

# The specific localization of (1→3,1→4)-β-glucanase isoenzymes EI and EII in germinated barley grain

C. E. Gibson<sup>A</sup>, R.A. Burton<sup>A</sup>, M. Sedgley<sup>B</sup>, S. Logue<sup>A</sup>, L.C. MacLeod<sup>C</sup> and G. B. Fincher<sup>A</sup>

<sup>A</sup>Department of Plant Science and <sup>B</sup>Department of Horticulture, Viticulture and Oenology, The University of Adelaide, Waite Campus, Glen Osmond, S.A. 5064. <sup>C</sup>Barrett Burston Malting Co. Pty. Ltd., Gough St, Richmond, Victoria 3121, Australia.

## Introduction

During commercial malting, barley is germinated under controlled conditions. The synthesis, secretion and migration of a large number of enzymes during germination are prerequisites for starchy endosperm modification and therefore, for successful malting (MacGregor, 1994). One of the most important events in the early stages of malting is the degradation of endosperm cell walls. This removes the physical barriers between hydrolytic enzymes that are released from the aleurone, and their polysaccharide and protein substrate reserves that are stored within the cells of the starchy endosperm. The major constituents of endosperm cell walls are (1→3,1→4)-β-D-glucans and arabinoxylans. Two (1→3,1→4)-β-D-glucan endohydrolases (EC 3.2.1.73) are important in the hydrolysis of (1→3,1→4)-β-D-glucans in germinated barley grain. These exist as two isoforms of (1→3,1→4)-β-D-glucanase, and are referred to as isoenzymes EI and EII. Both have been purified from germinated barley grain and characterized (Woodward and Fincher, 1982). The isoenzymes differ in their isoelectric points and molecular weights, but have similar substrate specificities and action patterns. Differential expression of the (1→3,1→4)-β-glucanase isoenzymes has been shown by northern blot analysis (Slakeski and Fincher, 1992). Isoenzyme EI mRNA transcripts appear to be more abundant in the scutellum, while both isoenzymes EI and EII can be found in the aleurone layer (Slakeski and Fincher, 1992).

In the majority of studies aimed at defining the location of enzyme synthesis and secretion during germination, isolated scutella and aleurone layers have been used. Problems exist with these types of studies, firstly because the tissue may be contaminated with other tissue types (Mares and Stone, 1973), and secondly because it is likely that such systems do not truly reflect *in vivo* events (Chrispeels and Varner, 1967). It would clearly be more appropriate to define the locations of enzyme expression in intact grain, using techniques such as *in situ* hybridization and immunostaining. *In situ* hybridization allows the detection of mRNAs encoding particular enzymes, through the use of specific complementary DNA (cDNA) or RNA probes, and the cells in which the corresponding genes are expressed can therefore be identified (McFadden *et al.*, 1988). Immunostaining techniques allow the locations of the enzymes themselves to be defined, through the use of specific monoclonal antibodies. It must be remembered, however, that while immunostaining will reveal where an enzyme is located at the time of tissue sectioning, it might not allow the identification of cells from which secreted enzymes originate.

Although the sites of (1→3,1→4)-β-glucanase gene expression in germinated barley grains were identified by *in situ* hybridization in an earlier study from our group (McFadden *et al.*, 1988), the cDNA probe used in that study could not distinguish between isoenzymes EI and EII. When full-length cDNAs for both isoenzymes were subsequently isolated, it was noted that the nucleotide sequences were more than 90% identical in the coding regions of the two

genes. However, in the 3' untranslated regions of the genes, considerable sequence divergence was apparent. This allowed the preparation of probes from the 3' ends of the cDNAs that would enable the two mRNAs to be identified individually (Slakeski *et al.*, 1990). These 3' probes will present an opportunity to re-examine the sites of (1→3,1→4)-β-glucanase gene expression in germinated barley grains by *in situ* hybridization, so that time expression of genes encoding the two isoenzymes can be separately defined.

Specific monoclonal antibodies have also been raised against the two (1→3,1→4)-β-D-glucanases from barley (Høj *et al.* 1990), but these have not been used to locate the specific isoenzymes in germinated grain. In the work described here, the isoenzyme-specific cDNA probes and monoclonal antibodies were used to examine the spatial and temporal coordination of expression patterns of (1→3,1→4)-β-glucanase isoenzymes EI and EII by *in situ* hybridization and immunostaining.

