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# Increase in Linolenate Contents by Expression of the *fad3* Gene in Transgenic Tobacco Plants

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**Abstract:** An 1.4 kb of the *fad3* cDNA encoding microsomal linoleic acid desaturase catalyzing the conversion of linoleic acid (18:2, ω-6) to linolenic acid (18:2, ω-3) was introduced into tobacco plants by the *Agrobacterium*-mediated plant transformation. Among the transgenic tobacco plants conferring kanamycin resistance, five transformants showing increment in unsaturated fatty acid contents were selected and further analyzed for the transgenecity. In genomic Southern blot analyses, copy numbers of the integrated *fad3* DNA in chromosomal DNA of the five transgenic tobacco plants were varied among the transgenic lines. By Northern blot analyses, the abundancy of the *fad3* mRNA transcript directed by Cauliflower Mosaic Virus 35S promoter was consistent with the relative copy number of the *fad3* DNA integrated in the chromosome of transgenic tobacco plants. When compared with the wild type, accumulation of linolenic acid in transgenic tobacco roots was elevated 3.7- to 4.7-fold showing a corresponding decrease in the linoleic acid contents; however, slight increments for linolenic acid were noticed in transgenic leaf tissues. These results indicated that the elevated level of *fad3* expression is achieved in transgenic tobacco plants.
**Key words:** *fad3*, microsomal ω-3 desaturase, polyunsaturated fatty acid.

Unsaturated fatty acids in the membrane lipids play a key role in the mechanisms of chilling resistance in plants. The increase of unsaturated fatty acid affords the living organism an adaptation to low temperature or protection from chilling injury. When a plant is exposed to low temperature, more unsaturated fatty acids and fewer saturated ones are detected in the cell membranes, suggesting their active roles in cold tolerance (Gerloff *et al.*, 1966; Graham and Patterson, 1982). Among unsaturated polar lipids in the cell membrane, linolenic acid is mainly associated with survival of plants at low temperatures by increasing membrane fluidity (Lyons *et al.*, 1964; Geroff *et al.*, 1966; Marr and Ingraham, 1982). The crucial role of linolenic acid synthesis for chilling resistance in cotton seedlings also has been elucidated in studies with an inhibitor for linolenic acid synthesis. (St. John and Christiansen, 1979).

Generally, α-linolenic acid (18:3 $^{\Delta 9,12,15}$) and hexadecatrienoic acid (16:3$^{\Delta 7,10,13}$) account for more than 80% of total fatty acids in plant membrane lipids. The synthesis of polyunsaturated fatty acids in plant cells are accomplished by sequential desaturation of saturated fatty acids. As an initial step for 18:3 fatty acid synthesis, first double bond is introduced into stearic acid (18:0) by a soluble stearoyl-acyl carrier protein desaturase found in chloroplasts (Iba *et al.*, 1993). In the second step, two distinct mechanisms are responsible for further desaturation of 18:1 to 18:3 *via* 18:2; one occurs in plastids and the other occurs in microsomes. In *Arabidopsis thaliana*, the chloroplast ω-6 and ω-3 fatty acid desaturases encoded by the *fad6* and *fad7* loci are involved in the desaturation of 18:1 and 18:2 fatty acids, respectively, and microsomal ω-6 and ω-3 fatty acid desaturases encoded by the *fad2* and *fad3* loci are responsible for the desaturation of 18:1 and 18:2 fatty acids, respectively (Browse and Somerville, 1991).

Recently, a cDNA clone for *fad3* was isolated from *Brassica napus* by map-base cloning (Arondel *et al.*, 1992), and cDNA for *fad7* was also characterized in *A. thaliana* (Iba *et al.*, 1993). In order to reveal the roles of 18:2 fatty acid desaturase genes in plant growth temperature, we produced transgenic tobacco plants as an initial step. Here we report the expression of the *fad3* gene in tobacco plants and its effect on fatty acid desaturation in leaves and roots of the transgenic tobacco.

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## Materials and Methods

### Plasmid construction

Plasmid pBNDES3 (Arondel *et al.*, 1992) containing the *fad3* gene was obtained from the *Arabidopsis* Biological Center (Columbus, USA). Plasmid pBI121 was purchased from Clontech (Palo Alto, USA) and plasmid pT7T319U was a product of Pharmacia (Milwaukee, USA).

