

Modification of Fatty Acid Composition in Rice Plants by Transformation with a Tobacco Microsomal ω -3 Fatty Acid Desaturase Gene (*NtFAD3*)

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Received 12 August 1999; accepted 26 October 1999

Abstract

A tobacco microsomal ω -3 fatty acid desaturase gene (*NtFAD3*) under the control of CaMV35S promoter was introduced into rice plants by the microprojectile-mediated transformation. The transgenic plants grew normally and showed high seed fertility and characteristics similar to those of non-transgenic plants. However, the fatty acid compositions in leaves, roots and seeds were modified by the transformation. The content of linoleic acid (18:2) was lower in the transgenic plants than in the non-transgenic plants. However, the content of linolenic acid (18:3) in the root and leaf tissues was, respectively, 1.8- and 1.1-fold higher in 4 transgenic plant lines of the homozygous R₃ progenies, than in the non-transgenic plants. The 18:3 content in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) that are the components of the extrachloroplast membrane, in the leaves of mature plants was higher in transgenic plants than in the non-transgenic plants. Thus, *NtFAD3* was expressed and it desaturated 18:2 to 18:3 in the endoplasmic reticulum in rice cells.

1. Introduction

The change in the degree of unsaturation of fatty acids is an important factor in metabolic adaptation of higher plants to environmental stresses, especially to temperature stress (Somerville and Browse, 1991). In cyanobacteria, cold tolerance was enhanced by the increase in the amount of unsaturated fatty acids in membrane lipids by genetic engineering (Wada *et al.*, 1990). Alterations of chilling sensitivities have been reported in the transgenic tobacco (Murata *et al.*, 1992), *Arabidopsis thaliana* (Browse *et al.*, 1986) and rice (Yokoi *et al.*, 1998) into which the glycerol-3-P acyltransferase gene was introduced.

An increased production of highly unsaturated fatty acids such as linolenic acid (18:3) at low temperature has been observed in connection with cold acclimation in many plants (Graham and Patterson, 1982). The *FAD7* gene, a gene for a chloroplast ω -3 fatty acid desaturase, which is the key enzyme catalyzing the conversion of hexadecadienoic (16:2) and linoleic (18:2) acids to hexadecatrienoic (16:3) and linolenic (18:3) acids in lipids in

leaf tissues, was isolated from *Arabidopsis thaliana* by chromosomal walking (Iba *et al.*, 1993). The transgenic tobacco plants in which the trienoic fatty acid content was increased by the introduction of *Arabidopsis FAD7* gene, showed lower chilling injury than nontransgenic plants (Kodama *et al.*, 1994). These findings indicate that the application of molecular genetic techniques to control the expression of the ω -3 fatty acid desaturase genes may effectively modify the fatty acid composition, and that the polyunsaturated fatty acids are important in the chilling tolerance of higher plants.

The ω -3 fatty acid desaturases are found in microsomes as well as in plastid envelopes (Mazliak, 1994). A *FAD3* gene corresponding to the microsomal ω -3 fatty acid desaturase was isolated by using a map-based approach from *Brassica napus* (Arondel *et al.*, 1992) and by heterologous probing from *Nicotiana tabacum* (Hamada *et al.*, 1994). Hamada *et al.* (1998) indicated that an increase in the 18:3 level was not directly involved in the chilling tolerance of *NtFAD3* transgenic tobacco plants.

We introduced the *FAD3* gene isolated from tobacco (*NtFAD3*) under the control of the CaMV

35S promoter into rice plants to investigate the effect of an increase in the 18:3 level within microsomal membrane lipids. We report here that the *NtFAD3* is expressed to desaturase 18:2 to 18:3 in leaves, roots and seeds of the transgenic rice plants in R₃ generation, although the transgenic plants with modified fatty acid composition were normal in other characteristics. We also preliminarily evaluated the chilling tolerance at the seedling stage in the transgenic plant lines.