## Materials and Methods

### *Germination of barley*

Barley grains (*Hordeum vulgare* L. cv. Sloop) were surface-sterilized in 0.2% (w/v) sodium hypochlorite for 20 min, washed thoroughly with 0.5 M NaCl and sterile distilled water, and imbibed for 24 h in sterile water containing 100 μg.ml<sup>-1</sup> neomycin, 10 μg.ml<sup>-1</sup> chloramphenicol, 100 units.ml<sup>-1</sup> penicillin and 100 units.ml<sup>-1</sup> nystatin (Hoy *et al.* 1981). Grains were incubated on filter paper moistened with the antibiotic solution, in darkness, at 20°C for up to 96 h.

### *Tissue preservation*

Grain was collected throughout germination, cut longitudinally and fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 (PBS) for 12 h at 4°C. The grain was washed in PBS, dehydrated through a graded ethanol series, embedded in Paraplast Plus (Sigma), sectioned (7 μm) with a rotary microtome and applied with the aid of adhesive tape onto Superfrost Plus (Menzel-Glaser) glass slides.

### *Modification pattern*

Calcofluor White (0.05% w/v) was applied to each barley section to provide an estimate of (1→3,1→4)-β-glucan levels in cell walls and hence to gauge the extent of grain modification. Each section was grouped into a modification (M) category depending on this pattern. For the barley variety Sloop, nine M points were chosen, representing grain modified from 8-107 h from the start of steeping. Only three points, M2, M5 and M9, are presented in this paper; M2 represents grain collected 16 h from the start of steeping, M5 at 56 h and M9 at 96 h.

### *In situ hybridization*

Single-stranded RNA (riboprobes) were used to detect messenger mRNA in the barley sections. The isoenzyme EI probe was from the 3' untranslated region of the (1→3,1→4)-β-glucanase isoenzyme EI cDNA and was about 400 nucleotides in length. The isoenzyme EII probe was from the 3' untranslated region of the (1→3,1→4)-β-glucanase isoenzyme EII cDNA and was about 250 nucleotides long. The "total" glucanase probe was obtained from the coding region of the (1→3,1→4)-β-glucanase isoenzyme EII cDNA and was about 1,200 nucleotides long. Each RNA probe was tested at least three times on grain in each time point. No signal above background was detected when a sense probe was used.

For the preparation of the riboprobes, the corresponding cDNAs were subcloned into pBluescript SK, linearised and used as templates for either T3 or T7 RNA polymerase, so that

sense and anti-sense riboprobes were obtained from each cDNA. Each riboprobe was labeled with Digoxigenin (DIG-11-UTP) (Boehringer-Mannheim). The isoenzyme EI riboprobe was degraded to a mean length of 250 nucleotides by incubating in alkali at 60°C.

For the *in situ* hybridizations, standard procedures using the DIG-labeled riboprobes was adopted (Braissant and Wahil, 1998; Komminoth, 1996). Following pre-hybridization and hybridization, tissue sections were washed to 0.1 x SSC to 0.5 x SSC, and DIG was observed with a monoclonal antibody conjugated with alkaline phosphatase and the alkaline phosphatase substrate (NBT/BCIP).

#### *Immunostaining*

Sections were incubated with monoclonal antibodies against (1→3,1→4)-β-glucanase isoenzymes EI or EII overnight at 4°C, followed by a secondary anti-mouse antibody conjugated to alkaline phosphatase and the same alkaline phosphatase substrate used for *in situ* hybridization. The immunostaining control sections were probed with pre-immune serum, but never showed positive staining.

