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Transformation of alfalfa with a bacterial fusion gene, *Mannheimia haemolytica* A1 *leukotoxin50-gfp*: Response with *Agrobacterium tumefaciens* strains LBA4404 and C58

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Abstract

Alfalfa transformed with a portion of the leukotoxin gene from *Mannheimia haemolytica* was produced to test the feasibility of developing an edible vaccine capable of protecting cattle from pneumonic pasteurellosis. Leukotoxin (Lkt), has been identified as an important protective antigen of *M. haemolytica*, and a fragment, Lkt50, was shown to produce toxin-neutralizing antibodies in rabbits. The construct chosen for introduction into alfalfa carried *lkt50* fused to a green fluorescent protein reporter gene, *mgfp5-ER*. The fusion gene was driven by either the cauliflower mosaic virus 35S promoter (35S) or the promoter from a rubisco small subunit (rbcS-3A) gene of pea. The constructs were introduced into alfalfa RSY27 germplasm using two *Agrobacterium tumefaciens* strains, LBA4404 and C58, producing a number of transformed lines with both *A.* strains. Although strain C58 had a slower initial response and produced less callus than strain LBA4404, it resulted in higher numbers of transformed embryos and plants. In total, 30 alfalfa lines (91% of those analyzed), each derived from a separate transformation event, produced detectable levels of Lkt50-GFP. Western analysis with anti-Lkt + 66 antiserum revealed the presence of both full-length and truncated polypeptides in plants kept in magenta boxes, while plants transferred to the greenhouse produced only the full-length product. Immunoblotting with anti-GFP antiserum provided evidence that part of the GFP moiety was lost in the truncated protein. Southern blot analysis indicated a low number of insertion sites per event.

Introduction

Plants expressing selected immunogenic proteins can be used to deliver edible vaccines (Mason and Arntzen, 1995). Our research has focused on the development of an edible vaccine against bovine pneumonic pasteurellosis, or shipping fever. *Mannheimia haemolytica* A1 is the principal microorganism associated with shipping fever, a major cause of sickness and death and significant economic loss in feedlots of North America. Leukotoxin (Lkt), a virulence factor, has been identified as an important protective antigen (Confer et al., 1995). We have demonstrated that an Lkt fragment (Lkt50) synthesized as a fusion protein with green fluorescent protein (GFP) in transgenic clover can induce an immune response in rabbits by eliciting antibodies that neutralize authentic Lkt (Lee et al., 2001). This fusion protein was also found to be stable in post-harvest processing and storage of field-grown clover (Lee et al., 2003).

Alfalfa is the premium forage for cattle, noted for its feeding value, high yields and palatability, and the technology for drying and forming it into cubes is well established. It is therefore the preferred crop to serve as the basis for an edible vaccine. Our initial efforts have focused on establishing an efficient *Agrobacterium tumefaciens*-mediated alfalfa transformation protocol to produce high number of transgenic alfalfa lines expressing the *M. haemolytica* A1 leukotoxin fragment (Lkt50).

Alfalfa transformation has been successful due largely to the use of highly regenerative genotypes and the selection of appropriate plant genotype/bacterial strain combinations (Samac, 1995). Critical to the transformation of the highly regenerative genotype RSY27 has been the use of *A. tumefaciens* strain LBA4404 (Austin et al., 1995; Micallef et al., 1995; Ziegelhoffer et al., 1999). When transforming the RSY27 genotype with *M. haemolytica* genes, we observed that *A. tumefaciens* strain C58, not previously associated with this germplasm, appeared to be non-responsive with the explant tissues for the first few months but eventually produced numerous embryos which developed into healthy transformed plants.

In this paper, we

- report the presence and expression of the *lkt50-gfp* fusion in alfalfa and
- discuss the implications of relatively efficient transformation with an *A. tumefaciens* strain not previously associated with the specific plant germplasm.