To transfer the gene for microsomal ω-3 desaturase (FAD3) into tobacco plants, the *fad3* cDNA comprising the entire open reading frame consisting of 384 amino acid residues of *Brassica napus* FAD3 in plasmid pBNDES3 was introduced into a plant binary vector, plasmid pBI121, as shown in Fig. 1. Plasmid pBNDES3 DNA was digested with *Kpn*I and an 1.4 kb *fad3* cDNA fragment was ligated into *Kpn*I-digested plasmid pT7T319U DNA. The *fad3* cDNA in the recombinant plasmid p19UDS3 was further excised by *Xba*I and *Sac*I double digestion and then replaced with a β-glucuronidase (GUS) gene located between the *Xba*I and *Sac*I sites in plasmid pBI121 in order to generate plasmid pFAD3. In plasmid pFAD3, the *fad3* cDNA was placed between a single Cauliflower Mosaic virus (CaMV) 35S promoter and a nopaline synthase terminator. Each of the steps in plasmid pFAD3 construction was monitored by agarose gel electrophoresis.

### Bacterial transformation

*Escherichia coli* HB101 transformation was performed by the calcium chloride method as described by Sambrook *et al.* (1989). The freeze-thaw method (Holster *et al.*, 1978) was used for *Agrobacterium tumefaciens* LBA4404 transformation.

### Plant transformation and regeneration

Tobacco (*Nicotiana tabacum* cv. Xanthi) plants, were grown *in vitro* on MS agar media (Murashige and Skoog, 1962) and leaf tissues were cut into small pieces and transformed by 2 days of cocultivation with *Agrobacterium tumefaciens* LBA4404 in the dark according to the leaf disk transformation method as described by Horsch *et al.* (1985). For regeneration, MS agar media supplemented with 1 mg/l benzylaminopurine (BAP), 0.1 mg/l α-naphthalene acetic acid (NAA), 200 mg/l kanamycin and 500 mg/l carbenicillin was used as a shooting media. For rooting, shooty parts were transferred to hormone-free MS agar media containing 200 mg/l kanamycin and 500 mg/l carbenicillin.

### Southern blot analysis

Genomic DNAs were isolated from leaf tissues from transformed or non-transformed plants as described by the method of Gawel and Jarret (1991). Plasmid pFAD3 DNA and tobacco genomic DNAs were double digested with *Pst*I and *Eco*RI, then separated on 0.7% agarose gel in TAE buffer, transferred to Gene Screen membrane (New England Nuclear, Boston, USA) by the method of Sambrook *et al.* (1989), and fixed by UV-irradiation using UV Crosslinker (Stratagene, La Jolla, USA). As a probe, a 959 bp of *fad3* cDNA fragment excised from *Bgl*II digested pBNDES3 DNA (Arondel *et al.*, 1992) was labeled with biotin. Labeling, hybridization and chemiluminescent detection were carried out according to the protocol from the Southern-Light™ Chemiluminescent Detection System (Tropix Inc., Bedford, USA).

### Northern blot analysis

Total RNA was isolated from tobacco tissues using the acid guanidium thiocyanate-phenol-chloroform extraction method as described by Chomezynski *et al.* (1987). 10 μg of total RNA was denatured at 65℃ for 15 min in 50% (v/v) formamide containing 2.2 M of formaldehyde and electrophoretically separated on a 2.2 M formaldehyde/1% agarose gel. After electrophoresis, RNA was transferred to a Gene Screen membrane and fixed by UV-irradiation as described above. A 959 bp *fad3* DNA fragment from *Bgl*II cleaved plasmid pBNDES3 DNA was labeled with biotin and used as a probe. Biotin labeling, hybridization and chemiluminescent detection were carried out by the same method as described in Southern blot analysis.

### Gas chromatography (GC) analysis

Fatty acid methyl esters were prepared from lipid extracts of root and leaf tissues (Chee *et al.*, 1990). GC analyses were performed using an HP5890 gas-chromatograph with a flame ionization detector and an HP 3396 integrator (Hewlett Packard, USA). The capillary column used was Supelco Omegawax 320 (0.32 mm inner diameter, 30 m in length). Oven temperature was held at 180℃, and 250℃ was maintained for the injector and detector. Fatty acid methyl esters were identified by comparison with the retention times of authentic standard fatty acids for GC grade from Sigma (St. Louis, USA).

