Chapter 7

# Development and Utilization of Transformation in *Medicago* Species

Deborah A. Samac
Stephen J. Temple

## *INTRODUCTION*

The genus *Medicago* includes both perennial and annual species that are important forage crops for livestock. Alfalfa *(Medicago sativa)* is a deep-rooted perennial forage legume grown extensively throughout the world. It is one of the most important crops in the United States, ranking fourth in terms of acreage and economic value following corn, soybeans, and wheat (Barnes et al., 1988). A number of the annual species are used in pastures, as cover crops, and for weed control. One of the annual species, *M. truncatula,* has become a model in legume biology and is the focus of a number of genome projects (Cook, 1999; Frugoli and Harris, 2001).

In contrast to many legumes and other forages, alfalfa and several of the annual *Medicago* species can be regenerated relatively easily from tissue culture and are thus amenable to transformation by *Agrobacterium*. This chapter will review the methods used to transform *Medicago* species, the expression patterns of promoters tested, and traits introduced for crop improvement, as well as provide a case study for posttransformation breeding required to develop a commercial transgenic variety of alfalfa.

## PLANT REGENERATION

### *Alfalfa Tissue Culture Systems*

Alfalfa regeneration in tissue culture occurs via somatic embryogenesis either indirectly from callus cells or directly from leaf explants. Indirect somatic embryogenesis in alfalfa was first described by Saunders and Bingham (1972) and shown to be genotype dependent. Because alfalfa is an open-pollinated plant, each variety is a highly heterogeneous and heterozygous population. Only a small percentage of plants within most varieties are able to form somatic embryos in tissue culture (Bingham et al., 1988). In a study of 35 alfalfa varieties that encompass the spectrum of alfalfa germplasm, Mitten and colleagues (1984) found regenerable genotypes in seven of nine germplasm sources. The varieties with the greatest number of individuals capable of regenerating in culture were from the germplasm sources 'Ladak' and 'Turkestan'. In a study of 76 varieties, Brown and Atanassov (1985) found that approximately 34 percent of the varieties had genotypes with some degree of somatic embryogenesis. The varieties with greater proportions of regenerating plants had strong genetic contributions from *M. sativa* ssp. *falcata,* the germplasm source 'Ladak', and *M. sativa* ssp.×*varia*. Several studies have shown that regenerating individuals can be obtained from most varieties by screening large numbers of plants (Desgagnés et al., 1995; Fuentes et al., 1993; Matheson et al., 1990; Saunders and Bingham, 1972). In fact, the highly regenerable variety 'Regen-S', in which approximately 67 percent of the genotypes will regenerate in culture, was developed by recurrent selection for regeneration from plants selected from the varieties 'DuPuits' and 'Saranac', which have relatively low proportions of regenerating genotypes (Bingham et al., 1975). Although direct regeneration can be a more rapid technique than indirect regeneration (Denchev et al., 1991), most transformation systems have utilized indirect embryogenesis protocols. Only recently a protocol was described for transformation using direct embryogenesis (Shao et al., 2000).

Several studies concluded that the ability of alfalfa genotypes to regenerate in culture is controlled by two independent dominant and complementary loci. Reisch and Bingham (1980) proposed that em-

bryo formation in a diploid alfalfa was controlled by two dominant genes. Similarly, embryogenesis was shown to be under the control of two dominant genes with complementary effects in tetraploid alfalfa genotypes selected from 'Ladak' and 'Lahontan' (Wan et al., 1988). These conclusions were confirmed with regenerating genotypes selected from 'Rangelander' (Hernández-Fernández and Christie, 1989) and 'Rangelander' and 'Regen-S' (Kielly and Bowley, 1992). Cloning and characterization of the loci controlling regeneration would provide significant insight into the nature of totipotency in alfalfa and other plants. A random amplified polymorphic DNA (RAPD) marker has been identified that is associated with somatic embryogenesis in alfalfa (Yu and Pauls, 1993). The development of chromosome maps and genomics tools should make it possible to map and clone the genes controlling regeneration and embryo formation.

Although certain varieties have a high regeneration capacity, the genotypes that regenerate in culture may not necessarily have all the desired agronomic traits. The genotypes most frequently reported in transformation experiments are derived from 'Rangelander', 'Regen-S', and 'Regen-SY' (Bingham, 1991). If these genotypes are used for transformation, backcrossing to other varieties is required to alter dormancy and increase yield, persistence, and disease resistance. Micallef and colleagues (1995) showed rapid improvements in yield in two to three backcrosses of transgenic lines to elite alfalfa lines. Alternatively, because the regeneration trait is highly heritable (Bingham et al., 1975), it can be introgressed into breeding lines and those plants used as stock plants in transformation experiments (Bowley et al., 1993). Transformation and regeneration of individuals selected from commercial breeding lines has also been reported (Desgagnés et al., 1995; Matheson et al., 1990; Tabe et al., 1995) and may facilitate development of improved transgenic alfalfa varieties.

Regeneration is highly influenced by the developmental state of explants, environmental conditions, and media components. Regeneration systems use either a two- or three-step procedure. In the two-step procedure, callus is initiated on a basal medium with 2,4-dichlorophenooxyacetic acid (2,4-D) and kinetin followed by transfer of calli to hormone-free medium for initiation and differentiation of embryos (Austin et al., 1995; Desgagnés et al., 1995; Hernández-

Fernández and Christie, 1989; Saunders and Bingham, 1972; Wan et al., 1988). In the three-step process, callus is initiated on a medium with either 2,4-D or napthaleneacetic acid (NAA) and kinetin, transferred to a medium with a high 2,4-D content for a short period, then transferred to hormone-free medium (Brown and Atanassov, 1985; Skokut et al., 1985; Walker et al., 1978).

The optimal medium for regeneration has been shown to vary with genotype, and several amendments to the basal medium increase embryo induction from regenerable genotypes. In all genotypes, incorporation of 2,4-D in the induction medium is critical for initiation of embryogenesis (Finstad et al., 1993). Optimal regeneration occurs on a medium without growth regulators containing 12.5 to 100 mM $NH_4^+$ (Walker and Sato, 1981) or amended with 30 mM alanine or proline (Skokut et al., 1985). Once embryos form, they are removed from the callus, usually at the cotyledonary stage, and placed on a hormone-free medium for conversion to plantlets. Some genotypes were shown to have a higher rate of conversion when exposed to a conditioning treatment that included abascisic acid (ABA), glutamine, and sucrose (Hindson et al., 1998). In transformation protocols, optimization of embryogenesis and conversion is of particular concern if the genotypes being used for production of transgenic plants have a relatively low efficiency of regeneration. In addition, cocultivation with *Agrobacterium* may negatively effect the efficiency of embryo formation (Desgagnés et al., 1995).

Wenzel and Brown (1991) concluded that alfalfa somatic embryos arise from epidermal and subepidermal cells of petiole explants. This suggests that the cells developing into somatic embryos can be easily accessed by *Agrobacterium* for T-DNA transfer during a cocultivation step. Embryos developing after cocultivation are most likely the result of a single transformation event since somatic embryos probably arise from a single cell. However, many genotypes are capable of secondary embryogenesis, as was first noted by Saunders and Bingham (1972). Thus, cultures should be managed to limit repetitive embryogenesis and plants evaluated to ensure that they are derived from independent transformation events. Alternatively, somatic embryos can be used as the explant material for cocultivation (Ninkovic et al., 1995).

## *Regeneration of Annual* Medicago *Species*

Somatic embryogenesis has been described for several annual *Medicago* species including *M. lupulina* (Li and Demarly, 1995), *M. polymorpha* (Scarpa et al., 1993), *M. suffruticosa* (Li and Demarly, 1996), and *M. truncatula* (Nolan et al., 1989); however, transformation systems have been described only for *M. truncatula*. Although the annual *Medicago* species are closely related to *M. sativa*, the protocols for somatic embryogenesis have distinct differences. Alfalfa regeneration protocols do not promote regeneration of *M. truncatula* (Nolan et al., 1989) or *M. lupulina* (Li and Demarly, 1995), and amendments to media that increase embryogenesis in alfalfa, such as addition of proline or 50 mM $NH_4^+$, increase embryo mortality of *M. truncatula* (das Neves et al., 1999). In contrast to alfalfa, in which auxin is necessary for induction of embryos, in the annual species, cytokinins are required for embryoid formation (Li and Demarly, 1995).