Materials and methods

Plant tissue

Alfalfa genotype RSY27, a highly regenerable clonal line, was kindly provided by Dr. S. Austin-Phillips (University of Wisconsin). Plants were multiplied *in vitro* by shoot-tip cuttings and maintained on MS medium (2% sucrose) supplemented with 1.0 μM indolebutyric acid. Cultures were maintained at 22 °C with a 16-h photoperiod and a light intensity of 80 μmol m^{-2} s^{-1}. Plants transferred to soil were grown in cabinets with 22/18 °C (day/night) temperatures, a 16 h photoperiod and light intensity of 300 μmol $m^{-2}s^{-1}$.

Plasmids and agrobacterium strains

Binary vector pBINmgfp5-ER (kindly provided by Dr J Haseloff, University of Cambridge, UK), uses the cauliflower mosaic virus 35S promoter (35S) to direct expression of the gene for green fluorescent protein (GFP) variant mGFP5, which contains three amino acid substitutions as well as ER targeting and retention signals (Haseloff et al., 1997). A truncated derivative of the leukotoxin gene, *lkt50*, lacking the moiety associated with cytotoxicity, was fused to *mgfp5* and subcloned back into pBINmgfp5-ER as described previously (Lee et al., 2001). The fusion gene includes sequences encoding the signal peptide and an ER-retention signal, and carries the polyadenylation signal from nopaline synthase. A second binary vector was also constructed in which the *Sca*I-*BamH*I fragment of 35S was removed and replaced with a 431 nucleotide promoter region directly upstream of the start codon of the pea rubisco small subunit (rbcS-3A) gene (Kuhlmeier et al., 1988). Binary vectors containing the fusion genes were introduced into *A. tumefaciens* strains LBA4404 or C58 via electroporation. *A. tumefaciens* strain LBA4404 contained the helper plasmid pAL4404 and was grown on YEP medium with kanamycin (50 mg l^{-1}) and streptomycin (50 mg l^{-1}), while C58 containing helper plasmid pMP90 was grown in LB medium with kanamycin (50 mg l^{-1}) and gentamycin (25 mg l^{-1}).

Plant transformation and regeneration

Plants maintained in growth cabinets (see Plant tissue section) were used for transformation according to the leaf-disk method of Austin et al. (1995) with some modifications. These included an incubation period of 15 min with agrobacteria, a co-cultivation period of 5 days in the dark on B5H medium (Brown and Atanassov, 1985), and the use of timentin (300 mg l^{-1}) to kill the agrobacteria. Media used were the following: co-cultivation- B5H (Growth regulators: 2,4-D (1.0 mg l^{-1}) + kinetin (0.1 mg l^{-1})); selection/induction-B5HKT (growth regulators as in B5H + kanamycin (25 mg l^{-1}) + timentin (300 mg l^{-1})); selection/ embryogenesis-B5OKT (B5HKT without growth regulators); and selection/regeneration- MSKT (MS medium with kanamycin (25 mg l^{-1}) + timentin (300 mg l^{-1})).

For all the plasmids/agrostrains tested, 12 explants per replication and three replicates were used. For response on selection media, data collected included explants producing embryos, numbers of embryos produced per explant and number of plants regenerated from independent transformation events. Data for explants producing embryos was analyzed by Analysis of Variance and Duncans Multiple Range test was used to detect significant differences between means (SAS, 1989).

For a positive regeneration control, nontransformed explants were placed on B5H medium for callus production and then transferred to B5H medium without growth regulators to induce embryo formation. As a negative control, nontransformed explants were placed on B5H medium with kanamycin (25 mg l^{-1}) to ensure effective selection of transformants.

Fluorescence microscopy

Putative transformants were examined by conventional fluorescence microscopy, using a Leica MZ 111 fluorescence stereomicroscope with a GFP3 filter set for excitation at 470/40 nm and emission at 525/50 nm.

