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# *AGROBACTERIUM* MEDIATED TOBACCO TRANSFORMATION WITH RICE FAE GENE AND SEGREGATION ANALYSIS OF T1 GENERATION

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**Abstract**

The transformation of plants through *Agrobacterium* is very important to impart desirable traits. Usually plants are subjected to various abiotic and biotic stresses, which affect the growth and metabolism. Plants evolve certain mechanisms to cope with the prevailing stressful conditions. Among them, cuticular waxy coating layer may serve as protective barrier to deter water loss as well as pathogens assault. The rice *OsFAE* encodes a protein involving elongation of fatty acids to form very long chain fatty acids (VLCFAs), necessary for cuticular wax biosynthesis. The *OsFAE* gene in the sense sequence was placed under the control CaMV35S promoter. The *Agrobacterium* mediated transgenic tobacco plants were obtained using tissue culture techniques. The PCR analysis of T2 generation confirmed the incorporation of transgene into the tobacco genome. The selection of transgenic plants was made on hygromycin. Segregation analysis of T1 generation revealed that most of the transgenic lines showed typical 3:1 Mendel's segregation ratio.

## Introduction

*Agrobacterium tumefaciens* is a Gram-negative soil pathogen to cause crown gall in a number of plants (DeCleene & DeLey, 1976). The bacterium transfers a part of DNA known as transfer DNA (T-DNA) along with virulence proteins after infecting the plant. The bacterium genetically transforms cells of several dicots, some monocots and gymnosperms (DeCleene & DeLey, 1976). It may even transform various fungal species as well as human cells (Bundock *et al*., 1995; de Groot *et al*., 1998; Gouka *et al*., 1999; Kunik *et al*., 2001). The *Agrobacterium* mediated genetic transformation of plants is an important means due to its natural capability to transfer foreign gene into the host plant genome. Even though there is an extensive use of transformation *via Agrobacterium*, very little information about the mechanisms of T-DNA integration into the host genome is yet available. Better understanding regarding the processes of T-DNA transfer would be helpful to improve transformation technology (Gelvin, 2003; Altman, 2003).

Plants carry out their life activities under prevailing environment by evolving specialized mechanisms and adaptation structures necessarily to cope successfully with harsh conditions. Among the most important traits, they secrete essentially waxy coating that covers nearly all plant parts interface to the environment (Jenks & Ashworth, 1999; Li *et al.*, 2008).

The combination of cutin, waxes, and possibly polysaccharides forms the cuticle, a hydrophobic covering at epidermis (Jeffree, 1996; Kolattukudy, 1996). The cuticular waxes are a mixture of lipophilic compounds that are chiefly composed of aliphatic monomers, glycerols, phenolics, very long-chain fatty acids (VLCFA) and their derivatives (Nawrath, 2002; Goodwin & Jenks, 2005; Clare *et al*., 2009). Different layers may frequently be distinguished in the cuticle of mature organs i.e., epicuticular and intracuticular waxes.

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The plant cuticle has been attributed to play an imperative role in protecting the plant from non-stomatal water loss (Riederer & Schreiber, 2001; Ristic & Jenks, 2002; Oliveira *et al*., 2003; Jung *et al*., 2006), UV irradiation (Long *et al*., 2003), mechanical injury (Eglinton & Hamilton, 1967), reduce water retention on the plant surfaces by amending surface wet-ability (Barthlott & Neinhuis, 1997; Beattie & Marcell, 2002).

With a little contribution of mitochondria in fatty acids synthesis (Wada *et al*., 1997), the den novo fatty acid (C16-C18) biosynthesis (Jung *et al*., 2006) occurs in plastids of leaf mesophyll tissue (Ohlrogge *et al*., 1979). The elongation of these fatty acids resulting to form very long chain fatty acids (VLCFAs) (Bach *et al*., 2008; Clare *et al*., 2009) ranging from C20 to C34 (Yu *et al*., 2008). VLCFAs form primary alcohols and wax esters via acyl reduction pathway or to synthesize aldehydes, alkanes, secondary alcohols and ketones through decarbonylation pathway (Kunst & Samuels, 2003; Millar *et al*., 1999). Previously, a number of wax related genes have been cloned as mutation in them, which resulted into abnormal morphology and malfunctions (Samuels *et al*., 2008). Among them, *Arabidopsis*' *FIDDLEHEAD* (*FDH*) (Yephremov *et al*., 1999; Pruitt *et al*., 2000) and *LACERATA* (*LCR*) (Wellesen *et al*., 2001; Jung *et al*., 2006) and *Zea mays*' *CRINKLY4* (*CR4*) (Becraft *et al*., 2001) are of vital interest.

