Transgene overexpression with cognate small interfering RNA in tobacco

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Abstract **Small interfering RNAs (siRNAs) are a key component of RNA silencing, including cosuppression. Here, we show an example in which siRNA does not serve in the downregulation of target genes. A tobacco endoplasmic reticulum ω-3 fatty acid desaturase (NtFAD3) catalyzes the formation of α-linolenate (18:3). Introduction of the *NtFAD3* gene into tobacco plants caused strong reduction of 18:3 content in leaf tissues, which is associated with the production of the *NtFAD3* siRNAs. However, this silencing effect was lacking in the root tissues. Both the introduced *NtFAD3* and endogenous *NtFAD3* genes were expressed successfully, and the roots showed increased 18:3 phenotype. Surprisingly, the *NtFAD3* siRNAs were produced even in the root tissues. Expression of a hairpin double-stranded RNA against the *NtFAD3* gene caused efficient reduction of 18:3 content in root tissues. Therefore, cosuppression of the *NtFAD3* gene in tobacco appears to include an as yet unidentified developmental stage and tissue-specific mechanism of regulation of siRNA function.**

Keywords: Cosuppression; Fatty acid desaturase; RNA interference; Short interfering RNA; α-Linolenate

1. Introduction

RNA silencing is a novel inhibitory mechanism for gene expression that includes reduction of the level of target transcripts in a sequence-specific manner [1,2]. RNA silencing can be induced artificially by introduction of double-stranded RNAs (dsRNAs) or hairpin dsRNAs into cells. This phenomenon termed RNA interference (RNAi) is observed in a number of organisms including animals and plants [3–5]. From the extensive investigation of RNAi mechanisms, we now know that small interfering RNAs (siRNAs) are a key component that determines the sequence specificity in RNA silencing [6]. These small RNAs are the cleavage product of an RNase III-like endonuclease, Dicer [7,8]. siRNAs assemble into a multiprotein RNA silencing complex (RISC) that retains either sense and antisense strands of the duplex. Base pairing between the antisense small RNA and the cognate mRNA in RISC directs mRNA cleavage [9,10].

This RNAi pathway accounts for the sense-transgene-induced RNA silencing, which has been initially found in plants and termed post-transcriptional gene silencing (PTGS) or cosuppression [11]. Within the plants transformed with the sense transgene sequence, several plants fail to express the introduced gene and the corresponding homologous endogenous genes. Current models of PTGS/cosuppression propose that aberrant RNA formation should be important in the initial step. Aberrancy can have either a quantitative or qualitative basis [12]. Aberrant RNAs would be produced though prematurely terminated transcription, transcription from cryptic flanking promoter [13], and improper processing [14]. These aberrant RNAs are then converted into dsRNAs by an RNA-dependent RNA polymerase [15,16], which is followed by the Dicer-mediated generation of duplex siRNAs. The resultant siRNAs are incorporated into the RNAi pathway as a guide of RNA degradation [17]. The components, including SGS2/SDE1, SDS3, AGO1, and HEN1, would play roles upstream of formation and dicing of dsRNA, and they are not required for the hairpin dsRNA-induced silencing activities [18]. Therefore, sense-transgene-induced RNA silencing requires additional components that are dispensable in the hairpin dsRNA-induced RNAi machinery.

Sense-transgene-induced gene silencing can also be found in the transformed plants with genes involved in the lipid metabolism [19,20]. The tobacco endoplasmic reticulum (ER) ω-3 fatty acid desaturase (NtFAD3) catalyzes the conversion of linoleate (18:2) to α-linolenate (18:3) in glycerolipids [21]. Overexpression of the *NtFAD3* gene in tobacco plants caused successful accumulation of 18:3 [22]. Here, we characterized a cosuppression strain of the *NtFAD3* transgenic plants. A strain designated S44 exhibited significantly lower 18:3 level than the wild-type (WT) plants, indicating that both introduced *NtFAD3* gene (*trans-NtFAD3*) and endogenous *NtFAD3* gene (*endo-NtFAD3*) are inactivated. Cosuppression of the *NtFAD3* gene is established in leaf tissues but is absent in root tissues. Unexpectedly, both the *trans-* and *endo-NtFAD3* genes were expressed even in the presence of cognate siRNAs, and the resultant roots show a phenotype in which the transgene is overexpressed. In contrast, the hairpin-dsRNA-mediated RNAi against the *NtFAD3* gene efficiently reduces the 18:3 content in root tissues. Therefore, cosuppression of the

