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Alfalfa

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The genus *Medicago* comprises over 60 annual and perennial species. Alfalfa (*Medicago sativa* L. and *Medicago falcata* L.) is a perennial species that has a long history of cultivation around the world (Russelle, 2001). The center of origin for alfalfa is likely the Middle East and the steppes of Central Asia. The record of its use by humans dates back to the 10th millennium BC at Abu Hureyra in present day Syria. By the 1st century BC, it had spread to Greece and China and the Roman Columella planted alfalfa in southern Spain by 100 AD. Alfalfa spread to North and South America by the 1700 s and to New Zealand and Australia by around 1800. The crop was so revered as an excellent source of nutrition for livestock that people carried alfalfa with them and established the crop on six out of the seven continents by the early 1800s.

1.2 Botanical Description

Alfalfa develops an extensive, well-branched root system that is capable of penetrating deep into the soil. Root growth rates of 1.8 m per year are typical in loose soils (Johnson *et al.*, 1996) and metabolically active alfalfa roots have been found 18 m or more below ground level (Kiesselbach *et al.*, 1929). This deep root system allows alfalfa plants to access water and nutrients that are not available to more shallowly rooted annual plants, enabling established alfalfa plants to produce adequate yields under less than optimal rainfall conditions. Alfalfa has a chromosome compliment of $x = 8$ with both diploid ($2n = 2x = 16$) and tetraploid forms ($2n = 4x = 32$) (Quiros and Bauchan, 1988).

1.3 Economic Importance

Alfalfa is widely grown for animal feed throughout the world. Alfalfa is known as the "Queen of the Forages" because it contains a high amount of crude protein, digestible fiber, and is an excellent source of vitamins and minerals for animals. In the United States, alfalfa is the fourth most widely grown crop with over 8.9 million hectares of alfalfa harvested in 2005 (USDA-NASS, 2006). In 2005, the US alfalfa cultivation for dry hay was over 70 million metric tons with a total direct value of over $7 billion. It is typically harvested for 4 years (an establishment year plus three subsequent years). Depending on location, alfalfa is harvested three or more times each year by cutting the stems near ground level. On average across the United States, alfalfa yields 7.6 Mg of dry matter (DM) per hectare each year although yields can

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vary by location from 4.5 (North Dakota) to 15.5 (California) Mg ha^{-1} (USDA-NASS, 2006). In 2005, the national harvest of alfalfa was over 68 million metric tons (USDA-NASS, 2006). The technology for cultivation, harvesting, and storing alfalfa is well established, machinery for harvesting alfalfa is widely available, and farmers are familiar with alfalfa production. There is a well-developed industry for alfalfa cultivar development, seed production, processing, and distribution.

The high biomass potential of alfalfa is based on underground, typically unobserved traits. Alfalfa roots engage in a symbiotic relationship with the soil bacterium *Sinorhizobium meliloti*. This partnership between the plant and bacterium results in the formation of a unique organ, the root nodule, in which the bacterium is localized. The bacteria in root nodules take up nitrogen gas (N_2) and "fix" it into ammonia. The ammonia is assimilated through the action of plant enzymes to form glutamine and glutamate. The nitrogen-containing amide group is subsequently transferred to aspartate and asparagine for transport throughout the plant. On average, alfalfa fixes approximately 152 kg N_2 ha^{-1} on an annual basis as a result of biological nitrogen fixation, which eliminates the need for applied nitrogen fertilizers (Russelle and Birr, 2004). Although a significant proportion of the fixed nitrogen is removed by forage harvest, fixed nitrogen is also returned to the soil for use by subsequent crops. This attribute of increasing soil fertility has made alfalfa and other plants in the legume family crucial components of agricultural systems worldwide. Cultivation of alfalfa has also been shown to improve soil quality, increase organic matter, and promote water penetration into soil. In addition, alfalfa sprouts are consumed by humans and alfalfa juice is being used in health food products for its flavonoid compounds.

