

# Systematic Spatial Analysis of Gene Expression during Wheat Caryopsis Development <sup>W</sup>

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**The cereal caryopsis is a complex tissue in which maternal and endosperm tissues follow distinct but coordinated developmental programs. Because of the hexaploid genome in wheat (*Triticum aestivum*), the identification of genes involved in key developmental processes by genetic approaches has been difficult. To bypass this limitation, we surveyed 888 genes that are expressed during caryopsis development using a novel high-throughput mRNA in situ hybridization method. This survey revealed novel distinct spatial expression patterns that either reflected the ontogeny of the developing caryopsis or indicated specialized cellular functions. We have identified both known and novel genes whose expression is cell cycle-dependent. We have identified the crease region as important in setting up the developmental patterning, because the transition from proliferation to differentiation spreads from this region to the rest of the endosperm. A comparison of this set of genes with the rice (*Oryza sativa*) genome shows that approximately two-thirds have rice counterparts but also suggests considerable divergence with regard to proteins involved in grain filling. We found that the wheat genes had significant homology with 350 *Arabidopsis thaliana* genes. At least 25 of these are already known to be essential for seed development in Arabidopsis, but many others remain to be characterized.**

## INTRODUCTION

The grass caryopsis is a single-seeded fruit that comprises the embryo and the carbohydrate-rich endosperm surrounded by the fused remnants of the integuments and the inner layer of the ovary (the pericarp). The caryopsis is characteristic of the Poaceae (grass family) and is the single major source of carbohydrates in the human diet. Therefore, the dissection of its development at the molecular and genetic levels is of considerable strategic significance for crop improvement. A comparison with other flowering plants will provide insight into fruit and endosperm development and evolution.

The caryopsis is derived from the products of the fertilized female gametophyte and surrounding maternal tissues. The gametophyte consists of the nucellus and the embryo sac and is surrounded by two distinct protective layers of maternal tissue, the inner and outer integuments. Outside of the integuments is the carpel wall, which later will develop into the pericarp. As caryopsis development proceeds after fertilization, all of the

nucellus except for its epidermis is reabsorbed. The outer integument disintegrates, and the inner integument and the nucellar epidermis form the hyaline layer in the mature caryopsis. Therefore, the outer protective layers of the mature caryopsis are derived from the nucellus, integuments, and carpel, which undergo a combination of wall thickening, cell death, and reabsorption during development (Percival, 1921; Esau, 1977).

The embryo is derived from the fertilized egg cell, whereas the triploid endosperm is derived from the fertilized polar nuclei. In wheat (*Triticum aestivum*), the endosperm nuclei undergo several rounds of mitosis in the absence of cytokinesis for ~4 to 5 d before cellularization occurs. Cellularization then begins on the outside (nearest the nucellus) and progresses inward (Evers, 1970; Mares et al., 1975; Morrison and O'Brien, 1976). In wheat, the outermost endosperm cell layer differentiates as aleurone, whereas the cells on the inside develop as starch- and protein-storing cells. However, the endosperm cell layers located adjacent to the nucellar projection (that portion of the nucellus overlying the vascular strands) are morphologically distinct (Morrison et al., 1978) and are known as the modified aleurone. The modified aleurone develops transfer cell morphology characteristic of cells active in apoplastic/symplastic solute transport (Thompson et al., 2001; Offler et al., 2003). Transfer cell morphology is also found in the nucellar cells adjacent to the aleurone transfer cells (Wang et al., 1994a). Together, the nucellar and aleurone transfer cells form a symplastic transport system connecting the phloem to the endosperm, thereby facilitating the transport of sucrose from the vascular system to the endosperm, where it is converted into starch (Wang et al., 1994b, 1995a, 1995b).

The functional analysis of developmental processes in wheat is difficult because of its hexaploid genome and because of its

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low efficiency of transformation. In model organisms such as *Arabidopsis thaliana*, enhancer traps and gene traps have been used to define genes involved in development (Sundaresan et al., 1995). Genetic analysis of cereal endosperm development has been largely restricted to diploid species such as barley (*Hordeum vulgare*) and maize (*Zea mays*) (reviewed in Olsen et al., 1999; Becraft, 2001; Olsen, 2001). Gene expression analysis using microarrays is a useful tool to survey gene expression within a tissue (Girke et al., 2000; Alba et al., 2004), but because it integrates data from all of the cell types used to make the RNA, most spatial information is lost, although to some extent this information can be restored by the approaches described by Birnbaum et al. (2003) and Wellmer et al. (2004). A complementary strategy for the expression patterns by in situ hybridization. In situ hybridization has been used to undertake large-scale surveys of gene expression in *Drosophila melanogaster* and other species (Kopczynski et al., 1998; Satou et al., 2001; Tomancak et al., 2002; Imai et al., 2004) and should be applicable to plants.

Here, we use this approach to determine the expression patterns of genes expressed during wheat caryopsis development. A set of 888 genes, whose expression changed during caryopsis development, was selected from microarray analysis. Procedures for probe preparation and mRNA in situ analysis were modified and partially automated to allow high throughput and reproducibility. The resulting images from 3, 6, and 9 d after anthesis (DAA) are available on a publicly accessible database at <http://bioinf.scri.sari.ac.uk/cgi-bin/insitu/home>. This survey revealed novel distinct spatial expression patterns that either reflected the ontogeny of the developing caryopsis or indicated specialized cellular functions. Our analysis of these results reveal novel cell cycle-regulated genes and show that a significant subset of genes, including some transcription factors, are restricted to individual cell types. This provides candidates for genes that control growth and development in the seed. Analysis of the patterning of genes involved in cell division and differentiation shows that the transition from division to differentiation is initiated in the region of the crease and spreads outward. Comparative analysis using wheat genes to search the rice (*Oryza sativa*) and Arabidopsis genomes suggests that a substantial subset is conserved through to Arabidopsis and that many of them play essential roles in seed development. This study highlights the similarities and differences across angiosperms in seed development and provides a framework for further functional analysis.

## RESULTS

### The Developing Caryopsis during the First 9 DAA

The size and shape of the wheat caryopsis change dramatically during early development (Percival, 1921). We characterized and documented these changes to form a firm basis for further analysis. A scheme of the caryopsis indicating the nomenclature is shown in Figure 1A. The outermost layer, the pericarp, is shown in green. Inside the pericarp is the outer integument (blue), the inner integument (cyan), and the nucellar epidermis (yellow).

At earlier stages, a nucellar lysate (light blue) is present. The nucellus forms a multilayered nucellar projection (gray at 3 DAA; subdivided into abaxial [dark gray] and adaxial [light gray] regions at 6 and 9 DAA), which lies adjacent to the vascular tissue of the floret (orange). This region is called the crease. Elsewhere, the nucellus forms a thin layer and is eventually crushed as the caryopsis develops, except for the nucellar epidermis, which persists in the mature seed. At 3 DAA, the endosperm (red) comprises a single layer of free nuclei surrounding the central vacuole. By 6 DAA, the peripheral endosperm begins to cellularize and nuclei are present throughout the central region previously occupied by the central vacuole. The remaining cell types within the endosperm begin to differentiate from 6 DAA onward, including the modified aleurone (at 6 and 9 DAA) and the aleurone (at 9 DAA only).

