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ORIGINAL ARTICLE

Cosuppression and RNAi induced by *Arabidopsis* ortholog gene sequences in tobacco

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Abstract The *Arabidopsis* ω-3 fatty acid desaturase (AtFAD7) catalyzes the synthesis of trienoic fatty acids (TA). A transgenic tobacco line, T15, was produced by a sense *AtFAD7* construct and showed a cosuppression-like phenotype, namely extremely low TA levels. The sequence similarity between *AtFAD7* and a tobacco ortholog gene, *NtFAD7*, was moderate (about 69%) in the coding sequences. *AtFAD7* siRNAs accumulated at a high level, and both *AtFAD7* and *NtFAD7* mRNAs are degraded in T15 plants. The low-TA phenotype in T15 was dependent on a tobacco RNA-dependent RNA polymerase6 (NtRDR6). We also produced tobacco RNAi plants targeting *AtFAD7* gene sequences. The *AtFAD7* siRNA level was trace, which was associated with a slight reduction in leaf TA level. Unexpectedly, this RNAi plant showed an increased *NtFAD7* transcript level. To investigate the effect of translational inhibition on stability of the *NtFAD7* mRNAs, leaves of the wild-type tobacco plants were treated with a translational inhibitor, cycloheximide. The level of *NtFAD7* mRNAs significantly increased after cycloheximde treatment. These results suggest that the translational inhibition by low levels of *AtFAD7* siRNAs or by cycloheximide increased stability of *NtFAD7* mRNA. The degree of silencing by an RNAi construct targeting the *AtFAD7* gene was increased by co-existence of the *AtFAD7* transgene, where NtRDR6-dependent amplification of siRNAs occurred. These results indicate that NtRDR6 can emphasize silencing effects in both cosuppression and RNAi.

Keywords Plastid ω-3 fatty acid desaturase · RNA-dependent RNA polymerase · RNA silencing · Transitivity · Translational repression

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Introduction

RNA silencing is a sequence-specific inhibitory mechanism of gene expression, in which 21- to 24-nucleotide (nt) small RNA molecules (called siRNAs and miRNAs) determine the specificity of target genes. RNA silencing can be manipulated by introduction of double-stranded RNA (dsRNA) molecules into plant cells, and this type of silencing is often called RNA interference (RNAi). dsRNAs are processed by DICER-like protein4 (DCL4), and small interfering RNAs (siRNAs) are generated. The 21-nt single-stranded siRNAs (ss-siRNA) are incorporated into ARGONAUTE1 (AGO1) protein, and the resulting complex guides cleavage of complementary mRNA molecules (Baumberger and Baulcombe 2005; Hammond 2005; Brodersen and Voinnet 2006; Vaucheret 2006; Ossowski et al. 2008). In contrast, 24-nt ss-siRNAs bound to AGO4 can induce cytosine methylation in the genome (RNA-directed DNA methylation, RdDM; Wassenegger et al. 1994). For induction of RNAi, plant cells are transformed with a vector in which target sequences are arranged in an inverted repeat manner (Brodersen and Voinnet 2006; Hirai and Kodama 2008). After transcription, the inverted repeat sequences form a dsRNA structure, and

then siRNAs are generated. The natural role of siRNAs is apparently involved in a defense mechanism against invasive nucleic acids, such as viruses and transposons (Robert et al. 2004; Voinnet 2005; Ding and Voinnet 2007). siRNAs harboring viral sequences are generated in the process of anti-viral plant responses. siRNAs originating from retrotransposon sequences are involved in the maintenance of the silent condition of transposable elements via the RdDM pathway (Qi et al. 2006; Zhang et al. 2007; Girard and Hannon 2008; Mosher et al. 2008).

RNA silencing often occurs in plants transformed with a sense transgene. The expression of both transgenes and homologous endogenous genes are simultaneously suppressed, and this type of RNA silencing has been called cosuppression (Napoli et al. 1990; van der Krol et al. 1990). In cosuppression, dsRNAs are synthesized by the plant-encoded, RNA-dependent RNA polymerase6 (RDR6) on templates of sense transgene transcripts (Dalmay et al. 2000; Mourrain et al. 2000; Brodersen and Voinnet 2006).

