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Agrobacterium-Mediated Transformation of *indica* Rice (*Oryza sativa* L.), IR64 with Mungbean LEA Protein Gene for Water-Stress Tolerance*

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Abstract: Late embryogenesis abundant proteins are associated with tolerance to water-stress resulting from desiccation and cold shock. A LEA protein gene, *emv2* from *Vigna radiata* was sense expressed into *indica* rice cultivar IR 64 to produce plants tolerant to limited water stress situations. Mature seed-derived embryogenic calli were transformed with *Agrobacterium* strain LBA 4404 harboring the binary vector pCEMV2 with mungbean *emv2* gene. Active proliferating resistant calli selected in hygromycin and cefotaxime were regenerated and transferred to half-MS rooting medium. The putative transgenic rice plants expressing *emv2* were analyzed for integration of the transgene through polymerase chain reaction and tested positive for *hph* and *emv2* genes.

Key words: *Agrobacterium*, *indica* rice, LEA, mature seed-derived calli, rice transformation, transgenic rice

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important food crop in the developing world with nearly one third of the population depending on it as the major source of nutrient calories (Tang *et al*., 2001). Rice crop suffers due to various biotic and abiotic stresses. Adaptation levels of plants to stress situations vary with the genotypes and the associated types of stress. Plants respond to stresses by displaying complex, quantitative traits that involve the expression of many stress-related genes. Expression of these genes alters the plant metabolism and the associated biochemical changes leading to accumulation of various low molecular weight organic compounds, referred to as compatible solutes or osmolytes, synthesis of late embryogenesis abundant (LEA) proteins. Our earlier work on the occurrence of LEA proteins in the embryonic axes of *Vigna radiata* (L.) Wilczek, referred as EMV proteins, the first ever report in the Fabaceae family (Manickam and Carlier, 1980). cDNA encoding this proteins were isolated, characterized (Manickam *et al*., 1996). *In silico* analysis of the 20-mer motif of this EMV2 categorize this protein to Group1 LEA and hypothesize to function as DNA/RNA binding proteins in stabilizing membranes/macromolecules at the time of dehydration process (Rajesh and Manickam, 2006; Gilles *et al*., 2007). Rice genetic transformation has taken rapid strides since the first transgenic rice plant produced 15 years ago (Bajaj and Mohanty, 2005). Research on *Agrobacterium tumefaciens* mediated genetic transformation has been successful and considerable progress has been made in making different rice genotypes amenable for regeneration and transformation (Azhakanandam *et al*., 2000; Jiang *et al*., 2000; Lin and Zhang, 2004; Rachmawati *et al*., 2004).

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The present study is perhaps the first report on transfer of a LEA protein coding gene isolated from leguminous plant, *Vigna radiata* (Manickam *et al*., 1996) to a recalcitrant monocot *indica* rice cultivar IR 64 through *Agrobacterium*-mediated transformation with a view of developing transgenic rice plants that can survive water-limited situations.

MATERIALS AND METHODS

Plant Materials Used

Mature seeds of rice cultivar IR64 (obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore) were manually dehusked, sterilized with 70% ethanol for 3 min and then with 0.1% mercuric chloride for 5 min, followed by 3 washes in sterile distilled water. Sterilized seeds were cultured on callus induction medium (MS salts (Murashige and Skoog, 1962) containing B5 vitamins, 30 g L^{-1} sucrose, 3.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid [2,4-D], 0.3 mg L^{-1} kinetin, 4.0 g L^{-1} phytagel and 2.0 g L^{-1} agar at 25°C in the dark). After 3-4 week, the proliferating calli were subcultured onto the same medium and cultured for another 3-4 week. Creamy yellow embryogenic calli of 5-6 mm diameter were subcultured onto the same medium 5 d before infection with *Agrobacterium*.

Plant Transformation Vector

The plant transformation vector, pCEMV2 (Fig. 1) is based on the backbone of pCAMBIA1301 with *emv2* as gene of interest at *Bam*H1 and *Hin*dIII restriction sites placed under the transcription control of a constitutive promoter, ubiquitin and *nos* poly A terminator (a gift from Dr. S. Muthukrishnan, Kansas State University, USA). Hygromycin and kanamycin serve as markers for plant and bacterial selection, respectively.