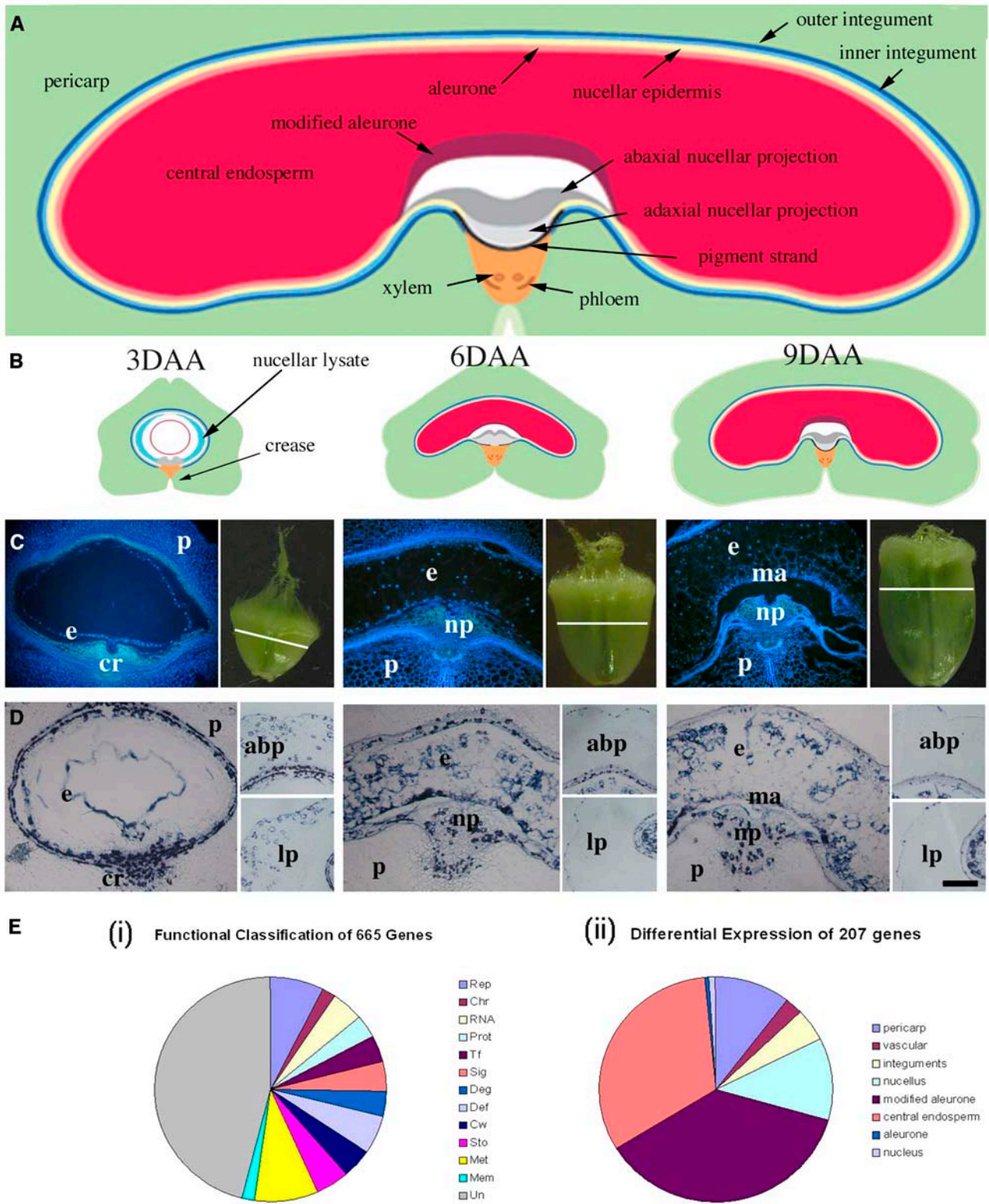
Before fertilization, the ovary resembles a blunt inverted cone (Figures 1B and 1C). Up to 3 DAA, this shape is largely maintained but the size increases. Then, the caryopsis lengthens significantly through 6 and 9 DAA.

The differentiation of cells with thickened cell walls (transfer cells) occurs in the crease region and nucellar projection throughout the early stages of caryopsis development. Calcofluor staining shows that the nucellar walls are thickened in the thin layer that persists around the developing endosperm at 3 DAA and in the cells of the nucellar projection (Figure 1C). Cell walls in the nucellar projection close to the endosperm (abaxial nucellar projection) are thicker than those near the vasculature (adaxial nucellar projection). Walls in the cells of the modified aleurone adjacent to the nucellar projection are also thickened by 9 DAA.

### Cell Cycle-Related Transcripts Reveal Cell Proliferation Patterns within the Developing Caryopsis

To define how cell cycle activity changes during early development, we used cell cycle-related transcripts as markers. Only cells in S phase of the cell cycle express histone H4 transcript, so in situ hybridization with a histone H4 probe can be used as an indicator of cell cycle activity (Fobert et al., 1994). Figure 1D shows, as an example, an in situ labeling with histone H4 at 3, 6, and 9 DAA. In each case, small insets show details of the abaxial (upper inset) and lateral (lower inset) pericarp. Further intermediate time points are shown in Supplemental Figure 1 online. At 3 DAA, there are high levels of histone H4 expression in the single layer of nuclear endosperm, the nucellar epidermis, the inner and outer integuments, and the outer regions of the pericarp. There are high levels of expression in the adaxial nucellar projection but less detectable expression in the abaxial nucellar projection (the layers directly adjacent to the ventral endosperm). The expression pattern of histone H4 in the thick-walled cells of the abaxial projection compared with the thinner walled cells of the adaxial projection indicates that the adaxial nucellar projection is a site of active cell proliferation. There are also large numbers of cells expressing histone H4 in the outer layers of the pericarp and in the pericarp epidermis, consistent with active growth in these regions.

By 6 DAA, cells expressing histone H4 are found in clusters throughout the endosperm, in the medial region of the nucellar



**Figure 1.** Structure of the Caryopsis at 3, 6, and 9 DAA.

**(A)** Scheme of the caryopsis in transverse section, showing the nomenclature of the cell types, based on the structure at 9 DAA.  
**(B)** Schemes of caryopsis development at 3, 6, and 9 DAA, with color coding as in **(A)**.

projection, and in the associated vascular bundle. In other tissues, including the inner and outer integuments and the pericarp, the number of histone H4-expressing cells has declined and become more scattered. This finding indicates that cell proliferation is substantially reduced in these tissues. The shape of the endosperm at 6 DAA is noticeably more flattened than at 3 DAA. Therefore, we examined the intermediate stages to investigate whether the change in shape was attributable to differential cell proliferation (see Supplemental Figure 1 online). However, histone H4 gene expression was uniformly distributed around the circumference of different tissues during the period of shape change (3 to 4 DAA). Thus, we conclude that differential cell proliferation is not directly responsible for the flattening of the grain.

By 9 DAA, the expression of histone H4 is almost totally restricted to the endosperm and the abaxial edge of the nucellar projection. This finding indicates that the site of cell cycle activity in the nucellar projection moves from the adaxial to the abaxial side between 6 and 9 DAA and that growth in the pericarp is largely attributable to cell expansion. The modified aleurone layer, overlying the nucellar projection, and the adjacent central endosperm region have reduced levels of histone expression by 6 to 7 DAA compared with the rest of the endosperm (see Supplemental Figure 1 online). The reduction in histone H4 transcript is already evident in the central region of the modified aleurone by 6 DAA and spreads laterally, coinciding with increased calcofluor staining of cellulose (Figure 1C). 4',6-Diamidino-2-phenylindole (DAPI) and calcofluor staining indicate that nuclear density is highest in the modified aleurone by 6 DAA and that cells have developed thickened cell walls by 9 DAA, showing that this part of the endosperm undergoes differentiation and cell cycle exit. At 13 DAA, histone H4 expression is highest in the aleurone layer and in the nucellar projection and vascular region, but the number of cells showing the expression is reduced (data not shown).

