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Pea DNA helicase 45 promotes salinity stress tolerance in IR64 rice with improved yield

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The helicases provide duplex unwinding function in an ATP-dependent manner and thereby play important role in almost all the nucleic acids transaction. Since stress reduces the protein synthesis by affecting the cellular gene expression machinery, so it is evident that molecules involved in nucleic acid processing including translation factors/helicases are likely to be affected. Earlier pea DNA helicase 45 (PDH45), a homolog of translation initiation factor 4A (eIF4A) was reported to play important role in salinity stress tolerance in tobacco and Bangladeshi rice variety Binnatoa. We report here the overexpression of *PDH45* gene in the indica rice variety IR64, via Agrobacterium-mediated transformation. Molecular analysis of the transgenics revealed stable integration of the transgene in the T1 generation. Enhanced tolerance to salinity was observed in the plants transformed with *PDH45* gene. Better physiological and yield performances including endogenous nutrient contents (N, P, K, Na) of the transgenics under salt treatment were observed as compared with wild type (WT), vector control and antisense transgenics. All these results indicated that the overexpression of *PDH45* in the IR64 rice transgenics enable them to perform better with enhanced salinity stress tolerance and improved physiological traits. Based on the homology of PDH45 protein with eIF4A protein we suggest that it may act at the translational level to enhance or stabilize protein synthesis under stress conditions.

Keywords: abiotic stress, DNA and RNA helicase, GM crop, PDH45, rice, salinity stress, transgenics

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Plants are exposed to various abiotic stresses including salinity, drought and extreme temperatures which influence plant physiology/metabolism, thereby inhibiting plant growth/development leading to decreased agricultural productivity and hence affecting food security.[1,2] Therefore, it is need of the hour to develop the stress tolerant variety by using tools of genetic engineering. The adaptation to stresses in plants is dependent upon the activation of networks involved in stress perception, signal transduction, and the expression of specific stress-related genes and metabolites.[3] In response to the stresses including salinity stress several changes occur in transcriptome, proteome and metabolome of plants.[4] The negative impacts of salinity stress include oxidative stress, membrane/cell wall damages, plasmolysis, imbalance of Na^+/K^+ ratio and inhibition of photosynthesis which lead to decreased yield.[5] The overexpression of several stress-induced genes including helicases have been shown to provide salinity stress tolerance in crop plants including rice.[6-9] DNA and RNA helicases are duplex unwinding enzymes and play important roles in almost all the nucleic acids transactions. The role of helicases in stress signaling is now emerging.[6,10] Earlier, we have reported the first direct evidence that overexpression of pea DNA helicase 45 (*PDH45*) gene in tobacco plant confers salinity stress tolerance without affecting yield which suggested a previously undescribed pathway for manipulating stress tolerance in crop plants.[6] The PDH45 is a member of the DEAD-box protein family and contains all the known canonical helicase motifs.[10] PDH45 is reported to be

localized in the nucleus and cytosol and contains 3' to 5' directional unwinding activity in an ATP-dependent manner.[11] We have reported that overexpression of *PDH45* gene in bacteria confers salinity stress tolerance.[12] Recently, we have shown that overexpression of *PDH45* gene in Bangladeshi rice variety Binnatoa confers salinity stress tolerance with enhanced yield.[8] Here, we report that overexpression of *PDH45* gene in another rice cultivar IR64 (*Oryza sativa* L, CV: IR64) also promotes salinity stress tolerance. In addition we also report a comparative study of various physiological parameters and nutrient analysis of PDH45 transgenic T1 rice (IR64) with WT, vector control and antisense transgenic rice.

