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Senescence-induced expression of cytokinin reverses pistil abortion during maize flower development

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Summary

Maize is a monoecious species that produces imperfect (unisexual), highly derived flowers called florets. Within the spikelet, the basic repeating unit of the maize inflorescence, the spikelet meristem gives rise to an upper and a lower floret. Although initially bisexual, floret unisexuality is established through selective organ elimination. In addition, the lower floret of each ear spikelet is aborted early in its development, leaving the upper floret to mature as the only pistillate floret. Expression from the cytokinin-synthesizing isopentenyl transferase (IPT) enzyme under the control of the *Arabidopsis* senescence-inducible promoter SAG (senescence associated gene)12 was observed during early maize floret development. Moreover, the lower floret was rescued from abortion, resulting in two functional florets per spikelet. The pistil in each floret was fertile, but the spikelet produced just one kernel composed of a fused endosperm with two viable embryos. The two embryos were genetically distinct, indicating that they had arisen from independent fertilization events. These results suggest that cytokinin can determine pistil cell fate during maize floret development.

Keywords: maize, pistil development, cytokinin, programmed cell death, kernel, embryo development.

Introduction

Programmed cell death is used extensively during plant development to discard tissues or organs that are no longer required, to modify existing organs or adapt to environmental changes. Programmed cell death is also employed during flower development, particularly for species with unisexual flowers. Although most angiosperms produce hermaphroditic (i.e. perfect) flowers, approximately 10% possess male- and female-specific flowers on the same individual (monoecy) or on separate individuals (dioecy; Yampolsky and Yampolsky, 1922). Regardless of their final state, early flower development in most of these cases is characterized by the initiation of male and female sex organs, and the development of unisexual flowers requires the selective abortion of either the developing pistil or stamens.

Although most grass species bear florets (reduced flowers) that are hermaphroditic, members of the Maydeae are monoecious, producing unisexual florets (Kellogg and Birchler, 1993). In maize, male- and female-specific florets are borne on separate inflorescences (reviewed by Bonnett, 1978; Cheng *et al.*, 1982; Weatherwax, 1916). The male (staminate) inflorescence (i.e. the tassel) develops from the vegetative shoot apical meristem and is responsible for producing pollen. The female (pistillate) inflorescence (i.e. the ear) arises from an axillary meristem and bears the ovaries that give rise to kernels following pollination. During their early development, the staminate and pistillate inflorescences are almost indistinguishable. The inflorescence meristem of both elaborates spikelet-pair meristems, each of which produce a pair of spikelets. Within each spikelet, the basic repeating unit of the maize inflorescence, the spikelet meristem, is determinate, producing only two florets, an upper and a lower, on axis called the rachilla (Clifford, 1987). Both florets are enclosed by a pair of subtending sterile bracts or glumes.

The floret meristem is determinate and produces one lemma, one palea, three stamens and lodicules, and a single pistil primordium. Unisexuality is established through the abortion of specific floret organs such that pistil initials are eliminated from florets in the tassel inflorescence, resulting in staminate (male) florets, whereas stamen initials are eliminated from florets in the ear

inflorescence, resulting in pistillate (female) florets (Dellaporta and Calderon-Urrea, 1993, 1994; Kellogg and Birchler, 1993). In addition to the selective organ elimination required to establish unisexuality, the lower floret from each ear spikelet aborts, leaving only one mature pistillate floret per spikelet. Arrest of pistil development in the lower floret is followed by degeneration, which initiates in the subepidermal cells of the pistil primordium (Calderon-Urrea and Dellaporta, 1999; Cheng *et al.*, 1982). The degeneration is characterized by loss of nuclear DNA that initiates at a specific developmental stage, suggesting that cell death is developmentally programmed (Calderon-Urrea and Dellaporta, 1999). Pistil abortion (in tassel and ear florets) requires *tasselseed* (*ts*) genes, such as *ts1* and *ts2*, and is suppressed by *silkless1* (*sk1*; Calderon-Urrea and Dellaporta, 1999; Dellaporta and Calderon-Urrea, 1994; Emerson, 1920; Irish, 1996). Loss of *sk1* results in the abortion of the upper floret pistil as well as the lower floret pistil in ear spikelets (Jones, 1925). In contrast, loss of *ts1* or *ts2* function results in the sexual maturation of pistils in tassel florets as well as the pistil in the lower floret of each ear spikelet. Expression of *ts2* coincides with the loss of nuclei in the subepidermal cells of the pistil primordium (Calderon-Urrea and Dellaporta, 1999). Although *ts2* is expressed in all pistil primordia, it is blocked from inducing cell death in the upper floret pistil of each ear spikelet by the action of *sk1* (Calderon-Urrea and Dellaporta, 1999; Veit *et al.*, 1993). *ts2* encodes a short-chain alcohol dehydrogenase (DeLong *et al.*, 1993), although how this might function in pistil abortion remains unclear. Mutation at two loci (*pi* (pistillata)*1 pi2*) also suppresses pistil abortion in the lower floret of ear spikelets but without affecting pistil abortion in tassel spikelets (Huelsen and Gillis, 1929; Veit *et al.*, 1993; Weatherwax, 1916). Although the identities of *pi1 pi2* are unknown, this mutant demonstrates that the rescue of the lower floret pistil from abortion in ear spikelets can occur independently of pistil abortion in tassel spikelets.

Pistil development and the arrest of stamen development also require gibberellic acid (GA). Exogenous application of GA results in sex reversal in tassels from staminate to pistillate florets (Hansen *et al.*, 1976; Nickerson, 1959; Nickerson and Dale, 1955), demonstrating that GA represses stamen development and prevents pistil abortion. Mutants deficient in GA exhibit perfect upper florets in the ear inflorescence, i.e. stamens and pistil are both present, whereas the lower floret contains stamens without a pistil (Bensen *et al.*, 1995; Phinney, 1961). Stamen development in these mutants could be suppressed by treatment with GA (Karpoff, 1983), demonstrating that GA is also involved in the repression of stamen development in ear florets. Although GA can arrest the early development of stamens, it is required during later developmental stages, e.g. during anther and pollen development (Huang *et al.*, 2003), indicating that its role in organ development is highly stage specific.

The role of cytokinin in regulating entry into a senescence program has been demonstrated in tobacco leaves in which the cytokinin-synthesizing isopentenyl transferase (*IPT*) gene was introduced under the control of the *Arabidopsis* senescence-inducible promoter from the cysteine protease gene *SAG* (senescence associated gene) *12* (Gan and Amasino, 1995). Although senescence and programmed cell death are developmentally distinct in that the former typically occurs as the last developmental stage of an organ whereas the latter can initiate even at early developmental stages, those hormones regulating entry into either program may be similar. For instance, ethylene promotes the senescence of leaves as well as the programmed cell death of root cortical cells in response to hypoxia (Grbic and Bleecker, 1995; He *et al.*, 1996). In order to investigate whether cytokinin may affect the abortion of maize floral organs, the *SAG12–IPT* construct was introduced into maize. *IPT* expression was observed in the ear during early floral development. Abortion of the lower floret pistil in the ear was suppressed, resulting in a spikelet with two florets, each containing a fertile pistil. Floret number or arrangement within a spikelet and organ number within each floret were unaltered demonstrating that *IPT* expression did not alter spikelet or floret determinacy. However, pollination resulted in the production of a single kernel from each ear spikelet that contained two normal size, genetically distinct embryos, indicating that fusion between the two pistils within a spikelet had occurred. The presence of two embryos in the kernel displaced growth of the endosperm, resulting in kernels with an increased ratio of embryo to endosperm content.