*Medicago truncatula* can be regenerated by indirect somatic embryogenesis or by direct shoot organogenesis. Embryogenesis is genotype specific, and most research has focused on optimizing regeneration from a few highly regenerable seed lines (Hoffmann et al., 1997; Trinh et al., 1998; Rose et al., 1999). Embryogenic callus of *M. truncatula* is initiated from explants on a medium containing NAA and benzylaminopurine (BAP) (Nolan et al., 1989), 2,4-D and BAP (Chabaud et al., 1996), or 2,4-D alone (Trinh et al., 1998) in darkness. Transfer of calli to a hormone-free medium stimulates embryogenesis (Nolan and Rose, 1998). Embryos emerging from calli are removed to fresh medium for plantlet conversion. The highly embryogenic lines of *M. truncatula* can form prolific secondary embryos that prevent conversion of the primary embryo. Efficient conversion of embryos has been the greatest challenge for optimizing regeneration systems in *M. truncatula*. If embryos develop in darkness, the rate of primary embryo conversion was found to be higher (Nolan and Rose, 1998). Furthermore, if embryos are removed from the callus at the torpedo stage and placed on fresh medium, secondary embryogenesis is decreased (das Neves et al., 1999). Production of plants from somatic embryos takes three to ten months depending on genotype. In contrast, shoot organogenesis is more rapid, requiring approximately 2.5 months of culture to produce plantlets, and is

highly efficient, avoiding the problem of formation of secondary embryos. Shoot organogenesis from cotyledonary explants has been demonstrated for plants from the cultivar Jemalong and can be adapted to *Agrobacterium*-mediated transformation (Trieu and Harrison, 1996).

## *PLANT TRANSFORMATION*

### *Transformation of Alfalfa*

#### Agrobacterium tumefaciens-*Mediated Transformation*

Alfalfa has been transformed using a number of different methods; however, the most common procedure involves cocultivation of explants with *Agrobacterium tumefaciens*. Early experiments focused on the parameters for efficient and rapid production of transgenic alfalfa plants. For many alfalfa genotypes, efficient transformation was found to depend significantly on the strain of *A. tumefaciens* used. The first report of transformation of *M. sativa* involved cocultivation of stem sections from plants of the variety CUF101 with the disarmed *A. tumefaciens* strain LBA4404 (Shahin et al., 1986). The first report of transformation of *M. sativa* ssp.×*varia* involved cocultivation of alfalfa stem explants with *A. tumefaciens* A281, an armed strain that retains the tumor-inducing genes (Deak et al., 1986). Chabaud and colleagues (1988) also transformed *M. sativa* ssp.×*varia* using either A281 or the disarmed strain LBA4404 and found that strain A281 increased the number of calli giving rise to transgenic embryos twofold compared to LBA4404. Du and colleagues (1994) evaluated four strains of *A. tumefaciens,* three armed strains (A281, C58, and C58-R1000) and one disarmed strain (GV3101) with three genotypes of alfalfa. Although each genotype was previously selected for efficient regeneration, cocultivation substantially reduced embryogenesis and only one strain-genotype combination resulted in transgenic plants. A strong strain-genotype interaction was observed in a similar study by Desgangés and colleagues (1995). All three genotypes tested produced transgenic embryos when transformed with strain LBA4404, two genotypes produced transgenic embryos after cocultivation with strain A281, while only one genotype produced transgenic embryos with strain C58. Clearly, the optimal genotype-strain combination is

a primary factor to consider in strain selection for a high transformation frequency. Cocultivation of explants above a feeder culture, a layer of alfalfa suspension culture cells on the agar medium covered by sterile filter paper moistened with liquid culture medium, has been used in alfalfa transformation and may reduce the negative responses of explants to *Agrobacterium* infection.

The length of the cocultivation period has also been shown to affect transformation frequency (Austin et al., 1995; Samac, 1995). Longer periods of cocultivation, up to eight days, may increase the frequency of transformation due to the delay in applying selection pressure. However, the optimal cocultivation period is most likely genotype dependent, and regrowth of some *Agrobacterium* strains may be more difficult to control following longer cocultivations.

The most widespread selectable marker in alfalfa transformation is the neomycin phosphotransferase *(nptII)* gene that confers kanamycin resistance; however, transgenic alfalfa with resistance to hygromycin or the herbicide phosphinothricin (PPT) has also been produced (D'Hulluin et al., 1990; Tabe et al., 1995). A concentration of 10 to 50 $mg \cdot L^{-1}$ kanamycin was found to prevent callus formation from explants that did not undergo transformation (Chabaud et al., 1988; Desgagnés et al., 1995), and most transformation systems use 25 to 100 $mg \cdot L^{-1}$ kanamycin in media for callus growth, embryo formation, and plantlet development. High amounts of kanamycin have been observed to inhibit transgenic callus growth and embryo production (Desgagnés et al., 1995) and may inhibit root formation (Austin et al., 1995). Transformation of alfalfa with the *bar* gene encoding phophinothricin acetyl transferase and direct selection on medium containing phophinothricin increases the frequency of transformation by practically eliminating nontransformed escapes (D'Hulluin et al., 1990; Tabe et al., 1995).

Although an array of variables must be evaluated to optimize *Agrobacterium*-mediated transformation, systems have been developed for rapid and efficient production of substantial numbers of transgenic alfalfa plants. Over the past seven years, scientists in the USDA-ARS-Plant Science Unit and collaborators have expressed more than 70 different constructs in alfalfa and produced thousands of transgenic plants using a relatively simple system based on that of Austin and colleagues (1995). Using this system, more than 75 per-

cent of the callus pieces produce multiple transgenic embryos, and transgenic plantlets can be generated within nine to 12 weeks. This system uses leaf explants from a highly embryogenic plant selected from 'Regen-SY' (Bingham, 1991). Although stem and petiole explants can be used, leaf explants give a more uniform and rapid callusing response and more rapid production of embryos. The stock plants are vegetatively propagated in vitro on Murashige and Skoog (MS) medium without hormones (Murashige and Skoog, 1962) and cultured at 25°C with a light intensity of approximately 100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. For production of explants, plants are transplanted to a soil mix and maintained in a growth chamber under conditions to maximize leaf production: 24°C/19°C day/night temperature, 16 hours of light per day with a light intensity of approximately 300 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. Plants are fertilized weekly with a complete fertilizer and watered daily. Leaves from the top second to fifth node are surface disinfested by a brief rinse in 70 percent ethanol followed by gentle agitation in a 20 percent bleach solution for 90 seconds. After three rinses in sterile water, leaflet margins are trimmed away with a scalpel and the leaflet piece is cut in half. In vitro grown plants can be used for explant material, however the tissues are fragile and more sensitive to handling and cocultivation. Leaflet pieces are placed in 12 ml of liquid SH medium without hormones (Schenk and Hildebrandt [SH], 1972). After sufficient pieces have been prepared, 3 ml of an overnight culture of *A. tumefaciens* strain LBA4404 containing the vector of interest is added to the leaf pieces and incubated at room temperature for 10 to 15 minutes. The leaf pieces are removed from the inoculum, blotted briefly on sterile filter paper, and placed on the surface of a callus-inducing medium. The medium contains B5 salts and vitamins (Gamborg et al., 1968), 30 $g \cdot L^{-1}$ sucrose, 0.5 $g \cdot L^{-1}$ $KNO_3$, 0.25 $g \cdot L^{-1}$ $MgSO_4$-$7H_2O$, 0.5 $g \cdot L^{-1}$ proline, 798 $mg \cdot L^{-1}$ L-glutamine, 99.6 $mg \cdot L^{-1}$ serine, 0.48 $mg \cdot L^{-1}$ adenine, 9.6 $mg \cdot L^{-1}$ glutathione, 1 $mg \cdot L^{-1}$ 2,4-D, 0.1 $mg \cdot L^{-1}$ kinetin, and 7 $g \cdot L^{-1}$ Phytagar (Gibco), pH 5.7. The plates are sealed with gas-permeable tape (#394 3M Venting Tape) and placed in an incubator at 24°C with a light intensity of approximately 100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. After seven days of cocultivation the leaf pieces are removed from the plate, rinsed three to four times in sterile distilled water, and placed on the surface of fresh callus-inducing plates containing 25 $mg \cdot L^{-1}$ kana-

mycin and 100 mg·L$^{-1}$ ticarcillin. Plates are sealed as before and returned to the incubator. After two to three weeks callus clumps are transferred to a modified hormone-free MS medium (MMS) containing MS salts, 30 g·L$^{-1}$ sucrose, vitamins as described by Nitsch and Nitsch (1969), 100 mg·L$^{-1}$ myo-inositol, 7 g·L$^{-1}$ Phytagar, plus the same antibiotics and cultured for an additional two to three weeks. As cotyledonary stage embryos arise from the calli, they are removed and transferred to fresh MMS medium with antibiotics for conversion to plantlets. When the plantlets have formed a primary leaf, they are moved to a Magenta vessel containing fresh MMS with ticarcillin but without kanamycin for further root and shoot development. Rooted plants are removed from the medium after two to five weeks and the roots placed in a test tube of water on the lab bench for 24 to 48 hours to condition the plants. The plants are then planted in a potting medium and placed in a growth chamber. The conditioning step reduces leaf desiccation, although some leaf loss occurs after transplanting.

