Female Reproductive Tissues Are the Primary Target of *Agrobacterium*-Mediated Transformation by the Arabidopsis Floral-Dip Method[1]

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The floral-dip method for *Agrobacterium*-mediated transformation of Arabidopsis allows efficient plant transformation without need for tissue culture. To facilitate use with other plant species, we investigated the mechanisms that underlie this method. In manual outcrossing experiments, application of *Agrobacterium tumefaciens* to pollen donor plants did not produce any transformed progeny, whereas application of *Agrobacterium* to pollen recipient plants yielded transformants at a rate of 0.48%. *Agrobacterium* strains with T-DNA carrying *gusA* (encoding β-glucuronidase [GUS]) under the control of 35S, LAT52, or ACT11 promoters revealed delivery of GUS activity to developing ovules, whereas no GUS staining of pollen or pollen tubes was observed. Transformants derived from the same seed pod contained independent T-DNA integration events. In Arabidopsis flowers, the gynoecium develops as an open, vase-like structure that fuses to form closed locules roughly 3 d prior to anthesis. In correlation with this fact, we found that the timing of *Agrobacterium* infection was critical. Transformants were obtained and GUS staining of ovules and embryo sacs was observed only if the *Agrobacterium* were applied 5 d or more prior to anthesis. A 6-fold higher rate of transformation was obtained with a CRABS-CLAW mutant that maintains an open gynoecium. Our results suggest that ovules are the site of productive transformation in the floral-dip method, and further suggest that *Agrobacterium* must be delivered to the interior of the developing gynoecium prior to locule closure if efficient transformation is to be achieved.

Plant transformation is a key methodology that has fostered diverse forms of scientific inquiry and technology development (Birch, 1997; Gelvin, 1998; Hansen and Wright, 1999). However, for many plant species, the generation of lines carrying stable heritable transformation events remains a technical challenge. Transformation can be a labor-intensive process that is plagued by low success rates, excessive mutagenesis (somaclonal variation), and/or the need for highly skilled practitioners. Research with Arabidopsis has benefited from the development of high-throughput transformation methods that avoid plant tissue culture (Azpiroz-Leehan and Feldmann, 1997). In particular the development of the *Agrobacterium tumefaciens*-mediated vacuum infiltration method (Bechtold et al., 1993) has had a major impact on Arabidopsis research. Arabidopsis transformation can now be performed routinely in laboratories that have little or no expertise in plant tissue culture and transformation, allowing in planta analysis of multiple DNA constructs (Azpiroz-Leehan and Feldmann, 1997; Clough and Bent, 1998). A number of academic and industrial laboratories have carried out large-scale transformation projects, generating thousands of independent transformed Arabidopsis lines from which T-DNA tagged mutants can be identified (e.g. Mollier et al., 1995; Azpiroz-Leehan and Feldmann, 1997; Hirsch et al., 1998; Richardson et al., 1998). Large-scale tagged populations can even be used for reverse-genetic identification of plant lines that carry a mutation in a gene of known DNA sequence (Krysan et al., 1996; http://www.biotech.wisc.edu/Arabidopsis/).

Germ-line transformation is the common feature that allows avoidance of tissue culture and regeneration in the vacuum infiltration, seed transformation, in planta transformation, and floral-dip methods (Feldmann and Marks, 1987; Feldmann, 1992; Bechtold et al., 1993; Chang et al., 1994; Katavic et al., 1994; Clough and Bent, 1998). With all of these methods, selection with antibiotic or herbicide is not applied to the *Agrobacterium*-treated T_0 plant. Instead, progeny seed are harvested and selection is applied to the resultant seedlings as they germinate. Previous studies have shown that T_1 transformants are typically hemizygous, carrying T-DNA at only one of two alleles of a given locus (Feldmann, 1991; Bechtold et al., 1993). Transformation events occurring prior to or early in floral development of the T_0 plant would be expected to give rise to identically transformed male and female gametophytes, which upon self-fertilization could produce a significant number of T_1 plants that are homozygous for the T-DNA insertion. The rarity or total absence of such lines indicates that the relevant transformation events usually occur during germ-line development after divergence of male and female gametophyte cell lineages,

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or possibly soon after fertilization of the T_1 embryo. Separate transformants from a single plant carry independent T-DNA insertions even in methods that apply *Agrobacterium* to T_0 seed early in the growth of the plant, again suggesting that transformation occurs late in floral development (Bechtold et al., 1993; Azpiroz-Leehan and Feldmann, 1997).

Despite the above, the cell type that is transformed and the timing of transformation have remained unknown. The success of the floral-dip method, in which flowering plants are simply dipped into a solution of *Agrobacterium*, suggested that the cellular targets are present on the exterior of the plant. Transformants are often derived at high frequency (as high as 4% of all T_1 seed; Clough and Bent, 1998), again suggesting that the transformed germ-line plant cells are readily accessible to *Agrobacterium*.