Results

Generation of transgenic maize expressing IPT *during leaf senescence and floret development*

Eight independent, fertile T_0 *SAG12–IPT* maize lines were generated and crossed with the inbred B73. Fifty-two per cent of the T_1 progeny (157/300) inherited the transgene as determined by PCR analysis, indicating a single insertion site. Progeny were grown from self-pollinated T_1 plants. *SAG12–IPT* transgene expression was not observed in young T_2 leaves but was observed in leaves entering into the senescence program (Figure 1). Thus, the *SAG12* promoter is induced during senescence in maize as it is in *Arabidopsis*, suggesting that its developmental regulation is conserved in maize. Abortion of the pistil from the lower floret of ear spikelets occurs early during lower floret development and progresses acropetally, in the same manner as the progression of spikelet and floret development. *IPT*

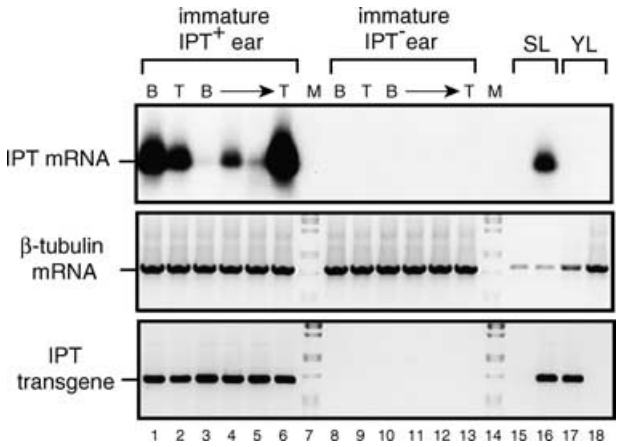


Figure 1. *SAG12–IPT* expression in transgenic maize.
Top panel, RT-PCR analysis of *IPT* mRNA expression in each 1-cm segment of developing T_2 ears 2 cm in length (lanes 1, 2, 8, and 9) when floret development has initiated in the basal two-thirds of the ear and 4 cm in length (lanes 3–6 and 10–13) when floret development is limited to the tip of the ear in *SAG12–IPT* (lanes 1–6) and control (lanes 8–13) plants. B, basal 1-cm section; and T, top 1-cm section of ear. RT-PCR analysis of *IPT* mRNA expression in senescing (SL; lanes 15 and 16) and young (YL; lanes 17 and 18) leaves of *SAG12–IPT* (lanes 16 and 17), and control (lanes 15 and 18) plants. Successful amplification was detected by Southern hybridization (using radiolabeled *IPT* DNA as the probe) following resolution by agarose gel electrophoresis. Middle panel, RT-PCR analysis of β-tubulin mRNA expression was performed with the same samples to serve as a control. Bottom panel, the presence of the *IPT* transgene was determined by PCR from genomic DNA of the same samples.

Figure 2. Leaf senescence is not delayed in *SAG12–IPT* maize.
Leaves from *SAG12–IPT* and syngenic maize were collected at tassel emergence. Leaves were arranged from youngest (left) to oldest (right) and were representative.

expression was observed in developing *SAG12–IPT* ears but not in the absence of the transgene (Figure 1). *IPT* expression correlated with the acropetal development of floret organs, i.e. it was induced first in the basal region of the ear, but was transient, disappearing shortly following floret development.

Leaf senescence is not delayed in SAG12–IPT *maize*

As IPT expression was observed in maize leaves entering senescence and expression of IPT delayed leaf senescence in tobacco (Gan and Amasino, 1995), we examined whether leaf senescence was delayed in *SAG12–IPT* maize. Leaves from *SAG12–IPT* and syngenic maize grown until tassel emergence were collected for analysis. No difference in entry into the senescence program was observed (Figure 2). These results suggest that the expression of IPT in leaves entering the senescence program observed in Figure 1 does not function in or is insufficient to delay the onset of leaf senescence.

Rescue of the lower floret pistil from abortion in SAG12–IPT *ear spikelets*

Maize spikelets are determinate, producing just two florets. To determine whether expression from the *IPT* transgene alters spikelet or floret development in the ear inflorescence, spikelets were examined following silk emergence. *SAG12–IPT* ear spikelets were observed to produce two silks (Figure 3a). The appearance of two silks from *SAG12–IPT* ear spikelets correlated with the presence of the *SAG12–IPT* transgene: only one silk emerged from ear spikelets of syngenic control plants that had not inherited the transgene from the self-pollination of a parent that was hemizygous for the transgene (Figure 3b). *ts2* mutants exhibit rescue of the lower floret from abortion and thus a pistil and silk develop in each floret, resulting in two silks per spikelet (DeLong *et al.*, 1993; Irish and Nelson, 1993; Nickerson and Dale, 1955). *pi1 pi2* is similar to *ts2* in that both florets of ear spikelets develop to maturity (Huelsen and Gillis, 1929; Veit *et al.*, 1993; Weatherwax, 1916). The number and position of silks in *SAG12–IPT* ear spikelets were similar to those observed in *ts2* (Figure 3c) and *pi1 pi2* (Figure 3d) mutant spikelets. Removal of the glumes showed that palea separated the two pistils in the *SAG12–IPT* ear spikelets (Figure 3e,f, also see below), indicating that the additional pistil represented a separate floret and was not a result of extra carpels in the upper floret. The additional floret was basipetally positioned (Figure 3e,f) consistent with the notion that it represented rescue of the lower floret. Removal of lemma and palea as well as the glumes from *SAG12–IPT* ear spikelets revealed that the silk of the upper floret emerged from a pistil of normal size and the second silk emerged from the additional pistil that ranged from normal size to the one that was attenuated (Figure 3g–i), the latter of which was observed most often. A spikelet containing two florets, each with one pistil, is similar to *pi1 pi2* spikelets, although the pistils in this latter mutant are nearly equal in size (Figure 3j). The *SAG12–IPT* ear spikelets differ from syngenic control spikelets in which only one pistil is present (Figure 3k), which contains a single ovule (Figure 3m). Removal of the carpels from

