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GENETIC TRANSFORMATION AND HYBRIDIZATION

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A tobacco microsomal ω-3 fatty acid desaturase gene increases the linolenic acid content in transgenic sweet potato (*Ipomoea batatas*)

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Abstract A tobacco microsomal ω-3 fatty acid desaturase gene (*NtFAD3*) under the control of the CaMV 35S promoter or an improved CaMV 35S promoter (El2Ω) was introduced into sweet potato. Transformed sweet potato plants were obtained from embryogenic calli following *Agrobacterium tumefaciens*-mediated transformation. The transgenic plants grew normally to form storage roots and showed properties similar to those of the non-transgenic plants. The fatty acid composition in the transgenic line with a *NtFAD3* gene driven by the CaMV 35S promoter was similar to that in the non-transformant. However, in the transgenic line that had a *NtFAD3* gene driven by the El2Ω promoter, linoleic acid (18:2) and linolenic acid (18:3) contents were 47.7 mol% and 24.8 mol%, respectively, which were significantly different from the 53.6 mol% and 11.3 mol%, respectively, in the non-transformant. The *NtFAD3* gene driven by the El2Ω promoter was expressed more strongly than that driven by the CaMV 35S promoter, thereby increasing the linolenic acid content in the transgenic sweet potato plants.

Keywords El2Ω-1 · Fatty acid composition · ω-3 Fatty acid desaturase · Sweet potato · Transgenic plants

Introduction

Sweet potato [*Ipomoea batatas* (L.) Lam.] ranks seventh in the world among food crops with respect to annual production (Jabsson and Raman 1991). However, conventional breeding programs based on sexual hybridization of sweet potato remain poorly developed because of its sterility and cross incompatibility. To overcome these limitations, breeders must incorporate novel approaches such as somatic hybridization and genetic transformation into conventional sweet potato breeding programs. A few reports are available on transformation in sweet potato using *Agrobacterium rhizogenes*- and *A. tumefaciens*-mediated systems (Dodds et al. 1991; Otani et al. 1993; Newell et al. 1995; Gama et al. 1996; Morán et al. 1998) and the direct gene delivery systems containing bombarded leaves and petioles and transformed protoplasts (Parakash and Varadarajan 1992; Murata et al. 1997). Recently, we established an efficient method for the production of transgenic sweet potato plants through *A. tumefaciens*-mediated transformation using embryogenic calli (Otani et al. 1998).

The genes that have been previously introduced into sweet potato are marker and/or selectable marker genes and genes for resistance to insect and virus diseases (Newell et al. 1995; Murata et al. 1997; Morán et al. 1998). On the other hand, modification of plant metabolism by the transformation technique is an attractive way of generating new cultivars that would have novel traits such as an improved production of metabolites and the production of novel compounds. We introduced foreign DNA related to fatty acid metabolism in order to modify the fatty acid composition of the lipids, expecting thereby a functional or nutritional improvement. The ω-3 fatty acid desaturases are membrane-bound enzymes catalyzing the conversion of dienoic fatty acids (16:2 and 18:2) to trienoic fatty acids (16:3 and 18:3) in lipids, and they are found in microsome and plasmid membranes (reviewed by Mazliak 1994). Since any change in the degree of the unsaturation of fatty acids is an important factor in the metabolic adaptation of higher plants to temperature stress (reviewed by Somerville and Brouse 1991), the application of molecular genetic techniques as a means of modifying the fatty acid composition of sweet potato might contribute to its adaptation to low temperature.

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We have introduced the microsomal ω-3 fatty acid desaturase cDNA isolated from tobacco (*NtFAD3*, Hamada et al. 1994) into the embryogenic calli via *A. tumefaciens* and produced transgenic sweet potato plants. In this paper, we describe the expression of the *NtFAD3* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter or the modified CaMV 35S promoter, the El2Ω promoter (Mitsuhara et al. 1996), and the subsequent modification of the fatty acid composition in the transgenic sweet potato plants.

