were grown in 3-mL cultures (LB medium supplemented with 100 μg/mL spectinomycin) for 2 d at 28°C, and then inoculated into 20 mL fresh culture medium. After culture for 2 d, cells were precipitated and resuspended to a final concentration of A_{600} =1.0 in a solution containing 10 mM $MgCl_2$, 10 mM MES, pH 5.6, 0.15 mM acetosyringone, and 0.025% silwet-77. With a 5-mL-volume syringe, the suspension of *Agrobacterium* cells was injected into the tobacco leaves. The infiltrated leaves were covered with a transparent plastic bag until ribonucleic acids were extracted.

Extraction of small RNA and Northern blot analysis

The small RNA-enriched fraction was prepared from the infiltrated leaves according to the protocol described by Goto et al. (2003). About 70 μg of small-RNA-enriched nucleic acids were separated on a 18% (w/v) polyacrylamide

gel containing 7 M urea, and then transferred onto nylon membranes.

We designated three parts of *NtFAD3* cDNA as the upstream (U), target (T) and downstream (D) regions in reference to the target sequence of pTF1AGS. The riboprobes corresponding to these regions (probes U, T and D) were prepared as previously described (Shimamura et al., 2007).

In addition, we prepared four riboprobes as follows (probes I to IV). The nucleotide sequence of T7 promoter was added to 5′ ends of the *NtFAD3* fragments corresponding to regions I to IV by means of PCR (Fig. 1). Each digoxigenin-labeled probe was prepared by transcription with T7 RNA polymerase. Since the small RNA-enriched fraction contains tRNAs, 5S RNAs and small RNAs, we used the rice tRNA-Gly probe to elucidate the loading amounts of RNA samples (Kodama et al., 1994). The hybridization and visualization of hybridized probes were performed as previously described (Hirai et al., 2007). The positions for 20- and 30-nt RNA oligomers (DynaMarker small RNA II, BioDynamics Laboratory Inc.) were visualized by staining gels with ethidium bromide.

RESULTS AND DISCUSSION

The *NtFAD3* RNAi plants (R11 line) were produced by introduction of an RNAi vector, pTF1AGS (Fig. 1). In these plants, the *NtFAD3* primary siRNAs cleaved endogenous *NtFAD3* mRNA, but the *NtFAD3* transcripts did not serve as a template for the secondary siRNA production in the R11 plants. Secondary siRNAs were generated by using the transgene-originated *NtFAD3* transcripts as a template for RdRP, and they can be observed typically in the crossbred plants (S24×R11) (Shimamura et al., 2007). To rapidly delineate the mechanisms involved in generation of secondary siRNAs, we examined, at first, whether or not transitive RNAi is induced by agroinfiltration-mediated introduction of the *NtFAD3* sense constructs, pSO-NtFAD3 (Fig. 1), into the R11 leaves. Since transient expression of the infiltrated plasmids reached a maximum at 4 d post infection (data not shown), we extracted ribonucleic acids 4 d after the RNAi vectors were introduced. The primary siRNAs were detected by using the T-probe which covered the target region of pTF1AGS (nucleotide position 181-678 of the *NtFAD3* cDNA). Transient expression of pSO-NtFAD3 in the R11 leaves triggered synthesis of the *NtFAD3* secondary siRNAs. The resultant secondary siRNAs were detected by hybridization with the D-probe covering the 3 downstream region relative to the inducer sequence (Fig. 2). When pSO-NtFAD3 was introduced into the R11 plants transformed with an RNAi construct against the *NtRDR6* gene (R11-ΔRDR6), the *NtFAD3* primary siRNAs were generated but the secondary ones were not (Fig. 2). Thus, transient expression of the *NtFAD3* sense transgene in the R11 leaves can trigger transitive RNAi as in the case of crossbred plants (S24 ×R11), and generation of secondary siRNAs was dependent on the *NtRDR6* activity.