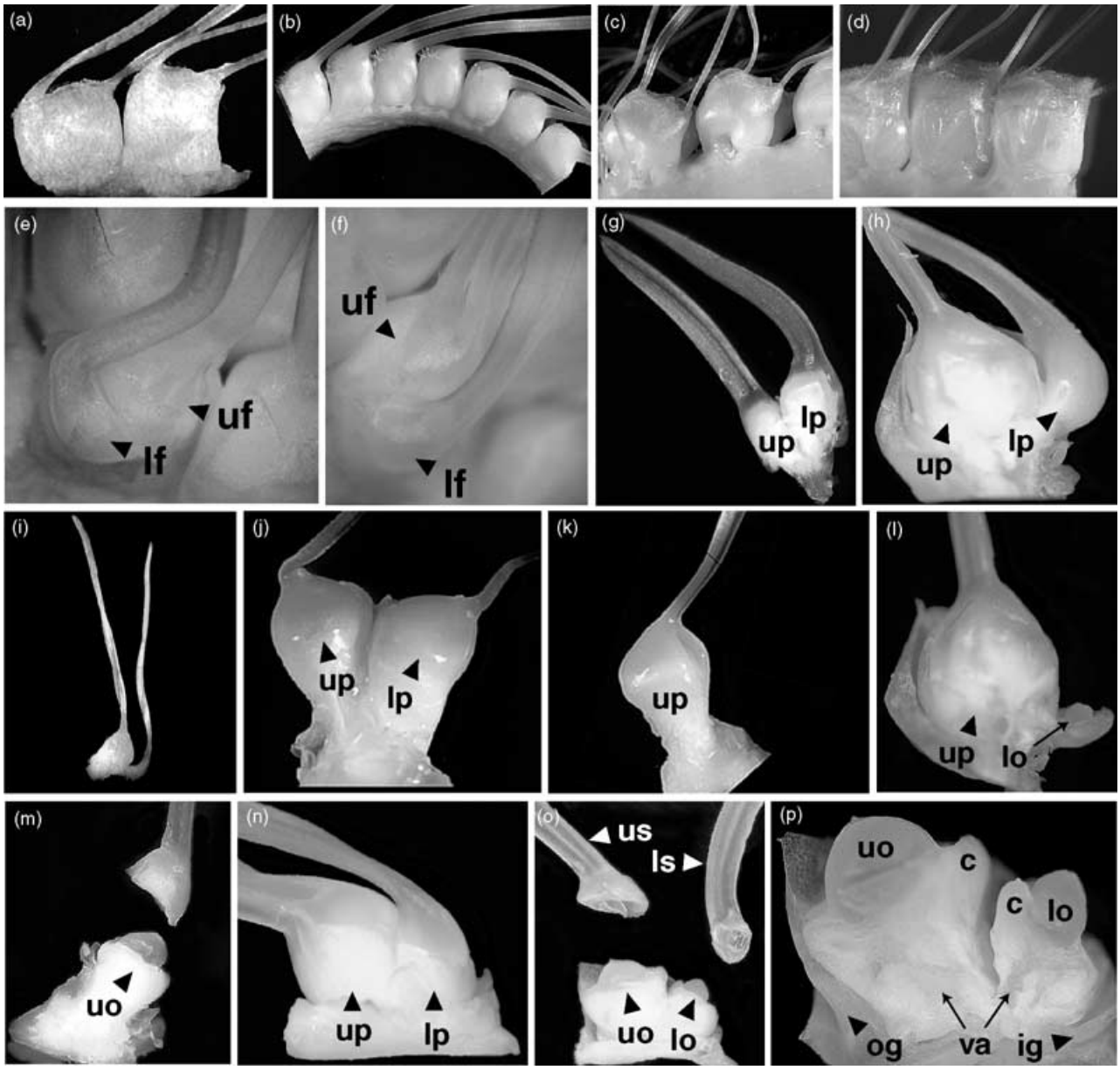


Figure 3. Rescue of the lower floret pistil in *SAG12–IPT* pistillate florets.
(a) *SAG12–IPT* spikelets with two silks per spikelet.
(b) Control spikelets with a single silk per spikelet.
(c) *ts2* spikelets with two silks per spikelet.
(d) *pi1 pi2* spikelets with two silks per spikelet.
(e, f) An *SAG12–IPT* spikelet with glumes removed to reveal the upper floret (uf) and lower floret (lf). The lemma and palea are present in each floret.
(g–i) A single *SAG12–IPT* spikelet with glumes, lemma, and palea removed to reveal the presence of the upper floret pistil (up) and lower floret pistils (lp).
(j) A *pi1 pi2* spikelet with glumes, lemma, and palea removed to show the up and lp.
(k) A control spikelet with glumes, lemma, and palea removed to show the up. No lf is present.
(l) A single *SAG12–IPT* spikelet with glumes, lemma, and palea removed. Carpels of the lp were removed to reveal the lower floret ovule (lo).
(m) A control spikelet with glumes, lemma, and palea removed and the upper portion of the carpel walls removed to show the upper floret ovule (uo).
(n) An *SAG12–IPT* spikelet with glumes removed to show the up and lp.
(o) *SAG12–IPT* spikelet in (n) with the upper portion of the carpel walls removed to reveal the uo and lo. The lemma and palea were also removed. ls, lower silk; and us, upper silk.
(p) Longitudinal hand section of an *SAG12–IPT* spikelet showing the vasculature (va) and branch point within the spikelet. og, outer glume; and ig, inner glume.

the attenuated pistil of the lower floret in an *SAG12–IPT* spikelet revealed the presence of a small ovule (Figure 3l). Removal of the carpels from an *SAG12–IPT* spikelet containing two florets in which the glumes were removed (Figure 3n) revealed the presence of a single ovule in the upper floret and a single smaller ovule in the lower floret (Figure 3o). Silks indistinguishable from the wild type were observed for each floret (Figure 3o). A longitudinal hand section revealed the branch point between the upper and lower florets as well as the spikelet vasculature. Up to 40% of the spikelets from *SAG12–IPT* ears contained two florets in which the upper floret pistil was of normal size whereas the lower floret pistil was smaller. No more than two florets per *SAG12–IPT* spikelet were ever observed, suggesting that expression from the *SAG12–IPT* transgene did not alter spikelet determinacy.

Although early floret development was unaltered in *SAG12–IPT* spikelets (Figure 4a,b), the delayed growth of the lower floret in *SAG12–IPT* spikelets could be seen relative to the upper floret from which the growth of the silk is observed (Figure 4c–e). Rescue of the lower floret pistil from abortion can be seen relative to the pistil in a control lower floret pistil in which growth arrest had occurred (Figure 4f). The position of the lower floret in *SAG12–IPT* spikelets was identical to the arrested floret in control spikelets (compare Figure 4e and Figure 4f). Moreover, the lower floret pistil was positioned between the lemma and palea (Figure 4c–e), consistent with the conclusion that the lower floret pistil had been rescued from abortion. Following growth of the lower floret pistil, separate silks emerged from the lower and upper floret pistil (Figure 4g) as was observed in Figure 3 and in contrast to a typical control spikelet in which only the upper floret had developed (Figure 4h). At this stage, the upper and lower floret pistils were separated by palea (Figure 4g), consistent with the conclusion that each had developed from separate florets.

The position of the palea and lemma on either side of a single pistil distinguishes normal floret development from that of mutants such as *silky1* (*si1*), *zea agamous1* (*zag1*, encoding the maize homolog of AGAMOUS), *thick tassel dwarf1* (*td1*), *indeterminate floral apex1* (*ifa1*), or *knotted1* (*kn1*) loss-of-function in which extra carpels develop within the upper floret (Ambrose *et al.*, 2000; Fraser, 1933; Kerstetter *et al.*, 1997; Laudencia-Chingcuanco and Hake, 2002; McSteen *et al.*, 2000; Mena *et al.*, 1996). The multiple silks observed in these mutant florets arise from the extra carpels and therefore do not constitute a separate floret. *SAG12–IPT* ear spikelets differ from those of *zag1*, *td1*, *ifa1*, and *kn1* mutants in that the two silks present in an *SAG12–IPT* spikelet emerge from separate florets (Figures 3e,f,n and 4g). Moreover, the pistil from the upper floret and the smaller, basipetally positioned pistil in *SAG12–IPT* ear spikelets were each bounded by a palea and lemma consistent

