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RESEARCH ARTICLE

Competence of Immature Maize Embryos for Agrobacterium-Mediated Gene Transfer

Michael Schläppi[1] and Barbara Hohn

Friedrich Miescher-Institut, CH-4002 Basel, Switzerland

Agrobacterium-mediated transfer of viral sequences to plant cells (agroinfection) was applied to study the susceptibility of immature maize embryos to the pathogen. The shoot apical meristem of immature embryos 10 to 20 days after pollination from four different maize genotypes was investigated for competence for agroinfection. There was a direct correlation between different morphological stages of the unwounded immature embryos and their competence for agroinfection. Agroinfection frequency was highest in the embryogenic line A188. All developmental stages tested showed Agrobacterium virulence gene-inducing activity, whereas bacteriocidal substances were produced at stages of the immature embryos competent for agroinfection. The results suggested that Agrobacterium may require differentiated tissue in the maize shoot apical meristem before wounding for successful T-DNA transfer. This requirement for the young maize embryo has implications for the possible use of Agrobacterium for maize transformation.

INTRODUCTION

The soil bacterium *Agrobacterium tumefaciens* has developed a sophisticated mechanism for stably integrating part of its extrachromosomal DNA, the T-DNA, into the nuclear genome of receptive plants (for reviews, see Koukolíková-Nicola et al., 1987; Zambryski, 1988; Hooykaas, 1989). Upon T-DNA–specific gene expression, plant metabolic resources are used for the overproduction of growth hormones as well as amino acid derivatives, opines, which the inciting Agrobacterium can catabolize. The successful invasion of Agrobacterium leads to the formation of crown gall tumors. The host range of Agrobacterium, as judged by tumor formation, is limited to most dicotyledonous plants (DeCleene and DeLey, 1976), to a few members of monocotyledonous plants of the species asparagus, narcissus, gladiolus, and yam, and to woody tree species (McGranahan et al., 1988; Hohn et al., 1989; Loopstra et al., 1990). Maize has remained refractory to tumor formation, although the production of specific opines after Agrobacterium inoculation was reported for young maize seedlings (Graves and Goldman, 1986) as well as for certain rice cultivars (Raineri et al., 1990). Currently, the only clearcut evidence for T-DNA transfer from Agrobacterium to graminaceous monocots stems from agroinfection, a technique using Agrobacterium-mediated transfer of viral sequences to plant cells (Grimsley et al., 1991).

Agroinfection had been developed previously for dicotyledonous plants (Gardner and Knauf, 1986; Grimsley et al., 1986). The first graminaceous monocot to be agroinfected was maize (Grimsley et al., 1987). Dimers of the geminivirus maize streak virus (MSV) were cloned between the 25 bp border repeats derived from the Ti plasmid and were introduced into the Agrobacterium nopaline strain C58. Viral symptoms were obtained routinely 7 to 14 days after inoculation of 3-day-old maize seedlings, with frequencies as high as 98%, independent of the maize genotype used (Grimsley et al., 1988). In contrast, naked MSV DNA as well as viral particles inoculated onto or injected into maize were not infectious. Agroinfection depended strictly on functional border repeats delimiting the cloned viral sequences as *cis*-elements, and on functional virulence (*vir*) genes as *trans*-acting factors (Grimsley et al., 1987). Furthermore, agroinfection of maize was Agrobacterium strain specific (Boulton et al., 1989; Grimsley et al., 1991).

For 3-day-old maize seedlings, it was demonstrated that only the coleoptilar node was competent for agroinfection (Grimsley et al., 1988). The three important steps preceding T-DNA processing and delivery (Koukolíková-Nicola et al., 1987), chemotaxis, Agrobacterium attachment, and *vir* gene induction, do not seem to be limiting factors. Agrobacterium attaches efficiently to vascular bundles of cut mesocotyl and hypocotyl sections of young maize seedlings (Graves et al., 1988), whereas extracts of every maize tissue tested can induce virulence promoters of Agrobacterium (Grimsley et al., 1989). Chemotaxis can possibly be neglected because the bacteria are injected directly into wound sites. Thus, the requirement to inject agrobacterial cells into the coleoptilar

[1] To whom correspondence should be addressed at the Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, MD 21210.

node must reflect the dependence of T-DNA transfer (or a later step) on the activity of the meristematic tissue inside this node.

In this study, we investigated the competence of meristematic tissue of immature maize embryos for agroinfection with MSV. Varying ages of immature embryos, four different maize strains, and different in vitro manipulations prior to the application of the bacteria were compared. Between 10 and 20 days after pollination (DAP), immature embryos provide several distinct developmental stages of meristematic tissue that can be analyzed for the competence for agroinfection. At 10 DAP, the shoot apical meristem is undifferentiated, whereas at 20 DAP, two to three leaf initials have differentiated (Sheridan and Clark, 1987).

We present evidence that the undifferentiated meristematic tissue of very young immature maize embryos is not competent for agroinfection. Meristematic tissue becomes competent at developmental stages that correlate with the differentiation of the first one to two leaf initials. Factors possibly influencing the developmental regulation of competence are proposed, and implications for the possible integration of T-DNA into the maize genome are discussed.