The plasmid construct pCEMV2 isolated from the *Agrobacterium* transconjugant strain, LBA4404 was back transformed into freshly prepared competent cells of *E. coli*, DH 5α by heat shock and the presence of constructs was confirmed by restriction digestion (Fig. 2).

Agrobacterium-Mediated Transformation

A super-virulent *Agrobacterium* strain LBA 4404 was transformed with pCEMV2 by triparental mating (Ditta *et al*., 1980). *Agrobacterium* LBA 4404 (pCEMV2) was grown to an Optical Density

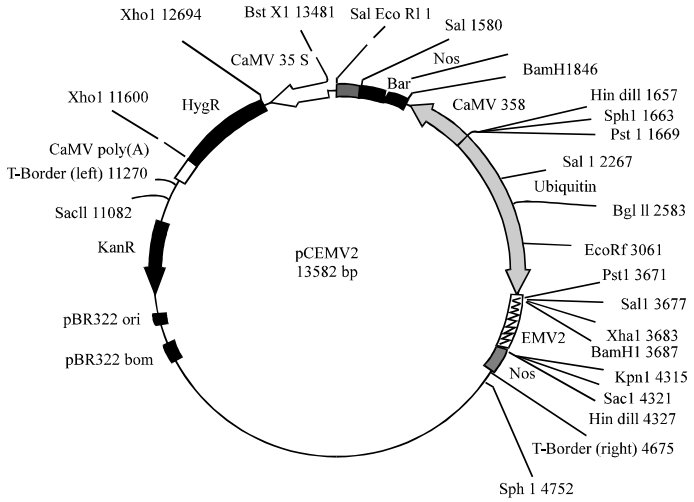

Fig. 1: Physical map of pCEMV2 harboring *emv2*, mung bean LEA protein gene

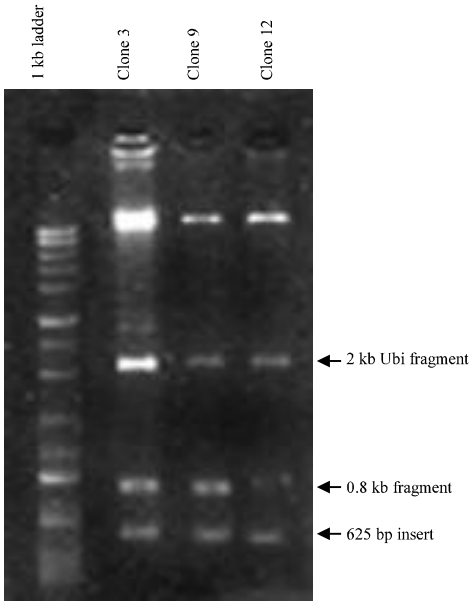

Fig. 2: Restriction digestion of pCEMV2 construct with *Bam* H1 and *Hin*d III

(OD) of 1.0 in AB medium (Chilton *et al*., 1974) containing 100 mg L^{-1} kanamycin and 10 mg L^{-1} rifampicin. The culture was centrifuged at 3220 g for 10 min and the pellet was resuspended in equal volume of AA-AS medium (AA medium containing 20 g L^{-1} sucrose, 10 g L^{-1} glucose, 1.0 g L^{-1} caesamino acid, 100 μM acetosyringone, pH 5.6; Toriyama and Hinata, 1985). The calli were immersed in bacterial suspension for 1 min and the excess bacterial suspension was removed by blotting on sterile tissue paper. Infected calli were transferred onto an MS co-cultivation medium (MS salts, B5 vitamins, 30 g L^{-1} sucrose, 10 g L^{-1} glucose, 100 μM acetosyringone, 3.0 g L^{-1} phytagel [pH 5.6]) overlaid with Whatman No. 1 filter paper (Rashid *et al*., 1996). Before transfer of calli, the Whatman No. 1 filter paper was wetted with 1 mL of AA-AS medium. Infected calli were incubated at 25°C for 3 day in the dark.

