Surface Position, Not Signaling from Surrounding Maternal Tissues, Specifies Aleurone Epidermal Cell Fate in Maize

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Maize (Zea mays) endosperm consists of an epidermal-like surface layer of aleurone cells, an underlying body of starchy endosperm cells, and a basal layer of transfer cells. To determine whether surrounding maternal tissues perform a role in specifying endosperm cell fates, a maize endosperm organ culture technique was established whereby the developing endosperm is completely removed from surrounding maternal tissues. Using cell type-specific fluorescence markers, we show that aleurone cell fate specification occurs exclusively in response to surface position and does not require specific, continued maternal signal input. The starchy endosperm and aleurone cell fates are freely interchangeable throughout the lifespan of the endosperm, with internalized aleurone cells converting to starchy endosperm cells and with starchy endosperm cells that become positioned at the surface converting to aleurone cells. In contrast to aleurone and starchy endosperm cells, transfer cells fail to develop in in vitro-grown endosperm, supporting earlier indications that maternal tissue interaction is required to fully differentiate this cell type. Several parameters confirm that the maize endosperm organ cultures described herein retain the main developmental features of in planta endosperm, including fidelity of aleurone mutant phenotypes, temporal and spatial control of cell type-specific fluorescence markers, specificity of cell type transcripts, and control of mitotic cell divisions.

Cereal endosperms are the product of the second fertilization event in double fertilization, representing a filial generation developing within the maternal tissues of the ovary (Friedman, 1998). The fully developed maize (Zea mays) endosperm consists of three different cell types, an epidermal layer of aleurone cells covering a body of starchy endosperm and a basal layer of transfer cells (see Fig. 1A; Olsen, 2004b). Research has shown that positional signaling plays a pivotal role in determining aleurone cell fate specification. Observations from wheat (Triticum aestivum), and later from maize, have provided indications that positional signaling is involved as periclinal (inner) daughters of dividing aleurone cells converted to become starchy endosperm cells upon internalization (Morrison et al., 1975; Bercraft and Asuncion-Crabb, 2000). Defective kernel 1 (Dek1) revertant sector analysis reinforced this conclusion, supporting the notion that aleurone cell fate specification is independent of cell lineage and dependent on positional signaling (Bercraft and Asuncion-Crabb, 2000). In addition, these authors also concluded that Dek1 activity is essential for aleurone cell fate specification and maintenance throughout the lifespan of the endosperm and that Dek1 function is cell autonomous. Additional data for the importance of position for aleurone cell formation come from two recent studies of maize mutants with perturbed endosperm development. First, studies of the glo1 mutant led Costa and coworkers to conclude that acquisition of aleurone cell fate does not occur solely in response to signaling from the surrounding maternal tissues, but also depends on communication from within the endosperm itself (Costa et al., 2003). In the second study, aleurone cell formation was studied in maize mutants with small, irregular masses of endosperm cells (Olsen, 2004a). In these mutants, all endosperm surfaces were covered by a single layer of aleurone cells regardless of the size and shape of the endosperm cell mass or whether the endosperm was in direct contact with the surrounding maternal seed tissues. Finally, supporting the conclusion that aleurone cells form in response to surface positions, aleurone cells of two fused or connated ovaries convert from aleurone cells to starchy endosperm cells at the interface of the two endosperms (Geisler-Lee and Gallie, 2005). Together, these reports establish that aleurone cell differentiation is position dependent and not lineage dependent.

In accord with positional signaling, all three genes thus far identified in aleurone cell fate specification in maize, Crinkly4 (Cr4), Dek1, and supernumerary aleurone layers 1 (sal1), encode proteins characteristic of those that function in cell-to-cell signaling networks in animals. Cr4 encodes a protein receptor-like kinase with similarity to tumor necrosis factor receptors (Bercraft et al., 1996; Chan et al., 2000). Homozygous cr4 endosperms lack patches of aleurone cells, and leaves of homozygous mutant plants are crinkly. Dek1 encodes a protein with 21 predicted transmembrane segments...
Aleurone in Maize Endosperm Organ Cultures

In this article, we studied aleurone and starchy endosperm cell fate specification using a novel maize endosperm in vitro organ culture methodology that supports cell fate specification in the absence of maternal tissues. Identification of cell types was aided by and a calpain-like protease in the C terminus (Lid et al., 2002; Wang et al., 2003). Homozygous dek1 endosperms almost entirely lack aleurone cells, and embryos are arrested at the globular stage of development (Lid et al., 2002). The third gene, sal1, encodes a 204-amino acid protein that is homologous to the human charged vesicular body protein 1 (Chmp1) protein, a member of the conserved family of E-class vacuolar sorting proteins (Shen et al., 2003). Homozygous sal1 endosperms have multiple layers of aleurone cells, suggesting a role for Sal1 in inhibiting aleurone cell fate specification (Shen et al., 2003). Exactly how these three proteins operate in a signaling cascade leading to endosperm cell differentiation remains unknown.

Interestingly, the signaling cascade leading to aleurone cell fate specification in maize endosperms may operate more broadly. Recently, Cr4 and Dek1 have also been implicated in epidermal cell fate specification in different organs of Arabidopsis (Arabidopsis thaliana) and tobacco (Nicotiana tabacum; Johnson and Russell, 1982; Tanaka et al., 2002; Gifford et al., 2003, 2005; Ahn et al., 2004; Cao et al., 2005; Lid et al., 2005). It therefore appears likely that epidermal cell fate specification in plants, including aleurone cells, occurs through a process that is conserved among all organs and between monocots and dicots.

Whereas positional signaling and identification of key proteins involved in the signal transduction cascade leading to aleurone cell fate have now been identified, no report has clearly established the source of the signal from which these cells are taking their developmental cues. A serious limitation in the design of experiments addressing questions regarding endosperm cell fate specification is the close contact between the endosperm and maternal tissues, making it difficult to separate maternal and zygotic effects. Efforts to resolve this problem in maize have included in vitro fertilization of maize central cells (Kranz et al., 1998) and in vitro cultures of maize endosperm (Chu and Shannon, 1975; Felker and Muhitch, 1990). In the first case, a requirement for barley (Hordeum vulgare) feeder cells and the early developmental arrest of in vitro-fertilized endosperm have so far led to limited new insight. Considerable efforts have been invested in endosperm in vitro cultures over the last 30 years. Although procedures have varied over time, usually, 10 d after pollination (DAP), isolated endosperm has been grown on medium with 3% Suc. Whereas these cultures retained some characteristics of in planta endosperm, including some degree of starch, storage protein, and anthocyanin accumulation (Chu and Shannon, 1975; Shimamoto et al., 1983; Racchi and Manzocchi, 1988), their lack of histological organization has limited their value for developmental biology studies (Felker, 1987).

