European Journal of Plant Pathology **103:** 695–710, 1997.

Coat protein-mediated resistance to isolates of barley yellow dwarf in oats and barley

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Accepted 15 July 1997

Key words: ELISA, transgenic, pathogen-derived resistance

Abstract

Tissue cultures of GAF30/Park oats were biolistically co-transformed with constructs containing the coat protein (CP) genes of the P-PAV, MAV-PS1 or NY-RPV isolates of barley yellow dwarf virus (BYDV), together with a construct containing the *bar* gene for herbicide resistance and the *uidA* reporter gene. Transformed, herbicide-resistant tissue cultures were screened by PCR for the presence of the CP genes. Fertile regenerated plants were recovered from some CP-transformed tissue cultures. T_1 progeny of these plants were screened for resistance to the BYDV isolate corresponding to the introduced gene by inoculation with viruliferous aphids followed by ELISA tests. Variation in ELISA values for GAF30/Park control plants made interpretation of the data difficult, but oat plants resistant to each of the three isolates of BYDV (ELISA values less than 0.3; virus titers equivalent to less than 25% of infected controls) were identified in T_1 generations. Further testing of MAV-PS1 CP-transformed lines to the T_2 generation, NY-RPV CP-transformed lines to the T_3 generation and P-PAV CP-transformed lines to the T_4 generation identified further resistant plants.

Similarly, immature embryos and calli of the barley cultivar Golden Promise were biolistically bombarded with constructs containing the CP gene of the P-PAV isolate of BYDV and the *bar* and *uidA* reporter genes, lines of self-fertile P-PAV CP-transformed barley plants were developed, and T_1plants were screened for resistance to P-PAV. Eight plants from six lines showed moderate to high levels of resistance to P-PAV that correlated with the presence of the CP gene. Plants giving low ELISA values were also found in other lines, even though the CP gene was not detected in these plants. Some T_2 plants derived from resistant parents that contained the CP gene were themselves highly resistant.

Introduction

Barley yellow dwarf is regarded as the most serious viral disease of cereals worldwide (Lister and Ranieri, 1995). The associated barley yellow dwarf viruses (BYDVs) comprise a group of several kinds of luteoviruses and strains thereof, differing in biological and genomic properties. Five variously interrelated strains are currently recognized. These were originally differentiated by and named for their aphid vectors (Rochow, 1969): MAV, PAV and SGV in Subgroup 1 and RPV and RMV in Subgroup 2 (Waterhouse et al., 1988, Martin et al., 1990, Martin and D'Arcy, 1995). The most widespread serotype is PAV (Lister

and Ranieri, 1995) or '*padi-avenae* virus', named for its primary vectors *Rhopalosiphum padi* and *Sitobion avenae* (Rochow, 1969). The host range of BYDVs includes most members of the Gramineae, including all the widely grown grass and cereal crops, and yields may be seriously affected (Lister and Ranieri, 1995). Present virus control strategies include cultural practices, such as varying the sowing date to avoid immigrations of viruliferous aphid vectors and applications of insecticides to reduce the spread of aphids within crops. Neither of these methods is very satisfactory, and currently the development and use of cereal varieties exhibiting 'resistance' – i.e. usually tolerance, *sensu* Cooper and Jones (1983) – is the preferred approach for control (Plumb and Johnstone, 1995; Burnett et al., 1995).

Unfortunately, tolerance is difficult to assess, and sources of true resistance (i.e. relatively low virus production) are scarce and difficult to use as their inheritance is complex (Burnett et al., 1995). Therefore, we have investigated prospects for developing such resistance via genetic engineering, specifically through the use of viral coat protein (CP)-mediated resistance, whereby the gene encoding the coat protein of a particular virus is inserted into the genome of the host plant. This technique has been successfully applied with many dicotyledonous host/virus systems (for reviews see Beachy et al., 1990; Nelson et al., 1990; Wilson, 1993), including a luteovirus system, potato leafroll virus (PLRV) in potato (Kawchuk et al., 1990; van der Wilk et al., 1991; Barker et al., 1992), but with only few monocotyledonous host/virus systems due to difficulties with transformation and regeneration. However, techniques are now available to produce fertile transformants of the major cereal crops, including rice (Wen et al., 1991), maize (Murry et al., 1993), oat (Somers et al., 1992), wheat (Weeks et al., 1993) and barley (Wan and Lemaux, 1994).

In this paper, we describe the transformation of oat (*Avena sativa* L.) with the CP genes from isolates of the PAV, MAV and RPV strains of BYDV, and the transformation of barley (*Hordeum vulgare* L.) with the CP gene of the PAV isolate. These strains were chosen because of their importance in various regions of the world (Lister and Ranieri, 1995), and because they represent the diversity of the BYDVs. Transformants were challenged by aphid inoculation with the identical virus isolates of BYDV used to clone the CP genes. Transformed individuals and lines were identified that showed resistance, manifested as a reduced virus titer assessed by ELISA. Viral CP gene constructs were developed at Purdue University. Transformation of oats and subsequent seed production was carried out at the University of Minnesota, while barley transformation and T_1 seed production was done at the Plant Gene Expression Center, Albany. All challenge inoculations, resistance evaluations and further seed production were done at Purdue University. Abstracts of some of these data have been published (Lister et al., 1994a, b).

Materials and methods

cDNA cloning and constructs

The BYDV isolates used were P-PAV (Hammond et al., 1983), NY-RPV (Rochow, 1969), and MAV-PS1 (Lister and Sward, 1988). cDNAs representing the CP genes of the NY-RPV, P-PAV and MAV-PS1 were cloned in pGEM-3Z (Promega, WI, USA) between the CaMV 35S promoter and the NOS polyadenylation signal sequences derived from pBI221 (Clontech, CA, USA) (Figure 1). For NY-RPV, the CP coding regions were cloned as an *Acc*I fragment from the cDNA 11A, representing the nucleotides 3646 – 4217 of the NY-RPV genome (Vincent et al., 1991). MAV-PS1 and P-PAV CP cDNA were cloned as PCR products using primers which represented conserved sequences flanking the CP coding regions. The forward primer was 5′ GGCTCTAGACGTATTTTATTTAC-3′; the 3′ 14 nucleotides (RNA form) are conserved in the 5′ untranslated region upstream of the MAV and PAV CP genes and are identical to the MAV-PS1 sequence in that region; there is a single nucleotide difference in the P-PAV sequence (Vincent et al., 1990; Ueng et al., 1992). The reverse PCR primer (5′-GGATCCGTCTACCTATTTGG -3′) (Lei et al., 1995), which was similar to that designed by Robertson et al. (1991), represented a 14 nucleotide sequence which is located (RNA form) at the 3′ end of the CP coding regions. The forward and reverse primers contained restriction enzyme sites to facilitate directional cloning of the CP gene into the expression cassette (Figure 1); an *Xba*I site in the forward primer and a *Bam*HI site in the reverse primer (underlined).

