Molecular basis of natural tolerance to glyphosate in *Convolvulus arvensis*

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*Convolvulus arvensis* is a troublesome weed that is naturally tolerant to glyphosate. This weed tolerates glyphosate at a rate 5.1 times higher than that of glyphosate-susceptible *Calystegia hederacea*. Glyphosate-treated *C. arvensis* plants accumulated less shikimic acid than *C. hederacea* plants. The overexpression of *EPSPS* genes from the two species in transgenic *Arabidopsis thaliana* resulted in similar glyphosate tolerance levels. qPCR of genomic DNA revealed that the *EPSPS* copy number in *C. arvensis* was approximately 2 times higher than that in *C. hederacea*. Moreover, glyphosate treatment caused a marked increase in *EPSPS* mRNA in *C. arvensis* compared to *C. hederacea*. GUS activity analysis showed that the promoter of *CaEPSPS* (*CaEPSPS*-P) highly improved GUS expression after glyphosate treatment, while no obvious differential GUS expression was observed in *ChEPSPS*-P transgenic *A. thaliana* in the presence or absence of glyphosate. Based on the obtained results, two coexisting mechanisms may explain the natural glyphosate tolerance in *C. arvensis*: (i) high *EPSPS* copy number and (ii) specific promoter-mediated overexpression of *EPSPS* after glyphosate treatment.

Glyphosate is a nonselective, foil-applied herbicide that has been used to manage annual, perennial, and biennial herbaceous species of grasses, sedges, and broadleaf weeds². It affects aromatic amino acid biosynthesis by inhibiting 5-enolpyruvyl-shikimate-3-phosphate synthase (*EPSPS*), a nuclear-encoded, plastid-localized enzyme in the shikimate pathway³. Glyphosate has become the most widely used herbicide in the world due to its advantage of broad-spectrum, low toxicity, and low soil residual activity⁴. However, the widespread and intensive use of glyphosate over years imposes selective pressure on weeds⁵,⁶. Since glyphosate resistance was first found in rigid ryegrass (*Lolium rigidum*)⁷ in Australia in 1996, 43 weed species with resistance to glyphosate have been detected⁸.

Mechanisms of glyphosate resistance are classified as target-site and non-target site. Target-site resistance is caused by mutations in *EPSPS* that decrease its binding affinity for glyphosate, or by *EPSPS* overexpression, which allows the plant to produce adequate *EPSPS* to maintain the synthesis of aromatic amino acids. Single amino acid substitutions in *EPSPS* at position 106 from proline to serine (*P106S*), alanine (*P106A*), threonine (*P106T*), or leucine (*P106L*) have been identified in *Eleusine indica*⁹,¹⁰, *Amaranthus palmeri*¹⁰,¹¹, *Amaranthus tuberculatus*¹², *Echinochloa crus-galli*¹³, and *Amaranthus tuberculatus*¹⁴. Additionally, a double amino acid substitution (*T102I + P106S*) in *E. indica*¹⁵,¹⁶ and *Bidens pilosa*¹⁷ in certain populations was found and reported to confer a higher glyphosate resistance level than that conferred by the single *P106S* mutation.

*EPSPS* overexpression through increased *EPSPS* copy number confers glyphosate resistance in *A. palmeri*¹⁸, *L. rigidum*¹⁹, *A. spinosus*²⁰, and *A. tuberculatus*²¹. For glyphosate-resistant *A. palmeri*, increased *EPSPS* copy number produces abundant enzymes to maintain the shikimate pathway²². Furthermore, *EPSPS* overexpression through elevated *EPSPS* transcript levels after glyphosate treatment is associated with glyphosate tolerance in *Diplotria chinensis*²³ and *Ophiopogon japonicus*²⁴.

Reduced glyphosate absorption, translocation²⁵,²⁶, and vascular sequestration²⁷ are the main non-target glyphosate resistance mechanisms. To protect the young meristematic tissue, resistant plants sequester glyphosate within the vacuoles of the leaves²⁸.²⁹. Maintaining glyphosate in vacuolar tissues by ABC transporters to avoid

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damage was identified to be responsible for glyphosate resistance. Furthermore, studies have reported that chloroplast proteins played an important role in glyphosate resistance in *Conyza canadensis*.

