Emerging Importance of Helicases in Plant Stress Tolerance: Characterization of *Oryza sativa* Repair Helicase XPB2 Promoter and Its Functional Validation in Tobacco under Multiple Stresses

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Genetic material always remains at the risk of spontaneous or induced damage which challenges the normal functioning of DNA molecule, thus, DNA repair is vital to protect the organisms against genetic damage. Helicases, the unique molecular motors, are emerged as prospective molecules to engineer stress tolerance in plants and are involved in nucleic acid metabolism including DNA repair. The repair helicase, XPB is an evolutionary conserved protein present in different organisms, including plants. Availability of few efficient promoters for gene expression in plants provoked us to study the promoter of XPB for better understanding of gene regulation under stress conditions. Here, we report the *in silico* analysis of novel stress inducible promoter of *Oryza sativa* XPB2 (OsXPB2). The *in vivo* validation of functionality/activity of OsXPB2 promoter under abiotic and hormonal stress conditions was performed by *Agrobacterium*-mediated transient assay in tobacco leaves using OsXPB2::GUS chimeric construct. The present research revealed that OsXPB2 promoter contains cis-elements accounting for various abiotic stresses (salt, dehydration, or cold) and hormone (Auxin, ABA, or MeJA) induced GUS expression/activity in the promoter-reporter assay. The promoter region of OsXPB2 contains CACG, GTAACG, CACGTG, CGTCA CCGCCGCGCT cis acting-elements which are reported to be salt, dehydration, cold, MeJA, or ABA responsive, respectively. Functional analysis was done by *Agrobacterium*-mediated transient assay using agroinfiltration in tobacco leaves, followed by GUS staining and fluorescence quantitative analyses. The results revealed high induction of GUS activity under multiple abiotic stresses as compared to mock treated control. The present findings suggest that OsXPB2 promoter is a multi-stress inducible promoter and has potential applications in sustainable crop production under abiotic stresses by regulating desirable pattern of gene expression.

**Keywords:** agroinfiltration, rice, helicases, OsXPB2 promoter, abiotic stress, tobacco
INTRODUCTION

Essentially vital for all living organisms, the unique molecular motors, helicases unwind the duplex nucleic acids (i.e., DNA, RNA, or RNA-DNA hybrid) by using the free energy of ATP-binding/hydrolysis. Helicases remains present everywhere during the processing of nucleic acid in the cell and also emerged as potential candidate molecules for engineering abiotic stress tolerance in plants. Environmental cues continuously threaten the genomic integrity of all living organisms therefore in order to maintain the integrity of genome almost all the organisms throughout evolution contain robust DNA repair and recombination pathways to repair/remove or to tolerate lesions (Singh et al., 2011). Recent helicase research supports the potential of DNA/RNA helicases to counteract the adverse effect of various abiotic stress factors (Gill et al., 2014). OsXPB2 is a member of highly conserved helicase super family 2 (SF2), in eukaryotes and it plays a vital role in DNA metabolism such as transcription and repair (Umate et al., 2011). XPB also known as ERCC3 and RAD25 is a 3′–5′ DNA helicase and it is an essential subunit of the eukaryotic basal transcription factor complex TFIIH [contains seven subunits (XPB, XPD, p62, p52, p44, p34, and TTDA)] (Schaefler et al., 1993). XPB facilitates initiation of RNA polymerase II transcription and nucleotide excision repair (NER) by unwinding dsDNA around a DNA lesion. It has been reported that helicases play important roles in cell metabolic processes, including plant growth and development (Ribeiro et al., 1998; Costa et al., 2001). Various helicases have been known to function in providing abiotic stress tolerance to plants and few of them like PDH45, MCM6, and p68 have been reported to contain stress inducible promoters (Sanan-Mishra et al., 2005; Luo et al., 2009; Dang et al., 2011a,b; Tajirishi and Tuteja, 2011; Gill et al., 2013; Tuteja et al., 2013; Banu et al., 2014). Therefore, exploitation of stress inducible promoters of candidate helicase genes can further complement the stress tolerance potential of crop plants.