Although the benefits of vacuum infiltration and/or floral-dip transformation methods are evident, efforts to apply these methods to other plant species have generally been unsuccessful. However, reports of success with *Brassica campestris* subsp. *Chinensis* and *Medicago truncatula* suggest that the method is not uniquely restricted to Arabidopsis (Liu et al., 1998; M. Harrison, personal communication). A mechanistic understanding of the successful Arabidopsis method should facilitate further improvement of Arabidopsis transformation and should foster application of similar transformation methods to other plant species. Accordingly, we investigated the site and timing of transformation in the *Agrobacterium* floral-dip method. Transformants were isolated following seed production from crosses between *Agrobacterium*-inoculated female (pollen recipient) and noninoculated male (pollen donor) plants, or vice versa. In addition, *Agrobacterium*-mediated delivery of *gusA* (β-glucuronidase)-reporter gene constructs was monitored in reproductive tissues. We also tested whether transformed plants originating from the same flower result from independent transformation events and examined the transformation rate for a plant line with altered floral morphology. Our group and two other research groups concurrently studied Arabidopsis transformation by independent approaches and now report similar overall conclusions (Ye et al., 1999; Bechtold et al., 2000; the present work). Our results indicate that female tissues such as developing ovules within the gynoecium of young flowers are the primary target of *Agrobacterium*-mediated floral-dip transformation of Arabidopsis.

RESULTS

Crosses between Plants Inoculated with *Agrobacterium* and Noninoculated Plants

As a first step toward identifying the primary sites of productive transformation in the floral-dip procedure, we sought to apply *Agrobacterium* to only the male or female germ line. Standard floral-dip transformation procedures were used for plant growth and inoculation (see "Materials and Methods"); plants were inoculated by dipping only (no vacuum infiltration). Candidate T_1 transformant seed lots were then generated by performing crosses in which only the male parent (pollen donor) or female parent (pollen recipient) had been inoculated. Individual siliques from these crosses were harvested separately and seeds were plated on selective media to identify transformed lines.

To accumulate a meaningful volume of data, we performed more than twelve separate sets of inoculation/crossing experiments that generated over 29,000 progeny seed from crosses (Fig. 1). Out of more than 14,300 seeds screened from 405 successful crosses in which the pollen donor plant was inoculated with *Agrobacterium*, we recovered zero transformants. In contrast, 71 transformants were recovered from 40 separate crosses out of a total of roughly 14,800 seeds from 498 successful crosses in which the pollen recipient plant had been treated with *Agrobacterium*. These results suggest that productive transformation events occur on female floral structures,

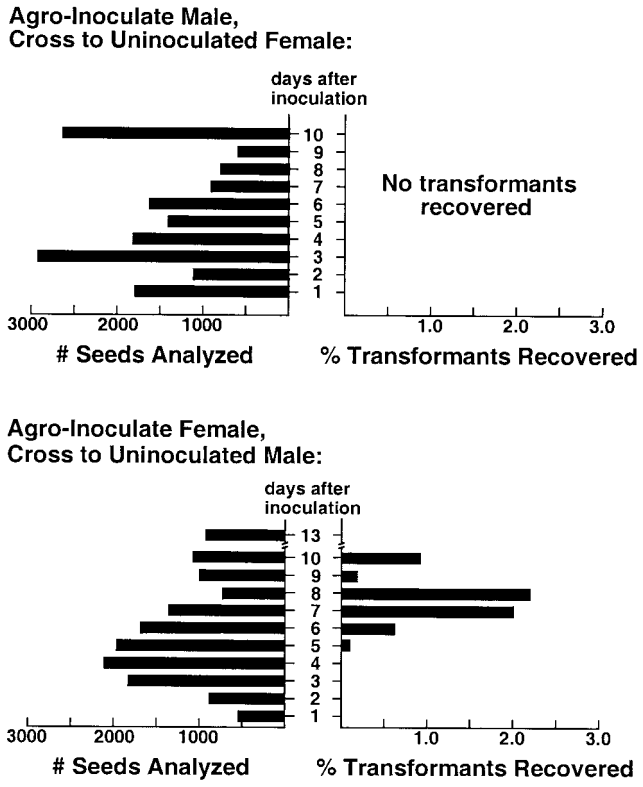

Figure 1. Transformation rate in flowers pollinated by crossing at different times after the pollen-donor or pollen-recipient was inoculated by *Agrobacterium* floral dip. Left histograms show number of F_1 seeds subjected to selection for transformants, right histograms show rate at which transformants were obtained from corresponding crosses. All crosses were performed 1 d prior to anthesis; *y* axis shows number of days between bacterial inoculation and performance of cross.

and that they do not occur during early stages of anther or pollen/microspore development prior to pollen release.

Arabidopsis plants form an indeterminate inflorescence that typically carries flowers of a wide range of developmental ages. In the experiment reported above and in Figure 1, crosses were made as individual flowers reached anthesis 1 to 13 d after plants had been dipped in *Agrobacterium*. This produced a data set in which the developmental stage at the time of *Agrobacterium* inoculation could be inferred for the parental flower of each transformant. Again, a striking discontinuity was observed in the data set (Fig. 1). Productive transformation events occurred in flowers that were developmentally young at the time of *Agrobacterium* treatment (6–11 d away from anthesis; equivalent to 5–10 d after inoculation in Fig. 1). Flowers that were 5, 4, 3, or 2 d away from anthesis at the time of inoculation failed to produce transformants, and very few transformants were obtained from flowers that had been inoculated 6 d prior to anthesis. Correlation of these results with the time line for development of Arabidopsis flowers is pursued in the "Discussion."

Although 21 of the crosses reported above produced a single transformant, 19 crosses produced more than one transformant, with up to seven transformants obtained from a single silique.