1.4 Traditional Breeding

Alfalfa, a perennial with perfect flowers is naturally cross-pollinated by bees, tolerates comparatively little inbreeding, and can be vegetatively propagated by stem cuttings. All cultivars are autotetraploids and the inheritance of economic traits is, therefore, quite complex. The introgression of *M. falcata* into *M. sativa* increased the genetic variation and the range of adaptation of this crop to temperate climates of all continents (Rumbaugh *et al.*, 1988). Nine highly diverse, distinct germplasm sources were introduced into North America from different regions of the world (Barnes *et al.*, 1977). Since the 1920s, geneticists have employed recurrent phenotypic selection taking advantage of additive genetic variance to successfully improve alfalfa for resistance to diseases and insects, and extend geographic adaptability. However, more complex quantitatively inherited traits, such as forage yield, have essentially not changed for the past 60 years (Brummer, 1999; Lamb *et al.*, 2006).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Comprehensive genomic analysis of alfalfa is limited due to the large size of the genome and obligate outcrossing autotetraploid nature of alfalfa. Thus, varieties are synthetic populations, consisting of a heterogeneous mixture of heterozygous genotypes. Although improved varieties of alfalfa have been developed, little is known about the genes controlling desirable traits due to the plant's complex genetic system. The traits needed to improve alfalfa are complex and require a multifaceted approach to first understand the molecular underpinnings of the characteristic and subsequently to select or engineer improved plants. To increase the crop value and utility of alfalfa a number of characteristics need to be enhanced. In areas of severe winters, winter hardiness needs to be improved to extend stand life and retain high yields over multiple years. In areas with low rainfall, water use efficiency and tolerance to salinity need to be increased. In acid soils, tolerance to aluminum is needed as well as increased tolerance to waterlogged soils. Improving forage fiber digestibility would enhance nutritional quality for ruminant animals and decrease processing costs when using alfalfa fiber as a feedstock for bioethanol production.

The basic tools required for modern genome analysis are largely not available in alfalfa. Direct study of alfalfa in isolation is unlikely to yield rapid improvements for these characteristics. The use of an annual relative of alfalfa, *Medicago truncatula*, is an ideal candidate for parallel analysis of agronomically important genes in alfalfa.

2. DEVELOPMENT OF TRANSGENIC ALFALFA

2.1 Promoters for Transgenic Alfalfa

The cauliflower mosaic virus 35S (CaMV 35S) promoter is the most widely used promoter in alfalfa transgenic work. In an attempt to find alternative constitutive promoters for transgenic alfalfa, several authors evaluated many promoters for their efficacy driving the reporter gene β-glucuronidase (*gusA*) or green fluorescent protein (GFP). In one such work, Winicov *et al.* (2004) generated transgenic alfalfa that contained the MsPRP2 (proline rich protein 2) gene promoter from alfalfa driving GFP and NOS 3′ transcription terminator. Transformation of alfalfa was accomplished by co-cultivation of alfalfa leaf discs with *Agrobacterium tumefaciens*. Kanamycin resistance conferred by a constitutive neomycin phosphotransferase (*npt*II) gene was used as a selectable marker. The MsPRP2 promoter showed root-specific reporter gene expression in transgenic alfalfa plants (Winicov *et al.*, 2004).

Samac *et al.* (2004b) generated transgenic alfalfa that contained the CaMV 35S promoter, the cassava vein mosaic virus (CsVMV) promoter, or the sugarcane bacilliform badnavirus (ScBV) promoter. Each promoter was fused separately to the reporter *gusA* gene and NOS 3′ transcription terminator. Transformation of alfalfa was done by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Kanamycin resistance conferred by the *npt*II gene was used as a selectable marker. The highest enzyme activity as well as *in situ* staining for β-glucuronidase (GUS) was obtained in transgenic alfalfa plants that contained the CsVMV promoter. In transgenic alfalfa leaves, GUS activity was observed in the order of CsVMV promoter > CaMV 35S promoter > ScBV promoter. The CsVMV promoter was expressed in all tissues examined. The 35S promoter was expressed in leaves, roots, and stems at moderate levels, but the promoter was not active in stem pith cells, root cortical cells, or in the symbiotic zones of nodules. The ScBV promoter was active primarily in vascular tissues throughout transgenic alfalfa plants studied.

The potato protease inhibitor II (PinII) promoter fused to the *gusA* gene and NOS 3′ transcription terminator was introduced into alfalfa by *Agrobacterium*-mediated transformation of leaf discs (Samac and Smigocki, 2003). Transformant alfalfa was selected by kanamycin resistance conferred by the *npt*II gene. Constitutive GUS expression was observed in leaf and root vascular tissues of transgenic alfalfa. In some transgenic alfalfa plants, GUS expression was observed in leaf mesophyll cells. Approximately twofold more GUS expression was observed following mechanical wounding of leaves compared to unwounded control alfalfa leaves (Samac and Smigocki, 2003).