In the present scenario, the in silico analysis of sequenced plant genome has become a routine to study and predict the promoter sequences (upstream of the 5′ end of the gene) and their contributing cis-acting elements. However, the demonstration of promoter activity is essential in order to confirm the functions of putative cis-elements. It is well-known that inducible promoters have broad biotechnological applications in the regulation of stress-related genes that are activated as a result of abiotic and biotic stresses (Kasuga et al., 1999; Oettgen, 2001). The inducible plant promoters based on their responsiveness, can be categorized as responsive to endogenous signals (plant hormones), external stimuli (biotic and abiotic stresses), and chemical stimuli. The promoters harbor various cis-regulatory elements and play vital role in the plant gene expression and regulation. Gene regulation can occur during different stages of gene expression and the most important point of control is RNA transcription. The promoter of Cauliflower mosaic virus (CaMV) 35S and its derivatives are used frequently for constitutive expression of transgene in plants and to achieve higher transgene expression (Odell et al., 1985; Battraw and Hall, 1990). However, the constitutive expression of functional genes/transcription factors in genetically engineered plants sometimes results in undesirable phenotype like growth inhibition or significant yield penalty (Capell et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Hsieh et al., 2002). Therefore, the inducible promoters which can drive the expression of foreign genes under specific stresses can be of prime importance in engineering tolerance potential of crop plants (Kasuga et al., 1999). These inducible and tissue-specific promoters are central to the study of gene regulatory networks in plant (Huda et al., 2013; Oettgen, 2001). Different helicases like PDH45, MCM6, SUV3, p68, and BAT1 are shown to be upregulated by abiotic stresses including salinity, dehydration, wounding, and low temperature and their overexpression conferred stress tolerance in plants (Sanan-Mishra et al., 2005; Tran et al., 2010; Tuteja et al., 2013, 2014a,b; Manjulatha et al., 2014).

Helicases are an intriguing aspect of the plant response to various stress factors but their potential has so far been poorly explored. Therefore, the functional validation of the upstream regulatory part or promoter of the DNA repair helicase OsXPB2 gene is important for understanding its regulation under stress conditions. Thus, the isolation and functional characterization of OsXPB2 promoter with respect to abiotic stresses and hormonal treatments may be of potential importance for engineering stress tolerance. The results presented in this report suggest that OsXPB2 promoter can be a convincing tool that can be used as stress-inducible promoter for engineering crops with higher tolerance against abiotic stresses.

MATERIALS AND METHODS

In Silico Analysis of Promoter

The 1000 bp promoter sequence upstream of the start codon of the OsXPB2 gene (ID: Os01g49680; http://rice.plantbiology.msu.edu/) was retrieved from the rice genome database and cis-elements in the promoter were analyzed using PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.afrc.go.jp/PLACE/) database.

Amplification of OsXPB2 Promoter and Development of Chimeric Promoter-Reporter Construct

Genomic DNA was isolated from the leaves of Oryza sativa (Var. IR 64) by CTAB method and 30× dilution of genomic DNA was used as template for the amplification of OsXPB2 promoter. Sequences of DNA adaptors and primers used for promoter amplification are OsXPB2FW: AA CTGCAGAGACCCAGTGAAGCCACCCCATTA, OsXPB2RV: ATGGATCCACCAT GCC CGG AAG CCC TGG AGC. The amplified fragment was cloned into pJET2.1 vector (Thermo Scientific). Subsequently the promoter was cloned into pCAMBIA-1391Z (promoter less vector) at PstI and BamHI restriction sites (Figure 1A, Supplementary Figure 1). The OsXPB2 promoter cloned in pCAMBIA-1391Z was transformed in Agrobacterium tumefaciens (LBA4404) and confirmed by colony PCR using OsXPB2 promoter specific primers.
**Agrobacterium-Mediated Transient Assay**

*Agrobacterium*-mediated transient assay was performed to study the expression of *OsXPB2* promoter using the method described by Yang et al. (2000). Fully expanded leaves of tobacco (*Nicotiana tabacum* cv. USA) plants were agro-infiltrated by using 500 µl of bacterial suspension with 1 ml syringe into the abaxial surface of intact leaf. After 3 days, leaves were used for the stress and mock treatment analysis.

**Stress Treatments**

Agro-infiltrated leaf discs were soaked in petri dishes filled with 200 mM NaCl, 20% PEG (Polyethylene glycol), 5 µM ABA, 10 µM MeJA, or 10 µM NAA, respectively, and incubated for 1, 6, 12, or 24 h at room temperature. For cold stress, agro-infiltrated leaf discs were incubated at 4°C and the samples were collected at 1, 6, 12, and 24 h. Similarly, CaMV35S::GUS fusion construct transformed leaf discs were treated with H₂O and used as mock treated control.