Plasmid pAHC25 (provided by P.H. Quail, Plant Gene Expression Center, Albany, CA) was used in the co-transformation of barley. It contained both *bar*, encoding resistance to the herbicide phosphophinothricin (PPT) and *uidA*, encoding GUS activity, each controlled by the maize (Ubi-1) ubiquitin promoter and first intron, and the polyadenylation sig-

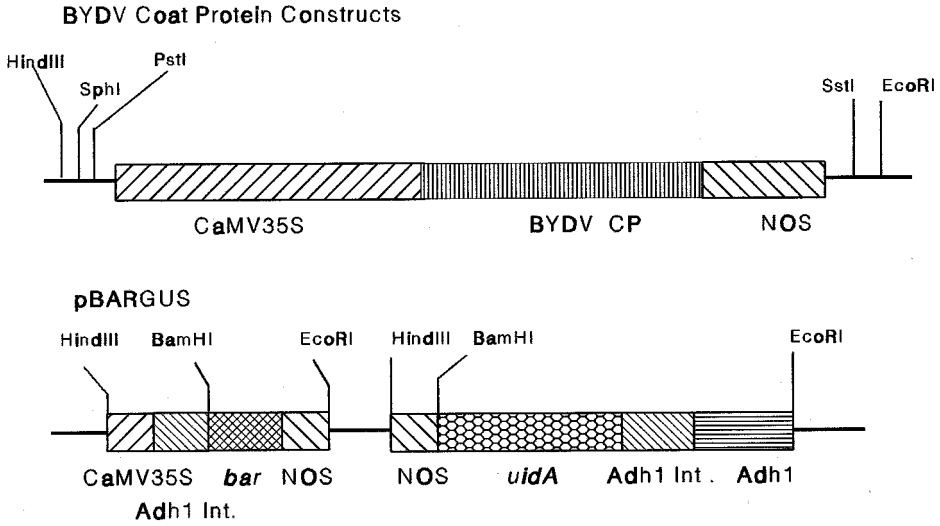

Figure 1. Diagram of the constructs used to transform GAF30/Park oats showing the relative positions of the genes involved, their promoter and termination sequences and the important restriction enzyme sites. Adh 1 Int. = Adh 1 Intron.

nal sequences from the nopaline synthase gene (*nos*) of *Agrobacterium tumefasciens*. A similar construct, pBARGUS, containing the PPT resistance (*bar*) and *uidA* reporter genes, was used in the co-transformation of oats.

PCR of CP genes

For cDNA clones prepared by the polymerase chain reaction (PCR), 1 μg of viral genomic RNA was reverse transcribed (Superscript, Life Technologies Inc., MD, USA) and then treated with RNAse H (Life Technologies Inc.). The DNA product was ethanol precipitated and dissolved in 20 μl of distilled water. The PCR was performed under the following conditions: 1 μl of the cDNA template, 200 μM dNTPs, 100 pM each of the primers, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% (w/v) gelatin and 2.5 units of *Taq* polymerase (Perkin Elmer Cetus, CA, USA) in 100 μl. The reaction mixture was covered with 50 μl mineral oil and after an initial 5 min incubation at 95 °C was subjected to 25 or 30 cycles of 95 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min. PCR products were visualized by ethidium bromide staining of 0.8% agarose gels and the isolated PCR product was digested with *Xba*I and *Bam*HI restriction enzymes and cloned into pGEM-3Z. All PCR clones were analyzed to verify their nucleotide sequences.

RNA was extracted from 0.5 g callus tissue of each of the putative transgenic oat lines by a guanidine isothyocyanate-based RNA purification system (GlassMAX RNA Microisolation Spin Cartridge System, Life Technologies Inc.) according to the manufacturer's protocol. RNA samples were incubated for 15 min at room temperature with Amplification Grade DNAse I (Life Technologies Inc.) to remove residual genomic DNA. One μg of RNA was used as the template for reverse transcription (20 μl reaction volume) for 1 h at 37 °C with 50 ng of random hexamers (Promega) and 200 units of SuperScript reverse transcriptase (Life Technologies Inc.) in the reaction performed according to the instructions for SuperScript. A similar reaction but without the addition of reverse transcriptase was used as a negative control. The PCR amplification was performed with a Perkin Elmer Cetus kit by including the following in the reaction mixture: 20 μl of the first strand cDNA reaction mixture, 1X PCR buffer, 800 μM dNTP mixture, 1 μM CP gene flanking primers, 1 mM $MgCl_2$, 2.5 units *Taq* polymerase in a 100 μl reaction volume. The denaturing, annealing and synthezising temperatures for PCR were performed for 35 cycles as described above.

For PCR analysis of putative transformed oats and barley, DNA was extracted by a modification of the procedure of McCouch et al. (1988). In a 1.5 ml microcentrifuge tube, 20–50 mg of leaf tissue was ground to a fine powder in liquid nitrogen, followed by the addition of 450 μl extraction buffer (7 M urea, 350 mM NaCl, 50 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 0.01% sarcosyl, 5% TE (10 mM Tris, 1 mM EDTA,

pH 8.0) equlibrated phenol, 0.2% sodium bisulphite) and 15 μl of 20% SDS. The samples were mixed and incubated at 65 °C for 30–60 min. Phenol: chloroform (1:1) (500 μl) was added and the samples were mixed and shaken for 15 min at room temperature. The aqueous phase was recovered after centrifugation and extracted with phenol: chloroform followed by chloroform: isoamyl alcohol (24:1). The DNA was precipitated with 1/10 volume 3M sodium acetate, pH 5.2, and two volumes of ethanol, after which the DNA pellet was washed repeatedly with 70% ethanol, air dried and dissolved in 100 μl TE. The sample was then treated with 1 μl of 10 mgml^{-1} RNAse, phenol: chloroform extracted as described above and suspended in a final volume of 20 μl of TE. PCR to detect the introgressed transgenes utilized the programme and the CP gene flanking sense and antisense primers described above. The following reagents were included: 300 ng of the DNA template, 200 μm dNTPs, 100 pM each of the primers, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% (w/v) gelatin and 2.5 units of *Taq* polymerase (Perkin Elmer Cetus) in 100 μl.

Plant material, transformation and regeneration

Tissue cultures of the only genotype of oat transformable to date, namely GAF30/Park (Rines and Luke, 1985), were initiated and maintained at the University of Minnesota, as described in Somers et al. (1992). For transformation, pBARGUS and the BYDV-CP constructs, were coated onto tungsten particles using procedures described by Somers et al. (1992). A 25 μl aliquot of a coat protein cDNA-containing plasmid (1 μg/μl) and 25 μl pBARGUS (1 μg/μl) DNA mixture was added to 1.3 mg prewashed tungsten particles resuspended in 250 μl ddH_2O. Particles coated only with pBARGUS were used to create transformation control lines (500 series). Friable, embryogenic 2-week old callus was plated onto solid MS2D medium lacking in asparagine and solidified with 0.2% Gelrite. Tissue (500–800 mg) was placed in the center of the petri plate creating a 3.0 cm diameter circle. The plates were positioned and bombarded as described by Somers et al. (1992).

After bombardment, callus tissue was incubated for 5 to 7 d at 20 °C in the dark. The tissue was then divided in half and placed on Whatman No. 1 7.0 cm diameter filter paper or directly onto a selection medium (Gelrite solidified MS2D medium lacking asparagine and containing 3mgl^{-1} of PPT; Crescent Chemical Co. Inc., Hauppauge, NY). Tissue was subcultured at 2 wk intervals by transferring filter papers plus overlying callus or by subculturing callus directly onto fresh medium. PPT-resistant colonies began to appear approximately 4 to 8 wk post bombardment, and were then subcultured directly onto fresh selection medium. Only calli shown by PCR to contain the CP gene were used to regenerate plants and the CP gene was later found among certain plants from all T_1 lines tested. A transformation control line (500 series) contained the pBARGUS plasmid alone. The expression of GUS activity in transformed tissue was performed as described by Jefferson (1987), using PPT-resistant tissue cultures and endosperm of dehulled seeds bisected transversely.

Growth chamber conditions were as described in Somers et al. (1992) and Torbert et al. (1995). Shoots (i.e. T_0 plants, see below) regenerated from the tissue cultures were transferred to Magenta boxes containing hormone-free MS medium with PPT. After 7–14 d, young plants were transferred to pots containing two parts soil to one part Fison's LC1 mix (Bellevue, WA) wetted with 1/10th strength MS salts. The plants were then maintained in growth chambers at 20 °C day and 15 °C night with a 12 h daylength and a light intensity of 300–400 μEm^{-2}sec^{-1} at the canopy top. After the plants had tillered, the day-length was increased to 16 h to promote flowering, self-fertilization and the production of seed for the T_1 generation of plants.

