Functional validation of a water deficit stress responsive AP2/ERF family transcription factor-encoding gene in *Oryza sativa*

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Cloning of drought responsive genes and validating their function are essential to crop improvement. In the present study, a drought responsive AP2/ERF family transcription factor was isolated from drought-tolerant *Oryza sativa* L. cv N-22 (AP2/ERF-‘N-22’). Embryogenic calli produced *in vitro* from dehusked mature seeds of rice were bombarded with a gene construct containing AP2/ERF-N22, driven under inducible promoter RD29A from Arabidopsis using the Biolistic method. The bombarded calli were selected on hygromycin-containing selection medium. Molecular analysis of regenerated plants confirmed the integration and enhanced expression of the gene under water deficit stress (WDS). Transgenics showed 1.4 fold more expression as compared to wild-type (WT) under control condition and up to 2 fold more expression of AP2/ERF-‘N-22’ under water deficit stress as compared to WT. Molecular analysis of regenerated plants confirmed the integration and enhanced expression of the gene under water deficit stress. The transformation efficiency was found to be nil, 0.97% and 3.11% for overstored seeds ≥ a year, ≥ a year and fresh seeds respectively. Transgenics showed 1.4 fold more expression under control condition and up to 2 fold more expression under WDS as compared to wild-type (WT). About 90% of the plants reached maturity and showed no negative phenotypic effects or aberrations as observed earlier under a constitutive promoter from that of the WT. Physiobiochemical analysis of transgenics showed enhanced drought tolerance.

**Keywords**: AP2/ERF, Calli, Drought, Gene bombardment, Regeneration, Transcription factor, Transformation, Transgenics

Rice is one of the world’s most important crops and caters the food grain requirement of more than half of the world population. However, rice is relatively sensitive to salinity and water stress. In order to adapt and grow in different environments, plants have developed a complex signaling network. At the molecular level, various transcription factors (TFs) regulate expression of multiple stress response genes. In the last few years, various transcription factors have been characterized by their roles in plant stress responses. Among different TFs, AP2/ethylene-responsive element-binding protein (AP2/EREBP) family plays an important role in the regulation of diverse biological processes such as plant growth, development, and response to abiotic stresses. To understand the regulatory networks and mechanisms of drought tolerance, researchers are manipulating the activity of these genes in transgenic plants to ameliorate crop yield under stressful conditions. The literature studies have shown that over expression under a constitutive promoter may have a negative effect on the plants. An earlier study of a differentially expressed AP2/ERF-N22 from drought-tolerant *Oryza sativa* sp. Indica cv N22, when over expressed in Arabidopsis under a constitutive promoter, caused phenotypic aberrations though it improved drought tolerance when compared to the wild-type (WT).

Plant regeneration from embryo-derived callus of rice was first reported by Nishi et al. Since then, many methods using various explants such as protoplast, immature seeds, and embryogenic tissues have been reported for efficient gene expression in rice. Ge et al. successfully standardized rice transformation by using anther explants. Apart from the exploration of the use of various explants, researchers have studied various factors that affect and influence the transformation efficiency. Ramesh and Gupta showed that time of incubation of callus prior to bombardment greatly affected the efficiency. Li et al. reported a substantial increase in transformation efficiency after bombardment by excising the resistant calluses followed by continuous sub-culturing during regeneration and plant growth.
Mandal et al.\textsuperscript{13} showed that the size and age of the callus before bombardment influences the rate of transformation in rice. Jadhav et al.\textsuperscript{14} investigated the efficiency of transformation based on the type of callus and induction medium. Partial desiccation of callus prior to and after bombardment influenced shoot and bud initiation and ultimately regeneration efficiency over the non-desiccated calluses irrespective of rice genotypes\textsuperscript{15-17}. Age of the seeds has never been considered seriously as one of the factors that influence transformation. No matter how good the storage condition may be, the viability of seeds decreases with time.

Taking this into account, we have optimized a rice transformation protocol using AP2/ERF family transcription factor and studied the effect of age of seeds on transformation efficiency as one of the factors by the biolistic method. Further, over expression of AP2/ERF-N22 in rice was taken up under drought-inducible promoter RD29A in this study to validate the function of a gene in imparting enhanced drought tolerance.