### *Transformation by Particle Bombardment*

Due to the ease of *Agrobacterium*-mediated transformation of alfalfa, biolistic transformation (particle bombardment) has received less attention than in crops that are recalcitrant to *Agrobacterium* transformation. Nonetheless, direct delivery of DNA into alfalfa cells has several important applications. One potential avenue to circumvent tissue culture steps and decrease the time and labor involved in generating transgenic plants is particle bombardment of pollen. Ramaiah and Skinner (1997) described a method of bombarding alfalfa pollen that was subsequently used to pollinate male sterile flowers. Approximately 27 percent of the plants obtained showed integration of the *gus* gene by DNA blot hybridization. Blotting results indicated that some plants contained multiple inserts as well as truncated copies of the *gus* gene, a frequent consequence of particle bombardment. Unexpectedly, after vegetative propagation, some lines appeared to lose the integrated *gus* gene and in others the copy number decreased.

Transformation of chloroplasts has been accomplished in several model systems using particle bombardment of leaf explants and has several advantages over nuclear transformation (Bogorad, 2000). Foreign proteins can accumulate to very high amounts because multi-

ple copies of the gene can be introduced. Integration into the chloroplast genome occurs by homologous recombination, which eliminates the "position effect" frequently observed in nuclear transformants. In many crops, plastid transformation also addresses concerns about gene escape through pollen or gene expression in pollen. Alfalfa would not have this advantage because plastid inheritance in alfalfa is biparental with a strong paternal bias (Smith et al., 1986). Nevertheless, chloroplast transformation would greatly facilitate projects in which expression of a large amount of a target protein is desired, such as for production of industrial enzymes or biodegradable plastics. Pereira and Erickson (1995) described stable nuclear transformation of alfalfa by particle bombardment, indicating that chloroplast transformation of alfalfa can be achieved.

## *Transformation of* Medicago truncatula

### *Transformation by* Agrobacterium *Cocultivation*

The first report of transformation of *M. truncatula* showed that transformation could be achieved using either *A. tumefaciens* or *A. rhizogenes* (Thomas et al., 1992). However, transformation frequencies using *A. rhizogenes* were low and most embryos did not develop past the globular stage, suggesting that the genes on the *A. rhizogenes* plasmid (pRi) have a negative effect on somatic embryogenesis. Transformation of several genotypes has been achieved by cocultivation of leaf explants with *A. tumefaciens* (Chabaud et al., 1996; Trinh et al., 1998; Wang et al., 1996). Trinh and colleagues (1998) investigated different growth regulators to develop a rapid and highly efficient transformation protocol. A suspension of the disarmed *A. tumefaciens* strain EHA101 or GV3101 is vacuum infiltrated into explants of the highly regenerable line, R108-1(c3), and cocultivated on agar medium with the virulence gene inducer acetosyringone in the dark for three days. Explants are cultured for five weeks in darkness for callus and embryoid induction, then transferred to medium in the light for embryo development. With this protocol, large numbers of somatic embryos can be obtained from leaf (Trinh et al., 1998) and floral explants (Kamaté et al., 2000) and up to 80 percent of the embryos regenerate into plants three to four months after culture initia-

tion. This highly efficient transformation method has enabled initiation of a T-DNA insertional mutagenesis program for *Medicago truncatula* (Scholte et al., 2002).

Rapid transformation of *M. truncatula* has been described based on cocultivation of *A. tumefaciens* with cotyledonary explants followed by culture to induce shoot organogenesis (Trieu and Harrison, 1996). Multiple shoots developed from the explants; however, not all shoots were derived from independent transformation events, and the transformation efficiency was low. Use of vectors containing the *bar* gene conferring phosphinothricin resistance coupled with culture of explants on media containing PPT was found to be more efficient for selection of transformants than selection for kanamycin resistance. Transgenic plantlets were produced with the system in 2.5 months.

Two methods have been described that eliminate tissue culture steps in *M. truncatula* transformation. Trieu and colleagues (2000) described a method based on infiltration of flowers with *Agrobacterium,* similar to the *Arabidopsis* flower infiltration protocol (Clough and Bent, 1998), and a method based on infiltration of seedlings. After infiltration of flowers, seed production was fairly low but transformation frequency was relatively high, from 21 to 76 percent of the seeds recovered. The seedling infiltration method allows for the simultaneous transformation of a large number of individuals. A high frequency of transformation was reported, with 9.4 percent of resulting seedlings showing resistance to PPT. Although promising, these results have not been repeated or further extended by this group and they have yet to be corroborated by other laboratories.

## *PROMOTERS FOR CONSTITUTIVE AND TISSUE-SPECIFIC EXPRESSION OF TRANSGENES IN* MEDICAGO

Transformation in *Medicago* species has been used to characterize genes from a range of legumes and for crop improvement. For some traits, the expression pattern desired is constitutive, whole-plant expression. For other traits, expression limited to specific organs or upon certain environmental cues may be required. A wide variety of promoters have been described from crop species, but relatively few have been tested for use in alfalfa.

### *Constitutive Promoters*

Undoubtedly the most frequently used promoter in genetically modified plants is the 35S promoter from the cauliflower mosaic virus (CaMV). Early studies showed that in virus-infected plant cells, this promoter directs high constitutive expression of a viral gene encoding a 35S RNA without requiring additional viral products (Guilley et al., 1982). When 343 bp of sequence upstream of the protein coding region of the 35S gene is fused to a marker gene such as β-glucuronidase *(gus)* and inserted into a plant chromosome, the gene is strongly expressed in nominally all cells. In contrast to the strong activity in other plant species, several reports suggest that the 35S promoter may have less activity in alfalfa (Khoudi et al., 1997; Narváez-Vásquez et al., 1992; Tabe et al., 1995). In leaves of tobacco plants containing a 35S::*gus* gene, GUS activity is reported to range from 5,000 to 200,000 pmol 4-MU·min$^{-1}$·mg$^{-1}$ protein. As shown in Figure 7.1, the amounts of GUS activity in alfalfa leaves with a 35S::*gus* gene are considerably lower than the GUS activity in tobacco, even though alfalfa leaves contain approximately five to ten times more soluble protein per unit fresh weight than tobacco leaves. In addition, not all alfalfa cells express the 35S::*gus* gene. In stems,

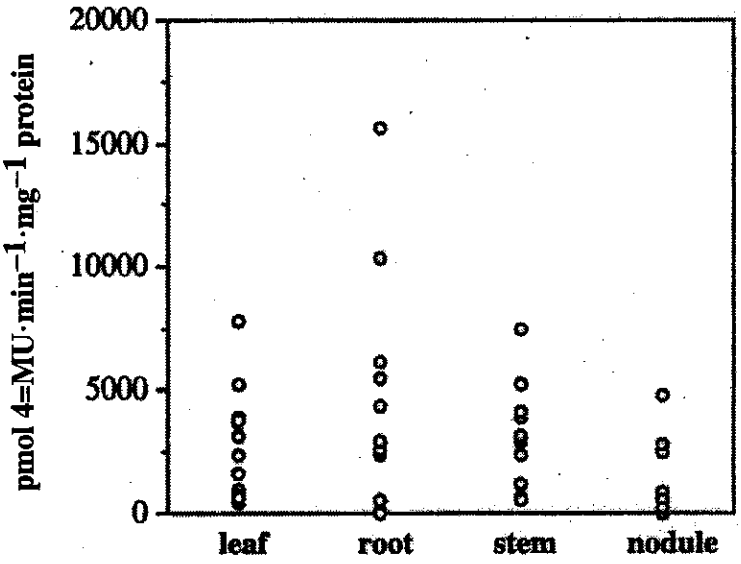


FIGURE 7.1. GUS expression in alfalfa plants containing a 35s::*gus* transgene. Each circle represents the mean value obtained from an individual transgenic plant.

activity is found in the epidermis, chlorenchyma, phloem, and cambium but rarely in xylem or pith cells (Figure 7.2A). In roots and nodules, activity is limited in most plants to vascular tissues. The Mac promoter, a chimeric promoter with elements from the 35S promoter and mannopine synthetase promoter, has been shown to have greater activity than the 35S promoter in model systems (Comai et al., 1990). It has been used for production of proteins that accumulate to high levels in alfalfa (Austin-Phillips and Ziegelhoffer, 2001). Although differences in expression may be dependent on the gene product, additional constitutive promoters should be investigated for expression of genes in alfalfa for biotechnological applications.

