Isolation and Characterization of an Endosperm-Specific Promoter from Wheat (*Triticum aestivum* L.)

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Genes coding for avenin-like proteins (ALP) represent a new family of wheat storage protein genes. To find a wheat endosperm-specific promoter, a 1644-bp fragment upstream of the *ALP type-B* gene (GenBank accession number JN622144) was isolated. The important promoter elements of the *ALP type-B* gene were ascertained through sequence analysis which revealed that this fragment contains the TATA and CAAT boxes, which are important elements in gene expression. A prolamin box containing an endosperm motif and a GCN4-like motif (GLM) is present at about 300 bp upstream of the translation start site. The promoter sequence has two ESP-like elements and one of them is followed by an RY motif with the nucleotides CATG overlapping. The RY motif is considered the core functional sequence in a promoter. In an attempt to confirm the promoter activity, a series of 5′-deletions of the promoter were fused with the *β*-glucuronidase (GUS) gene, and the constructs were stably introduced into tobacco plants. GUS staining confirmed that the *AVL type-B* promoter is an endosperm-specific promoter in tobacco seeds. Quantitative analysis of GUS expression in transgenic plants showed that even the shortest 5′-deletion, *i.e.* a 290-bp promoter sequence within the prolamin box, was sufficient to drive GUS expression in the endosperm. The highest expression level was found in transgenic plants containing the 5′-deletion vector construct pALP-8. This suggests that the ESP-like element overlapping with the RY motif may play a crucial role in the regulatory function of the promoter.

Key words: Wheat, Endosperm-Specific Promoter, Tobacco

Introduction

The endosperm is the storage organ of starch and proteins in cereal seeds. Thus, proteins expressed in the endosperm can be kept indefinitely in mature seeds in various organelles depending on the type of crop (Tandang-Silvas *et al.*, 2011). Wheat provides staple food for about 35% of the human population. Due to wheat's rheological properties, wheat flour is unique for the production of leavened bread and a wider range of foodstuffs which take advantage of these attributes (Gianibelli *et al.*, 2001). Cereal seed storage proteins (SSP) are classified into: globulins, albumins, glutelins, and prolamins (Tandang-Silvas *et al.*, 2011). The unique properties of the wheat grain reside primarily in the gluten-forming storage proteins of its endosperm, the prolamins (gliadins and glutenins) (Shewry *et al.*, 1997; Vasil and Anderson, 1997; Gianibelli *et al.*, 2001; Caballero *et al.*, 2004). Prolamins are the largest known protein polymers with molecular masses ranging into tens of millions of Daltons (Wrigley, 1996). The bread-making quality of wheat flour is determined particularly by the high-molecular weight glutenin subunits (HMW-GS) (Branlard and Dardevet, 1985; Flavell *et al.*, 1989), which are often primary targets for the biotechnological improvement of the bread-making quality of wheat flour (Blechl *et al.*, 2007). A number of glutenin

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genes have been introduced into bread wheat (*Triticum aestivum* L.) and pasta wheat (*Triticum turgidum* L.) by genetic transformation (Stöger *et al.*, 2001; Matta *et al.*, 2009).

Avenin-like proteins (ALP) are a small family of wheat storage proteins, so named because of the similarity of their sequence to the avenin proteins of oats. They have two isoforms: ALP type-A and ALP type-B. The distinguishing feature of these proteins is that they contain high levels of cysteine residues, which aid in correct folding and maintaining the tertiary structure of proteins (De Caro *et al.*, 2010). In dough mixing studies, the ALP type-B protein increased the mixing time and peak dough resistance and decreased the breakdown in resistance (Chen *et al.*, 2010). Genes of *ALP type-B* have been characterized in 23 species of Triticeae, including 18 species of *Aegilops*, barley, and wheat. In wheat, the presence of ALP type-B proteins and their respective mRNAs in endosperm have been confirmed by Western blotting and reversed transcription-polymerase chain reaction (RT-PCR), respectively, but not in roots, stems or leaves, suggesting that the promoter has endosperm specificity (Chen *et al.*, 2008).

