ORIGINAL PAPER

Estimating the role of maize *Y-EPSPS* gene in glyphosate resistance in Arabidopsis transgenic lines

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Abstract

The herbicide glyphosate inhibits the key enzyme 5-enolpyruvate shikimate-3-phosphate synthase (EPSPS) in the aromatic amino acid synthesis pathway of plants. This study aims to explore the *Y-EPSPS* gene derived from maize through codon optimization and validate its glyphosate resistance in Arabidopsis Thaliana. Germination rates of seeds under different glyphosate concentrations revealed that seeds overexpressing the *Y-EPSPS* gene exhibited higher germination rates compared to wild-type seeds. DAB and NBT staining methods were used to measure ROS levels in Arabidopsis plants under 0.8 mM glyphosate stress, showing that plants overexpressing *Y-EPSPS* had lower ROS levels compared to wild-type plants. Soluble sugar and malondialdehyde (MDA) content were higher in *Y-EPSPS* overexpressing plants, whereas MDA content was lower, indicating a potential stress response to glyphosate. Chlorophyll content and FV/FW ratio were higher in plants overexpressing *Y-EPSPS* compared to wild-type plants, suggesting reduced susceptibility to glyphosate. Enzyme activity and gene expression analysis further demonstrated significant increases in POD, SOD, and CAT enzyme activities in *Y-EPSPS* overexpressing plants compared to wild-type, while SD enzyme activity decreased significantly. Expression levels of ROS detoxification-related genes (*AtCAT3* and *AtSOD1*) and stress defense-related genes (*AtLTP3*, *AtSOS1*, and *DQSD*) were also elevated to varying degrees in *Y-EPSPS* overexpressing plants compared to wild-type plants. These results indicate that the optimized *Y-EPSPS* gene confers certain resistance to glyphosate.

Keywords *Y-EPSPS* · Glyphosate · Physiological and biochemical indicators · qRT-PCR

Introduction

Aromatic amino acids (AAA), including tryptophan, tyrosine, and phenylalanine, serve as precursors for numerous natural products (Parthasarathy et al. 2018) and play crucial roles in plant growth and development (Less et al. 2010). They are involved in various physiological processes such as auxin biosynthesis, enhancing drought resistance, promoting

lignin synthesis, participating in signal transduction, and responding to biotic and abiotic stresses (Lynch et al. 2020; Pandey et al. 2017).

Shikimic acid serves as a precursor in the synthesis of AAA, also known as the shikimate pathway. Shikimic acid is synthesized through a series of enzyme-catalyzed reactions involving phosphoenolpyruvate (PEP) and erythros-4-phosphate, including 3-deoxyarabinoheptulose-7-phosphate, 5-enolpyruvate shikimate-3-phosphate synthase (EPSPS), dehydroguanic acid synthase, dehydroguanic acid dehydrase and shikimate dehydrogenase (Maeda et al. 2012; Tzin and Galli 2010; Wu et al. 2022; Suh et al. 1993). Shikimic acid is then used in enzyme-catalyzed reactions involving shikimate kinase, EPSPS enzyme, cladonic acid synthase, AAA, and other enzymes to produce secondary metabolites.

EPSPS enzyme is a key enzyme in the shikimate pathway and the sole target of the herbicide glyphosate (Okumu et al. 2019). Glyphosate is the most widely used broad-spectrum herbicide worldwide, making research on glyphosate and EPSPS enzyme in plants highly significant (Duke 2011).

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Based on the inherent sensitivity and catalytic efficiency of EPSPS enzyme towards glyphosate, EPSPS enzymes can be classified into two major classes. Class I EPSPS enzymes are found in all plants and certain bacterial species such as Escherichia coli, Klebsiella pneumoniae, and Salmonella typhimurium, which are relatively sensitive to glyphosate inhibition. Class II EPSPS enzymes are only present in bacteria such as Staphylococcus aureus and Agrobacterium tumefaciens. Currently, the most widely used *EPSPS* gene is derived from Bacillus subtilis and has been extensively studied and applied in plants (Funke et al. 2006; Herrmann and Weaver 1999; Dill et al. 2008; Chhapekar et al. 2015).

The mechanism of action of glyphosate involves mimicking the carbon state of PEP. Glyphosate competitively binds reversibly with EPSPS enzyme to form a stable but noncovalent ternary complex EPSPS-S3P-glyphosate, leading to the loss of EPSPS enzyme activity. This diversion causes a substantial flow of carbon sources towards S3P, resulting in rapid accumulation of shikimic acid within tissues. Consequently, the synthesis of essential aromatic amino acids (AAA) necessary for protein biosynthesis is severely hindered, ultimately inhibiting plant growth (Duke and Powles 2009; Wiersma et al. 2015).

Frequent use of glyphosate can lead to the development of glyphosate resistance in weeds, with amplification of the *EPSPS* gene being the primary mechanism conferring glyphosate resistance in Amaranthus populations (Mahajan et al. 2018). Chao Ouyang discovered a naturally evolved TIPS-EPSPS mutation that has been shown to confer high resistance to glyphosate in goosegrass, with resistance levels 180 times that of the wild type (WT) (Ouyang et al. 2021). Overexpression of the *EPSPS* gene and site-specific mutations can enhance plant resistance to glyphosate to varying degrees. When the *EPSPS* gene is overexpressed, EPSPS enzyme synthesis increases, and recommended field doses of glyphosate do not kill the plants, thereby enhancing glyphosate resistance (Vázquez-García et al. 2020; Chandi et al. 2012; Alcántara-de la Cruz et al. 2016).

Although EPSPS enzyme is the sole key enzyme in the shikimate pathway (Ge et al. 2012), so far, the Class II *EPSPS* genes sourced from bacteria have been most widely studied and utilized in plants, while research on Class I *EPSPS* genes from plants remains insufficiently explored. Further investigation is needed to determine whether overexpressing endogenous *EPSPS* genes or optimizing them via codon usage can enhance plant resistance to glyphosate. Glyphosate reduces photosynthetic efficiency, increases chlorophyll degradation, inhibits chlorophyll function, carotenoid synthesis, ferredoxin enzyme activity, auxin transduction, and increases auxin oxidation (Ozturk et al. 2008; Vivancos et al. 2011). This study measured changes in chlorophyll content, soluble sugars, malondialdehyde (MDA), relative conductivity, and ROS levels before and after glyphosate treatment in overexpressing and wild-type plants to assess glyphosate's impact on photosynthesis and plant stress resistance. The study explores whether codon optimization of maize's endogenous *EPSPS* gene can enhance glyphosate resistance, optimizing the gene's impact on the shikimate pathway, and the effects of glyphosate treatment on plants. This research lays a theoretical foundation for breeding glyphosate-resistant maize.

Materials and methods

Optimization of EPSPS gene and construction of overexpression vector

Using the online software GeneScript (https://www.genscript.com.cn/codon-opt.html), the codons of the endogenous *EPSPS* gene in maize were optimized to construct an expression vector.

Plant materials and growth conditions

Materials included WT Arabidopsis thaliana seeds and T_2 seeds overexpressing the *Y-EPSPS* gene. Seeds were sterilized in 75% ethanol for 1 min, followed by 1% sodium hypochlorite for 10 min, and then rinsed 5–6 times with sterile water. Seeds were placed on 1/2 MS solid medium without glyphosate or with glyphosate, and incubated at 4 °C in the dark for 3 days before being transferred to continuous light for germination. T_2 Arabidopsis seeds were germinated in vermiculite under conditions of 220 mmolm-2s-1 light intensity, a 16 h photoperiod, 8 h dark period, temperature maintained at 22–24 °C, relative humidity at 65%, and nutrient solution sprayed every 15 days.