After 4 day, infected calli were washed 4-5 times with sterile distilled water and finally once with aqueous solution containing cefotaxime (250 mg L^{-1}), blotted on sterile tissue paper and transferred to MS selection medium (MS callus induction medium containing 50 mg L^{-1} hygromycin, 250 mg L^{-1} cefotaxime. After 12 day in the selection medium, healthy portions of the calli (2-3 mm) were subcultured onto a fresh selection medium, twice, at 3 to 4 week intervals. After three rounds of selection, actively growing pieces of calli were transferred to MS regeneration medium (MS medium containing 300 mg L^{-1} caesamino acid, 3.0 mg L^{-1} 6-benzylaminopurine [BAP], 1.5 mg L^{-1} α-naphthaleneacetic acid [NAA], 30 mg L^{-1} hygromycin, 250 mg L^{-1} cefotaxime and 4 g L^{-1} phytagel) and kept in the dark for 2 week. After that, the calli were transferred to fresh regeneration medium and incubated under a 16 h light (110-130 μE m^{-2} sec^{-1}) and 8 h dark photoperiod. Shoot regeneration was observed after 2-4 week. The regenerated shoots were transferred to the rooting medium (1/2 MS salt containing 15 g L^{-1} sucrose, 40 mg L^{-1} hygromycin, 250 mg L^{-1} cefotaxime and 4.0 g L^{-1} phytagel). After 2 week, rooted plants were kept in sterile water in a test tube covered with aluminium foil for 2-3 day and without aluminum foil another 2 day for hardening. The hardened plants were transferred to plastic pots and placed in the transgenic greenhouse.

Molecular Analyses of Putative Transgenics

Small leaf bits (2-3 cm) of putative transgenic rice plants were ground in a 1.5 mL microfuge tube containing 300 µL of extraction buffer (200 mM Tris-HCl pH 7.5, 200 mM NaCl, 25 mM EDTA and 0.5% SDS) and acid-washed sand using a pestle. The homogenate was centrifuged at 12,000 rpm for 10 min Equal volume of isopropanol was added to the supernatant and incubated at -20 °C for 20-30 min. The crude DNA was pelleted by centrifugation at 12000 rpm for 10 min, pellets were air dried at room temp and dissolved in 30 µL of 0.1X TE buffer (1 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA pH 8.0). For each PCR reaction, 1 µL (50-100 ng) of this DNA preparation was used as template.

PCR was performed with the total DNA isolated from leaf tissues of putative transformants (T_0) and a non transgenic control plant to amplify *hph* and *emv2 gene* as described by Sambrook *et al.* (1989). Reactions were performed in a final volume of 20 µL and the mixture contained 50-100 ng of genomic DNA, 2.0 µL of 10X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM of each of dNTPs, 70 ng of upstream and downstream primers specific to *hph* (HygF 5' CGTCTGTCGAGAAGTTTC 3' and HygR 5' TACTTCTACACAGCCATC 3') were used to amplify a 1215 bp-long internal fragment of the *hph* gene and *emv2* (emv2F-5' AGGGATCCAAACATGGCAAGTCGTC 3' and emv2R-5'GGGAAGCTTACAATATAACATCTAG 3') and 2 units of *Taq* DNA polymerase. PCR amplification was performed with a program of initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min and holded at 4°C for 30 min and the amplified products were resolved in 1.5% agarose gel. Hygromycin resistant gene was amplified at the annealing temperature of 55°C for 1 min.

RESULTS AND DISCUSSION

***In vitro* Culture of Rice**

Genetic transformation depends mainly on the availability of routine and efficient tissue culture protocols amenable for incorporating the genes of desired traits in crop plants. Further, problems pertaining to regeneration of plants from target cells used for gene transfer remain limiting factor in the development of transgenic crops. This can be overcome by optimization of callus induction and regeneration protocols, which shall be inherently more amenable for genetic manipulation. Generally *indica* rice lines are recalcitrant compared to *japonica* lines (Abe and Futsuhara, 1986) and show low regeneration potential. Plant regeneration mainly depends on the hormonal level, genotypic variability, culture media constituents and the choice of explants.

In the present study, mature seed-derived calli of IR64 was used as explants for genetic transformation. Calli were invariably initiated in the scutellum region and was visible within 7-10 days of seed inoculation. Globular, healthy, friable, cream-colored calli were obtained in 15-20 days old cultures (Fig. 3a). Reports on choice of explants include immature embryos (Christou *et al*., 1992), mature seed-derived calli (Hiei *et al*., 1994; Kumar *et al*., 2005), meristem discs (Chair *et al*., 1996), embryonic suspension cultures (Jain *et al*., 1996) and shoot meristems (Park *et al*., 1996). However, scutellum-derived embryogenic calli remain the most acceptable explants, since high transformation and regeneration frequencies have been achieved with this material as initial explants (Hiei *et al*., 1994).

