Journal of Experimental Botany, Vol. 54, No. 381, pp. 279–290, January 2003
DOI: 10.1093/jxb/erg031

Journal of Experimental Botany

RESEARCH PAPER

Isolation of GUS marker lines for genes expressed in *Arabidopsis* endosperm, embryo and maternal tissues

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Received 19 June 2002; Accepted 11 September 2002

Abstract

In order to identify marker lines expressing GUS in various endosperm compartments and at different developmental stages, a collection of *Arabidopsis thaliana* (L.) Heynh. promoter trap lines were screened. The screen identified 16 lines displaying GUS-reporter gene expression in the endosperm, embryo and other seed organs. The distinctive patterns of GUS expression in these lines provide molecular markers for most cell compartments in the endosperm of *Arabidopsis* seeds at all developmental stages, and represent a valuable research tool for characterizing present and future *Arabidopsis* seed mutants. GUS expression patterns of these 16 lines are presented here. One line showed chalazal endosperm-specific GUS activity at the heart stage of embryo development. In six lines embryo-specific GUS activity was detected. Six lines exhibited GUS activity predominantly in the endosperm and embryo while two lines showed strong GUS activity in all seed organs. In one line GUS activity was detected in integuments and syncytial endosperm, while the GUS activity at the cotyledonary stage of the embryo was seed coat-specific. In addition, two funiculus markers and two silique markers expressed in the abscission zone and the guard cells are also presented.

Key words: *Arabidopsis*, clearing, embryo, endosperm, GUS, seed, promoter, trapping.

Introduction

Phenotypic analysis of mutant plants, derived either from forward or reverse genetics approaches, is one of the richest sources for information on gene function. However, the task of unravelling the function of a gene is not always straightforward. In particular, seed lethal mutants in *Arabidopsis* can be challenging, and the stage of developmental arrest can be difficult to determine. Examples illustrating this include mutants affected in cytokinesis (Assaad *et al*., 2001; Lauber *et al*., 1997; Lukowitz *et al*., 1996; Strompen *et al*., 2002) and early endosperm development (Liu and Meinke, 1998; Mayer *et al*., 1999; Tzafrir *et al*., 2002). In such studies, molecular markers for cell types and tissues within the embryo and endosperm are very useful; the difference in marker expression between mutant and wild-type plants being able to detect differences that could otherwise have remained undetected. An example that illustrates the usefulness of such molecular markers is the analysis of *pilz* mutants (Mayer *et al*., 1999). In this study, expression patterns of several cell-cycle-dependent GUS reporters were followed during the development of *pilz* mutant embryos. Other examples of marker line studies include examinations of eight *emb* mutants, where the seed-specific promoters ABI3, 2S1 and Em1 were used to drive the GUS reporter gene (*gusA*) in a study of the effects of morphogenesis on storage proteins and Lea gene expression (Devic *et al*., 1996).

Several laboratories have reported the identification of molecular marker lines generated by promoter trapping, including the lines POLARIS (embryonic and seedling root tip), EXORDIUM (cotyledons and shoot and root

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apices) and COLUMELLA (root cap), which were used in the characterization of *gnom* and *hydra* mutants (Topping and Lindsey, 1997). In these studies, insertional mutagenesis and promoter trapping were combined in order to dissect polar development in *Arabidopsis* embryos and seedlings. Characterization of the *topless* mutation, causing shoot to root transformation during embryogenesis, was carried out by crossing the mutants to the enhancer trap lines J1092 and LENNY (root specific reporter), and the auxin-responsive reporter DR5 made by GUS promoter fusion (Long *et al*., 2001; JA Long, personal communication). This analysis showed that the LENNY reporter was expressed at both poles in *tpl* embryos while DR5 was misexpressed.

An alternative approach to the use of marker lines is *in situ* hybridization, in which the patterns of expression of known genes are studied. In this way, an analysis of five new MADS-box genes from *Arabidopsis*, termed AGL (agamous like), revealed that AGL18 and AGL16 are specifically expressed in the endosperm and in guard cells and trichomes, respectively (Alvarez-Buylla *et al*., 2000). In addition, several recent reports utilize this technique in the analysis of embryo and endosperm mutants of the *pilz* group (Mayer *et al*., 1999), *knolle* (Volker *et al*., 2001) and *prolifera* (Holding and Springer, 2002). Although highly efficient, this technique is time-consuming and requires knowledge of the gene product. In addition, in the *Arabidopsis* endosperm, identification of stage and compartment-specific developmental markers through differential screening is hampered by the small size of the *Arabidopsis* seed. In particular, the minute size and fragility of the syncytial endosperm do not allow dissection.

To date, few attempts have been made systematically to identify marker lines with GUS preferred pattern of expression in the *Arabidopsis* endosperm, although several endosperm marker lines have been derived from promoter and enhancer trapping experiments designed to detect GUS activity in embryos (Topping *et al*., 1994) and ovules in early seed development (Vielle-Calzada *et al*., 2000). For example, two lines, 17G2 and 19C1, were labelled as endosperm-specific in the promoter trap screening for molecular markers of embryogenesis. Using enhancer trapping, three endosperm-specific genes were mentioned, but their patterns of GUS expression have not been published (Vielle-Calzada *et al*., 2000). Studies of *Arabidopsis* mutants that exhibit autonomous endosperm development in the absence of fertilization *medea*/*fis1* (*fertilization independent seed*), *fis*/*fis2* and *fie* (*fertilization independent endosperm*)/*fis3* have also led to the identification of endosperm-specific markers (Chaudhury *et al*., 1997; Grossniklaus *et al*., 1998; Luo *et al*. 2000; Ohad *et al*., 1996). The endosperm-specific GFP reporter KS117 isolated by enhancer trapping showed expression patterns similar to *FIS1*/*MEA* and *FIS2* (Sørensen *et al*., 2001). This marker line features enhancer trapping of the *FORMIN* gene that most likely mediates actin polymerization (MB Sørensen, personal communication).