For barley transformation, immature embryos and immature embryo-derived callus of the cultivar Golden Promise were biolistically bombarded with the P-PAV-CP construct together with pAHC25, as described by Wan and Lemaux (1994). Transgenic lines (T_0) were selected on medium containing bialophos herbicide. T_0 plants, derived from calli from bombarded immature embryos, were grown to maturity and T_1 seeds harvested as described (Wan and Lemaux, 1994).

Nomenclature of regenerants

Each GAF30/Park oat callus that was biolistically bombarded was numbered, e.g. 804, and the plants regenerated from each callus (T_0 plants) were given a suffix number, e.g. 804-1, 804-2 etc. The seed from these T_0 plants then became the T_1 plants, which were numbered similarly, e.g. 804-1-1, and so on. The prefix 'P' indicates that the plant was grown at Purdue University; all other plants were grown at the University of Minnesota. Accurate identification of each seed from each line was critical in these tests, as in contrast to other CP-mediated resistance studies with dicotyledo-

nous plants, cereal plants cannot readily be clonally propagated e.g. by taking cuttings of vegetative material.

Transformed, regenerated Golden Promise barley plants were given the prefix 'GP' together with other characters to identify the callus from which they originated, e.g. GP1016-C1. T_1 plants grown at Purdue University were labeled according to the callus and T_0 plant from which they were derived, together with a number prefixed by 'P', indicating 'Purdue'. For example, plant GP1016B-C1-2-P39 was one of 39 or more plants derived from callus GP1016B-C1 and from the second ('-2-') of at least two T_0 parents.

The use of the word 'line' in this paper refers to the progeny from a single parent plant, whether at the T_1, T_2, T_3 or T_4 level. Sometimes these lines were sub-divided on the basis of the presence or absence of GUS expression.

Inoculation and evaluation of transformants

Viruliferous aphids were maintained on Clintland-64 oats in growth chambers as above. Isolates P-PAV and NY-RPV were cultured using *Rhopalosiphum padi*, and isolate MAV-PS1 was cultured using *Sitobion avenae*. Regular ELISA tests using specific polyclonal antisera (Webby et al., 1993) ensured that there was no cross-contamination. Transformed test plants at the two to four leaf stage, together with non-transformed controls (GAF30/Park in the case of oats, or Golden Promise in the case of barley) were placed in constant environment chambers at 20 °C, and appropriate viruliferous aphids were applied for a 24–48 h inoculation access period, i.e. plants transformed with P-PAV CP were inoculated with P-PAV only etc. After several hours each plant was checked to ensure that at least five to ten aphids were feeding. Experiments with barley also included plants from transformation control lines, GP1016B-4 series. In later experiments with oats , T_2 plants transformed with the pBARGUS construct only (500 series) were also included as a control.

After the inoculation period, the aphids were killed with Malathion and the plants grown on in an aphid-free greenhouse. Fourteen days later, the distal halves of two to three leaves of each plant were extracted at 1:10 (w/v) in 0.1 M phosphate buffer pH 7.0. Two replicates of 100 μl of each extract were applied to a microtiter plate coated with the respective BYDV antiserum for a comparative assessment of virus titer by double antibody sandwich (DAS)-ELISA (Lister and Rochow, 1979). Dilutions of non-transformed control extracts were included to give an indication of the relative level of resistance in plants giving low ELISA scores. After incubation with the homologous conjugates, followed by p-nitrophenyl phosphate substrate in diethanolamine buffer, pH 9.6, A_{405} readings of substrate reactions were taken after 30, 60, 90 and 120 min. Where possible, plants showing very low ELISA scores (i.e. < 0.1) were retested after a further 7 to 14 d to ensure that the plant was infected. A small sample was also taken from certain plants for PCR analysis to verify the presence of the BYDV CP gene. Plants giving low ELISA values (i.e. putative virus resistance) were maintained in the greenhouse until seed was harvested. Among oat lines, certain of those transformed with the MAV-CP gene were tested to the T_2 generation, certain of those transformed with the RPV-CP gene were tested to the T_3 generation, and certain of those transformed with the PAV-CP gene were tested to the T_4 generation. Several barley lines transformed with the PAV-CP gene were also tested to the T_2 generation. Also, in some plants of these lines, one new, fully expanded leaf was treated with PPT herbicide (Basta) and assessed for necrosis 7 d later to determine if *bar* was being expressed.

Determination of resistance

BYDV-infected GAF30/Park control plants often showed a range of virus contents while the response of the 500-series transformation control line was much more uniform. Likewise, Golden Promise barley plants gave a reasonably uniform response to infection. Plants showing low ELISA values after inoculation were identified (Tables 1, 2, 3 and 4). Transformants with A_{405} values less than 0.1 were categorised as 'highly resistant', although this was not necessarily correlated with the presence of the CP gene. This value indicated about 10% of the virus content of the plants giving typically high ELISA values, as determined by comparing dilutions of extracts with those of control plants. Similarly, ELISA values between 0.101 and 0.3 indicated about a 75% reduction in virus content and plants giving such low values were considered 'resistant'. Plants with ELISA values between 0.301 and 0.6 (oats) or between 0.301 and 0.4 (barley) were considered 'moderately resistant'. A small proportion of plants which showed very poor or otherwise abnormal growth were not included in the analyses.

Table 1. Summary of RPV-CP-transformed lines and plants showing moderate resistance ('MR', ELISA values between 0.301 and 0.6), resistance ('R', ELISA values between 0.101 and 0.3) or high levels of resistance ('HR', ELISA values less than 0.1)

Lines	No. of lines tested	No. of plants tested	No. (%) of MR plants	No. (%) of R plants	No. (%) of HR plants	No. (%) of lines with 1 or more plants R or HR
T_1 Generation						
601	7	58	4 (6.9)	0	0	0
903[1]	2	23	6 (26.1)	0	9[2](39.1)	1 (50.0)
Controls						
GAF30/Park	–	8	0	0	0	–
T_2 Generation						
601-6	16[3]	153	13 (8.5)	3 (2.0)	1 (0.07)	2 (12.5)
Controls						
GAF30/Park	–	20	1(5.0)	1 (5.0)	1 (5.0)	–
500 series	1	9	2 (22.2)	0	0	0
T_3 Generation						
903-1	6	46	18 (39.1)	3 (6.5)	3[4](6.5)	3 (50.0)
Controls						
GAF30/Park	–	8	1 (12.5)	1 (12.5)	1 (12.5)	–
500 series	1	9	2 (22.2)	0	0	0

[1]903-2: 22 plants and 903-2 GUS-: 1 plant.
[2]All nine resistant plants were from 903-2.
[3]16 lines tested in three separate experiments.
[4]Highly resistant plants = 903-1-1-2-P3 and 903-1-1-3-P3 and -P5 (Figure 2).

Results

Co-transformation

A total of 97 oat tissue cultures were selected on PPT-containing medium and thus were assumed to be expressing the *bar* gene. Of these, 39/53 calli co-transformed with the MAV-CP construct were shown by PCR to contain the MAV-CP gene, 10/12 calli co-transformed with the PAV-CP construct contained the PAV-CP gene, and 14/32 calli co-transformed with the RPV-CP construct contained the RPV-CP gene.

RT-PCR using the CP gene-flanking primers detected transcribed RNA corresponding to the introgressed CP gene in oat calli in cases where the CP gene had been shown to be present by PCR (data not shown). Likewise, Southern blotting (Southern, 1975; Wan and Lemaux, 1994) confirmed that 85% of the regenerated Golden Promise barley lines contained both *bar* and P-PAV CP genes. Twenty four T_0 plants regenerated from five transgenic callus lines containing both *bar* and the CP gene were used in this study. Other lines contained *bar*, but not the CP gene. Three T_0 plants derived from one such line were used in the resistance screening assays as the transformation control lines, namely GP106B-4-1, -4-2 and -4-3.

