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TRANSGENIC TOBACCO WITH RICE *FAE* GENE EXHIBITS HIGHER WATER USE EFFICIENCY

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Abstract

The rice *FAE* encodes protein for fatty acids elongation to form very long chain fatty acids (VLCFAs), which are the intermediates for wax biosynthesis. *Agrobacterium*- mediated transgenic tobacco plants bear rice fatty acid elongation gene (*OsFAE*), which has been incorporated into their genome. Amino acids multiple sequences alignment analysis reveals that rice FAE protein has sequence similarity with other fatty acids elongation and wax related proteins, especially corn FAE. Phylogenetic tree, a bioinformatics tool shows that OsFAE has a close evolutionary origin with that of maize FAE. Sense sequence of rice FAE gene incorporation to transgenic rice has consequently resulted into relatively more cuticular wax on leaf surface. Scanning electron microscopy (SEM) illustrates that transgenic tobacco leaves have phenotypically higher cuticular waxes than of control. Our findings also suggest that the transgenic tobacco exhibits more water use efficiency (WUE) at both the 90% and 35% field capacity (FC) levels under non-stress and stressful conditions, respectively.

Introduction

Plants are though sessile, yet they perform their life activities efficiently under sever stressful conditions by evolving numerous specialized mechanisms and adaptive structures compatible with their lifestyle (Pecinka *et al.*, 2009). Among most of the stresses, drought is considered very important stress as it impairs not only growth, but also productivity of plants. Of the most important adaptation traits, plants secrete essentially waxy layer that surrounds nearly all plant parts interface to the environment (Luo *et al.*, 2007). Combination of cutin, waxes, and possibly polysaccharides form the cuticle, a hydrophobic covering over the epidermis (Yukihiro *et al.*, 2011).

The cuticular waxes are a blend of different lipophilic compounds that are predominantly composed of aliphatic monomers, glycerols, phenolics, very long-chain fatty acids (VLCFAs) and their derivatives (Bach *et al.*, 2008). Additionally, waxes include triterpenoids (Vogg *et al.*, 2004) and phenylpropanoids (Goodwin & Jenks, 2005; Kunst & Samuels, 2003). Accumulating findings suggest that the wax load of a leaf fluctuates between not only abaxial and adaxial surfaces, but also intracuticular and epicuticular waxes (Gniwotta *et al.*, 2005; Vogg *et al.*, 2004). Furthermore, wax deposition may vary amongst different epidermal cells, such as guard cells and trichomes (Schreiber, 2005). Thickness of the cuticle may vary (0.02–200 um) among diverse plant species and different organs of the same plant (Zheng *et al.*, 2005).

The cuticles have been attributed to protect the plants from non-stomatal water loss (Raffaele *et al.*, 2009; Yukihiro *et al.*, 2011), UV irradiation (Long *et al.*, 2003), mechanical injury (Knight *et al.*, 2004)), frost damage (Teece *et al.*, 2008), chemicals and biotic invaders like, insects (Eigenbrode, 1996; Eigenbrode & Espelie, 1995) and other pathogens assault (Raffaele *et al.*, 2009).

Very little contribution of mitochondria towards fatty acids synthesis and the *de novo* synthesis of fatty acids (C16 - C18) occurs in plastids of leaf mesophyll tissue (Qiang *et al.*, 2009). After leaving from the plastids, elongation of these fatty acids results to form VLCFAs ranging from C24 to C36 (Clare *et al.*, 2009). From these VLCFAs various alcohols, aldehydes, alkanes, ketones and wax esters are formed via different pathways (Beaudoin *et al.*, 2009; Raffaele *et al.*, 2009).

The phenomenon of VLCFAs biosynthesis has been explored to some extent, but knowledge about the genes involvement in VLCFAs elongation and modification is limited enough that needs continuous strides (Kunst & Samuels, 2003). Many VLCFAEs have been characterized in plants such as *Arabidopsis thaliana* FAE1 (Millar & Kunst, 1997), KCS1 (Todd *et al.*, 1999), CER6 (Millar *et al.*, 1999), LCR (Wellesen, *et al.*, 2001), CUT1 (Millar *et al.*, 1999) and *Brassica napus* FAE1 (Han *et al.*, 2001).