2.2 Alfalfa Forage Quality Improvement

2.2.1 Manipulation of lignin and plant cell wall composition for forage quality improvement

Lignin is a polymer of monolignols that is found in secondarily thickened plant cell walls and is critical for structural integrity of the wall and the strength of stems. Alfalfa contains two major monolignols, monomethoxylated guaiacyl (G) and dimethoxylated syringyl (S) units. Increased lignin content and S/G ratio resulting from stem maturity have been linked with reduced forage digestibility by grazing animals and hence represent poor forage quality variables. Over the years, considerable effort has been made toward improving forage quality by suppressing the expression of plant genes that are thought to play a role in lignin content or modifying lignin composition in alfalfa. Improving forage digestibility provides a means to enhance animal production.

A cytochrome P450 gene from *M. truncatula* in antisense orientation, driven by the vascular-specific bean phenylalanine ammonia lyase (PAL2) promoter and NOS 3′ transcription terminator was used to generate transgenic alfalfa (Reddy *et al.*, 2005). Transformation of alfalfa leaf pieces was following co-cultivation with *A. tumefaciens*. Kanamycin resistance conferred by the *npt*II gene was used as a selectable marker. Transgenic alfalfa showed a range of differences in lignin content and composition. In cytochrome P450 transgenic alfalfa, lignin content was negatively related to rumen digestibility, but no relationship was observed between lignin composition and rumen digestibility (Reddy *et al.*, 2005).

Transgenic alfalfa plants containing the coumaroyl shikimate 3-hydroxylase (C3H) gene from *M. truncatula* in the antisense orientation were generated (Reddy *et al.*, 2005; Ralph *et al.*, 2006). The antisense C3H clone was driven by the vascular-specific bean PAL2 promoter and NOS 3′ transcription terminator. *Agrobacterium*-mediated transformation was used to transform alfalfa leaf pieces. Selection of alfalfa transformants was achieved by kanamycin resistance conferred by the *npt*II gene. Down-regulation of C3H transcript in alfalfa altered lignin composition as the proportion of p-hydroxyphenyl (P) units was increased compared to the usually dominant G and S units of lignin. Transgenic alfalfa plants displayed improved digestibility values (Reddy *et al.*, 2005; Ralph *et al.*, 2006).

In a separate study, transgenic alfalfa contained caffeic acid 3-*O*-methyltransferase (COMT) and caffeoyl coenzyme A 3-O-methyltransferase (CCoAOMT) genes from alfalfa in antisense orientation (Guo *et al.*, 2001; Marita *et al.*, 2003). The transgenes were driven by the vascular-specific bean PAL2 promoter and NOS 3′ transcription terminator. Alfalfa transformation was by co-cultivation of leaf pieces with *A. tumefaciens*. Kanamycin resistance conferred by the *npt*II gene was used as the selectable marker. Transgenic plants down-regulated for COMT showed reduced lignin content and altered lignin composition. Down-regulation of COMT transcript in transgenic alfalfa was accompanied by an increase in cellulose, resulting on average a 30% increase in cellulose:lignin ratio. Digestibility of transgenic alfalfa was also improved. On the other hand, lignin content in transgenic alfalfa down-regulated in the expression of CCoAOMT was reduced, but the structure of lignin was similar to that of nontransformed control plants. Nevertheless, a much greater improvement in digestibility values of alfalfa forage was reported for transgenic alfalfa down-regulated in CCoAOMT (Guo *et al.*, 2001).

Uridine diphosphate (UDP)-glucose dehydrogenase is a key enzyme in the biosynthesis of uronic acids. Uronic acids represent a large proportion of the primary cell wall matrix in plants. Samac *et al.* (2004a) reported the generation of transgenic alfalfa overexpressing a soybean UDP-glucose dehydrogenase gene in the sense direction. The transgene was driven by an Arabidopsis class III chitinase promoter or an alfalfa phosphoenolpyruvate carboxylase promoter and NOS 3′ transcription terminator. Transformation was following co-cultivation of leaf pieces with *A. tumefaciens* LBA 4404. Plant transformation vectors contained the *npt*II gene for selection of transformants by resistance to kanamycin. UDP-glucose dehydrogenase enzyme activity in transgenic lines grown in the greenhouse was up to sevenfold greater than in nontransformed control plants. However, enzyme activity of field-grown transgenic plants showed a maximum of 1.9-fold more enzyme activity than the nontransformed control plants. No significant increase in pectin or uronic acids was seen in the polysaccharide fraction of field-grown transgenic plants. However, lower cell wall polysaccharide content and mannose concentrations were accompanied by higher Klason lignin content in transgenic plants. Ectopic expression of UDP-glucose dehydrogenase showed a 15% increase in xylose content in stem tissues of most transgenic alfalfa lines.