Variation in virus titer within control plants

The infestation procedure led to infection of every regenerated plant tested. Typically, virus titers for infected Clintland-64 control oat plants were uniformly high (ELISA values greater than 1.0, data not shown). By contrast, in several experiments some GAF30/Park control plants gave relatively low or intermediate ELISA values (Tables 1, 2 and 3; Figures 2, 3 and 4). Possibly the presence of wild oat in the parentage of GAF30/Park (Rines and Luke, 1985) introduced a variable resistance response into this line. However, the percentage of such GAF30/Park controls giving low ELISA values, and therefore considered to display a resistant phenotype, was typically much lower than the

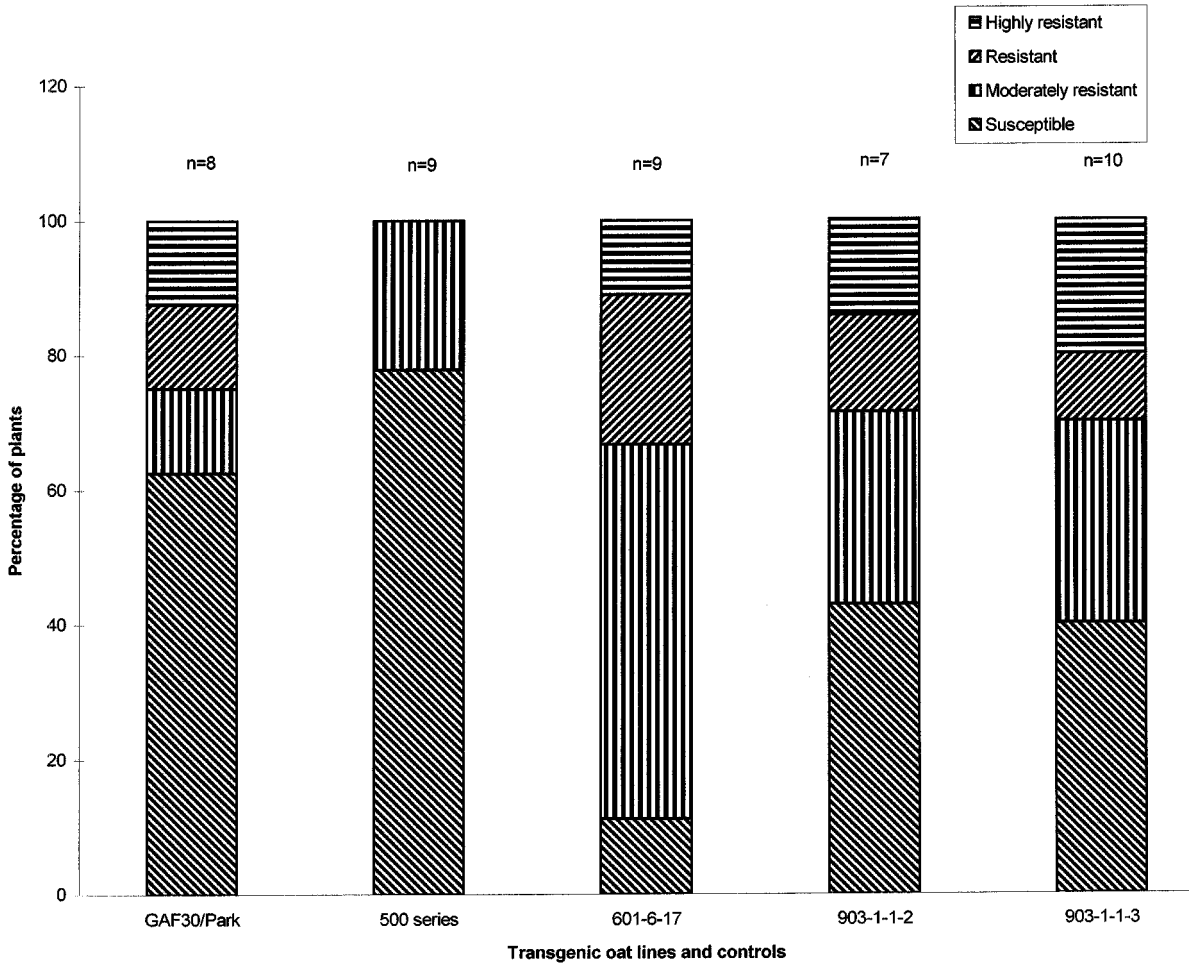

Figure 2. Percentage of susceptible (ELISA values greater than 0.601), moderately resistant (ELISA values between 0.301 and 0.6), resistant (ELISA values between 0.101 and 0.3) and highly resistant (ELISA values less than 0.1) from T_2 and T_3 RPV-CP-transformed lines 601 and 903 respectively. GAF30/Park are untransformed controls and 500 series plants were transformed with pBARGUS only.

percentage of transformants showing resistance (see below). Only in two instances, one involving a plant that may have escaped infection (Table 3), was a value of less than 0.1 observed for a GAF30/Park control plant.

Golden Promise control barley plants gave ELISA values ranging between 0.768 and 1.964. ELISA values of the transformation control lines ranged between 0.373 and 2.0 for GP1016B-4-1, between 0.406 and 1.784 for GP1016B-4-2, and between 0.509 and 2.0 for GP1016B-4-3. No plants from the Golden Promise or the transformation control lines were regarded as being resistant (ELISA values less than 0.3) and only one transformation control plant had an ELISA value less than 0.4 (Table 4). ELISA values among the CP-transformed lines ranged from 0.00 to 2.0, and values for healthy, uninoculated control tissue were typically around 0.01.

RPV-CP-transformed GAF30/Park oats

Regenerated plants from the tissue cultures transformed with the RPV-CP plasmid were tested for resistance to the T_3 generation. Seven 601 lines from three T_0 plants and two 903 lines from one T_0 plant were tested at the T_1 stage (Table 1). In the 601 T_1 lines tested, none of 58 plants showed greater than moderate resistance to RPV (Table 1). However, nine of the 23 plants tested from line 903-2 showed a high degree of resistance to NY-RPV 2 wk after inoculation (ELISA values less than 0.1; Table 1).