**Materials and Methods**

**Designing of the construct**

The AP2/ERF-N22 (Accession no. EF362638) driven by the CaMV35S promoter and Nos terminator was restricted out from the binary construct pBI121: CaMV35S-AP2-Nos\textsuperscript{6} (Fig. 1A) and cloned in pCambia1200. The recombinant construct named as pC1200: CaMV35S-AP2-Nos (Fig. 1B) was restricted to remove CaMV35S and replaced by inducible RD29A promoter to obtain recombinant binary construct, now named as pC1200: RD29A-AP2-Nos (Fig. 1C). Primers used for amplification of AP2/ERF-‘N-22’ were as follows:

\textbf{Forward} 5’ ATG GGA CAG TCG AAG AAG \textbf{Reverse} 5’ TCA GAT GAC GAG GCT AC

Primers were designed from the internal region of RD 29 A promoter with an expected amplicon size of 450 bp. Primers used for amplification of RD 29 A promoters region were as follows:

RD29A Forward- 5’ ATT CGA ATG AGA AGG ATG TC
RD29A Reverse - 5’ CCC TTT ATT CCT GAT CAT TG

**Culture media and conditions**

Culture media employed in these experiments were adjusted to pH 5.8 with NaOH. Callus induction medium (CIM) consisted of MS\textsuperscript{18} powder with sucrose 30 g/L, Casein hydrolysate 200 mg/L, proline 500 mg/L, 2,4-D (1 mg/mL) 2 mL/L and 2.5 g/L of phytagel. Osmotic media in addition to CIM consisted of sorbitol 0.2 M and mannitol 0.2 M. Hygromycin selection media in addition to CIM consisted of 50 mg/L hygromycin. Regeneration media in addition to CIM consisted of 300 mg/L glutamate, 6 benzylaminopurine (BAP) (1 mg/mL) 2.5 mL/L, α naphthalene acetic acid (NAA) (1 mg/mL) 0.75 mL/L, gelrite (in place of Phytagel) 3 g/L and hygromycin (50 mg/L). Solid rooting media

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Fig. 1—Binary construct pBI121: CaMV35S-AP2-Nos (A); pC1200: CaMV35S-AP2-Nos (B); and pC1200: RD29A-AP2-Nos (C) used for rice transformation
composed of MS powder 2.2 g/L, sucrose 15 g/L, FeSO$_4$ solution 2.5 mL and gelrite 3g/L. Liquid rooting media composition is same as solid rooting media with the exception of gelrite.

**Experimental plant tissues**

The dehusked mature seeds (200-500) of Pusa Sugandh 2 were surface sterilized and inoculated into CIM (Fig. 2A) for 1-2 weeks at 28°C under complete darkness. Nodular structures appeared from the general area of the scutellum. The callus was separated from the endosperm, cut into small pieces and arranged in clumps on osmotic medium plates as shown in (Fig. 2B). Prior to bombardment, these small calluses were incubated at 28°C under complete darkness for 4 h after which calluses were subjected to gold particle bombardment. After 48 h of incubation in the dark at 28°C, each callus was transferred into selection media containing hygromycin (50 mg/L), (Fig 2C & D) and incubated at 28°C under complete darkness. After 15 days the surviving calluses were transferred into fresh selection media. The rapidly proliferated calluses displayed somatic embryos in the form of whitish granular sectors. Some partially necrotic (brown sectors) and non-proliferating calluses were also observed, which were removed from each subculture. At the time of transfer, individual callusing embryos or callus pieces were broken using forceps into several small pieces which were maintained separately. During the subsequent two to three selection passages, callus pieces showing evidence of more vigorous growth were transferred to new selection plates. All callus tissue developed originally from each piece of embryo or callus was defined as a single line. There were many plantlets obtained from each callus defined as a line.

**Microprojectile bombardment**

All bombardments were conducted with biolistic Bio-Rad PDS-1000/HeTM system. 30 mg of gold particles (0.6 µm) was washed with absolute alcohol.
thrice. 50 µL of 2.5 M CaCl$_2$, 20 µL of 0.1 M spermidine and 10 µL of plasmid pC1200: RD29A-AP2-Nos (1 µg/mL) was added to the gold suspension of 10 µL, followed by 2 min incubation in ice, and short spin at 10000 rpm. The pellet was suspended in 90 µL ethanol, out of which 12 µL was evenly spread on macro carrier cat#1652335, Bio Rad, India). Each experiment was bombarded at a pressure of 1100 psi. The distance from the stopping plate to the target was maintained 9 cm from the ruptured disc(cat#1652329, Bio Rad).