2.2.2 Reducing protein loss for forage quality improvement

Ensiling is a popular method of preserving forage crops. Wounding of forage crop leaves during crop harvest and ensiling releases cellular proteases resulting in degradation of plant protein to small peptides and amino acids. Red clover (*Trifolium pratense* L.) experiences less protein loss than alfalfa when ensiled. Proteolytic inhibition of ensiled forage seen in red clover was hypothesized to occur through the activity of polyphenol oxidase (PPO) on endogenous *o*-diphenols. To test this idea, the *PPO* gene from red clover was placed under the control of a constitutive CsVMV promoter and NOS 3′ transcription terminator to produce transgenic alfalfa (Sullivan *et al.*, 2004). Transformation of alfalfa leaf pieces was mediated by co-cultivation with *Agrobacterium* strain LBA 4404. Kanamycin resistance conferred by the *npt*II gene was used to select transformed alfalfa. The red clover PPO was expressed in transgenic alfalfa and the protein was shown to be active in alfalfa extracts, as evidenced by *o*-diphenol-dependant extract browning and quantitative assays of PPO activity (Sullivan *et al.*, 2004; Sullivan and Hatfield, 2006).

Leaf extracts of transgenic alfalfa showed a nearly fivefold *o*-diphenol-dependent decrease in proteolysis compared to those of nontransformed control alfalfa (Sullivan and Hatfield, 2006). In small-scale ensiling experiments, proteolysis was reduced for PPO-expressing transgenic alfalfa (Sullivan and Hatfield, 2006).

2.2.3 Improving dietary proteins in alfalfa forage

Most dietary proteins ingested by ruminants are rapidly degraded by rumen bacteria. Rumen microbial proteins in turn become sources of protein for the animals. However, microbial proteins are poor in sulfur amino acids. A small number of proteins appear to be relatively resistant to degradation in the rumen and presumably are assimilated by ruminants in the lower gastrointestinal tract. Ovalbumin, one such protein that is found to be resistant to degradation by rumen bacteria, is also regarded as nutritionally valuable. Schroeder *et al.* (1991) placed a chicken ovalbumin gene under the control of the CaMV35S promoter and NOS 3′ transcription terminator to generate transgenic alfalfa plants. Transformation of alfalfa leaf pieces was done by co-cultivation with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *npt*II gene. Transgenic alfalfa plants expressed the recombinant protein in leaf tissues and ovalbumin expression was estimated from 0.001% to 0.01% of total soluble protein. Recombinant ovalbumin expression was stable and was detected in leaf tissues of all ages (Schroeder *et al.*, 1991).

2.3 Abiotic Stress Tolerance

2.3.1 Overexpression of transcription factor to improve salinity tolerance

Transcription factors modulate gene expression patterns through sequence-specific DNA binding and/or protein–protein interactions. They are capable of acting as switches of gene expression regulatory cascades by activating or repressing transcription of target genes. One such gene, *Alfin1*, encodes a putative transcription factor in alfalfa, and the recombinant protein binds DNA in a sequence-specific manner, including the promoter region of the salt (NaCl) inducible gene *MsPRP2* (Winicov and Bastola, 1999). Alfinl-binding sites were shown to occur in promoters of genes expressed in roots of a wide variety of plant species (Winicov, 2000). Transgenic alfalfa plants containing the alfalfa *Alfin1* cDNA (complementary DNA), in the sense or antisense direction, placed under the control of the CaMV 35S promoter and NOS 3′ transcription terminator were generated (Winicov and Bastola, 1999). Transformation of alfalfa leaf pieces was *Agrobacterium*-mediated. Selection of transformant alfalfa was by kanamycin resistance conferred by the *npt*II gene. Transgenic calli and plant roots overexpressing *Alfin1* showed enhanced levels of endogenous *MsPRP2* mRNA (messenger RNA) accumulation. Calli overexpressing *Alfin1* were more resistant to growth inhibition by 171 mM NaCl than vector-transformed controls, whereas calli expressing *Alfin1* in the antisense orientation were more sensitive to NaCl inhibition. Similarly, transgenic plants overexpressing *Alfin1* showed salinity tolerance comparable to a NaCl-tolerant control plant (Winicov and Bastola, 1999). Further more, *Alfinl*-overexpressing transgenic alfalfa plants showed increased root growth under normal and salt stress conditions (Winicov, 2000). In contrast, the antisense transgenic plants grew poorly in soil, demonstrating that Alfin1 expression is also essential for normal plant root development.

