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Agrobacterium mediated transformation of tobacco with *LEW1* from *Pennisetum glaucum*

Anoosha Thota, Varalaxmi Yellisetty and Maheswari Mandapaka

Abstract
LEW1 a gene encoding a long-chain Cis-Prenyltransferase in dolichol biosynthesis. Dolichols play an important role in protein glycosylation process and unfolded protein response (UPR) pathway. In the present study *LEW1* gene was isolated from *Pennisetum glaucum*. The *LEW1* was cloned into pRT100 vector to mobilize the promoter and terminator. The cassette containing *LEW1* gene was mobilized into plant expression vector pCAMBIA1300 with hygromycin phosphotransferase (*hpt*) as the selectable marker. The construct pCAMBIA1300-*PgLEW1* was then mobilized into *Agrobacterium tumifaciens* strain LBA4404. Transgenic tobacco plants expressing *PgLEW1* were generated and confirmed by PCR analysis using *hpt* and gene specific primers.

Keywords: *Agrobacterium tumefaciens*, *PgLEW1*, transformation, tobacco

Introduction
To survive, sessile plants must cope with environmental stress factors such as drought, high temperatures and salinity stress and also a combination of them [1]. Abiotic stress reduces agricultural yield, so that novel crop genotypes adapted to environmental stress need to be developed [2, 3, 4]. Restriction of plant growth by retarding cell extension is often the earliest visible effect of stress. Plants have developed a range of morphological, physiological and biochemical mechanisms that enable them to avoid and/or tolerate stress factors and survive [5]. Rapid expansion of water-stressed areas necessitates improvement of crops with traits such as drought tolerance and adaptation, through conventional breeding and/or genetic manipulation [6]. Plants induce expression of a number of genes in response to water limitation. The early response at the cellular level results partly from cell damage, and corresponds partly to adaptive processes that initiate changes in the metabolism and structure of the cell that allows it to function under low water potential [7] Advances in cellular and molecular biology have made it possible to clone important genes and mobilize them in any organism across barriers of sexual hybridization for stable expression and transmission. The enhanced level of compatible osmolytes, radical scavengers and other transgene products correlated with the degree of tolerance. Plants producing crucial enzymes or proteins from various organisms involved in abiotic stress tolerance mechanisms have shown significant advantage over their wild type controls under stressed environment. Further understanding of the molecular mechanisms of stress perception, signal transduction and response by plants and other organisms may help to engineer plants with high levels of tolerance to multiple stresses. Perspectives and additional approaches for further improving the tolerance to abiotic stresses through genetic engineering [8]. *LEW1* a gene encoding a long-chain cis-Prenyltransferase in dolichol biosynthesis in Arabidopsis. *LEW1* (Leaf wilting) gene plays a predominant role in catalyzing the biosynthesis of dolichol C_{75} to C_{80} in Arabidopsis [9]. Which play critical role in protein glycosylation process and UPR stress response (unfolded protein response path way). Osmotic stress may cause ER stress and activate UPR (unfolded protein response) pathway [10] and part of the drought responses (e.g., induction of *RD29A* and *COR47*) may be mediated by the UPR pathway [11, 12, 13]. The enhanced inducibility of UPR genes and drought-responsive genes in *plants* may contribute to the drought resistance and salt/osmotic tolerance.
Members of the genus Agrobacterium are soil-borne, Gram-negative bacteria, which belongs to the family of a Rhizobium. Agrobacterium is an oncogenic agent, which genetically transforms plant cells and directs the resulting tumours to synthesize special nutrients that support the growth of the bacteria [14]. Agrobacterium Ti (Tumor inducing) plasmid is being used as a vector for plant genetic engineering. The present study was aimed at developing transgenic tobacco plants expressing *Pg LEW1* gene through Agrobacterium-mediated transformation. The T_0 transgenics were confirmed by PCR and RT-PCR analysis using *hpt* and *PgLEW1*gene-specific primers.