Materials and methods

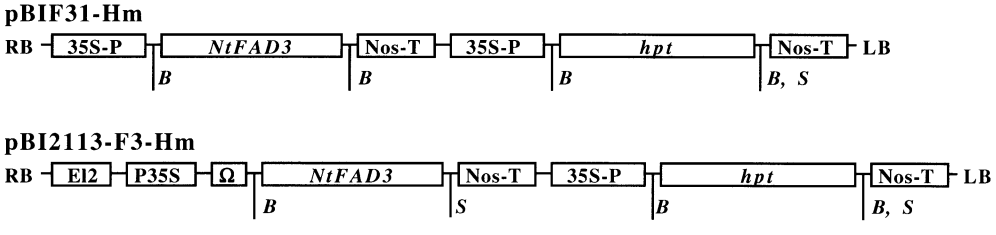
Bacterial strain and plasmid

Agrobacterium tumefaciens strains EHA101/pBIF31-Hm or EHA101/pBE2113-F3-Hm used in the present study contained a binary vector with the gene for hygromycin resistance (*hpt*) (Fig. 1). Plasmid pBIF31-Hm, a derivative of pTF1SN (Wakita et al. 1998), contained the *NtFAD3* cDNA fragment from nucleotide 52 to 1,381 (Accession no. D26509) driven by the CaMV 35S promoter. Plasmid pBE2113-F3-Hm, a derivative of pTF1SIIn (Hamada et al. 1998), contained the *NtFAD3* cDNA fragment driven by the El2Ω promoter, which is a new CaMV 35S promoter containing two extra-enhancer sequences; one is the El2 sequence, which is present in the upstream region of the CaMV 35S promoter, and the other is the Ω sequence, which is in the 5′-untranslated region of tobacco mosaic virus genomic RNA (Mitsuhara et al. 1996).

Transformation, selection and plant regeneration

Embryogenic calli were induced from shoot meristems of sweet potato cv. Kokei 14 on 4F1 medium, which is LS medium (Linsmaier and Skoog 1965) supplemented with 1 mg/l 4-fluorophenoxyacetic acid (4FA), 3% (w/v) sucrose and 0.32% (w/v) gellan gum (Otani and Shimada 1996). Transformation was performed according to the method of Otani et al. (1998) with some modifications. The colony of bacteria was transferred to liquid 4F1 medium supplemented with 10 mg/l acetosyringone and shaken at 100 rev/min for 1 h at 26 C in the dark. The embryogenic calli were then immersed in a bacterial suspension for 2 min and blotted dry with sterile filter paper to remove excess bacteria. The calli were subsequently transferred onto co-culture medium (4F1 medium supplemented with 10 mg/l acetosyringone). After 3 days of co-cultivation at 23 C in the dark, the infected calli were washed three times with sterile distilled water supplemented with 500 mg/l carbenicillin and then transferred onto selection medium (4F1 medium supplemented with 25 mg/l hygromycin and 500 mg/l carbenicillin). The calli were cultured at 26 C in the dark and sub-cultured onto fresh medium every 2 weeks. After 6 weeks of culture on the selection medium, the calli were transferred onto somatic embryo formation medium [LS medium supplemented with 4 mg/l abscisic acid (ABA), 1 mg/l gibberellic acid (GA_3), 25 mg/l hygromycin, 500 mg/l carbenicillin, 3% (w/v) sucrose and 0.32% (w/v) gellan gum] and cultured at 26 C under a 16-h (day)/8-h (night) photoperiod. Somatic embryos formed from hygromycin-resistant calli were transferred onto LS medium supplemented with 0.05 mg/l ABA, 25 mg/l hygromycin, 500 mg/l carbenicillin, 3% (w/v) sucrose and 0.32% (w/v) gellan gum after 21 days of culture on the somatic embryo formation medium. Regenerated plants derived from somatic embryos were cultured on LS medium supplemented with 25 mg/l hygromycin, 500 mg/l carbenicillin, 3% (w/v) sucrose and 0.32% (w/v) gellan gum.

