Recent Advances in Legume Transformation

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WHY TRANSFORM LEGUMES?

Legumes are a large, diverse family ranging from herbaceous annuals to woody perennials that, because of their capacity to fix nitrogen, are essential components in natural and managed terrestrial ecosystems. Legumes have been domesticated for the production of food, feed, forage, fiber, industrial and medicinal compounds, flowers, and other end uses. Understanding the molecular basis of nitrogen fixation and the unique metabolic pathways that result in the myriad of end uses of legumes is both a matter of scientific curiosity and of economic necessity because of their importance in the biosphere and to the sustainability of the human race. In accordance, model legumes are being rapidly developed as experimental systems to pursue a number of important biological questions unique to these plants using molecular tools including genomics. A key component of most functional genomics approaches is a high-throughput transformation system useful for developing various gene identification strategies. Transformation also is emerging as an important crop improvement tool. This is particularly evident in soybean (*Glycine max*), in which Roundup Ready soybean cultivars have captured a major stake in market share of soybeans planted in the U.S. and Argentina. Transformation theoretically expands the sources of genes for plant improvement to all organisms, far beyond the gene pool accessible via sexual hybridization. Transformation also offers strategies for overexpressing or suppressing endogenous genes. Thus, introducing new genes or manipulating endogenous gene expression via transformation generates new phenotypic variation useful for investigating gene function and for crop improvement.

ARE LEGUMES DIFFICULT TO TRANSFORM?

The answer to this question is, of course, that some legume species are much more difficult to transform than others. Legume transformation systems, like transformation in all organisms, require development of: (a) a source of totipotent cells or gametes that serve as recipients of delivered DNA, (b) a means of delivering DNA into the target cells, and (c) a system for selecting or identifying transformed cells. For legumes that have been regarded as recalcitrant to transformation, regeneration in vitro is highly genotype specific and only rarely are cultivated varieties amenable to regeneration. In these cases, plant regeneration remains an "art" that requires considerable training of the practitioner to develop the skills needed to generate sufficient transgenic plants for a thesis or publication. In addition, regeneration is often slow and the frequency of transformation (no. of transformed plants generated from each explant) is often low. In species that are amenable to in vitro somatic embryogenesis such as alfalfa, (lucerne; *Medicago sativa*), relatively rapid and efficient transformation methods have been developed based on cocultivation of tissue pieces (explants) with *Agrobacterium tumefaciens*. Because inducing somatic embryogenesis or organogenesis in many legume species is difficult, a variety of transformation methods have been reported that use cultures of meristematic cells as sources of totipotent cells. Most commonly, transformation has been based on infection by *A. tumefaciens*, although *Agrobacterium rhizogenes* is used for transformation of some species. Regeneration of shoots from the cotyledonary node or from other meristematic explants after *Agrobacterium* infection is emerging as a rapid and relatively efficient method of transformation in a number of legume species including soybean (Olhoft and Somers, 2002), *Lotus japonicus* (Øger et al., 1996), barrel medic (*Medicago truncatula*; Trieu and Harrison, 1996), and *Trifolium repens* (Larkin et al., 1996). A number of legume species also have been transformed by direct DNA transfer methods including microinjection, electroporation, and microprojectile bombardment (for review, see Christou, 1997; Atkins and Smith, 1997; Babaoglu et al., 2000).

In some species, the difficulty in regenerating transgenic plants has been circumvented by development of rapid and efficient transformation protocols using *A. rhizogenes* to produce hairy roots on "composite" plants (an untransformed plantlet with hairy roots). These composite plants have been used in studies focused on root characteristics such as nod-

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ulation and root diseases. Examples have been reported in *L. japonicus* (Stiller et al., 1997; Martirani et al., 1999), soybean (Narayanan et al., 1999), and barrel medic (Boisson-Dernier et al., 2001). Composite plants do not transmit the transgenic trait to their progeny and, thus, are of little use in crop improvement efforts.