Although a few mismatches in the siRNA–mRNA pair are expected to be permissive for triggering RNA silencing in *Drosophila* and mammalian cells (Elbashir et al. 2001; Jackson et al. 2003; Saxena et al. 2003), sequence identity with at least 22-nt continuous stretches is required for off-target silencing in plants (Xu et al. 2006). Generally, a high sequence similarity between inverted repeat sequences of RNAi constructs and their target sequences are required for elicitation of RNAi. For example, an RNAi vector targeting the *OsRac1* gene that encodes a rice small GTP-binding protein inhibits the expression of the *OsRac1* gene, but it does not inhibit the expression of six other genes which are the members of the *OsRac* gene family. The sequence identities among the members are 72–82% (Miki et al. 2005). In the case of cosuppression, however, RNA silencing could be elicited by introduction of a sense transgene which shows a low sequence similarity (<90%) with corresponding endogenous genes. For instance, a tobacco β-1,3-glucanase transgene could induce the repression of another class I glucanase gene (*GN1*) which shows moderate sequence identity (81%) with the transgene (de Carvalho Niebel et al. 1995). Although siRNAs are a key component of the determination of sequence-specificity in both the RNAi and cosuppression pathways, it is not clear whether or not differences in silencing specificity exist between conventional RNAi and cosuppression.

Introduction of a heterologous sense transgene often causes a cosuppression-like phenomenon in which the sense transgene and endogenous counterpart orthologous to the transgenes are simultaneously inactivated. Kodama et al. (1994) produced several transgenic lines in which *Arabidopsis* plastid ω-3 fatty acid desaturase gene (*AtFAD7*) was overexpressed. Plastidial hexadecadienoic acid (16:2) and linoleic acid (18:2) were desaturated by *AtFAD7*, and contents of hexadecatrienoic acid (16:3) and α-linolenic acid (18:3) were increased in the overexpressed lines. Representatives of such overexpressed line are T1 and T6 lines (Kodama et al. 1994). The same transgene can trigger a cosuppression-like inhibition of production of trienoic fatty acids (TA, 16:3 and 18:3) in several transgenic lines such as T15 and T23 (Murakami et al. 2000). The sequence identity between the *AtFAD7* gene and its tobacco ortholog gene (*NtFAD7*; Hamada et al. 1996) is 68.7% at the nucleotide level for the entire coding region. If the low-TA phenotype in the T15 line is established by RNA silencing, the *AtFAD7* siRNAs should interfere with expression of the *NtFAD7* gene. To investigate the silencing ability of *AtFAD7* siRNAs, we introduced an RNAi vector targeting the *AtFAD7* gene into tobacco plants. The resulting RNAi tobacco plants (a representative line, M47) showed a significantly weak silencing phenotype when compared with the T15 plants. Interestingly, the *AtFAD7* siRNAs inhibited *NtFAD7* expression at the translational level. In addition, a tobacco RDR6 (NtRDR6) is also important not only to establish the sense-transgene induced silencing but also to increase the degree of silencing in RNAi.

Materials and methods

Plasmid construction

pBI121 binary vector (Clontech) was used in the construction of pAthpKM (Fig. S1). *Bam*HI and *Xba*I sites were added to the 5′ and 3′ ends of a 744-bp sense-orientated *AtFAD7* cDNA fragment (nucleotide position 380–1123, GenBank accession no. NM_111953) by means of PCR with primers harboring these restriction enzyme sites. Similarly, *Xba*I and *Sac*I sites were created at the 5′ and 3′ ends of a 623-bp *AtFAD7* antisense fragment (nucleotide position 380–1002) by PCR. The 744-bp *Bam*HI–*Xba*I fragment *AtFAD7* sense fragment and the 623-bp *Xba*I–*Sac*I antisense fragment were ligated into the *Bam*HI/*Sac*I sites of pBI121 to produce a plasmid, pAthpKM. pSH-hp-RDR6 was constructed as previously described (Oka et al. 2008). This plasmid contains two 669-bp *NtRDR6* sequences (nucleotide position 1890–2599, GenBank accession no. AB361628) in an inverted repeat manner. These two fragments are separated by a 1,007-bp *GUS* fragment (nucleotide position 783–1,789).

