Turkish Journal of Botany

Volume 24 | Number 4 Article 2

1-1-2000

Reduction of Lignin in Tobacco Through the Expression of an Antisense Caffeic Acid O-methyltransferase

TİJEN TALAS OĞRAŞ

KEMAL KAZAN

NERMİN GÖZÜKIRMIZI

Turk J Bot
24 (2000) 221-226
@ TÜBİTAK

Research Article

Reduction of Lignin in Tobacco Through the Expression of an Antisense Caffeic Acid *O*-methyltransferase

Tijen Talas OĞRAŞ
TUBITAK, Marmara Research Centre, Research Institute of Genetic Engineering and Biotechnology
Gebze-Kocaeli 41470 - TURKEY
Kemal KAZAN
CSIRO (Commonwealth Scientific and Technical Research Organization) Cunningham Laboratory
306 Carmody Rd., Brisbane QLD 4067- AUSTRALIA
Nermin GÖZÜKIRMIZI
University of Istanbul, Faculty of Science, Department of Biology, Istanbul 34459 - TURKEY

Received: 22.07.1999
Accepted: 07.03.2000

Abstract : Caffeic acid O-methyltransferase (COMT) is a methylation enzyme involved in the early stages of lignin biosynthesis. To examine the effect of antisense expression of a heterologous COMT gene on lignification, an antisense construct of a cDNA encoding a caffeic acid *O*-methyltransferase (*pOMT8*) from *Stylosanthes humilis* Humb., Bonpl. & Kunth *Fabaceae* was transferred into tobacco via *Agrobacterium tumefaciens*. A large number of kanamycin-resistant shoots were recovered after transformation, and randomly selected putative transgenics were analysed for stable integration of the transgene into their genomes. The analyses demonstrated that antisense expression of *pOMT8* resulted in reductions in total COMT activity, which was correlated with reduction in the lignin content of the transgenic plants. The amount of lignin in one of the transgenic lines decreased by 62% compared with untransformed control plants.

Key Words: Antisense expression, caffeic acid *O*-methyltransferase, lignin biosynthesis.

Tütündeki Ligninin Kafeik Asit *O*-metiltransferaz'ın Antisens Anlatımı ile Azalması

Özet : Kafeik asit O-metiltransferaz (COMT), lignin biyosentezinin erken aşamalarında görev alan bir metilasyon enzimidir. Lignifikasyonda heterolog kafeik asit O-metiltransferaz (COMT) geninin antisens anlatımının incelenmesi için, *Stylosanthes humilis* Humb., Bonpl. & Kunth *Fabaceae*'den izole edilen COMT cDNA'sının antisens formunu kodlayan gen (*pOMT8*) *Agrobacterium tumefaciens* aracılığı ile tütüne aktarılmıştır. Transformasyon sonrası kanamisine dayanıklı birçok bitkicik elde edilmiş, rastgele seçilen ve transgenik olduğu varsayılan bitkicikler genomlarındaki COMT varlığının belirlenmesi için analiz edilmiştir. Transgenik bitkilerde yapılan analizler, *pOMT8*'in antisens anlatımı sonucu COMT aktivitesinde gözlenen azalmanın lignin miktarındaki azalma ile ilişkili olduğunu göstermiştir. Transgenik bitkilerden birinde lignin miktarında kontrol bitkiye kıyasla %62 azalma gözlenmiştir.

Anahtar Sözcükler: Antisens anlatım, kafeik asit *O*-metiltransferaz, lignin biyosentezi.

Introduction

The phenylpropanoid pathway plays a key role in plant growth, development and interaction with the environment. One of the products of this pathway is lignin, which is a major component of secondary cell walls. This three-dimensional aromatic polymer arises from the polymerization of *p*-coumaryl, coniferyl and sinapyl alcohols catalyzed by peroxidases (1). It also plays essential roles in the strengthening and impermeabilization of the cell walls and provides chemical barriers against pathogenes (2).

