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Absorption and translocation of [^{14}C]2,4-dichlorophenoxyacetic acid in herbicide-tolerant chromosome substitution lines of *Gossypium hirsutum* L.

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Abstract: Upland cotton is sensitive to 2,4-dichlorophenoxyacetic acid (2,4-D), and the identification of potentially 2,4-D tolerant cotton chromosome substitution (CS) lines and understanding tolerance mechanisms provide a significant step into the development and genetic improvement of upland cotton to reduce yield loss caused by 2,4-D herbicide effects including the drifts. Experiments were conducted to understand the possible mechanism of herbicide tolerance in CS-T04-15, CS-T07, and CS-B15sh, 2,4-D herbicide-tolerant cotton CS lines compared with TM-1, the 2,4-D herbicide susceptible recurrent parent of the CS line as control, using [^{14}C]2,4-D. Percent absorption rate and translocation patterns of the ^{14}C-labeled herbicide application at 5.17 kBq at 6 to 48 hours after treatment (HAT) were determined. The tolerant cotton CS lines showed 15-19% [^{14}C]2,4-D uptake while TM-1 exhibited a reduced uptake of only 1.4% [^{14}C]2,4-D at 24 HAT. Distribution of the absorbed [^{14}C]2,4-D showed that 2-5% was translocated outside the treated leaf. In TM-1, 77% of the herbicide was translocated above and below the treated leaf, contrasting with the reduced translocation of ^{14}C-labeled herbicide observed in the tolerant CS lines. Interestingly, CS-T04-15 showed a restricted movement of ^{14}C below the treated leaf at 6 to 48 HAT, suggesting a novel mechanism of herbicide tolerance. This finding is the first report on upland cotton demonstrating a complex differential uptake and translocation associated with herbicide tolerance for [^{14}C]2,4-D in cotton CS lines.

Keywords: 2,4-D; upland cotton; chromosome substitution lines; herbicide tolerance; 2,4-D absorption and translocation

1. Introduction

Herbicides used in production agriculture are typically synthetic chemicals used to control the growth of undesired plants. In cotton, herbicides are the most widely used weed management tool because of their speed, flexibility, and low cost. These compounds

are grouped into families based on a specific mode of action, including growth regulation, cell membrane disruption, and inhibitions of amino acid synthesis, lipid synthesis, photosynthesis, pigment formation, and seedling growth [1]. Since most herbicides are small molecules designed to inhibit specific target sites critical to plant biochemical pathways and physiological processes, understanding the mode of action of herbicide chemistries and genetics are essential for developing appropriate weed management control in agricultural production systems [2].

The development and discovery of 2,4-dichlorophenoxyacetic acid (2,4-D) initiated the revolution of chemical weed control in the 1940s [3]. The toxic properties of this growth-promoting substance in plants and its potential use as a chemical warfare agent during World War II prompted the development of 2,4-D as an herbicide [4]. 2,4-D is a synthetic auxin that contains phenoxyalkanoic acid as a major chemical group and mimics the natural auxin in plants known as indole-3-acetic acid (IAA) [5]. Specifically, auxin herbicides like 2,4-D mimic the overdose effects of IAA, producing the herbicide syndrome mediated by the interaction of this compound with ethylene and abscisic acid (ABA) biosynthesis [6,7]. This herbicide syndrome is characterized by the complex symptomology observed in sensitive crops and weeds when 2,4-D is applied, including leaf epinasty, tissue swelling, stem curling, tissue necrosis, senescence, and plant death [8,9].