A promoter is a regulatory region of DNA located upstream of a gene, providing a control point in the regulation of gene transcription. For expression of a protein in a host organism, a promoter can be selected based on the desired outcome, and may include constitutive, tissue-specific, inducible or other promoters (Hensel *et al.*, 2011). Constitutive promoters may be active in most environmental conditions, developmental and/or cell differentiation stages (Kim *et al.*, 2006). A tissue-specific or developmentally regulated promoter regulates directly the expression of a DNA sequence selectively in the cells/tissues of a plant (Hensel *et al.*, 2011). Many seed-specific promoters have been identified, such as those of the genes coding for zein (maize endosperm), phaseolin (bean cotyledon), phytohemagglutinin (bean cotyledon), *β*-conglycinin and glycinin (soybean cotyledon), and glutenin and gliadin (wheat endosperm). In order to understand the regulatory mechanisms of endosperm-specific genes in wheat, an *ALP type-B* promoter was isolated by inverse PCR (IPCR), and its heterologous expression specificity was investigated in transgenic tobacco (*Nicotiana tabacum* L.).

Material and Methods

Isolation of ALP type-B promoter by IPCR

IPCR was performed according to Digeon *et al.* (1999). Three rounds of IPCR were carried out. The restriction enzymes *Pvu*II, *Bcl*I, and *Nco*I were used to digest the genomic DNA of wheat (*Triticum aestivum* L.) cultivar EN1 isolated according to Stacey and Isaac (1994) and then circularized as template for each round of IPCR. The two pairs of nested primers (PVA1, 5′-GAAGCCCTGGCTACATGTGG-3′, and PVS1, 5′-AGCAGATGAGGCCGTGTGTG-3′; PVA2, 5′-CGAGGAGAGCCAGGATGAAGA-3′, and PVS2, 5′-CTGAGAATGCCGTTCCTCCA-3′) used in the first round of IPCR were designed according to the sequence of the *ALP type-B* gene (GenBank accession number, EU096549). Primers used in the second round of IPCR (BCA1, 5′-CTCTGATGTGATGGAACCGAAC-3′, and BCS1, 5′-TGTAAAGGGCATCCTACGAGTC-3′; BCA2, 5′-GGTCATGTGCTATGAGTCTGTAA-3′, and BCS2, 5′-GGAACACCTCTTCACACCTAAT-3′) were designed according to the sequence obtained from the product of the first round of IPCR. In the third round, IPCR primers (NCA1, 5′-CAAGACGAACGGTGATT-3′, and NCS1, 5′-TGTCCCTTCTAGTTGATCG-3′; NCA2, 5′-AGAACTATCTCAGAGGCTTG-3′, and NCS2, 5′-TATTACCGACCGACAAAG-3′) were designed in the same way. For each round of nested PCR, the reaction mixture was diluted 20-fold after the first PCR, and 1 μl was used as the template for the second PCR. The PCR products were purified and cloned into the vector pMD18-T (TaKaRa, Shiga, Japan). Then the Top10 competent cells were transformed with this vector construct. The positive recombinant clones were selected by PCR and sequenced by AuGCT Biotechnology Co. (Beijing, China).

Sequence analysis

With the combination of the three sequences isolated by IPCR, a 1664-bp sequence, upstream of the ATG translation start site was obtained (Fig. 1). Putative functional promoter elements were analysed by the PlantCARE (plant *cis*-acting regulatory elements) (Lescot *et al.*, 2002) and PLACE (Higo *et al.*, 1999) databases. Based on the locations of the motifs, different 5′-dele-

Fig. 1. Analysis of the sequence upstream of the translation initiation site of the *ALP type-B* gene. Various elements of the promoter likely related to its endosperm specificity are underlined and their names are given on top. The sites where the promoter sequence was shortened are marked by arrows. The overlapping sequence between the ESP-like element and the RY motif is boxed and italicized. The start codon ATG is boxed as well.

tion fragments were selected and amplified to construct the expression vectors.

