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Identification of genes specifically expressed in maternal and filial tissues of barley caryopses: a cDNA array analysis

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Abstract Developing seeds consist of genetically distinct maternal and filial tissues, whose interactions during development are largely unknown. To better understand the molecular physiology of developing seed tissues in barley, we created a high-density cDNA macroarray bearing 711 cDNA fragments from 691 clones representing at least 620 unique genes mainly derived from a cDNA library constructed with mRNA from the early stages of caryopsis development. This array has been used to compare gene expression patterns in maternal pericarp and filial embryo sac tissues of caryopses sampled 1–7 days after flowering (DAF). The profiles obtained for both tissues revealed that at least 26 genes in pericarp and 12 genes in embryo sac tissues were up-regulated by more than a factor of two during this period. RNAs expressed at high levels in the pericarp mainly encode enzymes involved in carbohydrate and lipid metabolism, but also include mRNA for a transcription factor related to FILAMENTOUS FLOWER (FIL). Genes preferentially expressed in the embryo sac are mainly related to degradation and/or processing of proteins or are involved in the process of starch accumulation, which begins in the seed at this time. Some of the most conspicuously regulated genes were studied in more detail by Northern analysis and in situ hybridization. The mRNA with the highest apparent signal intensity encodes a methionine synthase (MSY). MSY is highly expressed throughout the pericarp and to a lower extent in the transfer cell layer of the endosperm. Of special interest is a gene of unknown function because its high-level expression is restricted to the nucellar projection, the maternal transfer tissue of the caryopsis. This gene, represented by clone HY09L21, may play a central role in transport processes and thus in embryo growth.

Keywords Barley (*Hordeum vulgare*) · cDNA macroarray · Differential gene expression · Seed development

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Introduction

Developing seeds comprise several organ and tissue types, either derived from the mother plant or formed as a result of gene activity after fertilization. In barley grains the major tissues are the diploid embryo, the triploid endosperm, and the maternal pericarp surrounding the former. Whereas the vast majority of published research has been devonted to the endosperm and its specialized tissue the aleurone (for review, see e.g. Ritchie et al. 2000), much less is known about the pericarp, which makes up most of the early seed weight in caryopses before the storage phase. The pericarp is regarded as nourishing the growing embryo/endosperm, and maternal mutants, of the *seg* type, for instance (Felker et al.1985), underline the importance of the maternal tissue for proper embryo development. Nutrients from source tissues destined for the grain reach the pericarp through the main vascular bundle, which runs across the whole length of the grain at the bottom of the crease. After being unloaded from the vascular tissue they are further transported symplastically through the maternal pericarp tissues to the cells of the nucellar projection. A second unloading step into the endospermal cavity is required to allow uptake of the nutrients by the filial endosperm tissue (Patrick and Offler 1995; Weschke et al. 2000). During early seed development the embryo sac represents only a weak sink for nutrients, and thus the pericarp probably functions as a transient storage tissue. However, details of this process in monocotyledonous plants are unknown. In legume (dicotyledonous) seeds, in contrast, a close interrelationship between carbohydrate

metabolism in maternal tissues and embryo development has been convincingly demonstrated. Specifically during early development of faba bean seeds a cell wall-bound invertase is active only in the maternal seed coat. There the enzyme controls the glucose level, a key determinant of embryo development (Weber et al. 1995; Wobus and Weber 1999). If the tightly regulated glucose levels are perturbed, embryo development is disrupted (Weber et al. 1998). Later during seed development sucrose is crucial for initiating starch accumulation (Weber et al. 1996). Soluble sugars are, however, not the only components in the large regulatory network that exists within seed tissues and affects their interactions (see e.g. Wobus and Weber 1999, for seeds, and Cowan et al. 2001, for fruits). Most probably the activity of this regulatory network will also be reflected by the expression pattern of genes at the mRNA level. Therefore, extensive gene expression analysis is an important experimental approach in the quest for a more detailed understanding of seed development.

During the last few years technologies have been developed, e.g. cDNA arrays (Schena et al. 1995), which allow the parallel and comparative analysis of the expression of many genes. However, this powerful technique has not been used until recently to analyse seed development in detail, because of a lack of cDNA clones derived from plant seeds. More recently, a large set of randomly chosen, partially sequenced cDNAs from seeds has been reported from *Arabidopsis* (White et al. 2000), and a microarray with approximately 2600 seed-expressed genes has been employed to analyse their expression levels in seeds versus leaves and roots (Girke et al. 2000). Array-based expression analyses of developing cereal seeds, which, in contrast to Arabidopsis seedlings, mainly store carbohydrates in the endosperm instead of oil in the cotyledons, have not yet been reported. Therefore, we have used cDNA clones from developing barley caryopses and EST (Expressed Sequence Tag) data derived therefrom (Michalek et al. 2001) to establish the cDNA array technology and analyse gene expression in maternal and filial tissues during the pre-storage phase of the barley grain.

Almost 700 clones representing mostly unique genes were selected preferentially from a caryopsis cDNA library and the amplified inserts were spotted at high density onto nylon membranes. These macroarrays were hybridized with radioactively labelled total cDNA from pooled tissue samples of either maternal pericarp or filial embryo sac tissues collected during the pre-storage phase of the caryopsis, 1–7 days after flowering (DAF). These analyses provide an eclectic overview of gene expression in the maternal and filial tissues during early caryopsis development, and we have identified sets of genes that are preferentially expressed in one or the other tissue. Some of the most highly regulated genes were studied in more detail by Northern analysis and in situ hybridization, in order to obtain initial hints as to their functions in the context of seed development.

Materials and methods

Plant material and tissue preparation

Hordeum vulgare cv. Barke, a two-rowed, Spring barley cultivar, was cultivated in growth chambers at 20°C/18°C on a 16 h light/8 h dark cycle. The young developing seeds were harvested from the mid-region of the ear at 1-day intervals after flowering, and the developmental stage was determined in the centre of the spike as described by Weschke et al. (2000).

Selection of cDNA clones

The cDNA clones from barley were obtained from the EST programme of the IPK Plant Genome Resources Center (Michalek et al. 2001). Clones were selected from a cDNA library of developing caryopses (517 clones) and smaller samples were chosen from libraries constructed with RNA from etiolated seedlings (70 clones) and roots (104 clones), giving a total of 691. All sequences, except for a few of lesser quality, have been deposited in the EMBL sequence database, and can also be obtained from the Web server at the IPK (http://pgrc.ipk-gatersleben.de).

