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EMBRYONIC FACTOR 1 encodes an AMP deaminase and is essential for the zygote to embryo transition in Arabidopsis

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Summary

Fusion of the egg and the sperm cells in plants produces a zygote that develops into an embryo. Screening of ethyl methanesulfonate-mutagenized populations of Arabidopsis led to the identification of *EMBRYONIC FACTOR 1* (*FAC1*), a locus that gives a zygote-lethal phenotype when mutated. The *FAC1* gene was identified by positional cloning and confirmed by a genetic complementation test against a T-DNA insertion allele. It encodes an AMP deaminase (AMPD) that is known in human and yeast to convert AMP to IMP to maintain the energy potential. Expression of *FAC1* in a yeast *AMPD* mutant after removal of its N-terminal putative transmembrane domain complemented the mutant phenotype, suggesting a functional conservancy but a structural divergence through evolution. Although a low level of *FAC1* expression was observed in all organs tested, using a reporter construct we observed a significantly increased *FAC1* expression in the zygote, early embryo and endosperm. Furthermore, during somatic embryogenesis, a high level of *FAC1* expression was observed in developing embryos including putative embryogenic cells. *FAC1*, therefore, represents one of the earliest expressed genes known in plants. It may act through AMP depletion to provide sufficient energy for the zygote to proceed through development.

Keywords: ***FAC1*, zygote, embryogenesis, AMP deaminase.**

Introduction

The unfertilized egg cell is metabolically quiescent. At fertilization, the sperm activates various physiological, biochemical and morphological events in the egg cell, stimulating it to re-enter the cell cycle and begin embryo development (for review, see Ciapa and Chiri, 2000). In higher plants, a so-called 'double fertilization' sets in motion two parallel developmental events that produce a seed: the fusion of the egg and one sperm cell leads to the formation of a zygote and then a diploid embryo; the fusion of the central nuclei with the other sperm nucleus leads to a triploid endosperm (for review, see Berger, 2003; Mordhorst *et al.*, 1997). The events behind fertilization-activated zygotic embryogenesis, such as a Ca^{2+} influx, seem, to a certain degree, to be conserved between plants and animals (Antoine *et al.*, 2001; Ciapa and Chiri, 2000; Digonnet *et al.*, 1997). In maize, a rapid rise of cytosolic free Ca^{2+} caused by influx of extracellular Ca^{2+} has been observed during *in vitro* fertilization (Antoine *et al.*, 2001; Digonnet *et al.*, 1997). Moreover, it is likely that certain genes are expressed in the gametes before fertilization to suppress the sporophytic pathway, and/or another set of genes are expressed after fertilization to activate the pathway. Indeed, several genes, *MEDEA*, *FIE*, *MSI1* and *FIS2*, have been identified that suppress endosperm development before fertilization (Grossniklaus *et al.*, 1998; Kohler *et al.*, 2003; Luo *et al.*, 1999; Ohad *et al.*, 1999). MEDEA acts specifically to suppress the maternally supplied MADS-box gene *PHERS1*, but not the paternal equivalent (Kohler *et al.*, 2005). As many independent alleles have been identified at these loci, it is likely that the screens have been saturated. It is interesting, however, to note that no gene has been identified in these screens, whose product suppresses fertilization-independent embryo development. For the

initiation of embryogenesis, it is more likely that some developmental and metabolic pathways need to be activated or to be introduced through fertilization. Unfortunately, very little is known about fertilization-activated or -introduced genes. In contrast, when 20 genes were tested recently for differential expression between two parental alleles (Vielle-Calzada *et al.*, 2000), none of them showed detectable expression up to 80 h after pollination, by then embryo development should have reached the heart-shaped stage (Mansfield and Briarty, 1991). It is not clear yet how common the paternal repression is as a few exceptions have already been reported (Baroux *et al.*, 2001; Kohler *et al.*, 2005; Springer *et al.*, 2000; Weijers *et al.*, 2001).

Plant embryo development can also be induced from somatic cells. Differentiated cells have been induced to de-differentiate and then to re-initiate an embryogenic program using 2,4-D (for reviews, see Dodeman *et al.*, 1997; Mordhorst *et al.*, 1997). Several genes such as *BBM*, *LEC1*, *LEC2* and *WUS* are known to be able to promote somatic embryogenesis in the absence of external hormonal inducers in transgenic plants (Boutilier *et al.*, 2002; Lotan *et al.*, 1998; Stone *et al.*, 2001; Zuo *et al.*, 2002). Molecular studies with these genes provide a substantial contribution toward our understanding of somatic embryogenesis in plants, although the molecular link between somatic and zygotic embryogenesis remains to be established.

In the present study, we have used a genetic approach to identify genes that are essential for the transition from zygote to embryo. We describe a recessive mutation, *embryonic factor 1* (*fac1*), in which embryo development was arrested at the zygote stage. The combined study of molecular cloning, expression and yeast complementation analyses indicates a crucial role for *FAC1* in early embryo development, most likely in providing sufficient ATP for embryo development to proceed beyond the zygote stage.

Results

Screening for zygote-lethal mutants

In order to identify genes essential for early zygotic embryo development, we generated an ethyl methanesulfonate (EMS)-mutagenized population of Arabidopsis (*Ler*). About 3000 fertile M1 plants carrying M2 seeds were screened for zygote-lethal phenotype. We expected such mutants would only be obtained from selfing heterozygotes in which one quarter of the ovules in the silique would be shriveled at from an early stage, and would bear tiny, aborted ovules. Our preliminary experiments showed that post-fertilization aborted ovules could be distinguished from unfertilized ovules by their brownish color (Figure 1a), the latter being whitish. Lines showing 25% early ovule abortion were backcrossed at least three times to *Ler* to partially purify the background. We noted, however, that many lines showing early ovule abortion were not arrested at the zygote stage. Embryo development in these lines, as revealed by microscopy, was able to proceed for several division cycles of to produce pre-globular proembryos.

From the screen, we obtained three lines with a zygote-lethal phenotype. For this study, we focused our attention on one allele, namely *embryonic factor 1-1* (*fac1-1*). As a recessive zygotic lethal mutant, *fac1-1* was maintained in a heterozygous form. One quarter of seeds were aborted in the *FAC1-1*/*fac1-1* plants, demonstrating that the *fac1-1* mutation did not interfere with megasporogenesis nor microsporogenesis (Table 1). When wild-type pollen was used to pollinate *FAC1-1*/*fac1-1* plants, or vice versa, no phenotype was observed (Table 1), confirming that *FAC1* did not have any pre-fertilization maternal or paternal effects. This conclusion was confirmed by the examination of pollen and ovule development where no visible defect was observed.

