BIOTECHNOLOGY IN PASTURE PLANT IMPROVEMENT: METHODS AND PROSPECTS

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ABSTRACT

In recent years, significant progress has been made in establishing the methodological basis required for the genetic manipulation of the key pasture species which form the foundation of forage and grassland agriculture in temperate climates throughout the world. Reproducible and efficient plant regeneration systems that allow the recovery of fertile and genetically stable plants have been developed. Symmetric and asymmetric protoplast fusion has led to the recovery of interspecific and intergeneric somatic hybrid pasture plants. Transgenic forage grasses and legumes have been obtained by direct gene transfer to protoplasts, microprojectile bombardment technology, and *Agrobacterium*-mediated transformation. These methodologies have opened up opportunities for evaluating novel approaches to pasture plant improvement. These approaches include nutritional improvements through altered biosynthesis of lignin and soluble carbohydrates, the regulated expression of "rumen by-pass" proteins rich in essential amino acids, protection against pathogens and pests through engineered virus resistance and the regulated expression of antifungal and pesticidal proteins, the manipulation of growth and development for improved persistence, improved tolerance to abiotic stresses, and down-regulation of pollen allergens, as well as the use of transgenic plants as bioreactors for the production of heterologous proteins and peptides.

KEYWORDS

forage grasses, forage legumes, *in vitro* culture, plant regeneration, somaclonal variation, somatic hybrids, transgenic plants, molecular markers

INTRODUCTION

Genetic improvement of allogamous self-incompatible pasture plants by conventional plant breeding is slow. Allogamy and self-incompatibility represent a great challenge to the breeder because plants are usually heterozygous, and self-incompatibility limits inbreeding to concentrate desired genes for the rapid development of new cultivars. Biotechnological methods such as anther culture for the generation of haploids, somatic hybridization for combining germplasm from sexually incompatible species and for transferring organellar-coded traits, genetic transformation for the direct introduction of agronomically useful genes, and molecular markers as tools in cultivar identification and marker assisted selection, show promise when considered as part of pasture plant improvement programs. These methods are expected to complement, accelerate or enhance conventional breeding, since they extend the range of sources from which genetic information may be obtained, and offer additional tools for cultivar identification, protection of breeder rights and determination of seed purity.

TISSUE CULTURE

Plant regeneration systems from *in vitro* cultured organs, tissues and cells are now available for economically important forage grasses and legumes. Procedures for meristem and anther cultures, callus cultures, cell suspension cultures and protoplast cultures have been developed over the last 25 years for a wide range of forage species. In particular, significant progress has been made in the establishment of a complement of tissue culture techniques for ryegrasses (*Lolium*), fescues (*Festuca*), alfalfa (*Medicago sativa*) and clovers (*Trifolium*).

Meristem culture. Efficient methods for the *in vitro* culture of vegetative and floral apices have been developed for different forage grasses and legumes: cocksfoot (*Dactylis glomerata*) (Dale, 1977a; Dale and Dalton, 1983), fescues (Dale, 1977a; Dale and Dalton, 1983), ryegrasses (Dale, 1975, 1977a; Dale and Dalton, 1983; Dalton and Dale, 1985), timothy (*Phleum pratense*) (Dale, 1977a; Dale and Dalton, 1983), alfalfa (Cheyne and Dale, 1980), clovers (Barnett et al., 1975; Cheyne and Dale, 1980; Parrott and Collins, 1980; Skucinska and Miszke, 1980) and bird's-foot trefoil (*Lotus corniculatus*) (Tomes, 1979). Although undoubtedly not as commercially compelling as in vegetatively propagated crops, these tissue culture techniques are of potential value in breeding and seed production in forage crops. They show potential for virus eradication, *in vitro* germplasm storage, and clonal propagation. Eradication of viruses through *in vitro* culture of meristem tips has been reported for Italian ryegrass (*L. multiflorum*) (Dale, 1977b), white clover (*T. repens*) (Barnett et al. 1975) and red clover (*T. pratense*) (Phillips and Collins, 1979a). Meristem culture and *in vitro* tiller induction can be used for the propagation of cultivar mother plants stored *in vitro* in order to increase, upon their transfer to soil, the amount of basic seed produced (Dalton and Dale, 1985).

On a routine commercial scale, germplasm storage and plant propagation in forage grasses and legumes is by seeds. However, for certain purposes the maintenance over several years and the vegetative propagation of healthy genotypes is needed. Apical meristems or corresponding plantlets of Italian ryegrass, red clover and white clover have been maintained alive at low temperature (2-6°C) for one to eight years (Dale, 1980; Dale and Webb, 1985; Cheyne and Dale, 1980; Yamada and Okumura, 1997). Cryogenic techniques have been developed for the long-term storage of white clover meristems (Yamada and Okumura, 1997). The maintenance of sexually sterile plants such as haploids or sterile mutants, and of rare aneuploids or plants with unusual and meiotically unstable chromosomal compositions, represent further potential applications for meristem culture.

Severe problems of maintenance - particularly for the short-lived pasture plants - as well as problems of disease accumulation encountered when plants are conventionally field- or greenhouse-propagated can be overcome through the use of meristem culture, *in vitro* storage of aseptic plantlets and clonal propagation (Phillips and Collins, 1984; Dale and Webb, 1985). The effective virus elimination from infected plants, the limited scope for newly induced genetic variation, and an accelerated tiller production by the precocious development of shoots from axillary buds makes the *in vitro* culture of vegetative and floral apices an attractive tool. Furthermore, *in vitro* cultured vegetative and floral apices may be used as targets for genetic transformation using micro-ballistic methods (Pérez-Vicente et al., 1993).

Anther culture and production of haploids. Anther culture and the regeneration of anther culture-derived plants for the production of androgenetic haploids and doubled haploids have been reported for forage grasses belonging to the genera *Bromus* (Saito et al., 1973), *Dactylis* (Saito et al., 1973), *Festuca* (Kasperbauer et al., 1980),

Lolium (Olesen et al., 1988; Boppenmeier et al., 1989; Bante et al., 1990), *Pennisetum* (Haydu and Vasil, 1981), *Phleum* (Abdullah et al., 1994), *Setaria* (Ban et al., 1971) and *Thinopyrum* (Marburger and Wang, 1988). The production of haploid plants via anther culture in forage legumes has only been convincingly demonstrated for *M. sativa* (Zagorska et al., 1990; Zagorska and Dimitrov, 1995). For most forage grass and legume species, the development of anther culture methods is still largely at its infancy, allowing only an erratic and genotype-dependent regeneration of few haploid green plants. If perfected, however, haploid techniques are expected to be more attractive in forage crop breeding in the future. The application of anther culture in forage plant breeding will only be successful if the technique allows for the recovery of sufficient numbers of doubled haploid green plants from a broad range of commercially-useful germplasm. Progress on these lines has been made for perennial ryegrass (*L. perenne*) anther culture (Olesen et al., 1988; Halberg et al., 1990), where knowledge gained on the genetic control of the androgenetic response has opened up opportunities for the incorporation of inducer genotypes in ongoing breeding programs (Opsahl-Ferstad et al., 1994; Madsen et al., 1995). Recent results indicate an improved rate of green plant production in hybrids with breeding lines (Madsen et al., 1995). Thus, the requirement for an increased responsiveness to anther culture in agronomically acceptable germplasm appears in principle to be feasible. If fertile, highly inbred lines from doubled haploids can be established in *L. perenne*, it seems likely that synthetic varieties or F_1-hybrids based on these doubled haploids may be released in the near future.