The experiments described in the present paper were initiated to identify additional molecular marker lines that complemented existing marker lines. In particular, the markers were useful for the studies of *Arabidopsis* endosperm (Brown *et al*., 1999). To achieve this goal, a collection of *Arabidopsis* promoter trap lines was systematically screened (Mandal *et al*., 1995) using an improved clearing method for GUS staining of the seed compartments (Stangeland and Salehian, 2002). The frequency of GUS positive lines obtained in this experiment accords well with that reported by Topping *et al*. (1994), 13% and 16%, respectively, although the two experiments may not be directly comparable due to the use of different clearing techniques.

Materials and methods

Plant material and growth conditions

In order to isolate molecular markers for endosperm cells and tissues in *Arabidopsis,* a collection of T-DNA promoter trap lines was screened. These transgenic lines contained the pMHA2 vector, harbouring a promoterless GUS reporter gene at the right T-DNA border (Mandal *et al*., 1995). The 5′ end of the *gusA* gene was placed approximately 40 bp from the right end of the T-DNA (Mandal *et al*., 1995). The plant transformation vector contained a *pnos-nptII* plant selectable marker gene. T-DNA lines were generated in the C24 ecotype of *Arabidopsis thaliana* by root transformation. Progeny seeds were selected on MS medium (Murashige and Skoog, 1962) containing 50 mg l^{-1} kanamycin. Plants were transferred to soil and grown at 23±3 °C and 70% humidity, under 40 W cool white fluorescent lights (16/8 h light/dark). Between six and nine plants from each family were assayed for GUS activity. Up to six subsequent generations were regularly screened for GUS activity in seeds.

GUS assay and image processing

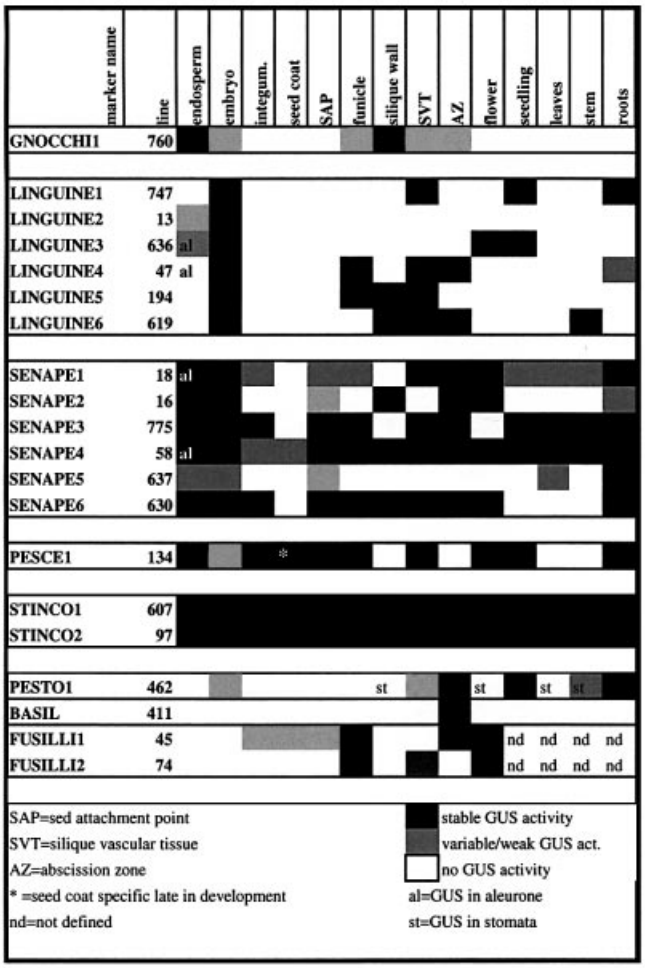
GUS assay (Jefferson *et al*., 1987) was performed on dissected, non-fixed *Arabidopsis* siliques at different developmental stages. Harvested siliques were slit twice longitudinally prior to the GUS assay and clearing (Stangeland and Salehian, 2002).

Results

The microscopy screen of 309 independent lines from the pMHA2 promoter trap collection (Mandal *et al*., 1995) was carried out on flowers, siliques and different vegetative organs that had been stained for GUS expression. Siliques were harvested from 0–10 DAP (days after pollination) and 16 lines were identified showing stable Mendelian inheritance of the GUS activity in seeds through several generations. GUS expression patterns in each of these lines behaved as single Mendelian traits and are presented in Table 1. Among these lines, one line showed staining only in the endosperm at a specific developmental stage (GNOCCHI1). Six lines showed embryo-specific activity (LINGUINE1-6), six in the endosperm, embryo and seed

Table 1. *Summary of GUS expression patterns of marker lines in* Arabidopsis *reproductive and vegetative organs*

Seed organs are indicated as endosperm, embryo, integuments, seed coat and seed attachment point. Parts of the siliques are indicated as funiculus, silique wall, silique vascular tissue (SVT) and the abscission zone of the silique (AZ). GUS specific activities in stomata (st) and aleurone layer (al) are indicated.



attachment point (SENAPE1-6), one in the endosperm, integuments and seed coat (PESCE1), and two in all seed organs (STINCO1, 2). In one line, GUS activity was observed in hydathodes of leaves, embryos and endosperm (SENAPE5). Of these 16 lines, 80% had detectable GUS activity in siliques and 60% in flowers. In addition, GUS activity in vegetative organs, especially, roots was not uncommon (Table 1).

Additionally, four silique markers were identified including an abscission zone marker (BASIL), a guard cell marker (PESTO) and two lines that expressed *gusA* in the funiculus (FUSILLI).

Endosperm expressed GUS reporters

The endosperm preferred GUS reporter line named GNOCCHI1 (GUS in nodule, chalazal chamber and integuments) showed specific staining of the chalazal endosperm and the adjacent integument tissue. Strong GUS activity was detected in the upper part of the siliques, bearing seeds at the globular stage of embryo development. At this stage, GUS staining was detected in the chalazal endosperm and in the embryo. At the heart stage of embryo development, GUS activity was detected exclusively in the chalazal endosperm (Fig. 1A). In addition to consistent staining in the chalazal chamber (ChC), staining of the surrounding tissues varied and was absent or distributed in a gradient-like fashion (Fig. 1A), as confirmed by studies of hundreds of seeds of this type. This gave the impression that the primary site of GUS activity was in the chalazal cyst and the nodule, and that the additional staining in the surrounding tissues might be caused by diffusion of the CIBr-indigo stain. Staining of the heart stage embryo was detected only in intensively stained seeds, suggesting that diffusion or uptake of CIBr-indigo produced in the ChC could be the reason for embryo staining at this stage. However, this is very difficult to verify. Whether or not *gusA* is truly expressed in the embryo at the globular stage of development, the GNOCCHI1 marker is specific for chalazal endosperm at the heart stage of embryo development. At later developmental stages (torpedo-walking stick) GUS activity could not be detected in seeds and siliques. Weak GUS activity was detected in the finiculus at the heart stage of embryo development.