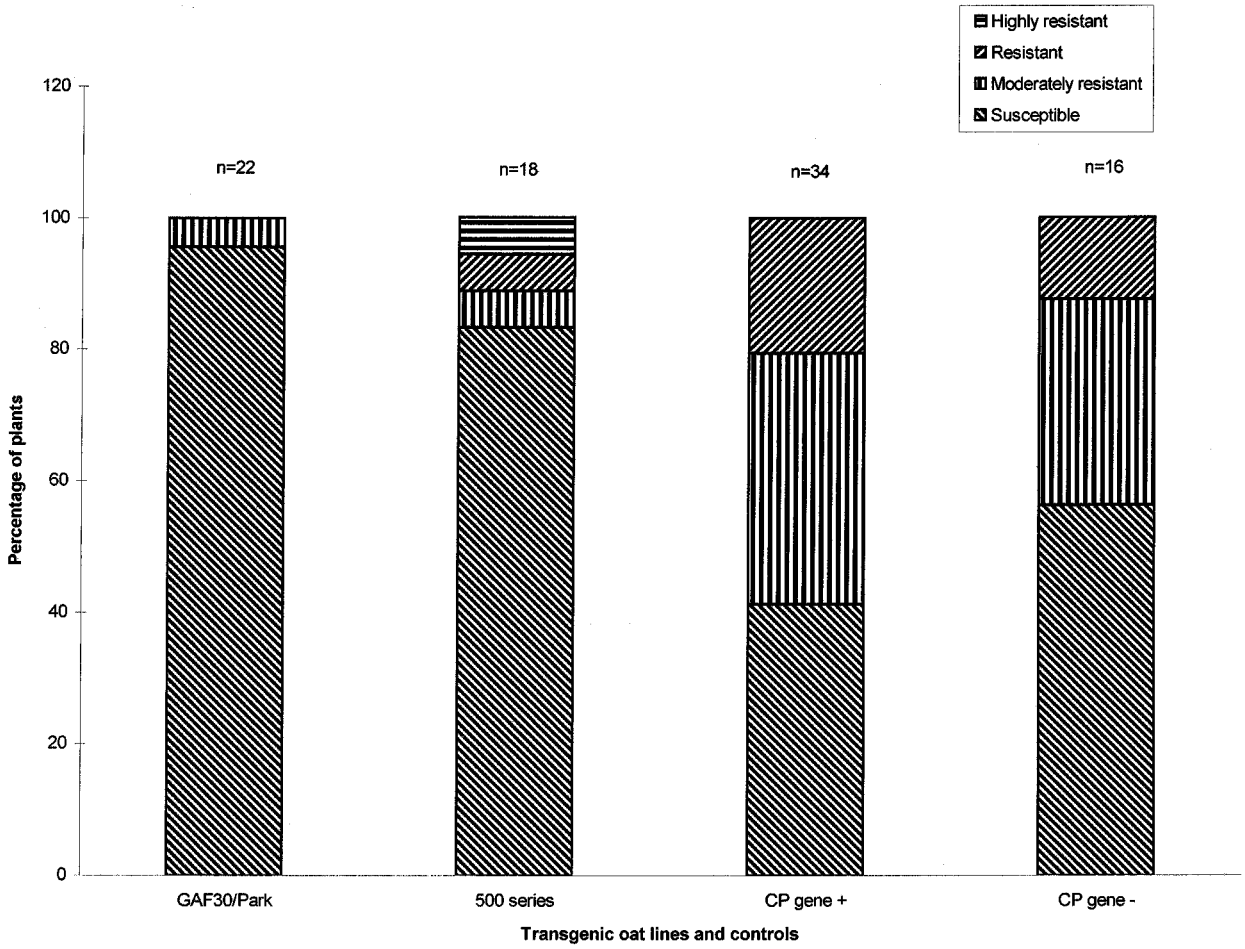

Figure 3. Percentage of MAV-CP-transformed plants (line 1001, T_2) showing susceptibility, moderate resistance, resistance and high resistance (see Figure 2 legend for details) to MAV-PS1. Plants have been divided on the basis of the presence (CP+) or absence (CP–) of the CP gene as determined by PCR. GAF30/Park are untransformed controls and 500 series are transformed with pBARGUS only.

Table 2. Summary of MAV-CP-transformed lines and plants showing moderate resistance ('MR', ELISA values between 0.301 and 0.6), resistance ('R', ELISA values between 0.101 and 0.3) or high levels of resistance ('HR', ELISA values less than 0.1)

Lines	No. of lines tested	No. of plants tested	No. (%) of MR plants	No. (%) of R plants	No. (%) of HR plants	No. (%) of lines with 1 or more plants R or HR
T_1 Generation	12[1]	106	8 (7.5)	1[2](0.9)	0	1 (8.3)
T_2 Generation[3]	4	46	18 (39.1)	9 (19.6)	0	4 (100)
Controls						
GAF30/Park	–	22	1 (4.5)	0	0	0
500 series	1	18	1 (5.6)	1 (5.6)	1 (5.6)	1 (100)

[1]Includes lines 1001, 1011, 1015 and 1017.
[2]Plant 1015-1-P4.
[3]All lines derived from 1001-2: the MAV CP gene was detected in all four lines (see Figure 3).

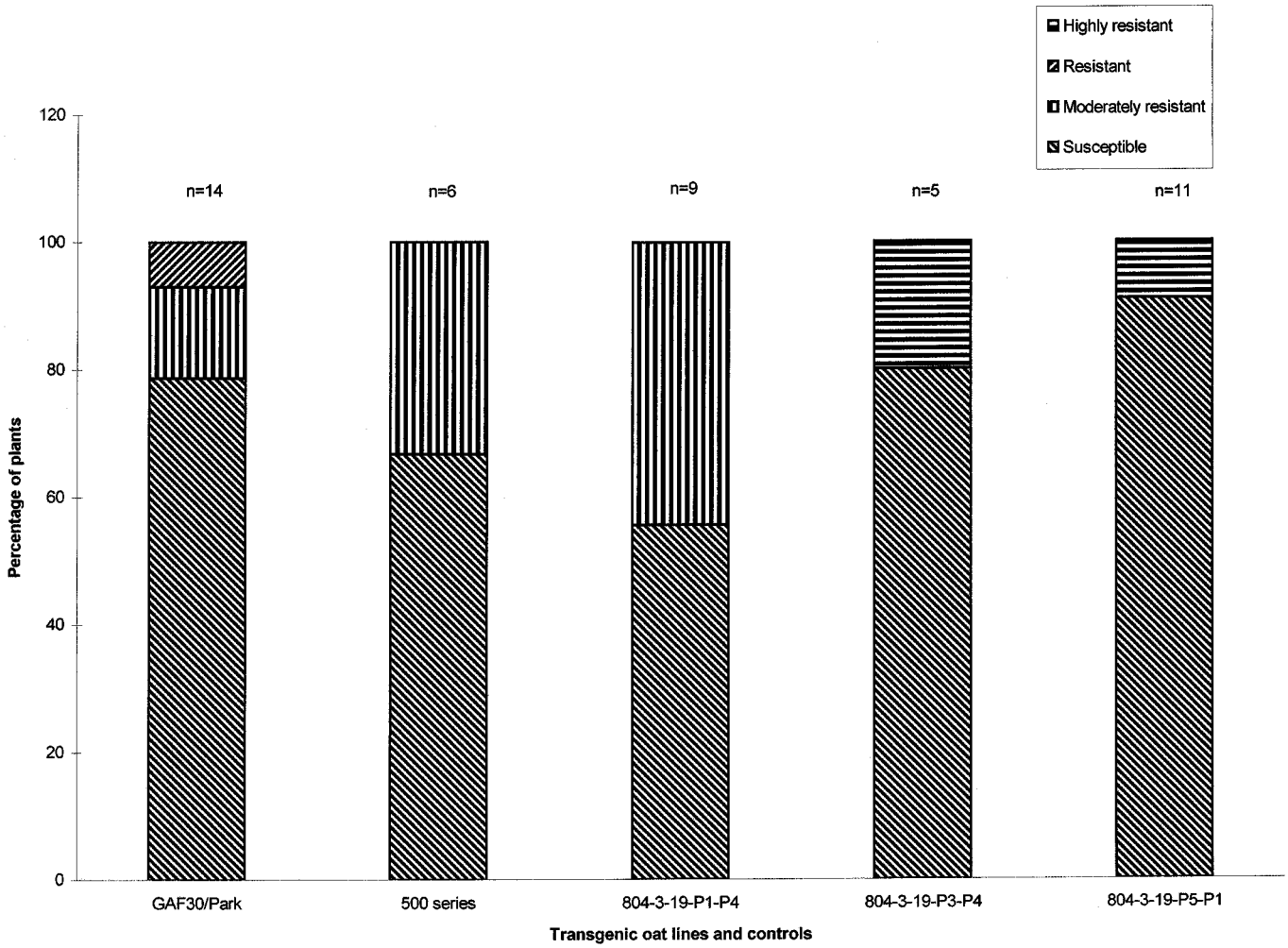

Figure 4. Percentage of susceptible, moderately resistant, resistant and highly resistant (see Figure 2 legend for details) T_4 PAV-CP-transformed plants after inoculation with P-PAV. GAF30/Park plants are untransformed controls and 500-series plants were transformed with pBARGUS only.

