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Short Communication

Isolation and Expression of a Barley β-1,3-Glucanase Isoenzyme II Gene

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A β-1,3-glucanase gene from *Hordeum vulgare* was isolated by a PCR strategy, cloned and subsequently sequenced. The amplified sequence contained the entire coding region of the isoenzyme II, which is interrupted by a 165 bp intron at 73 bp downstream the starting codon. This intron contains all the elements required for the processing mechanism in monocots : a high A + U content, the appropriate splice sites in the 5′ and 3′ ends and four typical YUNAN consensus sequences. Transient transformation of wheat protoplasts with the complete β-1,3-glucanase gene under the control of maize polyubiquitin promoter revealed that the intron sequence was spliced out. The gene was also expressed at high levels, probably due to an enhancer-like sequence found near the 3′ end of the intron.

Keywords: β-1, 3-glucanase, *Hordeum vulgare*, monocot intron, Pathogenesis-related, transient expression, wheat protoplasts

Plant β-1,3-glucanases (EC 3.2.1.39) are present in seeds and markedly increase during germination (Leah *et al.*, 1991; Cordero *et al.*, 1994). The high levels of the enzyme found in germinating seeds are difficult to conciliate with the quite low levels of endogenous β-1,3-glucans present in the grain (MacGregor *et al.*, 1989). As β-1,3-glucans are the major structural polysaccharides of fungal cell walls (Boller, 1987), the plant β-1,3-glucanases are classified with the pathogenesis-related proteins that are expressed in response to pathogen invasion (Boller, 1987) and were shown, together with chitinases, to be effective in increasing resistance to fungal aggression (Anuratha *et al.*, 1996). In monocots β-1,3-glucanases have been studied in barley, rice and maize (Hoj *et al.*, 1989; Linthorst, 1991; Leah *et al.*, 1991; Wu *et al.*, 1994). In barley, β-1,3-glucanases are encoded by a small gene family (Li et al., 1996) and the products of these genes are seven isoenzymes designated GI-GVII. There is increasing evidence that the isoenzyme GII is particularly important in the plant response to pathogen attack (Roulin *et al.*, 1997). This isoenzyme GII is extracellular and was purified from germinated barley seeds and its cDNA sequenced (Hoj *et al.*, 1989; Leah *et al.* 1991). Screening of a barley genomic library with this isoenzyme GII cDNA probe lead to the isolation of a β-1,3-glucanase isoenzyme GI gene (Burton *et al.*, 1998). Therefore, the gene of GII remained to be isolated.

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TABLE I Primers designed for PCR amplification of β-1,3-glucanase isoenzyme G II gene

Primer set and sequences	*Nucleotide position in cDNA (M36992)*	*Length of PCR product (bp)*
Set 1		
O1: 5′-CTCGAATTCCTCCGTGTGTGCACCAATGGCTAG-3′	33–55	
O2: 5′-CTCGGATCCTCCACCATCGCGTCGAACAGGGA-3′	742–764	896*
Set 2		
O3: 5′-GGGAATTCAACGAGGTGCAGGGCGGCGCCA-3′	409–430	
O4: 5′-CTTGGATCCGCATTACGTACGTACGTGCAGCTT-3′	990–1113	704
Set 3		
O5: 5′-CTCCGTGTGTGCACCAATGGCTAG-3′	1–24†	
O6: 5′-ACTTGATGTTCACGGCAGGGTAGT-3′	504–527†	527

*. The expected length from cDNA was 731 bp
†. Nucleotide position in the isolated gene (AF030771)

In the present work we designed a PCR strategy to isolate the β-1,3-glucanase isoenzyme GII gene from barley DNA based on the sequence of the cDNA encoding the isoenzyme II. Two oligonucleotide pairs containing flanking recognition sequences of *Bam*HI and *Eco*RI were synthesized to amplify the genomic barley DNA as forward (O1, O3) and reverse (O2, O4) primers (Table I, set 1 and 2) of the cDNA (Leah et al., 1991, M36992). PCR amplifications were carried out in a final volume of 50 µl using 2 units *Taq* DNA polymerase (Promega), 1x Promega buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 80 pmol each primer and 0.3 µg genomic barley DNA (previously denatured for 10 min at 95°C) for 35 cycles at 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. The amplified fragments were cloned into pUC119 and doublestranded sequenced using *Taq* DNA Polymerase (Promega) according to manufacturer's instructions. Initial sequences were obtained using either universal or reverse primers. Sequences were then extended by using specific primers. Both isolated DNA fragments contained the *Sma*I recognition site corresponding to nucleotides 661–666 of the original nucleotide sequence of the cDNA (Leah et al., 1991, M36992). Therefore, in order to obtain the full length gene, each isolated DNA fragment was cut with *Eco*RI-*Sma*I or *Sma*I-*Bam*HI and joined through the *Sma*I recognition site contained in both fragments. This new DNA consisted of 1242 bp and encoded the full length β-1,3-glucanase isoenzyme GII of barley (AF030771). Analysis of this nucleotide sequence revealed the presence of an additional sequence of 165 nt at 73 bp downstream the initial ATG. This sequence contains a 51% of A + U and the GT and AG sequences in the 5′ and 3′ ends (Fig. 1). These features indicate that the intron possess not only the required AU-content (Goodall and Filipowicz, 1991) but also the appropriate splice site sequences and length (Goodall and Filipowicz, 1990; White *et al.* 1992). Plant introns are significantly greater than that in fungi and insects and the minimum functional intron length for monocots and dicots was found to be between 70 and 73 nt. They have a high A+U content, reflecting an unique processing mechanism of monocots (Goodall and Filipowicz 1991).

