Decreased GA₁ Content Caused by the Overexpression of OSH1 Is Accompanied by Suppression of GA 20-Oxidase Gene Expression

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We previously reported that overexpression of the rice homeobox gene OSH1 led to altered morphology and hormone levels in transgenic tobacco (Nicotiana tabacum L.) plants. Among the hormones whose levels were changed, GA₁ was dramatically reduced. Here we report the results of our analysis on the regulatory mechanism(s) of OSH1 on GA metabolism. GA₅₃ and GA₂₀, precursors of GA₁, were applied separately to transgenic tobacco plants exhibiting severely changed morphology due to overexpression of OSH1. Only treatment with the end product of GA 20-oxidase, GA₂₀, resulted in a striking promotion of stem elongation in transgenic tobacco plants. The internal GA₁ and GA₂₀ contents in OSH1-transformed tobacco were dramatically reduced compared with those of wild-type plants, whereas the level of GA₁₉, a mid-product of GA 20-oxidase, was 25% of the wild-type level. We have isolated a cDNA encoding a putative tobacco GA 20-oxidase, which is mainly expressed in vegetative stem tissue. RNA-blot analysis revealed that GA 20-oxidase gene expression was suppressed in stem tissue of OSH1-transformed tobacco plants. Based on these results, we conclude that overexpression of OSH1 causes a reduction of the level of GA₁ by suppressing GA 20-oxidase expression.

The regulatory mechanisms controlling plant morphogenesis constitute one of the most important questions in plant biology. The homeobox gene knotted-1, which is involved in maize leaf development, was isolated in 1989 (Hake et al., 1989). Many plant homeobox genes have subsequently been isolated and it is believed that these genes play a role in regulating morphogenesis (Kerstetter et al., 1994). The homeobox gene products share a unique and homologous structure, the homeodomain (Gehring, 1987). Homeodomain proteins possess a helix-turn-helix motif, and recognize and bind to specific DNA sequences, resulting in altered expression of the target gene (Scott et al., 1989). Accordingly, plant homeobox genes are thought to control plant morphogenesis through the regulation of expression of genes involved in plant development.

It has been reported that ectopic expression of the rice homeobox gene OSH1 causes morphological changes in rice, Arabidopsis, tobacco (Nicotiana tabacum L.), and kiwi-fruit (Kano-Murakami et al., 1993; Matsuoka et al., 1993; Kusaba et al., 1995). For example, OSH1-transformed tobacco plants exhibit abnormal-shaped leaves and flowers, and loss of apical dominance. These observations suggest that the OSH1 gene product may regulate the expression of genes involved in plant morphogenesis. Kano-Murakami et al. (1993) suggested that OSH1 need not be expressed continuously or throughout the entire plant to result in morphological aberrations. These results indicate that OSH1 may be a morphological regulator acting at an early stage of tissue or organ differentiation. However, the molecular mechanism(s) by which OSH1 regulates plant morphogenesis are unknown.

Plant morphogenesis is thought to be regulated by various physiological factors, including gene expression and plant hormones. It is well known that different plant hormones have distinct influences on plant growth and development. Our recent results indicate that ectopic expression of OSH1 causes morphological changes in transgenic tobacco plants by affecting plant hormone metabolism (Kusaba et al., 1998). In OSH1-transformed tobacco plants showing dwarfism, GA₁ levels were drastically reduced. From the fact that ectopic expression of OSH1 causes morphological changes and the product of OSH1 contains a putative DNA-binding domain, it is possible that OSH1 regulates the expression of gene(s) involved in hormone metabolism or sensitivity of plants. In the present study we report results that implicate OSH1 in the regulation of expression of a gene involved in GA biosynthesis in transgenic tobacco plants.

MATERIALS AND METHODS

Plant Materials

The preparation of OSH1-transformed tobacco (Nicotiana tabacum cv Samsun NN) plants was as described in Kano-Murakami et al. (1993). T₂ seedlings of 35S-OSH1 transformants and wild-type seedlings were grown under greenhouse conditions at 25°C.