2.3.2 Overexpression of transcription factor to improve drought tolerance

The *WXP1* gene, an AP2 domain-containing putative transcription factor from *M. truncatula*, placed under the control of the CaMV 35S promoter and NOS 3′ transcription terminator was used to transform alfalfa (Zhang *et al.*, 2005). Transformation of alfalfa leaf pieces was following co-cultivation with *A. tumefaciens* strain C58C1. Transformed lines were selected for resistance to the herbicide Basta conferred by the *bar* gene. Cuticular wax loading on leaves of transgenic alfalfa was significantly increased. The total leaf wax accumulation per surface area increased approximately 30–38% in the transgenic alfalfa

lines. Transgenic alfalfa leaves showed reduced water loss and chlorophyll leaching, as well as delayed wilting after watering was ceased and quicker and better recovery when the dehydrated plants were rewatered (Zhang *et al.*, 2005).

2.3.3 Improvement of tolerance to aluminum phytotoxicity in acid soils

Development of acid soils that limit crop production is an increasing problem worldwide. Many factors contribute to phytotoxicity of these soils, however, in acid soils with a high mineral content, aluminum (Al) is the major cause of toxicity. Toxicity can be reduced through lime application to raise soil pH, however, this amendment does not remedy subsoil acidity, and liming may not always be practical or cost effective. Addition of organic acids to plant nutrient solutions alleviates phytotoxic Al effects, presumably by chelating Al and rendering it less toxic.

In an effort to increase organic acid secretion and thereby enhance Al tolerance in transgenic plants, Tesfaye *et al.* (2001) produced transgenic alfalfa using the nodule-enhanced form of malate dehydrogenase (*neMDH*) gene from alfalfa. The *neMDH* gene in the sense direction was placed under the control of the CaMV 35S promoter and NOS 3′ transcription terminator. Transformation of alfalfa leaf discs was *Agrobacterium*-mediated. Kanamycin resistance conferred by the *npt*II gene was used as the selectable marker. Malate dehydrogenase enzyme specific activity in root tips of selected transgenic alfalfa was 1.6-fold higher than in nontransformed control plants. A 4.2-fold increase in root concentration as well as a 7.1-fold increase in root exudation of citrate, oxalate, malate, succinate, and acetate was measured in transgenic alfalfa lines compared with nontransformed control alfalfa plants. In acid soils and hydroponic culture, transgenic alfalfa plants showed increased biomass accumulation compared to nontransformed control alfalfa.

2.3.4 Winter freezing tolerance

Transgenic alfalfa plants that overexpress manganese superoxide dismutase (Mn-SOD) cDNA from alfalfa were produced (McKersie *et al.*, 1999). The transgene was driven by the CaMV 35S promoter and NOS 3′ transcription terminator in the sense direction. Alfalfa petioles were transformed by co-cultivation with *A. tumefaciens*. Kanamycin resistance conferred by the *npt*II gene was used as the selection agent. In two field experiments, transgenic alfalfa plants showed over 25% better winter survival than the nontransgenic alfalfa controls (McKersie *et al.*, 1999).

Similarly, the Arabidopsis iron-superoxide dismutate (Fe-SOD) was expressed in transgenic alfalfa (McKersie *et al.*, 2000). The Fe-SOD cDNA was under the control of the CaMV 35S promoter and NOS 3′ transcription terminator. Alfalfa petioles were transformed by co-cultivation with *A. tumefaciens*. Kanamycin resistance conferred by the *npt*II gene was used as the selection agent. Transgenic alfalfa plants overexpressing the Arabidopsis Fe-SOD transcripts showed greater survival after two winter seasons than the nontransformed control alfalfa plants (McKersie *et al.*, 2000).

2.4 Herbicide Resistance and Weed Control

In fall 2005, the first biotechnology-enhanced alfalfa, Roundup Ready® alfalfa, was commercialized in the United States. The plant transformation vector for this transgenic alfalfa contained an *Agrobacterium* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) fused to a transit peptide (Samac and Temple, 2004). The transgene was driven by a strong constitutive promoter and NOS 3′ terminator (Samac and Temple, 2004). Transformation of alfalfa leaf discs was achieved by co-cultivation with *A. tumefaciens*. The transgenic alfalfa is resistant to glyphosate (active ingredient of Roundup herbicide). This trait is expected to provide growers with improved weed control and increased flexibility in timing of herbicide application.