Materials and Methods

Construction of plant expression vector

The plasmid pTZ57R-*PgLEW1* was digested with *Kpn*I and *Bam*HI, and cloned into the corresponding sites of pRT100 for mobilization of promoter and terminator. The gene cassette was then sub-cloned into plant expression vector pCAMBIA1300. The construct pCAMBIA1300 35S: *PgLEW1* and pCAMBIA 1300 only vector were mobilized into *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw method [15]. The transformed colonies were subjected to colony PCR, plasmid isolation and restriction digestion. The positive clones were used for *Agrobacterium* mediated transformation of tobacco.

Agrobacterium mediated genetic transformation of tobacco with *PgLEW1* gene

The transgenic tobacco plants were generated using pCAMBIA1300 35S:PgLEW1 and pCAMBIA1300 vector alone. Seeds of *Nicotiana benthamiana*, were surface sterilized by 70% ethanol for 1 min.. Subsequently they were rinsed four times with sterile distilled water. The seeds were germinated on half strength MS medium (1.5% sucrose(W/V) and pH 5.8) under12h light cycle for 1 month at 24 °C as a source for obtaining leaf disc explants. The leaf bits were precultured on regeneration media ((MS + 1.0 mg BAP/l + 0.1 mg NAA/l + 3 % sucrose (w/v) and pH 5.8) for two days. The precultured explants were infected with *A. tumefaciens* strain LBA4404 carrying the pCAMBIA1300*PgLEW1* construct and pCAMBIA1300only vector, which were grown overnight in YEP broth. The infected leaf bits were co-cultivated for two days at 24 ºC on plant regeneration medium with 100 µM acetosyringone to allow efficient T-DNA transfer. After the co cultivation, the explants were washed with water containing cefotaxime (250mg/l) and transferred onto plant regeneration media containing hygromycin @ 25mg/l for selection of transformants and cefotaxime @250mg/l for elimination of *Agrobacterium* cells. Subsequent sub-culturing was done at every two weeks intervals and individual shoots which were actively growing on selection plates were transferred to ½ strength MS medium which contain 1.0 mg IBA/l and 25 mg hygromycin/l. Elongated shoots were then transferred to rooting media containing 0.1mg/l NAA and maintained for 15d. Hygromycin selection pressure was maintained at all the stages. The rooted plantlets were transferred to cocopeat for hardening and then finally to pots maintained in the transgenic glass house. The plants were allowed to grow to maturity and T_0 seed were collected.

Molecular characterization of putative *PgLEW1* tobacco transgenics

The putative *PgLEW1* tobacco transgenics growing in the pots were subjected to molecular analysis using PCR and semi quantitative RT-PCR. Genomic DNA and total RNA were isolated from putative transgenics, vector control and untransformed control plants. For PCR analysis genomic DNA was isolated using cTAB method [16], for semi quantitative RT-PCR analysis, RNA isolation was carried out by Trizol method (Sigma, USA) in according to the manufacturer's instructions and Five µg of total RNA was used to synthesize first strand cDNA using Superscript II reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions.

PCR and RT-PCR analysis was carried out by using *hpt* and *PgLEW1*gene specific primers are as follows: *hpt* Forward primer: 5´GATGTAGGAGGGCGTGGATA3´, Reverse Primer: 5´ATTTGTGTACGCCCGACAGT3´and optimized PCR conditions: 94ºC for 5min for initial denaturation, followed by 30 cycles of 94ºC for 30s, 60ºC for 30s, 72ºC for 1min and final extension at 72ºC for 5min. For *PgLEW1* Forward primer 5´ATGGATTCCAAATCCATG3′ Reverse primer 5′ TCATTTCCCATAATTTTGG 3′. and optimized conditions were initial denaturation for 5 min at 94ºC followed by 30 cycles of 94ºC for 30s, 52ºC for 30s, 72ºC for 1min and final extension at 72ºC for 5min. PCR amplification *was* carried out using 5-10ng of template, 0.2mM dNTP mix, 0.8 u/µl *Taq* DNA polymerase, 100 pmol/µl of forward and reverse gene specific primers in PCR thermal cycler (Applied Biosystems). Genomic DNA from the plants which are transformed with pCAMBIA 1300 only vector used as vector control. The genomic DNA from the untransformed control plants and plasmid DNA of pCAMBIA1300 *PgLEW1* were used as negative and positive controls, respectively. The amplified PCR products were resolved on 0.8% agarose gel, visualized on UV transilluminator upon ethidium bromide staining.