Detection of overexpression positive plants

T_2 generation Arabidopsis thaliana overexpressing the *Y-EPSPS* gene was screened with a 1/1000 concentration of glyphosate, sprayed every 2 days. Surviving seedlings were transplanted into soil (nutrient soil:vermiculite = 8:3). Arabidopsis young leaves were ground with a pestle in 1.5 mL centrifuge tubes containing 200 μL buffer, followed by incubation of Bar test strips in the tubes for 5 min, and subsequent PCR detection. After covering the entire plastic tray with leaves from overexpressing plants, samples were collected for genomic extraction, and PCR detection of the screened marker gene Bar was conducted (primer sequences in Supplementary Material Table 1). Positive plants were utilized for subsequent physiological and biochemical indicator assays.

Glyphosate resistance of overexpressed positive seeds and plants

Wild-type Arabidopsis seeds and seeds overexpressing the *Y-EPSPS* gene were separately placed on 1/2 MS solid medium without glyphosate or supplemented with 0.5 and 0.8 mM/L glyphosate, and their germination rates were assessed. Arabidopsis plants overexpressing the *Y-EPSPS* gene were cultivated in nutrient soil. When these plants reached 4 weeks of age, they were subjected to 0.8 mM/L glyphosate stress. One week later, agronomic traits of the plants were statistically analyzed and photographed.

Expression analysis of *Y-EPSPS* gene under glyphosate stress

Four-week-old T_2 generation Arabidopsis overexpressing the *Y-EPSPS* gene were subjected to 0.8 mM/L glyphosate stress. Leaf samples were collected 3 days post-treatment, rapidly frozen in liquid nitrogen, and RNA was extracted for fluorescence quantitative PCR. The top two positive plants with the highest expression levels of the *Y-EPSPS* gene were selected for subsequent experiments.

Detection of H_2O_2 and O_2^- content

DAB staining buffer was prepared (0.02 g DAB dissolved in 50 mL water) and placed in 50 mL centrifuge tubes. The fifth leaf of 4-week-old Arabidopsis thaliana was immersed in DAB staining solution and vacuum-infiltrated for 30 min, followed by overnight incubation at room temperature. Chlorophyll was removed in 95% ethanol at 80 °C, and photographs of the staining results were taken (Wang et al. 2022).

NBT staining buffer was prepared (0.5 g NBT, 500 μL sodium azide solution, 500 μL 1 M sodium phosphate buffer, diluted to 50 mL with deionized water) and placed in 50 mL centrifuge tubes. The fifth leaf of 4-week-old Arabidopsis thaliana was soaked in NBT staining buffer for 60 min, washed in 80 °C water containing 95% ethanol until chlorophyll was completely removed, and then photographed for staining results (Geerts and Roels 1981).

Measurement of physiological and biochemical indicators

Hydrogen peroxide, superoxide anion, soluble sugars, malondialdehyde, chlorophyll content, and activities of peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and shikimate dehydrogenase (SD) were measured. Test kits were purchased from boxbio (http://www.boxbiological.com/) and experiments were conducted according to the manufacturer's instructions.

Fluorescence quantitative PCR

Total RNA was extracted from maize leaves using the Trizol method. cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Takara). qRT-PCR was performed using SYBR PreMix Ex Taq (Takara) with amplification conditions of: 95 °C for 10 min pre-denaturation, followed by 40 cycles of 95 °C denaturation for 10 s, 58 °C annealing for 20 s, and 72 °C extension for 15 s, with a melt curve analysis. Data were analyzed using the $2^{-\Delta\Delta t}$ method with *AtActin1* gene as an internal reference, and each sample was biologically replicated three times. Primers used in this study are listed in Supplementary Material Table 1.

Statistical analysis

All results in this study were repeated at least three times. Experimental quantitative data were statistically analyzed using SPSS 19.0 software (SPSS Inc.Chicago, IL, USA), and differences in treatment outcomes were confirmed using t tests. Significance levels were denoted as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Results

Codon optimization results and expression cassette frame in overexpression vectors

We optimized the codons of the *EPSPS* gene using online software and observed changes in codon preferences favoring maize compared to the original sequence (Fig. 1). The expression cassette is illustrated in Fig. 2.
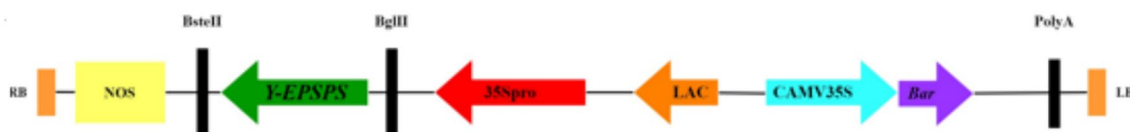