In the above crosses, different genetically marked plant lines were used as pollen donor and recipient. All putative transformants carried the genetic marker of the pollen donor, indicating successful crossing. In all cases examined by PCR or by hybridization of genomic DNA blots (reported below; Clough and Bent, 1998), we never observed a kanamycin-resistant putative transformant that did not carry a transgene insertion. In 87% of the crosses reported in Figure 1, the female parent was homozygous for the temperature-sensitive *ap3-1* allele. This *ap3-1* mutation causes partial male sterility and facilitates the performance of a large number of crosses by reducing the occurrence of anthers that must be removed prior to pollination. Use of the *ap3-1* mutation was unlikely to affect experimental outcomes given that crossing with other genotypes requires removal of anthers from pollen-recipient flowers. Equal rates of transformation were obtained in crosses to Landsburg *erecta* (Ler) *ap3-1* pollen-recipient plants (0.48%) and in crosses for which the pollen recipient was male-fertile and of the Columbia (Col) genetic background (0.47%). Mutant *ap3-1* plants were never used as the pollen donor.

As a positive control in the above crossing experiments, transformation rates were determined for seed derived from self-fertilization of the same *Agrobacterium*-inoculated plants that were used as pollen donor or pollen recipient. The overall transformation rate for seeds from self-fertilization was 1.0%, ranging from 0.06% to 2.6% among experiments. For the six experiments where both Ler-0 plants and Col background plants were included, we found that the average transformation rate for Col plants was 47 times higher than that of Ler-0 plants. The discrepancy between this result and the similar transformation rate of Col and Ler pollen recipients in crossing experiments (preceding paragraph) suggests that the low rate of Ler transformation may be due to self-pollination/fertilization deficiencies in *Agrobacterium*-inoculated Ler plants. Consistent with this hypothesis, we observed very poor pollen quality on *Agrobacterium*-inoculated Ler plants when attempting to use these plants for crossing experiments.

It also bears mention that high levels of Silwet L-77 (OSi Specialties, Danbury, CT; 0.05%–0.1%) caused notable flower mortality in some experiments (Clough and Bent, 1998). Most experiments utilized L-77 at 0.03%, but even at that level we occasionally noted deformities in the pistils of flowers used for crosses in the first few days after inoculation.

Delivery by *Agrobacterium* of *gusA* Reporter Gene Constructs

The success of transformation following mere dipping of flowers into *Agrobacterium* had suggested that the targets of transformation are exposed on the plant surface. The crossing experiments reported above and in Figure 1 indicated that productive transformation events occur on the pollen-recipient flower and not on developing anthers, but did not distinguish between transformation of female germ-line tissues as opposed to transformation of pollen after it germinates on the stigmatic surface. To explore further the target site(s) and the possible timing of the transformation, we sought to detect expression of an *Agrobacterium*-delivered *gusA* transgene as early as possible after transformation.

When plants were inoculated with *Agrobacterium* carrying a 35S-*gusA*/intron fusion in the T-DNA, no GUS staining was observed in flowers collected 4 to 14 d after inoculation. However, in control experiments using six independent lines stably transformed with a 35S-*gusA* construct, staining seldom occurred in the pistil (stigma, style, or ovary) and was never observed in the stamens (anther or filament). Staining in these control flowers was commonly observed in sepals, petals, and peduncle, and was occasionally observed in the side walls of the ovary. In one flower, we observed very faint blue staining in ovules but not in other parts of the ovary. These results confirmed and extended the work of others, indicating that the 35S promoter directs very little gene expression in stamen, pistil, or gametophyte tissues. Although the 35S-*gusA*/intron construct could not be used to detect transformation of pollen or pistil cells, this work indicated that transformation of other developing flower parts was rare following *Agrobacterium* floral dip.

LAT52-*gusA*

To monitor transformation events in reproductive tissues, experiments were performed that utilized LAT52-*gusA*, ACT11-*gusA*, and ACT11-*gusA*-intron promoter-reporter gene fusions. LAT52 is a promoter specifically expressed in pollen (Twell et al., 1990). It is also expressed briefly in embryos after fertilization, but it is not expressed in developing, unfertilized ovules. Following inoculation of plants with *Agrobacterium* carrying the LAT52-*gusA* fusion, we collected flowers 3 to 10 d after inoculation and analyzed 20 or more flowers per day. No GUS staining was observed in the pollen of *Agrobacterium*-inoculated plants. In contrast, staining of multiple pollen tubes was readily apparent in control plants stably transformed with the LAT52-*gusA* promoter (not shown). GUS activity was evident in a small number of developing embryos starting 5 d after dip-inoculation, suggesting that successful transformation events were occurring despite the absence of observable pollen transformation.

ACT11-*gusA* and ACT11-*gusA*-Intron

Parallel experiments utilized a *gusA* reporter gene driven by the ACT11 promoter (Huang et al., 1997). According to Huang et al. (1997), ACT11-*gusA* expression can be observed throughout floral development in the floral primordia, in the emerging floral buds, in ovules (with notably high expression in the young pistil in the final few days prior to anthesis), in pollen near the time of anthesis, and subsequently, with a gradual reduction of expression, in the carpel tissues. Huang et al. found that ACT11-*gusA* activity was detectable in ovules from the time of their initiation. Our observation of flowers of control T_2 plants stably transformed with the ACT11-*gusA* construct confirmed the strong activity in the pollen, but we found that the timing of GUS activity in the pistil and the ovules was more subtle. In transformants generated and grown in our laboratory, we found that GUS expression in the ovules was activated somewhat after their initiation, after activity was detectable in the pollen. In addition, in weakly expressing ACT11-*gusA* lines, GUS staining was not uniform in the whole ovule but, rather, was more prominent within the embryo sac. We also found that ACT11-*gusA* expression intensified in the embryo in the first few days after fertilization and then became stronger in the rest of the ovule (data not shown).

