BIOLOGIA PLANTARUM 48 (3): 361-366, 2004

Characterization of transgenic rice plants expressing an *Arabidopsis FAD7*

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Abstract

Fatty acid ω-3 desaturase (FAD) is the key enzyme catalyzing the formation of trienoic fatty acids. We utilized an *Arabidopsis FAD7* gene and the seven independent transgenic rice plants harbouring 1 to 3 copies of this gene were generated. The expression of *FAD7* mRNA was different among independent transgenic lines regardless of the copy number. The total linolenic acid (18:3) contents reduced by about 7 - 32 % in transgenic rice plants but the linoleic acid (18:2) content increased accordingly. With or without wounding treatments, the jasmonate content was higher in transgenic lines than in wild-type rice plant. The transgenic lines overproducing jasmonate also showed increased expression of PR1b mRNA and allene oxide synthase in response to wounding.

Additional key words: fatty acid desaturase, jasmonic acid, linoleic acid, linolenic acid, malondialdehyde.

Introduction

The membrane desaturation is closely associated with the maintenance of chloroplast function, the pollen development, the tolerance to cold, and the production of plant growth hormone jasmonic acid (Gibson *et al*. 1994, Kodama *et al*. 1995, McConn and Browse 1996, Routaboul *et al*. 2000).

The enzyme desaturase catalyzes the formation of double bond into fatty acyl chains. The substrate specificity, subcellular location, and regulation pattern of eight desaturase genes were characterized (Napier *et al*. 1999, Somerville *et al*. 2000). Among these, *FAD7* gene which encodes the chloroplast ω-3 fatty acid desaturase responsible for the trienoic fatty acids accumulation has been studied extensively because it is involved in cold tolerance and production of linolenic acid, a substrate for jasmonic acid (Browse *et al*. 1986, Kodama *et al*. 1994, Martin *et al*. 1999).

So far, two transgenic plants regarding *FAD7* gene expression were developed to evaluate its physiological role in plant cells. One plant was transgenic tobacco expressing heterologously the *Arabidopsis FAD7* (Kodama *et al*. 1994), the other was a cosuppression line of potato transgenic plants expressing a potato antisense *FAD7* gene (Martin *et al*. 1999).

The transgenic tobacco overexpressing the *Arabidopsis FAD7* gene showed that the increased production of trienoic fatty acids (16:3, 18:3) was in parallel with the enhancement of cold tolerance (Kodama *et al*. 1994). Transgenic potato plants which lower linolenic acid by expressing a potato *FAD7* gene in an antisense orientation revealed a marked reduction of trienoic acids leading to a corresponding decrease of jasmonate content and proteinase inhibitor II expression upon wounding (Martin *et al*. 1999).

To see whether the previously observed roles of *FAD7* gene were also functioning in monocotyledonous rice plant, we generated seven independent transgenic rice plants expressing the *Arabidopsis FAD7* gene under the constitutive ubiquitin promoter.

Received 23 June 2003, *accepted* 12 February 2004.
Abbreviations: FAD - fatty acid desaturase; JA - jasmonic acid; MDA - malondialdehyde; PR - pathogenesis related.
Acknowledgements: This work was supported by the Korea Ministry of Agriculture and Forestry and the Korea Science and Engineering Foundation (R11-2001-09203001-0).
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Materials and methods