Production of transgenic plants

Two transgenic lines, T6 and T15, were produced by introduction of a sense *AtFAD7* transgene (pTiDES7) as previously described (Kodama et al. 1994; Murakami et al.

2000). *Nicotiana tabacum* cv. SR1 was transformed by the leaf-disc method (Horsch et al. 1985) by using *Agrobacterium tumefaciens* C58 containing pAthpKM to generate the M47 plants. Transformants were selected on Murashige–Skoog medium containing 100 mg/L kanamycin sulfate. pSH-hp-RDR6 was introduced into the homozygous T15 plants by *Agrobacterium*-mediated transformation. The sequentially transformed plants were selected on a culture medium containing 20 mg/L hygromycin B, and a resulting representative line was designated T15-ΔRDR6. An RNAi construct targeting *NtFAD7* gene was introduced into tobacco plants to generate the N7-2 plants as previously described by Hamada et al. (2006). The N7-2 plants showed an extremely low TA phenotype in leaves and the *NtFAD7* siRNAs accumulated (Hamada et al., unpublished results). The homozygous T6 plants were crossed with the homozygous M47 plants.

Fatty acid analysis

Fatty acid composition was determined as previously described (Kodama et al. 1994).

Extraction of total RNA and northern blot analysis

Total RNA was extracted from tobacco leaves with TriPure Isolation Reagent (Roche) according to the manufacturer's protocols. The digoxigenin-labeled DNA probes for the *NtFAD7* and *AtFAD7* gene were prepared by the manufacturer's protocol. An 895-bp fragment of *NtFAD7* cDNA (nucleotide position 107-1001, GenBank accession no. D79979) and a 722-bp fragment of *AtFAD7* cDNA (nucleotide position 792–1513) were used for producing both DNA probes. Total RNAs (10 μg) were electrophoresed on a 1% agarose gel as previously described (Kodama et al. 1991), and then they were transferred to nylon membranes. The blots were hybridized with DNA probes at 50°C in DIG Easy Hyb solution (Roche). The membrane was washed twice with 2 × SSC, 0.1% SDS, and twice with 0.1 × SSC, 0.1% SDS at 50°C. Hybridized probes were detected using CDP-star reagent (GE Healthcare).

Extraction of small RNA and northern blot analysis

The small RNA-enriched fraction was prepared from leaves according to the protocol described by Goto et al. (2003). About 30 μg of small-RNA-enriched nucleic acids were separated on an 18% (w/v) polyacrylamide gel containing 7 M urea, and then transferred onto nylon membranes. Hybridization and visualization of hybridized probes were performed as previously described (Hirai et al. 2007). We prepared three riboprobes. A riboprobe that covered a 502-bp region of *NtFAD7* cDNA (nucleotide position 786–1287) was used to detect the *NtFAD7* siRNA. The *AtFAD7* riboprobe that covered a 1,156-bp *AtFAD7* cDNA region (nucleotide position 405–1560) was used to detect *AtFAD7* siRNAs in T15 and M47 plants. The *AtFAD7* riboprobe covered a 314-bp fragment of *AtFAD7* cDNA (nucleotide position 1247–1560) and was used to detect secondary *AtFAD7* siRNAs in M47 × T6 plants.

Cycloheximide (CHX) treatment

Young leaves were detached from plants by cutting them off at the base of the petiole with a razor blade. Cut ends were immersed in aqueous solution supplemented with 0, 3.5 μM and 35 μM CHX as previously described by Nishiuchi et al. (2002). Aliquots of leaves were collected at 30, 60, and 180 min after the treatment was started, and they were subjected to northern analysis.