Treatment with GA Derivatives

Ten microliters of a 10 or 100 μM solution of GA₂₀ or GA₅₃ in 5% acetone was applied to the shoot apex of
severe-phenotype transformants once a week. GA_20_ and GA_3 used in this study were prepared as described in a previous report (Murofushi et al., 1982).

Analysis of GA Derivatives

Analysis of GA_1, GA_20, and GA_19 was performed by ELISA using antibodies raised against GA_4 (Nakajima et al., 1991), GA_20 methyl-ester (Yamaguchi et al., 1987), and GA_24 (Yamaguchi et al., 1992), respectively. Extraction of GA derivatives and ELISA procedures were performed as described in Kusaba et al. (1998) with some modifications to the HPLC conditions. HPLC analyses of extracts were performed using an ODS column (6×150-mm i.d.; Pegasil ODS, Senshu Kagaku, Tokyo, Japan). Samples were eluted with 0.5% acetic acid in 10% aqueous acetonitrile (solvent A) and 0.5% acetic acid in 80% aqueous acetonitrile (solvent B) at room temperature as follows: 0 to 30 min, linear gradient of 0% solvent B to 50% solvent B; 30 to 35 min, linear gradient of 50% solvent B to 100% solvent B; and 35 to 50 min, isocratic elution with solvent B. The flow rate of the solvent was 1.5 mL min⁻¹ and fractions were collected every minute. The retention times of GA_1, GA_19, and GA_20 were 20 to 21 min, 20 to 22 min, and 21 to 23 min, respectively. Fractions containing each GA (retention time ±3 min) were divided into three parts and assayed by ELISA. The cross-reactivity of the antibodies to other GAs was less than 1%.

Cloning of Tobacco GA 20-Oxidase PCR Fragment

First-strand cDNA was synthesized using a reverse transcription-PCR Kit (Takara Shuzo, Otsu, Shiga, Japan) with random primers. Total RNA extracted from young leaves of wild-type tobacco was used as a template. PCR was carried out with primers (5'-CA[AG]T[CT]AT[ACT]TGCGCNA-3' and 5'-CTGACGGAGCGCCCAT TCGTTG-3') using the first-strand cDNA as a template. Samples were heated to 94°C for 2 min, then subjected to 28 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The reaction was completed by a 10-min incubation at 72°C. The resulting 720-bp DNA fragment was cloned into the vector pCRII (Invitrogen, San Diego, CA).

Isolation of cDNA Clones

A cDNA library was constructed from RNA isolated from stem tissue of mature tobacco plants. Poly(A⁺)-enriched RNA was purified by two passages through an oligo d(T) cellulose column (Type 7, Pharmacia Biotech). Double-stranded cDNA was synthesized from poly(A⁺) RNA and EcoRI adapters were added using a cDNA synthesis kit (Pharmacia Biotech). The products were ligated into AZAP II (Stratagene) that had been digested with EcoRI and dephosphorylated. Ligation products were packaged using Gigapack II (Stratagene) and the resulting cDNA library of 2.4 × 10⁸ recombinants was amplified by passage through Escherichia coli XL1 Blue. Screening was performed in 6× SSC, 5× Denhardt’s solution, 0.1% SDS, and 100 μg mL⁻¹ salmon-sperm DNA at 57°C for 16 h using the PCR product described above as a probe. Filters were washed in 2× SSC and 0.1% SDS at room temperature and then further washed in 0.2× SSC and 0.2% SDS at 57°C.

Sequence Analysis

Nucleotide sequences were determined by the dideoxynucleotide chain-termination method using an automated sequencing system (ALF DNA Sequencer II, Pharmacia Biotech). Analysis of cDNA and inferred amino acid sequences were carried out using Lasergene computer software (DNASTAR, Inc., Madison, WI).

RNA-Blot Analysis

Total RNA was prepared from various organs for gel-blot analysis. Ten micrograms of each RNA preparation was separated on agarose gels in the presence of formaldehyde, followed by transfer to Hybond-N membrane (Amersham). The tobacco GA 20-oxidase cDNA or a XbaI/SacI fragment of the OSH1 cDNA was labeled with [α-³²P]dCTP using the Rediprime DNA-labeling system (Amersham). Hybridization was carried out at 42°C in a solution containing 50% formamide, 5× SSC, 0.2% SDS, 0.1% N-lauroylsarcosine, 1% blocking reagent (Boehringer Mannheim), 10% dextran sulfate, and 100 μg mL⁻¹ salmon-sperm DNA. Blots were hybridized for 14 h, washed in 2× SSC and 0.1% SDS at room temperature, then in 0.2× SSC and 0.1% SDS at 65°C, and then exposed to Kodak XAR film.

