Freezing Tolerance of Transgenic Tobacco with Increased Content of Unsaturated Fatty Acid by Expressing the CvFad3 Gene

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Freezing Tolerance of Transgenic Tobacco with Increased Content of Unsaturated Fatty Acid by Expressing the CvFad2 or CvFad3 Gene

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Two fatty acid desaturases were independently expressed in tobacco plants and freezing tolerance of the transgenic plants was investigated: the two desaturases were CvFAD2, which desaturates microsomal C18:1 (oleic acid) to C18:2 (linoleic acid), and CvFAD3, which desaturates microsomal C18:2 to C18:3 (linolenic acid). Fatty acid composition analysis showed that the C18:2 contents in the plants transformed with pBE21131CvFad2 did not increase compared with that of the wild-type plant. However, an increase in the C18:3 content by 2.40% was observed in the line of No. 19 although the increase was not statistically significant. In the plant transformed with pBE21131CvFad3, the C18:3 content increased by 6.40% compared with that of the wild-type plant. Measurement of the electrolyte leakage of the leaves showed that freezing tolerance of the CvFad2 plant was a little higher than that of the wild-type plant at all temperatures investigated (from −1 to −4°C). Contrary to the case of the CvFad2, the freezing tolerance of the CvFad3 plant tended to be slightly lower than that of the wild-type at all temperatures tested despite the unsaturation levels of the plant were higher than those of the CvFad2 plants.

ABBREVIATIONS
FAD, fatty acid desaturase; G6PDH, glucose 6-phosphate dehydrogenase; GPx, glutathione peroxidase; PC, phosphatidylcholine; SOD, superoxide dismutase

INTRODUCTION

Freezing stress is one of the most severe environmental stresses and cold-sensitive plants suffer serious damage by freezing. But some plant species develop freezing tolerance by cold acclimation when they are exposed to non-freezing low temperatures and survive freezing (Wanner and Junttila, 1999, Webb et al., 1994). During the acclimation, expressions of many genes are induced and various biochemical and physiological changes occur (Jaglo-Ottosen et al., 1998, Seki et al., 2001, Guy, 1990). Among these
changes, increase in the level of polyunsaturated fatty acids such as C18:3 is a major change and observed in many plants (Graham and Patterson, 1982).

We have studied the development of freezing tolerance in plants using Chlorella vulgaris C-27, which is useful as an eukaryotic model. Hardened cells of C. vulgaris C-27 can survive slow freezing to -196°C (Hatano et al., 1976). During this hardening period, several changes including up-regulation of CvFad2 and CvFad3 genes (Suga et al., 2002), accumulation of HIC6 protein (Honjoh et al., 1995) and an increase in G6PDH activity (Sadakane et al., 1980) occur. CvFAD2 and CvFAD3 desaturate microsomal C18:1 to C18:2 and microsomal C18:2 to C18:3, respectively.

The increase in unsaturation level of fatty acids is thought to be important for development of cold tolerance in plants. The fad2 mutant of Arabidopsis, which is deficient in the activity of the microsomal C18:1 desaturase, failed to undergo stem elongation at 12°C and gradually died at 6°C although it showed very similar growth to a wild-type (Miquel et al., 1993). On the other hand, FAD7 transgenic tobacco, having increased contents of C16:3 and C18:3 in plastid membrane lipids, showed decreased leaf chlorosis induced by low temperature treatment (Kodama et al., 1995). As well as increasing cold tolerance, fatty acids desaturation seems to be important for development of freezing tolerance. Freezing tolerance of protoplasts isolated from nonacclimated rye leaves was increased by an artificial enrichment of the plasma membrane with 18:2/18:2-PC or 18:3/18:3-PC (Steponkus et al., 1988). However, definite evidence that fatty acids desaturation increases freezing tolerance in plants has not been shown until now.

In this study, in order to make clear the contribution of increase of unsaturated fatty acids in plasma membrane to freezing tolerance in plants, we tried to make tobacco plants with high content of unsaturated fatty acids by introduction of the CvFad2 or CvFad3 gene.

