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# Increasing Expression of an Anti-Picloram Single-Chain Variable Fragment (ScFv) Antibody and Resistance to Picloram in Transgenic Tobacco (*Nicotiana tabacum*)

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Systematic research involving four chimeric gene constructions designed to express the same anti-picloram single-chain variable fragment (scFv) antibody is described. *Agrobacterium*-mediated transformation produced at least 25 transgenic tobacco plants with each of these, and the number of T-DNA loci in each plant was determined using kanamycin-resistance segregation assays. The relative amounts of active and total scFv in each plant were evaluated using quantitative enzyme-linked immunosorbent assay and immunoblot technologies, respectively. No significant differences in scFv activity were found among the four groups of single-locus plants, although the 35S/M construct was found to produce significantly more total anti-picloram scFv than the other three constructs. A dose−response bioassay involving $T_1$ seedlings from several of the highest expressers of active scFv demonstrated resistance to a constant exposure of picloram at $5 \times 10^{-8}$ M. Other approaches for increasing antibody-based herbicide resistance are discussed, as further improvements are needed before practical application of this technology.

**KEYWORDS: Single-chain variable fragment antibody; scFv; picloram; herbicide resistance; immunomodulation; transgenic plant; tobacco; *Nicotiana tabacum***

## INTRODUCTION

Herbicide resistance in plants can be acquired by three general mechanisms: metabolic detoxification of the herbicide, modification of the site of action where the herbicide molecule acts, and prevention of the herbicide from reaching its site of action. The International Survey of Herbicide Resistant Weeds reports 295 cases of weeds resistant to herbicides, including 106 dicot and 71 monocot species (*1*). Resistance to herbicides can also be conferred to plants via conventional breeding, for example, imazethapyr-resistant crops (*2*), or by transgenic techniques (*3*), for example, glyphosate-resistant canola, corn, cotton, and soybean. In most cases, alteration of the target site has been the mechanism used to confer herbicide resistance to crops.

Immunomodulation, that is, the expression of an antibody in an organism to affect its phenotype by altering the function of the target molecule in vivo (*4*), has also been used to create herbicide resistance in plants. Transgenic tobacco and *Arabidopsis* plants that express herbicide-specific single-chain variable fragments (scFvs) have been made that are resistant to picloram and chlorpropham, respectively (*5*, *6*). These resistances were achieved by expressing specific antiherbicide scFvs, and in both cases resistance correlated with scFv expression levels. There are limitations to these models, as the levels of resistance obtained against the two specific herbicides are low: in both cases herbicide tolerance increased only 2−3-fold above that of wild-type plants. Almquist et al. (*5*) found the highest dose that transgenic tobacco plants expressing anti-picloram-specific scFv could resist was equivalent only to 0.5 g of active ingredient (ai)/ha, which is much lower than the 35−50 g of ai/ha field dose of picloram typically applied to cereal crops (*7*). Similarly, Eto et al. (*6*) reported that the highest dose of chlorpropham resisted by transgenic *Arabidopsis* was a constant exposure equivalent to 0.2 g/ha, which is several orders of magnitude less than its field dose.

A priori remedies for increasing immunomodulation-based resistance involve increasing the level of antibody expression, improving antibody targeting to intracellular locations where the herbicide is active, and/or increasing the antibody affinity for the specific herbicide. Eto et al. (*6*) targeted an anti-chlorpropham scFv to four intracellular locations and found that targeting to the endoplasmic reticulum (ER) provided the greatest expression and concomitant herbicide resistance. This resistance is likely due to ER targeting being associated with higher expression of recombinant proteins in planta (*8*, *9*), as

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opposed to the ER being the optimal intracellular location for effecting herbicide resistance.

Increases in the quantities of transgene products in plants have been achieved with stronger promoters (*10*−*12*) and through the use of genetic control elements such as translational enhancers (*13*, *14*) and matrix attachment regions (*15*). Suppression of post-transcriptional gene silencing mechanisms can also improve transgene product accumulation, either by coexpression with a viral suppressor of gene silencing (*16*) or by transgene expression in gene silencing mutants (*15*). In other cases, transgenes that are particularly difficult to express in plants have required coding sequence modification to reflect the codon usage of the intended host species for improved transgene expression (*11*, *17*).

In this study, we attempted to improve the resistance of transgenic tobacco plants to picloram by increasing the expression of anti-picloram scFv in planta. Four chimeric gene constructions were produced with one of two promoters, either the tCUP3 (*18*) or a double-enhancer CaMV 35S promoter (*19*), combined with one of two scFv coding sequences, the original (O; *5*), which was of murine origin (*20*, *21*), or a modified (M) version. Both the O and M sequences encoded identical polypeptides, but the M sequence was synthesized to mimic tobacco codon usage and eliminate the potential for problems in transcription, transcript stability, and translation. Four groups of transgenic plants were produced with these constructs, and these were analyzed for relative total and active anti-picloram scFv, as well as for number of T-DNA loci. Offspring of four primary transgenic plants that were among the highest expressers of active scFv were subjected to a picloram dose−response bioassay, and one line showed resistance to a constant dose of $5 \times 10^{-8}$ M picloram, which was an ~3-fold improvement compared with the resistance reported by Almquist et al. (*5*). Increased scFv expression may lead to eventual development of this mechanism as a practical model for creating antibody-based herbicide resistance through immunomodulation.