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Abbreviations: 18:2, linoleate; 18:3, α-linolenate; CaMV, cauliflower mosaic virus; dsRNA, double-stranded RNA; ER, endoplasmic reticulum; GUS, β-glucuronidase; PTGS, post-transcriptional gene silencing; RISC, RNA silencing complex; RNAi, RNA interference; RT, reverse transcription; siRNA, small interfering RNA

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NtFAD3 gene includes the developmental regulation in which siRNAs function or do not function as a guide of RNA silencing.

2. Materials and methods

2.1. Plasmid construction

pTF1SIIn was constructed as previously described [22]. This plasmid contains the *NtFAD3* cDNA fragment from 52 to 1381 (see the GenBank Accession No.D26509) in sense orientation relative to the promoter sequences. In an RNAi construct, the 1005-bp β-glucuronidase (GUS) fragment was used as a spacer between the *NtFAD3* fragments in the antisense and sense orientation. pTF1AGS contained two 497-bp sequences (corresponding to nucleotide positions 181–678) of the *NtFAD3* cDNA.

2.2. Plant transformation

Nicotiana tabacum cv. SR1 was transformed by leaf disc method [23] using *Agrobacterium tumefaciens* LBA4404 containing pTF1SIIn or pTF1AGS. The kanamycin-resistant R_1 seedlings were transferred to soil.

2.3. Fatty acid analysis

Fatty acid compositions were determined as previously described [24].

2.4. Preparation of total RNA and small-RNA-enriched fractions

The total RNA was isolated with TriZOL reagent (Life technology) according to the manufacturer's protocols. When small RNAs were collected, high-molecular-weight RNAs were removed by precipitation with 6% PEG 6000 in 0.6 M NaCl. The small RNAs in the resultant supernatant were precipitated using ethanol.

2.5. Northern blot analysis of total RNAs

The *NtFAD3* cDNA was subcloned into pGEM3Zf (Promega). The digoxigenin-labeled riboprobe was prepared according to the manufacturer's protocol. Twenty micrograms of total RNAs was denatured and separated on 1% (w/v) agarose gel as previously described [24]. Separated RNAs were transferred to nylon membranes (Pall Ultrafine Filtration) by capillary blotting and hybridized with the riboprobes at 68 °C in the DIG Easy Hyb solution (Roshe). The membrane was washed twice with 2× SSC, 0.1% SDS and twice with 0.1× SSC, 0.1% SDS at 68 °C. Hybridized probes were visualized using CDP-star reagent (Amersham Biotechnology).

2.6. Northern blot analysis of small RNAs

Fifty micrograms of small RNA-enriched nucleic acids was resolved on 18% polyacrylamide/7 M urea gels and transferred onto nylon membranes by semidry electroblotting. The digoxigenin-labeled antisense and sense *NtFAD3* riboprobes were hybridized to small RNAs at 37 °C in the DIG Easy Hyb solution. The membrane was washed twice with 0.2× SSC, 0.1% SDS at room temperature and then twice at 50 °C. The same hybridization patterns were obtained with the antisense and sense *NtFAD3* probes.

2.7. Reverse transcription (RT)-PCR analysis

One microgram of total RNA was analyzed with TaKaRa One Step RNA PCR Kit (TaKaRa Biochemicals). By using primers corresponding to the Ω sequence [25] or to the 5′ untranslated region of the *endo-NtFAD3* mRNA, transcripts from the *trans-* and *endo-NtFAD3* gene can be distinguished.