***In vitro* plant regeneration, callus cultures and somaclonal variation.** Efficient and reproducible protocols for the *in vitro* plant regeneration from young meristematic tissues and the establishment of regenerable callus cultures have been developed for different forage grasses and legumes. Plants have been regenerated from callus cultures in different grass species belonging to the genera *Agropyron* (Lo et al., 1980; Gyulai et al., 1992), *Agrostis* (Krans et al., 1982), *Alopecurus* (Lo et al., 1980), *Andropogon* (Chen et al., 1977), *Bothriochloa* (Metzinger et al., 1987; Franklin et al., 1990), *Bromus* (Lo et al., 1980), *Cynodon* (Ahn et al., 1985), *Dactylis* (Conger et al., 1983), *Digitaria* (Gonzales and Franks, 1987), *Echinochloa* (Wang and Yan, 1984; Takahashi et al., 1984; Samantaray et al., 1995), *Eleusine* (Rangan, 1976), *Elymus* (Park and Walton, 1989a and b), *Festuca* (Lowe and Conger, 1979; Eizenga and Dahleen, 1990; Wang et al., 1993a; Spangenberg et al., 1994a), *Lolium* (Creemers-Molenaar et al., 1988; Wang et al., 1993b), *Panicum* (Lu and Vasil, 1981; Rangan and Vasil, 1983; Denchev and Conger, 1994), *Paspalum* (Bovo and Mroginski, 1986; Akashi and Adachi, 1992a), *Pennisetum* (Vasil and Vasil, 1981; Haydu and Vasil, 1981), *Poa* (McDonnell and Conger, 1984), *Setaria* (Osuna-Avila et al., 1995), *Stenotaphrum* (Kuo and Smith, 1993), *Vetiveria* (Mucciarelli et al., 1993), and *Zoysia* (Asano, 1989). Plants have been regenerated from *in vitro* tissue cultures for different pasture legumes belonging to the genera *Lotus* (Orshinsky and Tomes, 1985; Damiani et al., 1990; Pofelis et al., 1992), *Medicago* (Saunders and Bingham, 1972; Bingham et al., 1975; Walker et al., 1978, 1979; Groose and Bingham, 1984), *Stylosanthes* (Manners, 1988), and *Trifolium* (Oswald et al., 1977; Beach and Smith, 1979; Phillips and Collins, 1979b; Gresshoff, 1980; Parrott and Collins, 1982; Bond and Webb, 1989; White and Voisey, 1994; Beattie and Garrett, 1995).

For the initiation of regenerable callus cultures in forage grasses, different explants containing meristematic cells or showing a developmental proximity to the embryo have been used, for example, isolated mature and immature embryos (Lu and Vasil, 1981; Vasil and Vasil, 1981; Boyd and Dale, 1986), caryopses (Eapen and George, 1990; Franklin et al., 1990), immature inflorescences (Chen et al., 1977; Lo et al., 1980; Vasil and Vasil, 1981; Wang and Vasil, 1982; Tyagi et al., 1985; Bovo and Mroginski, 1986), shoot apices (Wu and Antonovics, 1978; Osuna-Avila et al., 1995), young leaf bases (Haydu and Vasil, 1981; Lu and Vasil, 1981; Conger et al., 1983; Mucciarelli et al., 1993), mesocotyls and leaf stem segments (Rangan, 1976; Cobb et al., 1985; Fladung and Hesselbach, 1986; Shatters et al., 1994). In forage legumes, it has been observed that immature tissue sources generally respond more rapidly than mature tissues, and meristematic tissues tend to regenerate plants more efficiently than non-meristematic ones (Phllips and Collins, 1984). Explants used for *in vitro* plant regeneration in forage legumes were mainly cotyledonary-staged embryos (Maheswaran and Williams, 1984, 1985; Parrott, 1991; Weissinger and Parrott, 1993; White and Voisey, 1994; Beattie and Garrett, 1995), hypocotyls (Mokhtarzadeh and Constantin, 1978; Beach and Smith, 1979; Yamada, 1989), seedling sections (Gresshof, 1980; Phillips and Collins, 1979b), leaves (Damiani et al., 1985), petioles (Phillips and Collins, 1979b) and stolon segments (Bond and Webb, 1989).

In vitro tissue cultures in forage grasses and legumes have been shown to produce adventitious shoots, and somatic embryos that finally lead to the recovery of whole plants. Regeneration from somatic cells in grass callus cultures was found to take place via organogenesis by adventitious shoot formation and subsequent root formation from meristematic regions or by somatic embryogenesis through the formation of embryoids which were capable of germination (Ahloowalia, 1983). In forage legumes, regeneration of alfalfa plants from *in vitro* cultures is almost exclusively via somatic embryogenesis, while regeneration, in other forage legumes such as widely studied species of *Trifolium* and *Lotus*, has been achieved most often via shoot organogenesis (McKersie and Brown, 1997).

Somaclonal variation, the recovery of genetic changes in plants regenerated from tissue culture, has been observed among *in vitro* regenerants for forage grasses and legumes (Phillips and Collins, 1984; Damiani et al., 1990; Duncan, 1996). Regenerants may show considerable variation in morphology, developmental capabilities, and genetic stability. Although plant regeneration from callus cultures offers an opportunity to uncover natural variability and to potentially use this variability as a random, undirected form of mutation breeding for the development of improved cultivars, the potential to exploit the genetically heritable variation to develop novel breeding material in forage crops has been limited. So far only one cultivar, namely *Cynodon dactylon* cv. Brazos R3 with improved fall armyworm resistance has been released in forage grasses (Croughan et al., 1994). Nevertheless, callus cultures show potential to generate useful variation, particularly in apomictic grass species, where the reproductive mode represents a barrier to recombination but allows one to fix agronomically interesting variant genotypes arising from tissue cultures.

Embryogenic cell suspension and protoplast cultures. In the grass species, embryogenic cell suspensions have proven to be a unique source of totipotent protoplasts. Regeneration of soil-grown plants from embryogenic cell suspension cultures and protoplasts have been described for grasses in the genera *Agrostis* (Asano and Sugiura, 1990; Terakawa et al., 1992), *Dactylis* (Horn et al., 1988a), *Festuca* (Dalton, 1988a and b; Takamizo et al., 1990; Wang et al., 1993a, 1994; Spangenberg et al., 1994a), *Lolium* (Creemers-Molenaar et al., 1989; Wang et al., 1993b, 1995), *Paspalum* (Akashi and Adachi, 1992b; Akashi et al., 1993) and *Poa* (Nielsen et al., 1993a and b).