Field bindweed (*Convolvulus arvensis*) is a perennial weed in the morning-glory family. It is considered one of the most troublesome weeds threatening wheat and cotton production in China. *C. arvensis* was the first weed reported to be naturally tolerant to glyphosate. Previous studies aimed at illuminating the glyphosate tolerance mechanism in *C. arvensis* have mainly focused on glyphosate absorption and translocation. However, there were no obvious differences in absorption and translocation. Until recently, the tolerance mechanism has not been fully understood. As *C. arvensis* is naturally tolerant to glyphosate, and a susceptible population in China was not obtained in our previous studies. Therefore, glyphosate-susceptible *Calystegia hederacea* was used as a control because *C. hederacea* belongs to the Convolvulaceae family and shares similar biological characteristics with *C. arvensis* in many aspects, such as perennial, vine climbing, and rapid growth. In this article, we investigated the mechanism of glyphosate tolerance in *C. arvensis* with physiological (shikimic acid accumulation) and molecular (EPSPS cloning, overexpression of EPSPS gene, and EPSPS gene expression pattern) approaches. We cloned the EPSPS genes of *C. hederacea* and *C. arvensis* and inserted the EPSPS gene into the common model plant *Arabidopsis thaliana*, which is an excellent tool for research in plant biology. We examined the glyphosate tolerance of EPSPS transgenic *A. thaliana*. We also compared the basal and glyphosate-induced mRNA levels of EPSPS from the two species.

**Materials and Methods**

**Plant material and growth conditions.** Seeds of *C. arvensis* and *C. hederacea* collected in Beijing, China were germinated in Petri dishes with moist filter paper in an illumination incubator (25 °C day/night temperature). Individual seedlings in the cotyledon growth stage were transplanted into pots (5 cm radius; 6 seedlings per pot) containing a 1:1 (V/V) peat: sand sterile potting mix. The plants were placed in a greenhouse with an average day/night temperature of 25/20 °C and a 12-h photoperiod under artificial illumination (300 μmol m⁻² s⁻¹). The plants were watered as needed.

**Glyphosate dose–response assay.** Plants at the 5–6 leaf stage were sprayed with glyphosate (Roundup Ultra, 41% glyphosate isopropylammonium, Monsanto, USA) at doses of 0, 250, 500, 1000, 2000, 4000 and 8000 g ha⁻¹ using a research track sprayer (3WPSH-500D), which delivered 450 L ha⁻¹ spray solution at 0.3 MPa. All treatments contained 3 replicate pots (6 plants per pot). Plants were assessed 14 days after treatment (DAT). All aboveground plant materials were cut and dried at 60 °C for 72 h. Dry weight was measured when constant weight was achieved. The experiment was arranged in a completely randomized design and was repeated two times with three replications each.

**Shikimate accumulation in vivo assay.** Plants at the 5–6 leaf stage sprayed with 1000 g ha⁻¹ glyphosate were harvested at 2, 4, 6, 8, 10 and 12 DAT, and foliar tissue samples were stored at –80 °C until further processed. Determination of shikimate accumulation in *C. hederacea* and *C. arvensis* tissue was conducted spectrophotometrically according to Chen. Shikimic acid was detected using a double-beam spectrophotometer at 380 nm. The determination of the shikimic acid concentration was based on a shikimate standard curve.

**EPSPS gene cloning and sequence analysis.** Leaves of *C. hederacea* and *C. arvensis* were sampled and ground to fine powders in liquid nitrogen, and the total RNA was extracted with the RNAprep Pure Plant Kit (Tiangen Biotech Co., Ltd., China) following the manufacturer's protocol. First-strand complementary DNA (cDNA) was amplified with random primers using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China). The final cDNA was stored at –20 °C.

The primer pair EPSPS-cf and EPSPS-cr was designed from plant EPSPS gene sequences in NCBI. PCR was performed in a thermal cycler as follows: 5 min at 95 °C; 30 s at 95 °C; 30 s at 57 °C; 35 s at 72 °C (35 cycles); and 10 min at 72 °C. The amplified product was purified and cloned into the pMD19-T vector (Takara, Japan) for sequencing. The sequence obtained from the conserved region was used to design the 5'-end and the 3'-end primers. Fragments amplified by 5' and 3' RACE were purified, cloned into the pMD19-T vector and sequenced. Because of their high homology, ChEPSPS-f and ChEPSPS-r were designed to amplify the full-length EPSPS gene of *C. hederacea* according to that of *C. arvensis*. Sequence assembly and comparative analyses of the EPSPS genes from the two species were conducted using DNAMAN (Version 5.0).