2.8. Real-time RT-PCR assay

cDNAs were synthesized from 1 μg of total RNA with a reverse transcriptase (QIAGEN) and primers specific for each tested gene. The cDNAs for the *endo-NtFAD3* and actin mRNAs were amplified in separate tubes. Each PCR mixture contained 1/40 of reverse transcription mixture and cDNAs were amplified with Tbr EXT DNA polymerase (Finnzymes). Amplification and quantitation of PCR products were performed with a Roter-Gene (Corbett Research) by staining with SYBR Green (Molecular Probe).

3. Results and discussion

In this report, two sets of plant lines harboring the *NtFAD3* sequence were generated. When a sense construct (pTF1SIIn) was introduced (Fig. 1), most transgenic plants showed a markedly increased 18:3 level in both root and leaf tissues as previously reported [22]. The S24 line was used here as a typical plant for the successful overexpression of the *NtFAD3* gene. Within this set, two independently cosuppressed plant lines, S2 and S44, were obtained. These two plants showed a similar phenotype, and here we characterized the S44 plants in detail. For comparison, an RNAi construct (pTF1AGS) which produces the hairpin dsRNA for the *NtFAD3* sequence was also introduced into tobacco plants (Fig. 1). Of the 12 independent transformants in which the transgene was inserted at a single locus, four transgenic lines showed a decreased 18:3 phenotype in leaf tissues. One such RNAi line, the R11, was used as a representative of them.

In the S44 line, the low-18:3 phenotype was cosegregated with antibiotic resistance and was reproducible in at least subsequent three generations. The S44 plants hemizygous for the transgene showed the moderately reduced leaf 18:3 levels and the homozygous S44 plants exhibited the strikingly decreased 18:3 levels in leaf tissues (Fig. 2A). This decreased 18:3 level was associated with a concomitant increase of 18:2 (data not shown), indicating that the ω-3 fatty acid desaturase activity from both endogene and transgene was cosuppressed. Interestingly, the *trans-NtFAD3* gene induced cosuppression in an organ-specific manner. In root tissues, the 18:3 level of the S44 plants increased to a similar level as that seen in the S24 plants (Fig. 2A). An RT-PCR analysis was performed to determine the levels of the *trans-* and *endo-NtFAD3* mRNAs. In the S44 leaf tissues, cDNAs for the *trans-NtFAD3* mRNA were detected but cDNAs for the *endo-NtFAD3* mRNA were barely amplified (Fig. 2B). Such stronger suppression of endogene than the suppression of the transgene has been also described in cosuppression of the polygalacturonase gene [26] and chalcone synthase gene [27]. In the S44 root tissues, cDNAs for the *trans-* and *endo-NtFAD3* mRNAs were clearly detected (Fig. 2B). The real-time RT-PCR analysis showed that the level of *endo-NtFAD3* mRNA in the leaves of the S44 plants decreased to about 10% of the corresponding level in the WT plants, but no discernible silencing effects were seen in the roots of the S44 plants (Fig. 2C). These results indicate that

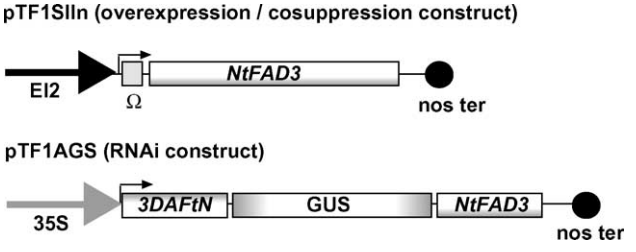

Fig. 1. Vector design for the expression of a sense mRNA that mediates overexpression/cosuppression (pTF1SIIn) and for the expression of a hairpin dsRNA that mediates RNAi against the *NtFAD3* gene (pTF1AGS). The EI2 means the enhanced cauliflower mosaic virus (CaMV) promoter sequence [25]. The 35S means the CaMV 35S promoter sequence. Arrows indicate the transcriptional start position. The nos ter means the terminator sequence from the nopaline synthase gene.

