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The ability to move DNA into an organism and thereby alter its phenotype is central to both basic and applied molecular biology. Transformation is a simple task with Escherichia coli or Saccharomyces cerevisiae, but is usually more difficult with multicellular eukaryotes and can be particularly challenging with some important plant species. However, for Arabidopsis, in planta transformation methods have been developed that are incredibly simple. Attempts to apply in planta transformation methods to other plant species have often failed. This may be due in part to a poor understanding of the mechanisms that underlie the successful Arabidopsis transformation method. Studies of Arabidopsis transformation have accordingly been pursued, and three groups have recently published relevant findings. Successful in planta transformation of the legume Medicago truncatula was also reported recently, showing that the method can be adapted to other species. The cellular target for transformation of M. truncatula may differ somewhat from the target in Arabidopsis. The above findings may guide future efforts to improve transformation of other plant species.

This update opens by briefly reviewing transformation protocols that avoid tissue culture, and their impressive utility. Recent findings concerning Arabidopsis and M. truncatula transformation are then described. The review closes by commenting on possible avenues for improvement of transformation in other plant species.

BACKGROUND

Genetic transformation of plants occurs naturally (Hooykaas and Schilperoort, 1992). Scientists have been able to carry out controlled plant transformation with specific genes since the mid-1970s. The most common methods for introduction of DNA into plant cells use Agrobacterium tumefaciens bacteria or rapidly propelled tungsten microprojectiles that have been coated with DNA (Birch, 1997; Hansen and Wright, 1999). Other methods such as electroporation, microinjection, or delivery by virus have also been exploited. To allow physiological selection of cells that have been successfully transformed, the DNA of interest is typically cloned adjacent to DNA for a selectable marker gene such as nptII (encoding kanamycin antibiotic resistance).

Genetic transformation can be transient or stable, and transformed cells may or may not give rise to gametes that pass genetic material on to subsequent generations. Transformation of protoplasts, callus culture cells, or other isolated plant cells is usually straightforward and can be used for short-term studies of gene function (Gelvin and Schilperoort, 1998). Transformation of leaf mesophyll cells or other cells within intact plants may in some cases broaden the utility of single-cell assays (e.g. Tang et al., 1996). Exciting new approaches such as virus-induced gene silencing may also be applicable for some studies (Baulcombe, 1999). In the era of genomics these short-term assays will become increasingly important. However, in many cases it is desirable or necessary to produce a uniformly transformed plant that carries the transgene in the nuclear genome as a single Mendelian locus.

The generation of genetically homogeneous plants carrying the same transformation event in all cells has typically presented two separate hurdles: transformation of plant cells and regeneration of intact, reproducitively competent plants from those transformed cells (Birch, 1997; Hansen and Wright, 1999). Although many successful plant regeneration methods have been developed, these methods often require a great deal of protocol refinement and the focused effort of expert practitioners. It is unfortunate that plant regeneration from single transformed cells often produces mutations ranging from single base changes or small rearrangements to the loss of entire chromosomes. In addition, significant epigenetic changes (for example, in DNA methylation) can also occur (Phillips et al., 1994). It is often necessary to generate and screen a dozen or more independent plant lines transformed with the same construct to find lines that have suffered minimal genetic damage and that carry a simple insertion event (Birch, 1997; Hansen and Wright, 1999). Transformation is feasible

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in many plant species, but has required acceptance of the above limitations.

TRANSFORMATION METHODS THAT AVOID TISSUE CULTURE

A number of laboratories have pursued plant transformation methods that avoid tissue culture or regeneration. In many cases these methods have targeted meristems or other tissues that will ultimately give rise to gametes (Chee and Slighton, 1995; Birch, 1997). The same is true of popular tissue culture-based transformation methods for corn, rice, wheat, and soybean, which target young apical meristems for transformation (Birch, 1997). For those methods, excised or partially disrupted meristems are transformed, subjected to antibiotic or herbicide selection, and then carried through tissue culture to regenerate shoots and roots from the transformed tissues. For non-tissue culture approaches, Agrobacterium or tungsten particles have been used in a number of species to transform cells in or around the apical meristems that are subsequently allowed to grow into plants and produce seeds (Chee and Slighton, 1995; Birch, 1997). However, transformed sectors have typically not persisted into gametes at reasonable frequencies, or the methods have been difficult to reproduce (Birch, 1997). Injection of naked DNA into ovaries has also been reported to produce transformed progeny (Zhou et al., 1983). Variations of this method and “pollen tube pathway” delivery of DNA are still practiced in China (Hu and Wang, 1999). Electroporation-mediated gene transfer into intact meristems in planta and a variety of pollen transformation procedures have also been reported (Chowrira et al., 1995; Touraev et al., 1997 and refs. therein). However, most of these methods have been difficult to reproduce and have not gained widespread acceptance.