Drought is a deficit of adequate moisture, critically necessary for a plant to grow normally to complete its life cycle (Zhu, 2002). The paucity of optimum moisture leads to drought stress, which is very common in the arid and semi-arid rain-fed regions across the globe especially, where erratic rains and poor irrigation prevails (Lawlor, 2002). Habitually, plants are tolerant to water stress, though its level varies among the species (Chaitanya *et al.*, 2003). Every year, drought hits many parts of the world that often inflicts worse impact over the crop production (Thomas, 2008; Ludlow & Muchow, 1990). Therefore, it has been predicted that worldwide losses in crop yield because of water deficit, might surpass the losses incur from all the combined causes (Litsinger, 2009).

Progress regarding genetic improvement in crops for water stress is dawdling and more limited (Evenson & Gollin, 2003), owing to poor understanding of water stress tolerance mechanisms, and dearth of proficient techniques for selection breeding resources for drought tolerance (Khush, 2001). Development of stress tolerant crops is the usual outcome of the most plant breeding projects/programs. Speedy development of genetic engineering and molecular breeding has offered a practical approach to improve stress tolerance in crops (Collard *et al.*, 2008; Ramanjulu & Bartels, 2002).

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Recently, a number of successful endeavors to incorporate stress-tolerant genes into plants for developing enhanced tolerance against water and other stresses have been well documented (Bressan *et al.*, 2009; Bhattacharya *et al.,* 2004; Chandra et *al.*, 2004). Previously, we introduced rice *FAE* gene into tobacco via *Agrobacterium*-mediated transformation system to raise transgenic plants (Bhatti & He, 2009). We studied some of the morphology, growth and physiological parameters to expound the biological role of *OsFAE* in the transformed tobacco.

In the present work, we focused on SEM and relevant aspects of water stress such as actual water use, biomass increment and WUE (water use efficiency) of tobacco plants per plant at non-stress and stress conditions at 90% and 35% FC (field capacity) respectively.

Materials and Methods

Plant material and growth conditions: *Agrobacterium*-mediated tobacco plants with rice *FAE* gene have been generated according to method described by Bhatti & He (2009). The T_2 transgenic tobacco and the control (wild type SR-1) plants were used for experimentation, which were maintained under green-house conditions.

PCR analysis of T2 transgenic tobacco: Total genomic DNA was isolated from the control and hygromycin-resistant transgenic tobacco leaves using CTAB method (Reichardt & Rogers, 1994). PCR analysis of T_2 transgenic and control plants was essentially performed as described by Bhatti & He (2009).

Sequences alignment and phylogenetic analysis: The amino acid sequences of proteins encoding for wax and the intermediate synthesis products in rice and other plant species were aligned using software ClustalW 1.83, according to method (Chenna *et al*., 2003; Thompson *et al.,* 1994) with the following parameters: gap open penalty (5.00), gap extension penalty (0.05). Then, the alignment was adjusted manually. The phylogenetic tree was constructed by the neighbor-joining method using (MEGA 3.1) software as described by (Fujita *et al.*, 2004). The confidence level of monophyletic groups was estimated by bootstrap analysis with 1000 replicates.

Scanning electron microscopy (SEM): The leaves of T_2 transgenic and control tobacco were collected and prepared for SEM by adopting protocol with some modification reported by Bensalem *et al.*, (2009). The leaves were cut into 1-cm^2 pieces, and dried in shade. Adaxial as well as abaxial surfaces of matured leaves were used for SEM. Fragments of the leaves were first fixed in 6% glutaraldehyde and then mounted them on stubs. Samples were coated with 15-20 A° grain-size gold particles for 20 min by using an appropriate coater. The coated samples were used for scanning electron microscopy at 70 KV (Hitachi, Japan).