Use of the ACT11-*gusA* construct to track plant transformation events was confounded by the fact that this was not a *gusA*/intron construct. In particular, we observed strong GUS expression in pure cultures of *Agrobacterium* that carried the ACT11-*gusA* construct. Although this led to construction and use of a *gusA*/intron construct (below), the bacterial GUS expression from the ACT11-*gusA* construct with no intron was exploited to track the presence of *Agrobacterium* in floral tissues.

When previously non-transformed plants were treated by floral dip with *Agrobacterium* carrying the ACT11-*gusA* construct (no intron), limited staining was occasionally observed in very young flowers. In flowers that were 1 d away from anthesis (i.e. ready for crossing), we observed staining only 5 or more d after inoculation. Note that these positively stained flowers were still unpollinated. Examination of the more strongly stained flowers revealed that in some ovaries, or sometimes in just one locule of the ovary, GUS expression was very strong in the entire cavity (Fig. 2A). This result suggested an overwhelming presence of GUS-expressing bacteria. In other cases, GUS staining was limited to one or several spots in the ovary (Fig. 2, B and D), suggesting staining of transformed plant tissues. The possibility remains that this discrete and localized staining resulted from expression of ACT11-*gusA* (no intron) from within colonizing bacteria. However, this localized staining was only observed at sites for which plants cells are known to drive ACT11 expression, including entire ovules (and the funiculus in many cases), or at a location near the micropyle of the ovule, and not at other locations within the flower (Fig. 2D). Regardless of bacterial or plant source of expression, flowers of this type provided clear evidence that *Agrobacterium* was penetrating to the ovary of Arabidopsis flowers.

Another striking but perhaps not surprising observation was the discontinuity of the transformation rate between separate flowers. Overall, plant transformation rates by the floral-dip method are often roughly 1%, but most flowers remain completely non-transformed, whereas others are transformed at a high rate. In experiments with the ACT11-*gusA* construct or the ACT11-*gusA*-intron construct, most flowers were not stained and some were stained at a small number of ovules, but in some flowers, one-quarter or more of the ovules displayed GUS staining (Fig. 2, B and C). Note that, due to the possibility of *gusA* expression from either stable integration events or transiently delivered T-DNAs, the rate of GUS staining overestimates the rate of productive transformation events in these and all other GUS-staining experiments.

To monitor ACT11-*gusA* expression from plant cells and not from bacteria, the binary vector pCD1301-ACT11 was constructed in which the ACT11 promoter drives expression of a *gusA*-intron gene. Using this construct in our floral-dip inoculations of previously non-transformed plants, we detected floral GUS expression as isolated foci of blue staining within the ovaries (Fig. 2C). Although this staining was localized to developing ovules, the patterns of GUS staining were variable. In some cases, staining of entire ovules was observed, whereas in other cases, staining was very localized within the

