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ORIGINAL PAPER

Agroinfiltration as a technique for rapid assays for evaluating candidate insect resistance transgenes in plants

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Abstract Functional analysis of candidate transgenes for insect resistance in stably transformed plants is a time-consuming task that can take months to achieve in even the fastest of plant models. In this study, a rapid screening technique is described, which employs candidate transgene transient expression using agroinfiltration in *Nicotiana benthamiana* combined with a simple insect bioassay. Using this system the known insecticidal protein Cry1Ac is demonstrated to effectively control *Helicoverpa zea*. Insects fed tissue with synthesized GFP (green fluorescent protein) as a positive control were shown to have enhanced growth and development. Additionally, a *Brassica oleracea* proteinase inhibitor (BoPI), a less characterized insect resistance candidate, demonstrated effectiveness to decrease the growth and development of *H. zea* at high levels of transient expression. Bioassays performed on stable transformants showed that BoPI had a low level of insect resistance at the more typical levels of gene transcription found in stably transformed plants. This agroinfiltration-insect bioassay procedure can give a rapid assessment of insect resistance significantly decreasing the time needed for evaluation of candidate genes.

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Introduction

The evaluation of potential insect resistance genes in transgenic plants can be arduous, given that the generation of stably transgenic plants is costly and labor intensive and can take several months to achieve with tobacco or Arabidopsis, two of the most facile plants to transform. In addition, transgenic events usually vary with regards to transgene expression because of gene insertion (position) effects and copy numbers of inserted genes (Wroblewski et al. 2005). The combination of a robust transient transgene expression assay and a reliable screening protocol could decrease the time required for initial transgene evaluation to determine whether an appropriate phenotype warrants production of stably transgenic plants. The use of transient expression through infiltration of *Agrobacterium tumefaciens* (agroinfiltration) harboring the transgene and promoter of interest should substantially decrease the time required to test candidate insecticidal genes and might provide a better platform to assess the potential of these gene products.

Transient expression through agroinfiltration is a relatively simple procedure. The most time-consuming step is cloning a transgene construct under the control of a tissue-active or constitutive promoter into a binary vector. Agroinfiltration is simple and effective, involving the injection of *A. tumefaciens* into leaves or organs of interest, and then monitoring transient transgene expression within the infiltrated tissue during the next few days (Sparkes et al. 2006). Agroinfiltration has been demonstrated to be effective for transient expression in many plant species

including tobacco (Sheludko et al. 2006), grapevine (Santos-Rosa et al. 2008), lettuce, tomato, *Arabidopsis* (Wroblewski et al. 2005), switchgrass (VanderGheynst et al. 2008), radish, pea, lupine, and flax (Van der Hoorn et al. 2000), but has never been used in conjunction with bioassays for screening transgenes that could confer possible insect resistance. Since there is a wide range of plant species susceptible to *A. tumefaciens* infection, the use of agroinfiltration for the evaluation of candidate insect resistance genes has great potential for rapid screening on numerous target insects and host plants. This report describes the application of agroinfiltration coupled with a bioassay procedure to determine the predictive effects of overexpressing two different candidate insect resistance genes in plants: one that is a documented strong insecticidal gene (Bt *Cry1Ac*) (for reviews, see Estruch et al. 1997; Shelton et al. 2002), and another, a *Brassica oleracea* proteinase inhibitor (BoPI) that has shown moderate effectiveness in transgenic tobacco plants on both *Helicoverpa zea* and *Heliothis virescens* (Pulliam et al. 2001).

Materials and methods

Agroinfiltration

Agroinfiltration of leaves

Agroinfiltration experiments were performed on *N. benthamiana*. *N. benthamiana* seeds were planted and grown in a growth chamber at 25°C under a 16 h light and 8 h dark photoperiod. Plants were grown for 8 weeks before infiltration and subsequent bioassays.

Transformed and non-transformed *A. tumefaciens* strain GV3850 was used in all infiltrations. Binary vectors in transformed bacteria included pBin/BoPI (containing *Brassica oleracea* serine proteinase inhibitor under the control of the CaMV 35S promoter) (Pulliam et al. 2001), pH602SBt (containing synthetic Bt *Cry1Ac* under the control of the CaMV 35S promoter) (Stewart et al. 1996), pBin-mGFP5-ER (containing the GFP5-ER gene under the control of the CaMV 35S promoter) and pSAM12 (containing both GFP5-ER and synthetic Bt *Cry1Ac* genes under the control of the CaMV 35S promoter) (Harper et al. 1999). The mGFP5-ER is an endoplasmic reticulum-targeted GFP (green fluorescent protein) gene that has the spectral property of equal UV and blue light excitation (Harper et al. 1999). GFP was used as a visual marker to gauge relative transgene expression and temporal and spatial patterns; co-infiltration of GFP with the candidate transgene plasmid facilitated subsequent precise insect bioassays.