## MATERIALS AND METHODS

**Anti-Picloram scFv Antibody Coding Sequence Modification.** The anti-picloram scFv coding sequence assembled by Almquist et al. (*5*) was virtually modified prior to synthesis (see below). The first goal of the modification was to make the coding sequence more similar to that of tobacco (*Nicotiana tabacum*) than to mouse (*Mus musculus*) DNA, from which the sequence was originally obtained (*20*). Codon optimization of the 885 bp sequence was performed utilizing the Protein Back Translation program (*22*) and *N. tabacum* coding sequence preferences. Codons with a frequency lower than 10 per thousand in the tobacco genome (*23*) were replaced in the scFv coding sequence by more preferred tobacco codons. Furthermore, potential intervening sequence splice-site acceptor and donor motifs were identified (*24*, *25*) and subsequently removed by replacement with nucleotides that resulted in codons encoding the same amino acids. Inverted repeat sequences were analyzed using the Genebee RNA Secondary Structure software package (*26*, *27*); nucleotides were changed to reduce the free energy (kilocalories per mole) of potential secondary structure while maintaining the polypeptide sequence. Repeated elements were analyzed (*28*) and replaced when present. Potential methylation sites (i.e., CXG and CpG; *29*) were replaced where possible and always without changing the encoded amino acid sequence. Plant polyadenylation sites (i.e., AATAAA, AATGAA, AAATGGAAA, and AATGGAAATG; *30*, *31*) and ATTTA RNA instability elements (*32*) were analyzed, but none were found in the O or M sequence. Finally, restriction enzyme sites required for subcloning were ensured as unique within the M sequence.

**Synthesis of the Modified Anti-Picloram scFv Coding Sequence.** Polymerase Chain Reaction-mediated gene synthesis was performed (*33*). The virtual M coding and anti-coding sequences were divided among 24 overlapping and complementary oligonucleotides, each not longer than 80 bases (see Supporting Information Table 1), which were synthesized, 5′-phosphorylated, and purified (Sigma Genosys, Oakville, ON). Volumes of 1 μL of each oligonucleotide from stock concentrations (25 μM) were pooled in a final volume of 30 μL in 1× ligase buffer (MBI Fermentas, Burlington, ON, Canada). The reaction tube was placed in a water bath, which was heated to 90 °C for 5 min and cooled (6 h) to room temperature inside a Styrofoam box. Two microliters of ligase (5 Weiss units/μL; MBI Fermentas) was added and incubated at 14 °C for 16 h. PCR was performed to amplify the M coding sequence using outer oligonucleotides as primers (PICPCR1 and PIC-12A; Supporting Information Table 1) and 2.5 μL of ligation product as template in a 25-μL reaction that contained a final concentration of 2.5 mM $MgCl_2$, 0.25 mM of each deoxyribonucleoside triphosphate, and 1.25 units of Platinum *Pfx* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). The reaction conditions were 5 min at 94 °C and 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension of 7 min at 72 °C. An additional 10 min extension period at 72 °C using 5 units of *Taq* polymerase (MBI Fermentas) was performed before subcloning the 900 base-pair PCR product into pCR2.1 vector (Invitrogen) and sequencing at Laboratory Services Division, University of Guelph. Repairs were made by cloning correct sequences from two different clones into the same plasmid. To do this, two *Xba*I restriction enzyme sites, located at the multicloning site of the vector plasmid and at 682 bp of the coding sequence, were taken advantage of for combining the correct sequences between two complementary clones. The final sequence of the corrected plasmid was verified by DNA sequencing.

**Table 1.** Comparison of Original (O) and Modified (M) Anti-Picloram scFv Coding Sequences

| parameter | O anti-picloram scFv | M anti-picloram scFv |
|---|---|---|
| % nucleotide identity to O scFv | 100 | 73 |
| % G+C content | 49 | 41 |
| rare (<10‰) tobacco codon usage | 27 | 20 |
| medium (10–20‰) tobacco codon usage | 155 | 122 |
| abundant (>20‰) tobacco codon usage | 113 | 153 |
| potential splice sites (*24*) | 12 | 4 |
| plant polyadenylation sites (*31*) | 0 | 0 |
| consecutive identical codons | 8 | 6 |
| RNA predicted secondary structure (kcal/mol; *26*) | −179.7 | −103.4 |
| CXG potential methylation sites | 71 | 27 |
| CpG potential methylation sites | 15 | 15 |

**Binary Vector Assembly.** Four chimeric gene constructs were assembled using one of two promoters, the tobacco cryptic constitutive promoter 3 (tCUP3; *18*) or a double-enhancer version of the CaMV 35S promoter (taken from pFF19G; *19*), and one of two anti-picloram scFv coding sequences, O, which was of murine origin, or M. One of these, a binary plasmid utilizing the tCUP3 promoter to drive transcription of the O sequence (i.e., tCUP3/O), has been published as pBIN/tCUP3/PICscFv (*5*). A second binary plasmid (i.e., tCUP3/M) was produced by replacing the O coding sequence from tCUP3/O with the M sequence after both DNAs had been treated with *Eco*RI and *Sac*I. A third binary plasmid (pCAMterX/2X35S/PICscFV, unpublished), containing the O sequence between the double-enhancer version of the CaMV 35S promoter and the *nos* terminator, was provided by Kirk Brown (Agriculture and Agri-Food Canada, London, ON, Canada) and is hereafter referred to as 35S/O. A fourth binary plasmid (i.e., 35S/M) was also produced by replacement but after introduction of an *Eco*RI site at the 3′ end of the sense strand of the M coding sequence by PCR mutagenesis using the ECOPIC-A and PICPCR1 primers (Supporting Information Table 1). The newly amplified M sequence was cloned between the 35S promoter and the *nos* terminator of the 35S/O construct after treatment of both DNAs with *Eco*RI. For both replacements, that is to produce the tCUP3/M and 35S/M constructs, the tCUP3/O and 35S/O vector fragments were treated with shrimp alkaline phosphatase (MBI Fermentas) prior to ligation with the respective M fragments and subsequent introduction into *Escherichia*