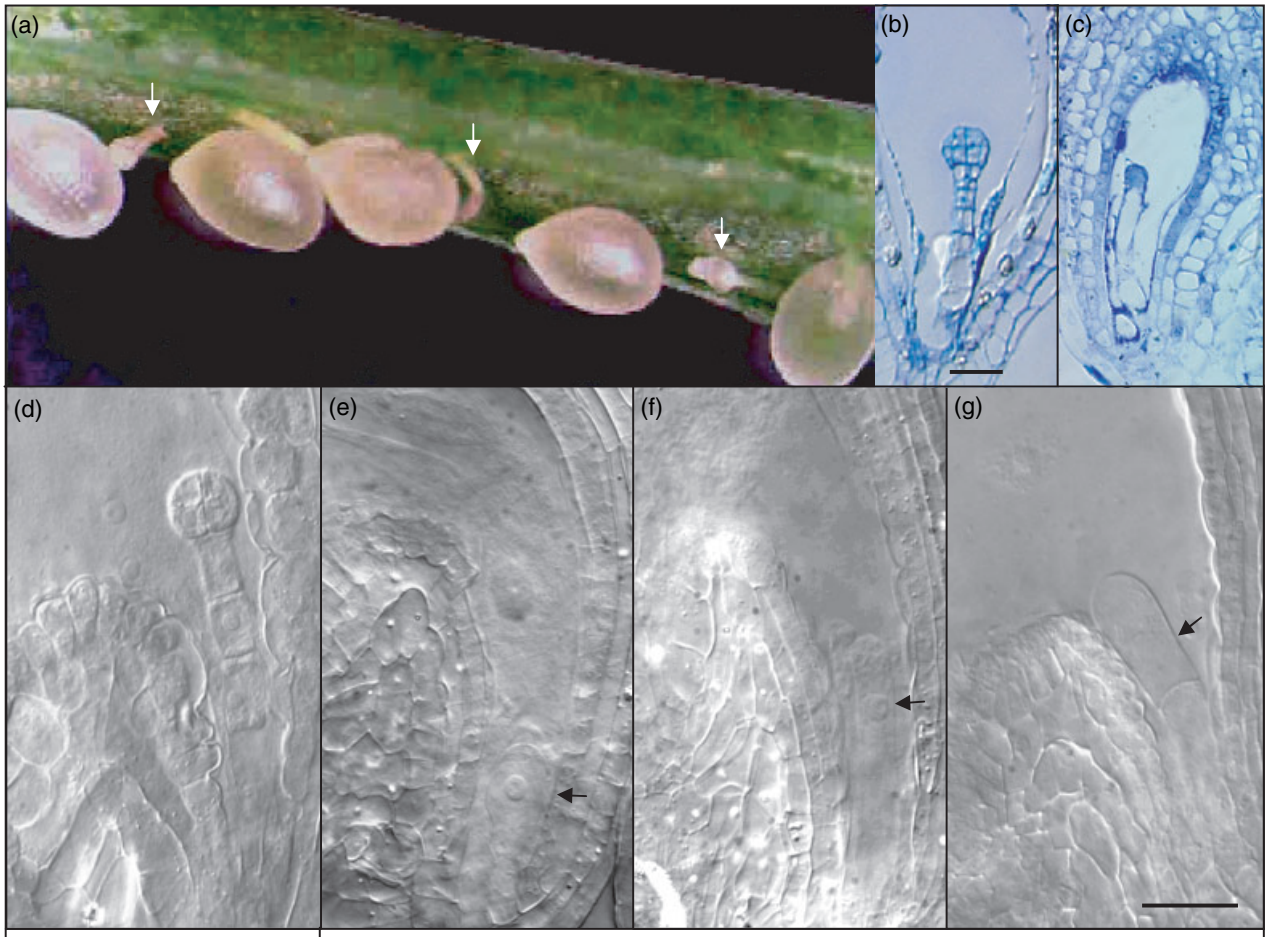
The fac1-1 *phenotype*

Ovules carrying *fac1-1* mutant embryos started to shrivel 2–3 days after fertilization (Figure 1a). At this stage the wild-type embryos in the same silique had reached the 8- to 16-cell stage (Figure 1b,c). Examination of the aborted ovules showed that 8.1% embryos were arrested at the zygote stage (prior to elongation, Figure 1e), 56.3% were arrested after zygote elongation (Figure 1c,f) and 35.6% were at the stage of the first division (Figure 1g, $n = 87$).

The sizes of nuclei in the mutant embryos and endosperm were similar to that in the wild type, suggesting that no major endoreduplication event had occurred. Endosperm development in the mutant progressed slightly further than in the embryos. Examination of the endosperm showed on average 18.8 nuclei ($n = 23$) in each ovule, indicating that four to five cycles of nuclear division have taken place. Nuclear migration toward the chalazal end was observed in most mutant ovules (data not shown), indicative of normal endosperm patterning (Berger, 2003).

Map-based cloning of the FAC1 *gene*

To map the *FAC1* gene, *FAC1-1*/*fac1-1* was crossed to the wild type (*Col-0*). Using a population of 250 F_2 plants, we were able to position *FAC1* to the lower arm of chromosome 2, near *ER*. We used the *er* phenotype that is in the *fac1-1* background to assist the selection of more recombinants in a population of 4500 F_2 plants. The Cereon SNP database (http://www.arabidopsis.org/Cereon) was used to generate additional markers in this region (Figure 2a). As such we were able to narrow down the position of *FAC1* to a 40-kb region between markers *HY17* and *HY19* (in BAC F16M14, Figure 2a). The open reading frames (ORFs) of all 20 genes in this region were amplified by PCR from a *Ler* and an *FAC1-1*/*fac1-1* plant



fac1-1	8.1%	56.3%	35.6%
fac1-2	78.2%	14.5%	7.9%

Figure 1. The *fac1* phenotype.
(a) Ovules in an open silique from a selfed *FAC1-1*/*fac1-1* heterozygous plant showing the aborted ovules (indicated by arrows).
(b–g) The wild type (b, d) and the arrested *fac1-1* embryos were observed after sectioning and staining with toluidine blue (b, c), or after Nomarski microscopy of cleared whole-mount ovules (d–g). The drawings underneath (d)–(g) illustrate the embryo form in pictures above. The numbers indicate the percentages of *fac1-1* and *fac1-2* mutant embryos arrested at a particular stage. The data were collected from 87 *fac1-1* and 55 *fac1-2* ovules at the stage when wild-type embryos were at their octant stage (d, eight cells in the embryo proper plus four to five cells in the suspensor). Bar in (b) = 25 μm for (b) and (c), in (g) = 25 μm for (d–g).

and sequenced to identify point mutations. One single base change (G to A) was identified (as a double peak in the sequencing output) in the 13th exon of gene At2g38280 (Figures 2b and 3), which led to the amino acid (598) change from aspartic acid (D) to asparagine (N, Figure 3a). Sequencing of two parental lines (*Col* and *Ler*) confirmed that it was a true mutation. One full-length Arabidopsis cDNA sequence (2847 bp) was available in the NCBI database (accession no. AY056301) that encodes an 839-amino acid (AA) polypeptide with the calculated molecular mass of 95 128 Da (Figure 3a). Alignment of the genomic DNA and the cDNA sequence showed that *FAC1* encodes a large gene with 20 exons. The coding region was distributed among the first 19 exons, and the last one represents its 3′-UTR (Figure 2b).

Identification of the knockout allele and complementation analysis

To confirm whether the point mutation was indeed causing the *fac1-1* phenotype, we identified a transgenic line in

Table 1 Genetic analysis of the *fac1* mutant

	FAC1-1/fac1-1 selfing	*FAC1-1/fac1-1*[a] × *Col*	*Col*[a] × *FAC1-1/fac1-1*	*FAC1-2/fac1-2* selfing	*FAC1-2/fac1-2*[a] × *FAC1-1/fac1-1*	*FAC1-1/fac1-1*[a] × *FAC1-2/fac1-2*
No. of ovules examined	392	212	141	1505	85	88
No. of aborted ovules[b]	100	6	3	401	26	27
Abortion frequency (%)	25.5[c]	2.8[d]	2.1[d]	26.6[c]	30.6[c]	30.7[c]

[a]The female partner for the cross.
[b]Ovule abortion was examined under a dissection microscope for small, brownish ovules. Where these could not be identified readily, ovules were collected at an earlier stage and examined under Nomarski optics.
[c]The proportion of aborted ovules was not significantly different from the theoretical proportion of 25% for a single recessive mutation ($P > 0.05$).
[d]It is normal for a low percentage (<4%) of seed abortion to occur in wild-type plants.

which a T-DNA was inserted in gene At2g38280 (line *N555006* from the SIGnAL database http://signal.salk.edu/cgi-bin/tdnaexpress). The T-DNA was inserted in the 10th exon (Figures 2b and 3a), as confirmed by PCR analysis (data not shown). The insertion resulted in an interruption from amino acid 504 onward (Figure 3a). Phenotype analysis of the insertion line (named *fac1-2*) showed a *fac1*-like zygote-lethal phenotype. Detailed analyses demonstrated that *fac1-2* was a stronger allele than *fac1-1*, with 78.2% mutant embryos arrested at the non-elongated zygote stage rather than the 8.1% in *fac1-1* (Figure 1). Only a small portion of mutant embryos was able to reach the elongated zygote (14.5%) or the first division stage (7.9%, $n = 55$). In the aborted ovules, there were, on average, 8.2 endosperm nuclei ($n = 22$), suggesting that approximately one less cycle of nuclear division occurred in the *fac1-2* endosperm when compared with that in *fac1-1*. The phenotype segregated in a typical Mendelian fashion, producing 26.6% lethal ovules ($n = 1505$, Table 1). Plating of 100 seeds from a *FAC1-2/fac1-2* heterozygous plant on kanamycin selection media resulted in 63 resistant and 37 sensitive seedlings (2:1, $P > 0.05$), indicating the presence of a single T-DNA insertion with a homozygous lethal phenotype. All 63 K_m-resistant plants segregated the *fac1*-like phenotypes, demonstrating that the T-DNA was tightly linked to the phenotype.