Fig. 1 Genetic transposition of the expression cassette

```
Total amino acid number: 506, MW=53357
Max ORF starts at AA pos 1(may be DNA pos 1) for 506 AA(1518 bases), MW=53357
            10        20        30        40        50        60        70        80        90       100       110       120       130       140       150
1    ATGGCAGCAATGGCAACTAAAGCCGCTGCTGGCACAGTTAGCCTTGACCTGGCCGCCCCAAGCAGGAGGCACCACCGTCCCAGCAGTGCAAGGCCACCAGCCCGGCCGGCAGTGAGAGGTCTTCGCGCGCCAGGACGCCGCGGTTATTGCA
1      M  A  A  M  A  T  K  A  A  A  G  T  V  S  L  D  L  A  A  P  S  R  R  H  H  R  P  S  S  A  R  P  P  A  R  P  A  V  R  G  L  R  A  P  G  R  R  V  I  A

            160       170       180       190       200       210       220       230       240       250       260       270       280       290       300
151  GCGCCTCCAGCAGCCGCGGCTGCAGCTGCAGTCCAGGCAGGGGCTGAGGAGATTGTGCTCCAGCCAATCAAGGAAATCAGTGGGACAGTGAAATTGCCTGGGAGTAAGTCTCTCAGCAACAGGATCCTGTTGCTGGCTGCCTTGAGTGAG
51     A  P  P  A  A  A  A  A  A  A  V  Q  A  G  A  E  E  I  V  L  Q  P  I  K  E  I  S  G  T  V  K  L  P  G  S  K  S  L  S  N  R  I  L  L  L  A  A  L  S  E

            310       320       330       340       350       360       370       380       390       400       410       420       430       440       450
301  GGCCACCACCGTGGTGGATAACTTGCTCAATTCCGAGGACGTCCACTACATGCTCGGTGCGCTCCGGACGCTCGGTCTGTCAGTGGAAGCAGATAAGGCGGCCAAGAGGGCCGTCGTTGTCGGGTGCGGCGGGAAGTTCCCTGTGGAGGAT
101    G  T  T  V  V  D  N  L  L  N  S  E  D  V  H  Y  M  L  G  A  L  R  T  L  G  L  S  V  E  A  D  K  A  A  K  R  A  V  V  V  G  C  G  G  K  F  P  V  E  D

            460       470       480       490       500       510       520       530       540       550       560       570       580       590       600
451  GCGAAAGAGGAGGTGCAGCTTTTCTTGGGAAACGCCGGGACTGCGATGCGGCCACTTACTGCGGCCGTGACCGCCGCTGGCGGCAACGCAACATACGTGTTGGATGGCGTCCCCAGGATGCGCGGAGAGCCCATCGGCGACCTTGTGGTC
151    A  K  E  E  V  Q  L  F  L  G  N  A  G  T  A  M  R  P  L  T  A  A  V  T  A  A  G  G  N  A  T  Y  V  L  D  G  V  P  R  M  R  E  R  P  I  G  D  L  V  V

            610       620       630       640       650       660       670       680       690       700       710       720       730       740       750
601  GGCCTCAAGCAACTCGGAGCGGATGTTGATTGCTTCCTCGGGACCGATTGCCCACCCGTGAGGGTGAACGGTATTGGTGGTTTGCCAGGCGGTAAGGTCAAGTTGTCAGGATCTATCAGTAGTCAGTACCTCAGTGCACTCCTCATGGCC
201    G  L  K  Q  L  G  A  D  V  D  C  F  L  G  T  D  C  P  P  V  R  V  N  G  I  G  G  L  P  G  G  K  V  K  L  S  G  S  I  S  S  Q  Y  L  S  A  L  L  M  A

            760       770       780       790       800       810       820       830       840       850       860       870       880       890       900
751  GCTCCTTTGGCTCTCGGAGACGTGGAAATCGAGATTATAGATAAGCTCATAAGCATCCCATATGTGGGAAATGACCCTGCGGCTTATGGAGCGCTTTGGCGTGAAGGCAGAGCACTCAGATTCCTGGGACCGCTTTTACATAAAGGGCGGG
251    A  P  L  A  L  G  D  V  E  I  E  I  I  D  K  L  I  S  I  P  Y  V  E  M  T  L  R  L  M  E  R  F  G  V  K  A  E  H  S  D  S  W  D  R  F  Y  I  K  G  G

            910       920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040      1050
901  CAGAAGTACAAGTCACCGAAGAATGCATACGTCGAGGGGGACGCCAGTTCCGCGTCATACTTCCTTGCCGGCGCAGCTATCACAGGTGGTACCGTTACGGTGGAGGGGTGCGGCACAACATCCTTGCAGGGAGACGTTAAGTTCGCCGAA
301    Q  K  Y  K  S  P  K  N  A  Y  V  E  G  D  A  S  S  A  S  Y  F  L  A  G  A  A  I  T  G  G  T  V  T  V  E  G  C  G  T  T  S  L  Q  G  D  V  K  F  A  E

           1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
1051 GTTCTCGAGATGATGGGAGCTAAAGTGACCTGGACGGAGACATCGGTGACAGTGACGGGGCCGCCCAGGGAGCCCTTCGGAAGGAAGCATCTGAAGGCCATCGACGTGAATATGAATAAGATGCCAGACGTGGCCATGACGCTCGCGGTG
351    V  L  E  M  M  G  A  K  V  T  W  T  E  T  S  V  T  V  T  G  P  P  R  E  P  F  G  R  K  H  L  K  A  I  D  V  N  M  N  K  M  P  D  V  A  M  T  L  A  V

           1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320      1330      1340      1350
1201 GTGGCGCTGTTCGCGGATGGTCCGACAGCCATTCGCGATGTGGCATCTTGGCGTGTTAAGGAAACAGAACGCATGGTGGCTATTCGCACTGAGCTGACAAAGCTCGGCGCCAGCGTCGAGGAGGGGCCCGACTACTGTATTATCACTCCT
401    V  A  L  F  A  D  G  P  T  A  I  R  D  V  A  S  W  R  V  K  E  T  E  R  M  V  A  I  R  T  E  L  T  K  L  G  A  S  V  E  E  G  P  D  Y  C  I  I  T  P

           1360      1370      1380      1390      1400      1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
1351 CCCGAAAAACTCAACGTTACGGCCATCGACACTTACGATGACCACCGGATGGCGATGGCCTTCTCTCTCGCAGCCTGCGCTGAAGTCCCGGTCACAATCAGAGATCCCGGTTGCACCCGCAAGACATTCCCAGATTACTTCGACGTGCTT
451    P  E  K  L  N  V  T  A  I  D  T  Y  D  D  H  R  M  A  M  A  F  S  L  A  A  C  A  E  V  P  V  T  I  R  D  P  G  C  T  R  K  T  F  P  D  Y  F  D  V  L

           1510      1520
1501 TCTACGTTCGTCAAAAACTAA
501    S  T  F  V  K  N  *
```

Fig. 2 *Y-EPSPS* Amino acid sequence

Results of overexpression in transgenic plants

Following PCR validation and strip tests (Fig. 3A, C), we successfully obtained 15 positive overexpression transgenic plants. Fluorescence quantitative PCR indicated that plants OE4 and OE13 exhibited the highest gene expression levels (Fig. 3B). Consequently, we selected these strains for propagation and subsequent experiments.

Glyphosate resistance in *Y-EPSPS* overexpressing plants

EPSPS enzyme is the sole target of glyphosate action (Ge et al. 2014), reacting to glyphosate. *Y-EPSPS* overexpressing and wild-type (WT) plants were separately inoculated on 1/2MS solid media containing different concentrations of glyphosate, and seed germination was observed after 8 days. We found that under glyphosate-free conditions, germination in overexpressing plants was similar to WT plants and remained unaffected. However, in media containing 0.5 mM glyphosate, both overexpressing and WT plants showed reduced germination, with higher germination rates observed in overexpressing plants: WT 54.02%, E-OE4 74.44%, E-OE13 80% (Fig. 4A, C). In media with 0.8 mM glyphosate, significant inhibition of germination was observed in all strains: WT 34.48%, E-OE4 52.22%, E-OE13 43.48% (Fig. 4A, C). After spraying 0.8 mM glyphosate solution on overexpressing and WT plants, overexpressing plants remained viable while WT plants perished (Fig. 4B). These results demonstrate that the *Y-EPSPS* gene can reduce sensitivity of Arabidopsis to glyphosate, enhancing its resistance.