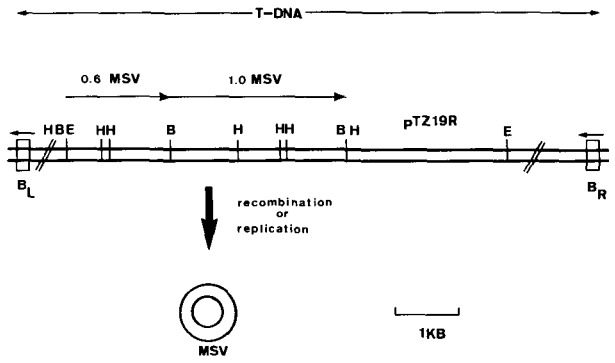

Figure 1. Schematic Representation of the 1.6-mer MSV Construct Used for Agroinfection.

After T-DNA transfer to maize cells, MSV excises by recombination or replication, replicates, and spreads from cell to cell. B_L, left border of T-DNA; B_R, Right border of T-DNA; H, HindIII; B, BamHI; E, EcoRI.

RESULTS

Germinated Mature Embryos Can Be Agroinfected In Vitro

Maize seedlings are competent for agroinfection 3 days after germination (Grimsley et al., 1987). To test whether viral symptoms would develop in in vitro conditions, mature embryos of the four different maize lines A188, bx/bx (a mutant that lacks 2,4-dihydroxy-7-methoxy-^{2}H-1,4-benzoxazin-3(^{4}H)-one [DIMBOA], a cyclic hydroxamate toxic for Agrobacterium [Sahi et al., 1990]), W23, and the McClintock line 880254A were excised from the kernels and germinated in vitro for 3 days. The coleoptilar node was inoculated with Agrobacterium C58 harboring MSV sequences on a binary vector (see Methods). In Figure 1, the MSV construct used is shown. Table 1 shows that mature embryos of the four maize lines were competent for agroinfection in in vitro conditions. We do not know whether the differences in agroinfection efficiencies of the tested lines reflect differences in susceptibility to Agrobacterium or the virus.

Immature Maize Embryos Are Differentially Susceptible to Agrobacterium

A systematic analysis of the competence of different developmental stages of immature embryos for Agrobacterium-mediated gene transfer was done. The excised immature embryos of each series were subjected to different in vitro manipulations prior to inoculation with the bacteria (see Methods). The first viral symptoms were observed 1 week after inoculation, on the first or second leaf of developing plantlets, with the subsequent appearance of more infected plantlets up to 3 weeks, in agreement with results published for seedlings inoculated 3 days after germination (Hohn et al., 1987; Grimsley et al., 1988).

Different series of immature embryos isolated between 10 and 22 DAP from the four described maize lines were tested for their competence for agroinfection. Interestingly, the whole series of bx/bx immature embryos obtained 14 DAP did not survive the application of Agrobacterium. The coleoptiles developed normally for 3 days but turned dark red. Only three of 120 immature embryos survived the treatment. The results suggest that the lack of bacteriotoxic substances like DIMBOA in this maize mutant allows the elicitation of a response that is lethal for the immature embryos inoculated with Agrobacterium. This response was dependent on the presence of Agrobacterium and was not seen when bx/bx control embryos from the same batch were mock inoculated

Table 1. Agroinfection Frequencies[a] of Seedlings and In Vitro-Germinated Mature Embryos

Maize Lines	In Vitro-Germinated Excised Mature Embryos, %	3-Day-Old Seedlings, %
A188	81	90
bx/bx	57	52
W23	33	53
880254A	53	30

[a] Frequencies of MSV symptom formation were determined 3 weeks after inoculation by calculating the fraction of shoots showing MSV symptoms per total surviving (germinating) shoots.

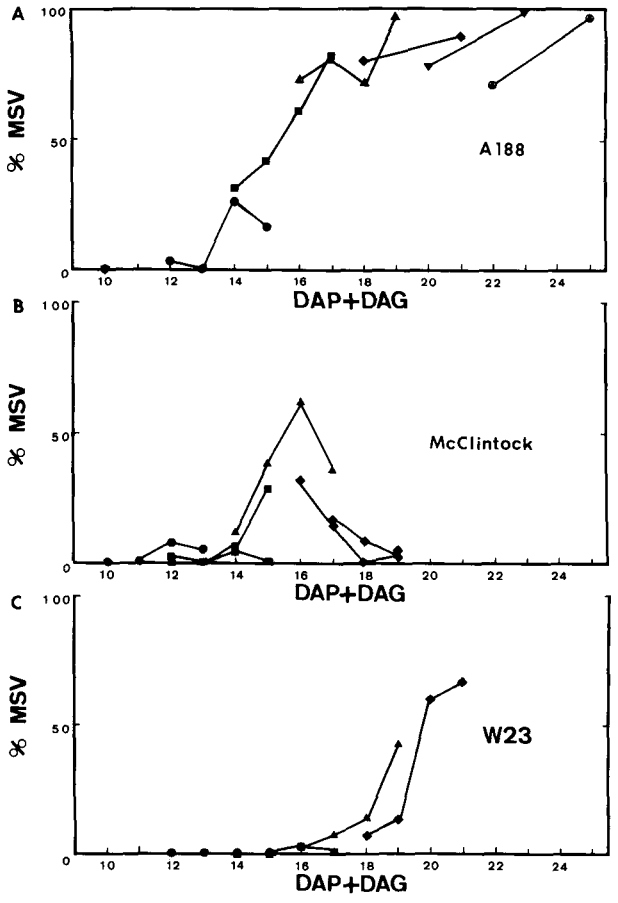

Figure 2. Agroinfection Frequencies (%MSV) of Immature Embryos.