Fig. 1 Schematic representation of the plasmids used in the experiment. *RB* Right border, *LB* left border, *B Bam*HI site, *S Sac*I site, *35S-P* CaMV 35S promoter, *Nos-T* Nos terminator, *El2* 5′-upstream sequence of CaMV 35S promoter (–419 to –90) × 2, *P35S* 5′-upstream sequence of CaMV 35S promoter (–90 to –1), *Ω* 5′-upstream sequence of TMV



Southern blot analysis of the transgenic plants

The total DNAs were prepared from leaf tissues harvested from transgenic sweet potato plants using the MagExtractor-Plant Genome DNA extraction kit (Toyobo Biochemicals). Five micrograms of the DNAs was digested with restriction endonucleases *Bam*HI and *Sac*I and then fractionated by electrophoresis on a 0.8% (w/v) agarose gel. The fractionated DNA segments were transferred to nylon membranes (Biodyne Plus, Pall Ultrafine Filtration) and hybridized with a polymerase chain reaction (PCR)-labeled probe. The 615-bp product of the *hpt* gene and the 490-bp product of the *NtFAD3* gene after PCR amplification were used as probes. Primers NF3–1, 5′-GCTCTCCTCCTCTATCTCTG-3′, and NF3–2, 5′-TGTTTCTCACTCATTCATCC-3′, were designed and synthesized according to the sequence of the *NtFAD3* gene; they were expected to produce a 490-bp product. Primers HPT-F, 5′-GACCTGCCTGAAACCGAACT-3′, and HPT-R, 5′-TTCTGCGGGCGATTTGTGTA-3′ were designed and synthesized according to the sequence of the *hpt* gene; they were expected to produce a 615-bp product. Southern blot analysis was performed as described in the protocol of AlkPhos Detect systems (Amersham Pharmacia Biotech).

Northern blot analysis of the transgenic plants

In our experiments, gene expression was examined using leaf tissues because the RNAs of the leaf tissues were easy to prepare and both the CaMV 35S and El2Ω promoters functioned constantly. RNAs were prepared from leaf tissues using the ISOGEN system (Nippon Gene). Twenty micrograms of the total RNAs was denatured and fractionated by electrophoresis on a 1.2% (w/v) agarose gel. The RNAs were transferred to positively charged nylon membranes (Boehringer Mannheim) and hybridized with a PCR-labeled probe. The major fragments were identified by Northern hybridization against probes of the *hpt* or *NtFAD3* genes using the DIG systems (Boehringer Mannheim) (Wakita et al. 1998).

To estimate the level of *NtFAD3* gene expression, we performed Northern blot analysis by changing the concentration of RNAs prepared from transgenic plants: 2 μg RNAs of transformants plus 18 μg RNAs of non-transformants (total 20 μg), 5 μg RNAs of transformants plus 15 μg RNAs of non-transformants (total 20 μg), 10 μg RNAs of transformants plus 10 μg RNAs of non-transformants (total 20 μg) and 20 μg RNAs of transformants, respectively, were loaded on a 2.0% (w/v) agarose gel.

Fatty acid analysis of the transgenic plants

The fatty acid compositions of whole lipids extracted from the leaves, roots and root tubers of transgenic sweet potato plants were analyzed as described by Kodama et al. (1994). The fatty acid compositions were determined by gas chromatography.

Results and discussion

Regeneration of hygromycin-resistant plants

The embryogenic calli infected with *Agrobacterium tumefaciens* were cultured on hygromycin-containing media for 60 days, and several hygromycin-resistant calli producing numerous somatic embryos were obtained. Hygromycin-resistant plantlets were developed from these somatic embryos on plant formation medium. All of the regenerated plants grew and rooted on the plant growth regulator-free LS medium supplemented with 25 mg/l hygromycin; these were subsequently examined for fatty acid composition. Regenerated plants were transferred to pots containing a mixture of vermiculite and perlite (3:1) and maintained at 26 C under a 16-h (day)/8-h (night) photoperiod in a growth chamber for 14 days. The regenerated plants were then grown in a greenhouse and analyzed for DNA integration by Southern blot analysis and RNA expression by Northern blot analysis. These transgenic sweet potato plants grew normally and formed storage roots after 3 months.