Only plants from the T_2 generation of line 601-6 were inoculated. One transformant gave an ELISA value of less than 0.1, while several other gave ELISA values between 0.101 and 0.6 (Figure 2). However, in these tests low ELISA values were occasionally obtained from GAF30/Park controls, although the 500-series transformation controls gave more uniformly high ELISA values.

T_3 progeny from RPV-CP-transformed plants multiplied at Minnesota were also screened for resistance at Purdue. Among these, a total of 46 plants from six 903-1 lines were evaluated (Table 1). Although some GAF30/Park controls gave low ELISA values, there was evidence of resistance in the T_3 generation of line 903-1. Among 17 plants derived from lines 903-1-2 and -3, three were highly resistant, with ELISA values less than 0.1, and a further two were resistant (ELISA values between 0.101 and 0.3) (Figure 2).

MAV-CP-transformed GAF30/Park oats

Plants regenerated from tissue cultures transformed with the MAV-PS1 CP plasmid were tested for resistance at the T_1 and T_2 generations. Of 106 T_1 plants tested, no resistance was observed in lines derived from calli numbers 1001, 1011 or 1017 (Table 2). However, several plants derived from each of these lines gave moderately low (0.301 to 0.6) ELISA values as com-

Table 3. Summary of PAV-CP-transformed lines and plants showing moderate resistance ('MR', ELISA values between 0.301 and 0.6), resistance ('R', ELISA values between 0.101 and 0.3) or high levels of resistance ('HR', ELISA values less than 0.1)

Lines	No. of lines tested	No. of plants tested	No. (%) of MR plants	No. (%) of R plants	No. (%) of HR plants	No. (%) of lines with 1 or more plants R or HR
T_1 Generation						
804/806/904	7[1]	103	3 (2.9)	0	8[2] (7.8)	4 (5.7)
Controls						
GAF30/Park	–	12	0	0	0	–
T_2 Generation						
803-2	5	48	4 (8.3)	0	0	0
Controls						
GAF30/Park	–	12	0	0	0	–
500 series	1	10	0	0	0	–
804-3-X[3]	16	107	12 (11.2)	5 (4.7)	5 (4.7)	8 (50.0)
804-3-19[4]	1	8	3 (37.5)	2 (25.0)	0	1
804-3-23 GUS+	1	3	0	0	0	0
804-3-23 GUS–	1	4	0	1 (25.0)	0	1
804-3-23[5]	1	17	4 (23.5)	6 (24.9)	6 (35.3)	1 (100)
804-3-31[6]	1	16	3 (18.8)	2 (12.5)	2 (12.5)	1 (100)
Controls						
GAF30/Park	–	20	6 (30.0)	2 (10.0)	1[7] (5.0)	–
T_4 Generation						
804-3[8]	40	241	19 (7.9)	8 (3.3)	3 (1.2)	9 (22.5)
Controls						
GAF30/Park	–	39	3 (7.7)	3 (7.7)	0	–
500 series	3	34	1 (2.9)	0	0	0

[1]Includes three lines from 804, three lines from 806 and one line from 904.
[2]806 lines contained 83 plants including seven of the eight plants showing reduced ELISA values.
[3]Includes all 804-3 lines except 804-3-19, -23 and -31, i.e. 804-3-4, -7, -9, -12, -13, -14, -15, -17, -18, -20, -21, -25, -27, -28 and -33.
[4]PAV CP gene found in all five plants tested by PCR.
[5]Includes lines 804-3-23 not tested for GUS activity.
[6]PAV CP gene detected in all four 'R' or 'HR' plants in this line.
[7]This plant was not re-tested by ELISA and therefore it is possible that it escaped infection.
[8]Includes three lines derived from 804-3-1, two lines from 804-3-7, three lines from 804-3-14, nine lines from 804-3-19, twelve lines from 804-3-23 and eleven lines from 804-3-31. For further details of resistance in lines 804-3-19, see Figure 4.

pared to GAF30/Park controls, which gave a mean ELISA value of 1.451 ± 0.315. The only resistant T_1 plant was one of 13 from line 1015, which gave an ELISA value of 0.118.

Four T_2 lines derived from 1001-2 were tested (Table 2) and each contained at least one plant which gave an ELISA value of less than 0.3. Three of the four lines (1001-2-2, 1001-2 -7, 1001-2-8) produced several resistant plants, with mean ELISA values considerably lower than those of GAF30/Park and the 500-series controls. Among the control infections, two plants gave low ELISA readings (i.e. less than 0.3), but the proportion of GAF30/Park and 500-series plants which gave ELISA values corresponding to moderate

Table 4. Summary of results of virus resistance screening and herbicide resistance among the T_1 generation of control Golden Promise, transformation control lines (GP1016B-4-) and P-PAV transformed test lines. Moderately resistant ('MR', ELISA values 0.301 to 0.4), resistant ('R', ELISA values 0.101 to 0.3) or highly resistant ('HR', ELISA values less than 0.1) plants in which the presence of the CP gene was verified are in **bold**, and plants in which the CP gene was not detected are underlined

Line	No. of plants tested	ELISA Tests			P-PAV Gene[1]	Herbicide resistance[2]
		No. plants MR	No. plants R	No. plants HR		
Controls						
Golden Promise	41	0	0	0	NT	susceptible
GP1016B-4-1	36	1 (P8)	0	0	NT	1/5
GP1016B-4-2	11	0	0	0	NT	0/5
GP1016B-4-3	11	0	0	0	NT	0/3
Test lines						
GP1016B-C1-1	36	0	0	0		36/36
GP1016B-C1-2	55	0	0	1 (**P39**)	1/1	32/40
GP1016B-C1-3	57	1 (**P18**)	0	0	1/1	37/42
GP1016B-C1-4	30	0	0	0	NT	29/30
GP1016B-C1-9	23	0	0	0	NT	NT[3]
GP1016B-C2-1	9	2 (P1, **P8**)	1 (P3)	0	1/3	7/8
GP1016B-C2-3	9	1 (P2)	1 (P7)	0	0/1	6/9
GP1016B-C2-4	10	1 (P6)	0	1 (P5)	1/2	4/10
GP1016B-C2-5	10	1 (P7)	0	1 (**P8**)	1/3	8/10
GP1016B-C2-6	10	1 (**P8**)	1 (P5)	0	1/3	7/10
GP1016B-C2-7	7	0	0	0	NT	5/7
GP1016B-C2-8	9	1 (P8)	3 (P1, P7, P9)	0	0/1	4/7
GP1016B-C2-9	18	0	1 (P5)	1 (P3)	0/3	6/6
GP1016B-C2-10	23	2 (**P2, P9**)	1 (**P5**)	1 (P3)	5/5	7/9
GP1016B-C2-12	9	0	3 (P1, P2, P4)	0	0/5	0/9
GP1016B-3-1	14	0	0	1 (P1)	0/3	1/4
GP1016B-3-2	25	1 (P13)	2 (P3, P4)	2 (P10, P14)	0/4	0/10
GP1016B-3-3	39	2 (P3, P6)	3 (P5, P8, P37)	1 (P7)	0/5	0/10
GP1016B-8-9	21	0	0	0	NT	NT
GP1016B-8-11	19	0	0	0	NT	NT
GP23B-4-1	21	0	0	0	NT	NT
GP23B-4-3	17	0	0	1 (P16)	NT	NT
GP23B-4-5	20	0	0	0	NT	NT
GP23B-4-6	18	0	0	0	NT	NT

[1]Number of plants with gene present / Number tested by PCR.
[2]Number of plants resistant to PPT / Number tested.
[3] NT = not tested.

resistance (0.301–0.6), namely 4.5% and 5.6% respectively, was considerably less than in those lines containing resistant individuals; 1001-2-2 (18.8%), 1001-2-7 (14.0%), 1001-2-8 (54.5%) and 1001-2-9 (16.7%). Plants showing both high and low ELISA values were detected throughout these four lines, although a greater proportion of resistant and moderately resistant plants occurred among those in which the CP gene was detected by PCR than in those in which the gene was not detected (Figure 3).