A high lignin content in plant material causes monetary and environmental costs in its removal from wood in the paper and pulp industries. In addition, the presence of lignin limits the digestibility of forage crops (3). The reduction of lignin content and changes in its composition by manipulation of some enzymes involved in this pathway have been widely investigated. Down-

regulation of phenylalanine ammonia lyase (PAL) (4), caffeic acid *O*-methyltransfrase (COMT, EC 2.1.1.68.) (5, 6), and cinnamyl alcohol dehydrogenase (CAD) (7) have resulted in changes in the content and composition of lignin in transgenic plants.

COMT is a bifunctional enzyme responsible for catalysing methoxylation of the lignin precursors to form ferulic and sinapic acids in the lignin biosynthesis pathway (1). In dicotyledonous plant species, such as poplar (8), tobacco (6) and alfalfa (9), COMT sequences of *Stylosanthes humilis* Humb., Bonpl. & Kunth are highly conserved (10). For instance, a COMT cDNA clone from a tropical pasture legume, *S. humilis (pOMT8)*, shows 74% DNA sequence homology to the corresponding tobacco OMT genes (tobacco1 and tobacco2) (10,11). Therefore, heterologous antisense constructs from these species can be potentially employed to suppress the expression of COMT and alter lignin monolignol synthesis in transformed tobacco plants (8).

Using a heterologous gene construct we investigated the effect of antisense inhibition of tobacco COMT on lignin content. We report here that antisense expression of 35S-*pOMT8* reduced the COMT activity and modified the content of lignin in the stems of transgenic tobacco plants.

Materials and methods

Binary vector and plant transformation

A binary vector construct (35S-*pOMT8*) was kindly provided by Dr. John M. Manners, CRC, for Tropical Plant Pathology, the University of Queensland, Australia. The construct carries a cDNA clone (1.2 kb) of the *Stylosanthes humilis* COMT gene (*pOMT8* Genbank accession no: 236109) in antisense orientation between the CaMV 35S RNA promoter and the polyadenylation signal (3') of the *tmr* gene of *Agrobacterium tumefaciens* in pGA643 (Figure 1; 12).

The binary vector was mobilized into *A. tumefaciens* strain LBA4404 via triparental mating using pRK 2013 as a helper plasmid (13). This *Agrobacterium* was then used to infect leaf discs of tobacco (*Nicotiana tabacum* L. cv. Samsun) according to Horsch et al. (14). The regeneration and selection of transformed shoots were performed on Murashige-Skoog (MS) medium (15) containing 1 mg/l BAP, 0.1 mg/l NAA, 100 mg/l kanamycin and 400 mg/l cefotaxime. Regenerated shoots were grown in a growth cabinet under a 16-h photoperiod at 26°C. The untransformed control tobacco leaf discs were also regenerated under the same conditions without antibiotics.

Molecular analyses

Genomic DNA was extracted from transformed and untransformed plant leaves according to Edwards et al. (16). The presence of the transgene in the genome of putative transformants was confirmed by polymerase chain reaction (PCR). A forward primer (5' TGA GCA AAG AAG CTC GCA TGG GAG 3'), which anneals internally to the 35S promoter, and a reverse primer (5' GTC GAA CAT CTG TTA CTC TTA CTC TG 3'), which anneals internally to the *tmr* terminator, were used to amplify the *pOMT8* gene located on the T-DNA region of pGA643. PCR was performed in a volume of 25 µl and included an initial denaturation step at 94°C for 4 min, followed by 35 cycles at 94°C (1 min), 52°C (1 min), 72°C (1.5 min) and ended with a 10 min extention step at 72°C. For Southern blot analysis, total genomic DNA (6 µg) from transformed and untransformed control plants was digested with *EcoR*V, fractionated on a 0.9% (w/v) agarose gel and subsequently transferred to a positively charged nylon membrane (Boehringer Mannheim, Germany) by electroblotting. The DNA was immobilized to the membrane by UV exposure for 10 min. A PCR amplified DNA fragment corresponding to the full length of the *pOMT8* gene was labelled with digoxigenin-dUTP (DIG-dUTP) using a non-radioactive DIG DNA labelling kit