Reciprocal crosses between *fac1-1* and *fac1-2* heterozygous plants showed that these two mutations failed to complement each other, revealing that the two mutations were allelic (Table 1). We conclude, therefore, that At2g38280 is the gene that is responsible for the *fac1* mutant phenotype.

FAC1 *encodes an AMP deaminase*

FAC1 encodes a putative AMP deaminase (AMPD; EC:3.5.4.6). A search of the Arabidopsis genome sequence showed that *FAC1* is a single-copy gene in this species. The closest homolog is an ARF-GTPase-activating domain-containing protein (At1g10870) that shares only 30% identity in a 70 AA region. Yeast (*Saccharomyces cerevisiae*) *AMD1* is a putative ortholog of the *FAC1* gene, which is also a single copy in its genome. The AMD1 protein that shares 41% overall sequence identity with FAC1 has AMPD activity (Meyer *et al.*, 1989).

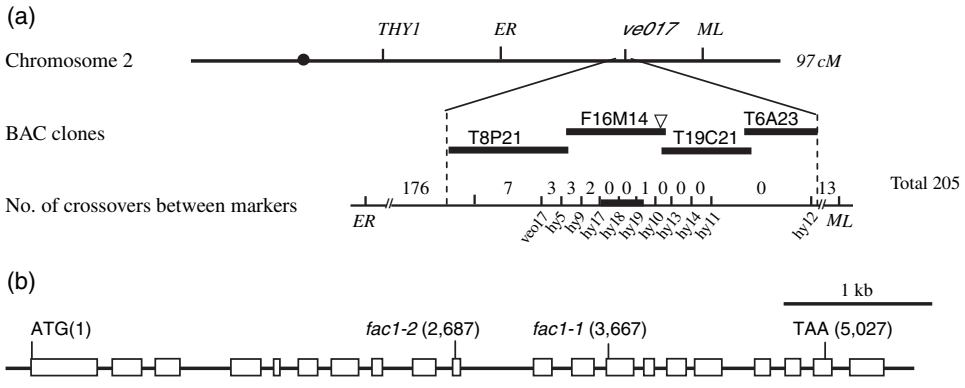


Figure 2. Map-based cloning of *FAC1*.
(a) Schematic representation of the map-based cloning of *FAC1*, showing the chromosomal position, BAC contig in this region, and detected crossovers between markers. The fine mapping results allowed us to locate *FAC1* to a 40-kb region between *hy17* and *hy19* (indicated by a thick bar).
(b) The *FAC1* gene structure, containing 20 exons (boxes) and 19 introns. The EMS-generated *fac1* point mutation (*fac1-1*) occurred in the 13th exon, with a G to A nucleotide change. In the allelic mutant, *fac1-2*, a T-DNA was inserted in the 10th exon.

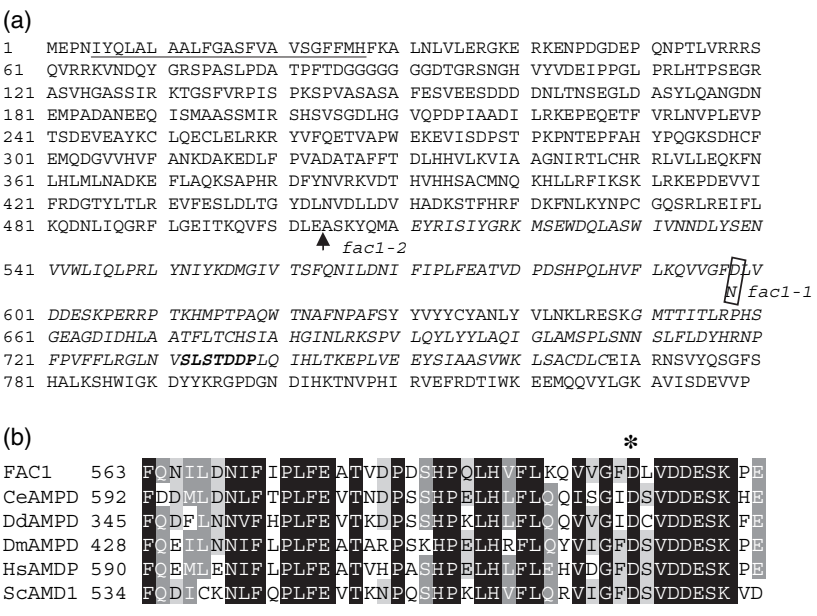


Figure 3. The FAC1 protein and its functional motifs and mutation sites.
(a) The FAC1 peptide in which a predicted trans-membrane domain is underlined. The exact positions of disruption by the point mutation in *fac1-1* and by the T-DNA insertion in *fac1-2* (N555006) are marked with a box and an arrow, respectively. Two PH domains are in italic and the deaminase signature motif is highlighted in bold.
(b) Alignment of amino acid sequences around the first PH domain (the *fac1* point mutation is marked with*), with AMPD identified from *Caenorhabditis elegans* (CeAMPD), *Dictyostelium discoideum* (DdAMP), *Drosophila melanogaster* (DmAMP), *Homo sapiens* (HsAMP) and *Saccharomyces cerevisiae* (ScAMD1). Identical amino acids are in black and similar ones in gray.

The deduced FAC1 protein contains two pleckstrin homology (PH) domains that are shared by all eukaryotic AMPDs (Figure 3a, in italics; Sims *et al.*, 1999). Using the TMHMM 2.0 prediction software from the CBS server (http://www.cbs.dtu.dk/services/), we found that FAC1 has 99% probability of carrying a hydrophobic trans-membrane motif near its N-terminus (amino acids 5–27, underlined in Figure 3a). SignalP analysis using the same server indicated that it is unlikely to be a secretion signal. The same prediction was made for the rice AMPD (data not shown). The putative membrane-binding motif, possibly involved in sub-cellular targeting, seems to be plant-specific, as it was not found in any non-plant AMPDs that we examined.

The D^{598} to N^{598} mutation in *fac1-1* was located in the first PH domain (Figure 3a). This particular amino acid is highly conserved among AMPDs from different origins (Figure 3b) and thus the point mutation could potentially damage enzyme function as it replaced a negatively charged amino acid to a polar, but uncharged one. However, from the NCBI blast search, we did observe that one of the two AMPD cDNAs of zebra fish (accession no. AAH44154) encodes an N instead of D on the same position. Careful examination of all sequences available for zebra fish suggested that it was most likely to be a sequencing error, as the same codon in the corresponding genomic sequence (ctg10949) encodes a D. The T-DNA insertion in *fac1-2* caused a changed peptide sequence from A^{504} onward, including most of the PH domains and the whole deaminase domain (Figure 3a).

Post-embryonic expression of the FAC1 *gene*

Northern blot analysis was used to detect the expression of *FAC1* in Arabidopsis. A single band with the size of ca. 3 kb was observed after hybridization of the RNA blot with an *FAC1* cDNA probe. The results showed that *FAC1* was expressed in all organs tested, at a relatively high level in 7-day-old seedlings, roots, leaves, flowers and siliques, but at a lower level in stems (Figure 4a). The low level of constitutive expression was confirmed by digital Northern analysis, using the Arabidopsis Affymetrix GeneChip® data set compiled by the Gruissem Laboratory (http://www.genevestigator.ethz.ch, see Figure S1).