In recent years, the establishment of reproducible and efficient plant

regeneration systems from suspension cells and protoplasts in different pasture grasses, particularly, for fescues and ryegrasses has been greatly advanced (Takamizo et al., 1990; Wang et al., 1993a and b, 1994, 1995; Spangenberg et al., 1994a). Limited progress towards establishing reproducible systems had been obtained from previous studies when a wide range of media constituents were evaluated. In contrast, the use of essentially similar, simple culture media and selected single genotype-derived embryogenic suspension cultures allowed the reproducible regeneration of plants from suspension cells and protoplasts for a wide range of cultivars and genotypes (Takamizo et al., 1990; Wang et al., 1993a and b, 1994, 1995; Spangenberg et al., 1994a). This suggests that the morphogenic competence of the cells, rather than the medium composition holds the key to the successful culture of these graminaceous protoplasts. Efficient plant regeneration systems are based on: a) a genotype screening of approximately 200-2000 seeds or embryos per cultivar for the identification of genotypes producing friable and highly embryogenic callus of single-seed origin; b) the establishment of single genotype-derived highly embryogenic cell suspensions; c) the cryopreservation of established embryogenic suspension cultures in liquid nitrogen for their long-term storage; d) the isolation of morphogenic protoplasts from young highly embryogenic cell suspensions before their ability to regenerate green plants has ceased, and e) the culture of protoplasts in a bead-type system including non-morphogenic nurse cells.

In the forage legume species, where protoplasts isolated from differentiated plant tissues have the ability to de-differentiate and to re-enter the cell cycle, the establishment of embryogenic suspension cultures has been mainly used for the production of artificial seeds (McKersie and Brown, 1997) as well as for *in vitro* selection (Phillips and Collins, 1984; Chaudhary et al., 1994). Totipotent protoplasts have been isolated from different sources, e.g. leaves, cotyledons, hypocotyls, cell suspensions and calluses (Gilmour et al., 1989; Arcioni et al., 1990). Protoplast-derived plants have been obtained in *Lotus* (Ahuja et al., 1983; Webb et al., 1987; Wright et al., 1987; Nizeki et al., 1993), *Medicago* (Kao and Michayluk, 1980; Dos Santos et al. 1980; Arcioni et al., 1982; Mariotti et al., 1984; Gilmour et al., 1989a; Scarpa et al., 1993), *Onobrychis (*Pupilli et al., 1989) and *Trifolium* (Gresshoff, 1980; Grosser and Collins, 1984; Webb et al., 1987; Honkanen and Ryoppy, 1989; Yamada, 1989; Radionenko et al., 1994).

The plant regeneration systems established for forage grasses and legumes provide the experimental basis needed for performing genetic manipulations at the cellular level. They facilitate manipulations such as protoplast fusion for the production of somatic hybrids and cybrids (cytoplasmic hybrids), direct gene transfer to protoplasts and gene transfer via biolistic™ transformation of embryogenic suspension cells for the generation of transgenic plants.

SOMATIC HYBRIDIZATION

Due to the difficulties faced over many years in the culture and regeneration of graminaceous monocot protoplasts (Vasil, 1987), very few attempts have been made in the past to obtain somatic hybrid and cybrid plants in forage grasses (Table 1). Only with the development of efficient protoplast-to-plant regeneration systems, progress in somatic hybridization research was possible (Takamizo and Spangenberg, 1994; Spangenberg et al., 1995a).

Genotypically and phenotypically different intergeneric somatic *Festulolium* hybrid plants have been regenerated from symmetric (for the combination of whole genomes) and asymmetric (for the transfer of partial genomes) protoplast fusions between tall fescue (*F. arundinacea*) and Italian ryegrass (*L. multiflorum*) (Takamizo et al., 1991; Spangenberg et al., 1994b). Protoplasts isolated from morphogenic cell suspensions of *F. arundinacea* and non-morphogenic suspension cultures of *L. multiflorum* were used in these studies (Takamizo and Spangenberg, 1994). A selection scheme based on metabolically inactivated totipotent protoplasts of tall fescue and unirradiated (for symmetric hybridization) or X-ray irradiated (for asymmetric hybridization) non-morphogenic protoplasts of Italian ryegrass was found suitable for the enrichment of protoplast fusion products to generate symmetric and asymmetric somatic hybrids (Spangenberg et al., 1995a). Cloned interdispersed repetitive DNA sequences specific to tall fescue and Italian ryegrass have been successfully used for analyzing the genomic composition of the somatic hybrids (Spangenberg et al., 1995a).

In addition, protocols for the isolation of sperm and egg cells have been partially optimized in perennial ryegrass (Van Ark et al., 1992; Van der Maas et al., 1993a and b, 1994b). Thus, new opportunities for modifying the organellar composition and generating novel nuclear-organellar interactions have arisen.

Among the forage legumes, most effort to obtain somatic hybrid plants has focused on *Medicago* and *Lotus* (Table 1). The lack of somatic hybrid plants recovered in *Trifolium* is related to difficulties in regenerating whole plants from protoplasts (Honkanen and Ryoppy, 1989).

Interspecific somatic hybrid plants have been regenerated in the fusion combinations *L. corniculatus + L. conimbrensis* (Wright et al., 1987), *L. corniculatus + L. tenuis* (Aziz et al., 1990), *M. sativa + M. arborea* (Nenz et al., 1996a), *M. sativa + M. coerulea* (Pupilli et al., 1992) and *M. sativa + M. falcata* (Téoulé, 1983; Mendis et al., 1991), while intergeneric asymmetric somatic hybrids have only been obtained in *M. sativa + Onobrychis viciifolia* (Li et al., 1993). Fusion products were selected based on visual identification, antibiotic resistance as well as the complementation in the hybrids of parental traits such as chlorophyll synthesis, resistance to toxic compounds, and the ability of colony formation (Arcioni et al., 1997). Somatic hybrids were identified and characterized by isozymes, cytological features, DNA markers and morphological traits (Arcioni et al., 1997). While all *Lotus* somatic hybrid plants were completely sterile, the *Medicago* somatic hybrids showed differing levels of auto- and cross-fertility (Arcioni et al., 1997).

Although further efforts are needed for generating and evaluating somatic hybrids in wider combinations, the results achieved have demonstrated that symmetric and asymmetric protoplast fusions provide a powerful tool for directed one-step nuclear genome transfer in intra- or inter-generic combinations in forage grasses and legumes. These techniques show potential for facilitating limited alien gene transfer between sexually incompatible species to complement or enhance conventional wide hybridization programs in forage plants.