Herbicide tolerance in crops grown at a commercial scale is primarily based on transgenic cultivars expressing specific genes for enzymes or proteins that block the action or degrade the active ingredient of the herbicide. The most important example of this is the glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) transgene from a soil bacterium that confers resistance to glyphosate in the widely grown transgenic Roundup Ready™ soybean and cotton cultivars [10,11]. Tolerance to auxin herbicides in crop cultivars and weed populations has also been reported [12-17]. Understanding the tolerance mechanism to herbicides is central to identifying and developing appropriate weed management control measures based on response to specific modes of action. In general, herbicides traverse the cell wall, plasma membrane, and different organelles of the plant cell to reach their particular site of action and induce toxicity in plants [18]. As early as the 1950s, experiments using carbon-14 [^{14}C] labeled herbicides, including 2,4-D, were conducted to investigate the translocation, distribution, and metabolism of these compounds in plants [19-21]. For example, when radiolabeled 2,4-D was spotted on the bean leaf, movement of the compound was shown to be higher when spots were on the midrib compared to spots on the edge or tip of the leaf [22]. Translocation of [^{14}C]2,4-D was shown to be slower in monocots (i.e., barley, oats, corn) compared to dicots species [23-25]. Transgenic corn expressing aryloxyalkanoate dioxygenase-1 (AAD-1) exhibited reduced 2,4-D uptake that provided enhanced resistance to the herbicide [26]. Interestingly, translocation patterns of radiolabeled 2,4-D were the same in both herbicide-resistant and susceptible (non-transgenic) corn [26]. In 2,4-D resistant and susceptible waterhemp (*Amaranthus tuberculatus*), herbicide uptake and translocation patterns were the same, while an increased metabolism of the compound was observed in 2,4-D resistant waterhemp [27]. In contrast to the above findings, 2,4-D resistance in mustard has been associated with a reduced translocation from the treated leaf [28]. Similarly, in wild radish (*Raphanus raphanistrum* L.), 2,4-D resistance was due to reduced herbicide translocation via alteration of the cellular transport system and not due to sequestration of the herbicide or uptake by mesophyll cells [29]. The same pattern was observed in glyphosate-resistant plants in which translocation of ^{14}C-labeled glyphosate was lower among herbicide-resistant *Lolium rigidum*, but higher in untreated young leaves of susceptible plants [30]. Other factors, including temperature regimes, also impacted the efficacy, absorption, and translocation of 2,4-D in both resistant and susceptible types of ragweed species (*Ambrosia artemisiifolia* and *Ambrosia trifida*) [31].

Petiolar absorption of [phenyl-^{14}C]-2,4-D has been examined in herbicide-tolerant cotton expressing the tfdA transgene [32]. The tfdA transgene isolated from the soil bacterium, *Alcaligenes eutrophus* (strain JMP134), encodes a 2,4-dicholorophenoxyacetate monooxygenase that degrades 2,4-D herbicide into non-phytotoxic dichlorophenol (DCP)

[33,34]. In the transgenic herbicide-tolerant cotton, 2,4-D was degraded to 2,4-dichlorophenol (2,4-DCP) which was then converted into various polar metabolites, including 2,4-DCP glucoside conjugate (2,4-DCP-β-O-glucoside) and the complex glucosides, 2,4-DCP-(6-O-malonyl)glucoside and 2,4-DCP-(6-O-sulfate)glucoside [32]. However, it is only recently that 2,4-D tolerant cotton varieties using the Enlist™ technology (Dow Agrosciences, Indianapolis, IN) have been introduced to the commercial landscape. The Enlist™ technology uses an aryloxyalkanoate dioxygenase, AAD-12, from *Delftia acidovorans* [12,35] to degrade 2,4-D into non-phytotoxic compounds, and this transgene technology provides resistance as well to several additional herbicides, including triclopyr and fluroxypyr [12]. Other than the AAD transgenes, no other forms of tolerance to auxin herbicides, particularly 2,4-D, has been developed in upland cotton.

The narrow genetic base of cotton limits the genetic improvement of Upland cotton varieties. Exotic unadapted genetic resources have contributed beneficial alleles for many crop species. While it is widely recognized in cotton that exotic germplasm contains potentially valuable genes, the exotic gene pools from *Gossypium* species other than *G. hirsutum* L. mostly remain untapped, uncharacterized, and underutilized due to the paucity of information about the beneficial alleles in these species and the biological and technical challenges associated with interspecific introgression of valuable traits. Solutions for the weed problems are best addressed through the breeding of herbicide-resistant or tolerant varieties. Genetic diversity exists in the relatives of Upland cotton, such as the wild accessions of *G. hirsutum* L. and the tetraploid species, *G. barbadense* L., *G. tomentosum* Nuttal ex Seeman, and *G. mustelinum* Meers ex Watt. Although these wild accessions offer great potential solutions, commercial breeders do not readily use wild or unadapted germplasm because of associated adverse genetic linkages and genetic drag. A set of chromosome substitution (CS) lines were developed in the genetic background of Upland cotton, TM-1 with introgressions from *G. barbadense*, *G. tomentosum*, and *G. mustelinum* [36]. We have documented in previous studies that the CS lines are a novel breeding tool for targeted introgression of many valuable traits such as drought resistance, nematode, and *Fusarium* wilt resistance genes, and improved agronomic and fiber traits in Upland cotton [37,38]. In our previous work, we identified 2,4-D tolerant and susceptible CS lines of *G. hirsutum* containing specific chromosome introgressions from *G. barbadense*, *G. tomentosum*, and/or *G. mustelinum*. Specifically, we identified CS-T04-15 (301-8) and CS-B15sh (31-4) with possible tolerance to 2,4-D in both greenhouse and field experiments [39]. We also confirmed the genetic inheritance of the 2,4-D tolerance trait through the progeny test. This paper reports on the physiological mechanisms associated with absorption and translocation of 2,4-D by comparing the selected 2,4-D tolerant non-transgenic CS lines with the susceptible non-transgenic CS lines and the recurrent CS line's parent TM-1.