Construction of the expression vectors and deletion analysis

The full-length *ALP type-B* promoter sequence and four 5′-deletions were selected for the evaluation of their ability to direct expression. These five promoter sequences were cloned in the vector pBI121, making sure that they replaced the constitutive promoter *CaMV* in each of the respective constructs. Five sense primers named p3S (5′-TGTAAAGTGATGATTGATGAG-3′), p6S (5′-GCACTACTAATGGATGTTTATC-3′), p8S (5′-CCCAATGAACACTAAATATGG-3′), p10S (5′-GATTTATTACCGACCGACA-3′), and p17S (5′-TCCACACAACCTACAAGT-3′) were designed, corresponding to the upstream sequences beginning at -290, -550, -785, -999, and -1664 bp, respectively. Paired with the sense primers, an anti-sense primer designated pA (5′-GGTGGCTAGCCTCAAGT-3′) was designed. To replace the *CaMV* promoter region in pBI121 at the *Hin*dIII/*Bam*HI site, a *Hin*dIII site was added to the 5′ end of each sense primer, and a *Bam*HI site was added to the 5′ end of the anti-sense primer. These five fragments were digested with *Hin*dIII and *Bam*HI and inserted into the pBI121 vector (Jefferson *et al.*, 1987). The resulting vectors were named pALP-3, pALP-6, pALP-8, pALP-10, and

pALP17. All inserted fragments were sequenced on both strands, and no mutations were found.

Transformation and selection of the transgenic tobacco plants

The five vector constructs and the control vector pBI121 were introduced into competent cells of *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method (Höfgen and Willmitzer, 1988). Then these bacterial cells were introduced into tobacco leaf discs (Horsch *et al.*, 1985). The transformants were selected by their resistance to kanamycin, and the transgenic plants were confirmed by PCR. Five plants for pALP-3, four for pALP-6, five for pALP-8, five for pALP-10, and nineteen for pALP-17 were found to be transgenic.

Histological β-glucuronidase (GUS) staining

GUS staining was performed as described by Jefferson *et al.* (1987). Tissues from transgenic and wild-type tobacco plants were washed briefly in 75% (v/v) ethanol and then rinsed with sterile distilled water. All tissues were incubated in X-gluc buffer for 16 h at 37 °C and 24 h at 26 °C. Chlorophyll was extracted from green tissues to allow the visualization of GUS activity by washing tissues in 70% ethanol, followed by several changes in 100% ethanol. After GUS reaction, the samples were viewed under a dissecting microscope.

Quantitative GUS analysis

For the quantitative GUS activity analysis, mature seeds were collected from the transgenic or wild-type plants and ground in GUS extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% SDS, 0.1% Triton X-100, and 10 mM *β*-mercaptoethanol). The mixture was then centrifuged at 12000 x *g* for 15 min at 4 °C. GUS activity in the supernatant was determined in the extraction buffer, containing 1 mM 4-methylumbelliferyl-*β*-D-glucuronide (MUG). After incubating at 37 °C for 24 h, the reaction was terminated by adding 0.2 M Na_2CO_3. Fluorescence was measured in a Perkin Elmer (Kyoto, Japan) luminescence spectrometer LS55 with excitation at 365 nm and emission at 455 nm. The protein concentration of extracts was determined as described by Bradford (1976). For each transgenic plant, independent assays were performed at least three times and the average was calculated.

Results and Discussion

Promoters play a crucial role in determining tissue, expression stage, and/or cell specificity in a transgenic organism (Kim *et al.*, 2006). The type of promoter, rather than the chromosomal site of integration, is critical for transgene expression (Jackson *et al.*, 2001). Dicot promoters exhibit relatively lower activity in monocots (Kim *et al.*, 2006), and heterologous promoters do not always maintain tissue specificity and activity (Chen *et al.*, 1998). Thus, currently there is a lack of well characterized wheat-derived promoters for transformation. To mitigate the effects of various complications faced in the functional analysis of transgenic plants, endosperm-specific promoters are needed to target expression and enhance yield and quality. Though some wheat-derived endosperm-specific promoters have been used in wheat transformation (Wiley *et al.*, 2007; Pistón *et al.*, 2008), many studies have demonstrated that repeated use of the same or similar promoters may lead to more frequent transgene silencing caused by a high degree of sequence homology or by a higher chance of rearrangement among duplicated promoter fragments (Bhullar *et al.*, 2003). Therefore, more endosperm-specific promoters are needed for application in cereal transformation.