Western immunoblot analysis

Protein expression in plants was evaluated by western immunoblotting. Total protein extracts were prepared by grinding plant material with 2 ml phosphate-buffered saline containing 0.5% (w/v) Tween 20 per g fresh weight. Insoluble material was removed by centrifugation in a microfuge at maximal speed (18,000 rpm) for 10 min at 4 °C. Proteins were separated by SDS-PAGE and immunoblotting was performed with a rabbit anti-Lkt66 antiserum (derived from a less truncated form of leukotoxin) as described previously (Lee et al., 2001).

DNA isolation and Southern hybridization

Total genomic DNA was extracted from transformed and control alfalfa leaf tissue using a commercially available kit (DNeasy Plant Mini Kit, Qiagen, Mississauga, ON). Ten micrograms of genomic DNA was digested overnight with *Hin*dIII, concentrated by ethanol precipitation and separated on a 0.7% agarose gel. A downward alkaline transfer of DNA to a nylon membrane (Maximum Strength Nytran, Schleicher and Schuell, Keene, NH) was performed as described by Meng (1994). A digoxigenin-labeled probe to *gfp* (the generous gift of Dr A. Nassuth, University of Guelph, Guelph, ON) was hybridized to the blot. Subsequent washes and chemiluminescent detection were performed according to standard techniques (DIG System User's Guide for Filter Hybridization, Boehringer Mannheim). The chemiluminescent signal was recorded with a FlourChem 8800 imaging system (Alpha Innotech, San Leandro, CA).

Results

Response in explants

The response of explants to *A. tumefaciens* strain LBA4404 was rapid with all three constructs. Callus formation initiated at cut edges of leaves within 2 weeks of inoculation. After 4 weeks on induction medium there was proliferation of callus that covered a major portion of the explant and a few embryos had formed (Table 1; Figure 1a, b). However, the majority of embryos formed after the transfer of explants to embryogenesis selection medium (B5OKT).

In comparison, at the end of 4 weeks explants transformed with strain C58 had produced no callus and the tissue appeared dead. Instead of discarding these plates, we subcultured explants on fresh induction medium (B5HKT) every 4 weeks. Two months after inoculation, small foci of callus and a large number of bright green embryos could be seen (Table 1; Figure 1c, d). Embryos forming on explants transformed with both *A. tumefaciens* strains were transferred to regeneration medium (MSKT), on which a number germinated under selection (Figure 1e). Those with sufficient root and shoot growth were transferred to magenta boxes. A few of these lines were propagated and eventually transferred to pots in the greenhouse (Figure 1f). Even though the initial response with strain C58 was slow, the total time required for regenerating transformed plants was approximately 12 weeks for both strains.

A summary of the transformation results with different constructs and *A. tumefaciens* strains is

Table 1. Response of alfalfa leaf disks transformed with *A. tumefaciens* C58 and LBA4404 containing *lkt50-mgfp5* fusions

A. tumefaciens strain	Time after inoculation (weeks)	Response in explants
LBA4404	2	Callus formation at leaf edges
	4	Lots of callus and few embryos. Subcultured to B5OKT
	8–10	Explants with callus, embryos. Embryos transferred to MSKT
	~12	Plantlets produced
C58	2	No response
	4	Senescence of explants. Subcultured on B5HKT
	8–10	Explants brown in colour except for bright green embryo and very small amounts of callus. Embryos transferred to MSKT
	~12	Plantlets produced

B5HKT = B5 medium + growth regulators + kanamycin + timentin; B5OKT = B5 medium + kanamycin + timentin but no growth regulators; MSKT = MS medium + kanamycin + timentin.

given in Table 2. Only bright green embryos with normal development were included in the data sets. For all constructs, the number of explants producing embryos was slightly higher for C58-exposed leaf disks. Differences were even more pronounced in the numbers of embryos that formed on each explant; explants transformed with the C58 strain produced as many as 30 individual embryos per disk.

