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PCR Amplification, Sequencing and *In Silico* Sequence Analysis of Germin-like Protein Gene Promoter in *Indica* Rice

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Abstract—*Germin and germin-like proteins (GLPs) are extracellular water soluble proteins expressed in response to some developmental and environmental signals. These proteins represent a large family of plant proteins which are also used as germination markers owing to their high expression levels during germination/early growth. These proteins are known to be resent in many stress associated processes as well, but their biochemical functions and physiological roles have not been fully described. Detection of transcription related regulatory elements in promoter regions of the concern gene coding for germin/GLPs is of great interest for biologists as these elements regulate gene expression. The information about core elements of promoters can reveal the function/regulation of the gene. With this understanding the present study was carried out on 'PCR amplification, sequencing and in silico sequence analysis of Germin-like protein gene promoter in Satabdi variety of indica rice.*

Keywords*: Germin-like proteins, Oryza sativa, promoter.*

1. INTRODUCTION

Rice (*Oryza sativa* L.) is considered as an important staple food in the world as well as in our country. Around two billion Asians obtain 60-70% of their energy intake from rice and its derivatives. It is also an excellent model species in plant biology, especially for studies on monocotyledonous plants, because of its small genome size (430 Mb), availability of complete genome sequence as well as efficient genetic transformation technology and vast genetic resources. Rice plants are frequently exposed to a plethora of biotic stress conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. So there is ample scope for developing transgenic plants with tolerance to various stress factors which necessitates particular transgenes and its specific promoters. Germin was initially identified as a specific marker for the start of germination in wheat embryos, from which function it was given the name 'germin' [21]. Germins also constitute a group of homologous proteins only found in "true cereals [13] providing the name 'Germin-like protein' (GLP). Germin and GLPs, 130 kDa homologomeric glycoprotein composed of 26 kDa subunit, which was originally identified in monocots [14] are encoded by a family of genes found in all plants.

The subgroups of GLPs have different enzyme functions that include the two hydrogen peroxide-generating enzymes, oxalate oxidase (OxO) and superoxide dismutase (SOD) which are known to be associated with the biotic and abiotic stresses. Additionally they help in osmotic regulation, photoperiodic oscillation, defence, a poptosis and are also founded to be associated with cell wall deposition [3, 15, 16]. Germin relatives have also been identified in fern spores, prokaryotes and animals. Over expression of wheat germin in sunflowers (*Helianthus annuus*) influences some defence-related transcripts and increases resistance to pathogens [11]. Moreover, tobacco leaf apoplast proteome showed changes in response to salt stress and level of a GLP increased significantly along with chitinases [5]. But functions of many GLPs remain biochemically uncharacterized. In order to gain insight into the functions and regulation of a rice GLP gene (OSRGLP1), about 1.27 Kb of its upstream region was amplified, sequenced and analyzed by Yasmin *et al*. [25].

Most GLPs have been identified from cDNA sequences. Currently, there are 58 complete cDNAs or genes from 16 plant species in the databases defining GLPs [4]. The structural and integral activity of the promoter can only be unveiled through a functional insight into GLPs and the analysis of corresponding promoter regions. Identification of transcriptional regulatory elements within promoter regions is of great interest for biologists since these elements govern the regulation of gene expression. Knowledge about different core elements of promoters can unveil the function/regulation of genes. The main rationale behind the present study was amplification and sequencing of a rice GLP gene promoter followed by computational analysis for identification/ characterization of possible putative regulatory elements.

2. MATERIAL AND METHODS

The rice variety Satabdi (IET 4786) was considered as experimental material which was collected from Chinsurah Rice Research Station, Hooghly.

The young healthy healthy and diseased free seedlings with two tender leaves were screened and taken for extraction of genomic DNA to be used as a template for amplification of the promoter region. Plant genomic DNA was extracted following the method of Doyle and Doyle [6] slightly modified by Bhattacharyya and Mandal [2].

Two sets of primers wear designed using Primer 3 software to amplify the upstream sequence of *Oryza sativa* GLP promoter gene and synthesized from Xcelris genomica, Ahmedabad. The sequences for the oligo primers are as follows:

OGL2 (F): 5′GGAATCGAA TTCATGCCACCTGGACGATG ACATCG -3′and UTROGL2(R): 5′GGAATCAAGCTTGG TGGATTACTAGTGTCGCTAGG-3′and UTROGL1(F):

5′-GCTCAGTGCAACGCCACTGG-3′and **Rev** OGL1(R):

5′-TTGTGATCTGATACGATTAATCACC-3′.

The PCR mixture of 25 µl volume contained 4 µl (20 ng) diluted DNA (Satabdi rice), 2.5 µl 10X PCR buffer, 1µl 2.5 mM dNTPs, 2 µl primer (100 ng), 0.5 µl Taq DNA Polymerase enzyme and 15 µl double distilled sterile water. Reaction condition was programmed as initial hold at 94°C for 5 min and then 35 cycles for denaturation at 94°C for 45s, annealing of primer at 62°C for 45s, extension at 72°C for 1.0 min. Further extension was allowed at 72°C for 5 min, followed by holding the samples at 4°C for 5 min. On completion of reaction, 4 µl of PCR product were loaded on 1% agarose gel. After electrophoresis the PCR product was purified by the PCR product purification kit from Xcelris genomics and sent to Xcelris genomics, Ahmedabad for sequencing.

