



# Selective fertilization with phosphite allows unhindered growth of cotton plants expressing the *ptxD* gene while suppressing weeds

Devendra Pandeya<sup>a,1</sup>, Damar L. López-Arredondo<sup>b,1</sup>, Madhusudhana R. Janga<sup>a</sup>, LeAnne M. Campbell<sup>a</sup>, Priscila Estrella-Hernández<sup>b</sup>, Muthukumar V. Bagavathiannan<sup>c</sup>, Luis Herrera-Estrella<sup>d,2</sup>, and Keerti S. Rathore<sup>a,c,2</sup>

<sup>a</sup>Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, TX 77843-2123; <sup>b</sup>StelaGenomics México, 36821 Irapuato, Guanajuato, México; <sup>c</sup>Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843-2474; and <sup>d</sup>Laboratorio Nacional de Genómica para la Biodiversidad, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, 36624 Irapuato, Guanajuato, México

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Weeds, which have been the bane of agriculture since the beginning of civilization, are managed manually, mechanically, and, more recently, by chemicals. However, chemical control options are rapidly shrinking due to the recent rise in the number of herbicide-resistant weeds in crop fields, with few alternatives on the horizon. Therefore, there is an urgent need for alternative weed suppression systems to sustain crop productivity while reducing our dependence on herbicides and tillage. Such a development will also allay some of the negative perceptions associated with the use of herbicide-resistance genes and heavy dependence on herbicides. Transgenic plants expressing the bacterial phosphite dehydrogenase (*ptxD*) gene gain an ability to convert phosphite (Phi) into orthophosphate [Pi, the metabolizable form of phosphorus (P)]. Such plants allow for a selective fertilization scheme, based on Phi as the sole source of P for the crop, while offering an effective alternative for suppressing weed growth. Here, we show that, when P is supplied in the form of Phi, *ptxD*-expressing cotton (*Gossypium hirsutum* L.) plants outcompete, in both artificial substrates and natural soils from agricultural fields, three different monocot and dicot weed species intentionally introduced in the experiments, as well as weeds naturally present in the tested soils. Importantly, the *ptxD*/Phi system proved highly efficacious in inhibiting the growth of glyphosate-resistant Palmer amaranth. With over 250 weed species resistant to currently available herbicides, *ptxD*-transgenic plants fertilized with Phi could provide an effective alternative to suppressing the growth of these weeds while providing adequate nutrition to the crop.

weed suppression | phosphite | phosphite dehydrogenase | herbicide-resistant weeds | cotton

Unjudicious use of herbicides has paralleled the widespread evolution of herbicide-resistant weeds in the United States and several other countries (1). Herbicide resistance has so far been confirmed in 254 weed species globally (1), and its widespread occurrence poses a serious threat to crop production (2, 3). In particular, resistance to glyphosate (the active ingredient in the herbicide Roundup) has become a serious global issue with at least 41 weed species reported to have evolved resistance to this herbicide to date (1). Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri* [S.] Wats.) is among the major concerns in the United States (4), as it has the potential to reduce yields up to 54% in cotton (5); 79% in soybean (*Glycine max*) (6); 91% in corn (*Zea mays*) (7); and 28% in peanut (*Arachis hypogaea*) (8), depending upon the soil type and weed density. The rise in herbicide-resistant weeds has coincided with the increased adoption of herbicide-resistant genetically modified (GM) crops (9), which is further compounded by a critical reduction in the discovery of new herbicide active ingredients (10). In particular, the widespread occurrence of weed resistance to glyphosate has diminished the usefulness of this “once-in-a-century” herbicide (11) and has prompted the deployment of weed control strategies based on the use of two or more stacked herbicide-resistant

genes to control some of the highly noxious weeds. Multiple resistance to more than one herbicide is also a growing concern in *A. palmeri* (12, 13) and many other weed species (1). In addition to the herbicide resistance issues, some poorly conducted studies on Roundup-Ready crops, which have been discredited since their publication, have created the perception that herbicide-resistant GM crops themselves and/or the glyphosate residue have negative health consequences (14, 15). Thus, there is an urgent need to develop novel tools, especially nonherbicidal ones, that can be deployed to manage the weeds in an effective manner.

The phosphite dehydrogenase (*ptxD*) gene derived from *Pseudomonas stutzeri* WM88 encoding an enzyme that catalyzes the oxidation of phosphite (Phi) into orthophosphate (Pi) (16), when expressed in transgenic plants, has been shown to confer the ability to use Phi as a sole source of phosphorus (P). Moreover, *ptxD*-expressing rice and tobacco plants, in combination with Phi as a P source, provided a nonherbicidal mechanism for weed suppression (17–19). In low-Pi soils, which comprise about 70%

## Significance

An increasing number of herbicide-resistant weeds are being reported in the United States, Argentina, and Brazil. This is becoming a global challenge for the production of several major crops, such as cotton, maize, and soybean. New strategies for weed control are required to sustain agricultural production while reducing our dependence on herbicides. Here, we report that selective fertilization of transgenic cotton, expressing a bacterial phosphite dehydrogenase (PTXD), with phosphite provides an effective way to suppress weed growth. Importantly, we show that the *ptxD*-transgenic cotton plants successfully outcompete a highly aggressive glyphosate-resistant weed. The *ptxD*/phosphite system represents one of the most promising technologies of recent times with potential to solve many of the agricultural and environmental problems that we encounter currently.

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See Commentary on page 7456.

<sup>1</sup>D.P. and D.L.L.-A. contributed equally to this work.

<sup>2</sup>To whom correspondence may be addressed. Email: lherrerae@cinvestav.mx or rathore@tamu.edu.

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of the world's arable land, selective fertilization with Phi should allow transgenic plants to outcompete weeds, the growth of which will be limited by the lack of sufficient levels of available Pi. The combination of *ptxD* with Phi selection has also proved to be an effective alternative system for generating transgenic plants without the use of antibiotic/herbicide resistance genes in cotton (20), maize (21), tobacco, and *Arabidopsis* (17). Here we report that *ptxD*-transgenic cotton plants grow normally when fertilized with Phi as the sole source of P. More importantly, growth chamber and greenhouse studies, using both an artificial substrate and natural, low-P soils from agricultural fields, show clearly that the *ptxD*-expressing cotton plants, when provided with P in the form of Phi, can easily and effectively outcompete aggressive weeds including tall morning glory [*Ipomoea purpurea* (L.) Roth.], purple false brome [*Brachypodium distachyon* (L.) P. Beauv.], and a glyphosate-resistant biotype of *A. palmeri*, as well as weeds that are naturally present in different soil types. The results from competition experiments conducted in various growth media with a wide variety of weed species establish the robustness of the *ptxD*/Phi technology as an excellent system for weed suppression.