RESULTS

We previously demonstrated that the morphology of transgenic tobacco plants expressing OSH1 under the control of the cauliflower mosaic virus 35S promoter could be divided into three categories ranging from a mild to a severe phenotype (Kano-Murakami et al., 1993). In these transformants severe-phenotype plants were dwarf and the axillary buds developed into vegetative stems; these buds were dormant in wild-type plants (Fig. 1). We have analyzed the hormone contents of OSH1-transformed tobacco plants to investigate the regulatory mechanism(s) through which OSH1 alters plant morphogenesis, and have shown that the morphological changes are accompanied by a decrease of GA_1 content (Kusaba et al., 1998).

Treatment of Severe-Phenotype Tobacco Plants with GA_1, Precursors

We have previously reported that stem elongation in severe-phenotype OSH1-transformed tobacco plants was restored by GA_1 treatment (Kusaba et al., 1998). This suggests that the dwarfism observed in plants expressing OSH1 at a high level could be caused by the suppression of GA_1 biosynthesis by OSH1 rather than changes in the GA_1 signal transduction pathway. If this is the case, precursors of GA_1 that are formed after the OSH1 block point in the GA biosynthetic pathway should be able to restore stem elongation in severe-phenotype plants. When GA_3, a sub-
strate of GA 20-oxidase, and GA 20, an end product of GA 20-oxidase, were applied to the shoot apex of severe-phenotype transgenic tobacco plants, only GA 20 could restore stem elongation in a dose-dependent manner (Fig. 2). This result indicates that in _OSH1_ overexpressing plants the GA biosynthetic pathway is blocked between GA 53 and GA 20.

**Contents of GA 1 Precursors in Severe-Phenotype Tobacco Plants**

The GA 19, GA 20, and GA 1 contents of wild-type and severe-phenotype transgenic tobacco plants were analyzed to confirm that _OSH1_ could suppress GA 20-oxidase activity in transformants (Fig. 3). The content of GA 19, a mid-product of GA 20-oxidase, was decreased to 25% of that observed in wild-type plants. In contrast, GA 20, an end product of GA 20-oxidase, was reduced to a very low level, similar to that of GA 1. These observations strongly suggest that _OSH1_ overexpression leads to a decrease in GA 1 content by suppressing GA 20-oxidase activity in transgenic tobacco.

**Cloning of a Tobacco GA 20-Oxidase cDNA**

Plant GA 20-oxidase genes have recently been isolated from several species, e.g. pumpkin (Lange et al., 1994), Arabidopsis (Phillips et al., 1995; Xu et al., 1995), spinach (Wu et al., 1996), pea (Martin et al., 1996), and French bean (García-Martínez et al., 1997). We used degenerate primers based on conserved regions of the GA 20-oxidase genes (Fig. 4) to amplify a fragment of the GA 20-oxidase gene from tobacco. Sequence analysis revealed that the 720-bp fragment obtained by PCR encoded a polypeptide that was 77% identical and 88% similar to the GA 20-oxidase inferred from the French bean gene (García-Martínez et al., 1997). The 720-bp PCR product was used to screen a tobacco cDNA library constructed using mRNA isolated from vegetative stem tissue. Several positive clones were identified from the 2.4 × 10^5 recombinant library. Plasmids containing the inserts of the clones were obtained by in vivo rescue. Restriction endonuclease digestion showed that one of these clones contained a 1.5-kb insert, the expected size for a full-length GA 20-oxidase cDNA clone. DNA sequencing revealed that the insert of this cDNA clone contained the sequence of the 720-bp PCR product and possessed an open reading frame encoding 379 amino acids (Fig. 4), indicating that it represented a full-length clone. The deduced amino acid sequence of this cDNA showed 74%, 66%, and 50% identity to those of GA 20-oxidase cloned from French bean (Pv15–11; García-Martínez et al., 1997), Arabidopsis (At2301; Phillips et al., 1995), and pumpkin (Cm20ox; Lange et al., 1994), respectively. From these results, the 1.5-kb cDNA appeared to represent a full-length clone of tobacco GA 20-oxidase.