Integration of foreign DNA in the genome of transgenic plants

We obtained 32 independent hygromycin-resistant lines, 14 transgenic lines transformed with pBIF31-Hm harboring the CaMV 35S promoter-*NtFAD3* and 18 transgenic lines with pBE2113-F3-Hm harboring the El2Ω promoter-*NtFAD3*. Eight lines, four with the CaMV 35S promoter-*NtFAD3* (35S-1, 35S-2, 35S-3, 35S-4) and four with the El2Ω promoter-*NtFAD3* (El2Ω-1, El2Ω-2, El2Ω-3, El2Ω-4) were investigated for the integration of the introduced genes by Southern blot analysis. Figure 2 shows the results of Southern blot analysis of DNAs after digestion with *Bam*HI and *Sac*I from the untransformed control plants and hygromycin-resistant plants. All of the hygromycin-resistant plants showed the hybridization signal for the *hpt* gene (Fig. 2a), and all of the hygromycin-resistant lines showed the hybridizing bands for the *NtFAD3* gene, i.e. 1.4-kb cDNA of *NtFAD3* (Fig. 2b). Those hybridization bands over 4.5 kb in length in the non-transgenic plants and transgenic sweet potato plants were considered to be endogenous sweet potato *FAD3* genes (Fig. 2b).

Expression of foreign DNA in the transgenic plants

Eight transgenic lines were examined by Northern blot analysis for expression of the *NtFAD3* gene in the total

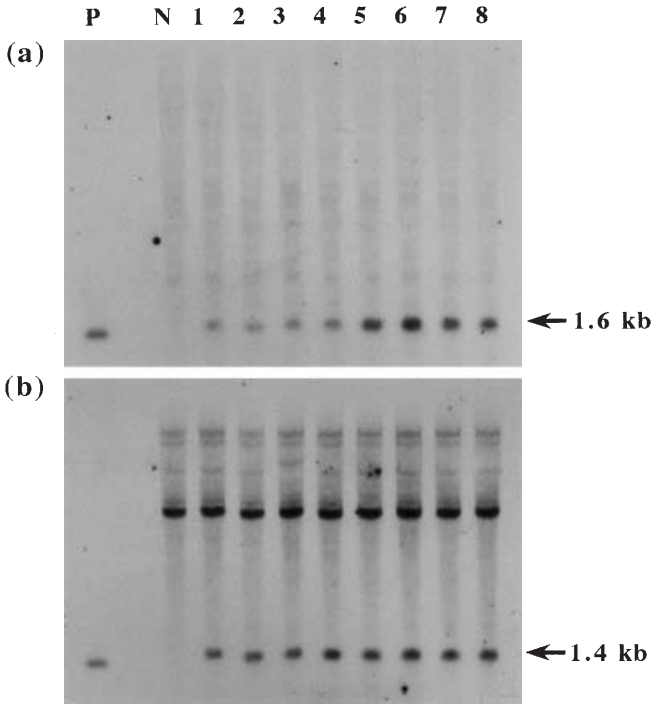


Fig. 2a, b Southern blot hybridization of DNAs after digestion with *Bam*HI and *Sac*I from the transgenic sweet potato plants which were probed with the 615-bp product of the *hpt* gene (**a**) and the 490-bp product of the *NtFAD3* gene (**b**) after PCR amplification. *P* pBIF31-Hm (100 pg) as a positive control, *N* non-transformant as a negative control, *lanes 1–4* transgenic lines with CaMV 35S promoter-*NtFAD3* (35S-1 to -4), *lanes 5–8* transgenic lines with El2Ω promoter-*NtFAD3* (El2Ω-1 to -4)