Vector construction and transformation: Binary vector pCAMBIA 1380 was obtained from the *CAMBIA* center (Canberra, Australia). The *Arabidopsis FAD7* cDNA and the maize ubiquitin promoter were provided by Dr. B.H. Cho (Chonnam National University, Korea) and Dr. G. An (Pohang University, Korea). pCAMBIA 1380 predigested with *BamHI*/*BstEII* was ligated with the 1.9 kb maize ubiquitin promoter (*BamHI*/*SacI* fragment) and the 1.3 kb *Arabidopsis FAD7* gene (*SacI*/*BstEII* fragment) so as to express the *Arabidopsis FAD7* under the control of the maize ubiquitin promoter. This construct was transformed into *Agrobacterium tumefaciens* strain LBA4404 by a freeze and thaw method. *A. tumefaciens* LBA4404 harbouring pCAMBIA1380-*FAD7* was grown overnight at 28 °C in YEP (Rashid *et al*. 1996) medium supplemented with 50 μg cm^{-3} kanamycin and 20 μg cm^{-3} hygromycin. The cultures were spun down and pellets were resuspended in an equal volume of AA medium containing 100 μM acetosyringone. The calli were induced from scutellum of rice (cv. Nackdong) seed on N_6 media (Rashid *et al*. 1996, Hiei *et al*. 1997). The 3- to 4-week-old compact calli were soaked in bacterial suspension for 3 min, blotted dry with a sterile filter paper to remove any excess bacteria. Then the calli were transferred onto a co-culture medium for 2 - 3 d, kept in darkness at 25 °C. The co-cultivated calli were washed with sterile water containing 250 mg dm^{-3} cefotaxime to remove bacteria and then transferred onto N_6 media containing 250 mg dm^{-3} cefotaxime and 50 mg dm^{-3} hygromycin. After a selection for 3 - 4 weeks, the calli were transferred to the regeneration media for shoot and root development. Once the roots had sufficiently developed, the transgenic plants were transferred to a greenhouse and grown to maturity.

DNA and RNA blot analysis: Genomic DNA was isolated according to standard methods (Ausubel *et al*. 1987). Five microgram of genomic DNA was digested with *Sac*I restriction enzyme, size-fractionated by electrophoresis in 0.8 % (m/v) agarose gels, blotted to nylon membranes (*Nylon 66 plus*, *Pharmacia Biotech*., UK), and hybridized with the full length of *Arabidopsis FAD7*. Total RNA was isolated by using TRI Reagent (*Sigma*, St. Louis, USA). Total RNA (10 μg) was fractionated on 1 % agarose gels containing formaldehyde using 20 mM 3-(N-morpho-lino)-propanesulfonic acid (Mops) solution as a running buffer. The gel was blotted to a nylon membrane and hybridized with the full length of the *Arabidopsis FAD7*. Equal loading of RNA samples was checked by ethidium bromide staining prior to blotting. The full-length rice *PR1b* gene is amplified through PCR using one set of oligonuleotide primers on the basis of the nucleotide sequence of rice *PR1b* cDNA (Agrawal *et al*. 2000). The primer sequences were as follows: 5´-ATGGAGGTATCCAAG-3´ (forward) and 5´-TTAGTAAGGCCTCTGTCC-3´ (reverse). A PCR was performed with a genomic DNA of rice under the standard condition. The 495-bp PCR product was ligated into pBluescript (*Stratagene*, La Jolla, USA) and its *PR1b* insert was confirmed by the dideoxynucleotide chain-termination method with Sequenase 2.2 (*Amersham*, Piscataway, USA). Rice allene oxide synthase (*AOS*) cDNA was from Ha *et al*. (2002). DNA and RNA blot hybridization were performed as previously described (Shin *et al*. 2001).

Total fatty acid analysis: For analysis of fatty acid composition, total lipids were extracted from 0.5 g of an 8-week-old transgenic rice leaf (T_1) grown at field by a modified Bligh and Dyer medium containing 0.15 M acetic acid and chloroform:methanol (1:2 v/v). The chloroform phase was evaporated to dryness under N_2 and fatty acid methyl esters were formed in dry methanol with 2.5 % sulfuric acid. The fatty acid composition was quantified by gas chromatography using heptadecanoic acid as internal standard (Griffiths *et al*. 1997).