Advancement of molecular genetics in legumes, e.g. gene overexpression, gene suppression, promoter analysis, T-DNA tagging, and expression of genes for crop improvement, requires efficient transformation systems that produce low frequencies of tissue culture-induced phenotypic abnormalities in the transgenic plants. The development of the in planta transformation system for Arabidopsis (Clough and Bent, 1998) radically accelerated research in basic plant molecular biology. By analogy, development of simple, rapid transformation systems in legumes that require the minimum amount of "art" will have a similar impact on legume biology. In this *Update*, we report recent advances in transformation of forage and pasture, grain and pulse, and tree legumes updating the excellent summaries of Babaoglu et al. (2000) and Atkins and Smith (1997). This information is summarized in Table I such that the DNA delivery method, source of totipotent target cells, and selection system is presented for each species. Aspects of transformation system components that have resulted in improvements in transformation efficiency of legumes will also be discussed. Finally, we speculate on possible avenues for developing non-tissue culture transformation systems for legumes.

PROGRESS IN LEGUME TRANSFORMATION

Forage and Pasture Legumes

In the past decade, considerable success has been achieved in transformation of forage and pasture legumes. Efficient transformation protocols have been developed for alfalfa and *T. repens* that have enabled research to advance from expression of marker genes to evaluation of genes for crop improvement. Commercialization of the first transgenic forage crop, Roundup Ready alfalfa, is slated for 2004 (http://www.foragegenetics.com/biotechnology.htm). Efficient transformation methods have been critical to the rapid adoption of *L. japonicus* and barrel medic as model systems in plant biology. A summary of protocols for transformation of bird's foot trefoil, *L. japonicus*, *Lotononis bainesii*, alfalfa, barrel medic, *Medicago varia*, *Medicago arborea*, *Onobrychis viciifolia*, *Stylosanthes humilis*, *Stylosanthes guianensis*, *T. repens*, and *Trifolium subterraneum* was provided by Atkins and Smith (1997).

Recent advances in transformation of forage species since that review (Atkins and Smith, 1997) are shown in Table I. Chinese milk vetch is grown as a green manure, for animal fodder, as a nectar source for bees, and can be used to volatilize selenium from soil. *A. rhizogenes* inoculation of seedlings in vitro results in formation of hairy root, which spontaneously produce shoots in culture (Cho et al., 1998). Similarly, a number of protocols using *A. rhizogenes* for production of transgenic *Lotus corniculatus* have been described (Atkins and Smith, 1997). Transformation of *L. corniculatus* via cocultivation of leaf explants with *A. tumefaciens* followed by callus formation and shoot organogenesis was reported by Webb et al. (1996). In contrast, transformation of red clover is based on regeneration via somatic embryogenesis after cocultivation of petiole explants with *A. tumefaciens* using genotypes selected for high frequency of this culture response (Quesenberry et al., 1996).

L. japonicus was suggested as a model system for legume genomics by Handberg and Stougaard (1992). In addition to other positive attributes as a model system, transformation of hypocotyls with *A. tumefaciens* is relatively efficient via shoot organogenesis. This method was further optimized and the time to produce whole plants reduced by Stiller et al. (1997). Somaclonal variation and sterility were significantly reduced by use of the *bar* gene and selection with PPT (Lohar et al., 2001).

A highly efficient transformation method has enabled initiation of a T-DNA insertional mutagenesis program for barrel medic (Scholte et al., 2002). Each explant of line R108-1(C3), a genotype selected for superior regeneration, produces large numbers of somatic embryos, and up to 80% of the embryos regenerate into plants 3 to 4 months after culture initiation (Trinh et al., 1998). Methods with the potential to reduce tissue culture manipulations for transformation of barrel medic have been reported. Trieu and Harrison (1996) described a method based on cocultivation of *A. tumefaciens* with cotyledonary node explants followed by culture to induce multiple shoots from explants. Transgenic plantlets were produced in 2.5 months. Two in planta transformation systems were described by Trieu et al. (2000); one method is based on infiltration of flowers with *A. tumefaciens*, similar to the Arabidopsis flower infiltration protocol, and the other on infiltration of seedlings. Both methods were reported to result in high transformation frequencies. Although promising, these results have not been repeated or further extended by this group, nor have they been corroborated by other laboratories.