## Results and Discussion

### Transfer of the *fad3* gene to tobacco plants

Plasmid pFAD3 DNA in *E. coli* HB101 (Fig. 1) as described in Materials and Methods was isolated to transfer into *Agrobacterium tumefaciens* LBA4404. *A. tumefaciens* transformants were selected on YEP agar containing 20 μg/ml of kanamycin and the presence of plasmid pFAD3 in the transformant was also identi-

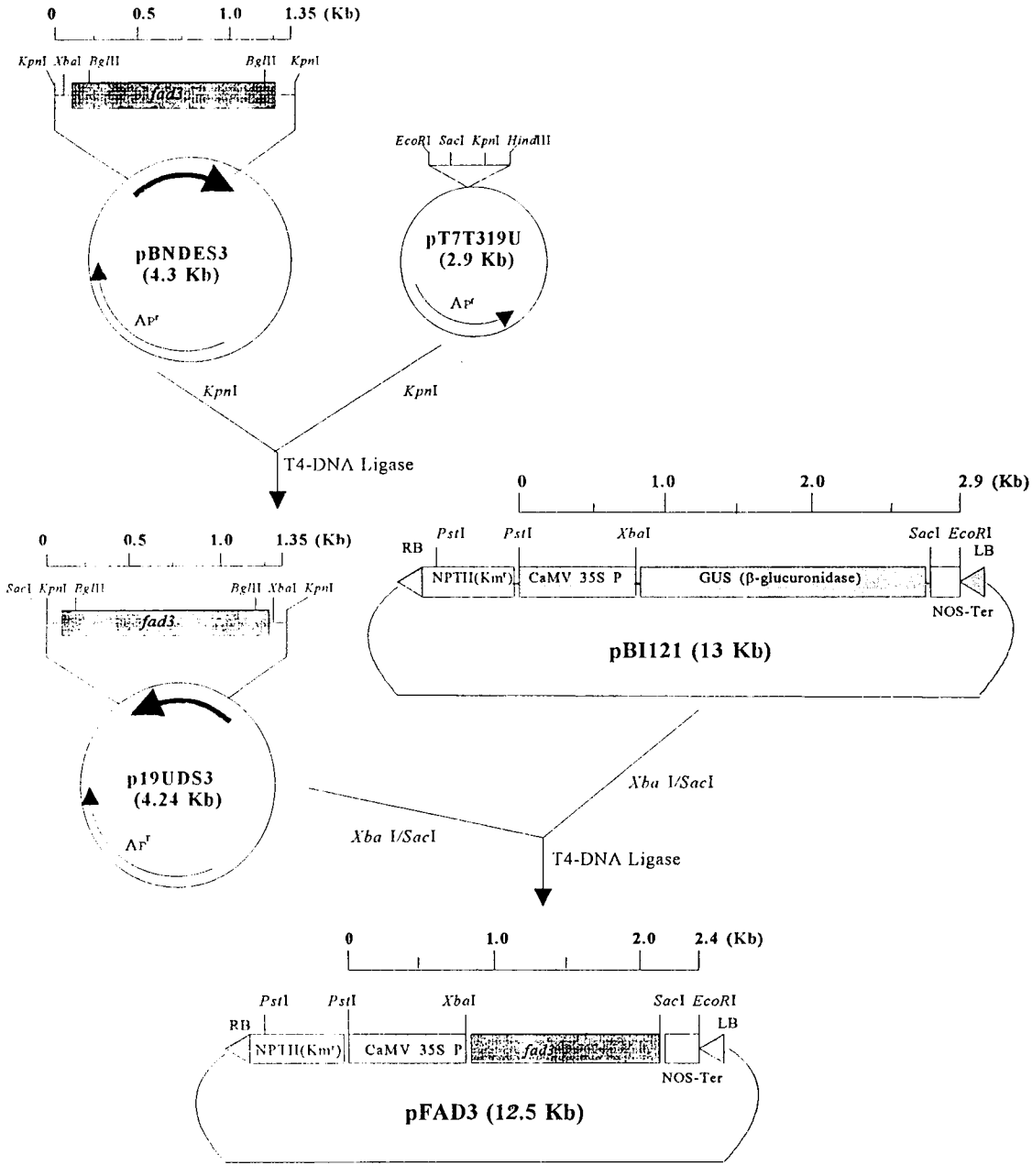


**Fig. 1.** The construction of plasmid pFAD3. The 1.4 kb of *fad3* cDNA in plasmid pBNDES3 was excised by the digestion of restriction endonuclease *Kpn*I and inserted into the same cohesive end of plasmid pT7T319 to generate plasmid p19UDS3. The plasmid p19UDS3 was double digested with of *Xba*I/*Sac*I and isolated fad3 cDNA was replaced with GUS gene in plant binary vector pBI121 to construct pFAD3. The open reading frame of each gene is indicated by the shaded box.

fied by agarose gel electrophoresis (data not shown). For the gene transfer into tobacco, leaf disks from *in vitro* grown tobacco were cocultivated with *A. tumefaciens* transformants harboring plasmid pFAD3 for 2 days, and then transferred to solid shooting media containing kanamycin (200 μg/ml) and carbenicillin (500 μg/ml) for plant regeneration.

After 4 weeks, healthy looking shoots were developed from the periphery of the leaf disks (data not shown). For rooting, shoots were transferred to hormone-free MS agar media supplemented with antibiotics as in the shooting media. Completely regenerated transgenic tobacco plants grown *in vitro* were hardened, transferred to soils and used for analyzing the transgenesis.

### Identification of transgenic tobacco plants

In order to isolate the transgenic tobacco plants, seven independent lines from regenerated plants conferring kanamycin resistance were further grown on soil for 30

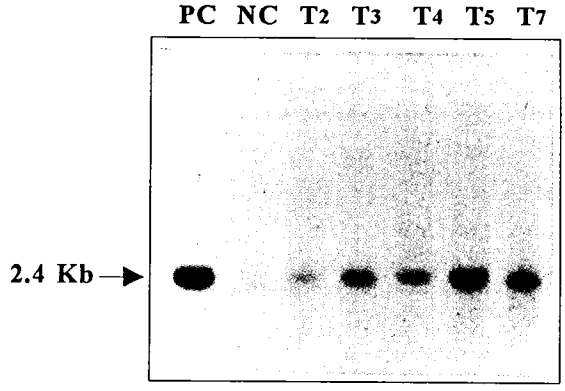