For all three constructs tested we were able to regenerate more plants in transformations with *A. tumefaciens* strain C58 than with LBA4404 (Table 2). The plant regeneration data include only numbers from clearly independent transformation events. Explants transformed with the C58 strain produced a number of well-separated embryos with little intervening callus, such that it was possible to identify multiple transformation events per explant. On leaf disks transformed with the LBA4404 strain, in contrast, embryos generally formed within massive beds of callus, rendering it difficult to distinguish independent transformation events.

GFP expression in plants

Leaves from nontransformed plants failed to exhibit green fluorescence. Bright green fluorescence was detected in roots, stems, petioles and leaves of plants transformed with *mgfp5-ER*, the positive control construct. Plants transformed with *lkt50-gfp* fusion constructs exhibited either no, or low levels of, fluorescence in meristematic areas such as nodes and apical meristems. There were no obvious differences in the fluorescence patterns of plants transformed with the two *A. tumefaciens* strains.

Molecular analysis of transformants

Plants surviving selection on kanamycin and maintained in magenta boxes were used for analysis of gene expression by western immunoblotting. All the plants analyzed from independent transformation events had detectable levels of Lkt-GFP expression. Western immunoblot data for three lines of alfalfa from the selection medium (2-3B, 1-5F, 4-2A), along with field-grown transgenic clover (Lkt-2) as a positive control, are shown in Figure 2A. Rabbit antisera against Lkt66 (Figure 2A) immunostained proteins in all the putatively transformed alfalfa plants, indicating that they possessed the Lkt epitope. Lines 1-5F and 2-3B exhibited the 80 kDa protein predicted from the nucleotide sequence, while line 4-2a exhibited only a 66 kDa band that was also seen in 1-5F and 2-3B. The 66 kDa band immunostained with anti-Lkt (Figure 2A) but not with anti-GFP (Figure 2B). Blots treated with anti-GFP produced secondary bands of about 29 kDa. Extracts from untransformed wild type alfalfa plants (wt) do not produce immunoreactive bands with either antibody (Figure 2B).

The plants from magenta boxes were transferred to soil, grown until well established, then reanalyzed by western immunoblotting. In propagated plants transferred to the greenhouse before testing (Figure 3, gh), the 80 kDa protein was present in greater amounts than in the same plants grown under sterile tissue culture conditions in