### **Results and Discussion**

#### *In situ hybridization*

The *in situ* hybridization results (Table 1) demonstrated that the expression of mRNAs for both isoenzymes EI and EII first appears in the scutellar epithelium and embryo early in the germination process (16 h from the start of steeping). This pattern of expression was also observed by McFadden *et al.* (1988) using non-specific probes for (1→3,1→4)-β-glucanases, and by Sugimoto *et al.* (1998) for α-amylase expression. An interesting observation from the present study was the appearance of isoenzyme EII mRNA transcripts early in germination (16 h from the start of steeping) and the presence of mRNA for both isoenzymes at the tip of the embryo, near the acrospire; isoenzyme EI mRNA appeared to be slightly more abundant than mRNA for isoenzyme EII at this early stage. Slakeski and Fincher (1992), previously documented the presence of isoenzyme EI mRNA in the scutellum early in germination, but did not detect the presence of isoenzyme EII transcripts. Low levels of isoenzyme EII mRNA and slightly less isoenzyme EI mRNA were detected in the aleurone at the M2 stage; these effects were not reported by McFadden *et al.* (1988).

At the later, M5 stage, isoenzyme EI mRNA was seen at very high levels in both the scutellar epithelium and embryo, while in the aleurone high levels of mRNA for both isoenzymes were observed along the dorsal side of the grain.

By the M9 stage, most of the mRNA for the two isoenzymes was in the aleurone layers, particularly along the dorsal side of the grain. This is not unexpected, because it follows the pattern of cell wall breakdown in germinated barley grain (Briggs and MacDonald, 1983) and also reflected the Calcofluor White staining patterns during germination (data not shown).

**Table 1.** *In situ* hybridization with specific probes on tissue from germinated barley grain.

<b>Probes</b>	<b>E1</b>	<b>E11</b>	<b>T</b>	<b>E1</b>	<b>E11</b>	<b>T</b>	<b>E1</b>	<b>E11</b>	<b>T</b>
	<b>M2</b>	<b>M2</b>	<b>M2</b>	<b>M5</b>	<b>M5</b>	<b>M5</b>	<b>M9</b>	<b>M9</b>	<b>M9</b>
Time <sup>&amp;</sup>	<b>16</b>	<b>16h</b>	<b>16h</b>	<b>56h</b>	<b>56h</b>	<b>56h</b>	<b>96h</b>	<b>96h</b>	<b>96h</b>
acrospire, rootlets	++	+	+++	+++	+	+++	-	-	+/-
scutellum	-	-	+	++	-	++	-	-	+/-
scutellum epi	++ <b>a</b>	++ <b>a</b>	++ <b>a</b>	+++ <b>a, r</b>	++ <b>a</b>	+++ <b>a</b>	-	-	+/-
dorsal aleurone	+/-	+	++	++	++*	++	+++	+++	+++
ventral aleurone	+/-	+	++	+*	+/-*	+*	++*	++*	++
aleurone tip	+	+	++	-	-	+ <sup>#</sup>	+ <sup>#</sup>	-	+ <sup>#</sup>

**Note** +: Positive staining, +++: max staining, +/- minimum staining, -: no staining, T: total glucanase, epi: epithelium, time<sup>&</sup>: from start of steeping, **a**: near acrospire, **r**: near rootlet, \* staining is detected only ¼ way down the aleurone from the scutellum, <sup>#</sup>: staining only at the dorsal side of the aleurone tip.

### *Immunostaining*

The results of immunostaining of tissue sections from the M2 and M9 stages with monoclonal antibodies against (1→3,1→4)-β-glucanases isoenzymes are shown in Table 2. Isoenzyme EII staining is somewhat stronger than isoenzyme EI staining at the M2 point, but both enzymes are found predominantly in the scutellum and embryo. This result may be contrasted to those of Stuart *et al.* (1986) and Edmonds *et al.* (1994), who found only isoenzyme EI in secretions from the scutellum. At the M9 stage, the staining increases in the aleurone for both isoenzymes EI and EII, and decreases in the scutellum.

**Table 2.** Immunostaining with specific monoclonal antibodies.

<b>Antibodies</b>	<b>E1</b>	<b>E11</b>	<b>E1</b>	<b>E11</b>
	<b>M2</b>	<b>M2</b>	<b>M9</b>	<b>M9</b>
Time <sup>&amp;</sup>	<b>16h</b>	<b>16h</b>	<b>96h</b>	<b>96h</b>
acrospire, rootlets	+/-	+	+/-	+/-
scutellum	++	++	+	+/-
scutellum epi	++ <b>a</b>	++ <b>a</b>	-	+/-
dorsal aleurone	-/+*	-/+*	+	+++
ventral aleurone	-	-	+	+++
aleurone tip	-	-	+	+++

Same notes as for table 1.