PAV-CP-transformed GAF30/Park oats

Plants derived from calli transformed with the P-PAV CP gene were tested for virus resistance at the T_1 and T_2 generations. From these, several resistant plants of the T_3 generation were used only to multiply seed for further testing of T_4 progeny. T_1 plants tested were derived from three transformed calli, 804, 806 and 904 (Table 3). Seven of 83 plants from the 806 lines 806-2, 806-3 and 806-9 were identified as highly resistant to P-PAV. Line 904-3 produced no resistant individuals, and one plant (804-4 GUS-P1) of 14 of the 804-derived progeny gave an ELISA value of less than 0.1.

In the T_2 generation, PAV-CP lines 803-2 and 900-13 were tested. A total of 48 progeny were tested from the 803-2 source. Of the ten plants tested from line 803-2-1, two gave ELISA values of between 0.301 and 0.6 (designated as moderately resistant). No other plants within the five 803-2 derived lines had ELISA values less than 0.5, and ELISA values for all control plants were uniformly high (Table 3). In tests of 900-13 derived progeny, several plants had a ELISA values less than 0.3 (12/59, 20.3%), but three of 14 GAF30/Park control plants (21%) also gave low ELISA values.

Among 16 T_2 lines derived from 804-3 (designated 804-3-X), the proportion of plants (4.7%) giving low ELISA values (less than 0.1) was similar to that among the GAF30/Park controls (5%, Table 3). The PAV CP gene was detected in only 4/15 plants tested from five lines of this 804-3-X series. Two of these plants were from line 804-3-20 (determined to be homozygous for GUS expression) and two were from line 804-3-33. All four had high ELISA values (greater than 1.4). Another line, 804-3-12, was also homozygous for GUS expression, but the CP gene was not detected in PCR tests of any of six plants from this line, all of which were susceptible to PAV. However, in tests of plants from three other T_1 parents (804-3-19, 804-3-23 and 804-3-31), a larger proportion of individuals (25%, 64.7% and 25% respectively) displayed low virus titers (ELISA values less than 0.3, Table 3) including some plants with ELISA values of less than 0.1. Plants in lines 804-3-19 and 804-3-23 segregated with respect to GUS expression. Within line 804-3-19, the PAV CP gene was detected by PCR in five plants tested, all of which had low ELISA values (less than 0.41). In all, 12 of 24 plants from line 804-3-23 gave ELISA values below 0.3. PCR tests of a few of these plants showed that the CP gene did not co-segregate with GUS expression. In line 804-3-31, four plants of 16 were also identified as resistant, two of the four plants giving ELISA values less than 0.1 and therefore classed as being highly resistant (Figure 5). The CP gene was identified in all four plants tested from this line.

PAV-CP-transformed GAF30/Park oats T_4 generation

241 T_4 plants derived from plant 804-3 were tested for their response to infection (Table 3). Ninety plants were tested from 11 resistant lines derived from 804-3-31. Two of eighty plants from 10 lines derived from 804-3-31-P10 gave low ELISA values (A_{405} = 0.013 and 0.241). One of eight plants from another line (804-3-31-P13) showed moderate resistance (A_{405} = 0.467).

Among the progeny of plant 804-3-19, nine lines were tested, three plants of which gave ELISA values less than 0.3, including two with values less than 0.1, one from each of lines 804-3-19-P3-P4 and 804-3-19-P5-P1 (Figure 4). The CP gene was detected in the T_2 parent of one of these lines, but not tested for in the other. Also, from line 804-3-19-P1-P4, of which nine plants were tested, four plants were identified as being moderately resistant (Figure 4). The CP gene was present in the T_2 parent of this line. Eleven lines (74 plants) derived from 804-3-23 were tested, but only two of these plants gave low ELISA values.

PAV-CP-transformed Golden Promise barley

Golden Promise barley plants derived from calli transformed with the P-PAV CP gene were tested for resistance to P-PAV inoculation at the T_1 and generation. In five experiments, 41 Golden Promise plants and 58 transformation controls were inoculated together with 509 T_1 plants derived from 24 lines from 5 transgenic calli, namely GP1016B-C1, GP1016B-C2, GP1016B-3, GP1016B-8 and GP23B-4. Progeny of some of these plants were further tested for virus resistance in the T_2 generation.

Among the transformed lines tested, of 264 plants from 15 lines, ten (3.8%) gave ELISA values less than 0.1 and were regarded as being highly resistant, and a further 16 (6.1%) gave ELISA values between 0.101 and 0.3 (resistant). Several other plants were also regarded as being moderately resistant, for their ELISA values were between 0.301 and 0.4 (Table 4). No resistant plants were identified in the other nine P-PAV-CP transformed lines tested, accounting for almost half (245) of the plants tested.

The CP gene was detected by PCR in 11 of 40 regenerated T_1 barley plants tested (Table 4). However, not all plants in which the CP gene was detect-

ed were resistant, and the CP gene was not detected in all resistant plants. In fact, the CP gene was detected in only 3 of 15 plants tested with ELISA values less than 0.3 (GP1016B-C1-2-P39, GP1016B-C2-5-P8, GP1016B-C2-10-P5) (Table 4), and also in several plants with ELISA values less than 0.4 (GP1016B-C1-3-P18, GP1016B-C2-1-P8, GP1016B-C2-6-P8, GP1016B-C2-10-P2 and -P9). The CP gene was not detected in any of 12 plants from the GP1016B-3 lines although twelve of 78 plants had ELISA values below 0.4 (Table 4).

Forty T_1 barley plants were tested for the presence of the CP gene by PCR and also for herbicide resistance by swabbing a new, fully expanded leaf with PPT. Whenever the CP gene was detected (11 cases), the plant was resistant to PPT. However, of 30 cases in which the CP gene was not detected, seven were resistant to PPT while the remaining 23 were susceptible. Certain lines, including two of three of the transformation control lines, displayed no herbicide resistance (Table 4), despite being selected on PPT-containing medium at the T_0 stage. No experiments were done to determine directly if the *bar* gene was present but not expressed in these PPT-susceptible plants. All eight P-PAV resistant or moderately resistant plants in which the CP gene was detected were from lines expressing PPT resistance.

Seed from three resistant, P-PAV CP-containing T_1 plants were selected for resistance screening in the T_2 generation, namely GP1016B-C1-2-P39, GP1016B-C2-5-P8 and GP1016B-C2-10-P2 (Table 4). Nine plants derived from -C1-2-P39 were tested, two of which were moderately resistant, one resistant, and one highly resistant. Among the 14 plants from -C2-5-P8, one was moderately resistant, two were resistant and two others highly resistant. From line -C2-10-P2, 15 plants were tested, two were moderately resistant and a third was highly resistant to P-PAV infection. Line GP1016B-C2-5-P7, in which the CP gene was not detected, was also tested in the T_2 generation and two of 11 plants showed moderate resistance and one plant showed resistance. No plants in this line were highly resistant.