To further analyze *FAC1* expression, we made reporter constructs with different genomic fragments of *FAC1* fused to reporter genes and transformed them into Arabidopsis. *FAC1* is located in a relative gene-dense region, as only 736 bp of non-coding sequence is available before its translation start codon and 663 bp after its stop codon, before the flanking genes are reached. Transgenic plants were made with a construct carrying all the upstream and the downstream non-coding sequences fused to a *GFP-GUS* reporter gene in between (P_{FAC1}:*GFP-GUS*:T_{FAC1}, Figure 4b, top). GUS assays showed that these plants had only weak GUS expression in leaf hydathodes, root vascular bundles and the receptacles of the flowers and siliques, but not in the cotyledons (Figure 4c), leaf blades and stems, which was inconsistent with the expression pattern observed in Northern blot. Another fusion construct was made with an additional 1281 bp genomic sequence downstream of the ATG (including the first three introns/exons and a small part of the fourth exon) fused in-frame to *GFP-GUS* (named P_{FAC1}*IE:GFP-GUS*:T_{FAC1}, Figure 4b, bottom). All nine transgenic plants showed low levels of constitutive expression in roots, leaves, stems and flowers (data not shown). In seedlings, GUS expression was stronger in the root tip and the hypocotyl (Figure 4d). This result was consistent with the Northern blotting results and

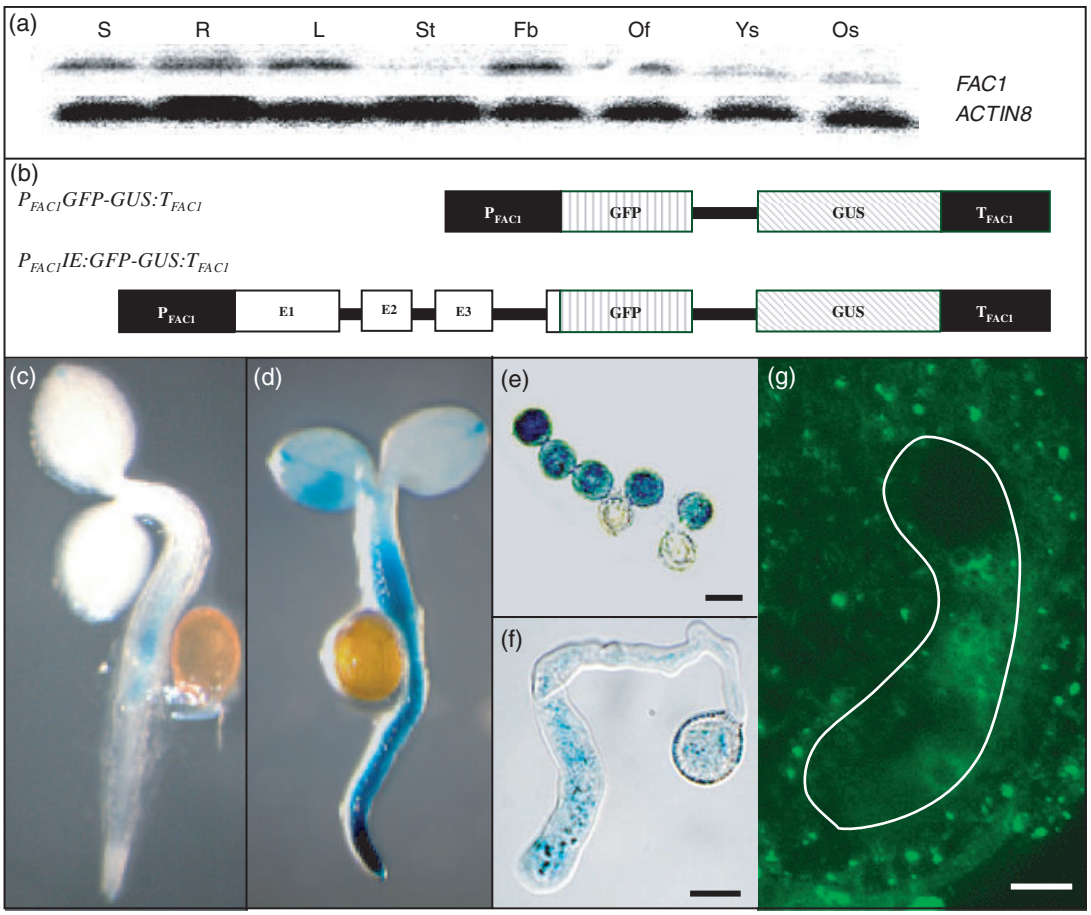


Figure 4. Expression analysis of *FAC1*.
(a) Northern blot analysis of *FAC1* expression by RT-PCR. *ACTIN8* was used as quantitative control. S, 7-day-old seedlings; R, roots; L, leaves; St, stems; Fb, flower buds; OF, open flowers; Ys, young siliques; Os, old siliques.
(b) Schematic representation of the reporter constructs of P_{FAC1}:*GFP-GUS*:T_{FAC1} (top) and P_{FAC1}*IE:GFP-GUS*:T_{FAC1} (bottom) (for details, see Experimental procedures).
(c, d) GUS expression in transgenic seedlings carrying P_{FAC1}: *GFP-GUS*:T_{FAC1} (c) and P_{FAC1}*IE:GFP-GUS*:T_{FAC1} (d) constructs. The GUS assay was carried out 3 days after germination.
(e–g) GUS expression in male and female gametophytes as revealed by transgenic plants carrying the P_{FAC1}*IE:GFP-GUS*:T_{FAC1} construct. Mature pollen (e) was isolated from a transgenic plant carrying a single copy of the transgene for GUS assay. GUS assay was also performed in *in vitro* germinated pollen (f). (g) A weak GFP expression was observed in the mature embryo sac, as examined by confocal microscopy. The white line shows the edge of the embryo sac.
Bar = 20 μm.

the digital Northern analysis based on microarray data (see Figure S2), which indicated that some regulatory elements are present in the first three introns and exons.

During the reproductive phase, weak *FAC1* expression was observed in both male and female gametophytes, as revealed by the GUS assay. In heterozygous transgenic plants carrying P_{FAC1}*IE:GFP-GUS*:T_{FAC1}, GUS expression was observed in both mature pollen grains (Figure 4e) and in pollen tubes germinated *in vitro* (Figure 4f). In both cases, approximately 50% of them were GUS positive. In the mature embryo sac, weak *GFP* expression was observed in the cytoplasmic regions surrounding the nuclei (Figure 4g). Most likely these nuclei were from the egg cell and the central nuclei based on their positions, although the exact identity remains to be defined.

Expression of the FAC1 *reporter gene during zygotic embryogenesis*

Transgenic plants carrying the P_{FAC1}*IE:GFP-GUS*:T_{FAC1} construct showed strong GUS expression during early embryo development, and weaker expression in the seed coat (Figure 5c; the embryo was viewed in a seed where the seed coat also expressed the GUS gene). The GUS reaction was reduced, therefore, to 1 h, which allowed us to observe expression in the zygote. The same results were observed when transgenic plants were pollinated with wild-type pollen (data not shown), suggesting that the maternal *FAC1* gene was expressed at the zygote stage. To define when paternally supplied *FAC1* was activated in the embryos, we introduced the P_{FAC1}*IE:GFP-GUS*:T_{FAC1} transgene into

