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Picloram Resistance in Transgenic Tobacco Expressing an Anti-Picloram scFv Antibody Is Due to Reduced Translocation

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Picloram resistance exhibited by transgenic tobacco (*Nicotiana tabacum*) plants expressing an anti-picloram single-chain variable fragment (scFv) antibody was investigated through the study of homozygous lines expressing the antibody. Dose−response bioassays, using foliar application of picloram, showed that these homozygous transgenic plants were resistant to at least 5 g of ai ha^{-1} picloram and grew normally to produce seed, whereas wild-type plants did not survive. Although these lines had improved resistance compared with those previously reported, significant improvements are still required to achieve field-level resistance. Uptake and translocation studies demonstrated that [^{14}C]picloram translocation from treated leaves to the apical meristem was reduced in transgenic versus wild-type plants. The presence of [^{14}C]picloram, visualized by autoradiography and quantified by liquid scintillation spectrometry, demonstrated the distribution of more picloram in the treated leaf and less in the apical meristem of transgenic plants when compared to wild-type plants. No differences between transgenic and wild-type plants were found in the distribution of [^{14}C]clopyralid, a herbicide with structural similarity to picloram as well as the same mechanism of action. No differences were found in the metabolism of [^{14}C]picloram. Taken together, these results suggest that reduced translocation to the site of action is a major mechanism responsible for picloram resistance in tobacco plants expressing this anti-picloram antibody.

KEYWORDS: Herbicide resistance mechanism; immunomodulation; picloram; single-chain variable fragment antibody; herbicide translocation; auxin

INTRODUCTION

Herbicide resistance (HR) is the trait most frequently introduced into genetically modified (GM) crops, as approximately 80% of the global GM crops planted in 2004 were HR varieties (*1*, *2*). At least six mechanisms can be used by plants to achieve HR: (i) inhibition of herbicide uptake, due to impermeability or active pumping of herbicide from cells; (ii) sequestration; (iii) reduced translocation to the site of action; (iv) production of an additional or hyperproduction of a native herbicide-binding target; (v) structural alteration of the binding target; and (vi) herbicide detoxification or degradation. Currently, glyphosate and glufosinate are the two broad-spectrum herbicides widely used for weed control in GM crops (*3*). Glyphosate blocks 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase and hence interferes with aromatic amino acid biosynthesis (*4*, *5*). GM crop resistance to glyphosate is most often due to expression of a mutated maize EPSP synthase (*6*) or an *Ochrobactrum anthropi* glyphosate oxidase (GOX), which detoxifies the herbicide, in combination with a glyphosate-insensitive EPSP synthase (*7*, *8*). Glufosinate is a competitive inhibitor of glutamine synthetase (GS) and hence interferes with glutamine synthesis (*9*). GM crop resistance to glufosinate is conferred by transgenic expression of *Streptomyces hygroscopicus* phosphinothricin acetyltransferase (PAT; *10*), which inactivates the herbicide (*11*).

The expression of antibodies (Abs) or Ab fragments in plants may be developed as a novel strategy toward HR by buffering herbicide effects in planta through specific binding and sequestration (*12*). Abs are glycoproteins produced by immune systems of vertebrates in response to pathogenic organisms and foreign substances including proteins, polysaccharides, nucleic acids, and in some cases small molecules such as organic contaminants and herbicides. Antiherbicide Abs have traditionally been used in the field of weed science for in vitro detection of herbicides in soil, water, plants, urine, and blood using immunoassays (*13*, *14*).

Hiatt et al. (*15*) were the first to express an Ab in transgenic plants. Since that demonstration, much of the Ab research performed in plants has focused on large-scale production of therapeutic Abs (*16*−*18*). Some research on transgenic Ab expression for modulation of plant physiology (*19*) and control of abscisic acid in planta (*20*, *21*) has been reported. Eto et al.

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(*22*) demonstrated limited resistance to the herbicide chlorpropham, which inhibits mitosis by interfering with microtubule organization, by expressing an anti-chlorpropham single-chain variable fragment (scFv) Ab in *Arabidopsis*. An Ab against the auxinic herbicide picloram (4-amino-3, 5, 6-trichloro-2-pyridinecarboxylic acid) has been characterized (*23*−*25*) and expressed in transgenic tobacco (*Nicotiana tabacum* L.) as a scFv, conferring picloram-specific HR (*26*). Picloram resistance was subsequently improved in transgenic tobacco by increasing the expression of the anti-picloram scFv through the introduction of a stronger promoter and changing the coding sequence to incorporate preferred tobacco codons (*27*).