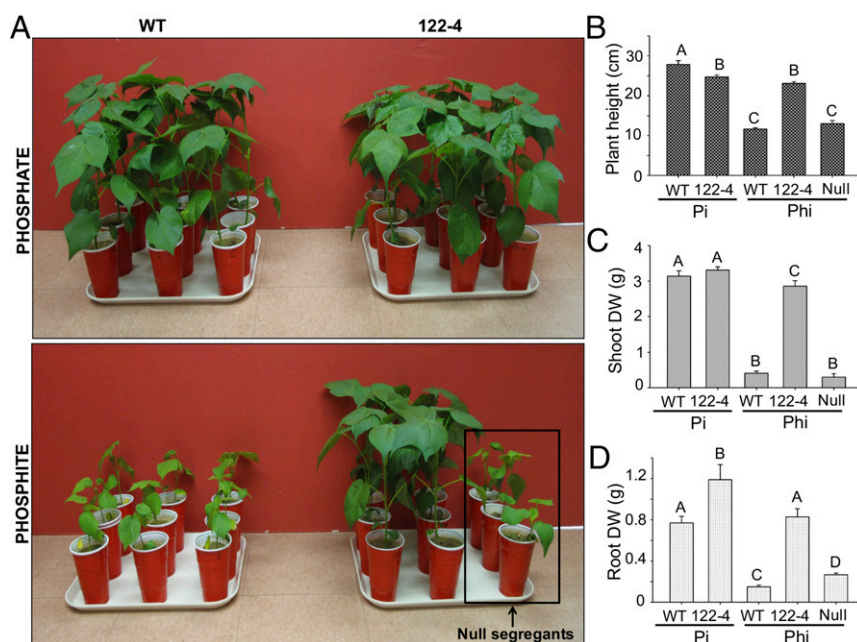
## Results

***ptxD*-Expressing Cotton Plants Can Utilize Phosphite as a Sole Source of Phosphorus.** We previously published a report on the use of *ptxD* as a selectable marker gene to produce transgenic cotton plants (20). Having LCT122- and LCT123-transgenic cotton and their progeny available, we used three independent lines to evaluate the *ptxD*/Phi system for weed suppression and P fertilization. LCT122 lines harbor a T-DNA containing a *bar* gene cassette, the *ptxD* gene under the control of CaMV35S promoter, and the *GFP*-coding sequence under the control of *rolD* promoter. The LCT123 lines contained the same T-DNA but lack the *GFP* expression cassette. Southern blot analyses using specific *ptxD* and Nos terminator probes showed that most of the

LCT123 lines had a single-copy transgene insertion (*SI Appendix, Fig. S1*). Some of the LCT122 and LCT123 lines were analyzed to determine *ptxD* expression levels, enzymatic activities, and/or number of inserts. qRT-PCR analysis verified the presence of *ptxD* transcripts at varying levels in the lines, which correlated with PTXD activity determination (*SI Appendix, Fig. S1*).

To examine the capacity of the *ptxD* transgenic cotton plants to utilize Phi as the sole source of P, we performed a sand-culture hydroponics experiment on T<sub>1</sub> progeny obtained from the line 122-4. The mineral nutrients were supplied to the cotton plants as per the Hoagland solution recipe (*SI Appendix*), with the exception of P, which was supplemented either in the form of Pi or Phi. When the plants were fertigated with Pi, both wild type (WT, parental cultivar Coker-312) and the T<sub>1</sub> transgenics grew equally well (Fig. 1). However, when Phi was the sole source of P, there was an approximately sevenfold reduction in the total biomass of the WT plants, as well as a significant reduction in plant height (Fig. 1). On the other hand, the T<sub>1</sub> transgenic plants that carried the *ptxD* gene had growth under Phi treatment similar to that observed with Pi. Since the progeny of transgenic line (line 122-4) used in this experiment were of the T<sub>1</sub> generation, some null segregant plants were also present, and these were unable to use Phi as a sole source of P and responded in a manner similar to that of the WT plants under the Phi fertilization regimen (Fig. 1).

***ptxD*-Expressing Cotton Plants Are Able to Outcompete Weeds When Fertilized with Phosphite Under Hydroponic Conditions.** To test the efficacy of the *ptxD*/Phi system to suppress broadleaf as well as grass types of weeds by providing P nutrition in the form of Phi to the transgenic cotton plants, we performed competition experiments between *I. purpurea* (broadleaf weed) or *B. distachyon* (grass weed) and the transformants expressing the *ptxD* gene (Competition Experiment 1). The T<sub>3</sub> generation homozygous seeds from two independent cotton transgenic events, 122-2 and



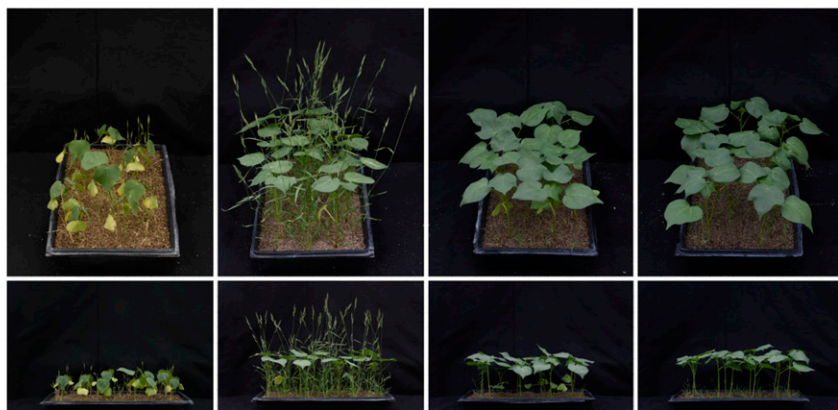
**Fig. 1.** Sand culture hydroponics study to assess the effect of Pi or Phi fertilizer regimen on the growth of cotton plants. (A) Photographic image of WT (cv. Coker 312) plants and 122-4 line (T<sub>1</sub> generation) grown in sand culture for 45 d under Pi or Phi treatment. The plants highlighted with the black box are the null segregating plants identified by the lack of GFP expression. (B–D) Bar graphs depicting plant height, shoot dry weight (DW), and root DW for WT cotton plants and T<sub>1</sub> generation *ptxD* transformants (line 122-4) under Pi or Phi fertilizer regimens. Data represent mean  $\pm$  SE;  $n = 10$  (WT cotton plants and line 122-4 under Pi),  $n = 10$  (WT cotton plants under Phi),  $n = 7$  (line 122-4 under Phi),  $n = 3$  (null segregants under Phi). The values indicated by bars within a group are significantly different at  $P \leq 0.05$  if labeled with different letters.

122–4, were germinated along with *I. purpurea* or *B. distachyon* in a nonsterile, inert substrate [sand and vermiculite (1:1)]. The substrate was amended with Pi or Phi (80 or 120 mg·kg<sup>-1</sup>) before sowing the seeds. In the control treatment in which no P was applied, both the weed and cotton plants showed limited growth (Fig. 2). Under Pi-fertilization regimes, the grass weeds grew vigorously and produced abundant tillers and seeds after 6 wk, while the broadleaf weed also grew quite rapidly and clearly outcompeted the transgenic cotton plants (Fig. 2A and *SI Appendix*, Fig. S2). In contrast, under Phi treatment, the *ptxD*-expressing cotton plants clearly outgrew the weeds, under both 80 and 120 mg·kg<sup>-1</sup> of Phi treatments. Weeds were stunted from the beginning of the experiment and showed leaf damage, which was more severe at 6 wk after sowing. Line 122–2 transformants fertilized with 80 or 120 mg·kg<sup>-1</sup> Phi in competition with *I. purpurea* accumulated 330 or 480% more biomass, respectively, compared with those in competition with the same weed but fertilized with Pi (Fig. 2B). Likewise, transgenic cotton plants fertilized with 80 or 120 mg·kg<sup>-1</sup> Phi in competition with *B. distachyon* accumulated between 25 or 41% more biomass, respectively, compared with those in competition with the same weed but grown on Pi-amended substrate (Fig. 2A). More importantly, *B. distachyon* biomass production was reduced by over

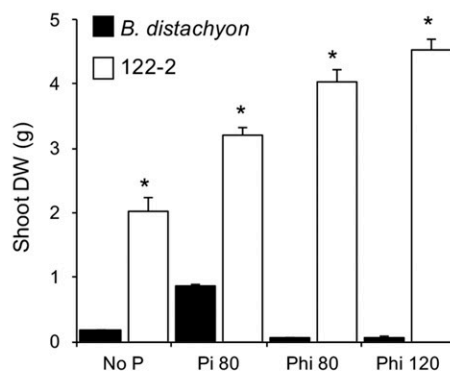
10-fold and that of *I. purpurea* by about 8-fold when plants were grown on either 80 or 120 mg·kg<sup>-1</sup> Phi-amended substrate, compared with the biomass accumulation under the Pi treatment (Fig. 2A and B). Similar results were observed in competition experiments conducted with another independent transgenic cotton line (122-4) in competition with *I. purpurea* (*SI Appendix*, Fig. S2). These results show clearly that *ptxD* expression enables the transgenic cotton plants to grow normally when Phi is used as the sole source of P and that Phi fertilization offers an effective and lasting control of weed growth.