Glyphosate treatment increases H_2O_2 and O_2^- levels

Reactive oxygen species (ROS) are crucial signaling molecules in response to various stress processes. Changes in hydrogen peroxide and superoxide anion levels play a vital role in plant stress (Sedigheh et al. 2011). Therefore, assessing ROS levels in *Y-EPSPS* overexpressing and WT plants is crucial. The sixth leaf of control and glyphosate-treated Arabidopsis plants was isolated and immersed in NBT and DAB staining solutions to reveal hydrogen peroxide and superoxide anion levels. Results showed no significant difference between overexpressing plants and WT under normal conditions. However, under glyphosate treatment, overexpressing plants exhibited lighter leaf color compared to WT (Fig. 5A, B). Quantitative results of H_2O_2 and O_2^- were consistent with chemical staining results (Fig. 5C, D). These findings indicate that *Y-EPSPS* overexpression reduces ROS accumulation in Arabidopsis leaves, thereby conferring glyphosate resistance.
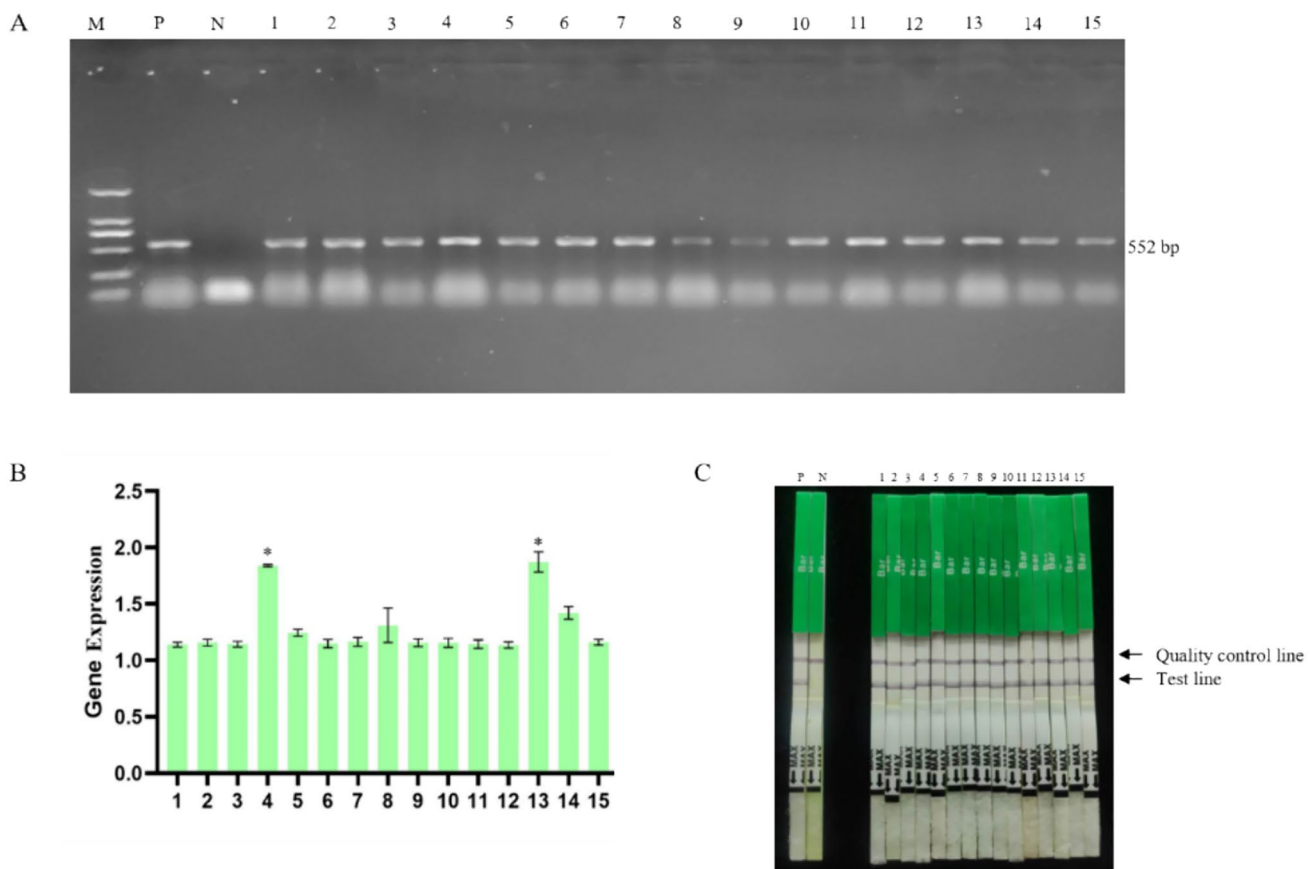
Fig. 3 Results of overexpressed positive plants. M: DNA Marker DL 2000; P: positive control; N: water; 1–15: Overexpressed positive plants. **A** PCR test results. **B** qPCR test results. **C** Test result of bar gene test strip. P: Positive control; N: Negative control; 1–15: Over-expressed positive plants. The student's test was used, with asterisks indicating statistically significant differences (*p < 0.05; **p < 0.01). The data is shown as the mean ± SD from three independent experiments
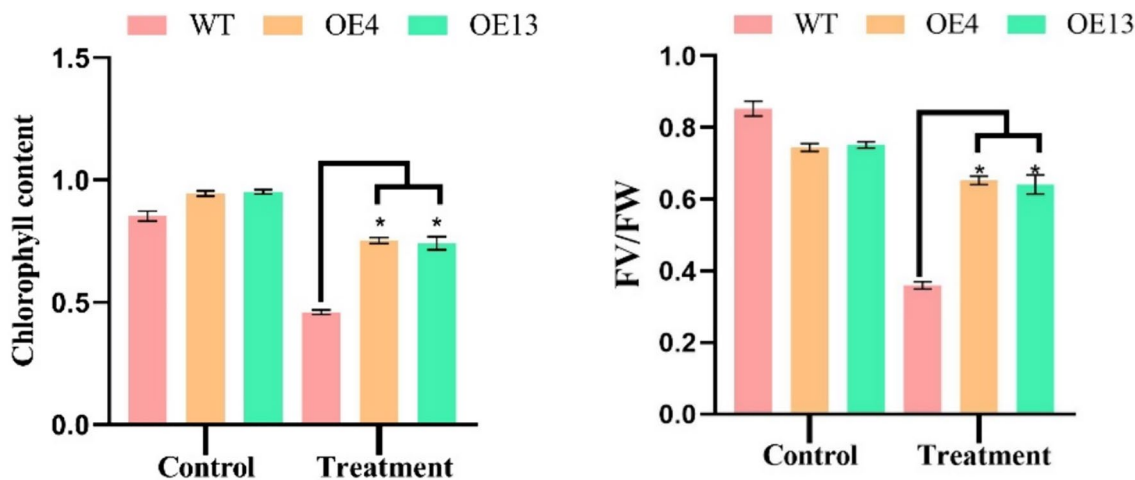
Impact of *Y-EPSPS* gene on chlorophyll content and Fv/Fm

To further understand the role of the *Y-EPSPS* gene in glyphosate resistance, two *Y-EPSPS* overexpressing Arabidopsis plants were genetically transformed, and their chlorophyll content and Fv/Fm were measured. Before glyphosate treatment, chlorophyll content and Fv/Fm levels were similar between overexpressing and WT plants. However, after glyphosate treatment, overexpressing plants showed significantly higher chlorophyll content and Fv/Fm compared to WT plants (Fig. 6). This suggests that overexpressing the *Y-EPSPS* gene reduces sensitivity of plants to glyphosate, mitigating its toxic effects.

Y-EPSPS gene enhances soluble sugar and MDA content

We measured the levels of soluble sugars and MDA in both *Y-EPSPS* overexpressing plants and wild-type plants before and after glyphosate treatment. Initially, there was nearly no difference in soluble sugar content between overexpressing and wild-type plants prior to glyphosate treatment. However, post-treatment, the soluble sugar content in overexpressing plants significantly exceeded that of WT plants. Similarly, before glyphosate treatment, there was minimal difference in MDA content between overexpressing and WT plants. Yet, after glyphosate treatment, MDA levels in overexpressing plants notably decreased compared to WT plants (Fig. 7), indicating enhanced stress resistance and antioxidant capability, thereby reducing glyphosate-induced damage and conferring resistance to glyphosate.