**Molecular analysis**

DNA was isolated using the CTAB method$^{19}$. For analysis of transgenic plantlets, very small quantity of leaf (a part of the leaf) sample from each line was taken with the help of sterile forceps, DNA was isolated by using a micropule. However, for molecular analysis of fully grown mature plants, 500 mg of leaf tissue was taken. Total RNA was isolated from WT and transgenic plants using TRI-Reagent® from Sigma under control and WDS. WDS was given by withholding the water from transgenics for three days. cDNA was synthesized by using Fermenta’s cDNA synthesis kit. The integration of the gene was confirmed by PCR and Southern blot analysis$^{19}$. For PCR analysis specific primers for RD29A, hpt gene and AP2/ERF-N22 were used. PCR products of promoter and AP2/ERF-N22 were used as probes for hybridizing the blots. 5 µg DNA DNA was digested with EcoRI. EcoRI sites are absent inside the gene and within the RD29A. The probe was labeled using the DecaLabel™ labeling kit (cat#KO62, Fermentas, India) with $^{32}$P labeled ATP (BARC, India). Labeling of the probe was done according to manufacturer’s protocol.

**Result and Discussion**

**Isolation and characterization of AP2/ERF-N22 gene**

AP2/ERF family transcription factor-encoding genes were searched from rice genome annotation project (Release 7). Further immediate upstream promoter region (500 bp upstream) of these genes were searched for the presence of an ABA-responsive element (ABRE) and drought responsive element (DRE) cis-acting elements. From TIGR rice database a specific AP2/ERF transcription factor gene having ABRE and DRE cis-acting elements within 500 bp upstream of the initiation codon was identified. *Oryza sativa* sp. Japonica LOC_Os06g40150 (chromosome 6) showed the presence of ABRE and DRE cis-acting elements within 500 bp upstream of the initiation codon suggesting the transcription factor gene might be responsive to ABA and drought. The annotated gene from *Oryza sativa* sp. Japonica LOC_Os06g40150 (chromosome 6) had a genomic sequence of 964 bp and cDNA of 732bp. Primers were then designed manually for this japonica gene (LOC_Os06g40150) and genomic DNA from indica cultivar was amplified using these primers. Genomic DNA corresponding to *AP2/ERF-N-22* from *Oryza sativa* L. cv N22 was isolated, characterized and submitted to Genbank (Accession no. EF362638)$^6$. Northern and RT-PCR analysis showed that transcript of *AP2/ERF-N22* accumulates in response to drought stress and the Southern analysis indicated the presence of a single copy of *AP2/ERF-N22* gene in *Oryza sativa* genome$^6$. Phylogenetic analysis of AP2/ERF family revealed that *AP2/ERF-N22* belongs to group Va along with SHN clade of AP2/ERF protein which activates wax biosynthesis$^6$. Homologous sequences to *AP2/ERF-N-22* (Accessions # EF 362638) were isolated from cultivars differing in drought tolerance such as IR 64 (Accession # KP231018), MTU (Accession # KP 207594) and Taipei 309 (Accession # EU 034698) to ascertain the importance of the gene in relation to drought tolerance. All the three gene sequences were submitted to Genebank. Polymorphism was observed among these three varieties. Substitution in base sequences was “observed at positions 17 and 771 while deletions were observed at positions 20-21 and 283 (Fig. 3). *AP2/ERF-N22*, when overexpressed in Arabidopsis under a constitutive promoter, showed improved turgor and less wilting as compared to wild-type plants under WDS. However, the transgenic plants showed phenotypic aberrations such as stunted growth, smaller silique size, reduced number of seeds and delayed flowering$^7$.

**Designing of Binary construct for rice transformation**

The binary construct pBI121:CaMV35S-AP2-Nos (Fig. 2A) carrying the gene AP2/ERF-N22 (Accession no. EF362638) driven under CaMV35S promoter and containing kanamycin selection marker gene used earlier for Arabidopsis transformation in our lab$^7$ was not used for rice transformation because of two reasons *i.e.* presence of constitutive CaMV35S (promoter) and kanamycin selection marker gene. The recombinant construct under the name pC1200: RD29A-AP2-Nos (Fig. 2B) designed for rice transformation was further confirmed with PCR using
gene-specific primers for AP2/ERF-N22. An expected amplicon size of 964 bp was obtained corresponding to AP2/ERF-N22. Presence of inducible promoter was confirmed by using RD29A promoter-specific primers. As primers were designed from the internal region, an expected size of 450 bp was obtained. RD29A (forward) and AP2/ERF-N22 (reverse) primers were used to confirm the sense orientation of the gene, using the following primers RD29A (forward) and AP2/ERF-N22 (reverse). An expected amplicon size of 1436 bp was obtained confirming that the gene was cloned in the sense orientation and was driven by an inducible promoter. The pC1200: RD29A-AP2-Nos (Fig. 2C) was further confirmed by sequencing.

