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Production of *Bean yellow mosaic virus* resistant subterranean clover (*Trifolium subterraneum*) plants by transformation with the virus coat protein gene

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Summary

Transgenic lines of subterranean clover were constructed that contained three different *Bean yellow mosaic virus* (BYMV) coat protein (CP) gene constructs; full-length CP, the core region of the CP, and full-length CP plus the 3' untranslated region of the viral genome. Transgenic plants containing the full-length and core CP gene constructs showed high and moderate levels of BYMV resistance. Resistance was measured as a lack or amelioration of viral disease symptoms, which was correlated with a reduction in virus levels and yield loss. A range of different resistance phenotypes was observed. They included reduced infection rates, delay and reduction in local lesion development, and delay and reduction in severity of systemic symptom development. Resistance levels were not correlated with transgene mRNA levels and no transgene-encoded protein was detected in any of the transgenic lines. This is the first example of genetically engineered virus resistance in a clover.

Key words: Bean yellow mosaic virus, subterranean clover, coat protein, virus resistance, genetic engineering, transgenic plants

Introduction

Subterranean clover (*Trifolium subterraneum* L., subclover) is the major pasture legume in Australia and is grown on more than 16 million ha of mainly acidic and infertile lands. *Bean yellow mosaic virus* (BYMV) is recognised as an economically significant pathogen of subclover (Johnstone & McLean, 1987; Jones, 1994, 1996). Studies have indicated that BYMV can induce subclover herbage and seed yield losses by up to 49% and 47%, respectively (Jones, 1994).

BYMV is a member of the Potyviridae in the Potyvirus genus (Barnett, 1991, 1992). The Potyviridae comprises the largest group of plant viruses including many economically important pathogens. Potyviruses have a single-stranded, positive sense RNA genome that is approximately 10 000 nucleotides (nt) long (Riechmann, Lain & Garcia, 1992). It contains one long open reading frame (ORF) that encodes a polyprotein that is proteolytically processed into individual viral proteins. The genome structure of all potyviruses is similar with the nucleotide sequence encoding the coat protein (CP) located at the 3' end of the genome.

There is a high degree of sequence similarity in the CP of viruses of the Potyviridae, especially among viruses belonging to the same virus group (Dougherty, Willis & Johnson, 1985; Shukla & Ward, 1988). The greater sequence variability that occurs between the CPs of viruses in different groups is in the N- and C-termini (Shukla & Ward, 1988, 1989). Several studies have shown that N- and C- terminal regions of the CP are exposed on the viral particle surface (Dougherty *et al.*, 1985, Shukla *et al.*, 1988). Mild treatment with trypsin removed the N- and C-terminal regions leaving a core region which contains the group-specific antigenic epitopes (Shukla *et al.*, 1988).

The sequence of the 3' region of the genome has been determined for several BYMV strains (Hammond & Hammond, 1989; Boye, Jensen, Stummann & Henningsen, 1990; Tracey *et al.*, 1992; Nakamura *et al.*, 1994; Mathews, Dwyer, Wylie & Jones, 1995; Guyatt, Proll, Menssen & Davidson, 1996). In the S strain of BYMV (BYMV-S) the CP ORF is 846 nt long (encoding a 30 816 Dalton protein of 282 amino acids) followed by a 173 nt long 3' untranslated region (UTR) (Tracey *et al.*, 1992). The core region lies between amino acid residues 39 and 255.

CP sequences are the most common region of the viral genome that has been selected to elicit pathogen derived resistance (PDR) (Sanford & Johnson, 1985;

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Nelson, Powell & Beachy, 1990; Fitchen & Beachy 1993). PDR is based on the stable integration of part of the viral genome into the genome of the host plant and results in the host acquiring resistance to the virus (Sanford & Johnson, 1985). CP-mediated resistance has been demonstrated in a number of potyviruses (Fitchen & Beachy, 1993; Lindbo, Silva-Rosales & Dougherty, 1993).

A transformation and regeneration system enabling genes to be introduced into subclover via *Agrobacterium tumefaciens*-mediated gene delivery has recently been developed (Heath, Chin, Spencer & Higgins, 1993; Khan *et al.*, 1994). In this paper we report the production of BYMV-resistant subclover plants following the introduction of either the BYMV CP or the CP core region, whereas plants transformed with the CP plus the 3' untranslated region did not show any resistance to BYMV. A range of resistance phenotypes was observed in the BYMV-resistant transgenic subclover plants.

Materials and Methods

Virus isolation, propagation and purification

BYMV-S (Randles, Davies, Gibbs & Hatta, 1980), kindly provided by J Randles, Waite Agricultural Research Institute, Adelaide, South Australia was maintained and propagated in *Vicia faba* cv. Aquadulce in an insect-proof glasshouse and purified by the method described by Samah (1982). Virus preparations were examined by electron microscopy (JOEL JEM 100S) for virus particle integrity after staining with 2% ammonium molybdate, pH 6.5. The concentration of purified virus preparations was estimated spectrophotometrically by assuming an A_{260} of 3 as equal to 1 mg ml^{-1} of virus.

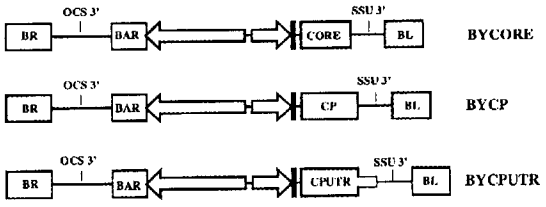

Fig. 1. Schematic diagram of the three BYMV CP gene constructs in the plant transformation vector pTAB10. The three constructs; BYCORE, BYCP and BYCPUTR, differ only by the regions of the BYMV genome that they contain. CORE corresponds to the core region of the CP, CP is the entire CP ORF, and CPUTR corresponds to the entire CP ORF plus the 3' untranslated region of the viral genome. The CP genes are flanked 5' by the CaMV 35S promoter (small arrow) and the AMV leader sequence (solid box), and 3' by the tobacco rubisco small subunit transcription termination sequence (SSU 3'). The *bar* gene (BAR) is flanked 5' by the CaMV 35S promoter (large arrow) and 3' by the octopine synthase transcription termination sequence (OCS 3'). BR and BL correspond to the right and left border sequences. The various regions of the constructs are drawn to scale.