GENETIC TRANSFORMATION

Considerable progress has been made in the development of protocols for the production of transgenic forage grasses and legumes over the last decade. Protoplast- and biolistic™-mediated transformation has been the main methods for generating transgenic plants in forage grasses, while *Agrobacterium*-mediated transformation has been widely used for producing transgenic forage legumes.

Direct gene transfer to protoplasts. Transient gene expression studies using transfected protoplasts and the recovery of transgenic plants from protoplasts have been reported for different grass species:

Agrostis alba (Asano et al. 1991; Asano and Ugaki, 1994), *Dactylis glomerata* (Horn et al. 1988b), *Festuca arundinacea, F. pratensis* and *F. rubra* (Wang et al., 1992; Spangenberg et al., 1994a, 1995b; Dalton et al., 1995), *Panicum maximum* (Hauptmann et al. 1987, 1988; Vasil et al. 1988b), *Pennisetum purpureum* and a trispecific hybrid (*P. purpureum* x *P. americanum*) x *P. squamulatum* (Hauptmann et al. 1987; Vasil et al. 1988b) (Table 2). The experimental basis required for the generation of transgenic plants from protoplasts, namely the establishment of: a) efficient protoplast-to-plant regeneration systems, b) conditions allowing for tight *in vitro* selection of transformed clones, and c) appropriate plant expression vectors tested for their functionality in transient expression studies with target protoplasts, is now in place.

Proven transgenic plants from protoplasts have been produced so far in *A. alba* (Asano and Ugaki, 1994), *D. glomerata* (Horn et al., 1988b), *F. arundinacea* (Wang et al., 1992; Dalton et al., 1995), *F. rubra* (Spangenberg et al., 1994a) and *F. pratensis* (Spangenberg et al., 1995b). No proven transgenic plant from protoplasts has yet been described for any *Lolium* species. Stably transformed calli have been recovered in *L. multiflorum* (Potrykus et al., 1985). Regenerated plants were considered transgenic when: a) molecular evidence (e.g. PCR-screening and Southern hybridization data) for the presence of transgene-homologous sequences in genomic DNA isolated from regenerants recovered from selected clones showing expected resistant phenotype; b) molecular evidence (e.g. Southern and *in situ* hybridization data) for the integration of transgene-homologous sequences in non-digested high molecular weight plant DNA; and c) functional assays (e.g. *in vitro* enzyme assay and herbicide spraying) for the transgene product, were provided.

Although transgenic plants from protoplasts have been recovered for different *Festuca* species, current protocols are still suboptimal. Methods allowing for the largely *genotype-independent* generation of *fertile* transgenic plants with mainly *single copy transgene integration* and *appropriate level of transgene expression* are needed. Some progress, such as the development of transfection protocols applicable to protoplasts prepared from single genotype-derived suspension cultures established for a wide range of cultivars, and the development of improved selection regimes allowing a tight selection of transformants with mainly single transgene copies, has been made. However, critical information such as the inheritance of transgenes in transgenic grass plants from protoplasts and corresponding meiotic stability of transgene expression is required. Until this is reproducibly demonstrated, direct gene transfer to protoplasts will largely remain a useful tool for transient gene expression studies.

Biolistic™ and silicon carbide whisker-mediated transformation. Problems encountered with protoplast-based methods for the generation of transgenic plants have prompted the development and application of two alternative techniques for gene transfer in grasses: the *biolistic*™ method and the *silicon carbide whisker*-mediated transformation. *Biolistics*™ or *particle bombardment* transformation technology involves gene delivery into intact plant cells and tissues by high-velocity microprojectiles coated with biologically active DNA (Sanford, 1988). Plasmid DNA can be delivered into intact suspension cultured cells by mixing these in the presence of needle-like whiskers. The collisions between the silicon carbide whiskers and the suspension cells lead to cell penetration, DNA uptake, and stable transformation of plant cells (Frame et al., 1994).

These protoplast-independent DNA delivery methods have been used for transient gene expression studies and the recovery of transgenic plants in different grass species (Table 2). Most reports deal with microprojectile bombardment-mediated delivery of foreign DNA. Morphogenic calli and suspension cells (Spangenberg et al., 1995c and d; Ye et al., 1997), non-morphogenic suspension cells (Hensgens et al., 1993; Van der Maas et al., 1994a), vegetative and floral meristem cells (Pérez-Vicente et al., 1993), and seedlings (Hensgens et al., 1993) have been used as targets for biolistic™ transformation. Transgenic forage grass plants have been obtained by microprojectile bombardment of embryogenic cells in *F. arundinacea* (Spangenberg et al., 1995c), *F. rubra* (Spangenberg et al., 1995c), *L. perenne* (Spangenberg et al., 1995d) and *L. multiflorum* (Ye et al., 1997). No report on the recovery of transgenic grass plants by whiskers-mediated transformation has yet been published.

Although many of the transgenic grasses produced by microprojectile bombardment technology show complex transgene integration patterns, transgenic plants with single copy integration have also been obtained. The stable inheritance of transferred genes in transgenic ryegrass obtained from biolistic™ transformation has been recently demonstrated (Spangenberg et al., 1997).

Surrogate transformation with transformed endophytes. Surrogate transformation of *L. perenne* and *F. arundinacea* by infection of wild type seedlings with transformed *Acremonium* endophyte has been reported (Murray et al., 1992; Tsai et al., 1992) as an additional method allowing the introduction of foreign genes into grass plants. Once the transformed *Acremonium* endophyte is established in the infected wild type grass plant, the foreign genes previously transferred to the endophytic fungus will be maternally transmitted as a consequence of the invasion of the ovule by the fungal hyphae. For biotechnological applications, this approach shows promise for the expression of protective proteins to confer pest resistance. It also allows the biological containment of transgenes due to the strict maternal transmission of the endophyte. However, due to the unfavourable fungal mass/plant mass ratio, and absence of endophyte hyphae in different plant parts (e.g. roots, male floral organs), limitations for other applications are expected. Furthermore, the method shows little scope for uses requiring either the down-regulation of plant gene expression, or the induction of tissue- and cell-specific transgene expression.

***Agrobacterium*-mediated transformation.** Methods for generating transgenic plants in forage legume species via *Agrobacterium tumefaciens*- or *A. rhizogenes*-mediated transformation have been developed for *Lotus angustissimus* (Nenz et al., 1996b), *L. corniculatus* (Jensen et al., 1986; Petit et al., 1987; Tabaeizadeh, 1993; Carsolio et al., 1994; Marsolier et al., 1995; Webb et al., 1994, 1996), *L. japonicus* (Handberg et al., 1994; Thykjaer et al., 1995; Oger et al., 1996), *Medicago arborea* (Damiani & Arcioni, 1991), *M. sativa* (Deak et al., 1986; Shahin et al., 1986; Spano et al., 1987; D'Halluin et al., 1990; Kuchuck et al., 1990; Golds et al., 1991; Du et al., 1994), *M. truncatula* (Thomas et al., 1992; Chabaud et al., 1996), *Onobrychis viciifolia* (Golds et al., 1991), *Stylosanthes humilis* (Manners, 1988; Sarria et al., 1994), *S. guianensis* (Sarria et al., 1994), *Trifolium pratense* (Quesenberry et al., 1996), *T. repens* (White & Greenwood, 1987; Voisey et al., 1994a; Larkin et al., 1996) and *T. subterraneum* (Khan et al., 1994) (Table 2). Protoplast- and biolistic™-mediated transformation has been explored for *Medicago* spp. (Kuchuk et al., 1990; Brown et al., 1994; Pereira and Erickson, 1995), but has not been widely adopted.