### **Conclusions**

Optimal endosperm modification during barley germination is fundamental to successful malting. The results of the present study are generally consistent with those of earlier work and show that (1→3,1→4)-β-glucanase gene expression is initiated in the scutellar epithelium. As germination proceeds, expression is observed in aleurone layers and

progresses from the proximal to the distal end of the grain. In addition, mRNA for isoenzyme EII is detected in higher amounts in the scutellum than in previous studies.

It should now be possible to use the *in situ* hybridization procedure to monitor the expression of genes encoding other important hydrolytic enzymes in germinated barley grain. In particular, specific cDNA probes are available for (1→4)- $\beta$ -endoxyylanases (Banik *et al.*, 1996) and the coordination of expression of all enzymes involved in cell wall degradation could eventually be defined. Similarly, cDNAs encoding limit dextrinase (MacGregor, 1987) and limit dextrinase inhibitor (Macri *et al.* 1993) could be used to examine recently reported anomalies in the synthesis and secretion of limit dextrinase from barley aleurone layers (Burton *et al.*, 1999; Schroeder and MacGregor, 1998), and to define more precisely the roles of the enzyme and its inhibitor in starch degradation in germinated grain.

### Acknowledgment

C. Gibson is supported by an Australian Postgraduate Award (Industry), which is sponsored by Barrett Burston Malting, Joe White Malting and Adelaide Malting.

### References

- Banik, M., Garrett, T. P. S., and Fincher, G. B. (1996). *Plant Mol. Biol.* 31, p1163.
- Braissant, O., and WahiL, W. (1998). *Biochemica*, Vol 1, Boehringer Mannheim.
- Briggs, D. E. and MacDonald, J. (1983). *J. Inst. Brew.* 89, p260-273.
- Burton, R. A., Zhang, X-Q., Hrmova, M. and Fincher, G. B. (1999) *Plant Physiology* 119, p859-871.
- Chrispeels, M. J. and Varner, J. E. (1967). *Plant Physiol.* 42, p398.
- Edmunds, M. D., Allan, G. R., Fincher, G. B. and Stuart, I. M. (1994) *Proc. 23<sup>rd</sup> conv. IOB* 79.
- Høj, P. B., Hoogenraad, N. J., Hartman, D. J., Yannakena, H. and Fincher, G. B. (1990). *J. Cereal Science* 11,p 261.
- Hoy, J. L., Macauley, B. J. and Fincher, G. B. (1981). *J. Institute of Brewing* 87, p77-80.
- Komminoth, P. (1996). *Nonradioactive In situ manual*. Boehringer Mannheim
- MacGregor, A. W., Macri, L. J., Schroeder, S. W. and Bazin, S. L. (1994). *J. Cereal Science* 20, p33
- MacGregor, A. W., Marci, L. J., Bazin, S. L. and Sadler, G. W. (1995) *EBC 25th Convention*. p185.
- MacGregor, A.W. (1987) *CRC Crit Rev. Biotechnol.* 5, 117-128.
- Macri, L. J., MacGregor, A. W., Schroeder, S. W., and Bazin, S. L. (1993). *J. Cereal Science* 18, p103.
- Mares, D. J. and Stone, B. A. (1973). *Aust. J. of Biol. Science.* 26, p813.
- McFadden, G. I., Ahluwalia, B., Clarke, A. E. and Fincher, G. B. (1988). *Planta.* 173, p500
- Schroeder, S. W., and MacGregor, A. W. (1998). *J. Am. Soc. Brew. Chem.* 56, p32-37.
- Slakeski, N., Baulcombe, D. C., Devos, K. M., Ahluwalia, B., Doan, D. N. P. and Fincher, G. B. (1990). *Mol. Gen. Genet.* 224, p437.
- Slakeski, N., and Fincher, G. B. (1992). *Plant Physiol.* 99 p1226.
- Sugimoto, N., Takeda, G., Nagato, Y. and Yamaguchi, J. (1998) *Plant cell Physiol* 39, p323-333.
- Stuart, M., Loi, L. and Fincher, G. B. (1986). *Plant Physiol.* 80, 310-314.
- Woodward, J. R. and Fincher, G. B. (1982). *Eur. J. Biochem.* 121, p663.