The levels of 2,4-D used in this experiment was optimized at 3.0 mg L^{-1} for IR 64 rice cultivar, for increased callusing from mature seeds. Because, the concentration of 2,4-D during callus induction was found to be inhibiting the regeneration potential of the calli and therefore needs a balance between the 2,4-D concentration used for callus induction and regeneration of calli (Morita *et al*., 1999). However, repeated subculturing of calli may lead to risk of somaclonal variations at a later stage of

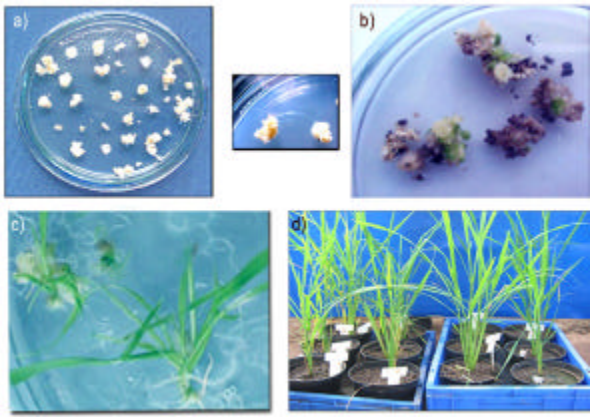

Fig. 3: *Agrobacterium*-mediated transformation of *indica* rice cultivar IR64. (a) 1-month-old mature seed-derived calli, (b) proliferating calli at the end of third round of selection on hygromycin (50 mg L^{-1}), (c) shoot regeneration on media containing hygromycin (30 mg L^{-1}) and (d) putative transgenics maintained in transgenic green house

regeneration. Co-cultivation of rice calli with *Agrobacterium* was optimized to obtain high transformation efficiency; however, it may vary with the genotypes and explants used. The embryogenic calli after infection for 10-15 min was blot-dried and co-cultivated in dark for 4 days on co-cultivation medium; callus induction medium supplemented with 100 µM acetosyringone, 10 g L^{-1} glucose and adjusted to pH, 5.8. Addition of acetosyringone in co-cultivation medium has been reported to induce *vir* genes, extend host range of *Agrobacterium* strains and found essential for rice transformation as a signaling compound (Hiei *et al*., 1994; Godwin *et al*., 1991).

***Agrobacterium*-Mediated Transformation of Rice**

The ability of *Agrobacterium* to transform monocots has been the subject of debate for sometime, since these plants are not natural hosts of this bacterium. However the controversy has been resolved with the clear demonstration of stable integration of foreign DNA in rice chromosomes in a process mediated by *A. tumefaciens* by Chan *et al*. (1993). Furthermore the evidence of Hiei *et al*. (1994) made clear the success strategy of exploiting *Agrobacterium* for genetic transformation of rice.

Embryogenic calli derived from the mature rice seeds of IR 64 were used as target tissue for incorporating *emv2*, a LEA protein gene through *Agrobacterium* mediated transformation. Mature seed-derived calli has been reported as excellent starting material for transformation of rice by *Agrobacterium* (Hiei *et al*., 1994). A binary vector system, pCAMBIA 1301was modified to construct pCEMV2, which harbors the *emv2* gene expression cassette driven under the control of ubiquitin promoter and *nos* terminator. The construct was mobilized into *Agrobacterium* strain LBA404 by triparental mating. Choice of vector and strains are important for transformation of recalcitrant genotypes. pCAMBIA 1301 series were successfully employed for transformation of monocot species and LBA4404 strain of *Agrobacterium* was proven to be efficient in genetic engineering of rice (Hoekema *et al*., 1983; Hiei *et al*., 1994) compared to the super virulent EHA101 strain (Hood *et al*., 1986).

Embryogenic Calli were Co-Cultivated with *Agrobacterium tumefaciens* LBA4404 (pCEMV 2) and the duration of co-cultivation was optimized to 4 days and was used for subsequent experiments.