### Systematic *In Situ* Analysis of Gene Expression during Caryopsis Development

We surveyed the expression patterns for those genes that displayed differential expression between different cell types. We used the output from a temporal microarray analysis of caryopsis development (unpublished data from Wheat Improvement Centre, Syngenta, Norwich, UK) to select cDNAs from genes that are expressed during the first 9 d of caryopsis development as candidates for high-resolution *in situ* hybridiza-

tion. We generated probes for 888 of these genes and obtained expression patterns for 665 genes (75%) at one or more stages of development (223 probes did not produce a detectable signal at any stage tested). A representative negative control (ID 702017929) is shown in Figure 2A. The corresponding clones for the 665 genes that gave detectable signals were end-sequenced and subjected to BLASTX analysis for functional annotation. Of these, 358 genes (54%) matched ( $P < e^{-15}$ ) previously identified proteins with defined functions. The remainder were similar to proteins with unknown functions, hypothetical proteins, or had no homology in BLAST searches (Figure 1E, i). We then classified the 665 genes based on the spatial expression patterns obtained by *in situ* analysis (Table 1; see also Supplemental Spreadsheet 1 online). A database has been established at <http://bioinf.scri.sari.ac.uk/cgi-bin/insitu/home> and is searchable by a unique nine-digit clone ID number and also by cell type.

### Cell Cycle-Regulated and Proliferation-Associated Gene Expression

Cells in growing plant tissues tend to be asynchronous with regard to their immediate neighbors; therefore, transcripts produced in a given phase of the cell cycle tend to label individual cells or small groups of cells discontinuously. On the other hand, other genes expressed in proliferating cells are not regulated during the cell cycle; therefore, neighboring cells tend to have similar levels of transcript. With this in mind, we identified two groups of expression patterns that are associated with proliferation in all cell types.

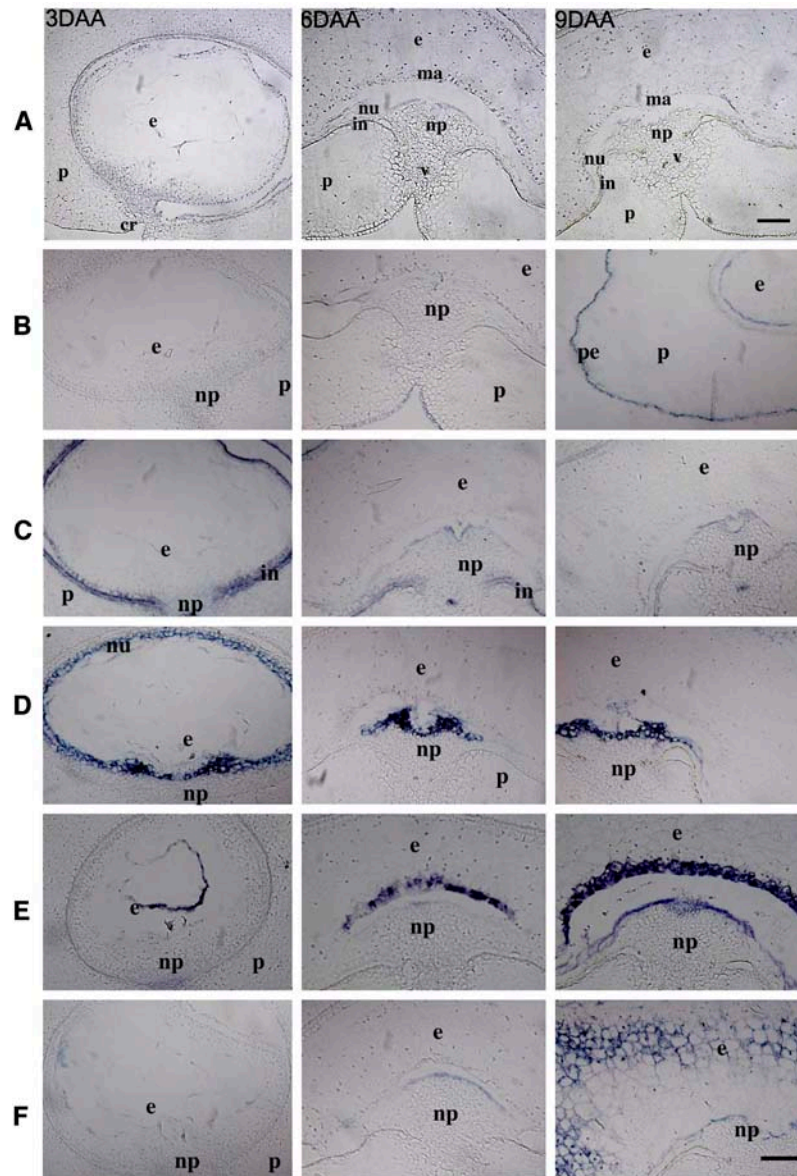
The first group, which we termed “spotty,” was expressed in all cell types but in a spatially discontinuous pattern and varying through time such that individual cells express highly whereas neighboring cells do not. This group comprises 65 genes and represents the pattern for cell cycle regulation. These include several genes with known S-phase function in other organisms, including most of the histone gene family, *met1*, *mcm6*, and *pcna* (IDs 701961163, 701997153, and 702015038). Counterstaining with the DNA dye DAPI indicates that these genes are not highly expressed in mitotic cells (data not shown). Others genes, such as EB1, kinesin, and MAP65 (IDs 701970465, 702011978, and 702014956) are implicated in spindle function (Chan et al., 2003; Yang et al., 2003; Wicker-Planquart et al., 2004) and are more likely to be expressed in G2/M. A novel group of microtubule-associated protein-related genes (ID 701998672) with similarity

**Figure 1.** (continued).

**(C)** Transverse sections (left panels) through the caryopsis (right panels) at 3, 6, and 9 DAA costained with calcofluor (indicating the positions of cell walls) and DAPI (indicating the positions of nuclei). The orientation of the section is indicated by the white bar on the caryopsis.

**(D)** Regions showing cell cycle activity as visualized by RNA *in situ* hybridization with the S-phase marker histone H4 (ID 701992946) at 3, 6, and 9 DAA. For **(C)** and **(D)**, the following cell types are annotated: pericarp (p), endosperm (e), nucellar projection (np), integuments (in), nucellus (nu), modified aleurone (ma); abaxial pericarp (abp), crease (cr), and lateral pericarp (lp). Bar = 0.3 mm for **(C)** and **(D)**.

**(E)** Pie charts summarizing the functional classifications of the 665 genes for which expression was detected by *in situ* hybridization (i) and the spatial distribution of the 207 differentially expressed genes (ii). Abbreviations for functional classes from top to bottom are as follows: Rep, cell cycle and cytoskeleton; Chr, chromatin binding, RNA, RNA binding; Prot, protein synthesis; Tf, transcription factor; Sig, signaling; Deg, degradation; Def, defense; Cw, cell wall; Sto, storage proteins; Met, metabolism (enzymes); Mem, transport and membrane proteins; Un, hypotheticals/unknowns/unknown function.



**Figure 2.** Cell Type-Specific Transcript Labeling at 3, 6, and 9 DAA as Shown by *In Situ* Hybridization of Transverse Sections of the Caryopsis Probed with Digoxigenin-Labeled Antisense RNA and Viewed under Bright-Field Optics.

**(A)** Negative control (ID 702017929); a gene encoding a protein of unknown function with no detectable expression in the caryopsis.