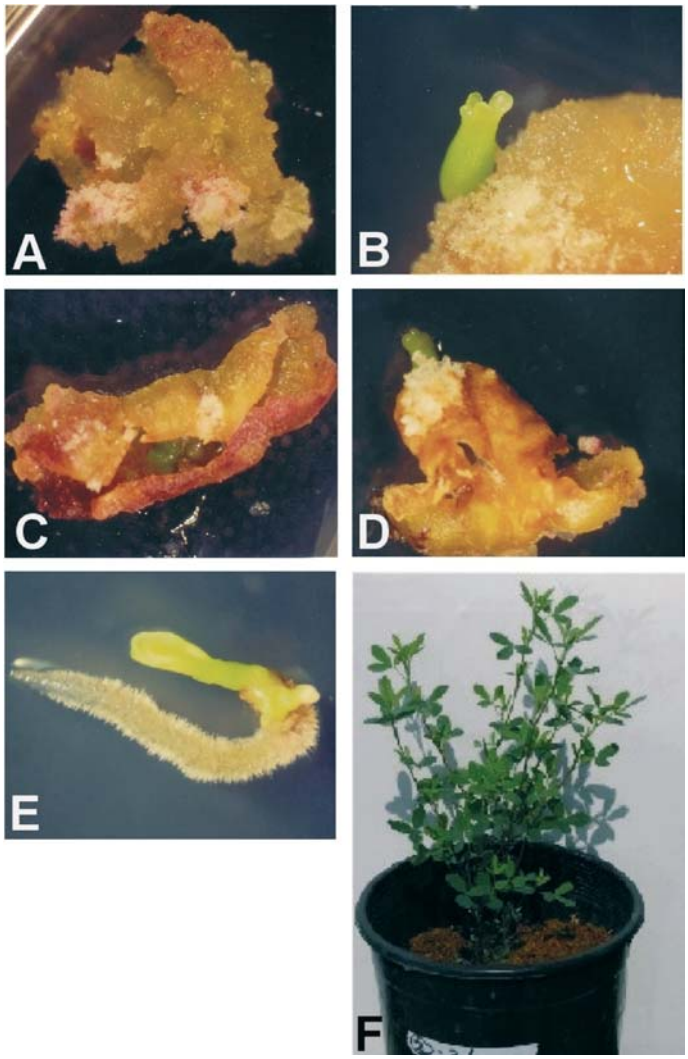

Figure 1. Leaf disks transformed with *A. tumefaciens* LBA4404 (A and B) or C58 (C and D). (A) Callus formation, 4 weeks; (B) embryo formed on callus; (C) embryos forming in centre of folded senescing leaf explant, 8 weeks; (D) embryo at the edge of a senescing leaf explant; (E) embryo germinating on selection medium; (F) transgenic alfalfa plant transferred to soil.

magenta boxes (Figure 3, tc), and the 66 kDa protein recognized by anti-Lkt was no longer detectable. The levels of Lkt-GFP expression were higher in some lines than others but there were no obvious differences between plants derived from the two different *A. tumefaciens* strains, and no apparent differences between transgenes driven by the 35S (Figure 3, 2-3B) and rbcs-3A (Figure 3, 1-5F) promoters.

Southern blot analysis of transgenic plants 1-5F and 2-3B probed with *gfp* indicated the presence of *gfp* sequences in the transformed lines but not controls (Figure 4). The Southern data also verify that the two lines are derived from separate transformation events, with two and one insertion site(s) respectively,

Discussion

Agrobacterium-mediated transformation of alfalfa has been successful largely due to matching precise *A. tumefaciens* strains with specific germplasm. The browning of explants and absence of callus formation, such as we observed with *A. tumefaciens* strain C58, are often used as criteria for

Table 2. Results of transformation with different constructs using *A. tumefaciens* C58 and LBA4404

Plasmids/agrostrains	Explants[a]	Explants producing embryos[b]	Range in numbers of embryos/explant	Plant regeneration[c]
pBin35S-mGFP5/C58	12	5.3 AB	1–10	10
p35S-Lkt50-mGFP5/C58	12	5.0 AB	1–9	20
pRbcs-Lkt50-mGFP5/C58	12	7.0 A	1–30	31
pBin35S-mGFP5/4404	12	2.7 B	1–6	5
p35S-Lkt50-mGFP5/4404	12	2.3 B	1–6	7
pRbcs-Lkt50-mGFP5/4404	12	3.7 B	1–6	3

[a]12 explants per replication.
[b]Means followed by the same letter are not significantly different.
[c]Plant with roots and shoots; total numbers from 3 replicates; only one embryo from cluster transferred for regeneration.