Agroinfection frequencies are presented as a function of the initial age, given as DAP, and the in vitro germination stage of immature embryos, given as days after germination (DAG; see Methods). Germination series, starting at a given DAP, and ending 3 DAG are connected with solid lines.

(**A**) Embryogenic inbred line A188: ▼, 10 DAP (no germination series); ●, 12 DAP; ■, 14 DAP; ▲, 16 DAP; ♦, 18 DAP; ▼, 20 DAP; ◬, 22 DAP.

(**B**) McClintock line 880254A: ●, 10 DAP; ■, 12 DAP; ▲, 14 DAP; ♦, 16 DAP.

(**C**) Inbred line W23: ●, 12 DAP; ■, 14 DAP; ▲, 16 DAP; ♦, 18 DAP.

with 10 mM $MgSO_4$; eight of 10 control inoculated immature embryos survived. Even coleoptilar node inoculated seedlings were sensitive to Agrobacterium: only 35% of the bx/bx seedlings continued to germinate after inoculation, whereas 70 to 80% from the other genotypes did.

The results of agroinfection experiments with the other maize lines are shown in Figure 2. Per time point, 30 to 50 immature embryos were inoculated. The three lines differed in susceptibility to Agrobacterium. Immature embryos of the embryogenic line A188 (Green and Phillips, 1975; Figure 2A) were competent for agroinfection at an early developmental stage, 12 to 14 DAP, and the competence increased up to 3 days after germination in vitro or at later embryonic stages, respectively, reaching a plateau of 90 to 100% at ~18 to 22 DAP. The line W23 (Figure 2C) was less competent for agroinfection than A188, following a similar pattern of increased susceptibility to Agrobacterium upon prolonged in vitro germination and especially at later embryonic stages. Immature embryos older than 18 DAP were not tested. In contrast, the 880254A line analyzed (Figure 2B) exhibited a "window of competence" for agroinfection. Immature embryo stages up to 14 DAP followed almost precisely the increase in competence for agroinfection observed with the line A188, whereas from 16 DAP on, there was a dramatic decrease in competence. However, this competence was regained at the seedling stage (see Table 1).

Immature Embryos Induce Virulence Genes but Produce Bacteriocidal Substances

A simple explanation for the lack of competence of early developmental stages of immature embryos would be that they fail to induce the Agrobacterium *vir* genes. Due to the limited amount of available immature embryos, this hypothesis was tested qualitatively as follows. Immature embryos were wounded and cocultivated overnight in a medium containing X-Gal with an Agrobacterium strain containing *lacZ* inserted by transposon mutagenesis into a gene inducible by plant phenolic compounds (Grimsley et al., 1989). Immature embryos of all embryonic stages tested produced inducing activity (data not shown).

An alternative explanation for the lack of competence could be that early embryonic stages of immature embryos produced factors that inhibit the *vir* gene induction of Agrobacterium. To exclude this possibility, bacteria were preinduced in liquid media with the potent virulence inducer acetosyringone (Stachel et al., 1985). For seedling inoculations, it was shown that acetosyringone induction had no influence on agroinfection frequencies (Grimsley et al., 1989). Immature embryos also of the control line B73 that were incompetent 12 DAP did not become competent upon inoculation with acetosyringone-induced Agrobacterium. (None of the 50 surviving plantlets showed MSV symptoms.)

On the other hand, an interesting inverse correlation was observed between the competence of immature embryos for agroinfection and their ability to produce bacteriocidal substances. Upon wounding, 10- to 12-DAP immature embryos of all lines tested were not able to control the growth of applied bacteria on the agar solidified Murashige and Skoog (1962) medium (MS medium). Ten-DAP immature embryos were overgrown completely by bacteria after 2 days of cocultivation, whereas from 12 DAP on, the wounded immature embryos seemed to secrete substances that were toxic for Agrobacterium. Even removal of the immature embryos after 2 days and further incubation of the empty plates at 25°C for

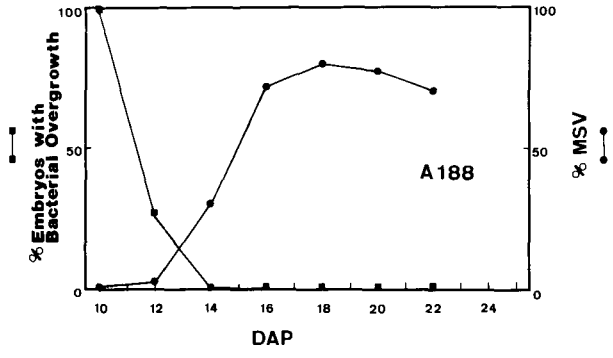

Figure 3. Inverse Correlation between Agroinfection Frequencies (%MSV) and the Ability of Agrobacterium to Survive the Cocultivation Period on the Agar with Wounded Immature Embryos of the Inbred Line A188.