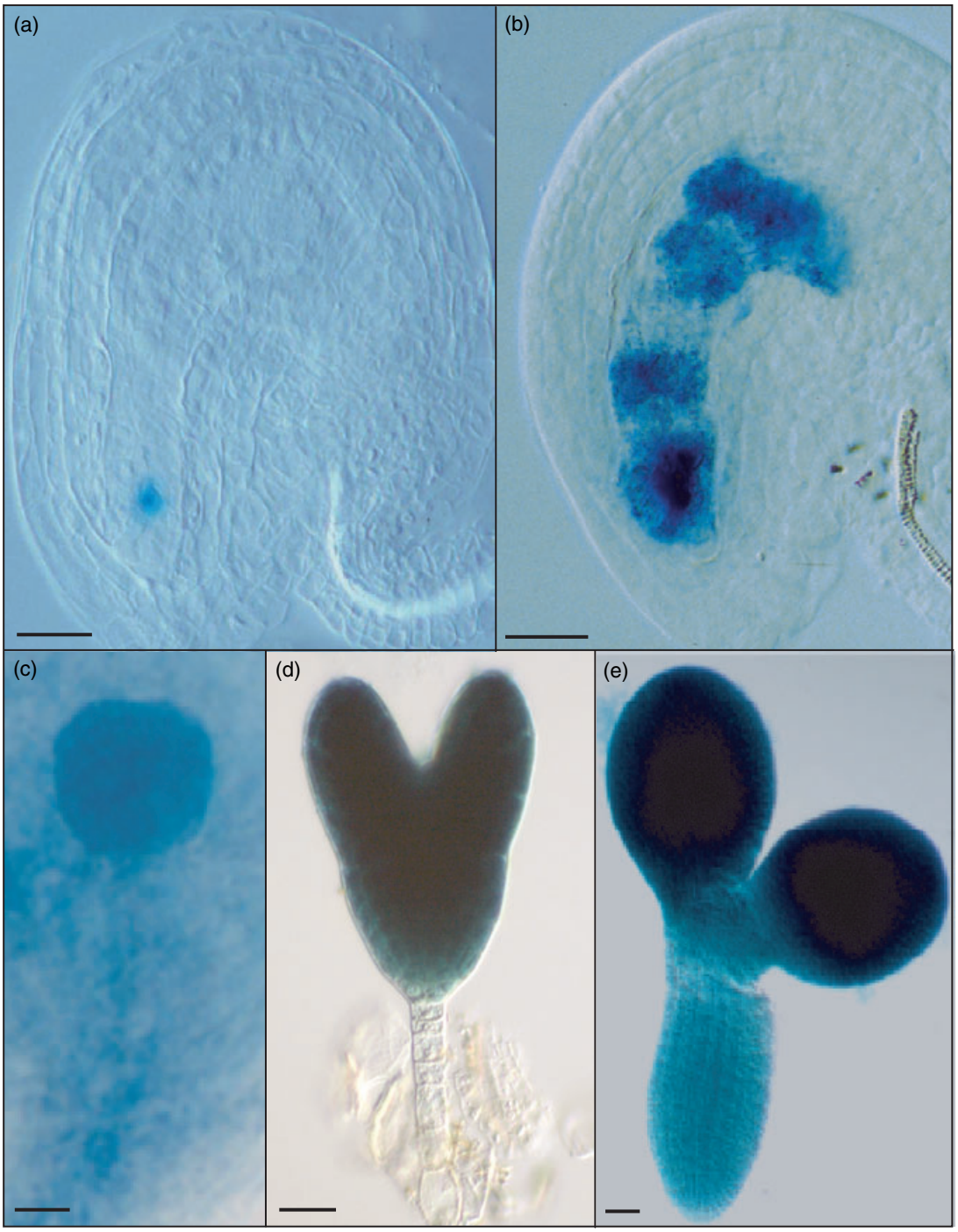


Figure 5. Expression of *FAC1* during zygotic embryogenesis as revealed by GUS assay using transgenic plants carrying P_{FAC1}*IE:GFP-GUS:*T_{FAC1}.
(a, b) GUS expression in the zygote and the endosperm, observed after a wild-type plant was pollinated with a transgenic plant carrying the P_{FAC1}*IE:GFP-GUS:*T_{FAC1} construct. GUS expression was first observed in the zygote (a, 3 HAF), and when the zygote starts to elongate, the expression was observed in both the zygote and the endosperm (b, 4 HAF).
(c–e) *FAC1* expression during later stages of zygotic embryo development. At the globular (c), torpedo-shaped (d) and cotyledonary (e) stages, GUS was strongly expressed in the embryo proper, but not in the suspensor (e). Note that these photos were either taken through the transgenic ovule (c) or dissected out of the ovules (d, e) from a selfed transgenic plant carrying the P_{FAC1}*IE:GFP-GUS:*T_{FAC1} construct.
Bar = 20 μm.

wild-type plants through pollination. GUS expression was first observed in non-elongated zygotes (Figure 5a, which represents ovules approximately 3 h after fertilization, HAF; Mansfield and Briarty, 1991). At the stage when the zygote was elongated (about 4 HAF), GUS expression was observed in both embryo and endosperm (Figure 5b). Within the endosperm, GUS expression was strongest in the cytoplasmic regions around the free nuclei. These results illustrate that the paternally supplied GUS reporter gene was active in both zygote and endosperm in the first few hours following fertilization.

From the globular to the torpedo-shaped stage, GUS expression was evenly distributed in the embryo proper (Figure 5c,d). No detectable GUS expression was observed in the suspensor (Figure 5d). In cotyledonary embryos a stronger GUS expression was observed in the cotyledons when compared with the basal part of the embryo (hypocotyl and root; Figure 5e), and the expression was decreased significantly from this stage onward. Mature embryos had a very low level of GUS expression (data not shown).

Expression of the FAC1 *reporter gene during somatic embryogenesis*

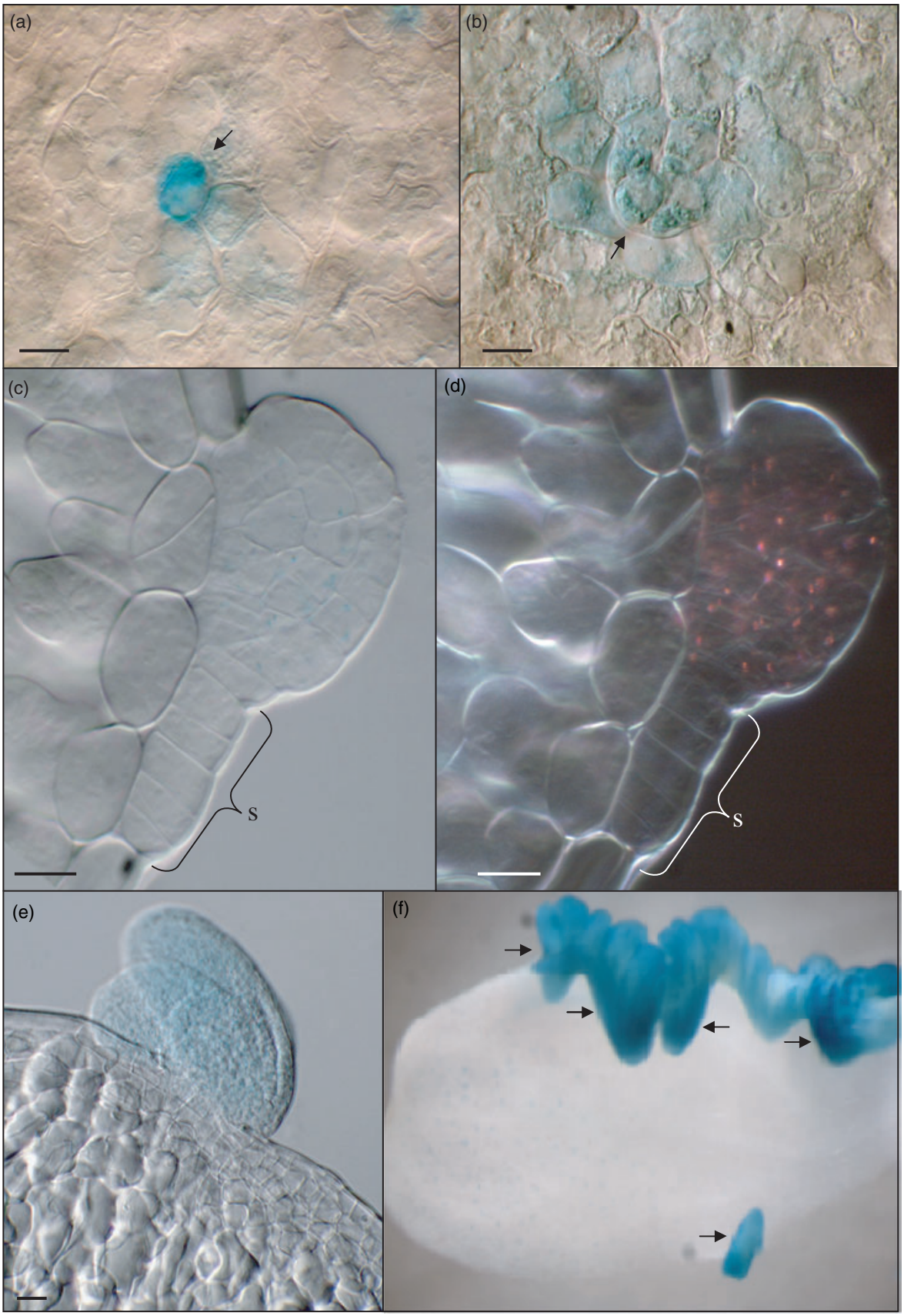
To determine whether *FAC1* expression was also associated with somatic embryos, we examined its expression during somatic embryo development. As reported previously, autonomous somatic embryogenesis can be induced by ectopic expression of the *BBM* gene in Arabidopsis (Boutilier *et al.*, 2002). We therefore studied the GUS expression in a hybrid plant carrying both P_{FAC1}*IE:GFP-GUS:*T_{FAC1} and *35S:BBM* constructs. Six- and 10-day old F_1 seedlings germinated on a hormone-free medium were examined for somatic embryogenesis and GUS expression. Somatic embryos were observed in almost all seedlings, mostly at the edges of the cotyledons where elevated GUS expression was also observed. These somatic embryos showed strong GUS expression compared with the weak GUS expression in the cotyledon explants (Figure 6f, the somatic embryos are indicated by arrows). Detailed observation of over 50 F_1 seedlings revealed that an elevated GUS expression was associated with different stages of embryo development (Figure 6). The early embryo characteristic was seen by the formation of a thick cuticle layer and a protodermal layer around the globular embryos (Figure 6b,c). More intriguingly, we also detected some GUS-positive and cytoplasm-rich single cells with a round shape in the epidermal and the mesophyll layer of the cotyledon (Figure 6a). In contrast, such GUS-positive single cells were never seen in the parental plant carrying only the P_{FAC1}*IE:GFP-GUS:*T_{FAC1} construct. These cells were mainly observed in 6-day-old seedlings, rarely in 10-day-old seedlings. We therefore hypothesized that these single cells are embryogenic and lead to the formation of somatic embryos. Suspensor formation was occasionally observed in somatic embryos. Similar to those in zygotic embryos, such suspensor cells showed no detectable GUS expression (Figure 6c,d).