ARABIDOPSIS TRANSFORMATION WITHOUT TISSUE CULTURE

Early stages of the revolution that transformed Arabidopsis transformation were carried out by Ken Feldmann and David Marks. They applied Agrobacterium to Arabidopsis seeds, grew plants to maturity in the absence of any selection, then collected progeny seeds and germinated them on antibiotic-containing media to identify transformed plants (Feldmann and Marks, 1987; Feldmann, 1992). Although the procedure was difficult to reproduce consistently, successful rounds produced transformants at a high enough rate that thousands of transformed lines were produced in a matter of a few years. These “insertional mutagenesis” lines helped speed gene cloning by the Arabidopsis community (Azpiroz-Leehan and Feldmann, 1997). The lines could be screened for mutant phenotypes of interest and the mutated gene responsible for the phenotype could often be identified by isolation of the Arabidopsis chromosomal DNA flanking the previously known T-DNA (transferred DNA from Agrobacterium).

Other laboratories later succeeded in generating transformed Arabidopsis lines by “clip ‘n squirt” methods (Chang et al., 1994; Katavic et al., 1994). Reproductive inflorescences were clipped off, Agrobacterium was applied to the center of the plant rosette, new inflorescences formed a few days later were again removed, Agrobacterium was re-applied, and plants were then allowed to develop and set seed. Transformants were obtained more reliably than with the seed treatment method, but the methods were only marginally more productive than traditional tissue-culture approaches to Arabidopsis transformation (e.g. Valvekens et al., 1988).

A third, crucial stage of the revolution in Arabidopsis transformation came when Georges Pelletier, Nicole Bechtold, and Jeff Ellis reported success at transformation by “vacuum infiltration” (Bechtold et al., 1993). Arabidopsis plants at the early stages of flowering were uprooted and placed en masse into a bell jar in a solution of Agrobacterium. A vacuum was applied and then released, causing air trapped within the plant to bubble off and be replaced with the Agrobacterium solution. Plants were transplanted back to soil, grown to seed, and in the next generation stably transformed lines could be selected using the antibiotic or herbicide appropriate for the selectable marker gene. Transformation rates often exceeded 1% of the seeds tested. Variations of this extremely simple new method (Fig. 1) have been widely adopted by Arabidopsis researchers. Tissue culture and plant regeneration are no longer necessary and the associated high rates of mutation are avoided.

THE UTILITY OF AN ACCESSIBLE TRANSFORMATION METHOD

The impact of the vacuum infiltration method on Arabidopsis research has been remarkable. Generation of transformed lines is simple and routine (Fig. 1; Bechtold and Pelletier, 1998; Clough and Bent, 1998). First and foremost, barriers to in planta testing of a gene of interest have been dramatically lowered. With minimal effort and in a matter of 3 to 6 months, multiple transgenic plant lines can be constructed and numerous DNA constructs can be tested.

A second example of this method’s utility can be seen in positional cloning projects, in which a gene of unknown structure is isolated based on its genetic map position (e.g. Clough et al., 2000). Once a gene has been mapped to a genetic interval of a few centiMorgans, an Arabidopsis chromosome walk can be accelerated by the use of publicly available bacterial
artificial chromosome collections. Bacterial artificial chromosome clones containing insert DNAs that span the genomic region can be subcloned into a transformation-competent binary vector, moved into Agrobacterium, and used for “focused shotgun complementation” of a mutant plant line. With the genome sequence available and anchored to genetic maps, researchers may even choose to target their effort to specific candidate genes. The gene of interest is identified by screening sets of transformed plants for individuals that exhibit a corrected phenotype.

Another important use of simple, high-throughput transformation returns to the insertional mutagenesis methods pioneered by Feldmann and others. Collections of Arabidopsis containing tens of thousands of independent transformed lines are now available for screening (Azpiroz-Leehan and Feldmann, 1997; Krysan et al., 1999). Researchers alternatively can pursue insertional mutagenesis of a unique plant line, for example to carry out screens for genetic suppressors of a particular mutation. Activation-tagging T-DNAs can be used that enhance the expression level of genes near the T-DNA insert, or one can use T-DNAs that place β-glucuronidase (GUS) or other marker genes under the control of host promoter or enhancer elements that may flank the T-DNA at the site of insertion (Weigel et al., 2000).