**(B)** An example of epidermal labeling as shown by lipid transfer protein (ID 701992241). Transcripts are restricted to epidermal cell types. At 6 DAA, transcript is detected only in the pericarp epidermis, but at 9 DAA, it is also present in the aleurone epidermis.

**(C)** An example of integument labeling as shown by a  $\gamma$ -thionin gene (ID 701961876). Transcripts are located in the integuments at 3 DAA, but levels decrease markedly by 6 DAA.

**(D)** An example of nucellar labeling by a C13 endopeptidase,  $\alpha$ -galactosidase gene (ID 701996036). Transcripts are located throughout the nucellus at 3 DAA and become restricted to the abaxial nucellar projection at 6 and 9 DAA.

**(E)** An example of modified aleurone labeling by an  $\alpha$ -galactosidase gene (ID 701966021). Transcripts are located in uncellularized endosperm at 3 DAA and become restricted to the modified aleurone at 6 and 9 DAA.

**(F)** An example of central endosperm labeling as shown by avenin (ID 701995490). Transcripts are located in the central starchy endosperm at 9 DAA. The main tissue types are annotated as follows: crease region (cr), nucellus (nu), integuments (in), nucellar projection (np), endosperm (e), pericarp (p), pericarp epidermis (pe), and aleurone (al). Bars = 0.3 mm.

**Table 1.** Summary of All Expression Patterns Detected in This Screen Assigned on the Basis of the Cell Type Showing the Highest Signal or Other Pattern

Pattern	Rep	Chr	RNA	Prot	Tf	Sig	Deg	Def	Cw	Sto	Met	Mem	Un	Total
Spotty	42	1		1	3	3	1	1				1	12	65
General proliferation-associated	3	4	14	9	7	6	3	6	1	1	13	5	73	145
Pericarp		1		2	2	1	1				1	1	6	15
Vascular tissues		1				1	1				1		1	5
Integuments					1		2	1			1		3	8
Nucellus						1	3	2	4		2		5	17
Modified aleurone		1	1	1		2	3	3	6		6	1	20	44
Aleurone						1								1
Central endosperm			1		3	3		5		9	2		3	26
Nuclear localization of transcript					2									2
Total cell type-specific					1	4	1	9	11	20	9	1	33	89
Signal in all tissues	5	5	14	8	7	7	9	9	5	2	25	1	151	248
Total detected	50	13	30	21	26	29	24	36	27	32	60	10	307	665

Functional classification was based on BLAST sequence homologies. Abbreviations for functional classes are as follows: Rep, cell cycle and cytoskeleton; Chr, chromatin binding; RNA, RNA binding; Prot, protein synthesis; Tf, transcription factor; Sig, signaling; Deg, degradation; Def, defense; Cw, cell wall; Sto, storage proteins; Met, metabolism (enzymes); Mem, transport and membrane proteins; Un, hypotheticals/unknowns/unknown function.

to Targeting Protein for Xklp2 in *Xenopus laevis* (Wittmann et al., 2000) also displayed phase-specific expression. Finally, other genes encode components of the E3 ubiquitin ligase pathway (Skp1-like; ID 701985938a) involved in regulating cell cycle transitions by the targeted degradation of cell cycle regulators (Connelly and Hieter, 1996). There was strong phase-specific expression of a wheat cyclin D2 (ID 701985938b), which has not been shown for D-type cyclins previously (Sorrell et al., 1999). In addition to processes known to be regulated by the cell cycle, cytosolic tRNA-Ala synthetase (ID 701988340) and sphingosine kinase (ID 701986779) were also expressed in a spotty pattern. The general areas in which this pattern was observed indicate regions of active cell proliferation.

A second class, which we termed “general proliferation-associated,” comprised 145 genes. In this class, the level of expression was not constant within a cell type but varied spatially from region to region within the cell type. However, the expression in neighboring cells tended to be similar, and comparison with spotty expression patterns indicated that the expression levels were highest in regions of active cell proliferation. Genes showing this general proliferation-associated pattern of expression included many involved in protein translation (such as ribosomal proteins L18 and S2 and an argonaute-like gene; IDs 702035544, 702010465, and 701991206).

### Cell Type-Specific Gene Expression

To identify cell type-specific genes, we examined the expression pattern of each gene in each cell type. We found that 207 genes showed differential gene expression, either being specifically or preferentially expressed in a single cell type, as classified in Table 2 and Figure 1E, ii. Eighty-nine of the 207 genes showed expression in a specific cell type. More than half of the differentially expressed genes, 118 of 207, were enriched in a single cell type but expressed in more than one cell type. Therefore, we classified these remaining genes based on the cell type showing

the highest expression level (Table 1). The greatest proportion of these was specific for endosperm tissues.

The genes expressed in a cell type-specific manner are listed in Table 2, and examples of each class are shown in Figure 2. Many genes characterized previously fall into this class, including cathepsin B in the modified aleurone layer (ID 702041754; Cejudo et al., 1992), a nucellain and a NUC1 ortholog in the nucellar tissues (IDs 701987242 and 701996036; Doan et al., 1996; Linnestad et al., 1998), and several in the starchy endosperm, including gluten storage proteins (ID 701996036; Lamacchia et al., 2001) and sucrose synthase (ID 701987242; Guerin and Carbonero, 1997).

Five genes were expressed specifically in the outermost layer of the caryopsis, the pericarp. A lipid transfer protein gene that is specifically expressed in the outer epidermis of both pericarp and endosperm is strongly upregulated from d 6 and may be involved in defense processes as the endosperm develops (ID 701992241; Figure 2B). Of the two integument-specific genes, one was related to a gene with defense functions (ID 701961876; Figure 2C) and one was of unknown function with no significant BLAST hit (ID 702007351).

Seven genes encoding proteins belonging to a variety of functional classes, including degrading enzymes, were found to be expressed specifically in the nucellus (nucellar lysate, nucellar projection, and nucellar epidermis), and of these, five were expressed in the abaxial nucellar projection and in the nucellar lysate. These included a gene (ID 701996036; Figure 2D) orthologous to nucellain in barley for which the same expression pattern has been shown (Linnestad et al., 1998).

Thirty-two genes were expressed specifically in the modified aleurone (Figure 2E), a tissue implicated in nutrient transfer into the developing endosperm and in signaling (Thompson et al., 2001; Offler et al., 2003). The largest class (12 of 32) of modified aleurone-specific genes found no related proteins in BLAST searches or in one case hit an unknown protein. This unknown protein has been found only in rice (ID 701970066). Six of the

**Table 2.** Genes Expressed Predominantly or Specifically in One Cell Type

Pattern	Rep	Chr	RNA	Prot	Tf	Sig	Deg	Def	Cw	Sto	Met	Mem	Un	Total
Pericarp-predominant		1		2	2	1	1				1	1	6	15
Pericarp-specific					1			1					5	7
														22
Vascular tissues		1				1	1				1		1	5
Integument-predominant					1		2	1			1		3	8
Integument-specific								1					1	2
														10
Nucellus-predominant						1	3	2	4		2		5	17
Nucellus-specific						1	1		2				3	7
														24
Modified aleurone-predominant		1	1	1		2	3	3	6		6	1	20	44
Modified aleurone-specific						1		1	7		3		20	32
														76
Aleurone + central endosperm + pericarp						1								1
Central endosperm-predominant			1		3	3		5		9	2	1	3	26
Central endosperm-specific						2		8		20	6		4	41
														67
Nuclear localization of transcript					2									2
Total differentially expressed	3	2	3		9	13	11	20	21	29	22	3	71	207

Abbreviations for functional classes are as in Table 1.