Transformed *A. tumefaciens* test tube starter cultures were grown in a shaker overnight at 24°C at 200 rpm in YEP media (1% peptone, 1% yeast extract, and 0.5% NaCl) (3 ml) containing 50 mg/l rifampicin and 50 mg/l kanamycin to select for transformed *Agrobacterium* cells. Optical density at 600 nm was taken on starter cultures with a Synergy HT plate reader (BioTek Instruments, Inc, Winooski, VT, USA). Starter cultures were used to inoculate two 25 ml cultures to an OD_{600} of 0.004 and grown overnight. Non-transformed *A. tumefaciens* grown without kanamycin as an antibiotic selection agent was used as a negative control.

Agrobacterium infiltrations were performed as described in Sparkes et al. (2006). Infiltration suspensions for each vector and non-transformed *Agrobacterium* were brought to OD_{600} 0.6. Mixtures of bacterial suspensions (1:1) were made for co-infiltrations with a final OD_{600} of 0.6. Co-infiltration suspensions included non-transformed GV3850 + pBin-mGFP5-ER (GV + GFP), non-transformed GV3850 + pSAM12 (GV + GFP/Bt), pBin-mGFP5-ER + pSG/Bt (GFP + Bt), pBin-mGFP5-ER + pBin/BoPI (GFP + BoPI), and non-transformed GV3850 + non-transformed GV3850 (GV + GV). The addition of non-transformed *Agrobacterium* in the GV + GV, GV + GFP, and GV + GFP/Bt treatments was used to control for the effect of the co-infiltration mixture. The addition of multiple infiltration points per infiltration spot was used to expand the area of each spot on a leaf to approximately 4 cm^2. GV + GV infiltration spots were outlined with a black marker for later identification. After infiltration, plants were placed back into the growth chamber. Three days after infiltration, spots were visualized with GFP expression under a BlackRay model B100 UV light (UVP, Upland, CA, USA) and excised with a scalpel (Fig. 1). Outlined spots for GV3850-only infiltrations were also excised.

Nicotiana benthamiana plants were grown and leaves were infiltrated as described above. A single plant was infiltrated in five leaves; each leaf had a different co-infiltration treatment in two spots (one on each side of the mid-vein). Three replicate plants were infiltrated for a total of six spots for each co-infiltration treatment. After 3 days, spots were excised and measured with a GFP meter for on-the-leaf GFP expression analysis (Opti-Sciences Inc., Hudson, NH, USA). Infiltrated leaf tissue was measured at four independent points and averaged according to a modified protocol described in Millwood et al. (2003).

The tissue was snap-frozen in liquid nitrogen and ground in a mortar and pestle with liquid nitrogen. The tissue was then divided into three parts: 100 mg of tissue was placed in RLT (Qiagen RNeasy mini prep) buffer for RNA extraction and stored in a −80°C freezer; one half of the remaining tissue was added to ice cold protein extraction buffer (100 μM Tris–HCl pH 7.5, 100 μM $CaCl_2$) in a

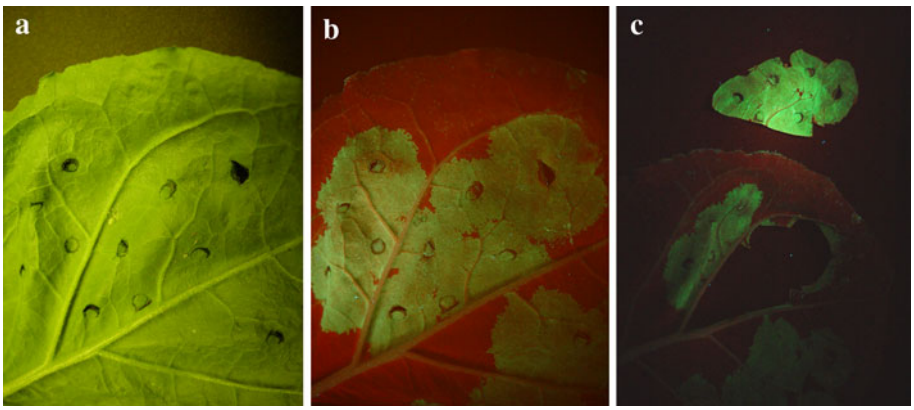

Fig. 1 Infiltrated *N. benthamiana* tissue expressing GFP + Bt **a** under white light **b** under ultraviolet light, and **c** excised under ultraviolet light. Pictures taken 3 days post-infiltration

1.5 ml microcentrifuge tube; the third portion was placed in a 2 ml cryo vial (Sarstedt Inc., Newton, NC, USA) for potential future experiments. All samples were stored in a −80°C freezer until needed.