**Fig. 2.** Genomic Southern hybridization analysis of transgenic tobacco plants. Each genomic DNA sample (10 μg) from five transgenic tobacco leaf tissues ($T_2$, $T_3$, $T_4$, $T_5$ and $T_7$) was double digested with *Pst*I and *Eco*RI, separated by 0.7% agarose gel electrophoresis, and then transferred to Gene Screen membrane. A 958 bp DNA containing the part of *fad3* cDNA was isolated by the *Bgl*II digestion of palsmid pFAD3 and used as a probe. The mixture of plasmid pFAD3 DNA and genomic DNA from non-transformed tobacco leaf were simultaneously digested with *Pst*I and *Eco*RI and then uses for positive control (PC) and size marker (2.4 kb). NC denotes *Pst*I and *Eco*RI double digested genomic DNA from the non-transformed tobacco plant leaf. $T_2$, $T_3$, $T_4$, $T_5$ and $T_7$ represented the transgenic tobacco plants. Arrow indicates the hybridized bands for *fad3* gene.

days in a growth chamber. The chromosomal DNAs from the leaf of the transformed tobacco plants were analyzed by Southern blot analysis with 959 bp biotin-labeled *fad3* cDNA (Fig. 1) as a probe. As shown in Fig. 2, *Pst*I and *Eco*RI double digested chromosomal DNAs from five transformed lines ($T_2$, $T_3$, $T_4$, $T_5$ and $T_7$) showed 2.4 kb DNA bands identical to the DNA size from *Pst*I and *Eco*RI double digested plasmid pFAD3 DNA comprising CaMV35S promoter-linked *fad3* gene with an NOS terminator (Fig. 1). These results indicated that *fad3* DNAs were integrated in the chromosome of five transgenic plants. On the other hand, no significant bands were hybridized in the either the non-transformed tobacco plant or two transformed lines ($T_1$ and $T_6$, data not shown). Among the five transgenic plants, the $T_5$ line showed the highest band intensity, thus indicating that the $T_5$ line contained the highest copy number of the *fad3* DNA in its chromosome. The five transgenic lines selected were further characterized in order to analyze their transgenicity.