Then we investigate the relationship between location of silencing target on the *NtFAD3* gene and the efficiency of secondary siRNA generation. We prepared four RNAi constructs in which the inverted repeat cassettes harbored the *NtFAD3* sequences corresponding to the following 4 regions (Fig. 1); 121-373 (pSO-hp-I construct), 451-700 (pSO-hp-II), 783-1040 (pSO-hp-III), and 1122-1358 (pSO-hp-IV) of the *NtFAD3* cDNA (GenBank accession no. D26509). We investigated the silencing capability of these newly constructed RNAi vectors by using hairy roots according to a method previously reported (Hirai et al., 2007). Hairy roots were produced by co-introduction of the Ri plasmid and the RNAi vector into the S24 leaves. Then, the 18:3 level of each hairy root was determined. The average 18:3 content was 60% of total fatty acids in the control hairy roots and decreased by about 25%~45% in hairy roots containing the

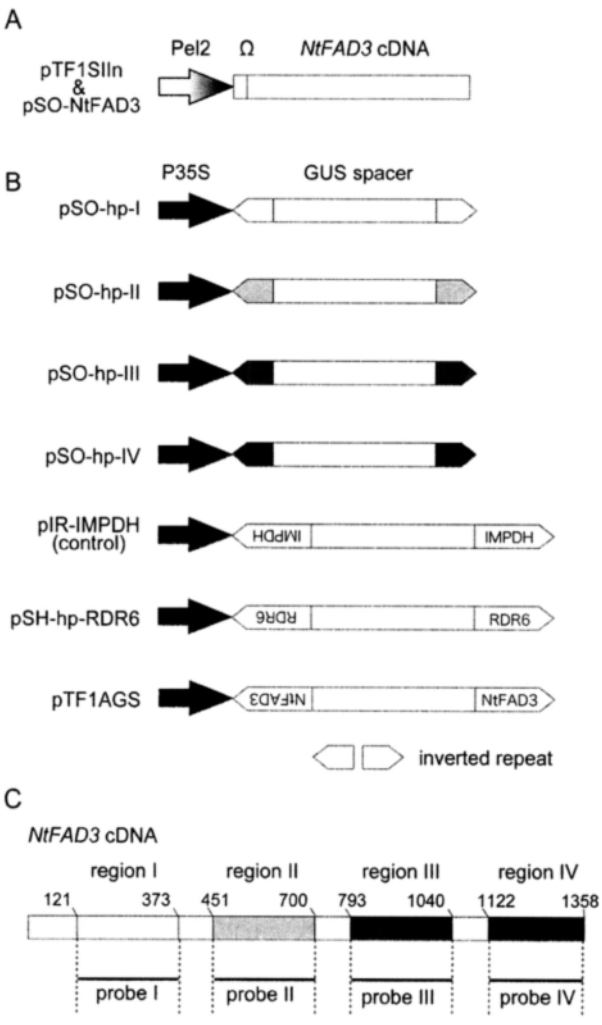

Figure 1. Schematic diagrams of the binary vectors and probes used. (A) The *NtFAD3* sense constructs. pTF1SIIn and pSO-NtFAD3 were used in production of the S24 plants and in the transient expression of the *NtFAD3* gene, respectively. Pel2, a tandem repeat of 5′-upstream sequence of CaMV 35S promoter (Mitsuhara et al., 1996); Ω, 5′-untranslated sequence of tobacco mosaic virus. (B) RNAi constructs used. Each RNAi vector contains the inverted repeat sequence corresponding to regions I to IV of the *NtFAD3* gene as indicated in (C). A control plasmid, pIR-IMPDH, harbored an RNAi cassette for the tea IMPDH gene. pSH-hp-RDR6 contained an inverted repeat sequence against the *NtRDR6* gene. pTF1AGS was used in the production of RNAi plants (R11 line) against the *NtFAD3* gene. P35S, CaMV 35S promoter. (C) Probes used. Probes I to IV were designed to detect the *NtFAD3* siRNAs corresponding to regions I to IV.