**MATERIALS AND METHODS**

**Construction of pBE2113/CvFad2 plasmid**

The CvFad2 was mutated, using PCR, to introduce a BamHI site just before the ATG start codon and a SacI site after the stop codon. For this purpose, two oligonucleotides, 5′-AGCAGGGGATCCATGGCTGCAACCCGG-3′ and 5′-CCGGAGAGCTCACGCTATACTC-CCAGCACC-3′, were synthesized as primers. The open reading frame of the CvFad2 was amplified by PCR, using KOD Dash DNA polymerase (Toyobo, Tokyo, Japan), then the amplified fragment was subcloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). After confirming the sequence of the pGEM-T Easy Vector/CvFad2, the plasmid was digested with a combination of BamHI and SacI, and the BamHI–SacI fragment containing CvFad2 was ligated into the BamHI–SacI–digested pBE2113 plasmid vector (Mitsuhara et al., 1996). *Escherichia coli* HB101 was used for the propagation of this plasmid.

pBE2113/CvFad3 was constructed as described previously. (Suga et al., 2002)

**Plant transformation and growth conditions**

The pBE2113/CvFad2 and pBE2113/CvFad3 transgenic tobacco plants (Nicotiana
Freezing Tolerance of CvFad2 or CvFad3 Transgenic Tobacco

Tabacum var. Samsun) were constructed via Agrobacterium tumefaciens LBA4404, as described previously (Honjoh et al., 2001). The wild-type tobacco plants were cultured on MS agar medium and the transgenic were on MS agar medium supplemented with 100 µg/ml kanamycin and 250 µg/ml carbenicillin, under the conditions described previously (Honjoh et al., 2001). For freezing tolerance tests, the tobacco plants were grown in pots filled with a mixture of perlite and vermiculite (1:1, v/v).

Southern hybridization analysis of CvFad2 and CvFad3 genes

Southern hybridization analysis was done as described previously (Honjoh et al., 2001). Thirty-five µg of genomic DNA were digested with HindIII (for CvFad2) or EcoRI (for CvFad3), then separated on a 1.0% agarose gel. The separated DNA was transferred onto a Hybond-N+ nylon membrane (Amersham, Buckinghamshire, England) and hybridized with the 32P-labeled CvFad2 or CvFad3 probe.

Northern hybridization analysis of CvFad2 and CvFad3 genes

Total RNAs were prepared from leaves by using “Sepasol RNA I Super” (Nacalai tesque, Kyoto, Japan), according to the manufacturer’s instruction. Forty µg of the RNAs were separated on a 1.2% agarose gel containing formaldehyde, transferred to a Hybond-N+ nylon membrane, and hybridized with the 32P-labeled CvFad2 or CvFad3 probe.

Fatty acid composition analysis of leaf tissue

For lipids extraction, healthy green leaves were placed in a 2 ml microtube and heated in a boiling bath for 3 min. Then the tube was immersed in liquid nitrogen and the leaves were ground. One point eight ml of chloroform/methanol (2:1, v/v) was added, and the sample was incubated at 40°C for 30 min in the dark and centrifuged at 13,800 × g for 20 min at 4°C. The supernatant was divided equally and transferred to two new microtubes, to each of which 220 µl of water was added. After centrifugation at 1,500 × g for 10 min at 4°C, each of the lower chloroform phases was transferred to a new tube, vacuum dried and redissolved in 100 µl of chloroform. The two solutions were combined and transferred to a screwcapped centrifuge tube. For lipids methanolation, the sample was mixed with 400 µl of 5% (v/v) HCl in methanol. The sample was boiled for 2 h, then mixed with 1 ml of 0.9% (w/v) NaCl solution and 400 µl of hexane were added and centrifuged at 1,500 × g for 10 min. The upper organic phase was transferred to a new microtube and the lower aqueous phase was re-extracted with 100 µl of hexane. The two extracts were combined, condensed by drying under N2 down to 250 µl, and then analyzed with a gas chromatograph (model GC-9A; Shimadzu, Kyoto, Japan), as described in Joh et al. (1993).