Y-EPSPS gene alters ROS-related enzyme activities

The activity of reactive oxygen species ROS-related enzymes is an important indicator of the plant's ability to scavenge ROS. Therefore, we assessed the activity of ROS-related enzymes. The results showed that before glyphosate treatment, the levels of POD, SOD, and CAT activities were similar between overexpressing plants and wild-type (WT) plants. After glyphosate treatment, however, the enzyme

Fig. 4 Results of positive seed germination experiment and glyphosate resistance. **A** Germination results of Arabidopsis overexpressed positive seeds with different concentrations of glyphosate. **B** Resistance results of overexpressed Arabidopsis positive plants to 0.8 mM glyphosate spray. **C** Seed germination of Arabidopsis thaliana under different concentrations of glyphosate. The student's test was used, with asterisks indicating statistically significant differences (*p < 0.05; **p < 0.01)
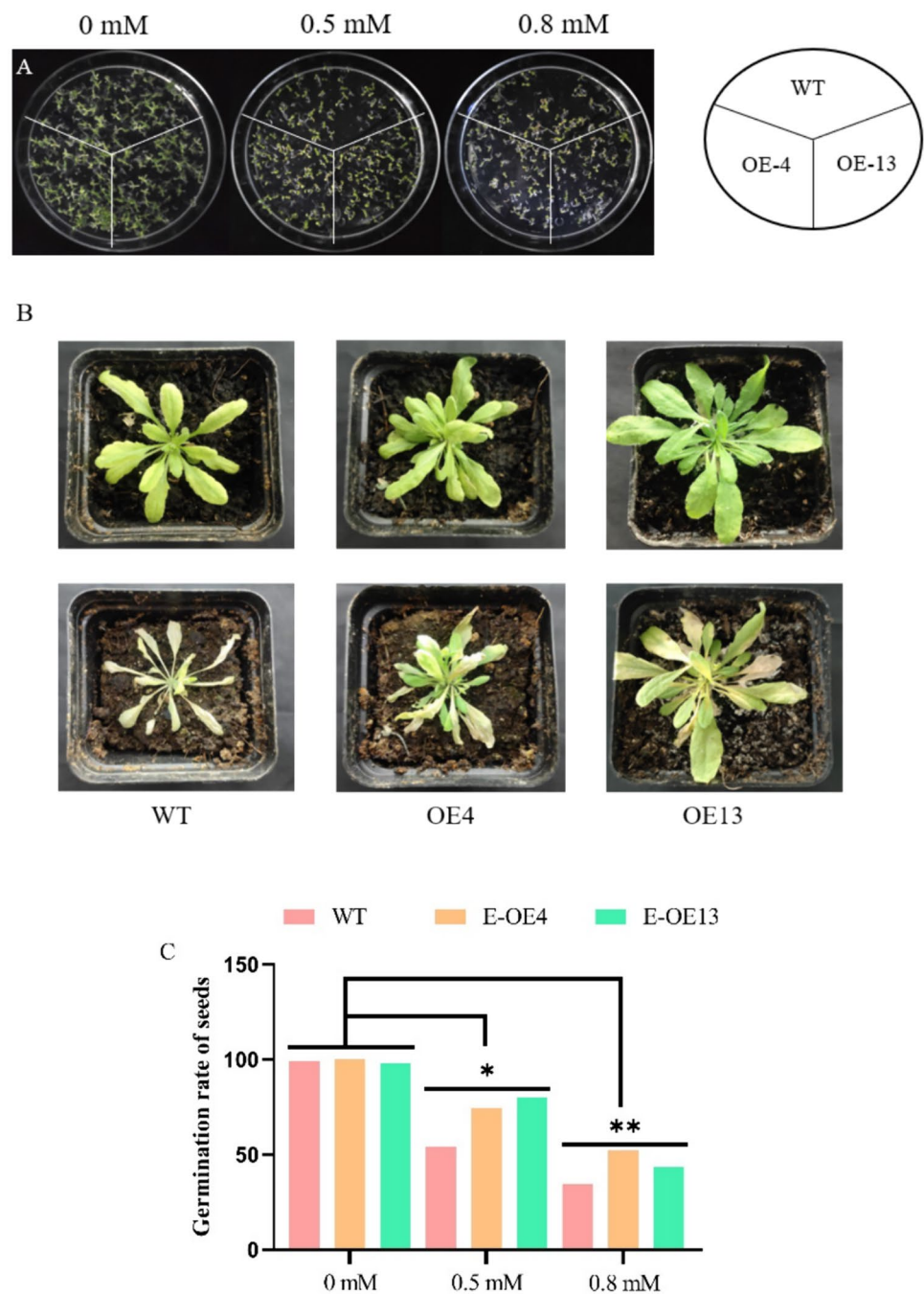
activities of POD, SOD, and CAT in overexpressing plants were significantly higher than those in WT plants (Fig. 8). This enables the breakdown of higher and more oxidative molecules produced in plants, reducing glyphosate's toxic effects on plants.

Y-EPSPS gene reduces shikimate dehydrogenase activity

We measured the activity of shikimate dehydrogenase (SD), a bifunctional enzyme essential for shikimate acid synthesis. Before glyphosate treatment, SD activity showed little difference between overexpressing and wild-type plants. However, post-treatment, SD activity decreased in both overexpressing and wild-type plants. Yet, SD activity in overexpressing plants was notably higher than in WT plants, reaching twice the level observed in WT plants (Fig. 9). This suggests that glyphosate reduces EPSPS enzyme activity, leading to excessive accumulation of shikimate acid in plants, thereby inhibiting SD enzyme activity.
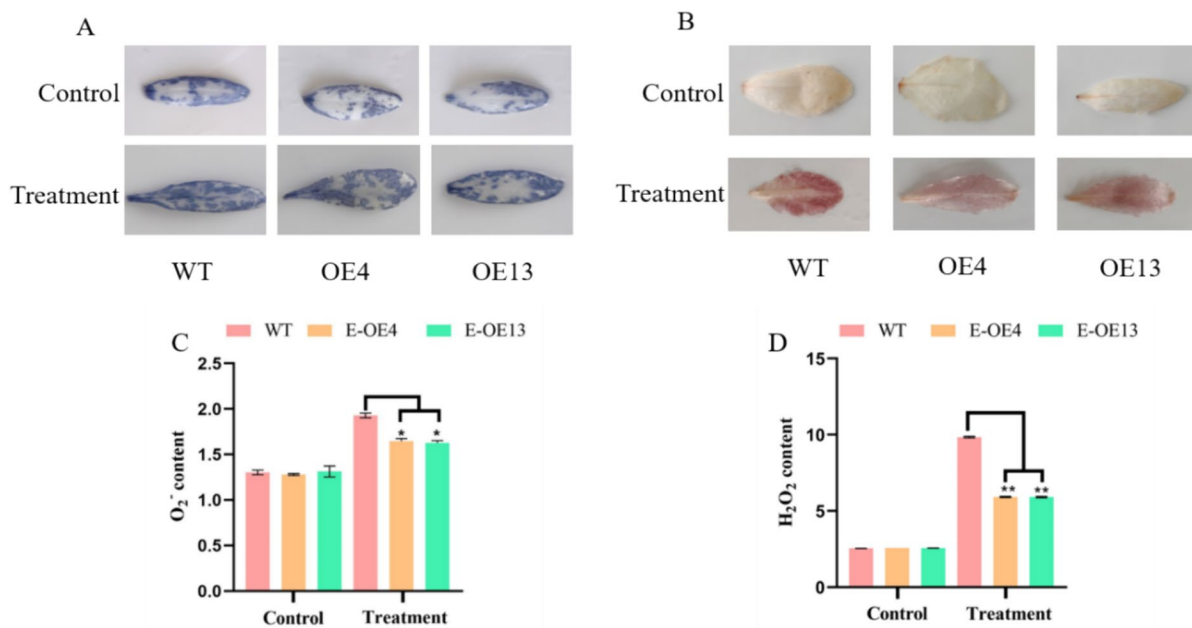
Fig. 5 H_2O_2 and O_2^- content detection results. **A, B** NBT, DAB staining. Overexpressed and positive plants at 4 weeks of age were stained. **C, D** The amount of H_2O_2 and O_2^- accumulated in plants. The student's test was used, with asterisks indicating statistically significant differences (*$p < 0.05$; **$p < 0.01$). The data is shown as the mean ± SD from three independent experiments

Fig. 6 Chlorophyll content and Fv/Fm detection results. The student's test was used, with asterisks indicating statistically significant differences (*$p < 0.05$; **$p < 0.01$). The data is shown as the mean ± SD from three independent experiments