In this study, the promoter of the endosperm-specific gene *ALP type-B* from wheat was cloned by IPCR, and its heterologous expression specificity in tobacco was studied. No homologous sequence was found by BLAST search, which means that the *ALP type-B* promoter is being reported here for the first time. A putative TATA box and CAAT box, the base sequences that assist in the regulation of gene expression, are present upstream of the translation initiation codon (ATG) at positions -86 and -140 bp, respectively. Through analysis based on the PLACE and PlantCARE databases, multiple potential *cis*-acting elements were found in the *AVL type-B* promoter (Table I), which has two endosperm motifs (5′-TGTAAAG-3′) at positions -290 and -579 bp. The former endosperm motif is followed by a GCN4-like motif (GLM, 5′-ATGAGTCAT-3′) at position -274 bp; together they form the putative prolamin box which was first called the -300-

Table I. Potentital endosperm-specific *cis*-acting elements found in the *ALP type-B* promoter.

Element	Sequence	Position	Reference
Endosperm motif	TGTAAAG	-290 -579	Halford and Shewry (2007)
ESP-like	CATGACCCATG CATGGATCATG	-677 -388	Vickers *et al.* (2006)
G box	CACGTG	-881	Ishige *et al.* (1999)
GLM	ATGAGTCAT	-274	Oñate *et al.* (1999)
RY motif	CATGCA	-690 -1406	Moreno-Risueno *et al.* (2007)

bp element. The *AVL type-B* promoter sequence has two potenital RY motifs (5′-CATGCA-3′) at positions -690 and -1406 bp. Two palindromic sequences, 5′-CATGACCCATG-3′ and 5′-CATGGATCATG-3′, are present at positions -677 and -388 bp, respectively. These two sequences are similar to the ESP element (endosperm specificity palindrome, 5′-ACATGTCATCATGT-3′) which was identified in an oat globulin promoter and found to be involved in endosperm specificity (Vickers *et al.*, 2006). There is a putative G box (5′-CACGTG-3′) in this sequence at -881 bp, which is considered to have long-distance positive effects.

To further ascertain the length of the promoter that is crucial for endosperm-specific expression, a series of 5′-deletions of the *AVL type-B* promoter were fused with the GUS gene and stably introduced into the tobacco plants. Tissues from all of the T_0 transformants positive for the transgene were analysed by histochemical staining. All plants harbouring either of the five vectors showed GUS activity only in the endosperm (Fig. 2), while GUS expression was not observed in any other organ, such as leaf, root, stem or flower, in either transgenic or wild-type plants (data not shown). The control plants transformed with the pBI121 vector harbouring the constitutive *CaMV* promoter showed GUS activity in all of the tested organs including the embryo and endosperm (Fig. 2). These results explicitly confirm the endosperm specificity of the *AVL type-B* promoter. However, different levels of GUS activity driven by the different 5′-deletion constructs were observed which indicates the existence of some functional regulatory elements in these fragments.