Molecular analysis

Real-time RT-PCR was performed to confirm expression of transgenes in all infiltrated tissues collected in the infiltration characterization experiment. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The cDNA for real-time PCR analysis was generated by reverse transcription of 5 µg of total RNA using the Superscript III first-strand synthesis kit (Invitrogen Corporation, Carlsbad, CA, USA) and oligo(dT) primers. Real-time RT-PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each tissue type was tested in triplicate. Real-time RT-PCR reactions were carried out in 20 µl reaction volumes consisting of gene-specific primers and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The gene-specific primers were BoPI (fwd) TCCCGTGAAATTCTCAAACTGG, BoPI (rev) ACTGA GCGCAGATCGTAGGTTC, Bt *Cry1Ac* (fwd) CGCTCTC TTTCCCAACTACGA, Bt *Cry1Ac* (rev) ACCGTCGAAG TTCTCGAGGACT and were designed with primer express software (Applied Biosystems, Foster City, CA, USA). GFP gene-specific primers (fwd) CAACTTCAAGACCCGCCA CA and (rev) TCTGGTAAAAGGACAGGGCCA were designed and provided by Laura Abercrombie. The reference gene used in real-time RT-PCR analysis was ubiquitin. The ubiquitin specific primers were described in Lacomme et al. (2003). C_t values were recorded for both the transgene and reference gene for further analysis. An analysis of variance (ANOVA) was performed on ΔC_t values using a general linear model in SAS 9.13 (SAS Institute 2003). Means separation was performed with Tukey's range test. Ratios of expression compared to the reference gene were determined using ΔCt as described in Yuan et al. (2008). Pearson correlations of expression data with GFP meter, GFP ELISA, and Bt ELISA were performed with the Proc CORR procedure of SAS 9.13 (SAS Institute 2003).

GFP and Bt Cry1Ac were quantified from the infiltration characterization using ELISA. For protein extraction, frozen samples were thawed on ice and allowed to incubate for 1 h. Tubes were clarified by centrifugation at 13,000×*g* for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and quantified using Coomassie plus staining (Pierce Biotechnology, Rockford, IL, USA). Protein from infiltrated tissue was adjusted to 10 µg/ml for GFP quantification and 20 µg/ml for Bt quantification. Protein was quantified for GFP using a Reacti-Bind Anti-GFP ELISA plate (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Bt Cry1Ac was quantified with a Cry1Ac QuantiPlate (EnviroLogix Inc., Portland, ME, USA) according to the manufacturer's instructions. Proteinase inhibitor activities of tissues transiently expressing BoPI were not quantified in this assay. We expect that expression of the BoPI transgene leads to the translation of active proteinase inhibitors as previous work with pBin/BoPI transformed plants has indicated (Pulliam et al. 2001).

Insect bioassays

Eggs of *H. zea* were obtained from Benzon Research (Carlisle, PA, USA). Insect eggs were placed in large plastic containers until they hatched. Neonate larvae were then transferred to 128-well insect trays (Bio-Serv Inc., Frenchtown, NJ, USA) containing synthetic fall armyworm diet (Bio-Serv Inc., Frenchtown, NJ, USA) and held for 3 days before bioassays were performed. The addition of an artificial diet-feeding interval was included for the agroinfiltration assays to increase the likelihood of surviving the initial transfer to the *N. benthamiana* tissue. Two tobacco plants were infiltrated on five leaves for each co-infiltration as described above. Three days post-infiltration the infiltrated

spots were excised and GFP quantified with the GFP meter. Ten excised infiltrated plant tissue pieces from each co-infiltration (Fig. 1) and ten excised plant tissue pieces from non-infiltrated *N. benthamiana* were placed into clear plastic cups (29.6 cm^3) (Bio-Serv Inc., Frenchtown, NJ, USA) with 1/4th of a moist #5 Whatman filter paper (Whatman International Ltd, Kent, UK). A second instar corn earworm was placed onto each infiltrated plant tissue piece. Containers were sealed with a lid and held at 24°C. Containers were arranged in a complete randomized design. The experiments were ended due to low amounts of remaining leaf tissue on day 6. Larval mortality, larval weight, and larval head capsule size were recorded at the end of the experiments. Head capsule size of larvae was measured with an eyepiece micrometer in a stereoscope (Olympus SZ40, Olympus Imaging America Inc., Center Valley, PA, USA) and larval weights were taken with a digital scale (Denver Instruments, Göttingen, Germany). This insect bioassay was replicated for a total of 20 insects per treatment.

Larval weight and head capsule size were analyzed by ANOVA using a general linear model in SAS 9.13 (SAS Institute 2003). Means separation was performed with Tukey's multiple range test. Larval mortality was analyzed with the Proc Glimmix procedure of SAS 9.13 (SAS Institute 2003) since the data had a bimodal distribution.