For rice transformation studies, hygromycin selection was preferred over kanamycin as it has been reported that protoplast-derived callus selected on kanamycin is very inefficient in terms of regeneration and that a large number of albino plants arise from such experiments. Hygromycin is more effective than kanamycin for transformation because rice shows a natural resistance to kanamycin. Also, hygromycin neither inhibits regeneration nor affects the fertility of transformants. The hpt conferring resistance to hygromycin has therefore been used as a marker gene in the selection of transformants generated by both Agrobacterium-mediated and particle bombardment.

**Effect of the age of seeds**

Many studies have been done to identify suitable explants for in vitro callus induction. Various explants such as mature and immature embryos, leaf blade, microspore, and anther, root, and coleoptile have been used as good resources for in vitro production of embryogenic callus. Embryogenic calluses with high regeneration capacity are required for efficient rice transformation. Though immature tissues, which contain a large number of meristematic cells are more suitable for the production of embryogenic callus as compared with mature seeds, especially for recalcitrant genotypes, the preparation of immature embryos on a large scale is tedious and has seasonal limitations. Whereas, mature seeds have the advantage of convenient storability and ready availability throughout the year. Further using mature seeds makes the protocol for rice transformation efficient and simple for easier production of improved genotypes by genetic engineering with the potential for gene function studies.

It was observed that seeds (explants) which were stored for a very long time showed a delay in callus formation, or no callus formation, while some showed delayed regeneration or no regeneration (Table 1). However, when fresh seeds (1-2 weeks after harvesting) were used there was neither a delay in callus formation nor in regeneration (Table 1). Further, it was observed that fresh seeds gave rise to healthy callus, which started to show initial greening, with regeneration within a period of one month (Fig 2E). These were transferred into bottles, to allow them to regenerate further. Four weeks after transferring into the regeneration medium and incubation under photoperiod (16 h light/8 h dark) in a growth room at 25°C, somatic embryos rapidly regenerated into shootlets with small roots (Fig. 2F). Each plantlet was separated and transferred into solid rooting media for enhancing full root growth. After a week or so, with the full development of primary and secondary roots, each plantlet was then transferred into a liquid rooting media which lead to the
development of normal roots (Fig. 2G). Each plantlet confirmed to be transgenic was transferred into small pots containing soilrite and covered using transparent plastic bags for acclimatizing the plants. To allow air exchange, small holes were made in plastic bags (Fig. 2H). After 7-12 days, the plastic bags were removed and plantlets were transferred into pots and allowed to grow in the greenhouse till maturity (Fig. 2I). This study indicates that the ability of callus formation is mainly dependent on the age of the seeds which in turn is a prerequisite for highly efficient rice transformation.

Transformation efficiency and survival rate of plants

Transformation efficiency and regeneration time are greatly influenced by the age of seeds in this protocol. In this experiment, the standardization of the protocol was based on the use of stored seeds and freshly harvested seeds. The transformation efficiency with stored seeds for ≥ a year was nil and increased to 0.97% when stored for ≥ a year. However, both showed delayed regeneration time (about 4-5 months) (Table 1) and some callus did not regenerate into plantlets. However, freshly harvested seeds (1-2 weeks after harvesting) showed a marked increase in the transformation efficiency (3.11%) and callus regenerated into a number of plantlets over a period of one month. Three experiments in Table 1 were compared using completely randomized design. ANOVA revealed that transformation efficiency with freshly harvested seeds was significantly higher from stored seeds. However, transformation efficiency with stored seeds for ≥ a year and ≥ a year are not significantly different.