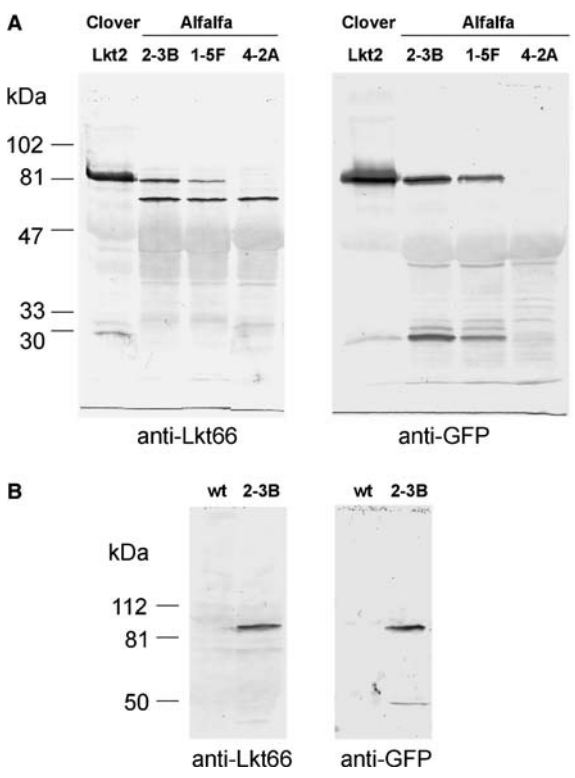

Figure 2. Fusion protein expression in three transgenic alfalfa lines. (A) Plants maintained in magenta boxes were analyzed by western immunoblotting with rabbit antisera against Lkt66 (anti-Lkt66) and GFP (anti-GFP). Three alfalfa lines 2-3B, 1-5F and 4-2A are shown along with a field-grown Lkt50-producing white clover line (Lkt2). Molecular markers in kDa are shown at the left. (B) Unlike the transgenic alfalfa line 2-3B (2-3B) which produces an 80 kDa protein containing both Lkt and GFP epitopes, untransformed wild type alfalfa (wt) do not result in any immunoreactive bands with either antibody (anti-Lkt66, anti-GFP) in immunoblots.

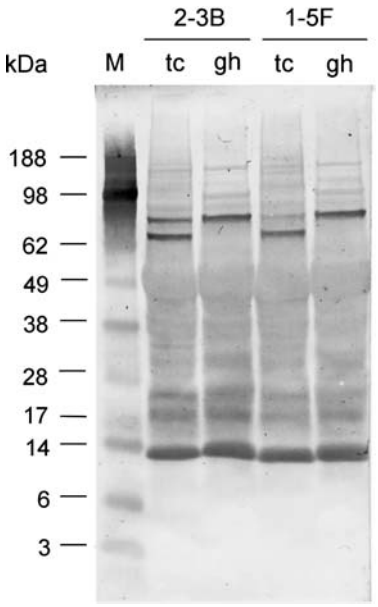

Figure 3. Changes in fusion protein levels in propagated alfalfa lines. Greenhouse-grown (gh) plants were compared with the same lines maintained in tissue culture (tc) in magenta boxes by western immunoblotting with rabbit antisera against Lkt66. Protein extracts from alfalfa lines 2-3B (2-3B), containing 35S-*lkt-gfp* and 1-5F, containing rbcS3A-*lkt-gfp* were used. The lane marked 'M' contains molecular markers and the kDa are shown at the left.

eliminating strains (Samac, 1995). Continued maintenance of senescing tissue via subculturing was critical for detecting the ultimate responsiveness of the C58 strain. By subculturing the apparently dead tissue we ultimately obtained more transformed plants from C58 than LBA4404.

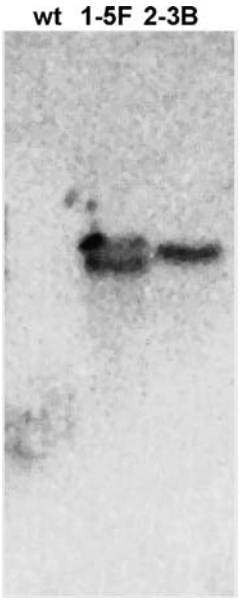

Figure 4. Southern blot analysis of transgenic alfalfa. Genomic DNA samples from untransformed wild type alfalfa (wt) and transgenic alfalfa lines 1-5F and 2-3B were digested with HindIII and analyzed by hybridization to a *gfp* probe. The blot shows evidence for *gfp* sequences only in the transformed lines and for recovery of the two lines from independent transformation events.

Thus, an *A. tumefaciens* strain apparently unresponsive with specific germplasm in the initial stages can ultimately prove to be more useful for plant transformation.