Grains and Pulses

Progress in transformation of large-seeded legumes has been extensively reviewed (Christou, 1997; Nagl et al., 1997; Trick et al., 1997), and more recent progress is presented in Table I. Historically, both microprojectile bombardment and *Agrobacterium* have been used for DNA delivery into either embry-

Table I. *Summary of legume transformation systems yielding transformed plants that transmitted the transgenic genotype to progeny reported since or in addition to Atkins and Smith (1997) and Babaoglu et al. (2000)*

Genotype, DNA delivery system, explant, selectable marker gene and agent, and citation are presented. N, Not identified; At, *A. tumefaciens*; Ar, *A. rhizogenes*; MB, microprojectile bombardment. *Agrobacterium* strain and tissue culture type: O, organogenesis; E, embryogenesis; and C, callus are indicated in parentheses.

Species, Genotype	DNA Delivery	Explant	Selection		Citation
			Marker	Agent	
Pasture and forage species					
Chinese milk vetch (*Astragalus sinicus*)					
Japan	Ar (DC-AR2)	Seedlings (O)	*nptII*	Kan	Cho et al. (1998)
Bird's foot trefoil (*Lotus corniculatus*)					
Leo	At (LBA4404)	Leaves (O)	*aphIV*	Hyg	Webb et al. (1996)
L. japonicus					
Gifu	At (LBA4404, C58C1, GV2260)	Hypocotyls (O)	*nptII*, *hpt*	Kan, Hyg	Handberg and Stougaard (1992)
	Ar (9402, AR10)	Seedlings (O)	*nptII*	Kan	Stiller et al. (1997)
	At (AGL1)	Hypocotyls (O)	*bar*	Phosphinothricin (PPT)	Lohar et al. (2001)
Barrel medic (*Medicago truncatula*)					
R108-1	At (A281, GV2260)	Leaves (E)	*nptII*, *hph*	Kan, Hyg	Hoffmann et al. (1997)
R108-1(C3)	At (EHA105, GV3101)	Leaves (E)	*nptII*, *hph*	Kan, Hyg	Trinh et al. (1998)
R108-1(C3), Jemalong J5	At (EHA105)	Floral organs (E)	*nptII*	Kan	Kamaté et al. (2000)
R108-1(C3)	At (EHA105)	Leaves (E)	*bar*, *nptII*	PPT, Kan	Scholte et al. (2002)
Jemalong	At (LBA4404)	Cotyledons (O)	*bar*	PPT	Trieu and Harrison (1996)
	At (EHA105, ASE1, GV3101)	Flowers, seedlings	*bar*	PPT	Trieu et al. (2000)
Red clover (*Trifolium pratense*)					
NEWRC germplasm	At (EHA101, A208)	Petiole pieces (O)	*nptII*	Kan	Quesenberry et al. (1996)
Grains, pulses, and other seed crops					
Peanut (*Arachis hypogaea*)					
Florunner/MARC-1/Georgia Runner	MB	Embryogenic cultures (E)	*hph*	Hyg	Wang et al. (1998)
Gajah and NC-7	MB	Somatic embryos (E)	*hph*	Hyg	Livingstone and Birch (1999)
AT120/VC1	MB	Embryogenic cultures (E)	*hph*	Hyg	Magbanua et al. (2000)
JL-24	At (C58)	Cotyledons (O)	*nptII*	Kan	Sharma and Anjaiah (2000)
TMV-2	At (LBA4404)	Embryo axes non-tissue culture	*gusA*	Visual	Rohini and Rao (2000)
Pigeon pea (*Cajanus cajan* L. Millsp.)					
N	At (GV2260)	Embryonic axis (O,C)	*nptII*	Kan	Lawrence and Koundal (2001)
Hyderabad					
	AT (EHA105)	Embryonic axes and cotyledonary nodes (O)	*nptII*	Kan	Satyavathi et al. (2003)
Chickpea (*Cicer arietinum*)					
PG1/PG12/Chafa/Turkey	At (C58C1/ EHA101)	Embryonic axis (O)	*pat*, *nptII*	PPT, Kan	Krishnamurthy et al. (2000)
Guar (*Cyamopsis tetragonoloba*)					
Lewis/ Santa Cruz	At (LBA4404)	Cotyledons (O)	*nptII*	Kan	Joersbo et al. (1999)