2.5 Disease and Pest Resistance

As a potential strategy to improve disease resistance in plants, Masoud *et al.* (1996) generated transgenic alfalfa utilizing constitutive expression of chitinase and glucanse genes separately or

in tandem for coexpression. The acidic glucanse (*Aglu1*) gene from alfalfa and the rice basic chitinase (*RCH10*) gene were placed under the control of CaMV 35S and NOS 3′ transcription terminator. Plant transformation vectors contained each transgene separately, or in tandem for coexpression in a single transgenic plant. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *npt*II gene. Both transgenes were expressed in transgenic alfalfa plants. However, the level of expression of each transgene by transgenic alfalfa plants with both transgenes in tandem was much less than transgenic alfalfa plants carrying a single transgene. Only transgenic plants expressing Aglu1 displayed reduced disease symptoms from *Phytophthora megasperma* f. sp. medicaginis inoculations. Transgenic alfalfa plants did not tolerate infections from other fungi infections including *Stemphylium alfalfae*, *Colletotrichum trifolii*, or *Phoma medicaginis* (Masoud *et al.*, 1996).

Alfalfa mosaic virus (AMV) can cause significant yield losses to forage legumes. An AMV RNA 4 gene encoding the coat protein of AMV was placed under the control of the CaMV 35S promoter and T-DNA ORF 25 RNA terminator (Hill *et al.*, 1991). Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *npt*II gene. Production of the coat protein by transgenic alfalfa plants ranged from <100 ng to nearly 500 ng coat protein per milligram soluble leaf protein. While nontransformed alfalfa plants showed systemic infections of the virus following inoculations with $10\,\mu g\,ml^{-1}$ AMV, transgenic alfalfa plants develop resistance to AMV with inoculations of up to $50\,\mu g\,m\,l^{-1}$ AMV (Hill *et al.*, 1991).

Phytocystatins, inhibitors of cysteine proteinases, are found in a number of plants where they may play a role in defense against pathogens and pests. The cDNAs of the phytocystatins of rice, oryzacystatin I (*OC-I*) and oryzacystatin II (*OC-II*), were expressed in transgenic alfalfa plants under the control of the *Pin*II promoter (Samac and Smigocki, 2003). Transformation was by co-cultivation of leaf discs with *A. tumefaciens*. Transformants were selected by kanamycin resistance conferred by the *npt*II gene. Populations of the root-lesion nematode (*Pratylenchus penetrans*) in alfalfa roots from one transgenic alfalfa line containing the *Pin*II*::OC-I* transgene and one transgenic line containing the *PinII::OC-II* transgene were reduced by 29% and 32%, respectively, compared with a vector only transgenic control line (Samac and Smigocki, 2003).

2.6 Transgenic Alfalfa for Industrial Applications

Plants are capable of carrying out acetylation, phosphorylation, and glycosylation as well as other post-translational protein modifications required for the biological activity of many recombinant proteins. Indeed, numerous recombinant proteins have been produced in transgenic plants. Alfalfa has primarily been used as a source of forage and animal feed. Improving the economic value of forage legumes could be achieved by developing new uses and value-added products of these crops. The ease of genetic transformation and large-scale cultivation makes alfalfa suitable for production of recombinant proteins.

2.6.1 Production of biodegradable plastic

A biodegradable polymer, polyhydroxybutyrate (PHB), is made naturally by many bacteria under nutrient-limiting conditions, and can be produced commercially by fermentation (Saruul *et al.*, 2002). Three genes from *Ralstonia eutropha* required for PHB synthesis, *pha*, *phb*, and *phc*, were each placed between the CaMV 35S promoter and NOS 3′ transcription terminator, in the same plant transformation vector. Alfalfa transformation was by co-cultivation of leaf discs with *A. tumefaciens*. Kanamycin resistance conferred by the *npt*II gene was used as the selectable marker. Polymer granules similar to bacterial PHB indicating the production of the bioplastic were observed in chloroplasts of transgenic alfalfa. The amount of PHB accumulation in transgenic alfalfa was estimated at approximately $0.025–1.8\,g\,kg^{-1}$ dry weight. Transgenic alfalfa exhibited growth similar to nontransformed plants, and F_1 hybrid progeny, obtained from crosses of PHB transgenic plants

with elite alfalfa germplasm exhibited leaf PHB levels similar to the transgenic parental line.

2.6.2 Phytoimmunogen production in transgenic alfalfa

Vaccines produced in transgenic plants offer a system for oral delivery of recombinant immunogens. This approach is especially relevant when used for enteric pathogens since oral immunization may be capable of eliciting appropriate immune mechanisms for the induction of protective responses.

