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TRANSGENIC TOBACCO WITH RICE FAE GENE SHOWS ENHANCED RESISTANCE TO DROUGHT STRESS

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Abstract

Plants have evolved various adaptative traits to cope successfully with the stresses. Among them, cuticular waxy coating layer may serve as protecting barrier to diminish water loss, which consequently imparts drought resistance in plants. In order to characterize the role of rice *FAE* in drought tolerance, the *OsFAE* transgene was incorporated into tobacco via *Agrobacterium*–mediated leaflets transformation with sense sequence orientation under control of constitutive promoter CaMV35S. PCR and RT-PCR assays suggest that the OsFAE transgene has incorporated in tobacco genome and over-expressed in the transformed tobacco leaves. The characterization assay revealed some correlation between OsFAE transgene expression and drought tolerance in transgenic tobacco. The drought parameters data reveal that the transformed tobacco lines exhibit relatively less wilting on withheld-water stress, early recovery from the stress, containing higher relative water contents. Additionally, the transgenic tobacco lines exhibit more protein contents after exposure to sub-lethal drought stress and relatively higher contents were measured in them as compared to control on re-watering after 48 hours. Proline contents were found higher in the transgenic lines as compared to control under drought on 6th day of with-held water stress. Data shows that leaf water potential was less negative in the selected transgenic lines as compared to control on both; 10th day of with-held water stress and after 24 hours rehydration. It is concluded form present study that the selected OsFAE transgenic tobacco lines showed enhanced resistance against drought stress conditions.

Introduction

Plants are sessile entities, which most of the time in their life cycle face the scarcity of water among other stresses. Among stressful conditions, water deficit is very important factor that adversely affects plant growth, development and productivity (Bhatnagar *et al.,* 2008; Azhar *et al*., 2011). Various metabolic processes are affected due to drought stress. Thus, since the last two decades, morphological, physiological and molecular aspects of plants response against drought remained a subject of various research programs (Shinozaki & Yamaguchi, 2000; Xiong *et al.,* 2002; Kidokoro *et al*., 2009; Moosavi, 2012).

Environmental stresses are the major cause of global crop losses that inflict more than 70% average yield losses in most of the crops every year (Wang *et al.,* 2003; Bray, 2004, Ahmed *et al.,* 2010; Shinwari *et al*., 1998). They also act as key factor to regulate geographic distribution of plant species. Among stresses, dehydration is the main factor that adversely affects plant growth, development, and productivity. Out of 1500 million hectares of global cropland, only 17% is irrigated land that provides about 40% of the world's food production, whilst remaining 60% land is rain-fed (Anonymous, 2002).

Thus, different physiological processes in plants are more or less affected by drought. However, a limited number of plants have been taken under consideration for biochemical and molecular studies for exploration of dehydration tolerance mechanisms. In recent years, physiological and molecular basis for plant responses to drought tolerance has been a subject of passionate research (Xiong *et al.,* 2002, Narusaka *et al*., 2003).

Drought stress responses involving physiological processes are operational in certain plants (Passioura, 2007; Nakashima *et al*., 2000). Usually, plants respond and adapt themselves to dehydration by altering their cellular metabolism and activating defense mechanisms. Characterization of physio-morphological changes in the transgenic material is very important to unearth mechanism of drought tolerance (Wang *et al.,* 2012). However, under stressful conditions, there has been a lack of physio-morphological characterization and analysis of transgenic material. The transgene potential against abiotic stresses especially drought has been largely measured through observation of phenotypes and endogenous chemicals (Zhang *et al.,* 2007; Mandhania *et al.,* 2011; Yang *et al*., 2012).

Generally, most of the transgenic research aimed at improving stress tolerance using model plant species or model cultivars/genotypes. Thus, this concept of testing seems valuable to prove functionality of the candidate genes in elite varieties is of prime importance for developing commercially viable materials. Our present study is aimed at to characterize the OsFAE gene potential in transgenic tobacco plants under mild drought stress.