***In silico* sequence analysis-** The sequence homology searching was carried out using BLAST. The promoter sequence was aligned with other GLP promoters using CLUSTAL W [22] available at www.ebi.ac.uk/clustalw to find out homology. To find out the regulatory elements, their types and positions, whole promoter sequence was analyzed using PLACE/Signal Scan [10] available at www.dna.affrc.go.jp. Many regulatory elements were traced by Signal Scan on both the strands of promoter; however, only cis elements without any ambiguous nucleotide were considered.

3. RESULTS AND DISCUSSION

Germins and germin-like proteins (GLPs) constitute a large and highly diverse family of ubiquitous plant cell wall proteins. These proteins seem to be involved in many developmental stages and stress-related processes, but their exact participation in these processes so also the function of the concerned gene(s) generally remains obscure. Significant insight into their role may be gained by analysis of gene functions especially their promoter. All these thus lead to carry out the present work in indica rice variety Satabdi.

After electrophoresis primer OGL-2 gave the PCR product of approximately 875 basepair (Fig.1), while the primer OGL-1 did not produce any amplicon. The amplified PCR product was purified by the PCR purification kit from Xcelris genomics and sent to the Xcelris genomics, Ahmedabad for sequencing.

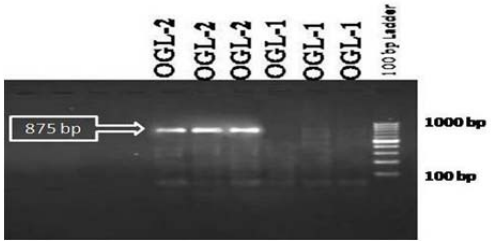

Fig.1. Agarose gel picture of PCR amplicon (approx. 875 bp) of Satabdi GLP Promoter gene (first three lanes from the ladder containing OGL1 PCR product and next three lanes containing OGL2 PCR product)

BLAST search results and multiple sequence alignment

In BLAST searching BCKV isolate showed maximum homology with Pakistani isolate (Accession No. EU74284.1) and minimum homology with another isolate of Pakistan (Accession No. KJ807471.1). Multiple sequence alignment using CLUSTAL W 2.1 of BCKV sample to the other available GLP promoter sequence from NCBI data base showed maximum homology with the Pakistani isolate (Accession No. EU 742684) and minimum homology with other Pakistani isolate (Accession No. KJ807471, KJ807473 and KJ807472) (Table 1).

Table 1: Pair-wise table showing homology percentage on the basis of CLUSTAL W 2.1 multiple sequence alignments

Sequence	KJ807471 Pakistan (1)	AP01494J apa (2)	KJ807P akistan(3)	AP00551J apan (4)	AP00505J apan (5)	KJ807473Pakistan (6)	KJ807472Pakistan (7)	KJ807475Pakistan (8)	**BCKV India (9)**	EU742684Pakistan
KJ807471Pakistan(1)	**100**	99.79	99.80	99.79	99.79	99.80	99.80	99.70	**97.35**	94.67
AP014964Japan(2)	99.79	**100**	100	100	100	99.79	99.79	99.89	97.58	94.89

KJ807747Pakistan (3)	99.80	100	**100**	100	100	99.80	99.80	99.90	97.58	94.89
AP005531Japan(4)	99.79	100	100	**100**	100	99.79	99.79	99.89	97.58	94.89
AP005505Japan(5)	99.79	100	100	100	**100**	99.79	99.79	99.89	97.58	94.89
KJ807473Pakistan (6)	99.80	99.79	99.80	99.79	99.79	**100**	99.80	99.90	97.35	94.67
KJ807472 Pakistan (7)	99.80	99.79	99.80	99.79	99.79	99.80	**100**	99.90	97.35	94.67
KJ807475 Pakistan (8)	99.70	99.89	99.90	99.89	99.89	99.90	99.90	**100**	97.47	94.78
BCKV India (9)	97.35	97.58	97.58	97.58	97.58	97.35	97.35	97.47	**100**	97.80
EU742684Pakistan(10)	94.67	94.89	94.89	94.89	94.89	94.67	94.67	94.78	**97.80**	**100**

Promoter sequence analysis

Many putative regulatory elements of variable lengths were identified by PLACE/Signal Scan (Table 2) and mapped on the promoter (Fig. 2). The putative elements were distributed densely in two main clusters (- 10 to -330 bp and -330 to -775 bp) (Table 3, Fig. 2). On whole promoter sequence except from -775 to -873 bp region where density of elements were very much low, suggesting that the promoter lies within -775 region of the sequence and that whole of it has been sequenced.