In an attempt to use alfalfa plants for vaccine production, a 96bp coding sequence for the bovine rotavirus VP4, eBRV4 peptide, fused with the *gusA* gene was driven by the CaMV 35S promoter and NOS 3′ transcription terminator (Wigdorovitz *et al.*, 2004). Transformation of alfalfa petioles was *Agrobacterium*-mediated. Kanamycin conferred by the expression of the *npt*II gene was used as a marker gene for selection of transformants. The recombinant protein was shown to induce protective lactogenic immunity against a virulent rotavirus in suckling mice born from immunized adult female mice (Wigdorovitz *et al.*, 2004).

2.6.3 Production of industrially important enzymes and phytopharmaceuticals

In an effort to utilize transgenic alfalfa for the production of industrially useful enzymes, Austin *et al.* (1995) used an α-amylase gene from the bacterium *Bacillus licheniformis* to transform transgenic alfalfa. α-amylase is involved in starch degradation and the production of a recombinant protein using plants has potential industrial-scale applications in starch processing. The transgene was driven by the MAC promoter and mannopine synthase transcription terminator from *Agrobacterium*. The MAC promoter is a hybrid fragment that contains some elements from the CaMV 35S promoter and the nopaline synthase promoter from *Agrobacterium*. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *npt*II gene. Transgenic alfalfa plants looked normal throughout the growth period, and recombinant protein expression was estimated in the range of 0.001–0.01% of total soluble protein extracts. Field-grown transgenic alfalfa plants also expressed the recombinant α-amylase enzyme with no effect on biomass production (Austin *et al.*, 1995).

Another recombinant enzyme that was produced using transgenic alfalfa was manganese-dependent lignin peroxidase (Mn-P) from the fungus *Phanerochaete chrysosporium* (Austin *et al.*, 1995). The objective was to produce a recombinant enzyme using plants for large-scale application as a bleaching agent in the biopulping process (Austin *et al.*, 1995). The transgenic alfalfa is also thought to improve digestibility of crude fiber as ruminant feed. The transgene was driven by the MAC promoter and mannopine synthase transcription terminator from *Agrobacterium*. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *npt*II gene. Expression of the recombinant protein was estimated in the range of 0.01–0.5% of total soluble protein extracts. Field-grown transgenic alfalfa plants also expressed the recombinant Mn-P protein with an estimated yield of up to 0.15% (high expressing plants) of total soluble protein (Austin *et al.*, 1995). Transgenic alfalfa plants expressing Mn-P looked normal for the first 2–3 weeks of growth, but high expressing plants showed foliar yellowing accompanied by stunted and slow growth rate.

Most phosphorus (P) in plants is stored as phytic acid, and phytic acid is not readily digested by monogastric animals, such as poultry and swine. To help overcome this problem, a phytase enzyme, secreted by *Aspergillus niger*, has been widely incorporated in animal feeds to help improve P availability by degradation of phytate. Ullah *et al.* (2002) reported the production of transgenic alfalfa carrying the phyA gene from *Aspergillus ficuum*. The transgene was driven by the MAC promoter and mannopine synthase transcription terminator from *Agrobacterium*. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *npt*II gene. The recombinant enzyme was recovered from alfalfa leaf tissues

and was found to be active and stable, with an approximate 17-fold increase in phytase enzyme activity values in leaves of transgenic alfalfa plants (Ullah *et al.*, 2002).

Production of transgenic plants that express cellulase enzymes are thought to have great potential for use in the conversion of lignocellulosic biomass to ethanol. Ziegelhoffer *et al.* (1999) produced transgenic alfalfa plants carrying the E2 and E3 cellulase genes of *Thermomonospora fusca* under the control of the MAC promoter and manopine synthase transcription terminator from *Agrobacterium*. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *npt*II gene. Recombinant cellulase expression by transgenic alfalfa plants ranged from 0.04% of total soluble protein for E3 cellulase to 0.1% of total soluble protein for E2 cellulase (Ziegelhoffer *et al.*, 1999).