In this article, we studied aleurone and starchy endosperm cell fate specification using a novel maize endosperm in vitro organ culture methodology that supports cell fate specification in the absence of maternal tissues. Identification of cell types was aided by

Figure 1. Anatomy and structure of in planta- and in vitro-grown maize endosperms. A, Cartoon showing three endosperm cell types in the colors of the transgenic maize triple-marker line. al, Aleurone; e, embryo; se, starchy endosperm; tc, transfer cells (same section plane as in B–D). B, Transverse section of 6-DAP ovule with fertilized embryo sac (arrow) embedded in nucellus parenchyma cells. C, Transverse section of 10-DAP grain. D, Transverse section of 15-DAP grain. E, Intact unstained in vitro endosperms harvested at 6 DAP and grown in vitro for various time intervals. F, Six-DAP endosperms grown for 2 DIV. G, Six-DAP endosperms grown for 4 DIV. H, Six-DAP endosperms grown for 10 DIV. In planta- or in vitro-grown endosperms are from the triple-marker line. I, γ-Zein:AmCyan expression in the starchy endosperm of 12-DAP in planta endosperms. J, γ-Zein:AmCyan in starchy endosperm cells of 6-DIV in vitro-grown endosperms. K, Ltp2:ZsYellow in the aleurone layer of 12-DAP in planta endosperms. L, Ltp2:ZsYellow in the surface aleurone cell layer of 6-DIV in vitro endosperms. M to P, Expression of Ltp2:ZsYellow in the surface layer of aleurone cells in vitro-grown endosperms harvested at 6 DAP (M) and grown for 2 (N), 4 (O), and 6 DIV (P). Q, End1:DsRed expression in the transfer cell layer of 12-DAP in planta endosperms. R, View of the basal exterior surface of 6-DIV in vitro-grown endosperms from the triple-marker line, with the area where transfer cells form in planta lacking red fluorences shown in black and adjoining aleurone cells with ZsYellow fluorescence. tca, Transfer cell area.
the use of a transgenic maize marker line in which fluorescent proteins labeled aleurone, starchy endosperm, and transfer cells in different colors. To assess the similarity between endosperms grown in planta and in vitro, we compare the mitotic index of surface cell layers and perform transcript profiling of total endosperm as well as dissected endosperm cell types. Finally, we study endosperm organ cultures from maize endosperm developmental mutants to assess to what extent mutant phenotypes are maintained in in vitro cultures.

RESULTS

Establishment of Maize in Vitro Endosperm Organ Cultures That Maintain the Ability to Specify Aleurone and Starchy Endosperm Cell Identities

Previous efforts to establish immortalized endosperm cultures typically resulted in media conditions that did not support in planta-like cell organization (Shannon and Batey, 1973; Felker, 1987). To examine cell fate specification, culture conditions supporting both endosperm growth and endosperm cell organization were required. Experimentation in our laboratory showed that a simple synthetic agar medium containing 15% Suc permitted the establishment of maize endosperm organ cultures that both divided and maintained properly differentiated aleurone and starchy endosperm cells. Such cultures could be established free of maternal tissues as soon as the developing endosperm could be mechanically separated from the nucellus (3–4 DAP). In the work described here, we routinely initiated cultures at 6 DAP by manually isolating embryo sacs, structures that comprise both the embryo and the endosperm (Fig. 1B, arrow). At this stage, the embryo sac floats freely in a lysate of nucellus parenchyma cells and can be routinely isolated intact free of contaminating maternal tissues. In planta, the maize endosperm expands rapidly, filling almost the entire maternal cavity initially occupied by nucellar parenchyma cells at 10 DAP (Fig. 1C). At 15 DAP, the starchy endosperm has expanded to fill the entire maternal cavity, and the embryo is clearly visible (Fig. 1D). At 6 DAP, the time of initiation of the in vitro cultures, isolated embryo sacs were pointed and appeared translucent (Fig. 1E). After 2 d in culture, at 2 d in in vitro culture of 6-DAP isolated endosperm (DIV), the endosperm appeared opaque (Fig. 1F) and, at 4 DIV, the shape had become more rounded (Fig. 1G). Similar to in planta endosperms, the surface of the in vitro endosperm stayed smooth up to 10 DIV (Fig. 1H). The overall expansion of the in vitro-grown endosperm was less than in planta, which we ascribe to a deficiency in the uptake of carbon and/or nitrogen compared to the in planta situation. In in vitro-cultured endosperm that were initiated without removing the embryo, embryos germinated precociously around 20 to 25 DIV, giving rise to viable plantlets. We did not observe differences in the morphology of in vitro-grown endosperms with or without the embryo. Under the conditions used, in vitro-grown endosperms grew actively for a period of time comparable to in planta, 30 to 40 d, at which time they became brown and necrotic.