Actual water-use, biomass production and water-use efficiency assay: Thirty-five days old independent transgenic tobacco plants (T_2 generation) expressing *OsFAE* gene and the control plants were maintained at 90 % and 35 % field capacity (FC). The soil moisture was measured according to method of Singh (1980) with slight modifications. To determine the field capacity, 15 cm plastic pots were filled with finely ground soil and green yard manure in 3:1 ratio (after passing through 3 mm sieve), leaving about 5 cm of the pot top unfilled, in a way that no air pockets left inside. The pots were watered until their saturation. The upper surface of the soil was then sealed with paraffin wax and covered with a watch glass to check evaporation from the soil surface. The pots were allowed to stand for 48 to 72 hrs. The soil samples were taken from the pots and then moisture contents at field capacity were determined after drying the soil sample in an oven at 70ºC.

The desired moisture levels were obtained by allowing the soil to dry until close to the specific moisture level that was determined gravimetrically on each pot (Galmes *et al.*, 2005). The pots were weighed on alternate days, and the required amount of water was added in order to maintain the correct moisture level. The pots were maintained at the respective field capacity for 30 days. The water loss was corrected after every two days by application of the calculated water for each of the respective required field capacity. Some pots were kept without plants as control for the determination of water loss through evaporation. The actual water applied, increment of biomass/plant and water use efficiency (biomass increment/kg water applied/per plant) were worked out for the transgenic and control plants. The experiments were repeated essentially twice with ten replicates for each parameter.

Statistical analysis: Student t-test was performed using MS Excel 2003 (Microsoft Corporation, Seattle, USA). Differences between results are described as being significant where $p \leq 0.001$, and not significant where $p \geq 0.05$.

Results and Discussion

The Sequence analyses: Various proteins relevant to cuticular wax biosynthesis have been identified, which were assumed to be characterized by sequence alignment. The sequence analysis gives a glimpse to predict a probable role of *OsFAE* in comparison with other related genes. The open reading frame (ORF) of rice *FAE* gene encodes a protein of 519 amino acids - the longest peptides amongst the transcription products for wax synthesis. The deduced amino acid sequence of FAE contains some conserved domains. Whilst, aligned with the characterized fatty acid elongation related protein sequences, the rice FAE shares high similarity with other plants' proteins like Zm (*Zea mays*), At (*Arabidopsis*), Mp (*Marchantia polymorpha*), Bn (*Brassica napus*) and Sb (*Sorghum bicolor*) (Fig. 1). The OsFAE shares identical sequences with ZmFAE (94%), AtKCS2 (78%), AtAcytf (61%), MpKCS (61%), BnKCS, AtCUT1 (61%) and SbKCS (64%), respectively.

The *OsFAE* transgene, under the control of constitutive promoter CaMV35S, has been incorporated into tobacco *via Agrobacterium*-mediated transformation system (Bhatti & He, 2009). The multiple protein alignment analysis revealed that OsFAE highly shares the homology with other fatty acids elongation proteins from different plant species, especially with ZmFAE (Fig. 1). The bioinformatics tools, especially the multiple sequence alignment, are very helpful to envisage the role of gene, which may further be employed for imparting the desired traits in the target organism through accessible transformation system (Yilmaz *et al.*, 2009; Thompson, 2003). The phylogenic analysis signifies that OsFAE and ZmFAE are from a very close origin (Fig. 2). In the sequence analysis, phylogeny inference regarding proteomic studies is very important (Edgar & Batzoglou, 2006).