A full-length cDNA (1.63 kb) encoding PDH45 was cloned (Accession number AY17186) from pea cDNA library as described earlier.[6] The cDNA consists of an open reading frame (ORF) of 1.22 kb, a 5' untranslated region (UTR) of 78 bp and a 3' UTR of 328 bp including a 18 bp poly(A) tail and it encodes a protein of 45 kDa.[11] To further establish the functional significance of the *PDH45* gene, the ORF of the *PDH45* gene was cloned at PstI sites in transformation vector (pCAMBIA-1301) as described earlier.[8] Briefly, the 1.2 kb *PDH45* coding sequence was cut out of the vector pBI-121-PDH45 [6] by using XbaI and ligated into the MCS of pRT100. The CaMV35S-PDH45-poly A fragment thus generated in pRT100 was then cut out with PstI and ligated into the MCS of the rice compatible pCAMBIA-1301 containing the hygromycin-phosphotransferase selectable marker and the reporter *GUS* gene (**Fig. 1A**). The same gene was inserted in pRT100 vector in reverse orientation (antisense) also by using the same enzyme (XbaI) followed by its cloning in the pCAMBIA-1301 as described above. A competent strain of *Agrobacterium tumefaciens* (LBA4404) was transformed with the construct pCAMBIA-1301/CaMV35S-PDH45 using standard protocols. Aliquots of the transformed cells were plated on YEM agar containing kanamycin and incubated for 48 h at 28°C. PDH45 positive colonies were selected as described earlier.[8] An improved Agrobacterium-mediated transformation technology[13] for mature seed-derived callus tissues of indica rice cultivar IR64 was used to transform the *PDH45* gene (both in sense and antisense orientations). As a control the empty vector (pCAMBIA-1301) was also transformed in rice by using an improved transformation technology recently developed by us.[13] Different stages of transformation from mature seeds of rice to mature transgenic rice plants are shown in the **Figure 1B-I.**

Analysis for the *PDH45* transgene integration was performed on the T1 generation of transgenic plants. As an initial test, the plants were analyzed by PCR for the presence of 1.2 kb *PDH45* gene. The integration was then confirmed by amplification of the expected sizes of 1.2 kb in randomly selected five transgenic lines (**Fig. 1J**). The transgenic lines were further confirmed by using another PCR with gene specific forward primer and vector specific reverse primer and the expected size of the fragment was observed (data not shown). The Gus activity was visualized in leaf tissue of all the five transgenic lines. The results show that all were Gus positive (**Fig. 1K**). To determine whether PDH45-imparted salinity tolerance is functionally and genetically stable, the T1 progeny was analyzed. Seeds from the T_0 plants, when plated onto hygromycin-containing medium, segregated in the expected 3:1 ratio of Hygr/Hygs (**Table 1**). Only the lines germinated on hygromycin plated were carries forward for growth, nutrient, stress tolerance and yield analysis. To test for salinity tolerance, leaf disks from T1 transgenic and wild type (WT) rice plants were floated separately on 300 mM NaCl and H_2O for 96 h. Salinity-induced loss of chlorophyll was lower in PDH45 overexpressing lines compared with those from the wild type plants (**Fig. 1L**). The damage caused by stress was reflected in the degree of bleaching observed in the leaf tissue after 96 h. It was evident that transgenic plants have a better ability to tolerate salinity stress.

The experiment was arranged in a randomized block design. For various growth, physiological and yield attributes of the H_2O grown WT, vector control, antisense and salt grown (200 mMNaCl) PDH45 T1 transgenic plants [Lines 1 to 5], values are presented as the mean of three replicates (each plant was considered as a replicate). Data was analyzed statistically and standard error was calculated. Analysis of variance (ANOVA) was performed on the data using SPSS (10.0 Inc., USA) to determine least significant difference (LSD) for significant data to identify difference in the mean of the treatment. The means were separated by Duncan's multiple range tests. Different letters indicate significant difference at $p < 0.05$.

The nutrient (N, P, K and Na) content in plant samples were estimated by modified Kjeldahl method stated by Jackson[14] and Gupta.[15] Agronomic performance and nutrient analysis of PDH45 transgenic T1 rice as compared with WT, vector control and antisense lines were performed and the results are shown in the **Table 1**. Overall, significant differences were observed between WT plants (without stress) and T1 transgenic plants after salinity stress under greenhouse conditions. The T1 transgenic (with stress) plants behaved better than the WT plants (without stress) in several vital growth parameters like plant height, number of tillers/plant, panicle length, panicle weight, number of filled grain/panicle, root length, root dry weight and total chlorophyll content (**Table 1**). WT plants under salt stress could not survive till harvest. No detrimental effects on overall physiological performance, growth and development, flowering pattern or seed production was observed due to introgression of *PDH45* gene. In all physiological parameters tested PDH45 transgenic T1 rice plants (with 200 mM NaCl stress) behaved better than wild type plants (without stress). Phenotypic observation has also been recorded, which showed normal growth of transgenic plants, whereas, WT experienced significant reduction in growth performance (data not shown).