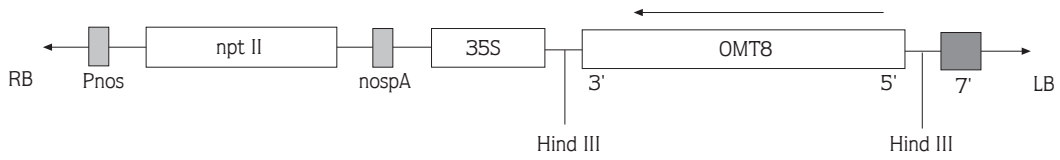

Figure 1. Schematic representation of the T-DNA region of the *pOMT8* antisense construct in the binary vector pGA643. 35S, 35S RNA promoter of cauliflower mosaic virus; nosP, promoter of the nopaline synthase gene; nospA, terminator of the nos gene. RB, LB are the right and left borders of the T-DNA.

(Boehringer Mannheim) and used as probe for Southern hybridization. Probe labelling, hybridization, washing and immunological detection of hybridized DNA were performed according to the manufacturer's instruction (Boehringer Mannheim). The washing procedure was performed under stringent conditions.

COMT enzyme assay

For COMT enzyme assays, total proteins from stem tissue taken from the top internode of the 4-month-old greenhouse grown tobacco plants were extracted by grinding in liquid nitrogen. The ground tissue was homogenized with cold extraction buffer (100 mM Tris.HCl, pH 7.5, 2 mM EDTA and 5 mM DTT). The homogenate was clarified twice by spinning in a microcentrifuge at 12 000 g at 4°C for 30 min and the supernatant was used for enzyme assays. The amount of protein was quantitated using bovine serum albumin as the standard according to the Bradford assay (17).

The COMT activity assay was performed with 100 µl of stem extract using 1 ml of sodium phosphate buffer (pH 7.5) containing 3 mM caffeic acid as substrate and 50 µM triated S-adenosyl-L-(methyl-^{14}C) methionine (3.7 kBq/assay) as methyl donor (18). The assay reaction was incubated at 37°C for 1 h and the reaction was stopped with 10 µl of 9 M sulphuric acid. The reaction mix was blotted onto Whatmann GF/C filters (glass microfibre) under vacuum and unused radioactivity was removed by a series of washes with 5-10% trichloroacetic acid. Finally the filters were air-dried and ^{14}C-labelled ferulic acid was measured by LKB Wallac 1212 Rackbeta liquid scintillation counter.

Determination of total lignin content

The amount of total soluble lignin was determined using the thioglycollic acid (TGA) method with stem extracts of transgenic and control plants (19). The plant tissue was ground in liquid nitrogen and dried for 4 h at 105°C, and then 750 µl of distilled H_2O, 250 µl of 37% HCl and 100 µl of TGA were added to 50 mg of baked plant material. The homogenate was incubated for 3 h at 80°C and centrifuged at 12 000 g for 10 min. The pellet was washed with distilled H_2O and resuspended in 1 ml of 1 M NaOH and shaken for 16 h at 4°C. The homogenate was centrifuged for 10 min, and the supernatant was collected and mixed with 200 µl concentrated HCl. The mixture was incubated at 4°C for 4 h and centrifuged as before. Following centrifugation, the pellet was dissolved again in 1 ml NaOH and the absorbance of the samples was measured at 280 nm. In this technique, the high phenolic material of plant cell wall is removed as a methanol-insoluble fraction by NaOH and TGA.

The insoluble lignin was assayed in stem samples using the Klason method (20). Two hundred mg of fresh tissue was ground with liquid nitrogen, mixed with 200 ml of H_2SO_4 and incubated for 1 h at 37°C. The homogenate was autoclaved for 1 h after the addition of 56 ml of dH_2O and dried for 4 h at 100°C. The dried material was mixed with KBr and the pellet disc was characterized by infrared spectroscopy (Perkin Elmer 983) between 400 and 4000 cm^{-1}.

Results and Discussion

Expression of heterologous antisense gene for COMT in tobacco

In this study, a heterologous antisense construct of the COMT gene from *S. humilis* was introduced into tobacco plants to assess the potential of this construct in reducing the total lignin content. Following inoculation with *Agrobacterium*, 210 kanamycin-resistant plants were recovered. Thirty-five putative transgenic tobacco plants were randomly selected and used for analyses of COMT activity and lignin content. All biochemical analyses were performed with 4-month-old transgenic and control plants grown under similar growth conditions. No significant phenotypic or developmental differences were observed in these plants.