Plants have acquired numerous characteristics that help them to adaptive to environments. For instance, nearly all the aerial plant surfaces are sheathed by cuticle waxy-coating (Bhatti & He, 2009; Islam *et al.,* 2009a; Wang *et al.,* 2012). The cuticular waxes are a blend of diversified compounds like glycerols, phenolics, very long-chain fatty acids (VLCFAs) and their derivatives (Goodwin & Jenks, 2005; Nawrath, 2002), triterpenoids (Vogg *et al.,* 2004) and phenylpropanoids (Goodwin & Jenks, 2005).

Plant cuticle has been found to be involved in protection against non-stomatal water loss (Oliveira *et al.,* 2003), UV irradiation (Long *et al.,* 2003), mechanical injury (Eglinton & Hamilton, 1967), frost damage (Thomas & Barber, 1974) and biotic stresses (Islam *et al.,*

2009a). Additionally, waxes are involved in preclusion of post-genital organ fusion, pollen dispersal, pollen–stigma interaction (Wolters-Arts *et al.,* 1998) and pollen rehydration (Piffanelli *et al.,* 1998).

Fatty acids synthesis occurs in mitochondria to some extent (Wada *et al.,* 1997); however, *de novo* synthesis of fatty acid (C16-C18) takes place in plastids of leaf mesophyll tissue (Ohlrogge *et al.,* 1979). After leaving from the plastids, these fatty acids elongate to form VLCFAs (very long chain fatty acids) ranging from C24 to C36. VLCFAs lead to form primary alcohols and wax esters via acyl reduction pathway (Kunst & Samuels, 2003).

So far, many plant *VLCFAEs* genes have been characterized such as *Arabidopsis thaliana FAE1* (Millar & Kunst, 1997), *KCS1* (Todd *et al.,* 1999), *KCS2* (Clemens & Kunst, 2001), *CER6* (Millar *et al.,* 1999), *Brassica napus FAE1* (Han *et al.,* 2001), *Zea mays CR4* (Becraft *et al.,* 2001), and *FAE2* from the moss *Marchantia polymorpha* (Kajikawa *et al.,* 2003).

Very little information is available about rice *FAE* (fatty acids elongation) gene (Bhatti & He, 2009). Additionally, drought responses in plants are very complex and thus, precise structural and functional modifications driven by scarcity of water are almost inadequately understood. Thus, discovery of the novel genes and understanding about their expressions and functions are very important to improve drought tolerance in plants. However, some of drought responsive genes are involved in protection of cells against water-deficit and regulation of signal transduction (Munns *et al.,* 2010).

Keeping in view, we generated transgenic tobacco harbouring rice FAE gene in its genome (Bhatti & He, 2009). This study was conducted to explore the performance of *OsFAE*-transgenic tobacco plants under drought stress in comparison with the control. Thus, in the present paper, we characterized *OsFAE* transgene in the selected transgenic tobacco lines through study of phenotypic and biochemical parameters regarding drought stress.

Materials and Methods

Plant material and growth conditions: The SR1 tobacco was transformed with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1301+*OsFAE*) with rice fatty acid eleongation gene OsFAE, using the leaf disc method (Bhatti & He, 2009). After fundamental screenings, two independent hygromycine resistant *OsFAE* transgenic tobacco lines were selected for onward study together with a control, WT. The T2 transgenic plants and the controls were propagated through healthy seeds. Well established plants were transferred into 6-inch pots filled with clay and farm yard manure (FYM) in 3:1 ratio. Plants were watered well in the green house under day/night length 16/8 h, 3000 flux light-intensity and day/ night temperature 30/22ºC. After 4 weeks of growth, plants of similar size were grouped into at least five replicates and plants were subjected to water withheld stress for upto 11 days until the control plants became almost dried if not intended for physiological measurement after re-watering. Experiments were repeated at least three times for all treatments. Two separate experiments were conducted for phenotypic and biochemical characterization.

Drought tolerance assay: In the greenhouse experiments, 30-days old the transgenic and control plants were kept at 28/30 °C under 16/8 hr photoperiod and subjected to withheld-water stress (drought) for 11 days. The wilting percentage of the transgenic lines and control plants was calculated. Thereafter, the plants were watered and on re-hydration, recovery percentage of tobacco plants from drought stress was recorded.