To monitor differentiation and growth of in vitro-grown endosperm at the cellular level, we developed a transgenic maize line expressing the cyan fluorescent protein (AmCFP1) in starchy endosperm cells under the control of the maize 27-kD γ-zein promoter (Ueda and Messing, 1991; Russell and Fromm, 1997), γ-Zein:AmCyan; the yellow fluorescence protein (ZsYellow1) in aleurone cells driven by the barley Ltp2 promoter (Kalla et al., 1994), Ltp2:ZsYellow; and the red fluorescent protein (DsRed2) in transfer cells directed by the maize End1 promoter (Doan et al., 1996), End1:DsRed. We refer to this line as the triple-marker line. In planta, the γ-Zein:AmCyan marker is expressed throughout the starchy endosperm, stronger toward the periphery, and with a less intense zone in the central endosperm (Fig. 1I). In in vitro-grown endosperm, a similar pattern was observed, although the fluorescence appeared weaker in the interior due to a less dense mass of cells (Fig. 1J). The Ltp2:ZsYellow marker is present exclusively in aleurone cells of in planta endosperms (Fig. 1K). The same also holds true for in vitro-grown endosperms with one layer of aleurone cells fluorescing in the periphery (Fig. 1L). In addition to maintaining the same spatial control over the Ltp2:ZsYellow marker, temporal control of this marker is also maintained in in vitro-grown endosperms. At the time of isolation at 6 DAP, no fluorescence was visible on the surface of the embryo sac (Fig. 1M). Similar to in planta, a weak yellow fluorescence appeared at 2 DIV, corresponding to 8 DAP in planta (Fig. 1N). The fluorescence remained strong and uniform after 4 and 6 DIV (Fig. 1, O and P). Finally, in in planta endosperms, transfer cells fluoresced red from the expression of the End1:DsRed construct (Fig. 1Q). In contrast, red fluorescence was never observed in the basal region of the in vitro-grown endosperm where transfer cells normally develop (Fig. 1R). Microscopic examination of the basal region failed to identify cells with transfer cell morphology. Cells in this region also did not fluoresce yellow (Fig. 1R), indicating that the cells on the surface of the basal region did not differentiate into aleurone cells either. The orientation of the endosperm on the culture medium (basal region in contact with media or facing away from media) had no additional effect on basal endosperm development. It is also unlikely that disruption of the basal endosperm region during the dissection process impeded development as fully intact endosperms always lacked red fluorescence in the basal endosperm region (data not shown).

In addition to cell type-specific fluorescence markers, we also used massively parallel signature sequencing (MPSS) to compare the steady-state levels of endosperm marker transcripts between in vitro- and in planta-grown endosperm (Table I). Examination of
the steady-state level of the 27-kD γ-zein transcript showed that the level first increased in the developing in planta endosperm, going from 101 $\times$ 10³ transcripts per million (tpm) in 12-DAP whole endosperms to 220 $\times$ 10³ tpm at 18 DAP. Following this initial increase, the 27-kD γ-zein transcript proceeded to drop on a tpm basis to 163 $\times$ 10³ in a sample of dissected endosperms (aleurone removed) at 27 DAP (Table I). These data are in accord with the results we observed in in planta endosperms of the AmCFP marker (27-kD γ-zein promoter) accumulation. As described above, the AmCFP marker becomes visible in starchy endosperm at 12 DAP, with the fluorescence signal increasing in intensity out to 20 DAP, after which the intensity remained stable (data not shown). MPSS data generated using in vitro-grown endosperms also contained high levels of 27-kD γ-zein transcript at 6 DIV (12 DAP), 147 $\times$ 10³ (Table I). Also similar to in planta endosperms, the 27-kD γ-zein transcript level in in vitro endosperms (15 DIV/21 DAP) decreased on a tpm basis at a later stage of development. To determine whether other storage protein transcripts are also expressed in in vitro-grown endosperms, we studied the 16-kD γ-zein transcript (Table I). Similar to 27-kD γ-zein, in vitro-grown endosperms contain high levels of this transcript, 206 $\times$ 10³ at 6 DIV, dropping to 23 $\times$ 10³ at 15 DIV (Table I).

The Ltp2:ZsYellow fluorescence in aleurone cells of in vitro-grown endosperm, together with their morphology, indicate that these cells are true aleurone cells. To provide further evidence supporting this conclusion, we compared the expression of two aleurone-specific or highly aleurone-preferred transcripts in the two types of endosperm, NLTP_MAIZE (Tchang et al., 1985) and GAMMA-ZEATHIONIN 2 (THZ2; Castro et al., 1996; Table I). In our assessment, these transcripts are aleurone specific, their presence in the starchy endosperm and basal endosperm samples most likely being caused by contaminating aleurone cells (Table I). In vitro-grown endosperms contain both transcripts at a comparable or higher level than in planta, lending support to the conclusion that in vitro endosperms are capable of differentiating true aleurone cells.

The lack of red fluorescence from the End1:DsRed construct in the basal endosperm region of in vitro-grown endosperms suggested that transfer cells are not formed in cultures (Fig. 1R). In the MPSS analysis, we used the basal endosperm-specific transcript LTP_895 as an indicator of the presence of transfer cells (Table I). This transcript was totally absent from both 6- and 15-DIV in vitro-grown endosperms, supporting the observation that lack of End1:DsRed fluorescence is caused by a lack of differentiated transfer cells.

From these observations, we conclude that in vitro-grown endosperms retain the ability to specify the aleurone cell fate on surfaces and starchy endosperms internally as revealed by the cell-specific fluorescent markers. The transcript profile analysis shows that in vitro endosperms contain major starchy endosperm and aleurone cell, but not transfer cell, molecular markers. These data support the conclusion that starchy endosperms and aleurone cells, but not transfer cells, develop normally in in vitro endosperm cultures. The observation that endosperm in vitro organ cultures have approximately the same lifespan as in planta endosperms suggests that the overall temporal control of endosperm development is endosperm autonomous.

To further investigate the indication that the in vitro-grown endosperm retains temporal control of developmental patterns similar to in planta, we compared the frequency of mitotic cell divisions in the periphery of the developing endosperms. In planta, the frequency of mitotic cell divisions in the periphery of the endosperm has been reported to peak at 8 to 10 DAP and then to decline to a low level 2 to 4 d later (Mangelsdorf, 1926). We confirmed this for our material by scoring the mitotic index of in planta endosperms (Fig. 2A) and observed both the expected peak at 8 DAP and the decline to a very low level by 12 DAP (Fig. 2B, top). Mitotic activity of in vitro-grown endosperms was recorded in cultures that were initiated from dissected endosperms harvested at 4, 6, 8, and 10 DAP. Observation of the frequency of mitotic divisions in this material was done by fixing samples in 2-d intervals following culture initiation (Fig. 2B, remaining images). Independent of the developmental stage for initiation of the in vitro endosperm cultures, the mitotic activity appeared to follow the same developmental pattern as observed in planta. For example, for endosperms placed in culture at 4 DAP, the mitotic

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Table 1. MPSS analysis of transcript abundance in in planta and in vitro endosperms