Construction of BYMV CP transformation vectors

A cDNA clone of the 3' terminal region of the BYMV-S genome (Tracey *et al.*, 1992) was used as the template for PCR amplification (Sambrook, Fritsch & Maniatis, 1989) of the three specific BYMV CP sequences. A schematic diagram of the BYMV CP constructs (BYCP, BYCORE, BYCPUTR) used in this study is shown in Fig. 1. The entire CP coding region (BYCP) was amplified by PCR using the 5' primer A (5' GAATTCGGATCCTAAACAATG GCTTCTGACCAAGAGAAGCCC) which contained *Eco*RI and *Bam*HI recognition sequences followed by an ATG translation initiation codon in the optimal context (Kozak, 1981), and the first 18 nt of the BYMV CP ORF (underlined), and the 3' primer B (5' GAATTCGTCGACCTAAACACGAACACC AAGCATGG) which contained *Eco*RI and *Sal*I recognition sequences and the last 20 nt of the CP ORF (underlined). The core region of the BYMV CP sequence (BYCORE; residues 39–255) was amplified using the 5' primer C (5' GAATTCGGATCCT AAACAATGGCTGATATCAATACAGGAACTG) which contained the same non-viral sequences as primer A and the first 19 nt of the CP core region (underlined), and the 3' primer D (5' GAATT CGTCGACCTATCTCTCTGTGTTCTCCTCG) that contained the same non-viral sequences as primer B, and the last 19 nt of the CP core region (underlined). The construct containing the BYMV CP cistron and the 3' UTR (BYCPUTR) was amplified with the 5' primer A, and the 3' primer E (5' GAATTC GTCGACCTCGCTCTACAAAGATCAAGC) which had the same non-viral sequences as primer B plus the 3' terminal 21 nt of the BYMV genome (underlined).

The PCR products were digested with *Bam*HI and *Sal*I and cloned into the intermediary plasmid pWM3 (Tabe *et al.*, 1995) digested with the same enzymes between the cauliflower mosaic virus (CaMV) 35S promoter (nt 6909–7439, Pietrzak, Shillito, Hohn & Potrykus, 1986), the alfalfa mosaic virus (AMV) 5' leader sequence (Jobling & Gehrke, 1987), and the tobacco small subunit (Tob SSU) transcription termination sequence (nt 1983–2433, Mazur & Chui, 1985). Each of the resultant BYMV coat protein gene constructs, comprising the 35S promoter, AMV 5' leader sequence, BYMV coat protein coding sequence, and 3' termination sequence (see Fig. 1) was excised from the recombinant pWM3 at the EcoRI sites, and then subcloned into the binary vector pTAB10 at an EcoRI site (Tabe *et al.*, 1995) to produce the constructs shown in Fig. 1. This vector contains the selectable marker gene, bar (De Block *et al.*, 1987) encoding phosphinothricin acetyl transferase (PAT) flanked by the CaMV 35S promoter and the transcription termination sequence from the octopine synthase (OCS) gene of Agrobacterium tumefaciens. The binary plasmid constructs were transformed into *A. tumefaciens* strain AGLI (Lazo, Stein & Ludwig, 1991) by

triparental mating (Ditta, Stanfield, Corbin & Helsinki, 1980).

Production and selection of transgenic plants

Transformation and regeneration of subterranean clover (cv. Larisa) was as described in Khan *et al.* (1994), using an *A. tumefaciens*-mediated gene delivery system. Transgenic plants appearing to contain the *bar* gene, identified by their ability to grow in the presence of phosphinothricin (PPT, 50 mg litre^{-1}) in tissue culture, were transferred to the glasshouse in autumn and acclimatised as described in Khan *et al.* (1994) except that the day/night temperature was 23°C/16°C. Expression of the *bar* gene in plants was tested by painting duplicate, young, fully expanded leaflets with 1 g litre^{-1} PPT and scoring 7 days after treatment. Transgenic plants expressing the *bar* gene were resistant to the PPT while those of non-transgenic plants were killed by the herbicide treatment (Khan *et al.*, 1994).

RNA isolation and northern blot analysis

Total RNA was isolated from young leaves of transgenic primary transformant subterranean clover plants before clonal propagation using the method described in Khan *et al.* (1994). Northern blot analyses were performed as outlined in Higgins & Spencer (1991). A BYMV CP probe was obtained by [^{32}P]-labelling of the core CP PCR generated fragment using an Amersham Megaprime DNA-labelling system according to the manufacturers instructions.

Challenge inoculation of transgenic plants

Twenty-five regenerated transformed subclover lines and eight non-transformed subclover seedlings, plus two regenerated but non-transformed lines were grown in a Biosafe glasshouse specifically designed for genetically modified plants. Plants were allowed to grow throughout winter to early spring to reach a size suitable for clonal propagation, which was standardised as follows. Cuttings were made from auxiliary shoots prior to stolon elongation and flowering. Cuttings were propagated in a mister using the John Innes CII potting mix. For each inoculation trial, sufficient cuttings were propagated from each line to generate 6–8 replicate clonal plants per inoculum level per line. Cuttings were transplanted after developing roots 3–4 wk later. When the cuttings were well established (~3–4 wk after root growth), they were cut back to one shoot with four–five fully expanded leaves and then challenged by mechanical inoculation with freshly purified BYMV-S preparations. Two or three inoculum levels, chosen from a set of concentrations of 25, 50, 100 and 200 μg ml^{-1} virus diluted in 10 mM phosphate buffer, pH 7.0, containing 1% carborundum were used for each trial. Inoculum (400 μl per plant) was applied onto the four youngest fully expanded leaves of each plant by rubbing the upper surface of each leaflet five times with the inoculum. Six to eight replicate plants per inoculum level, per line were used in each trial. Under these conditions infection of non-transformed control plants reached over 80% of inoculated plants in each trial. *Chenopodium amaranticolor* and *Vicia faba* L. cv. Aquadulce were used as local lesion and systemic infection indicator hosts, respectively. Plants were monitored daily for symptom development and local lesions were counted at 7 days post inoculation (PI). Leaf samples were harvested for ELISA before inoculation and at 1, 2, 3 and 6 wk PI as required to confirm infection and to measure virus levels. Leaves harvested after inoculation consisted of the two youngest fully expanded uninoculated (systemic) leaves of the main shoot and were stored at –20°C until ready for ELISA tests. At three weeks PI there was a good correlation between symptoms and ELISA infectivity. However 2–3 wk PI was needed to obtain reliable infectivity data. An inoculum level of 100 μg ml^{-1} was needed to obtain 100% infectivity. Symptom expression was a more reliable infectivity indicator than ELISA analysis for BYMV in the subclover cv. Larisa because of susceptibility and low ELISA values. Each putative resistant line was re-tested using three levels of fresh inoculum to confirm resistance.