### *Expression of Heterologous Promoters in Alfalfa*

Tissue-specific and inducible promoters from other plant species must be tested empirically in alfalfa as the expression pattern may differ from that in the original plant species. Table 7.1 lists the ex-

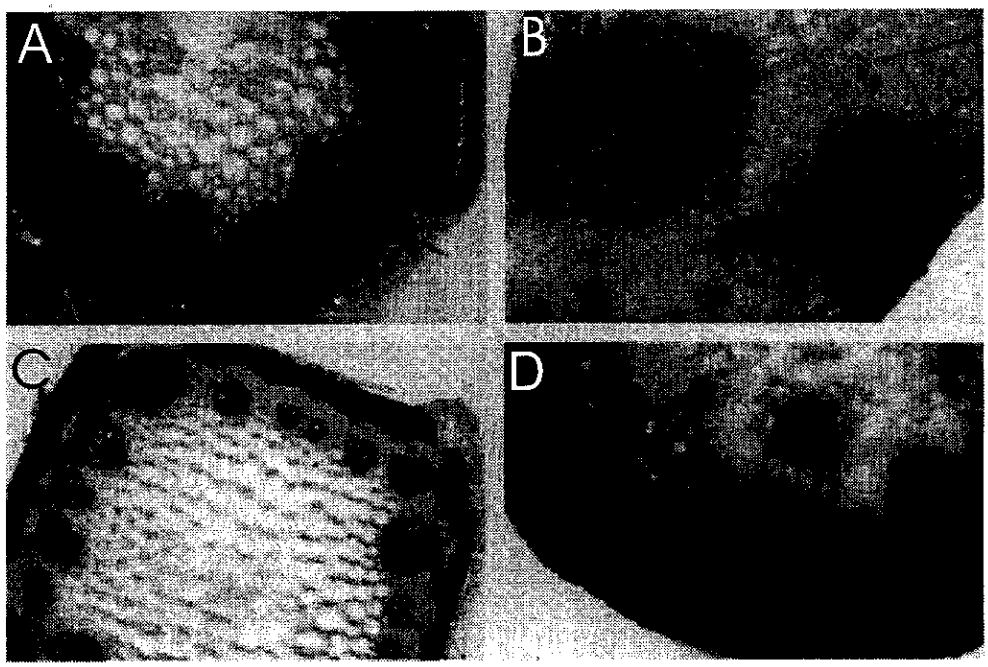


FIGURE 7.2. Expression patterns of three promoters in alfalfa: (A) constitutive expression of the 35S::*gus* gene in alfalfa stem; (B) constitutive expression of the *Arabidopsis* class III chitinase promoter in alfalfa stem; (C) induction of the *Arabidopsis* class III chitinase promoter in alfalfa leaves by infection with *Phoma medicaginis;* (D) constitutive expression of the PEPC-4 promoter in alfalfa stem.

pression patterns of heterologous promoters in alfalfa. The Blec4, PsUGT-1, PAL2, and TA29 promoters had expression patterns similar to those in the original plant species. For example, the TA29 promoter when fused to the *bar* gene resulted in production of barnase in alfalfa anthers, leading to male sterility (Rosellini et al., 2001). In contrast, the *Arabidopsis* class III chitinase promoter and potato *pin2* promoter had unexpected expression patterns in alfalfa. In *Arabidopsis,* the class III chitinase promoter has enhanced expression in roots and expression is induced by fungal pathogens (Samac and Shah, 1991). When the *Arabidopsis* class III chitinase promoter::*gus* gene is expressed in alfalfa, GUS activity is limited to vascular tissue, particularly phloem (Figure 7.2B-C). Interestingly, upon infection by the foliar fungal pathogen *Phoma medicaginis,* GUS activity occurs around lesions. The potato protease inhibitor II *(pin2)* gene promoter is expressed constitutively in root tips of potato. After wounding leaves strong expression occurs in vascular tissue throughout the plant (Keil et al., 1989). In alfalfa plants containing the *pin2-gus* gene, low constitutive activity occurred in leaf and root vascular tissues and root tips or plants had low constitutive activity in leaf mesophyll and root tips. Weak gene induction was seen 24 hours after wounding leaves (Samac and Smigocki, unpublished).

TABLE 7.1. Expression of heterologous promoters in alfalfa

| Gene promoter | Origin | Expression pattern | Reference |
|---|---|---|---|
| Blec4 | *Pisum* | Epidermal cells | Mandaci and Dobres, 1997 |
| PsUGT-1 | *Pisum* | Root meristems | Woo et al., 1999 |
| PAL2 | *Phaseolus* | Vascular tissue | Guo, Chen, Inoue, et al., 2001 |
| TA29 | *Nicotiana* | Anther tapetum | Rosellini et al., 2001 |
| Class III chitinase | *Arabidopsis* | Vascular tissue | This chapter |
| pin2 | *Solanum* | Vascular tissue and mesophyll | Samac and Smigocki, unpublished |

### *Expression of Homologous Promoters in Alfalfa*

Several promoters have been isolated and characterized from alfalfa, primarily from nodule-enhanced genes central to nitrogen and carbon cycling (Table 7.2). The expression patterns of the *gus* gene fusions of aspartate aminotransferase (AAT-1, AAT-2), phosphoenolpyruvate (PEP) carboxylase (PEPC), asparagine synthetase (AS), and NADH-glutamate synthase (GOGAT) were identical or highly similar to mRNA accumulation as determined by RNA blotting and in situ hybridization. Although the expression of these genes is enhanced in root nodules, expression is also observed in other organs and tissues. For example, the full-length promoter (1277 bp) of the PEPC nodule-enhanced gene directs expression in the nitrogen-fixing zone of nodules as well as in root tips, leaf pulvini, and pollen (Pathirana et al., 1997). In experiments to identify promoter elements

TABLE 7.2. Expression of promoters from alfalfa

| Gene promoter | Primary expression pattern | Reference |
|---|---|---|
| Rubisco | Light inducible, leaf tissue | Khoudi et al., 1997 |
| IFR | Root meristem and cortex, nodules; fungal induced | Oomen et al., 1994 |
| AAT-1 | Uninfected nodule cells | Yoshioka et al., 1999 |
| AAT-2 | Infected nodule cells | Yoshioka et al., 1999 |
| PEPC | Infected nodule cells, pulvinal cells | Pathirana et al., 1997 |
| PEPC-4 | Vàscular tissue, xylem cells | Pathirana et al., 1997 |
| AS | Infected and uninfected nodule cells | Shi et al., 1997 |
| GOGAT | Infected nodule cells | Trepp et al., 1999 |
| MAPK | Meristematic cells, glandular hairs | Schoenbeck et al., 1999 |

involved in nodule-enhanced expression, a 536 bp sequence of the PEPC promoter (PEPC-4) was found to be a strong promoter for expression in vascular tissue, particularly xylem (Figure 7.2D). Strong xylem-specific expression is relatively unusual and could be exploited for expression of specific genes in alfalfa for improving nutritional quality of stems for ruminant animals.