***ptxD* Gene/Phosphite System Is Highly Effective in Controlling the Growth of a Glyphosate-Resistant *A. palmeri* Biotype.** To examine the efficacy of the *ptxD*/Phi system to control glyphosate-resistant weeds when fertilized with Phi as the sole source of P, we conducted a competition experiment in sand and vermiculite (1:1) medium using a glyphosate-resistant biotype of *A. palmeri* that was found in a cotton production field near Somerville, Texas (Competition Experiment 2). Under the Pi-fertilizing regime, both sets of cotton plants (WT and transgenic) and the weeds grew well. In the Phi fertilizer regimen, on the other hand, the 122–4 *ptxD* transformants, with their ability to utilize Phi, were significantly taller, had more leaves and produced higher biomass

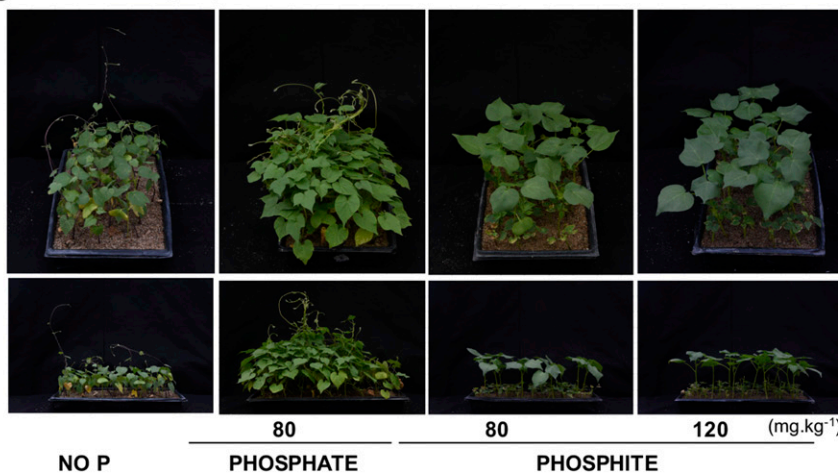
### A 122-2 vs *B. distachyon*



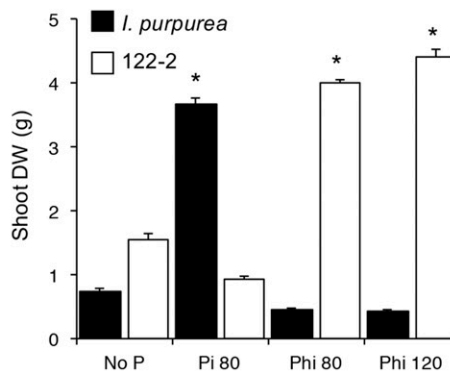
### B



### C 122-2 vs *I. purpurea*



### D



**Fig. 2.** Competition experiment between transgenic cotton and two different weeds in inert artificial substrate (Competition Experiment 1). Plants from the 122–2 ( $T_3$  progeny) line were grown in sand:vermiculite mixture in competition with (A) *B. distachyon* and (C) *I. purpurea* and fertilized with 80 and 120 mg·kg<sup>-1</sup> of P supplied as Phi or Pi. Treatment without added P (No P) was used as control. After 6 wk, representative plants were photographed and cut at the soil surface for biomass determination (B and D). Photographs taken from two different angles are provided for visualization of the growth differences. The bar graphs show the combined weight of three plants of each type. Data represent mean  $\pm$  SE;  $n = 10$  (cotton plants),  $n = 20$  (*I. purpurea* and *B. distachyon*). The single asterisk (\*) indicates values that are significantly different at  $P \leq 0.05$ .

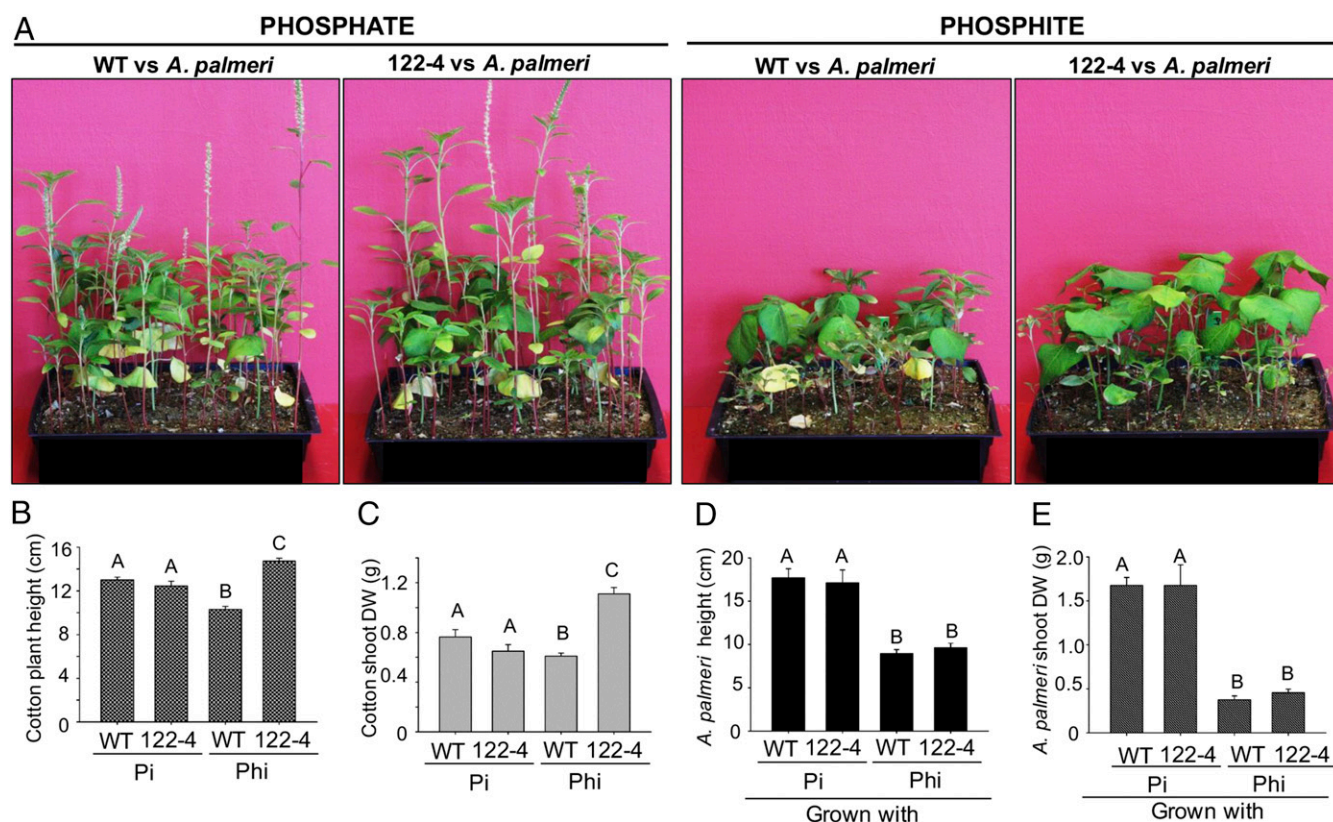


(~1.8-fold) compared with the WT cotton plants (Fig. 3 and *SI Appendix, Table S1*). The 122-4 transformants performed significantly better under Phi treatment than the same line and the WT cotton plants under Pi fertilizer regimen, benefiting from lesser competition with weakly growing weeds. The *A. palmeri* plants fertilized with Phi were severely stunted and exhibited typical symptoms of P deprivation in the form of leaf bleaching (Fig. 3). Growth parameters of the *A. palmeri* such as plant height and shoot dry weight under the Phi treatment were significantly reduced, compared with their counterparts grown under the Pi fertilizer regimen (Fig. 3). The shoot biomass of the *A. palmeri* plants fertilized with Phi were approximately fourfold lower compared with those fertilized with Pi (Fig. 3).