Stably transgenic plants

Transgenic plants

Five lines of T_1 transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) expressing BoPI (*Brassica oleracea* serine proteinase inhibitor) under the control of the CaMV 35S promoter (BoPI 2, BoPI 6, BoPI 7, BoPI 8, and BoPI 15) were used (Pulliam et al. 2001). BoPI lines used in this study had previously been characterized for proteinase inhibitor activity (Pulliam et al. 2001). Seeds from T_1 plants were surface-sterilized and transgenic segregants were selected on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 200 mg/l kanamycin. T_1 plants exhibiting kanamycin resistance were allowed to self-pollinate and were grown to maturity. T_2 seed was collected from T_1 plants. Homozygous lines were confirmed by screening sterilized T_2 seeds on MS medium containing 200 mg/l kanamycin, and the homozygous lines were used for further research. Non-transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) and a high expressing homozygous line of pSAM 12 transgenic tobacco (Bt/GFP) expressing both Cry1Ac and GFP5er under the control of the CaMV 35S promoter (Harper et al. 1999) were used as negative and positive controls, respectively.

All plants used in the testing of stable transformants were started from seed and grown in a growth chamber at 27°C with 16 h light and 8 h dark photoperiod. Plants were watered and fertilized as needed. Plants were grown for 2 months before bioassays were performed.

Molecular analysis

Tissue for transgene expression comparisons among each of the five BoPI lines was collected as 25 mg samples from the first true leaves of four plants per line and pooled for total RNA extraction. Tissue from the first true leaf of the three BoPI plants (BoPI 2, BoPI 7, and BoPI 15) used in the insect bioassay assays were collected as separate 100 mg samples to test for variation in transgene expression within lines. Tissue was frozen in liquid nitrogen and stored in a −80°C freezer until extraction. Extraction of total RNA, synthesis of cDNA, and real-time PCR were performed as described above.

Real-time RT-PCR was performed to compare the BoPI transgene expression from each of the pooled samples from the original five BoPI lines (BoPI 2, BoPI 6, BoPI 7, BoPI 8, and BoPI 15). The second experiment compared transgene expression among each of the three plants per line (BoPI 2, BoPI 7, and BoPI 15) that were used in the corn earworm feeding assay. Normalized C_t values from each of the real-time PCR experiments were analyzed in paired *t* tests using the mixed procedure of SAS 9.13 (SAS Institute 2003). Ratios of expression were determined as described in Yuan et al. (2008).

Insect bioassays

A single plant from the selected BoPI lines (BoPI2, BoPI7, and BoPI 15) and controls (Xanthi and Bt/GFP) were evaluated individually in corn earworm bioassays. The experimental design included 10 larvae per treatment, five treatments per replicate, one plant per line was tested in each of three replicate assays. Each plant was grown as described above. Ten leaf discs (5.8 cm^2) were obtained from a single leaf of each plant. Each leaf disc was placed in a plastic cup (29.6 cm^3) (Bio-Serv Inc., Frenchtown, NJ, USA) containing moist filter paper. Insects were obtained and reared as described above. A single early second instar corn earworm larva was placed on each leaf disc. Cups were sealed with a lid and held at room temperature. On the fourth day of the bioassay, leaf discs were collected to quantify insect herbivory, larval mortality and weights were also recorded. Larger pieces of leaf tissue from the respective plants were substituted for the removed piece. Plant tissue was frequently added to maintain a constant source of food for insects throughout the experiment. The bioassays were ended after the first insect was observed to

have entered the pre-pupal stage, this occurred on day 17 of the experiments. On day 17, larval mortalities, larval head capsule widths, and larval weights were recorded. An additional day 9 collection of larval mortality and larval weights was added to evaluate insects. Preliminary data had indicated that the experiments would last for 18 days and the day 9 collection of data was intended to fall at the midpoint of the experiment. Larval mortalities were analyzed as percentages of each replicate, corrected using Abbott's formula to account for control mortalities (Abbott 1925) and arcsine square-root transformed. Larval weight, head capsule size, and larval mortality were analyzed with analysis of variance using a general linear model in SAS 9.13 (SAS Institute 2003) and mean separation conducted using Tukey's multiple range test.

Leaf discs collected on day 4 of each experiment were scanned with an HP Photosmart C4100 series scanner at a resolution of 200 pixels per inch. Scanned images were imported into SigmaScan Pro (Systat Software, Inc., San Jose, CA, USA), where the area of each disc was recorded and converted to cm^2. Analysis of variance was performed on the remaining leaf areas using the mixed procedure of SAS 9.13 (SAS Institute 2003) and mean separation conducted using Tukey's multiple range test.

Results

Agroinfiltration-facilitated insect bioassay

Infiltration characterization

Real-time RT-PCR analysis confirmed the transient expression of all transgenes in infiltrated tissue. Levels of BoPI expression in infiltrated tissues were significantly higher than the expression levels of all other transgenes ($F = 255.78$; $df = 119,6$; $P < 0.0001$) (Fig. 2). The average level of BoPI expression was over 6 times that of ubiquitin, with the highest expression level observed at over 9 times that of ubiquitin. Large variation was observed in GFP expression and fluorescence among different infiltration types. The highest relative GFP expression was found in GV + GFP infiltrated spots (2.5 times the level of ubiquitin expression), which was significantly higher than levels of GFP in all other infiltration types. The lowest relative GFP expression (0.94 times the level of ubiquitin expression) was in GFP + Bt infiltrated spots. Bt *Cry1Ac* expression levels in all infiltrated spots were lower than the internal reference gene. Spots from GFP + Bt (0.45 times the level of ubiquitin expression) and GV + GFP/Bt (0.55 times the level of ubiquitin expression) infiltrations were found to have statistically similar levels of Bt expression, although they had significantly lower levels of expression than all other transgenes. No transgene expression was detected in GV + GV samples.