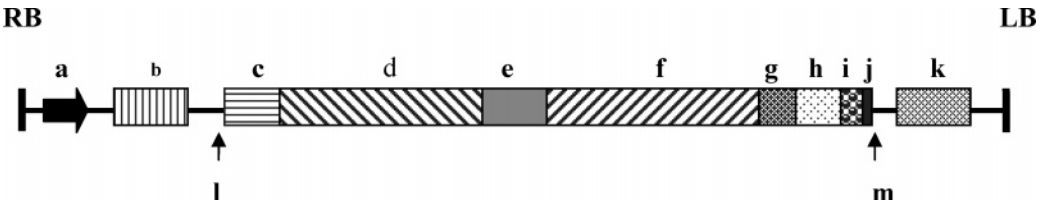


**Figure 1.** Schematic diagram of the anti-picloram scFv antibody gene transfer-DNAs (T-DNAs): **RB**, right border of T-DNA; **a**, neomycin phosphotransferase gene (*npt*II); **b**, tobacco cryptic constitutive promoter (tCUP3) or double-enhancer cauliflower mosaic virus (CaMV) 35S promoter; **c**, *Arabidopsis* basic chitinase signal sequence; **d**, heavy-chain variable fragment domain; **e**, polypeptide linker; **f**, light-chain variable fragment domain; **g**, c-MYC tag; **h**, hexahistidine tag; **i**, endoplasmic reticulum (ER) retrieval signal; **j**, stop codons; **k**, nopaline synthase (*nos)* terminator; **l**, *Eco*RI recognition site; **m**, *Sac*I recognition site; **LB**, left border of T-DNA.

*coli* strain DH5α (*34*). Both constructs containing the M sequence were sequenced at Laboratory Services Division, University of Guelph, to confirm the desired sequences.

***Agrobacterium*-Mediated Plant Transformation.** Binary plant expression vectors containing each of the four constructs were introduced into *Agrobacterium tumefasciens* At542 by electroporation, and respective T-DNAs were transferred into *N. tabacum* 81v9 (*5*, *35*). For each of the four constructs, at least 25 primary transgenic plants were produced. Enzyme-linked immunosorbent assay (ELISA) and quantitative immunoblots were performed on extracts from each plant to characterize levels of total and active scFv produced by the plants, respectively (see below).

**Preparation of Leaf Protein Extracts.** Protein extracts were prepared by grinding, for 5 min, 250 mg of fresh leaf tissue from each transgenic plant in 1 mL of cold (4 °C) extraction buffer (50 mM $Na_2HPO_4$, 500 mM NaCl, 50 mM ascorbate, 0.05% Tween 20; pH 5.8) using a vibration mill type MM300 (Retsch GmbH & Co., Haan, Germany) and three steel beads (Qiagen, Mississauga, ON, Canada) per sample. Samples were centrifuged for 30 min at 4°C, and the supernatant containing the total soluble protein (TSP) fraction was transferred to a new tube. Tubes containing the samples were kept on ice. The relative TSP concentration of each sample was determined utilizing the Bio-Rad (Hercules, CA) protein assay reagent according to the supplier's instructions. Three samples per plant were made, and normalization of TSP concentrations among all samples was performed by diluting the more concentrated samples to the lowest relative TSP concentration with extraction buffer. These normalized samples were directly used in the ELISA and quantitative immunoblot experiments (see below).

**Enzyme-Linked Immunosorbent Assay.** Measurements of active plant-produced anti-picloram scFv antibody were carried out using indirect ELISA following the protocol described by Almquist et al. (*5*). Briefly, microtiter plates were coated with picloram conjugated to ovalbumin (pic−OVA), blocked, and incubated with 100, 50, 25, 12.5, 6.25, 3.13, or 1.56 μL of normalized plant protein extracts containing the expressed scFv. Three independent, normalized protein samples per plant were analyzed. A $T_0$ transgenic plant (PIC21), produced by Almquist et al. (*5*), was maintained in a greenhouse, and its was extract was used as an internal control in all ELISA plates to normalize absorbance readings over all plates by transformation of the internal control readings to an absorbance of 0.1 for the 3.13 μL aliquots; experimental sample absorbances were transformed accordingly. The average active scFv produced by each plant was calculated by averaging the three sample repeats.

**Quantitative Immunoblot Analysis.** Normalized leaf extracts produced above were used to compare the total amount of scFv produced by each plant. Fifty microliter aliquots of the three protein-normalized extracts per plant were pooled, and immunoblotting was performed as described in Almquist et al. (*5*). Immunopositive bands were scanned and intensities were measured using Kodak (Rochester, NY) 1D version 3.5.4 software. Equal volumes of the internal control (PIC21) were loaded on each gel to allow comparison over all immunoblots, as described for the ELISA plates (above).