Measurements of the freezing tolerance of the leaves

Freezing tolerance of the tobacco leaves was assessed by measuring the electrolyte leakage (EL), as described previously (Honjoh et al., 2001).
RESULTS

Production of transformed tobacco with pBE2113/CvFad2

In order to obtain plants with high contents of polyunsaturated fatty acids, a gene coding for CvFAD2 (Suga et al., 2002), which desaturates C18:1 to C18:2, was introduced into tobacco plants. In this process, a pBE2113 vector (Mitsuhara et al., 1996), which has a highly efficient promoter, was used. Through the transformation with pBE2113/CvFad2, eight independent lines of plant were regenerated.

![Genomic Southern blot analysis](image)

Fig. 1. Genomic Southern blot analysis to show the integration of the CvFad2 gene into the genome of the tobacco plants transformed with pBE2113/CvFad2. Genomic DNA (35 μg) was digested with HindIII and detected with a 32P-labeled CvFad2 probe. The identification numbers of the transgenic lines are indicated on the top. W, wild-type tobacco; G, pBE2113-GUS transformed tobacco.

![Northern blot analysis](image)

Fig. 2. Northern blot analysis of CvFad2 in tobacco plants transformed with pBE2113/CvFad2. Total RNA (40 μg) was subjected to gel blot analysis using a 32P-labeled CvFad2 probe. Then blots were stripped and reprobed with the fragment of actin gene as a loading control. The identification numbers of the transgenic lines are indicated on the top. W, wild-type tobacco; G, pBE2113-GUS transformed tobacco.
To confirm the incorporation of the *CvFad2* gene into the genomic DNA of the transgenic tobacco plants, the plants were analyzed by genomic Southern hybridization analysis using a \(^{32}P\)-labeled *CvFad2* probe (Fig. 1). All transgenic plants showed at least one hybridization band; one hybridization band was observed in five clones (Nos. 4, 5, 10, 11 and 19), two bands in one clone (No. 7) and three bands in two clones (Nos. 6 and 8).

To make sure the expression of *CvFad2*, total RNAs were prepared from tobacco plants and northern hybridization analysis was done (Fig. 2). In all *CvFad2* transgenic plants, only a single band was detected with the \(^{32}P\)-labeled *CvFad2* probe. Especially, No. 19 line showed the highest expression level although this line had only one copy of the *CvFad2* gene. In contrast, lines of No. 6 and 8, each of which had three Southern hybridization bands, showed relatively low expression levels. These results showed that *CvFad2* was integrated into the genomic DNA and expressed in the transgenic tobacco plants.

To investigate the fatty acid composition of these plants, fatty acid methyl esters were prepared from the leaf tissues and analyzed by gas chromatography. The wild-type and pBE2113-GUS transgenic plants were used as the control plants. The result of the fatty acid composition analysis is shown in Table 1. In all *CvFad2* plants, there was no increase in the C18:2 content. But, in two clones (Nos. 5 and 19), the C18:1 content decreased and an increase in the C18:3 content was observed although any of the changes is not significant. So it was supposed that C18:2, which had been desaturated from C18:1 by CVFAD2, was desaturated into C18:3 by endogenous FAD3.

