Proceedings of the 10th Australian Barley Technical Symposium, 2001

Early embryo and endosperm genes of barley

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Abstract

In cereals, the starchy endosperm of the grain is the major site for the deposition of storage polymers that will support seedling growth following germination. Cellularisation of the starchy endosperm early in grain development involves `atypical' growth of cell walls around individual nuclei in a multinucleate syncytium. In this project the genes and enzymes responsible for the coordination of wall synthesis are being investigated, together with the changes that occur during aleurone differentiation. The genes involved in controlling the early stages of embryo development will also be examined, in particular the genes that control the major transitional stages that determine key structural features of the embryo. A wide range of novel genes have so far been identified through direct fingerprinting of mRNA prepared from early embryos and endosperm and sequencing of cDNA libraries. Several of these genes are now being characterised in order to reveal their function.

Introduction

In angiosperms double fertilisation generates simultaneously a single-celled zygote and a progenitor cell of the endosperm. Subsequently, the joint development of the zygotic embryo and the endosperm leads to formation of a viable seed. Embryogenesis is concerned primarily with establishing the basic shoot-root body pattern of the sporophyte, and thus impacts on the vigour and architecture of the post-embryonic sporophyte. The triploid endosperm provides nutrients to the developing embryo and the germinating seedling. The deposition of starch and storage proteins in the developing endosperm of cereals provides the major staple food of humans globally.

The key structural features of the cereal embryo and endosperm are determined early on in the development of the grain. Thus, the early phases of embryogenesis are

critical in defining the polar axis, specifying the root and shoot apices, and generating the embryonic tissue and organ systems. The first phase of endosperm development, occurring 1 to 5 days after pollination (DAP) in wheat and barley, is multiple divisions of the triploid nucleus to form a multinucleate cytoplasmic matrix around the periphery of the central cell (Figure 1a,c). At 6-13 DAP, a period of cell wall, starch granule and protein body formation in the endosperm occurs (Figure 1b,d). Embryo growth and endosperm cell division are completed two to four weeks after pollination, and this is followed by a period of seed dehydration and dormancy.

Large research efforts, going back several decades, have focussed on grain development but most studies to date have concentrated on the maturation stages due to the relative inaccessibility of the tissues of immature grain. Genetics approaches, using chemical or insertional mutagenesis, have yielded embryo and endosperm mutants in the model species *Arabidopsis* and maize, but the functions of many of these genes remain elusive. In recent years, genomics approaches have provided an alternative strategy for the identification of developmentally regulated genes in higher plants. In order to isolate genes which may be "master regulators" of early grain development, we have constructed cDNA libraries from the early stages of embryo and endosperm development. Genes will now be identified using either a random approach by nucleotide sequencing of cDNA clones, or a targeted approach utilising sequence information generated in other species. This project will further analyse these sequences to identify full length genes, characterise expression patterns and develop functional information on the genes and their promoters.

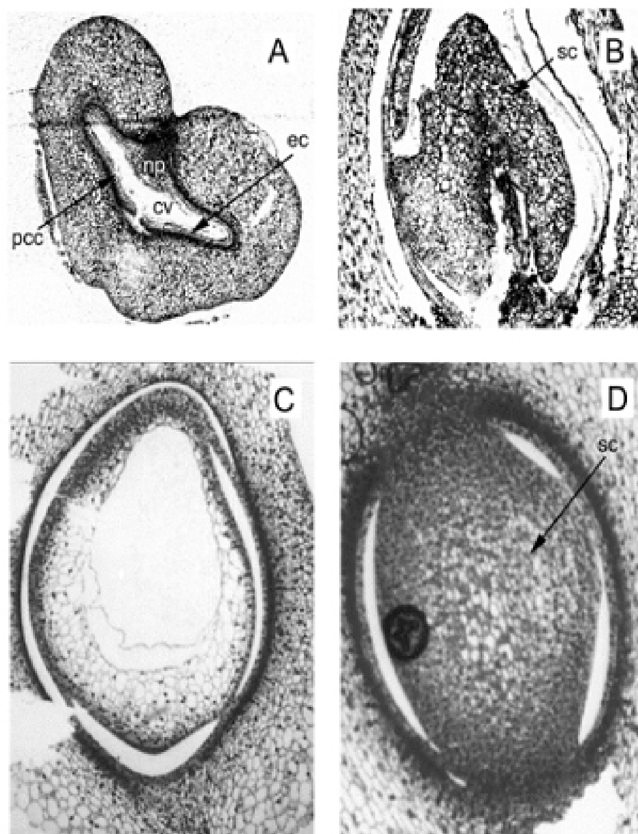


Figure 1. Light Micrographs Showing the Anatomy of Wheat and Barley Immature Grains. (A) Transverse section of wheat grain, 5 DAP: (cv) central vacuole; (np) nucellar projection; (pcc) photosynthetic cross cells; (ec) endosperm coenocyte. Longitudal sections of (B) 7 DAP wheat grain;

Materials and Methods

cDNA Library Construction

Zygotic embryos of wheat (*Triticum aestivum* L. cv. Chinese Spring) and barley (*Hordeum vulgare* L. cv. Sloop) were isolated at the globular and transition/coleoptilar stages for the extraction of mRNA. For endosperm isolation, flowers were emasculated and manually pollinated, and the liquid fraction of the central, multinuclear cell was collected at 3 to 5 DAP.