**Kanamycin Resistance Segregation Assays.** The number of T-DNA loci in each primary transgenic tobacco ($T_0$) plant was determined using kanamycin resistance segregation assays. Approximately 200 $T_1$ seeds obtained from each self-pollinated $T_0$ plant were surface-sterilized (10% bleach), washed, and placed on MS medium (Sigma) containing 50 mg/L of kanamycin. The number of plants resistant (R) and sensitive (S) to kanamycin were counted after 4 weeks. Mendelian segregation was evaluated with chi-square analyses ($\chi^2$; *36*, *37*), and probabilities >5% were considered to be adequate for accepting the null hypothesis tested in each case (Supporting Information Tables 2−5).

**Picloram Dose−Response Bioassays.** $T_1$ seeds from four self-pollinated $T_0$ plants, which were among the best expressers of active and total scFv (i.e., tCUP3/M/#09, 35S/O/#43, 35S/M/#02, and 35S/M/#30), were distributed on MS agar plates (as above) containing the auxinic herbicide picloram (Sigma) at concentrations of $1 \times 10^{-7}$, $5 \times 10^{-8}$, $2.5 \times 10^{-8}$, $1 \times 10^{-8}$, $5 \times 10^{-9}$, $2.5 \times 10^{-9}$, and $1 \times 10^{-9}$ M and grown for 4 weeks. Two controls were utilized: seeds from wild-type variety 81V9 (WT) and from a $T_0$ plant produced by Almquist et al. (*5*; i.e., PIC42). Plants were visually inspected, and auxinic herbicide symptoms such as epinasty and obvious growth reduction were monitored in comparison with the controls.

**Statistical Analyses.** Analysis of variance (ANOVA) at the 95% level of confidence was performed to determine whether the means among the four groups of $T_0$ plants were different in terms of relative total or active scFv. Duncan's test (*38*) was performed to separate the means. All analyses were done utilizing the SAS V.8 statistical program (SAS Institute Inc., Cary, NC).

## RESULTS

**Anti-Picloram scFv Antibody Coding Sequence Modification.** The 885 bp anti-picloram scFv coding sequence published previously (*5*) was modified for expression in *N. tabacum* plants in silico (GenBank accession AY710431). **Figure 1** shows a schematic diagram of the T-DNAs containing the scFv genes, which encode an amino-terminal signal sequence, followed by the anti-picloram heavy and light chains connected by the $(SGGGG)_3$ linker, the c-MYC, and hexahistidine tags, a KDEL endoplasmic reticulum retrieval signal, and two stop codons. Codon optimization and removal of instability elements were performed to make this coding sequence more similar to tobacco DNA (see Material and Methods). **Table 1** summarizes the differences between the O and M coding sequences. Twenty-seven percent of the nucleotides were changed from the original sequence assembled by Almquist et al. (*5*). The G+C content was reduced from 49 to 41%, closer to the 43% of *N. tabacum* (*23*). Codons with fewer than 10 per thousand frequencies in tobacco were reduced from 27 to 20 in the M coding sequence, whereas codons with 10−20 per thousand were reduced from 155 to 122. Abundant codons in tobacco, with frequencies >20 per thousand, were increased from 113 to 153 in the M coding sequence. Overall, 188 of 295 codons were changed to make the M sequence. Potential RNA splice sites (*24*) were reduced from 12 to 4. Consecutive identical codons were reduced from 8 to 6. The predicted secondary structure of the mRNA was reduced from −179.7 to −103.4 kcal/mol (*26*). Potential CXG methylation sites were reduced from 71 to 27, and the number