Yield loss assessment

The aboveground forage of all inoculated replicate plants of each line (including both infected and uninfected replicates) was harvested at 6 wk PI when the symptoms were well established and the average dry matter (DM) yields determined. The relative yield loss for each line is the difference in the average DM yields, expressed as a percentage, between the inoculated plants and the corresponding uninoculated control plants in each line.

ELISA analysis

ELISA was carried out using a direct DAS-ELISA system as described by Clark & Adams (1977), using BYMV-S specific polyclonal rabbit antiserum kindly provided by Dr J Randles. Leaf samples were extracted in five volumes of buffer containing PBS-Tween-20 and 0.02 M sodium diethyldithiocarbamate (McLaughlin, Barnett, Gibson & Burrows, 1984), using a sap extractor (Helms, Muller & Waterhouse, 1993). Each extract was applied onto duplicate wells of a Nunc maxisorb ELISA plate using an optimised concentration of coating α-globulin (1 μg ml^{-1}) and alkaline phosphatase conjugate (0.5 μg ml^{-1}) as described by Chu, Bing-sheng, Zhong-yi & Larkin (1993). Six uninoculated and four non-transgenic infected plant samples were used as controls in each plate. Absorbance readings following ELISA were measured using a Titertek Multiscan MC ELISA reader. Samples were considered positive when both duplicate values exceeded either the total value of the mean healthy background plus five times its standard deviation or two times the mean healthy background,

whichever was the greater.

Results

Northern and western blot analysis of transgenic plants

Total RNA isolated from primary transformant transgenic plants that had tested positive for expression of the *bar* gene was analysed for the presence of the BYMV CP transcript by northern blot analysis. Only plant lines containing the BYCP and BYCORE constructs were analysed (Fig. 2) because those that contained the BYCPUTR construct did not show any degree of virus resistance (see below).

The results presented in Fig. 2 show that control non-transgenic subclover plants do not contain any RNA molecules that are able to hybridise to the BYMV CP probe. Four out of six and seven out of eight of the plant lines containing the BYCP and BYCORE constructs, respectively, had a readily visible hybridising band of approximately the same size. The RNA levels, as judged from the intensity of the hybridisation signal, varied between the different lines of transgenic plants containing both constructs. BYCORE line 7 appeared to have the highest level, followed by BYCP lines 4b and 3b and BYCORE lines 2, 4 and 8a. An intermediate level of RNA was seen in BYCP lines 2b and BYCORE lines 3 and 6. Only a low level of RNA was detected in BYCORE line 1, and BYCP lines 1a, 2a and 3a, and BYCORE line 5 either had levels below the detectable limit or did not accumulate the BYMV derived transcript.

Western blot analysis (Towbin, Staehelin & Gordon, 1979) was carried out on total leaf protein extracts from all BYCP and BYCORE transgenic plant lines that were analysed by northern blot as described above. No BYMV CP was detected in any of these lines (data not presented).

Analysis of BYMV protection

Transgenic subclover lines were tested for virus resistance by inoculation with purified virus. The relative levels of resistance were assessed using a range of criteria including local lesions (number, time taken for development, visual appearance), virus accumulation (determined by ELISA analysis), systemic symptoms (presence, time taken for development, severity), yield loss and disease recovery. Typical symptoms of BYMV in the control plants consisted of the development of necrotic and/or hypersensitive spots on the inoculated leaves (local lesions) and veinal necrosis spreading from the necrotic spots to the veins at 5–10 days PI, followed in subsequent weeks (2 wk onwards) by the development of necrotic rings and veinal necrosis on the inoculated leaves and severe vein yellowing and stunting of systemic shoots with veinal necrosis on young leaves.

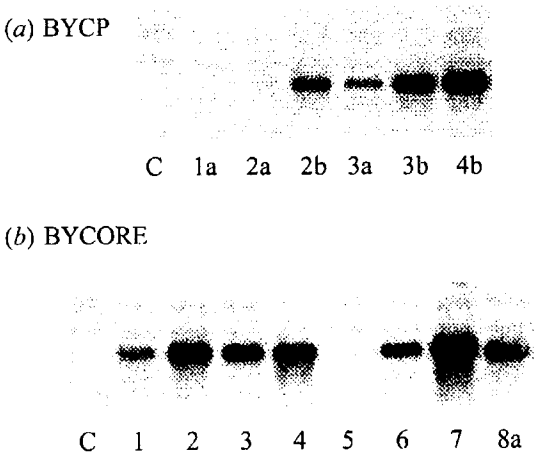

Fig. 2. Northern blot analysis of BYCP and BYCORE transgenic subclover lines. Ten micrograms of total RNA isolated from young leaves was analysed by denaturing agarose gel electrophoresis and blotted onto a nylon membrane which was then hybridised with a probe derived from the BYMV CP fragment. The individual BYCP (A) and BYCORE (B) lines are indicated below the appropriate lanes along with a control non-transgenic subclover sample (C).