The accumulation of ions in WT and transgenic plants, either untreated or salt treated (200 mM NaCl) were measured in the plant tissues. Salt treated transgenic plants showed high accumulation of nitrogen (N), potassium (K) and phosphorus (P) as compared with the WT untreated plants (**Table 1**). The Na^+ concentration was found statistically non significant in H_2O grown WT and PDH45 transgenic lines. These evidences suggest that the

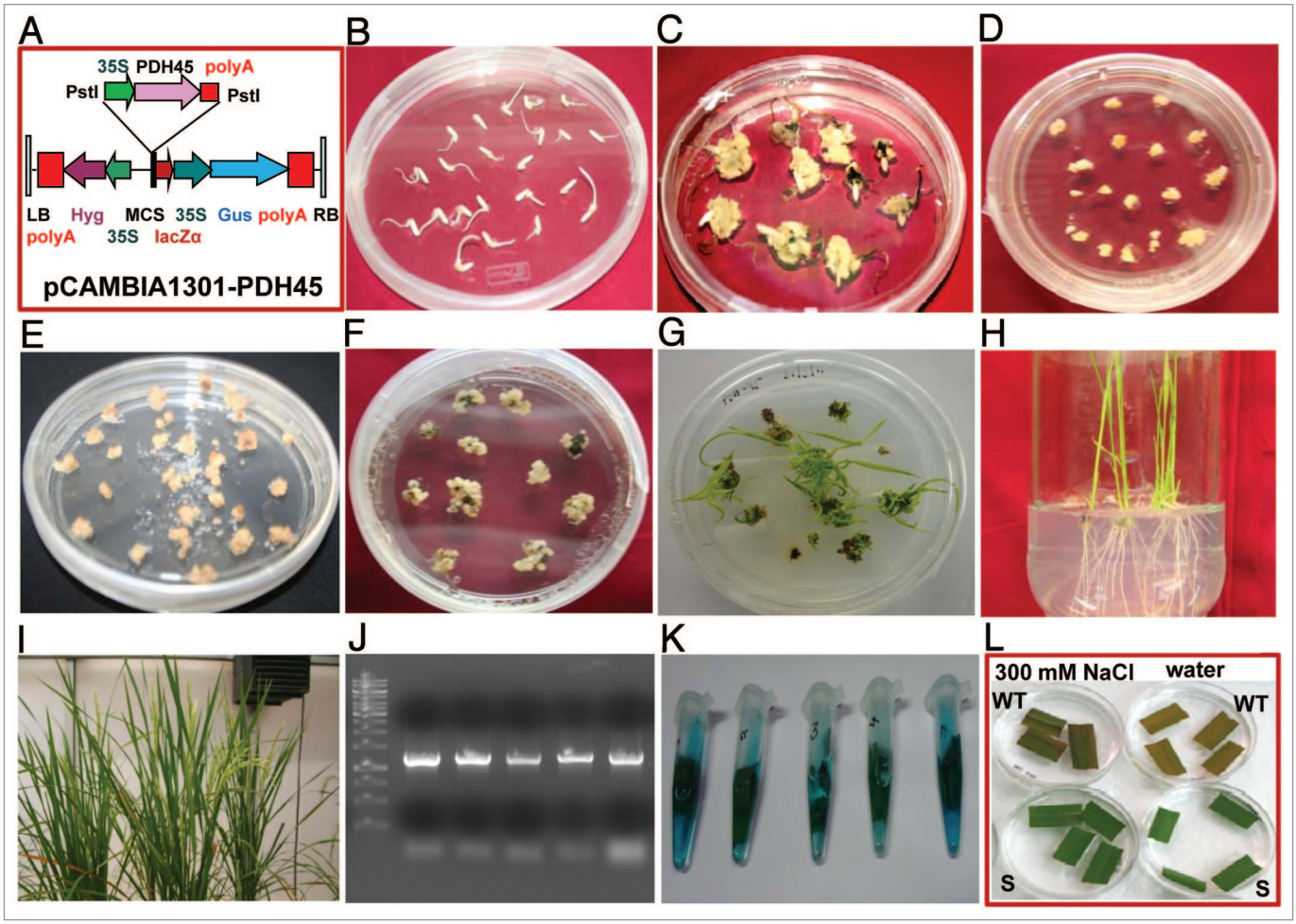

Figure 1. Different stages of developing transgenic *PDH45* plants from preparation of gene construct to plant regeneration in indica rice variety, IR64 and confirmation of gene (*PDH45*) integration by PCR, Gus assay and Leaf disc senescense assay. (A) Structure of T-DNA region of pCAMBIA1301 containing *PDH45* gene (1.2 kb) in PstI restriction enzyme site of MCS region with CaMV35S promoter and poly A terminator (pCAMBIA1301-*PDH45*). (B) Mature seeds of IR64 in callus induction medium for 20 d. (C) After 20 d of calli initiation. (D) Calli sub-cultured on fresh callus induction medium for 2 d. (E) Calli in co-cultivation media after transformation (F) Selection of transformed calli on selection medium having hygromycin 50 mg/l. (G) The resistant calli in MS regeneration medium and emerging shoot buds on regeneration medium. (H) Shoots in rooting medium. (I) Hardening of plant and transfer to soil. (J) PCR analysis of the transgenic (T1) lines by the use of gene (*PDH45*) specific forward and reverse primers. Lane M is 1 kb ladder. Lanes 1–5 are, rice transgenic lines (L1, L2, L3, L4 and L5 respectively) showing the required amplification (1.2 kb). (K) Visualization of gus activity in the leaf tissue of transgenic lines by using 1 mM 4-methyl-umbelliferyl β-D glucuronide. (L) Leaf disk senescence assay for salinity tolerance in transgenic rice plants in 300 mMNaCl and water. Transgenic lines showing high salinity tolerance as compared with WT.