Although a cultivar-dependent response has been observed with both *A. tumefaciens* (Du et al., 1994; Samac, 1995) and *A. rhizogenes* (Golds et al., 1991), *Agrobacterium*-mediated transformation has

become relatively routine for some major pasture legume species, e.g. alfalfa, white clover and subterranean clover.

In recent years, the recovery of transgenic cereals by *Agrobacterium* mediated-transformation has been unequivocally demonstrated, e.g. for rice and maize (Hiei et al., 1994; Ishida et al., 1996; Rashid et al., 1996). This success has encouraged the evaluation of this method for producing transgenic pasture grasses.

GENE MARKERS AND MAPPING

The identification of polymorphic loci in pasture plants has come about with the advent of isozymes and DNA-based markers (Hayward et al., 1994).

Isozymes as near-neutral genetic markers have provided a powerful means of cultivar identification in *Lolium* (Hayward and McAdam, 1977; Nielsen, 1980; Gilliland et al., 1982; Kennedy et al., 1985; Nielsen et al., 1985; Quaite and Camlin, 1986; Greneche et al., 1991; Lallemand et al., 1991), *Poa* (Wehner et al., 1976; Wu et al., 1984; Weeden and Emmo, 1985) and *Trifolium* (Collins et al., 1984). They have also proven useful in the identification of interspecific and intergeneric somatic hybrids of *F. arundinacea* + *L. multiflorum* (Takamizo et al., 1991), *L. corniculatus* + *L. conimbrensis* (Wright et al., 1987), *L. corniculatus* + *L. tenuis* (Aziz et al., 1990), *M. sativa* + *M. arborea* (Nenz et al., 1996a), *M. sativa* + *M. falcata* (Téoulé, 1983; Mendis et al., 1991) and *M. sativa* + *O. viciifolia* (Li et al., 1993). Furthermore, somaclonal variation in isozyme markers has been evaluated for tissue culture derived plants of *F. arundinacea* (Eizenga, 1987; Dahleen and Eizenga, 1990; Eizenga and Cornelius, 1991; Humphreys and Dalton, 1992; García et al., 1994).

However, the number of genetic markers provided by isozyme assays is limited. This limitation has been superseded by the use of DNA-based markers, such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs). These markers allow the detection of numerous polymorphisms at the molecular level (Helentjaris, 1992).

RAPDs have been used for the identification of cultivars in perennial ryegrass, alfalfa and red clover (De Loose et al., 1993; Yu and Pauls, 1993; Kongkiatngam et al., 1996), for the evaluation of genetic stability in plants regenerated from tissue culture in *Festuca* and *Lolium* (Vallés et al., 1993; Wang et al., 1993b), for fingerprinting alfalfa mutants (Barcaccia et al., 1995), for assessing genetic relatedness and phylogenetic relationships in the *Festuca-Lolium* complex, and in *Medicago* and *Stylosanthes* (Kazan et al., 1993a and b; Yu and Pauls, 1993; Brummer et al., 1995; Stammers et al., 1995), for the identification of cross-pollinated and self-pollinated progenies to estimate outcrossing rates in alfalfa (Gjuric and Smith, 1996), and for population genetic studies in *Medicago* (Crochemore et al., 1996).

RFLP markers have revealed genome divergence and allowed the detection of high levels of polymorphism in *Festuca* and *Lolium* (Xu and Sleper, 1991, 1994; Xu et al., 1992, 1994), and have been used to evaluate genetic relationships of alfalfa accessions (Kidwell et al., 1994). RFLP markers have proven useful to characterize the genetic make-up of *F. arundinacea* + *L. multiflorum* (Spangenberg et al., 1995a) and *M. sativa* + *M. coerulea* (Pupilli et al., 1995) somatic hybrids.

The development of DNA-based markers and genetic maps in pasture grasses has been relatively slow compared to some major cereal crops. Nevertheless, progress has been made in the construction of linkage maps for ryegrass, tall fescue and pearl millet (Evans et al., 1991; Xu and Sleper, 1991; Liu et al., 1994; Hayward et al., 1994). A relatively dense RFLP-based genetic map of pearl millet (*Pennisetum glaucum*), with an average distance of 2cM between markers, has been generated (Liu et al., 1994). In *Lolium*, thirteen linkage groups have been identified based on isozyme, RFLP and RAPD markers (Hayward et al., 1994).

Independent linkage maps of alfalfa, based on different diploid populations and different sets of molecular markers, have been developed (Brummer et al., 1993; Kiss et al., 1993; Echt et al., 1994). A prominent feature of these maps is the large proportion of marker loci that show significant distortion from expected segregation ratios, probably due to the uncovering of deleterious recessive alleles with inbreeding. These maps are currently being integrated, and this process should provide additional markers to help fill in the gaps of all three maps (Osborn et al., 1997). Although the segregation of RAPD markers has been described for a tetraploid F_1 population, a complete linkage map for tetraploid alfalfa has not yet been published (Osborn et al., 1997).

These linkage maps can now be used to locate the genomic positions of genes controlling agronomical traits. The generation of enhanced genetic maps for the major pasture plant species will be essential for trait linkage studies and marker assisted selection.

APPLICATIONS AND PROSPECTS

Significant progress has been made, in recent years, in establishing the genetic manipulation methodologies for forage grasses and legumes. The challenge now is to apply these technologies to generate novel germplasm and to efficiently incorporate this into breeding programs for the development of improved cultivars.

Forage quality, disease and pest resistance, tolerance to abiotic stresses, nitrogen fixation capacity, manipulation of growth and development, as well as the production of foreign proteins of industrial relevance, represent current targets for molecular breeding of pasture plants.

Biotechnological approaches to overcome limitations in forage quality include modification of lignin profile for improved digestibility, expression of 'rumen by-pass' proteins to enhance supply of essential amino acids, and the elimination or reduction of incidence of anti-quality factors and bloat. Since most quality and anti-quality parameters are associated with specific metabolic pathways, target enzymes can be identified, corresponding genes can be isolated, and their expression can be manipulated in transgenic pasture plants.