The promoters of EPSPS from *C. hederacea* and *C. arvensis* were amplified with the gwEPS-1, gwEPS-2, and gwEPS-3 primers of the Universal Genome Walker Kit (Clontech, USA) following the manufacturer's protocol. The sequences of primers used in the present study are listed in Table 1. The prediction of cis-acting elements in the promoters was performed by using the software Plant-CARE.

**Quantitative PCR (qPCR) analysis.** The relative EPSPS copy number was estimated using genomic DNA. Total DNA from young leaves (100 mg) of the two species from three plants of each replicate was extracted using the New Plant Genome Extraction Kit (Tiangen Biotech Co., Ltd., China). After eluting in double-distilled water, the genomic DNA quality and concentration were determined spectrophotometrically, and the DNA samples were stored at –20 °C.

The EPSPS expression level was determined using mRNA extracted from plants after glyphosate treatments. Plants sprayed with 1000 g a.e. ha⁻¹ glyphosate at the 5–6 leaf stage were harvested at 0.5, 1, 2, 4, 6 and 8 DAT. The leaves (the uppermost three leaves, 100 mg) of the two species were sampled from three plants of each replicate and ground to a fine powder in liquid nitrogen, and the total RNA was extracted by using the RNAprep Pure Plant Kit (Tiangen, China) following the manufacturer's protocol. After elution of total RNA in double-distilled water,
DNase I was added to digest any contaminating DNA and then removed. The cDNA was amplified with random primers using the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China). qPCR was performed in 96-well plates on the ABI 7500 real-time PCR system with the SYBR Green I Master Mix (Invitrogen, USA). To quantify the copy number and expression level of EPSPS, the housekeeping GAPDH gene was used as the internal control gene because the GAPDH gene did not vary across the samples based on our qPCR results (data not shown). The primer sequences used in this study are listed in Table 1. Melting curves were performed before each qPCR experiment to assess the specificity of the primers. The following two-step real-time PCR detection system was used: 15 s at 95 °C and 25 s at 62 °C. Relative gene copy number or expression level was obtained with the formula for fold induction, $2^{-\Delta\Delta CT}$.

Chimeric vector construction, plant transformation and overexpression of the EPSPS gene in *A. thaliana*. Total RNA was isolated, and cDNA was synthesized. The coding regions of EPSPS of *C. hederaceae* and *C. arvensis* were amplified using the EPSPS-1f and EPSPS-1r primer pair (Table 1), and the complete EPSPS gene was inserted into the pMD19-T. The vector was verified by sequencing and then digested using XbaI. The resulting product was cloned into the pBI121 vector, and the 3SS::EPSPS construct was obtained.

The expression vectors 3SS::CaEPS-P and 3SS::ChEPS-P were introduced into GV3101 Agrobacterium tumefaciens to infect *A. thaliana* by the floral-dipping method. T1 seeds were collected and grown under sterile conditions on media containing half-strength MS basal salt mixture, 1% sucrose and 40 mg L$^{-1}$ kanamycin. The surviving T1 seedlings showed a ratio of 3:1 KanR/KanS and were selected to produce T2 seeds. T2 lines containing the EPSPS gene were considered homozygous and used for further analysis. Three lines of each transgenic *A. thaliana* were used for glyphosate dose response analysis or GUS activity assay. Wild-type (WT) *A. thaliana* was used as a control.

To investigate the role of EPSPS in glyphosate, the seeds of transgenic EPSPS and WT *A. thaliana* were planted on plates containing half-strength MS salts and glyphosate (1.0 mM), respectively. The subsequent growth of these plants was assessed visually and photographed at 14 d after seeding.