2. Materials and Methods

2.1 Plant materials

Four fertile transgenic lines (#3, #11, #16 and #27) produced from a Japonica rice variety, Notohikari, by the microprojectile-mediated transformation, as previously described by Wakita *et al.* (1998), were used in this experiment. The transgenes were a microsomal ω -3 fatty acid desaturase gene isolated from *Nicotiana tabacum* (*NtFAD3*) controlled by CaMV 35S promoter and a bialaphos resistant gene (*bar*) driven by rice Actin 1 promoter. Selfed seeds (R₁) of the transgenic plants were sown on LS basal medium (Linsmaier and Skoog, 1965) containing 5 mg l⁻¹ bialaphos and then the bialaphos-resistant plants were grown in a chamber. The transgenic plants were selected by bialaphos resistance and the homozygous R₃ progenies of 4 independent transgenic lines were obtained. They were grown together with nontransformed plants in a closed greenhouse and some characteristics were compared with those of the nontransgenic plants.

The integration of the *NtFAD3* gene was confirmed by the Southern-blot analysis performed as described in the protocol of Alk Phos Detect systems (Amersham Pharmacia Biotech) (Wakita *et al.*, 1998).

2.2 Analysis of fatty acid composition

For analysis of the fatty acid compositions, the seeds of the homozygous R₃ transgenic lines were sown in aseptic conditions and were grown in growth chambers kept at 26 °C and 16-h photoperiod under fluorescent lamps with 67.2 $\mu\text{Mm}^{-2} \text{s}^{-1}$. At the third leaf stage, the third leaf and all roots were collected. The fatty acids were isolated from the leaves, roots and dehusked seeds, and analyzed by gas chromatography as described by Kodama *et al.* (1994). To investigate the fatty acid composition of individual lipid species, we performed two-dimensional TLC (Roughan *et al.*, 1978) for the lipids extracted from the mature leaves grown in a greenhouse and determined the fatty acid compositions of the major lipids by gas-

chromatography.

2.3 Evaluation of chilling tolerance

The seeds of transgenic lines and nontransgenic Notohikari were sown on a mixture of vermiculite and perlite (1:1) in a polyethylene pot, and the seedlings were grown in a growth chamber and kept at 26 °C with a 16-h photoperiod provided by fluorescent lamps. From the 11th day after germination, the seedlings at the third leaf stage were exposed to 5 °C under a 16-h photoperiod for 9 days in a growth chamber. After the low temperature treatment, the seedlings were transferred into another growth chamber furnished with fluorescent lamps and kept at 26 °C. Irradiance at 26 °C and 5 °C was 67 and 19 $\mu\text{Mm}^{-2} \text{s}^{-1}$, respectively, and the relative humidity in the chambers was 50-80%. Following the low temperature treatment, the degree of chilling injury was scored for each of 20 plants after 7 days of growth at 26 °C, as the percent of green area in the third leaf. Survival rate was calculated after 20 days of growth at 26 °C.

3. Results and Discussion

3.1 Characters of transgenic rice plants

We obtained four independent transgenic lines, #3, #11, #16 and #27, which integrated both *bar* and *NtFAD3* cDNA fragments. We selected the homozygous progenies having a modified fatty acid compositions from each of the four lines. The four transgenic lines in the R₃ generation germinated and grew normally in the greenhouse. **Fig. 1** and **Table 1** show the results of Southern-blot analysis of *Bam* H1-digested DNAs from eight plants in the four transgenic lines. All plants showed the hybridization signals for the *bar* (**Fig.1 a**) and *NtFAD3* (**Fig.1 b**) showing the same pattern within each line. The patterns of hybridizing bands were also similar to those from the R₀ generation (Wakita *et al.* 1998), indicating that integrated cDNA fragments were inherited stably and in a closely linked manner.