The lines PESCE1 and STINCO2 (see below) showed especially strong GUS activity in the endosperm nodule (Fig. 2A), as well as activity in the whole seed. In a fraction of the seeds of the PESCE1 line, GUS activity was detected exclusively in the nodule, micropylar endosperm and integuments (Fig. 2A, B).

Reporter lines with GUS activity in embryos

The embryo marker lines identified in this study were termed LINGUINE (line with GUS in embryo). Within the collection of six LINGUINE lines, GUS activity was present either only in the embryos (LINGUINE1), or also in trace amounts in the endosperm at an early developmental stage (LINGUINE2, LINGUINE6), or at a late developmental stage (LINGUINE3, LINGUINE5) (Table 1; Figs 1, 2).

In LINGUINE1, visible GUS expression was detected at the heart stage of embryo development. Staining was strongest in the basal embryo proper, corresponding to the cells that are developing into the hypocotyl and the root tip (Fig. 1B, C). At later developmental stages, GUS staining was still present in the same area of the torpedo stage embryo (Fig. 1B), but became more evenly distributed in the hypocotyl at the cotyledonary stage of embryo development (Fig. 1C). GUS activity was not detected in the endosperm.

LINGUINE2 showed GUS activity from the heart stage onwards. Some weak and diffuse activity was detected in

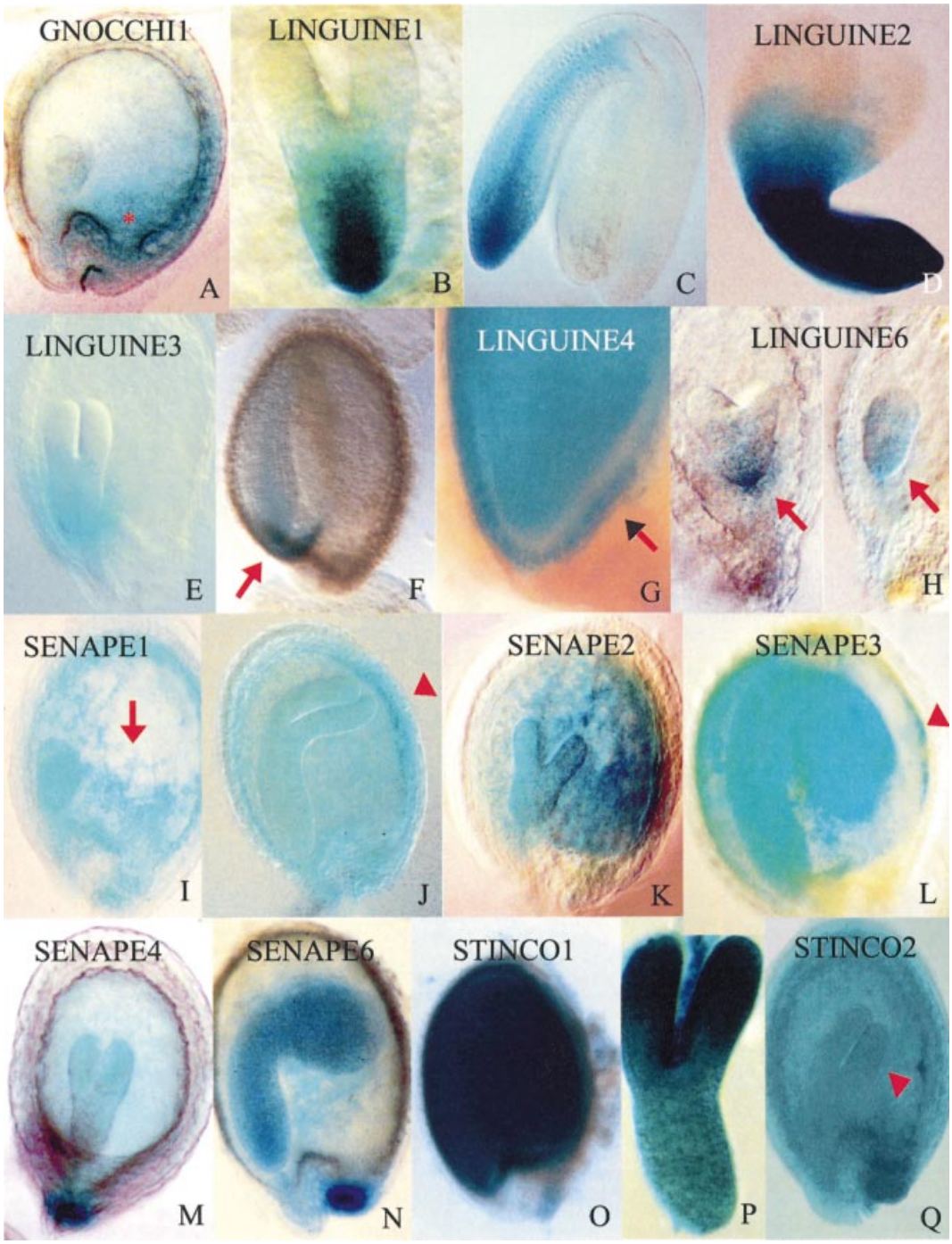


Fig. 1. GUS activity in promoter trap lines. GUS reporter lines expressing GUS in chalazal endosperm: GNOCCHI1 (A), embryo: LINGUINE1 (B, C), LINGUINE2 (D), LINGUINE3 (E, F), LINGUINE4 (G), LINGUINE6 (H), and endosperm, embryo and SAP: SENAPE1 (I, J), SENAPE2 (K) SENAPE3 (L) SENAPE4 (M), SENAPE6 (N), STINCO1 (O, P), and STINCO2 (Q) were presented. Arrows in (F) and (G) show staining of the aleurone layer. Arrows in (H) show GUS staining of the basal part of the embryo proper and the surrounding endosperm. Arrow in (I) points to the filamentous endosperm structures observed in this line. Arrowheads in (J) and (L) indicate staining of the aleurone and in (Q) staining of the endosperm nodule. Asterisk in (A) marks the chalazal endosperm cyst. See text for details.

the endosperm at earlier stages. GUS activity was also detected in the hypocotyls and cotyledons of the walking stick stage embryos (Fig. 1D).