The percent of embryos with bacterial overgrowth was calculated as the number of inoculated immature embryos revealing bacterial growth on the agar around the wounded immature embryos per total inoculated immature embryos.

up to 4 weeks did not reveal growth of Agrobacterium. Replica plating of the empty MS plates after 2 days' cocultivation onto fresh agar solidified Luria broth (LB) plates did not rescue any bacterial colony. The immature embryos most competent for agroinfection produced the highest amount of substances lethal for the applied bacteria. Figure 3 shows the very qualitative results obtained for A188 immature embryos. For instance, immature embryos exhibited 14 DAP an agroinfection frequency of 30% that did not allow the growth on the agar surface of any of the about 10^6 bacteria applied to each immature embryo (see Methods), whereas 100% of the immature embryos obtained 10 DAP allowed the bacteria to survive on the agar around the inoculated tissue. Interestingly, these bacteriotoxic substances, at least in part, seem unrelated to DIMBOA because bx/bx immature embryos wounded 14 DAP had the same effect on bacteria as A188 embryos of the same age (data not shown).

Competence for Agroinfection Is Correlated with Differentiation of the First Leaf Initials

Agroinfection frequencies of in vitro–germinated immature embryos coincided with the agroinfection frequencies of immature embryos that developed on the cob for the same amount of time (see Figure 2). Histological studies revealed that the earliest stages of competence for agroinfection coincided with the development of the first leaf initials, no matter whether the development occurred in vivo on the cob or during germination in vitro. (The in vitro–germinated material had slightly elongated coleoptiles, as compared to the in situ developing embryos.) The shoot apex of A188 (and 880254A) immature embryos, for instance, which started to develop the first leaf primordium 10 DAP, as seen in Figures 4A and 5A, exhibited 0% agroinfection frequency. Two days later, the leaf primordium was developing into a leaf initial, as seen in Figures 4B and 5B. Immature embryos inoculated at this stage showed only 0 to 3% agroinfection frequency. If allowed to germinate 2 days in vitro, however, one leaf initial and a second leaf primordium developed and the agroinfection frequency increased to 7% (880254A) or 28% (A188), respectively. The same is true for immature embryos that were allowed to develop for 14 DAP on the cob, as seen in Figure 5C (agroinfection frequency: 12% [880254A] or 32% [A188]). At 16 DAP, two leaf initials were formed, the coleoptile had fully covered the differentiating shoot meristem, as seen in Figure 5D, and the agroinfection frequencies increased to 32% (880254A) or 73% (A188), respectively. Interestingly, initiation of the first leaf primordium in the W23 line began 2 to 3 days later than in the A188 or 880254A lines, and increase in agroinfection frequency again coincided with this development.

The initial length of immature embryos obtained from the three maize lines, shown in Table 2, was different when measured at the same DAP. Immature embryos of line 880254A were the largest at all time points, and immature embryos of the W23 line the smallest. There was no correlation, however, between the size of immature embryos and their competence for agroinfection. A188 immature embryos measuring 2.6 mm in length 14 DAP exhibited 32% agroinfection frequency, whereas W23 embryos measuring 5.5 mm 18 DAP showed only 8% (see Figure 2).

DISCUSSION

Competence of Immature Embryos for Agroinfection Is Developmentally Regulated

The competence of the shoot apical meristem of immature embryos of different developmental stages and from different maize lines for Agrobacterium-mediated gene transfer was

Table 2. Length[a] of Immature Embryos of Different Maize Lines at Different Ages

	Age in DAP					
Maize Lines	10	12	14	16	18	20
A188	1.0[b]	1.9	2.6	4.1	6.0	6.5
880254A	1.5	2.0	3.4	4.6	ND[c]	ND
W23	ND	1.5	2.2	3.65	5.5	ND

[a] Lengths are given in millimeters.
[b] An average of 5 to 10 immature embryos per time point was measured.
[c] ND, not determined.