32 modified aleurone-specific genes hit a group of hypothetical proteins in both Arabidopsis and rice that are similar to a maize gene (Zm-EBE1) expressed in the modified aleurone (ID 701999018; Magnard et al., 2003). Another class of genes expressed in the modified aleurone (4 of 32) encoded proteins with high similarity to invertase and pectin methylesterase inhibitors (ID 702035913; Giovane et al., 2004; Rausch and Greiner, 2004). Two encode proteins belonging to a family of Cys-rich proteins in Arabidopsis predicted to be protease inhibitors/seed storage/lipid transfer proteins (a family defined by sequence homologies; ID 701989322). Of the eight remaining genes expressed specifically in the modified aleurone, three encoded proteins related to known cell wall biosynthetic genes, consistent with the formation of thickened walls in these specialized cells.

The final group of cell type-specific genes defined the central starchy endosperm (Figure 2F) and was the largest group in the cell type-specific set. Almost half of these (20 of 41) encode proteins involved in protein or carbohydrate storage, whereas the majority of those remaining encode proteins potentially involved in signaling and defense.

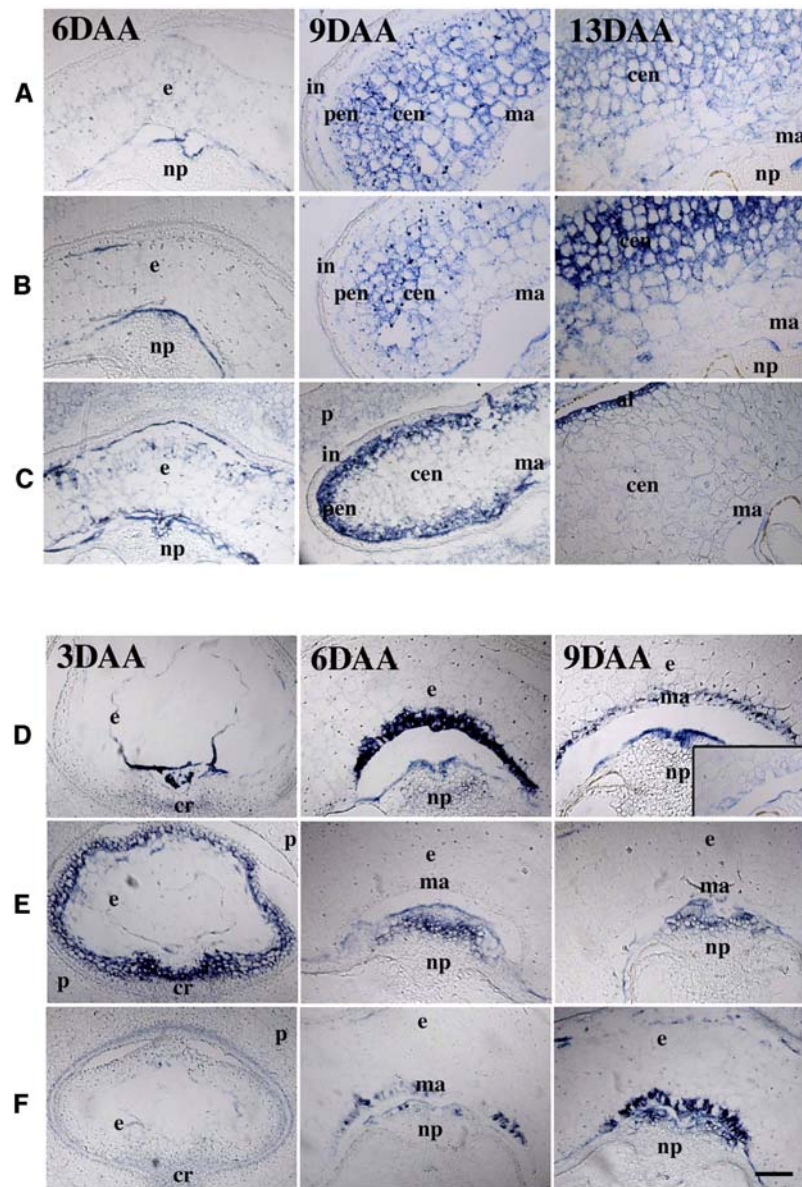
### Gene Expression in the Endosperm

The END1 gene (ID 702037156; see Supplemental Figure 2A online) was strongly and preferentially expressed in the modified aleurone. Expression was highly polarized, being strong in the ventral or adaxial endosperm overlying the crease at 3 DAA. END1 transcripts continued to accumulate as the modified aleurone cellularized and remained at a high level in the outer two cell layers until after 9 DAA. This expression pattern essentially matched the results obtained with the barley ortholog (Doan et al., 1996), in which it was shown that END1 is expressed from

syncytial stages. END1 is of unknown function but is similar to a family of Cys-rich proteins in Arabidopsis classed as proteinase inhibitors/storage proteins/lipid transfer proteins.

Several other genes have a similar spatial pattern of expression to END1 but vary temporally, differing in when expression is initiated or terminated (see Supplemental Figure 2 online). For example, expression of a gene encoding a  $\beta$ -expansin (ID 702008339; Figure 3D) occurred in a similar domain and at the same time as that of END1, but transcript levels reached a maximum at 6 DAA, at approximately the time of cellularization and differentiation, and decreased again by 9 DAA. The inset in Figure 3D shows that the decrease in expression levels continued until 13 DAA, suggesting that the role of this protein is not required after cells have differentiated. By contrast, expression of a gene encoding an ethylene-forming enzyme (ID 702006333; 1-aminocyclopropane-1-carboxylic acid oxidase; see Supplemental Figure 2B online) was not detected until 6 DAA, and levels increased at 9 and 13 DAA (inset). Several genes in the unknown function class were also expressed predominantly in the modified aleurone, with either early or late onset of expression. One example (ID 702038349; see Supplemental Figure 2C online) was expressed early but was expressed throughout the endosperm at 3 and 6 DAA and then became largely restricted to the modified aleurone cell layer at 9 DAA. Another example (ID 701965703; see Supplemental Figure 2D online) displayed highly polarized expression restricted to the ventral endosperm at 3 DAA. At 9 DAA, it was restricted to peripheral domains within the modified aleurone cell layer.

Genes expressed solely or predominantly in the starchy endosperm were detected from 6 DAA onward, and transcript levels tended to increase progressively at 9 and 13 DAA. Serial sections from the same caryopsis probed with these genes (Figures 3A to 3C, middle panels) indicated significant spatial



**Figure 3.** Gene Expression Patterns in the Central Endosperm and Coordinated Expression Patterns in Maternal and Endosperm Tissues.

**(A)**  $\alpha$ -Thionin (ID 701996413) transcript is present throughout the central endosperm at 9 and 13 DAA.

**(B)** Gliadin (ID 701994563) transcript is present in the central endosperm but reduced in the peripheral endosperm and aleurone layers.

**(C)** PPK (ID 701993242) is expressed in outer layers of the endosperm at 6 and 9 DAA and then is restricted to the aleurone layers at 13 DAA.

**(D)**  $\beta$ -Expansin (ID 702008339) transcript is present in the modified aleurone layer, reaching a peak at 6 DAA.

**(E)** A pectinesterase (ID 702008330) transcript is present throughout the nucellus at 3 DAA and is maintained in the abaxial nucellar projection from 6 to 9 DAA.

**(F)** A Ser carboxypeptidase (ID 702014362) transcript accumulates in both the modified aleurone and the nucellar projection from 6 DAA.