Measurement of leaf water potential: The leaf water potential was measured using pressure chamber technique according to method suggested by Ritchie & Hinckley (1975). The leaf samples were placed in a chamber with the cut end protruding through a rubber stopper sealing the chamber. The atmospheric pressure in the chamber was gradually increased by means of an external source of compressed air until sap just appears at the cut ends of the xylem elements, the readings were recorded. Mid-day leaf water potential of transgenic and control plants using Wescor Thermocouple Psychrometer (Wescor Inc., South Logan, UT, USA) on 10^{th} day of water-withheld and after 24 h on re-watering was measured with five replicates each.

Relative water contents assay: Relative water contents percent (RWC %) was determined according to method proposed by Ma *et al.,* (2004) and Yoshida *et al.,* (2002) with minor modifications. The fresh weight (FW) of leaves was recorded just after sampling and placed in covered Petri-dishes containing distilled water, so their cut ends were dipped in water. The Petri-dishes were kept at (4°C) for at least 4 h to achieve full turgidity of the leaves. After removing leaves from the Petri dishes, turgid fresh weight (TW) was determined. Finally, the leaves were oven-dried at 70°C for 2 days and their dry weight (DW) was recorded at each time point. The relative water contents (RWC) of leaves were calculated using formula [(FW - DW) / TW - DW) X100] according to Ritchie *et al.,* (1990).

Measurement of protein contents: Fresh leaves (0.5 g) were ground to a fine powder with a mortar and pestle under liquid nitrogen. Proteins were then extracted at 4°C by grinding with 50 mM of ice-cold phosphate buffer, pH 7.0, containing 0.1% (w/v) Ammonium acetate, 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinyl pyrrolidine. The homogenate was centrifuged at 4°C for 20 min at 12,000*g*. The clear supernatant fraction was used to determine the protein contents according to method proposed by Bradford (1976), using bovine serum albumin in an appropriate buffer as a standard.

Proline estimation: 0.5g of the leaves was homogenized in 5 mL of 5% aqueous sulphosalicylic acid. The homogenate was filtrated using Whatman No. 2 filter paper. 2mL of the filtrate was put in a test tube and added 2mL glacial acetic acid and acid ninhydrin each and put it in the boiling water for 1h. The reaction was terminated by placing it in ice tub. Then, 4mL toluene was added to the reaction mixture and stirred vigorously 30 sec. The toluene layer was separated at the

room temperature. Red color intensity was measured at 520 nm. A batch of standards of pure proline was run and obtained a standard curve. Thereafter, the amount of proline in the sample was determined accordingly. The proline contents were calculated on fresh-weight-basis using following formula:

$$\mu\text{moles per g tissue} = \frac{\mu\text{g proline/mL x mL toluene}}{115.5\text{x}5} \text{ X } \frac{5}{\text{g sample}}$$

Statistical analysis: A completely randomized design (CRD) was employed to arrange the experimental units in five replications. The data obtained was analyzed statistically by using Analysis of Variance (ANOVA) technique using the MSTAT-C computer package.

Results and Discussion

Transgenic tobacco plants with rice FAE gene were raised by Agrobacterium mediated transformation system (Bhatti & He, 2009). In this study, following physiological responses of two *OsFAE* transgenic T2 tobacco lines under both withheld water stress and recovery conditions have been studied.

PCR and RT-PCR assays: Agrobacterium-mediated tobacco plants were raised with rice *FAE* gene (Bhatti & He, 2009). The PCR assay (Fig. 1) showed that *OsFAE* gene has been integrated into tobacco lines. The gene expression assay by RT-PCR (Fig. 2) showed that *OSFAE* transcript has higher intensity in the selected transgenic lines, while it was absent in the control. This caused an enhanced level of wax development in the OsFAE transgenic tobacco leaves as compared to control (Bhatti, 2008). Consequently, the *OsFAE* transgenic lines exhibited more biomass production and higher water use efficiency (WUE) with less utilization of water (Bhatti, 2008).