Jasmonic acid (JA) analysis: JA in leaf tissue samples were measured using methods modified from Baldwin *et al.* (1997). Frozen tissues of a leaf (1 g) were ground under liquid N_2. The leaf powder was suspended in a solution of acetone and 50 mM citric acid (70:30 v/v). As an internal standard, [9,10-$^2H_2$]dihydro-JA (210 ng) was added. The organic solvent was allowed to evaporate overnight at room temperature to avoid losses of volatile fatty acids. The resulting aqueous solutions were extracted with 3 × 7 cm^3 of diethyl ether. The pooled extracts were then loaded onto a solid-phase extraction cartridge containing 360 mg of the sorbent aminopropyl. After the loading, the cartridges were washed with 10 cm^3 of a solvent mixture of trichloromethane: 2-propanol (2:1, v/v). Bound JA and the standard were eluted with 10 cm^3 diethyl ether:acetic acid (98:2, v/v). The solutions were analyzed by gas chromatography-mass spectrometry (GC-MS) without further purification. The silylated JA and the standard [9,10-$^2H_2$]dihydro-JA eluted separately. The amount of endogenous JA was calculated from the peak areas of JA and the standard.

Results

Transformation of rice: On average, 15 - 20 % calli from co-cultivation with *Agrobacterium tumefaciens* harbouring the construct pCAMBIA1380-*FAD7* gene survived the selection medium containing 50 μg cm^{-3} hygromycin. After transferring onto the regeneration medium, selected calli were regenerated into shoots at a rate of 3 - 5 %. As an initial stage of transformation, an initial 19 independent transgenic rice plantlets expecting to express the transgene were generated and grown to maturity in a field. Most of transgenic lines appeared to have normal phenotypes, but their T_1 seed production varied ranging from 200 to 1000 seeds depending on individual transgenic lines due to possible genetic alteration by either T-DNA or other somatic variation occurred through *in vitro* tissue culture procedure. Among these transgenic rice plants, 7 independent lines were selected and utilized for further analysis.

Genomic DNA gel blot analysis: To assess the stable integration of the *Arabidopsis FAD7* gene into the rice genome, DNA extracted separately from 7 transgenic T_0 and wild type rice plants was digested with *Sac*I and hybridized with ^{32}P-labeled *Arabidopsis FAD7*. Due to the absence of *Sac*I site within the probed transgene, the number of hybridized bands directly corresponds to the copy number of transgene in the genome of the transgenic plants. The majority of transgenic lines showed the single copy insertion with each above 5 kb in size except F13 line exhibiting 2 kb in size suggesting the transgene insertions at different locations in the rice genome (Fig. 1). In contrast, F10 line had three copies of transgene insertion in the rice genome. The number of transgene insertion loci was examined by counting hygromycin-resistant progeny (T_1) of the primary transgenic plants (T_0). All the transgenic lines turned out to be inserted at single locus showing 3:1 (hygromycin resistant versus sensitive) ratio segregation (data not shown).

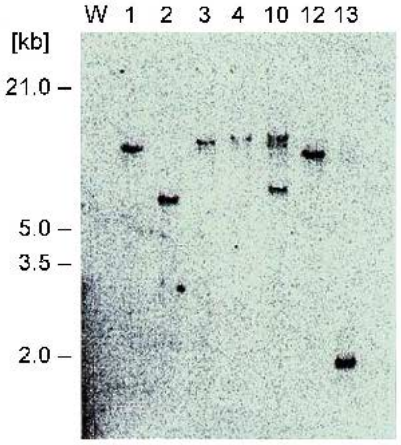

Fig. 1. Genomic DNA blot analysis of the *Arabidopsis FAD7* transgene in transgenic rice lines at T_0 generation. W - nontransgenic wild rice plants; 1 - 13 - transgenic lines.

RNA gel blot analysis: *Arabidopsis FAD7* mRNA was detected in total RNA isolated from the leaves of primary transgenic lines, whereas no *Arabidopsis FAD7* mRNA was present in total RNA prepared from the non-transgenic rice leaves (Fig. 2). All the lines tested could transcribe the *Arabidopsis FAD7* mRNA, in which F1, F2, and F4 lines exhibited the highest transgene expression. Other F3, F12, and F13 lines also showed relatively high transgene mRNA expression, however, F10 that had three copies showed very low expression.