Amplification of cDNA inserts

Plasmid DNAs were prepared in 96-well format (Qiaprep 96 Turbo Bio Robot Kit; Qiagen, Hilden, Germany). Inserts of cDNA clones were amplified by PCR using slightly modified M13 universal (5′-CGACGTTGTAAAACGACGGCCA-3′) and reverse primers (5′-ACAGGAAACAGCTATGACCTTG-3′) complementary to vector sequences flanking the cDNA inserts. For each cDNA clone, about 5 ng of plasmid DNA was used as template in a 50-µl PCR containing 10 mM TRIS-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, each primer at 1 µM, and 0.6 U of Taq polymerase (Amplitaq Gold). After initial denaturation at 94°C for 1 min, inserts were amplified (Master Cycler Gradient, Eppendorf, Hamburg, Germany) for 30 cycles of 30 s at 94°C, 30 s at 58°C and 2 min at 72°C. An aliquot (5 µl) of each reaction was fractionated on a 1.5% agarose gel to evaluate the quality of amplicons. PCRs that yielded more than one fragment or resulted in low amounts of product, were repeated using a slightly different annealing temperature. After purification on QIA-quick columns (Qiagen) according to the manufacturer's protocol, amplification products were again analysed on 1.5% agarose gels to estimate their DNA content and finally adjusted, if necessary, to concentrations of between 2.0 and 0.1 µg/µl.

Spotting of cDNA fragments

The concentration-adjusted PCR products were diluted 1:1 with 1 M NaOH/5 M NaCl and deposited in duplicate on positively charged 5×9 cm nylon membranes (BiodyneB, Pall, Dreieich, Germany) using a BioGrid robot equipped with spotting pins of 0.4 mm diameter (Biorobotics, Cambridge, UK). Three strokes with the spotting tool were used to transfer approximately 15 nl of each PCR product to the nylon membrane. After spotting was completed, the membranes were washed in 0.4 M NaOH/1.5 M NaCl, neutralized in 0.5 M TRIS-HCl (pH 7.5)/1.5 M NaCl, and the DNA was cross-linked to the membrane surface by a brief exposure to UV light (120 mJ, Stratalinker, Stratagene, La Jolla, Calif.). After washing in 2×SSC, arrays were dried for 1 h at 80°C and stored at room temperature until further use.

RNA extraction and synthesis of ^{33}P-labelled cDNA

Tissue samples (100–200 mg) from pericarp and embryo sac were prepared by manual dissection, and used to obtain total RNA as

described by Heim et al. (1993). For the synthesis of ^{33}P-labelled cDNA, poly(A)$^+$ RNA was extracted from 35 µg of total RNA using oligo(dT)-magnetic beads (Dynal, Hamburg, Germany) according to the manufacturer's recommendations. Poly(A)$^+$ RNA bound to the magnetic beads was used directly for synthesis of covalently bound total first-strand cDNA as described by Dynal, using Superscript reverse transcriptase (Life Technologies, Karlsruhe, Germany). ^{33}P-Labelled second-strand cDNA free of unincorporated [^{33}P]dCTP was obtained using a random priming reaction (Megaprime Labelling Kit, Amersham Pharmacia, Freiburg, Germany) with an increased amount of Klenow polymerase (10 units). After removal of the supernatant, ^{33}P-labelled cDNA was eluted from the magnetic beads in 50 µl of 2 mM EDTA by heat denaturation (3 min, 95°C), and filtered using a micro-centrifuge tube filter equipped with a 0.2-µm Anapore membrane (Whatman, Göttingen, Germany).

Hybridization procedures

Before the first hybridization experiment with labelled cDNA, every cDNA array was cycled through a mock hybridization including a probe removal procedure to detach loosely bound spotted DNA. After wetting in 2×SSC, the array was pre-hybridized for at least 3 h at 65°C in Church buffer (0.5 M sodium phosphate pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA) containing sheared salmon sperm DNA (10 µg/ml). Heat-denatured (3 min, 95°C) labelled cDNA was added together with fresh hybridization buffer and hybridized at 65°C for at least 12 h. cDNA arrays were washed three times with 40 mM sodium phosphate pH 7.2, 1% SDS, 2 mM EDTA for 20 min at 65°C, wrapped in plastic wrap and exposed to the imaging plate of a Fuji BAS2000 phosphoimager (Fuji Photo Film, Tokyo, Japan) for 2–4 h. Labelled probes were removed completely by successively treating the array with boiling wash solution, 0.4 M NaOH (for 15 min at 45°C), and a neutralizing solution (0.1×SSC; 0.1% SDS; 0.2 M TRIS-HCl pH 7.5). Successful removal of the labelled probe was confirmed by exposing the washed membrane to an imaging plate for at least 4 h.

Array evaluation

The image data obtained from the phosphoimager were imported into the programme package Array Vision (Imaging Research, St. Catharine's, Ont., Canada) for spot detection and quantification of hybridization signals. Local background intensities were determined around each subarray of nine spots and subtracted. After export of the values to a common spreadsheet programme, the signal intensities were normalized with respect to the total amount of radioactivity bound to the array and the signal intensities of the two spots representing each amplified cDNA fragment were averaged.

Northern blotting and in situ hybridization

Total RNA prepared from pericarp and embryo sac tissues of developing caryopses (0–8 DAF) harvested at 2-day intervals was fractionated on a 1% agarose gel and blotted onto HybondN$^+$ membranes (Amersham Pharmacia). Membranes were sequentially hybridized with the following probes derived from cDNA clones: HY09N16 (HvSUS2), a 312-bp PCR fragment derived from the 3′-untranslated region (UTR); HY03B06 (putative transcription factor FIL), a 412-bp *Pst*I fragment; HW02F11 (vacuolar invertase), a 283-bp PCR fragment of the 3′-UTR; HY09L21 (a previously unknown gene called NucPro), a 1.2-kb *Dra*I-*Pvu*I fragment. Signals were detected with the Fuji BAS2000 phosphoimager. Lanes were checked for equivalent loading of total RNA using a 26S rDNA probe (data not shown).

In situ hybridization was performed according to Panitz et al. (1995) using ^{33}P-labelled fragments specific for the unknown gene HY09L21 (see above) and the methionine synthase gene HY05K19 (1.5-kb *Xho*I-*Nco*I fragment). As a negative control, slides were treated with RNase before hybridization. No labelling of any cell type by any probe was seen in control experiments (data not shown).