**Expression Analysis of Tobacco GA 20-Oxidase**

To investigate the expression of the putative tobacco GA 20-oxidase gene, RNA-blot hybridization was performed. Ten micrograms of total RNA extracted from mature leaves, vegetative stems, develops flowers, and developing siliques was probed with the ^32_P-labeled full-length tobacco GA 20-oxidase cDNA. Accumulation of tobacco GA 20-oxidase mRNA was seen mainly in stem tissue, with relatively low levels detected in RNA from leaves, siliques, and flowers (Fig. 5a).

In stem tissue, treatment with GA$_3$ 8 h before RNA extraction reduced the abundance of tobacco GA 20-oxidase mRNA (Fig. 5b). Similar results have also been obtained in Arabidopsis (Phillips et al., 1995; Xu et al., 1995) and in pea (Martin et al., 1996).

Severe-phenotype transgenic tobacco plants expressing _OSH1_ showed extreme dwarfism. To confirm whether this dwarfism could be attributed to the suppression of GA 20-oxidase gene expression in stem tissue, we analyzed the abundance of GA 20-oxidase mRNA in stem tissue of severe-phenotype and wild-type tobacco plants. The GA 20-oxidase mRNA was substantially suppressed in severe-phenotype stems compared with wild-type stems (Fig. 5c).

**DISCUSSION**

Expression of the rice homeobox gene _OSH1_ causes morphological changes in transgenic tobacco, including dwarfism and loss of apical dominance (Kano-Murakami et al., 1993). In _OSH1_-transformed tobacco plants exhibiting a severe phenotype, hormone levels are altered, with a decrease of GA 1 and increased levels of ABA and trans-zeatin (Kusaba et al., 1998). Many GA-responsive mutants showing dwarfism have been isolated from several species. These dwarf mutants show decreased bioactive GA levels, and their growth can be restored with applied bioactive GA (Hedden and Kamiya, 1997). Our recent finding that...
exogenous GA$_3$ can correct the dwarfism in severe-phenotype tobacco plants expressing $OSH1$ indicates that these plants may represent GA-responsive dwarfs (Kusaba et al., 1998). The application of GA$_{53}$ and GA$_{20}$, precursors of GA$_1$, to severe-phenotype tobacco transformants indicated that the biosynthetic pathway between GA$_{53}$ and GA$_{20}$ appears to be blocked in these plants (Fig. 2). The conversion of GA$_{53}$ to GA$_{20}$ is catalyzed by the multifunctional 2-oxoglutarate-dependent dioxygenase GA $_{20}$-oxidase (Lange, 1994). In the GA-responsive semidwarf ga5 mutant of Arabidopsis (Koornneef and van der Veen, 1980), the contents of C19-GAs were reduced compared with the wild type (Talon et al., 1990). Xu et al. (1995) determined that the GA5 locus of Arabidopsis encodes GA 20-oxidase. Severe-phenotype tobacco plants also showed a drastic decrease of the C19-GAs GA$_{20}$ and GA$_1$ (Fig. 3). These observations suggest that overexpression of $OSH1$ in transgenic tobacco results in a suppression of GA 20-oxidase activity.

Because the $OSH1$ gene product contains a putative DNA-binding domain, the homeodomain, $OSH1$ is thought to control plant morphogenesis through the regulation of gene expression (Matsuoka et al., 1993). However, no target gene of $OSH1$ has yet been identified. These observations plus our current results indicate that overexpression of $OSH1$ may regulate GA 20-oxidase gene expression either directly or indirectly. To investigate this hypothesis, we cloned a tobacco cDNA encoding GA 20-oxidase. The amino acid sequence deduced from this full-length cDNA showed 74%, 66%, and 50% identity to those of GA 20-oxidase cloned from French bean (Pv15–11; García-Martínez et al., 1997), Arabidopsis (At2301; Phillips et al., 1995), and pumpkin (Cm20ox, Lange et al., 1994), respectively. GA 20-oxidases exhibit a relatively low degree of sequence conservation, with amino acid identities ranging from 50% to 75% (Hedden and Kamiya, 1997). Tobacco GA 20-oxidase gene expression was suppressed by GA application (Fig. 5b), as has been demonstrated in several other plant species (Phillips et al., 1995; Xu et al., 1995; Martin et al., 1996). These data indicate that the cDNA isolated from tobacco almost certainly encodes GA 20-oxidase.