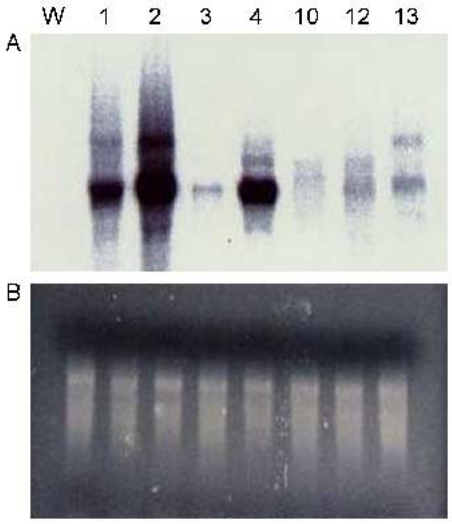

Fig. 2. RNA blot analysis of the *Arabidopsis FAD7* mRNA levels in transgenic rice lines and wild at T_0 generation (*A*). W - nontransgenic rice plants; 1 - 13 - transgenic lines. The equal loading of total RNA was shown by ethidium bromide staining (*B*).

Fatty acid analysis of the transgenic plants: The mature rice leaves had a large quantity of linolenic acid (18:3) over 75 % of the total fatty acids examined, but rice leaves contained a very low amount linoleic acid (18:2) (Table 1). All the transgenic lines with *FAD7* gene had substantially reduced amounts of linolenic acid ranging from 92 to 68 % compared with the wild type. In contrast, the relative amount of linoleic acid increased up to 2- to 7-fold in transgenic plants compared with that of wild type. Although we did not observe 16:2 and 16:3 fatty acids, our observation clearly showed that the introduction of the *Arabidopsis FAD7* into rice led to the suppression of linolenic acid and consequently increase of linoleic acid in all transgenic lines. When judged by Northern blot and fatty acid composition data, there was no close relation between the level of *Arabidopsis FAD7* mRNA expression and suppression of linolenic acid content.

Table 1. Fatty acid composition [%] of total lipids extracted from leaves of wild type and transgenic plants. Total lipid were extracted from 0.5 g leaf of 8 weeks old grown at field. Values are the mean percentage ± SD of three independent experiments. F1 - F13 - independent transgenic rice plants at T_1 generation.

	16:0	16:1	18:0	18:1	18:2	18:3	20:0
Wild	16 ± 2	3 ± 1	2 ± 1	1 ± 1	3 ± 1	75 ± 3	1 ± 0.2
F1	15 ± 2	3 ± 1	3 ± 1	2 ± 2	13 ± 1	64 ± 4	1 ± 0.1
F2	16 ± 3	3 ± 1	3 ± 2	2 ± 2	11 ± 3	66 ± 4	1 ± 0.2
F3	16 ± 2	2 ± 1	3 ± 2	3 ± 1	9 ± 2	66 ± 5	1 ± 0.1
F4	14 ± 1	2 ± 2	3 ± 1	2 ± 1	12 ± 2	68 ± 2	1 ± 0.1
F10	18 ± 2	3 ± 1	3 ± 2	3 ± 1	22 ± 2	51 ± 2	1 ± 0.2
F12	15 ± 3	2 ± 1	2 ± 1	2 ± 1	10 ± 2	69 ± 2	1 ± 0.1
F13	19 ± 2	1 ± 2	7 ± 3	3 ± 1	7 ± 2	63 ± 3	1 ± 0.6

Jasmonic acid analysis: Wild type rice plants synthesized 3 to 5 ng g^{-1}(f.m.) JA, but increased its synthesis up to 2-3 fold 24 h after wounding (Table 2). All transgenic rice plants tested showed a higher concentration of JA than in wild type prior to wounding. When these transgenic rice plants were exposed to wounding, JA content also increased by the similar fold induction as in the wild type rice, except for F1 transgenic lines.