Results and Discussion

pRT100 vector facilitates the cloning of the *PgLEW1* gene downstream the CaMV35S promoter. Double digested (*Kpn* I / *Bam*H I**)** pRT 100 vector and *PgLEW1* used for ligation reaction lead to transformed colonies on LB agar plate containing ampicillin(100mg/ml) Colony PCR analysis of randomly selected transformed colonies using *PgLEW1* gene specific primers resulted in the amplification of 822bp product confirming the presence of the *PgLEW1* gene. Further confirmation was done by plasmid isolation and restriction digestion of positive clones with *Pst* I resulted the 1533 bp fragment (Fig 1a) which confirmed the *PgLEW1* gene cassette with CaMV35S promoter and terminator. Dephosphorylated pCAMBIA1300 vector and *PgLEW1* gene cassette used for ligation reaction resulted in transformed colonies on LB agar plates containing kanamycin (50mg/ml). Colony PCR analysis of transformed colonies resulted in amplification of 822bp product confirming the presence of the *PgLEW1* gene in them. Restriction digestion of the plasmid DNA isolated from the positive clones with *Pst* I restriction enzyme had released the 1533bp gene cassette (Fig 1b) which confirmed ligaton of *PgLEW1* expression cassette into the binary vector. The recombinant vector maps of the *PgLEW1* gene cloned into the T-DNA region of pCAMBIA1300 vector is depicted in (Fig.2).

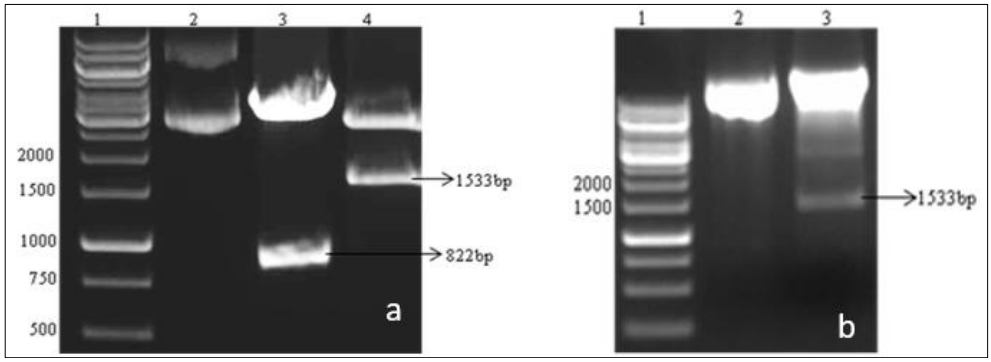

Fig 1: Restriction digestion analysis of (a) pRT100*PgLEW1* plasmid. Lane 1 -1kb ladder;2- undigested plasmid;3- plasmid DNA digested with *Kpn* I and *Bam* H I; 4- plasmid DNA digested with *Pst* I.(b) pCAMBIA1300*PgLEW1* plasmid. Lane 1-1 Kb ladder; 2- un digested plasmid DNA; 3-plasmid DNA digested with *Pst* I.