Results

Silencing of *NtFAD7* gene by *AtFAD7* siRNAs

A sense construct, pTiDES7, in which expression of the *AtFAD7* gene was controlled by the cauliflower mosaic virus (CaMV) 35S promoter (Fig. S1), was introduced into tobacco wild-type (WT) plants (Kodama et al. 1994). Most of them showed increased TA levels. The *AtFAD7* mRNAs accumulated in leaves of T6 plants (an overexpressor line of the *AtFAD7* gene). In contrast, 4 of 29 independent *AtFAD7* transformants showed a significant reduction in the TA level (Murakami et al. 2000). Here, the T15 line is used as a representative showing a cosuppression-like phenotype. Hemizygous T15 plants showed moderately reduced TA levels, and the plants homozygous for T-DNA plants showed more enhanced TA reduction (Fig. 1a). *AtFAD7* siRNAs were present in T15 plants, and levels of both *AtFAD7* and *NtFAD7* mRNAs were markedly low in T15 plants (Fig. 1b, c). We examined whether or not the *AtFAD7* siRNAs are responsible for inhibition of TA accumulation in T15 plants. An RNAi vector targeting the *NtRDR6* gene (Oka et al. 2008) was introduced into the homozygous T15 plants. This double-transformant (T15-ΔRDR6) showed a significant reduction of *AtFAD7* siRNA level (Fig. 1c), which resulted in increased TA levels similar to that of the homozygous T6 plants (Fig. 1a), and recovered expression of both *AtFAD7* and *NtFAD7* genes was observed (Fig. 1b). Therefore, the low-TA phenotype in T15 is dependent on a NtRDR6-dependent RNA silencing mechanism.

The sequence similarity between the *AtFAD7* and *NtFAD7* cDNAs is not so high (about 68.7% identity in the coding region), and a 22- and 24-nt continuous identical

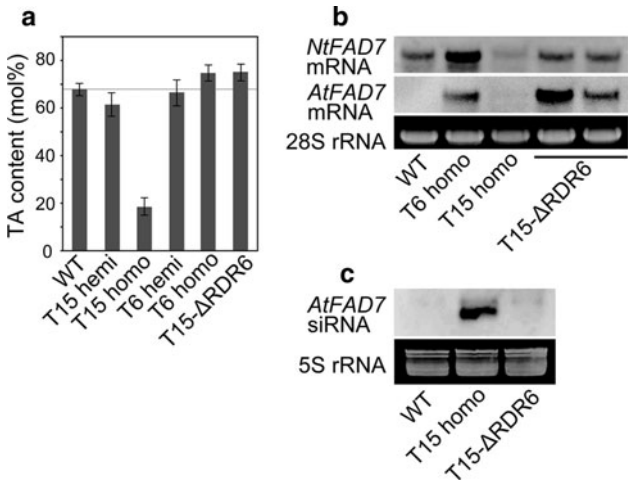

Fig. 1 The *NtRDR6*-dependent silencing in tobacco plants transformed with the *AtFAD7* sense transgene. **a** TA levels in total fatty acids of leaf tissues. *Vertical lines* SD ($n = 5$). **b** The amounts of *NtFAD7* and *AtFAD7* mRNAs in leaf tissues. **c** The amounts of *AtFAD7* siRNAs in leaf tissues. *Hemi* and *homo* hemizygous and homozygous T-DNA allele, respectively. The hygromycin-resistant seedlings were selected after self-pollination of hygromycin-resistant transformants (T15-ΔRDR6), and were subjected to these analyses