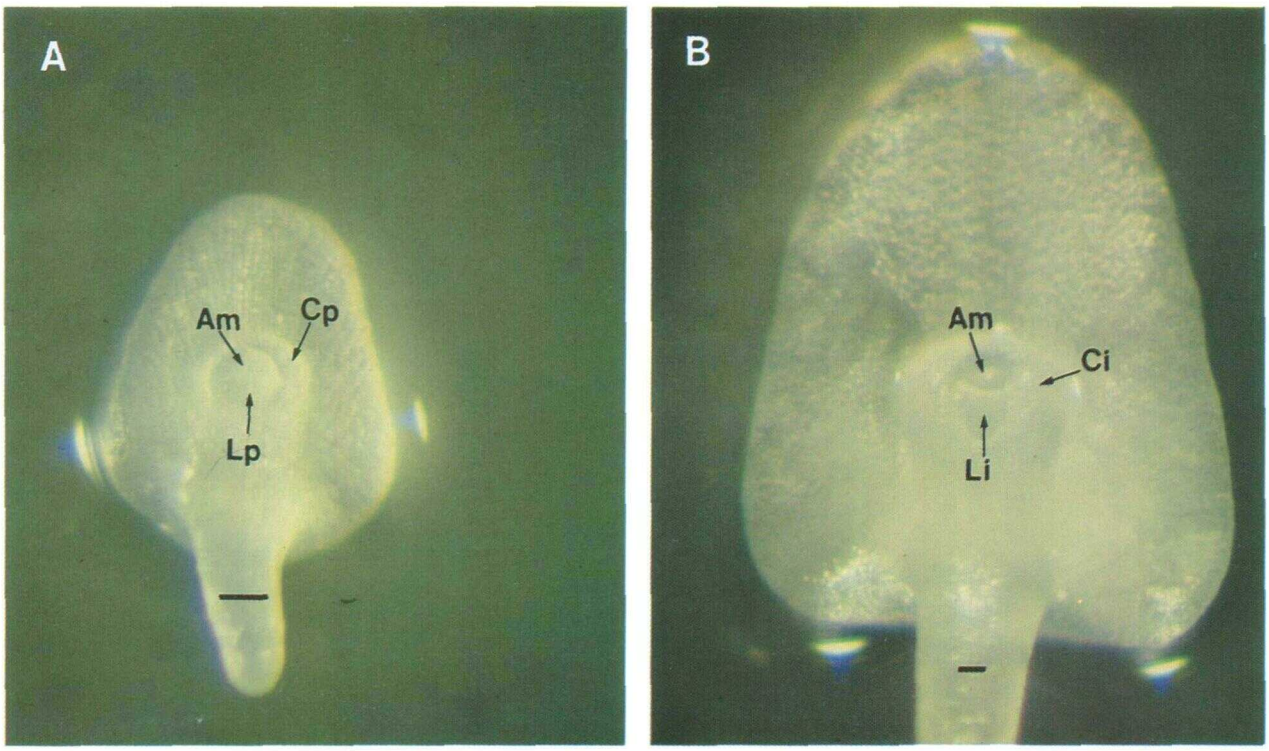

Figure 4. Immature Embryos Derived from the Embryogenic Line A188.

(**A**) Ten DAP.
(**B**) Twelve DAP.
Am, shoot apical meristem; Lp, first leaf primordium; Li, first leaf initial; Cp, coleoptile primordium; Ci, coleoptile initial. Bars = 100 μm.

investigated by means of agroinfection. We found a developmental regulation of competence. Early embryonic stages without differentiation of the shoot apical meristem were not competent for agroinfection. The beginning of competence was observed at later embryonic stages correlating with differentiation activity of the unwounded apical meristem. We also report that the observed competence of immature embryos for agroinfection differed dramatically between three different maize lines. Three different patterns were observed: a highly competent one (maize line A188), a weakly competent one (W23), and a differentially competent one (880254A), showing a "window of competence" (see Figure 2). Because the growth conditions were essentially the same, maize genotype–specific factors are proposed to be responsible for the observed differences in competence. Such factors may include (1) the availability of *vir* gene–inducing substances, (2) the production of bacteriotoxic substances, (3) endogenous hormone levels at the time of immature embryo excision, (4) induction of genes leading to differentiation, (5) embryo size, and (6) the availability of receptors for a productive attachment of Agrobacterium to the surface of meristematic cells.

Possible Factors That Influence Agrobacterium-Maize Interactions

Our working hypothesis was that the agroinfection approach for determining the earliest developmental stage of the apical meristem of immature embryos competent for Agrobacterium-mediated gene transfer correctly reflects the interaction of the bacterium with maize cells. This notion is based on reports that MSV replicates in dividing meristematic cells (Davies et al., 1987). Therefore, the incompetence of early embryonic stages cannot be explained with the requirement of differentiated cells for MSV replication. Viral particles, however, were found to be mainly phloem associated (Goodmann, 1981), thus revealing typical viral symptoms only when the first leaf had emerged from the coleoptile (i.e., the first symptoms appeared not before 1 week after germination of inoculated immature embryos).

The susceptibility of nongerminated immature embryos to Agrobacterium seemed to peak (line 880254A) or reach its maximum levels (line A188) at 14 to 16 DAP (see Figure 2). For line W23, this age correlated with the beginning of competence for agroinfection. Based on a qualitative assess-