transgenic plants are highly tolerant to NaCl stress. The accumulation of Na^+ in the transgenic plants in response to salinity was significantly less. This clearly reveals the capability of the transgenic plants to eliminate the deleterious effects of salinity stress to the endogenous membranes and protecting the photosynthetically active parts of the leaf by restricting the entry and distribution of Na^+ and loss of high level of K^+ from tissues. The high accumulation of nitrogen ion in transgenic plants indicates the high salinity tolerance of transgenic plants.

The increased leaching of chlorophyll under high NaCl concentration is due to the increased membrane permeability to salt.[16] Generally, Na^+ appears to reach a toxic level before the other ions do, and thus the control of Na^+ homeostasis is vital.[17] For right metabolic function it is important to maintain appropriate intracellular K^+/Na^+ ratio, as Na^+ cytotoxicity is mainly due to the competition with K^+ for binding sites in enzymes essential for cellular functions.[18] Usually, the higher K^+/Na^+ ratios are considered as determinants of salt tolerance itself. In response to salinity stress the accumulation of Na^+ in the transgenic plants was found to be less in leaves and the loss of K^+ was also much lesser, compared with the WT, vector control and antisense transgenics plants. Thus, the PDH45 transgenic rice (IR64) plants showed overall superior physiological traits under high salt conditions. Overall, this study reveals that PDH45 has the potentiality in conferring relatively high degree of tolerance against salinity conditions.

The exact mechanism of PDH45-mediated salinity stress tolerance is not known. However, based on its homology with eukaryotic translation initiation

Table 1. Physiological, yield attributes and nutrient analysis of *PDH45* transgenic T1 rice (*Oryza sativa* L, CV: IR64) plants