In LINGUINE3 (Fig. 1E, F), GUS activity was detectable in the central area of the embryo proper at the globular and heart stage. From late heart to the torpedo stage of embryo development, GUS activity could be detected in the whole embryo, with the strongest activity in the basal part of the embryo proper and weakest in the surrounding endosperm (Fig. 1E). GUS activity could also be detected

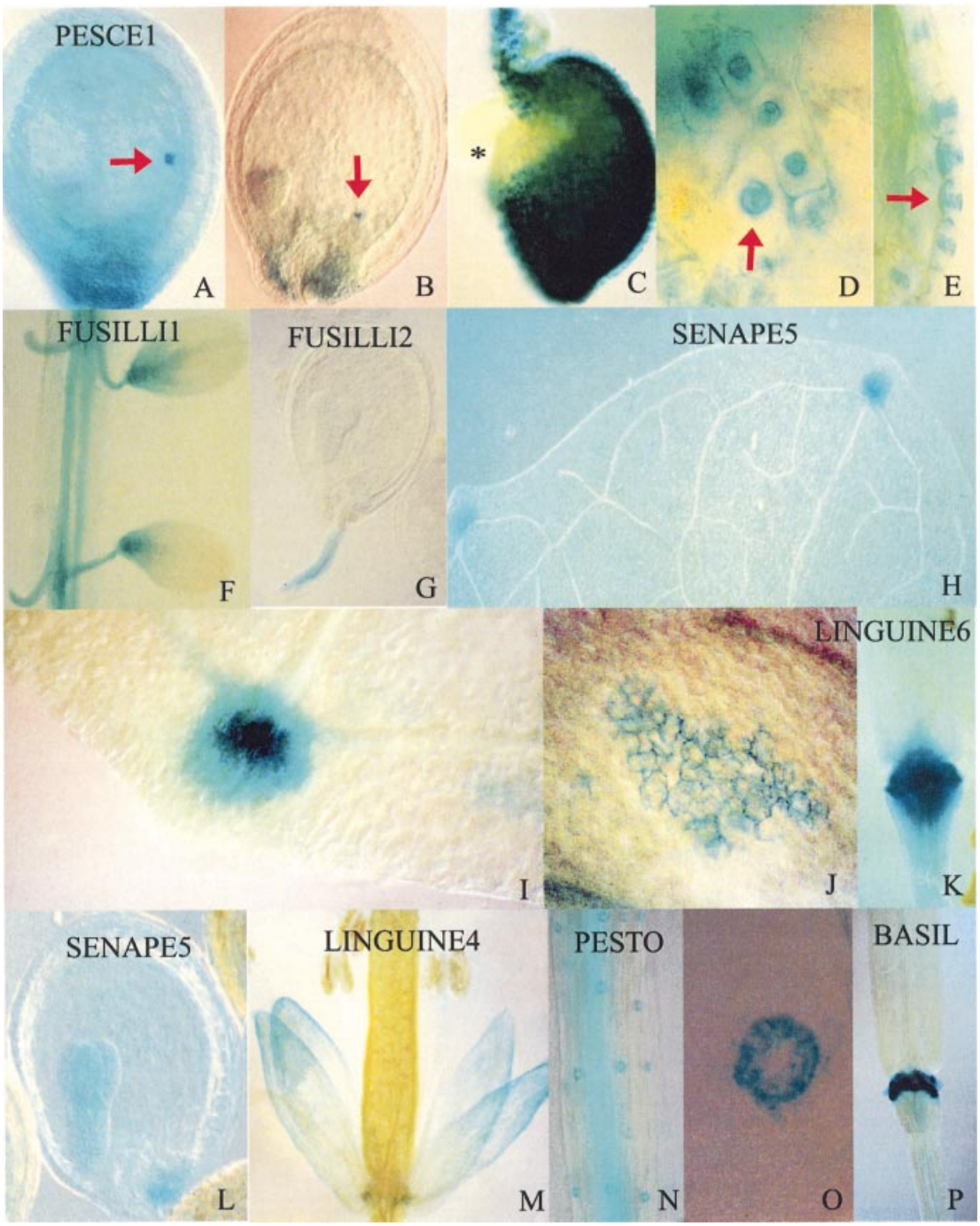


Fig. 2. GUS activity in promoter trap lines (continued). GUS staining in the marker line PESCE1 expressing GUS in integuments and endosperm at early stages (A, B), and exclusively in seed coat at later stages (C, E) is presented. In markers FUSILLI1 (F) and FUSILLI2 (G) GUS activity is detected in funiculus and silique. SENAPE5 is expressed in leaf hydathodes (H–J) and seeds (L). LINGUINE4 (K) is expressed in seeds (Fig. 1G) and in petals (M). Markers PESTO (L, M) and BASIL (N) are expressed predominantly in pedicel stomata (N, O) and in the abscission zone of the floral organs (P) respectively. Arrows in (A) and (B) indicate GUS staining of the endosperm nodule. Arrows in (D) and (E) point at the GUS stained columella, central area of the seed coat cells. Asterisk in (C) indicates late stage embryo that is not GUS stained.

in the few endosperm cells adjacent to the cotyledons (not shown). At the late torpedo stage, GUS activity could be detected in the embryo, suspensor, and the thin epidermal layer underneath the embryo corresponding to the aleurone layer (Fig. 1F).

In LINGUINE4, embryo and aleurone GUS expression patterns at the later stages were similar to LINGUINE3 (Fig. 1G). The GUS marker was also expressed in siliques and petals (Fig. 2M). Line LINGUINE5 showed GUS activity in the embryo, funiculus and siliques. Dissected

embryos from this line resembled embryos presented in Fig 1P, showing uniform GUS staining of the embryo. In LINGUINE6, very weak activity was present in the basal part of the embryo from the globular to the walking stick stage of development (Fig. 1H), whereas strong activity was present in the abscission zone of the siliques (Fig. 2K). Very weak activity was localized in the endosperm tissue surrounding the base of the embryo proper (Fig. 1H).