The extraction and purification of proteins from biochemically complex plant tissues is a laborious and expensive process that presents a major limitation to large-scale protein production using transgenic plants. Exudation of recombinant proteins from roots may offer an alternative for use of alfalfa in the production of value-added phytopharmaceuticals. Transgenic alfalfa plants were produced following transformation with a fungal cDNA from *Trichoderma atroviride* encoding an endochitinase gene (*ech42*) fused in frame to the signal peptide of a white lupin acid phosphatase and under the control of the CsVMV promoter and NOS 3′ terminator (Tesfaye *et al.*, 2005). Plant transformation vectors were introduced into alfalfa by *Agrobacterium*-mediated transformation. Kanamycin resistance was used as the selectable marker for transformed alfalfa. Chitinase mRNA and protein synthesis in transformed plants was accompanied by chitinase enzyme activity. The recombinant ech42 enzyme was secreted into the rhizosphere. Chitinase activity in root exudates of transgenic alfalfa was 7.5–25.7 times higher than in the nontransformed control alfalfa plants. The secreted recombinant endochitinase retained its lytic activity against glycol chitin substrate and also showed antifungal activity by inhibition of spore germination of two fungal pathogens.

2.6.4 Phytoremediation of herbicide contaminated soils

Atrazine is one of the most widely used herbicides in the United States. In specific Gram-negative soil bacteria, atrazine chlorohydrolase (atzA) is the first enzyme in a pathway that catalyses the hydrolytic dechlorination and detoxification of atrazine to hydroxyatrazine. The potential of transgenic alfalfa expressing an *atzA* gene to take up, dechlorinate, and detoxify atrazine was investigated (Wang *et al.*, 2005). Transgenic alfalfa plants were generated containing an *atzA* gene modified for plant expression, *p-atzA*, under the control of the CsVMV promoter and NOS 3′ transcription terminator. Plant transformation vectors were introduced into alfalfa following co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Kanamycin resistance conferred by the *npt*II gene was used as the selectable marker for transformed alfalfa. Transgenic alfalfa plants expressing *p-atzA* grew over a wide range of atrazine concentrations. Thin layer chromatography analyses indicated that *in planta* expression of *p-atzA* resulted in the production of hydroxyatrazine. Hydroponically grown transgenic alfalfa dechlorinated atrazine to hydroxyatrazine in leaves, stems, and roots (Wang *et al.*, 2005).

3. FUTURE ROAD MAP

3.1 Expected Products

The development and commercialization of Roundup Ready® alfalfa has paved the way for development of additional biotechnology-derived traits in alfalfa. However, the traits developed must be of high economic value in order to recover the considerable costs of developing and releasing transgenic crop varieties. Because alfalfa is used primarily for livestock feed, the direct value of a biotechnology-derived trait would be targeted to the producer of alfalfa or livestock, rather than to the general public. Traits of interest to livestock producers include increasing forage digestibility and decreasing the potential for bloat. Recently, significant advances have been made to reduce the lignin content and/or composition of alfalfa to increase digestibility (Guo *et al.*, 2001; Marita

et al., 2003; Reddy *et al.*, 2005; Ralph *et al.*, 2006). Additionally, a greater understanding of the genes for proanthocyanidin (condensed tannin) production has been achieved (Ray *et al.*, 2003; Xie *et al.*, 2003), which will accelerate the development of bloat-safe alfalfa. Forage yield is of primary importance to alfalfa producers. A significant decrease in leaf senescence, with a concomitant delay in plant flowering, has been achieved by controlled expression of isopentenyl transferase, an enzyme involved in cytokinin biosynthesis (Gan and Amasino, 1995; Sandman *et al.*, 2003). In addition to potentially increasing yields, a delay in flowering will provide producers with greater flexibility in crop harvest. Enhancing crop adaptation, such as tolerance to salt, cold, or acid soils using transgenic approaches may not have the economic return for commercializing these traits.

3.2 Risks and Concerns

Alfalfa is an insect-pollinated crop; therefore, safeguards are needed to ensure trait purity of the biotechnology-derived trait and to mitigate the dissemination of a regulated trait. Field studies were conducted in the seed-producing areas of Canyon County, Idaho using leaf cutter bees (Fitzpatrick *et al.*, 2003) and Kings County, California using honeybees (Teuber *et al.*, 2004) to measure pollen flow. Based on these studies, 1.6 km minimum isolation for production of Roundup Ready® alfalfa seed was established.

3.3 Expected Technologies

Transformation of alfalfa chloroplasts, via optimization of biolistic transformation, would facilitate development of specific biotechnology-derived traits. Chloroplast transformation in other crops has been shown to increase gene copy number and protein production. Enhanced product accumulation would be useful in production of vaccines and other high value proteins. In addition, retaining some protein products, such as cell wall hydrolases, within the chloroplast may protect the cell from deleterious effects of production. Finally, the chloroplast is an ideal location for synthesis of some biopolymers, such as polyhydroxyalkanoates. Because chloroplasts are found in alfalfa pollen, chloroplast transformation would not be a means of managing trait dissemination.

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