The presence of acetosyringone was found to be an essential factor for transformation. However, layering of liquid AAM medium over co-cultivation medium was not essential as reported by Rashid *et al*. (1996). Subculturing of calli 4 days prior to co-cultivation as reported by Hiei *et al*. (1994) was found to be very useful for the selection of fast growing calli for transformation.

Efficient plant selection during transformation requires a substantial level of expression of the selectable marker gene (Wang *et al*., 1997). In the present study, calli co-cultivated with LBA4404 (pCEMV2) were allowed to grow and subjected to three rounds of selection in the medium containing 50 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime. Hygromycin allowed clear distinction between transformed and non-transformed calli. Only few calli proliferated in the selection medium while others turned dark and eventually dried off (Fig. 3b). This was attributed to the expression of *hph* gene in the transformed calli. The *hph* gene has been used as an efficient marker for selection of transformed tissues (Hiei *et al*., 1994; Rashid *et al*., 1996; Dong *et al*., 1996). Thus antibiotic selection provides a continuous advantage to the transformed cells, which may otherwise be overgrown by the far greater number of proliferating non-transformed calli. In addition, *hph* gene/hygromycin selection system has been reported to be more efficient for *indica* rice transformation than the bar/bialophos system (Li *et al*., 1997).

Hygromycin-resistant calli that survived three rounds of selection was transferred to shoot regeneration medium (Fig. 3c), MS medium s upplemented with kinetin 3.0 mg L^{-1} and NAA 1.5 mg L^{-1}. Out of 430 calli co-cultivated in three different experiments, 108 hygromycin-resistant calli were obtained. The low regeneration frequency was attributed to stringent selection in the hygromycin for three rounds compared to two rounds of selection of co-cultivated calli by Mohanty *et al*. (1999).

Molecular Analysis of Rice Transgenics Expressing LEA Gene

Transgenic plants allow the expression of drought-related genes *in vivo* and are, therefore an excellent system to assess the function and tolerance conferred by the encoded proteins. LEA proteins are among the best-known of water stress-induced proteins. For transformation systems that generate substantial number of non-chimeric primary transformants, genes conferring resistance to a selective chemical agent (Wilmink and Dons, 1993), genes conferring a phenotype allowing visual or physical screening (Bower *et al*., 1996) or even PCR screening to identify the plants containing transferred genes (Christou *et al*., 1992) can all be used for the recovery of transformants.

In the present study, molecular analysis of the T_0 plants (Fig. 3d) provided evidence of the incorporation of T-DNA into rice chromosomes. The expression cassettes of *emv2* and *hph* gene were presumed to be incorporated in the rice chromosome of putative transformants. Hence, PCR amplification was performed with the genomic DNA isolated from the putative transgenic and non transformed control plants of IR 64 using gene specific primers to amplify *emv2* and *hph* genes. PCR analysis for *emv2* gene showed a product size of 625 bp in the positive plants equal to the size of the positive control and there was no amplification in the non transformed control (Fig. 4).

The analysis revealed three out of five plants in IR 64 to be positives for *emv2* gene. PCR analysis of the selectable marker gene, *hph* using gene specific primers gave a product of size 1215 bp in three out of five plants in IR 64 putative transformants (Fig. 5).

The absence of gene of interest (*emv2*) plants tested positive for *hph* gene shows chimerism in the transformants for the transfer of genes that lie between the border regions of T-DNA. This may be due to excision of *emv2* expression cassette from the construct before transfer to cells of those two lines or mere integration of the *hph* expression cassette as the case may be. Chimerism of this type is reported in the transgenic plants of tobacco produced through *Agrobacterium* gene transfer method (Schmulling and Schell, 1993). However, this has not been a limiting factor in the application of *Agrobacterium* technique for rice transformation.