To confirm that the *A. palmeri* biotype used in this research was resistant to glyphosate, an additional tray with WT cotton plants and *A. palmeri* growing side by side, under Pi treatment, was sprayed with a recommended field rate of glyphosate (870 g ae ha<sup>-1</sup>) using standard application procedures. Twelve days after glyphosate application, all of the cotton plants were dead, while the majority of the *A. palmeri* plants survived the treatment (*SI Appendix, Fig. S3*). Overall, the results confirmed the capability of *ptxD*-expressing cotton plants to use Phi as the sole source of P and, consequently, their ability to outcompete a glyphosate-resistant weed, which was effectively suppressed under the Phi treatment.

***ptxD* Gene/Phosphite Fertilizer Is Highly Effective in Suppressing Weeds in Low-Phosphorus, Natural Field Soils.** Although the results of the experiments described above related to competition

between the *ptxD*-expressing cotton plants and weeds were impressive, these studies were conducted under a sand:vermiculite (1:1) hydroponic culture. To evaluate the efficacy and applicability of this system to suppress weeds in real field soil that contains natural microbes and has the capacity to bind Pi, weed competition experiments were performed using three different nonsterile soils from agricultural fields with contrasting properties and Pi availability (*SI Appendix, Table S2*). One of these nonsterile soils was collected from a cotton-production field near San Angelo, Texas (18 ppm of available P, pH 7.8, and 1.8% organic matter content); the second from an agricultural subsistence farm in Guanajuato, Mexico (1.83 ppm of available P, pH 8.1, and 5.85% organic matter content); and the third from a farm near Eagle Lake, Texas (10 ppm of available P, pH 6.6, and 0.45% organic matter content). In all cases, the soil was supplemented with the nutrients at rates recommended by the soil-testing laboratory for optimal growth of cotton plants in the field, except for P, that was added in the form of Pi or Phi. In one such experiment (Competition Experiment 3), we used the San Angelo soil, which had very low levels of the natural weed seed bank (of 16 trays used in this experiment, only two showed emergence of a single weed seedling). Consistent with the previous experiments under hydroponic conditions, the *ptxD*-expressing cotton plants fertilized with Phi showed faster growth and an ~1.3-fold greater shoot biomass compared with that of their WT counterparts (Fig. 4 and *SI Appendix, Table S1*). In contrast, *A. palmeri* growing under the Phi fertilizer regime showed stunted growth and an ~12-fold lower shoot biomass compared with the weeds growing



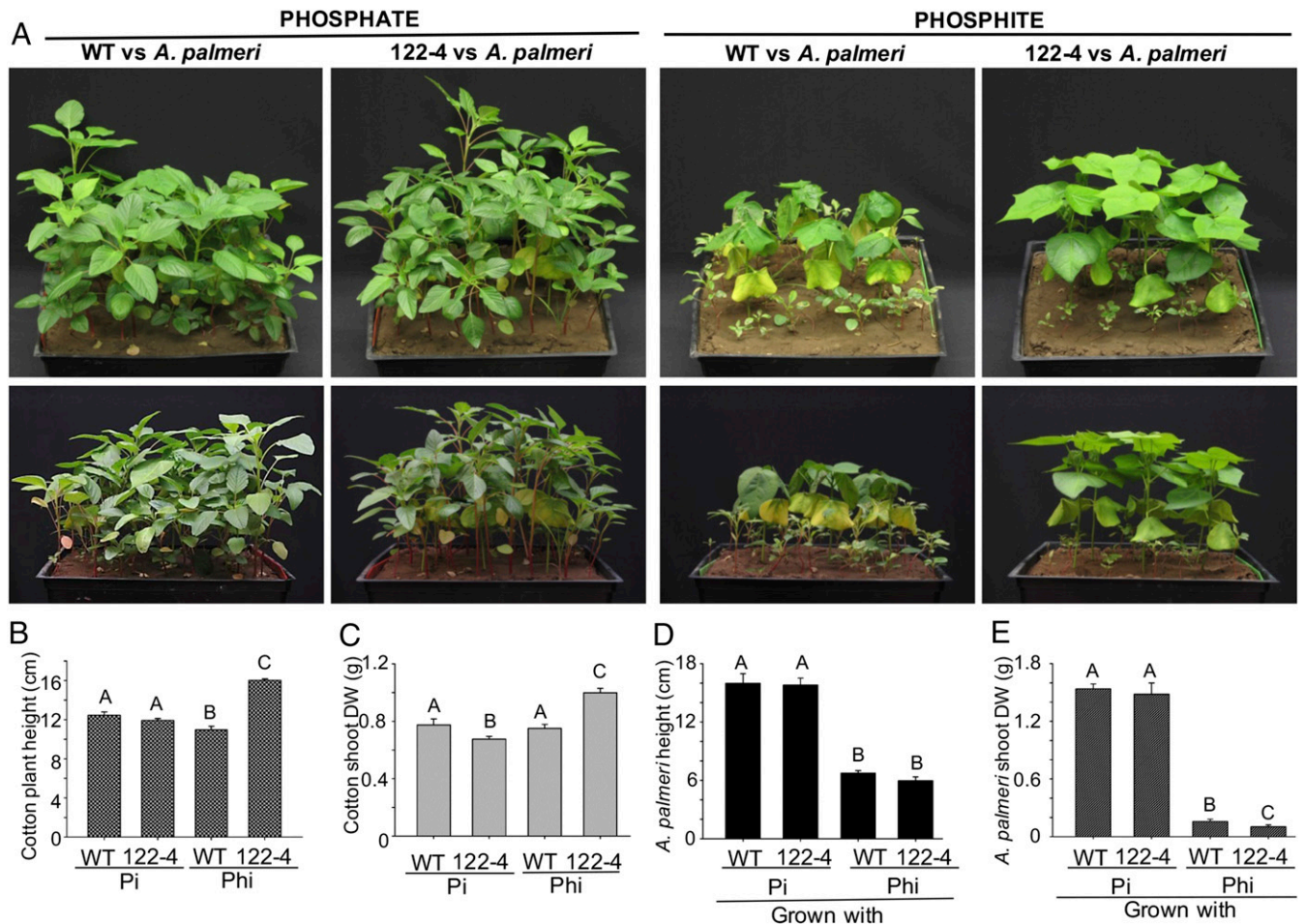
**Fig. 3.** Competition experiment between cotton plants and glyphosate-resistant Palmer amaranth (*A. palmeri*) in inert artificial substrate (Competition Experiment 2). WT (cv. Coker 312) cotton plants and T<sub>2</sub> progeny from line 122-4 were grown in competition with *A. palmeri* in sand:vermiculite mixture for 5 wk. (A) WT cotton plants and *ptxD* plants in competition with *A. palmeri* under Pi or Phi fertilizer regimen. (B and C) Plant height and shoot DW of cotton plants under Pi or Phi fertilizer regimens. (D and E) Plant height and shoot DW (combined weight for 10 plants from each row) of *A. palmeri* grown in competition with cotton plants under Pi or Phi fertilizing regimen. Data represent mean  $\pm$  SE;  $n = 6$  (cotton plants) and  $n = 9$  (*A. palmeri*). The values indicated by bars within a group are significantly different at  $P \leq 0.05$  if labeled with different letters.

in trays that contained Pi as the source of P (Fig. 4). Weed suppression aspect and better growth of the *ptxD*-expressing cotton plants under the Phi treatment were visibly obvious and statistically significantly different compared with the growth of their respective counterparts under Pi treatment in this field soil (Fig. 4). Toward the end of this experiment at 4 wk, the WT cotton plants under Phi treatment showed leaf burn symptoms and premature senescence in their cotyledons, typical of P deficiency (*SI Appendix, Fig. S4*). Thus, even though the soil used in this experiment contained 18 ppm P, the results obtained were qualitatively similar to the ones obtained from experiments conducted in the sand:vermiculite hydroponic system that totally lacked P.