Discussion

cDNAs representing the CP genes from three serologically distinct isolates of BYDV were successfully inserted into GAF30/Park oats, and self-fertile, BYDV-CP-transformed lines were regenerated for each of the CP genes. cDNA of the P-PAV CP gene was also successfully inserted into Golden Promise barley and self-fertile P-PAV-CP transformed lines were regenerated (Wan and Lemaux, 1994). Challenging the T_1 up to T_4 generations with the corresponding viruses identified plants with moderate to low ELISA values indicative of reduced virus titer and hence defined as resistant (*sensu* Cooper and Jones, 1983). Where such resistance is associated with tolerance, the yield of resistant plants (i.e. plants developing low virus titers when infected) should be increased over that of plants producing high virus titers. Also, studies with both BYDV and PLRV have demonstrated reduced transmission efficiency from plants with a reduced virus titer (Barker and Harrison, 1986; Gray et al., 1991; Gray et al., 1993; van den Heuvel et al., 1991), a factor which should lead to less rapid development of epidemics in the field.

An unforeseen problem in resistance evaluation was the variability among control plants of the GAF30/Park line in their response to infection, although this was not always apparent, e.g. the controls for the T_1 generation (Table 1). Other controls, i.e. Clintland-64 (data not shown), the 500 series transformation controls (Figures 2 and 4), and progeny of parents in which the CP gene was not detected by PCR (e.g. 804-3-12, see above), were typically more uniform in their response to infection. Despite this variation, in several lines a higher proportion of transformed plants gave relatively lower ELISA values compared to the controls. Many of these plants were highly resistant, i.e. they gave ELISA values of less than 0.1 (e.g. Figures 2, 3 and 4, Tables 1, 2, and 3). Only in two instances did control GAF30/Park plants give ELISA values of less than 0.1. At least one of these had probably escaped infection (Table 3).

Golden Promise barley control plants gave a more uniform response to P-PAV infection than GAF30/Park oats. However, Golden Promise transformation controls did vary in response, with one plant showing moderate resistance (ELISA value = 0.373). Also, in several resistant or highly resistant plants from P-PAV transformed lines, the CP gene was not detected (Table 4). This phenomenon, whereby it appears that the co-transforming plasmid (i.e. pAHC25) conferred resistance to virus infection, has also been reported with PLRV, with respect to transformed potatoes (Presting et al., 1995).

Inoculation of 24 T_1 barley lines and four T_2 barley lines with P-PAV also identified plants with low ELISA values, i.e. resistance. Some T_1 plants from

14 of 24 lines gave absorbency values in ELISA tests of less than 0.3, although in seven of these lines, the CP gene was not detected. A further line, GP23B-4-3, was not tested. Within the remaining six lines, five plants showing moderate resistance, one plant with resistance and two plants showing high levels of resistance all contained the P-PAV CP gene (Table 4). In other cases, susceptible individuals were also shown to contain the CP gene. This variation in expression between plants is comparable to that described for BYDV-CP mediated resistance tests in oats and also for *bar* expression in barley (Wan and Lemaux, 1994) where the presence of the *bar* gene did not necessarily lead to expression in all lines studied. BYDV-CP mediated resistance in barley also resembles that described for oats, in that the inheritance of the resistance trait seems unpredictable. Among 38 T_2 progeny derived from resistant, P-PAV CP-containing T_1 parents, 12 gave ELISA values below 0.4, four being assessed as highly resistant. None of the 11 T_2 plants derived from parents that apparently lacked the CP gene were highly resistant, indicating that at least for highly resistant plants, CP-mediated resistance was inherited.

We have no evidence as to how the resistance mechanism works. A sensitive monoclonal based ELISA technique which was able to detect virus in sap diluted 1:10000 (data not shown) was unable to detect expressed CP in several lines tested. However, the CP gene was routinely detected by PCR in lines showing moderate to strong resistance. We also observed that not all progeny of a low virus-titer parent plant containing the CP-gene were resistant (Figure 4). Stable inheritance of the transgenes is important in the genetic engineering of cereals, as unlike most dicotyledonous plants, they cannot readily be propagated vegetatively. For this reason, BYDV-CP mediated resistance was tested to the T_2 (MAV), T_3 (RPV) and T_4 (PAV) generations in oats and to the T_2 generation in barley (PAV). Resistant plants were found in all these generations, although less frequently than expected, for resistance was not expressed consistently (Figures 2, 3 and 4). The inheritance of the resistance phenotype seems to be unpredictable and may depend on several factors including transgene expression level, which can be affected by the number of transgene copies, by the number of rearranged or truncated transgenes, by the position in the genome or by methylation (Pawlowski and Somers (in press)). Transgene suppression has also been well documented in certain plant/transgene systems (e.g. Flavell, 1994; Matzke and Matzke, 1995), and may account for the observed low proportion of plants displaying BYDV resistance. In barley, *bar* expression varied from plant to plant and seemed to depend on the developmental stage of the plant (Wan and Lemaux, 1994). These authors also reported that in 25% of lines they studied, presence of the *bar* gene did not necessarily lead to expression, an effect paralleling our results with oats, where even when the CP gene was present, resistance was sometimes not detected. Indeed, other workers have also shown that the presence of a transgene in barley does not necessarily lead to expression (Ritala et al., 1995).

Little work has been done on CP-mediated resistance to luteoviruses, previous efforts having been concerned mainly with producing PLRV resistance in potatoes and *Nicotiana* species. In these studies (Kawchuk et al., 1990, 1991; van der Wilk et al., 1991; Barker et al., 1992), detection of expressed CP was also difficult and it varied from undetectable amounts to only 0.01% of the total protein content. None of the studies determined if expressed CP was the source of the resistance. However, as constructs containing antisense CP gene cDNA have been shown to be effective at mediating resistance to PLRV, it has been proposed that transcribed mRNA is responsible (Kawchuk et al., 1990, 1991; van der Wilk et al., 1991; Barker et al., 1993). This is consistent with current thinking as to the mechanisms of pathogen-derived disease resistance genes which seem to require low steady-state mRNA levels operating through an antisense mechanism or via a cytoplasmic surveillance system that targets specific RNAs for degradation (Smith et al., 1994; Lomonossoff, 1995).

We have demonstrated that it is possible to transform and regenerate oat with the CP genes of isolates representative of both Subgroups of BYDV, and that this resistance is heritable, although apparently not in a stable form in the oat line (GAF30/Park) selected for transformation. Likewise, Golden Promise barley was transformed with the PAV CP gene and T_1 and T_2 resistant plants were produced. Although, in barley, some evidence suggests that not all of the resistance observed was due to the P-PAV CP gene, and may be a result of the co-transforming plasmid (e.g. Presting et al., 1995), we believe that the strong resistance (ELISA values less than 0.1) observed in the T_2 generation is due to CP-mediated protection. If stable inheritance can be achieved then the resistance is likely to hold in field tests, as it was selected for by the use of aphid vector transmissions, simulating the natural situation, unlike those cases in which transgenic resistance to mechanical inoculation has proved ineffective against

vector inoculation (Lawson et al., 1990; Ploeg et al., 1993). However, GAF30/Park oat was developed for tissue culture and is not an agronomically useful variety (Rines and Luke, 1985). In order for the resistance we have generated to be confirmed and utilized, it must be back-crossed into elite oat lines. In contrast, Golden Promise is an elite barley cultivar, although to be agronomically useful, further work is needed to produce a uniform line of transformed, PAV-resistant Golden Promise barley that is not resistant to PPT. Herbicide resistance proved to be a convenient trait for selecting transformed tissue cultures in our experiments, but the use of the *bar* gene as a selectable marker, particularly in oat transformations, has practical limitations. The possibility of out-crossing into wild relatives makes herbicide resistance undesirable in the field. An alternative approach would be to produce virus resistant CP-transformed lines by selection for antibiotic resistance rather than herbicide resistance (Torbert et al., 1995).

Acknowledgments

We thank JoAnne Fisher for assistance with ELISA tests and Andrew Jackson for reviewing a previous draft of the manuscript. This work was funded by grants from The Quaker Oats Company and the Midwest Plant Biotechnology Consortium. This is journal paper number 15193 from the Purdue Experimental Research Station.

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