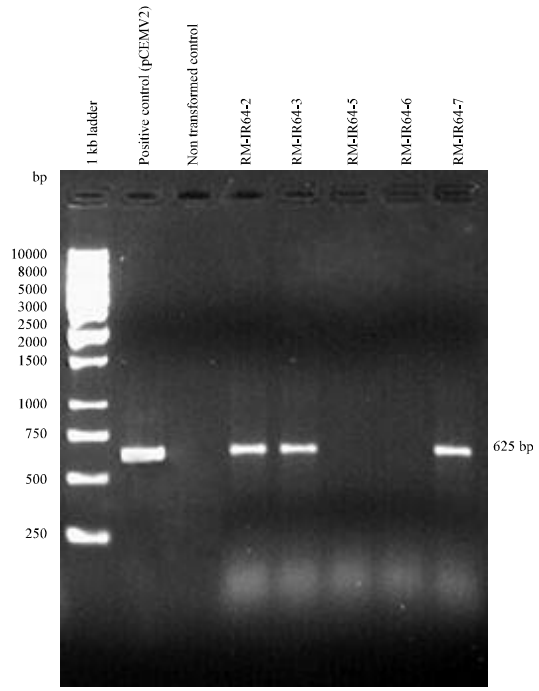

Fig. 4: PCR amplification of *emv2* gene in the putative transgenic (T_0) rice plants

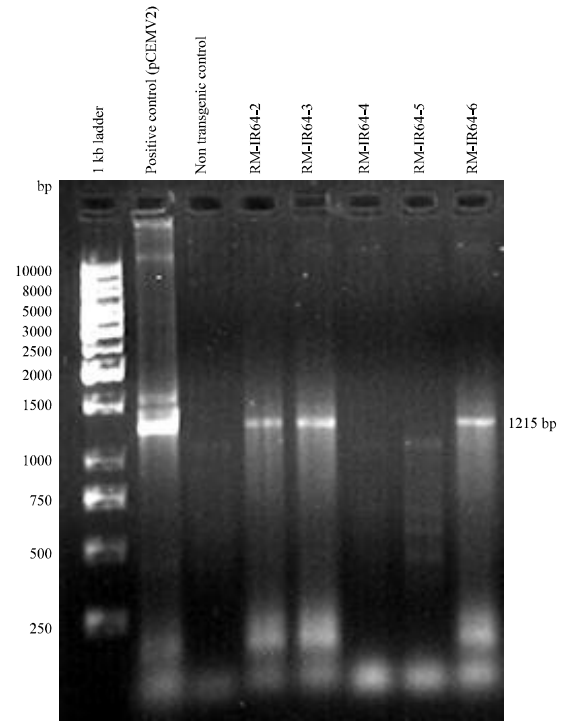

Fig. 5: PCR amplification of *hph* gene in the putative transgenic (T_0) rice plants

The first report of transgenic rice plants expressing HVA1, a group 3 late embryogenic abundant protein from *Hordeum vulgare* was shown to tolerate water deficit and salt stress situations (Xu *et al*., 1996). The plants showed enhanced accumulation of the HVA1 protein that delayed stress-related damage symptoms and improved recovery from the removal of stress conditions. Similarly, constitutive expression of the HVAI, in transgenic wheat plants resulted in improved growth characteristics under water deficit conditions (Sivamani *et al*., 2000). The precise mode of action of HVA1 under drought conditions is unclear.

Xu *et al*. (1996) produced transgenic rice plants expressing the barley HVA1 gene, driven by a constitutive promoter from the rice actin1 gene. In the present study, mungbean *emv2* gene was placed under the control of ubiquitin1, a constitutive promoter from maize. However high level of expression of stress-related genes can be achieved by use of stress inducible promoters like rd29a (or) ABRC promoter as supported by Rohila *et al*.(2002) for enhanced stress tolerance.

A significant finding regarding the function *Cor* gene, a LEA-related gene, has been reported. Constitutive expression of *Cor*15a in transgenic *Arabidopsis* enhances the freezing tolerance of both chloroplast and protoplasts indicating that the constitutive expressions of *Cor*15a affects the cryostability of the plasma membrane, possibly through the interaction of *Cor*15am polypeptide with lipid bilayers . Similarly screening of T_1 transgenic rice over-expressing OsLEA3-S and OsLEA3-H constructs at anthesis stage revealed significantly higher relative yield under drought conditions (Steponkus *et al*., 1998; Xiao *et al*., 2007).