Symptom development

BYCP lines 3a, 3b and 4b, and BYCORE lines 1, 2, 3, 4, 6, 7 and 8a were tested for BYMV resistance by inoculation with purified virus particles at 100 and 200 μg ml^{-1} and assessed by ELISA and a visual appraisal of systemic symptom development and severity at 2 wk PI. The bar graph presented in Fig. 3*a* shows the average ELISA values (means of all replicate plants inoculated) and proportion of replicate plants that developed systemic symptoms in the non-transgenic control, BYCP and BYCORE transgenic lines when inoculated with virus at 100 μg ml^{-1}. High levels of BYMV resistance, as judged by significantly lower ELISA values and systemic infection compared to the non-transgenic controls, were seen in BYCP line 3a and BYCORE lines 1 and 7. A lower level of BYMV resistance was seen in BYCORE lines 3 and 6, and no virus resistance was evident in BYCP lines 3b and 4b and BYCORE lines 2, 4 and 8a. Some transgenic lines (e.g. BYCORE 8a) appeared more susceptible than the non-transgenic controls. Comparison of these results with the northern blot results described in Fig. 2 above, indicates that resistance was not correlated with the level of RNA derived from the transgene, assuming that RNA levels have not altered as a result of clonal propagation.

When inoculated with virus at 200 μg ml^{-1}, the various lines showed a similar resistance pattern as that described above (data not presented). The control, non-transgenic lines showed 100% infection 14 days PI. A greater degree of disease development, as compared with plants inoculated at 100 μg ml^{-1}, was generally observed for all lines, i.e., more severe symptoms,

except among some replicates of the resistant lines BYCP 3a, and BYCORE 1 and 7. ELISA analysis of most replicates in all lines showed readings similar to the non-transgenic control samples. These data tend to suggest that the resistance seen in some of the transgenic subclover lines was inoculum-dependent and maximum protection was demonstrated at 100 μg ml^{-1}.

Yield loss

Pooled data (to include larger number of replicate plants and different genotypes) from the resistant and susceptible transgenic lines, and the control non-transgenic line inoculated at 100 μg ml^{-1} in the experiment described in Fig. 3*a* were harvested to estimate yield loss (Fig. 3*b*). Comparison of yield loss (Fig. 3*b*) showed that transgenic lines with resistance to BYMV (lines CO3, CO6, CO7, CO1 and CP3a combined [Fig. 3*a*]) resulted in substantially lower yield reduction than both the non-transgenic control line (line C [Fig. 3*a*]) and the susceptible transgenic lines (lines CO4, CO4b, CO8a, CP3b and CO2 combined [Fig. 3*a*]).

Symptom variation between different subclover lines

Marked visual differences were evident between the highly resistant, moderately resistant and susceptible transgenic subclover lines (Fig. 4). The lines that demonstrated high and moderate resistance at the 100

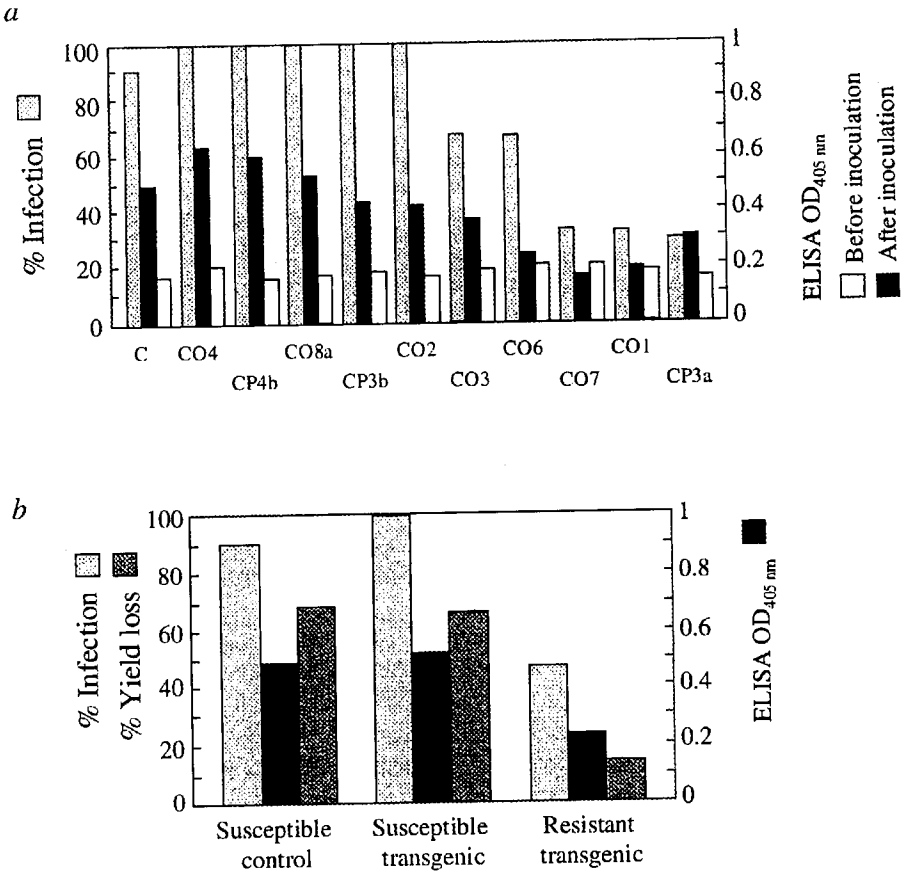

Fig. 3. BYMV accumulation (as measured by ELISA), percentage of plants showing systemic symptoms and yield loss of transgenic BYCP and BYCORE subclover lines after inoculation with BYMV-S. The various BYCP (CP), BYCORE (CO) and control non-transgenic (C) subclover lines were mechanically inoculated with BYMV virus particles at 100 μg ml^{-1}. *a*: Analysis of individual transgenic lines. The open bars represent average ELISA readings from leaf samples of all inoculated plants taken before inoculation, solid and shaded bars represent the average ELISA readings and percent of plants that showed visual signs of systemic viral infection at 2 wk PI, respectively. *b*: Percent yield loss at 6 wk PI relative to uninoculated plants. Pooled data for the control non-transgenic, susceptible and resistant transgenic lines are presented. The legends for the different bars representing average ELISA readings, % of plants showing systemic infection at 2 wk PI, and the % yield loss in each of the plant groups are shown along the y-axis.