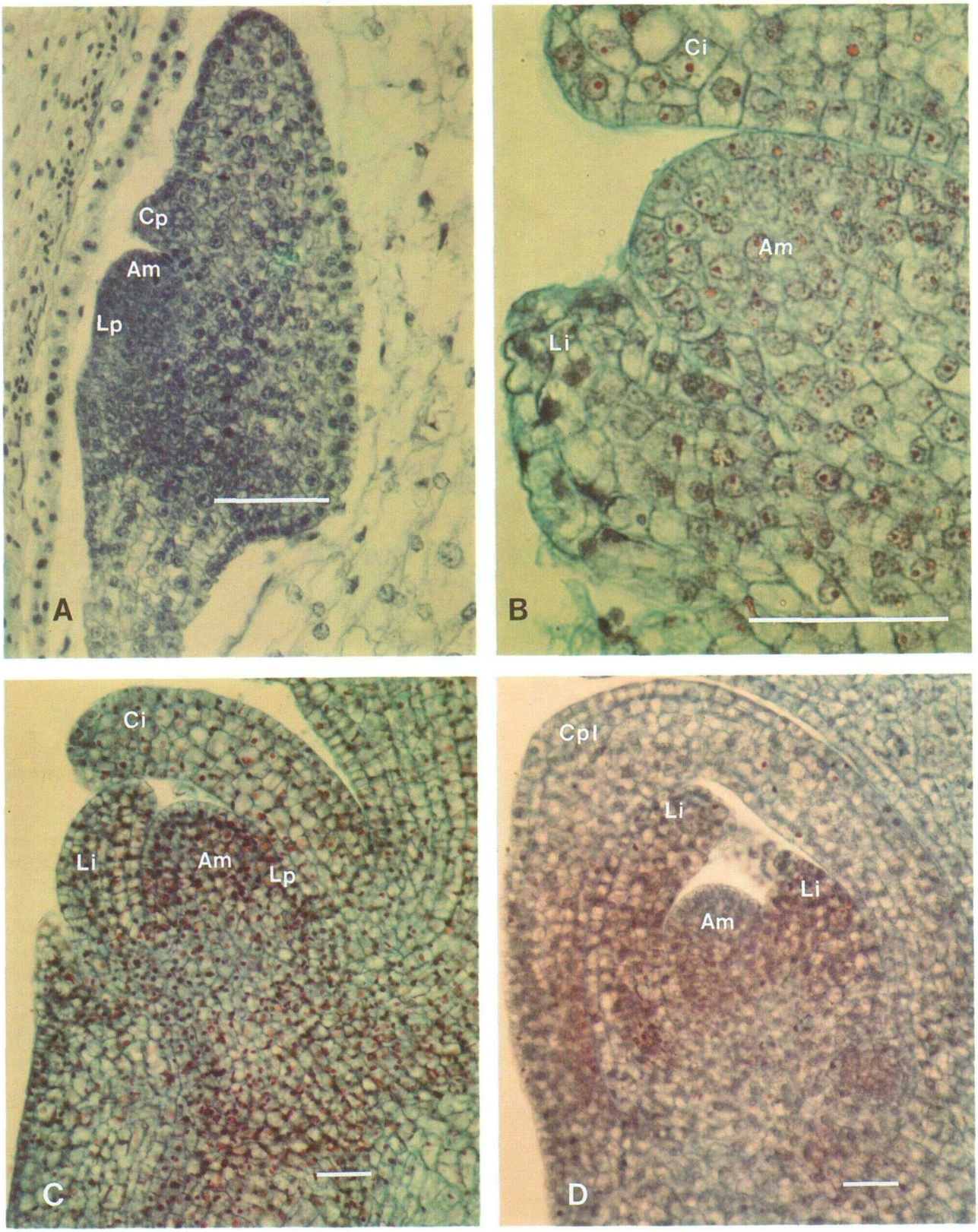

Figure 5. Histological Analysis of Immature Embryos of A188.

ment, there seemed to be no limitation in the availability of Agrobacterium *vir* gene-inducing substances even in immature embryos of earlier developmental stages (10 to 12 DAP) that were not competent for agroinfection. We cannot fully exclude the possibility, however, that small differences in the quantity of available inducer might account for the complete incompetence of young immature embryos. Furthermore, agroinfection-competent immature embryos produced bacteriotoxic substances not detectable yet in agroinfection–incompetent ones (see Figure 3). The lack of those substances might have exaggerated the *vir* gene induction results for agroinfection-incompetent immature embryos because older immature embryos might have killed, after being wounded, many agrobacteria before the *lacZ* gene product was made. The fact that induction of agrobacteria that were associated with older immature embryos could be measured at all in the presence of bacteriocidal substances may be attributed to a dilution effect in the liquid assay. In addition, induction and killing might follow different kinetics, thus possibly allowing the accumulation of a high enough amount of dye before bacterial cell death. There might not be a direct correlation, however, between the presence or absence of these bacteriotoxic substances and the competence of immature embryos for agroinfection because no data were obtained on the survival of bacteria within the wounded tissue. On the other hand, we cannot rule out that bacterial overgrowth actually prevented T-DNA transfer to very young immature embryos or that it killed or otherwise affected the cells already transformed, although we consider this possibility unlikely.

Immature embryos of three out of four maize genotypes tested survived the application of Agrobacterium into the shoot apical meristem. Only the DIMBOA-deficient bx/bx mutant exhibited a lethal response for the plant after application of the bacteria. A direct correlation, however, between the lack of bacteriotoxic DIMBOA (Sahi et al., 1990) and the fatal response cannot be made because no bx/bx line otherwise isogenic to the bx/bx one tested was available for comparison.

It has been reported that different maize lines contained significantly different hormone levels during embryo development (Carnes and Wright, 1988). Low levels of auxin and cytokinin in the line A188 were correlated with its potential to form embryogenic callus in vitro. Interestingly, this line was also the most competent one for agroinfection. Different endogenous hormone levels may have an influence on the timing of differentiation during embryo development. The competence of nongerminated immature embryos peaked (line 880254A) or reached its maximum level (A188) at 14 to 16 DAP. For W23 (the development of which was delayed with respect to the other two lines), this age correlated with the beginning of competence for agroinfection. Many genetic switches take place at this developmental stage in the embryo and in the kernel. Zein genes, for instance, start to be expressed, and starch grains are being initiated within the endosperm (see Lending and Larkins, 1989, and references therein). The endosperm starts to differentiate the outermost cell layer into the aleurone, and anthocyanin pigments are being deposited in it (Racchi and Manzocchi, 1988). Levy and Walbot (1990) were able to correlate the time of highest induction of transposition events with the onset of aleurone differentiation 14 to 16 DAP, independent of the transposon family investigated.

No direct correlation between immature embryo length at a particular age and the competence for agroinfection could be made (see Figure 2 and Table 2). A direct correlation was evident, however, between different morphological stages of immature embryos and the competence for agroinfection. Immature embryos with an undifferentiated shoot apical meristem were not competent, whereas differentiation of the first leaf initials coincided with the development of competence. This discovery is interesting because it has been reported that Agrobacterium binds specifically to vascular bundles of maize when applied to cut mesocotyl sections (Graves et al., 1988). Immature vascular bundles have been detected at the earliest in immature embryos in the first leaf initial (Sass, 1977). They thus might be a prerequisite for a productive interaction with Agrobacterium. Furthermore, they might transport viral DNA or particles to the meristem where MSV is thought to replicate and to systemically infect newly formed leaves (Davies et al., 1987).