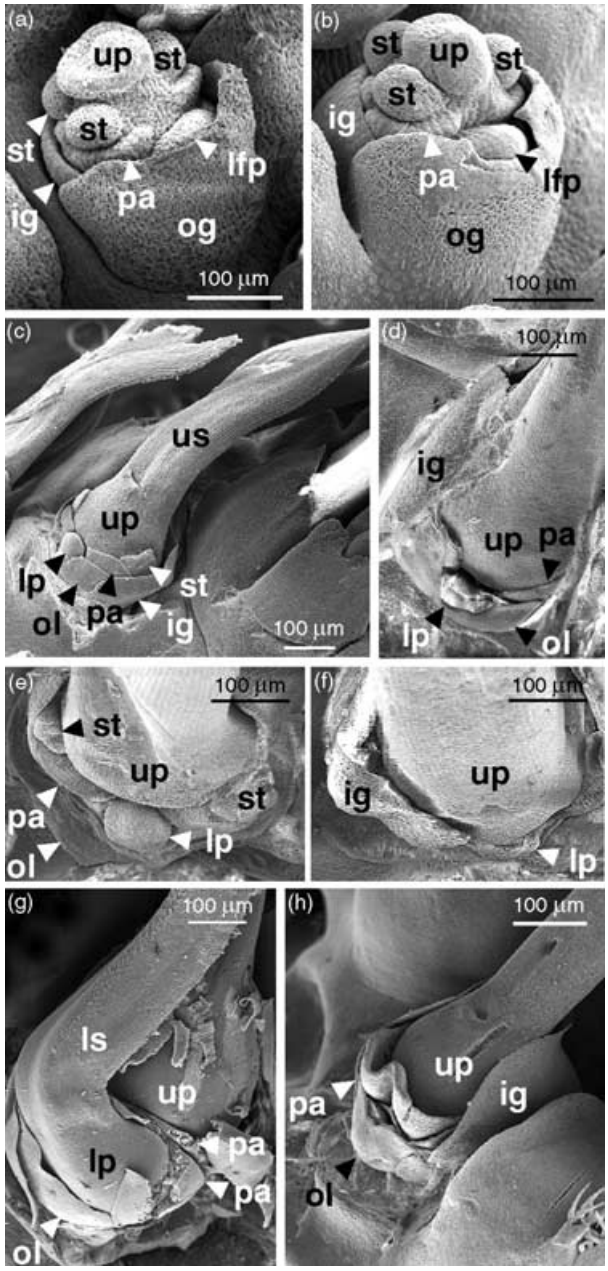


Figure 4. Organ initiation during floret development in *SAG12–IPT* pistillate florets.
(a, b) Early floret development in (a) *SAG12–IPT* and (b) control spikelets. lfp, lower floret primordium; and st, stamen.
(c–e) Delayed growth of the lower floret in *SAG12–IPT* spikelets relative to the upper floret from which the growth of the silk has initiated. In each example, the lower floret pistil is positioned between the lemma and palea. ol, outer lemma.
(f) Control spikelet in which growth arrest of the lower floret pistil had occurred.
(g) *SAG12–IPT* spikelet with one silk emerging from the upper floret pistil (up) and one emerging from the lower floret pistil (lp). The up and lp are separated by palea.
(h) Control spikelet in which only the upper floret has developed.

with the development of individual florets. Only one silk was observed to emerge from the pistil of the upper floret and one silk to emerge from the pistil of the lower floret (Figures 3e–i,n and 4g), indicating that each floret contained just a single pistil with no extra carpels.

The presence of one pistil per *SAG12–IPT* ear floret and the presence of a single ovule per pistil were also observed following dissection of *SAG12–IPT* florets. Longitudinal sections of *SAG12–IPT* ear spikelets confirmed the presence of only one ovule per pistil (Figure 5a), thus excluding the possibility that multiple ovules were present within the upper floret pistil that might have generated a kernel containing multiple embryos. The presence of one ovule in the upper floret pistil is in contrast to the one or more ovules observed in the upper floret pistil of *kn1* ear spikelets (Kerstetter *et al.*, 1997). A single embryo sac was observed in the upper floret *SAG12–IPT* ovule (Figure 5b), eliminating the possibility that multiple embryo sacs may have been present in the upper floret ovule.

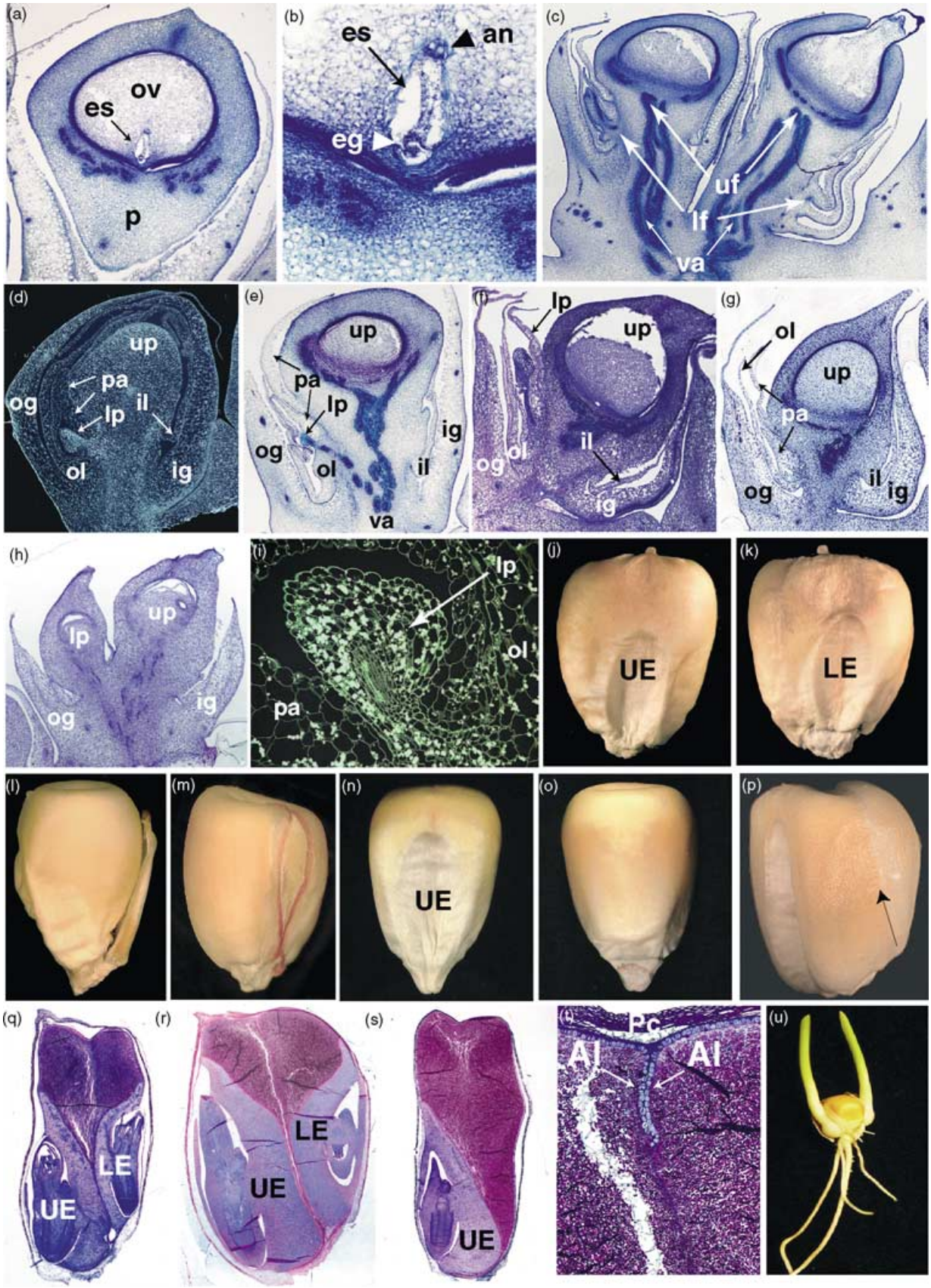
The two florets within a maize spikelet are arranged at defined positions along the rachilla. The lower floret branch point subtends the upper floret and the lower floret is basipetally positioned in the inflorescence. The remnants of the lower floret, i.e. the palea and lemma, in a control spikelet, can remain at ovule maturity and indicate the branch position of the aborted lower floret with respect to the upper floret (Figure 5g). In *SAG12–IPT* ear spikelets, the branch point of the rescued floret subtended the upper floret and was consistently basipetally oriented in the inflorescence, precisely what would be predicted if the lower floret had survived (Figure 5c–f). Thus, the position of the rescued floret with respect to the upper floret was identical to the position of the remnants of the lower floret in control spikelets. The observation that floret arrangement within the spikelet remained unchanged in that the branch point of the rescued floret subtended the upper floret and was basipetally positioned supports the notion that it represents the lower floret. These results indicate that *IPT* expression does not alter the arrangement of the two florets along the rachilla within the spikelet.