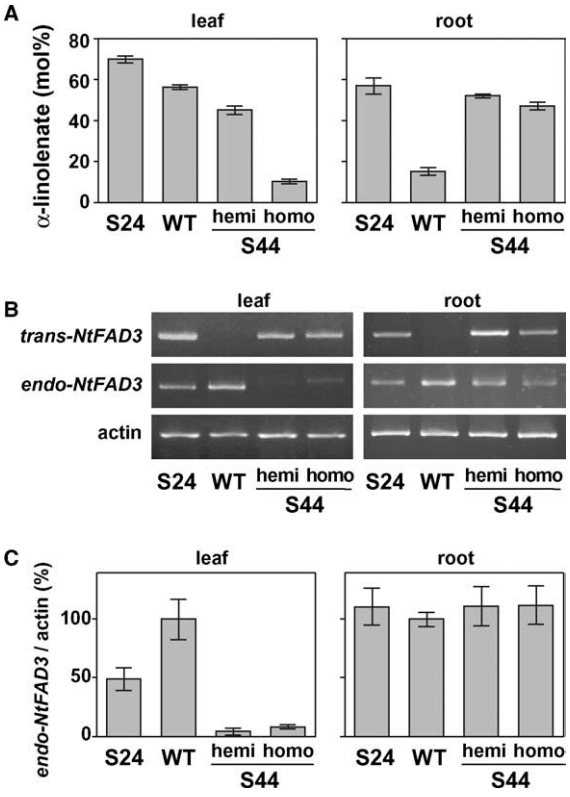

Fig. 2. Characterization of the transgenic plants with pTF1SIIn. (A) Levels of the 18:3 in total fatty acids of leaf and root tissues. Vertical lines indicate SD ($n = 10$). (B) Levels of the *NtFAD3* mRNAs in leaf and root tissues. The electrophoretograms were obtained following the RT-PCR by using total RNAs isolated from leaf and root tissues. (C) Relative levels of the *endo-NtFAD3* transcripts. The *endo-NtFAD3* mRNA level was measured by real-time RT-PCR and was normalized with the corresponding actin mRNA content. The normalized value for the WT plants was designated as 100%, and data for other plants were shown as a percentage of that of the WT plants. Hemi and homo indicate, respectively, hemizygous and homozygous T-DNA allele. The homozygous S24 plants were used.

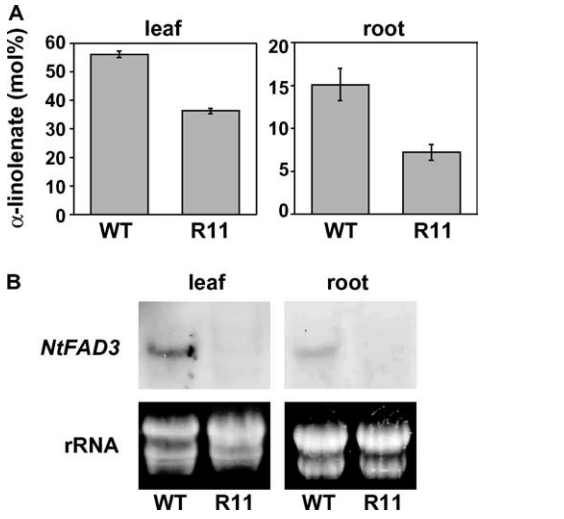

Fig. 3. Characterization of the transgenic plants with pTF1AGS. (A) Levels of 18:3 in total fatty acids of leaf and root tissues of the WT and R11 plants. Vertical lines indicate SD ($n = 5$). (B) Levels of the *endo-NtFAD3* mRNAs in leaf and root tissues. Total RNAs were analyzed by Northern hybridization with the *NtFAD3* riboprobe. The equivalence of RNA loading among lanes was demonstrated by ethidium bromide staining of rRNA bands.

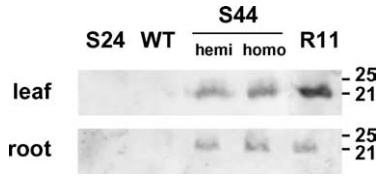

Fig. 4. siRNA accumulation. Small RNA species from leaves and roots was analyzed. The homozygous S24 and R11 plants were used. The positions of the 21- and 25-nucleotide-long oligodeoxyribonucleotides are indicated on the right.

the *trans-NtFAD3* and *endo-NtFAD3* genes are expressed successfully in the roots of the S44 plants.