Table 2: Regulatory elements found in promoter of rice (Satabdi) GLP promoter

Regulatory elements	No. of copies	Location	Functions	Reference
GTAC (core ofa CuRE-copper-responseelement)	1	-308	Involved in oxygen response of Cyc6 and Cpx1 genes	[17]
AAAG	3	-622, -41, -10	Binding site for Dof proteins	[24]
TGAC (core sequence in W box)	4	-771, -321, 756,-385	Pathogen inducibility, gibberellin signaling	[8]
AGAAA	1	-458	Pollen specific expression	[23, 9]
GATA	6	-611,-641,-652, -163, -267, -364	Light regulated and tissue specific gene expression	[12]
GTGA	5	-265, -471, -575,-660, -33	Found in promoter of tobacco late pollen gene g10	[18]
GATAA	2	-611, -163	Function in expression oflight-regulated genes	[20]
TTGAC/TTGACC (W box)	3/1	-384,-770,-320(TTGAC) -384 (TTGACC)	Gibberellin signaling pathway	[23]
CATGTG	3	-522, -324, -30	Involved in water stress	[19]
CACATG	2	-791, -520	Involved in water stress	[1]
CAAT	3	-345, -554,-730	Tissuespecificpromoter activity	[18]
ATATT	5	-129, -194,-203, -217, -229	Root specific expression	[7]
ACGT	4	-101, -336, 588,-687	Dark and senescence inducedexpression	[19]

Table 3: Distribution of regulatory elements found in promoter of rice (Satabdi) GLP promoter

Range of location of regulatory region		Locations	Total no. of locations where regulatory elements are found
A	(-10 to -330)	-10, -30, -33, -41, -129,-163, -163, -203, -101,-194, -217, 229, -265 , -267, -308,-320, -321,-324,	18
B	(-330 to -558)	-385, -384, -364, -345, -384, -458, -471, -336, -554, -520, -522	11
C	(-558 to -775)	-588, -575, -611, -622, -641, -652, -687, -660, -756, -771, -770, -730, -741	13
D	(-775 to -873)	-791, -822	2

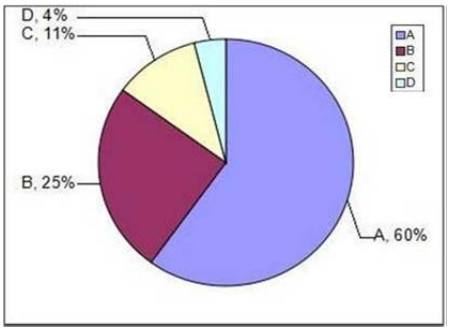

Fig. 2. Distribution percentage of 4 regulatory regions (A, B, C and D) reveals that promoter sequences are likely to be present in between -10 to -741 regions (A to C region)

Phylogenetic analysis

The sequence of BCKV sample was compared with the other reported isolates of GLP promoters through CLUSTAL W programme. An unrooted neighbor joining tree was generated which displayed that the BCKV isolate was genetically more close to Pakistani isolate (Accession No. EU742684) (Fig. 3), which is also evident from the data generated by BLAST search and multiple sequence alignment.

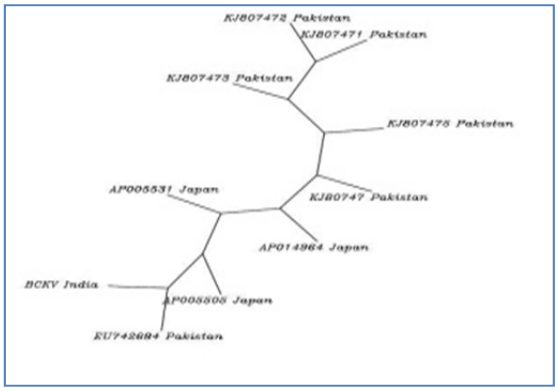

Fig. 3. Unrooted neighbor joining tree showing maximum similarity of BCKV isolate with Pakistani isolate EU 742684

The regulatory elements search by the Signal Scan resulted in number of notable elements like tissue and developmental stage specific elements, elements related to inducible expression and biotic or abiotic stress related elements (Table. 2).

The regulatory elements searched by the Signal Scan resulted innumber of notable elements like tissue and developmental stage specific elements, elements related to iducible expression and biotic and abiotic stress element elements (Table 2).

4. CONCLUSION

Germin and germin-like proteins (GLPs) are water soluble extracellular proteins reportedly expressed in response to some environmental and developmental signals. Some enzymatic activities have also been associated with germin/GLPs. However, the function or role of the concerned genes is not yet fully understood. Therefore, to know the function of the genes, researchers are motivated to identify, clone and characterize the promoters of germine/ GLP promoter in rice. The present study involving the identification of putative GLP promoter gene in *indica* rice is a primary work leading to cloning, characterization and specific expression of regulatory elements. Controlled transgene expression via a promoter may particularly trigger in response to biotic and abiotic stresses. This is significant for eliciting stress resistant features in crops through genetic engineering.

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