### Expression of the *fad3* gene in transgenic tobacco plants

The expression of *fad3* in transgenic tobacco plants was analyzed by Northern blot analyses. As shown in Fig. 3, one species of *fad3* mRNA transcript (1.3 kb) was detected in five transgenic lines whereas no transcript was observed in the non-transformed line. This indicated that the expressions of the *fad3* gene integrated in the chromosomes of transgenic plants are successfully achieved by the CaMV 35S promoter. Among the five transgenic lines, line $T_5$ was the most abundant in its *fad3* mRNA transcript. This result might be caused by the highest copy number of the *fad3* DNA integrated in the chromosome, as shown in Fig. 2.

### Changes of fatty acid composition in transgenic tobacco plants

In order to reveal the effect of *fad3* gene for fatty acid desaturation, changes in fatty acid composition were further examined in transgenic tobacco plants. To

**Table 1.** Fatty acid compositions of root and leaf tissues of wild type and transgenic tobacco plants

| Tissues | Fatty acid | wild type | $T_2$ | $T_3$ | $T_4$ | $T_5$ | $T_7$ |
|---|---|---|---|---|---|---|---|
| Roots | 16:0(%) | 29.34± 0.95 | 28.98± 1.42 | 29.07± 1.42 | 28.48± 0.30 | 29.23± 1.18 | 26.75± 0.58 |
| | 18:0(%) | 13.68± 1.11 | 4.73± 1.51 | 4.37± 0.68 | 6.97± 0.03 | 4.38± 0.47 | 6.29± 0.53 |
| | 18:1(%) | 8.43± 0.20 | 8.08± 1.03 | 6.63± 0.17 | 6.41± 0.02 | 5.80± 0.80 | 9.34± 0.96 |
| | 18:2(%) | 36.24± 0.60 | 11.57± 1.08 | 3.76± 0.53 | 7.69± 0.10 | 1.72± 0.18 | 2.29± 0.14 |
| | 18:3(%) | 12.34± 0.24 | 46.65± 2.20 | 56.18± 0.04 | 50.46± 0.24 | 58.89± 0.28 | 55.38± 0.10 |
| | 18:2+18:3(%) | 48.58± 0.37 | 58.22± 1.12 | 59.94± 0.57 | 58.15± 0.34 | 60.6± 0.10 | 57.66± 0.04 |
| | 18 total(%) | 70.68± 0.94 | 71.03± 1.42 | 70.93± 1.41 | 71.52± 0.30 | 70.78± 1.17 | 73.29± 0.40 |
| Leaves | 16:0(%) | 15.81± 1.06 | 15.45± 0.14 | 15.24± 0.28 | 17.13± 0.30 | 14.02± 0.19 | 16.14± 0.05 |
| | 18:0(%) | 4.16± 0.08 | 3.85± 0.01 | 4.10± 0.22 | 5.11± 0.08 | 4.24± 0.12 | 3.57± 0.09 |
| | 18:1(%) | 0.99± 0.01 | 1.03± 0.05 | 0.84± 0.14 | 1.04± 0.14 | 0.84± 0.14 | 0.71± 0.07 |
| | 18:2(%) | 10.96± 0.04 | 2.95± 0.24 | 1.80± 0.19 | 2.09± 0.04 | 1.56± 0.07 | 1.77± 0.17 |
| | 18:3(%) | 68.04± 0.17 | 76.74± 0.15 | 78.03± 0.27 | 74.64± 0.05 | 79.35± 0.14 | 77.81± 0.31 |
| | 18:2+18:3(%) | 79.00± 0.13 | 79.69± 0.10 | 79.83± 0.08 | 76.73± 0.09 | 80.91± 0.07 | 79.58± 0.14 |
| | 18 total(%) | 84.15± 0.05 | 84.56± 0.14 | 84.76± 0.28 | 82.87± 0.30 | 85.98± 0.19 | 83.86± 0.02 |

The values presented are the means of ± S.D. (n=3).
18 total(%) : 18:1+18:2+18:3.
$T_2$, $T_3$, $T_4$, $T_5$, $T_7$ : transgenic tobacco plants.