Table 1. shows some characteristics of the transgenic and nontransgenic plants. The length of culms and panicles in the four transgenic lines were similar to those in the nontransgenic plants, although the flowering date was about 2 days later in the transgenic plants than in the nontransgenic plants. Pollen fertility and seed fertility were higher than 95% in all transgenic and nontransgenic plants.

The seedlings of all transgenic lines, except for #27, grew normally on the bialaphos containing medium (**Table 1**). Although #27 integrated the *bar* gene (**Fig. 1**), this line did not grow on the bialaphos containing medium. This may have been due to the

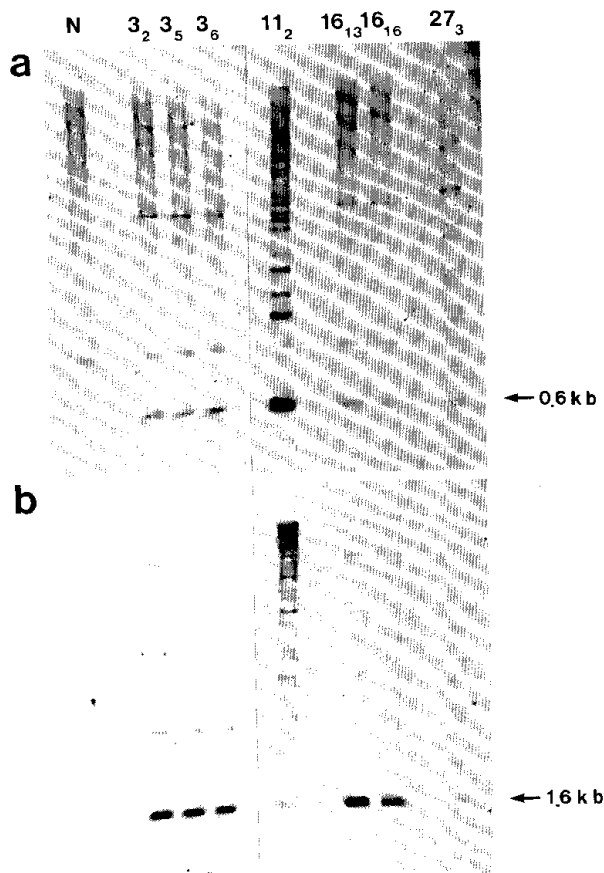


Fig. 1. Southern-blot hybridization of *Bam*H1-digested DNAs from the R_3 plants of four transgenic lines. (a) and (b) were probed with the 402 bp product of *bar* gene and 490 bp product of *NtFAD3* gene after PCR amplification, respectively. N: nontransformant as a negative control, #3-2, #3-5 and #3-6, are homozygous sib-lines of #3, and #16-13 and #16-16 are those of #16, and so on. The 0.6 kb and 1.6 kb show an estimated full length of *bar* and *NtFAD3* genes, respectively.

silencing of the *bar* gene.

3.2 Fatty acid compositions of transgenic plants in R_3

Lipids in the root tissues of nontransgenic Notohikari mainly consist of three kinds of fatty acids, 16:0, 18:2 and 18:3. The molar ratio of these fatty acids was 31.6, 34.7 and 26.7 %, respectively. The fatty acid compositions in the root tissues of the seedlings of the four transgenic lines was significantly different from those in Notohikari. The content of 18:2 in #3, #11, #16 and #27 was 14.4, 9.5, 10.6 and 12.9 mol%, respectively, although it was 34.7 mol% in nontransgenic plants (Table 2). The content of 18:3 in #3, #11, #16 and #27 was 44.2, 51.9, 49.6 and 46.0 mol%, respectively, although it was 26.7 mol% in nontransgenic plants (Table 2).

The content of 18:3 in the leaf tissues of nontransgenic seedlings was 66.2 mol%, although the contents in the four transgenic seedling leaves were higher than 71.9 mol% (Table 3). Thus, in both root and leaf tissues, 18:3 contents were significantly higher, and 18:2 contents were significantly lower in the four transgenic lines than in non-transgenic Notohikari.