Impact of *Y-EPSPS* gene on ROS detoxification genes, stress response genes, and shikimate synthesis enzyme gene expression under glyphosate stress

Under glyphosate stress, overexpression of the *Y-EPSPS* gene partially reduces H_2O_2 and O_2^- levels in plants. These findings indicate that *Y-EPSPS* is involved in ROS regulation and participates in plant growth and development. In this study, we analyzed the expression of several ROS detoxification genes (*AtCAT3*: Gene ID: 838651 and *AtSOD1*: Gene ID: 837405), stress defense-related genes (*AtLTP3*: Gene ID: 836051 and *AtSOS1*: Gene ID: 814729), and the *DQSD* gene (Gene ID: 819809) (primer sequences in Supplementary Table 1) to determine the role of *Y-EPSPS* in glyphosate resistance. Results showed that gene expression levels increased post glyphosate treatment (Fig. 10), in response to glyphosate stress, thereby reducing glyphosate toxicity.
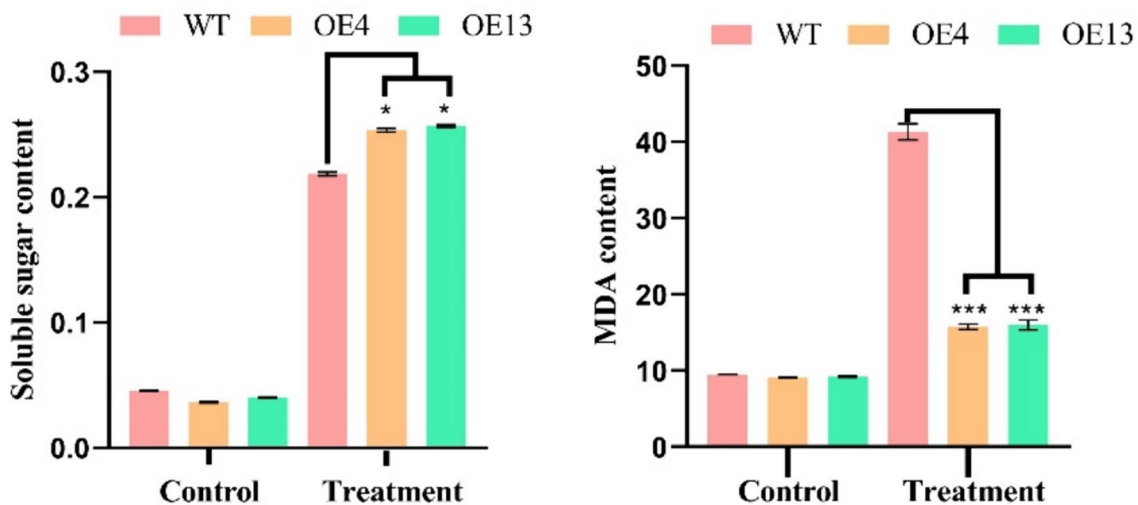
Fig. 7 The content of soluble sugar and MDA was detected. The student's test was used, with asterisks indicating statistically significant differences (*p < 0.05; **p < 0.01; ***p < 0.001). The data is shown as the mean ± SD from three independent experiments
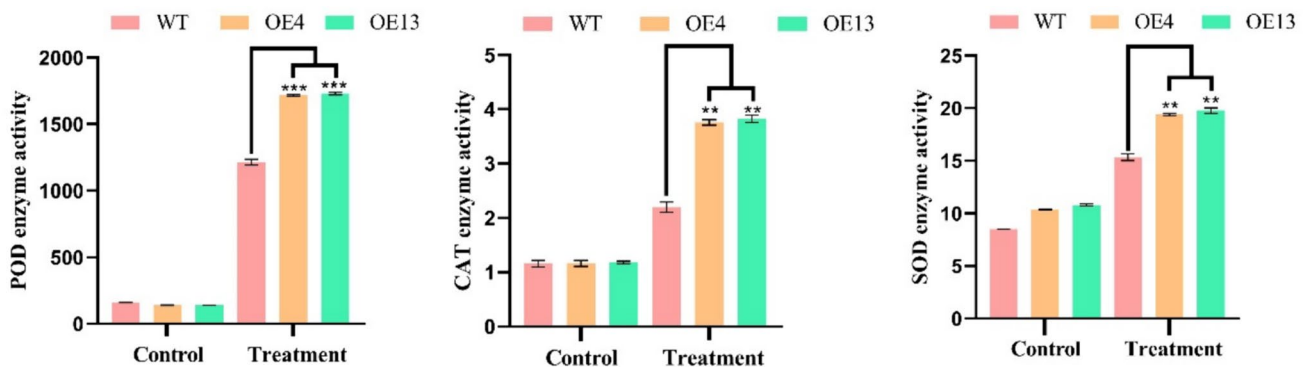
Fig. 8 Results of POD, SOD and CAT enzyme activity. The student's t test was used, with asterisks indicating statistically significant differences (*p < 0.05; **p < 0.01: ***p < 0.001). The data is shown as the mean ± SD from three independent experiments

Discussion

To produce high-quality glyphosate-resistant maize, we utilized the floral dip method to transfer the optimized *Y-EPSPS* gene into Arabidopsis thaliana (Ali et al. 2022; Zhang et al. 2006), resulting in 15 overexpressing plants. We assessed changes in chlorophyll, soluble sugars, MDA content, relative conductivity, and ROS levels in both overexpressing and wild-type plants before and after glyphosate treatment. Additionally, changes in enzyme activities such as POD, SOD, CAT, and SD were analyzed. Expression patterns of genes related to chlorophyll synthesis, ROS scavenging, and shikimate pathway were also examined.

Codon optimization involves altering gene sequences to enhance the expression of recombinant proteins. The degeneracy of the genetic code allows multiple codon sequences to encode the same protein. Codon usage bias prioritizes the use of specific synonymous codons (Gillen et al. 2021; Fu et al. 2020; López et al. 2020). Therefore, codon optimization can increase gene expression by altering the gene sequence without changing the amino acid sequence, thereby boosting protein content within cells. Traditionally, optimal codons are thought to enhance translation efficiency due to faster ribosomal movement and release along mRNA (Andersson and Kurland 1990; Ehrenberg and Kurland 1984; Sørensen and Pedersen 1991), potentially leading to faster growth rates through more efficient ribosome utilization. Another view suggests that optimal codon usage improves translation accuracy (Akashi 1994).

This study employed codon optimization to make the *EPSPS* gene sequence more favorable for maize-specific
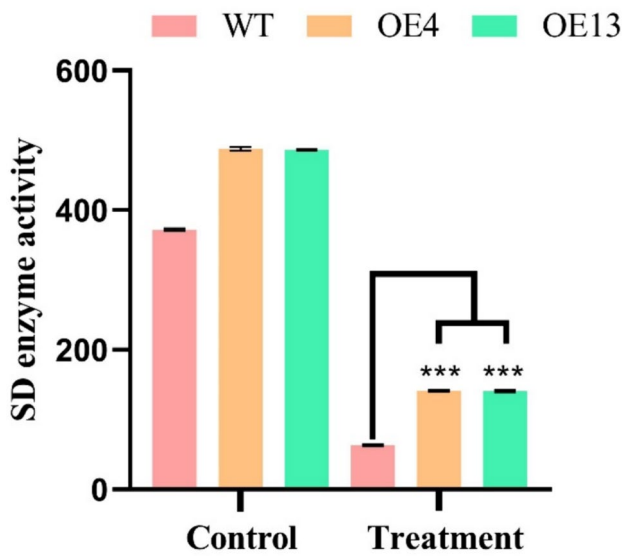
Fig. 9 Results of SD enzyme activity detection. The student's test was used, with asterisks indicating statistically significant differences (*p < 0.05; **p < 0.01: ***p < 0.001). The data is shown as the mean ± SD from three independent experiments

codon usage, enhancing *EPSPS* expression in plants. This increased the content of EPSP synthase expressed in Arabidopsis plants and verified its activity through indoor glyphosate tolerance experiments, aiming to acquire a novel *EPSPS* gene that enhances glyphosate tolerance.