Forward genetic approaches study phenotype first and then genotype, whereas reverse genetic strategies start with a DNA sequence and then seek a plant line mutated in that gene. Efficient transformation methods have facilitated reverse genetic screening in plants. Public collections have been created that allow PCR-based screening of ordered pools of DNA from thousands of transgenic lines (Krysan et al., 1999). Once the subpool of DNA carrying an insert in the gene of interest has been identified, progeny seeds corresponding to that pool can be requested. It is important to note that many of these insertional mutagenesis methods can also be accomplished using transposon mutagenesis (e.g. Tissier et al., 1999). A goal for many T-DNA and transposon-mutagenized seedbank collections is to obtain and compile in databases a small stretch of sequence data for the DNA that flanks each insertion (Tissier et al., 1999). This will allow researchers to simply request plant lines that carry a mutation within any DNA sequence in the genome.

The above strategies could be extremely useful in research with other plant species. For some species, the current transformation protocols are close to being sufficient (witness the recent production of more than 18,000 fertile transgenic rice lines to form an insertional tagged population; Jeon et al., 2000). For many important species, however, pursuit of the above strategies would be greatly facilitated by the availability of high-throughput/non-tissue culture transformation methods. After the success of the Arabidopsis vacuum infiltration protocol, a number of laboratories tried to use Agrobacterium vacuum infiltration with other plant species, but failed to obtain transformants. Why? In the absence of a suitable answer, study of the successful Arabidopsis methods was a logical next step.

HOW DO IN PLANTA ARABIDOPSIS TRANSFORMATION PROCEDURES WORK?

What is the cellular target of transformation? For the Arabidopsis seed transformation and vacuum infiltration methods, it was shown early on that most primary transformants carry hemizygous T-DNA insertion events (Feldmann, 1992; Bechtold et al., 1993). The presence of the T-DNA on only one of two homologous chromosomes implies that productive transformation occurs late in floral development, after the divergence of male and female germ lines (Arabidopsis self-pollinates within individual flowers, and if transformation occurred earlier, self-
fertilization would be expected to give rise to some homozygous transformants due to presence of the same T-DNA insert in pollen and embryo sac cells. The transformation target is further defined in that transformants obtained from a given plant usually carry independent T-DNA insertion events (Feldmann, 1992; Bechtold et al., 1993). This suggests that transformation occurs after the divergence of individual pollen or egg cell lineages within a flower. A developmental endpoint for the typical target of transformation can also be postulated. Although the result is not as well established, typical primary transformants apparently carry the transgene in all parts of the plant, suggesting that transformation occurred before the cell divisions in a fertilized embryo that establish independent meristems and other distinct adult plant cell lineages. Hence, transformation seems to occur in developing flowers after individual gametophyte cell lineages form, but before extensive development of the embryo. The next question was: Does transformation occur primarily in pollen, ovules, fertilized embryos, or any of the three?

It is an interesting historical sidelight that rather than addressing this key question, Arabidopsis researchers in the mid-1990s focused on empirical transformation protocol improvement. Practical motivation to proceed with the generation of transformants was understandably paramount, and overall satisfaction with the new transformation method delayed efforts to understand how it worked. Nevertheless, protocol modifications, ideas, and anecdotal observations were shared widely through meetings, word-of-mouth, and the Arabidopsis electronic newsgroup (http://www.bio.net/hypermail/Arabidopsis/). Significant findings resulting from this community effort included the discoveries that (a) Plants did not need to be uprooted, treated with Agrobacterium, and re-planted. Transformants could be obtained by treating only the protruding inflorescences; (b) inclusion of Silwet L-77, a strong surfactant that shows relatively low toxicity to plants, often enhanced transformation reliability; and (c) many different Arabidopsis ecotypes were transformable and many different Agrobacterium strains could be used, although notable differences in efficiency were observed. Most important, the popular name “vacuum infiltration” was superceded when a number of groups found that plants could be transformed when dipped in Agrobacterium solution with no vacuum infiltration. Some workers subsequently moved to spray application of Agrobacterium rather than dipping. A number of other mechanistic clues and procedural tips were shared (see http://www.bio.net/hypermail/Arabidopsis/; Clough and Bent, 1998). A simplified protocol for “floral dip” transformation of Arabidopsis is available at http://plantpath.wisc.edu/wisc.edu/~afb/protocol.html.

OVULES ARE THE PRIMARY TARGET FOR TRANSFORMATION

Returning to the question of the cellular target of transformation, three research groups worked in parallel to address this issue and have now published their results (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000). Given that transformation can occur by mere dipping of flowers in Agrobacterium solution and that anthers and pollen are exposed whereas ovules are not, it seemed likely that the male germ-line would be the target of transformation. However, all three groups found that the female germ-line is the primary target of transformation.