(*Table continues on facing page.*)

Table I. (*Continued from previous page.*)

Species, Genotype	DNA Delivery	Explant	Selection		Citation
			Marker	Agent	
Soybean					
Jack	MB	Immature embryos (E)	*hph*	Hyg	Santarem and Finer (1999)
A3237	EHA101/EHA105	Cotyledonary node (O)	*bar*	PPT	Zhang et al. (1999)
Jack	EHA105	Immature cotyledon (E)	*hpt*	Hyg	Yan et al. (2000)
BR-16/DokoPC/BR-19/Conquista	MB	Embryonic axis (O)	*ahas*	imazapyr	Aragão et al. (2000)
Bert	EHA101	Cotyledonary node (O)	*hph*	Hyg	Olhoft et al. (2003)
Lentil (*Lens culinaris* Medik)					
Laird/CDC599–23	MB	Cotyledonary node (O)	*als*	Chlorsulfuron	Gulati et al. (2002)
Lupin (*Lupinus angustifolius*)					
Unicrop/Merrit	At (AgL0)	Axillary shoot embryonic axis (O)	*bar*	PPT	Pigeaire et al. (1997)
Yellow lupin (*Lupinus luteus*)					
Wodjil/Popiel/Teo/Juno	At (AgL0)	Apical meristem (O)	*bar*	PPT	Li et al. (2000)
Tepary bean (*Phaseolus acutifolius* A. Gray)					
NI576	At (C58C1RifR)	Bud explants (O,C)	*nptII*	G418	De Clercq et al. (2002)
Bean (*Phaseolus vulgaris*)					
Olathe/Carioca	MB	Embryonic axes (O)	*bar*	PPT	Aragão et al. (2002)
Pea (*Pisum sativum*)					
94-A26/ Bolero/Hadlee/ Crown/ Courier/89T46.UK	At (AGL1)	Immature cotyledons (O)	*nptII*	Kan	Grant et al. (1998)
Laser, Heiga	At (EHA105; C58C1/LBA4404)	Cotyledons (O)	*nptII, bar*	Kan, PPT	Nadolska-Orczyk and Orczyk (2000)
Greenfeast/CDC Vienna/ S2–90–25E/ 93-4-18G/	At (EHA105)	Embryonic axis (O)	*bar, nptII*	PPT, Kan,	Polowick et al. (2000)
MP1338/MP1382/ AWPNZ66/ AWP1512			*als*	chlorsulfuron	
Fava bean (*Vicia faba*)					
Mythos	At (EHA101 and 105)	Epicotyls (O,C)	*nptII*	Kan	Böttinger et al. (2001)
		Internodal stem			
Narbon bean (*Vicia narbonensis*)					
Var. narbonensis	At (EHA101)	Epicotyls and shoot tips (E)	*nptII*	G418	Czihal et al. (1999)
Azuki bean (*Vigna angularis* Willd. Ohwi/Ohashi)					
Beni-dainagon	At (EHA105)	Elongated epicotyls (O,C)	*nptII*	Kan	Yamada et al. (2001)
Mung bean (*Vigna radiata* L. Wilczek)					
K-851	At (LBA4404)	Cotyledonary node (O)	*nptII*	Kan	Jaiwal et al. (2001)
Asparagus bean (*Vigna sesquipedalis*)					
Koern	At (EHA101)	Cotyledonary node (O)	*bar, nptII*		Ignacimuthu (2000)
Tree species					
Acacia mangium					
N	At (LBA4404)	Rejuvenated shoots (O)	*nptII*	G418	Xie and Hong (2002)
Black locust (*Robinia pseudoacacia*)					
N	Ar (RI601)	Hypocotyl segments (O)	*nptII*	Kan	Han et al. (1993)
N	At (GV3101)	Stem and leaf segments (O)	*hpt*	Hyg	Igasaki et al. (2000)