FAC1 *without its transmembrane motif can rescue a yeast* AMD1 *deletion mutant*

The *AMD1* deletion strain of yeast, Y7 (*Mat*^{a}; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YML035c::kanMX4*), has a slow-growth phenotype in a medium with 2% lactate compared with the wild-type strain Y0. As indicated above, the yeast AMD1 does not have the hydrophobic transmembrane domain observed in FAC1. Using the *pESC-HIS* vector, three constructs were made to express the full-length or modified *FAC1* cDNA in the yeast strains under the control of the *pGAL10*-inducible promoter. The construct, *pEF*, carried a full-length *FAC1* cDNA whereas *pET* carried a truncated *FAC1* cDNA in which the first 25 AA (predicted transmembrane) domain was deleted and *pEm* was the same as *pET*, but with the same point mutation as in *fac1-1* that led to a D-to-N change.

A spotting assay of strains expressing different versions of the *FAC1* cDNA showed that only *pET* can fully rescue the slow-growth phenotype of *Y7* (Figure 7a). This result suggests that the FAC1 protein without the hydrophobic domain is functional in *S. cerevisiae.* The same result was observed in a growth curve assay measured over a 9-day culture period (Figure 7b). It is interesting to note that *Y0* transformed with *pEF* showed even reduced growth when compared with the same strain transformed with an empty vector (Figure 7b). It is possible that the full-length FAC1 protein can interact with the yeast AMD1 or its related components and disrupt its function. *pEm* failed to complement the yeast mutant,

Figure 6. GUS expression during somatic embryogenesis in the F_1 seedlings carrying both the P_{FAC1}*IE:GFP-GUS:*T_{FAC1} and the *35S:BBM* constructs.
(a) A small individual cell (indicated by an arrow) in the sub-epidermal layer of the cotyledon showed GUS expression in 6-day-old seedlings. Such round, cytoplasm-dense cells were not normally found in the mesophyll of the cotyledon.
(b) A pro-embryo formed in the mesophyll of the cotyledon showed GUS expression. Note the cuticle layer (indicated by an arrow) surrounding the somatic embryo.
(c, d) A globular-like somatic embryo formed at the edge of the cotyledon from the epidermal layer (c) with a positive GUS staining, as showed by the blue dots in (c) and red dots in the dark field image in (d). Note the suspensor-like structure(s) were negative in GUS assay.
(e) GUS expression in a torpedo-staged somatic embryo formed at the edge of the cotyledon.
(f) A cotyledon from a 10-day-old seedling growing on a hormone-free medium, showing GUS-positive somatic embryos (indicated by arrows) on the surface of the cotyledon. Note the weak GUS expression in the cotyledon from the zygotic embryos.
Bar = 20 μm.


(a)
(b)
(c)
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(d)
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(e)
(f)

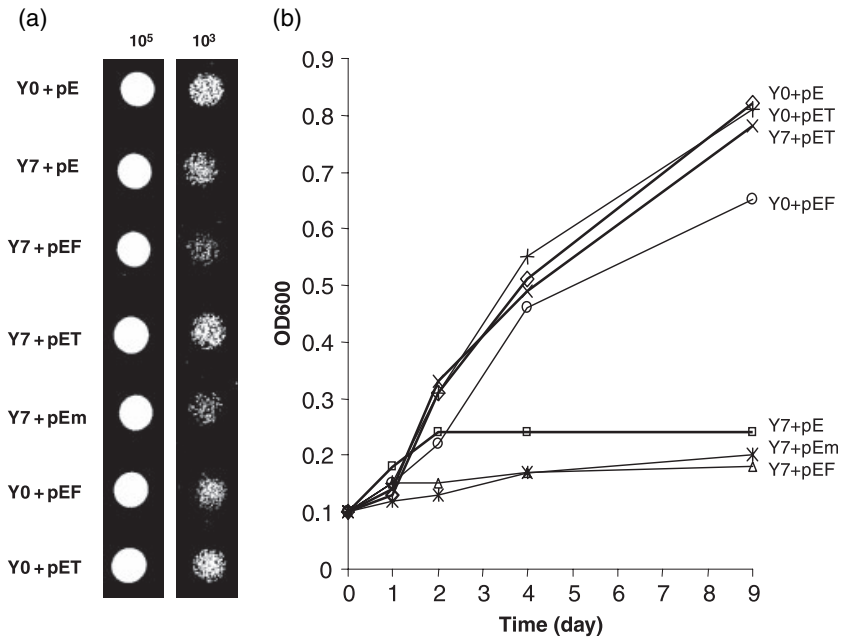

Figure 7. Complementation of the yeast *AMD1* mutant

Wild-type (*Y0*) and *AMD1* deletion strains (*Y7*) were transformed with different plasmids under the control of the inducible promoter *pGAL10*. *pEF*: full-length *FAC1* cDNA; *pET*: truncated *FAC1* cDNA from which 25 AA were removed from its N-terminal; *pEm*: truncated *FAC1* cDNA as in *pET*, but with the same point mutation as *fac1-1*.

(a) Spotting assay performed on an SG plate with 2% lactate from the starting yeast cells of 10^5 (left) and 10^3 (right). The pictures were taken 9 days after incubation. Note that only *pET* was able to complement the yeast AMD1 mutant, but not *pEF* nor *pEM*.

(b) Growth curve measurement at OD_{600} of the different strains mentioned in (a) cultured in liquid media during the course of a 9-day period.

indicating the importance of the particular amino acid altered in *fac1-1* for enzymatic activity and confirming that this point mutation resulted in a functional disruption of the FAC1 protein (Figure 7). As the P_{FAC1}*IE:GFP-GUS:*T_{FAC1} construct carries the transmembrane motif of FAC1, we examined whether the fusion protein was targeted to membranes in Arabidopsis. We observed by confocal microscopy the root tips of transgenic seedlings and the results showed that the GFP signal was not evenly localized in the cytoplasm, but was punctuated (see Figure S2), suggesting that the fusion protein was associated most probably with certain organelles, or was self-assembled.