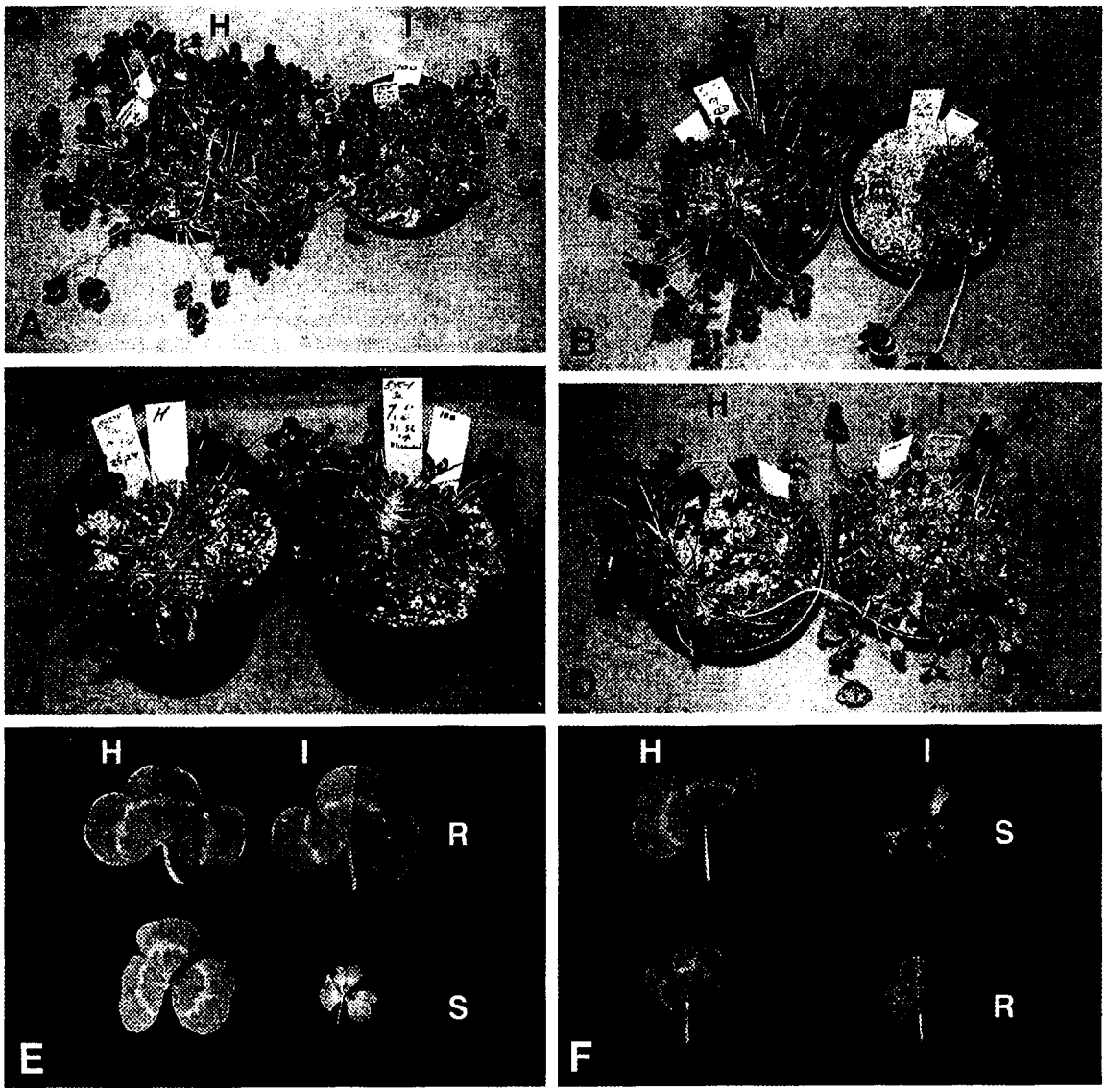

Fig. 4. Symptoms of BYMV infection on representative transgenic and non-transgenic subclover plants. Non-transgenic Control (Panel A), susceptible BYCP-4b (Panel B), highly resistant BYCP-3a (Panel C) and highly resistant BYCORE-1 (Panel D) lines are presented with an uninoculated plant (H) in the left pot and an inoculated (100 μg ml^{-1}) plant (I) in the right pot. Individual leaves from a highly and a moderately resistant BYCORE line are shown in Panels E and F, respectively. In Panel E the leaves on the top row are from a highly resistant line BYCORE-7 (R) while those on the bottom row are from a susceptible non-transgenic control plant (S). In Panel F the leaves on the top row are from a susceptible non-transgenic plant (S) and those on the bottom row from the moderately resistant transgenic line BYCORE-3 (R). In Panels E and F, leaves on the left are uninoculated (H) and those on the right inoculated (I) with virus at 100 μg ml^{-1}. Photos were taken at 2 wk PI. These photographs corresponded to the results presented in Fig. 3*a*.

μg ml^{-1} inoculum level had a range of phenotypes among the replicates of each line. In the highly resistant lines BYCP 3a, BYCORE 1 and BYCORE 7, 80%, 33% and 33% of replicates, respectively, were completely symptomless (Fig. 4*c*, *d* and *e*). The remaining replicates showed either no systemic symptoms or a delayed development of systemic symptoms compared with the non-transgenic control plants (Fig. 4*a* and *e*). In these plants there were fewer local lesions (see below) that consisted of chlorotic spots rather than necrotic spots and considerably milder systemic symptoms with no severe veinal necrosis or yellowing and only slight stunting. In the moderately resistant lines (BYCORE 3 and BYCORE 6) the infected replicates developed only milder systemic symptoms (Fig. 4*f*). However line BYCORE 6 showed a significant reduction in virus accumulation as judged by the ELISA analysis (Fig. 3*a*). The susceptible transgenic lines developed viral symptoms that were indistinguishable from the controls (Fig. 4*b*).