Quantitative analysis of GUS activity. To further investigate the EPSPS expression pattern, the EPSPS promoters from the two species were amplified using specific primers (ChEPS-P · F × ChEPS-P · R and CaEPS-P · F × CaEPS-P · R) (Table 1). The sequencing-verified promoters were isolated from pMD19-T using HindIII/XbaI digestion and then inserted into the pBI121 vector to generate EPSPS-P::GUS. The recombinant vectors were then verified by restriction digest. Expression vectors of ChEPS-P · F::GUS and CaEPS-P · F::GUS were finally introduced into *A. thaliana*. The method of plant transformation was described as above.

The GUS activity assay in transgenic *A. thaliana* seedlings used the methods described by Huang42. The data represent the means ± SD of triplicate measurements.

Table 1. Primers used in this study.

| Primer name | Primer sequence (5' to 3') | Purpose of the primers |
|-------------|---------------------------|------------------------|
| EPSPS-ct    | TGTTCTGAACACGCTTGGGCC    | Amplify the core of EPSPS |
| EPSPS-cr    | CACTGTGGCTCCACCTTCTT    | Amplify the full length of ChEPSPS |
| EPSPS-5     | GCCGCCAACGCTTAAAGCA     | 5' RACE |
| EPSPS-3     | GCAGGAAAGCAGAAGTGGCC    | 3' RACE |
| ChEPS-P-1f  | ATACCCACCCGAATCAGTAAGAGTT | Amplify the full length of ChEPSPS |
| gwEPS-1     | CCTCTCAAGCTGGTCGCTGATGTCC | TAIL-PCR of the EPSPS promoter |
| gW-2       | TGAAGAAAAACACAGAAGGAGAA | Amplify CaEPS-P |
| gW-3       | CACAATCTCCTCGTGGCCATGAC | Amplify CaEPS-P |
| EPS-1f      | TCCTAGAATGGGCCAATGAGCAACA | Amplify the full length of EPSPS |
| EPS-1r      | CCAGGCCTCAATCCTGGGAACCTTG | Amplify CaEPS-P |
| CaEPS-Pf    | TAAACCTTTAATGTAATTTT | Amplify CaEPS-P |
| CaEPS-Pr    | GGTATTTTTTAAAAGAGGGGTTG | Amplify CaEPS-P |
| ChEPS-Pf    | GGACTCCTAGCTAGTGGCA   | Amplify ChEPSPS-P |
| ChEPS-Pr    | GGTATTTTTGAAGAGGGGTG | Amplify ChEPSPS-P |
| Q-EPS-1f    | GGTCCCTTACCCGTAACAC    | qRT-PCR analysis of the EPSPS gene |
| Q-EPS-1r    | GGGGAGGTCAGAATACAA     | qRT-PCR analysis of the GAPDH gene |
| GAPDH-f     | AATGGTGGCTTGCTCGGGCTCA | qRT-PCR analysis of the GAPDH gene |
| GAPDH-r     | AGAACCTTCCACAGCCTTGCC | qRT-PCR analysis of the GAPDH gene |

Table 1. Primers used in this study.
was inhibited, and the cotyledons turned yellow and died. In contrast, the CaEPSPS transgenic ChEPSPS significantly different at P
the means were compared using Student’s t-test or Tukey’s multiple range tests. Means with different letters are
using SigmaPlot software (version 12.0), and Tukey’s multiple range tests were used for comparison.
(Fig. 3b).
CaEPSPS-P
ment, which is involved in defence and stress responsiveness, located in
cis
 cis-acting elements, such as spl, ARE, and GATA motifs. Furthermore, there was a
sis
sequence analysis of
PlantCARE analysis of
by genome walking and designated as promoter regions (named
CaEPSPS-P and CaEPSPS-P, respectively). PlantCARE analysis of ChEPSPS-P showed that a TATA box at −40 to −36 and three CAAT boxes at −350 to −152 were included in the promoter. Furthermore, a putative cis-acting sp1 element was found within the pro-
moter sequence (Fig. 3b). Sequence analysis of CaEPSPS-P with PlantCARE showed the presence of common
core promoter elements, including a “TATA-box” (−40 to −36), six “CAAT-box” (−379 to −156) and many
cis-acting elements, such as spl, ARE, and GATA motifs. Furthermore, there was a cis-acting TC-rich repeat
element, which is involved in defence and stress responsiveness, located in CaEPSPS-P (Fig. 3b).
Response to glyphosate in transgenic A. thaliana. To investigate the role of CaEPSPS and ChEPSPS in response to glyphosate, three independent transgenic A. thaliana lines expressing either EPSPS gene and WT were assayed. Because the three CaEPSPS or ChEPSPS transgenic A. thaliana lines showed similar tolerance to glyphosate (data not shown), one line of CaEPSPS or ChEPSPS transgenic A. thaliana was selected for imaging. Based on Fig. 4, there was no obvious difference in plant growth among the WT, CaEPSPS and ChEPSPS transgenic A. thaliana in the absence of glyphosate. However, in the presence of glyphosate (1 mg L−1), the WT growth was inhibited, and the cotyledons turned yellow and died. In contrast, the CaEPSPS and ChEPSPS transgenic A.