In this study, homozygous lines were developed from single T-DNA locus primary transgenic (T_0) plants produced by Olea-Popelka et al. (*27*) that expressed relatively high levels of anti-picloram scFv. These lines were used in whole-plant dose−response bioassays involving a field-spray simulation to quantify the level of picloram-specific HR conferred by the scFv. Picloram uptake, translocation, and metabolism were analyzed in these lines to investigate the mechanism involved in immunomodulation-mediated HR. The results of these investigations suggest that these plants are resistant to picloram at >5 g of ai ha^{-1} and that the HR mechanism involves specific Ab-mediated herbicide sequestration and reduced translocation in planta.

MATERIALS AND METHODS

Plant Material. Two primary transgenic (T_0) tobacco plants characterized previously (*27*) and their offspring (T_1 and T_2 progeny) were used in this study. Both T_0 plants expressed an anti-picloram scFv coding sequence from the cauliflower mosaic virus 35 S promoter; the T-DNA of one contained the original murine scFv coding sequence (35S/Original/#43, or T_0-O43) and the other, an optimized coding sequence (35S/Modified/#30, or T_0-M30). Offspring will be referred to as T_x-*Y*-*z*, where *x* represents the generation, *Y* represents either M30 or O43, and *z* represents the plant number from that generation. Homozygous plants in the T_2 generation were generally not given plant numbers, as these were considered to be identical to their siblings for the purposes of these experiments. Nontransgenic (wild-type; WT) *N. tabacum* cv. 81v9 (*28*) plants were used as negative control.

Kanamycin and Picloram Resistance Bioassays. Kanamycin resistance, picloram resistance, and picloram dose−response bioassays were performed on solid media according to the method of Olea-Popelka et al. (*27*). Dose−response bioassays were performed on plates containing media with 1−100 nM picloram.

A field-spray simulation dose−response bioassay involved picloram application on plants initially germinated in ProMix BX (Plant Products, Brampton, ON, Canada) in a growth room with a 16-h daylight cycle and diurnal temperatures of 23/18 °C for day/night. Six plants per replicate were sprayed with aqueous solutions of 0, 0.5, 1.0, 5.0, and 10.0 g of ai ha^{-1} of picloram in 10% ethanol and 0.5% Tween 20 (Sigma Chemical Co., St. Louis, MO) at the five-leaf developmental stage using an automatic hood sprayer (RC-5000-100EP, Mandel Scientific) equipped with a flat fan nozzle (SS80015E Spraying Systems). The spray volume was equivalent to 110 L ha^{-1} of spray solution delivered at 240 kPa. Photographs were taken and plant heights measured 21 days after treatment (DAT). Afterward, five replicate plants from each treatment were harvested and dried in an oven at 60 °C for 72 h prior to the determination of shoot dry weight 21 DAT. The remaining replicate for each treatment was grown until carpel formation and examined for indications of abnormal development.

Picloram Uptake and Translocation Study. Plants were germinated and grown in ProMix BX for 2 weeks and then transplanted to 8-cm square pots containing crushed particulate expanded baked clay (Turface MVP, Plant Products, Brampton, ON, Canada). Plants were watered every second day with Hoagland's solution (*29*). Once plants reached the four-leaf stage of development, the cotelydons, as well as the first, second, and third leaves, were removed. The fourth leaf of each plant was treated with 50000 dpm (840 Bq) of either radiolabeled [^{14}C]-picloram (2,6-^{14}C; 910.2 MBq $mmol^{-1}$; Dow Chemical Co., Midland, MI) or radiolabeled [^{14}C]clopyralid (3,6-dichloro-2-pyridinecarboxylic acid; 2,6-^{14}C; 351.87 MBq $mmol^{-1}$; Dow Chemical Co.) in 10 μL of water containing 10% ethanol and 0.5% Tween 20. The solution was adjusted to pH 2 using HCl, and herbicide was applied as described by Ramsey et al. (*30*): a Hamilton syringe (50 μL) was stabilized so that the end of the needle was directly in front of an air stream and the release of treatment solution into the air stream produced a fine spray. A template with a rectangular hole measuring 800 mm^2 (20 by 40 mm) was placed over a central area of the treated leaf prior to spraying to ensure each plant had the same treated area. Plants were harvested 6, 12, 24, 48, and 72 h after treatment (HAT). The treated leaves were removed, rinsed with 10% ethanol and 0.5% Tween 20 solution, and sectioned into treated and untreated portions by cutting across the leaf 1 cm below the treated zone. Thus, the untreated portion of the leaf was considered to be tissue >1 cm below the treatment zone, including the petiole. Tissue above the dissection line was considered to be the treated area. The remainder of each plant was sectioned into meristem and root tissue. All harvested plant tissues were wrapped in Kimwipe-sEX-L and dried at 60 °C for 48 h. Plant tissues were oxidized in a biological oxidizer (OX 500, R. J. Harvey Instrument, Hillsdale, NJ), where $^{14}CO_2$ was trapped in scintillation cocktail (R. J. Harvey Instrument) and measured by liquid scintillation spectrometry (LSS; Beckman LS6000SC, Mississauga, ON, Canada). The efficiency of combustion and recovery was >90%. Leaf rinses were collected, and ^{14}C content was quantified by LSS. Translocation data are expressed as a percentage of ^{14}C recovered from the plant.