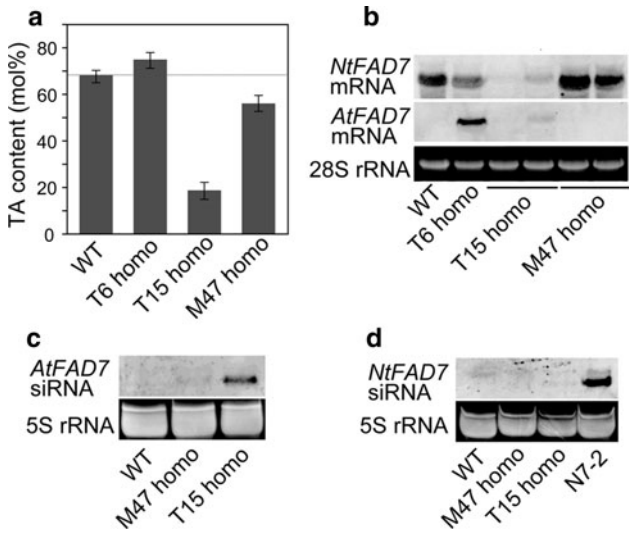

Fig. 2 Characterization of RNAi tobacco plants targeting the *AtFAD7* gene. **a** TA levels in total fatty acids of leaf tissues. *Vertical lines* SD ($n = 5$). **b** The amounts of *NtFAD7* and *AtFAD7* mRNAs in leaf tissues. **c** The amounts of *AtFAD7* siRNAs in leaf tissues. **d** The amounts of *NtFAD7* siRNAs in leaf tissues. The kanamycin-resistant seedlings were obtained from the T2 seeds of N7-2 plants (Hamada et al. 2006)

stretches are found in these two genes (Fig. S2). We questioned whether *AtFAD7* siRNAs can suppress expression of the *NtFAD7* gene. An RNAi vector targeting the *AtFAD7* gene was constructed (pAthpKM) and then introduced into tobacco WT plants. A 590-bp-fragment of the *AtFAD7* cDNA (nucleotide position, 408–997) was arranged in an inverted repeat structure. A 98-bp fragment (998–1095 of the *AtFAD7* cDNA) was used as a spacer (Fig. S1). The fragment used in a stem of this RNAi vector covers an *AtFAD7* cDNA region showing moderate similarity (about 78.0%) to the corresponding region of the *NtFAD7* cDNA. Both continuous identical stretches (22- and 24-nt long regions) found in these two cDNAs were included in this target region. We prepared 50 independent transgenic RNAi lines, and the M47 line showed the lowest TA level among the transgenic lines produced.

If *AtFAD7* siRNAs can efficiently guide silencing of the *NtFAD7* gene, a phenotype of M47 plants would be close to that of T15 plants. The leaf TA level of homozygous M47 plants was lower than that of the WT plants, but the degree of reduction was very limited (Fig. 2a). Interestingly, the *NtFAD7* mRNA level did not decrease and was rather elevated in the M47 plants (Fig. 2b). The *AtFAD7* siRNAs accumulated at only a trace level in M47 (Fig. 2c). We also examined generation of the *NtFAD7* siRNAs in the M47 and T15 plants. As a control, an RNAi plant targeting the *NtFAD7* gene (line name, N7-2) was used (Hamada et al. 2006). The *NtFAD7* siRNAs were present in the N7-2 plants but were under the detection level in T15 and M47 (Fig. 2d). These results suggest that a high level of *AtFAD7* siRNA was responsible for silencing of both *AtFAD7* transgene and *NtFAD7* endogenous gene in the T15 plants. Because the level of *NtFAD7* siRNAs was under the detection level, generation of the *NtFAD7* siRNAs was limited in the T15 plants.

Increase in the level of *AtFAD7* siRNAs by co-existence of their homologous target

The level of *AtFAD7* siRNA was very low in M47 plants even though transcription of a hairpin construct was controlled by the CaMV 35S promoter (Fig. 2c). The same promoter was used for transcription of the hairpin RNA targeting the *NtFAD7* gene in N7-2 plants, and the *NtFAD7* siRNAs could be clearly detected (Fig. 2d). We examined whether the level of siRNAs is affected by the presence of their homologous target transcripts. For this purpose, a homozygous M47 plant was crossed with a homozygous T6 plant. Because the *AtFAD7* transgene is successfully expressed in T6 plants, offspring of this cross (M47 × T6) should simultaneously express the *AtFAD7* mRNAs and *AtFAD7* siRNAs. The crossbred plants showed markedly increased levels of *AtFAD7* siRNAs than the hemizygous M47 plants (Fig. 3c), and the *AtFAD7* mRNAs originating from the T6 transgenic locus were degraded (Fig. 3b). The level of *NtFAD7* mRNA was up-regulated in the hemizygous M47 plants (Fig. 3b) as was observed in the homozygous M47 plants (Fig. 2b), whereas the M47 × T6

plants showed a significant decrease in the *NtFAD7* mRNAs (Fig. 3b).