Discussion

In this paper we describe the identification and molecular characterization of a zygote-lethal mutant in Arabidopsis, in which embryo development was arrested at the single-cell zygote stage or occasionally at the first zygotic division stage. We identified the *FAC1* gene via map-based cloning and confirmed this identity by complementation analysis with a T-DNA knockout allele. We showed that *FAC1* encodes a functional AMPD and is highly expressed in the zygote and in putative embryogenic somatic cells, where it may help in providing sufficient energy for embryogenesis.

FAC1 *is crucial for further development of the zygote*

Genetic studies of Arabidopsis have identified 167 loci that, when mutated, give rise to an embryo-lethal phenotype (Meinke *et al.*, 2003). Embryo development in these mutants is arrested at different stages of embryogenesis, from early globular to late seed maturation. Several of them, such as *gnom*, *titan* and the newly identified *yoda*, showed aberrant development from the first zygotic division stage (Liu and Meinke, 1998; Lukowitz *et al.*, 2004; Shevell *et al.*, 1994). Study in female gametophytic development in Arabidopsis leads to the identification of a large number of mutants with a distorted maternal transmission and arrested zygote development (Pagnussat *et al.*, 2005). In this work, we observed that although *FAC1* is expressed in both male and female gametophytes, the fact that *fac1* mutation could be transmitted perfectly to progeny suggests that the *fac1* mutation did not cause any gametophytic defect, or redundant pathways and maternal supplies are available to compensate for *fac1* mutations. As such, *fac1-1* is the only post-fertilization zygote-lethal mutant known in higher plants.

The fact that some embryos could reach the two-cell stage is most likely to be because this allele is weak, as the insertion mutant (*fac1-2*) showed a much stronger defect. Three to five cycles of mitosis were seen in the *fac1-1* endosperm, where the defect may be partially compensated for by the surrounding maternal tissues.

FAC1 *is expressed abundantly from the zygote stage*

Northern blot analysis showed that *FAC1* was weakly expressed in all organs tested. The zygote-lethal phenotype suggests that *FAC1* is expressed from the zygote stage. We were able to follow the expression of *FAC1* before and during embryogenesis using stable transgenic plants carrying the 5′ upstream sequence plus the first 3 introns/exons and 3′ downstream sequence fused to a *GFP-GUS* reporter gene

(P_{FAC1}*IE:GFP-GUS:*T_{FAC1}). As indicated by confocal microscopy, a low level of *GFP* signal was observed in the embryo sac. After fertilization, however, an elevated level of GUS expression was observed in both embryos and endosperm. Through the pollination of a wild-type plant with pollen from a hemizygous transgenic line carrying the reporter gene construct, we found that the paternally supplied GUS gene was expressed clearly in the non-elongated zygote, whereas expression in the endosperm was observed only after the elongation of the zygote. It is unlikely that GUS expression was from the transcripts delivered by the pollen tubes because, if that were the case, we would have expected that expression would be observed mainly in the embryo sac and the endosperm rather than the zygote. It is well accepted that sperm cells carry almost no cytoplasm (Morgensen, 1988). The fact that 50% of the ovules showed GUS expression revealed that the paternally supplied transgene was transmitted and expressed with efficiency close to 100%.

According to the study of Mansfield and Briarty (1991), the non-elongated and elongated zygotes occur about 3 and 4 HAF, respectively. GUS expression at such an early stage is striking as Vielle-Calzada *et al.* (2000) reported that of 20 genes tested through pollination, none of them were expressed prior to 80 HAF, suggesting a general delay in expression when genes were transmitted paternally. Similarly, Springer *et al.* (2000) observed that, although the paternally supplied *PRL* transcripts could be detected by PCR at the octant stage (a stage in which the embryo proper has eight cells and the suspensor has four to five cells), GUS expression could only be detected at the heart-shape stage when the *pPRL:GUS* transgene was transmitted paternally through pollen. Using reporter gene constructs, it has been shown that both *AtRPB5A* and the cyclin B promoters are active in two-cell embryos (Baroux *et al.*, 2001; Weijers *et al.*, 2001). A more recent report showed that although the maternally provided *PHERSE1* allele is repressed, the paternally supplied allele is expressed 1 day after fertilization (Kohler *et al.*, 2005). As such, *FAC1* is probably the only known paternally transmitted gene in plants expressed at the zygote stage. This is consistent with the zygote-lethal phenotype and the typical Mendelian segregation observed in *fac1* mutants.

FAC1 *is expressed in putative embryogenic cells*

As the expression of *FAC1* is elevated during early zygotic embryogenesis and its expression is crucial for the zygote to embryo transition, we examined whether *FAC1* expression is also associated with somatic embryogenesis. In many plant species, embryos can be induced from somatic cells using 2,4-D treatments through a process of de-differentiation and then the establishment of embryogenic competence (for a review, see Dodeman *et al.*, 1997). In addition, several genes – *BBM*, *LEC1*, *LEC2* and *WUS* – are known to promote somatic embryogenesis in transgenic plants (Boutilier *et al.*, 2002; Lotan *et al.*, 1998; Stone *et al.*, 2001; Zuo *et al.*, 2002). By crossing *35S:BBM* transgenic plants (Boutilier *et al.*, 2002) with plants carrying the P_{FAC1}*IE:GFP-GUS:*T_{FAC1} fusion construct, we demonstrated that *FAC1* is highly expressed in the early stages of somatic embryogenesis. The expression pattern also mimicked that in zygotic embryos, where suspensor cells were GUS negative. GUS expression allowed us to identify some cytoplasm-dense single cells in the epidermal and mesophyll layer of the cotyledon explants as being putative embryogenic cells. The term embryogenic cell is used to define those cells that have achieved the transition from a somatic cell to a stage where no further external stimuli are required to produce somatic embryos (Komamine *et al.*, 1990; Schmidt *et al.*, 1997). The only known gene that can be used as a marker to recognize embryogenic cells is the *SERK* (Schmidt *et al.*, 1997). It may now be possible to use *FAC1* as an additional marker. Cell tracking as performed by Schmidt *et al.* (1997), however, is required before the identity of the GUS-positive single cells observed here can be assigned conclusively.

FAC1 *encodes an AMPD*

FAC1 encodes a well-conserved AMPD in all eukaryotic organisms. AMPD forms a tetramer in human cells, for which the N-terminal sequence is essential (Bausch-Jurken and Sabina, 1995; Haas and Sabina, 2003; Mahnke-Zizelman *et al.*, 1998). It catalyzes the irreversible hydrolytic deamination of AMP to produce IMP and NH_3. By removing AMP from the adenylate pool, AMPD drives the equilibrium of the reaction catalyzed by adenylate kinase ($2ADP \leftrightarrow ATP + AMP$) toward ATP synthesis to create a high-energy potential (Bausch-Jurken and Sabina, 1995). In the yeast and Arabidopsis genomes, *AMPD* is a single-copy gene, whereas in the human and mouse genomes, three orthologs (*AMPD1*, *2* and *3*) have been identified. *In vitro* experiments showed that human oocytes failed to fertilize at a low ATP/ADP ratio (Slotte *et al.*, 1990). A recent report showed that ATP production is absolutely required for sperm-trigged Ca^{++} oscillations in mouse (Dumollard *et al.*, 2004). Cells with high-energy demand such as those in muscle create a high ATP availability by irreversibly degrading AMP using AMPD without accelerating the rate of mitochondrial respiration (Sims *et al.*, 1999). Interestingly, AMP depletion seems critical for plants to survive as inhibition of AMPD in pea leads to herbicidal effects (Dancer *et al.*, 1997). Our work shows that *FAC1*/*AMPD* is essential for the zygote to initiate embryo development, possibly through enhancing ATP availability to provide a high-energy state.

We have found that, in comparison with those from other organisms, plant AMPD (Arabidopsis and rice) has a putative transmembrane motif near its N-terminus. We showed that

the Arabidopsis *FAC1*/*AMPD* could complement the yeast *AMD1* mutant only when its transmembrane motif was removed, illustrating functional conservation and structural divergence between the yeast and plant proteins. Although human AMPD does not contain a transmembrane domain, the enzyme is associated with erythrocyte membranes through the interaction with PI(4,5)P2 (Sims *et al.*, 1999). It has been proposed that AMPD interacts with PI(4,5)P2 through two PH domains (Sims *et al.*, 1999). The point mutation in *fac1-1* is located in the first PH domain, and we showed in yeast that the mutation interrupts FAC1 function, indicating the essential role of this domain for enzyme activity.