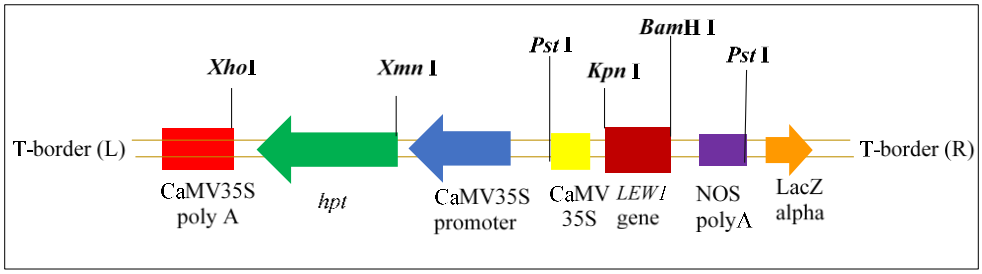

Fig 2: Recombinant vector map of pCAMBIA1300 *PgLEW1* T-DNA

A. tumefaciens strain LBA4404 transformed with constructs pCAMBIA 1300 *PgLEW1* and pCAMBIA1300 only vector, resulted in transformed colonies on YEP agar plates containing kanamycin (50µg/ml) (Fig.3).Colony PCR analysis of randomly selected transformed colonies using *hpt* forward and reverse primers for pCAMBIA1300 only vector colonies resulted in the amplification of 800bp product (Fig 4a), using *PgLEW1*forward and reverse primers resulted in the amplification of 822bp product(Fig.4b) confirming the transformation of *Agrobacterium* with the constructs. Further confirmation was done by plasmid isolation and restriction digestion of pCAMBIA1300*PgLEW1*plasmid DNA with *Pst* I had released the 1533bp gene cassette, which confirmed the transformation of LBA4404 strain with pCAMBIA1300 *PgLEW1* plasmid DNA (Fig 4c). These Transformed cultures were used for *Agrobacterium* mediated genetic transformation of tobacco.

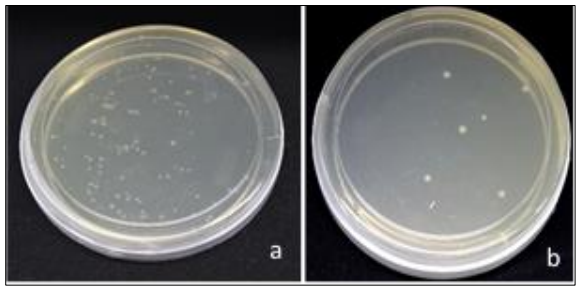

Fig 3: Transformed *Agrobacterium* strain LBA4404 colonies on YEP selection media. (a) pCAMBIA1300 vector alone; (b) pCAMBIA1300*PgLEW1*.

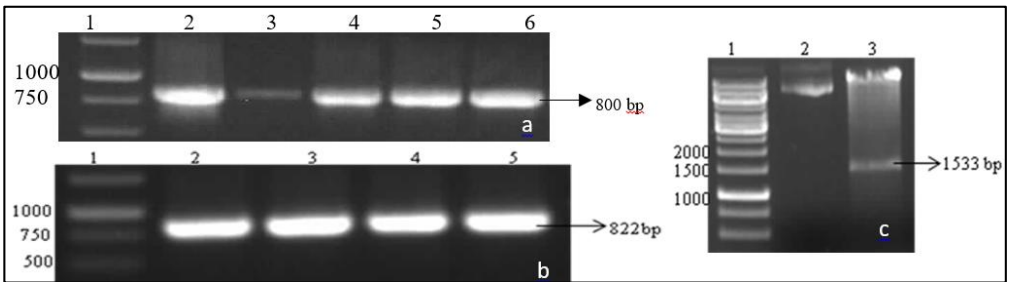

Fig 4: Colony PCR and restriction digestion analysis of *Agrobacterium* strain LBA4404 transformed clones. (a) pCAMBIA1300 vector alone with *hpt* primers Lane1:1kb DNA marker, 2-6 *Agrobacterium* colonies.(b) pCAMBIA1300*PgLEW1* with *PgLEW1* gene specific primers, lane1:1kb DNA marer,2-5 *Agrobacterium* recombinant colonies. (c)Restriction digestion analysis of LBA4404 transformed plasmid with pCAMBIA1300 *PgLEW1*.Lane 1:1 Kb DNA marker, 2 un digested plasmid DNA, 3 digested plasmid DNA with *Pst* I.

Agrobacterium* mediated genetic transformation of tobacco with gene *PgLEW1

The *PgLEW1* gene construct in pCAMBIA1300 vector was used to transform tobacco by tissue culture based *Agrobacterium* mediated leaf disc transformation method. *In vitro* grown tobacco leaf disks (Fig 5a) were transformed with pCAMBIA1300*PgLEW1* construct via *Agrobacterium tumefaciens*. Cocultivation of tobacco leaf disc explants with the *Agrobacterium* culture showed the formation of thin agrofilm around the explants after 5 days of incubation (Fig 5b). Subsequently the leaf discs were selected on hygromycin (10mg/l) containing medium (Fig 5c). Calli showing shoot initiation (Fig 5d, 5e) they were transferred to regeneration medium. The non-transformed explants turned brown and

were discarded. Regenerated plantlets were then transferred to rooting medium and maintained for 2 weeks. Rooted plantlets were transferred to coco peat in small pots. Over 90% of rooted plantlets were fertile after transfer to net house (Fig.6).