So far, progress in genetic improvement of crops for stress resistance is dawdling and more restricted (Clausen *et al*., 2000; Datta *et al*., 2001; Evenson & Gollin, 2003), owing to poor understanding of stress tolerance mechanism and dearth of proficient techniques for selection breeding resources for stress resistance (Melchers *et al*., 1993; Khush, 2001). With the speedy development of genetic engineering, molecular breeding has offered a promising approach to improve stress tolerance of crops and some progress has been made (Smirnoff & Bryant, 1999; Ramanjulu & Bartels, 2002, Islam *et al*., 2007). Recently, some successful endeavors to incorporate stress-tolerant genes among plants for enhanced tolerance to water and other stresses were reported (Bhattacharya *et al*., 2004; Chandra *et al*., 2004). To have insight about the biological role, we introduced the rice *FAE* gene in tobacco via *Agrobacterium tumefaciens*-mediated leaflets transformation.

In this work we focused on some aspects of tobacco transformation employing tissue culture techniques, analysis of *OsFAE* tansgene incorporation into tobacco genome and segregation analysis of T1 generation of transgenic lines.

## Materials and Methods

**Plant materials and growth conditions:** Tobacco (*Nicotiana tabacum*) cv. SR1 seeds were surface sterilized for 2 min with 70% ethanol and for 30 min with 1.0 % Sodium hypochlorite solution. Sterilized seeds were rinsed 5-7 times with sterile water, after drying; they were subsequently spread onto Petri dishes containing solid ½ MS medium (Murashige & Skoog, 1962) supplemented with sucrose (15 g/l). Plates were kept at 18°C for 4 days in dark and then at 22°C in 16/8 hour light. The seedling about 2-3 cm long were transferred aseptically to the glass bottles to get 5-8 young leaves.

**Construction of plant transformation vector (pCAMIA1301+ *OsFAE*):** For the construction of *OsFAE* sense vector, the entire *OsFAE* coding fragment from a rice cDNA plasmid library (He *et al.*, 1999) through PCR using forward primer 5'-GAAGATCTTCCAGAGATGGAGACCTCGG-3' containing a *Bgl II* recognition site (underlined) and reverse primer 5'-GGGTTACCCCACTCAACCCACCATACTTT-3' with a *BstEll* recognition site (underlined) was amplified. After digesting with *Bgl II* and

*Bst*Ell restriction enzymes, the resulting PCR product was inserted into the *Bgl II* and *Bst*Ell predigested modified expression vector pCAMBIA1301. The required PCR product was verified by sequencing and was re-confirmed by digestion with *Bgl II* and *BstEll* restriction enzymes. The resulting vector containing *OsFAE* gene was under the control of constitutive CaMV35S promoter with nopaline synthase terminator (Nos). The p*OsFAE*S construct was then allowed to introduce into *Agrobacterium tumefaciens* LBA4404 by electroporation. The transformed *Agrobacterium* colonies were chosen on solid LB medium supplemented with 50 mg/l kanamycin and 10 mg/l rifampicin by keeping for 2 days at 28°C in continuous darkness. The selected colonies were picked and cultured in 5 ml of liquid LB medium overnight shaken at 240 rpm at 28°C. The plasmids harboring *OsFAE* gene were isolated and digested with the restriction enzymes for further confirmation.

**Tobacco transformation:** Tobacco was transformed with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1301+*OsFAE*) using the leaf disc method (Horsch *et al.,* 1988). The bacterial culture was made to grow at OD600 to 0.4–0.6. The *Agrobacterium* and 1 $cm^2$ young tobacco leaf discs were co-cultured for 20-25 minutes. The infected leaf discs (upside down) were placed on co-culturing MS medium containing 6-BA (2mg/l) and IAA (1mg/l) at 25°C in the dark for 2 days. Then the infected explants still (upside down) were transferred to a fresh co-culturing solid MS medium supplemented with (50mg/l hygromycin and 500mg/l carbencillin) at 25°C in the light for 25-30 days for shoot regeneration. The shoots (2–3 cm tall), after excision were transferred to solid MS medium containing 6-BA (2mg/l) + IAA (0.1mg/l), supplemented with (50mg/l hygromycin and 500mg/l carbencillin) for root generation. The conditions for root generation were same as that for shoot generation. The shoots bearing roots were maintained under *In vitro* conditions and some of them were transferred to soil under growth room conditions for seed set.

**PCR analysis:** Total genomic DNA was isolated from control and hygromycin-resistant homozygous transgenic tobacco leaves from T2 generation using CTAB method (Reichardt & Rogers, 1994). The fragments of *OsFAE* transgene were amplified by using forward OsFA-F (5'-ATTGGTGTGGTTGTCGTCAA-3') and reverse OsFA-R (5'-TCTCCTTCGCTGGATTCACTG-3') primer pair. While, the fragments of *hygromycin* gene were amplified by employing forward Hyg-F (5'-TGCGCCCAAGCTGCATCAT-3') and reverse Hyg-R (5'-TGAACTCACCGCGACGTCTGT-3') primer pair. The PCR was performed in a 20 µl reaction volume of containing 1.0 µl DNA, 10 pmol each of primers, 10 mmol dNTPs, 1X PCR buffer, 1X $MgCl_2$ and 0.5 U Taq polymerase (Takara, China). The PCR reaction was carried out at 94°C for 3 min followed by 32 cycles of amplification (1 min at 94°C, 1 min at 58°C and 1 min at 72°C) with final extension at 72°C for 5 min.