In one set of experiments, transformants were produced by outcrossing after Agro-inoculation of only the pollen donor or pollen recipient. No transformants were observed among more than 14,000 seeds produced following inoculation of the pollen donor, but 71 transformants were recovered out of roughly 14,800 seeds produced following inoculation of the pollen recipient (Fig. 2; Desfeux et al., 2000). Ye and colleagues observed zero and 15 transformants, respectively, in a similar study (Ye et al., 1999). These findings seemingly to rule out transformation of pollen as it develops within anthers, but do not preclude the possibility that pollen is transformed after it ger-
minates on the stigmatic surface of the pollen recipient.

Ovule transformation was convincingly demonstrated when constructs containing a GUS marker gene were used to document sites of delivery of T-DNA (Fig. 3; Ye et al., 1999; Desfeux et al., 2000). 35S and other standard promoters are poorly expressed in gametophyte tissues, so additional promoters used for GUS fusions were Arabidopsis ACT11 (Desfeux et al., 2000), an oilseed rape Skp1-like promoter (Bechtold et al., 2000), or a Figwort mosaic virus promoter (Ye et al., 1999). Staining was observed in ovules in mature flowers and in younger flowers that had not yet reached pollination (Ye et al., 1999; Desfeux et al., 2000). Desfeux et al. (2000) and Bechtold et al. (2000) did not observe GUS staining of anthers or pollen (except in stably transformed positive controls), providing another line of evidence that pollen transformation is not common. Ye et al. (1999) reported frequent GUS staining of ovules and pollen, but also concluded that ovules are the primary target for transformation. It is curious that Bechtold et al. (2000) did not observe staining of ovules or embryos in their work.

A third, very different line of evidence also points to ovules as the primary site of productive transformation. Genetic linkage analysis with a marked chromosome demonstrated that most transformants (25 of 26 tested) carry T-DNA on the maternally derived chromosome set (Bechtold et al., 2000). For the one of 26 events associated with the paternal chromosome set, the most likely origin was pollen transformation or integration of T-DNA within the diploid genome of a fertilized embryo. However, the aggregate message from the efforts of these three labs seems convincing: developing ovules are the primary target of productive transformation in the Arabidopsis floral dip or vacuum infiltration transformation procedures (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000).

**GUS STAINING AND TRANSFORMANT GENERATION: SALIENT DETAILS**

In the above experiments GUS staining was often observed only within the embryo sac of ovules, indicating a time of transformation late in megagametophyte development (Fig. 3c; Ye et al., 1999; Desfeux et al., 2000). Late transformants were also obtained, albeit at a very low frequency, from inoculation of flowers that were sufficiently developed to contain trinucleate pollen and embryo sacs at the four-nuclei stage of development (Bechtold et al., 2000). Uniform blue staining of entire ovules was sometimes observed, suggesting that transformation can also occur earlier in the formation of the megagametophyte cell lineage (Fig. 3b). However, earlier transformation events that give rise to larger transformed sectors encompassing multiple ovules seem unlikely. Arabidopsis transformants from the same plant (Bechtold et al., 1993; Ye et al., 1999) or even from the same silique (seed pod; Desfeux et al., 2000) are usually independent. This latter point contrasts with recent results from the legume *M. truncatula* (discussed below).

Desfeux and colleagues tracked the presence of *Agrobacterium* by using GUS constructs that are expressed within *Agrobacterium* (Desfeux et al., 2000). Floral dip inoculation produced staining along the stigmatic surface and in various crevices of the flower (not shown), but also produced examples in which closed locules were filled with blue stain (Fig. 3d), suggesting that locules can harbor substantial colonies of *Agrobacterium*. Transformants have been obtained from siliques located at multiple sites across the inflorescence (Bechtold et al., 1993; Ye et al., 1999). However, transformants in one study were not randomly (Poisson) distributed on a per silique or per plant basis (Bechtold et al., 2000). In another study roughly one-half of the transformant-bearing siliques contained more than one and up to seven transformants (Desfeux et al., 2000). In addition, although most siliques on inoculated plants showed no GUS staining, a few siliques showed multiple stained embryos (Desfeux et al., 2000). It is apparent that some flowers are particularly amenable to transformation.

**TIME OF INOCULATION RELATIVE TO FLOWER DEVELOPMENT IS CRUCIAL**

If productive transformation events occur in the female germ-line, one is forced to wonder how *Agrobacterium* gains access to ovules that develop within enclosed locules. However, Arabidopsis locules are not always closed. The ovary develops as a ring of cells that protrude from the floral meristem,
The occurrence of sibling transformants is not desirable for applications such as insertional mutagenesis, but the overall high rates of transformation obtained (Trieu et al., 2000) indicate that this method will be of tremendous utility to researchers who study *M. truncatula*.