A mitogen-activated protein kinase (MAP kinase) gene promoter from alfalfa also has relatively specific activity. MAP kinases are expressed in dividing cells but are also associated with response to stress responses such as wounding, drought, cold, and fungal elicitors. The alfalfa nodule-enhanced MAP kinase promoter directed strong GUS expression in the meristematic cells of the root nodule, the root tip, and in glandular trichomes (Schoenbeck et al., 1999). The gene appears to be especially active in developing nodules and roots. Expression was induced in leaves by mechanical wounding and pathogen infection. With fairly specific and strong root tip expression, this promoter may also be useful for alfalfa improvement.

## *UTILIZATION OF TRANSFORMATION FOR CROP IMPROVEMENT*

### *Crop Plants As "Bioreactors"*

Genetic engineering has the potential to improve the economic value of alfalfa through introducing genes for value-added products such as industrial raw materials. Alfalfa is particularly well suited as a "bioreactor." Once established, alfalfa requires very few agronomic inputs, is well adapted to many environmental conditions, and is highly productive over many years. In addition, processes for fractionation are well established and do not interfere with subsequent use of the residue as an animal food. For a number of industrial materials, production in alfalfa would be a cost-effective alternative to bacterial fermentation. Austin and colleagues (1995) demonstrated expression of two industrial enzymes in alfalfa, α-amylase from *Bacillus licheniformis,* which can be used in starch degradation, and manganese-dependent lignin peroxidase (Mn-P), an enzyme with potential in biopulping processes, from the fungus *Phanerochaete chrysosporium.* Two bacterial cellulase genes, with potential for use in conversion of plant biomass to ethanol, have also been produced in alfalfa (Ziegel-

hoffer et al., 1999). These enzymes were produced at relatively low levels, from 0.01 percent to 0.5 percent of total soluble protein. However, when the gene encoding phytase from *Aspergillus niger* was expressed in alfalfa, a high amount of active enzyme, from 0.1 to 1.5 percent of total soluble protein, was recovered from fresh alfalfa juice and dried plant material (Austin-Phillips and Ziegelhoffer, 2001). Most phosphorus (P) in plant seeds, stored as phytic acid, is unavailable to monogastric animals such as poultry and swine. Phytase releases P from phytic acid and can be added to animal feeds to increase P availability. The enzyme can be delivered inexpensively as a crude alfalfa juice extract or as leaf meal. By eliminating the need to add P to animal diets and utilizing the endogenous phytic acid, the amount of P entering the waste stream is greatly reduced, providing both economic and environmental benefits.

Alfalfa plants have also been developed to produce nonenzymatic, high-value compounds. Wigdorovitz and colleagues (1999) expressed the structural protein VP1 of foot and mouth disease virus (FMDV) in alfalfa. When mice were fed freshly harvested alfalfa leaves from transgenic plants, or were immunized parenterally with leaf extracts, they developed a virus-specific immune response and were protected against challenge with the virus. Production of animal vaccines in a forage plant would be a convenient means to deliver the antigens.

Antihuman IgG is a widely used reagent in blood banks for phenotyping and cross-matching red blood cells. It is usually produced by large-scale culture of murine hybridomas. To develop a less costly source of this reagent, Khoudi and colleagues (1999) expressed full-length cDNAs for the heavy and light chains separately and then intercrossed plants. In plants expressing both chains, the antibody accumulated from 0.13 to 1.0 percent of total soluble protein. In diagnostic assays the antibody produced in alfalfa and the hybridoma-derived antibody reacted similarly. Under field conditions the antibody accumulated to 140 $\mu g \cdot g^{-1}$ dry hay and was stable in dried material for at least 12 weeks after harvest.

An additional nonenzymatic industrial material produced in alfalfa is the biodegradable plastic polymer polyhydroxybutyrate (PHB) (Saruul et al., 2002). This compound is made naturally by many bacteria under nutrient-limiting conditions and can be produced commercially by fermentation. The three genes required for PHB synthesis, *phba, phbb,* and *phbc,* have been expressed in several model plant

species, and PHB accumulates to high levels if the gene products are targeted to chloroplasts. In alfalfa the three genes were placed in the same vector, each controlled by the 35S promoter, and the gene products targeted to the chloroplast. Granules of PHB were observed in chloroplasts and PHB accumulated in leaves to 2 $g \cdot kg^{-1}$ dry weight (Saruul et al., 2002). Production of bioplastics in alfalfa leaves could be combined with use of alfalfa for feed and energy. In such a system, alfalfa leaves could be harvested to produce PHB, leaf by-product could be processed into a feed, and stems could be used for producing either electricity by burning or ethanol by fermentation.

### *Enhancing Nutritional Qualities*

Alfalfa is often called the "queen of forages" because of its excellent nutritional properties for ruminant animals. However, the protein in alfalfa leaves can be digested too rapidly in the rumen and the nitrogen lost by excretion as urea. While leaf tissues are easily broken down, lignin retards digestion of alfalfa stems, limiting feed intake value and negatively impacting milk and meat production. Additional problems include bloating of animals fed fresh alfalfa and insufficient digestible carbohydrates in alfalfa hay to balance the amount of available protein. One approach to improving protein quality is overexpression of genes for proteins resistant to digestion by rumen fluids. Schroeder and colleagues (1991) overexpressed the cDNA for chicken ovalbumin, a rumen-stable, sulfur amino acid rich protein in alfalfa using the CaMV 35S promoter. A similar strategy was used by Tabe and colleagues (1995) in which a cDNA encoding a sunflower seed storage albumin was expressed in alfalfa under the control of constitutive promoters. In plants with the highest expression, protein accumulated to approximately 0.11 percent of total soluble protein; however, the authors estimated that expression needs to be 25- to 50-fold higher to impact wool growth rate in grazing sheep.

Modification of the amount and type of lignin in alfalfa stems has been attempted using several antisense approaches. Baucher and colleagues (1999) decreased cinnamyl alcohol dehydrogenase (CAD) by antisense RNA expression using the CaMV 35S promoter. Activity in plants containing an antisense C-terminal CAD fragment was reduced to 30 percent of the activity in control plants. No significant change in total lignin content was observed, although lignin compo-

sition was altered significantly in several lines. In particular, the syringyl units were decreased compared to the control. In situ digestibility was greater in the transgenic lines compared to controls, indicating that modification of lignin composition can impact forage digestibility. Guo and colleagues (Guo, Chen, Inoue, et al., 2001; Guo, Chen, Wheeler, et al., 2001) found that down-regulation of two other genes in the lignin biosynthetic pathway, caffeic acid 3-*O*-methyltransferase (COMT) and caffeoyl CoA-*O*-methyltransferase (CCOMT) can change significantly the lignin composition in alfalfa stems. When the COMT cDNA was expressed in the antisense orientation under the control of the bean PAL2 promoter, syringyl units could be eliminated in xylem tissue while antisense expression of CCOMT reduced guaiacyl units and Klason lignin. Down-regulation of CCOMT led to a 2.8 to 6 percent increase in forage digestibility (Guo, Chen,Wheeler, et al., 2001). Because a 1 percent increase in digestibility can positively impact animal performance, this is a significant achievement in improving forage quality.

### *Enhancing Tolerance to Biotic and Abiotic Stresses*

Transgenic approaches are being applied to enhance tolerance in alfalfa to the biotic and abiotic stresses for which traditional approaches have had limited success. Alfalfa mosaic virus (AMV) infections can cause yield losses of up to 30 percent dry weight in alfalfa and 80 percent in *M. truncatula*. Hill and colleagues (1991) and Jayasena and colleagues (2001) used AMV coat protein expression to confer resistance to AMV in alfalfa and *M. truncatula,* respectively. In alfalfa, transgenic plants were resistant to the virus under greenhouse conditions; however, expression of the coat protein did not confer a yield advantage under field conditions (David Miller, personal communication). *Medicago truncatula* plants expressing the AMV coat protein demonstrated high levels of resistance or immunity to two strains under greenhouse growth conditions (Jayasena et al., 2001).

A number of phenolic compounds have potent antifungal activity. Hipskind and Paiva (2000) demonstrated that a unique antifungal compound, *trans*-resveratrol-3-*O*-D-glucopyranoside (Rgluc) accumulates in transgenic alfalfa expressing the resveratrol synthase gene from peanut. Growth of the fungal pathogen *Phoma medicaginis* was

inhibited in agar plate bioassays with purified Rgluc and resveratrol. When Rgluc-containing leaves were inoculated with *P. medicaginis,* disease symptoms were reduced compared to control leaves. Rgluc also reduced the number of spore-producing pycnidia in infected leaves, which could reduce spread and severity of the disease within a field. It is likely that Rgluc will also reduce damage to leaves by other foliar pathogens that directly impact yield and quality of alfalfa.