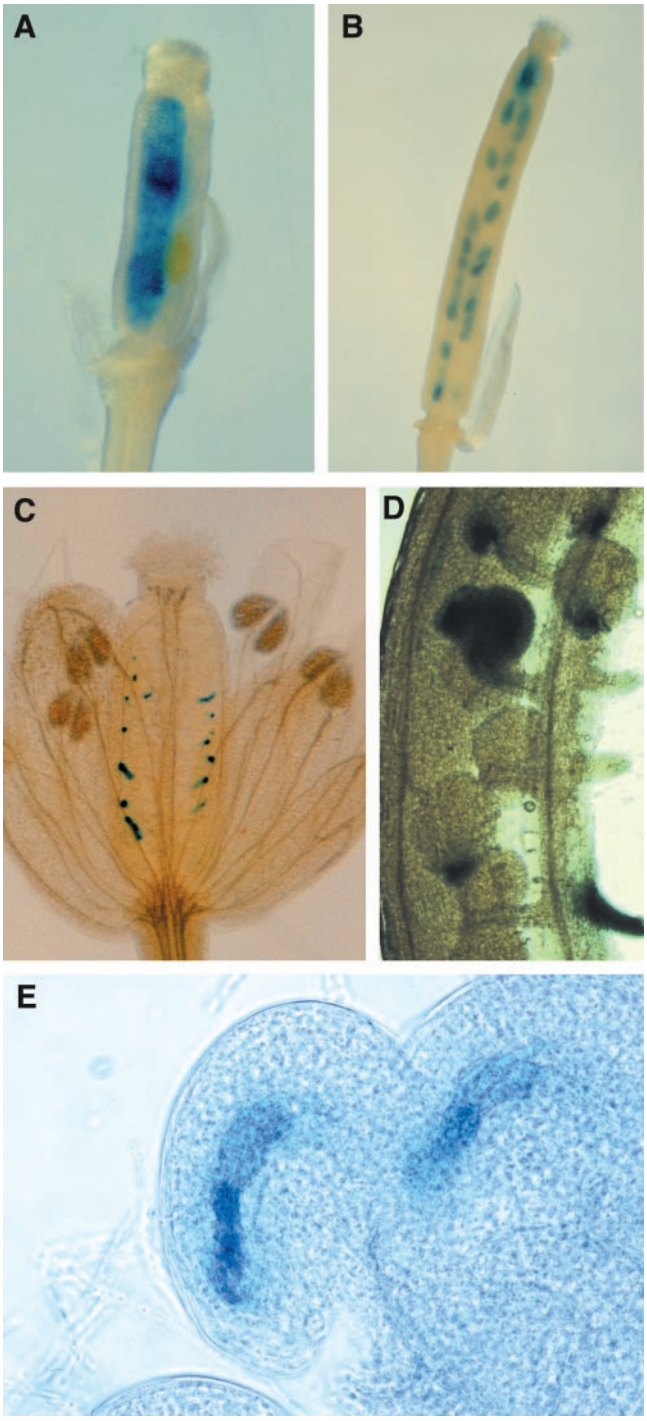

Figure 2. GUS expression in ovules/developing seeds of flowers from previously non-transformed plants dipped in *Agrobacterium* carrying ACT11-*gusA* T-DNA constructs. A, B, and D, ACT11-*gusA* (no intron) construct. C and E, ACT11-*gusA*-intron construct. A, Staining of an entire locule cavity, likely due to bacterial GUS expression from *Agrobacterium* colonizing the locule interior. B, Elongating seed pod from fertilized flower. C, Entire flower with staining of ovules only. D, Close-up of ovules in a segment of a dissected flower showing no staining, localized staining, or complete staining of individual ovules. E, Close-up of two ovules (partially overlapping in photo) showing staining of embryo/embryo sac rather than entire ovules.

ovule to the site of the embryo sac near the micropyle (Fig. 2E). Ovules that were completely stained and ovules that stained only at the site of the embryo could be found within the same locule.

Sampling and GUS staining of a given inflorescence yielded a set of flowers that were all the same number of days beyond inoculation, but at a variety of developmental stages. With the ACT11-*gusA*-intron construct, staining was first evident in unpollinated flowers collected at least 5 d after inoculation. GUS expression was only found in the ovaries of pollinated flowers collected at least 6 d after inoculation. No GUS staining was observed in flowers collected 4 or fewer d after inoculation. This time line for transformation was consistent with the results of the crossing experiments reported above and in Figure 1. Ten days after inoculation, the activity of the transgene could still be found in both pollinated and unpollinated flowers; this staining may have been due to some extent to residual GUS enzyme and not to new *gusA* gene expression. Twelve days after inoculation, we found some GUS staining only in pollinated flowers, and no staining of flowers was found 14 d after inoculation.

After floral-dip inoculation with *Agrobacterium* strains carrying either of the ACT11-*gusA* constructs, we did not observe any GUS expression in anthers, in pollen on the stigmatic surface, in germinated pollen tubes, or on any other parts of the flowers except the ovules. These results, together with those from work with the other *gusA* constructs discussed above and from the crossing experiments, showed that ovules are the primary target for productive transformation in the floral-dip method.

Independence of Separate Transformants

As a third means of addressing the site and timing of productive transformation, we examined the independence of T-DNA insertion events in transformed progeny derived from the same silique. Previous work had shown that T-DNA insertion events derived from the same plant are generally independent (Bechtold et al., 1993). However, with the achievement of higher transformation rates (in excess of 1% of all seed from a given plant) and with the identification of multiple transformed seeds within individual siliques, it remained a reasonable possibility that transformation early in floral ontogeny could give rise to multiple identically transformed progeny. Southern DNA-blot and inverse-PCR experiments were performed to examine the structure of the genomic DNA flanking T-DNA inserts. In five of five siliques examined using inverse PCR, separate transformants from a given silique contained T-DNAs that had inserted into distinct flanking sequences (Fig. 3). Similar results were obtained with progeny from these and other siliques in Southern DNA-blot studies (data not shown).

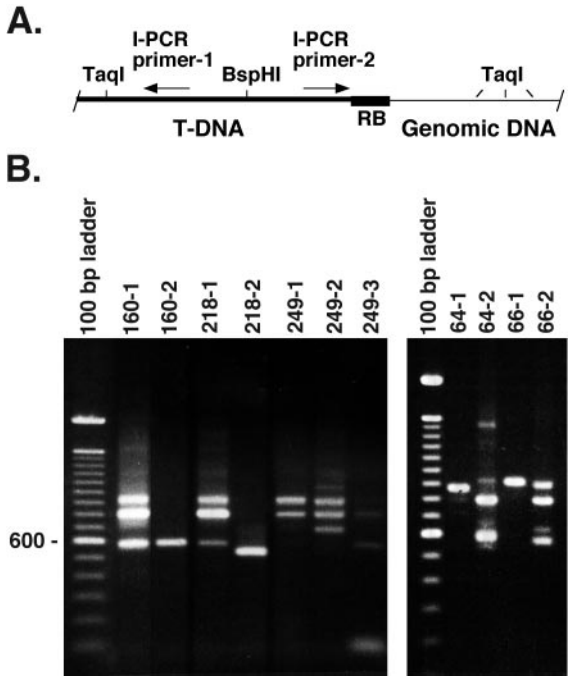

Figure 3. Distinct T-DNA insertions sites within separate transformants obtained from the same silique. Inverse PCR was used to amplify genomic DNA flanking the right border of the T-DNA. A, Diagram of inverse PCR strategy. Location of *Taq*I restriction sites in genomic DNA will vary according to site of T-DNA integration. Note that inverted repeats and other complex T-DNA integration structures are not depicted. RB, Right border. B, Inverse PCR products. Lanes marked with the same two- or three-digit prefix, but with a different suffix, are from separate transformants from the same silique. The pair of bands at approximately 800 and 940 bp that are common to many plant lines match the expected product for head-to-head (inverted repeat) T-DNA insert structures.