Total soluble protein levels (TSP) of GFP measured by ELISA were significantly higher in the GV + GFP (1.35%) infiltrated tissues than in all other infiltration types ($F = 15.14$; $df = 3,20$; $P < 0.0001$) (Table 1). The lowest level of expression detected by ELISA was in GFP + Bt infiltrated tissues, which had an average GFP total soluble protein of 0.57%. No GFP was detected in GV + GV samples.

Bt *Cry1Ac* levels in infiltrated spots showed no significant differences among those co-infiltrated in different vectors (GFP + Bt) (0.022% TSP) versus those in the same vector (GV + GFP/Bt) (0.031% TSP) (Table 1). The range of Bt Cry1Ac synthesized in individual spots ranged from 0.054 to 0.015% TSP. No significant associations were observed between Bt Cry1Ac % TSP and GFP % TSP for either infiltration types.

GFP meter values in the infiltration characterization experiment correlated strongly with GFP ELISA data ($r = 0.82$, $P < 0.0001$). Similar differences to those found in ELISA data were detected in average GFP meter readings ($F = 22.11$; $df = 4,25$; $P < 0.0001$) (Table 1). GV + GV infiltrated spots had a low level of background at 61 counts per second (CPS). Ratios of GFP expression were shown to have a moderate positive relationships with GFP meter readings ($r = 0.56$, $P < 0.01$) and GFP ELISA data ($r = 0.51$, $P < 0.01$). Ratios of Bt expression had no significant relationship with Bt ELISA data.

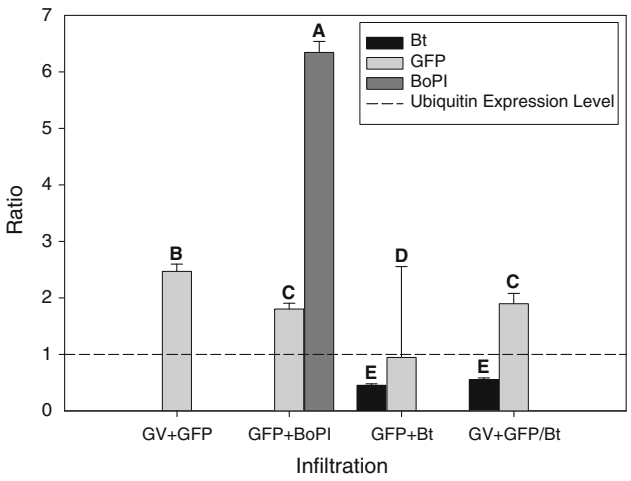

Fig. 2 Ratio of transcription of the mGFP5-er, Bt Cry1Ac, and BoPI transgene genes relative to the expression level of ubiquitin *N. benthamiana* infiltrations with *Agrobacterium tumefaciens* containing (GV + GFP), (GFP + BoPI), (GFP + Bt), or (GV + GFP/Bt). ANOVA was performed on ΔC_t values using a generalized linear model in SAS 9.13. Means were separated by Tukey's multiple range test. Ratios of expression were determined as described in Yuan et al. (2008). *Error bars* indicate standard error of the mean and *bars* with the *same letters* are not significantly different ($P < 0.05$)

Table 1 Average GFP meter reading (counts per second (CPS) are arbitrary units), GFP percent total soluble protein (%TSP), and Bt percent total soluble protein from *N. benthamiana* leaf tissue agroinfiltrated with GV + GV, GV + GFP, GFP + BoPI, GFP + Bt, and GV + GFP/Bt

Infiltration type[a]	GFP meter (CPS) (mean ± SE)		GFP ELISA (%TSP) (mean ± SE)		Bt ELISA (%TSP) (mean ± SE)
GV + GV	61 ± 19	C	NT		NT
GV + GFP	1395 ± 49	A	1.35 ± 0.09	A	NT
GFP + BoPI	977 ± 179	B	0.83 ± 0.11	B	NT
GFP + Bt	619 ± 77	B	0.57 ± 0.09	B	0.022 ± 0.003
GV + GFP/Bt	999 ± 126	B	0.71 ± 0.03	B	0.031 ± 0.017

ANOVA performed using a generalized linear model in SAS 9.13. Values followed by the same letter are not significantly different based on a Tukey's multiple range test ($P < 0.05$)

SE standard error of the mean, *NT* data not taken

[a] Tissue was snap-frozen 3 days post-infiltration

Insect bioassay using infiltrated tissue

GFP meter values in the insect bioassay revealed lower levels of GFP expression than those found in the infiltration characterization assay. Although there were significant differences found between infiltration types ($F = 15.51$; $df = 4,95$; $P < 0.0001$) (Table 2), they were different than those found in the infiltration characterization assay. Both GV + GFP and GV + GFP/Bt spots had significantly higher levels of GFP detected than all other treatments. GV + GV infiltrated spots had a low background of 23 CPS.