Results and discussion

Preparation of a cDNA macroarray

A programme designed to investigate the functional genomics of barley was started with the synthesis of cDNA libraries from developing caryopses, etiolated seedlings and roots. Subsequent single-pass sequencing of randomly selected clones from these libraries has provided about 13,000 ESTs derived from 5′- and 3′-ends of more than 7500 clones to date (Michalek et al. 2001). During the early phase of this programme 691 clones were selected to construct a cDNA array. A complete list of these clones, BlastX2 results and other data relevant to this publication are available from our WWW server (http://pgrc.ipk-gatersleben.de/screeni/exp.html). Based on current sequence and clustering data these clones represent more than 620 unique genes and therefore comprise the largest collection used for expression analysis of barley reported so far.

The cDNA inserts of all clones used for array preparation were amplified using vector-specific primers, purified, analysed on agarose gels, adjusted to concentrations between 2.0 and 0.1 µg/µl, if necessary, and spotted in duplicate onto nylon membranes as described in Materials and methods. The resulting cDNA array (5×9 cm) consists of 10×18 subarrays, each being a square of nine spots (see Fig. 1). The central spot in each subarray provides a blank control, while the remaining eight spots contain four different amplification products, each of them represented twice.

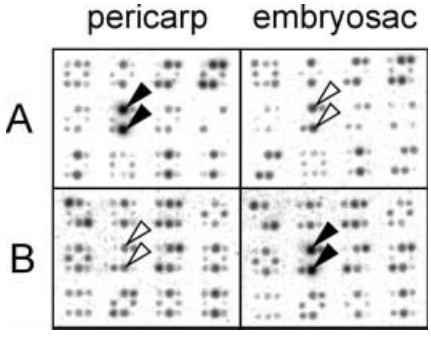

Fig. 1A, B Segments of a cDNA macroarray hybridized with ^{33}P-labelled second-strand cDNAs derived from pericarp and embryo sac tissues of the developing barley grain 1–7 DAF. Each panel shows 12 subarrays in a 4×3 arrangement, which are made up of a blank spot in their centre and eight surrounding spots representing four different cDNA-fragments, spotted in duplicate. Hybridization signals for the cDNA clones HY05K19 (**A**) and HY09L21 (**B**) which were used for Northern analysis and in situ hybridization are marked. The *filled triangles* indicate strong signals, the *open triangles*, weaker signals

Macroarray performance

After hybridization with ^{33}P-labelled second-strand cDNA, and three washing steps under highly stringent conditions, the signals on the array were detected using a phosphoimager. Resulting images were processed with a specialized software package for spot detection, and data files were exported to a standard spreadsheet programme. To allow comparison of data sets from different experiments (see Tables 1, 2 and 3), signals were normalized after background subtraction (see Materials and methods).

The ratio between the highest and the lowest signal (the latter defined as the average background intensity plus three standard deviations), was used to estimate the dynamic range of our array experiments. With background values ranging from 0.05–0.25 au (arbitrary units; standard deviation 0.08–0.12 au) and the most intense signals lying between 450 and 1000 au, the dynamic range was greater than 1000 in all our experiments. As a consequence of the weak influence of intense signals on neighbouring spots (data not shown), we did not fully exploit this dynamic range, but rather restricted our interpretations to clones which gave a signal intensity above 5 au in at least one of the two tissues examined (see Fig. 2). Internal control sets of 20 genes represented twice on the array but amplified independently (for example, in Table 1, HK03G06; in Table 3, HY02P18) or derived from different cDNA clones of the same gene (HY03B06 and HY10J06, and HK03G06 and HW01G04 in Table 1; HY05F13 and HY03B11 in Table 3) showed that expression ratios between pericarp and embryo sac can be reproduced with considerable accuracy within an array. In contrast to the ratios, the

Table 1 cDNA clones that are preferentially expressed in the pericarp

Clone ID[a]	Tissue 1/ Array 1			Tissue 2/ Array 1			Tissue 2/ Array 2			BlastX2 result[b]	Score (bits)	E-value
	Peri	Emb	P/E ratio	Peri	Emb	P/E ratio	Peri	Emb	P/E ratio			
HY10J06	21	1.9	**11**	24	0.7	**34**	20	0.5	**38**	Filamentous flower protein FIL, Arabidopsis	68	2E-11
HY03B06	15	1.4	**11**	14	0.3	**40**	12	0.4	**34**	Filamentous flower protein FIL, Arabidopsis	133	8E-31
HY01A03	17	3.0	**5.8**	23	1.0	**23**	25	1.1	**22**	β-Amylase	446	1E-125
HY02E15	20	3.5	**5.8**	17	1.9	**9.0**	29	2.1	**14**	Lipoxygenase 2	416	1E-116
HY05B22	12	2.9	**4.1**	17	1.5	**12**	18	1.7	**10**	Probable NADP-dependent oxidoreductase	205	2E-52
HK04H17	19	3.2	**6.0**	28	1.8	**16**	21	2.0	**10**	Probable glutathione S-transferase	187	7E-47
HY05O10	223	44	**5.1**	219	34	**6.4**	270	41	**6.7**	Fructokinase	254	2E-67
HY05K19	459	126	**3.6**	644	84	**7.6**	549	87	6.3	Methyltransferase/methionine synthase	357	4E-98
HY03J19	19	5.0	**3.8**	23	3.1	**7.4**	20	3.3	**6.0**	Hypothetical protein T8P19.200, Arabidopsis	166	1E-40
HK03G06	15	3.7	**4.0**	10	1.6	**6.2**	8.7	1.6	**5.4**	Cysteine proteinase 1	114	3E-30
HK03G06	8.9	2.0	**4.4**	7.4	1.5	**5.0**	6.4	1.3	**5.1**	Cysteine proteinase 1	114	3E-30
HY03G16	7.8	2.1	**3.8**	6.5	1.6	**4.1**	7.7	1.9	**4.1**	Hypothetical protein F26G5.50, Arabidopsis	122	2E-27
HW01K18	9.1	3.9	**2.3**	8.0	3.5	**2.3**	9.0	2.5	**3.5**	Tubulin α-chain	66	1E-16
HW02F11	28	8.3	**3.4**	20	4.1	**5.0**	18	5.3	**3.4**	Vacuolar invertase	247	4E-65
HY08P04	14	4.6	**3.0**	11.5	4.2	**2.7**	7.5	2.3	**3.2**	Membrane-anchored cellulase	312	1E-84
HY04F24	47	16	**3.0**	72	24	**2.9**	67	22	**3.0**	Hypothetical protein F28J12.210, Arabidopsis	156	1E-37
HY04H09	31	11	**2.8**	30	9.5	**3.1**	43	15	**3.0**	Auxin-responsive protein	213	9E-55
HY04N15	57	23	**2.5**	65	20	**3.2**	67	24	**2.9**	Cytoplasmic phosphoglucomutase	369	1E-101
HY07C03	37	12	**3.0**	33.8	17.4	**2.0**	40	16	**2.6**	Glucan endo-1,3-beta-glucosidase	98	1E-20
HY03C16	63	18	**3.5**	45	14	**3.1**	49	15	**2.6**	Acyl-CoA-binding protein	117	4E-26
HW01G04	48	21	**2.3**	29	12	**2.4**	22	9.3	**2.3**	Cysteine proteinase 1	282	2E-75
HW01F04	11	4.2	**2.7**	5.2	2.1	**2.4**	5.1	2.2	**2.3**	dTDP-glucose-4,6-dehydratase	350	1E-104
HY04F14	20	7.6	**2.6**	16	5.0	**3.2**	17	7.4	**2.4**	Glycine dehydrogenase	361	1E-103
HY10D17	32	8.4	**3.8**	21	8.7	**2.4**	31	14	**2.2**	Protein disulfide-isomerase	30	5.3
HY08K19	18	8.9	**2.0**	16	6.0	**2.8**	17	7.7	**2.2**	RF2 nuclear restorer protein	215	1E-55
HY09N04	50	23	**2.2**	49	17	**2.9**	29	13	**2.2**	Argonaute protein	256	4E-68