Enhanced Transformation Rate in *crc* Floral Mutant That Retains an Open Gynoecium

If transformation via the floral-dip method is limited by access of *Agrobacterium* to the interior of the gynoecium, one might predict that plant lines that retain an open gynoecium for a longer period during floral development would be transformable at an elevated rate. At the suggestion of Dr. John Bowman and colleagues (University of California, Davis), we performed transformation experiments with the CRABS-CLAW (*crc*) mutant of Arabidopsis and the near-isogenic parental line Ler-0. Flowers of the *crc-1* mutant line form a gynoecium in which carpel fusion is incomplete at the apex (Bowman, 1994). The vase-like gynoecium remains partially open when independent stigma form at the non-fused tips of the carpels, rather than becoming enclosed by stigmatic cap formation as occurs in wild-type plants. The *crc-1* mutation does not alter the development of other flower organs (Bowman, 1994). We grew Ler-0 and Ler *crc-1/crc-1* plants side-by-side and performed standard floral-dip transformation. The *crc-1* mutant line transformed at a 6-fold higher rate than wild-type Ler-0 (means ± SE = 1.20% ± 0.08% for *crc-1*; 0.21% ± 0.03 for wild type).

DISCUSSION

The primary goal of this study was to identify the sites of productive transformation in the floral-dip procedure for *Agrobacterium*-mediated transformation of Arabidopsis. In large-scale crossing experiments using plants for which only the maternal or paternal donor had been inoculated with *Agrobacterium*, transformed progeny were obtained only when the female (pollen-recipient) plant had been inoculated. Experiments that monitored delivery by *Agrobacterium* of T-DNA carrying a β-glucuronidase reporter gene revealed staining in individual ovules. No staining of pollen was observed. T-DNA insertion events were independent in separate transformed seedlings derived from the same silique. Taken together, these results indicate that ovules are the primary site of transformation.

Our work identified a range of probable target cell types for productive *Agrobacterium*-mediated transformation. In some cases, staining of entire ovules was observed, whereas in other cases, staining was very localized within the ovule to the site of the embryo sac near the micropyle. It is possible that multiple developmental stages serve as productive targets, ranging from the ovule primordia that will give rise to the megasporocyte, through any stage of megagametophyte development, to the recently fertilized embryo. Although we saw no staining of pollen before or after germination and growth through the pistil, our data also do not rule out transformation of the generative cell nucleus of pollen just prior to fertilization. Bechtold et al. (2000) have recently performed experiments to identify the genome (megagametophyte or microgametoyphyte) that receives the T-DNA insert in floral transformation of Arabidopsis. Their findings are consistent with our work, indicating that the predominant target is the female genome. However, they did observe apparent T-DNA integration into the male genome in one of the 26 cases examined. These rarer transformation events may occur within pollen or within the fertilized embryo.

While our work was in progress, Ye et al. (1999) also examined *Agrobacterium*-mediated floral transformation of Arabidopsis. In a smaller crossing study that did not monitor timing of floral development, they observed 15 transformants when *Agrobacterium* was applied to the pollen-recipient flower and zero transformants when *Agrobacterium* was applied to the pollen-donor. In T-DNA delivery GUS-staining experiments that used a Figwort mosaic virus promoter-*gusA*-intron construct (expression pattern in stable transformants not reported), they observed staining of all or some parts of ovules, as well as staining of seed coats and/or parts of interior seed

tissues. Those data are consistent with transformation events in the female germ line, but in contrast to our work, Ye et al. also reported GUS staining of pollen. Their data could not rule out transformation of the male germ line, but taken together with our work and the work of Bechtold et al. (2000), it is apparent that male germ-line transformation is rare. Ye et al. also explored macro-scale sites of transformation by mapping flower locations on a single plant, finding no particular bias in that regard. Last, Ye et al. observed independent transformation events when progeny from a given plant were checked, as was also found in the original work of Bechtold et al. (1993). We extended this last result by observing independence of T-DNA insertion events in transformants from the same silique.

We observed a reduction of transformation events and a reduced occurrence of GUS-positive flowers 12 to 14 d after plants were inoculated with *Agrobacterium*. This observation suggests that *Agrobacterium* persists for a limited period at levels high enough to achieve reasonable rates of transformation in newly forming flower buds, and it is consistent with our earlier finding that the number of transformants obtained on a plant could be increased by a second floral-dip application of *Agrobacterium* roughly 1 week after the first application (Clough and Bent, 1998).

The identification of ovules and/or megagametophytes as the primary target for transformation brings up the question of how *Agrobacterium* gains access to the interior of the ovary. The success of transformation after gentle dipping of inflorescences into *Agrobacterium* solution had suggested that the transformed tissues were exposed at or near the surface of the plant. Developing anthers present one such target, as do germinating pollen grains present on the stigmatic surface. However, in extensive studies we did not obtain evidence for pollen transformation. The possibility remains that germinated pollen tubes occasionally carry *Agrobacterium* from the stigma down the style to mature ovules, but our crossing experiments did not produce transformants in flowers pollinated during the first 4 d after inoculation. Pollen transformation or pollen-tube mediated delivery of *Agrobacterium* to the embryo sac also do not account for the uniformly GUS-stained ovules that were frequently observed in non-pollinated flowers in our study and in the work of Ye et al. (1999). These delivery methods are also not consistent with the recent findings of Bechtold et al. (2000) implicating the female genome as the primary target of transformation.