To investigate the expression of the *NtFAD3* gene in the leaf tissues of transgenic plants, we extracted lipids from the leaves of mature plants at the booting stage and analyzed the fatty acid compositions. The fatty acid compositions of the major chloroplast lipids, monogalactosyldiacylglycerol (MGD) and digalactosyldiacylglycerol (DGD), isolated from leaves of transgenic line #11, were similar to those in the nontransgenic plant. In contrast, the fatty acid composition of the phospholipids in transgenic plant cells such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) that are the components of the extrachloroplast membranes, differed from those in Notohikari. The 18:2 and 18:3 contents of MGD, DGD, PC and PE

Table 1. Characteristics of transgenic plants (R_3)

Lines	Bialaphos resistance ¹⁾	Transgene ²⁾		Flowering Date ³⁾	Culm length (cm)	Panicle length (cm)	Pollen fertility (%)	Seed fertility (%)
		<i>bar</i>	<i>NtFAD3</i>					
Notohikari	S	—	—	0	74.2	23.1	97.8	97.7
#3-2	R	+	+	+2	73.1	25.2	98.2	96.4
#11-2	R	+	+	+1	75.1	21.4	97.4	93.5
#16-13	R	+	+	+2	72.5	21.2	97.8	96.7
#27-3	S	+	+	+2	74.5	23.6	97.3	97.6

¹⁾ S: susceptible, R: resistant

²⁾ Southern-blot hybridization

³⁾ Flowering date of Notohikari was regarded as 0

Table 2. Fatty acid composition of total lipids extracted from the seedling root of nontransgenic and transgenic plant lines (R_3)

Fatty acids	16:0	18:0	18:1	18:2	18:3
Notohikari	31.6 ± 0.3	2.3 ± 0.3	4.9 ± 0.4	34.7 ± 0.3	26.7 ± 0.2
#3-2	34.7 ± 0.9	3.0 ± 0.9	3.8 ± 0.5	14.4 ± 4.1	44.2 ± 6.3
#11-2	32.9 ± 0.3	1.9 ± 0.1	3.8 ± 0.2	9.5 ± 1.4	51.9 ± 2.0
#16-13	34.1 ± 1.3	2.2 ± 0.1	3.8 ± 0.8	10.6 ± 4.7	49.6 ± 6.9
#27-3	32.9 ± 1.5	2.0 ± 0.2	6.2 ± 4.6	12.9 ± 3.1	46.0 ± 3.1

Values are mol% ± S. D.

16:0, hexadecanoic acid (palmitic acid); 18:0, octadecanoic acid (stearic acid); 18:1, Δ 9-octadecenoic acid (oleic acid); 18:2, Δ 9, 12-octadecadienoic acid (linoleic acid); 18:3, Δ 9, 12, 15-octadecatrienoic aci (α -linolenic acid).

Table 3. Fatty acid composition of total lipids extracted from the seedling leaves of nontransgenic and transgenic plant lines (R_3)

Fatty acids	16:0	16:1	18:0	18:1	18:2	18:3
Notohiakari	17.0 ± 0.8	0.2 ± 0.1	1.6 ± 0.2	1.0 ± 0.2	14.1 ± 1.7	66.2 ± 2.6
#3-2	17.1 ± 0	0.5 ± 0.1	1.6 ± 0.1	1.1 ± 0.2	6.1 ± 0.6	73.7 ± 0.6
#11-2	17.1 ± 0.9	0.2 ± 0.1	1.6 ± 0.1	1.2 ± 0.2	8.1 ± 0.1	71.9 ± 0.7
#16-13	18.3 ± 0.1	0.2 ± 0	1.7 ± 0.3	0.7 ± 0.1	4.9 ± 1.7	74.3 ± 2.1
#27-3	16.9 ± 0.4	0.2 ± 0	1.6 ± 0	1.2 ± 0.2	7.7 ± 1.8	72.5 ± 1.5

Values are mol% ± S. D.