Table 2. Jasmonic acid content [ng g^{-1}(f.m.)] in transgenic rice leaf with or without wounding treatments. Wounding was applied by cutting leaves of rice. T_1 transgenic rice leaf segments around 0.5 cm in length were floating on distilled water for 24 h. One gram of rice leaf (fresh mass) was taken and subjected to GC/MS analysis. Values are the mean percentage ± SD of three independent experiments. F1 - F12 - independent transgenic rice plants at T_1 generation.

	Control	Wound
Wild	2.7 ± 1.1	7.0 ± 0.9
F1	4.5 ± 0.5	1.9 ± 0.7
F2	5.8 ± 0.4	8.7 ± 2.4
F10	6.2 ± 0.4	8.7 ± 2.6
F12	7.0 ± 0.4	15.5 ± 1.8

Expression pattern of JA responsive genes: We chose two genes, a pathogenesis-related (PR) gene and allene oxide synthase (AOS) gene because these were closely associated with JA content in plant (Agrawal *et al.* 2003). The *PR1b* gene, which was induced by JA or PP2a inhibitors (Agrawal *et al.* 2000), was employed to measure the transcriptional pattern. *PR1b* mRNA was not detected in unwounded leaves of all rice tested, but was induced 24 h after wounding (Fig. 3). The inducibility of *PR1b* mRNA was higher in transgenic plants than in wild type (W) or transgenic control (Tc) which expressed only vector, suggesting that the higher synthesis of JA was in parallel with the strong induction of JA responsive gene in transgenic rice. *AOS* mRNA was expressed constitutively in both wild and transgenic rice. However, its level of transcription was enhanced only in transgenic rice, not in wild and transgenic control lines in response to wounding. Therefore, the increased synthesis of JA in transgenic rice was accompanied with the upregulated expression of *AOS*.

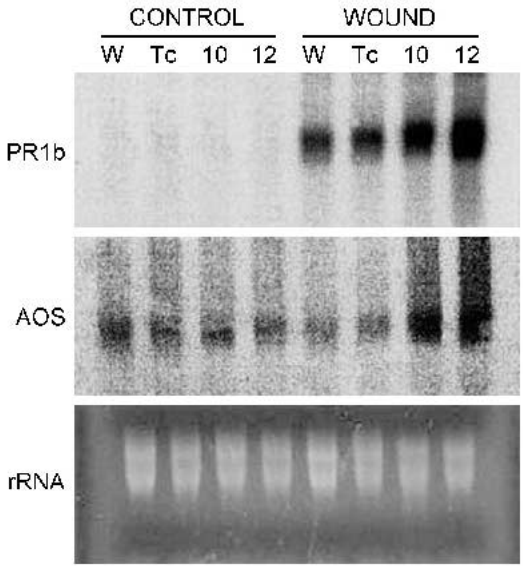

Fig. 3. Induction pattern of jasmonic acid responsive *PR1b* and *AOS* mRNA in leaves after wounding. Rice leaves were subjected to wounding as did in Fig. 3 and harvested for 12 h. Total RNA was extracted from the leaves. The blots were hybridized to the [α-^{32}P]dCTP-labeled rice *PR1b* and rice *AOS* cDNA probes. Equal loading (10 μg total RNA) was shown by staining with ethidium bromide in the lowermost panel. W - wild type, Tc - transgenic control, 10, 12 - transgenic lines.