Examination of the cellular organization of the lower floret pistil revealed that it survived the developmental stage at which it normally enters the cell death program (Figure 5d). Vasculature developed up to the ovule in the lower floret as it did to the ovule of the upper floret (Figure 5e), similar to that observed for *pi1 pi2* florets (Figure 5h). The pistil from the lower floret of *SAG12–IPT* ear spikelets contained starch-containing cells (Figure 5d,i) typical of the gynoecium, supporting the notion that it was functional as was the pistil of the upper floret. The lemma and palea surrounding the lower floret pistil were readily observed (Figure 5c–f) and no evidence of mature stamens was observed. No evidence of additional florets or organs was observed supporting the conclusion that spikelet and floret determinacy were unchanged in *SAG12–IPT* maize. Other than the small size of the pistil in the lower floret, the development of *SAG12–IPT* pistillate florets was similar to that observed in *ts2* or *pi1 pi2* mutants.

SAG12–IPT *spikelets produce a single kernel with two embryos following pollination*

To determine whether the lower floret in *SAG12–IPT* maize was functional, T_2 ears from eight independent transformed lines were pollinated and the resulting progeny were examined. Because florets initiate in a distichous pattern in which florets alternate 180° from one another along the spikelet branch (Chuck *et al.*, 1998; Weatherwax, 1916), the arrangement of the floral organs within the lower floret (if it were to develop) is opposite to that in the upper floret. Consequently, the embryo of the kernel produced from the upper floret is abaxial and acropetal, whereas the embryo of the kernel produced from the lower floret (when rescued from abortion in mutants such as *ts2* or *pi1 pi2*) is abaxial but basipetal. Thus, the embryo from kernels produced from the upper and lower florets face in opposite directions, and the fusion of developing pistils would be expected to result in a kernel with an embryo on the abaxial side and an embryo on the adaxial side. This prediction was borne out in the *SAG12–IPT* double embryo kernels where the two embryos were positioned directly opposite to one another (Figure 5j,k), consistent with the fusion of pistils in florets from opposing sides of the spikelet rachilla. The branch point between kernels can be seen in occasional instances when development of the lower kernel aborts leaving an empty caryopsis, which often does not fuse with the upper kernel (Figure 5l), but in some cases does completely or partially, and can result in a split in the pericarp upon kernel drying (Figure 5m).

Longitudinal sections of *SAG12–IPT* kernels revealed two embryos on opposing sides of an endosperm that was bounded by a single continuous pericarp (Figure 5q,r). The contributions of the two independent pistils to the endosperm were most evident following self pollination of an *SAG12–IPT* Hill (light yellow aleurone layer) and B73 (darker yellow aleurone layer) heterozygote. The endosperm contributed by each pistil following pollination is evident by the presence of a light yellow aleurone layer on one side of the junction and a dark yellow aleurone layer on the other (Figure 5p). In this example, the kernel from the lower floret is fused piggyback on the kernel from the upper floret. The junction between the two endosperms at which the aleurone layers from each are observed to fuse can be seen in longitudinal sections of a double embryo kernel (Figure 5t). Interestingly, the aleurone failed to differentiate along the entire length of the fused endosperm. Moreover, the presence of the embryo from the lower floret replaced the endosperm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that would normally be present in that portion of the kernel from the upper floret (compare Figure 5q,r to Figure 5s), thus resulting in a kernel with reduced endosperm but two times the embryo content.

To determine whether both embryos were functional, *SAG12–IPT* kernels were germinated. Both embryos germinated and produced seedlings (Figure 5u), which grew normally and produced fertile plants. As the seedlings represented T_2 progeny from the self-pollination of T_1 *SAG12–IPT* hemizygotes, segregation of the *IPT* transgene in T_2 seedlings from a double embryo kernel would be predicted if the embryos resulted from independent pollination of the upper and lower pistils. PCR analysis of plants grown from *SAG12–IPT* kernels revealed that the two embryos present in each kernel were genetically distinct in that they inherited the transgene independently and with a frequency of approximately 69% (i.e. containing at least one copy of the transgene) in good agreement to what would be predicted (i.e. 75%) for progeny from a self-pollination of a hemizygous transgenic parent. The observation that the two embryos within a single kernel are genetically distinct supports the conclusion that the double embryo kernels represent a fusion of the upper and lower pistils following their pollination.

Staminate spikelet and floret development is unaltered in SAG12–IPT *maize*

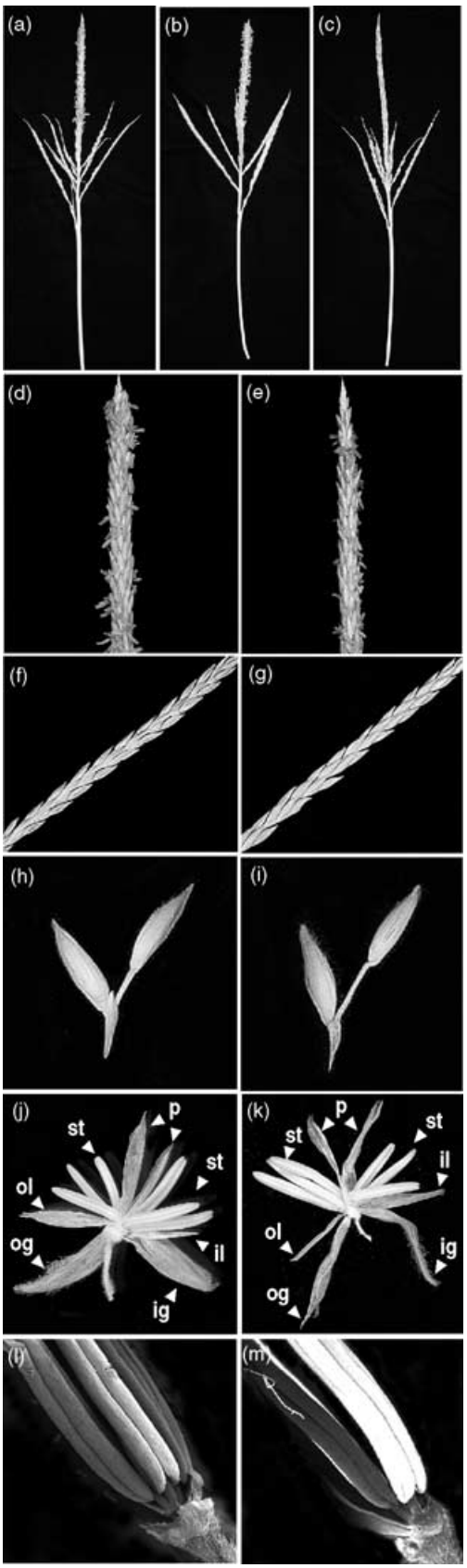
Branching within the tassel inflorescence in *SAG12–IPT* maize (Figure 6a) remained unaltered relative to inflorescences from vector-only (Figure 6b) and syngenic control maize (Figure 6c). Moreover, spikelet pair production in the *SAG12–IPT* inflorescence (Figure 6d,f) was unchanged relative to syngenic maize (Figure 6e,g), suggesting that inflorescence branching was not affected by the transgene. Only two florets were observed in *SAG12–IPT* spikelets (Figure 6h) identical to the number in syngenic control spikelets (Figure 6i), supporting the conclusion that spikelet determinacy was not altered. Organ development in *SAG12–IPT* tassel florets (Figure 6j) was indistinguishable from control florets (Figure 6k) in that both contained a lemma, a palea, and three stamens, and that the two tassel florets present in a spikelet were subtended by a pair of glumes. No evidence of pistil development was observed in florets of *SAG12–IPT* (Figure 6l) or control tassel spikelets (Figure 6m).