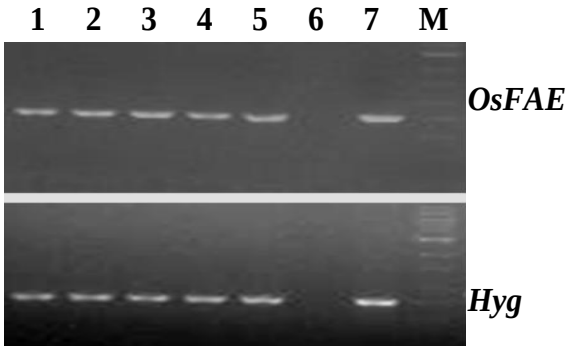

Fig. 1. Showing PCR reaction for rice FAE and Hygromycin gene integration into T2 transgenic tobacco plants genome and control. Where, 1-5 OsFAE transgenic lines; 6, negative control; 7, positive control OsFAE plasmid vector ad M, 1-Kb Marker DNA.

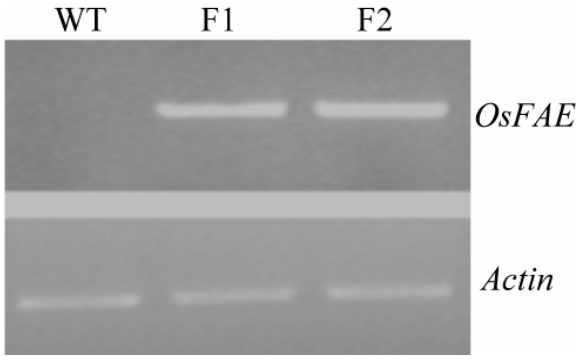

Fig. 2. RT- PCR analysis of the transcript obtained from transgenic lines and control. Where, WT, control; F7 and F11 OsFAE transgenic tobacco lines.

Wilting resistance assay: Wilting percentage data (Fig. 3) indicate that the randomly selected transgenic tobacco lines were more resistant to wilting than control on all the treatment days. However, higher significant resistance against withheld water stress was shown by both the transgenic lines from 7th to 11th day in comparison with control. However, the line F7, showed relatively higher resistance against the wilting as compared to control (p=0.001) on all the days of withheld water stress except 11th day, when it exhibited moderate resistance (p=0.01). This might be due to less evaporation of water through cuticle due to more wax. Our findings are harmonious with the results presented by Jiang *et al.,* (2010) for the transgenic white clover. The results of the present study can also be supported with those of Islam *et al*., (2009b) in which Glossy 1 (GL1) is one of the important genes controlling wax biosynthesis in rice plant under drought stress.

Drought stress recovery assay: The transgenic tobacco lines showed an early from withheld water stress as compare to control plants on re-watering (Fig. 4). However, the difference was moderately significant (p=0.01) among transgenic and control tobacco on 1st DAR (day after rehydration). On 2nd DAR, the difference was highly significant (p=0.001) among the transgenic lines and control plants. Our results coincide with the findings of Meiri *et al.,* (2006). Less wilting symptoms may be attributed to FAE transgene integration into tobacco genome. Results of the present study correlate with the findings of Bigang *et al.,* (2011) where WSL2 gene in transgenic rice synthesis leaf cuticle under drought conditions.

Protein contents estimation: Data regarding protein contents (Fig. 5) show that proteins contents were found more in OsFAE transgenic lines as compared to control. The difference on AWS (after water stress) was highly significant (p=0.001) among transgenic and control plants. However, on re-watering, increment in the protein contents was recorded more in the transgenic plants than in control however, the difference was highly significant between F7 and control plants (p=0.001). Conversely, the difference was recorded insignificant between the transgenic lines. Changes in proteins are caused by different stresses including water stress (Yordanova *et al.,* 2004). Our findings correspond with the results proposed by Fazeli *et al.,* (2007) that the drought susceptible plants can have less protein contents under drought stress.

Relative water contents: The data (Fig. 6) shows that on the 6th DWW (day withheld water) the transgenic lines exhibit more relative water contents than of the control. However, difference between the transgenic line F7 and control was less significant (p=0.05) and it was significant between F11 and control plants on the same day. However, on 11th DWW, both the transgenic lines showed higher relative water contents than that of the control and the difference was highly significant (p=0.001) between them. Our findings are in agreement with the findings of Medici *et al.,* (2003). This response might be due to better osmotic adjustment capacity of the transgenic tobacco over control plants under stressful condition (Gholinezhad *et al.,* 2009).