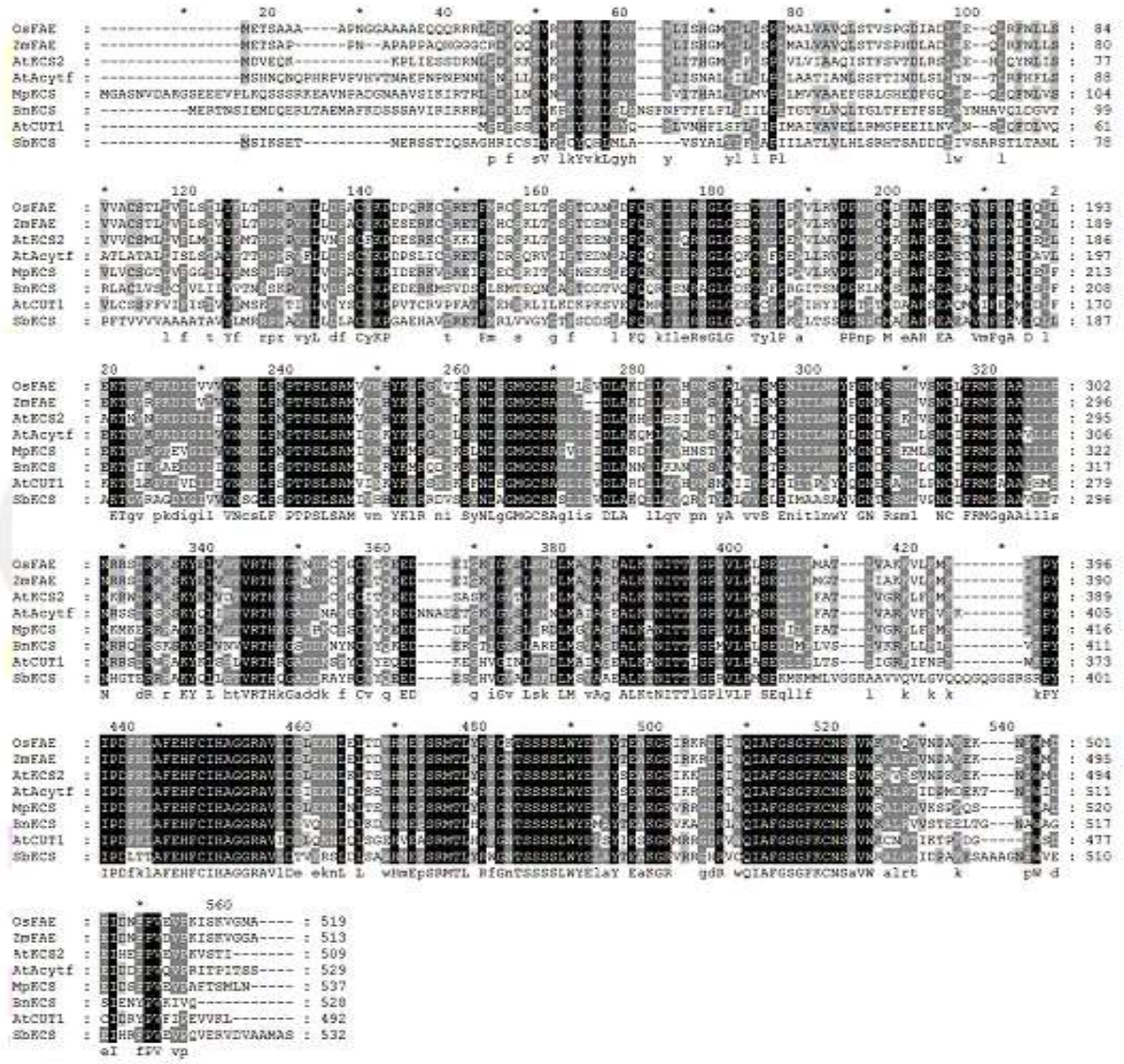

Fig. 1. Multiple alignments of plant proteins showing high similarity with the rice protein *OsFAE*. (GenBank accession numbers are indicated in parentheses). *OsFAE*, *Oryza sativa* fatty acid elongase (NP_001057996); ZmFAE, *Zea mays* FAE (CAC01441); AtKCS2, *Arabidopsis thaliana* 3-ketoacyl-CoA synthase 11 (O48780); AtAcytf, *Arabidopsis thaliana* acyltransferase (NP_195178); MpKCS, *Marchantia polymorpha* beta-ketoacyl-CoA-synthase (AAO48425); BnKCS, *Brassica napus* beta-ketoacyl-CoA-synthase (AAT65207); AtCUT1, *Arabidopsis thaliana* cuticular 1 (NP_177020); SbKCS, *Sorghum bicolor* beta-ketoacyl-CoA-synthase (AAD27560). Sequences were aligned with CLUSTAL W. Black background indicates identical amino acid residues and gray background designates similar amino acids. Gaps required for optimal alignment are shown by dashes. Asterisks indicate perfectly matched amino acids amongst these eight proteins. The identity and similarity of the aligned proteins for *OsFAE* are shown at the last part of the alignments.