Discussion

In maize expressing the *SAG12–IPT* transgene, we have observed the development of two florets per ear spikelet, that once pollinated, generate a kernel with two embryos. The conclusion that the lower floret was rescued from abortion in *SAG12–IPT* ear spikelets is based on the observation that floret determinacy remained unaltered with no additional pistils or carpels observed, spikelets remained determinate with only two florets per spikelet each of which is composed of a palea, a lemma, and one pistil, and branching within a spikelet remained unchanged in that the rescued lower floret subtended the upper floret and was basipetally positioned within the inflorescence.

SAG12–IPT ear spikelets were observed to contain two pistils each with one silk. The presence of multiple pistils was also observed in *si1*, *zag1*, *td1*, *ifa1*, and *kn1* loss-of-function ear spikelets (Fraser, 1933; Kerstetter *et al.*, 1997; Laudencia-Chingcuanco and Hake, 2002; McSteen

Figure 5. Expression of *IPT* results in rescue of the lower floret pistil in pistillate florets and the production of double embryo kernels.
(a) Longitudinal section of the upper floret of an *SAG12–IPT* spikelet stained with Toluidine Blue O showing the single ovule (ov) with embryo sac (es) present in the pistil (p).
(b) High magnification of the embryo sac in the ov of the upper floret (uf) showing a single es with antipodal cells (an) and egg cell and synergids (eg).
(c) Cross-section of an *SAG12–IPT* spikelet pair stained with Toluidine Blue O showing survival of the p in the lower floret (lf) of the left spikelet and the arrest of the p in the uf of the right spikelet. The vasculature (va) within the spikelet pair is indicated by the dark-staining strands.
(d) Longitudinal section of an *SAG12–IPT* spikelet stained with Periodic acid Schiff's reagent (inverted image) showing starch accumulation in the ovule of the upper floret pistil (up) and lower floret pistil (lp). og, outer glume; ig, inner glume; ol, outer lemma; il, inner lemma; and pa, palea.
(e, f) Longitudinal section of *SAG12–IPT* spikelets stained with Toluidine Blue O showing the large up and small lp.
(g) Longitudinal section of a syngenic control spikelet stained with Toluidine Blue O showing the position of the aborted lp as indicated by the lemma (ol) and palea (pa) of the lf.
(h) Longitudinal section of a *pi1 pi2* spikelet stained with Toluidine Blue O showing the up and lp.
(i) High magnification of a longitudinal section of an *SAG12–IPT* lf stained with Periodic acid Schiff's reagent (inverted image) showing starch accumulation in the p of the rescued lf.
(j, k) Representative double embryo kernel from *SAG12–IPT* maize. Image of each side of kernel is shown. UE, upper embryo; and LE, lower embryo.
(l) Incomplete development of a kernel from the lf of an *SAG12–IPT* spikelet showing a fully developed upper kernel and an aborted, unfused lower kernel in which the branch point in the spikelet can be seen.
(m) Incomplete development of a kernel from the lf of an *SAG12–IPT* spikelet showing a fully developed upper kernel and aborted lower kernel, which had partially fused with the upper kernel, causing a split in the pericarp.
(n, o) Control maize kernel from the uf with a single embryo. Image of each side of kernel is shown.
(p) Double embryo *SAG12–IPT* kernel from a self pollination of an *SAG12–IPT* Hill (light yellow aleurone layer) and B73 (darker yellow aleurone layer) heterozygote, illustrating the fusion point (arrow) between the endosperm of each kernel. Side view of the kernel is shown.
(q, r) Longitudinal section of typical *SAG12–IPT* double embryo kernels showing the embryo from the uf and lf.
(s) Longitudinal section of control kernel with a single embryo.
(t) Longitudinal section of an *SAG12–IPT* double embryo kernel showing the junction of the two fused endosperms. Al, aleurone layer; and Pc, pericarp.
(u) *SAG12–IPT* double embryo kernel 5 days following germination. Both seedlings emerge simultaneously and grow into normal fertile plants.



et al., 2000; Mena *et al.*, 1996). However, comparison of ear florets from *SAG12–IPT* maize with those from *si1*, *zag1*, *td1*, *ifa1*, and *kn1* mutants reveals substantial and significant differences. Although multiple silks are present in *si1*, *zag1*, and *kn1* mutant ear florets, they arise from the development of extra carpels, that can fuse to enclose the ovule. In some cases, the extra carpels formed a partial extra pistil that lacked an ovule. The extra pistil in *zag1* and *kn1* was adjacent to and fused with the apparently normal pistil, which itself contained one or more ovules (Kerstetter *et al.*, 1997; Mena *et al.*, 1996). Protrusion of nucellar material from incompletely fused carpels was also observed with two or three silks associated with the pistil (Kerstetter *et al.*, 1997; Mena *et al.*, 1996). The additional carpels present in *si1* pistillate florets result from the homeotic transformation of stamens to carpels (Ambrose *et al.*, 2000). In each of these examples, the extra carpels or pistils develop within the same floret, (i.e. the upper floret) and were bounded by the same palea and lemma that enclosed the main pistil. In contrast, ear spikelets in *SAG12–IPT* maize show none of these abnormalities. Rather than multiple silks emerging from one floret, the two silks of *SAG12–IPT* spikelets emerged from separate florets. The *SAG12–IPT* spikelet contained just two florets, each of which was composed of a palea, a lemma, and one pistil. No more than one pistil was present within each floret and the pistils from the upper and lower florets were not fused prior to pollination. Consequently, only one silk was ever observed to emerge from each pistil, consistent with the observation that *SAG12–IPT* florets do not contain extra carpels. Only a single ovule was present per pistil and incomplete fusion of carpels or protrusion of nucellar material was not observed. Thus, whereas loss of ZAG1 or KN1 specifically affects floret determinacy, expression from the *SAG12–IPT* transgene does not alter floret determinacy.

Mutation of *indeterminate spikelet1* (*ids1*), *Tasselseed6* (*Ts6*), or *reverse germ orientation1* (*rgo1*) results in a loss of spikelet determinacy, producing additional florets on an elongating rachilla (Chuck *et al.*, 1998; Irish, 1997; McSteen *et al.*, 2000). Multiple silks emerge from *ids1* spikelets at ectopic positions but usually do not develop normally and are often unfused (Chuck *et al.*, 1998). *SAG12–IPT* ear spikelets differ from *ids1*, *Ts6*, and *rgo1* mutant

Figure 6. Development of the male inflorescence and staminate florets remains unaltered in *SAG12–IPT* maize.
Tassel from (a) *SAG12–IPT*, (b) syngenic, and (c) vector-only maize at anthesis. Main spike of staminate inflorescence from (d) *SAG12–IPT* and (e) syngenic tassels. Lateral branch from (f) *SAG12–IPT* and (g) syngenic tassels showing spikelet branching. Representative spikelet pair from (h) *SAG12–IPT* and (i) syngenic tassels showing spikelet determinacy. Representative floret from (j) *SAG12–IPT* and (k) syngenic tassels showing floret determinacy. og, outer glume; ig, inner glume; ol, outer lemma; il, inner lemma; pa, palea; st, stamen; and p, pistil. Representative staminate floret from (l) *SAG12–IPT* and (m) syngenic spikelets showing the lack of pistil development. The glumes, lemma, and palea were removed.

spikelets in that they remain determinate with only two florets per spikelet and the florets are present in the same relative positions as they are in *ts2* or *pi1 pi2* mutants where the lower floret is rescued from abortion.