Results
Whole-plant bioassay. The responses of C. hederacea and C. arvensis to glyphosate were different (Fig. 1).
At the glyphosate field rate (1000 g ha−1), the growth of C. hederacea was reduced by approximately 70%, whereas
the growth of C. arvensis was reduced by nearly 30%. The C. arvensis plants were not completely controlled
by a glyphosate rate of up to 4000 g ha−1. The GR50 values for C. hederacea and C. arvensis were 562.1 and
2,866.3 g ha−1, respectively, and the calculated tolerant index was 5.1.
Shikimic acid accumulation. Basal shikimate acid levels were similar (55.1–59.2 µg g−1 FW) for C. heder-
acea and C. arvensis in our study. Shikimic acid accumulation exceeded the initial levels of untreated plants after
glyphosate application (1000 g ha−1), and both species accumulated shikimate acid until 6 DAT. However, the
two species thereafter differed in shikimate accumulation at 6 DAT, accumulation decreased in
C. arvensis but fluctuated in C. hederacea (Fig. 2). Shikimic acid accumulation in C. hederacea (with a peak of 326.2 µg g−1 FW at
6 DAT) was 3.5 times higher than that in C. arvensis at 6 DAT.
Sequence analysis of EPSPS. Full-length EPSPS cDNAs were isolated from C. hederacea and C. arven-
sis (ChEPSPS, EU526078; CaEPSPS, EU698030) using specific primers. Sequence analysis revealed that both
ChEPSPS and CaEPSPS consisted of a 1,563 bp open reading frame (ORF) encoding a polypeptide of 520 amino
acids. The deduced amino acid sequences shared high similarity (identity was 97.31%). There are 14 different
mutations, such as those mainly found at positions 102 or 106 in EPSPS, which have previously been associated
with glyphosate resistance.
Fragments of 1,077 bp and 1,142 bp upstream of the ChEPSPS and CaEPSPS genes, respectively, were obtained
by genome walking and designated as promoter regions (named ChEPSPS-P and CaEPSPS-P, respectively).

\[ Y = \frac{a}{1 + e^{-(X - GR_{50})/b}} \]

In this equation, \(a\) is the difference between the upper and lower response limits, \(GR_{50}\) is the glyphosate dose that results in a 50% growth reduction, and \(b\) is the slope of the curve around \(GR_{50}\). The estimates were obtained using SigmaPlot software (version 12.0), and Tukey’s multiple range tests were used for comparison.

Data from the EPSPS copy number analysis and other experiment results were subjected to ANOVA, and the
means were compared using Student’s t-test or Tukey’s multiple range tests. Means with different letters are
significantly different at \(P = 0.05\). All statistical analyses were performed using SPSS software (SPSS 17.0, SPSS
Institute Inc.).

Figure 1. Dose–response assay of C. hederacea and C. arvensis treated with different glyphosate doses. Dry
weight was expressed as a percentage of the untreated control. Each data point represents the mean ± SE of
twice-repeated experiments containing three replicates each, and vertical bars represent the standard error.
*thaliana* produced normal plants on Petri dishes and showed similar growth. Thus, the *CaEPSPS* and *ChEPSPS* genes similarly conferred the ability to withstand higher glyphosate treatments in transgenic *A. thaliana*. These results indicate that the amino acid differences in EPSPS were not the cause of glyphosate tolerance in *C. arvensis*.