Autoradiography. Plants were grown, pruned, and treated with radiolabeled [^{14}C]picloram or [^{14}C]clopyralid as described for the uptake and translocation study. Plants were harvested 24 and 72 HAT, and unabsorbed picloram and clopyralid were washed from leaf surfaces with 10% ethanol and 0.5% Tween 20 solution. Plants were dissected into treated leaf, meristem, and roots and then placed between sheets of paper and cardboard to dry at 60 °C for 48 h. A general-purpose storage phosphor screen (Amersham Biosciences, Piscataway, NJ) was exposed to the plant sections for 9 days at room temperature. A Typhoon 9400 Variable Mode Imager (Amersham Biosciences) was used to read the storage phosphor screen using the 200 μm setting. Color contrast of the images was produced using ImageQuant 5.2 (Molecular Dynamics, Piscataway, NJ).

Metabolism Study. Wild-type and T_2-O43 plants were grown under the same conditions and pruned as described for the uptake and translocation study. A treatment of radiolabeled [^{14}C]picloram (50000 dpm; 840 Bq) was applied as 10 1-μL droplets to the surface of the fourth leaf of each plant. Plants were harvested 72 HAT, and the unabsorbed picloram was washed off the leaf surface using 10% ethanol and 0.5% Tween 20 solution. Plants were processed and analyzed according to methods developed by Hall and Vanden Born (*31*). Plant material was wrapped in aluminum foil and stored in the freezer at −20 °C. Each plant was removed from the freezer, cut into small pieces, and homogenized in 90% acetone (10 mL/g of fresh weight) using a 20 mm diameter Polytron probe homogenizer (Kinematica, GmbH, Switzerland). The homogenate was filtered and the remaining residue washed with acetone and dried. The residue was combusted to determine the amount of ^{14}C present. The filtrate was concentrated by evaporating the acetone at 60 °C under a stream of nitrogen gas. The remaining aqueous extract was frozen at −20 °C. A 25 μL aliquot of each aqueous sample was applied to aluminum-backed silica gel thin-layer plates (Whatman, Florham Park, NJ), and chromatography was performed with CH_2Cl_2/methanol/acetone/acetic acid (8:1:1:1) until the solvent front reached 150 mm. The plates were divided into 10 mm wide lanes, which were subdivided into 15 mm sections and assayed for radioactivity by LSS.

Statistical Analyses. The uptake and translocation data were treated as a split-plot design with the whole-plot being time and the subplot being each plant. First, the data were compared by a simple contrast at each time point. A test of residuals was performed to ensure the data were homogeneous and normally distributed. Nonlinear regression was performed followed by a likelihood ratio test to compare the full model to the reduced model. The type I error rate was set at 0.05. All statistical

Table 1. Picloram Sensitivity Segregations on 25 nM Solid Medium among T_1 Plants from Self-Pollinated 35S/Original/#43 and 35S/Modified/#30 T_0 Plants

		picloram sensitivity[a]				
T_0 progenitor	total T_1 plants classified	resistant	moderate	susceptible	χ^2 [b]	P [b]
35S/Original/#43	116[c]	28	59	29	0.0517	0.9745
35S/Modified/#30	99[c]	22	53	24	0.5758	0.7499

[a] T_1 seeds were surface-sterilized, plated on agar medium containing 25 nM picloram, and classified as resistant, moderately resistant, or susceptible (see Materials and Methods). [b] Chi-square values (χ^2, df = 2) and associated probabilities (P) were the result of testing the goodness of fit to a 1:2:1 (resistant/moderately resistant/susceptible) segregation ratio. The type I error rate (α) was set at 0.05 (*38*). [c] Heterogeneity tests indicated that the data from three replicates of T_1 plants from T_0 progenitor plants 35S/Original/#43 ($\chi^2 = 6.8115$, df = 4, $P = 0.14619$) and 35S/Modified/#30 ($\chi^2 = 2.5712$, df = 4, $P = 0.63193$) could be pooled (*38*).

analyses were performed using SAS V.8 (SAS Institute Inc., Cary, NC). Regression lines plotted in **Figure 4** were drawn using Graphpad Prism.