ogenic or organogenic cultures of some species that have been subjects of extensive research. However, the majority of the most recent reports are focused on *A. tumefaciens*-mediated transformation. This trend is evident for *Arachis hypogaea* and soybean. On the other hand, pea transformation systems historically have been based mostly on *A. tumefaciens*. In contrast, we could find no reports of transgenic bean plants produced via *Agrobacterium* (Table I). This latter observation suggests inefficient transformation due to problems with *Agrobacterium* infection, T-DNA delivery, or both in this species.

Cowpea (*Vigna unguiculata*) appears to be the most recalcitrant large-seeded legume. Although there is a report of successful production of transgenic plants (Muthukumar et al., 1996), further evidence of transmission of the transgene genotype to progeny has not been reported.

Trees

Leguminous trees are a rich source of wood, paper pulp, and animal fodder in many locations around the world. Recently, transformation methods have been developed for *Acacia mangium* (Xie and Hong, 2002) and *Robinia pseudoacacia* (Han et al., 1993; Igasaki et al., 2000). For transformation of *A. mangium*, rejuvenated shoots were cultured from axillary buds and shoot apices of mature trees and shoot pieces cocultured with *A. tumefaciens*. Regeneration and culture of shoots required approximately 13 months (Xie and Hong, 2002). Transgenic *R. pseudoacacia* plants were obtained approximately 12 weeks after inoculation of hypocotyl segments with *A. rhizogenes*. Shoots arose spontaneously from hairy root cultures. Regenerated plants showed phenotypic abnormalities (Han et al., 1993). In contrast, phenotypically normal plants were obtained approximately 2 months after cocultivation of stem segments with *A. tumefaciens* (Igasaki et al., 2000).

AVENUES FOR TRANSFORMATION SYSTEM IMPROVEMENT

Somatic embryogenic and organogenic tissue cultures are the primary sources of totipotent target cells used in legume transformation systems (Table I). For some species a range of genotypes, including cultivars, are amenable to a specific tissue culture type, whereas in others, only a specific genotype or wild relative can be used for tissue culture initiation and, therefore, transformation. Expanding the range of genotypes within a species that undergo the requisite tissue culture process would provide a major contribution to improving the transformation system. This may be accomplished by the conventional empirical approach of manipulating culture media composition, phytohormones, explant source, and tissue culture environment. Alternatively, ectopic expression of genes or legume homologs that promote vegetative-to-embryogenesis transitions such as *WUSCHEL* (Zuo et al., 2002) or BABY BOOM (Boutilier et al., 2002) may enhance embryogenic response in specific legumes and improve their regeneration capacity. Finally, extending the current trend in legume transformation of using meristems as sources of totipotent cells is also likely to be productive. Development of soybean transformation systems from meristems provides an interesting case study illustrating this point. The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee et al., 1988) and shoot multiplication from apical meristems (McCabe et al., 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of axillary meristems in the node (Hinchee et al., 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente et al., 2000; Olhoft et al., 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe et al., 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell et al., 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). It is postulated that the shoot multiplication method is even less limited to specific genotypes compared with the cotyledonary node method. Thus, further exploration of meristem culture systems as targets for transformation in other legumes likely will be productive in expanding the range of genotypes that can be transformed.