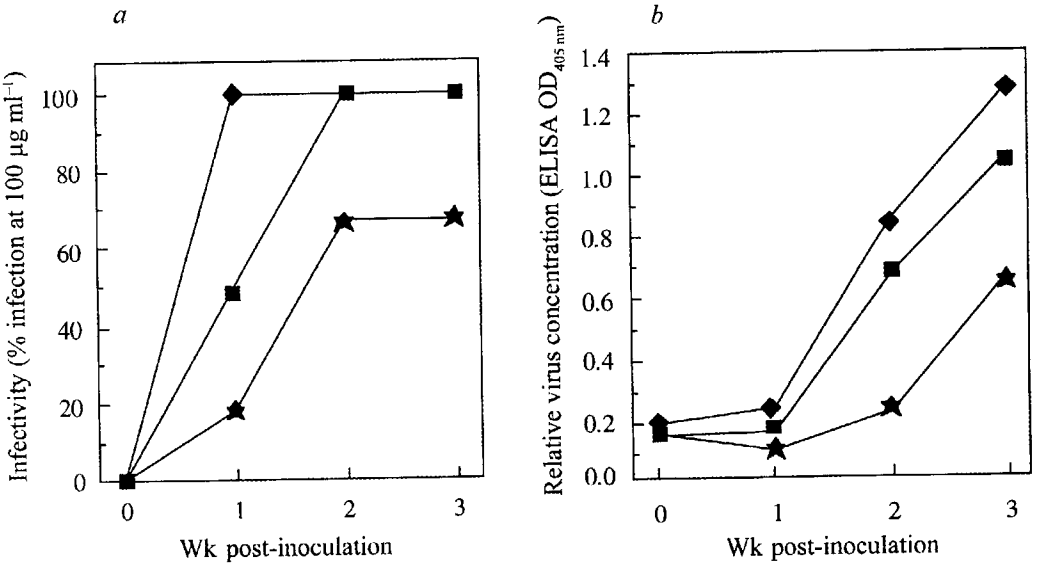

Fig. 5. Monitoring BYMV infection and virus accumulation in susceptible (BYCORE 4) and resistant (BYCORE 7) transgenic lines over 3 wk PI. Plants were assessed following inoculation at 100 µg ml^{-1} by determining the percent of replicates that showed symptoms of infection (5*a*) and by ELISA analysis (5*b*). Both graphs present data for the non-transgenic control (■) and the transgenic subclover lines BYCORE 4 (◆) and BYCORE 7 (★).

Percent infection and ELISA readings

The percent infection and average ELISA readings (means of all replicate plants inoculated) were monitored over the first three wk PI for two of the transgenic subclover lines inoculated at 100 µg ml^{-1} (Fig. 5). The two lines chosen were the susceptible line BYCORE 4 and the resistant line BYCORE 7. At 1 wk PI 100% of the replicates of the susceptible line BYCORE 4 were infected (Fig. 5*a*), whereas less than 50% and 18% of replicates from the non-transgenic controls and line BYCORE 7, respectively, were infected at 1 wk PI. At 2 wk PI 100% of the control non-transgenic plants were infected, but less than 70% of the replicates of line BYCORE 7 were showing infection symptoms. This level of infectivity in line BYCORE 7 remained the maximum level of infection that was observed. The average ELISA readings are shown in Fig. 5*b*. No detection of virus by ELISA analysis was evident until after 1 wk PI. During the second and third wk PI the ELISA readings increased sharply for the non-transgenic control and susceptible BYCORE 4 lines, with those of BYCORE 4 being slightly greater than the controls. In the resistant line BYCORE 7 the ELISA readings increased slightly between 1 and 2 wk PI and then increased to a level just over half that measured in the non-transgenic controls.

Recovery phenotype

Recovery from virus infection was observed for some of the transgenic (resistant and susceptible) and non-transgenic subclover lines between 7–10 wk PI at both the 100 and 200 µg ml^{-1} inoculation levels. These lines showed either slight or partial recovery with no lines completely recovering from infection. Slight recovery was characterised by a slight elongation of petioles and reduced leaf necrosis on some new leaves. Partial recovery was seen as increased leaf size, reduced leaf distortion and chlorosis, and

Table 1. *Local lesion numbers and percent of plants infected at 14 days PI in control, BYCP and BYCORE transgenic subclover lines*

	Inoculum levels (µg ml^{-1})			
	25		50	
	No. local lesions[a]	% infection[b]	No. local lesions	% infection
Plant Lines				
Regenerant[c]	50 ±13	100	96 ±61	100
Control[d] 1, 2	–	–	91 ±25	67
Control 3, 4	54 ±16	100	83 ±31	100
Control 5, 6	–	–	70 ±37	100
Control 7, 8	32 ±12	100	92 ±39	100
BYCP 3a	14 ±8	88	37 ±13	100
BYCP 3b	106 ±44	100	195 ±94	100
BYCORE 1	34 ±19	88	99 ±41	100
BYCORE 4	146 ±60	100	303 ±79	100
BYCORE 6	22 ±7	75	20 ±7	37
BYCORE 7	22 ±6	88	63 ±24	100

[a] Average local lesion numbers of all replicates of each line indicating the standard deviation shown by individual lines

[b] Percent of replicates infected at 14 days PI

[c] Non-transformed subclover plants regenerated through tissue culture

[d] Non-transgenic subclover plants

Table 2. *Proportion of control and transgenic subclover lines showing BYMV resistance*

Plant Lines	Resistant lines/total number of lines tested
Regenerant[a]	0/2
Control[b]	0/8
BYCP	3/5
BYCORE	5/13
BYCPUTR	0/6

[a] Non-transformed subclover plants regenerated through tissue culture

[b] Non-transgenic subclover plants

complete elongation of petioles on some new leaves. Non-transgenic control lines showed only slight recovery and this was also seen in lines BYCORE 2 and 7 and BYCP 3a. Partial recovery was evident in lines BYCORE 4 and 8a and BYCP 3b and 4b. No signs of recovery were seen in lines BYCORE 1, 3 and 6. These results indicate that in some lines the presence of the transgene may have enhanced recovery from BYMV infection.