RESULTS

Development of Homozygous Transgenic Lines. Two of the highest scFv-expressing T_0 plants produced by Olea-Popelka et al. (*27*) showing resistance to picloram on solid medium at 25 nM were used to create homozygous lines for use in this study. Kanamycin resistance segregation assays were performed to reconfirm single T-DNA loci in these T_0 plants (not shown). Picloram dose−response experiments indicated a possible gene dosage effect on solid medium containing 25 nM picloram (not shown); thus, it was hypothesized that the phenotypes of resistant, moderately resistant, and susceptible plants among T_1 seedlings would segregate 1:2:1 for T-DNA genotypes of +//+ : +//− : −//−. Twenty-four days after seeds had been placed on 25 nM picloram, resistant plants showed no auxinic herbicide symptoms, moderately resistant plants showed petiole elongation and leaf cupping, and susceptible plants showed symptoms identical to those of wild-type plants including epinasty and hypertrophy (not shown). **Table 1** presents statistically significant phenotypic segregation ratios of resistant, moderately resistant, and susceptible plants that fit expected genotypic segregation ratios of 1:2:1.

For homozygous line selection, four T_1 plants from T_0-O43 (T_1-O43-1 to T_1-O43-4) and seven T_1 plants from T_0-M30 (T_1-M30-1 to T_1-M30-7) showing no auxinic herbicide symptoms were rescued from 25 nM picloram plates, transplanted to soil, grown to maturity, and self-pollinated. T_2 seeds were harvested, and kanamycin and picloram resistance bioassays were performed as above (not shown). All T_2 plants tested were resistant to kanamycin and picloram, demonstrating that the T_1 progenitors were homozygous at their transgene loci.

All homozygous plant lines were further examined to evaluate the effect of anti-picloram scFv expression for HR. Plantlets from lines T_1-O43-1 through -4 and T_1-M30-1 through -7 showed similar picloram resistance levels: auxinic herbicide symptoms were not observed on 25 nM plates, but symptoms such as petiole elongation and leaf cupping were observed on 50 nM plates (not shown). Because of the homogeneity of resistance observed among these lines, only T_2 seeds from line T_1-O43-1 were used in further experiments; these will be referred to as T_2-O43 seeds or plants.

Quantification of Picloram Resistance in a Field-Spray Simulation. A dose−response bioassay was performed as a field-spray simulation, in which both T_2-O43 and wild-type plants were treated with picloram at 0, 0.5, 1.0, 5.0, and 10.0 g of ai ha^{-1} during the five-leaf developmental stage, using six plants per treatment per genotype. All wild-type plants showed symptoms of picloram injury 7 DAT at all applied doses (not shown). At 21 DAT, wild-type plants treated with the lowest dose, 0.5 g of ai ha^{-1}, showed petiole elongation and leaf cupping, whereas those treated with higher doses showed more severe auxinic herbicide symptoms (**Figure 1A**). Major differences between T_2-O43 and wild-type plants were observed at 5.0 g of ai ha^{-1} 21 DAT: wild-type plants showed height reduction, epinasty, and damage at the apical meristem; transgenic plants showed slight height reduction compared with untreated controls and no damage at the apical meristem (**Figure 1A**). T_2-O43 plants treated with 0.5 and 1.0 g of ai ha^{-1} were indistinguishable from untreated plants, and those treated with 5.0 g of ai ha^{-1} were only slightly smaller 21 DAT (**Figure 1A**).

At 21 DAT, five of the six plants from each of these treatment groups were harvested for shoot dry weight and plant height measurements. These are plotted versus the logarithm of the dose in **Figure 2**, panels **A** and **B**, respectively. Both dose−response curves for T_2-O43 plants had steeper slopes than those of wild-type plants, because these showed no symptoms to picloram below 5 g of ai ha^{-1}. Wild-type plants showed sustained phytotoxicity over a broad range of lower doses. From these plots, the concentrations of picloram that would make a 50% reduction in growth (GR_{50}) for shoot weight and plant height, compared with untreated controls, were determined. GR_{50} values for shoot dry weight were 4.8 and 1.7 g of ai ha^{-1} for T_2-O43 and wild-type plants, respectively (**Figure 2A**), yielding a dry weight index of 2.8 for the GR_{50} of the T_2 plants over that of the wild-type plants (i.e., GR_{50}-T_2-O43/GR_{50}-wild-type). GR_{50} values for plant height were 4.5 and 0.72 g of ai ha^{-1} for T_2-O43 and wild-type plants, respectively (**Figure 2B**), yielding a plant height index of 6.2.

By 32 DAT, the remaining T_2-O43 plant treated with 5.0 g of ai ha^{-1} showed height reduction compared with the untreated T_2-O43 plant, whereas the wild-type plant treated with this dose did not survive (**Figure 1B**). Carpel development was monitored 75 DAT, and all T_2-O43 plants had normal seed development with the exception of the plant treated with 10 g of ai ha^{-1} of picloram, which was lethal. Wild-type plants treated at the lower concentrations of 0.5 and 1.0 g of ai ha^{-1} of picloram had asymmetrical carpel development (not shown), whereas those treated at the two higher concentrations did not survive 32 DAT (**Figure 1B**).