Insects that were fed tobacco tissue with transiently expressed synthetic *Cry1Ac* had high larval mortality ($F = 772.42$; $df = 5,18$; $P < 0.0001$) (Table 2). Both treatments GFP + Bt and GV + GFP/Bt resulted in 100% larval mortality. No insect mortality was observed in larvae feeding on all other treatments.

Larval weights were recorded for all treatments except those containing synthetic *Cry1Ac* (GFP + Bt and GFP/Bt), which had no larvae remaining on day 6. The largest average larval weight was observed in those insects feeding on the GV + GFP treatment (45 mg) ($F = 6.16$; $df = 3,76$; $P < 0.001$) (Table 2). Insects feeding on all other treatments had significantly lower average larval weights. The lowest average larval weight was recorded in insects fed the non-infiltrated *N. benthamiana* tissue (26.1 mg).

Average larval head capsule size was similar to average larval weight with values recorded for all treatments except those fed synthetic *Cry1Ac* (GFP + Bt and GFP/Bt). Insects fed tissue from the GV + GFP treatment resulted in the largest average larval head capsule size (1.6 mm) ($F = 2.65$; $df = 3,76$; $P = 0.055$), indicating that these insects had developed to both the 4th and 5th instars (Neunzig 1969). While there were no significant differences in average larval head capsule size at an alpha level of 0.05, at an alpha level of 0.1 the head capsules of GV + GFP fed insects were significantly larger than those insects fed the non-transformed control. Insects fed non-infiltrated tobacco tissue were observed to have the smallest average larval head capsule size (1.3 mm).

Table 2 Average GFP meter reading of agroinfiltrated tissues used in bioassays and fed to larval *Helicoverpa zea* and the resulting larval weights, head capsule size and percent mortality

Infiltration type	GFP meter[a] (CPS) (mean ± SE)		Larval weight[b] (mg) (mean ± SE)		Head capsule size[b] (mm) (mean ± SE)[c]		Mortality[b] (%)[d]	
Non-transformed	NT		26.1 ± 2.0	B	1.3 ± 0.1	B	0	B
GV + GV	23 ± 3	C	31.1 ± 3.7	B	1.4 ± 0.1	AB	0	B
GV + GFP	475 ± 65	A	45.1 ± 3.1	A	1.6 ± 0.1	A	0	B
GFP + BoPI	289 ± 42	B	32.4 ± 3.9	B	1.4 ± 0.1	AB	0	B
GFP + Bt	345 ± 36	B	NA		NA		100	A
GV + GFP/Bt	469 ± 60	A	NA		NA		100	A

ANOVA performed using a generalized linear model in SAS 9.13. Values followed by the same letter are not significantly different based on a Tukey's range test ($P < 0.05$)

SE standard error of the mean, *CPS* counts per second, *NT* data not taken, *NA* data not available because of a lack of living insects

[a] Readings taken 3 days post-infiltration

[b] Data recorded 6 days after exposure to leaf tissue (9 days post-infiltration)

[c] Head capsule size evaluated at $P < 0.1$

[d] ANOVA performed using Proc Glimmix procedure of SAS 9.13

Stable transformants

Real-time RT-PCR of stable transformants

Comparisons of normalized C_t values of transgenic BoPI lines revealed no significant differences among lines. When compared to the internal reference gene ubiquitin, BoPI 15 plants had the largest average ratio of expression (2.14) (Table 3), while BoPI 2 plants had the lowest average ratio of expression (1.52). Comparisons within lines of plants used in corn earworm bioassays showed no significant differences. Wild-type samples in all tests were negative for transgene amplification.

Corn earworm bioassay on stable transformants

Tissue from tobacco lines expressing the BoPI proteinase inhibitor and non-transgenic (Xanthi) tobacco fed to larval *H. zea* resulted in significantly lower insect mortality than insects fed transgenic tobacco tissue expressing the insecticidal protein *Cry1Ac* (Bt/GFP)) ($F = 16.7$; $df = 4,8$; $P < 0.001$) (Table 3). As expected, feeding insects Bt/GFP tobacco tissue resulted in high mortality (100%) with all insects dying by day 4 (data not shown). Of the other treatments, only insects fed tissue from BoPI line 7 showed an increased level of mortality (29.7%) compared to the wild-type tobacco (0%).