SD is a key enzyme catalyzing the fourth step of the shikimate biosynthetic pathway (Yuan et al. 2022). In plants, SDH forms a DQD/SDH complex through its N-terminal to enhance metabolic cycling efficiency, and its activity analysis is crucial for shikimate pathway analysis (Akhlaghi et al. 2018). SD enzyme catalyzes shikimate to 3-dehydrogenated shikimic acid, while EPSPS catalyzes shikimate to 5-enol-shikimic acid, eventually synthesizing the aromatic amino acid precursor chorismate (Choi Si-Sun et al. 2019). Glyphosate competitively binds to EPSPS reversibly, forming a stable but non-covalent ternary complex EPSPS-S3P-glyphosate, leading to loss of EPSPS enzyme activity. This diverts substantial carbon sources towards shikimic acid, resulting in its rapid accumulation within tissues and severely hindering the synthesis of aromatic amino acids essential for protein biosynthesis, ultimately inhibiting plant growth (Duke and Powles 2009; Wiersma et al. 2015). Glyphosate spraying inhibits SD activity in plants. It is speculated that glyphosate
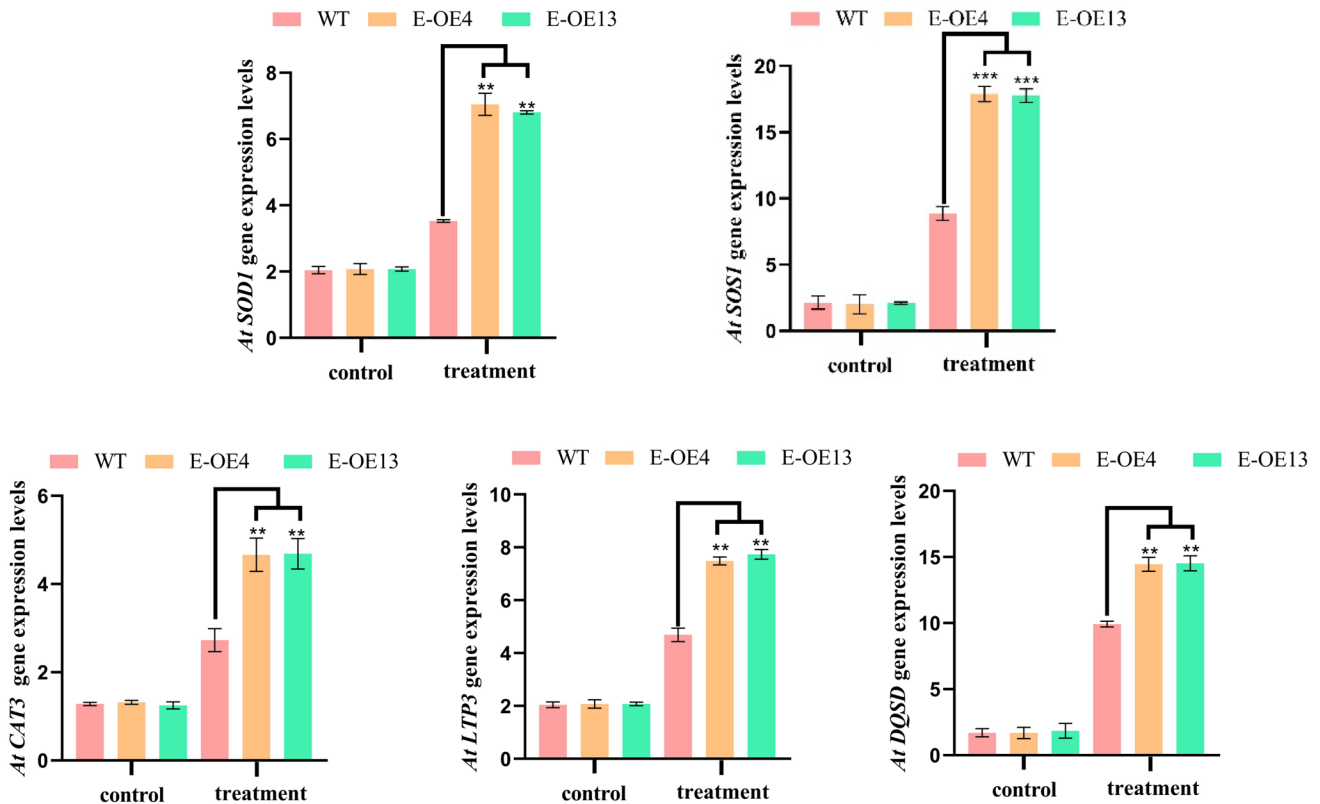
Fig. 10 Effects of *Y-EPSPS* gene on the expression of active oxygen-related genes, stress response genes and shikimic acid pathway genes. Expression analysis of ROS (**A**, **B**), stress response (**C**, **D**) and shikimic acid pathway related genes (**E**) in Col-0, transgenic lines OE4 and OE13 under control and glyphosate stress conditions. The student's test was used, with asterisks indicating statistically significant differences (*p < 0.05; **p < 0.01: ***p < 0.001). The data is shown as the mean ± SD from three independent experiments

forms a ternary complex with EPSPS, blocking the shikimate pathway and causing accumulation of shikimic acid, which in turn downregulates SD activity due to feedback regulation by shikimic acid, leading to decreased enzyme activity.

Previous studies have found that glyphosate can promote the formation of plant ROS (Jalal et al. 2021). In this study, following glyphosate stress, accumulations of H_2O_2 and O_2^- were observed in the leaves of both overexpressing plants and wild-type (WT) plants, though the accumulation in overexpressing plants was lower than in WT plants. Although the accumulation of O_2^- increased, it was not as significant as the accumulation of the ROS H_2O_2, which may be due to biochemical differences between these two ROS. Superoxide is a transient molecule, whereas hydrogen peroxide is the most stable ROS, making it easier to accumulate (Shaner et al. 2005). POD, CAT, and SOD constitute the most important protective enzyme systems in plants and are crucial for scavenging reactive oxygen species (ROS) (Shafiq et al. 2021). The activities of POD, CAT, and SOD indirectly reflect the vigor of plants. SOD is responsible for converting O_2^- to H_2O_2, while CAT and POD degrade H_2O_2 to H_2O and O_2, thereby clearing ROS generated by glyphosate stress and maintaining dynamic balance of ROS in plants (Hu et al. 2018; Jiang et al. 2023; Zhao et al. 2005). This study found that the enzyme activities of POD, SOD, and CAT decreased in plants subjected to glyphosate stress, with overexpressing plants showing higher enzyme activities compared to WT plants, suggesting that the *Y-EPSPS* gene can mitigate ROS accumulation and oxidative damage caused by glyphosate stress to some extent. After glyphosate stress, both malondialdehyde (MDA) and soluble sugar content increased in both overexpressing and wild-type plants. In this study, the soluble sugar content in overexpressing plants was higher than in wild-type plants, whereas the MDA content was lower (You et al. 2012). MDA, an active metabolic byproduct, is often used as an indicator of oxidative stress. When plant cells undergo oxidative damage, MDA accumulates inside the cells and may react with molecules such as proteins, nucleic acids, and lipids, leading to abnormal cellular functions or even cell death (Mao et al. 2023; Gill and Tuteja 2010; Noctor 2006). Numerous studies have indicated that adverse environments enhance lipid peroxidation in leaf tissues of plants, resulting in increased MDA content and relative conductivity. Lipid peroxidation is a secondary effect of oxidative stress, which has been observed following glyphosate stress in various plants, including soybean, rice (*Oryza sativa* L.), tobacco (*Nicotiana tabacum* L.), and peanut (*Arachis hypogaea* L.) (Li et al. 2019; Vennapusa et al. 2022; Daramola et al. 2023). The content of soluble sugars in plants typically reflects their resistance to stress because sugars serve as both an energy source and antioxidants, aiding plants in coping with adverse