The study reported here is perhaps the first on transfer of a LEA protein coding gene from a leguminous plant, *Vigna radiata* to a recalcitrant *indica* rice plant that can survive under limited water situations. Our earlier studies on the mungbean LEA protein structures using bioinformatics tools showed consistent spatial arrangements and clearly demonstrated its possibility to have a functional role in dehydration tolerance (Rajesh and Manickam, 2006). The transgenic approaches for water-stress tolerance described were on the basis of insights gained from the physiological and biochemical studies in different crop plants. Interestingly, any results gained from transgenic studies, will help us to better understand the physiological functions of different stress-related genes.

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REFERENCES

Abe, T. and Y. Futsuhara, 1986. Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). Theor. Applied Genet., 72: 3-10.

Azhakanandam, K., M.S. McCabe, J.B. Power, K.C. Lowe, E.C. Cocking and M.R. Davey, 2000. T-DNA transfer, integration, expression and inheritance in rice: Effects of plant genotype and *Agrobacterium* super-virulence. J. Plant Physiol., 157: 429-439.

Bajaj, S. and A. Mohanty, 2005. Recent advances in rice biotechnology-towards genetically superior transgenic rice. Plant Biotechnol. J., 3: 275-307.

Bower, R., A.R. Elliott, B.A.M. Potier and R.G. Birch, 1996. High efficiency, microprojectile-mediated co-transformation of sugarcane using visible or selectable markers. Mol. Breed., 2: 239-249.

Chair, H., T. Legavre and E. Guiderdoni, 1996. Transformation of haploid, microspore-derived cell suspension protoplasts of rice (*Oryza sativa* L.). Plant Cell Rep., 15: 766-770.

Chan, M.T., H.H. Chang, S.L. Ho, W.F. Tong and S.M. Yu, 1993. *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/β-glucuronidase gene. Plant Mol. Biol., 22: 491-506.

Chilton, M.D., T.C. Currier, S.K. Farrand, A.J. Bendich, M.P. Gordon and E.W. Nester, 1974. *Agrobacterium tumefaciens* DNA and P58 bacteriophage DNA not detected in crown gall tumours. Proc. Natl. Acad. Sci. USA, 71: 3672-3676.

Christou, P., T.L. Ford and M. Kofron, 1992. The development of a variety-independent gene transfer method for rice. Trends Biotechnol., 10: 239-246.

Ditta, G., S. Stanfield, D. Corbin and D. Helinski, 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA, 77: 7347-7351.

Dong, J., W. Teng, W.G. Buchholz and T.C. Hall, 1996. *Agrobacterium*-mediated transformation of Javanica rice. Mol. Breed., 2: 267-276.

Gilles, G.J., K.M. Hines, A.J. Manfre and W.R. Marcotte, 2007. A predicted N-terminal helical domain of a group 1 LEA protein is required for protection of enzyme activity from drying. Plant Physiol. Biochem., 45: 389-399.

Godwin, I., T. Gordon, B. Ford-Lloyd and H.J. Newbury, 1999. The effect of acetosyrigone and pH on *Agrobacterium mediated* transformation vary according to plant species. Plant Cell Rep., 9: 671-675.

Hiei, Y., S. Ohta, T. Komari and T. Kumashiro, 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J., 6: 271-282.

Hoekema, A., R.R. Hirsch, P.J.J. Hooykaas and R.A. Schilperoort, 1983. A binary plant vector strategy based on separation of vir- and T-region of *Agrobacterium tumefaciens* Ti plasmid. Nature, 303: 177-180.

Hood, E.E., G.L. Helmer, R.T. Fraley and M.D. Chilton, 1986. The hyper virulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J. Bacteriol., 168: 1291-1301.

Jain, R.K., S. Jain, B. Wang and R. Wu, 1996. Optimization of biolistic method for transient gene expression and production of agronomically useful Basmati rice plants. Plant Cell Rep., 15: 963-968.

Jiang, J.D., S.D. Linscombe, J.L. Wang and J.H. Oard, 2000. High efficiency transformation of US rice lines from mature seed-derived calli and segregation of glufosinate resistance under field conditions. Crop Sci., 40: 1729-1741.

Kumar, K.K., S. Maruthasalam, M. Loganathan, D. Sudhakar and P. Balasubramanian, 2005. An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite *indica* rice cultivars. Plant Mol. Biol. Rep., 23: 67-73.

Li, Z., N.M. Upadhyaya, S. Meena, A.J. Gibbs and P.M. Waterhouse, 1997. Comparison of promoters and selectable marker genes for the use in *indica* rice transformation. Mol. Breed., 3: 1-14.