Other seed GUS reporters

In several of the lines studied, GUS activity was localized predominantly in the endosperm and embryo, but was also detectable in maternal tissues, including the seed attachment point and the integuments. This group of markers was termed SENAPE (staining in endosperm, attachment point and embryo). In SENAPE1, GUS activity was strongest in the endosperm and embryo, though weak activity in the integuments at later developmental stages could also be observed (Fig. 1I, J). In this line, GUS activity was detected shortly after fertilization in the central cell, in the endosperm, as well as in the embryo at the globular stage. At the heart stage of embryo development, filament-like patterns could be observed in the central endosperm chamber (Fig. 1I). Detailed analysis showed that these patterns represent intensively stained endosperm nuclei. Occasionally, GUS activity in the embryo was masked by blue staining in the endosperm, and was difficult to resolve. Later in development, GUS activity was detectable in torpedo stage embryos and the aleurone layer (Fig. 1J). In SENAPE1, GUS activity was also present in petals and in transport tissues and the abscission zone of the silique.

In SENAPE2, GUS activity could be detected in siliques (not shown) and seeds (Fig. 1K). At the globular and heart stages of embryo development, GUS activity was predominantly localized to the peripheral endosperm with some activity in the embryos. At the late heart/early torpedo stage, GUS activity could be detected both in embryos and in the endosperm (Fig. 1K), staining being especially strong in the chalazal chamber (Fig. 1K). At the walking stick and cotyledonary stages of embryo development, staining of the embryo was especially strong in the protoderm, the shoot apical meristem (SAM), and the embryonic root (not shown). In vegetative organs, none or only weak GUS activity was detected (not shown).

In the marker line SENAPE3, intensive GUS staining was detected in siliques and seeds (Fig. 1L). At the heart stages of embryo development, very weak GUS activity was detected in the embryo. At the torpedo and walking stick stages, embryo staining became intense (Fig. 1L). At this stage, GUS activity could be detected in the cellular endosperm and the epidermal layer corresponding to the aleurone layer (Fig. 1L, arrowhead). In integuments, GUS activity was either very weak or absent. Intense GUS activity was also present in the cotyledonary embryo, seedlings and flower. In SENAPE4, GUS activity was present in the endosperm, embryo and seed attachment points (Fig. 1M).

In seeds of the line SENAPE5, GUS activity was detected in the embryo, endosperm, funiculus, and hydathodes (Fig. 2H–J, L), and also in the area between leaf veins (Fig. 2J). While the GUS staining of the hydathodes (Fig. 2H, I) was strong and stable, GUS activity of the embryo and endosperm showed variable intensity (Fig. 2L). In SENAPE6, GUS activity was strongest in the embryo, endosperm and seed attachment points (Fig. 1N).

In other lines, including STINCO (staining intense and constitutive) 1 and 2, GUS activity was ubiquitous. In STINCO1 the GUS reporter was strongly expressed in all vegetative and reproductive tissues (Fig. 1O, P). In dissected embryos the strongest GUS activity was detected in cotyledons (Fig. 1P). GUS activity in STINCO2 was also intense and ubiquitous (Fig. 1Q). Particularly strong activity was detected in the endosperm nodule (Fig. 1Q, arrowhead), thus resembling marker PESCE1 (Fig. 2A). Later in seed development, GUS activity was detected predominantly in embryos.

Intense GUS staining, resembling the STINCO lines, was also found in PESCE1 (preferentially expressed in seed coat and endosperm). However, at later developmental stages this marker was seed-coat-specific (Fig. 2C–E), in contrast to the STINCO lines. GUS staining could be detected in the integuments immediately after fertilization. At all later stages, strong GUS activity was detected in the integuments and seed coat (Fig. 2A–E). At the globular and heart stages of embryo development, GUS activity could also be detected in the endosperm, but was absent or weak in embryos (Fig. 1A–C). The strong GUS staining of the chalazal endosperm nodule was especially noticeable (Fig. 1A, B, arrows). Stained endosperm nodules were attached to the chalazal integument wall, but their local position varied. A fraction of seeds showed GUS activity only in the nodule, micropylar chamber and the chalazal proliferating tissue (Fig. 2B). It is thought that the GUS expression patterns in this line could, at least partially, be caused by the diffusion of the CIBr-indigo stain from the chalazal endosperm to the adjacent seed and maternal tissues. At later stages, GUS staining was localized to the seed coat, precisely to the cytoplasm and cell walls of the mucilage cells (Fig. 2D, E, arrows). The morphology of this cell layer has been described in detail (Western *et al*., 2000; Windsor *et al*., 2000). Based on the information from these reports it was concluded that the GUS stain is localized to the central elevation, columella, harbouring the cytoplasm of these cells (Fig. 2D, E). Dissected embryos did not show GUS staining (Fig. 2C, asterisk). GUS activity in siliques was detected at the stages earlier than the heart-torpedo stage of embryo development. At later stages GUS activity was detected only in seeds.

Silique GUS reporter lines

In the lines FUSILLI 1 and 2 (funiculus and siliques line), GUS activity was detected in the funiculus, SAP and vascular tissue of the silique (Fig. 2F, G). In BASIL (basis of the silique), GUS activity was detected only in the basal part of the siliques that corresponds to the zone of abscission (Fig. 2P). In PESTO (pedicel stomata), GUS activity was localized to the guard cells (Fig. 2N, O), silique vascular tissue, auxiliary meristems, and the hypocotyls of seedlings. Staining of guard cells was especially strong in pedicels and stems. In leaves, either none or only weak staining of stomata, often in the vicinity of vascular tissue, was observed.

Several marker lines show imprinting but none of the lines showed visible phenotypes

Even though weak endosperm defects were occasionally observed in the original isolates of the lines described above, mutant seed phenotypes were not identified. By counting seeds from 6–9 plants per line; 5–10 siliques (up to 500 seeds) per plant, frequencies of non-pollinated and aborted seeds were estimated to be similar in transgenic lines and the wild-type plants growing in the same growth chamber. between 0.5% and 1% aborted seeds were registered in all lines. The number of non-pollinated ovules per silique varied a lot, but was, on average, about a few per cent. All marker lines showed stable patterns of inheritance of the GUS expression pattern over several generations. Neither lethality, nor a deficiency of homozygous genotypes was observed.