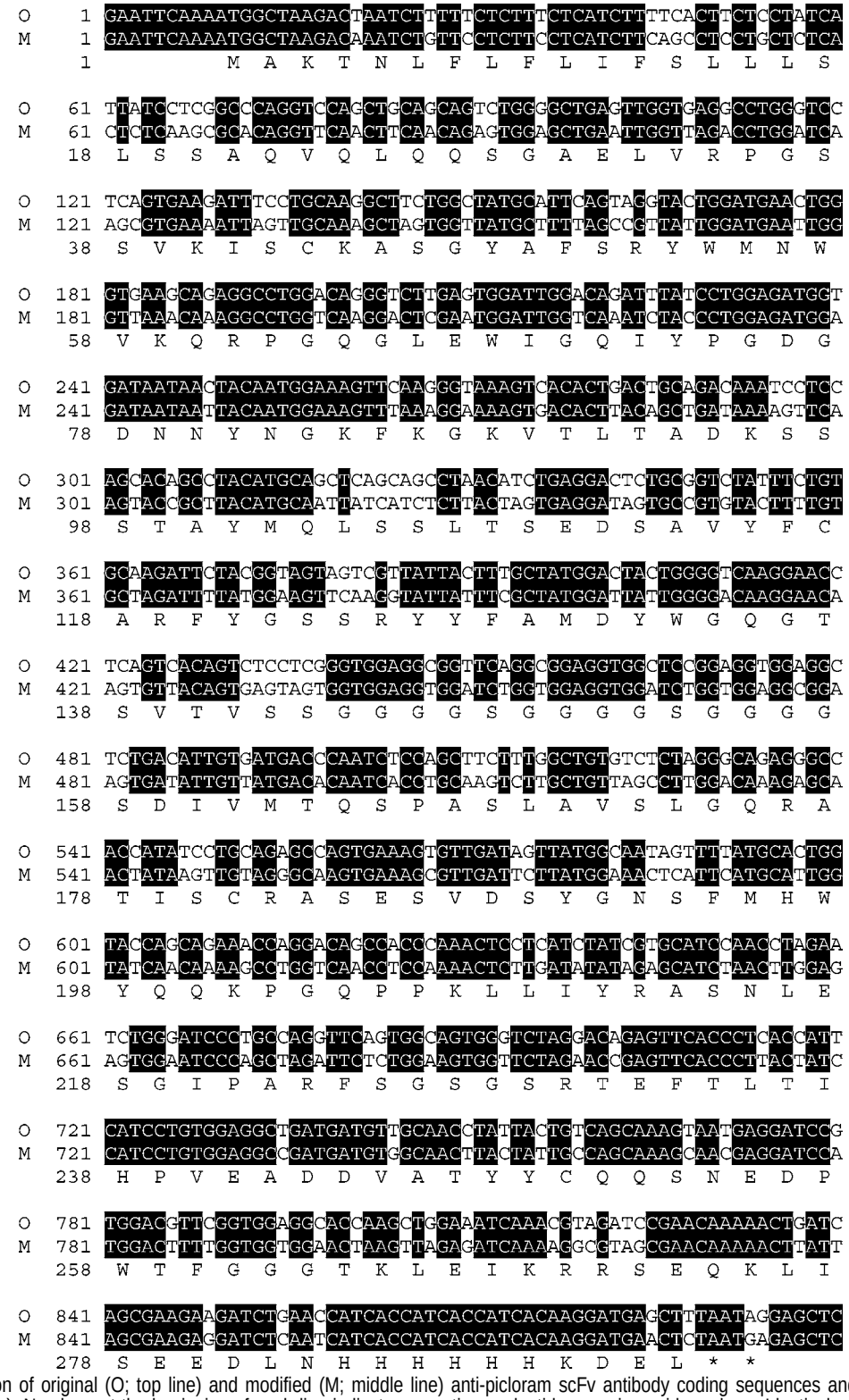


**Figure 2.** Comparison of original (O; top line) and modified (M; middle line) anti-picloram scFv antibody coding sequences and the scFv polypeptide sequence (bottom line). Numbers at the beginning of each line indicate respective nucleotide or amino acid numbers. Identical nucleotides are boxed in black. Amino acids: 1–21, *Arabidopsis* basic chitinase signal sequence; 22–141, heavy-chain variable fragment; 142–158, polypeptide linker; 159–271, light-chain variable fragment; 272–283, c-MYC tag; 284–289, hexahistidine tag; 290–294, ER retrieval signal; 295 and 296, stop codons. Note that the nucleotide sequences begin with *Eco*RI restriction sites and end with *Sac*I restriction sites. The GenBank accession number for the M sequence is AY710431.

of CpG sites remained the same in both the O and M sequences, that is, 15. Plant polyadenylation sites (*30*, *31*) and instability elements (ATTTA; *32*) were not found in either sequence. An alignment of both DNA sequences, plus the polypeptide sequence that they encode, is shown in **Figure 2**, where identical nucleotides are boxed in black.

**Transgenic Plant Production and Determination of T-DNA Locus Number.** One hundred and eight transgenic *N. tabacum* plants, variety 81V9, were produced: 28 contained the tCUP3/O construct; 28, tCUP3/M; 27, 35S/O; and 25, 35S/M. All $T_0$ plants were analyzed for number of T-DNA loci by kanamycin resistance segregation analysis.

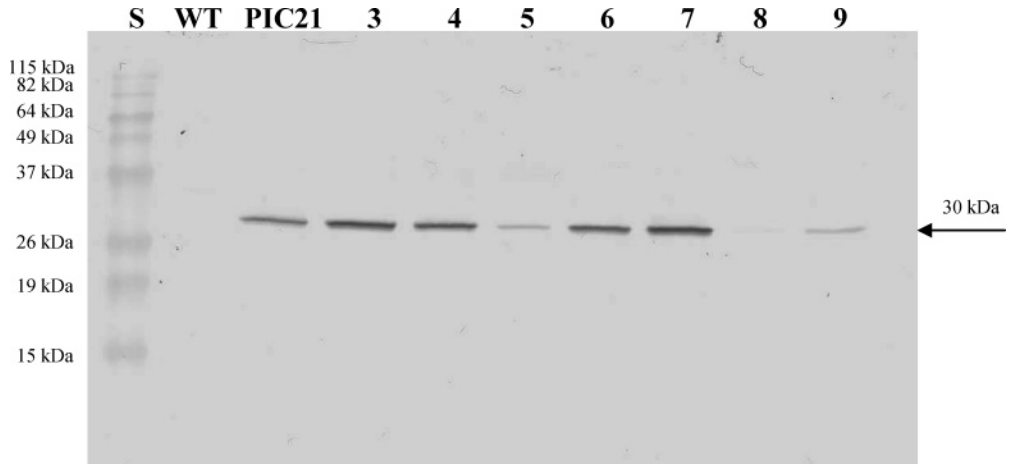


**Figure 3.** Representative immunoblot probed with anti c-MYC antibody to detect anti-picloram scFv bands. Samples are indicated along the top. Migration of molecular weight protein standards is indicated along the left side. The migration and size of the immunopositive scFv protein bands are indicated on the right side. S, Bench Mark (Invitrogen) prestained protein ladder; WT, wild-type tobacco negative control; PIC21, internal positive control; 3–9, seven independent $T_0$ samples from this study.