**Histochemical GUS Staining and GUS Activity Quantification**

GUS histochemical staining was performed using the method described earlier (Jefferson et al., 1987). The protein extraction (Bradford, 1976) and GUS fluorometric analysis was done using the method described earlier (Huda et al., 2013).

**Statistical Analysis**

Statistically significant differences between mean values were analyzed by Student's *t*-test (*P* ≤ 0.05).

**RESULTS**

**Isolation of *OsXPB2*** Promoter from Rice and Analysis of CIS-Acting Elements

*OsXPB2* promoter was amplified using promoter specific primer pairs as described earlier (Supplementary Figures 1A–C). Cis-acting elements present in the *OsXPB2* promoter

| Element Position Database ID Strand Expected function |
|----------------|--------------------------|-------------------------------|
| CCGTCC-box 427 PC | -- | Meristem specific activation |
| CGTCA-motif 811 PC | + | MeJA-responsiveness |
| TGACG-motif 628 PC | + | MeJA-responsiveness |
| Skn-1_motif 627 PC | -- | Endosperm expression |
| Circadian 204 PC | + | circadian control |
| AC-1 119 PC | + | Enhanced xylem expression and repressed phloem |
| CAAT-box 60 PC | + | Promoter and enhancer regions |
| TC-rich repeats 52 PC | + | Defense and stress responsiveness |
| CACTFTPPCA1 517 (P)SO00449 | + | Mesophyll expression module |
| CGACGOSAMY3 718 (P)SO00205 | + | Expression during sugar starvation |
| box S 459 PC | + | Elicitation; wounding and pathogen response |
| ABRELATERD1 46 (P) S000414 | -- | Early responsive to dehydration |
| ARFAT 824 (P) S000270 | -- | Auxin response factor (ARF) |
| ASF1MOTIFCAMV 629 (P) S000024 | + | ASF-1 binding site in CaMV 35S promoter |
| BS1EGCCR 882 (P) S000352 | + | Vascular expression |
| CBFHV 49 (P) S000497 | -- | Dehydration-responsive element (DRE) |
| CCAATBOX1 165 (P) S000030 | + | Heat shock protein genes |
| GCCCORE 612 (P) S000430 | + | Ethylene-responsive element |
| GT1CONSENSUS 861 (P) S000198 | + | SA-inducible gene expression |
| MYB2CONSENSUSAT 70 (P) S000409 | + | Dehydration-response |
| MYBCCORE 152 (P) S000176 | + | Responsive to water stress |
| MYCCONSENSUSAT 211 (P) S000407 | + | Cold response |
| PREATPRODH 647 (P) S000450 | -- | Hyposmolality-responsive element |
| RGATAOS 254 (P) S000191 | + | Phloem-specific |
| TAAAGSKST1 863 (P) S000387 | + | Guard cell-specific |
| TATCCACHVAL21 42 (P) S000416 | + | GA response |
| WRKY71OS 629 (P) S000447 | + | Transcriptional repressor of the gibberellins signaling pathway |
| Motif lib 961 PC | + | Abscisic acid responsive element |
region as identified by in silico analysis are listed in Table 1. The promoter region has a transcription start site TATA (TACAAA, consensus TTCC) and CCAAT box at position −55 and −61 base pair, respectively (Table 1). The sequence analysis suggests that several cis-elements including defense and stress responsiveness (TC-rich repeats), early responsive to dehydration (ABRELATERD1), dehydration responsive elements (CBFHV), heat shock protein responsive element (CCAATBOX1), cold response (MYCCONSENSUSAT), and element responsive to water stress (MYBCORE) are present in the OsXPB2 promoter sequence (Table 1). The sequence also contains hormone responsive cis-acting elements like MeJ responsive CGTCA-motif, GCCCORE ethylene responsive element, GT1CONSENSUS SA response element and ABA responsive CGTCA-motif, GCCCORE ethylene responsive element, TAAAGSTKST1, and mesophyll expression elements (CACTFTPPCA1) are also present in the sequence (Table 1).