In order to characterize inheritance patterns of molecular markers further, reciprocal crosses were also carried out involving the marker lines and wild-type plants (Table 2). In this analysis, wild-type flowers were pollinated with pollen from the marker lines SENAPE1, SENAPE2, GNOCCHI1, and PESCE1. Genetic crosses were performed by pollinating 15–20 wild-type flowers per transgenic line. Directly after the genetic cross 2–3 siliques were harvested every day and seeds were tested for GUS activity (20–30 seeds per line daily). seeds were analysed from the very early stages up to and including the cotyledonary stage of embryo development (7 DAP, days after pollination). In total, up to 100–200 seeds per marker line (1–7 DAP) were tested for GUS activity. In parallel, a GUS test was performed on self-pollinated seeds using the same assay chemicals. As shown in Table 2, GUS activity was never detected in the out-cross progeny, indicating that GUS expression was either extremely low or completely absent. These data suggest that these genes might be imprinted.

Discussion

Endosperm-specific markers

During early endosperm development, three basic compartments of the *Arabidopsis* ovule can be distinguished, namely the micropylar chamber (MC), the central chamber (CC) and the chalazal chamber (ChC) (Boisnard-Lorig *et al*., 2001; Brown *et al*., 1999) (Fig. 3). The micropylar endosperm that surrounds the embryo is the compartment where cellularization of the free nuclear endosperm starts (Brown *et al*., 1999; Otegui and Staehelin, 2000; Van Lammeren *et al*., 1996). The central endosperm chamber, also termed the peripheral endosperm, represents the main body of the endosperm harbouring the majority of the endosperm nuclei distributed as a thin layer around the central vacuole (Fig. 3). The chalazal endosperm cyst (CEC) remains free nuclear long after the rest of the endosperm became cellular (Brown *et al*., 1999; Nguyen *et al*., 2000).

In addition to this compartmentalization, the main body of cellular endosperm consists of two cell types, a peripheral layer of cells that appears to be similar to the aleurone layer of cereal endosperm (Brown *et al*., 1999; Kuang *et al*., 1996), and a central mass of cells that temporarily accumulates starch grains (Eastmond *et al*.,

Table 2. *Several GUS reporter genes seem to be imprinted when introduced as a pollen parent*

GUS activity was performed on seeds from self-pollinated progeny of marker lines SENAPE1, SENAPE2, GNOCCHI1 and PESCE1 and genetic crosses where the wild-type flowers were pollinated with the pollen from these marker lines.

Genetic cross	Stage of embryo development					
	Early stages	Globular	Heart	Torpedo	Walking stick	Cotyledonary
	GUS activity					
SENAPE1×SENAPE1	Yes	Yes	Yes	Yes	Yes	Yes/no
WT×SENAPE1	No	No	No	No	No	No
SENAPE2×SENAPE2	No	Yes	Yes	Yes	Yes	Yes
WT×SENAPE2	No	No	No	No	No	No
GNOCCHI1×GNOCCHI1	No	Yes	Yes	No	No	No
WT×GNOCCHI1	No	No	No	No	No	No
PESCE1×PESCE1	Yes	Yes	Yes	Yes	Yes	Yes
WT×PESCE1	No	No	No	No	No	No