Co-existence of the transgene mRNAs and the siRNAs complementary to the transgene mRNAs frequently induces synthesis of secondary siRNAs (Vaistij et al. 2002; Himber et al. 2003; Kościańska et al. 2005; Miki et al. 2005; Petersen and Albrechtsen 2005; Shimamura et al. 2007). Formation of the siRNA/mRNA duplex recruits RDR6, which is followed by synthesis of complementary antisense RNAs on the template of sense transgene mRNAs (Voinnet 2008). The resultant long dsRNAs are processed by DCL4, and secondary siRNAs are generated. In several cases, the degree of RNA silencing is enhanced when secondary siRNAs are synthesized (Bleys et al. 2006; Shimamura et al. 2007). Interestingly, the degree of reduction in the TA level was also strengthened in the M47 × T6 plants when compared with those of the hemizygous M47 plants (Fig. 3a) and hemizygous T15 plants (Fig. 1a). We investigated the generation of secondary *AtFAD7* siRNAs in the M47 × T6 plants. Since secondary siRNAs have been detected at the 3′ downstream region of the transgene sequences relative to the target region of the primary siRNAs (Oka et al. 2008), we used the *AtFAD7* riboprobe covering the 3′ most downstream region (nucleotide position 1247–1560 of the *AtFAD7* cDNA). As expected, the *AtFAD7* siRNAs corresponding to the 3′ most downstream region were present in the M47 × T6 plants but not in the hemizygous M47 plants (Fig. 3e). These results indicate that NtRDR6 was recruited on the mRNAs produced from the *AtFAD7* transgene and that secondary *AtFAD7* siRNAs were produced in M47 × T6 plants. Since the level of *NtFAD7* siRNAs was still trace in the M47 × T6 plants (Fig. 3d), generation of secondary siRNAs remained limited on the template of *NtFAD7* transcripts.

Stability of *NtFAD7* transcripts was increased by CHX treatment

The TA level was slightly lower in the M47 plants than in the WT plants (Figs. 2a and 3a). The M47 plants did not show any decrease in the *NtFAD7* mRNA level, rather an increase in the *NtFAD7* mRNA level was observed (Figs. 2b and 3b). Therefore, the low TA phenotype in M47 should be accounted by translational inhibition of the *NtFAD7* gene via the trace level of *AtFAD7* siRNAs. We questioned whether or not stability of the *NtFAD7* mRNAs is affected when translation was compromised by treatment with a protein synthesis inhibitor, CHX. The level of *NtFAD7* mRNAs in tobacco leaves increased 1 h after treatment of CHX. After 3-h treatment, a significant increase of *NtFAD7* mRNA level was observed. This effect of CHX on the *NtFAD7* mRNA level was dose-dependent, and more enhanced accumulation of the *NtFAD7* mRNAs