Further analysis of selected subclover lines

Further tests for virus resistance were carried out on selected lines that demonstrated high (BYCP 3a and BYCORE 1 and 7) and moderate (BYCORE 6) resistance, and susceptibility (BYCP 3b and BYCORE 4) to infection. Included in this experiment were eight non-transgenic control lines in order to assess whether there was evidence of any natural BYMV resistance in subclover. These plants were inoculated with lower amounts of virus (25 and 50 μg ml^{-1}) which should have allowed lines with low to moderate resistance to be more easily identified. The plants were assessed by counting local lesions and by evaluating the number of replicates that became infected at 2 wk PI (Table 1). Infectivity remained the same at 6 wk PI (data not presented).

The non-transgenic control lines were all susceptible to BYMV infection and demonstrated 100% infection except for control lines 1 and 2 in which some replicates were uninfected after inoculation at 50 μg ml^{-1} virus inoculum. Transgenic lines that had previously shown resistance generally had lower local lesion numbers and/or infection levels compared to the non-transgenic controls at the 25 μg ml^{-1} level of inoculation. At 50 μg ml^{-1} inoculum level, line BYCORE 6 remained highly resistant while line BYCP 3a appeared moderately resistant compared to the controls. The results also showed that previously susceptible lines (BYCP 3b and BYCORE 4) were clearly susceptible to infection and in fact had greater numbers of local lesions than the control lines. Line BYCORE 6 had previously only shown a moderate degree of resistance. However now it appeared as resistant, if not more resistant, than the previously highly resistant lines, displaying lower local lesion numbers and percent infection even at 50 g ml^{-1}.

The plants were also assessed for symptom severity and the timing of onset of symptoms. All the control lines developed moderate to severe systemic symptoms mostly by 2 wk PI. The two previously susceptible transgenic lines (BYCP 3b and BYCORE 4) developed severe systemic symptoms within 2 wk PI. Of the previously resistant transgenic lines, BYCP 3a developed moderate systemic symptoms within 2.5 wk PI, BYCORE 1 developed moderate to severe systemic symptoms within 2 wk PI, and BYCORE 7 displayed a range of systemic symptoms (mild to severe) in different replicates within 2.5 wk PI. BYCORE 6, the transgenic line that had previously displayed a moderate level of viral resistance developed predominantly mild systemic symptoms from 2.5 wk PI.

Transgenic lines that initially showed high levels of resistance at inoculum levels of 100 and 150 μg ml^{-1} did not show the same degree of resistance in the second experiment even though lower inoculum levels were used. Line BYCORE 1 in particular appeared to be as susceptible to infection as the non-transgenic control plants at both 25 and 50 μg ml^{-1}. Lines BYCP 3a and BYCORE 7 did show a moderate degree of resistance but there was a significantly higher incidence of systemic infection compared to clonal plants from the same lines previously inoculated at 100 μg ml^{-1}. The only line that had a high level of resistance was line BYCORE 6.

Additional transgenic subclover lines containing the BYCP, BYCORE and BYCPUTR constructs were tested for virus resistance at inoculum levels of 25, 50 and 100 μg ml^{-1} and assessed visually. A summary of the results covering all the lines tested is presented in Table 2. Another two lines containing the BYCP construct were found to be strongly resistant which along with BYCP 3a made a total of three resistant BYCP lines. In lines containing the BYCORE construct, two additional lines had significant levels of resistance, which gave a total of five lines for this construct including BYCORE 1, 6 and 7. Lines that were classified as resistant showed a lack or significant amelioration of symptoms at the three inoculum levels. All of the six lines, which contained the BYCPUTR construct, were susceptible to infection (Table 2).

Discussion

BYMV resistance has been demonstrated in transgenic subclover lines containing either the full-length CP or the CP core region. Susceptibility to BYMV infection and either moderate or high level resistance was not correlated with the presence of the transgene encoded protein or mRNA levels, assuming RNA levels were not altered as a result of clonal propagation. Resistant subclover lines showed a range

of different phenotypes, even among those transformed with the same BYMV construct. This is the first report of potyvirus resistance in a genetically engineered annual pasture legume.

The different phenotypes that were seen among lines containing either the BYCP or BYCORE constructs were possibly a reflection of the different types of resistance that may be operating against the virus. These phenotypes consisted of delayed development of viral symptoms, restriction of symptoms to the inoculated leaves with no systemic symptoms, milder local lesions and systemic symptoms and reduced virus levels in systemic tissue. Similar variation in resistant phenotypes of transgenic plants containing translatable potyviral CP sequences has been reported previously (Dinant *et al.*, 1993; Hammond & Kamo, 1993; Kollar *et al.*, 1993; Tennant *et al.*, 1994). The different phenotypes suggest that resistance may be operating at either the level of viral replication, or cell to cell, or long distance movement or a combination of these mechanisms. This variation in symptom phenotype is different to that mediated by non-translatable CP constructs, in which the phenotypes are of three distinct types; susceptible, susceptibility followed by recovery and immunity (Smith *et al.*, 1994 and references therein). None of the transgenic subclover lines we generated were totally immune to BYMV. However, immunity is often correlated with no detectable transgene mRNA and because we tested only lines that had a detectable CP mRNA, immune lines may have been unknowingly discarded.

A lack of correlation between the levels of virus resistance and the amounts of transgene encoded CP and mRNA levels has been observed in many other studies on a wide range of different viruses (Stark & Beachy, 1989; Lawson *et al.*, 1990; Kollar *et al.*, 1993; Lindbo *et al.*, 1993; Scholthof, Scholthof & Jackson, 1993; Wilson, 1993). This contrasts with those studies in which virus resistance is dependent upon the presence of viral CP (van Dun, Overduin, van Vloten-Doting & Bol, 1988; Powell *et al.*, 1990). Such observations have contributed to the belief that PDR may be protein and/or RNA mediated. In our study no BYMV CP was detected in any of the transgenic plants. However all of these plants were resistant to phosphinothricin (due to the *bar* marker gene) and most had readily detectable transgene mRNA. Either the CP was not being translated or it did not accumulate to a detectable level. As some of the plants had high levels of transgene mRNA it is possible that translation was occurring and the viral CP was being rapidly degraded or inefficiently recovered from transgenic tissue. Potyviral CP is released from a long viral polyprotein by proteolytic cleavage during viral infection (Riechmann *et al.*, 1992) and it is possible that translation of an individual protein from the transgene did not permit normal folding and hence increased the instability of the protein.