The conclusion that the lower floret was rescued from abortion in *SAG12–IPT* spikelets was supported by the branch position and orientation of the rescued floret within the spikelet. The rescued floret subtended the upper floret, suggesting that its development had initiated prior to that of the upper floret. Moreover, its position with respect to the upper floret was identical to its position in control spikelets, in which the remnants of the lower floret, i.e. the palea and lemma, indicate the position of the lower floret. Thus, the branch position and orientation of the rescued floret within an *SAG12–IPT* ear spikelet is consistent with survival of the lower floret.

The development of the lower floret and its subsequent fusion with the upper floret in *SAG12–IPT* maize is similar to the connation observed in *Zea mays* var. *polysperma* (Blaringhem, 1920; Stratton, 1923) where, as in *ts2* or *pi1 pi2* mutants, the upper and lower florets developed fully and independently but the pistils from each fused, resulting in a two-seeded fruit in which the two kernels were fused back to back and enclosed under a common pericarp. As observed with *SAG12–IPT* maize, the degree of fusion between the two kernels varied from partial to complete connation. Thus, *Z. mays* var. *polysperma* demonstrates that pistil fusion between the two florets in a maize ear spikelet can occur. In this respect, the pistil fusion and connation observed in *SAG12–IPT* maize largely phenocopies *Z. mays* var. *polysperma*. However, there are significant differences in the resulting kernels. Whereas the fusion of the pistils in *SAG12–IPT* ear spikelets results in a double embryo kernel in which the embryo from the lower floret displaces an equivalent volume of endosperm from the otherwise normal-sized kernel of the upper floret, in the fused kernels of *Z. mays* var. *polysperma*, there was no reduction in endosperm but rather the connate seeds were simply back-to-back fusions of the two kernels produced from a single ear spikelet (Stratton, 1923). In addition, the position of the embryos within the double embryo kernels of *SAG12–IPT* maize differed from those in *Z. mays* var. *polysperma* in that those of the former were arranged adaxially and abaxially with respect to the cob (i.e. opposite each other on the two flat sides of the kernel) whereas those of the latter were arranged opposite each other but at the right and left edges of the kernel (Stratton, 1923). Moreover, three florets were occasionally seen in *Z. mays* var. *polysperma* (Stratton, 1923) whereas only two were observed in *SAG12–IPT* maize. The smaller size of the lower floret in *SAG12–IPT* maize may account for the differences in endosperm to embryo ratio and in the position of the embryos observed for *SAG12–IPT* maize and *Z. mays* var. *polysperma*.

Although smaller, pollination of the lower floret pistil from *SAG12–IPT* spikelets produced full-size embryos and some endosperm, suggesting that despite its small size, the ovule was fully functional. Thus, expression from the *SAG12–IPT* transgene rescued the lower floret pistil from abortion but did not fully promote its growth prior to pollination. This suggestion is consistent with the known regulatory activity of the *SAG12* promoter: initial entry into the senescence program induces *SAG12* promoter activity, but synthesis of cytokinin from the promoter reverses entry and represses further expression from the promoter (Gan and Amasino, 1995). This autoregulation was observed when the construct was introduced in heterologous species (Gan and Amasino, 1995; McCabe *et al.*, 2001). The autoregulated nature of the *SAG12–IPT* transgene also prevented the use of *in situ* localization to detect *IPT* expression. In addition to cytokinin, *SAG12* promoter activity is also repressed by auxin, which would likely be present during early ovule development, and by sugars, such as sucrose, glucose, and fructose (Noh and Amasino, 1999) that are used for the generation of starch grains in the ovule.

SAG12 was originally identified following its induction in senescing *Arabidopsis* leaves (Lohman *et al.*, 1994) and was specifically activated by developmentally controlled senescence pathways (Noh and Amasino, 1999). Expression from *SAG12* is undetectable in non-senescent leaves, but once induced during senescence, it becomes one of the most abundant mRNAs in the senescing leaf (Lohman *et al.*, 1994). The developmental regulation of *SAG12* promoter activity is conserved in non-related species such as tobacco, petunia, and lettuce (Chang *et al.*, 2003; Gan and Amasino, 1995; McCabe *et al.*, 2001). The observation that *SAG12* promoter activity is also induced in senescing leaves but not in young leaves of maize indicates that this promoter is developmentally regulated in a similar manner in this monocot species.

The induction of *SAG12* promoter activity that was observed during the early development of the ear inflorescence is inconsistent with a senescence-related developmental program. However, abortion of the lower floret in ear spikelets that includes the nuclear degeneration of subepidermal cells of the lower floret pistil (Calderon-Urrea and Dellaporta, 1999) demonstrates that cell death does occur during the early development of the ear inflorescence. Induction of *SAG12* promoter activity during floret development suggests a degree of similarity between this programmed cell death-related induction and the senescence-related induction observed in maize leaves.

GA has been implicated in maize pistil development, at least in tassel florets where the exogenous application of GA results in the generation of pistillate tassel florets (Hansen *et al.*, 1976; Nickerson, 1959). Growth under low light or cool temperatures also promotes pistil development in the tassel where a substantial increase in

GA is observed (Richey and Sprague, 1932; Rood *et al.*, 1980; Schaffner, 1930). Despite the effect of GA on pistil development in tassel florets, neither the exogenous application of GA nor growth under low light or cool conditions altered floret development in the ear (Hansen *et al.*, 1976), suggesting that the presence of GA may not be sufficient to prevent abortion of the lower floret pistil.

Cytokinin and GA have been shown recently to be required for maize anther and pollen development (Huang *et al.*, 2003). Our observation that expression of IPT in the developing ear inflorescence reversed the normal abortion of the lower floret pistil indicates that the developmental program resulting in the arrest and degeneration of the lower floret pistil can be prevented by cytokinin and suggests that, as in anther and pollen development, pistil development in the ear inflorescence may require cytokinin in addition to GA. These data suggest that cytokinin may regulate entry into programmed cell death and senescence. Only one hormone, ethylene, has been demonstrated to date to play a role in regulating entry into senescence and programmed cell death (Johnson and Ecker, 1998; Young and Gallie, 2000). However, whereas ethylene serves to promote entry into both programs, cytokinin would have the opposite effect.

Reversal of lower floret pistil abortion was the only phenotype observed in *SAG12–IPT* florets. In wild-type pistillate florets, stamen development is arrested following the initiation of stamen primordia. Arrest of stamen development was unaltered in *SAG12–IPT* transgenic pistillate florets. The lack of change in stamen development suggests either that no IPT was expressed during stamen arrest or that cytokinin is not involved in reversing its developmental arrest.

Expression from *SAG12–IPT* has been shown to delay leaf senescence in tobacco (Gan and Amasino, 1995), to delay flowering in lettuce (McCabe *et al.*, 2001), and to delay corolla senescence in petunia (Chang *et al.*, 2003). No delay in leaf senescence was observed in *SAG12–IPT* transgenic maize despite the observed expression of IPT in senescing leaves. Moreover, no alteration in the development of vegetative organs was observed in *SAG12–IPT* transgenic maize in contrast to other mutations that affect floret development, such as *kn1* loss-of-function mutants, in which extra portions of leaf fused with a vegetative leaf (Kerstetter *et al.*, 1997). Branching within the ear or tassel inflorescence was unaltered in *SAG12–IPT* transgenic maize in contrast to *kn1* mutants in which a reduction in the branching of the staminate and pistillate inflorescences was observed (Kerstetter *et al.*, 1997). Moreover, no alteration in tassel floret development was observed: staminate-bearing upper and lower florets lacking pistils developed normally. This suggests that the level of cytokinin produced in the pistils of tassel florets was not sufficient to prevent their abortion or was not involved in determining their fate.