Poly(A)$^+$ RNA was obtained using an mRNA Direct Kit (Dynal, Norway), and cDNA was synthesised and amplified using the SMART cDNA Library Construction Kit (Clontech, USA). Endosperm cDNA was ligated into pGEM-Teasy (Promega, UK), a TA-cloning plasmid vector, and transformed into the *E. coli* strain XL10-Gold (Stratagene, UK). Embryo libraries were prepared in the phage vector _TriplEx2 (Clontech) and packaged using the GigaPackIII Gold Packaging Extract (Stratagene). For EST sequencing, *in vivo* excision of pTriplEx2 plasmids was performed in the *E. coli* strain BM25.8.

Nucleotide Sequence Determination

Plasmid DNA for nucleotide sequencing was prepared using DNA purification columns (QIAprep Spin Miniprep Kit, QIAGEN, Australia). The length of inserts was estimated by PCR using vector-specific primers. Partial 5′ and 3′ nucleotide sequences of random cDNA inserts were determined by the fluorescence detection method using an ABI 373A autosequencer. The homology of each partial cDNA sequence was searched against the GenBank nucleotide and SwissProt protein databases using the BLAST algorithm (NCBI, USA).

Results and Discussion

cDNA Library Construction

We have constructed PCR-based cDNA libraries from limited amounts of starting material: the contents of the multinucleate central cell of wheat and barley endosperms at 3 to 5 DAP, and zygotic embryos as small as 200 _m in diameter. The long distance PCR amplification strategy (Barnes, 1994) we employed made possible the preparation of libraries from as little as 25 ng of mRNA and has been used previously for the successful preparation of cDNA libraries from early human embryos (Adjaye *et al.*, 1999). The use of the SMART oligonucleotide enables PCR amplification of full length cDNAs and eliminates the need for conventional second-strand cDNA synthesis and adaptor ligation. After PCR amplification the size of cDNA products was in the range 0.2 to 7.0 kb.

Early Endosperm Genes

The complexities of the early endosperm libraries (wheat and barley) were 10^5 clones each. These libraries are expected to be fully representative of the genes transcribed in the central cell before cellularization, as the number of genes estimated to be active in endosperm does not exceed 8000 (Clarke *et al.*, 2000). The insert sizes of 20 clones randomly picked from each library were in the range 0.2 to 2.3 kb. Sequencing demonstrated that all the clones have poly(A) tails and many of the cDNA clones bear the translation initiation codon ATG and 80-200 bp of 5′-untranslated region.

Sixty clones (30 from each library) with a size >0.6 kb were sequenced in both orientations. BLAST searching revealed that twelve clones (20%) were found only in EST libraries prepared from wheat, barley or maize endosperm, the whole caryopsis, or from the spike after pollination, suggesting the gene products are endosperm-specific. A further seven sequences matched hypothetical and putative proteins from *Arabidopsis*. Remarkably, the sequencing of only 60 clones revealed endosperm-specific genes from the same family in both libraries: two wheat homologues and one barley homologue to END1. Expression of barley END1 is localised to the nucellar projection in the layer of free nuclei of the central cell and can be detected by northern blot hybridization at 4 to 5 DAP, continuing for one to two more weeks until the end of starchy cell maturation (Hueros *et al.*, 1999). Using a yeast two-hybrid system, we have shown that the product of one wheat END1-like gene interacts with an up-regulated nucleopore protein in the endosperm (data not shown), potentially providing stage-specific regulation of nucleopore trafficking. High abundance of such factors is not unexpected, taking into consideration the active and specific export and import communication between the cytoplasm and multiple nuclei of the giant central cell before anticlinal cell wall formation. This data is supported by the detection of a clone encoding another nucleopore protein, RanBP1b GTPase, which converts Ran-GTP to Ran-GDP, releasing nuclear cargo into the cytoplasm. An additional sequenced clone encodes a nucleic acid-binding glycine-rich protein, which may be involved in the export of nuclear mRNA. Participation in such nuclear export has been demonstrated for the RNA binding glycine-rich protein A1 from mammals (Michael *et al.*, 1995).