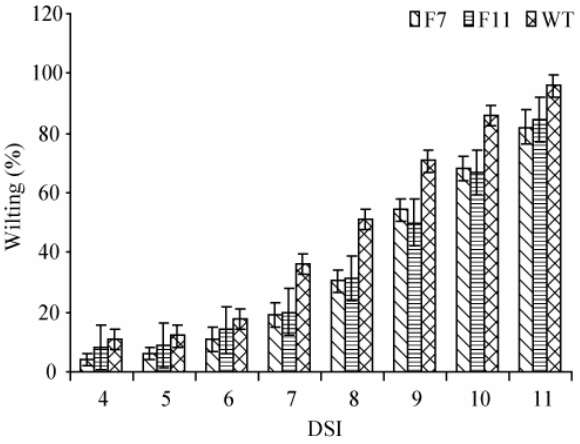

Fig. 3. Wilting percentage of tobacco leaves from 4th to 11th day of withheld-water stress. Values are means ± standard error (n=5). Where, F7 and F11, *OsFAE* transgenic lines; WT control, SR-1 tobacco plants; DSI, days of stress induction.

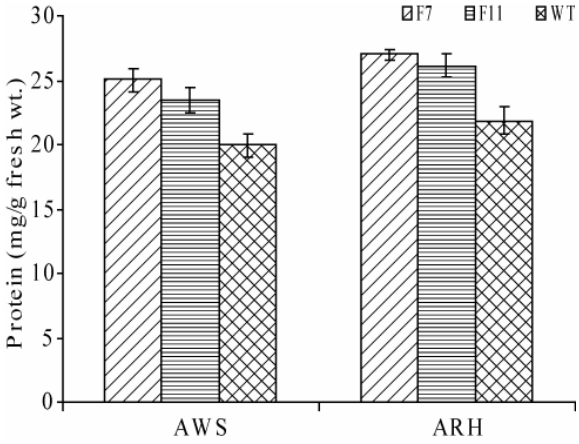

Fig. 5. Protein contents mg/g fresh weight in tobacco leaves on 8th day subjected to sub-lethal drought stress and on the second day of re-watering. Values are means ± standard error, (n=6); F7 and F11, *OsFAE* transgenic lines; WT control, SR-1 tobacco plants; AWS, after water stress; ARH, after re-hydration

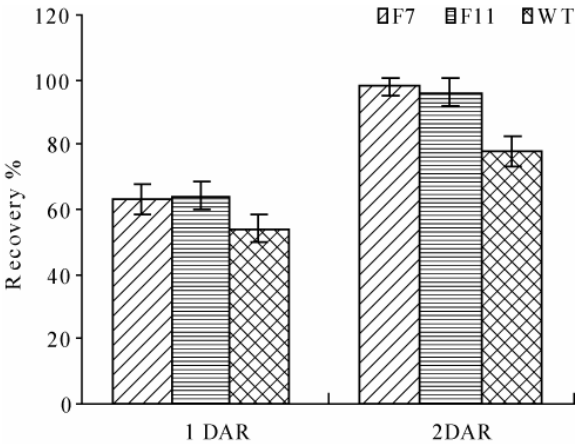

Fig. 4. Representing the tobacco plants' recovery percentage from withheld-water stress after re-watering. Values are means ± standard error (n=5). Where, F7 and F11, *OsFAE* transgenic lines; WT control, SR-1 tobacco plants; DAR, days after re-hydration.

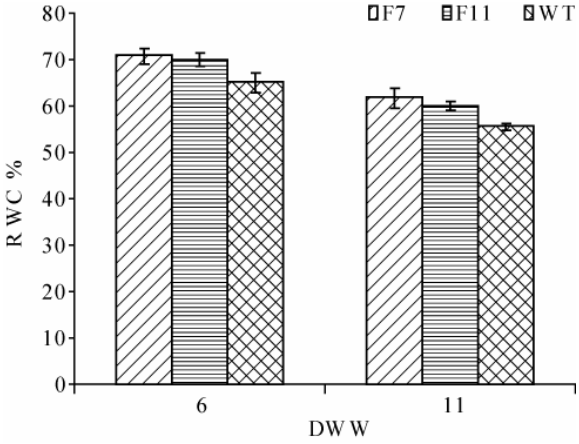

Fig. 6. Relative water contents (RWC %) in tobacco leaves, on 6th and 11th DWW (day of withheld-water). Values are means ± standard error (n=5); F7 and F11, *OsFAE* transgenic lines; WT control, SR-1 tobacco plants.