In order to confirm the transgenic status of the selected plants, tobacco plants were first analyzed by PCR for the presence of the *pOMT8* gene. Observation of a PCR product with an expected size (1.2 kb) of the *pOMT8* gene on an agarose gel suggested that the gene was integrated into the genome of the transgenic tobacco plants. No PCR product was observed when DNA from untransformed control plant was used as template (Figure 2). Stable integration of the *pOMT8* into the transformed tobacco plants was also confirmed by Southern blot analysis. This analysis, which was performed using DIG-dUTP labelled *pOMT8* as probe, confirmed that few copies (one or two) of the transgene were stably integrated into the genome of the tobacco plants (Figure 3).

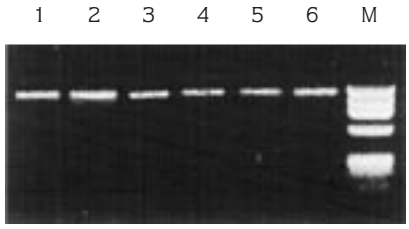

Figure 2. Transgene-specific amplification products (1.2 kb) were obtained by PCR analyses of transformed tobacco lines (lanes 1-6) with the specific primers of 35S promoter and *tmr* terminator. *Hae* III digested ϕX174 was used as DNA size marker (M); 1353, 1078, 872, 603…(Promega Coorporation).

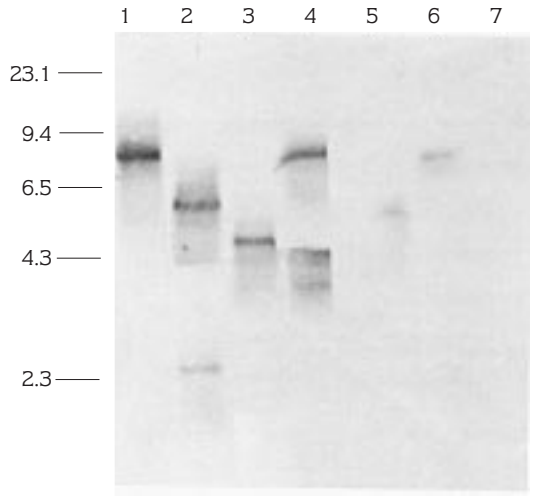

Figure 3. Southern blot analysis of transgenic tobacco (lanes 1-6) and untransformed control (lane 7) plants. Genomic DNA (6 µg) from each plant was digested with *EcoRV*, blotted onto nylon membrane and hybridized with non-radioactive digoxigenin labelled *pOMT8* as probe. *Hind* III digested lambda was used as size marker.

Reduced COMT activity is correlated with reduced lignin content in transgenic plants

Randomly selected tobacco plants transformed with an antisense construct of *35S-pOMT8* was analysed for any change in endogenous COMT activity. The COMT activity was determined using ^{14}C-labelled ferulic acid in the stem extracts of tobacco plants. These analyses showed that the total COMT activity was reduced in most of the transgenic plants but the level of reduction varied from one plant to another (Figure 4). The highest reduction in COMT activity was measured in transgenic line 03, the COMT activity of which was 68% less than that of the control plants (Figure 4). Analysis of lignin by the TGA method showed that the total lignin in this line was also 62% lower than that of untransformed control plants (Figure 5). The levels of lignin in transgenic lines transformed with *pOMT8* differed from all transformants. In total, the reduction of the amount of lignin in the transformed plants ranged from 17% to 62% of the average lignin content measured in control plants. The correlation between reduced COMT activity and reduced lignin content was highly significant ($r=78$, $p>0.01$). These results suggested that the inhibition of COMT activity resulted in reduced lignin in the stems of transgenic plants. However, it is not known whether the monomeric composition of the lignin in these transgenic plants also changed. These results are also in agreement with those of a previous study by Ni et al. (21), who reported that expression of chimeric antisense lucerne COMT genes caused a 15% reduction in the amount of lignin in transgenic tobacco plants. However, other studies have reported changes only in the monomeric composition of lignin as a result of inhibition of COMT

