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Enhanced drought and salinity tolerance in transgenic mustard [*Brassica juncea* (L.) Czern & Coss.] overexpressing *Arabidopsis* group 4 late embryogenesis abundant gene (*AtLEA4-1*)

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ABSTRACT

Indian mustard [*Brassica juncea* (L.) Czern & Coss.] is one of the most important oilseed crop worldwide, yet relatively mild drought and salinity stress significantly reduce its growth and yield. Expression of LEA (Late Embryogenesis Abundant) proteins was previously shown to alleviate osmotic stress by stabilizing water status, protecting cytosolic structures and cell through increased membrane and protein stability. We overexpressed *Arabidopsis* group 4 LEA protein *At*LEA4-1 in mustard, resulting the transgenic lines to show enhanced drought and salinity tolerance. Southern hybridization showed the copy number of the gene inserted into the transgenics while, qRT PCR determined the transcript level of the overexpressed gene. Leaf senescence assay showed better adaptability of the transgenics to both salt and PEG stress, as revealed by increased chlorophyll content in the transgenic plants when compared to wild type. The transgenic mustard plants showed better survivability under both drought and salinity stress in soil. Physiological analysis revealed lower levels of lipid peroxidation, hydrogen peroxide and oxygen radical production but higher levels of relative water content (RWC) in T_1 transgenic *AtLEA4-1* lines with not so significant increase in proline content. Antioxidative enzyme activities showed better adaptation of transgenic plants to drought stress. Even when the biochemical parameters were analysed as a function of RWC, the transgenic plant tissues showed improved adaptation to drought and salinity stress depicting, the functions of LEA proteins as antioxidant and molecular chaperone exerting equivalent impact as that of hydration buffer towards abiotic stress tolerance.

1. Introduction

Brassica juncea is considered one of the most important source of vegetable oil worldwide including India where it is cultivated in more than 6 million hectares of land with high susceptibility to abiotic stress impacting its overall production (Singh et al., 2009). Exponential increase in population and rapid industrialization have resulted in decline of arable land. To cope up with the ever-increasing demand of oilseed it has become very much essential to work on mustard crop improvement (Dutta et al., 2008). Development of Indian mustard genotypes with enhanced abiotic stress tolerance is expected to increase its growth and production under limiting environmental conditions. Although diverse strategies have been adapted to alleviate drought stress, genetic engineering seems to be the most prominent for re-tailoring drought and salinity tolerance because of its targeted and less arduous approach.

Late Embryogenesis Abundant (LEA) proteins, canonically known for its accumulation in latter stages of seed development, are also known to be involved in protecting plants from abiotic stress by acting as osmoprotectant, membrane stabilizers, antioxidant and molecular chaperones (Mowla et al., 2006; Kovacs et al., 2008). The LEA genes encoding these proteins are induced by abiotic stress through abscisic acid mediated signal transduction pathway (Shinozaki and Yamaguchi-Shinozaki, 2007). The LEA proteins have been subdivided into nine different groups based on distinct conserved motifs (Hundertmark and Hincha 2008; Bies-Etheve et al., 2008) out of which group 1, 2 and 3 are the most extensively studied groups. For Group 4 proteins few functional

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analysis has been reported till date. Dalal et al. (2009) has been successful in isolating an *Arabidopsis* homolog of *LEA4-5*, *BnLEA4-1* from *Brassica napus* and found that overexpression of this gene confers enhanced drought and salinity stress tolerance. Olvera-Carrillo et al. (2010) then reported the relevance of three *Arabidopsis* group 4 proteins *LEA4-1, LEA4-2, LEA4-5* for survival in water deficit conditions. Liu et al. (2011) revealed that two soyabean group 4 LEA protein act as ion scavengers, depicting their activity also as antioxidants. Gu et al. (2012) isolated group 4 LEA family gene from chickpea and reported its high induction rate in response to drought, salt, heat, cold, ABA, IAA, GA_3 and MeJA.

Here we report the overexpression of an *Arabidopsis thaliana* group 4 LEA gene, *AtLEA4-1* in an economically important oilseed crop, Indian mustard and its characterization for drought and salt stress. This is the first report showing enhanced drought tolerance in *B. juncea* through overexpression of a candidate gene by transgenic approach. The transgenic plants showed enhanced ability to survive under both drought and saline condition without much adverse effect on the normal growth physiology as depicted by pot stress assays.

2. Materials and methods

2.1. Plant material and explant preparation

Seeds of Indian mustard (*Brassica juncea* L. Czern and Coss) cultivar Pusa Jaikisan, obtained from Indian Agriculture Research Institute, New Delhi were used. Seeds were surface sterilized with 0.05% $HgCl_2$ (w/v) for 10 min and subsequently rinsed three times with sterile distilled water before being cultured on basal half strength MS medium (Murashige and Skoog, 1962). The hypocotyl explants (see Supplementary 1a) were excised from 5 day old seedlings.