Approximately 200 $T_1$ seeds from self-pollinated $T_0$ plants were tested for kanamycin resistance or sensitivity by distributing them on MS agar plates containing kanamycin (50 mg/L). Resistant (green) and sensitive (white) plants were counted after 4 weeks, and the segregation ratio for each $T_1$ seedling set was compared with three different null hypotheses corresponding to one, two, and three T-DNA loci (3:1, 15:1, and 63:1) according to Mendelian segregation of a dominant trait. Chi-square ($\chi^2$; *36*, *37*) statistics were calculated, using the 0.05 probability value as the cutoff for acceptance of a null hypothesis (Supporting Information Tables 2−5). In the tCUP3/O group there were 17 plants with one T-DNA locus, 7 with two, and 2 with three; 1 did not have progeny resistant to kanamycin, and another died before seeds were collected. In the tCUP3/M group, there were 10 plants with one T-DNA locus, 14 with two, and 3 with three; 1 died before seeds were collected. In the 35S/O group, there were 18 plants with one T-DNA locus, 4 with two, and 1 with three; 2 had kanamycin resistant progeny with frequencies that did not fit a Mendelian segregation model; 2 died before seeds were collected. In the 35S/M group, there were 15 plants with one T-DNA locus, 5 with two, and 2 with three; 3 died before seeds were collected.

**Quantification of Total and Active Anti-Picloram scFv.** All transgenic $T_0$ plants were analyzed for expression of total and active anti-picloram scFv utilizing quantitative immunoblotting and ELISA technologies, respectively (Supporting Information Tables 2−5). **Figure 3** shows a representative immunoblot, where the total scFv produced by seven $T_0$ plants was compared to that of the PIC21 control plant. **Figure 4** presents the average total scFv, with standard errors, for only those plants determined to possess single T-DNA loci for each of the four T-DNA expression constructs. In this comparison, the intensity of the immunopositive band of the PIC21 internal control (PIC21), which was present on all immunoblots, was set at 1.00, whereas the band intensity for each $T_0$ plant was adjusted accordingly. The average relative total scFv among single T-DNA locus plants possessing the tCUP3/O, tCUP3/M, 35S/O, and 35S/M constructs was $0.97 \pm 0.08$, $0.85 \pm 0.14$, $1.20 \pm 0.12$, and $1.60 \pm 0.17$, respectively. Duncan's test (*38*) at the 95% confidence level showed that the 35S/M construct produced significantly more total scFv than the other three T-DNA constructs in single-locus $T_0$ plants.

**Figure 4** also presents the average active scFv, with standard errors, for single T-DNA locus plants for the four groups. In this comparison, ELISA absorbances were adjusted to that of the PIC21 internal control, which was set at 1.00 as above. The average relative scFv activities for plants possessing the tCUP3/O, tCUP3/M, 35S/O, and 35S/M constructs were $1.78 \pm 0.36$, $1.44 \pm 0.67$, $2.28 \pm 0.36$, and $2.65 \pm 0.48$, respectively. There were no significant differences among these means using Duncan's test (*38*) at the 95% confidence level.

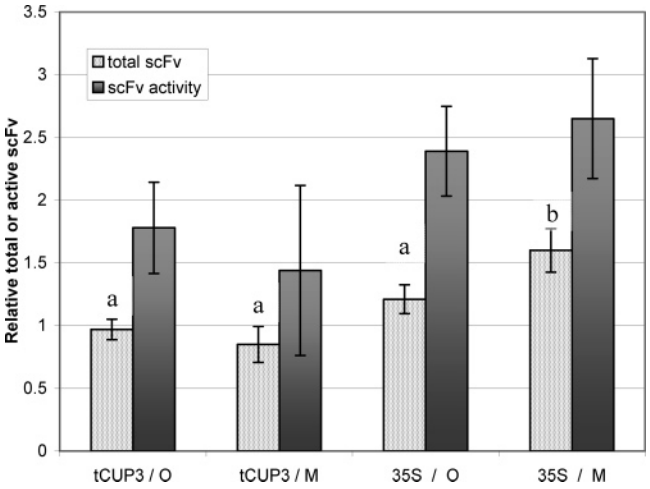


**Figure 4.** Average total and active anti-picloram scFv, ± standard errors, for four groups of transgenic plants relative to a common internal control (PIC21). Values for total and active scFv of the PIC21 control were arbitrarily set to 1. Relative total or active scFv is shown on the left. The four groups of plants are indicated on the bottom, with the promoter indicated before the slash and the coding sequence after the slash. Only transgenic plants possessing single T-DNA loci, as determined by segregation of kanamycin resistance in the $T_1$ generation, were included in this analysis. The inset box shows the key for total and active protein. Letters a and b indicate groups that are statistically different for total scFv by Duncan's test at the 95% level of confidence; there were no statistical differences for scFv activity among the four groups.