In the Competition Experiment 4, we used the natural soil collected from a subsistence farm in Guanajuato, Mexico, which had a significant number of naturally occurring seeds of a broadleaf and a grass weed species. In addition to the naturally occurring weeds, 50 *B. distachyon* and 10 cotton seeds were sown in each tray. When the plants were grown in the soil with no P amendment, all plants exhibited poor growth (Fig. 5). However, when the plants were grown in the soil amended with 80 ppm Pi, the growth of cotton and weed plants was higher compared with the plants grown in the no-P soil. The biomass accumu-

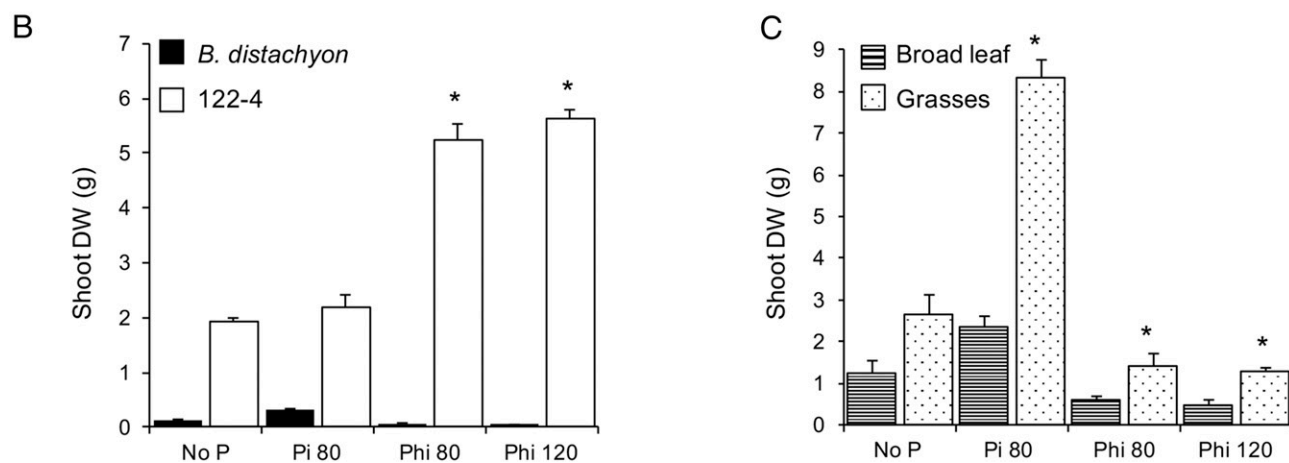
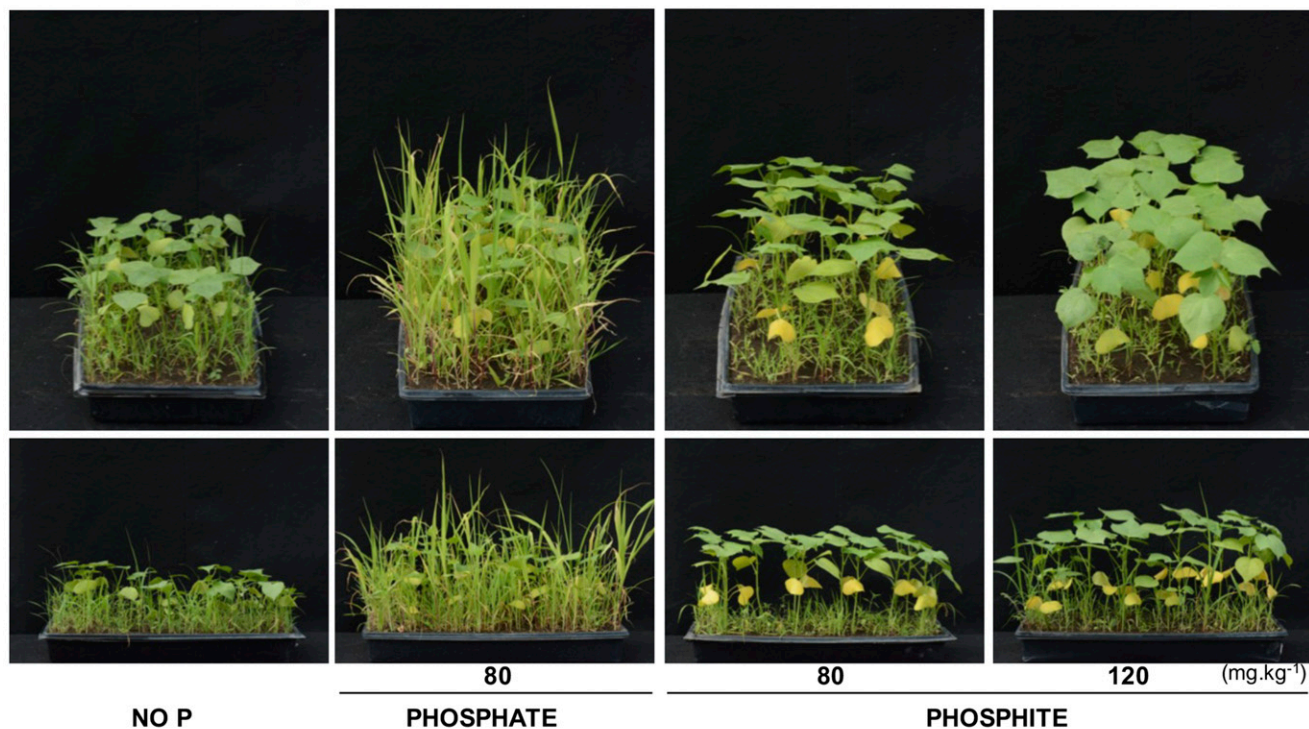
lation of the naturally occurring weeds was increased between two- to threefold, while cotton and *B. distachyon* had a modest increase. In contrast, when plants were grown in the soil with 80 ppm of Phi, the biomass accumulation of cotton plants increased 2.5-fold in comparison with the plants grown in the soil with 80 ppm Pi. The weed growth was suppressed by Phi to about 50% of that observed when the soil had no P amendment and was two- to threefold lower than that observed when the soil was fertilized with 80 ppm Pi (Fig. 5). Similar results were obtained, in terms of enhancement of cotton growth and suppression of weed growth, when the soil was amended with 120 ppm Phi and for a different transgenic cotton line (*SI Appendix, Fig. S5*).