2. Materials and Methods

2.1 Plant Materials and Experimental Design

Selected cotton CS lines, including CS-T04-15 (301-8), CS-T07 (206-2), CS-B15sh (31-4), and the susceptible cultivar Texas Marker 1 (TM-1), were used as genetic materials in the study. In previous experiments, we conducted screening of cotton CS lines for tolerance to 2,4-D at 1x field application rate (1.12 kg ae ha^{-1}) under both greenhouse and field conditions. Two CS lines, CS-T04-15 and CS-T07, were identified as having a very high tolerance to 2,4-D in the field experiment. In contrast, CS-B15sh was identified tolerant, showing reduced injury to 2,4-D compared with the susceptible cultivars in the greenhouse. All three CS lines are in the *G. hirsutum* L. (TM-1) genetic background, meaning that the CS lines carry the same chromosome segments of *G. hirsutum* L. TM-1 except for specific substituted chromosome or chromosome segments from *G. tomentosum*, an wild tetraploid species endemic to Hawaii and *G. barbadense,* a cultivated tetraploid species with high fiber qualities respectively. Cotton CS line CS-T04-15 has substituted segments of chromosomes 4 and 15 pair, and CS-T07 have a complete pair of chromosome 7 from

G. tomentosum in TM-1 genetic background. At the same time, CS-B15sh contains a chromosome 15 short arm segment pair from *G. barbadense* in TM-1 genetic background. Comparative analysis of the CS lines with their almost isogenic recurrent parent, the inbred TM-1 (*G. hirsutum*), provided a method to detect and discover important traits with specific substituted chromosome or chromosome segments from the alien species [40,41].

Seeds were sown directly into 18-cell Landmark plastic inserts, size 53 cm length and 26 cm width (Landmark Plastic, Ohio, USA), containing soil (BX PROMIX Growing Medium 10280, BWI Companies, Inc., Nash, TX 75569, USA), mixed with 1-2 g basal fertilizer, (Osmocote Plus, The Scotts Company, Marysville, OH 43041, USA), so that each cell contained one cotton seedling. The experimental design for both uptake and translocation experiments followed a completely randomized design with four replications and one plant per replication. The plants were maintained in the greenhouse from November to December 2020, where mean temperature and relative humidities were 25°C and 58%, respectively.

2.2 Treatment of radiolabeled 2,4-D solution on cotton seedlings

Cotton seedlings used for the [^{14}C]2,4-D treatment were at the four-leaf growth stage. Before the treatment, the plants were transferred to a controlled growth chamber for 3-days of acclimatization to a 12-hour photoperiod (07:00 to 19:00), 30°C/25°C day/night temperatures, and 60% relative humidity. A radiolabeled 2,4-D solution was prepared at 0.517 kBq μl^{-1} working concentration from a commercial stock of 2,4-dichlorophenoxy acetic acid [carboxyl-^{14}C] containing 0.1 mCi mL^{-1}, the specific activity of 50 mCi $mmol^{-1}$, and >98% radiochemical purity (ARC 0722, American Radiolabeled Chemicals, Inc., St. Louis MO 63146, USA). The working stock of [^{14}C]2,4-D used for experiments was prepared using distilled water as a diluent and was maintained at -20°C before the treatment.