16:0, hexadecanoic acid (palmitic acid); 16:1, Δ 3-trans-hexadecenoic acid; 18:0, octadecanoic acid (stearic acid); 18:1, Δ 9-octadecenoic acid (oleic acid); 18:2, Δ 9, 12-octadecadienoic acid (linoleic acid); 18:3, Δ 9, 12, 15-octadecatrienoic aci (α -linolenic acid).

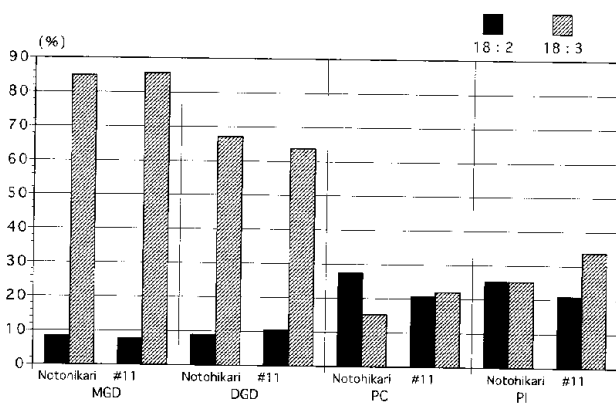


Fig. 2. Linoleic acid (18:2) and linolenic acid (18:3) contents in the lipids extracted from leaf tissues of mature plants. MGD: monogalactyldiacylglycerol, DGD: digalactosyldiacylglycerol, PC: phosphatidylcholine, PE: phosphatidylethanolamine

in the transgenic line #11 and nontransgenic Notohikari are shown in **Fig. 2**. The 18:2 and 18:3 contents of both MGD and DGD in #11 were similar to those in Notohikari. However, the 18:2 contents of PC and PE in #11 were lower and the 18:3 contents of PC and PE in #11 were higher than those in Notohikari. *NtFAD3* was expressed in the

leaf tissues of transgenic rice plants, and it desaturated 18:2 to 18:3 in the endoplasmic reticulum.

Table 4 shows the fatty acid composition of the total lipids extracted from seeds (R_3). The 18:3 content was significantly higher in the transgenic lines than in the non-transgenic plants, although the content was only 2.5-4.2 mol%.

NtFAD3 under the control of CaMV35S promoter was stably expressed in the transgenic rice plants, and it desaturated 18:2 to 18:3 effectively in microsomal membranes of rice tissues. These results are complementarily in agreement with the study on *Arabidopsis* *fad3* mutant, which is deficient in linoleate desaturation. In this mutant, the amount of 18:3 in all the major extrachloroplast phospholipids of seeds, leaves, and roots was lower than in the wild type (Browse *et al.* 1993).

3.3 Chilling injury at the seedling stage

Fig. 3 shows the chilling injury after 5 °C-treatment at the seedling stage. At the 7th day after the end of the low temperature treatment, half of the area of the third leaf remained green in transgenic #3-2, although the third leaf withered in 74%, on

Table 4. Fatty acid composition of total lipids extracted from the seeds of nontransgenic and transgenic plant lines (R₃).

Fatty acids	16:0	16:1	18:0	18:1	18:2	18:3
Notohiakari	22.6 ± 1.2	0.2 ± 0	3.3 ± 0.2	30.6 ± 1.3	41.7 ± 1.2	1.7 ± 0
#3-2	23.7 ± 0.3	0.2 ± 0	2.9 ± 0.3	29.6 ± 0.3	41.1 ± 0.3	2.5 ± 0.3**
#11-2	23.7 ± 0.6	0.2 ± 0.1	2.9 ± 0.4	30.0 ± 1.3	39.0 ± 0.4**	4.2 ± 1.3**
#16-13	23.1 ± 0.5	0.2 ± 0	2.9 ± 0.2	28.5 ± 0.6	42.7 ± 0.7	2.7 ± 0.2**

Values are mol% ± S. D.