Apart from considering the age of seeds as an influential factor for enhancing the transformation efficiency, there are other factors too. As seen in previous studies, the procedure used for precipitation of DNA onto gold micro-particles prior to biolistic transformation may impact transformation success and efficiency. Typically, DNA is usually precipitated onto micro-particles in the presence of spermidine and CaCl₂ for biolistic transformation of plants, which was used in the present experiment.
Sivamani et al\textsuperscript{32} reported that using a cationic polymer protamine instead of spermidine for biolistic transfer of full plasmids to rice and maize resulted in over a five-fold increase in transient transformation efficiency and a 3.3-fold increase in stable transformation efficiency. Stable transformation of sugarcane, by biolistic transfer using protamine, spermidine and Seashell protocols were equally effective\textsuperscript{33}. Interestingly Sivamani and group in their observations showed spermidine resulted in significantly higher transient expression than the other two precipitation protocols. Xiong et al. \textsuperscript{34} however, found that protamine protects plasmid DNA for a longer period from DNAse degradation than spermidine. Various studies have shown different ways to increase the transformation efficiency. Thus no single protocol is said to be the best, as it depends on many factors which need to be studied.

The rate of regeneration is greatly influenced, by the quality of seeds and is very important for successful tissue culture. The survival rate of plants was 87\%, after their transfer to soil in the greenhouse and more than 90\% of plants reached maturity. Most T\textsubscript{0} plants had normal morphology and were self-fertile.

Molecular analysis

PCR analysis was done, to confirm the integration of the gene before plantlets were transferred into soilrite. Each plantlet was analyzed with specific primers for the gene, promoter and hpt marker and only those giving the required amplicon size of 964, 450 and 160 bp respectively were raised to maturity in the greenhouse, the rest were discarded. The four plants (obtained from the 1\textsuperscript{st} batch) grown to maturity in the greenhouse were again confirmed by PCR for the promoter (Fig. 4A), the gene (Fig. 4B) and the hpt marker (Fig. 4C).

Southern blot analysis for the integration of promoter was done for the four plants using RD29A as a probe (Fig. 4D). Southern analysis showed the integration of the RD29A gene in only three transgenic lines. As expected no band was observed in WT. RD29A is an inducible promoter which is not present in wild-type. Although PCR analysis showed the integration of RD29A in all four, southern analysis showed bands only in three lines. It did not confirm the same as PCR analysis because this transgenic plant might have been chimerically transformed. Southern blot analysis was also used to characterize the transgenic status of the plant and to estimate the copy number. The integration of the gene was analysed using gene product as a probe. The intensity and number of hybridizing bands give a rough estimate of copy number. (Fig. 4E) showed the integration of the AP2/ERF gene with 3 copy number in all the four transgenic lines. The same pattern was observed with all the four transgenics, suggesting that these plants probably originated from single integration event.

The copy number following biolistic gene transfer depends on the quantity of the DNA that is delivered to the nucleus\textsuperscript{35-37}. The microprojectile bombardment has commonly resulted in complex integration patterns\textsuperscript{38}. Several studies have been made to correlate expression and copy number of the transgene. Contradictory results were obtained, with some studies reporting positive correlation\textsuperscript{39} and others no correlation\textsuperscript{40-43} or negative correlation\textsuperscript{44,45}. However, in the present study, we observed a positive

\begin{figure}[h]
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\caption{PCR analysis using RD29A (A); AP2/ERF-N22 primers (B); hpt primers (C); Southern blot analysis showing four transformed rice lines hybridized with labeled RD29A (D); AP2/ERF-N22 (E), and Expression study of AP2/ERF-N22 in four transgenic lines under control and water deficit stress (F) [M, 100 bp ladder; WT, Wild type; 1-4, four different transgenic lines; 5, Positive control (Binary construct)]}
\end{figure}
correlation between expression and copy number of the transgene.

Comparison of the expression level of AP2/ERF-N22 between the WT and transgenics under control and water deficit stress conditions was carried out using qRT-PCR. The fold change in the expression was determined using actin as the internal control. In expression study, WT under water deficit stress showed 1.2 fold more expression when compared to control. Transgenics showed ~ 1.4 fold more expression as compared to WT under control condition; whereas under water deficit stress transgenics showed up to 2 fold more expression as compared to WT (Fig. 4F).

Conclusion

Transgenic lines of different crop plants have been successfully obtained by particle bombardment method. Agrobacterium-mediated transformation systems have also been used successfully in recent years for developing transgenic rice, barley and wheat. However, particle bombardment remains preferred method for developing transgenics of monocot species, because at least in rice it is variety-independent, efficient and simple. In our experiments, we have found the age of seed to be an important factor that influenced the rate of regeneration and transformation efficiency. The transformation efficiency was found to be nil, 0.97% and 3.11% for overstored seeds ≥ a year, ≥a year and fresh seeds respectively. Several features of the transformation protocol reported in this study will facilitate its adoption by other laboratories.

References