[a]Clones that are preferentially expressed in the pericarp are defined as those that give pericarp/embryo sac signal intensity ratios larger than 2 and absolute signal intensities greater than 5 au in three array experiments. Normalized signal intensities for pericarp (Peri) and embryo sac (Emb) tissues, as well as the corresponding Peri/Emb (P/E) ratios are listed for three experiments involving two different tissue prepeparations (Tissues 1 and 2) for probe synthesis, and two different array filters (Arrays 1 and 2)

[b]Top scores from a BlastX2 search against the protein databases SwissProt and PIR (June 2001) provide hints as to the potential functions of the respective genes

Table 2 cDNA clones preferentially expressed in the embryo sac

Clone ID	Tissue 1/ Array 1			Tissue 2/ Array 1			Tissue 2/ Array 2			BlastX2 result	Score (bits)	E-value
	Peri	Emb	E/P ratio	Peri	Emb	E/P ratio	Peri	Emb	E/P ratio			
HY03M02	1.3	5.4	**4.2**	3.5	96	**27**	4.2	114	**27**	α-Hordothionin	272	2E-72
HY09L21	100	932	**9.4**	28	541	**19**	20	518	**26**	Nuclear transition protein 2	38	3.8E-2
HY06E14	4.8	16	**3.4**	1.8	46	**26**	2.0	50	**24**	Proteinase inhibitor	78	1E-14
HY09N16	14	34	**2.5**	9.4	174	**18**	11	169	**16**	Sucrose synthase 2	331	2E-90
HY04N22	6.1	17	**2.8**	2.9	58	**20**	4.0	63	**15**	None		
HY04E07	19	73	**3.9**	1.8	26	**14**	1.5	23	**15**	Cysteine proteinase	213	7E-55
HY01O19	6.5	21	**3.2**	3.1	54	**17**	3.3	48	**15**	α-Amylase/ subtilisin inhibitor	69	2E-11
HY07E21	25	141	**5.7**	9.1	64	**7.0**	8.2	67	**8.2**	Replication factor C 38-kDa subunit	142	2E-33
HY03O04	1.4	9.4	**6.6**	1.0	8.6	**8.9**	1.5	11	**7.2**	None		
HY09L18	22	110	**4.9**	12	77	**6.6**	12	65	**5.5**	Probable aspartic proteinase	296	5E-80
HY02B16	6.8	35	**5.1**	1.8	11	**6.4**	2.4	11	**4.6**	Serine carboxypeptidase I precursor	390	1E-113
HY02F04	57	122	**2.1**	12	82	**6.8**	27	84	**3.2**	none		

[a]Clones preferentially expressed in the embryo sac are defined as those with embryo sac to pericarp signal intensity ratios larger than 2, and absolute signal intensities greater than 5 au in three array experiments. Normalized signal intensities for embryo sac (Emb) and pericarp (Peri) tissues, as well as the corresponding Emb/Peri (E/P) ratios are listed for three experiments. For further details see footnotes to Table 1

Table 3 Examples of cDNA clones expressed at about equal levels in pericarp and embryo sac tissues