Parameter	H_2O grown wild type	H_2O grown vector control	H_2O grown antisense	Progeny	Salt grown PDH45 sense transgenics (200 mM NaCl)				
					Line 1	Line 2	Line 3	Line 4	Line 5
Segregation ratio	0	0	0	T1	3:1 [163]	2.91:1 [158]	3.1:1 [149]	3.0:1 [150]	3.2:1 [147]
Seedlings survival (%)	97 ± 3.9[a]	94 ± 3.7[a]	96 ± 4.1[a]	T1	97 ± 4.2[a]	98 ± 4.1[a]	94 ± 3.8[a]	97 ± 4.1[a]	96 ± 3.7[a]
Days for flowering	90 ± 2.6[a]	90 ± 2.8[a]	90 ± 2.8[a]	T1	90 ± 2.5[a]	90 ± 2.6[a]	90 ± 2.1[a]	90 ± 2.7[a]	90 ± 2.8[a]
Plant height (cm)	69 ± 3.1[b]	65 ± 3.0[b]	63 ± 3.7[b]	T1	76 ± 3.8[a]	76 ± 3.9[a]	79 ± 3.5[a]	78 ± 3.0[a]	79 ± 2.9[a]
No. of tillers/plant	25 ± 1.0[c]	25 ± 0.9[c]	27 ± 1.2[c]	T1	33 ± 1.1[b]	33 ± 1.2[b]	37 ± 1.0[a]	32 ± 0.9[b]	34 ± 1.1[b]
No. of panicle/plant	21 ± 0.7[b]	20 ± 0.8[b]	23 ± 0.7[b]	T1	30 ± 1.0[a]	29 ± 1.0[a]	35 ± 1.2[a]	30 ± 1.1[a]	32 ± 1.0[a]
No. of filled grain/panicle	75 ± 3.2[b]	74 ± 3.0[b]	69 ± 2.5[b]	T1	105 ± 4.1[a]	108 ± 4.3[a]	102 ± 4.9[a]	101 ± 4.5[a]	108 ± 4.7[a]
No. of chaffy grains/panicle	11 ± 0.33[a]	10 ± 0.31[a]	12 ± 0.29[a]	T1	06 ± 0.21[b]	05 ± 0.20[b]	04 ± 0.11[b]	06 ± 0.17[b]	05 ± 0.15[b]
Straw dry weight (g)	55 ± 2.1[b]	54 ± 2.0[b]	50 ± 0.95[b]	T1	68 ± 2.5[a]	69 ± 2.1[a]	72 ± 2.6[a]	74 ± 2.5[a]	74 ± 2.2[a]
100 grain weight (g)	2.83 ± 0.10[a]	2.82 ± 0.11[a]	2.9 ± 0.10[a]	T1	3.3 ± 0.12[a]	3.4 ± 0.10[a]	3.5 ± 0.11[a]	3.5 ± 0.12[a]	3.7 ± 0.12[a]
Root length (cm)	21 ± 0.98[a]	20 ± 0.87[a]	20 ± 0.99[a]	T1	22 ± 1.0[a]	23 ± 1.2[a]	21 ± 1.1[a]	22 ± 1.0[a]	24 ± 1.1[a]
Root dry weight (g)	1.79 ± 0.06[a]	1.69 ± 0.07[a]	1.64 ± 0.06[a]	T1	3.7 ± 0.15[a]	3.6 ± 0.15[a]	3.9 ± 0.16[a]	3.6 ± 0.15[a]	3.8 ± 0.18[a]
Total protein (mg/g FW)	13.08 ± 0.51[b]	13.17 ± 0.55[b]	14.56 ± 0.4[b]	T1	20.25 ± 0.87[a]	21.28 ± 0.85[a]	20.61 ± 0.88[a]	22.23 ± 0.95[a]	21.13 ± 0.91[a]
Chlorophyll (mg/g FW)	8.18 ± 0.39[c]	9.07 ± 0.41[b]	9.10 ± 0.40[b]	T1	9.73 ± 0.42[a]	9.75 ± 0.38[a]	9.84 ± 0.35[a]	9.72 ± 0.39[a]	9.71 ± 0.37[a]
Nitrogen (%)	0.329 ± 0.012[b]	0.347 ± 0.015[b]	0.327 ± 0.011[b]	T1	0.415 ± 0.015[a]	0.439 ± 0.012[a]	0.442 ± 0.014[a]	0.427 ± 0.013[a]	0.457 ± 0.016[a]
Phosphorus (%)	0.353 ± 0.010[b]	0.337 ± 0.013[b]	0.353 ± 0.012[b]	T1	0.391 ± 0.012[a]	0.384 ± 0.011[a]	0.373 ± 0.012[a]	0.381 ± 0.010[a]	0.382 ± 0.012[a]
Potassium (%)	0.165 ± 0.005[a]	0.163 ± 0.006[a]	0.163 ± 0.005[a]	T1	0.179 ± 0.005[a]	0.172 ± 0.004[a]	0.173 ± 0.005[a]	0.172 ± 0.006[a]	0.175 ± 0.005[a]
Sodium (%)	0.001 ± 0.0001[a]	0.001 ± 0.0001[a]	0.001 ± 0.0001[a]	T1	0.001 ± 0.0001[a]	0.001 ± 0.0001[a]	0.001 ± 0.0001[a]	0.001 ± 0.0001[a]	0.001 ± 0.0001[a]

Data followed by the same letter in the same row are significantly not different at $p < 0.05$ as determined by Duncan's multiple range test (n = 3). Three plants from each lines were analyzed to study the effect of salinity on various growth, nutrient analysis and yield parameters.

factor (eIF4A),[6,11] we suggest that it may act at the translation level to enhance or stabilize protein synthesis which is affected in response to stress. Generally, in response to stress the extra secondary structures could be formed in the 5' UTR region in the mRNA of many essential genes, which could be inhibitory for translation. These inhibitory secondary structures need to be resolved in order to have active translation. In the PDH45 overexpressing lines the non-functional secondary structures are resolved by its RNA helicase activity, which permit the translation initiation to proceed. Overall, these stress-induced helicases help in recovering the functions of the genes for stress adaptation, which were stopped previously because of the negative impact of the stress. The data presented here suggest that *PDH45* could be an ideal candidate for developing a variety of salinity stress tolerant crops.