In considering access of *Agrobacterium* to the ovary interior, it is relevant to correlate our findings with previous observations of Arabidopsis floral development (Bowman, 1994). In Arabidopsis flowers, the gynoecium initially develops as a ring of cells that protrude from the floral meristem. This ring is surrounded by separate discrete mounds of cells that form stamens, and by young developing petals and sepals. As floral development progresses, the gynoecium extends to form a vase-like structure that remains open at the top. It is only at a stage roughly 3 d prior to anthesis that the stigmatic cap forms over the top of the elongated gynoecium, sealing off the interior to form enclosed locules. This is in contrast to other plants such as soybean, where locule closure occurs more than 10 d prior to anthesis (Johns and Palmer, 1982). Arabidopsis ovule primordia arise roughly 1 d prior to closure of the gynoecium, and megasporocyte formation is not estimated to occur until roughly 1 d after closure of the gynoecium (Bowman, 1994). In our crossing experiments, transformants were only obtained from developmentally young flowers that were still 5 or more d from anthesis at the time of inoculation. We hypothesize that no transformants were obtained from flowers that were more mature at the time of inoculation because these flowers carried closed locules, preventing access of *Agrobacterium* to the developing ovules and megaspores. In younger flowers and especially with the aid of a surfactant such as Silwet L-77, *Agrobacterium* applied to the plant surface is able to penetrate inside the developing flower where the open gynoecium apparently allows access to the relevant cell types at sites of ovule and megagametophyte development.

GUS staining experiments and studies with the *crc-1* mutant provided additional support for the above model regarding access to the interior of the gynoecium. Experiments that monitored GUS expression by *Agrobacterium* only revealed staining in locules of flowers that had been inoculated 6 or more d prior to anthesis. ACT11-*gusA*-intron staining of ovules was not detected unless flowers reached anthesis at least 5 d after inoculation. Some ovules were not uniformly stained, rather they exhibited GUS staining only at the site of the megasporocyte that forms 2 or 3 d prior to anthesis. *Agrobacterium* applied to flowers at or near the time that these megasprocytes were developing might conceivably have generated transformants, but we only obtained transformants from flowers that had been inoculated with *Agrobacterium* 5 or more d prior to anthesis.

These results carry significant implications for attempts to transform other plant species by *Agrobacterium* floral dip or related methods. Delivery of *Agrobacterium* to the interior of the gynoecium and transformation of developing ovules are apparently key benchmarks for successful transformation by these methods.

MATERIALS AND METHODS

Plant Growth

Arabidopsis plants used for crosses and inoculations with *Agrobacterium tumefaciens* were grown in moist pot-

ting mix (Sunshine mix no. 1, Sun Gro Horticulture, Bellevue, WA) in a 24-h light growth chamber at 22°C (five plants per 25-cm^2 pot). The ecotypes and genetic markers used were Col-0, Col *gl1/gl1* (glabrous; Herman and Marks, 1989), Col *rps2-201/rps2-201* (a non-functional allele of the resistance gene *RPS2*; Kunkel et al., 1993), Ler-0, and Ler *ap3-1/ap3-1* (a male-sterile mutant; Bowman et al., 1989). When the primary inflorescence reached 5 to 10 cm, plants were clipped to favor the growth of multiple secondary bolts.

Inoculation with *Agrobacterium*, Crossing, and Selection

About one-half of the plants for each genotype were dipped in *Agrobacterium* when most secondary bolts were 1 to 10 cm tall and carried multiple young floral buds (typically 5–8 d after clipping); the remaining plants were left uninoculated. Plants were inoculated with *A. tumefaciens* strain GV3101 (pMP90) carrying pBIN-mgfp5-ER (Koncz and Schell, 1986; Haselhoff et al., 1997); pBIN-mgfp5-ER was used primarily for delivery of kanamycin resistance rather than green fluorescent protein in these experiments. Inoculations were performed by dipping aerial parts of the plants for a few seconds in 300 mL of a solution containing 5% (w/v) Suc, 10 mM $MgCl_2$ (optional), resuspended *Agrobacterium* cells from a 150-mL overnight culture, and 0.03% (300 μL/L) of the surfactant Silwet L-77 (OSi Specialties; Clough and Bent, 1998). In some early experiments, the concentration of Silwet L-77 was varied between 0.005% and 0.1%, with higher levels causing plant damage. After the inoculation plants were left in a low-light or dark location and covered with a transparent plastic dome to maintain humidity; the dome was removed and the plants returned to the growth chamber 12 to 24 h after inoculation.

Crosses were performed by standard methods using genetically marked lines. Flowers roughly 1 d from anthesis were chosen as pollen recipients and all anthers were removed prior to outcrossing. The temperature sensitive male-sterility of the *ap3-1* line is nearly but not completely penetrant at 22°C, so *ap3-1* flowers were checked for the presence of anthers prior to use as pollen recipient. Siliques (seed pods) from crosses were collected individually in microfuge tubes 2 to 3 weeks after pollination.

Seeds were surface-sterilized by liquid or vapor-phase methods. For liquid sterilization, seeds were first treated for 30 to 60 s with isopropanol, then with a 50% bleach: 0.05% Tween 20 solution (v/v) for 5 min, and then rinsed three times with sterile water. Vapor-phase sterilization was used as a more efficient alternative when dealing with multiple tubes containing a small amount of seed. Open microfuge tubes marked with pencil rather than pen were placed in an approximately 8-L bell jar (under a fume hood) containing a beaker with 100 mL of household bleach (5.25% (w/v) sodium hypochlorite); 3.3 mL of glacial hydrochloric acid was then carefully added to the bleach and the jar was quickly sealed. Seed remained in contact with chlorine gas for several hours (4 h–overnight). After sterilization, the open tubes were placed for a least 20 min in a sterile hood to disperse trace chlorine gas, and tubes were then closed for storage or seeds were plated out for selection.