We asked whether the lack of silencing phenotype in the roots of the S44 plants resulted from a defect of the RNAi pathway in this organ. Differential efficiency of RNA silencing was reported between the root and leaf tissues. In the PTGS seen in the plants producing antibody fragments, downregulation of antibody content was much less pronounced in roots than in leaves [28]. In contrast, sense-transgene-induced silencing of nitrite reductase genes occurred in both leaves and roots [29]. To investigate whether or not the RNAi pathway functions in root tissues, the phenotype of plants that produce the hairpin dsRNA for the *NtFAD3* gene was analyzed. The 18:3 content of the R11 plant is reduced by up to 50% in both leaf and root tissues (Fig. 3A). In fact, accumulation of the *endo-NtFAD3* mRNA was severely inhibited in the leaf and root tissues of the R11 plants (Fig. 3B). Thus, synthesis of the hairpin dsRNA brings effective removal of the *endo-NtFAD3* mRNA, indicating that the RNAi pathway can also be made to function in root tissues.

We next asked whether the defect of RNA silencing by co-suppression in roots of the S44 plants was due to the limited siRNA production. Hybridization of the leaf small RNA with a *NtFAD3* riboprobe showed that siRNAs were present in the S44 and R11 plants and not in the WT and S24 plants (Fig. 4). However, there is less correlation between quantity of the siRNA and degree of 18:3 reduction, since the siRNA levels are almost the same between the hemizygous and homozygous S44 plants. In our example, it is difficult to explain the severe cosuppressed phenotype in the homozygous plants only by the level of the *NtFAD3* siRNA. Then, we also hybridized the small RNAs extracted from roots with the *NtFAD3* probe. Surprisingly, the *NtFAD3* siRNAs accumulated at a similar level in root tissues of the R11 plants, hemizygous and homozygous S44 plants, indicating that in the S44 roots the overexpressed phenotype (increased 18:3 content) was achieved in the presence of siRNAs. In root tissues of the S44 plants, siRNAs appear to be synthesized via cleavage of dsRNAs converted from the *trans-NtFAD3* mRNA, but they did not trigger the degradation of *endo-NtFAD3* mRNA and did not interfere with the expression of residual *trans-NtFAD3* mRNA.

The RNAi pathway itself functions in all tissues where the promoter used for production of the hairpin dsRNA is active, as seen in the case of R11 plants. In contrast, cosuppression/PTGS mechanisms have been shown to include the

developmental and spatial control of its silencing process, but this control has been little delineated [30,31]. One such control includes the interaction between transgene and endogenous gene transcripts. In cosuppression of the polygalacturonase gene and chalcone synthase gene, the endogenous mRNA is involved in the initiation step of cosuppression [26,27]. The expression of these two genes is developmentally regulated, and high level of expression of the endogenous genes appears to be essential for RNA silencing. However, the expression of *endo-NtFAD3* gene is constitutive. Therefore, it is unlikely that cosuppression is triggered by quantitative changes of the *endo-NtFAD3* mRNA. The developmental or/and tissue-specific control of cosuppression of the *NtFAD3* gene may include the different intracellular sites of the siRNA production. Since the *NtFAD3* protein is an integral membrane protein, the *NtFAD3* mRNAs should be translated on the rough ER. Thus, it is possible that the *NtFAD3* siRNA is produced on the ER membrane surface in root tissues of the S44 plants. Indeed, presence of Dicer and other RNAi components in the ER-membrane-associated fraction has been reported [32]. In this case, accessibility of RNAi machineries on the ER membrane surface to other *NtFAD3* mRNAs may be limited when compared with that of the cytoplasmic RNAi machineries. In contrast, the hairpin dsRNAs for the *NtFAD3* gene would be processed into siRNA in the cytoplasm because hairpin dsRNAs are unlikely to be incorporated into the translational equipments on the rough ER. In conclusion, our results indicate the presence of additional control steps in the action of siRNA. These steps might include developmental factors and regulate whether the *trans-* and *endo-NtFAD3* genes are expressed or cosuppressed.

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