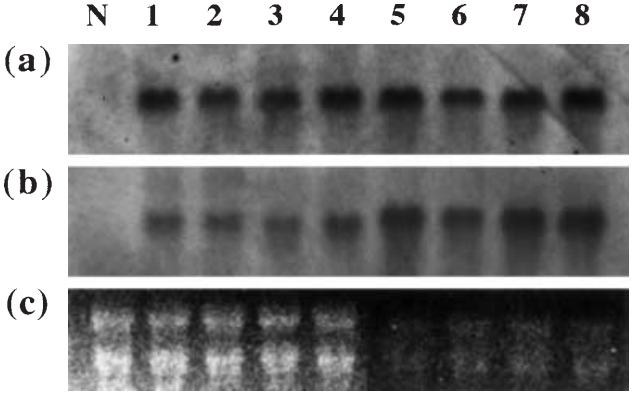


Fig. 3a–c Northern blot hybridization of mRNA from transgenic sweet potato plants which were probed with the 615-bp product of the *hpt* gene (**a**) and the 490-bp product of the *NtFAD3* gene (**b**) after PCR amplification. **c** Ethidium bromide-stained total RNA as a loading control. *N* Non-transformant as a negative control, *lanes 1–4* transgenic lines with CaMV 35S promoter-*NtFAD3* (35S-1 to -4), *lanes 5–8* transgenic lines with El2Ω promoter-*NtFAD3* (El2Ω-1 to -4)

RNAs isolated from leaves (Fig. 3). The *hpt* mRNA was observed in all transgenic lines that had the *hpt* gene driven by the CaMV 35S promoter (Fig. 3a). Expression of the introduced *NtFAD3* gene was also confirmed in all of the transgenic lines (Fig. 3b). However, the expression

Table 1 Fatty acid composition[a] of total lipids from leaves of sweet potato

	16:0	16:1	18:0	18:1	18:2	18:3
Non-transformant	15.3±0.5	1.9±0.2	2.7±0.2	0.9±0.1	10.1±0.4	69.2±0.9
Transformant[b]						
35S-1	14.6±0.1	2.2±0.1	2.4±0.1	0.8±0.1	11.4±0.6	68.6±0.7
35S-2	14.7±0.5	2.0±0.2	2.6±0.1	1.0±0.1	11.6±0.5	68.2±0.4
35S-3	15.0±0.2	1.9±0.1	2.9±0.1	0.8±0.1	10.0±0.9	69.3±1.0
35S-4	14.3±0.1	2.0±0.1	2.4±0.1	1.1±0.1	12.1±0.5 *	68.1±0.5
El2Ω-1	14.7±0.1	2.0±0.1	2.9±0.1	1.0±0.1	9.6±0.7	69.8±0.7
El2Ω-2	15.3±0.5	2.2±0.2	2.8±0.1	0.9±0.1	7.4±1.2	71.5±0.8
El2Ω-3	15.2±0.5	2.1±0.1	2.8±0.1	1.0±0.1	10.7±0.5	68.2±0.9
El2Ω-4	15.0±0.2	2.1±0.1	2.6±0.2	0.9±0.1	10.2±0.6	69.1±0.7

*$P \leq 0.05$
[a] The values are in mol% ± SD (n=5)
[b] 35S-1 to -4, Transgenic lines with a *NtFAD3* gene driven by the CaMV 35S promoter; El2Ω-1 to -4, transgenic lines with a *NtFAD3* gene driven by the El2Ω promoter