GUS fluorometric assays were performed to determine the strength of GUS expression in mature seeds of the transgenic plants. As shown in Fig. 3, although the vector pALP-3 with the

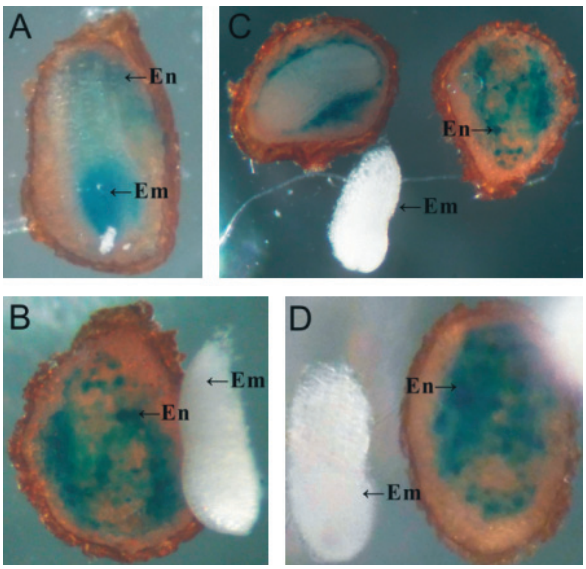

Fig. 2. Histochemical localization of GUS activity in mature seeds of tobacco plants transformed with *AVL type-B* promoter-GUS constructs. En, endosperm; Em, embryo. (A) Positive control, *i.e.* seeds transformed with pBI121, in which GUS expression is driven by the *CaMV* promoter. (B)–(D) Seeds from different lines transformed with the vector pALP-8 upstream sequence beginning with -785 bp.

-290-bp promoter region supported a weak basal GUS activity as compared to the vectors pALP-6 and pALP-8, the activity was still nearly 20-fold higher than that of the negative control and could initiate endosperm-specific expression. Amongst the five deletions, pALP-6 provided the second highest expression, although there was no significant difference compared to pALP-3 and pALP-10. The expression level increased by 42%, when the promoter was extended from -550 (pALP-6) to 785 bp (pALP-8). In comparison, expression levels in pALP-10 plants were much lower, almost

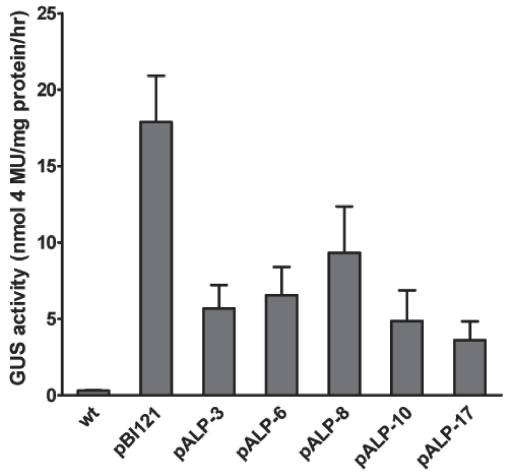

Fig. 3. Deletion analysis of the *AVL type-B* promoter by the quantitative measurement of GUS for plants obtained from the seeds of individual tobacco plants transformed with vector constructs containing the various deletions of the promoter sequence. wt, wild-type plants. The error bars represent the standard error of the means (SEM), $n = 3$.

half of those in pALP-8 plants. In all five lines of transgenic plants, lowest expression was observed in plants harbouring the pALP-17 vector.

According to the results of the quantitative GUS analysis and the distribution of the putative elements (shown in Fig. 1, endosperm motifs at -290 and -579 bp, GLM at -274 bp, RY motifs at -670 and -1406 bp, ESP-like elements at -388 and -677 bp, and G-box at -881 bp), which are required for endosperm-specific expression, candidate functional motifs were confirmed in some regions. The severe decrease in transcriptional activity observed, when the length of the promoter is extended from -785 to -999 bp, indicates that there could be some negative *cis*-elements in this 214-bp sequence, so more detailed deletion experiments are still needed. It should be noted that in this region there is a G box, which is considered to have long-distance positive effects. The promoter sequence up to the -290 bp position contains a prolamin box which consists of an endosperm motif and GLM that can drive a basal level of reporter gene expression and maintain the endosperm specificity. The prolamin box was identified through comparison of promoter sequences of several wheat gliadin and barley hordein genes (Forde *et al.*, 1985). Prolamin boxes exist in the promoters of a wide range of prolamin genes in oats, barley, rye, and wheat. Though there are more endosperm motifs and GLMs upstream of the prolamin box, Müller and Knudsen (1993) found that, when a prolamin box drives endosperm-specific expression, any additional putative endosperm motifs and GLMs upstream of the prolamin box have very limited effects on expression. This is also the case with the promoter investigated in this study. A functional analysis of the prolamin box in the gene coding for the low-molecular weight subunit in transgenic tobacco showed that both the endosperm motif and GLM were required for seed-specific expression (Colot *et al.*, 1987). Transcription factors that recognize the GLM have been cloned from wheat and are known as SPA (Albani *et al.*, 1997).