In the same soil, another competition experiment between the *ptxD* transgenic cotton and *I. purpurea* was performed and subjected to the following treatments: no P fertilization, 80 ppm Pi, 80 ppm Phi, and 120 ppm Phi. In this case, cotton plants fertilized with Phi had a biomass 2.3-fold higher than when Pi was applied. The biomass of *I. purpurea* under Phi was reduced threefold and that of naturally occurring grass-type and broadleaf weeds, sixfold and threefold, respectively, compared with that under the Pi treatment (*SI Appendix, Fig. S6*). Among the



**Fig. 4.** Competition experiment between cotton plants and glyphosate-resistant Palmer amaranth (*A. palmeri*) in natural soil from a cotton field in the San Angelo area in Texas (Competition Experiment 3). WT (cv. Coker 312) cotton plants and T<sub>2</sub> progeny from line 122-4 were grown in competition with *A. palmeri* for 4 wk. (A) WT cotton plants and *ptxD* plants in competition with *A. palmeri* in soil amended with Pi or Phi. Photographs taken from two different angles are provided for visualization of the growth differences. (B and C) Plant height and shoot DW (combined weight for five plants from each row) of cotton plants under Pi or Phi treatment. (D and E) Plant height and shoot DW (combined weight for five plants from each row) of *A. palmeri* grown in competition with cotton plants under Pi or Phi treatment. Data represent mean  $\pm$  SE;  $n = 8$  (cotton plants) and  $n = 12$  (Palmer amaranth). The values indicated by bars within a group are significantly different at  $P \leq 0.05$  if labeled with different letters.



A 122-4 vs *B. distachyon*

**Fig. 5.** Competition experiments between transgenic cotton and grass weed *B. distachyon* in natural soil from a subsistence farm in Celaya, Guanajuato (Competition Experiment 4). The soil also contained a large number of viable seeds of several weeds. T<sub>3</sub> progeny from line 122-2 were grown in competition with (A) *B. distachyon* and fertilized with 80 and 120 mg·kg<sup>-1</sup> of P supplied as Phi or Pi. Treatment without added P (No P) was used as control. After 6 wk, representative plants were photographed and cut at the soil surface for shoot biomass determination. Photographs taken from two different angles are provided for visualization of the growth differences. The bar graphs show the combined weight of three plants of each type. (B) Graphs represent dry weight per three plants of *B. distachyon* or cotton 122-2. (C) Graphs represent dry weight of all grasses and broadleaved weeds per tray. Data represent mean ± SE; *n* = 10 (cotton plants), *n* = 20 (*B. distachyon*) *n* = 4 (weeds). A single asterisk (\*) indicates values that are significantly different at *P* ≤ 0.05.

weeds present in the Celaya soil and suppressed by Phi treatments are the following: *Urochloa meziana*, *U. panicoides*, *Cynodon hirsutus*, *Trichloris pluriflora*, *Chloris virgata*, *Malvastrum* sp., *Physalis* sp., *Dyssodia* sp., *Viola* sp., *Matricaria* sp., *Heliopsis* sp., and *Chenopodium* sp.

We conducted an additional experiment (Competition Experiment 5) using nonsterile, natural field soil obtained from Eagle Lake, Texas. Rice is a major crop in this location, although some cotton production is also observed in the area. Preliminary tests with this soil in pot culture showed high levels of soil crusting, resulting in poor emergence of cotton seedlings. To

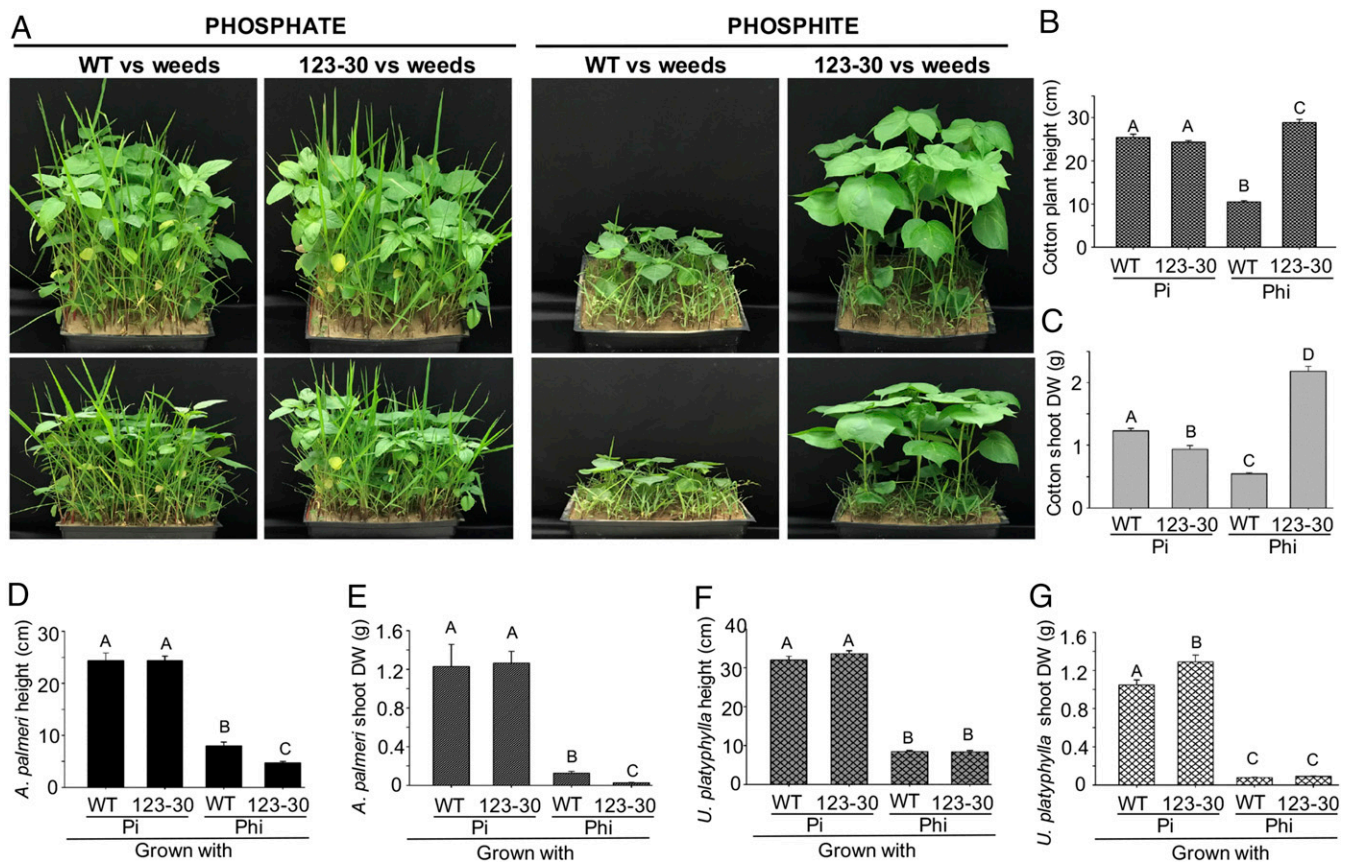
overcome this, the soil was thoroughly mixed with an equal volume of pure sand that allowed normal emergence of cotton seedlings and subsequent growth. Unlike the soil from San Angelo that contained very few indigenous weeds, the Eagle Lake soil contained high densities of viable, broadleaf signalgrass [*Urochloa platyphylla* (Munro ex C. Wright) R. D. Webster] seeds and some (one to two seedlings/tray) smell melon (*Dudaim melon*, *Cucumis melo* var. *dudaim*) seeds. Interseeding *A. palmeri* provided an ideal setup to monitor the impact of competition with both grass and broadleaf weeds on cotton. To further corroborate that the expression of the phosphite dehydrogenase

(PTXD) confers a robust phenotype that is independent of the T-DNA insertion site, in this experiment we used a new independent *ptxD*-expressing transgenic cotton line (123-30), derived from an independent transformation experiment using the LCT123 construct. Within a few days of initiating the experiment, the *U. platyphylla* seedlings began to emerge in each tray, followed by *A. palmeri* and small melon. Under the Pi treatment, all plants including cotton and weeds grew well (Fig. 6). In fact, the Eagle Lake soil produced the best growth results of all three competition experiments between cotton and *A. palmeri*. By the end of the experiment at 4 wk under Pi treatment, the cotton plants were overwhelmed due to severe competition from the weeds (particularly *A. palmeri* and *U. platyphylla*) and were barely visible among the rapidly growing weeds. When Phi was the sole source of P, the biomass accumulation of plants from the transgenic line 123-30 was increased by approximately fourfold compared with the respective WT plants (Fig. 6 and *SI Appendix, Table S1*), whereas the growth and development of all weeds was significantly suppressed with a decrease in biomass accumulation of ~18- and ~14-fold in *A. palmeri* and *U. platyphylla*, respectively (Fig. 6 and *SI Appendix, Table S3*). Within a week after initiating the experiment, weeds were growing noticeably slower and had pale, smaller leaves under Phi treatment compared with that under the Pi treatment. By the end of the experiment, several weeds in the Phi treatment showed severe leaf bleaching symp-

oms (*SI Appendix, Fig. S7*). The small melon plants that were randomly present in the trays also exhibited dramatic growth suppression under the Phi treatment (*SI Appendix, Fig. S7D*). The visual observations described here are also reflected in the growth parameter measurements (Fig. 6 and *SI Appendix, Table S3*). As expected, without the addition of P, the average growth parameters of the WT cotton plants were certainly lower compared with their counterparts in the Pi treatment (*SI Appendix, Fig. S8 and Table S2*).