**Comparison of EPSPS gene copy number and expression level.** As we found that the amino acid differences did not account for glyphosate tolerance in *C. arvensis*, the EPSPS gene copy number in both species was...
evaluated by qPCR using GAPDH as a normalization gene. The EPSPS copy number in the glyphosate-susceptible C. hederacea ranged from 0.64 to 0.75; however, the glyphosate-tolerant C. arvensis had higher relative EPSPS copy numbers, varying from 1.41 to 1.63 (Fig. 5), showing approximately 2 times higher copy number expression than that of C. hederacea. A higher EPSPS copy number indicated that C. arvensis could produce adequate EPSPS to bind glyphosate, thus conferring higher tolerance compared to C. hederacea.

To examine the expression level of the EPSPS transcript in C. arvensis and C. hederacea, we carried out qPCR analysis with template cDNA derived from plants induced by 1000 g ha\(^{-1}\) glyphosate for different times. As shown in Fig. 6, glyphosate treatment induced a remarkable and steady increase of EPSPS expression in C. arvensis from 0.5 to 1 DAT with nearly 12 times higher peak induction than that of the untreated control, and then the EPSPS transcript level declined. In comparison, glyphosate caused a longer but weaker induction of EPSPS in C. hederacea. The induction began at 0.5 DAT and declined at 2 DAT. The peak induction in C. hederacea was much lower than that in C. arvensis (Fig. 6).
GUS expression from the EPSPS promoter. As the expression levels of the EPSPS genes induced by glyphosate in *C. arvensis* and *C. hederacea* were obviously different (Fig. 6), we assumed that the specific promoter was likely associated with the differences in EPSPS expression. Hence, we fused the EPSPS promoters to the GUS gene and transformed the recombinant vectors into *A. thaliana* plants to further investigate the EPSPS expression regulatory mechanism. The GUS activity in three transgenic *A. thaliana* lines expressing *ChEPSPS-P* or *CaEPSPS-P* was examined at 0.5, 1, 2 and 4 days after glyphosate application. The results showed that there was no significant difference of the GUS activity in the *ChEPSPS-P* transgenic *A. thaliana* throughout the experiment. In contrast, the GUS activity of the *CaEPSPS-P* transgenic *A. thaliana* was induced at much higher levels by glyphosate from 0.5 to 1 days. The peak induction was detected at 1 day after glyphosate application (Fig. 7). These results indicated that some cis-elements likely exist in *CaEPSPS-P* that are induced by glyphosate and drive GUS overexpression. This result was consistent with our hypothesis that the overexpression of EPSPS after glyphosate treatment in *C. arvensis* was likely mediated by a specific EPSPS promoter.

Discussion

Several weeds, including *C. arvensis*, have been identified with different glyphosate tolerance levels. The recommended glyphosate field doses are commonly 900 to 1500 g ha⁻¹, although these doses vary according to the agronomic management and product marketing of the crops. Thus, *C. arvensis* (at GR₅₀ level) is tolerant to glyphosate at 1.9–3.2 times the field dose and 5.1 times the level of the glyphosate-susceptible *C. hederacea* (Fig. 1). To achieve the complete control of *C. arvensis*, at least double the GR₅₀ rate of glyphosate should be applied; however, this application rate will increase the selection pressure and accelerate the resistance evolution to glyphosate.

Glyphosate affects aromatic amino acid biosynthesis by inhibiting EPSPS, which is a critical enzyme in the shikimate pathway. Previous studies have employed shikimic acid accumulation as a parameter for discriminating glyphosate resistance. For example, the inhibition of EPSPS by glyphosate in susceptible weeds usually results in shikimic acid accumulation. Furthermore, glyphosate-tolerant or glyphosate-resistant weeds accumulate shikimate at much lower levels than susceptible plants. In our study, growth setback and eventual death were observed in *C. hederacea* owing to shikimic acid accumulation. This effect was due to the complete binding
of EPSPS by glyphosate in *C. hederacea*, resulting in the accumulation of shikimic acid, whereas EPSPS in *C. arvensis* was not fully inhibited and would still maintain the shikimic pathway, thus leading to normal growth with slight developmental anomalies, such as deformed leaves and shortened internodes. Shikimate accumulation assays indicated that the glyphosate targeting of EPSPS plays a critical role in glyphosate tolerance in *C. arvensis*. Therefore, EPSPS alteration (mutation or amplification) is likely the major mechanism underlying glyphosate tolerance in *C. arvensis*.