The main tissue types are annotated as follows: crease region (cr), nucellar projection (np), integuments (in), central endosperm (cen), peripheral endosperm (pen), pericarp (p), modified aleurone (ma), and aleurone (al). Bar = 0.3 mm.

variation in the patterns of gene expression that became more pronounced as the endosperm developed. Some, like the formate dehydrogenase gene (ID 701973313), were expressed throughout the central endosperm, including the peripheral and modified aleurone, and this pattern was maintained until at least

13 DAA, when all of the major cell types had differentiated. Others, such as sucrose synthase (ID 701995555), were expressed throughout the endosperm at 6 DAA, but by 9 DAA the central endosperm and aleurone contained significantly higher levels of transcript than the modified aleurone. Some

transcripts were strongly downregulated in the aleurone while being maintained in the central regions; for example, the gene encoding  $\alpha$ -thionin (ID 701996413; Figure 3A) was detected only in the central starchy endosperm by 9 DAA. This pattern is similar to that of gliadin (Figure 3B), a storage protein whose transcript began to accumulate in the central starchy endosperm by 9 DAA. At 13 DAA, gliadin expression was still excluded from the peripheral and modified aleurone layers. By contrast, expression of the PPKK (for pyruvate orthophosphate dikinase) gene (ID 701993242; Figure 3C) was progressively restricted to the outer layers. PPKK transcript was detectable at a low level throughout the central endosperm at 6 DAA but became localized to the peripheral endosperm by 9 DAA. By 13 DAA, PPKK transcript was localized solely in the aleurone layer.

### Gene Expression in Maternal Tissues

Several genes were expressed predominantly in the nucellar layers during early stages and then became restricted to the nucellar projection by 6 DAA. This restriction to the nucellar projection coincided with the collapse and death of the innermost nucellar lysate tissue surrounding the endosperm. These genes include an ortholog of the C13 endopeptidase gene, which shows a similar pattern of expression in the nucellar tissues of barley (ID 701996036; Linnestad et al., 1998) (Figure 2D), and a nucellin ortholog (ID 702041135; Chen and Foolad, 1997). Other genes, such as that encoding a wheat allergen (ID 701968577; see Supplemental Figure 3A online), have a more general pattern of expression during early stages but are preferentially expressed in the nucellar projection even at 3 DAA and eventually become restricted to that region by 9 DAA. A gene similar to dehydration response genes in rice and Arabidopsis is expressed in the integuments, nucellar projection, and modified aleurone, with the strongest expression at 3 DAA (ID 702013671; see Supplemental Figure 3B online).

In addition to cell layer-specific gene expression, five genes are also expressed within the nucellar vascular bundle; among these, a gene encoding an unknown protein showed extremely high expression in the ventral layers of the nucellar projection and in the cells surrounding the vascular tissue (ID 702040719; see Supplemental Figure 3C online). At 3 DAA, this extended to the xylem bundles in the pericarp, but by 6 and 9 DAA, transcript became progressively concentrated in the ventral region of the nucellar projection and the vascular region. Another example is shown in Supplemental Figure 4A online (ID 702015496) of a gene encoding a proteinase inhibitor gene that was expressed both in the integuments and surrounding the xylem vessels of the vascular bundle at 3 DAA. Preferential expression in the integuments was also seen for other genes. A gene encoding a GDSL-lipase (ID 701965571; see Supplemental Figure 4B online) was strongly expressed in the inner integument and showed general low expression in the pericarp and endosperm, whereas a gene encoding a Ser proteinase (ID 702011770; see Supplemental Figure 4C online) was expressed in the integuments and the tip of the nucellar projection. Finally, the transcript for a chalcone synthase gene (ID 702001184; see Supplemental Figure 4D online) was detected in the pigment strand crossing the nucellar projection.

### Coordinated Expression of Cell Wall-Related Genes in Maternal and Endosperm Tissues

Using the predicted functional annotation (see Supplemental Spreadsheet 1 online), we examined the expression patterns of genes encoding related cellular functions in different tissues. This indicated coordinated expression of cell wall-metabolizing genes in both maternal and endosperm cells in the region of the crease. Figure 3D shows the expression of a  $\beta$ -expansin gene (ID 702008339) in the modified aleurone, and Figure 3E shows that of a pectinesterase gene (ID 702008330) in the nucellar lysate and adaxial nucellar projection. A Ser carboxypeptidase (ID 702014362; Figure 3F) was expressed in both the cells of the modified aleurone (endosperm) and the tip of the nucellar projection (maternal) beginning at 6 DAA. Many other cell wall-metabolizing genes were also expressed in this region; for example, another  $\beta$ -expansin gene (ID 701965357) was expressed in the adaxial nucellar projection.

### Transcription Factor Gene Expression

Within the collection of expressed genes, we found 21 that had significant homology with transcription factors. Fourteen of these had spatially restricted patterns of gene expression. Supplemental Figures 5A and 5B online show the expression of a wheat *MADS13* ortholog in the outer integument and the vascular tissue (phloem) at 3 and 9 DAA. The *OsMADS13* and *ZAG2* genes have been implicated in ovule development before anthesis and in early seed development based on their expression patterns and on functional studies of the related transcription factors FBP7 and FBP11 in *Petunia hybrida* (Schmidt et al., 1993; Colombo et al., 1995; Lopez-Dee et al., 1999).

Other transcription factors had varied patterns of expression that may provide clues to their function or regulation. A gene encoding a high-mobility group (ID 701990762; Grasser et al., 1996) (see Supplemental Figure 5C online) was expressed stochastically in a pattern consistent with cell cycle-dependent transcription. A homolog of an uncharacterized transcription factor in maize (ID 701990488; Yao et al., 2002) (see Supplemental Figure 5D online) was expressed in a similar spatial and temporal pattern to the high-mobility group but was not regulated by the cell cycle. The transcript of a putative basic domain/leucine zipper transcription factor was restricted to the nuclei of the endosperm (ID 701769401; see Supplemental Figure 5E online). Finally, a gene encoding an RCD1-like protein was expressed in the central starchy endosperm (ID 702012812; see Supplemental Figure 5F online); RCD1 is a differentiation factor in yeast (Okazaki et al., 1998) and mammals (Hiroi et al., 2002).

### DISCUSSION

This study shows the feasibility of systematic spatial analysis of gene expression at cellular resolution in a plant species. We surveyed nearly 900 genes and obtained a clear and interpretable pattern for 75% using a consistent and robust method. Genetic resources for many species are rapidly being developed, and this approach could feasibly be extended to entire genomes. Tilling for functional analysis is well established in barley

(Caldwell et al., 2004) and is being developed for wheat (Slade et al., 2005). Our study has identified many candidate genes that can be characterized in the future by such genetic approaches. Even with the resources available at present in wheat, a comparative bioinformatics approach with *Arabidopsis* and rice is likely to confirm function for at least a subset of the genes we have characterized.

Our analysis, combined with functional predictions for the genes, has provided insights into the regulation of cellular specialization, cell cycle regulation of gene expression, coordination of endosperm and maternal development, and evolutionary conservation of seed-related functions.