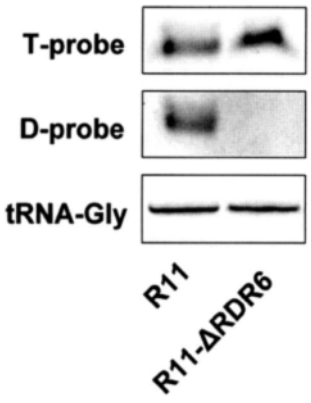

Figure 2. Detection of the siRNAs in RNAi plants infiltrated with the *NtFAD3*-sense construct. The primary *NtFAD3* siRNAs generated from the R11 locus were detected by a sense-specific riboprobe covering target regions (T-probe). The secondary *NtFAD3* siRNAs were detected by a riboprobe (D-probe) covering the 3′ downstream region adjacent to the target region as previously reported (Shimamura et al., 2007). pSO-NtFAD3 was introduced into the R11 and R11-ΔRDR6 leaves. The blot was reprobed with a gene for tRNA-Gly (GCC) to verify that approximately the same amount of small RNA was loaded per lane.

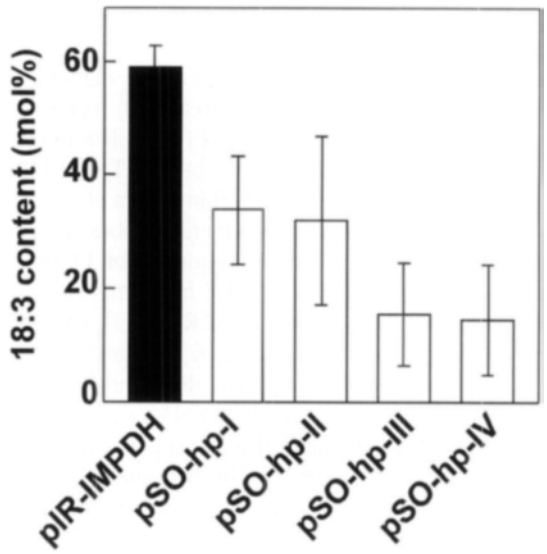

Figure 3. Effects of different RNAi target sequences on RNAi efficiency. The levels of 18:3 in total fatty acids of hairy roots are shown. Each RNAi vector was introduced into the S24 plants with an Ri plasmid. pIR-IMPDH was used as a control. All RNAi constructs against the *NtFAD3* gene showed the RNAi phenotype, namely decreased level in 18:3. Vertical line indicates SD (n=16).

RNAi constructs (pSO-hp-I to -IV) (Fig. 3). The silencing phenotype by pSO-hp-III and -IV was apparently intense relative to the phenotype by pSO-hp-I and -II. The precise reason of this enhancement of silencing by pSO-hp-III and -IV is unclear. These results indicated that the RNAi vectors tested were effective to induce RNA silencing of the *NtFAD3* gene.

Next, we investigated the secondary siRNA generation induced by these four RNAi vectors. The hairpin dsRNAs were transiently expressed in the *NtFAD3*-overexpressing S24 leaves. As a control, pIR-IMPDH was also introduced. This plasmid harbored a tea cDNA fragment against the IMPDH gene in an inverted repeat manner. The generated *NtFAD3* siRNAs in the infiltrated leaves were detected by using sense riboprobes corresponding to regions I to IV (Fig. 1).

When pSO-hp-I was introduced into the S24 leaves, we observed the production of *NtFAD3* siRNAs. No *NtFAD3* siRNAs were generated in the leaves infiltrated with a control plasmid, pIR-IMPDH. About 21-nt-long primary siRNAs derived from pSO-hp-I could be detected by using probe I, and the secondary *NtFAD3* siRNAs corresponding to regions II to IV were successfully detected in the S24 leaves (Fig. 4). Therefore, RdRP synthesized the *NtFAD3* cRNAs covering the 3′ downstream region corresponding to the nucleotide position from 451 to 1358 of *NtFAD3* cDNA.