**EPSPS** point mutations have been well established as major mechanisms of glyphosate resistance. Some weeds displaying glyphosate resistance have a site mutation (particularly at the Pro106 codon) in the **EPSPS** gene. Recently, **E. indica** and **Bidens pilosa** with a double mutation reported as TIPS (Thr102-Ile + Pro106-Ser), have been found to have a high degree of glyphosate resistance. Three amino acid residues (Asp71-Met, Ala112-Ile, and Val201-Met) and a 91Glu deletion in **EPSPS** were reported to be associated with natural tolerance to glyphosate in three lilyturf species. In our study, six different amino acid substitutions were discovered in **EPSPS** in *C. arvensis*. To investigate the response of different **EPSPS** proteins to glyphosate, **EPSPS** genes were inserted into *A. thaliana*. Glyphosate response assays showed that the two transgenic *A. thaliana* shared similar glyphosate tolerance levels (Fig. 4). Therefore, target-site mutations are unlikely to account for glyphosate tolerance in *C. arvensis*.

To examine the possibility of **EPSPS** overexpression contributing to glyphosate tolerance in *C. arvensis*, both the basal and induced **EPSPS** mRNA levels were determined for the two species in this study. The **EPSPS** copy number for *C. arvensis* was 2 times higher than that of *C. hederacea* (Fig. 5). This result alone is not sufficient to explain the tolerance of *C. arvensis* at the whole plant level. However, the glyphosate-induced expression of the **EPSPS** gene in *C. arvensis* was highly enhanced after treatment compared to that in *C. hederacea* (Fig. 6). Multiple **EPSPS** copy numbers and/or increased expression of **EPSPS** have also been reported in other weed species, such as *D. chinensis*, *O. japonica*, *A. palmeri*, and *Conyza* species. Therefore, a higher **EPSPS** copy number together with increased **EPSPS** expression likely play an important role in glyphosate tolerance in *C. arvensis*.

Gene expression is mostly regulated by the promoter. Different promoter regions may have distinctive regulatory functions. In our study, there was a 77 bp extension in the **EPSPS** promoter of *C. arvensis*, which includes three CAAT-boxes. CAAT boxes are known to play important roles in enhancing the transcriptional level of the gene. Moreover, TC-rich repeats, which are involved in defence and stress responsiveness, are located in *CaEPSPS-P* (Fig. 3b). Thus, cis-elements, such as CAAT-boxes or TC-rich repeats, are likely induced by glyphosate treatment and improve the capacity to respond to glyphosate treatment via feedback regulation. In combination with **EPSPS** gene amplification, the **EPSPS** promoter containing specific cis-elements or increased transcription factor activity may increase **EPSPS** expression and confer glyphosate tolerance in *C. arvensis*. Further study will be necessary to detect the function of these cis-elements in the glyphosate feedback regulatory mechanism.

**Conclusion** We have demonstrated that *C. arvensis* is naturally tolerant to glyphosate at a much higher dose than glyphosate-susceptible *C. hederacea*. *C. arvensis* accumulated less shikimic acids when treated with glyphosate. The **EPSPS** of *C. arvensis* shares high similarity with that of *C. hederacea*, with six different conserved amino acids; however, the response to glyphosate in **EPSPS** transgenic *Arabidopsis* assays showed that these plants shared similar glyphosate tolerance levels. We also observed that the **EPSPS** copy number in *C. arvensis* was approximately 2 times higher than that of *C. hederacea*, and the **EPSPS** mRNA in *C. arvensis* could be highly induced by glyphosate. We conclude that the underlying basis for the glyphosate tolerance of *C. arvensis* is primarily due to high **EPSPS** gene copy numbers and specific promoter-mediated **EPSPS** overexpression after glyphosate treatment. This study could be of increased importance in weed management if the weeds share a similar glyphosate tolerance mechanism. Our future studies will focus on identifying the putative cis-elements of *CaEPSPS-P* in the glyphosate feedback regulatory mechanism.

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Author Contributions
C.Z. and Z.H. designed the experiments; Z.H., H.H., C.J. and S.W. did the experiments; Z.H., L.J., Y.L. and S.W. analyzed data and wrote the manuscript.

Additional Information
Competing Interests: The authors declare no competing interests.

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