How cytokinin may prevent pistil abortion in the lower florets of the ear inflorescence remains to be determined. The effect of cytokinin may involve inhibition of the action of pro-death genes, such as *ts* or *pi1 pi2* genes (Calderon-Urrea and Dellaporta, 1999; DeLong *et al.*, 1993; Huelsen and Gillis, 1929; Weatherwax, 1916). Alternatively, cytokinin may induce expression of pro-survival genes, such as *sk*, which would be predicted to prevent the action of a pro-death gene, such as *ts2* (Calderon-Urrea and Dellaporta, 1999; DeLong *et al.*, 1993). As GA treatment suppresses pistil abortion in tassel florets, it is also possible that *IPT* expression might function by increasing the level of GA in the pistil of the lower ear floret. Given our observations with kernel development in *SAG12–IPT* maize, the rescue of the lower floret and connation observed in *Z. mays* var. *polysperma* (Stratton, 1923) may also result from an increase in the production of cytokinin during floret development or altered expression of a cytokinin-signaling intermediate or a downstream effector that is responsible for inhibiting pistil cell death.

The observations presented here illustrate that one potential outcome of the development of both pistils in a maize spikelet is a single kernel with two embryos, suggesting that death of the lower floret pistil ensures the competitiveness of the progeny by preventing this outcome. This conclusion is supported by the connation observed in *Z. mays* var. *polysperma* (Stratton, 1923). Mutations in other genes controlling the survival of the lower floret pistil illustrate other possible outcomes. In *ts2* and *pi1 pi2* mutants, abortion of the lower floret pistil is suppressed, resulting in the full development of both pistils in each spikelet, producing independent, non-fused kernels following pollination (Huelsen and Gillis, 1929; Irish and Nelson, 1989; Weatherwax, 1916). The lack of kernel fusion in these mutants may be because of the fact that the pistil of the lower floret develops to nearly the same size as that of the upper floret (Weatherwax, 1916). However, the increase in grain number in *pi1 pi2* ears deforms the kernels such that they are slender and elongated (Weatherwax, 1916). Postgenital organ fusion occurs widely in plants, e.g. fusion of carpels during flower development (reviewed by Verbeke, 1992) as well as the fusion between two florets in *Z. mays* var. *polysperma* or two flowers in *Mitchella reprens*, which, in each case, results in a two-seeded fruit enclosed in a common pericarp (Stratton, 1923). In maize, fusion between florets may be inhibited by growth of the palea, lemma, and glumes, which normally enclose the pistils. The fused kernel phenotype in *SAG12–IPT* maize may result from the substantially smaller size and delayed development of the lower floret, which together with the close proximity of the pistil in the upper floret may make fusion more likely. As much of the protein and oil reserves present in maize grain are stored in the embryo, this fusion event to generate kernels composed of two embryos with reduced

endosperm content increases the contribution of these storage reserves, thus improving the value of this economically important food.

Experimental procedures

Generation of transgenic lines

Embryogenic callus from Hill (derived from A188 × B73) was used for transformation by particle bombardment (Gordon-Kamm *et al.*, 1990). The ubiquitin promoter-*bar* (bialaphos resistance) herbicide-resistance plasmid pAHC20 (Christensen and Quail, 1996) was co-delivered with the *SAG12–IPT* construct (Gan and Amasino, 1995) in order to select for transformants on bialaphos (De Block *et al.*, 1987). One hundred and sixty-eight plants were regenerated from 18 transformation events as described by Armstrong (1994). Transgene-containing plants were crossed to B73 and the progeny were selfed. Rescue of the lower floret was examined in T_3 kernels of self-pollinated T_2 plants.

PCR analysis of transgenic plants

DNA was isolated from a seedling leaf of each transgenic plant. Leaf samples were frozen in liquid nitrogen and ground to a fine powder. Six hundred microliters of extraction buffer (100 mM Tris (pH 8.0), 50 mM EDTA, 200 mM NaCl, 1% SDS, 10 μl ml^{-1} β-mercaptoethanol) was added immediately and the mixture vortexed thoroughly. Seven hundred microliters of phenol:chloroform (1 : 1) was added, and the sample was vortexed and centrifuged for 10 min at 12 000 ***g***. Nucleic acid was precipitated from 500 μl of the supernatant following the addition of 1/10 volume of 3 M sodium acetate and 1 volume of isopropanol. The nucleic acid was pelleted by centrifugation at 12 000 r.p.m., washed three times with 75% ethanol and re-suspended in 500 μl of H_2O.

The presence of the *IPT* transgene was determined by PCR using HotStarTaq (Qiagen, Valencia, CA, USA) in a reaction containing 200 μM of each dNTP (deoxynucleotide 5′-triphosphate), 3 mM $MgCl_2$, 0.25 μM forward primer (SAG12F2; CGTACGTATCCCTCTTGTCGTCTAATGA), 0.25 μM reverse primer (IPTR1; CGTTCCTTTCAGTTCTTCCACTGTTGGT), 0.25 μl of HotStarTaq and 1.5 μl of total nucleic acid as a template in a total reaction volume of 25 μl. Reaction conditions were as follows: 95°C for 15 min (1 cycle); 95°C for 1 min, 62°C for 1 min, 72°C for 2 min (35 cycles); and 72°C for 5 min (1 cycle). Samples were separated on a 1% agarose gel, and the products were visualized following staining with ethidium bromide.

Expression analysis

Immature ears, seedling leaves, and older leaves with visible signs of senescence were collected from control and T_2 plants. Immature ears were collected at 2 and 4 cm and dissected into 1-cm sections. *IPT* expression was detected in each ear segment and in leaves by RT-PCR using oligo-dT_{20} to prime cDNA synthesis (using the Omniscript RT) from poly(A) RNA isolated from total RNA treated with RNA-qualified (RQ1) DNase. One microliter of the reverse transcription reaction was used for PCR analysis using primers to *IPT*. RT-PCR samples were separated on a 1% agarose gel and transferred to Hybond-N membrane according to manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA). A PCR-generated *IPT* fragment was radiolabeled with dCTP (deoxycytidine 5′-triphosphate) using Prime-a-Gene labeling system (Promega, Madison, WI, USA) and used for hybridization with the membrane overnight at 68°C in 5× SSPE (150 mM NaCl, 10 mM $NaH_2PO_4{\cdot}H_2O$, 1 mM EDTA), 5× Denhardt's solution, and 1.5% SDS. Blots were washed five times at 68°C in 0.1× SSPE and 0.1% SDS and exposed to film for 2 h at 80°C with an intensifier screen. RT-PCR analysis of β-tubulin was performed as a control using reaction conditions the same as those for *IPT* except that 0.1 μl of the reverse transcription reaction was used, the cycles were reduced to 25, and the primers were ZmBTubF1 (ACCAGATCGGCGCCAAGTTCT) and ZmBTubR1 (CATCATGTTCTTGGCATCCCACA).

Microscopy

Tissue was fixed in FAA (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) at 4°C and dehydrated through a graded ethanol series to 100%. The samples were embedded in Paraplast or resin. Sections were stained with either toluidine blue O, periodic acid Schiff's reagent, or aniline blue black, and counter-stained with periodic acid Schiff's reagent, and images were collected using a compound microscope. Images of whole florets and kernels were collected using a dissecting microscope.

Tassel and ear samples were prepared for scanning electron microscopic (SEM) analysis by fixing the samples in 100 mM sodium cacodylate, 2% glutaraldehyde, pH 7.4, solution overnight, followed by post-fixation in 1% OsO_4 in 100 mM sodium cacodylate for 6 h, and dehydration by passage through an ethanol series. Samples were subjected to critical point drying, sputter-coated with gold/palladium, and viewed in a Philips XL30 Field Emission Electron Gun SEM with an accelerating voltage of 10 kV.

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