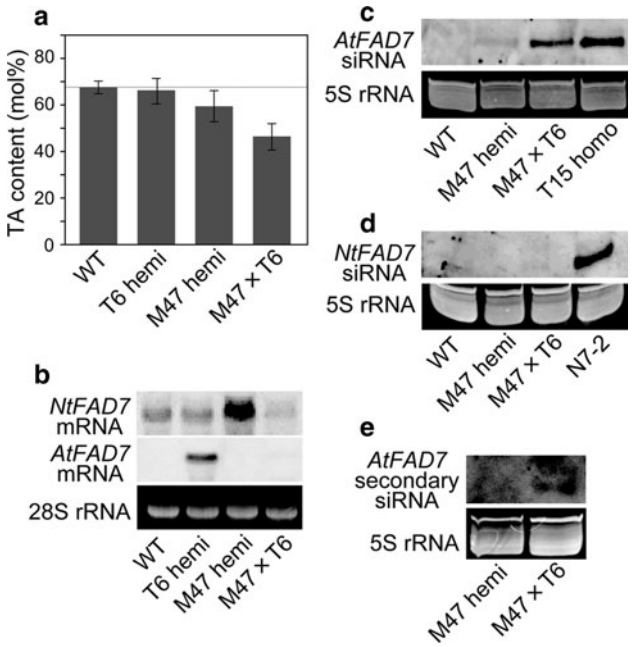

Fig. 3 Enhanced silencing in the crossbred line between M47 and T6 plants. **a** TA levels in total fatty acids of leaf tissues. *Vertical lines* SD ($n = 5$). **b** The amounts of *NtFAD7* and *AtFAD7* mRNAs in leaf tissues. **c** The amounts of *AtFAD7* siRNAs in leaf tissues. **d** The amounts of *NtFAD7* siRNAs in leaf tissues. **e** Detection of secondary *AtFAD7* siRNAs in the crossbred plants

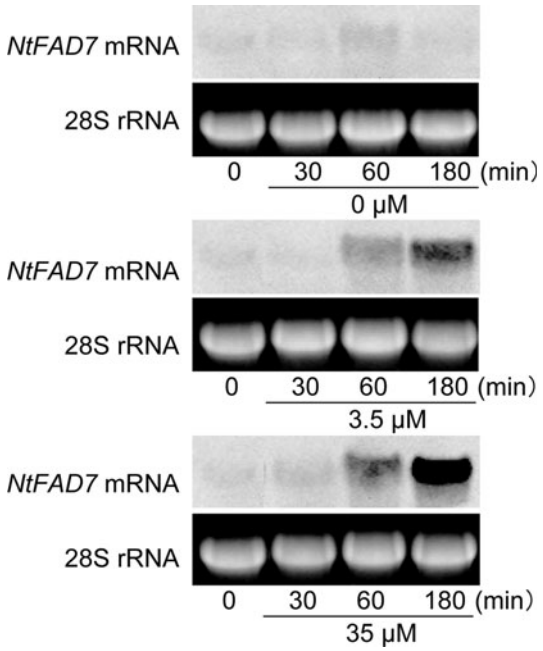

Fig. 4 Effects of CHX on the stability of *NtFAD7* mRNA in tobacco leaves. Detached WT leaves were treated with 0, 3.5 or 35 μM CHX. After treatment for 30, 60, and 180 min, total RNA was extracted. Total RNA (10 μg) was subjected to RNA gel blot analysis

was obtained in a high-dose experimental group (Fig. 4). This observation is in good agreement with increased stability of the *NtFAD7* mRNAs in M47 plants.

Discussion

Sense-transgene induced silencing is now widely recognized as one of the variations in RNA silencing (Tomita et al. 2004; Luo and Chen 2007; Voinnet 2008). However, detailed analysis of silencing-like phenomena has been limited when ortholog and paralog genes are used as a sense transgene. The moderate similarity between the ortholog transgene and corresponding endogenous genes blocks straightforward interpretation in which siRNAs originating from ortholog transcripts guide the silencing. We showed that the low-TA phenotype in T15 was cancelled by knock-down of the *NtRDR6* gene (Fig. 1). Therefore, the phenomenon in T15 was clearly accounted for by the sense transgene-induced gene silencing, namely cosuppression.