For the uptake experiments, [^{14}C]2,4-D was applied to the adaxial surface (upper surface) of the 2nd true leaf of the cotton seedling. A total of 5.17 kBq was applied to each leaf, similar to the procedure described by Johnston et al. [42], where ten 1 µl spots (total volume of 10 µl per treated leaf) were delivered using a micropipette (P-10, Eppendorf, Hamburg, Germany). The plants were allowed to air-dry for 30-60 minutes on the bench to allow absorption of the radiolabeled herbicide on the treated leaf. The treated plants were moved to and maintained in the growth chamber under the same conditions described above until the collection and processing of tissues for analysis. At 24 hours after treatment (HAT), the treated leaves were cut from individual plants and immediately washed in a 50 mL Falcon tube containing 5 mL 1:1 water: ethanol solution for 60 seconds with gentle mixing using speed no. 7 (5-35 rpm) on a Roto-Shake Genie (Scientific Industries Inc., Bohemia, NY 11716, USA) to remove non-adsorbed [^{14}C]2,4-D from the leaf surface. The wash solutions were mixed with Ultima Gold Liquid Scintillation (PerkinElmer, Waltham, MA 02451, USA) solution at a ratio of 5:14 (5 mL wash solution and 14 mL Ultima Gold) and placed in 20-mL scintillation vials (VWR, Radnor, PA 19087, USA). Residual [^{14}C]2,4-D after 24 hours was counted and analyzed using a liquid scintillation counter (Perkin Elmer Tri-Carb 2900TR, Waltham, MA 02451, USA) to determine the percentage absorption of the [^{14}C]2,4-D compound by the plant.

The same conditions and method were used for [^{14}C]2,4-D application to cotton seedlings for the translocation experiment. To determine the movement of [^{14}C]2,4-D from the treated leaf to other parts of the plant, tissues were collected from the individual treated plants at 6, 12, 24, and 48 HAT [42]. Tissues collected from each plant were divided into three categories: treated leaf, including the petiole (T), above the treated leaf (AT), including all leaf and stem tissues collected from above sample T, and below the treated leaf (BT), including all leaf, cotyledon, and stem tissues below sample T. Collected tissues were individually wrapped in paper wipes (Kimwipes, VWR, Radnor, PA 19087, USA), labeled and dried overnight (~12 hours) in an oven at 60-70°C. After drying, the tissues were removed and processed in a biological oxidizer (PerkinElmer Model 307, Waltham, MA 02451, USA) using 0.5 min combustion time. Radioactivity in terms of disintegration per minute (DPM) of [^{14}C] in each sample was quantified using a liquid scintillation counter

(PerkinElmer Tri-Carb 2900TR, Waltham, MA 02451, USA). The overall method was followed as per the previous report of a similar experiment with weeds investigating how application timing influences translocation of auxinic herbicides in Palmer amaranth [42].

2.3 Data analysis

Percent absorption was calculated as the total [^{14}C]2,4-D in DPM obtained from leaf washes at 24 HAT divided by the total [^{14}C]2,4-D recovered from all tissue fractions of each plant, including AT, T, and BT tissues. For this experiment, percent absorption of radiolabeled 2,4-D was conducted only at 24 HAT. Percent translocation was calculated as the amount (in DPM) of [^{14}C]2,4-D obtained from each tissue fraction (i.e., AT) divided by the total [^{14}C]2,4-D recovered from all tissue samples analyzed. Analysis of variance and comparison of means using the Tukey-Kramer HSD and Dunnett's Method was applied to percent absorption, percent translocation, and distribution of [^{14}C]2,4-D at 6 to 48 HAT using the JMP 14 statistical package (SAS Institute, Cary, NC 27513-2414, USA).

3. Results

The overall absorption of [^{14}C]2,4-D was a modest 15-23% among the cotton CS lines, while TM-1 showed uptake of only 1.4% 24 HAT with radiolabeled herbicide solution (Figure 1). Thus, the percent absorption observed among the cotton CS lines, CS-T04-15, CS-T07, and CS-B15sh was significantly increased compared with TM-1. Complementary amounts of [^{14}C]2,4-D were detected in the leaf surface washes where 98.6% of the labeled material was recovered from TM-1 compared to 77-85% of the total [^{14}C]2,4-D recovered from the cotton CS lines after 24 hours.

The translocation patterns of absorbed [^{14}C]2,4-D among the different tissue fractions after 24 hours are presented in Figure 2. The data reflects how much [^{14}C]2,4-D was retained in the treated leaf versus the proportion that was translocated into tissues above or below the treated leaf. All of the cotton CS lines exhibited a similar pattern in which 91-95% of the absorbed [^{14}C]2,4-D remained within the treated leaf tissue. In contrast, only 23% of the radioactivity entering the TM-1 plants remained in the treated leaf fraction (Figure 2). Conversely, 77% of the radiolabeled herbicide entering TM-1 plants was translocated beyond the treated leaf, while only 2-5% entering the cotton CS line plants left the treated leaves.