The introns of dicot as well as in monocot plant genes are known to have a marked influence upon expression. In maize *GapA1* gene an octameric sequence was identified in intron 1 (CGTGCCGC), which may play a role in intron-mediated enhancement (Donath *et al.* 1995). In this barley β-1,3-glucanase isoenzyme GII intron we found a highly homologue sequence (TGCGCCGC) near the 3′ end (Fig. 1).

5′gtacgtgcttacgtttctctcgtgctcttgtagtattttctaccgtatcgctttgcatgcatgcacctgctggccggtcattcacgtcgactacgc

tacgaacaggtaataaacggatgccact CTTAT CTGAG ta CTTAT CTTAT ccgcaTGCGCCGCatgcag3′

FIGURE 1 Intron sequence of β-1,3-glucanase II gene. Schematic representation of the intron sequence showing the consensus nucleotide at 5′ and 3′ splicing sites (underlined), the enhancer (in bold) and the YUNAN sequences (in upper case)

It is in the appropriate position within the intron to function as an enhancer. In fact, Luehrsen and Walbot, (1994) reported that an enhancer must be near the splicing site. We also found four typical YUNAN consensus sequences along this intron (Fig. 1). It was demonstrated that in plants, as in other eukaryotes, such sequences can be an important determinant of intron splicing activity (Simpson *et al.*, 1996).

To investigate if this isolated β-1,3-glucanase isoenzyme GII gene was expressed *in vivo*, we performed transient expression experiments in wheat protoplasts. For this purpose the gene was placed under the control of the ubiquitin promoter from maize (Christensen et al., 1992; S94464) by subcloning in the *SmaI-BamHI* site of pUbiNos resulting in the plasmid pUbiHvGlu. Wheat protoplasts were prepared from 10 day-old leaves (*Triticum aestivum*, Buck Ombú cultivar) and transient transformation was carried out by the PEG-$CaCl_2$ method essentially as described (Saul *et al.* 1988) in 450 µl of protoplast suspension (1.5×10^6 cells/ml) with or without the addition of 9 µg of pUbiHvGlu. Total RNA was extracted and Northern analysis performed as shown in Fig. 2. The isolated β-1,3 glucanase isoenzyme GII gene was transcribed in wheat protoplasts when placed under control of the ubiquitin promoter (Fig. 2, lane 4). The signal corresponds to an approximate 1000 bp mRNA. Absence of transcription was observed at zero time of incubation or when the plasmid was not added (Fig. 2, lanes 1–3), indicating the absence of the endogenous transcript. To investigate if the intron was spliced out in this system, Northern blot hybridization experiments were also performed using total RNA from 2 h pUbiHvGlu transformed wheat protoplast (see Fig. 2) and probed with an oligonucleotide complementary to the intron nucleotide sequence 5′-CGTAGTC-GACGTGAATGACCGGCCAGCAG-3′ and (^{32}P) labeled by reacting with [γ-^{32}P] ATP and

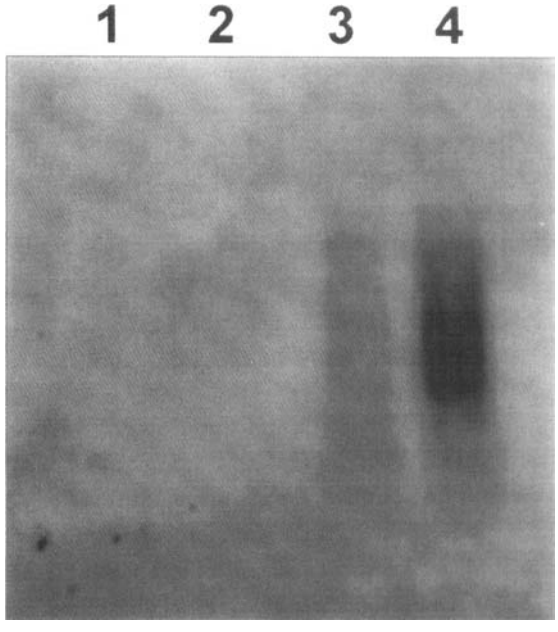

FIGURE 2 Northern blot analysis of wheat protoplasts transformed with pUbiHvGlu. Total RNA was obtained from wheat protoplasts according to Wadsworth *et al.*, (1988), fractionated by formaldehyde agarose (1%) gel electrophoresis and transferred to Hybond N-membranes. For the hybridization procedure a 527 bp probe was used, which was obtained by PCR amplification using the isolated β-1,3-glucanase isoenzyme GII gene (AF 030771) as template and the primers O5 and O6 (Table I, set 3) as forward and reverse oligonucleotides, respectively. The amplification product was purified from agarose gel and labeled with [α-^{32}P]dATP by random priming according to manufacturer's procedure (Boehringer Mannheim). Total RNA was from control (lanes 1 and 2) or transformed (lanes 3 and 4) protoplasts at 0 (lanes 1 and 3) or 2 h after transformation (lanes 2 and 4)

T4-polynucleotide kinase. As a control experiment ^{32}P labeled oligonucleotide O2 (Table I) was used as hybridization probe. No hybridization signal was observed when the membranes were probed with the oligonucleotide complementary to the intron sequence. As expected, a hybridization signal was observed when the membranes were probed with oligonucleotide O2 (data not shown). These results clearly indicate that the isolated barley β-1,3-glucanase isoenzyme GII gene was actively transcribed in wheat protoplasts and that the intron is spliced out in this system. Moreover, they suggest that the barley β-1,3-glucanase isoenzyme GII intron posses all the elements needed for appropriate processing in monocots. Further analysis on the mechanism of mRNA accumulation will make clear the stimulating effect of consensus sequences found on β-1,3-glucanase II intron on plant gene expression.

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