Tissue specific promoters from rice and wheat for modifying grain characteristics

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INTRODUCTION

Wheat, rice and barley are important agricultural crops and major sources of human food and animal feed. An important application of genetic engineering is expected to be an improvement of grain size, quality and yield by the modulation of the levels of expression of particular gene(s), without losing the useful agronomic qualities of a cultivar[1,2,3]. An essential tool in grain biotechnology is therefore the development of grain-specific promoters, which help to overcome undesirable phenotypes generated by constitutive over-expression of a transgene[4]. Most of the endosperm-specific promoters from monocots described to date have been shown to be activated in the late phases of grain development, at least two weeks after fertilisation, when cell proliferation and differentiation are mostly complete[5,6,7,8]. However, for the manipulation of grain size and shape, it is probable that the expression of transgenes will be needed before or during cellularisation and in tissues involved in nutrient transfer from the maternal tissue to the endosperm and from the endosperm to the embryo.

In this study we cloned and tested the activity of wheat and rice promoters using stable transformation of wheat, barley and rice with promoter-GUS fusion constructs. The spatial and temporal activities of the promoters were studied using whole-mount and histological analysis. It was shown that all six tested promoters are activated either a short time before fertilisation or within 9 days after fertilisation (DAP), and that they have very different spatial regulation patterns. Five from six analysed promoters show strong expression. Possible applications of promoters for the improvement of grain quality are discussed.

MATERIALS AND METHODS

Either full length cDNA sequences or 3'UTRs were used as probes to screen a BAC library prepared from the genomic DNA of *Triticum durum* cv. Langdon[9] using Southern blot hybridisation as described elsewhere[10]. For each probe, plasmid DNA from two strongly hybridising BAC clones was isolated using the Large Construct Kit (QIAGEN). The *T. durum* homologs were confirmed by PCR using BAC DNA as a template and primers derived from the ends of the coding region of cDNAs. The promoter sequences were first identified on the BAC clone by several consecutive sequencing reactions. As a result of such 'walking' along the DNA, about 2.5-3.0 kb of sequence upstream from the translation start codon was obtained. These sequences were subsequently used to design forward and reverse primers for the isolation of the promoter fragment. Sequences of promoters with a full-length 5'-untranslated region were amplified by PCR using AccuPrime™ Pfx DNA polymerase (Invitrogen) and DNA of the respective BAC clone as a template. Promoter fragments were cloned into the pENTR-D-TOPO vector (Invitrogen), verified by sequencing and then subcloned into the pMDC164 vector[11] using recombination-mediated cloning. The pMDC164 vector includes selectable marker genes for hygromycin resistance in plants and kanamycin resistance in bacteria. The recombinant binary vectors were introduced into *Agrobacterium tumefaciens* AGL1 strain by electroporation.

The constructs were transformed into rice and barley using *Agrobacterium*-mediated transformation [12] [13]. Rice (*Oryza sativa* L. ssp. Japonica cv. Nipponbare) and barley (*Hordeum vulgare* L. cv. Golden Promise) were used as donor plants. Wheat (*Triticum aestivum* L. cv. Bobwhite) was transformed using biolistic bombardment [14]. Histochemical and histological GUS assays were performed [15] using T_0, T_1 and some cases T_2 transgenic plants.

Quantitative real-time PCR analysis was performed using primers spanning the coding and 3' untranslated regions of genes. cDNAs prepared from wheat (cv. Chinise Spring) grain at different days after fertilsiation were used as a template[16]. Northern blot hybridisation was performed as described elsewhere[10]. The same membrane was hybridised consecutively with full length cDNA or 3' UTR probes.

RESULTS AND DISCUSSION

The purpose of this work was to clone grain and endosperm-specific promoters which are active in a window from just before to 2-3 weeks after pollination. Accordingly, cDNA libraries were prepared from the liquid part of the syncytial and cellularising endosperm (3-6 DAP) and the whole wheat grain (0-6 DAP)[17] and several potentially grain specific cDNAs were identified using databases and literature data and their specificity was confirmed either by Q-PCR or northern blot hybridization. Using these cDNAs we cloned several promoters from wheat, which are active either only in grain tissues or in grain and flowers shortly before fertilization. Transcriptional GUS fusion constructs were prepared using the pMDC164 vector[11] and transformed either into rice and barley using *Agrobacterium*

tumefaciens-mediated transformation, or into wheat using microprojectile bombardment.

Two full length cDNAs, designated *TaPR60* and *TaPR61*, were identified among sequenced inserts of about 200 library clones, which were randomly selected from the cDNA library prepared from liquid endosperm. Both genes belong to the same family of small cysteine-rich proteins with hydrophobic signal peptides. *TaPR60* is a wheat orthologue of *END1* from barley[18]. It was demonstrated by *in situ* hybridization that *END1* is specifically expressed in the coenocyte above the nucellar projection during the free-nuclear division stage. After cellularisation, *END1* transcripts were detected over the nucellar projection. From 8 DAP a low level of expression was observed in the modified aleurone and the neighbouring starchy endosperm[10]. Later the activity of the promoter of *OsPR602*, the rice homologue of *END1* and *TaPR60*, was analysed in transgenic rice and barley[15]. In transgenic rice the activity of the promoter was detected in the endosperm transfer cells, the transfer cells of maternal tissue and in several floral tissues shortly before pollination. *OsPR602* promoter activity was similar in the heterologous species barley, except that GUS expression was exclusively in the endosperm transfer cells and differed in timing of activation relative to rice.