Clone ID[a]	Tissue 1/ Array 1			Tissue 2/ Array 1			Tissue 2/ Array 2			BlastX2 result[b]	Score (bits)	E-value
	Peri	Emb	P/E ratio	Peri	Emb	P/E ratio	Peri	Emb	P/E ratio			
HY04L20	106	98	**1.1**	93	88	**1.1**	107	100	**1.1**	Elongation factor 1α	420	1E-117
HY01E21	84	63	**1.3**	71	81	**0.9**	72	87	**0.8**	Elongation factor 1α	459	1E-129
HY04G04	6.9	5.3	**1.3**	2.8	3.7	**0.8**	3.6	4.5	**0.8**	Elongation factor 1α	73	4E-13
HY02L19	41	41	**1.0**	27	33	**0.8**	27	31	**0.8**	Elongation factor 1β	162	4E-45
HY02A19	54	48	**1.2**	55	56	**1.0**	73	66	**1.1**	Elongation factor 2	300	4E-81
HY04L07	64	64	**1.0**	52	56	**0.9**	67	67	**1.0**	Histone H2b	184	4E-46
HY01M11	3.5	3.4	**1.0**	2.5	2.2	**1.1**	3.3	3.5	**0.9**	Tubulin α-3 chain	381	1E-105
HY05E03	5.3	5.5	**0.9**	6.5	6.0	**1.1**	7.3	7.2	**1.0**	SAR DNA binding protein	282	2E-75
HY02D20	8.8	8.0	**1.1**	4.2	5.3	**0.8**	5.0	5.9	**0.8**	Ribosomal protein S25	119	1E-26
HY05F13	115	112	**1.0**	92	81	**1.1**	84	83	**1.0**	Heat shock cognate protein 70 kD (HSC 70)	317	5E-86
HY03B11	122	117	**1.0**	115	95	**1.2**	110	90	**1.2**	Heat shock cognate protein 70 kD (HSC 70)	427	1E-119
HY05N18	5.4	5.7	**0.9**	4.2	3.9	**1.1**	3.2	3.5	**0.9**	Heat shock protein 82	312	3E-94
HY02P18	61	76	**0.8**	89	74	**1.2**	69	87	**0.8**	Granule-bound starch synthase	173	1E-69
HY02P18	36	33	**1.1**	29	23	**1.3**	29	30	**1.0**	Granule-bound starch synthase	173	1E-69
HY05H18	156	156	**1.0**	113	109	**1.0**	106	99	**1.1**	Maltase	307	1E-102
HY01B03	76	72	**1.1**	45	42	**1.1**	46	47	**1.0**	Adenine nucleotide translocator	183	1E-46
HY07L24	65	70	**0.9**	77	83	**0.9**	75	80	**0.9**	X-Pro aminopeptidase, pig	111	6E-26
HY02F14	58	57	**1.0**	79	81	**0.9**	72	75	**1.0**	Protoporphyrin IX magnesium chelatase	164	2E-46
HY08G11	31	30	**1.0**	23	23	**1.0**	24	26	**0.9**	Phosphatase-like protein	133	1E-51
HY02N18	19	20	**0.9**	20	20	**1.0**	20	19	**1.1**	Nucleoside diphosphate kinase	257	3E-68
HY01I03	12	11	**1.1**	16	17	**0.9**	13	13	**1.0**	Peptidylprolyl isomerase	290	9E-78
HW02G19	11	12	**0.9**	13	12	**1.1**	12	12	**1.0**	Hexokinase	32	2.8E-1
HY09A06	7.2	7.6	**0.9**	6.2	5.9	**1.1**	5.0	5.5	**0.9**	Nascent polypeptide-associated complex α-chain	133	8E-31

[a]For further details see footnotes to Table 1

signal intensities themselves cannot be used as reliable indicators of the expression level of a certain gene, because their values depend strongly on the amount and quality of the spotted amplification product. Reproducibility from array to array was evaluated by hybridizing two independently spotted arrays with the same labelled cDNA. A comparison of the results is shown in a scatter plot (Fig. 2A) which clearly demonstrates that significant deviations between the two arrays occur only at low signal intensity, when the accuracy of the spot-finding algorithm is reduced and the influence of background noise increases considerably.

For gene families it is expected that homologous sequences will lead to cross-hybridization, which may obscure the data for individual members. Even though the overall sequence identity may be as low as 70%, more highly conserved segments do result in considerable cross-hybridization (Girke et al. 2000; U. Hähnel, unpublished). We do not know how cross-hybridization might influence our data set in general, or in special cases where we have clearly identified gene families, e.g. the three different members of the elongation factor 1α (eEF1A) family present on our cDNA array (HY04L20, HY01E21, HY04G04 in Table 3). Protein eEF1A is multifunctional, and in maize endosperm, where five isoforms have been found, it forms part of a cytoskeletal network involved in zein storage protein biosynthesis and its level is highly correlated with the lysine content of the kernel (see Wang et al. 2001). In addition, several cDNAs that showed wide variability between hybridizations with tissue preparations 1 and 2 have been observed, e.g. clone HY09N16, representing the gene for sucrose synthase 2 (HvSUS2), yields embryo sac/pericarp ratios of 2.5 and 18 (see Table 2), while for the two clones HY10J06 and HY03B06, which are both derived from the one gene, pericarp/embryo sac ratios vary from 11 to 40, see Table 1) between tissue preparations. We confirmed that this variability is not a result of hybridization artefacts, but rather a consequence of differences between the two tissue preparations (for further explanation, see below).

Barley seed morphology and tissue preparation

For synthesis of hybridization probes, we prepared two tissue fractions, which should represent the maternal pericarp and the filial embryo sac (Fig. 3). Detailed knowledge of the tissue and cell types present in the two samples is of particular importance for the proper evaluation of the hybridization results. In the young barley caryopsis, the well-developed green pericarp represents the largest part of the grain. Conspicuous elements of the pericarp are the main vascular tissue bundle (VT), through which assimilates reach the grain; the cells of the nucellar projection (NP), which release the assimilates into the endospermal cavity; the maternal outer and inner integument (Fig. 3B), each composed of two cell layers; and the monolayered nucellar epidermis. These cell layers surround the diploid embryo and the triploid endosperm forming the embryo sac. Two cuticles, one between the two integuments and the second between the inner integument and the nucellar epidermis, separate the filial endosperm/embryo from the maternal tissue, except in the area of the main vascular bundle. To allow uptake of assimilates into the filial tissues, specific endospermal transfer cells (ET) differentiate (for an electron microscopic study of this process, see Weschke et al. 2000). These cells, localized in front of the nucellar projection cells, are part of the maternal-filial boundary of the caryopses (see below).

Because of the presence of the two cuticles, a predisposition exists for an easy separation of maternal and filial tissues. However, two tissues of maternal origin, the main vascular bundle (VT) and the nucellar projection (NP), tend to adhere to the embryo sac (Fig. 3C, D), and outer integument cells are always found in both fractions. This explains the finding that the mRNA that gave the highest hybridization signal in the embryo sac preparation is actually the product of a gene expressed in the maternal nucellar projection (see below). Therefore, the assignment of gene expression to a specific tissue or cell may require techniques with higher spatial resolution, such as in situ hybridization.