The lack of detectable CP in resistant subclover plants suggests that the resistance could be RNA mediated. There have been numerous recent studies on RNA mediated viral resistance using non-translatable potyviral CP constructs (Lindbo & Dougherty, 1992*a,b*; van der Vlugt, Ruiter & Goldbach, 1992; Smith *et al.*, 1994; Cassidy & Nelson, 1995; Tanzer *et al.*, 1997). This resistance is characterised by the two phenotypes mentioned above (susceptibility followed by recovery and immunity), tends to be correlated with mRNA expression levels and is not inoculum dependent. We did not see resistance phenotypes typical of RNA mediated resistance in our study and resistance to BYMV was inoculum dependent. Some of the transgenic plants showed signs of recovery but we cannot be sure that this is related to a possible RNA mediated resistance mechanism until further studies are conducted, since some control non-transgenic plants also showed signs of recovery, although to a lesser extent than the transgenic plants.

BYMV resistance was observed in transgenic subclover plants that contained the BYCP and BYCORE constructs but not in transgenic plants transformed with the BYCPUTR construct. The lack of resistance in plants transformed with the BYCPUTR construct may be due to rearrangements or deletions in the transferred DNA resulting in loss of the viral gene, or may indicate that this construct does not confer viral resistance efficiently. There have been several other studies assessing the ability of modified potyviral CP sequences to impart virus resistance. Fang & Grumet (1993) inserted the full-length and core portion of the CP of zucchini yellow mosaic virus (ZYMV) into transgenic plants. Plants containing the full-length CP construct exhibited apparent immunity to ZYMV, while those containing the core construct showed a delay of several days in symptom development and a reduction in virus titer. Both constructs contained the 3' UTR of the viral genome. In contrast, Lindbo & Dougherty (1992*a*) demonstrated that tobacco etch virus (TEV) CP constructs lacking nucleotide sequences encoding the C-terminal or C- and N-terminal amino acids provided much better protection against TEV than did full-length TEV CP. Their constructs did not contain the viral 3' UTR. Cassidy & Nelson (1995) found a similar degree of virus resistance in plants containing the peanut stripe virus (PStV) full-length CP gene, and CP genes with amino terminal deletions of 16 or 106 amino acids. All constructs contained the viral 3' UTR. Two modified plum pox virus (PPV) CP gene constructs (containing either a deletion of the DAG amino acid triplet or the first 420 nt of the CP gene), including the viral 3' UTR region, also imparted a similar high level of virus resistance (Jacquet, Ravelonandro, Bachelier & Dunez, 1998).

The core region of the potyviral CP is thought to be responsible for virion assembly and contain regions involved in CP-CP and CP-RNA interactions (Dougherty *et al.*, 1985; Shukla *et al.*, 1988). Although

the exact mechanism of CP mediated resistance is not known, functions associated with the core region have been implicated (Fitchen & Beachy, 1993 and references therein) and hence the CP core would be expected to elicit resistance (Fang & Grumet, 1993). Our study and those described above indicate that the core region is able to impart virus resistance which is at least as effective as that imparted by the full-length CP. The variation in resistance levels of plants containing full-length *versus* deleted CP constructs is probably related to different resistance mechanisms.

Virus resistance has been successfully achieved with full-length CP constructs from many potyviruses both with and without the viral 3' UTR, e.g., potato virus Y (Lawson *et al.*, 1990), PPV (Regner *et al.*, 1992), papaya ringspot virus (Fitch *et al.*, 1992), ZYMV (Fung & Grumet, 1993), TEV (Lindbro & Dougherty, 1992a), watermelon mosaic virus 2 (Fuchs & Gonsalves, 1995), PStV (Cassidy & Nelson, 1995), vanilla necrosis virus (Wang, Gardner & Pearson, 1997), and lettuce mosaic virus (Dinant *et al.*, 1997). Hence it is surprising that our BYCPUTR construct did not appear to elicit resistance. We may well have detected resistance if we had examined additional transgenic lines containing this construct. If this were so, then the frequency of obtaining a resistant line would be significantly lower than for the other two constructs (see Table 2).

Several studies have demonstrated that transgenic plants containing potyviral CP constructs provide cross protection against other potyviruses (Stark & Beachy, 1989; Ling *et al.*, 1991; Namba *et al.*, 1992; Dinant *et al.*, 1993; Fuchs & Gonsalves, 1995). This may be related to the high degree of sequence conservation of the CP core region that exists between different potyviruses (Shukla & Ward, 1988, 1989). Therefore, the use of transgenic plants containing core constructs, such as the ones we have generated, may be a mechanism of providing broader virus resistance.

Our study has demonstrated that the level of virus resistance may vary from one experiment to the next. Transgenic subclover lines that initially appeared to have strong resistance to BYMV appeared less resistant in a second experiment and *vice versa*. There are a number of possible explanations for this variability, which are probably related to the genetic stability of the transgenic plants and the conditions of the infectivity trials. Since we only examined the primary transgenics it is possible that there may be some genetic instability that may result in lines becoming less or more virus resistant between batches of clonal plants that were propagated for independent trials at different times. Production of R1 and R2 generations and selection for homozygosity may overcome this potential problem. The important experimental conditions associated with the infectivity trials that could have resulted in variable resistance include quality of the viral inoculum and physiological status of the transgenic plants. Our results demonstrate the need for retesting and verifying virus resistance in transgenic plants.

Following the production of subsequent generations that are homozygous for the transgene, we are interested in crossing transgenic plants that display different resistance phenotypes. Analysis of resistance phenotypes in progeny plants could lead to further studies on the molecular basis of the resistance mechanisms and provide new BYMV resistant subclover cultivars.

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