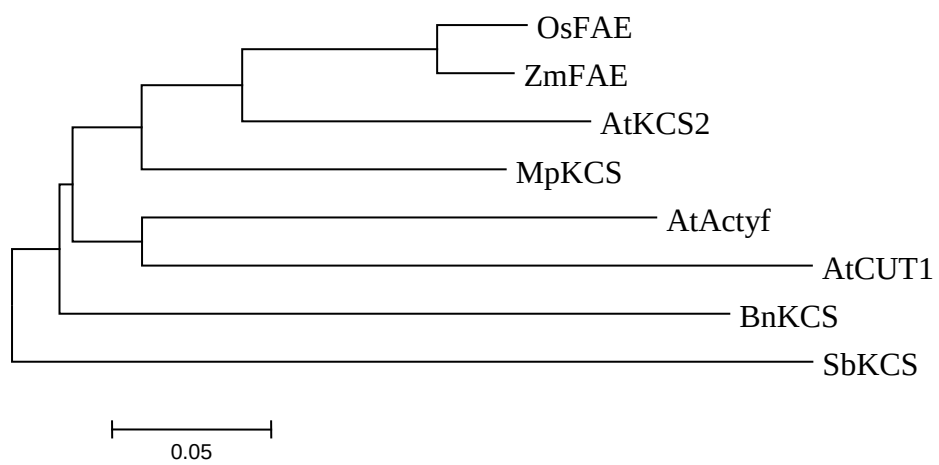

Fig. 2. Phylogenic analysis of proteins similar to *OsFAE*. Phylogenic analysis by MEGA 3.1 was performed using the neighbor-joining tree with 1000 replicates; the handling gap option was pair-wise deletion.

The PCR analysis of T2 transgenic tobacco: PCR gel electrophoresis results of the selected T_2 transgenic tobacco lines reveal that transformed tobacco genome contains the both, *OsFAE* a transgene and *hygromycin* a marker gene (Fig. 3). The results were similar to Gao & Hu (2008) and our previous findings for PCR analysis regarding molecular studies of putative transgenic plants (Bhatti & He, 2009). It suggests that the transgene has been integrated into tobacco genome. However, the role of the gene products and the phenomenon of gene expression are not initially predictable, however, it can be traced to fully grasp the molecular response to drought stress (Karaba *et al.*, 2007).

Scanning electron microscopy: The SEM was carried out of the selected T_2 *OsFAE* transgenic lines and control plants. Both adaxial and abaxial leaf surfaces showed altered cuticular wax morphology. SEM micrographs of the adaxial surfaces are shown (Fig. 4). Our SEM results found coherent with the finding reported by Islam *et al.*, (2009) and Zhang *et al.*, (2007). As the rice FAE gene was placed under a constitutive promoter CAMV35S in the transforming vector Bhatti & He (2009), subsequent to transformation its expression probably led to an enhanced accumulation of wax over the leaves of transgenic plants. The accumulation of waxes over the aerating surfaces of plants frequently leads to induction of protection against various abiotic and biotic stresses (Pinto & Yephremov, 2009). However, SEM micrographs of abaxial surfaces of transgenic leaves showed not substantially different from that of control for cuticular wax morphology (data not shown), and therefore, it possibly be accredited to the differential gene expression among various tissues (Nouar *et al.*, 2003).

The actual water use, biomass increment and water use efficiency (WUE**):** The actual water used by the transgenic lines was relatively less than that of the control at both the 90% and 35% FCs. However, the difference was significant (P=0.01) among transgenic and control plants at 35% FC, but it was found insignificant in them at 90% FC (Fig. 5). Nevertheless, the difference among the control plants and the transgenic lines was found also insignificant at both the 35% and 90% FCs (data not shown). Relatively, less amount of actual water utilization by the transgenic lines as compared to control at both the 90% and 35% field capacities (FCs) is a good indicator for drought resistance. The lower amount of actual water utilization might be due to smaller and/ or less number of stomata per unit area and more cuticular waxes may lower the transpiration rate to adjust water economy budget (Chen *et al.*, 2005). It was assumed that lesser root permeability might contribute to less water utilization (Anders & Jens, 2009).