Leaf punches collected from containers with living insects on day 4 had no significant differences in the remaining total leaf punch areas ($F = 0.21$; $df = 3,81$; $P = 0.89$) (data not shown). Punches from Xanthi plants (5.41 cm^2) had the smallest remaining mean leaf disc area. The largest average total leaf punch area measured was from BoPI 2 plants (5.52 cm^2).

Weights of *H. zea* larvae were similar at the beginning of the experiment but over time insects fed the plant lines expressing proteinase inhibitors grew larger than those insects fed wild-type tissue (Table 3). Larval *H. zea* weights recorded on Day 4 revealed that there were no significant differences ($F = 2.4$; $df = 3,81$; $P = 0.07$) between treatments. *H. zea* larval weights taken on Day 9 showed that insects fed tissue from BoPI 15 (32.2 mg) plants had greater larval weights ($F = 3.2$; $df = 3,72$; $P < 0.05$) than insects fed the wild-type (12.3 mg) but was statistically similar to all other treatments. At the conclusion of the experiment (day 17), insects fed tissue from BoPI 15 (369.2 mg) weighed significantly more than insects fed wild-type (233.8 mg) plants. Insects fed BoPI 15 plant leaves weighed more than 1.5 times those insects that were fed wild-type plants ($F = 3.7$; $df = 3,67$; $P < 0.05$).

Larval *H. zea* feeding on the transgenic and wild-type plant lines showed no significant differences in head capsule size at the conclusion of the experiment (data not shown). All mean head capsule sizes for insects ranged from 2.6 mm to 2.8 mm indicating that these larvae were in their 5th and 6th instars (Neunzig 1969).

Discussion

These results demonstrate that a system combining agroinfiltration and insect bioassays can be used as a powerful tool for the rapid assessment of potential toxicity and efficacy of products from candidate insect resistance genes. This method of screening was effective at evaluating genes for insecticidal activity, as insects feeding on tissue infiltrated with a synthetic Bt *Cry1Ac* gene suffered 100% mortality. Similar results were found with insects fed stable

Table 3 Weights and percentage of mortality of larval *Helicoverpa zea* fed wild-type (Xanthi) and transgenic (BoPI lines 2, 7, and 15) tobacco leaf tissue

Plant line	Ratio of expression[a]	Larval weight (mg) (mean ± SE)					Corrected mortality (%)[b]	
		Day 4	Day 9		Day 17			
Xanthi	–[c]	2.2 ± 0.2	12.3 ± 2.9	B	233.8 ± 27.3	B	0 ± 0	C
BoPI 2	1.53	2.8 ± 0.4	15.0 ± 4.7	AB	288.6 ± 34.0	AB	20.9 ± 14.2	BC
BoPI 7	1.54	2.9 ± 0.4	18.1 ± 5.4	AB	349.4 ± 33.1	AB	29.7 ± 12.9	B
BoPI 15	2.14	3.7 ± 0.5	32.2 ± 6.7	A	369.2 ± 35.3	A	12.2 ± 11.4	BC
GFP/Bt	NT	NA	NA		NA		100 ± 0	A

ANOVA performed using a generalized linear model in SAS 9.13. Values followed by the same letter are not significantly different based on a Tukey's range test ($P < 0.05$)

SE standard error of the mean, *NT* data not taken, *NA* data not available because of a lack of living insects

[a] Level of BoPI expression relative to that of the internal reference gene, ubiquitin

[b] Percentage of mortality on day 17

[c] No expression of BoPI detected

transgenic material expressing Bt. The effects on insects fed tissues transiently expressing genes with the potential to affect growth and development were less predictable as levels of gene expression were higher than seen in stable transgenics.

Levels of transient expression have been reported to be up to 1,000-fold higher than that of stable transformants (Janssen and Gardner 1989). While these extreme levels of increased expression were not achieved in our assays, we did see the levels of BoPI expression rise from two times the level of ubiquitin expression in stably transformed plants to over six times the level of ubiquitin in infiltrated tissues. While proteinase inhibitor activity of the agroinfiltrated tissues was not taken, proteinase inhibitor activity has previously been characterized from plants stably transformed with this BoPI construct (Pulliam et al. 2001). As this study reveals a positive relationship between GFP expression ratios and GFP data, we expect that increased levels of mRNA seen in the BoPI-infiltrated tissues would translate to higher levels of proteinase inhibitors.