Lin, Y.J. and Q. Zhang, 2004. Optimising the tissue culture conditions for high efficiency transformation of *indica* rice. Plant Cell Rep., 23: 540-547.

Manickam, A. and A.R. Carlier, 1980. Isolation and function of a low molecular weight protein of mung bean embryonic axes. Planta, 149: 234-240.

Manickam, A., E.J.M. Van Damme, K. Kalaiselvi, P.W. Verhaert and W.J. Peumans, 1996. Isolation and cDNA cloning of an Em-like protein from mung bean (*Vigna radiata*) axes. Physiol. Plant., 97: 524-530.

Mohanty, A., N.P. Sarma and A.K. Tyagi, 1999. *Agrobacterium* mediated high frequency transformation of *indica* rice variety, Pusa Basmati and transmission of the transgenes to R2 progeny. Plant Sci., 147: 127-137.

Morita, M., X.H. Xing and H. Unno, 1999. Synchronized shoot regeneration of rice (*Oryza sativa* L.) calli on solid medium by adjustment of intracellular 2, 4-Dichloro phenoxy acetic acid concentration. Plant Cell Rep., 18: 633-639.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15: 473-497.

Park, S.H., S.R.M. Pinson and R.H. Smith, 1996. T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Plant Mol. Biol., 32: 1135-1148.

Rachmawati, D., T. Hosaka E. Inoue and H. Anzai, 2004. *Agrobacteria*-mediated transformation of javanica rice cv. Rojolele. Biosci. Biotechnol. Bioch., 68: 1193-1200.

Rajesh and Manickam, 2006. Prediction of functions for two LEA proteins from mungbean. Bioinformation, 1: 133-138.

Rashid, H., S.I. Yokoi, K. Toriyama and K. Hinanta, 1996. Transgenic plant production mediated by *Agrobacterium* in *indica* rice. Plant Cell Rep., 15: 727-730.

Rohila, J.S., R.K. Jain and R. Wu, 2002. Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley *Hva1* cDNA. Plant Sci., 163: 525-532.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular cloning. A laboratory manual. 1st Edn. Cold Spring Harbor Laboratory Press, Cold Spring harbor, New York, .

Schmulling, T. and J. Schell, 1993. Transgenic tobacco plants regenerated from leaf disks can be preclinical chimeras. Plant Mol. Biol., 21: 705-708.

Sivamani, E., A. Bahieldin, J.M. Wraith, T. Al-Niemi, W.E. Dyer, T.H.D. Ho and R.D. Qu, 2000. Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley *HVA1* gene. Plant Sci., 155: 1-9.

Steponkus, P.I., M. Uemura, R.A. Joseph, S. Gilmour and M.F. Thomashow, 1998. Mode of action of the *COR15a* gene on the freezing tolerance of *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA, 95: 14570-14575.

Tang, K., X. Sun, Q. Hu, A. Wu, C.H. Lin, H.J. Lin, R.M. Twyman, P. Christou and T. Feng, 2001. Transgenic rice plants expressing the ferredoxin-like protein (AP1) from sweet pepper show enhanced resistance to *Xanthomonas oryzae* pv. *oryzae*. Plant Sci., 160: 1035-1042.

Toriyama, K. and K. Hinata, 1985. Cell suspension and protoplast culture in rice. Plant Sci., 41: 179-183.

Wang, M.B., Z.Y. Li, N.M. Upadhyaya, R.I.S. Brettel and P.M. Waterhouse, 1997. Intron-mediated improvement of a selectable marker gene for plant transformation using *Agrobacterium tumifaciens*. J. Genet. Plant Breed, 51: 325-334.

Wilmink, A. and J.M. Dons, 1993. Selective agents and marker genes for use in transformation of monocotyledonous plants. Plant Mol. Biol. Rep., 11: 165-185.

Xiao, B., Y. Huang, N. Tang and L. Xiong, 2007. Over-expression of a LEA gene in rice improves drought resistance under the field conditions. Theor. Applied Genet., 115: 35-46.

Xu, D., X. Duan, B. Wang, B. Hong, T.D. Ho and R. Wu, 1996. Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol., 110: 249-257.