The primary siRNAs can induce the generation of secondary siRNAs. This amplification of siRNAs often occurs outside the primary target region of the transcripts, and this spreading of the target region by siRNA amplification is called transitivity (Brodersen and Voinnet 2006). Transitivity is preferentially observed along the transgenes (Miki et al. 2005; Shimamura et al. 2007) and only one report showed transitivity on endogenous plant genes (Sanders et al. 2002). Consistent with this, we found that the *NtFAD7* gene did not serve as the template of secondary siRNA synthesis in T15 and M47 plants (Figs. 2d and 3d). At present, we cannot rule out the possibility in which a very low level of *NtFAD7* siRNAs was generated in these plants. If secondary siRNAs are generated from the *NtFAD7* mRNAs, the resulting siRNAs have perfect complementarity and should degrade the homologous *NtFAD7* mRNAs. In this respect, we prefer an explanation in which the *AtFAD7* siRNAs, and not the *NtFAD7* siRNAs, are a key component of the RNA silencing in M47 plants. One conspicuous result is the quite low level of the *AtFAD7* siRNAs in both homozygous and hemizygous M47 plants. In *Caenorhabditis elegans*, release of a miRNA molecule from the miRNA–AGO complex and subsequent degradation of free miRNA molecules can both be inhibited in vitro by the addition of target mRNA molecules (Chatterjee and Grosshans 2009). If similar mechanisms exist in plant cells, stability of siRNAs should decrease in the absence of their target mRNA molecules as was observed in the M47 plants (Figs. 2c and 3c).

The increased level of the *NtFAD7* mRNA in M47 plants and also in the CHX-treated WT plants indicated that stability of the *NtFAD7* mRNA was increased by translational repression. These observations reveal two important aspects of RNA silencing. One is the siRNA-mediated translational repression. Translational repression has been known as one of the silencing mechanisms by miRNAs. The degree of miRNA–mRNA complementarity is a key determinant of translational repression. Central mismatches in the miRNA–mRNA duplex promote translational repression (Hutvágner and Zamore 2002; Song et al. 2004). In contrast, silencing by siRNAs with perfect complementarity is always associated with mRNA degradation. Brodersen et al. (2008) demonstrated by using *ago1-27* plants that siRNAs can mediate translational repression in addition to mRNA degradation. Here, we further showed that low level of siRNAs with moderate similarity to the target mRNA can preferentially mediate translational repression without mRNA degradation. Translational repression is apparently established when the siRNA population with moderate similarity to the target was very low level as seen in M47 plants (Figs. 2c and 3c). An increased level of *AtFAD7* siRNA population should mediate degradation of the *NtFAD7* mRNA as was the case of M47 × T6 plants (Fig. 3b). In the latter case, the level of 21-nt siRNA molecules with perfect complementarity should increase and guide degradation of *NtFAD7* mRNA. The second one is that more attention should be paid to off-target phenomena. The potential target of the *AtFAD7* siRNAs should include the *NtFAD7* ortholog gene. However, an increased level of the *NtFAD7* mRNA would compromise the identification as potential targets of *AtFAD7* siRNAs. CHX has been shown to increase mRNA stability of several plant genes including the soybean *SAUR* genes, rice α-amylase gene, alfalfa histone H3 gene, and *Arabidopsis MYB2* genes (Franco et al. 1990; Gil et al. 1994; Sheu et al. 1994; Kapros et al. 1995; Hoeren et al. 1998). In addition, 2.1% (78/3,700) of all the normalized cotton ovule ESTs were up-regulated by CHX treatment (Wu et al. 2005). These previous reports imply that a small, but significant, proportion of genes may increase the abundance of their transcripts after translational repression.

The silencing by *AtFAD7* siRNAs was enhanced in M47 × T6 plants (Fig. 3a). Although the primary cause of the enhanced silencing should be accounted for by amplification of the *AtFAD7* siRNAs, another possibility can be provided. Recent studies suggested that primary siRNAs produced from plant viruses in the absence of RDR6 cannot guide efficient silencing and that more active silencing was associated with the RDR6-dependent secondary siRNAs (Vaistij and Jones 2009; Wang et al. 2009). In RNAi plants, hairpin RNAs were processed into siRNAs by the RDR6-independent pathway as was the case of M47 plants. In contrast, secondary *AtFAD7* siRNAs were produced by the RDR6-dependent pathway in M47 × T6 plants, and active degradation of both *AtFAD7* and

NtFAD7 mRNAs was observed. Therefore, it is also possible that primary siRNAs generated from the hairpin RNA and secondary siRNAs have different activities of RNA silencing. The role of RDR6 in the establishment of efficient silencing remains to be solved.

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