Different sets of genes are preferentially expressed in maternal and filial seed tissues

To monitor tissue-specific gene expression, second-strand ^{33}P-labelled cDNAs were synthesized from two completely independent preparations of pericarp and embryo sac tissues (tissue preparations 1 and 2) and used

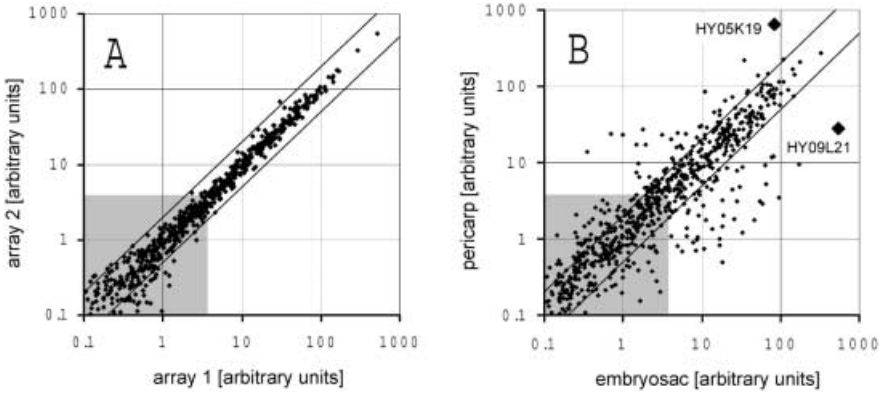

Fig. 2A, B Comparison of the normalized signal intensities obtained from two independently spotted arrays hybridized with the same labelled cDNA (**A**) and from one array hybridized successively with labelled cDNA from embryo sac and pericarp tissues of the developing barley grain 1–7 DAF (**B**). Signals outside the *diagonal lines* differ by more than a factor of two between the two hybridization experiments. The cDNA clones HY05K19 and HY09L21 (*enlarged symbols* in **B**) were used for Northern analysis and in situ hybridization. Signals within the *shaded areas* were excluded from further evaluation because of their low signal intensities

for hybridization first with array 1. In addition, tissue preparation 2 was hybridized to a second membrane (array 2) to check the consistency of results between different arrays. Fig. 2B shows the plotted results of a representative experiment (tissue 2/array 1) in which the membrane was hybridized first with cDNA from pericarp and, after probe removal, with cDNA from embryo sac tissue. cDNAs that showed more than a two-fold difference in signal intensity between the two tissues fall outside of the two parallel lines in Fig. 2B. In this experiment, 48 cDNAs appeared to be expressed preferentially in the pericarp and 42 genes were more highly expressed in the embryo sac that gave a signal intensity above 5 au in at least one of the two tissues examined and at levels at least two-fold higher than in the other tissue. If all three experiments (tissue 1/array 1; tissue 2/array 1; tissue 2/array 2) are taken into consideration, 38 clones, representing 34 different genes, consistently showed a more than two-fold difference between the two tissues (Tables 1 and 2). We would like to add at this point that, among the clone inserts representing the 620 unique genes used for array preparation, approximately 36 sequences had no significant match in the databases, and of those unknown genes at least six were found to be up-regulated in pericarp and four in the embryo sac. One of the latter (HY09L21) is described in more detail below.

Table 3 lists examples of genes that are expressed at similar levels in maternal and filial tissues, including members of the elongation factor gene family, chaperonin genes and genes for proteins involved in various biochemical pathways.

Genes preferentially expressed in the pericarp

As outlined above, during early grain development the pericarp's main function is assimilate transport, and transient storage of starch (Weschke et al. 2000) and possibly lipids (Molina and Garcia-Olmedo 1993), to allow continuous feeding of the filial tissues. This is at least partially reflected in the expression profiles, which revealed significant signals from pericarp (Table 1) for some mRNAs that can be assigned to either carbohydrate (HY01A03, HY05O10, HW02F11, HY07C03, HW01F04) or lipid (HY02E15, HY03C16) metabolism. Among the genes of carbohydrate metabolism that are up-regulated in the pericarp tissue, we note the increased level of transcripts for vacuolar invertase (HWO2F11). This enzyme and a related isoform, cell wall-bound invertase (HvCWINV2), the mRNA for which was found to be up-regulated 1.8-fold in the pericarp (not shown), are involved in the irreversible cleavage of sucrose into the hexoses fructose and glucose. It is assumed that the cleavage products can be transported through the cells of the nucellar projection, released into the endospermal cavity and used for feeding of the filial tissues. However, as indicated by the high levels of a fructokinase mRNA (HY05O10), fructose can also be phosphorylated and the resulting fructose phosphates can either be further metabolized via glycolysis or used for starch production. Fructokinase may play a specific role in this process in sink tissues, as suggested by Schaffer and Petreikov (1997), and could also function as a sugar sensor (Pego

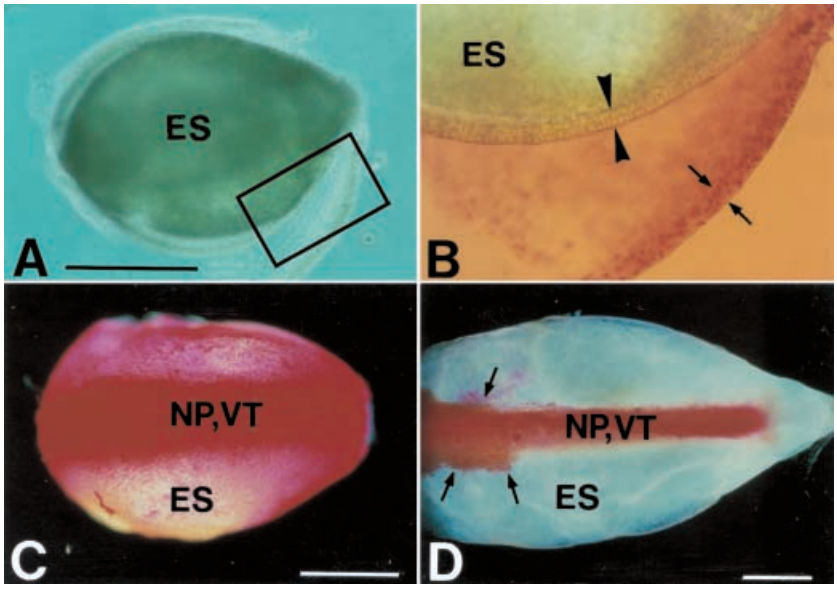

Fig. 3A–D Embryo sacs at different developmental stages, obtained by manual dissection. **A** Embryo sac (ES) at 2 DAF (phase-contrast image) with loose patches of the outer integument (*boxed region*). These adherent fragments of maternal tissue "contaminate" the pooled preparations of filial tissue. **B** The part of the embryo sac shown *boxed* in **A,** after staining with acetocarmine (bright-field image) to highlight the inner (*arrowhead*) and outer integuments (*arrows*). **C** Embryo sac at 3 DAF. The outer integument has been peeled off from the inner integument. Nucellar projection cells (NP) and the main vascular bundle (VT) adhere to the embryo sac, as indicated by strong acetocarmine staining (dark-field image). **D** Embryo sac at 5 DAF after removal of the outer integument; adhering residual parts of the outer integument are indicated by *arrows* (acetocarmine staining, dark field image). *Bars* = 500μm

and Smeekens 2000). Also involved in starch synthesis is a granule-bound starch synthase that is transcribed at moderate levels in both pericarp and embryo sac tissues (HY02P18; Table 3). As mentioned above, pericarp-specific starch is accumulated only transiently and mobilized later during development to feed the filial tissues. Mobilization of starch is catalysed by enzymes such as β-amylase, the RNA for which is almost exclusively found in tissues of the pericarp fraction (HY01A03; Table 1).