Table 2 Fatty acid composition[a] of total lipids from roots of sweet potato

	16:0	18:0	18:1	18:2	18:3
Non-transformant	35.6±1.0	6.7±0.6	2.0±0.1	45.3±1.5	10.6±1.0
Transformant[b]					
35S-1	37.8±1.5	5.9±0.6	2.9±0.3	41.8±1.4	11.5±0.7
35S-2	30.6±1.4	6.0±0.6	1.7±0.1	49.2±1.0	12.5±0.5
35S-3	30.9±0.8	4.6±0.3	2.6±0.3	48.3±0.9	13.6±0.6*
35S-4	32.9±1.0	5.4±0.2	2.4±0.3	46.4±1.1	13.0±03*
El2Ω-1	35.7±1.8	6.1±0.6	2.6±0.4	33.5±1.3**	22.1±2.2**
E12Ω-2	40.8±1.8	9.6±1.4	2.5±0.2	28.8±3.0**	18.3±2.1*
El2Ω-3	34.5±2.2	8.1±0.9	2.1±0.2	31.2±3.6*	24.1±4.7*
El2Ω-4	39.6±1.4	8.0±0.4	2.6±0.3	31.6±1.4**	18.2±1.7**

*$P \leq 0.05$, **$P \leq 0.01$
[a] The values are mol% ± SD (n=5)
[b] 35S-1 to -4, Transgenic lines with a *NtFAD3* gene driven by the CaMV 35S promoter; El2Ω-1 to -4, transgenic lines with a *NtFAD3* gene driven by the El2Ω promoter

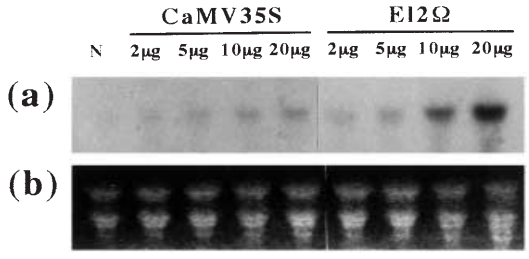


Fig. 4 a Comparison of the *NtFAD3* gene expression level between transgenic lines under the control of the CaMV 35S promoter and El2Ω promoter. **b** Ethidium bromide-stained total RNA shown as a loading control. *CaMV 35S* Transgenic line with CaMV 35S promoter-*NtFAD3* (35S-1), *El2Ω* transgenic line with El2Ω promoter-*NtFAD3* (El2Ω-2). *2 μg:2 μg* RNAs of transformants plus 18 μg RNAs of non-transformants (total 20 μg), *5 μg:5 μg* RNAs of transformants plus 15 μg RNAs of non-transformants (total 20 μg), *10 μg:10 μg* RNAs of transformants plus 10 μg RNAs of non-transformants (total 20 μg), *20 μg:20 μg* RNAs of transformants

level of the *NtFAD3* gene under the control of the El2Ω promoter seemed to be higher than that under the control of the CaMV 35S promoter on the basis of the Northern blot analysis performed using the above-mentioned gradient concentration of RNAs prepared from transgenic plants; the expression level of the *NtFAD3* gene driven by the El2Ω promoter was estimated to be about two to four times higher than that driven by the CaMV 35S promoter (Fig. 4). This indicates that the enhancer sequence of the CaMV 3 S promoter and the Ω sequence from TMV were effective and could enhance *NtFAD3* gene expression in sweet potato plants just like the β-glucuronidase (GUS) gene expression in tobacco plants reported previously (Mitsuhara et al. 1996).

Fatty acid composition of the transgenic plants

The fatty acid compositions of whole lipids extracted from leaves, roots and root tubers were analyzed (Tables 1, 2, 3). In the leaf tissues of sweet potato plants, linolenic acid (18:3) content was higher than 68 mol%, and no significant differences in fatty acid composition were observed between transgenic and non-transgenic plants (Table 1), suggesting that in leaf cells the expression of *NtFAD3* in the microsomal membrane could not significantly modify total fatty acid composition. However, the fatty acid composition in roots of the transgenic plants differed from that in the roots of the non-transgenic plants: linolenic acid (18:3) content was 10.6 mol% in non-transgenic root tissues, while in the transgenic plants with *NtFAD3* driven by the CaMV 35S promoter (the 35S transgenic lines) and El2Ω promoter (the El2Ω transgenic lines) it was more than 11.5 mol% and 18.2 mol%, respectively (Table 2). In the root tubers, the fatty acid composition of the 35S transgenic lines was similar to that present in the non-transformant, but that in the El2Ω transgenic line was significantly different: linoleic acid (18:2) and linolenic acid (18:3) contents in the El2Ω transgenic lines ranged from 38.6 mol% to 45.1 mol% and from 20.5 mol% to 27.2 mol%, respectively, while those in non-transgenic plants were 62.8 mol% and 6.8 mol%, respectively (Table 3). The tobacco microsomal ω-3 fatty acid desaturase gene