Time course induction pattern of JA responsive genes: The detailed time-course analyses of the level of JA responsive genes over a period of 48 h after wounding were further studied. The *PR1b* mRNA began to induce

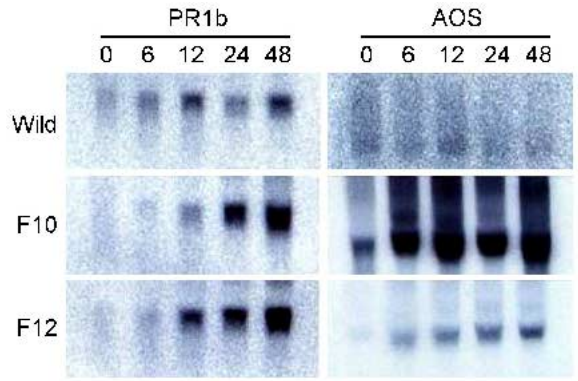

Fig. 4. Time course expression of *PR1b* and *AOS* mRNA. Rice leaves were applied to wounding and then harvested for various times. Total RNA extraction and blot analysis were the same as in Fig. 3.

6 h after wounding in both wild and transgenic lines (Fig. 4). However, the relative content of *PR1b* transcript was higher in transgenic lines than that of wild type after wounding which were consistent with the data of Fig. 3. Accordingly, *AOS* transcript in wild type was not accumulated in response to wounding during the entire time course up to 48 h, which was identical to prior data (Fig. 3). In contrast, the *AOS* mRNA was induced upon wounding treatment in transgenic lines. Wounding induced an approximate three fold *AOS* mRNA increase when measured by the *Phospho Image 1.61* program. Induction of *AOS* mRNA in transgenic lines appeared 6 h after wounding and remained constant until 48 h. This suggested that the JA increase upon wounding in transgenic lines is accountable for the higher expression of *AOS* mRNA.

Discussion

The transgenic rice plants expressing the *Arabidopsis FAD7* gene had shown that the total content of linolenic acid (18:3) was diminished by 7 - 32 % in transgenic lines compared to wild control plants. In contrast, the change in corresponding linoleic acid (18:2) showed 3- to 7-fold higher in the transgenic lines. This reduction of linolenic acid (18:3) was possibly ascribed to the gene-silencing whose phenomenon was also observed in transgenic tobacco plants expressing the *Arabidopsis FAD7* gene under the control of the cauliflower mosaic virus 35S promoter (Kodama *et al*. 1994, Murakami *et al*. 2000).

The broad level of sequence homology of *FAD7* in plants may give birth to the sense suppression of FAD7 in transgenic tobacco and rice, but the mechanism by which the gene-silencing occurred in our transgenic rice remains to be elucidated (Kodama *et al*. 1997, Lyer *et al*. 2000).

Another important role of linolenic acid in plant cells is its involvement in plant hormone jasmonic acid synthesis. When plants are subjected to wounding, linolenic acid of plant membrane lipid is released and provides for a substrate of jasmonic acid synthesis (Wasternack and Parthier 1997). Transgenic plants showed higher JA content in all the lines tested in comparison with wild-type rice plants before the wounding treatment. In response to wounding, JA content increased both in wild-type rice plant and in transgenic rice plants, except line F1. However, the JA content was much higher in transgenic rice plants than in wild-type rice plants. The high JA synthesis in transgenic rice plants upon wounding were in accord with the upregulation of *PR1b* and *AOS*, a JA responsive marker gene and a JA biosynthetic gene, respectively. The high JA content, prior to wounding, was also observed in transgenic potato expressing the antisense ω-3 fatty acid desaturase in which the content of linolenic acid (18:3) was decreased (Martin *et al*. 1999).

As yet, the reasons for the upregulation of jasmonic acid synthesis in transgenic plants remain unclear, but this may be attributed to either the production of signal molecules derived from altered synthesis of fatty acid composition (Vollenweider *et al*. 2000) or the linoleic acid (keto form) itself having the role of a stress signal (Kachroo *et al*. 2001).

Currently, we are investigating whether or not the upregulation of jasmonic acid synthesis, *AOS*, and *PR1b* in transgenic rice plants is coupled with the activation of mitogen activated protein kinase (MAP kinase).

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