Next we introduced pSO-hp-II into the S24 leaves. The primary *NtFAD3* siRNAs detected by probe II accumulated, and secondary siRNAs corresponding to regions III and IV were detected. As expected from our previous results (Shimamura et al., 2007), we failed to detected secondary siRNAs generated from the 5′ upstream region (namely region I). There were some faint hybridized signals with the probe I (Fig. 4). These signals are expected to have originated from nonspecific hybridization since there was no significant difference in the signal intensities between the RNA sample infiltrated with pIR-IMPDH and that with pSO-hp-II.

Then, we transiently expressed the RNAi cassette of pSO-hp-III in the S24 leaves. The primary and secondary *NtFAD3* siRNAs corresponding to regions III and IV, respectively, were generated in the infiltrated S24 leaves. Although the secondary siRNAs against region II were observed in the leaves infiltrated with pSO-hp-I, the siRNAs corresponding to region II apparently lacked in the S24 leaves infiltrated with pSO-hp-III (Fig. 4).

By introducing pSO-hp-IV into the S24 leaves, we detected the primary *NtFAD3* siRNAs corresponding to region IV. However, the *NtFAD3* siRNAs derived from regions I to III were deficient in the infiltrated S24 leaves (Fig. 4). These results indicated that generation of *NtFAD3* secondary siRNAs corresponding to the 5′ upstream region relative to the inducer sequence was severely limited irrespective of the region of inducer sequence.

Our results suggest that RdRP does not participate in cRNA synthesis corresponding to the 5′ upstream region regardless of the distance between the inducer sequence and 3′ end of the *NtFAD3* transgene template, which is in agreement with the RDR6-dependent synthesis of a 3′ downstream region of the *trans*-acting siRNAs (Ronemus et al., 2006). The possibility in which the secondary *NtFAD3* siRNAs are slightly synthesized from the 5′ upstream region adjacent to the inducer sequences cannot be excluded, but there was a significant difference between the amounts of the secondary siRNAs from the 5′ upstream and those from the 3′ downstream regions. Several early reports clearly demonstrated efficient spreading into a 5′ upstream region adjacent to the inducer sequence (Van Houdt et al., 2003; Garcia-Perez et al., 2004; Bleys et al., 2006). In such cases, another RdRP, not RDR6, may synthesize cRNAs by using 5′ upstream transgene fragments as a template.

The factors required for recruitment of another RdRP onto 5′ upstream regions in the spreading of RNA silencing are still unclear. The green fluorescent protein (GFP) transgene