Detection of two cDNA clones of the same translation initiation factor EIF-4A6 and of three ribosomal proteins is evidence of high translational activity in the central cell a short time before the rapid formation of multiple cell walls. Unexpectedly abundant, though, were four clones encoding components of the ubiquitin-related protein degradation machinery. These were the already known wheat ubiquitin-activating enzyme E1 and ubiquitin-conjugating enzyme E2, and two novel proteins: the ubiquitin carrier E2 protein with homology to the yeast *RAD6* gene product, and a partial clone with significant homology to the C-terminus of the ubiquitin-protein ligase E3. Activation of protein synthesis and degradation suggests rapid turnover of at least some proteins in the central cell. One of the possible groups of proteins subject to such rapid turnover could be the nucleopore proteins. During multiple nuclear divisions the process of assembly and disassembly of nuclear pores is repeated many times and might require repeated degradation and synthesis of the nuclear pore components.

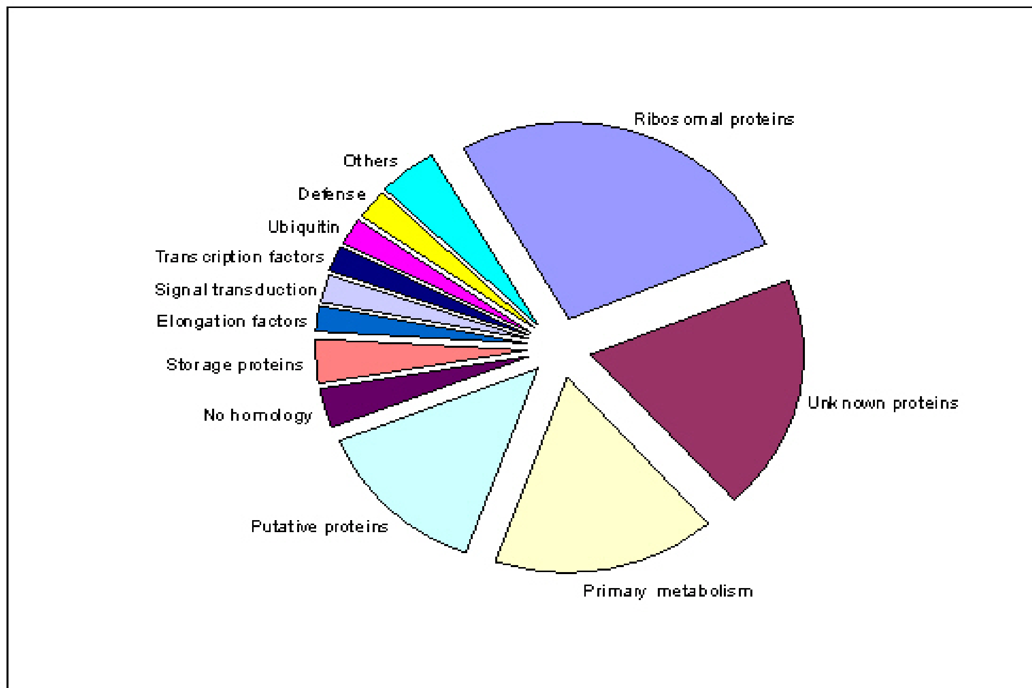
A clone encoding a homologue of the specific basal endosperm transfer layer protein 3 (BETL3) from maize (Hueros *et al.*, 1999) was isolated from wheat endosperm.

Such proteins exhibit potent broad-range activity against a range of filamentous fungi, including several plant pathogens (Serna *et al.*, 2001). The reason of the specific expression of such proteins in endosperm is still unclear. Three further interesting proteins, novel for plants, match cDNA clones of the TRANSLIN-binding protein (TRAX), the GTBP-like DNA mismatch recognition and binding protein, and a nuclear protein with homology to the suppressor of apoptosis in human B Lymphoma cells. A cDNA clone of starch synthase, an enzyme with a role in the second phase of endosperm development, was also found.

Early Embryo Genes

To date, 90 EST sequences have been obtained from the barley transition/coleoptilar stage library, which comprises 1.5×10^6 independent clones with an average insert size of around 800 bp. This library was most abundant in cDNAs encoding proteins with translational activity (30%), in particular ribosomal proteins and elongation factors (Figure 2). A further one third of the cDNA clones could not be assigned a putative identity, aligning only to EST sequences (unknown proteins) or to genomic DNA sequences from rice or *Arabidopsis* (putative proteins). Of the remaining sequences, those clones with a potential role in signal transduction or transcriptional activation provide the best candidates for regulators of embryogenesis. For example, homologues to the maize CRINKLY4 gene, a putative receptor kinase with a potential role in differentiation signals, and to the *Arabidopsis* homeotic protein APETALA2, important in gene regulation during flower and seed development, were found. Large-scale sequencing of the early embryo libraries, following a pre-screen to remove ribosomal protein sequences, should reveal more such candidates for functional analysis.

Figure 2. Summary of ESTs Isolated from a cDNA Library Prepared from Transition/Coleoptilar Stage Barley Embryos



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