## Discussion

Over the past 10 y, several weed species have evolved resistance to multiple herbicides, including glyphosate, the most commonly used herbicide globally. Among these, *A. palmeri* is the most noxious glyphosate-resistant weed affecting cotton production throughout the southern United States (4), and, for populations resistant to multiple herbicides, it is an increasingly important problem. Infestation of *A. palmeri* in the cotton fields not only reduces lint yield and quality, but also slows down mechanical harvesting by 2- to 3.5-fold (22, 23). With the rapid loss of effective herbicide options, growers are turning toward old, expensive practices, such as hand weeding as well as environmentally destructive practices such as tillage cultivation. To ascertain the feasibility of using the *ptxD*/Phi system to control weeds, we conducted experiments in a growth chamber and greenhouse using cotton *ptxD* transformants in competition with different weeds including



**Fig. 6.** Competition experiment between cotton plants and glyphosate-resistant Palmer amaranth (*A. palmeri*) in natural soil from a field in the Eagle Lake area in Texas (Competition Experiment 5). The soil also contained a large number of viable seeds of broadleaf signalgrass (*U. platyphylla*). (A) WT (cv. Coker 312) cotton plants and T<sub>2</sub> progeny from line 123-30 were grown in competition with *A. palmeri* and *U. platyphylla* for 4 wk in soil amended with Pi or Phi. Photographs taken from two different angles are provided for visualization of the growth differences. (B and C) Plant height and shoot DW of cotton plants under Pi or Phi treatment. (D and E) Plant height and shoot DW (combined weight for five plants from each row) of *A. palmeri* grown in competition with cotton plants under Pi or Phi treatment. (F and G) Plant height and shoot DW (combined weight for 10 plants from each row) of *U. platyphylla* growing in competition with cotton plants under Pi or Phi treatment. Data represent mean  $\pm$  SE;  $n = 8$  (cotton plants),  $n = 12$  (*A. palmeri*),  $n = 12$  (*U. platyphylla*). The values indicated by bars within a group are significantly different at  $P \leq 0.05$  if labeled with different letters.



*I. purpurea*, *B. distachyon*, *U. platyphylla* (naturally present in one of the field soils), and a glyphosate-resistant biotype of *A. palmeri*, which severely affect cotton production. Competition experiments were conducted using a hydroponic system composed of a sand:vermiculite mixture as the substrate and three very different types of agricultural field soils. As expected, based on the nature of the growth conditions, both cotton plants and weeds showed some differences in their respective growth and development. However, in all these experiments, three independent *ptxD* transgenic lines clearly outperformed the weeds when Phi was the sole source of P. Moreover, the system proved to be effective in soils of neutral, alkaline, and acidic nature, containing limiting (1.83 ppm) to suboptimal (10–18 ppm) levels of P and different organic matter content. Under natural field conditions, uptake, conversion, transportation, and utilization of nutrients by the plant are affected by several factors such as the abundance of microorganisms in the soil, presence of organic matter, soil texture, soil pH, mineral content, and nutrient composition (24). Our studies demonstrate a high degree of efficacy of the *ptxD*/Phi system in controlling various types of weeds irrespective of the growth medium, inert substrate mixtures, or “live” field soils with different levels of P content (as long as P was limiting in the soil). This technology is robust enough to allow normal growth and development of cotton while providing an effective means to manage weeds in different types of soils characterized by low levels of Pi availability.

Competition experiments between the 122–2 transgenic cotton line and *I. purpurea* and *B. distachyon* under Pi fertilization permitted us to observe clearly the higher capacity of the broadleaf weed *I. purpurea* to compete with cotton for Pi than that observed for the grass-type weed *B. distachyon* (Fig. 2 and *SI Appendix*, Fig. S2). In contrast, under Phi fertilization, transgenic cotton plants were more competitive compared with both weed species (Fig. 2 and *SI Appendix*, Fig. S2). A similar scenario is evident under real-life conditions in the field, wherein crops compete with a variety of aggressive weeds where adequate weed management, rather than a correct fertilization regime, directly affects crop yield. The *ptxD*/Phi system could serve as an effective alternative to address these issues in agriculture.

The weed control aspect of the *ptxD*/Phi system has previously been demonstrated for two other plant species. Tobacco plants expressing the *ptxD* gene were able to outgrow several weeds such as *B. distachyon*, *I. purpurea*, *Brachiaria plantaginea*, and *Amaranthus hybridus* when P was supplied in the form of Phi (18). In another recent study on rice, Manna et al. (19) demonstrated successfully the use of Phi as a selective, pre-emergent weed control tool against *Amaranthus spinosus*. Moreover, they showed that foliar (postemergence) application of Phi was effective in controlling broadleaf weeds, including *Phyllanthus niruri*, *Euphorbia hirta*, *Portulaca oleracea*, and a monocot weed, *Chloris barbata*. These results suggest that foliar application of Phi can also be used as an effective over-the-top weed control agent and also as a way to minimize potential alterations in soil microbiota associated with Phi application to the soil.

The severe stunting and leaf damage observed in all of the weeds under the Phi treatment may not be entirely due to P deficiency, at least in the early stages of growth. In all of the experiments conducted in the growth chamber and greenhouse, in sand:vermiculite cultures as well as in field soils (with 1.8, 10, and 18 ppm P), the weeds in the Phi treatment showed extensive damage to the leaves and severe growth reduction. In fact, under Phi treatment in the Eagle Lake soil (10 ppm P) and the Celaya soil (1.8 ppm P), the weeds exhibited more extensive damage and growth inhibition compared with the weeds growing in the soil that was not supplemented with additional P (no-P treatment; Fig. 5 and *SI Appendix*, Table S3). It has been suggested that Phi application further exacerbates the impact of P deficiency by “tricking” the Pi transport and sensing mechanism of the plant

cells, and thus the plant’s response system to scavenge Pi from the soil is not activated (25–27). Therefore, the severe damage suffered by the weeds in the low- or moderate-P field soils could be due to a severe P deprivation caused indirectly by the Phi treatment. Cotton plants expressing the *ptxD* gene are not affected by Phi as their P needs are met by the oxidation of the absorbed Phi to Pi within their cells. Thus, while Phi is not a herbicide, it acts in a manner that can be used to selectively promote the growth of the crop of interest while suppressing the growth of the weeds. Once the crop plants expressing the *ptxD* gene have gained a competitive advantage over the weeds and outgrow them, the shading effect is likely to further suppress weed growth.