**WILL THE ABOVE INFORMATION HELP WITH TRANSFORMATION OF OTHER SPECIES?**

Transformation by infiltration of adult plants with *Agrobacterium* has also been reported for pakchoi (Liu et al., 1998) and has been informally reported for other Brassicaceae beyond pakchoi and Arabidopsis. Thus multiple plant species have now been successfully transformed using *Agrobacterium* in planta approaches. In addition, although the methods have not been widely reproduced or adapted, *Agrobacterium*-mediated shoot apex transformation and related methods that minimize tissue culture have been reported for a number of other plant species (Chee and Slighton, 1995 and refs. therein). Development of robust in planta transformation protocols for other plant species should be within reach.

Transformation technology development has been regarded by some as art as much as science, but success is most likely to come from efforts informed by the scientific literature and past experiences (e.g. see the reviews of Hansen and Wright, 1999 and Birchen, 1997). This must be coupled with a willingness to try different approaches and to tolerate failures along the way.

It is very probable that success will be easier to achieve with some species and than with others, but what are some of the criteria that may contribute to success? Arabidopsis and *M. truncatula* are relatively small plants with rapid generation times, but that may enhance ease of effort more than ultimate success at transformation. A high seed set is also likely to help, but is not an ultimate determinant: although single Arabidopsis plants commonly produce 5,000 to 10,000 seeds, only 33 or fewer seeds per Agro-inoculated plant were collected in the successful transformation of *M. truncatula* (Trieu et al., 2000).

Aspects of experimental design such as planting configuration and mode of Agro-inoculation (i.e. inoculating seedlings as opposed to flowering plants) can allow dramatic shifts in the number of specimens processed. Large numbers can be important if rates of success are low, but can also be a trap if quality of treatment is more important than quantity. Prime examples of this are the low success rates and/or poor reproducibility that were achieved with Arabidopsis in planta transformation procedures until inoculation of plants in full flower was attempted. The need for vernalization of *M. truncatula* to achieve efficient transformation offers another example. Trying a larger variety of approaches may be more productive than trying very large-scale attempts with a narrow set of methods.
The discovery of the ovule as the site of productive Arabidopsis transformation produces specific suggestions for floral transformation efforts with other species. Application of Agrobacterium to flowering tissues very early in their development and prior to locule closure is likely to be important. In an alternate manner, with some species it may be possible to deliver Agrobacterium by microinjection of ovaries, or by shooting Agrobacterium into flowers using microprojectiles or high-pressure air guns (e.g., see U.S. patent no. 5,994,624). As a further alternative, plant lines such as the Arabidopsis CRABS-CLAW mutants that bear a more accessible locule may provide an improved target for transformation (Desfeux et al., 2000). Work with M. truncatula and Arabidopsis suggests that younger plants can also be treated, although onset of flowering soon after Agrobacterial inoculation appears to be preferable.

In numerous plant transformation systems, the choice of host genotype and/or Agrobacterium genotype has been an important parameter (Birch, 1997). If recent findings with Arabidopsis are any indication, surveys for compatible host and bacterial genotypes might best be focused on assays that monitor transformation of ovules or ovule progenitor tissues. With some plant species the use of anti-oxidants or other necrosis-reducing approaches has improved transformation rates, and many other modifications can be considered (Birch, 1997; Hansen and Wright, 1999). A better understanding of T-DNA transfer and other aspects of Agrobacterium/plant interactions (e.g., Hooykaas and Schilperoort, 1992; Mysore et al., 2000) may also allow engineering of better host/bacteria combinations.

Other substantially different transformation methods also must be kept in mind (e.g., Chowriria et al., 1995; Chen et al., 1998). Who would have dreamed, 20 years ago, that coating DNA on to little metal particles and then shooting it into plants (Klein et al., 1995; Chen et al., 1998) could be so successful?

Agrobacterium floral transformation procedures have been a tremendous success with Arabidopsis, and similar success now seems likely for M. truncatula. Such successes, along with the recent information about the targets for Arabidopsis transformation, should inspire a renewal of efforts to adapt these methods to the transformation of other plant species. The benefits are clear: transformation without tissue culture can provide a high throughput method that requires minimal labor, expense, and expertise. Rates of unintended mutagenesis are reduced. More important, simplified transformation protocols facilitate positional cloning, insertional mutagenesis, and other transformation-intensive procedures, reducing the effort required to test any given DNA construct within plants.

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LITERATURE CITED


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