**Cloning of OsXPB2 Promoter and Its Activity in Tobacco Leaves**

The OsXPB2 promoter was cloned into pCAMBIA-1391Z and the clones were confirmed by PCR and restriction analysis (Supplementary Figures 1A–C). Different stress responsive cis-elements are shown in the OsXPB2 promoter sequence (Supplementary Figure 2). The fusion construct OsXPB2::GUS was transiently expressed in tobacco leaves and it was used to check the promoter inducibility under different abiotic and hormonal stress conditions at different time points to study time course of GUS activity. To check whether the isolated promoter region of OsXPB2 possesses active promoter functions, the tobacco leaves agro-infiltrated with OsXPB2::GUS or CaMV35S::GUS (as control) were mock treated (Figures 1B,C). There was no blue color development in the mock treated OsXPB2 promoter and very low level of GUS expression and activity was recorded (Figures 1B,J). The CaMV35S::GUS was also given mock treatment and an intense blue coloration developed at different time points suggesting very high GUS activity (Figures 1C,J). The activity of OsXPB2 promoter was analyzed under different abiotic stress (Salt, PEG, or cold) conditions by transient assay (Figures 1D–F). Histochemical staining revealed that the GUS expression increased at 12–24 h as compared to 1 and 6 h (Figure 1D). The effect of PEG stress varied for the OsXPB2 promoter; the blue staining was detected at 1–6 h of stress treatment, but the intensity of the blue color gradually increased from 12 to 24 h, and similar trend was also observed in the quantitative GUS activity (Figures 1E,J). Under cold stress treatment at the early time period 1–6 h, slight blue coloration was detected and increase in the blue color intensity was present at 24 h (Figures 1E,J). These results reveal that OsXPB2 promoter is a stress inducible promoter and it mainly responds to osmotic and cold stresses.

Leaf disks were also incubated with different hormones (NAA, ABA, or MeJA) and differential pattern in GUS activity was noted (Figures 1G–I). The GUS expression driven by OsXPB2 promoter under NAA treatment showed moderate blue color at 1–12 h and slight increase was noted at 24 h and the corresponding GUS activity was also recorded (Figures 1G,J). It is interesting to note that in the ABA treatment the blue color was intense in the initial time period and it sustained up to 24 h and the GUS activity recorded was also high (Figures 1H,J). Furthermore, in the MeJA treated leaves the blue color developed but the variation in the blue color did not differ much up to 24 h, and the corresponding GUS activity was recorded (Figures 1I,J). The observed variation in GUS expression levels may be due to difference in response of cis-acting elements of OsXPB2 promoter.

**DISCUSSION**

Helicases, the motor proteins have vast potential as modulators of stress responses in plants. The new emerging role of helicases in engineering plant abiotic stress tolerance has encouraged studying the associated promoters for better understanding of gene regulation under stressful conditions. At present, constitutive and inducible promoters are widely used for the expression of candidate genes and their functional analysis. However, the constitutive expression of transgene may lead to homology-dependent gene silencing. Therefore, the exploitation of inducible promoters may be a vital tool for spatial and temporal gene expression under stress. In this study, we have presented important information regarding the complex regulation of rice helicase promoter in response to different abiotic stresses. Recent report regarding the function of OsXPB2 gene in DNA damage and the concomitant activation of TC-NER pathway in response to γ-radiation and salinity stress emphasizes the importance of helicases in abiotic stress tolerance (Macovei et al., 2014). Therefore, the identification and functional validation of cis-elements is crucial in understanding the regulation of promoter and its possible exploitation in transgenic research. It is well-established that the putative regulatory elements in plant promoters can be easily identified using in silico analysis (Pujade-Renaud et al., 2005; Wei-Min et al., 2005; Huda et al., 2013). The analysis of cis-regulatory elements present in the promoter regions have received special attention as they provide insights into gene regulation and plant signaling under stress conditions. Further, Agrobacterium-mediated transient expression assay is a widely accepted method for in vivo quantitative analysis of plant promoters and cis-element/trans-factor interactions (Yang et al., 2000). The analysis of the expression of GUS reporter gene in OsXPB2::GUS revealed abiotic and hormonal regulation of GUS expression.