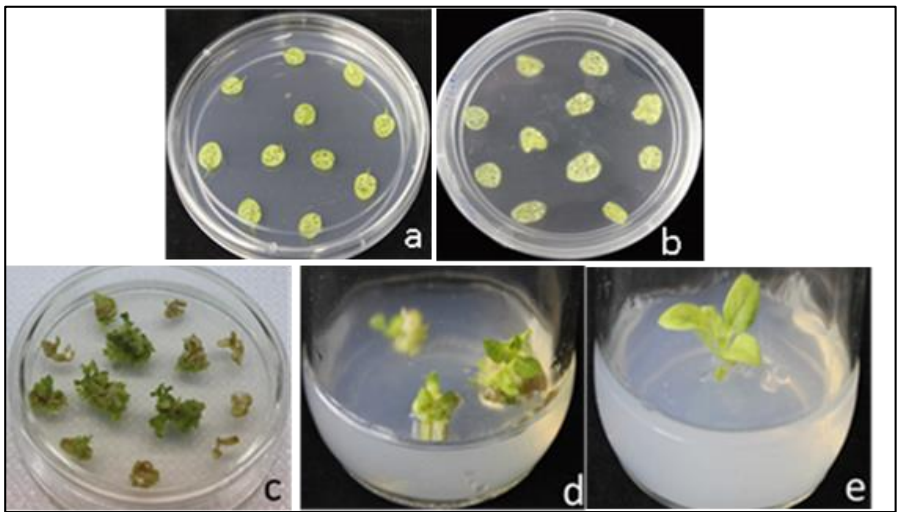

Fig 5: Regeneration of *PgLEW1* tobacco transgenic plants from leaf disc explants. (a) Pre-condition (b) agro infected leaf discs growing on cocultivation media (c) selection media d) elongation.

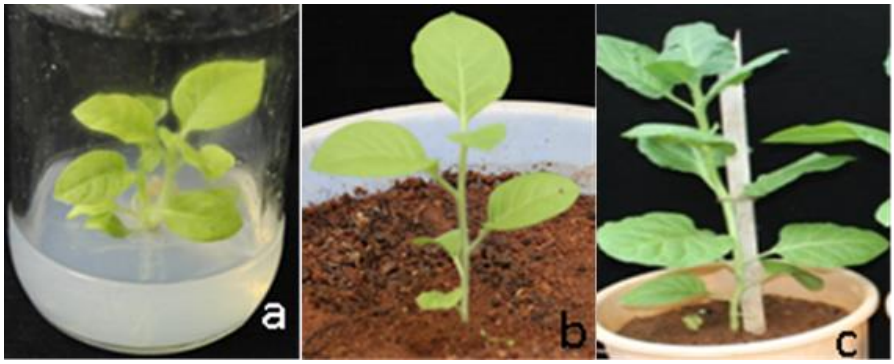

Fig 6: Acclimatization of the putative tobacco *PgLEW1* transgenics in transgenic glass house (a) rooted plantlet (b) growing in soil pots (c) maturity stage.