**Selection of transgenic plants:** The seeds from T0 generation of putative transgenic plants were made to germinate on ½ MS medium in Petri dishes lacking antibiotic under growth room conditions. The T1 generation 2-4 leaved plantlets were then transferred to the Petri dishes containing MS medium supplemented with hygromycin (50 mg/l). The selection of transgenic tobacco lines was made on the basis of hygromycin tolerance. The segregation analysis of T1 generation of transgenic lines was obtained from independent plants.

## Results and Discussion

**Vector construct:** The full length of *OsFAE* gene encoding sequence was amplified through PCR using rice cDNA library. The *OsFAE* gene is involved in fatty acid elongation necessary for cuticular wax formation covering the aerial surfaces of plants. To envisage the physiological role of *OsFAE*, in tobacco plants *OsFAE* gene encoding sequence in sense orientation was placed under the control of CaMV35S promoter. The pCAMBIA 1301+*OsFAE* vector construct is shown in (Fig. 1). The generation of transgenic plants is prerequisite for the improvement of plants to impart desired traits (Zupan *et al*., 2000; Jaime & Teixeira, 2003; Yang *et al*., 2008).

**Shoot generation:** The tobacco leaf discs infected with *Agrobacterium* on MS medium with selectable hygromycin showed callus formation. The callus formation initiated from only the putative transformed leaf discs and the untransformed leaf discs were began to yellow after 3-5 days. Later on, during 5-10 days they became brown and did not initiate callus formation. The transformed leaflets developed into the bright green callus and then the shoots were emerged with 3 to 5 leaflets in 25-30 days (Fig. 2). Accumulating evidences suggested that *In vitro* tissue culture and micropropagation are necessary for transformation experiments to remodel the aesthetic and growth characteristics of the plants (Jaime & Teixeira, 2003**;** Siemens & Schieder, 1996; Tang *et al*., 1999).

**Root generation:** The emerging shoots with 3-5 leaflets showed root formation in 20-25 days (Fig. 3). The roots continued to grow in the putative transgenic plants, but the roots in untransformed plants did not develop (data not showed), which kept on growing. A number of independent hygromycin resistant *OsFAE* transgenic tobacco lines have been generated. All these hygromycin resistant tobacco plants showed normal phenotype. It has been reported that fine balance of *Agrobacterium* and selective agents might be helpful to raise antibiotics resistant transgenic plants (Silva & Fukai, 2001).

**PCR analysis:** Hygromycin resistant *OsFAE* transgenic tobacco lines were generated from independent transgenic plants. PCR analysis of homozygous transgenic lines of T2 generation results confirmed that the transformed tobacco genome stably contained both, the *OsFAE* transgene and plant selectable *hygromycin* marker gene. However, some of the T1 plants containing hygromycin gene were not positive for *OsFAE* transgene (data not shown). Nonetheless, PCR results showed that SR1, the control plants were negative for *OsFAE* as well as for hygromycin (Fig. 4). The hygromycin phosphotransferase (*hpt*) gene has been used as a selectable marker aimed with raising transformed plants (Punja & Chen, 2003).

**Selection of transgenic plants:** The plants of T1 generation of putative transformed plantlets were transferred from the ½ MS medium to selectable hygromycin supplemented MS medium. The plantlets remained green and developed elongated roots as they were resistant to hygromycin, while the untransformed plants became yellow and roots ceased to elongate because of their sensitiveness (Fig. 5). The similar findings were reported for transgenic tobacco plants resistant to antibiotic by Zhang *et al*., (2007).

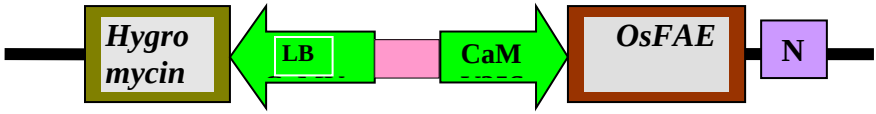


Fig. 1. Schematic representation of sense vector pCAMBIA1301 containing *OsFAE* transgene. LB, left border; RB, right border; PS; polylinkers site; CaMV35S, cauliflower mosaic virus 35S RNA promoter; Nos , nopaline synthase terminator.

Fig. 2. Represents the generation of transgenic tobacco plantlets from callus induced on MS medium supplemented with plants' selectable antibiotic hygromycin .