To select for transformed plants, liquid-sterilized seeds were resuspended into approximately 150 μL of 0.1% (w/v) agarose and plated on kanamycin selection plates. For gas-sterilized seed, 150 μL of 0.1% (w/v) agarose containing 50 μL/L (0.005% w/v) Silwet L-77 was added into the tube, the seed was allowed to imbibe for 15 to 45 min and it was then resuspended and plated. Selection plates contained one-half-strength Murashige-Skoog medium (M-5519, Sigma Chemical, St. Louis), 0.8% (w/v) agar, and 50 μg/mL of kanamycin. Typically, all of the seeds from twelve siliques were placed on a 100 × 25-mm plate. Plates were dried briefly to remove excess moisture, the number of seeds for each silique was recorded, plates were shifted to 4°C for 2 d, and they were then moved to a growth chamber at 24°C with 24 h of light. After roughly 10 d at 24°C, transformants were transplanted into soil and placed in a 9-h daylength growth chamber. Leaves were collected from those plants for DNA analysis and plants were then moved to a 24-h daylength growth chamber to set T_2 seed. In all cases, transformation rates were expressed as (no. of kanamycin-resistant plants/total no. of seeds plated) × 100.

As a positive control for transformation efficiency in each experiment, seeds from self-pollinated flowers were collected from *Agrobacterium*-treated plants used in the experiment. These seeds were plated on selective medium at a density of 3,000 seeds per 150- × 15-mm plates. Male-sterile *ap3-1* plants were excluded from this analysis.

Southern Blots and Inverse PCR

Leaf tissue for genomic DNA was collected from the T_1 plant and from T_2 progeny. Junction fragments containing T-DNA from pBIN-mgfp5-ER and flanking genomic DNA were detected by probing blots of *Bam*HI-digested Arabidopsis genomic DNA with a *nptII* gene probe, using standard methods (Ausubel et al., 1997). Alternatively, DNA sequences flanking the right border of T-DNA inserts were amplified by inverse PCR (Does et al., 1991) by digestion of genomic DNA with *Taq*I, ligation to circularize products, and performance of the PCR (annealing temperature of 62°C) using T-DNA Right Border oligonucleotide primers 5′-CGTTTCCCGCCTTCAGTTTA-3′ and 5′-GGAACTGAC-A-GAACCGCAAC-3′. In some cases, circularized products were linearized prior to PCR by digestion with *Bsp*HI at a T-DNA site that is 5′ to both primers (i.e. outside of the amplified region). Phenol/chloroform extraction and ethanol precipitation were performed between each of the above steps for inverse PCR.

Agrobacterium-Delivery of GUS Reporters That Are Expressed in Reproductive Tissues

Plants used in this experiment were either grown from seed in a growth chamber (at 22°C with 24 h of light) or grown in the greenhouse (at 24°C during the day and at

20°C during the night, with an 18-h daylength) and moved before flowering to 24 h of light in the laboratory. Inoculations took place at the same developmental stage and followed the procedure described above. We used *Agrobacterium* strain GV3101 (pMP90) carrying one of the following binary vectors: pAL145 (carrying a LAT52 promoter—*gusA* fusion; Twell et al., 1990), pBI-ACT11 (carrying an ACT11 promoter—*gusA* fusion; Huang et al., 1997), pCD1301-ACT11 (carrying an ACT11 promoter—*gusA*/intron fusion; construct described below), or pCAMBIA2301 (carrying a 35S promoter—*gusA*/intron fusion; http://www.cambia.org.au/). Plasmids were moved into *Agrobacterium* GV3101 (pMP90) by mating either using *Escherichia coli* S17–1 (Simon et al., 1983) or the helper plasmid pRK2013 (Figurski and Helinski, 1979). *Agrobacterium* were selected on AB medium (Lichtenstein and Draper, 1986) containing 0.2% (w/v) mannitol and 50 mg/L kanamycin.

pCD1301-ACT11 was constructed by replacing the 35S promoter region of the binary vector pCAMBIA1301 (http://www.cambia.org.au/) with the ACT11 promoter region of pBI-ACT11. The ACT11 promoter region (2.5 kb) was amplified by the PCR using TaqPrecision Plus polymerase (Stratagene, La Jolla, CA) and the following primers: 5′-ACACAGGAAACAGCTATGACC-3′ and 5′-CTGCCATGGGAACCATTCCGGTTCCATTGT-3′. PCR products were digested with *Pst*I and *Nco*I, ligated into the vector pPO28 (a modified pUC18 with a *Nco*I site in its polylinker, courtesy of P. Oger, University of Illinois, Urbana-Champaign) previously digested by the same enzymes, and confirmed by DNA sequencing. The ACT11 fragment was then recut by *Pst*I and *Nco*I and inserted into pCAMBIA1301 cut by the same enzymes to create pCD1301-ACT11. Two independently derived versions of pCD1301-ACT11 produced similar results.

To monitor the expression of *gusA* genes, flowers or floral clusters were collected after inoculation and incubated in the dark at room temperature in a 5-bromo-4-chloro-3-indolyl β-D-glucuronide-staining solution (Jefferson et al., 1987; U. Grossniklaus, personal communication). In some experiments, the solution was forced into the plant tissues by vacuum infiltration. After 3 d, the solution was replaced by a clearing solution containing 20% (w/v) lactic acid, 20% (v/v) glycerol in 1× PBS (20 mM sodium phosphate, pH 7.2, and 150 mM sodium chloride).

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