Molecular breeding for improved digestibility based on modification of lignin profile by down-regulating, through antisense and sense suppression in transgenic plants, the expression of genes encoding enzymes involved in lignin biosynthesis, is being explored. Main target enzymes considered are caffeic acid *O*-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), 4-coumarate:CoA ligase (4CL), cinnamoyl CoA reductase (CCR) and peroxidases (PER). Experiments with the model plant tobacco have demonstrated that the down-regulation of the expression of genes encoding COMT, CAD and 4CL lead to a reduction of lignin content or changes in lignin composition (Ni et al., 1994; Halpin et al., 1994; Atanassova et al., 1995; Boudet et al. 1996; Dixon et al., 1996; Kajita et al., 1996; Bernard Vailhé et al., 1996). cDNA homologues of COMT, CAD and PER have been isolated from *Stylosanthes humilis*, *M. sativa* and *L. perenne* and are being used to down-regulate the

expression of corresponding genes in transgenic pasture plants (Ni et al., 1994; McIntyre et al., 1995; Heath et al., in preparation; J. Watson, pers. comm.).

An alternative approach to improve digestibility and nutritive value in pasture plants has been undertaken based on manipulation of fructan metabolism through the expression of fructosyl-transferase genes to increase levels of soluble carbohydrates and to offset the decline in digestibility due to lignification (Ye et al., in preparation). This modification has also potential to enhance performance under drought-stress (Pilon-Smits et al., 1995).

Different 'rumen-by pass' proteins rich in sulphur containing amino acids have been transgenically expressed in forage legumes (Schroeder et al. 1991; Ealing et al. 1994; Khan et al., 1996) to develop materials for an enhanced supply of limiting essential amino acids for ruminant nutrition. Transgenic alfalfa and white clover plants expressing chimeric chicken ovalbumin and pea albumin 1 genes, respectively, have been generated (Schroeder et al., 1991; Ealing et al., 1994). In both cases, the 'rumen by-pass' protein acccumulated to levels lower than 0.01% of total soluble protein (Schroeder et al., 1991; Ealing et al., 1994). Transgenic subterranean clover plants expressing chimeric genes encoding a methionine-rich rumen stable sunflower seed albumin at levels up to 0.3% of the total extractable protein were produced (Khan et al., 1996). Transgenic forage grass plants expressing this sunflower seed albumin have also been obtained (Wang et al., in preparation). However, further work to optimize transgene expression is required in order to achieve nutritionally relevant levels of accumulation of the 'rumen by-pass proteins' to influence animal production.

In alfalfa, white and red clovers, a reduction in bloating propensity may be improved by the expression of the tannin biosynthetic pathway in foliar tissue. Biotechnological approaches based on asymmetric somatic hybridization to transfer this trait from high-tannin legume species, such as *Onobrychis* and *Lotus*, to the tannin-free legumes have been reported (Li et al., 1993). An improved understanding of the regulation of tannin biosynthesis will enable gene technology-based strategies to modify the amount, structure and tissue distribution of condensed tannins (Morris and Robbins, 1997; Larkin et al., these proceedings).

An armory of genes and strategies for engineering pest and disease resistance in transgenic plants has been developed and tested over the last decade: *Bacillus thuringiensis* (Bt) toxins, proteinase inhibitors, α-amylase inhibitors, glucanases, chitinases, plant defensins, phytoalexins, ribosome-inactivating proteins, viral coat proteins, etc. Some of them have been applied to the development of pasture plants for enhanced disease and pest resistance.

Transgenic alfalfa plants expressing the coat protein gene of alfalfa mosaic virus (AMV) and showing resistance to AMV infection have been obtained (Hill et al., 1991). Transgenic white clover plants expressing the whole of the subgenomic RNA4, or just the coat protein gene, of AMV and shown to be highly resistant or immune to AMV infection are currently being field tested (Garrett and Chu, 1997).

Overexpression of a chimeric *Aglu1* glucanase gene in alfalfa led to a significant symptom reduction when the transgenic alfalfa plants were challenged with *Phytophthora megasperma* (Masoud et al., 1996).

Transgenic white clover plants expressing a *B. thuringiensis cry*IA(b) gene have been produced and shown to reduce survival of the test insect *Orocrambus flexuosellus* (Voisey et al., 1994b). Improved expression of a synthetic *B. thuringiensis cry*IC gene in transgenic alfalfa plants provided protection against the Egyptian cotton leafworm (*Spodoptera littoralis*) and beet armyworm (*Spodoptera exigua*) (Strizhov et al., 1996).

Molecular techniques provide a powerful means to dissect the molecular basis of plant adaptation to its environment, to assess the link between specific biochemical changes and the plant phenotype, and to contribute to plant improvement for tolerance to abiotic stresses. Transgenic alfalfa plants expressing a Mn-superoxide dismutase (Mn-SOD), an enzyme involved in prevention and repair of cell damage following stresses such as freezing and icing, were produced (McKersie et al., 1993). Progenies from the transgenic alfalfa plants expressing a functional Mn-SOD cDNA from *Nicotiana plumbaginifolia* showed more rapid regrowth following freezing stress and water-deficit than those lacking the transgene (McKersie et al., 1993, 1996). A three year field trial indicated that yield and survival of the Mn-SOD transgenic alfalfa plants were significantly improved (McKersie et al., 1996). Many cold regulated cDNAs have been isolated from alfalfa and their sequence analysis has provided clues regarding their putative products (Castonguay et al., 1997). However, the real functions of the corresponding genes can only be elucidated by generating transgenic plants.

Herbicide resistant transgenic pasture legumes and grasses have been produced, mainly due to the selectable marker genes chosen for *in vitro* selection of transformants (Deak et al., 1986; D'Halluin et al., 1990; Wang et al., 1992; Khan et al., 1994).

The wind-pollinated species of grasses are among the most important etiological agents of seasonal atopic allergy. Grass pollen allergy afflicts about 20% of the population living in cool temperate climates. Some of the allergenic proteins from ryegrass pollen have been characterized and corresponding cDNAs isolated. Transgenic ryegrass plants bearing antisense vectors for the main pollen allergenic proteins *Lol p*I and *Lol p*II have been generated (Wu et al., these proceedings). These plants will allow to study the functional role *in planta* of main pollen allergens, and to explore the potential for the generation of hypo-allergenic ryegrass cultivars.

Nitrogen fixation in forage legumes has been studied in great detail at the biochemical and molecular level. Due to the complexity of the symbiosis, which requires the consideration of multiple genes from two separate organisms, so far no single example of N_2 fixation improvement through genetic engineering has been reported (Vance, 1997).