In summary, our work demonstrates that *FAC1*, which encodes a functional AMPD, is crucial for further development of the zygote. It is possible that the enzyme is required for AMP depletion to cope with the high-energy demand needed for the initiation of embryogenesis.

Experimental procedures

Plants and growth conditions

All Arabidopsis plants, ecotypes (*Col-0*) and *Ler*, were grown in a greenhouse with a 16 h light, (100–200 μmol photons sec^{-1} m^{-2} at 22°C) and 8 h dark (18°C) period. The T-DNA insertion line N555006 (*fac1-2*, *Col*) was obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK).

EMS mutagenesis and mutant screening

For mutagenesis, dry seeds of Arabidopsis (*Ler*) were first imbibed on a wet filter paper in a sealed petri dish at 4°C, for 5 days, and then dried for 1 day at 24°C. These seeds were transferred to a 50-ml tube containing a solution of 60 mM EMS (Sigma, Zwijndrecht, the Netherlands) and 66 mM phosphate buffer (pH 7.0) for 5 h. The seeds were washed five times with tap water for 10 min each, before sowing them on soil. The fourth and fifth siliques (counting from bottom) of the primary inflorescence of each M1 plant were screened for 1/4 seed abortion under a dissection microscope. The browning of the ovules was used as an indicator of fertilization.

To confirm T-DNA insertion, DNA was extracted from the progeny of a heterozygous *fac1-2* plant and PCR was performed using a T-DNA and a *FAC1* primer for genotyping.

Microscopic observation

Ovules were excised from siliques and mounted with a clearing solution (Sabatini *et al.*, 1999). After 15–60 min, samples were examined under a microscope (Nikon E600, Tokyo, Japan) with Nomarski optics. For histological analysis, ovules were isolated and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde mixed with a buffer containing 50 mM PIPES, 5 mM EGTA, 5 mM MgSO4 (pH 7) and embedded in a butyl methacrylate and methyl methacrylate (BMM) mixture followed by polymerization under UV light at −20°C. Sections (3 μm) were prepared and stained with toluidine blue (Sigma).

Positional cloning of FAC1

Rough mapping was carried out by establishing linkage with markers from different chromosomes in an F_2 population made between *FAC1-1*/*fac1-1* (*Ler*) and wild type (*Col*). From 250 F_2 plants, 17 DNA pools of homozygous wild-type plants (five each) were made and PCR reactions were carried out to look for linked markers using 25 SSLP and CAPS markers distributed evenly across the whole genome.

For fine mapping, a population of 4500 F_2 plants with the same parent combination was established. As *FAC1* is linked to *ER*, screening for surviving *er*/*er* plants allowed us to identify recombinants with crossovers between *FAC1* and *ER*. Polymorphic SSLP and CAPS markers were designed based on the sequence differences between *Col* and *Ler* deposited at the Cereon database (http://www.arabidopsis.org/Cereon/index.html).

To identify the mutation in *fac1-1*, genomic DNA was extracted from a *FAC1-1*/*fac1-1* and a wild-type *Ler* plant, and PCR primers were designed to amplify the ORFs of all 20 genes in the assigned *FAC1* region. PCR products were subjected to direct sequence analysis with the BigDye Mix and ABI PRISM 310 Genetic Analyzer (ABI, Foster City, CA, USA).

Northern blot analysis

RNA from different organs of Arabidopsis (*Col*) was extracted by the RNeasy kit (Qiagen, Venlo, the Netherlands). For hybridization a 614-bp *FAC1* fragment was amplified from cDNA by PCR using primers of 5′-GTTATGCTAACCTCTATGTG-3′ and 5′-GGAACAA-CTTCATCAGAG-ATAAC-3′. An *ACTIN8* fragment generated by PCR was used as a qualitative control.

Transgenic analysis

To visualize the expression pattern of the *FAC1* gene, a 736-bp fragment in the promoter region (from the terminator of the upper stream gene of At2g38290 to the ATG of *FAC1*, denoted as P_{FAC1}) was cloned by PCR and fused to a *GFP-GUS* fusion construct (Quaedvlieg *et al.*, 1998). Afterward, a 663-bp fragment of the *FAC1* downstream sequence (from *FAC1* stop codon to the transcription starting site of the gene At2g38270, T_{FAC1}) was amplified and fused to the stop codon of *GFP-GUS*. The whole fusion construct was then inserted into the T-DNA of the *pGreen II* vector (Hellens *et al.*, 2000), resulting in the construct of P_{FAC1}:*GFP-GUS*:T_{FAC1}. Subsequently, P_{FAC1}*IE*:*GFP-GUS*:T_{FAC1} was made by replacing P_{FAC1} with the P_{FAC1}*IE* fragment that has, in addition to P_{FAC1}, the first three introns/exons and a small part of the fourth exon of *FAC1*. The resulted constructs were transformed to Arabidopsis (*Col*) via *Agrobacterium tumefaciens* strain *C58C1 pGV3101* (*pMP90*) carrying the helper plasmid *pJIC Sa_RepA* (Hellens *et al.*, 2000).

To examine *FAC1* expression in somatic embryos, F_1 hybrid plants were obtained by crossing transgenic plants carrying P_{FAC1}*IE*:*GFP-GUS*:T_{FAC1} and *35S:BBM* constructs. Six- and 10-day-old seedlings germinating on a hormone-free medium were examined for somatic embryogenesis and GUS expression.

GUS assay

Excised organs of Arabidopsis were immersed in a staining solution containing 10 mM NaHPO4 buffer (pH 7), 0.5% Triton X-100, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$ and 1 mg ml^{-1} X-Gluc at 37°C for

overnight. For GUS staining in zygotic and somatic embryos, the reaction was performed in 6- and 10-day old seedlings in the staining solution for 1 h.

Complementation of yeast AMD1 *mutants*

The wild type, Y0, and the AMD1 deletion strain, Y7, of yeast (*S. cerevisiae*) were obtained from EUROSCARF (http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/). Full-length and truncated cDNAs of *FAC1* were amplified by RT-PCR. Mutated cDNA was obtained through megaprimer mutagenesis according to Lai *et al.* (2003). The cDNA fragments thus obtained were cloned, sequenced and inserted into *pESC-HIS* vector under the control of the inducible promoter *pGAL* (Stratagene, La Jolla, CA, USA). Both yeast strains were transformed with these vectors using the TRAFO protocol (Gietz and Woods, 2002).

Log phase yeast cells in a SG-His minimal medium (Stratagene) were counted, and washed once in sterile water, and then diluted to 1×10^7 cells ml^{-1} ($OD_{600} = 0.5$). Ten-fold serial dilutions of these cells were spotted onto a plate of SG-His + 2% lactate, with a 10-μl aliquot on each spot. The result was observed after incubation at 30°C for 9 days. Liquid culture with the log phase cells (dilute to $OD_{600} = 0.1$) was carried out at 30°C on a shaker, and growth measured at OD_{600} at different time points. These experiments were repeated twice.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2411/TPJ2411sm.htm

Figure S1. Digital Northern blot analysis showing the low level of constitutive expression in different organs of *FAC1* (green) using Arabidopsis Affymetrix GeneChip® data, in comparison with *ACTIN8* (red).

Data source: http://www.genevestigator.ethz.ch (Zimmermann *et al.*, 2004)

Linear *x*-scale was used for the analysis.

Figure S2. Expression of *FAC1* in the root tip.

Left: localization of GFP in roots carrying the P_{FAC1}*IE:GFP-GUS:*T_{FAC1} construct, observed under confocal microscopy. The results showed that GFP was localized in dots in the cytoplasm. It is not defined yet to which membrane the GFP is associated.

Right: digital *in situ* analysis showing the expression of FAC1 in the root tip, which is rather consistent with the expression pattern observed in the confocal data at the left. Data were extracted from Benfey's microarray data base (http://www.arexdb.org, Birnbaum *et al.*, 2003).

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