**Picloram Dose−Response Bioassay.** A picloram dose−response bioassay was used to determine the resistance levels among $T_1$ seedlings from four $T_0$ plants that were among the best expressers of active scFv (i.e., tCUP3/M/#09, 35S/M/#02, 35S/M/#3, and 35S/O/#43; Supporting Information Tables 2−5). Seedlings were grown on MS agar media containing picloram at seven different concentrations (i.e., $1 \times 10^{-7}$, $5 \times$

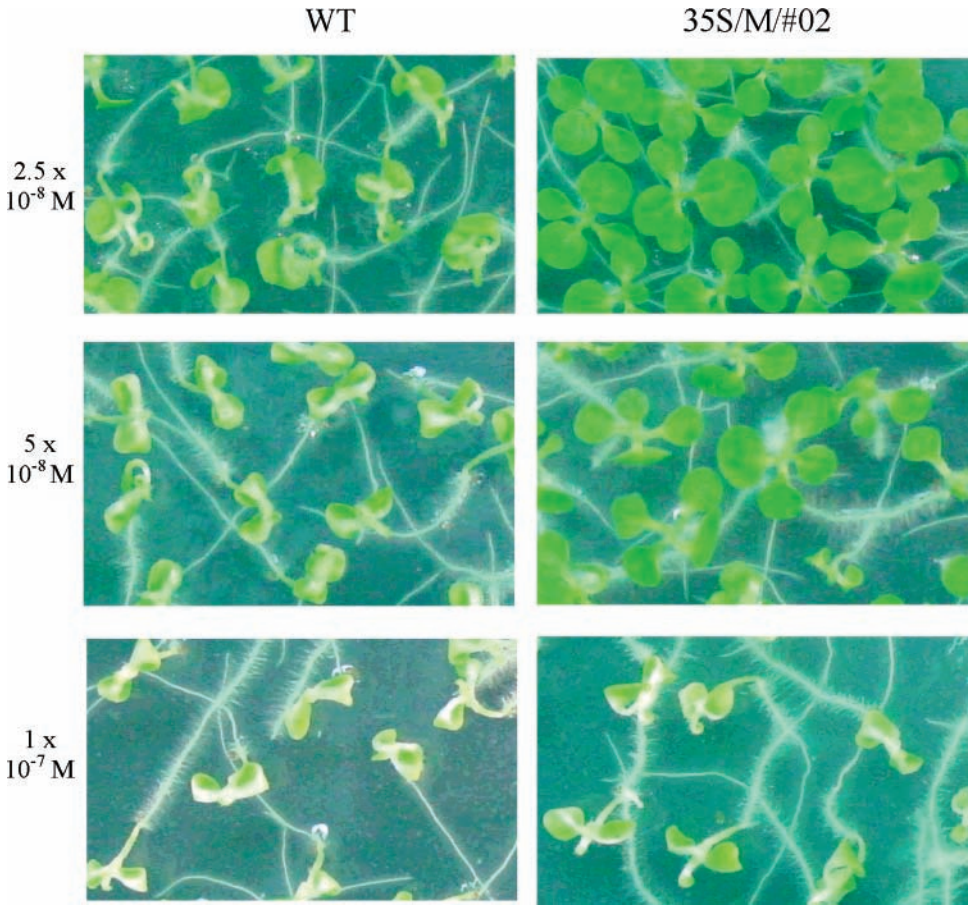


**Figure 5.** Picloram dose–response bioassay: representative photographs of wild-type (WT; left column) and $T_1$ seedlings of primary transgenic plant 35S/M/#02 (right column) treated with $2.5 \times 10^{-8}$ M (top row), $5 \times 10^{-8}$ M (middle row), and $1 \times 10^{-7}$ M (bottom row) picloram on agar media (see Materials and Methods).

$10^{-8}$, $2.5 \times 10^{-8}$, $1 \times 10^{-8}$, $5 \times 10^{-9}$, $2.5 \times 10^{-9}$, and $1 \times 10^{-9}$ M). It was found that $T_1$ progeny of $T_0$ plant 35S/M/#02 had the highest level of resistance to picloram. **Figure 5** shows this group of seedlings to be resistant to doses up to and including $5 \times 10^{-8}$ M when compared with the wild-type control (i.e., 81v9). $T_1$ progeny of $T_0$ plants 35S/M/#30 and 35S/O/#43 were resistant to $2.5 \times 10^{-8}$ M picloram (not shown). $T_1$ progeny of $T_0$ plant tCUP3/M#09 were not resistant to picloram (not shown) due to transgene silencing (*39*) and, like the wild-type control seedlings (**Figure 5**), showed typical auxinic herbicide symptoms, such as epinasty, hypertrophy, and obvious growth reduction of true leaves at concentrations $>1 \times 10^{-9}$ M. These findings confirm the in vitro biochemical assays, where the $T_0$ plant 35S/M/#02 was among the best $T_0$ plants expressing active anti-picloram scFv (Supporting Information Tables 2−5).

## DISCUSSION

The goal of this project was to increase the amount of recombinant anti-picloram scFv produced by transgenic tobacco plants in order to improve our previous model for conferring herbicide resistance to plants by immunomodulation (*5*). This goal was achieved by utilizing two different promoters and coding sequences in four combinations to produce plants that all directed synthesis of the same anti-picloram scFv. Offspring from the best expressers of scFv among these were subjected to a picloram resistance dose−response bioassay to test for levels of resistance to picloram.