Uptake and Translocation of [^{14}C]Picloram in Transgenic and Wild-Type Plants. Applications of [^{14}C]picloram to single leaves of wild-type and T_2-O43 plants was performed to study the distribution of [^{14}C]picloram in whole plants. Autoradiography showed that more ^{14}C remained in the treated leaf and was transported to the roots of T_2-O43 plants than in wild-type plants 72 HAT, whereas more ^{14}C accumulated in the apical meristem of the wild-type (**Figure 3**, left panels). In contrast, there was no difference between T_2-O43 and wild-type plants in the distribution of [^{14}C]clopyralid (**Figure 3**, right panels), a structural analogue of picloram that does not cross-react with the scFv (*26*).

A similar experiment involving applications of [^{14}C]picloram to single leaves of wild-type and T_2-O43 plants was performed, but the treated and untreated zones of leaves, apical meristems,

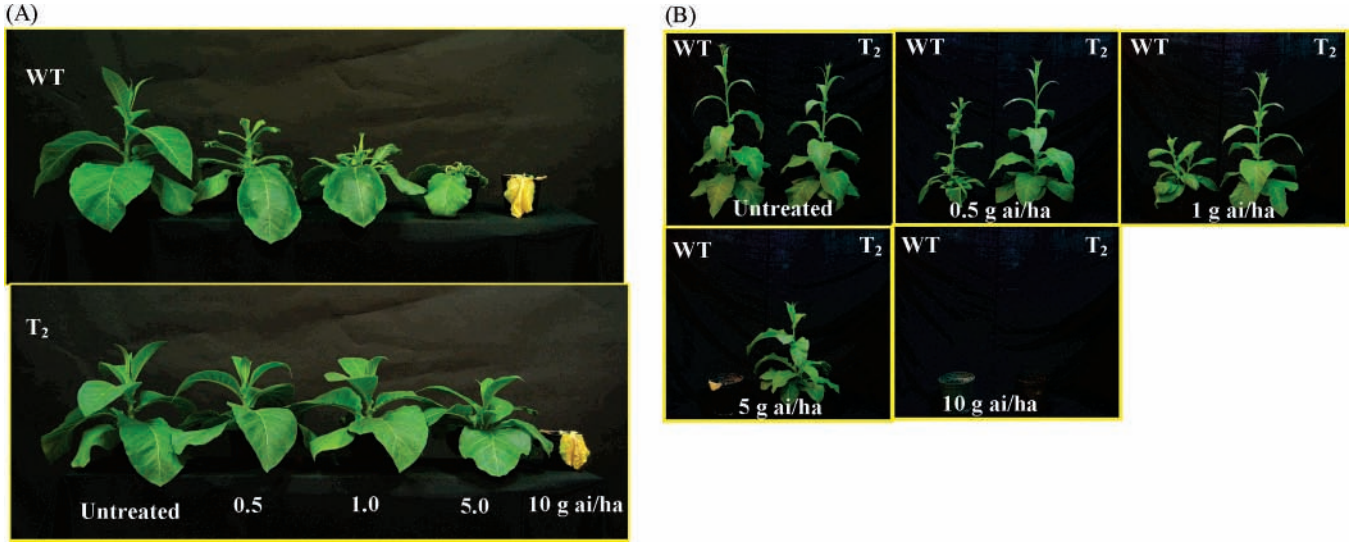

Figure 1. Dose–response bioassay on T_2 progeny of T_1 plant 35S/Original/#43-1 (T_2) and wild-type (WT) with picloram applied at 0, 0.5, 1.0, 5.0, or 10 g of ai/ha: series comparison 21 days after treatment (**A**) and individual comparisons 32 days after treatment (**B**). Six replicate plants of each genotype were treated with each picloram concentration.

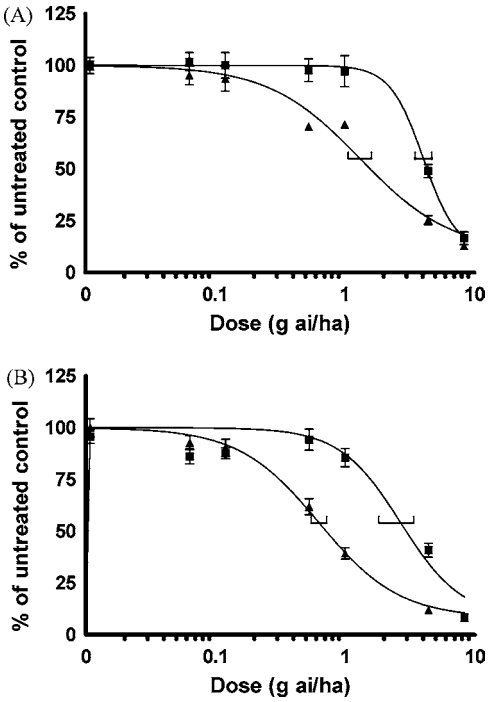

Figure 2. Dose–response curves with standard errors following foliar application of picloram on T_2 progeny of T_1 plant 35S/Original/#43-1 (■) and wild-type (▲). Five plants from each treatment were analyzed 21 days after treatment for shoot dry weight (**A**) and plant height (**B**). Growth reduction by 50% (GR_{50}) values are shown and bracketed by their 95% confidence intervals. Dose–response curves and GR_{50} values were generated using GraphPad Prism 4.0.