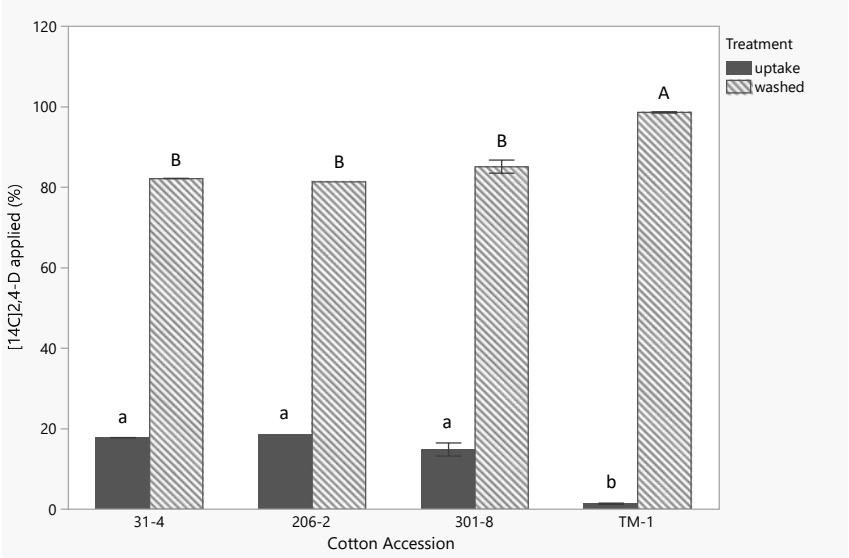

Figure 1. Percent absorption of [^{14}C]2,4-D on cotton seedlings of CS-B15sh (31-4), CS-T07 (206-2), CS-T04-15 (301-8), and TM-1 at 24 hours after treatment (HAT). Means are separated with Tukey-Kramer HSD (α = 0.05). Means of the same letter within treatment (i.e., uptake) indicate not significantly different.

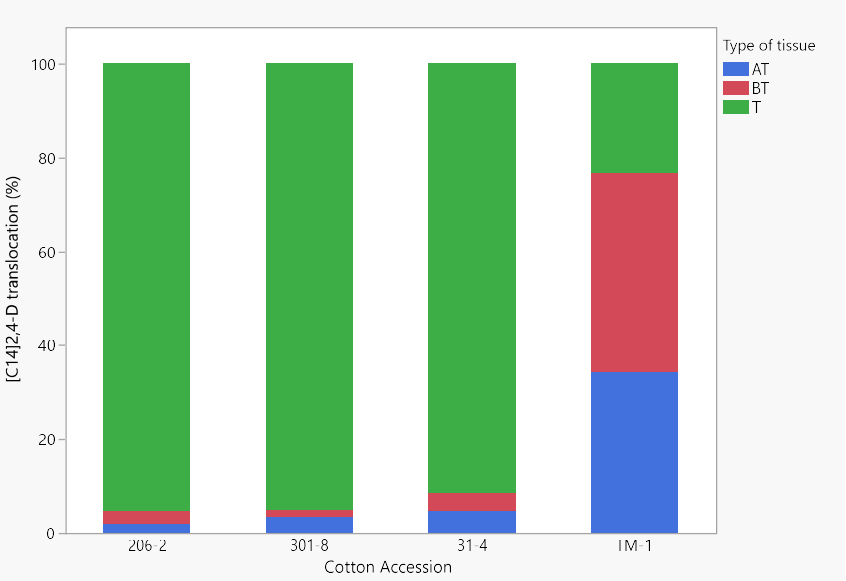

Figure 2. Percent translocation of [^{14}C]2,4-D on cotton seedlings of CS-B15sh (31-4), CS-T07 (206-2), CS-T04-15 (301-8), and TM-1 at 24 hours after treatment (HAT). Data is an average of three replications per cotton accession (variety) and tissue type (AT = Above the treated leaf , BT = Below the treated leaf , and T = Treated leaf).