Winter hardiness is a critical trait in a perennial crop for maintaining stand density and dry matter yield. However, it is a very complex trait, involving tolerance to a multitude of stresses. Because generation of superoxide is a common factor in many plant stresses, expression of genes for superoxide dismutase (SOD) has been investigated as a means of reducing oxidative stress and enhancing winter hardiness in alfalfa (McKersie et al., 1999, 2000). Alfalfa plants were transformed with constructs for Mn-SOD targeted to either mitochondria or chloroplasts (McKersie et al., 1999). In most tissues the SOD activity in the highest expressing transgenic plants was approximately twofold higher than in control plants. In laboratory freezing tolerance assays, the most tolerant transgenic plant had only 1°C more freezing tolerance than the control. Nonetheless, in field trials, most transgenic lines had greater survival after one winter and greater total shoot dry matter yield than nontransgenic control lines. Similarly, expression of Fe-SOD in alfalfa was associated with increased winter survival over two years (McKersie et al., 2000).

Tolerance to salt and acid soils will become important in the coming decades. Many agricultural soils, especially those under irrigation, have plant growth inhibiting concentrations of salt. Salt tolerance is also of key importance in developing crops that can be used for disposal of manure or municipal waste water. Winicov (2000) found that constitutive overexpression of *Alfin1,* a putative zinc-finger transcription factor, increases root growth of alfalfa under normal and saline conditions. Because tolerance to salt is most likely a complex trait, modifying expression of multiple genes by controlling expression of key regulatory factors is a promising strategy for enhancing salt tolerance.

Poor crop growth in mineral acid soils is primarily due to aluminum (Al) toxicity, although toxicity to other minerals and nutrient limitations can occur. In an effort to increase Al tolerance in alfalfa,

Tesfaye and colleagues (2001) generated transgenic alfalfa with gene constructs designed to increase synthesis of organic acids to chelate Al. Several lines overexpressing an alfalfa nodule-enhanced malate dehydrogenase had significantly higher amounts of organic acids in root tissues compared to controls and secreted more malate and citrate from roots. In hydroponic assays and in acid soil, these plants had significantly greater root and shoot growth compared to the controls. Development of varieties using this approach would provide alternative methods for managing soil acidity.

## *DEVELOPMENT OF ROUNDUP READY ALFALFA: A CASE STUDY*

The development of crops that are tolerant to glyphosate started in the 1980s and has resulted in the successful commercial release of Roundup Ready corn, soybean, cotton, and canola. Forage Genetics International (FGI) and Monsanto are jointly developing Roundup Ready (RR) alfalfa. The project was initiated in 1997 when an elite FGI alfalfa clone was transformed with a series of Monsanto gene constructs. RR alfalfa will represent the first application for regulatory approval of a genetically modified trait in alfalfa. After regulatory approval has been obtained, which is anticipated for late 2004 in the United States, FGI intends to release RR cultivars adapted to all major U.S. alfalfa markets. The RR alfalfa trait and trademark purity standard will be 90 percent (i.e., 90 percent of the plants in a RR alfalfa variety will demonstrate the RR phenotype). It is anticipated that RR alfalfa will provide producers with an effective weed management tool while retaining a high-quality harvest. A summary of key steps in the development of RR alfalfa including product development, a unique marker-assisted breeding strategy, and issues of pollen flow are discussed.

### *Background*

Glyphosate (*N*-[phosphonomethyl]glycine) is the active ingredient in the foliar-applied, postemergent herbicide Roundup. Glyphosate provides excellent weed-control capabilities and has very favorable environmental and safety characteristics (Padgette et al., 1996). The target for glyphosate is the chloroplast-localized enzyme, 5-enolpyruvyl-

shikimate-3-phosphate synthase (EPSPS). Although EPSPS enzymes from higher plants are sensitive to glyphosate, enzymes isolated from a number of bacterial sources exhibit tolerance. An EPSPS with high tolerance was identified in the *Agrobacterium* sp. strain CP4 (CP4 EPSPS) (Barry et al., 1992; Padgette et al., 1996). The EPSPS gene was cloned from *Agrobacterium* sp. strain CP4 and its suitability for conferring glyphosate tolerance in plants was examined.

The CP4 EPSPS gene was fused to a chloroplast transit sequence (CTP) and placed under the control of the CaMV 35S promoter (P-E35S) and nopaline synthase transcription terminator (NOS 3') (Padgette et al., 1995). Progeny from a selected soybean line transformed with this vector showed no visual injury after application of up to 1.0 gallon/acre of commercial Roundup formulation (Padgette et al., 1995, 1996). Molecular analysis of this line showed it to contain a single insert of DNA with the glyphosate tolerance trait segregating in a Mendelian fashion. The presence of the transgene resulted in no significant yield reduction under field conditions (Padgette et al., 1995, 1996; Delannay et al., 1995). This work led to the development and commercial release of Roundup Ready soybeans in 1996. During 2002 Roundup Ready soybeans were planted on 54.75 million acres or 75 percent of the soybean acreage (http://usda.mannlib.cornell. edu/reports/nassr/field/pcp-bba/).

### *Alfalfa Transformation and Event Sorting*

A vector containing a version of the CP4 EPSPS gene and CTP targeting sequence under the control of a strong constitutive promoter was used to transform the FGI regenerable alfalfa clone R2336. The line R2336 was selected from an elite high-yielding fall dormant FGI breeding population using a tissue culture screen for callus formation and somatic embryo induction. Transformation and plant regeneration was carried out on SH growth media (Schenk and Hilderbrandt, 1972) using standard *A. tumefaciens*-mediated alfalfa transformation and regeneration conditions employing the two-step procedure described previously. A total of 212 $T_0$ plants derived from 122 embryogenic lines were recovered from tissue culture. Plants established from stem cuttings from the $T_0$ lines were evaluated for tolerance to glyphosate, and the best plant from each line was used in an $F_1$ cross to elite FGI material. In a greenhouse screen the progeny from the $F_1$ cross were sprayed with Roundup Ultra at a rate equivalent to 1.0 gal-

lon/acre. Eighty-five of the lines exhibited excellent tolerance to glyphosate, of which approximately 75 demonstrated $F_1$ segregation ratios indicative of single locus T-DNA insertions.

During 1999 an aggressive event-sorting program based on agronomic performance and plant phenotype under glyphosate selection in field and greenhouse conditions was carried out with the remaining lines. Figure 7.3 shows a section of the event-sorting field evaluation. Concurrent with this evaluation a modified back-crossing program was used to introgress the RR transgene into numerous diverse FGI breeding populations. DNA blot analyses combined with $F_1$ segregation ratios were used to identify lines containing single T-DNA insertions. As a final step in the event sorting process a comprehensive molecular analysis of the transgene region in the remaining elite lines allowed the selection of approximately 12 lead events. From these 12 lead events, four lines were subsequently selected for use in the marker-assisted breeding program and product development program.