The GA 20-oxidases are encoded by multiple genes in Arabidopsis (Phillips et al., 1995), pea, and French bean.
The GA 20-oxidase genes in Arabidopsis exhibit tissue-specific expression, leading to the belief that the various genes are responsible for GA biosynthesis associated with different aspects of plant development. The gene corresponding to the GA5 locus shows stem-specific expression, indicating that stem-specific GA 20-oxidases may be involved in stem elongation in Arabidopsis. The tobacco GA 20-oxidase gene was expressed mainly in developing stem tissue, with relatively low-level expression in leaves and siliques (Fig. 5a). Taken together, these results indicate that tobacco GA 20-oxidase may also be involved in stem elongation. We propose that the dwarfism of severe-phenotype transgenic tobacco plants may be due to the suppression of GA 20-oxidase gene expression (Fig. 5c).

Recently, a tobacco homeobox gene termed NTH15 (Nicotiana tabacum homeobox 15) was isolated and its homeodomain sequence shows 88% identity to that of OSH1 (Tamaoki et al., 1997). Ectopic expression of NTH15 in transgenic tobacco causes morphological changes that are in large part similar to those seen in OSH1 transformants. In transgenic tobacco expressing NTH15, a drastic decrease of GA1 content was also observed. In wild-type tobacco NTH15 gene expression was strongly expressed in vegetative stems and weakly expressed in shoot apices, flower buds, and flowers. These results imply that expression of NTH15 in stem tissue may be involved in stem elongation by regulating the expression of GA 20-oxidase.

The deduced amino acid sequence of OSH1 contains a homeodomain, leading us to propose that the OSH1 gene product controls plant morphogenesis through regulation of expression of certain target gene(s). However, little is known about the target genes of plant homeodomain-containing proteins. Our recent results suggest that OSH1 affects plant hormone metabolism either directly or indirectly, thereby causing changes in plant development (Kusaba et al., 1998). Our present results indicate that a developmental signal from the OSH1 protein may act to suppress GA 20-oxidase expression in transgenic tobacco. GA 20-oxidase gene expression has been reported to be developmental stage and organ specific, and to be regulated by end products and photoperiod (Hedden and Kamiya, 1997). However, the analysis of cis-elements and trans-factor(s) that regulate the expression of GA 20-oxidase genes has not yet been reported. Further work is needed to elucidate the regulatory mechanisms controlling GA 20-oxidase gene expression.

Figure 4. Nucleotide and deduced amino acid sequences of tobacco GA 20-oxidase cDNA. Regions corresponding to the degenerate primers used in PCR amplification are underlined.

OSH1 Suppresses GA 20-Oxidase Gene Expression
ACKNOWLEDGMENTS

We would like to thank Y. Ohashi (National Institute of Agrobiological Resources, Tsukuba, Japan) for kindly supplying us with wild-type tobacco plants, M. Nakajima and M. Hasegawa (University of Tokyo) for skillful technical assistance, and T. Mato- tani (National Institute of Fruit Tree Science) for helpful comments.

Received February 12, 1998; accepted April 25, 1998.

Copyright Clearance Center: 0032-0889/98/117/1179/06.
The accession number for the nucleotide sequence of tobacco GA 20-oxidase described in this article is AB012856.

LITERATURE CITED


Talon M, Koornneef M, Zeevaart JAD (1990) Endogenous gibberellins in Arabidopsis thaliana and possible steps blocked in the biosynthetic pathways of the semidwarf ga4 and ga5 mutants. Proc Natl Acad Sci USA 87: 7983–7987