Our analysis has revealed numerous novel cell cycle-regulated transcripts, including some that encode cytoskeletal functions, nuclear structure, protein translation, and signaling. Orthologs of several of these genes are also differentially regulated during the cell cycle in *Arabidopsis* suspension cells (Menges et al., 2002), indicating that transcriptional control of cell cycle gene expression is largely conserved in different groups of plants. However, others appear to be novel, and their cell cycle regulation may be specific to endosperm. Sphingosine kinase is an interesting example because this protein is implicated in cell cycle reentry in animal cells (Olivera et al., 1999). Sphingosine kinase is a key enzyme catalyzing the formation of an important bioactive lipid messenger, sphingosine 1-phosphate, and has been implicated in the regulation of cell proliferation and anti-apoptotic processes in mammalian cells (Olivera et al., 1999), but its role in plants is unknown. More surprising, perhaps, is the observation that tRNA-Ala synthetase is regulated by the cell cycle. However, other tRNA synthetases have been reported to be regulated by the cell cycle in *Arabidopsis* culture cells (Menges et al., 2002), and other aspects of protein translation are coupled to cell cycle progression in animal cells (Pyronnet et al., 2001). CDKA, the major cell cycle regulatory kinase, binds to translation initiation factors in a proliferation-dependant manner (Hutchins et al., 2004). The regulated expression of these genes involved in protein translation and signaling may couple growth with cell proliferation.

The spatial regulation of cell division plays a central role in plant development (Meyerowitz et al., 1998). Our analysis shows that the cessation of cell cycle activity in the endosperm begins in the modified aleurone layer, in the vicinity of the crease. The expression of >65 genes that display cell cycle-regulated gene expression is downregulated first in this region. The downregulation then spreads into the central region as the endosperm genes become active ~9 DAA and finally spread around the periphery as the aleurone cells differentiate. Second, many cell type-specific and differentially expressed genes display the opposite pattern: expression is initiated in the vicinity of the crease. Expression then spreads outward through the modified aleurone or through the entire endosperm. Where the function of these genes can be predicted, they tend to encode proteins that are involved in cell differentiation. Together, these two general observations suggest that a signal emanates from the crease, switching off cell proliferation and inducing cell differentiation. The nature of this signal is unknown, but an ethylene biosynthetic enzyme is expressed in the modified aleurone at the same stage at which cellularization begins. Ethylene signaling controls sev-

eral aspects of plant cell differentiation and development and plays a central role in cell specification of epidermal cell types in the root (Dolan et al., 1998). The aleurone is the epidermal cell layer of the endosperm, and cellular differentiation may involve similar mechanisms in the two tissues. These results are consistent with the idea that the modified aleurone is not only involved in nutrient transfer but may also be a conduit for regulatory signals.

The nucellus and aleurone layers represent the interface between maternal and endosperm tissue, two organs whose growth and development must be orchestrated during seed development. Maternal and endosperm cell types can coordinately become specialized, and this is reflected by temporally coordinated transcription in different cell types. For example, we found that both the nucellar projection and the adjacent modified aleurone layer preferentially express genes encoding several cell wall-modifying enzymes. Although most of the nucellar cells die and begin to break down soon after fertilization (Norstog, 1974; Chen and Foolad, 1997; Dominguez et al., 2001), the maternal nucellar projection and neighboring modified aleurone in the endosperm establish complex transfer cell morphology that involves the elaboration of specialized cell walls (Morrison et al., 1978; Offler et al., 2003; Barron et al., 2005). The spectra of differentially expressed genes specific to particular cell types, therefore, can reveal their metabolic or functional specialization.

By contrast, we show that defense-related functions are expressed widely in the caryopsis, both spatially and temporally dispersed. For example, defensins and thaumatin are expressed early in the integuments and vasculature, defensins are expressed in the aleurone layer from 6 DAA, and later, thionins and  $\alpha$ -amylase inhibitors are expressed in the starchy endosperm, correlating with the beginning of storage product deposition. These results are consistent with a role for these genes in protecting the main nutritional resource of the developing caryopsis from exterior microbial attack.

A comparative analysis suggests that a large number of endosperm functions may have diverged within monocots, because approximately one-third of the wheat genes we surveyed produce no significant BLAST hits in the rice genome. Notably, the genes that do not produce hits include the wheat storage protein gene sequences, highlighting the variation in the predominant storage protein type in cereals, which are gliadins (prolamins) in wheat and glutelins (globulins) in rice (Shewry and Halford, 2002; Gibbings et al., 2003). In agreement with this, the largest classes of wheat genes analyzed in this study with no rice hit at the nucleotide level are those with patterns in the central endosperm and the modified aleurone. However, many other functions seem to have been conserved across the seed plants. BLASTP interrogation indicated that 400 wheat genes have significant similarity with *Arabidopsis* counterparts. In recently published descriptions of *Arabidopsis* seed mutants (Tzafrir et al., 2003; Pagnussat et al., 2005), at least 25 showed significant similarity with genes expressed in the caryopsis (see Supplemental Spreadsheet 2 online). The seed phenotypes of many other *Arabidopsis* genes related to caryopsis-expressed genes have not been examined, but they provide good candidates for future phenotypic analysis.

Much of the value of analyses such as this is in providing a body of knowledge to suggest hypotheses that can inform and direct subsequent investigations. For example, our results clearly suggest the importance of the region of the modified aleurone layer near the crease in the change from division to differentiation in the endosperm. We also show that the peripheral and central endosperm are already distinct at the molecular layer before cellular differentiation. We have identified several coregulated groups of genes that may have functional significance for wheat caryopsis development and cellular specialization and cell proliferation. Cross-species comparisons with the model plants rice and *Arabidopsis* can elucidate the functional significance of the expression patterns we have identified. Together with data from model species, the genetic and genomic resources currently being developed for cereals (Ogihara et al., 2003; Close et al., 2004; Wilson et al., 2004) will allow studies such as ours to provide a descriptive and predictive framework to understand this complex and important system.

## METHODS

### Plant Material Preparation

Wheat (*Triticum aestivum* cv Savannah) was grown from seed in soil-based compost in a controlled-environment room under conditions of 16 h of light and 8 h of darkness at 18°C and 70% RH. After 4 to 5 weeks, seedlings were vernalized for 6 to 8 weeks by growth under conditions of short days (8-h photoperiod) and low temperature (10°C) and then returned to the controlled-environment room. Ears were tagged daily at anthesis. The in situ protocol was derived from the methods described by Coen et al. (1990) and Fobert et al. (1994) with modifications described below to support a high-throughput capacity. Wheat caryopses harvested at 3, 6, and 9 DAA were trimmed and fixed in 4% (w/v) formaldehyde (freshly made from paraformaldehyde) in PBS (2.48 g of NaH<sub>2</sub>PO<sub>4</sub>, 21.36 g of Na<sub>2</sub>HPO<sub>4</sub>, and 87.66 g of NaCl), then transferred to the Tissue Tek vacuum infiltration processor supplied by Bayer (Newbury, UK) for an automated fixation/dehydration/infiltration process as follows: 4% formaldehyde in PBS for 6 h at 35°C; 70% ethanol for 1 h at 35°C; 80% ethanol for 1.5 h at 35°C; 90% ethanol for 2 h at 35°C; 100% ethanol for 1 h at 35°C; 100% ethanol for 1.5 h at 35°C; 100% ethanol for 2 h at 35°C; 100% xylene for 0.5 h at 35°C; 100% xylene for 1.0 h at 35°C; 100% xylene for 1.5 h at 35°C; and molten paraffin wax (supplied by VWR International, Poole, UK) for 1, 1, 2, and 2 h at 60°C. All steps were performed under vacuum (>50 cm Hg). Samples were then transferred to the Tissue Tek embedding console (Bayer) for embedding in paraffin blocks.