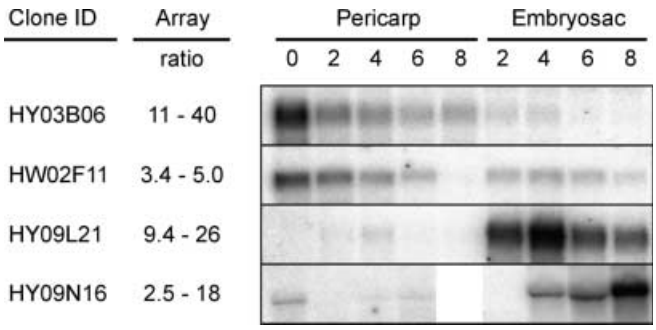

Fig. 4 Levels of transcripts differentially accumulated in pericarp and embryo sac of developing caryopses (Northern analysis) compared to their range of pericarp to embryo sac ratios (HY03B06 and HW02F11) or embryo sac to pericarp ratios of expression (HY09L21 and HY09N16) as determined by cDNA array analysis. The numbers 0, 2, 4, 6, and 8 indicate the time (in days after flowering) at which tissue samples were taken for the isolation of total RNA. HY03B06: FIL-related transcription factor; HW02F11: vacuolar invertase (HvVCINV); HY09L21: gene of unknown function termed Nucpro; HY09N16: sucrose synthase isoform 2 (HvSUS2)

Genes preferentially expressed in the embryo sac

During the first 6 days after pollination the endosperm expands dramatically and changes from a syncytial to a cellularized state (Olsen et al. 1992). Processes such as cell division and cell elongation are typical for this developmental state, whereas appreciable storage product accumulation is not expected. This situation is partly reflected in the results obtained with the embryo sac tissue probe. Signals from mRNAs for various storage proteins were near background levels (data not shown). However, significant signals were detected for some mRNAs coding for protein-degrading/processing enzymes (see HY03M02, HY06E14, HY04E07, HY09L18 and HY02B16; Table 2). This suggests that proteins, as well as starch and oils (see above), may accumulate transiently in the embryo sac, as has been observed during early developmental stages of faba bean seeds (Panitz et al. 1995).

Further characterization of selected genes

We selected five genes for further characterization by Northern analysis (Fig. 4) and in situ hybridization (Fig. 5), mainly to corroborate the array data and to localize precisely the sites of transcription of highly expressed genes. HY05K19 (MSY) and HY09L21 were selected because they gave strikingly high hybridization signals (see Tables 1 and 2), HY03B06 (FIL) shows the highest pericarp to embryo sac expression ratio (see Table 1), HY09N16A (HvSUS2) shows one of the highest differences in pericarp to embryo sac ratios between the two tissue preparations (see Table 2), and HW02F11 (vacuolar invertase, HvVCINV) shows a rather low expression level but a fairly constant pericarp to embryo sac ratio in all three experiments (see Table 1).

Methionine synthase (MSY)

The cDNA which gave the highest absolute hybridization signal in our analysis (HY05K19; Table 1) encodes a methionine synthase (EC2.1.1.14) with 87–89% homology to genes from *Solanum tuberosum*, *Mesembryanthemum crystallinum*, *Arabidopsis thaliana* and *Catharanthus roseus*. The enzyme catalyses the formation of methionine by the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine. This is the last step in the cobalamin-independent, de novo biosynthesis of L-methionine. The enzyme also serves to regenerate the methyl group of S-adenosylmethionine used for methylation reactions (Eichel et al. 1995). Therefore, if the high level of expression of MSY mRNA is indicative of high enzyme activity, the pericarp is either a site of intensive methylation reactions and/or the tissue produces large amounts of methionine. In situ hybridization localized the major sites of expression to the outer part of the pericarp, especially in the micropylar region but also in endospermal transfer cells (Fig. 5E, F). Eckermann et al. (2000) detected the enzyme by immunohistochemistry in cotyledons of C. *roseus* seedlings in the upper epidermis and suggested an involvement mainly in methylation reactions. We favour a similar interpretation for the growing young barley caryopsis in which massive storage protein synthesis has not yet started.

FIL-related transcription factor (FIL)

Among the genes with low transcript levels but highly specific expression in the pericarp is one represented by two ESTs, HY10J06 and HY03B06 (see Table 1). The sequence is related to that of an *Arabidopsis* gene for the transcription factor FIL (FILAMENTOUS FLOWER) and *FIL* in turn shows homology to the *CRABS CLAW* genes, founding members of the *Arabidopsis YABBY* gene family (Bowman and Smyth 1999). In *Arabidopsis*, members of the *YABBY* gene family are involved in various aspects of flower formation and abaxial cell fate specification (see, e.g., Sawa et al. 1999; Siegfried et al. 1999), i.e. the RNA is expressed in maternal tissues, as is shown here for barley. However, the function of the gene represented by the two clones HY10J06 and HY03B06