The successful T-DNA transfer into the shoot apical meristem of maize might require a specific plant cell receptor. Cells of the undifferentiated apical meristem may not provide enough receptors for a productive attachment of Agrobacterium. The existence of a specific receptor in the cell wall of competent cells, possibly a glycoprotein, has been proposed (Neff and Binns, 1985; Gurlitz, 1987). Although the nature of such a receptor is not known yet, we suggest that its production is induced at a specific developmental stage of the differentiating shoot apical meristem of maize immature embryos. Interestingly, a developmental regulation of specific cell wall receptors for Agrobacterium has been suggested for germinating pinto bean, a dicotyledonous plant competent for tumor formation (Lippincott and Lippincott, 1978). In this light, the "window of competence" observed for line 880254A may be explained by assuming that at later developmental stages a competitive inhibitor for productive attachment

Figure 5. (continued)

(A) Longitudinal section through an immature embryo stained with hematoxylin, 10 DAP.

(B) Longitudinal section through the shoot apical meristem of an immature embryo stained with safranin/fast-green, 12 DAP.

(C) Longitudinal section through an immature embryo, stained with safranin/fast-green, 14 DAP.

(D) Longitudinal section through an immature embryo, stained with safranin/fast-green, 16 DAP.

Am, shoot apical meristem; Lp, leaf primordium; Li, leaf initial; Cp, coleoptile primordium; Ci, coleoptile initial; Cpl, coleoptile. Bars = 100 μm.

might be produced, leading to the observed dramatic decrease in competence (see Figure 2). However, this explanation would imply a different spatial and temporal regulation of factors for productive attachment during seedling germination because the competence for agroinfection was regained for 3-day-old seedlings (see Table 1).

Implications for the Possible Use of T-DNA Integration into the Maize Genome

We have demonstrated that immature embryos of maize are competent for Agrobacterium-mediated gene transfer and that competence depends on genotype and developmental stage. We have not looked for possible T-DNA integration because initial transformation events are difficult to detect by means of agroinfection. Although oncogenic C58 Agrobacterium was used as the helper strain for T-DNA transfer, we did not observe any differences between Agrobacterium and mock inoculated controls such as "tumorigenic callus" described for inoculated rice (Raineri et al., 1990).

It is well known that the wound response of most monocots is very different from that of most dicots. Wounded dicot cells respond with the production of wound callus; this cell proliferation event might be necessary for T-DNA integration and oncogene expression (Binns and Thomashow, 1988). Most wounded monocot cells, on the other hand, do not divide but differentiate into lignified or sclerified cells, thus producing a ring of hardened cells around the initial wound site. If T-DNA integrated into such terminally differentiated cells, it would be difficult to determine the initial transformation events in the cells of immature embryos. The inoculation procedure might disturb the differentiation program of the apex, however, and a new cell lineage, with possibly integrated T-DNA, might originate from such an event.

Determination of the competent stage of immature embryos for Agrobacterium-mediated gene transfer by means of agroinfection can now be followed by the analysis of nonviral marker genes such as β-glucuronidase. Such studies would allow the monitoring of initial transformation events because transformed sectors on leaves and on the stem of developing plantlets give information about the apparent number and type of cells that were transformed (McDaniel and Poethig, 1988). This research might allow the determination of the types of maize apical cells that are competent for stable transformation with Agrobacterium for a possible germ line propagation.

METHODS

Bacterial Strains, Plasmids, and Plasmid Constructs

Escherichia coli DH1 and JM109 were used for maize streak virus (MSV)-containing plasmid transformations. Other plasmids were maintained in the following *E. coli* strains: the binary vector pCIB200 (Rothstein et al., 1987) in JM109 and the broad host range plasmid pRK2073 as a helper strain for bacterial conjugation in HB101. *E. coli* was grown at 37°C in Luria broth (LB) medium (Miller, 1972) or on plates containing LB solidified with 1.5% Difco agar. Agroinfection was done with the nopaline C58 wild-type strain of *Agrobacterium tumefaciens*. *A. tumefaciens* was grown at 28°C in YEB medium (Grimsley et al., 1986) or on plates containing YEB solidified with 1.5% Difco agar.

The 1.6-mer partial dimer of MSV is described in Hohn et al. (1989). Handling of Agrobacterium containing MSV sequences was done in BL3 containment.

Maize Strains

The inbred line A188 (Green and Phillips, 1975) was obtained from T. Hein (Friedrich Miescher-Institut, Basel, Switzerland) and was maintained for several generations by selfing in the greenhouse. Inbred bx/bx, a mutant deficient in cyclic hydroxamates including DIMBOA (Coe et al., 1988; Sahi et al., 1990), was obtained by P. King (Friedrich Miescher-Institut). Inbred W23 (*b*, *r-g* background) was obtained from V. Chandler (University of Oregon, Eugene [Chandler et al., 1989]). The McClintock line 880254A (*su1*, *b*, *R-r* background) was provided by B. Burr (Brookhaven National Laboratory, Upton, NY).