In TM-1 plants, 35% of the herbicide taken into the plants moved into tissues above the treated leaves, while 42% was transported to tissues below the treated leaves (Figure 2, Table 1). The movement of ^{14}C above and below the treated leaves exhibited similar patterns in two of the three 2,4-D tolerant genotypes. In CS-T07, equal amounts of absorbed [^{14}C]2,4-D were translocated above and below the treated leaf tissue, while in CS-B15sh, 5% of the absorbed [^{14}C]2,4-D was translocated to tissues above the treated leaves, and 4% was translocated into tissues below the treated leaves. Line CS-T04-15 displayed a similar pattern for translocation, with 4% of the absorbed label was translocated to tissues above the treated leaf; while a slightly lower amount of the herbicide (2%) was translocated to tissues below the treated leaf at 24HAT (Figure 2, Table 1). It should be noted that CS-B15sh consistently exhibited reduced 2,4-D injury compared to CS-T04-15 and CS-T07 that showed complete tolerance to the herbicide (data not shown). The data presented here only accounts for the distribution of [^{14}C]2,4-D in the aerial tissues of treated plants 24 HAT. It will be interesting to expand these observations to additional time points and belowground tissues.

Table 1. Translocation of [^{14}C]2,4-D in the cotton accessions after 24 hours of treatment.

[1] T, treated leaf; AT, above the treated leaf; BT, below the treated leaf; [2] absolute radioactivity detected on each type of tissue; [3] [^{14}C]2,4-D was absorbed and translocated to the different tissues at 24 hours after treatment (HAT); Comparison of means by Tukey-Kramer HSD (honestly significance difference).

In an attempt to gain mechanistic insights relating to 2,4-D movement and herbicide tolerance in the cotton CS lines, we examined time courses for the appearance of radiolabel in aerial tissues above and below the treated leaves in control and tolerant plants. The four tested lines showed different patterns of radiolabel movement over 48 hours. In CS-T04-15, radiolabel movement into tissues above the treated leaf increased between 12 and 24 hours. It leveled off between 24 and 48 hours, whereas no significant change in the movement of label into tissues below the treated leaf was seen over the entire time course (Figure 3A). This data for CS-T04-15 suggests a restricted basipetal movement of [^{14}C]2,4-D into tissues below the treated leaf, which may point to a mechanism of herbicide tolerance distinct from the mechanism(s) underpinning tolerance in CS-T07 and CS-B15sh. Radiolabel slowly moved into tissues above and below the treatment site at roughly equivalent rates in TM-1 and CS-T07 (Figures 3B & 3D). Radiolabel movement in CS-B15sh was similar to TM-1 and CS-T07 through the first 24 hours, but the movement of [^{14}C]2,4-D into tissues above the treated leaves increased markedly from 24 to 48 HAT (Figure 3C).

Accession	Type of tissue[1]	DPM1[2]	Translocation, %[3]
TM-1	T	878 a	23.0 a
	AT	1184 a	34.8 a
	BT	1446 a	42.2 a
CS-B15sh	T	57292 a	90.7 a
	AT	3218 b	5.1 b
	BT	2648 b	4.2 b
CS-T04-15	T	50728 a	94.3 a
	AT	2408 b	4.1 b
	BT	814 b	1.6 b
CS-T07	T	63899 a	94.6
	AT	1818 b	2.7
	BT	1841 b	2.7