The biomass increment per plant reveals that in the transgenic lines, it was produced relatively more than that of control at both the field capacity levels i.e., 90% and 35% FC, respectively (Fig. 6). It was significantly higher (P= 0.01) in the transgenic plants in comparison with control at 90% FC. It indicates that the transgenic lines are relatively more productive and their higher biomass increment might be due to more photosynthetic efficiency Cheng *et al.*, (2009). Our results are coherent with the findings of Ge *et al.*, (2004), and the more biomass production depends on internal and physical factors involvement Kanno *et al.*, (2009). However, the difference in terms of biomass increment among the transgenic lines was recorded insignificant at both the 35% and 90% FCs (data not shown).

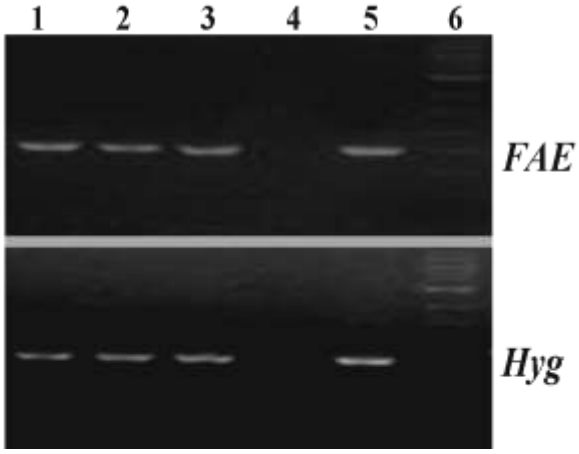

Fig. 3. Representatives of PCR analysis of T2 transgenic tobacco bearing rice *FAE* and *hygromycin* gene. Where, 1, 2 and 3 are the transgenic lines; 4, negative control (Wild-type SR-1); 5 positive control (plasmid) and 6, 1-Kb fragment size marker.

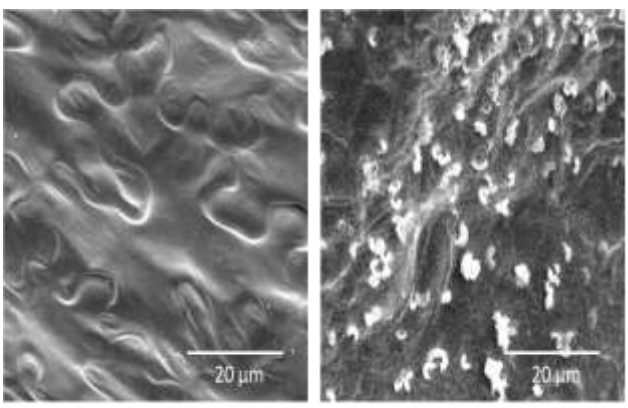

Fig. 4. Representatives of scanning electron microscopy (SEM) on the adaxial surfaces for cuticular wax morphology of the transgenic and control (Wild- type SR-1) leaves.

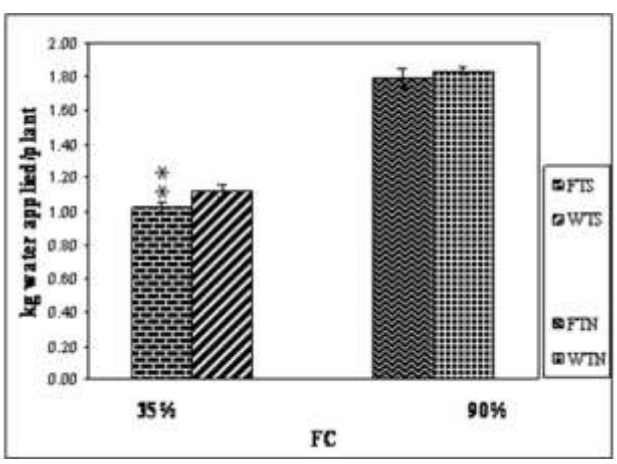

Fig. 5. Representative of the actual water used per plant by transgenic and control plants at 90% and 35% FC (field capacity). The actual water applied/plant was determined by subtracting the water lost by evaporation through soil. The bars represent the standard deviation (n=10). Where, FTN, non-stressed transgenic; FTS, stressed transgenic; WTN, non-stressed control (Wild-type SR-1) and WTS, stressed control (Wild-type SR-1) plants.