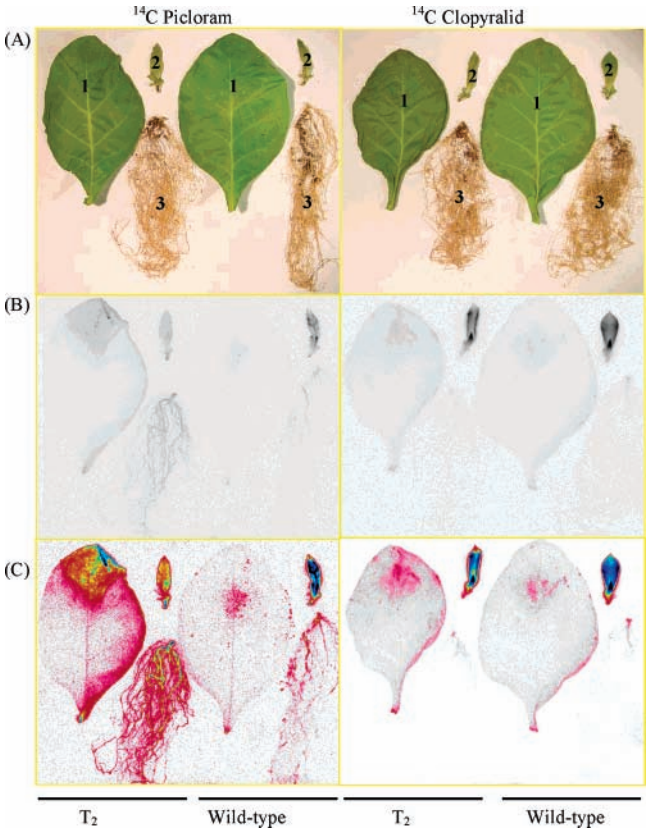

Figure 3. Distribution of ^{14}C in a T_2 progeny plant from T_1 plant 35S/Original/#43-1 (T_2) and wild-type tobacco 72 h after treatment with [^{14}C]-picloram (left panels) and [^{14}C]clopyralid (right panels). Plant parts are labeled in the top photographs (**A**) as treated leaf (1), apical meristem (2), and roots (3). Tissue was exposed to a phosphor storage screen for 9 days, then read by an image reader to give a raw digital image (**B**) and an image manipulated by ImageQuant 5.2 software (Molecular Dynamics; C, violet is most intense; red, least intense).

and roots were dissected, combusted, and assayed by scintillation counting. There were no differences in foliar uptake of [^{14}C]-picloram between wild-type and T_2-O43 plants (not shown). Thus, as absorption of [^{14}C]picloram was the same for both genotypes, any difference in ^{14}C found in the treatment zones was attributed to differences in translocation of ^{14}C out of the treatment zone (**Figure 4A**). At all harvest times, significantly more ^{14}C remained in the treatment zones of T_2-O43 leaves than in those of wild-type leaves. For example, 24 HAT the treatment zones of T_2-O43 leaves had approximately 2.5 times the radioactivity of wild-type treatment zones (**Figure 4A**). The relationship between the quantity of radioactivity found in the treatment zones of T_2-O43 and wild-type leaves was significantly different at all times sampled, as compared by nonlinear regression (not shown).

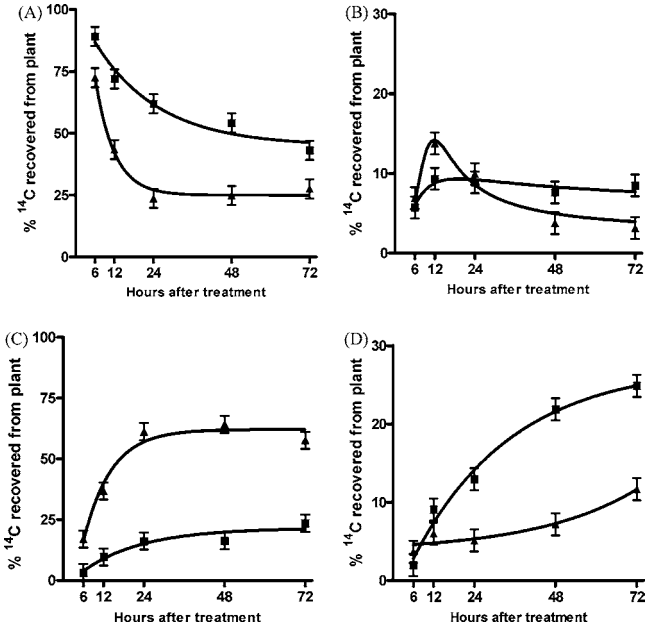

Figure 4. Translocation of [^{14}C]picloram from the treatment zone of single leaves of T_2 progeny of T_1 plant 35S/Original/#43-1 (■) and wild-type (▲) plants. Each treated leaf was sectioned 1 cm below the treatment zone into treatment zone of treated leaf and untreated portion of the treated leaf (including petiole). The quantity of [^{14}C]picloram remaining in the treatment zone of the treated leaf (**A**), the untreated portion of the treated leaf (**B**), at the meristem (**C**), and in the roots (**D**) is expressed as a percentage of total ^{14}C recovered from the treated plant in each panel.