Brosché et al. (2002) reported that the promoter of XPD helicase of Arabidopsis thaliana contains multiple cis-elements [AGCT, ACCAA, H-box, myeloblastosis (Myb), Myb recognition element (MRE), SET binding factor 1 (SBF-1) and TCA-element, salicylic acid-responsive element] and has implication in light
regulation and in UV stress response. It has also been reported that AtXPD gene was among some DNA repair genes that are hypomethylated in the promoter region (Boyko et al., 2010). Hypomethylation was reported to be correlated with permissive chromatin histone modification and increased AtXPD expression (Boyko et al., 2010). The significance of few cis-regulatory elements like G-box and ABREs combinations have also shown that stress-responsive genes are regulated by multiple transription factors (Abe et al., 1997; Liu et al., 2014; Wang et al., 2014). Therefore, functional analysis of cis-regulatory elements is crucial to understand the regulatory gene networks in stress-responsive pathways. Our present study demonstrates the presence of different stress responsive cis-elements in OsXPB2 promoter that are associated with tissue-specific expression, meristem specific, endosperm specific expression, defense and stress responsiveness, vascular expression, phloem specific, guard cell specific, and mesophyll expression module. The presence of these tissue-specific expression regulatory elements indicates the association of OsXPB2 gene to a wide range of cellular processes which still requires validation. In addition, the in silico analysis of OsXPB2 promoter suggests the presence of salt or dehydration responsive cis-acting elements in the sequence.

In vivo analysis of OsXPB2::GUS construct revealed that GUS expression was induced by different abiotic stresses and OsXPB2 promoter was able to drive GUS expression when agro-infiltrated in tobacco leaves treated with NaCl, PEG or cold stress. The presence of multiple copies of the NAC like element (5′-CACG-3′) in the upstream region of OsXPB2 gene might be responsible for salt induced expression. Tran et al. (2004) reported that NAC–type transcription factors regulate salt responsive genes in an ABA-dependent manner. Salt stress was also shown to induce several NAC genes in rice (Hu et al., 2006). It has been reported that OsNAC5 salt inducible NAC transcription factor which binds to the NAC recognition core sequence (CACG) of OsLEA3 promoter, when overexpressed, showed improved salt tolerance (Takasaki et al., 2010).

Furthermore, the PEG and ABA treatment leads to higher GUS activity. A significant increase in ABA levels has been observed in response to dehydration stress. Previous reports also support that most dehydration-inducible genes are induced by ABA (Chandler and Robertson, 1994; Shinozaki et al., 2003). The upstream region of OsXPB2 gene also contains multiple cold responsive elements, e.g., MYCCONSENSUSAT (CACGTG). Chinnusamy et al. (2003) reported that cold stress induced ICE1 binds to MYC cis-elements of the CBF promoter in Arabidopsis and it induces the expression of CBF, which regulates the COR genes and imparts cold acclimation.
Plant hormones are known to mediate the defense processes against pathogenic attack and herbivors (Ohshima et al., 1990). Furthermore, phytohormones like salicylate, jasmonates, and ethylene are reported to be involved in plant responses to various stresses (Ohshima et al., 1990). It is well-established that phytohormone auxin regulates several physiological processes such as apical dominance, shoot elongation, lateral root initiation, vascular differentiation, embryo patterning etc. (Davies, 1995) and enhances the transcription of various genes (Aux/IAA, GH3, and SAUR gene family members) (Abel and Theologis, 1996). In the present study, we have identified auxin responsive cis-regulatory elements in OsXPB2 promoter sequence and high GUS expression was observed in the agro-infiltrated tobacco leaves. Jasmonates including MeJA and JA are also key signaling molecules for diverse developmental processes from seed germination to fruit ripening and senescence (Wasternack and Hause, 2002). The GCC or G-box elements, CGTCA motif and TGACG motif are required for MeJA-inducible expression of different genes. The role of JA in response to various abiotic stresses has been reported in a number of studies (Clarke et al., 2009; Yoon et al., 2009; Brossa et al., 2011). The analysis of GUS expression in response to MeJA indicates that the cis-elements present in OsXPB2 promoter may have positive regulatory role toward stress tolerance.

In the present study, using histochemical analysis (qualitative and quantitative) we have demonstrated that the OsXPB2 promoter is able to drive GUS reporter gene expression in response to abiotic stress and hormonal treatments. The cis-elements identified in OsXPB2 promoter together with the data from GUS reporter gene expression profiles under different abiotic stresses, support that OsXPB2 promoter is stress responsive. The transient assay results along with GUS fluorometric assay results show that the OsXPB2 promoter triggers high levels of GUS expression under abiotic and hormonal treatment stresses. Our data collectively suggest that the OsXPB2 promoter analyzed in the present study could be potentially used to drive transgenes based on its responsiveness to different abiotic stresses including the genotoxic stress for the crop improvement.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01094

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