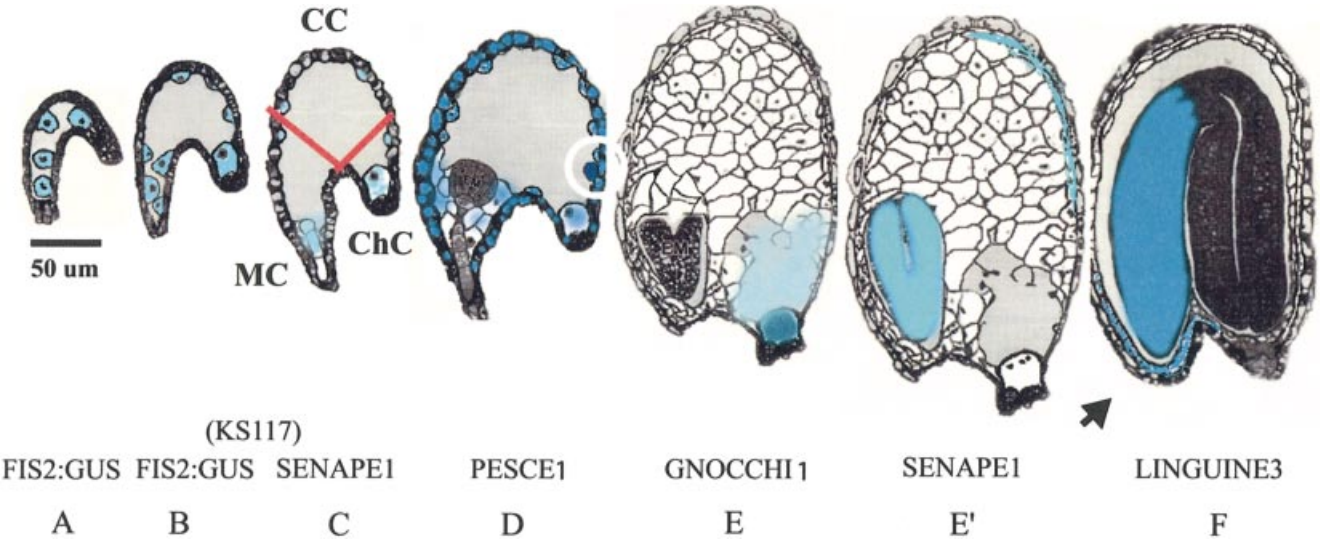


Fig. 3. Outline of endosperm development in *Arabidopsis* and pattern of expression of this and previously published endosperm marker lines. Graphic presentations are based on longitudinal sections of ovules (Brown *et al*., 1999). Stages of endosperm development are indicated as in (A) to (F). Stage (A) shows four-nucleate endosperm. In marker lines endosperm nuclei are indicated in blue and the central vacuole (CV) in grey. Intensities and nuances of the blue colour are corresponding to the histological GUS staining. Stage (B) represents an early peripheral (syncytial) stage of endosperm development. The syncytial endosperm consists of a thin layer of nuclei. Early marker of the endosperm development is FIS2:GUS fusion that shows endosperm-specific expression at the four nucleate (A) and early peripheral stage (B). Endosperm marker KS117 is expressed from the early peripheral stage (Sørensen *et al*., 2001). Stage (C) represents a mid-syncytial stage of endosperm development. A thin layer of syncytial endosperm surrounds the central vacuole in the 2-cell stage of embryo development. At this stage of endosperm development three compartments (indicated by two red lines) can be clearly identified: micropylar chamber (MC), central chamber (CC) and the chalazal chamber (ChC). Endosperm markers SENAPE1-2 are expressed in the endosperm and embryo at this stage. Stage (D) represents the late syncytial stage of endosperm development. In the ovule with late globular/transition stage of embryo, syncytial endosperm of the MC surrounds the embryo. Endosperm- and integument-specific GUS reporter PESCE1 (D) and the chalazal GUS reporter GNOCCHI expressed in the chalazal endosperm at late syncytial (not shown) stage are markers of this stage. White circle indicates strong staining of the endosperm nodule. Stages (E) and (E′) mark endosperm cellularization corresponding to the heart (E) and torpedo (E′) stages of embryo. Chalazal endosperm remains syncytial. At the cellularization stage marker GNOCCHI1 is expressed (E). SENAPE1 marker (E′) is expressed in the endosperm and the presumptive aleurone layer. Stage (F) marks maturation. In *Arabidopsis*, the persistent epidermal layer, corresponding to the aleurone layer (arrow), is the only part of the endosperm that can be found in the ovule at the cotyledonary stage of embryo development. Marker LINGUINE3 (F) is expressed in the embryo and aleurone at this late developmental stage.

2002; Vinkenoog *et al*., 2000). Unlike grass grains, where the endosperm is persistent, the *Arabidopsis* endosperm is consumed before seed maturity (Berger, 1999; Lopes and Larkins, 1993; Olsen *et al*., 1999; Olsen, 2001).

The present work was motivated by the low availability of *Arabidopsis* endosperm GUS-marker lines displaying GUS expression at different development stages as well as endosperm compartments. An overview of the authors' own and previously published endosperm markers is presented in Fig. 3. GUS fusions to the FIS1/MEA and FIS/FIS2 promoters represent specific markers of early nuclear endosperm development (Luo *et al*., 2000) (Fig. 3). GUS activity from these constructs can already be detected in the polar nuclei, the central cell nucleus of unpollinated ovules and in the syncytial endosperm (Luo *et al*., 2000). After cellularization, activity ceases in the micropylar and peripheral endosperm, being restricted to the chalazal chamber. The GFP reporter of the enhancer trap line KS117 is expressed in the chalazal cyst at the heart stage of embryo development, but not in the endosperm nodule (Sørensen *et al*., 2001). Thus, the GNOCCHI1 line described here represents a supplement to this marker line, displaying GUS expression exclusively in the ChC at the heart stage of embryo development. GFP activity of KS117 precedes cellularization and marks out the formation of an anterior–posterior (A–P) polar axis during endosperm development (Sørensen *et al*., 2001). Similar patterns were observed with GUS:FIS1/MEA and GUS:FIS2 fusions. In contrast, the GNOCCHI1 represents a marker that is specific to the chalazal endosperm.

Many known endosperm mutants are defective in the chalazal endosperm, including several *titan/pilz* mutants, in which CEC is either absent or enlarged (Liu *et al*., 2002; Liu and Meinke, 1998; Mayer *et al*., 1999; McElver *et al*., 2000; Tzafrir *et al*., 2002). Confocal studies of *fis1/mea, fis2,* and *fis3/fie* mutants revealed that CEC is usually enlarged and can also be detached from the maternal tissue (Sørensen *et al*., 2001). Further analysis of chalazal endosperm in these and other mutants using GNOCCHI1 will hopefully help to unravel the function of this interesting region of the *Arabidopsis* endosperm.

The endosperm nodule, another specialization of the chalazal endosperm, has not been extensively studied. Nodule-specific expression has been shown for the AGL18

gene (Alvarez-Buylla *et al*., 2000). AGL18 promoter fusions to *gusA* and GFP would be expected to create a compartment-specific marker for the endosperm nodule. In three transgenic lines from this collection, GUS activity was strong in the nodule. These lines include GNOCCHI1 (Figs 1A, 3E), PESCE1 (Figs 2A, B, 3D) and STINCO2 (Fig. 1Q). Of these, the latter two showed strong nodule expression, as well as expression in integuments and in the remainder of the endosperm. In some seeds of the line PESCE1, GUS activity was detected almost exclusively in nodules, chalazal proliferating tissue and the micropylar endosperm, indicating that these might be the sources of the GUS. It remains to be determined if the eventual spreading is caused by the diffusion of the dye or the secretion of the *gusA* product from the nodule to other seed organs. Performing numerous GUS assays in *Arabidopsis* seeds, it was observed that the diffusion could be a slight problem in some cases. Alternatively, endosperm could be secreting proteins into adjacent maternal tissue as already reported in maize (Serna *et al*., 2001). In order to confirm these results, *in situ* hybridization and immunostaining of the GUS product could be used.

Several of the markers lines have GUS activity in the MC and CC endosperm compartments. Of these markers, SENAPE2 represents a CC-specific endosperm marker, being expressed only in the central chamber at the globular stage of embryo development (Fig. 1K). This promoter trap line is highly endosperm- and embryo-specific, with none or only very weak GUS activity in vegetative organs. As mentioned above, a fraction of the seeds of the PESCE1 line, in addition to endosperm nodule, also shows expression in the micropylar endosperm (Fig. 2B). Embryo marker LINGUINE6 also exhibited GUS activity in the lower part of the embryo proper and the surrounding MC (Fig. 1H). It would be interesting to cross these markers to some of the endosperm mutants *titan/pilz,* that either contain reduced number of the endosperm nuclei in the CC or show delayed or abolished cellularization (Liu *et al*., 2002; Liu and Meinke, 1998; Mayer *et al*., 1999; McElver *et al*., 2000; Tzafrir *et al*., 2002).