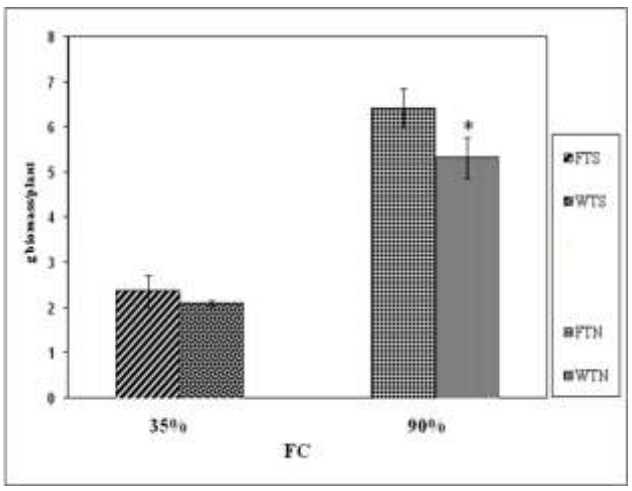

Fig. 6. Representative of the biomass increment per plant by transgenic and control plants at 90% and 35% field capacity (FC) after 30 days' water treatment. The bars represent the standard deviation (n=10). Where, FTN, non-stressed transgenic; FTS, stressed transgenic; WTN, non-stressed control (Wild-type SR-1) and WTS, stressed control (Wild-type SR-1) plants.

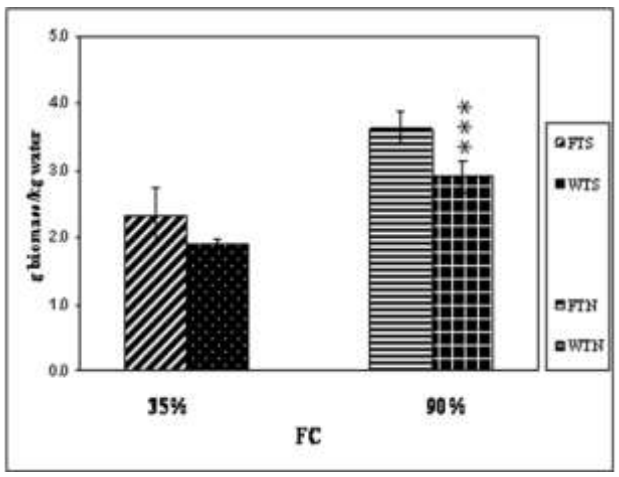

Fig. 7. Representative of WUE of transgenic and control plant at 35% and 90% FC after 30 days' water treatment. The WUE was calculated as biomass increment production per plant by dividing actual water applied (kg). The bars represent the standard deviation (n=10). Where, FTN, non-stressed transgenic; FTS, stressed transgenic; WTN, non-stressed control (Wild-type SR-1) and WTS, stressed control (Wild-type SR-1) plants.

Water use efficiency of transgenic lines was found generally higher than of t he control at the both 90% and 35% field capacity levels (Fig. 7). It was significantly higher (P=0.001) in the transgenic lines than of control at 90% FC. These results are quite similar with the findings reported by Karaba *et al.,* (2007) and Ge *et al.,* (2004). However, there was insignificant difference (P= 0.05) at 35% FC in both the transgenic and control plants for WUE.

In conclusion, *OsFAE* is a rice gene that is involved in regulation of very long chain fatty acids (VLCFAs) elongation. The rice *FAE* gene integration into tobacco genome subsequently showed its expression in the form of altered leaf cuticular wax morphology on the leaf surfaces. Because of more cuticular wax deposition, there was an enhanced biomass production with low water utilization and consequently more WUE in the transgenic plants due to rice FAE gene integration. Thus, the transgenic tobacco with rice *FAE* gene exhibits a higher WUE at both the field capacity levels (90% and 35% FC). However, the precise role of *OsFAE* gene in various agronomic important plants during biotic and abiotic stress conditions would remain an open venue for future research.

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