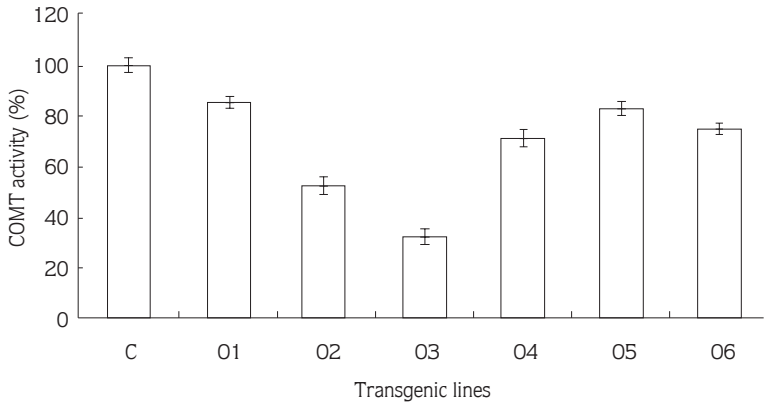

Figure 4. COMT activity towards caffeic acid determined in 4-month-old tobacco lines (01-06) transformed with *35S-pOMT8* and untransformed control (C) plants. The COMT activity is expressed as a percentage of the mean value of control plants. Each bar is the mean of triplicate assays done on the same plant. The error bars represent standard deviations.

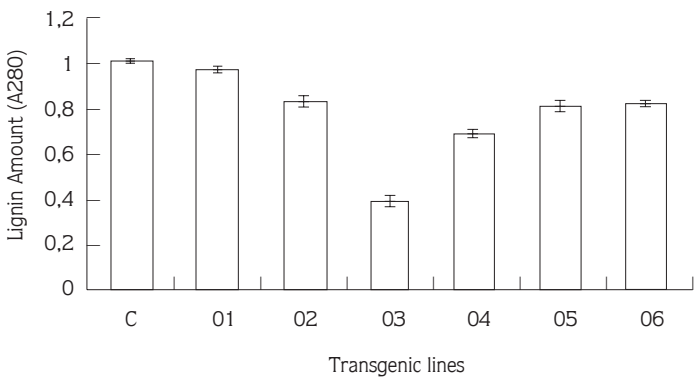

Figure 5. Amount of total lignin determined by TGA method in *35S-pOMT8* transformed (01-06) and untransformed tobacco (C) plants. Each bar is the mean of triplicate assays done on the same plant. The error bars represent standard deviations.

activity without significant changes in total lignin content (6, 22). Similarly, Zhong et al. (23) reported that reduction of OMTs by antisense constructs altered both lignin content and composition in tobacco plants.

We also examined stem extracts of transgenic and untransformed control plants by infrared (IR) spectroscopy to determine whether the subunit composition of lignin had changed. These analyses did not show any detectable differences in the composition of lignin between transgenic and untransformed control plants (data not shown). More lignin reduction in lignin content in transgenic plants may be necessary to obtain differences by IR spectroscopy analysis.

Our results indicate the feasibility of the inhibition of tobacco COMT activity by antisense expression of a heterologous COMT gene. It is possible that more substantial reductions in lignin content can be achieved by inhibition of the activities of other enzymes involved in lignification. However, a certain amount of lignin is essential for the plant to maintain its normal physiological functions, and therefore reduction of lignin content below a certain level may not be attainable.

Acknowledgements

This work was supported by TÜBITAK, MRC, Plant Biotech Project Grant No. 62-1-001.