The perennial growth habit and the availability of processing technology for alfalfa, makes it particularly suitable for molecular farming, the production of industrial enzymes, pharmaceuticals, vaccines and antibodies in transgenic plants. Transgenic alfalfa plants expressing a manganese-dependent lignin peroxidase (Mn-P) from *Phanerochaete chrysosporium* and an α-amylase from *Bacillus licheniformis* have been generated and field-tested (Austin et al., 1995; Austin and Bingham, 1997). The production of Mn-P adversely affected plant growth and development, while the expression of the α-amylase gene showed no effect on plant performance and led to accumulation levels of active α-amylase in the range of 0.001-0.015% of total soluble protein (Austin and Bingham, 1997). Multidisciplinary efforts are needed for identifying further targets, generating transgenic plants with suitable expression levels, and developing efficient processing methods. Molecular farming for the

production of industrial compounds in value-added forage crops is likely to be achievable in a not too distant future.

The enabling tissue culture and genetic transformation methods for the application of gene technology to the improvement of key pasture grasses and legumes have been developed. The first transgenic pasture plants with 'engineered' traits have reached the stage of field-evaluation. Gaps in our understanding of the underlying genetics, physiology and biochemistry of many complex plant processes are, however, likely to delay progress in other applications of gene technology to pasture plant improvement. Molecular markers offer prospects for more precisely identifying superior individuals and enhancing selection efficiency. The use of markers is likely to have its greatest impact in the manipulation of quantitative characters for pasture plant improvement. Future efforts in the development and deployment of molecular marker technology in pasture plants are thus expected to be focussed on the identification and location of quantitative traits loci by the presence of one or more linked markers.

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Table 1
Somatic hybridization in forage grasses and legumes

Plant Species Combination		Fusion Product	References
Festuca arundinacea	***Lolium multiflorum***	Symmetric Somatic Hybrid Plants	Takamizo et al., 1991
Festuca arundinacea	***Lolium multiflorum***	Asymmetric Somatic Hybrid Plants	Spangenberg et al., 1994b
Lolium perenne	***Lolium perenne***	Cybrid Calli	Creemers-Molenaar et al.,1992
Pennisetum americanum	***Panicum maximum***	Somatic Hybrid Calli	Ozias-Atkins et al., 1986
Pennisetum americanum	***Saccharum officinarum***	Somatic Hybrid Calli	Tabaeizadeh et al., 1986
Pennisetum americanum	***Triticum monococcum***	Somatic Hybrid Calli	Vasil et al., 1988a
Lotus corniculatus	***Lotus conimbrensis***	Symmetric Somatic Hybrid Plants	Wright et al., 1987
Lotus corniculatus	***Lotus tenuis***	Symmetric Somatic Hybrid Plants	Aziz et al., 1990
Lotus corniculatus	***Glycine max***	Somatic Hybrid Calli	Kihara et al., 1992
Medicago sativa	***Medicago arborea***	Somatic Hybrid Calli Symmetric Somatic Hybrid Plants	Dimiani et al., 1988 Nenz et al., 1996a
Medicago sativa	***Medicago borealis***	Somatic Hybrid Calli	Gilmour et al., 1989b
Medicago sativa	***Medicago coerulea***	Symmetric Somatic Hybrid Plants	Pupilli et al., 1992
Medicago sativa	***Medicago falcata***	Symmetric Somatic Hybrid Plants Somatic Hybrid Calli Symmetric Somatic Hybrid Plants	Téoulé, 1983 Gilmour et al., 1987 Mendis et al., 1991
Medicago sativa	***Medicago intertexta***	Somatic Hybrid Calli	Thomas et al., 1990
Medicago sativa	***Medicago quasifalcata***	Somatic Hybrid Calli	Gilmour et al., 1987
Medicago sativa	***Medicago scutellata***	Somatic Hybrid Calli	Thomas et al., 1990
Medicago sativa	***Medicago varia***	Somatic Hybrid Calli	Deak et al., 1988
Medicago sativa	***Medicago sativa***	Somatic Hybrid Calli	Pupilli et al., 1991
Medicago sativa	***Onobrychis viciifolia***	Asymmetric Somatic Hybrid Plants	Li et al., 1993
Trifolium pratense	***Trifolium hybridum***	Somatic Hybrid Calli	Honkanen & Ryoppy, 1989

Table 2
Genetic transformation of forage grasses and legumes

Plant Species	Transgene	Method	Outcome	References
Agrostis alba (Redtop)	*gusA*	Protoplasts	Transfected protoplasts	Asano et al., 1991
	npt2	Protoplasts	Transgenic plants	Asano & Ugaki, 1994
	gusA	Whiskers	Transient expression	Asano et al., 1991
Dactylis glomerata (Cocksfoot)	*hph*	Protoplasts	Transgenic plants	Horn et al., 1988b
Festuca arundinacea (Tall Fescue)	*hph, bar*	Protoplasts	Transgenic plants	Wang et al., 1992
	gusA	Protoplasts	Transfected protoplasts	Penmetsa & Ha, 1994
	hph, bar	Protoplasts	Transgenic plants	Spangenberg et al., 1995b
	hph	Protoplasts	Transgenic plants	Dalton et al., 1995
	hph	Protoplasts	Transformed endophyte and surrogate transformed plants	Tsai et al., 1992
	hph	Biolistics	Transgenic plants	Spangenberg et al., 1995c
	gusA	Biolistics	Transient expression	Spangenberg et al., 1995c
Festuca pratensis (Meadow Fescue)	*bar*	Protoplasts	Transgenic plantlets	Spangenberg et al., 1995b
Festuca rubra (Red Fescue)	*bar*	Protoplasts	Transgenic plants	Spangenberg et al., 1994a
	hph	Biolistics	Transgenic plants	Spangenberg et al., 1995c
	gusA	Biolistics	Transient expression	Spangenberg et al., 1995c
Lolium multiflorum (Italian Ryegrass)	*npt2*	Protoplasts	Transformed calli	Potrykus et al., 1985
	gusA	Biolistics	Transient expression	Pérez-Vicente et al., 1993
	hph	Biolistics	Transgenic plants	Ye et al., 1997
	gusA	Biolistics	Transient expression	Ye et al., 1997
Lolium perenne (Perennial Ryegrass)	*hph, gusA*	Protoplasts	Transformed endophyte and surrogate transformed plants	Murray et al., 1992
	hph	Biolistics	Transformed calli	Hensgens et al., 1993
	gusA	Biolistics	Transient expression	Hensgens et al., 1993
	gusA	Biolistics	Transient expression	Pérez-Vicente et al., 1993
	hph, gusA	Biolistics	Transformed calli	Van der Maas et al., 1994a
	hph	Biolistics	Transgenic plants	Spangenberg et al., 1995d
	gusA	Biolistics	Transient expression	Spangenberg et al., 1995d
Panicum maximum (Guinea grass)	*cat*	Protoplasts	Transfected protoplasts	Hauptmann et al., 1987
	cat	Protoplasts	Transfected protoplasts	Vasil et al., 1988b
	dhfr	Protoplasts	Transformed calli	Hauptmann et al., 1988
Pennisetum glaucum (Pearl Millet)	*gusA*	Biolistics	Transient expression	Taylor & Vasil, 1991
Pennisetum purpureum (Napier grass)	*cat*	Protoplasts	Transfected protoplasts	Hauptmann et al., 1987
Lotus angustissimus	*npt2, gusA*	*A. rhizogenes*	Transgenic plants	Nenz et al., 1996b
Lotus corniculatus (Bird's-foot Trefoil)	*cat*	*A. rhizogenes*	Transgenic plants	Jensen et al., 1986
	Ri T-DNA	*A. rhizogenes*	Transgenic plants	Petit et al., 1987
	Ri T-DNA	*A. rhizogenes*	Hairy roots	Shen et al., 1988
	Ri T-DNA	*A. rhizogenes*	Transgenic plants	Webb et al., 1990
	cat, npt2	*A. rhizogenes*	Transgenic plants	Tabaeizadeh, 1993
	dfr	*A. rhizogenes*	Hairy roots	Carron et al., 1994
	gusA, hph	*A. rhizogenes*	Transgenic plants	Webb et al., 1994
	gusA, npt2	*A. rhizogenes*	Transgenic plants	Carsolio et al., 1994
	gusA, npt2	*A. rhizogenes*	Transgenic plants	Marsolier et al., 1995
	gusA, hph	*A. tumefaciens*	Transgenic plants	Webb et al., 1996