### Table 1. Fatty acid composition of leaf tissues from *CvFad2* transgenic tobacco plants

<table>
<thead>
<tr>
<th>Clone</th>
<th>16:0</th>
<th>16:1</th>
<th>16:2</th>
<th>16:3</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>14.3±0.6</td>
<td>12.0±0.1</td>
<td>1.9±0.2</td>
<td>7.1±0.6</td>
<td>2.3±0.4</td>
<td>1.6±0.2</td>
<td>15.0±1.5</td>
<td>56.7±1.5</td>
</tr>
<tr>
<td>pBE2113-GUS</td>
<td>18.5±2.4</td>
<td>0.6±0.1</td>
<td>0.8±0.2</td>
<td>3.9±0.6</td>
<td>2.9±0.2</td>
<td>1.7±0.3</td>
<td>16.9±0.1</td>
<td>54.9±3.5</td>
</tr>
<tr>
<td>pBE2113/CvFad2 No. 4</td>
<td>14.3±0.3</td>
<td>1.8±0.0</td>
<td>1.5±0.0</td>
<td>8.5±0.2</td>
<td>1.5±0.0</td>
<td>1.4±0.0</td>
<td>14.5±0.2</td>
<td>55.9±0.1</td>
</tr>
<tr>
<td>pBE2113/CvFad2 No. 5</td>
<td>15.1±0.8</td>
<td>1.4±0.2</td>
<td>1.2±0.2</td>
<td>5.6±0.3</td>
<td>1.5±0.2</td>
<td>1.1±0.1</td>
<td>14.8±1.4</td>
<td>59.0±3.3</td>
</tr>
<tr>
<td>pBE2113/CvFad2 No. 6</td>
<td>14.7±0.4</td>
<td>2.2±0.0</td>
<td>1.6±0.1</td>
<td>8.5±0.3</td>
<td>1.4±0.1</td>
<td>1.0±0.1</td>
<td>13.7±0.2</td>
<td>56.8±0.5</td>
</tr>
<tr>
<td>pBE2113/CvFad2 No. 7</td>
<td>15.2±0.1</td>
<td>1.5±0.0</td>
<td>1.2±0.0</td>
<td>5.9±0.1</td>
<td>1.6±0.0</td>
<td>1.3±0.0</td>
<td>15.0±0.1</td>
<td>58.0±0.6</td>
</tr>
<tr>
<td>pBE2113/CvFad2 No. 8</td>
<td>15.5±0.5</td>
<td>1.5±0.1</td>
<td>1.6±0.1</td>
<td>7.0±0.2</td>
<td>2.5±0.3</td>
<td>2.3±0.3</td>
<td>13.6±1.7</td>
<td>55.9±3.1</td>
</tr>
<tr>
<td>pBE2113/CvFad2 No. 10</td>
<td>16.2±0.8</td>
<td>0.8±0.1</td>
<td>1.5±0.2</td>
<td>6.0±0.1</td>
<td>2.8±0.2</td>
<td>2.8±0.1</td>
<td>16.7±0.2</td>
<td>53.1±0.4</td>
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<tr>
<td>pBE2113/CvFad2 No. 11</td>
<td>17.0±0.5</td>
<td>1.1±0.2</td>
<td>1.2±0.1</td>
<td>5.3±0.1</td>
<td>1.7±0.2</td>
<td>1.4±0.1</td>
<td>16.4±0.2</td>
<td>55.8±0.2</td>
</tr>
<tr>
<td>pBE2113/CvFad2 No. 19</td>
<td>14.3±0.7</td>
<td>1.6±0.1</td>
<td>1.8±0.0</td>
<td>7.7±0.6</td>
<td>2.3±0.0</td>
<td>0.9±0.0</td>
<td>12.3±1.0</td>
<td>69.1±1.1</td>
</tr>
</tbody>
</table>

The values are mol%±SD (n=3)

### Production of transformed tobacco with pBE2113/CvFad3

Introduction of a gene coding for CvFAD3 (Suga et al., 2002), which desaturates C18:2 to C18:3, into tobacco plants was also done in the same way as *CvFad2* introduction. By the transformation with pBE2113/CvFad3, ten independent lines of plants were obtained.

Incorporation of the *CvFad3* gene into the genomic DNA of the tobacco plants was
confirmed by genomic Southern hybridization analysis using a $^{32}$P-labeled $CvFad3$ probe (Fig. 3). Unlike the case of the $CvFad2$ plants, a hybridization band of 2.7 kb was detected commonly in all plants including the wild-type and pBE2113-GUS plants. This band was probably due to an endogenous $fad3$ gene which was detected by cross-hybridization with the probe. Except the common band, other bands were observed only in the $CvFad3$ transgenic plants; one hybridization band was observed in six clones (Nos. 4, 5, 9, 11, 14 and 15), two bands in three clones (Nos. 7, 13 and 17) and three in one clone (No. 12).