<table>
<thead>
<tr>
<th>Transcript</th>
<th>27-kD γ-Zein</th>
<th>16-kD γ-Zein</th>
<th>NLTP_MAIZE</th>
<th>THZ2_MAIZE</th>
<th>LTP_895</th>
</tr>
</thead>
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<tr>
<td>Total endosperm</td>
<td>101</td>
<td>220</td>
<td>ND</td>
<td>73</td>
<td>274</td>
</tr>
<tr>
<td>Aleurone</td>
<td>ND</td>
<td>81</td>
<td>11</td>
<td>ND</td>
<td>38</td>
</tr>
<tr>
<td>Starchy endosperm</td>
<td>ND</td>
<td>486</td>
<td>163</td>
<td>ND</td>
<td>346</td>
</tr>
<tr>
<td>Basal</td>
<td>11</td>
<td>ND</td>
<td>62</td>
<td>ND</td>
<td>11</td>
</tr>
<tr>
<td>In vitro endosperm</td>
<td>147</td>
<td>41</td>
<td>206</td>
<td>23</td>
<td>82</td>
</tr>
</tbody>
</table>

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Aleurone in Maize Endosperm Organ Cultures

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index peaked after 4 d in culture, corresponding well with the in planta data. Also similar to in planta, the mitotic index reached very low levels after a total of 12 d of growth after fertilization (Fig. 2B). The observed mitotic values for endosperms placed in culture at 6, 8, and 10 DAP also followed the pattern from in planta endosperms (Fig. 2B). From these experiments, we conclude that the in vitro-grown endosperm is actively dividing under the culture conditions used and that the temporal control of aleurone cell mitosis during the cell division phase is similar to that of the in planta endosperm.

The Previously Published Method for Maize Endosperm in Vitro Culture Retains Some Degree of Endosperm Cell Identity But Not Organ Identity

Whereas maize endosperm in vitro cultures were established more than 30 years ago, studies of cell fate specification have not been possible due to the lack of cell organization under the established conditions (Shannon and Batey, 1973; Felker, 1987). To compare the level of tissue organization in the endosperm cultures described here with those previously reported in the literature, we initiated cultures from a maize Ltp2:ZsYellow line at 6 DAP on the medium of Shannon and coworkers (Shannon and Batey, 1973). The most notable difference in the media composition is the concentration of Suc (3% compared to 15%). After 15 DIV, significantly more cell proliferation had occurred in the basal part of the endosperm compared to endosperms grown on our medium (Fig. 3A). Overall, the 3% Suc cultures appeared more heterogeneous.

Figure 2. Mitotic activity in the surface layer of in planta- and in vitro-grown endosperms. A, Light micrograph of aceto-orcein-stained aleurone layer at 6 DAP showing cells in mitosis (arrows) and interphase. B, Remaining images, Mitotic index of in vitro-grown endosperms harvested at 4, 6, 8, and 10 DAP grown in vitro culture for 8, 6, 4, and 2 d, respectively, and examined at 2-d intervals during culture. y axis indicates mitotic index (MI) and x axis indicates net DAP.

Figure 3. Endosperms from suspension cultures grown in medium with 3% Suc contain a high proportion of nondifferentiated cells and lack internal organization. A, Endosperms grown in vitro for 15 DIV showing growth of endosperm cells on the basal surface. B, Light micrograph of callus at 45 DIV with firm surface. C, Transverse section of callus in B showing irregular pattern of Ltp2:ZsYellow fluorescence in internal cells intermingled with nonfluorescing high-vacuolated cells. D, Phase contrast micrograph of spherical structure from B with aleurone-like layer surface cells and highly vacuolated internal cells. E, Same as in D shown in epifluorescence and visible light. F, Loosely organized callus of unspecialized cells. G, Higher magnification of cells in callus type shown in F.
than the cultures described in this article, with some endosperms growing into dense structures with relatively strong ZsYellow fluorescence, suggesting the presence of aleurone cells (Fig. 3B). However, transverse sections of these structures demonstrated that the strict organization into an aleurone layer on the surface is lacking, and that nonfluorescing, highly vacuolated cells resembling cells typically seen in rapidly growing callus tissue cultures are predominating (Fig. 3C). Similarly, a contiguous surface layer of aleurone cells was always lacking and internal cells were vacuolated with no identifiable internal components, such as starch granules (Fig. 3, D and E). A second type of callus in these cultures consisted of cells that were only loosely connected (Fig. 3, F and G), lacking visible markers typical of in planta endosperms and resembling typical undifferentiated callus cells normally found in highly proliferate plant tissue cultures. Importantly, in contrast to cultures grown on the medium used in this article, where growth stops after approximately 40 DIV, these cultures continued to proliferate and were capable of giving rise to continuous suspension cultures as described previously (Shannon and Batey, 1973). These experiments confirm the lack of organ identity described previously (Felker, 1987) and suggest that Suc concentration is an important factor for maintaining endosperm cell fate specification and organ identity.

Analysis of Cultured Normal Endosperms and Mini-Endosperms Shows That Surface Position Is Sufficient to Establish Aleurone Cell Fate

Previous research has shown that maize aleurone cell fate specification in planta occurs via active positional signaling on all endosperm surfaces regardless of endosperm shape and that aleurone cell formation does not require direct physical contact with surrounding maternal tissues (Olsen, 2004a; Geisler-Lee and Gallie, 2005). However, these in planta studies were unable to determine what role continued diffusible maternal signaling may be performing in directing aleurone differentiation. Results presented here thus far indicate that diffusible maternal signaling is not specifically directing endosperm cell specification. Additionally, two specific observations of the expression pattern of Ltp2:ZsYellow in in vitro-cultured endosperms confirm and expand on this conclusion. First, whereas a strong and uniform yellow fluorescence was present in the aleurone cell layer of in vitro-grown endosperms (Fig. 4A), cells of a second layer in from the surface often fluoresced, but to a much lesser intensity (Fig. 4B). Our interpretation of these results was that the weakly fluorescing internal cells represented inner daughter cells of periclinaly dividing aleurone cells. Such cells, upon translocation to a nonsurface position, lost their aleurone cell identity and hence their Ltp2:ZsYellow fluorescence as they converted to starchy endosperm cells. This confirms that an active process exists for aleurone cells to