References

1. Whetten, R., Sederoff , R., Lignin biosynthesis. Plant Cell 7:1001-1013 (1995).
2. Lewis, N.G., Yamamoto, E., Lignin: occurrence, biogenesis and biodegradation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 41, 455-496 (1990).
3. Jung, H.J.G., Forage lignins and their effects on fiber digestibility. Agron. J. 81, 33-38 (1986).
4. Elkind, Y., Edwards, R., Mavandad, M., Hedric, S.A., Ribak, O., Dixon, R.A., Lamb, C.J., Abnormal plant development and down-regulation of phenylpropanoid biosynthesis and transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc. Natl. Acad. Sci. USA 87, 9057-9061 (1990).
5. Dwivedi, U.N., Campbell, W.H., Yu, J., Datla, R.S.S., Bugos, R.C., Chiang, V.L., Podila, G.K., Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense *O*-methyltransferase gene from *Populus*. Plant Mol. Bio. 26, 61-71 (1994).
6. Atanassova, R., Favet, N., Martz, F., Chabbert, B., Tollier, M-T., Monties, B., Fritig, B., Legrand, M., Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequences in sense and antisense orientation. The Plant J. 8, 465-477 (1995).
7. Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert, B., Tollier, B., Schuch, W., Manipulation of lignin quality by downregulation of cinamyl alcohol dehydrogenase.The Plant J. 6, 339-350 (1994).
8. Dumas, B., Van Doorsselaere, J., Gielen, J., Legrand, M., Fritig, B., Van Montagu, M., Inze, D., Nucleotide sequence of a complementary DNA encoding O- metyltransferase from poplar. Plant Physiol. 98, 796-797 (1992).
9. Gowri, G., Bugos, R.C., Campbell, W.H., Maxwell, C.A., Dixon, R.A., Stress responses in alfalfa (*Medicago sativa* L.) X. methionine: caffeic acid 3-*O*-methyltransferase, a key enzyme of lignin biosynthesis. Plant Physiol. 97, 7-14 (1991).

10. McIntyre, C.L., Rae, A.L., Curtis, M.D., Manners, J.M., Sequence and expression of a caffeic acid O-methyltransferase cDNA homologue in the tropical forage legume *Stylosanthes humilis*. Aust. J Plant Physiol. 22, 471-478 (1995).

11. Jaeck, E., Martz, F., Stiefel, V., Fritig, B., Legrand, M., Expression of Class I *O*-methyltransferase in healthy and TMV-infected tobacco. Mol. Plant Microbe Interact. 9, 681-688 (1992).

12. An, G., Ebert, P.R., Mitra, A., Ha, S.B., Binary vectors. In: Plant Molecular Biology Manual A3, Gelvin and Schilperoort (eds), Kluwer Academic Publishers, Dordrecht (1988).

13. Rogers, S.G., Horsch, R.B., Fraley, R., Gene transfer in plants; production of transformed plants using Ti-plasmid vectors. Methods in Enzymol. 118, 627-641 (1986).

14. Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eicholtz, D., Rogers, S.G., Fraley, T.A., A simple and general method for transferring genes into plants. Science 227, 1229-1231 (1985).

15. Murashige, T., Skoog, F., A revised medium for rapid growth and bioassay with tobacco tissue cultures. Plant Physiol. 15, 473-479 (1962).

16. Edwards, K., Johnstone, C., Thompson, C., A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nuc. Acid Res. 19, 1349 (1991).

17. Bradford, M.M., A rapid and sensitive method for the quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72, 248-254 (1976).

18. Edwards, R., Dixon, A., Purification and characterisation of S-adenosyl-l- methionine: caffeic acid 3-O-methyltransferase from suspension cultures of alfalfa (*Medicago sativa* L.). Arch. Biochem. and Biophys. 287, 372-379 (1991).

19. Campbell, M.M., Ellis, B.E., Elicited phenylpropanoid metabolism in pine cultures. Planta 186, 409-417 (1992) .

20. Effland, M.J., Modified procedure to determine acid-insoluble lignin in wood and pulp. TAPPI 60, 143-144 (1977).

21. Ni, W., Paiva, N.L., Dixon, R.A., Reduced lignin in transgenic plants containing a caffeic acid O-methyltransferase antisense gene. Trans. Res. 3, 120-126 (1994).

22. Van Doorsselaere, J., Baucher, M., Chognot, E., Chabbert, B., Tollier, M-T., Petit-Conil, M., Leple, J-C., Pilate, G., Cornu, D., Monties, B., et al., A novel lignin in poplar trees with a reduced O-methyltransferase activity. Plant J. 8, 855-864 (1995).

23. Zhong, R., Morrison III, W.H., Negrel, J., Ye, Z-H., Dual methylation pathways in lignin biosynthesis. Plant Cell 10, 2033-2045 (1998).