Three variables that affect scFv transgene expression levels are the number of T-DNA loci, the number of T-DNA insertions, and the position of an insertion within the host plant's genome. To reduce these effects, at least 25 $T_0$ plants per each of the four T-DNA constructs were produced and subsequently analyzed for numbers of T-DNA loci. Only those $T_0$ plants that possessed single T-DNA loci were used in our analysis of T-DNA construct performance (**Figure 4**; also see Supporting Information Tables 2−5), where it was assumed that each of the four groups possessed similar average numbers of transgene insertions. It was also assumed that at least 10 plants per group would provide sufficient data to overcome variable expression within a group due to position effects resulting from the random integration of T-DNAs within host genomes. We felt that these assumptions would lead to minimal variation within each group as only one tobacco host (81v9) and one *Agrobacterium* strain (At542) was used for all transformations, which were performed during the same period of time.

There were no significant differences in average anti-picloram scFv activity among the four groups of transgenic plants, although a trend in **Figure 4** suggests that the double-enhancer version of the CaMV 35S promoter may have produced more active scFv than the tCUP3 promoter did. Indeed, transgenic plants possessing the 35S promoter consistently produced higher amounts of active scFv. For example, the tCUP3/O, tCUP3/M, 35S/O, and 35S/M groups contained 10, 2, 14, and 11 plants, respectively, with single T-DNA loci that expressed anti-picloram scFv with activities >3 times that of the internal control (PIC21).

In contrast with the results for overall activity, statistical analyses determined a significant difference for the total anti-picloram scFv produced by single T-DNA locus plants, as plants with the 35S/M T-DNA construct produced the most total scFv. **Figure 4** shows that single-locus 35S/M plants produced an average of 1.6 times the total scFv of the internal control (PIC21), which was significantly better than the other three groups at the 0.05 level of confidence. The bars in **Figure 4** indicating the total scFv for each group also support the trend that the 35S promoter may have outperformed the tCUP3 promoter, as both 35S groups produced more total scFv.

To understand more completely how to improve transgene expression levels, more synthetic constructs should be studied to determine whether coding sequence modification has a significant and consistent effect on active scFv expression levels. Accumulation and stability of scFv transcripts, as well as amounts of total and active scFv protein, should also be studied; however, because the goal of this project was aimed at increasing active scFv expression, RNA levels were not studied. A better experimental design would incorporate more sequence modifications, with each one intended to affect only one of transcription, translation, or RNA stability, thereby allowing for simpler analyses of the effects coding sequence modification may have on these components of transgene expression. Parameters such as the order of $V_H$ and $V_L$ sequences within a scFv (*40*), and other linkers, should also be investigated.

The offspring of four $T_0$ plants that were among those that expressed the highest active anti-picloram scFv, that is, tCUP3/M/#09, 35S/O/#43, 35S/M/#02, and 35S/M/#30, were tested in a picloram dose−response bioassay. The seedlings among three of these sets of $T_1$ plants were resistant to picloram at a concentration of at least $2.5 \times 10^{-8}$ M (**Figure 5**; not shown for 35S/O/#43 and 35S/M/#30). Furthermore, $T_1$ seedlings of 35S/M/#02 were resistant to a constant dose of $5 \times 10^{-8}$ M picloram (**Figure 5**). Although it can be seen in **Figure 5** that some of the $T_1$ seedlings of 35S/M/#02 showed sensitivity to the herbicide at this concentration, their frequency was consistent with that of null T-DNA segregants. That is, $T_0$ plant 35S/M/#02 possesses two T-DNA loci; fewer than 10% of its $T_1$ offspring were sensitive to kanamycin, which is consistent with a 15:1 phenotypic ratio of $T\text{-}DNA^{+}$:$T\text{-}DNA^{null}$. The resistance achieved by the 35S/M/#02 $T_1$ seedlings was greater, by ~3-fold, than the resistance of the best anti-picloram scFv expressing plants reported in our first publication (*5*), as those plants showed resistance to picloram over a range of 5−20 nM on a similar agar medium bioassay. Because those plants had a 2−3-fold greater resistance than wild-type plants (*5*), this improvement brings the resistance achieved by this model to ~10 times that of wild-type tobacco. However, there is still a need for at least a 20−100-fold resistance improvement above this resistance level to attempt a practical application of this technology.

Immunomodulation is based on one-to-one binding of antibody to antigen. Attempts to improve immunomodulation-mediated herbicide resistance have included targeting the protein to subcellular compartments (*6*) and utilization of better promoter and/or improved coding sequences (this work). A method for improvement that has not yet been attempted is to increase the affinity of the antibody for the specific antigen. It should be possible to improve the specific affinity of the anti-picloram scFv (*41*), which has an equilibrium dissociation constant ($K_d$) of 600 nM (*5*), making this the next logical step toward improving this immunomodulation model.

The herbicide industry has produced hundreds of herbicide molecules for use in the control of weed and pest species (*7*), and immunomodulation-mediated resistances to these could allow for the production of plants for use in bioremediation programs, as novel herbicide-resistant crops and as biological reagents for use in the study of herbicide physiology in planta. Although the development of plants as crops with resistance against herbicides would be desirable, further increases in the resistance levels imparted by the expression of herbicide-specific recombinant antibodies are required before immunomodulation can be employed as a practical resistance mechanism. Current research in our laboratory is underway to derive homozygous lines from several of the transgenic plants presented in this work, which will be used to investigate the mechanism of immunomodulation-based herbicide resistance.

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**Supporting Information Available:** Oligonucleotides utilized for the synthesis of modified anti-picloram scFv coding sequence, primers utilized for its amplication and cloning, and scFv activities in transgenic tobacco plants. This material is available free of charge via the Internet at http://pubs.acs.org.

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