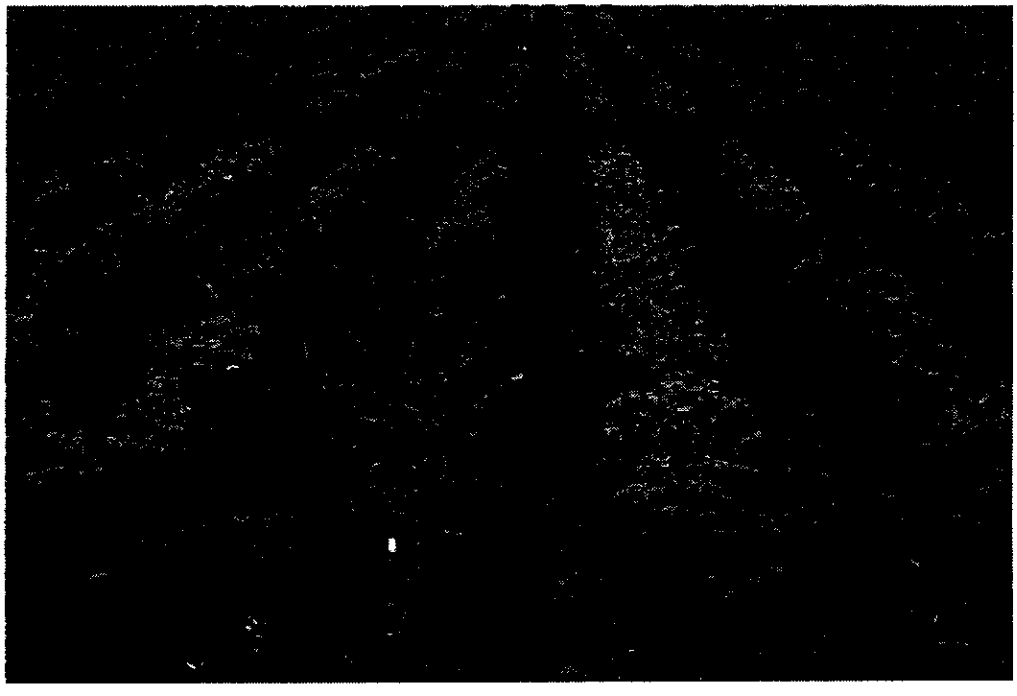

FIGURE 7.3. Field evaluation of Roundup Ready alfalfa lines. In May 1999 selected alfalfa lines were transplanted to the field for evaluation. During the initial growing season Roundup Ultra was applied twice at 1 gallon/acre. A row of control nontransgenic alfalfa has been killed. (*Source:* Sharie Fitzpatrick, Forage Genetics International, September 1999.)

### *Roundup Ready Alfalfa Breeding Strategy*

Genetically modified crop cultivars typically carry a transgene from a single transgenic event (A) such that the transgene is present in the hemizygous state at a single location within the genome of a diploid plant species (i.e., A-). After back-crossing the $T_0$ line to superior agronomic types, high trait purity is easily achieved with the single transgenic event by inbreeding to homozygosity (AA). If these homozygous lines are used as parents in the production of either $F_1$ hybrids or cultivars, 100 percent of resultant plants will have the transgenic phenotype (A- or AA, respectively). By contrast, alfalfa is an insect pollinated, out-crossing autotetraploid ($2n = 4x$). To obtain very high levels of transgene transmission (> 90 to 95 percent) requires an intercrossing of parents with the transgene in duplex (AA--), triplex (AAA-), and/or quadriplex (AAAA) state at the transgenic locus. Although these genotypes can be generated with multiple cycles of phenotypic recurrent selection (PRS), no high throughput laboratory assay can accurately and precisely distinguish between plants with varying doses of a transgene at a single locus [e.g., simplex (A---) versus triplex (AAA-) or quadriplex (AAAA)]. Therefore, progeny testing is required to identify and discriminate between individuals that share the same transgenic phenotype. The intensive selection/progeny testing activities involved in such a program introduces a significant risk of inbreeding depression and/or genetic drift. Such a program presents a genetic funnel that will likely narrow the germplasm base of RR alfalfa. Consequently, significant financial and product performance risk factors are associated with such a breeding program.

An alternative product development approach based on the use of molecular markers has been developed that overcomes many of the challenges discussed earlier. Implementation of this approach also represents a significant reduction in required resources. The approach uses transgenic plants that contain two independent transgenic events (i.e., A---B---) to achieve high levels of transmission of the transgenic trait in autotetraploid alfalfa cultivars. We propose using the term *multihomogenic* to describe concomitant presence of more than one copy of a single transgene at multiple independent loci within the genome of a single plant. Plants that carry at least one copy

of two transgenic events are termed *dihomogenic.* Dihomogenic plants are produced by an $F_1$ cross between a transgenic plant containing only event A and a transgenic plant containing only event B, each in the simplex condition. Under this convention plants with a single allele at each of two loci (A and B) are classified as 1,1-dihomogenic (i.e., A---B---). A plant that is duplex for the transgene at one locus and triplex at another would be 2,3-dihomogenic (AA--BBB-).

For each of the four elite single-copy RR transgenic alfalfa lines described (designated as events A, B, C, and D), the flanking genomic sequence of the T-DNA insertion position was determined. This was achieved using GenomeWalker technology (Clontech, Palo Alto, California) that utilizes suppressor polymerase chain reaction (PCR) technology to walk from a known point within the transgene cassette into flanking alfalfa sequence. The amplification products were then sequenced and a robust event-specific PCR (ES-PCR) assay was developed for each of the four events where one primer anneals to a sequence within the 5' or 3' region of the transgene, while the other anneals within the corresponding flanking region of the plant genome. The ES-PCR assays were coupled to a high-throughput DNA isolation procedure and have been applied to a breeding program for RR alfalfa variety development using pair-wise combinations of the four transgenic events. This high-throughput technique has been used to rapidly screen thousands of RR plants to identify the desired dihomogenic genotype (A---B---) for use as Syn1 parents. Plants identified using the PCR-aided genotypic recurrent selection procedure (GRS) are referred to as $GRS_1Syn0$ population. Plants identified as being dihomogenic can be intercrossed and the Roundup tolerance of the progeny ($GRS_1Syn1$ population) determined. A second cycle of genotypic recurrent selection using ES-PCR can then be used to produce $GRS_2Syn0$ plants. Cycles of phenotypic recurrent selection prior to intercrossing of the events followed by the identification of dihomogenic progeny ($PRS_nGRS_1Syn0$) can be used to increase trait purity. As with the example described, a second cycle of intercrossing and subsequent use of ES-PCR on the progeny can be used to identify a $PRS_nGRS_2Syn0$ population that will give a further increase in trait purity in the subsequent synthetic generations.

A computer model was developed to predict trait purity and inheritance of a dominant transgene in a two-event autotetraploid system (i.e., two loci). This allows the prediction of both genotypic and phenotypic frequencies in Syn1, Syn2, and Syn3 populations resulting from various crosses and selection programs (Table 7.3). For example, it is predicted that 96.8 percent of the Syn3 (certified class seed) plants will exhibit the RR phenotype if the parental line was selected using two cycles of genotypic recurrent selection ($GRS_2$). This example assumes phenotypic selection for Roundup tolerance during the Syn2 and Syn3 production cycles.

### *Validation of the Two-Event RR Breeding Model*

Populations of Roundup-tolerant alfalfa plants were derived from $F_1$ crosses between populations of nondormant plants that had a single copy of an independent RR transgene event (e.g., A--- × B---). In

TABLE 7.3. Comparison of single- and two-event RR product development strategies

| | | Percent RR progeny (w/selection) | | |
|---|---|---|---|---|
| **Selection** | **Syn1 parental genotype** | **Syn1** | **Syn2** | **Syn3** |
| | | *Single-event strategy* | | |
| $PRS_1PT_1$ | Duplex | 93.7 | 94.6 | 95.4 |
| $PRS_3PT_1$ | Triplex/quadriplex | 100.0 | 99.6 | 99.4 |
| | | *Two-event strategy* | | |
| $GRS_1$ | Dihomogenic | 93.7 | 92.7 | 93.8 |
| $PRS_1GRS_1$ | Dihomogenic | 95.3 | 94.5 | 95.3 |
| $PRS_3GRS_1$ | Dihomogenic | 96.8 | 96.3 | 96.6 |
| $GRS_2$ | Dihomogenic | 97.0 | 96.4 | 96.8 |
| $PRS_1GRS_2$ | Dihomogenic | 97.8 | 97.3 | 97.5 |

*Note:* Percent RR phenotype in advanced Syn generations produced by various combinations and number ($n$) of generations of genotypic recurrent selection ($GRS_n$) and/or phenotypic recurrent selection ($PRS_n$). $PT_1$ indicates one generation of progeny testing is required.

this example, three pair-wise event combinations were produced. Approximately two weeks after germination the seedling progeny from the $F_1$ crosses were sprayed with Roundup Ultra and Roundup tolerance values were obtained ($F_1$ percent RR). ES-PCR was used to identify 1,1-dihomogenic plants ($GRS_1Syn0$ plants). The plants identified as being dihomogenic were intercrossed and the Roundup tolerance of the progeny ($GRS_1Syn1$ populations) was determined (Table 7.4). ES-PCR was used to identify dihomogenic plants in the $GRS_1Syn1$ populations. These plants ($GRS_2Syn0$) were intercrossed and Roundup tolerance of the $GRS_2Syn1$ determined similarly and $GRS_3Syn0$ plants were generated. Table 7.4 presents the values predicted by the computer model and experimental data for three event combinations for three cycles of genotypic recurrent selection.