Table 3 Fatty acid composition[a] of total lipids from root tubers of sweet potato

	16:0	18:0	18:1	18:2	18:3
Non-transformant	24.1±0.5	3.9±0.5	2.4±0.3	62.8±0.3	6.8±0.7
Transformant[b]					
35S-1 to -4	24.8±0.6	10.9±1.4	3.0±0.4	55.2±1.1 **	6.0±0.3
El2Ω-1	25.4±0.4	5.0±0.1	2.7±0.1	43.9±0.8 **	23.1±0.6 **
El2Ω-2	27.5±0.5	4.4±0.5	2.3±0.5	38.6±1.6 **	27.2±2.2 **
El2Ω-3	28.3±0.4	3.8±0.1	2.1±0.3	40.4±1.2 **	25.4±1.1 **
El2Ω-4	26.4±0.5	5.4±0.3	2.5±0.3	45.1±1.8 **	20.5±1.8 **

** $P \leq 0.01$

[a] The values are mol% ± SD (n=5)

[b] 35S-1 to-4, Transgenic lines with a *NtFAD3* gene driven by the CaMV 35S promoter; El2Ω-1 to -4, transgenic lines with a *NtFAD3* gene driven by the El2Ω promoter

(*NtFAD3*) under the control of the El2Ω promoter was strongly expressed in the transgenic sweet potato plants, thereby causing desaturation of linoleic acid (18:2) to linolenic acid (18:3) in the microsomal membrane. Moreover, the fatty acid composition of whole lipids in roots and root tubers may reflect the expression of *NtFAD3*, which is localized in the extra-chloroplast membrane, given that root cells contain more microsomal membrane than chloroplast membrane.

The strength of the promoter driving the introduced gene is one of the most important factors affecting the expression of a foreign gene in sweet potato cells. In the present study, the CaMV 35S promoter driving the *hpt* gene was sufficient for the production of hygromycin-resistant transgenic plants, while the expression level of the *NtFAD3* gene under the control of the CaMV 35S promoter was not high enough to significantly change the composition of fatty acids in the transgenic lines. The expression level of the foreign *NtFAD3* gene under the control of El2Ω promoter was estimated to be two to four times higher than that under the control of the CaMV 35S promoter, and this expression level was reflected in the linolenic acid content of the transgenic sweet potato plants.

Kodama et al. (l994) introduced a chloroplast ω-3 fatty acid desaturase gene isolated from *Arabidopsis thariana* (*AtFAD7*) into tobacco plants. The transgenic tobacco plants in which the trienoic fatty acid content was increased showed lower chilling injury than non-transgenic tobacco (Kodama et al. 1994). Furthermore, when the *NtFAD3* gene was introduced into rice plants, the resultant transgenic plants had elevated levels of linolenic acid (18:3) in the root and leaf tissues of seedlings and showed a slightly enhanced chilling tolerance (Shimada et al. 2000). We are now examining the chilling tolerance of the transgenic sweet potato plants. Plant lipids have a variety of industrial and nutritional uses, and the modification of fatty acid composition in lipids through genetic manipulation should contribute to the improvement of oil quality (Shen et al. 1999). Although the lipid content of root tubers of sweet potato is only 0.2% (Kagawa 1998), modification of the fatty acid composition of the root tubers may be useful for nutritional improvement.

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