More [^{14}C]picloram was found outside the treatment zones (i.e., at least 1 cm below the treatment zones) of wild-type compared with T_2-O43 12 HAT (**Figure 4B**). However, significantly less ^{14}C was found outside the treatment zones of wild-type plants 48 and 72 HAT. This relationship between quantity of radioactivity outside the treatment zones of both plant types versus time was also significantly different by nonlinear regression (not shown).

The majority of the [^{14}C]picloram that left the treatment zone of the wild-type plants translocated to the apical meristem, where >50% of the recovered ^{14}C was detected 24 HAT (**Figure 4C**). In the T_2-O43 plants, most of the ^{14}C remained in the treated leaf, where approximately 50% remained 72 HAT (**Figure 4A**,**B**). Approximately 25% of the ^{14}C recovered from the wild-type plants remained in the treatment zone by 72 HAT, whereas very little ^{14}C remained outside the treatment zone of these leaves (**Figure 4B**). T_2-O43 plants showed gradual accumulations of ^{14}C in the apical meristem (**Figure 4C**) and in the root (**Figure 4D**), as approximately 25% of the ^{14}C recovered from these plants was detected by 72 HAT at both of these locations. The relationships between quantities of radioactivity that accumulated in both the apical meristems and roots of the two plant types, versus time, were again significantly different by nonlinear regression (not shown).

Metabolism of [^{14}C]Picloram. There were no differences in [^{14}C]picloram metabolism between T_2-O43 and wild-type plants as assayed with a thin-layer chromatographic (TLC) method previously developed by Hall and Vanden Born (*31*). ^{14}C-Labeled compounds from both types of tobacco plant that were identified as peaks on TLC plates had retardation factor (R_f) values similar to that of the [^{14}C]picloram standard (R_f = 0.7; **Table 2**). Rapeseed, which is known to metabolize picloram (*31*, *32*), was used as a control to ensure that metabolites of picloram could be detected using this TLC method. Two ^{14}C peaks found in a rapeseed extract had R_f values similar to those of the picloram standard (R_f = 0.65) and water-soluble metabolites of picloram (R_f = 0.33) (**Table 2**). Extractions from plants were made 72 HAT and recovered >90% of the applied ^{14}C (not shown).

Table 2. Retardation Factor Values of ^{14}C-Labeled Compounds Detected on Thin-Layer Chromatographic Separations of Extracts from Plants 72 h after Treatment with [^{14}C]Picloram

sample	R_f value[a]	% of sample ^{14}C[b]
T_2-O43	0.7	74 ± 16
wild-type	0.7	60 ± 10
rapeseed	0.65	76
	0.33	22
[^{14}C]picloram standard	0.7	78

[a] Retardation factor (R_f value): the ratio between the distance migrated by a metabolite and the distance migrated by the solvent. [b] The amount of ^{14}C identified as a peak in a separation is presented as a percentage of sample applied to the TLC plate from a plant extract. Standard errors of the mean are given for samples run in triplicate. Peak values are <100% due to background radioactivity along the chromatographic separations.

DISCUSSION

Immunomodulation-mediated HR is the result of specific binding between an Ab—or Ab fragment—and its cognate small molecule herbicide in planta, thereby attenuating the physiological effect of the herbicide. The level of specific HR exhibited by a transgenic plant expressing an antiherbicide Ab has been found to be proportional to the amount of Ab that is expressed by the plant (*26*, *27*). This is because the resistance mechanism involved with immunomodulation-mediated HR relies on one-to-one binding between the herbicide molecule and the Ab-binding domain.

To assess the potential for immunomodulation-mediated HR as an effective agricultural technology, resistance must be quantified via field-based dose—response experiments. Because these assays require homogeneous sets of herbicide-resistant plants, we chose to develop homozygous seed lines from T_0 plants that were relatively high expressers of an anti-picloram scFv and known to have single T-DNA loci (*27*). Also, it was anticipated that optimal scFv expression would correlate with homozygosity at the transgene loci (*33*—*35*). Picloram resistance assays performed on solid media containing 25 nM picloram were effective for the selection of homozygous transgenic plants, suggesting that the anti-picloram scFv gene could be developed as a selectable marker for genetic transformations of tobacco or other plant species that are sensitive to picloram.