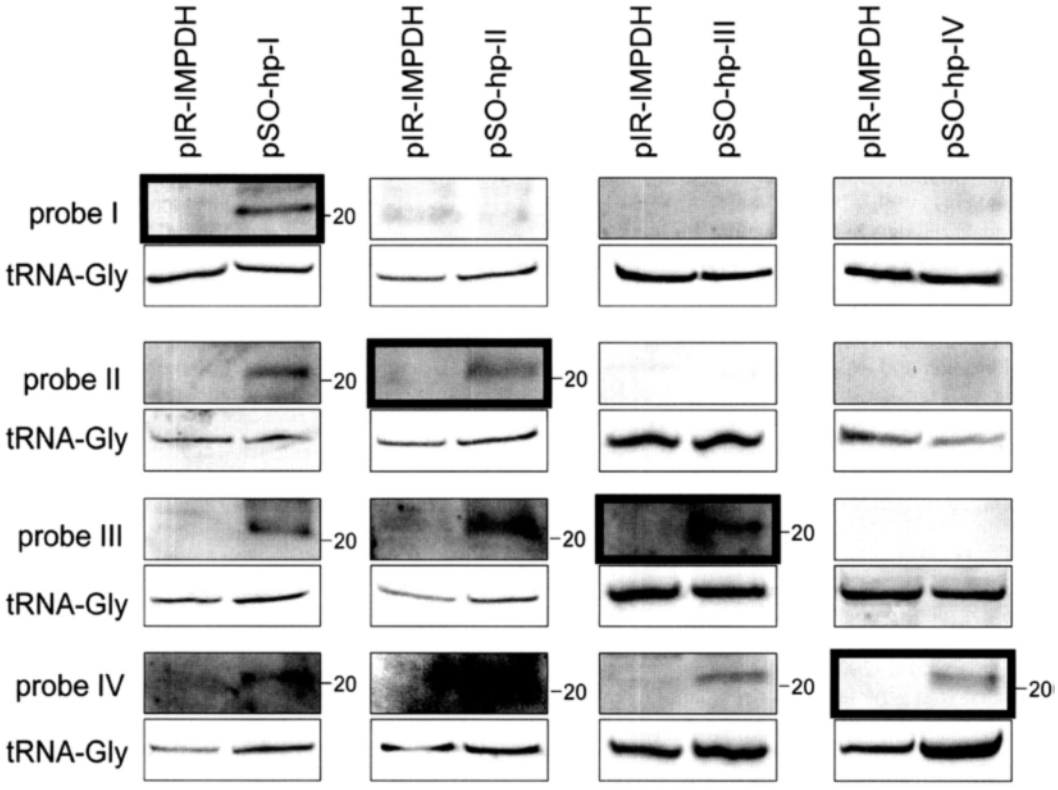

Figure 4. Detection of the *NtFAD3* siRNAs in the S24 leaves infiltrated with RNAi vectors. Four distinct sense riboprobes (probes I to IV) covering regions I to IV of the *NtFAD3* cDNA (see Fig. 1) were used. The blot was reprobed with a gene for tRNA-Gly (GCC) to verify that approximately the same amount of small RNA was loaded per lane. Little *NtFAD3* secondary siRNAs was detected in the S24 leaves infiltrated with pIR-IMPDH (left lane). The figures enclosed with a bold line show the accumulation of primary *NtFAD3* siRNAs originated from the RNAi constructs. The position of the 20-nt long ribonucleotides is shown on the right.

allowed the spreading of RNA silencing into a 5′ upstream region (Klahre et al., 2002; Himber et al., 2003; Miki et al., 2005), but preferential generation of secondary siRNAs from the 3′ downstream region of the GFP transgene was also reported in grafting-induced systemic gene silencing (Brosnan et al., 2007). Thus, the spread into a 5′ upstream region would not be regulated in a gene-specific manner. There would be unveiled cues determining the recruitment of RdRP onto the 5′ upstream fragments of the transgene. The elucidation of such factors would be important to clarify the molecular mechanisms of the transitive RNAi.

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LITERATURE CITED

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. Cell 121: 207-221

Bleys A, Vermeersch L, Van Houdt H, Depicker A (2006) The frequency and efficiency of endogene suppression by transitive silencing signals is influenced by the length of sequence homology. Plant Physiol 142: 788-796

Bonnet E, Van de Peer Y, Rouzé P (2006) The small RNA world of plants. New Phytol 171: 451-468

Brosnan CA, Mitter N, Christie M, Smith NA, Waterhouse PM, Carroll BJ (2007) Nuclear gene silencing directs reception of long-distance mRNA silencing in *Arabidopsis*. Proc Natl Acad Sci USA. 104: 14741-14746

García-Pérez RD, Houdt HV, Depicker A (2004) Spreading of post-transcriptional gene silen

Goto K, Kanazawa A, Kusaba M, Masuta C (2003) A simple and rapid method to detect plant siRNA using nonradioactive probes. Plant Mol Biol Rep 21: 51-58

Hamada T, Kodama H (2006) Phenotype of the transgene in plants: expression and silencing, in: J.A. Teixeira da Silva (Eds.), Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues, 1st edn. Vol. II, Grobal Science Books, London UK, pp 98-107