Expression of SSP genes is under the tight tissue-specific and temporal transcriptional control during the seed maturation phase. Two *cis*-regulatory elements (CREs) conferring seed-specific expression, *i.e.* an RY motif and a G box, were identified in the promoter of the *NapA* gene from *Brassica napus* (Ezcurra *et al.*, 1999). These CREs are also conserved and functionally relevant in the promoters of genes encoding 2S albumins in *Arabidopsis thaliana*. Extending this analysis to the promoter of an SSP gene isolated from wheat, rice, maize, and *Coix* (Job's tears, Chinese pearl barley), it was shown that RY motifs appear to be conserved in most of them. These results demonstrate that the RY motif is a key promoter element for endosperm-specific gene expression (Moreno-Risueno *et al.*, 2007).

Previous studies indicated that transcription factors binding to a promoter sequence as a dimer always recognize palindromic sequences (De Pater *et al.*, 1994; Ribeiro *et al.*, 1995; Xue, 2005; Xue *et al.*, 2006). Two ESP-like elements are present in the *AVL type-B* promoter at positions -677 and -388 bp which are similar to an ESP element. An ESP element involved in endosperm specificity was identified in a globulin promoter in oats (Vickers *et al.*, 2006). It is worth mentioning that the ESP-like element at position -677 bp overlaps with one RY motif by four base pairs, *i.e.* CATG, which is considered a core sequence of the RY motif (Reidt *et al.*, 2000).

The quantitative GUS analysis revealed that the GUS activity was markedly enhanced when the promoter sequence with the extensions from -290 to -550 bp and then from -550 to -785 bp

was used. Two ESP-like elements are also found in these two regions. The sequence with the second extension (from -550 to -785 bp) is especially important, because here the ESP-like element overlaps with the RY motif which might be playing a more positive role in endosperm-specific expression. Moreno-Risueno *et al.* (2007) found that barley FUSCA3 (HvFUS3) specifically binds to and activates the *Hor2* and *Itr1* genes through the RY motifs in their promoters. Though little is known about the *trans*-factors of the ESP element, further research is needed to elaborate the interactions between the *trans*-factors of the ESP-like element and the overlapping RY motif. Meanwhile, less spacing between the two palindromic half-sites CATG in ESP-like elements when compared with ESP elements is also a crucial factor, because spacing can determine the specificity of *trans*-factors (Seidel *et al.*, 1995; Xue *et al.*, 2006). RY motifs upstream of the position -999 do not seem to make any observable difference in the strength of expression.

Conclusions

Wheat yield must be doubled by the year 2025 to meet the increasing world demand (Vasil, 2007). Therefore, it is imperative to introduce novel genes into commercial wheat varieties by genetic transformation to obtain transgenic varieties with desired attributes. Exploiting tissue-specific promoters would make a marked difference in production quantity and quality of wheat. The presence of B-type AVL proteins and their respective mRNAs exclusively in the endosperm were previously confirmed by Western blotting and RT-PCR, which affirms the endosperm specificity of this promoter. In this study, an endosperm-specific promoter from wheat was successfully cloned and found to maintain its endosperm-specific activity in tobacco. Our results also suggest that an ESP-like element overlapping with the core sequence of the RY motif might be important for maintaining a high expression level. Regulatory attributes of a negative *cis*-element in the promoter sequence could not be sufficiently elaborated in this study, therefore further research involving site-specific mutagenesis and gain of function experiments are needed.

Acknowledgements

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