Proline assay: The proline contents (Fig. 7) were relatively higher in the transgenic lines as compared to control however, between the transgenic lines the difference was insignificant (p=0.5). On 6th day of WWS, the transgenic lines F7 and F71 exhibited higher proline contents than WT and the difference was highly significant (p=0.001). On ARH (after rehydration), the decline in proline contents was recorded on 8th day in the transgenic and control plants. However, difference among the transgenic lines and control was highly significant (p=0.001) while, it was insignificant between the transgenic lines. The proline acting as osmoregulatory in function helps plants to resist against drought stress (Mandhania *et al.,* 2011).

Leaf water potential: Data (Fig. 8) shows that on 10th day of withheld-water stress, leaf water potential was found less negative in both the transgenic lines as compared to control and the difference was exceedingly significant (p=0.001) between them. However, on re-watering, the difference was less significant at (p=0.01) between F7 and the control and was highly significant (p=0.001) between transgenic line F11 and WT tobacco plants. Our findings are coherent with the results presented by Gaxiola *et al.,* (2001). Thus, drought-tolerant plants maintain their turgor at low water potential by increasing the number of solutes in the cell (Bray *et al.,* 2000).

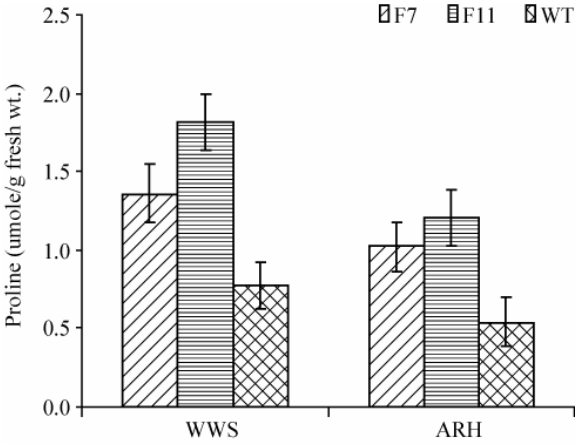

Fig. 7. Proline contents (µmoles per gram fresh wt.) in tobacco leaves, on 6th WWS (withheld water stress) and 8th ARH (after rehydration). Values are means ± standard error (n=5); F7 and F11, *OsFAE* transgenic lines; WT control, SR-1 tobacco plants; WWS, with-held water stress; ARH, after re-hydration.

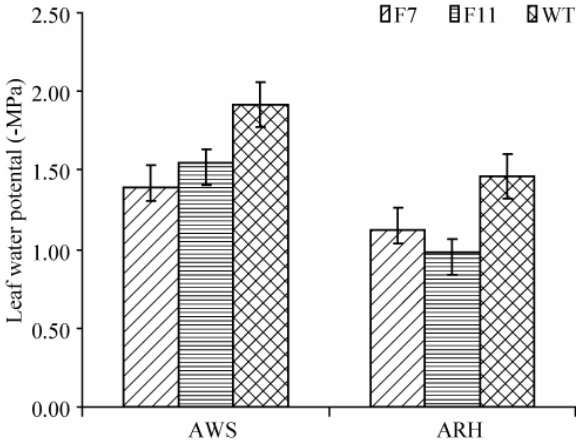

Fig. 8. Leaf water potential (-MPa) of transgenic tobacco lines (F7 and F11) and wild-type control (WT) after withheld-water stressed (AWS) applied on 10th day and after 24 hours re-hydration (ARH) conditions. Values are the means ± s.d.

Conclusion

The OsFAE transgenic transgenic tobacco lines exhibited high level of resistance against drought stress. Thus, the candidate gene may be incorporated to induce drought resistance in other plants for better growth and development.

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