Numerous reports document the impact of *A. palmeri* on crop cultivation. *A. palmeri* is a highly competitive weed due to its rapid growth rate, large accumulation of biomass, and production of large quantities of seeds. It has been reported that the presence of only one *A. palmeri* plant per 10-m row can reduce lint yield by 6–11.5% (23). In addition, this study also reported that for every kilogram of *A. palmeri* dry biomass per plot (36.4 m<sup>2</sup>), cotton lint yield is reduced by 5–9%. The growth stage of cotton plants and the period of weed control are critical during cotton cultivation to reduce the competitive interference from *A. palmeri*. When *A. palmeri* establishment is delayed until after the 12- or 17-leaf stage of the cotton plant, the impact on the yield can be negligible (28). Although our studies were conducted under greenhouse and growth chamber conditions and the plants were not taken to maturity to ascertain their yields, our results demonstrate that the *ptxD*/Phi system can be a very useful tool that can be integrated in a management scheme to selectively inhibit weed growth in cotton fields. Implementation of this technology should provide similar benefits to other major crops such as soybean, maize, and sorghum, which are also affected by *A. palmeri* infestation in the fields.

*A. palmeri* has been reported to have acquired resistance to six different herbicide sites of action (acetolactate synthase inhibitors, photosystem-II inhibitors, 5-enolpyruvylshikimate-3-phosphate synthase inhibitors, 4-hydroxyphenylpyruvate dioxygenase inhibitors, protoporphyrinogen oxidase inhibitors, and dinitroanilines), and some populations are resistant to more than one herbicide site of action (1). Moreover, some populations have apparently evolved different and independent mechanisms for resistance to glyphosate. It is less likely that weeds will develop resistance to Phi because the only way that weeds can escape the suppressing effect of Phi in a low-P environment is by gaining an ability to oxidize it into Pi, the metabolizable form of P. This would require evolution of a new enzyme activity in the plant, and, although there are a lot of dehydrogenase enzymes in all plant species, a complex array of multiple mutations is probably required to gain phosphite dehydrogenase activity. Thus, the ability of the *ptxD*/Phi system to allow successful growth of transgenic cotton plants at the expense of a glyphosate-resistant *A. palmeri* biotype offers a powerful alternative for crop production using a nontoxic chemical compound.

In addition to being a highly promising strategy to suppress weed growth, it has been reported that lower amounts of P fertilizer are required to achieve equivalent yields from transgenic plants capable of metabolizing Phi when it is supplied in the fertilization regimen instead of the usual Pi (18). This additional benefit is probably due to various factors, but the main reason is lower fixation of Phi in the soil compared with Pi and also the fact that few microorganisms can use Phi as a source of P (26, 29–31). Although most microorganisms, including microalgae, are unable to metabolize Phi, this reduced chemical form of P is nontoxic to microorganisms (32). Therefore, field application of Phi should, in principle, not cause major changes to the soil microbiota. In fact, a higher solubility of Phi, coupled with its lower tendency to bind to soil contents, when applied in proper formulation to prevent leaching, should allow for a lower overall



use of P without sacrificing crop yields. Even if some of the Phi from the fields ends up in the water bodies, the algal species will be incapable of utilizing it as a source of P, thus reducing the impact of P fertilization in promoting toxic algal blooms (32). Water eutrophication and toxic algal blooms have caused more than 400 dead zones in different parts of the world's oceans that cover over 250,000 square kilometers of ocean surface (33, 34). Therefore, the *ptxD*/Phi system should provide important ecological benefits by reducing water eutrophication and hypoxia caused by the runoff of Pi and nitrogen fertilizers into water bodies.

The *ptxD*/Phi system has been implemented in tobacco under field conditions, and it proved to be an effective technology to control weeds in trials conducted in Argentina (31). Our data, taken together with the results from these studies, strongly suggest that the *ptxD* transgene in combination with Phi treatment provides us with a highly effective weed-suppression mechanism that can help us not only manage the weeds, including those that are resistant to herbicides, but also reduce toxic algal blooms in lakes and the oceans that are caused by the release of fertilizers into water bodies.

The results presented in this paper demonstrate clearly that the *ptxD*/Phi system can serve as a highly effective system to suppress weeds under natural, low-phosphorus soils, including those that are resistant to the herbicide glyphosate, while allowing better growth of the *ptxD*-expressing cotton plants due to lesser competition from the debilitated weeds. Future studies will focus on testing *ptxD* transformants in the fields that are low in P content as well as on evaluating the utility of Phi as an over-the-top "herbicide." Also, the long-term impact of the use of Phi as a source of P on the soil microflora under field conditions needs to be investigated. Research is underway in our laboratory to isolate and characterize root-specific promoters from cotton that can be used to drive the *ptxD* gene. The efficacy of such transformants will be tested against the plants that constitutively express the *ptxD* gene. In conclusion, the *ptxD*/Phi system represents one of the most promising technologies of recent times that can help solve many of the agricultural, biotechnological, and environmental problems that we encounter currently.

## Materials and Methods

LCT122-2, 122-4, and 123-30 *ptxD*-expressing cotton lines generated by Pandeya et al. (20) were used in this study. Glyphosate-resistant *A. palmeri* seeds used in some of the experiments were obtained from a naturally occurring, resistant population near Somerville, Texas. *I. purpurea* and *B. distachyon* seeds were obtained from Cinvestav Irapuato and Universidad Autónoma de Chapingo, respectively.

**Southern Blot Hybridizations, qRT-PCR, and Enzymatic Activity.** Southern blot hybridizations, qRT-PCR, and enzymatic activity analysis were performed to

characterize the transgenic lines. Southern blot analysis was performed on genomic DNA extracted from leaves following a protocol published previously (20).

Total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma), treated with DNase (RNase-free DNase Kit; Qiagen), and then used for qRT-PCR analysis. For reverse transcription, 1  $\mu$ g of total RNA was used to synthesize cDNA in a 20- $\mu$ L reaction using the Taqman Reverse Transcription Kit (Applied Biosystems). Real-time PCR was then carried out by a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with Power SYBR Green PCR Master Mix (Applied Biosystems). *Gh histone 3A* was used as an internal control.

Total protein was extracted by mixing frozen tissue with a lysis buffer, and, after centrifugation, the supernatant fraction was passed through an equilibrated desalting column (Zeba Spin Desalting Column, Thermo Scientific). Protein extract was then used to determine PTXD activity according to Berkowitz et al. (35).

**Growth of Transgenic Cotton and Competition Experiments with Weeds.** To examine the ability of the *ptxD* transformants to utilize Phi as a source of P, T<sub>1</sub> progeny were grown in pure sand in plastic cups and irrigated with nutrient solution containing either Phi or Pi as the P source.

To test the efficacy of the *ptxD*/Phi system to control weeds, including a glyphosate-resistant one, when fertilized with Phi as the sole source of P, we conducted competition experiments using nonsterile, sand:vermiculite artificial substrate and three different natural agricultural soils with low-P content. In different experiments, three weed species, *B. distachyon*, *I. purpurea*, and the glyphosate-resistant *A. palmeri* were intentionally sown in the trays to compete with T<sub>2</sub> or T<sub>3</sub> homozygous transgenic cotton plants. Trays were arranged in a completely randomized manner either in a growth chamber under controlled conditions [32/28 °C (day/night) and a 16-h photoperiod] or a greenhouse. To apply Phi- or Pi-based treatments, the desired ionic form was provided either through irrigation or as a single, direct application to amend the soil. In trays with artificial substrate, only those weeds grew that were intentionally sown, whereas in trays with agricultural soils, viable weed seeds that were naturally present were also allowed to germinate and grow in competition with the cotton plants.

In all of the competition experiments, a representative tray from each treatment was photographed on the day of termination of the experiment, and plant growth parameters were determined. All parameters were subjected to statistical analysis using ANOVA and Tukey's test ( $P < 0.05$ ) using the R software package.

A detailed description of all materials and methods is provided in *SI Appendix, SI Materials and Methods*.

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