We have isolated the promoter of *TdPR60*, the *T. durum* orthologue of the bread wheat gene *TaPR60*, and tested its activity in transgenic wheat, barley and rice. The *TdPR60* promoter was activated specifically in endosperm transfer cells and adjacent starchy endosperm in wheat and barley starting from the ninth day after pollination (DAP). The GUS activity was detectable until 35-40 DAP. The activity and specificity of the *TdPR60* promoter in wheat and barley is therefore very similar to the activity and specificity of *OsPR602* promoter[15] in barley. However, the patterns of expression of the *OsPR602* and *TdPR60* promoters in rice are very different. Whereas the *OsPR602* promoter was active in endosperm transfer cells, maternal vascular tissue and vascular tissue of the lemma and palea, the relatively strong *TdPR60* promoter activity was detected only inside the starchy endosperm starting from 6 DAP, the end of endosperm cellularisation in rice. This promoter can be very useful for biotechnological manipulations of rice endosperm, as to our knowledge there have been no reports of promoters shown to be active so early and specifically in rice endosperm development. However, a promoter which is active only in endosperm transfer cells in rice remains to be discovered. The cell specificities of the *OsPR602* and *TdPR60* promoters suggest that they might be useful tools for the improvement of cereal grain quality via modification of nutrient transfer, both quality and quantity, to the grain. They may also be of value in enhancing the capability of this tissue to prevent pathogen movement from maternal tissue to the developing endosperm.

Another homologue of *END1*, *TaPR61*, was used as a probe to isolate the gene and promoter of *TdPR61*. The *TdPR61* promoter has been tested so far only in barley. The activity of the promoter was localised to the region of endosperm which surrounds the embryo as early as 5 DAP, and in the embryo at later stages of grain development. Maximum activity in the endosperm was detected at 8 DAP and then it was slowly diminished, but still could be seen at 50 DAP. In the embryo, the promoter remained active at least until the middle of the desiccation phase of grain development. In the isolated embryo GUS staining was observed as two very strong foci either side of the embryonic axis and two larger and slightly diffused points in the scutellum. In longitudinal sections of embryo, it could be seen that the activity of the *TdPR61* promoter was localised at the bases of the shoot apex and radicle, where these organs are attached to the scutellum. The activity was found to extend further in the radicle, but was confined to the epidermal cell layer. While the *TdPR60* promoter is active in cells responsible for the transfer of nutrients from the maternal tissue to the endosperm, the *TdPR61* promoter seems to be active in cells which might be involved in the transfer of nutrients from the endosperm to the embryo. Considering that *TdPR60* and *TdPR61* are close homologues, it is expected that their products have the same or similar functions. Although the roles of these genes still remain unknown, we can suggest that these proteins might be involved in transport of either sugars or lipids, or in signal transduction. *TdPR61* can be a valuable molecular marker for understanding the relations (cross-talk, nutrients and signal transfer) between the embryo and endosperm. The *TdPR61* promoter is very strong and could be used to engineer sterility through early embryo abortion.

TaPRPI was cloned in a yeast two-hybrid (Y2H) screen using TaPR60 as bait. The sequence of this gene was used to identify and clone its homologue from rice, designated *OsPRPI*. It was also used as a probe to isolate the gene and promoter of *TdPRPI*, its orthologue from *T. durum*. In rice, the patterns of expression of the GUS gene driven by the *OsPRPI* and *TdPRPI* promoters are very similar. Both promoters are active in a range of floral tissues, particularly in the male and female gametophyte before fertilisation. After fertilization the promoters are active only in grain, with weak activity in the aleurone and strong activity in maternal tissue cell layers adjacent to the aleurone. However, no activity is observed in the epidermis. Promoter activity reaches a maximum between 5 and 7 DAP and strongly decreases at 15 DAP. No GUS staining was observed after 15 DAP. In wheat, *TdPRPI* shows a very similar pattern of expression, except that before fertilization promoter activity was found exclusively in gametophytes, with no activity in other floral tissues. The *OsPRPI* and *TdPRPI* promoters will be useful for improvement of disease resistance at early stages of grain development.

TaPRGL7 and *TaPRGL9* encode homeodomain-containing transcription factors, which were cloned using a Y2H screen of a cDNA library prepared from whole grain at 0-6 DAP. 3'UTRs of these cDNAs were used as probes to isolate genes and promoters of close

homologues/orthologues of *TaPRGL7* and *TaPRGL9* from the BAC library of *T. durum*. The genes were designated *TdPRGL7* and *TdPRGL9*, respectively.

The *TdPRGL7* promoter was tested in both wheat and barley. Strong GUS expression was observed in both species, with similar patterns of expression. The promoter is activated in the female gametophyte shortly before fertilization. No promoter activity was observed in anthers and other flower tissues. In contrast to the *TdPRPI* promoter, the GUS expression was detected in the gametophyte and later in all grain tissues. The promoter was active until 40 DAP, at which point the GUS activity was detected mainly in embryo tissues.

The *TdPRGL9* promoter was analysed in barley. Relatively weak promoter activity was detected between 4 and 7 DAP in the embryo or close to the embryo. A more thorough analysis of the promoter activity is in progress.

The *OsPR602*, *TdPR61* and *TdPRGL7* promoters have been cloned into the pMDC32 vector, replacing the 35Sx2 promoter, and these new vectors are available for easy gene cloning by homologous recombination. Each construct is compatible with transformation of rice, barley or wheat to target gene expression to different grain tissues, with the aim of manipulating grain size and quality or tolerance to abiotic and biotic stresses.

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