When plants were co-infiltrated with both GFP and the BoPI serine protease inhibitor and fed to larval *H. zea*, a significant decrease in larval weights was detected compared to infiltrations containing only GFP. In addition, the larval head capsule size was smaller, although not significantly so, compared with those insects fed GFP-only infiltrated tissue. These results could be the result of lower levels of GFP total soluble protein in BoPI-infiltrated lines (Table 2.) While a decrease in the level of GFP would explain the decreased growth rate, the expression of BoPI should have a positive effect on growth rate similar to the enhanced growth seen in insects fed BoPI stable transformants (Table 3). Weight gains in insects fed transgenic plants expressing protease inhibitors have previously been documented (De Leo et al. 1998; Girard et al. 1998; McManus et al. 1994) and are thought to be the result of the insects adapting to the inhibitor. Adaptation of insects to transgenic plants expressing proteinase inhibitors have been attributed to an overproduction of inhibitor sensitive/insensitive proteases or the production of proteases that inactivate the recombinant protein (Zhu-Salzman and Zeng 2008). An alternative explanation for the decrease in larval weights of insects fed (GFP + BoPI) could be that the levels of BoPI expression in infiltrated tissue were extremely high compared to the levels seen in the stably transformed BoPI plants. This high titer of BoPI might have mitigated the efficacy of the insect's natural adaptive processes in response to both the endogenous plant proteases and BoPI, resulting in lower weight gains. The ability to evaluate candidate insect resistance genes at levels higher than those found in typical stable transformants is a major advantage to this screening system.

An interesting result from these experiments was that insects feeding on tissue infiltrated with GFP as the only transgene experienced enhanced levels of growth and development (Table 2). This is evidenced by the increased larval head capsule size seen in insects fed tissue infiltrated with GV + GFP. Additionally, GV + GFP fed insects had increased larval weight gains, which have previously been used to determine developmental effects on insect development (Broadway 1995; Daly 1985; De Leo et al. 1998). The increase in growth is, to our knowledge, the first report of transgenic plants expressing GFP having a positive effect on an insect herbivore. This enhanced rate of growth may be explained by a boost in protein nutrition from the high overexpression of GFP resulting in an increase of total soluble protein.

Intermediate levels of mortality were observed in insects feeding on one of the stably transformed BoPI lines. These results are different from those reported in Pulliam et al. (Pulliam et al. 2001), where *H. zea* experienced high levels of mortality when fed transgenic BoPI lines. We expected to see improvements in insect control compared to the previous study since Pulliam et al. (2001) used both hemi- and homozygous lines that were bulked together in their assays. A number of factors could have played into the observed reduction of insect mortality. First, we allowed neonates to feed on artificial diets until they reached the second instar of development, this step was added to decrease the levels of background mortality associated with the initial transfer of insects to the leaf tissue (data not shown). While this waiting period might have decreased background mortality, it might also have allowed the neonate larvae to adapt to proteinase inhibitors, which would serve to decrease their apparent efficacy. Another factor that potentially improved our insect survivorship was the addition of only one larva per experimental unit. *H. zea* has been shown to exhibit high levels of cannibalism (Barber 1936; Chilcutt 2006). As the previous study used three insects per container there is a high likelihood that a large amount of insect mortality might have been the result of direct cannibalism or wounding of insects in that study and not from plant-based defense.

The use of this system to evaluate insect resistance genes has several benefits when compared to other systems. As mentioned previously, the position effects of transgene insertion is a potential confounding factor, which is eliminated by the new method. The time for preparation of transgenic tissue is drastically reduced: days instead of months (Wroblewski et al. 2005), and also eliminating the possibility of escape of transgenes into the environment (Li et al. 2007). When compared with previously described viral based screening systems (Lawrence and Novak 2001), the time for expression of genes is halved. As viral vectors are not used to generate stable transformants, a system employing

agroinfiltration can be rapidly altered for the production of stable transgenic lines. Additionally, viral vectors have size constraints in the transgenes they can produce (Gleba et al. 2007) making it a less attractive system.

There are constraints within the current system. Large variation in expression was observed among infiltrations. Many factors can influence transient expression from agroinfiltration hence influencing observed bioassays. One of the major considerations is the OD of the infiltration, which has been documented to have a large effect on the expression of the transgene (Santos-Rosa et al. 2008; Sparkes et al. 2006). While this study used a standardized co-infiltration OD, it might be advantageous to adjust these ODs to suit a specific construct. In addition, species, leaf position, and age of the plant can all have impacts on transgene expression (Sheludko et al. 2006). Another issue with the high levels of transcription found in agroinfiltrated tissue is post-transcriptional gene silencing, by which the plants endogenous defenses against viruses can hinder the amount and duration of transgene expression (Voinnet et al. 2003). This problem might be alleviated by the addition of a silencing suppressor, such as P19 (Voinnet et al. 2003; Wroblewski et al. 2005), which could significantly increase the level and longevity of transgene expression. P19 was not used in our experiments as it would add an increased level of complexity to the assays, as P19 might have independent effects on the growth and development of assayed insects.

The use of agroinfiltration in tandem with insect bioassays could be very useful in discovering new insect resistance genes through systematic screens that are coupled with large-scale genomics and phenomics studies. This system allows for enhanced characterization of not only insecticidal genes, but also genes, which alter insect growth and development, which might have elusive modes of action. In addition to testing insect resistance genes, this agroinfiltration could have potential utility in the screening of pathogen and abiotic stress tolerance genes, traits in which the system has yet to be tested.

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