Figure 4. Expression of the Ltp2:ZsYellow fluorescence marker confirms that cells in surface position assume aleurone cell fate. A, Mixed light and epifluorescence micrograph of transverse section of 6-DIV in vitro endosperms showing ZsYellow fluorescence in the surface layer as well as in some areas in the interior of the starchy endosperm (one example indicated by arrow). B, Mixed light and epifluorescence micrograph of the peripheral endosperm layer of section shown in A with strong fluorescence in the outer layer and weaker fluorescence in the second cell layer. Arrow identifies a cell in the second layer that shows strong fluorescence. C, Mixed phase contrast and epifluorescence micrograph showing Ltp2:ZsYellow fluorescence in internal cells adjacent to a void (V) in the endosperm identified by an arrow in A. D, Same as in C shown in epifluorescence microscopy. E, Laser confocal microscopy image showing the periphery of in vitro-grown endosperms at 15 DIV showing Ltp2:ZsYellow in aleurone cells and γ-Zein:AmCyan in starchy endosperms. Arrow identifies aleurone cells around an internal void. F, Higher magnification of interior aleurone cells in E. G, Transverse section of in vitro-grown endosperms at 15 DIV where the interior starchy endosperms failed to develop showing partial aleurone layers on the interior side of the endosperm (arrow).
differentiate into starchy endosperm cells without the presence of continued maternal influence. The second line of evidence supporting the role of active surface position sensing in aleurone cell formation was based on the observation of occasional formation of voids in the interior of the in vitro-grown endosperm (Fig. 4A; arrow identifies spots of internal yellow fluorescence). Frequently, cells surrounding such voids displayed Ltp2:ZsYellow fluorescence, suggesting that they had assumed the aleurone cell fate. At higher magnification, it could be seen that these cells fluoresced brightly with Ltp2:ZsYellow (Fig. 4, C and D). In addition to Ltp2:ZsYellow fluorescence, such cells also possessed aleurone cell morphology (Fig. 4, E and F). A more dramatic display of conversion from starchy endosperm cell fate to aleurone cell fate occurred when the interior starchy endosperm cells of some of the in vitro-grown endosperms failed to develop (Fig. 4G). In these cases, the surface on the interior side, originally starchy endosperms, had become covered with aleurone cells (Fig. 4G). Interestingly, this appeared to result in the development of spherical bodies of endosperms consisting of one layer of aleurone cells covering an inner mass of starchy endosperm cells similar to that previously observed in planta (Olsen, 2004a). From these experiments, we conclude that aleurone cell fate is defined by active sensing of surface position only, and that endosperm cells can change cell fate upon sensing surface or interior position. Importantly, the ability to sense and respond to positional cues is an intrinsic property of the endosperm that is independent of specific short-range signal input from maternal tissues.

As mentioned earlier, one property of the in vitro-cultured endosperm as it grows beyond 8 DIV is that the surfaces develop bulges that ultimately cover the majority of the exterior surface by 15 DIV (Fig. 5A). The first indication of these bulges was observed as sectors on the surface of the endosperm with varying intensity of Ltp2:ZsYellow fluorescence (Fig. 5B). Soon thereafter, distinct bulges develop from these sectors (Fig. 5, C and D). Sections of 15-DIV endosperms show that individual bulges consist of a surface layer of Ltp2:ZsYellow-fluorescing cells with aleurone cell morphology, covering an interior mass of γ-Zein:AmCyan fluorescing starchy endosperm cells (Fig. 5, E and F). The presence of a single layer of aleurone cells can be clearly seen in three-dimensional (3-D) reconstructions of such bulges, which we refer to as mini-endosperms (Fig. 5, G and H).

To better understand the origin and development of mini-endosperms, plasmids carrying the Ltp2:ZsYellow...
construct were introduced into young in vitro-grown endosperms by particle bombardment. In these experiments, we observed that a low number of fluorescent spots developed into large sectors (Fig. 5, I and J), and occasionally into mini-endosperms (Fig. 5K). Frequently, when observed over time, these sectors did not develop with mitosis occurring at a constant rate. Rather, they appeared to result from a sudden burst of mitotic activity over a short period of time. From these experiments, we conclude that the ability of the endosperm to self-organize into structures with a surface layer of aleurone cells and an interior mass of starchy endosperm cells is retained throughout the lifespan of the in vitro endosperm cultures. Furthermore, it appears that mini-endosperm formation results from localized mitotic activity in the surface layers of the endosperm.

Maize dek1 and sal1 Mutant Endosperm Phenotypes Are Retained in in Vitro Organ Cultures

To investigate whether the phenotype of mutants in aleurone-signaling genes are faithfully reproduced in in vitro endosperm cultures, we grew homozygous dek1 endosperms expressing the triple-fluorescent marker construct (Fig. 6, A–H). Previous studies of dek1 have shown that homozygous mutant endosperms lack aleurone cells, except for a low frequency of cells expressing the β-glucuronidase marker under the control of the barley Ltp2 promoter indicating aleurone cell formation (Lid et al., 2002). In line with this observation, we found that most dek1 endosperms grown in vitro lack aleurone cells and only occasionally contain aleurone cells that are Ltp2::ZsYellow positive (Fig. 6, B–D). In addition to these typical dek1 mutant endosperms, we also found a low frequency of mutant endosperms that appeared more highly developed (Fig. 6E). In spite of the fact that these endosperms displayed a surface layer of aleurone-like cells, very few of these cells were Ltp2::ZsYellow positive (Fig. 6, F and G). Notably, this type of dek1 mutant endosperm also formed mini-endosperms (Fig. 6H), demonstrating that a fully differentiated aleurone cell layer was not a requirement for mini-endosperm formation. Although cell proliferation that potentially could lead to mini-endosperm formation occurred in the aleurone-like cells of the dek1 mutant endosperm (Fig. 6, F and G, arrow), the exact cellular origin of mini-endosperms was difficult to determine. Notably, at least one of the mini-endosperms shown in Figure 6H had an organized Ltp2::ZsYellow-negative cell layer on the surface; the rest of the mini-endosperms lacked this level of organization. In our interpretation, these data showed that the Ltp2 promoter is a late-stage molecular marker for aleurone cell differentiation. In addition to dek1, we also cultured homozygous sal1-2 (Shen et al., 2003) endosperms in vitro (Fig. 6I). The result showed that the double aleurone layer phenotype of sal1-2 is also faithfully reproduced in in vitro culture. These experiments support the hypothesis that the molecular mechanisms responsible for dek1 and sal1 phenotypes are fully recapitulated in vitro.