### Section Preparation

Fourteen-micrometer sections from wheat samples at the required stages were cut on a Leica Microtome (RM2125RT; Wetzlar, Germany) and organized on poly-Lys-coated slides overlaid with eight-well silicone isolators (from Grace Biolabs and supplied by Stratech Scientific, Soham, UK). The isolators facilitate multiple hybridizations on a single slide. After drying down at 42°C overnight, the isolators were removed and the slides were checked by visual inspection using a dissecting microscope.

To remove wax and prepare the slides for hybridization, pretreatment steps were performed using the VP2000 slide processor (from Vysis and supplied by Abbott Diagnostics, Maidenhead, UK) using the following program: two washes in xylene for 20 min each; 100% ethanol for 10 min, then through a 95, 85, 50, and 30%, ethanol series (2 min each); PBS for 3

to 4 min; proteinase K treatment (2 to 3 μg/mL in 100 mM Tris and 10 mM EDTA, pH 7.5) for 30 min at 37°C; Gly (0.2%, w/v) for 2 min; PBS for 3 to 4 min; acetic anhydride (0.5% [v/v] in 0.1 M triethanolamine, pH 8.0) for 10 min; PBS for 3 to 4 min; and then back through 30, 50, 85, 95, and 100% ethanol. Slides were dried at room temperature and stored at 4°C until hybridization.

### Generating Templates, Labeling Probes, and Sequence Analysis

Wheat cDNAs for screening were obtained from Syngenta as inserts in the pINCY vector, derived from pSPORT1. The cDNA library was constructed using an oligo(dT) primer with poly(A) RNA. For in situ probe labeling by in vitro transcription, primers were designed to append a T7 RNA polymerase site to the 3' end of the insert with the other primer nested inside the native vector T7 RNA polymerase site: T7.2, 5'-GAATT GTAATACGACTCACTATAGGGCCAGTGAATTGAATTTAGG-3'; R7.2, 5'-AGGGAAAGCTGGTACGCCTGC-3' (the T7 promoter binding site is underlined). PCR and subsequent manipulation of probes was done in 96-well format. PCR was performed with the following cycles: 94°C for 3 min; then 30 cycles of 94°C for 45 s, 63°C for 45 s, and 72°C for 1.5 min; and a final extension of 72°C for 6 min. For 96-well plates, PCR product purification was performed using the Montage cleanup kit (Millipore, Bedford, MA). PCR products were assessed on ethidium bromide-stained precast agarose gels (1% E-gels from Invitrogen, Paisley, UK) before end sequencing with primer R7.2. Because of the nature of the library's construction, all inserts contained the 3' untranslated region but were not full-length cDNAs. Sequences were subjected to systematic BLASTX analysis to identify homologs. Because of the incomplete nature of the sequences, BLASTN searches were conducted against wheat genes to identify longer cDNA or EST cognate sequences that could be subsequently included in BLASTX searches. Sequences were then compared using BLASTN from the National Center for Biotechnology Information with the rice (*Oryza sativa*) transcription units distributed in The Institute for Genomic Research rice all.seqs file dated October 2, 2005. For those having a significant hit ( $e10^{-5}$  or greater), the full-length rice protein sequence (taken from The Institute for Genomic Research rice all.pep file dated October 2, 2005) was compared using BLASTP from the National Center for Biotechnology Information with the *Arabidopsis thaliana* protein sequences (ATH1\_pep\_cm file from The Arabidopsis Information Resource dated February 28, 2004) to find the best hit in *Arabidopsis* ( $e10^{-5}$  or greater). Using the protein sequences identified, we then obtained protein domain and functional annotation information from the Protein Families and Gene Ontology Annotation databases using the Uniprot identifiers corresponding to the rice and *Arabidopsis* proteins.

In vitro transcription was performed in 10-μL reactions for 2 h at 37°C in the presence of digoxigenin-UTP nucleotides (0.35 mM) as described by Coen et al. (1993) but using 96-well plates. Hydrolysis was performed immediately in 100 mM carbonate buffer, pH 10.2, at 60°C for 30 min, and products were precipitated in 2.5 M ammonium acetate and 3 volumes of 100% ethanol for 1 h at 4°C. Plates were centrifuged at 4000 rpm for 30 min, and pellets were resuspended in 30 μL of 100 mM Tris, 10 mM EDTA buffer. Dilutions (100-fold) were made in water to assess the incorporation of digoxigenin-UTP. One microliter of each diluted probe was spotted on nitrocellulose for a dot blot and processed as follows: 30 min in blocking buffer (Sigma-Aldrich, St. Louis, MO); 30 min in anti-digoxigenin-alkaline phosphatase (Roche, Herts, UK); 5-min wash in Tris-buffered saline (TBS; 10 mM Tris and 250 mM NaCl); 5 min in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, pH 9.5, and 50 mM MgCl<sub>2</sub>); and developed in alkaline phosphatase buffer containing nitroblue tetrazolium (0.1 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate salt (0.075 mg/mL) for ~10 min. For use in hybridization, all probes were then diluted 100 times in hybridization solution (300 mM NaCl, 10 mM Tris, pH 6.8, 10 mM NaPO<sub>4</sub>, 5 mM EDTA, 50% [v/v] formamide, 5% [w/v] dextran sulfate,

0.5 mg/mL tRNA, 1× Denhardt's solution [1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA], and 0.1 mg/mL salmon testis DNA) and stored at −20°C until use.

### Hybridization and Washing

Hybridization chambers of the same dimensions as the silicone isolators (from Grace Biolabs and supplied by Stratech Scientific) were applied securely to the slides (after pretreatment), and a different probe (diluted in hybridization solution) was applied to each well for the three stages individually. Cover slips were placed on the chambers to prevent evaporation, and hybridization was performed overnight in a 50°C incubator.

Chambers were removed and the slides were immediately placed in the VP2000 slide processor for washing using the following program: 15 min in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 50% (v/v) formamide at 40°C; 40 min in same buffer at 50°C; 20 min in 1 × SSC and 50% (v/v) formamide at 50°C (all steps to this point with constant agitation); 5 min in 1 × SSC at room temperature; and 5 min in 1 × TBS at room temperature. The slides were then transferred into trays for anti-digoxigenin staining as follows: 1% blocking solution (Roche) in TBS for 1 h; 1 × TBS containing a 1:3000 dilution of anti-digoxigenin–alkaline phosphatase and 0.05% Tween 20 for 1 h; four 10-min washes in 1 × TBS; and 5 min in alkaline phosphatase buffer (0.1 M Tris, 0.1 M NaCl, and 50 mM MgCl<sub>2</sub>, pH 9.5). Then, the color reaction was developed in alkaline phosphatase buffer containing nitroblue tetrazolium (0.1 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine salt (0.075 mg/mL) for up to 24 h. In each 96-probe experiment, a selection of control probes was monitored to assess its sensitivity: histone H4 (high expression with distinctive spotty pattern; ID 701992946), a glutenin storage protein (as a negative control for the early stages of development; ID 701993554), and a gene expressed in the nucellar lysate (nucellain ortholog [ID 701996036] as a negative control for the later stages). Slides were then washed several times in water to stop the reaction, followed by sequential washes in 70 and 100% ethanol. Slides were allowed to dry and permanently mounted in Entellan (Merck, Rahway, NJ).

### Image Capture and Analysis

Representative sections for each probe were photographed with a Nikon E800 microscope (Tokyo, Japan) using a digital camera under bright-field conditions. These images were then entered in a Web-accessible database (<http://bioinf.scri.sari.ac.uk/cgi-bin/insitu/home>; user, guest; password, wheatinsitu) that is searchable by spatial expression pattern or by a nine-digit ID number for each gene. Magnifications and camera settings remained unchanged for all images through all stages for wheat sections.

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