Then, expression of $CvFad3$ was investigated by northern hybridization analysis (Fig. 4).
Eight clones (Nos. 4, 5, 7, 9, 11, 13, 14 and 15) showed single hybridization bands. But two clones (Nos. 12 and 17) revealed no hybridization band in spite of CvFad3 incorporation into the clones.

As shown in Table 2, an increase in the level of C18:3 was observed in four plants (Nos. 4, 7, 9 and 15). In No. 7 line, which showed the highest content of C18:3 (63.1%), the C18:3 content increased by 6.4% compared with the wild-type. In connection with the increase, the level of C18:2 decreased by 5.2%.

<p>| Table 2. Fatty acid composition of leaf tissues from CvFad3 transgenic tobacco plants |</p>
<table>
<thead>
<tr>
<th>Clone</th>
<th>Fatty acid composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
</tr>
<tr>
<td>Wild-type</td>
<td>14.3±0.6</td>
</tr>
<tr>
<td>pBE2113-GUS</td>
<td>18.5±2.4</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 4</td>
<td>15.5±1.5</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 5</td>
<td>15.9±0.8</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 7</td>
<td>14.6±2.0</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 9</td>
<td>16.3±0.3</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 11</td>
<td>14.4±3.1</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 12</td>
<td>16.4±1.1</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 13</td>
<td>14.7±2.3</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 14</td>
<td>16.9±1.4</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 15</td>
<td>14.0±1.0</td>
</tr>
<tr>
<td>pBE2113/CvFad3 No. 17</td>
<td>18.0±0.8</td>
</tr>
</tbody>
</table>

The values are mol%±SD (n=3).

Fig. 5. Freezing tolerance of leaves of CvFad2 transgenic tobacco plant, assessed by measuring electrolyte leakage. The results shown are the average of at least three different experiments; three samples were used for each experiment.
Measurements of freezing tolerance of the leaves

To assess freezing tolerance of the CvFad2 and CvFad3 transgenic tobacco plants, the electrolyte leakage (EL) of the leaves was measured after a freeze/thaw treatment, as described previously (Honjoh et al., 2001). Out of the CvFad2 transgenic lines, No. 19 line, which showed the highest unsaturation level, was selected as a representative of these plants. For the CvFad3 lines, No. 7 line was selected for the same reason.

The %EL values of the CvFad2 plant were slightly lower than those of the wild-type plant at all temperatures investigated (−1 to −4°C) (Fig. 5). Thus, the freezing tolerance of the CvFad2 plant was a little higher than that of the wild-type plant, although no significant differences in the %EL were observed at all temperatures tested. Contrary to the case of CvFad2, the freezing tolerance of the CvFad3 plant tended to be slightly but not significantly lower than that of the wild-type at all the temperatures (Fig. 6).

DISCUSSION

For introduction of the CvFad2 gene into tobacco plants, we used a pBE2113 vector (Mitsuhara et al., 1996) with a strong promoter. Nevertheless, the levels of unsaturated fatty acids in the CvFad2 transgenic plants increased only slightly. According to the result of northern hybridization analysis, the CvFad2 gene was certainly expressed in the transgenic plants. The original, very low content of C18:1, the substrate for CvFAD2, in the wild-type may be a reason for the slight change in the fatty acid composition of the CvFad2 plants.
In No. 7 line of CvFad3 transgenic plants, C18:3 content increased by 6.4% and C18:2 decreased by 5.2% compared with wild-type. Different from C18:2, which is diminished by desaturation into C18:3, C18:3 seems not to be desaturated further (Ohlrogge and Browse, 1995). In addition, the content of C18:2, the substrate for CvFAD3, was much higher than that of C18:1 originally in the tobacco plant. So the content of C18:3 might be easy to increase more than that of C18:2.