Figure 6. dek1 and sal1-2 mutant endosperms grown in vitro have similar phenotypes as in planta. A, Phase contrast micrograph of typical dek1 endosperms cultured in vitro. B, Same as in A shown in epifluorescence showing a surface layer of cuboidal cells. Arrow identifies peripheral cell that expresses the Ltp2::ZsYellow marker. C and D, Enlargement of the Ltp2::ZsYellow positive cell of B in phase contrast (C) and in epifluorescence microscopy (D). E, Phase contrast micrograph of transverse section of a typical dek1 mutant endosperm with surface layer of aleurone-like cells and mini-endosperms lacking aleurone cells. Arrow identifies area with three Ltp2::ZsYellow positive cells of section E shown in F and G at higher magnification. F, Phase contrast and epifluorescence micrograph of peripheral area identified by arrow in E. G, Phase contrast micrograph of area shown in F. H, Autofluorescence image showing enlargement of mini-endosperms in section shown in E. Arrow identifies mini-endosperms with organized layers of surface cells. I, Transverse section of sal1-2 mutant endosperms with double layers of aleurone cells (identified by arrows) from in vitro culture.
DISCUSSION

Since the first establishment of maize endosperm in vitro cultures, studies of starch, storage protein, and anthocyanin biosynthesis have been reported (Chu and Shannon, 1975; Shimamoto et al., 1983; Racchi and Manzocchi, 1988). However, these studies have provided limited insight into mechanisms in endosperm development. In our view, the in vitro culture system presented here overcomes many of the previous limitations. First, the endosperm maintains tissue organization with aleurone and starchy endosperm cells similar to the in planta endosperm. Second, the timing of the onset of aleurone and starchy endosperm cell marker transcripts in in vitro and in planta-grown endosperms is similar. For example, Ltp2:ZsYellow fluorescence becomes visible in aleurone cells in vitro around 9 DAP, the γ-Zein:AmCyan fluorescent marker in starchy endosperm cells at 12 DAP. Third, high levels of zein storage protein transcript accumulation as well as the presence of starch granules firmly support the conclusion that in vitro-grown endosperms retain the main features of in planta endosperms. In vitro starchy endosperms grew considerably more slowly than their in planta counterparts. The reason for this is not fully understood, but we infer that the assimilation of sugars and nitrogen from the medium performs an important role in regulating the overall growth of starchy endosperm cells as described in planta (Miller and Chourey, 1992). In our opinion, the size difference between the in vitro and the in planta endosperms of modern corns reflects the highly efficient system for transfer of carbon from the source to the sink tissues. Fourth, control of mitotic division activity during early developmental stages in the surface layer of the endosperm is similar in vitro and in planta. Fifth, both types of endosperm also retain a low activity of mitosis in the peripheral cell layers. In planta, these divisions, occurring as late as 42 DAP (Mangelsdorf, 1926), function to expand the surface of the aleurone layer to accommodate an increasing volume of underlying starchy endosperm cells. In vitro-grown endosperms form mini-endosperms after 10 DIV. Whether the mitotic activity that leads to mini-endosperm formation reflects the same intrinsic developmental program as the continued mitotic activity in the in planta endosperm remains to be determined. However, if a developmental program for continued mitotic activity exists in surface layer cells, mini-endosperm formation could result from this activity combined with the less expanded inner mass of starchy endosperm cells in vitro compared to in planta. A comparison of the total number of mitotic divisions in the surface layers of the two types of endosperm is needed to address this question. Finally, mutant endosperm phenotypes are fully recapitulated in vitro, confirming that perturbations of cell fate specification can also be studied in in vitro-grown endosperms. The in vitro endosperm system presented here provides novel opportunities to elucidate developmental mechanisms as well as the study of metabolic control in maize endosperms because this method opens the endosperm to dynamic observation, transgenic manipulation, and adjustment of carbon and nitrogen signaling sources. Also, the in vitro endosperm system should offer an interesting opportunity to identify the separate contributions of sink and source on grain yield.

The data presented here demonstrate that aleurone and starchy endosperm cell fate specification in maize endosperms occurs through an intrinsic developmental program that discriminates between surface and internal cell positions. Furthermore, we infer that this program does not rely on direct signaling from the surrounding maternal tissues for orienting cell positions. Although contaminating maternal tissues adhering to the isolated embryo sac cannot be completely ruled out, the amount of such tissue was very small due to autolysis of the juxtaposed nucellus parenchyma cells, which leaves the embryo sac suspended in liquid at the time of isolation at 6 DAP. Adhesion of maternal molecules that play a role in aleurone cell fate determination is also, at least formally, a possibility. However, considering the expansion in the size of in vitro-grown endosperms combined with the fact that new mini-endosperms form after many days of growth in culture, such molecules would have to work in highly diluted concentrations. In addition, endosperm organ cultures can also grow and differentiate in the same medium in liquid cultures (data not shown), which in our opinion makes it very unlikely that maternal signal molecules for aleurone cell fate specification adhered to the surface of isolated embryo sacs.