2.2. Plant expression cassette construction and Agrobacterium strain

The gene *AtLEA4-1* plant expression cassette was subcloned into plant binary expression vector pCAMBIA2300 (CAMBIA, Australia) by digesting with PstI from pRT101 (Fig. 1). The gene *AtLEA4-1* was earlier cloned into pRT100 with XbaI and SalI from *Arabidopsis*. The T-DNA of pCAMBIA2300 contains neomycin phosphotransferase gene as selectable marker driven by cauliflower mosaic virus (CaMV) 35S promoter and polyA terminator (Fig. 1). *AtLEA4-1* had CaMV35S as promoter for constitutive overexpression and polyA as terminator. pCAMBIA2300:CaMV35S: *AtLEA4-1*: PolyA was mobilized into *Agrobacterium tumefaciens* EHA105 by triparental mating (Bevan, 1984). The *A. tumefaciens* harbouring the construct was maintained on solid LB medium supplemented with 10 mg/L rifampicin, and 50 mg/L kanamycin.

2.3. Plant transformation

The explants were pre-cultured in SIM [MS medium supplemented with 1 g/L casein hydrolysate, 5 g/L $AgNO_3$, 2 mg/L 6-Benzylaminopurine (BAP) and 0.2 mg/L 1-Napthaleneacetic acid (NAA)] for two days (see Supplementary 1b). Single colony of *A. tumefaciens* EHA105 harbouring the construct from freshly streaked plate was inoculated in 25 mL of liquid AB minimal medium (Chilton et al., 1974) with appropriate antibiotics and grown overnight at 28 °C. When OD_{600} reached, 0.6–0.8 the cells were harvested by centrifuging at 5000 rpm for 5 min and was resuspended in co-cultivation medium, CM (liquid half strength MS, pH-5.5, supplemented with 100 μM acetosyringone) for inoculation. The two days precultured explants were inoculated in the suspension for 5 min with occasional shaking, blot dried and co-cultivated on Petri dish in CM moistened sterile filter paper for 1 day in dark at 22 °C. Following co-cultivation, the explants were washed with sterile distilled water, blot dried and transferred to post culture medium, PCM (MS medium containing 1 g/L casein hydrolysate, 5 g/L $AgNO_3$, 2 mg/L BAP, 0.2 mg/L NAA and 250 mg/L Cefotaxime) for 5 days (see Supplementary 1b). The explants were then cultured on shoot induction and selection medium, SISM (MS medium containing 1 g/L casein hydrolysate, 5 g/L $AgNO_3$, 2 mg/L BAP, 0.2 mg/L NAA, 250 mg/L cefotaxime and 15 mg/L kanamycin) for two 10 days cycle (see Supplementary 1c, d). The regenerated and selected shoots were rooted in basal full strength MS supplemented with 0.2 mg/L NAA, until roots emerged (see Supplementary 1e). The rooted transformed plants were acclimatised and established in transgenic containment greenhouse for further analysis (see Supplementary 1f).

2.4. Confirmation of transgenic events through polymerase chain reaction and southern hybridization

2.4.1. PCR analysis

Genomic DNA was isolated from both non-transformed and T_0 transformed putative *AtLEA4-1* overexpression lines using the CTAB (cetyltrimethylammonium bromide) method (Rogers and Bendich, 1994). The presence of *nptII* and *AtLEA4-1* was detected by polymerase chain reaction (PCR) in T_0 transformed putative plants. Gene specific primers (forward primer 5′-CCACCATGATATTCGG-CAAC-3′ and reverse primer 5′-GTGGAGAGGCTATTCGGCTA-3′) were used to amplify a 0.54-kb fragment of *nptII*. The PCR condition was 95 °C for 10 min; 35 cycles of 95 °C for 1 min, 58 °C for 30 s and 72 °C for 30 s; and a final extension of 72 °C for 10 min. Similarly, 1.0-kb fragment of 35SP:*AtLEA4.1*: polyATer cassette was amplified using gene specific primers (forward primer 5′-AACATGGTGGAGCACGACACTCTC-3′ and reverse primer 5′-TTTTTTTATTTTAAAAAGATAGTAGGT-3′). The PCR condition was set as 95 °C for 10 min; 35 cycles of 95 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min; and a final extension of 72 °C for 10 min. The PCR fragments were analysed on 1% agarose gel.

2.4.2. Southern hybridization

Analysis for transgene integration and copy number was done by southern hybridization in T_1 generation. 40 μg of genomic DNA from four transgenic T_1 lines ($T_1$1, $T_1$4, $T_1$5, $T_1$6) and non-transformed control (WT) were digested with restriction

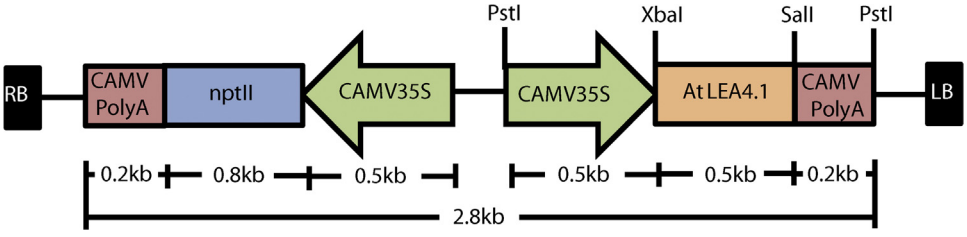

Fig. 1. T-DNA region (2.8 kb) of binary vector pCAMBIA2300. RB, right border; LB, left border; CaMV 35S promoter; CaMV poly A terminator; *AtLEA4-1* gene; *npt*II, neomycin phosphotransferase. Also highlighted are the positions of PstI, XbaI, SalI restriction sites, and the approximate sizes of promoters terminators and genes.