Hamada T, Kodama H, Takeshita K, Utsumi H, Iba K (1998) Characterization of transgenic tobacco with an increased α-linolenic acid level. Plant Physiol 118: 591-598

Hannon GJ (2002) RNA interference. Nature 418: 244-251

Haque AK, Tanaka Y, Sonoda S, Nishiguchi M (2007) Analysis of transitive RNA silencing after grafting in transgenic plants with the coat protein gene of *Sweet potato feathery mottle virus*. Plant Mol Biol 63: 35-47

Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O (2003) Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO J 22: 4523-4533

Hirai S, Oka S, Adachi E, Kodama H (2007) The effects of spacer

sequences on silencing efficiency of plant RNAi vectors. Plant Cell Rep 26: 651-659

Horiguchi G (2004) RNA silencing in plants: a shortcut to functional analysis. Differentiation 72: 65-73

Klahre U, Crété P, Leuenberger SA, Iglesias VA, Meins Jr F (2002) High molecular weight RNAs and small interfering RNAs induce systemic posttranscriptional gene silencing in plants. Proc Natl Acad Sci USA 99: 11981-11986

Kodama H, Hamada T, Horiguchi G, Nishimura M, Iba K (1994) Genetic enhancement of cold tolerance by expression of a gene for chloroplast ω-3 fatty acid desaturase in transgenic tobacco. Plant Physiol 105: 601-605

Kodama H, Akagi H, Kusumi K, Fujiwara T, Iba K (1997) Structure, chromosomal location and expression of a rice gene encoding the microsome ω-3 fatty acid desaturase. Plant Mol Biol 33: 493-502

Kościańska E, Kalantidis K, Wypijewski K, Sadowski J, Tabler M (2005) Analysis of RNA silencing in agroinfiltrated leaves of *Nicotiana benthamiana* and *Nicotiana tabacum*. Plant Mol Biol 59: 647-661

Llave C, Kasschau KD, Carrington JC (2000) Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proc Natl Acad Sci USA 97: 13401-13406

Miki D, Itoh R, Shimamoto K (2005) RNA silencing of single and multiple members in a gene family of rice. Plant Physiol 138: 1903-1913

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Physiol 37: 49-59

Petersen BO, Albrechtsen M (2005) Evidence implying only unprimed RdRP activity during transitive gene silencing in plants, Plant Mol. Biol 58: 575-583

Ronemus M, Vaughn MW, Martienssen RA (2006) MicroRNA-targeted and small interfering RNA-mediated mRNA degradation is regulated by argonaute, dicer, and RNA-dependent RNA polymerase in *Arabidopsis*. Plant Cell 18: 1559-1574

Shimamura K, Oka S, Shimotori Y, Ohmori T, Kodama H (2007) Generation of secondary small interfering RNA in cell-autonomous and non-cell autonomous RNA silencing in tobacco. Plant Mol Biol 63: 803-813

Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RH, Fire A (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107: 465-476

Tang G, Reinhart BJ, Bartel DP, Zamore PD (2003) A biochemical framework for RNA silencing in plants. Genes Dev 19: 49-63

Tomita R, Hamada T, Horiguchi G, Iba K, Kodama H (2004) Transgene overexpression with cognate small interfering RNA in tobacco. FEBS Lett 573: 117-120

Vaistij FE, Jones L, Baulcombe DC (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell 14: 857-867

Van Houdt H, Bleys A, Depicker A (2003) RNA target sequences promote spreading of RNA silencing. Plant Physiol 131: 245-253

Wassenegger M, Krczal G (2006) Nomenclature and functions of RNA-directed RNA polymerases. Trends Plant Sci 11: 142-151

Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of *trans*-acting siRNAs in *Arabidopsis*. Genes Dev 19: 2164-2175

Zhao MM, An DR, Huang GH, He ZH, Chen JY (2005) A viral protein suppresses siRNA-directed interference in tobacco mosaic virus infection. Acta Biochim Biophys Sin (Shanghai) 37: 248-253