The two-event marker-assisted breeding development program may have an additional advantage when compared to a single-event program using triplex and quadriplex plants containing a single event. A slight reduction in the average gene copy number per plant is required to achieve the desired trait purity; this may reduce the occurrence of homology-dependent gene silencing (Vaucheret et al., 1998). By comparing the experimental phenotypic and genotypic values obtained as part of the breeding program with the model predictive values (Table 7.4) we should be able to identify unstable event combinations that are likely to be silenced in subsequent synthetic generations. A careful analysis of the data generated thus far in the alfalfa RR development program shows no evidence for gene silencing with any of the four elite events through multiple back-crossing cycles and through either PRS or GRS cycles.

### *Using Roundup Ready Alfalfa to Estimate Pollen Flow*

Potential pollen flow between adjacent alfalfa seed production fields is a key factor in determining isolation distances required for commercial seed production. The current isolation requirements are based on measurements made using pest resistance genes to monitor pollen flow (Brown et al., 1986). RR alfalfa provides an excellent analytical tool for monitoring pollen-mediated gene flow between transgenic and nontransgenic seed production fields and also potential flow to feral alfalfa populations. In 2000 the FGI in association with

TABLE 7.4. Experimental Roundup tolerance and event-specific data for nondormant alfalfa populations using the two-event breeding scheme for three cycles of genotypic recurrent selection

| $F_1$ cross | $F_1$ % RR | $GRS_1Syn0$ % dihomogenic | $GRS_1Syn_1$ % RR | $GRS_2Syn0$ % dihomogenic | $GRS_2Syn_1$ % RR | $GRS_3Syn0$ % dihomogenic |
|---|---|---|---|---|---|---|
| A × B | 75.7 | 35.6 | 87.9 | 58.0 | 96.5 | 69.6 |
| B × C | 75.6 | 31.5 | 91.5 | 57.2 | 95.0 | 71.4 |
| A × C | 76.2 | 31.6 | 92.3 | 59.1 | 95.4 | 70.0 |
| Predicted value | 75.0 | 33.3 | 93.7 | 60.1 | 97.0 | 70.1 |

*Note:* Values predicted by the computer model are given for comparison. At the time of publication $GRS_3Syn_1$ data were not available.

the regulatory affairs committee of the North American Alfalfa Improvement Conference (NAAIC) conducted a pollen flow study near Nampa, Idaho (Fitzpatrick et al., 2001). The study measured pollen-mediated gene flow using leafcutter bees as pollinators under seed production conditions typical to the region.

Pollen-mediated gene flow was measured using the CP4 EPSPS gene as a dominant marker in a one-acre pollen source plot to replicated trap plots of conventional alfalfa planted at 500-foot increments (i.e., 500 feet, 1,000 feet, and 1,500 feet) from the source plot. The area between all plots was maintained fallow to provide conditions that would theoretically maximize bee movement. Total pollen-mediated gene flow was 1.39, 0.32, and 0.07 percent at 500, 1,000, and 1,500 feet, respectively. No pollen-mediated gene flow was detected at an additional larger trap plot at 2,000 feet where the intervening land was planted to onions and wheat (Fitzpatrick et al., 2001). A plot of mean gene flow against distance indicates a nonlinear decline (Figure 7.4). However, if the pollen flow values at 500 and 1000 feet are used and the assumption is made that a linear decline in pollen flow occurred between these two points, then it is possible to estimate gene flow at 900 feet (the current isolation distance for foundation class seed). Based on this, pollen flow at 900 feet would be predicted to be 0.53 percent based on a linear decline rate of –0.00214 percent per foot. If pollen-mediated gene flow from a point source decays to zero in a nonlinear manner, then the 0.53 percent predicted value would represent a small overestimation. The calculated value is somewhat lower than the 0.73 percent figure calculated based on a best-fit linear prediction equation (Fitzpatrick et al., 2001). The data from this study support the current foundation class seed field isolation distance of 900 feet as an effective limit for minimal gene flow. As part of the development program for RR alfalfa, FGI will be conducting additional pollen flow studies to determine the required isolation distance for commercial RR seed production.

In another recent alfalfa pollen flow study researchers monitored gene flow from source blocks to alfalfa plants at various distances using a combination of RAPD and gene-specific polymorphic markers (St. Amand et al., 2000). Although it is not possible to compare the results of the two studies directly due to differences in experimental design, several important observations were made in this study. Leaf-

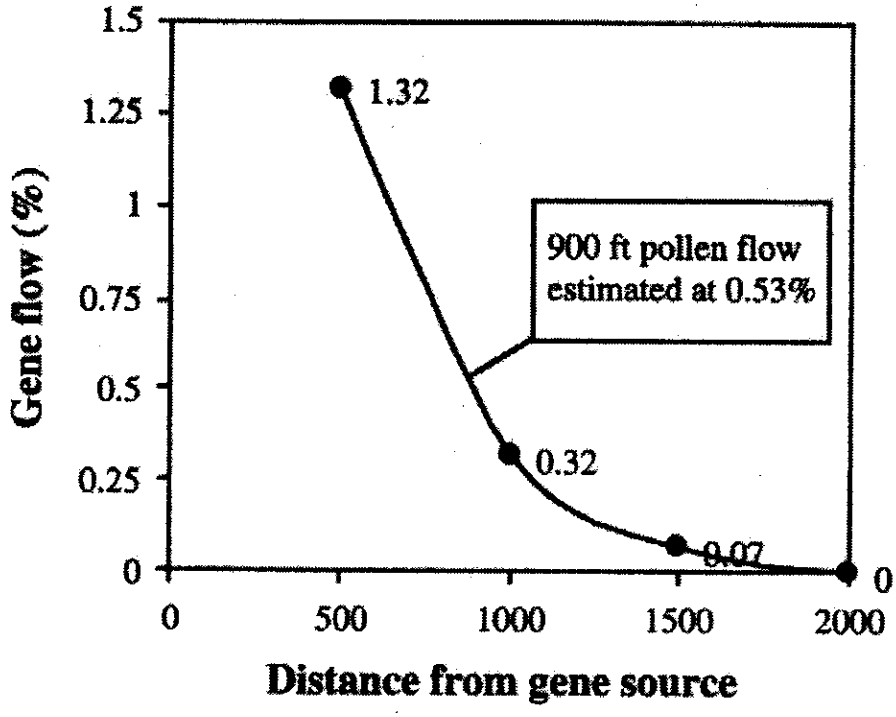


FIGURE 7.4. Plot of gene flow from a Roundup Ready alfalfa source plot to trap alfalfa plots planted at defined distances from the source plot. Pollen flow at 900 feet, the current isolation distance for foundation class seed, is estimated to be 0.53 percent.

cutter bees (*Megachile* spp.) showed a directional, nonrandom bias when pollinating within fields. Within-field movement of pollen was detected only over short distances. Only 0.2 percent gene flow was measured at 4 m and zero at 6 m. Interestingly, 84.6 percent of progeny carrying the marker were located directly north or south of the marker plants transecting the bee domicile, with the majority of pollen movement (61.5 percent) directed toward the domicile. Although the source plot in this study was extremely small, the study does suggest that within-field pollen flow is limited and that as the authors of the study indicated, border areas may provide an effective buffer zone to limit pollen flow (St. Amand et al., 2000). These findings may also affect bee domicile placement. This study also monitored pollen movement from alfalfa fields to small trap plots representing feral alfalfa populations that were planted at regularly spaced distances of up to 1,000 m from the source plot. Under these conditions pollen flow

was measured out to 1,000 m from large production fields (St. Amand et al., 2000). The design of this experiment with regularly spaced unidirectional plants may have created a somewhat artificial situation with the trap plots acting as bee bridges between source and more distant trap plots resulting in a worst-case scenario for pollen flow.

## *CONCLUSIONS*

From studies initiated more than 30 years ago to develop tissue culture systems, more recent research has resulted in the evolution of transformation systems for *M. sativa* and *M. truncatula* as tools to express genes that increase the value of crops and improve crop characteristics. The highly efficient transformation methods for *M. truncatula* will accelerate functional genomics approaches to gene discovery that can be applied to improvement of other legume species. Breeding strategies have been developed and validated for production of commercial genetically modified alfalfa varieties. Development of these tools opens up new avenues for variety development and increases the scope of utilization of forages in agricultural systems.

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