**Significant at 1% level of probability

16:0, hexadecanoic acid (palmitic acid); 16:1, Δ 3-trans-hexadecenoic acid; 18:0, octadecanoic acid (stearic acid); 18:1, Δ 9-octadecenoic acid (oleic acid); 18:2, Δ 9, 12-octadecadienoic acid (linoleic acid); 18:3, Δ 9, 12, 15-octadecatrienoic acid (α -linolenic acid).

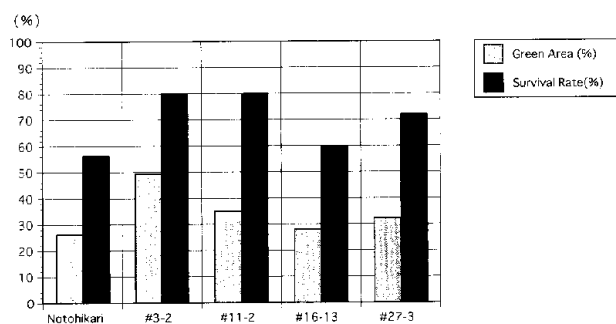


Fig. 3. Chilling injury at the seedling stage of nontransgenic Notohiakari and transgenic lines. The green area was measured in the third leaf 7 days after the end of the low temperature treatment. Survival rates were counted 20 days after the end of chilling.

average, in the nontransgenic plants. On the other hand, at 20 days after the low temperature treatment, the survival rates of transgenic lines #3, #11, #16 and #27 were 80, 80, 60 and 72%, respectively, while that of nontransgenic plants was 56%.

A rapid and stable method for the evaluation of chilling injury at the seedling stage has been reported by Nagamine and Nakagahra (1990); they exposed 12-day-old seedlings to 5°C for 4 days and examined the withering of the seedlings. Since the Japanese variety, Notohiakari is highly tolerant to low temperature, the difference in the responses to chilling of transgenic and nontransgenic plants was not observed by their method. In the present experiment, we prolonged the period of low temperature treatment to 9 days, and observed the tolerance to low temperature in transgenic plants, especially #3 and #11. The survival rate at the 20 days after the end of treatment was closely correlated with the green area rate of the third leaf on the 7th day.

Yokoi *et al.* (1998) obtained the transgenic rice plants to which a cDNA for *Arabidopsis* glycerol-3-phosphate acyltransferase (GPAT) was introduced, and found that those plants had a larger amount of

unsaturated fatty acids in phosphatidylglycerol (PG). They also found chilling tolerance in photosynthesis of the transgenic rice plants. Kodama *et al.* (1994) produced transgenic tobacco plants with increased amounts of trienoic fatty acids (16:3 and 18:3) in leaf tissues, by the introduction of the *Arabidopsis thaliana* *FAD7* gene, that corresponds to the gene for chloroplast ω -3 fatty acid desaturase. In their transgenic tobacco, the low temperature induced damage was effectively alleviated. This indicates the importance of polyunsaturated fatty acids in the chilling tolerance of higher plants.

In both experiments mentioned above, the increased levels of unsaturated fatty acids in the plastid membrane was considered to increase the chilling tolerance of plants. The transgenic rice plants we obtained also contained a larger amount of the polyunsaturated fatty acid in the microsomal membrane and some transgenic lines showed lower chilling injury than nontransgenic plants. However, two transgenic lines, #16 and #27 which contained high amount of 18:3 showed little differences of chilling tolerance. Hamada *et al.* (1998) did not observe differences in growth response to temperature between the wild type and high 18:3 transgenic tobacco plants overexpressing the *NtFAD3*. Further experiments are in progress to determine the chilling tolerance of the transgenic rice plants.

Acknowledgments

This study was supported by Grant RFTF96L00602 from the Japan Society for Promotion of Science. We are grateful to Dr. H. Anzai for supplying bialaphos and to Dr. T. Hamada for his technical advice in the analysis of fatty acids.

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