There is a current trend toward increasing the use of *A. tumefaciens* for DNA delivery in crop improvement programs compared with microprojectile bombardment. This is driven by recent development of highly virulent strains and binary vectors that are useful for legume transformation and its ease of use and researcher familiarity. There is also the consensus that because *A. tumefaciens* generally only delivers the T-DNA, transgene loci resulting from *A. tumefaciens* infection are less complex than those produced via direct DNA delivery methods. A large number of studies characterizing the infectivity, and thereby the ability to transfer T-DNA, of *A. tumefaciens* strains to different legume genotypes indicate

that there are strain by genotype interactions. This general result indicates that more research into matching *Agrobacterium* strains with legume genotypes will improve transformation efficiency.

Progress in improving legume transformation has also been achieved by increasing *Agrobacterium*-mediated T-DNA delivery via reducing or overcoming factors that inhibit the host-pathogen interaction. The development of super-binary strains with enhanced virulence and the addition of acetosyringone have increased transformation efficiencies. More recently, addition of various thiol compounds to the soybean cotyledonary node cocultivation medium was shown to dramatically increase the number of cells transiently transformed with T-DNA (Olhoft and Somers, 2001; Olhoft et al., 2001) and the production of transgenic plants (Olhoft et al., 2003). This increase appears to be mediated via thiol inhibition of peroxidase and polyphenol oxidase in the explant because iron and copper chelators, inhibitors of the respective enzymes, also increased T-DNA transformation. It will be interesting to learn if thiol compounds improve transformation of other legumes.

The optimization of selection and identification systems is crucial for improving transformation efficiency. For example, in soybean, development of a selection system based on hygromycin B greatly increased transgenic plant production and reduced both the number of non-transformed escapes and time in culture (Olhoft et al., 2003). Extensive evaluation of selection systems for legumes is reflected in the array of selectable marker genes and selective agents shown in Table I. There is no overall trend evident from reviewing the data, suggesting strong interactions between the selection system, culture type, and genotype within a species that require substantial experimentation to optimize.

Certainly, eliminating the requisite tissue culture step for legume transformation would be a great boost in progress toward development of high-throughput systems. However, is the development of non-tissue culture systems for legumes feasible? There are several reports describing legume transformation systems that require reduced or no tissue culture. Chowrira et al. (1996) reported on electroporation of nodal axillary buds in a range of large-seeded legumes resulting in production of transgenic progeny. Trieu et al. (2000) described a seedling and flower infiltration method using *A. tumefaciens* for barrel medic that would be extremely useful in genomics studies. Unfortunately, these methods have not been widely adopted, apparently because they are difficult to reproduce.

At least two approaches for development of non-tissue culture transformation systems can be pursued. Either the floral dip method for Arabidopsis is adapted to legumes or a novel legume-specific system is developed. Certainly, the development of a floral or seedling dip method has merit based on the remarkable success of the Arabidopsis system. However, attempts to use non-tissue culture methods for transformation of other legumes such as soybean have not been successful (Li et al., 2002; A. Bent, personal communication). This may be because the mechanism of the Arabidopsis floral dip method, although well characterized for Arabidopsis (Desfeux et al., 2000), is difficult to translate to species with different floral growth and development characteristics. Thus, further investigations of floral development and gametogenesis in the context of investigating floral dip methods seem necessary for successful transfer of this technology to the legumes.

Novel legume-specific non-tissue culture systems are already being developed in a number of species. The recalcitrance of many legumes to tissue culture initiation and plant regeneration has driven researchers to develop transformation systems that target apical and axillary meristems in the embryonic axis as sources of totipotent target cells. Further development of meristem culture systems and experience in production of transgenic plants from them will likely provide researchers with insights to bypass the tissue culture phase. Minimal tissue culture is required in the meristem multiplication method described by McCabe et al. (1988) for soybean as is the *A. tumefaciens*-based method described for peanut by Rohini and Rao (2000). Further research in developing such new sources of totipotent cells as targets for transformation, especially those that are less dedifferentiated, will require concomitant improvements in DNA delivery and methods for selection or identification of transgenic plants. Substantial progress in those areas has been achieved in legumes, suggesting that non-tissue culture methods for most legumes based on meristem cultures may be feasible. Recent progress in legume transformation suggests that some systems will achieve the transformation efficiencies required for functional genomics applications in the near future.

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