There is some evidence that the specific DNA sequences being introduced, along with the plant germplasm and *A. tumefaciens* strain, may be an important determinant of transformation efficiency (Desgagnes et al., 1995). In our hands, transformations with these and other constructs (data not shown) have occurred with similar frequencies.

Explants transformed with strain C58 developed a number of bright green embryos but very little callus. Limited callus formation can be beneficial for a number of reasons. Normally only one transformant is selected per explant, to ensure that each is derived from an independent event. Based on the location of the embryos on the senescing explant with very little callus, however, independent transformation events can be recognized with relative ease, allowing for higher efficiency in recovering transgenic plants. This may have contributed to, but does not fully explain, the higher transformation efficiencies reported here for strain C58.

Use of the green fluorescent reporter protein (GFP) facilitates direct visualization and provides a non-destructive method for assaying transformants. Chimaeric proteins, however, may or may not produce GFP fluorescence: alfalfa transformed with GFP exhibited bright fluorescence but those transformed with Lkt50-GFP exhibited little or none, although presence of the fusion protein was confirmed by western immunoblotting. A similar pattern was observed with white clover transformation (Lee et al., 2001), for which the more powerful technique of laser scanning confocal microscopy was required to visualize the low intensity fluorescence. We cannot determine the extent to which lower production of fusion protein or its improper folding reduced the level of GFP fluorescence. Whatever the explanation, fluorescence of fusion proteins would have to be higher than that seen in our Lkt50-GFP constructs to permit screening of putative transformants by fluorescence microscopy. An alternative form of GFP such as GFP S65C, which has been reported to fluoresce more strongly than mGFP5 in alfalfa (Bellucci et al., 2003), might permit fluorescence-based detection of our fusion proteins.

All the independent transformants analyzed by western immunoblotting had detectable levels of Lkt-GFP protein, indicating effective selection in our transformation system. However, expression levels in alfalfa were generally lower than those observed earlier in white clover (Lee et al., 2001, 2003). Levels of fusion protein in LBA4404-derived transformants were similar to those derived from C58. Some researchers have attributed poor levels of expression in alfalfa to use of the 35S (CaMV) promoter (Narvez-Vasquez et al., 1992). In our hands Lkt50-GFP expression levels were similar for 35S and pea rubisco small subunit (rbcS-3A) promoters. The fusion gene driven by 35S was also tested in tobacco, where transgene expression was significantly higher (data not shown). Levels of transgene expression driven by a hybrid Mac promoter have been reported to be lower in alfalfa than tobacco as well (Ziegelhoffer et al., 1999). Identification of an appropriate alfalfa promoter, from studies such as that described by Khoudi et al. (1997), would greatly facilitate efforts such as ours.

The availability of two antibodies for protein detection, anti-GFP and anti-Lkt66, was useful for providing evidence about the aberrant polypeptide produced by transformants harvested from magenta boxes. The protein immunostained with anti-Lkt66 but not anti-GFP, suggesting that part of the GFP was lost from the shorter protein. The samples containing the shorter anti-Lkt66-reacting fragment shorter fragments produced a 29 kDa band recognized by anti-GFP. We have seen a similar phenomenon with other chimaeric genes (data not shown). The simplest explanation is that some fraction of the chimaeric proteins have been cleaved close to the fusion site between the *M. haemolytica* antigen and GFP. The shift in banding patterns seen in the older plants subjected to a greenhouse environment was unexpected, and underscores the importance of continued monitoring of transgene expression.

Using the RSY27 germplasm we have been able to produce a number of alfalfa lines expressing the *lkt50-gfp* fusion gene. The lines analyzed so far indicate generally a low number of transgene insertion sites and verify our recovery of independent transformation events. There are no obvious phenotypic differences between plants producing the fusion protein and controls. Selected lines that are expressing higher levels of the fusion protein are being propagated in the greenhouse in preparation for calf-feeding tests to assess their potential for use in a plant-based edible vaccine.

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