Previous papers investigating mechanisms in aleurone cell fate specification have proposed an involvement of maternal signaling (Olsen et al., 1998), a role for positional signaling (Becraft and Asuncion-Crabb, 2000; Olsen, 2004a; Geisler-Lee and Gallie, 2005), no requirement for direct contact between maternal tissues and the endosperm (Olsen, 2004a), as well as a role for signaling between starchy endosperm cells and aleurone cells (Costa et al., 2003). Interestingly, the data presented here suggest a model for aleurone cell fate specification involving all of the proposed components. First, our data confirm and expand on the importance of surface position for aleurone cell fate specification. In accordance with observations from wheat and maize (Morrison et al., 1975; Becraft and Asuncion-Crabb, 2000), inner daughter cells of periclinally dividing aleurone cells assumed starchy endosperm cell fate as demonstrated by the rapid loss of Ltp2:ZsYellow fluorescence. Also, we demonstrate that the endosperm has full flexibility to respond to positional information by converting between starchy endosperm and aleurone cell fate in response to positional throughout the endosperm. This was shown by the conversion of starchy endosperm cells to aleurone cells around voids internally in the starchy endosperm, as well as in endosperms lacking an inner
body of starchy endosperm cells. In the latter case, aleurone layers developed on both sides of the endosperm. Second, because aleurone cells formed on all surfaces in isolated endosperms in the absence of direct maternal signaling (except for the basal transfer cell region), position must be sensed by cell-to-cell signaling between endosperm cells, as suggested by Costa et al. (2003). Last, short-range signaling from maternal tissues (Olsen et al., 1998) can also be ruled out because in vitro-grown endosperms form aleurone cells both in liquid and on solid medium in the absence of maternal tissues. On the other hand, the requirement for high Suc in the medium for proper organ differentiation identifies Suc as a maternal signaling component in endosperm development. This observation appears to be in accord with other reports describing carbon sources as having influence on aspects of endosperm development (Miller and Chourey, 1992; Weber et al., 2005). Although sugar sensing is an emerging area of plant research (e.g. see Franco-Zorrilla et al., 2005; Rolland and Sheen, 2005), it is still premature to speculate about the direct molecular basis for this phenomenon. Based on these observations, we propose a model for aleurone cell fate specification that predicts that aleurone cell fate is suppressed in the interior of the endosperm via inhibitory signaling from neighboring cells. One possible mechanism for this is that positive signaling for aleurone cell fate specification occurs by membrane proteins that are ubiquitously distributed, but only active (uninhibited) in surface membranes of endosperm surface cells. Although the molecules responsible for the proposed signaling are yet to be identified, Cr4 and Dek1 are attractive candidates, both proteins being predicted to be located in plasma membranes and both having extracellular domains capable of mediating cell-to-cell interactions (Becraft et al., 1996; Lid et al., 2002; Gifford et al., 2005). Additional observations that make Dek1 an attractive candidate for a direct role in aleurone cell fate specification is that it acts cell autonomously; starchy endosperm cells that lack Dek1 are unable to respond to surface position by converting to the aleurone cell fate (Becraft and Asuncion-Crabb, 2000). In addition, in situ hybridization experiments show that Dek1 is ubiquitously expressed, thus having the flexibility to respond to surface position upon cell repositioning. Dek1 has recently been implicated in epidermal cell fate specification in Arabidopsis embryos, leaves, and meristems (Johnson et al., 2005; Lid et al., 2005), suggesting that the mechanisms involved in surface position recognition described here for endosperms is universal to epidermal cell formation in all plants. Further studies using the maize endosperm in vitro organ should lead to a testable hypothesis for Dek1, Cr4, and Sal1 function in other systems, including Arabidopsis.

In contrast to the yellow and cyan fluorescence-marking aleurone and starchy endosperm cells in the triple marker of in planta endosperms, red fluorescence-marking transfer cells of in planta endosperms are missing in in vitro-grown endosperms. Furthermore, the basal endosperm-specific LTP_895 transcript, as well as cells with clear transfer cell morphology, is missing. We therefore tentatively conclude that transfer cells require interaction from maternal tissues to develop. This conclusion is in line with previous suggestions that maternal signaling plays a role in initiating transfer cell development after fertilization (Thompson et al., 2001; Gomez et al., 2002; Costa et al., 2003; for review, see Olsen, 2004b). The exact role of maternal tissues in transfer cell development remains to be determined, but one possibility deserves special mention. As proposed by Gomez et al. (2002), proteins from the maternal pedicel may be activated by high turgor pressure and diffuse into the transfer cell region where they transactivate ZmMRP1, which in turn sets off a cascade of Betl genes required for transfer cell development (Gomez et al., 2002). The in vitro endosperm organ culture system provides an opportunity to test whether ZmMRP1 is sufficient to induce transfer cell development by ectopically expressing the protein under the control of an endosperm autonomous promoter. Further experiments are clearly required to reveal the mechanisms of maternal influence in transfer cell development.

MATERIALS AND METHODS

Plant Genotypes and Growth Conditions

The triple-marker line is a transgenic maize (Zea mays) line expressing endosperm cell-type molecular markers that was created using Agrobacterium tumefaciens-mediated transformation and immortal high-II embryos as described (Zhao et al., 2001). Binary vectors were created containing the following promoters and molecular markers: Ltp2:ZsYellow-lflLtp2 promoter (Kalla et al., 1994) linked to ZsYellow (BD Biosciences CLONTECH; Matz et al., 1999; Lukyanov et al., 2000), γ-Zein:AmCyan-27-kD γ-Zein promoter (Ueda and Messing, 1991; Russell and Fromm, 1997) linked to AmCyan1 (BD Biosciences CLONTECH); and End1:DsRed-End1 promoter (U.S. patent no. 6,903,205 B2) linked to DsRed2 (BD Biosciences CLONTECH). The dek1-mum1 and sal1-2 mutants were originally isolated from Pioneer Hi-Bred International’s Trait Utility System for Corn (TUSC; Lid et al., 2002; Shen et al., 2003). For Dek1, hemizygous transgenic T2 events were self-pollinated to obtain lines homozygous at the transgenic loci. Homozygous lines expressing the transgenes were self-pollinated to generate endosperm materials. The self-pollinated F2 of a cross between heterozygous dek/+ plants and the triple line was created to introgress endosperm cell-type molecular markers. Plants used for isolation of endosperm were grown under typical greenhouse conditions (68°F–82°F, 16-h light, 1,800 photosynthetically active radiation) using a commercial potting medium (Metro-Mix 70; Scotts-Sierra) and fertilized as needed with a standard fertilizer mixture (20-10-20, N-P-K). Two to 3 d after the first silks appeared, pollinations were made to prepare material for initiating in vitro endosperm culture.

Initiation and Growth of in Vitro Endosperm Cultures

Typically, 6-DAP (unless otherwise specifically noted in text) freshly harvested ears were surface sterilized by incubation in 70% (v/v) ethanol for at least 5 min prior to dissection. A scalpel was used to slice open the tip of the kernel longitudinally and then forceps were used to split and remove the maternal tissues (pericarp and nucellus) at the tip of the kernel to expose the embryo sac. The embryo sac was then carefully lifted out of the surrounding nucellar tissue with a fine-tip forceps and placed immediately on culture medium containing 4.3 g/L Murashige and Skoog salts (no. 11117; Gibco-BRL), 0.5% (v/v) Murashige and Skoog vitamin stock solution, 5 mg/L thiamine HCl, 400 mg/L Asn, 10 μg/L 6-benzylaminopurine, 15 g/L Suc, and 800 mg/L threonine. Cultures were maintained at 25°C, 16-h light, 1,800 photosynthetically active radiation, and a 2,000 ppm CO2 atmosphere.
Microscopy

Endosperm tissue was fixed by incubating for 1 h at 4°C in freshly prepared 2% paraformaldehyde buffer (1 × phosphate-buffered saline; no. 20012; Invitrogen). Subsequently, materials were washed thoroughly with 0.5 × phosphate-buffered saline and stored at 4°C. In planta tissues were hand sectioned and mounted on glass slides using Fluoromount G (no. 17984–25; Electron Microscopy Sciences). In vitro cultures were prepared by embedding tissue (Tissue-Tek O.C.T. compound no. 4583; Sakura Finetek) at room temperature for 24 h prior to freeze sectioning (18- to 20-μm thickness, –11°C for the object and –15°C for the incubator) using a Reichert-Jung model 2800 Frigocut (Cambridge Instruments). Microscopy was performed using an epifluorescence-equipped Nikon Eclipse E800. Confocal microscopy was carried out with a Zeiss LSM410 (Carl Zeiss) or a Nikon Confocal C1 (Nikon Instruments) or a CARV (BD Biosciences, Bioimaging Systems).