Molecular characterization of *PgLEW1* transgenics

The transgenic nature of putative *PgLEW1* transgenics developed by tissue culture based *Agrobacterium* mediated transformation method was verified by PCR analysis. PCR analysis was carried out with the leaf genomic DNA isolated from the putative *PgLEW1*transgenics, untransformed control and vector control using *hpt* gene(hygromycin phosphor transferase) primers and *PgLEW1* gene specific primers resulted in 800bp and 822bp products, respectively confirmed the presence of *hpt* and *PgLEW1* genes in transgenics (Fig 6). while no such amplification was noticed in the untransformed control. Vector control plants were positive for the amplification of *hpt* gene only, no amplification has been observed with *PgLEW1* gene specific primers. Of the 20 T_0 transgenics, 18 were found to be PCR positive among them 4 plants were selected for further analysis. Semi-quantitative RT-PCR analysis of these 4 (L2,L7, L11 and L15) transgenic lines were found to be positive for the amplification of 800bp (Fig 7a) and 822 bp (Fig 7b) products of *hpt* and *PgLEW1* genes, respectively, which confirmed the carrying of transgene. No such amplification was observed in untransformed control plants. Vector control plants were positive for the amplification of *hpt* gene only, no amplification has been observed with *PgLEW1* gene specific primers. These lines can be used to develop plants with multiple tolerance.

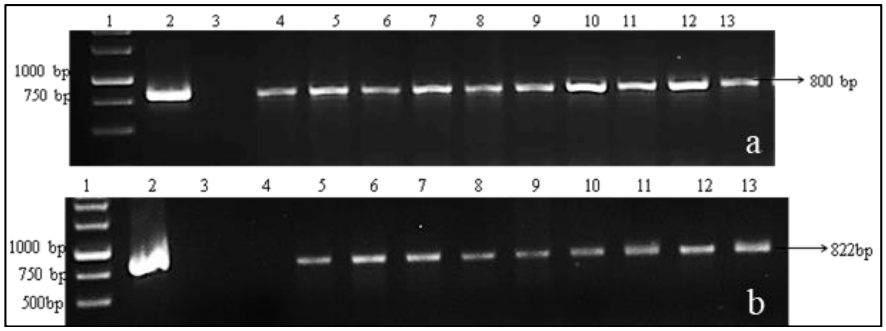

Fig 7: Molecular characterization of putative *PgLEW1* tobacco transgenics through PCR (a) using *hpt* gene specific primers(b)*PgLEW1* gene specific primers. Lanes1: 1kb DNA marker, 2: positive control plasmid pCAMBIA1300*PgLEW1*, 3: untransformed control, 4- Vector control,5-13: *PgLEW1* tobacco transgenics.

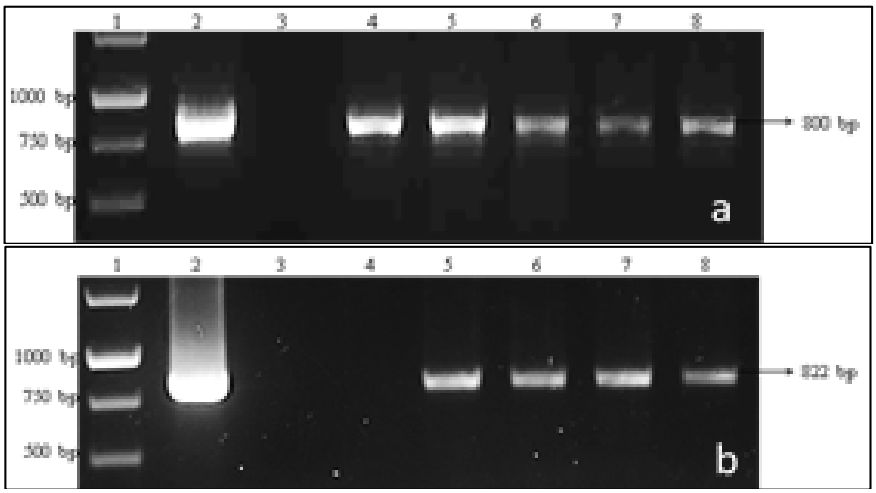

Fig 8: RT- PCR verification of *PgLEW1* tobacco transgenics (a) using *hpt* primers (b) using *PgLEW1* gene specific primers Lanes1: 1kb DNA marker, 2: positive control plasmid pCAMBIA1300*PgLEW1*, 3: untransformed control, 4- Vector control, 5-8: *PgLEW1* tobacco transgenics.

Conclusion

In this study, we successfully transformed the tobacco (*Nicotiana benthamiana*) plants with the *PgLEW1* genes using *Agrobacterium* and confirmed by PCR and RT -PCR. This gene can be used for developing transgenic crop plants with multiple stress tolerance.

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