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References

1. Mahajan S, Tuteja N. Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 2005; 444:139-58; PMID:16309626; http://dx.doi.org/10.1016/j.abb.2005.10.018.
2. Atkinson NJ, Urwin PE. The interaction of plant biotic and abiotic stresses: from genes to the field [Epub ahead of print]. J Exp Bot 2012; PMID:22467407; http://dx.doi.org/10.1093/jxb/ers100.
3. Huang GT, Ma SL, Bai LP, Zhang L, Ma H, Jia P, et al. Signal transduction during cold, salt, and drought stresses in plants. Mol Biol Rep 2012; 39:969-87; PMID:21573796; http://dx.doi.org/10.1007/s11033-011-0823-1.
4. Saeed M, Dahab Ah, Wangzhen G, Tianzhen Z. A cascade of recently discovered molecular mechanisms involved in abiotic stress tolerance of plants. OMICS 2012; 16:188-99; PMID:22433075; http://dx.doi.org/10.1089/omi.2011.0109.
5. Tuteja N. Mechanisms of high salinity tolerance in plants. Methods Enzymol 2007; 428:419-38; PMID:17875432; http://dx.doi.org/10.1016/S0076-6879(07)28024-3.
6. Sanan-Mishra N, Pham XH, Sopory SK, Tuteja N. Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. Proc Natl Acad Sci USA 2005; 102:509-14; PMID:15630095; http://dx.doi.org/10.1073/pnas.0406485102.
7. Hennig L. Plant gene regulation in response to abiotic stress. Biochim Biophys Acta 2012; 1819:85; PMID:22309844.
8. Amin M, Elias SM, Hossain A, Ferdousi A, Rahman MS, Tuteja N, et al. Overexpression of a DEAD box helicase, PDH45, confers both seedling and reproductive stage salinity tolerance to rice (*Oryza sativa* L.). Mol Breed 2012; http://dx.doi.org/10.1007/s11032-011-9625-3; In press.
9. Datta K, Baisakh N, Ganguly M, Krishnan S, Yamaguchi Shinozaki K, Datta SK. Overexpression of Arabidopsis and Rice stress genes' inducible transcription factor confers drought and salinity tolerance to rice. Plant Biotechnol J 2012; 10:579-86; PMID:22385556; http://dx.doi.org/10.1111/j.1467-7652.2012.00688.x.
10. Tuteja N, Gill SS, Tuteja R. Helicases in Improving Abiotic Stress Tolerance in Crop Plants. In: Improving Crop Resistance to Abiotic Stress, Tuteja, et al. (Eds.), Wiley-Blackwell, Wiley-VCH Verlag GmbH & Co., Germany 1:435-49.
11. Pham XH, Reddy MK, Ehtesham NZ, Matta B, Tuteja N. A DNA helicase from *Pisum sativum* is homologous to translation initiation factor and stimulates topoisomerase I activity. Plant J 2000; 24:1-13; PMID:11029699; http://dx.doi.org/10.1046/j.1365-313x.2000.00869.x.
12. Tajrishi MM, Vaid N, Tuteja R, Tuteja N. Overexpression of a pea DNA helicase 45 in bacteria confers salinity stress tolerance. Plant Signal Behav 2011; 6:1271-5; PMID:21847021; http://dx.doi.org/10.4161/psb.6.9.16726.
13. Sahoo RK, Tuteja N. Development of Agrobacterium-mediated transformation technology for mature seed-derived callus tissues of indica rice cultivar IR64. GM Crops Food 2012; 3; PMID:22538224; http://dx.doi.org/10.4161/gmcr.20032; In press.
14. Jackson ML. Soil chemical analysis, Prentice Hall of India Pvt. Ltd. New Delhi 1973; 2:1-498.
15. Gupta PK. Methods in environmental analysis water, soil and air. Agrobios, India 2004; 242-5.
16. Dhindsa RS, Dhindsa PP, Thorpe TA. Leaf senescence correlated with increased level of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 1981; 32:93-101; http://dx.doi.org/10.1093/jxb/32.1.93.
17. Tester MR, Davenport R. Na^+ tolerance and Na^+ transport in higher plants. Ann Bot 2003; 91:503-27; PMID:12646496; http://dx.doi.org/10.1093/aob/mcg058.
18. Serrano R. Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int Rev Cytol 1996; 165:1-52; PMID:8900956; http://dx.doi.org/10.1016/S0074-7696(08)62219-6.