Mitotic Index Measurement of In Vitro-Grown Endosperms

For each time point examined, three endosperms from each of three ears were assayed. In planta endosperms were carefully dissected at 4, 6, 8, 10, and 12 DAP. In vitro-cultured endosperms were initiated 4, 6, 8, 10, and 12 DAP and grown on solid medium as described. After harvesting, endosperms were immediately fixed with 50% glacial acetic acid (v/v in distilled, deionized water) for at least 2 h at room temperature. Samples were stored up to 48 h at room temperature in fixative or in 70% alcohol at 4°C for subsequent analysis. Assays were conducted using squash preparation on slides with one drop of aceto-orcein stain (10 mg/mL orcein dissolved in 55% boiling glacial acetic acid) and samples were dried using a 40°C incubator, followed by destaining with 5% glacial acetic acid. Mitotic activity was evaluated by counting the number of cells undergoing active mitosis from a random view of 100 cells. Two views were randomly selected for examination. Views for each sample were then averaged.

Particle-Mediated Transformation of In Vitro Endosperm Cultures

Endosperm cultures (3 DIV) grown on solid media were targeted with the PDS-1000 helium gun from Bio-Rad at one shot per sample (each sample comprising six endosperms) using 650 PSI rupture discs. Approximately 200 ng of DNA (Kltc2p2 promoter [Kalla et al., 1994] linked to ZsYellow; BD Biosciences CLONTECH) were delivered per shot.

Material for MPSS Libraries

Tissue samples from manually dissected whole endosperm at 12 and 18 DAP, dissected aleurone from endosperm at 18 and 27 DAP, dissected basal endosperm at 12 and 27 DAP, and starchy endosperm at 18 and 27 DAP were collected, RNA prepared, and mRNA isolated. Dissection of the aleurone, basal endosperm, and starchy endosperm was aided by tissue-specific marker genes, either anthocyanin expression (27-DAP aleurone and starchy endosperm) or fluorescent protein expression (see preceding description of the triple line). Cultured endosperm samples grown on solid media for 6 and 15 DIV were collected and mRNA isolated. Each of these mRNA samples was submitted to MPSS expression profiling (Solexa).

Normalization of MPSS Data

Counts reported for each 17-bp signature sequence detected in a sample were divided by a normalization term and multiplied by 1 million to generate a tpm expression value. The normalization term is the total number of beads sequenced minus the sum of the counts of the 10 most common signatures. In the case of the starchy endosperm cells that have a few genes with very high expression values, this normalization is superior to simply normalizing by the total number of beads because it mitigates the systematic reduction in counts of the non-seed storage protein genes and facilitates comparisons across tissue types.

Calculation of Statistical Significance Values for MPSS Data

A set of six samples that consisted of three pairs of biological replicates was used to construct a function relating the mean counts and the sd of the mean. The curve derived from this function was similar to that reported by Stolovitzky and colleagues for human MPSS data (Stolovitzky et al., 2005). Based on this function, t values were computed for each signature sequence in a pairwise sample comparison. By comparing the t value distribution for a pairwise comparison to that of a replicate sample comparison, false discovery rates were computed for each of the t values.

Defining Endosperm Cell-Type Markers from MPSS Expression Data

Data from the six dissected endosperm samples (aleurone at 18 and 27 DAP; basal endosperm at 12 and 27 DAP; and starchy endosperm at 18 and 27 DAP) were compared such that each sample was compared to two different tissue samples at similar developmental stages (the 12-DAP sample was compared to the two 18-DAP samples). To derive a set of highly significant, differentially expressed genes, we applied the criteria that a gene was considered preferential to a tissue (aleurone, basal endosperm, or starchy endosperm) if the expression value of the gene in both samples enriched in that tissue was higher than in the corresponding samples from the other two tissues and the false discovery rates of the four comparisons between the tissue samples with the highest expression and the two others was ≤0.1. These criteria produced a list of signatures that were further divided into two classes based on expression in the two nonpreferred tissues. Tissue-specific genes were those with 0 counts in the other two tissues and preferred genes were those with nonzero counts in either of the other tissues. This division is somewhat arbitrary in that the tissue samples are not 100% pure, and, thus, genes with very high levels of expression may be detected in the other two tissues due to contamination of a few cells of other tissues. The 17-bp signature sequences were subsequently mapped by exact sequence identity to a set of corn transcript sequences to produce the final gene list. From this list, three genes preferentially expressed in aleurone and starchy endosperm and one specific for basal endosperm were chosen based on detection in nondissected samples.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY104780, CD995889, AF371262, AF371261, J04176, AY110929, and X69793.

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LITERATURE CITED

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CORRECTIONS

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The authors regret two errors in this article. All absolute fluxes reported in $\mu$mol d$^{-1}$ cotyledon$^{-1}$ (in Fig. 6 and text) should be multiplied by the integer 3. This error occurred because the combined dry weight for three cotyledons (instead of that for a single cotyledon) was used while converting relative fluxes output by the program NMR2Flux to absolute fluxes. The relative fluxes (reported in carbon mol per 100 carbon mol of Suc uptake) and reaction reversibilities (reported in %) remain unchanged. None of the interpretations of data or conclusions in this article are altered by this error since they were based on relative flux values.

The minor hexose hydrolysis product referred to as “5-hydroxymethyl furfural” (HMF; p. 3045) should be “hydroxyacetone” (HyA). This error also does not affect any of the results, conclusions, or interpretations of the data in the article.


The authors regret that in the “Materials and Methods” section of this article, under the heading “Initiation and Growth of in Vitro Endosperm Cultures,” the amount of Suc in the growth medium is incorrectly stated as 10-fold lower than the correct concentration. The correct value is 150 g/L Suc.