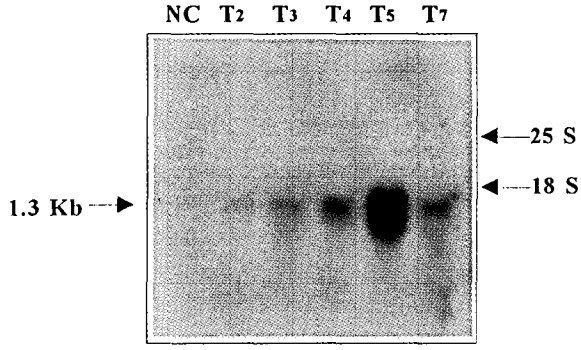


**Fig. 3.** RNA Northern blot analysis for *fad3* expression in transgenic tobacco plants. Total RNA were isolated from tobacco leaf tissues and denatured RNA samples (10 μg/well) were electrophoretically separated on a 2.2 M formaldehyde/1% agarose gel, transferred to Gene Screen membrane, and then hybridized with biotin labeled *fad3* DNA fragment (959 bp) as described in Materials and Methods section. The left arrow represents the hybridized mRNA transcripts for *fad3*.

measure the fatty acid composition, fatty acid methyl esters were prepared from lipid extracted from leaves and roots of tobacco plants and subjected to GC analysis.

In non-transformed tobacco plants, ratios for 18:2 fatty acids (linoleic acid) to 18:3 fatty acids (linolenic acid) in roots were maintained at about 3:1, whereas it was approximately 1:6 in leaves (Table 1). This result was consistent with the report that *fad3* mRNA transcripts are more abundant in leaf tissue than root tissue (Yadav *et al.*, 1993). In five independent transformants containing the cloned *fad3* gene, substantially increased accumulation of 18:3 fatty acids with corresponding decreases in the amount of 18:2 fatty acids were noticed with some variations as shown in Table 1. The remarkable decreases of 18:2 fatty acid contents in the leaves and roots of transgenic tobacco plants indicated that the levels of active microsomal ω-3 fatty acid desaturase were elevated by the cloned *fad3* genes.

In root tissues from transgenic tobacco plants, the contents of the 18:3 fatty acids were elevated from 3.7- to 4.7-fold when compared with non-transformed tobacco plants. Among the five transgenic tobacco plants, line $T_5$ showed the highest content of 18:3 fatty acid due possibly to the abundance of the *fad3* gene copy and, as a consequence, its high level of mRNA transcript production in transgenic tobacco plants (Fig. 2 and 3). In comparison with our transgenic tobacco plants, rooty tumors from the transgenic *Arabidopsis thaliana* (Arondel *et al.*, 1992) and transgenic carrot (Yadav *et al.*, 1993) showed 1.6- and 7-fold increments in the amount 18:3 fatty acids, respectively.

In transgenic leaf tissue, by contrast, only slight increases (1.1-fold) in 18:3 fatty acid synthesis were observed, which may be due to the large amount of linolenic acid as a basal level detected in nontransformed leaf tissues. The expression of the *fad3* gene did not change the total sum for the unsaturated fatty acids (oleic acid, linoleic acid and linolenic acid) content in root tissue, although a meaningful increment in the sum of linoleic acid and linolenic acid were noticed. The contents of oleic acid (18:1) in transgenic tobacco root tissue were not significantly changed, but that of stearic acid (18:0) was notably decreased. These results implied that the reduced level of 18:2 fatty acid owing to the activity of the cloned *fad3* gene acted as a cue to compensation for the overall fluxes of 18:0 fatty acid into 18:2 fatty acid *via* 18:1 fatty acid by the activities of the endogenous stearoyl-acyl carrier protein (Iba *et al.*, 1993) and ω-6 desaturase.