As all T_2 offspring from 11 homozygous T_1 lines had similar resistances to picloram on solid media, a field-spray simulation dose—response bioassay was performed to investigate the picloram resistance of homozygous T_2 plants from T_1-O43-1. Photographs taken 21 and 32 DAT show that T_2 progeny were resistant to picloram applied at 5 g of ai ha^{-1}, whereas wild-type plants were not (**Figure 1**). These T_2 plants developed flowers that produced normal seeds, whereas the wild-type plants treated with this dose did not survive by 32 DAT. A definitive quantification of resistance should take into account the production of seeds; thus, the resistance of these transgenic plants to picloram was somewhere between 5.0 and 10 g of ai ha^{-1} as T_2 plants treated with 5.0 g of ai ha^{-1} produced seeds and those treated with 10 g of ai ha^{-1} did not survive by 32 DAT.

Almquist et al. (*26*) performed a simpler field-spray simulation on T_1 plants that expressed the same anti-picloram scFv

and reported resistance to 0.5 g of ai ha^{-1} picloram, which was calculated to be 2.2 times more resistant than wild-type plants using affinity−absorption theory (*36*). The homozygous T_2 plants shown in **Figure 1** had at least 10 times the picloram resistance as the T_1 plants of Almquist et al. (*26*); therefore, we estimate the T_2 plants of this study were at least 20-fold more resistant to picloram than wild-type plants. This is a slight improvement in resistance to picloram compared with previous results (*26*, *27*), yet the resistance of these plants is still at least 1 order of magnitude less than the 35−50 g of ai ha^{-1} that can be applied as an effective field dose for this herbicide (*37*). However, because industrial or agricultural applications of picloram can be much higher, improvements in antibody-mediated HR must be achieved before practical application of this technology is possible.

Picloram is a phloem-mobile herbicide that can rapidly translocate out of a treated leaf and accumulate in the apical meristem, where it manifests auxinic herbicide symptoms (*26*, *27*, *31*, *37*). As expected, wild-type plants showed rapid translocation of [^{14}C]picloram out of treated leaves and accumulation in the apical meristem (**Figure 4A**,**C**). The flow of [^{14}C]picloram out of the treatment zone of wild-type plants stopped by 24 HAT (**Figure 4A**), and a change in flux out of the treated leaf of these plants can be seen by an inversion in the curve showing the amount of [^{14}C]picloram outside the treatment zone at about 12 HAT (**Figure 4B**). Translocation of [^{14}C]picloram out of the treated leaf of T_2-O43 plants was slower than that in wild-type plants (**Figure 4A**,**B**), due to sequestration of herbicide in the treated leaf by antiherbicide scFv engineered for retention in the endoplasmic reticulum of cells. Approximately twice the [^{14}C]picloram remained in the treated leaf of T_2-O43 plants compared with wild-type plants, resulting in the accumulation of less [^{14}C]picloram at the apical meristem of T_2-O43 plants (**Figure 4C**). These data suggest that immunomodulation-mediated HR is due to reduced translocation to the meristem. It is possible that antibody-mediated herbicide sequestration also occurs in the meristem, thereby buffering the effects of auxinic herbicide in that sensitive tissue.

Autoradiography of [^{14}C]clopyralid-treated plants (**Figure 3**) showed that this herbicide translocated mainly to the apical meristem of both wild-type and T_2-O43 plants. There was no appreciable sequestration of this herbicide in treated leaves because the anti-picloram scFv does not bind to it (*26*). Very little [^{14}C]clopyralid translocated to the roots, whereas more [^{14}C]picloram was seen in the roots of wild-type plants. Why the roots of T_2-O43 plants accumulated approximately twice the [^{14}C]picloram seen in the roots of wild-type plants by 72 HAT (**Figure 4D**) has not been determined; however, it may be due to antibody-mediated sequestration of the herbicide, thus prevending exudation by roots.

This study provides insight on the mechanism of immunomodulation-mediated HR, as reduced translocation and specific sequestration of herbicide protects the apical meristem from developing auxinic herbicide symptoms. Homozygous lines expressing anti-picloram scFv were developed and shown to have specific HR at >5.0 g of ai ha^{-1} in a field-spray simulation; however, these have not been studied in a true field test. The major limitation of this technology is due to the obligatory one-to-one binding between Ab and herbicide; thus, practical application of this HR model will require even greater expression of the anti-picloram scFv in planta and relatively low levels of herbicide application. Results of our translocation experiments suggest that high Ab expression in the leaf and possibly in the root might provide even greater protection. If expression levels of anti-herbicide Ab sufficient for practical HR cannot be achieved, development of an anti-picloram Ab with a stronger binding affinity, as was suggested in Almquist et al. (*26*), or with a catalytic (i.e., catabolic) property may be required.

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