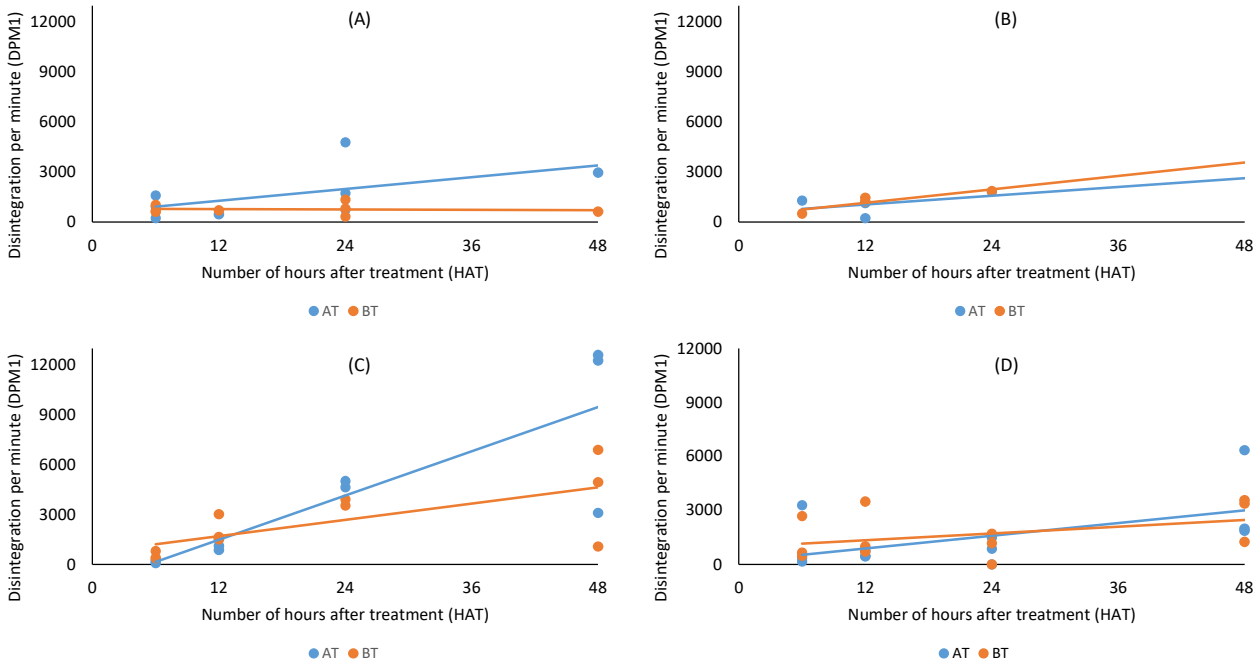

Figure 3. Distribution of [^{14}C]2,4-D detected in tissue fractions above the treated leaf (AT) and below the treated leaf (BT) in cotton seedlings of (A) CS-T04-15, AT(y = 59.025x + 561.41), BT (y = -1.6845x + 783.71); (B) CS-T07, AT (y = 43.974x + 515.11), BT (y = 67.456x + 344.84); (C) CS-B15sh, AT (y = 221.75x - 1178.3), BT (y = 81.648x + 728.15); and (D) TM-1, AT (y = 58.9x + 180), BT (y = 31.057x + 978.48), at 6 to 48 hours after treatment (HAT).

4. Discussion

The low absorption rates for ^{14}C-labeled 2,4-D in upland cotton seen in this study were similar to previous reports on ^{14}C uptake in a 2,4-D resistant Prickly lettuce treated with [^{14}C]2,4-D [43]. However, the increased uptake of [^{14}C]2,4-D among the cotton CS lines compared with the parental line, TM-1 control, suggests physiological differences in leaf surface components between the introgressed cotton CS lines and TM-1. Our data indicate that reduced absorption of [^{14}C]2,4-D is not associated with the mechanism of 2,4-D tolerance observed in the cotton CS lines. However, it is interesting to note that TM-1 greatly restricts the uptake of [^{14}C]2,4-D with only 1.4% of the radiolabeled herbicide taken up after 24 hours compared to the cotton CS lines. Since TM-1 is susceptible and exhibits high 2,4-D injury, it is possible that cells at the site of application die more quickly and therefore affecting the transport of 2,4-D. Our previous study showed that CS lines had significant variation in their root, shoot, and leaf morphology [44]. In addition, *G. tomentosum*, one of the donor parents of CS-T04-15 (301-8) and CS-T07 (206-2), is endemic to Hawaii and shows very distinct morphology from other *Gossypium* tetraploid species. It has hairy, silvery-green to gray-green, palmately veined leaves, sulfur-yellow corollas without petal spots, forms strongly exerted stigmas but devoid of leaf, bracteole, and extra floral nectarines [41]. It will be interesting to study in the future if leaf morphology differences, including leaf surface components such as cuticles, wax, and the presence of lysigenous gossypol glands with phenolic compounds, may be factors contributing to the reduced uptake of [^{14}C]2,4-D [45,46]. Our findings of low [^{14}C]2,4-D absorption in cotton contrast with the approximately 50% [^{14}C]2,4-D uptake by 2,4-D susceptible Palmer amaranth 24 hours after applying the same total amount of radiolabel (5.17 kBq) to treated leaves [42]. High rates of [^{14}C]2,4-D uptake were also observed in 2,4-D resistant wild radish with only 2-4% of the total of 3 kBq [^{14}C]2,4-D remaining on leaf surfaces 24 hours after treatment [29].