Immature Embryo Production, Isolation, and Cultivation

All maize seeds were surface sterilized in 1.4% sodium hypochlorite for 20 min and washed three times in sterile water. Aseptic seeds were germinated on wet filter paper in Petri dishes at 28°C for 3 days in the dark. Seedlings were propagated subsequently in small pots in a plant growth chamber under 16-hr light, 25°C, and 8-hr dark, 20°C, 20,000 lux light regime with 50% humidity. After 2 to 3 weeks, well-grown seedlings were transferred into 20-liter pots containing slow-release fertilizer and propagated under the same light/dark regime. Alternatively, seedlings were transferred into a greenhouse field and propagated under similar light conditions. After 2 to 2.5 months, emerging ears were bagged and the husks cut back as soon as the first silks appeared. One day later, newly emerging silks were pollinated with fresh pollen of either the same (selfed) or another plant of the same genotype (sibbed). Pollinated ears were protected with "Lawson" bags obtained from Funk Seeds (Bloomington, IL). Immature ears were harvested at the desired DAP, and immature kernels were removed, surface sterilized for 20 min in 1.4% sodium hypochlorite, and washed three times in sterile water.

Immature embryos were excised aseptically in a laminar flow bench using sterilized forceps and scalpels. Embryos smaller than 1 mm in length were removed with the help of a stereomicroscope. Excised embryos were placed with the scutellar side down onto 1% agar solidified MS medium (Murashige and Skoog, 1962) containing 3% sucrose and 1 mg/liter thiamine-HCl. Per plate, 30 to 50 immature embryos were either inoculated immediately after isolation or germinated up to 3 days in the dark at 25°C for 16 hr, followed by 8 hr at 20°C.

Immature embryos derived from one cob were always distributed onto four MS plates. One plate was inoculated immediately after embryo isolation, the others were inoculated 1, 2, and 3 days after germination, respectively. Excised mature embryos from sterilized seeds for control inoculations were treated in the same way.

Inoculation of Immature Embryos

The apical meristem or the shoot apex of immature embryos as small as 1 mm was punctured with a Microlance 26G3/8 0.45 × 10 fine needle, and the smaller embryos were punctured with a drawn out glass microcapillary. Immediately after puncturing, 2 to 4 μL of an overnight Agrobacterium culture containing the MSV construct (Figure 1) was applied. Bacteria were obtained with a titer of 10^9 cells per milliliter, washed, and resuspended in 10 mM $MgSO_4$ to the same concentration. Successive subcultivations were done up to 3 days for each germination series. After every subcultivation step, the presence of correct pLE1 sequences was tested by restriction analysis after plasmid isolation by an alkaline lysis procedure (Sambrook et al., 1989). Inoculated immature embryos were incubated with the apical side on the MS medium for 24 hr in the dark, then flipped over and incubated for another day on the same medium under 16-hr light, 10,000 lux, 25°C followed by an 8-hr dark, 20°C regime. Embryos were then transferred onto 0.8% agar solidified MS medium containing 3% sucrose, 1 mg/liter thiamine-HCl, 500 μg/mL cefotaxim (Hoechst, Frankfurt, Germany), and 500 μg/mL carbenicillin against Agrobacterium growth. After 1 week, immature plantlets were transferred into Magenta boxes containing MS medium solidified with 0.8% agar and containing 2% sucrose and 1 mg/liter thiamine-HCL. Twelve DAP immature embryos from the line B73 (Funk seeds) were inoculated with Agrobacterium preinduced with acetosyringone, as described previously (Stachel et al., 1985).

Scoring for MSV Symptoms: *vir* Gene Induction Assays

Immature shoots were scored 2 to 4 weeks after inoculation for MSV symptom formation. With the exception of the mutant bx/bx (see Results), between 45 and 95% of the inoculated immature embryos, depending on age and genotype, survived the inoculation procedure. Within a germination series, ungerminated immature embryos had higher survival rates than germinated embryos. Control inoculations with 10 mM $MgSO_4$ gave similar survival results. Therefore, the frequency of symptom formation was calculated as the fraction of shoots with MSV symptoms per total surviving shoots.

Induction of an Agrobacterium *vir* gene by immature embryos was measured qualitatively, as described by Grimsley et al. (1989). An Agrobacterium strain with a *lacZ* insertion in the *pinF* ("plant inducible") locus of the Ti-plasmid virulence region was grown in YEB medium supplemented with 100 μg/mL carbenicillin overnight at 28°C, subcultured into 1XM9 medium (Miller, 1972) supplemented with 100 μg/mL carbenicillin overnight at 28°C, then diluted to OD_{600} = 0.1 in 1XM9 medium. One microliter 2% 5-bromo-4-chloro-3-indole-galactose (X-Gal; Sigma) in dimethyl-formamide was added to 500 μL of bacterium suspension, and cocultivation with wounded immature embryos was done overnight at 28°C with 100 μL in a microtiter dish. Acetosyringone (Sigma) in a final concentration of 100 μM, added to the bacterium suspension, was used as a positive control. Blue spots on immature embryos and blue medium were scored as inducing the *pinF* gene.

Histological Analysis of Immature Embryos

Embedding, thin sectioning, and staining with hematoxylin or safranin/fast-green was done using standard procedures (Grimsley et al., 1988; M. Leupin and G. Neuhaus, unpublished data, Federal Institute of Technology, Zürich, Switzerland).

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