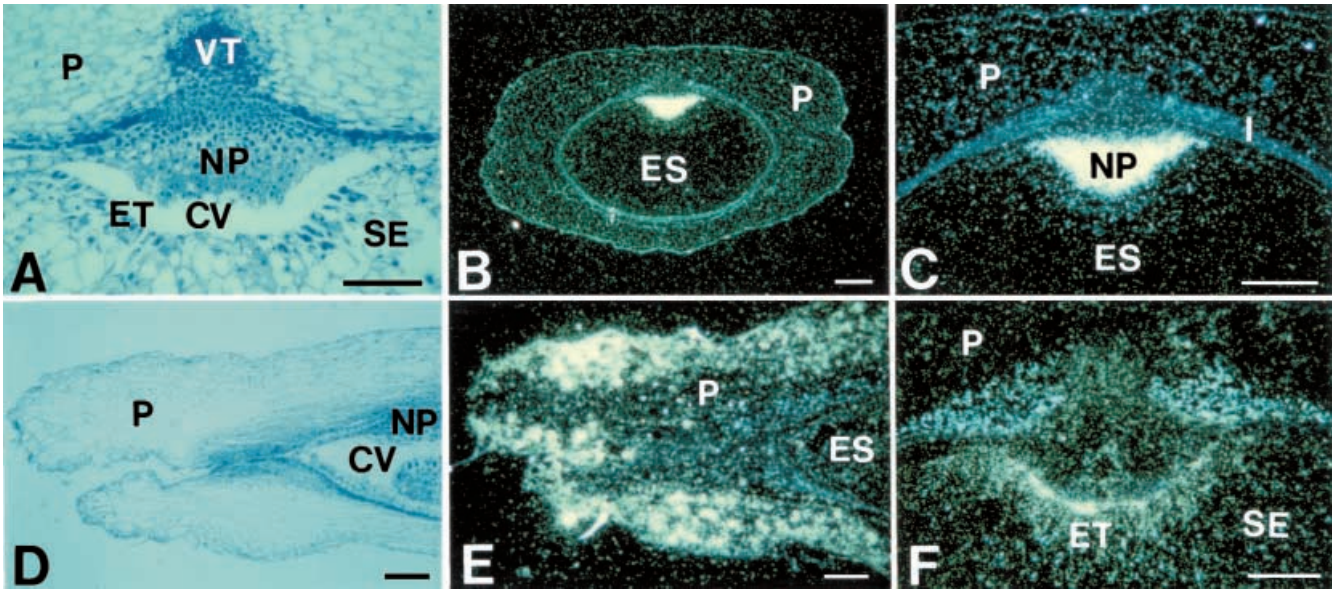

Fig. 5A–F Histological organization of the caryopsis (**A, D**), and in situ localization of HY09L2-Nucpro mRNA (**B, C**) and methionine synthase (MSY) mRNA (**E, F**) in developing barley caryopses. **A** Median-transverse section from the central part of a caryopsis showing its histological organization (6 DAF); toluidine blue staining. **B** Accumulation of HY09L21-Nucpro mRNA in the nucellar projection complex (4 DAF); dark-field image of a transverse section. **C** Higher magnification of the part of the nucellar projection area shown in **B**. **D** Median longitudinal section of the distal part of a caryopsis (6 DAF), toluidine blue staining. **E** Corresponding section showing the accumulation of MSY mRNA in the outer micropylar zone of the pericarp; dark-field image. **F** Accumulation of MSY mRNA in the endospermal transfer cells (4 DAF). Note the strongly labelled peripheral region contacting the central vacuole. The light blue colour of the maternal tissue flanking the nucellar projections is attributable to auto-fluorescence; median transverse section; dark-field image. *Bars* = 500 μm. Abbreviations: CV, central vacuole; ES, embryo sac; ET, endospermal transfer cells; I, integuments; NP, nucellar projection cells; P, pericarp; SE, starchy endosperm; VT, vascular tissue

in the barley pericarp is unknown. Northern analysis (Fig. 4) verified specific expression in the maternal pericarp with especially high levels at 0 DAF, the earliest developmental stage investigated.

HY09L21 (provisionally named *Nucpro*)

The highest hybridization signal found in experiments with probes for the embryo sac was given by clone HY09L21 (see Table 2). The available EST sequence did not exhibit significant homology to any gene of known function, i.e. we regard the BLASTX2 result "nuclear transition protein 2" listed in Table 2 as irrelevant. Expression of HY09L21 is highest at 4 DAF (Fig. 4). However, the transcript is also present in lower amounts at other developmental stages (2, 6 and 8 DAF; Fig. 4). Surprisingly, histological localization by in situ hybridization revealed expression exclusively in the cells of the maternal nucellar projection (Fig. 5B,C), leading to the provisional name Nucpro. This unexpected result can be explained by the tissue organization in the developing caryopsis, which leads to the adherence of maternal nucellar projection cells to the filial embryo sac (see above). It also points to the importance of using techniques with high spatial resolution, such as in situ hybridization, in gene expression studies. The massive expression of the gene in the unloading tissue for assimilates destined for the embryo suggests an important function in this process.

Sucrose synthase 2 (HvSUS2)

Northern analysis of HvSUS2 expression during early grain development (Fig. 4) revealed that mRNA levels in the embryo sac tissues rose from undetectable (2 DAF) to relatively low (4 and 6 DAF) and eventually very high levels (8 DAF). This increase can be correlated with the enzyme activity profile described earlier (Weschke et al. 2000).

A comparison of the mRNA profile of Fig. 4 with the different values obtained for HvSUS2 expression in cDNA array experiments 1 (ratio 2.4) and 2/3 (ratio 18/16, see Table 1) points to a problem in our analysis which becomes evident for genes like sucrose synthase 2. We studied early seed development from DAF 1 to 7. Since HvSUS2 mRNA levels rise dramatically at around DAF 7 (see Fig. 4) the signal intensity in the array is critically dependent on the exact developmental stage of the material collected for analysis. Generally, day 7 after flowering marks the beginning of an exponential increase in sucrose concentration in the whole caryopsis, a remarkable increase in the expression of the caryopsis-specific sucrose transporter HvSUT1 and a linear increase in sucrose synthase activity. All these parameters indicate the beginning of the final phase of starch accumulation in the starchy endosperm (Weschke et al.

2000). Therefore, small age differences between the caryopses used for pericarp and embryo sac tissue preparation will result in large differences in expression levels observed for genes related to carbohydrate metabolism, as was found for HvSUS2 (see above).

Sucrose synthase appears in monocot seeds usually in at least two isoforms. We identified by EST analysis and expression profiling four isoforms (data not shown), but only the HvSUS2 gene is expressed during the developmental time span we investigated here.

Vacuolar invertase (HvVCINV)

Northern analysis with an HvVCINV probe (see Fig. 4) in principle confirmed the array data discussed above and can also be related to enzyme activity profiles described before (Weschke et al. 2000). As in faba bean seeds (see Weber et al. 1995), the mRNA is preferentially found in maternal tissues especially during early developmental stages. We regard this fact also as the reason for the reproducibility of the pericarp to embryo sac expression ratio in all three experiments, which contrasts with the data for the *HvSUS2* expression pattern discussed above.

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