There was no correlation between the levels of CvFad genes transcription and those of fatty acid unsaturation. The No. 13 line of the CvFad3 transgenic plants, which showed the highest CvFad3 transcription level, did not increase the content of C18:3. So far the reason for this irreverence discrepancy remains to be elucidated.

A common hybridization band was detected in all plants including the wild-type and pBE2113-GUS plants by genomic Southern hybridization analysis using a CvFad3 probe. This band was probably derived from an endogenous fad3 gene. Because the hybridization was done in a relatively high stringent condition, the result suggests that the nucleic acid sequence of the CvFad3 is very similar to that of the tobacco endogenous fad3.

Measurement of the electrolyte leakage (EL) of the leaves showed that freezing tolerance of the CvFad2 plant was slightly higher than that of the wild-type plant. The EL method reflects the damage of cell membrane, so this result suggests that the increased unsaturated fatty acids content in cell membrane leads to the prevention of the plasma membrane from injury caused by freezing and thawing. Because the CvFad2 gene codes for microsomal FAD (Suga et al., 2002), increased unsaturated fatty acids would be derived from the plasma membrane.

The freeze-induced plasma membrane lesions can be classified mainly into two different forms dependent on freezing temperature (Uemura et al., 1995). Over the range from -2 to -4°C, the predominant membrane injury is "expansion-induced lysis", which is caused by the osmotic contraction and expansion cycle that occurs with freezing and thawing. At temperatures between -4 to -8°C, the predominant injury is freeze-induced lamellar to hexagonal II phase transitions, an interbilayer event that occurs when the plasma membrane is brought into close apposition with various endomembranes as a consequence of freeze-induced dehydration. In this study, a freezing tolerance test was done at -1 to -4°C, so the increased unsaturated fatty acids content seems to be a little protective against the expansion-induced lysis.

Freezing tolerance of the CvFad3 plant was slightly lower than that of the wild-type at all temperatures despite the unsaturation level of the plant being higher than that of the CvFad2 plant. In wheat, correlation of the acquisition of frost resistance and accumulation of linolenic acid was indicated (Willemot, 1977). Furthermore, when the plasma membrane of protoplasts isolated from nonacclimated rye leaves was artificially enriched with various PC species by adding them to the protoplasts in vitro, enrichment with 18:2/18:2-PC or 18:3/18:3-PC was the most effective in increasing the freezing tolerance, whereas fusion with saturated species had no effect (Steponkus et al., 1988). Therefore, desaturation of fatty acids in the plasma membrane is considered to be important for improving freezing tolerance. However, the freezing tolerance of CvFad3 plant did not increase but oppositely slightly decreased. Although it may not be applicable directly to the plant, in rat, an increase in the fatty acid double bond content in heart mitochondrial membranes leads to an increase in lipid peroxidation (Herrero et al., 2001). Because
oxidative stress is one of the major stresses induced by chilling in chilling-sensitive plants (Hodges et al., 1997, Pinheiro et al., 1997), unsaturated fatty acids might suffer too much oxidation, which leads to ion leakage from inside of the cell (Camp et al., 1996), during freezing tolerance tests. Simultaneous introduction of a gene encoding an enzyme, that protects plasma membrane lipids from peroxidation, such as GPx, G6PDH (Miki et al., 1996), SOD (Camp et al., 1996) or peroxidase (Yun et al., 2000) with CvFad3 may be effective in increasing freezing tolerance.

In this paper, we tried to investigate relationship between plasma membrane fatty acid desaturation and freezing tolerance. However, contribution of the desaturation to freezing tolerance has not yet been clarified. Freezing stress involves multiple stresses such as dehydration, low temperature and oxidation, and multiple genes are thought to be involved in freezing tolerance acquisition (Thomashow, 1998). So perhaps, it might be difficult to develop freezing tolerance of cold sensitive plants by introducing only a single gene. In other words, cooperation of multiple genes might be necessary to improve freezing tolerance. We hope that simultaneous introduction of gene(s), which concerns freezing tolerance, with CvFad gene(s) may help clarify the role(s) of the fatty acid desaturation.

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