Fig. 3. Representatitive of the induction of roots from transgenic tobacco plantlets on MS medium containing plants' selectable antibiotic hygromycin.

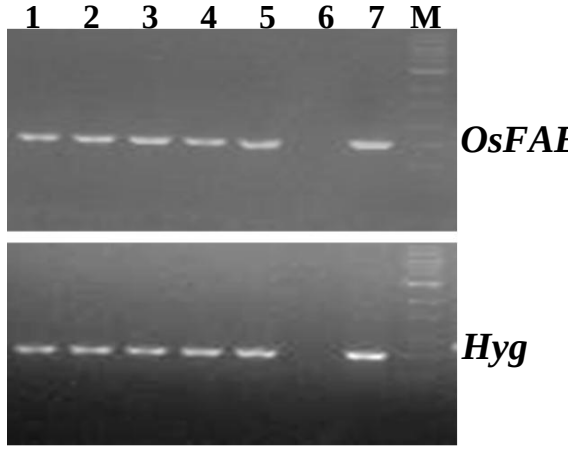


Fig. 4. PCR analysis of *OsFAE* transgene and plant selection marker resistance gene *Hyg* (hygromycin) of homozygous independent transgenic tobacco T2 generation. 1-5, the transgenic tobacco lines; 6, nontransgenic wild type tobacco as a negative control; 7, Vector p*OsFAE*S plasmid DNA as positive control; M, 1 kb DNA molecular weight marker .

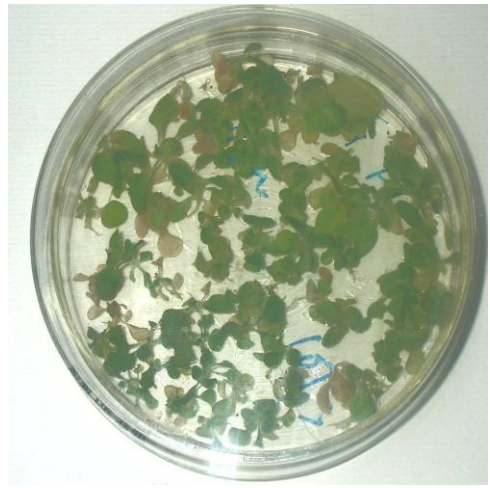

Fig. 5. Representative of the T1 generation of transgenic plants selection on MS medium supplemented with hygromycin. The flush green plantlets with elongated roots are transformed, which show resistance to hygromycin, and the yellowish plantlets with little roots are the nontransformed that display sensitivity to hygromycin.

**Segregation analysis:** The T1 progeny of T0 generation was allowed to segregate on hygromycin supplemented with MS medium in Petri dishes. The results presented in Table 1 revealed that in most of the transgenic lines, the hygromycin, a maker gene was segregated into 3:1 ratio. However, only one transgenic line showed the deviation from Mendel segregation ratio 3:1. The deviation may be related to multiple copy of transgene integration into host genome. Multiple copy insertion has been reported in transgenic tobacco transformed through *Agrobacterium* (Finnegan & McElroy, 1994; Islam *et al*., 2007).

**Table 1**. **Transgene segregation analysis of the T1 progeny of *OsFAE* transgenic tobacco lines.**

| Transgenic line | Total plants | Plants died | Plants survived | Ratio* |
|---|---|---|---|---|
| 02 | 82 | 23 | 59 | >3:1 |
| 03 | 90 | 28 | 62 | 3:1 |
| 14 | 87 | 29 | 58 | 3:1 |
| 17 | 79 | 23 | 56 | 3:1 |
| 20 | 84 | 25 | 59 | 3:1 |

* Values are the average of five replicates; >, greater than

The abiotic and biotic challenges cause the researchers to focus on the engineering of transgenic stress resistant plants worldwide. However, the advancement of transgenic stress tolerant plants is very slow, which might be owing to the little knowledge about the physiological and biochemical phenomenon of stress tolerance in plants. It needs an intricate genetic exploitation for this purpose. Since many years, the research accomplishments in engineering transgenic stress-tolerant crops are increasing (Schmitz & Schütte, 2000). Since last few years, the research orientation is focusing to reveal molecular basis of abiotic stress resistance (Krishna, 2002; Altman, 2003). We cloned the rice fatty acid elongation gene having assumption to impart constitutive cuticular waxy barrier to regulate of stress resistance from plants.

The introduction of genes involving various stress response into a single plant might be a base line for engineering stress tolerance among plants (Melchers *et al*., 1993; Holmberg & Bülow, 1998; Yu *et al*., 2008). The role of the gene products and the phenomenon of gene expression may be helpful to trace the molecular response to drought and disease resistance (Osusky *et al*., 2000; Iwai *et al*., 2002; Samuel *et al*., 2008).

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