Plant Species	Transgene	Method	Outcome	References
Lotus japonicus	*hph, npt2*	*A. tumefaciens*	Transgenic plants	Handberg et al., 1994
	Ac, gusA, aadA, npt2	*A. tumefaciens*	Transgenic plants	Thykjaer et al., 1995
	npt2	*A. tumefaciens*	Transgenic plants	Oger et al., 1996
Macroptilium atropureum (Siratro)	*Ri T-DNA*	*A. rhizogenes*	Hairy roots	Beach and Gresshoff, 1988
Medicago arborea	*hph*	*A. rhizogenes*	Transgenic plants	Damiani & Arcioni, 1991
Medicago borealis	*npt2*	Protoplasts	Transgenic plants	Kuchuk et al., 1990
Medicago sativa (Alfalfa)	*npt2*	*A. tumefaciens*	Transgenic plants	Shahin et al., 1986
	bar	*A. tumefaciens*	Transgenic plants	Deak et al., 1986
	Ri T-DNA	*A. rhizogenes*	Transgenic plants	Spano et al., 1987
	Ri T-DNA	*A. rhizogenes*	Hairy roots	Beach and Gresshoff, 1988
	Ti T-DNA	*A. tumefaciens*	Transgenic plants	Kuchuk et al., 1990
	bar	*A. tumefaciens*	Transgenic plants	D'Halluin et al., 1990
	AMV4-cDNA	*A. tumefaciens*	Transgenic plants	Hill et al., 1991
	Ri T-DNA	*A. rhizogenes*	Transgenic plants	Golds et al., 1991
	ova	*A. tumefaciens*	Transgenic plants	Schroeder et al., 1991
	Le-PI	*A. tumefaciens*	Transgenic plants	Narváez-Vásquez et al., 1992
	Mn-SOD	*A. tumefaciens*	Transgenic plants	McKersie et al., 1993
	npt2	*A. tumefaciens*	Transgenic plants	Du et al., 1994
	Ms-PI	*A. tumefaciens*	Transgenic plants	Thomas et al., 1994
	gusA, npt2	*A. tumefaciens*	Transgenic plants	Oommen et al., 1994
	npt2, gusA	*A. tumefaciens*	Transgenic plants	Ninkovic et al., 1995
	α-amylase, Mn-P	*A. tumefaciens*	Transgenic plants	Austin et al., 1995
	cryIC	*A. tumefaciens*	Transgenic plants	Strizhov et al., 1996
	Aglu1	*A. tumefaciens*	Transgenic plants	Masoud et al., 1996
	gusA	Biolistics	Transient expression	Brown et al., 1994
	npt2	Biolistics	Transgenic plants	Pereira and Erickson, 1995
	ruc	Protoplasts	Transient expression	Mayerhofer et al., 1995
Medicago truncatula (Barrel Medic)	*npt2*	*A. tumefaciens*	Transgenic plants	Thomas et al., 1992
	gusA, npt2	*A. tumefaciens*	Transgenic plants	Chabaud et al., 1996
Onobrychis viciifolia (Sainfoin)	*Ri T-DNA*	*A. rhizogenes*	Transgenic plants	Golds et al., 1991
Stylosanthes guianensis	*bar, npt2, gusA*	*A. tumefaciens*	Transgenic plants	Sarria et al., 1994
Stylosanthes humilis	*npt2*	*A. tumefaciens*	Transgenic plants	Manners, 1988
Trifolium repens (White Clover)	*npt2*	*A. tumefaciens*	Transgenic plants	White & Greenwood, 1987
	Ri T-DNA	*A. rhizogenes*	Hairy roots	Webb et al., 1990
	npt2, gusA	*A. tumefaciens*	Transgenic plants	Voisey et al., 1994a
	cryIA(b)	*A. tumefaciens*	Transgenic plants	Voisey et al., 1994b
	pa1	*A. tumefaciens*	Transgenic plants	Ealing et al., 1994
	bar, gusA	*A. tumefaciens*	Transgenic plants	Larkin et al., 1996
Trifolium pratense (Red Clover)	*Ri T-DNA*	*A. rhizogenes*	Hairy roots	Webb et al., 1990
	Ri T-DNA	*A. rhizogenes*	Hairy roots	Beach and Gresshoff, 1988
	npt2, gusA	*A. tumefaciens*	Transgenic plants	Quesenberry et al., 1996
Trifolium subterraneum (Subterranean Clover)	*α-ai, bar, gusA, npt2*	*A. tumefaciens*	Transgenic plants	Khan et al., 1994
	ssa	*A. tumefaciens*	Transgenic plants	Khan et al., 1996

α-*ai*: bean α-amylase inhibitor
aadA: aminoglycoside-3'-adenyltransferase
Ac: maize transposable element
Aglu1: acidic glucanase
AMV4-cDNA: alfalfa mosaic virus coat protein
bar: phosphinothricin acetyltransferase
cat: chloramphenicol acetyltransferase
cryIA(b), cryIC: Bacillus thuringiensis delta-endotoxins
dfr: dihydroflavonol reductase
dhfr: dihydrofolate reductase
gusA: β-glucuronidase
hph: hygromycin phosphotransferase
Le-PI: Lycopersicon esculentum proteinase inhibitor I
Mn-P: Manganese-dependent lignin peroxidase
Mn-SOD: Mn-superoxide dismutase
npt2: neomycin phosphotransferase II
ova: chicken ovalbumin
pa1: pea albumin 1
Ms-PI: Manduca sexta anti-elastase proteinase inhibitor
Ri T-DNA: wild-type *A. rhizogenes* T-DNA
ruc: Renilla luciferase
ssa: sunflower seed albumin
Ti T-DNA: wild-type *A. tumefaciens* T-DNA