Fatty acid composition has been reported to be an important factor for plant growth in dependence on temperature (Gerloff *et al.*, 1966; Graham and Patterson, 1982) and studies of mutants defective in polyunsaturated fatty acid synthesis have pointed out that polyunsaturated fatty acid contributes to a high extent of cold tolerance in bacteria and plants (St. John *et al.*, 1979; Somerville and Browse, 1991; Hugly and Somerville, 1992; Miquel *et al.*, 1993).

In a recent report by Kodama *et al.* (1994), the R1 seedling of the transgenic tobacco plant containing the cloned *fad7* gene showed a 1.1-fold increment of 18:3 fatty acid content when compared with the wild type. The increased levels of 16:3 and 18:3 fatty acid were suggested to be in relation to the enhanced cold tolerance of transgenic plants. The level of endogenous *fad7* gene expression in nontransformed tobacco plants, however, was not altered at a low temperature (Iba *et al.*, 1993). According to the results of the increment of 18:3 fatty acid synthesis by the cloned *fad3* gene (Table 1) or by *fad7* (Kodama *et al.*, 1994), ω-3 fatty acid sysnthesis is considered to occur in either microsomes or chloroplasts in tobacco leaves in a mode little influenced by shifting to a low temperature condition.

Despite numerous studies on the roles of unsaturated fatty acid in plant growth temperature, the molecular mechanisms of cold tolerance in plants have not been well established and thus remain to be further elucidated. Therefore, the transgenic tobacco plant produced in this experiment can be used as a tool for analyzing the roles of the *fad3* gene during the cold tolerance of plants.

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## References

Arondel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H. M. and Somerville, C. R., (1992) *Science* **258**, 1353.

Browse, J. and Somerville, C. R. (1991) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 467.

Chee, K. M., Gong, J. X., Meydani, M., Ausman, L., Johnson, J., Siguel, E. N. and Schaefer, E. F. (1990) *Lipids* **25**, 523.

Chomezynsky, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156.

Gawel, N. J. and Jarret, R. L. (1991) *Plant Mol. Biol. Reporter* **9**, 262.

Gerloff, E. D., Richardson, T. and Stahmann, M. A. (1966) *Plant Physiol.* **41**, 1280.

Graham, D. and Patterson, B. D. (1982) *Ann. Rev. Plant Physiol.* **33**, 347.

Holster, M., de Waele, D., Depicker, A., Messens, E., Van Montegue, M. and Schell, J. (1978) *Mol. Gen. Genet.* **163**, 181.

Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichnoltz, S., Rogers, S. C. and Farely, R. T. (1985) *Science* **277**, 1229.

Hugly, S. and Somerville, C. R. (1992) *Plant Physiol.* **99**, 197.

Iba, K., Gibson, S., Nishiuchi, T., Fuse, T., Nishimura, M., Arondel, V., Hugly, S. and Somerville, C. R. (1993) *J. Biol. Chem.* **268**, 24009.

Kodama, J., Hamada, T., Horiguchi, G., Nishimura, M. and Iba, K. (1994) *Plant Physiol.* **105**, 601.

Lyons, J. M., Wheaton, T. A. and Pratt, H. (1964) *Plant Physiol.* **39**, 262.

Marr, A. G. and Ingraham, J. L. (1982) *J. Bacteriol.* **84**, 1260.

Miquel, M., James, D. J., Dooner Jr., H. and Browse, J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6208.

Murashige, T. and Skoog, F. (1962) *Physiol. Plant* **15**, 437.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Somerville, C. R. and Browse, J. (1991) *Science* **252**, 80.

St. John, J. B., Christiansen, M. N., Ashworth, E. N. and Gentmer, W. A. (1979) *Crop Sci.* **19**, 65.

Yadav, N. S., Wierzbicki, A., Aegerter, M., Caster, C. S., Perez-Grau, L., Kinney, A. J., Hitz, W. D., Booth, J. R. B., Schweiger Jr., B., Stecca, K. L., Allen, S. M., Blackwell, M., Reiter, R. S., Carlson, T. J., Russel, S. H., Feldmann, K. A., Pierce, J. and Browse, J. (1993) *Plant Physiol.* **103**, 467.