At the torpedo stage of embryo development (Fig. 3E′), the main body of the endosperm is cellular. Later on, a thin epidermal layer corresponding to the aleurone layer and the small portion of the free nuclear chalazal endosperm, represents the remainder of the endosperm at this stage (Fig. 3F). Marker LINGUINE3 is expressed in the embryo and the adjacent aleurone (Fig. 3F, arrow). Two additional endosperm markers, SENAPE1 (Fig. 3E′) and SENAPE3 were expressed at later stages of development in the aleurone layer. Both lines also showed GUS activity in embryos. In cereals, the aleurone layer and the embryo are both involved in the synthesis and accumulation of lipids, desiccation tolerance and dormancy. Studies in barley showed that several genes are expressed both in embryos and in the aleurone layer (Aalen *et al*., 1994). The same pattern could be applied for *Arabidopsis* assuming that the thin epidermal layer is an orthologue of aleurone layer in cereals. In fact, the *Arabidopsis* homologue *AtPer1* of the aleurone and embryo expressed barley *Per1* gene is expressed in this layer as well as in the embryo in *Arabidopsis* seeds (Haslekås *et al*., 1998). Markers for the *Arabidopsis* aleurone layer will be used in the laboratories to probe for similarities with the cereal aleurone layer, and would be particularly valuable in characterization of the endosperm of *Arabidopsis dek1* mutants. In maize, homozygous *dek1* endosperm lacks aleurone layer, the *DEK1* gene encoding a member of the calpain gene superfamily (Lid *et al*., 2002). One of the previously identified marker lines 17G2, reported to be endosperm-specific (Topping *et al*., 1994), did not display an endosperm-specific pattern of GUS expression in this work. In addition to the endosperm, this marker also expressed GUS in the integuments (data not shown), thus resembling some of this study's SENAPE markers.

The expression of four of the endosperm GUS reporters was abolished when introduced paternally. In the light of the current debate addressing the contribution of the maternal versus paternal genomes in genetic crosses (Vielle-Calzada *et al*., 2000, 2001; Weijers *et al*., 2001), silencing of the pollen-introduced *gusA in vivo* fusions is an important issue. It has been documented that the zygotic translation of numerous paternal GUS reporters created by enhancer trapping has been delayed (Vielle-Calzada *et al*., 2000). It was not possible to detect any GUS activity 1–6 DAP, indicating that these markers are imprinted. However, a case has been reported where the GUS promoter fusion showed imprinting, while the paternal wild-type allele was transcribed (Springer *et al*., 2000). Whether the putative endogenous genes trapped in these lines show the same pattern of expression, as the promoter fusions must await further analysis.

It is concluded that the trapping experiment identified several additional endosperm markers, two of them being compartment-specific in a stage-specific manner, namely chalazal marker GNOCCHI1 and the CC marker SENAPE2. Marker LINGUINE6 was specifically expressed in MC and the lower part of the embryo proper (Fig. 1). Together, previously identified markers FIS2:GUS and KS117 and the markers identified by this study cover all stages of endosperm development. In order to achieve an even more specific MC reporter that is ideally not expressed in the embryo, further promoter or enhancer trapping lines should be analysed.

Embryo- and seed-coat-specific markers

Known embryo markers mostly include GUS reporters generated either by *in vivo* or *in vitro* promoter fusions. Two embryo-specific GUS reporters POLARIS/AtEM101 and EXORDIUM/AtEM201 were generated as *in vivo* fusions by promoter trapping (Topping *et al*., 1994).

Further embryo markers identified in gene and enhancer trapping screens were GT148/PROLIFERA (Springer *et al*., 1995) and J1092 and LENNY (JA Long, personal communication), respectively. Embryo marker lines created *in vitro* include *gusA* gene fusions to the promoters of the following genes: histone4, 2S1 (albumin storage protein), Em1 (desiccation programme), and ABI3 (Devic *et al*., 1996). These embryo GUS reporters are active from preglobular to the late cotyledonary stage of embryo (H4 and ABI3), during the mid-embryogenesis (2S1) or late in embryogenesis (Em1) (Devic *et al*., 1996). Known expression patterns of genes AtLTP1 and AtCYCB1 were utilized in construction of two additional embryo and endosperm GUS reporters (Baroux *et al*., 2001).

Six embryo expressed markers were identified in this screen. Most of these markers were quite specific showing only weak and localized endosperm activity. In all lines GUS was predominantly expressed in the lower segments of the embryo proper (LINGUINE1-3 and LINGUINE6) or in the whole embryo (LINGUINE4-5). These markers cover all developmental stages, and will be useful in further investigation of embryo and endosperm mutants. In addition, PESCE1 could be used in analysis of seed coat mutants (Penfield *et al*., 2001; Western *et al*., 2001).

The four silique markers identified in this screen could be used in versatile developmental studies. Mutation in the MADS box gene SEEDSTICK/AGL11, results in malfunctioning seed abscission and funiculus development (Pinyopich *et al*., 2001). Further characterization of this and other related mutants requires funiculus-specific markers, for example, the two funiculus specific markers (FUSILLI1-2) identified in this screen. According to present knowledge, no other funiculus-specific GUS reporters have been reported so far. The GUS reporter BASIL was also identified showing strong and specific GUS activity in the abscission zone. This marker can be used in the analysis of mutants that show delay in the abscission of the floral organs (M Butenko, S Patterson, R. Aalen, personal communication). Guard cells-specific GUS expression was detected in one of this study's lines (PESTO). GUS activity was strongest in pedicels and siliques. A similar GUS marker was recently described (Husebye *et al*., 2002). In this report *gusA* was fused to the myrosinase promoter resulting in the stomata-specific expression. Unpublished data show that the T-DNA present in the line PESTO was not inserted in the known myrosinase genes.

These results indicate that promoter trapping is an excellent method for the isolation of seed markers, because these insertions did not cause the death of homozygous genotypes or gametophytes. Identification of non-mutant seed markers is considered to be advantageous especially concerning propagation and analysis of known mutants where double mutant phenotypes are neither desirable nor easy to work with.

Acknowledgements

Grants to B Stangeland and Z Salehian have been provided by the Strategic University Program funded by the Research Council of Norway (project number 129525/420).

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