A pattern of increased translocation of ^{14}C-labeled 2,4-D outside treated leaves was reported for 2,4-D susceptible biotypes prickly lettuce weed, while reduced translocation was observed among 2,4-D resistant biotypes of this weed [43]. Reduced movement of ^{14}C-labeled 2,4-D beyond the treated leaf was also observed in 2,4-D resistant wild radish [29]. These patterns are associated with non-target site resistance mechanisms detected in weeds evolving to become weed-resistant species due to intense selection pressure combined with other biological processes such as vacuolar sequestration and detoxification of the toxic compounds [47,48]. Among the cotton CS lines, the reduced translocation outside the treated leaf may indicate sequestration of the [^{14}C]2,4-D and any derived metabolites within the treated leaves of the cotton CS lines. It is possible that genomic regions in CS-B15sh, CS-T04-15, and CS-T07 introgressed from the donor alien *Gossypium* species during CS line development could potentially carry genes that oxidize 2,4-D or add different conjugates necessary to inactivate and/or sequester 2,4-D and its derivatives [47]. The source of CS fragments in CS-T04-15 and CS-T07 is *G. tomentosum*, a wild allotetraploid *Gossypium* species native to the Hawaiian area. Although the two lines carry different chromosome introgressions (regions of chromosome 4 and 15 for CS-T04-15 and substitution on chromosome 7 for CS-T07), it is interesting to note that both exhibited high tolerance to the herbicide (data not shown).

On the other hand, CS line CS-B15sh could potentially carry unique genetic loci from the substitutions on the short arm of chromosome 15 from *G. barbadense* L. But with the increase of ^{14}C translocation observed on CS-B15sh outside the treated leaf compared with the limited translocation in CS-T04-15, we can infer that the reduced translocation mechanism is not involved in its response to 2,4-D. These translocated herbicide compounds detected in CS-B15sh may be inactivated or conjugated, becoming less toxic 2,4-D molecules [48] that could explain its reduced herbicide injury than the susceptible TM-1 cotton accession. This study is the first report on ^{14}C translocation in cotton from the treated leaf and beyond (above and below) the treated tissues to understand the potential mechanism of 2,4-D tolerance observed on the selected upland cotton CS lines.

Weeds are the most critical pests in US agriculture based on the consideration of the percentage of hectares treated with herbicides versus the percentage of hectares treated with other pesticides [49]. Weed management strategies in cotton often rely primarily on chemical herbicides due to their simplicity, ease of application, efficiency, and cost-effectiveness. Recently wide application of 2,4-D herbicide as a sole means of weed control caused the potential development of herbicide-resistant weeds. Fortunately, transgenic lines provided farmers a cost-effective method to control weeds in the cotton field. However, one of the important disadvantages of using 2,4-D is that cotton is extremely sensitive to this herbicide, and injury can occur when 2,4-D drifts onto neighboring cotton fields. Plants exhibit altered competitive and defense abilities during evolution in response to specific interference from other plants surrounding their environment. The cotton CS lines tolerant against 2,4-D provide a complementary genetic tool to solve the drift effects problem from 2,4-D in weed management based on non-transgenic cotton cultivars. The genes associated with the 2,4-D tolerance in the CS lines could be stacked as a new source of 2,4-D tolerant genes in a breeding program providing additional improvement to tackle weed management in cotton. The discovery of unique genetic loci in the cotton CS lines, which have been introgressed from the wild and unadapted genetic resource of cotton germplasm, suggests that the potential herbicide tolerance in the selected cotton CS lines can be transferred by breeding and hybridization in an upland cotton genetic improvement program. Ultimately, introgressing this useful trait into commercial cotton varieties while protecting non-GM cotton cultivars against off-target drift effects of auxin herbicides applied to neighboring fields will be a great benefit to cotton farmers.

There is almost no report available on the physiological mechanism associated with 2,4-D tolerance in cotton. Our results unveiled for the first time the novel mechanism of 2,4-D tolerance based on absorption and translocation comparing the 2,4-D tolerant CS

lines with the susceptible TM-1 line. The overall result from this research is essential from the following perspectives: 1) provided genetic information for the first time on the potential association of different chromosome or chromosome segments from the alien species of *G. barbadense* and *G. tomentosum* with 2,4-D tolerance, 2) results revealed that different 2,4-D tolerant CS lines suggested a complex 2,4-D tolerance mechanism involving different patterns of absorptions and translocations and, 3) our research for the first time provided a tool to develop natural (non-transgenic) herbicide-tolerant cotton CS lines and insights into genetic mechanisms involved that would connect future possibilities of metabolomics with genomic technologies in developing herbicide-tolerant cotton lines.

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