conditions (Barco-Antoñanzas et al. 2022). As signaling molecules for nutrition and metabolism, soluble sugars are crucial for maintaining plant structure, metabolism, growth, and cellular osmotic balance (Barco-Antoñanzas et al. 2022; Fernándes-Escalada et al. 2016). Therefore, the content of soluble sugars in plants serves as an important indicator for evaluating their physiological status and stress responses. The increase in soluble sugar content indicates that overexpressing plants exhibit greater tolerance to glyphosate (Li et al. 2020).

After spraying glyphosate, the chlorophyll content of both overexpressing and wild-type plants significantly decreased, while soluble sugars, MDA, relative conductivity, and ROS content markedly increased. The changes in overexpressing plants and wild-type plants were consistent with plant stress responses. Previous research has also indicated that glyphosate spraying reduces leaf area, subsequently lowering photosynthetic rates and aboveground biomass (Hoagland et al. 1980; Ding et al. 2008). Following glyphosate treatment, chlorophyll content in overexpressing plants initially declined but reached levels similar to untreated WT plants by day 30. This suggests glyphosate impacts plants, even those tolerant to it. Additionally, soluble sugars, MDA, relative conductivity, and ROS content in overexpressing plant leaves showed significant changes post-glyphosate treatment, albeit lower than WT plants. Therefore, these overexpressing plants exhibit significant resistance to glyphosate.

In this study, glyphosate treatment affected MDA, soluble sugars, and ROS production. As demonstrated, chlorophyll content decreased in both overexpressing and wild-type Arabidopsis. Glyphosate likely induces oxidative stress, leading to several minor or indirect effects on plant physiology (Gomes et al. 2014). As a result of oxidative stress, glyphosate damages plants, prompting increased production of soluble sugars in response to the damage. Glyphosate disrupts chlorophyll, impacting photosynthetic rates and metabolism. It affects the shikimate pathway, hindering plant growth and development. Ultimately, glyphosate can kill plants. Glyphosate increases ROS activity within plant tissues, and the plant's ability to scavenge ROS is crucial for enhancing glyphosate tolerance. Overexpressing the *Y-EPSPS* gene promotes synthesis of aromatic amino acids, enhancing the plant's ability to clear active substances. Previous studies utilized exogenous *EPSPS* genes sourced from other plants. This study seeks to identify endogenous *EPSPS* genes in plants and optimize their codons to enhance glyphosate tolerance. Optimizing endogenous genes can stabilize genetic traits and reduce responses to the biotic environment. To further explore the biological function of the *Y-EPSPS* gene, future work will involve yeast hybridization, dual luciferase assays, and HPLC detection. A series of experiments will also be conducted on maize to observe phenotypic changes in transgenic maize and measure related

physiological and biochemical indicators. This research lays a solid foundation for understanding the herbicide resistance mechanisms in maize, thereby benefiting maize breeding efforts.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval and consent to participate Not applicable.

References

Akashi H (1994) Synonymous codon usage in Drosophila melanogaster: natural selection and translational accuracy. Genetics 136(3):927–935. https://doi.org/10.1093/genetics/136.3.927

Akhlaghi M, Ghobadi S, Mohammad Hosseini M, Gholami Z, Mohammadian F (2018) Flavanols are potential anti-obesity agents, a systematic review and meta-analysis of controlled clinical trials. Nutr Metab Cardiovasc Dis 28(7):675–690. https://doi.org/10.1016/j.numecd.2018.04.001

Alcántara-de la Cruz R, Barro F, Domínguez-Valenzuela JA et al (2016) Target and non-target site mechanisms developed by glyphosate-resistant hairy beggarticks (*Bidens pilosa* L.). Planta 244:365–375. https://doi.org/10.3389/fpls.2016.01492

Ali I, Sher H, Ali A, Hussain S, Ullah Z (2022) Simplified floral dip transformation method of *Arabidopsis thaliana*. J Microbiol Methods 197:106492. https://doi.org/10.1016/j.mimet.2022.106492

Andersson SG, Kurland CG (1990) Codon preferences in free-living microorganisms. Microbiol Rev 54(2):198–210. https://doi.org/10.1128/mr.54.2.198-210.1990

Barco-Antoñanzas M, Gil-Monreal M, Eceiza MV, Royuela M, Zabalza A (2022) Primary metabolism in an *Amaranthus palmeri* population with multiple resistance to glyphosate and pyrithiobac herbicides. Plant Sci 318:111212. https://doi.org/10.1016/j.plantsci.2022.111212

Chandi A, Milla-Lewis SR, Giacomini D, Westra P, Preston C, Jordan DL et al (2012) Inheritance of evolved glyphosate resistance in a North Carolina Palmer Amaranth (*Amaranthus palmeri*) Biotype. Int J Agron 2012:176108. https://doi.org/10.1155/2012/176108

Chhapekar S, Raghavendrarao S, Pavan G, Ramakrishna C, Singh VK, Phanindra ML, Dhandapani G et al (2015) Transgenic rice expressing a codon-modified synthetic CP4-EPSPS confers tolerance to broad-spectrum herbicide, glyphosate. Plant Cell Rep 34:721–731. https://doi.org/10.1007/s00299-014-1732-2

Daramola OS, Kharel P, Iboyi JE, Devkota P (2023) Response of peanut (*Arachis hypogaea* L.) to sublethal rates of dicamba plus glyphosate at different growth stages. Agron J 115(4):1694–1704. https://doi.org/10.1002/agj2.21372

Dill GM, Cajacob CA, Padgette SR (2008) Glyphosate-resistant crops: adoption, use and future considerations. Pest Manag Sci 64:326–331. https://doi.org/10.1002/ps.1501

Ding X, Cao Y, Huang L, Zhao J, Xu C, Li X, Wang S (2008) Activation of the indole-3-acetic acid-amido synthetase GH3-8 suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice. Plant Cell 20(1):228–240. https://doi.org/10.1105/tpc.107.055657

Duke SO (2011) Glyphosate degradation in glyphosate-resistant and susceptible crops and weeds. J Agric Food Chem 59(11):5835–5841. https://doi.org/10.1021/jf102704x

Duke SO, Powles SB (2009) Glyphosate: a once-in-a-century herbicide. Pest Manag Sci 65:319–327. https://doi.org/10.1002/ps.1518

Ehrenberg M, Kurland CG (1984) Costs of accuracy determined by a maximal growth rate constraint. Q Rev Biophys 17(1):45–82. https://doi.org/10.1017/s0033583500005254

Fernandez-Escalada M, Gil-Monreal M, Zabalza A, Royuela M (2016) Characterization of the *Amaranthus palmeri* physiological response to glyphosate in susceptible and resistant populations. J Agric Food Chem 64:95–106. https://doi.org/10.1021/acs.jafc.5b04916

Fu H, Liang Y, Zhong X et al (2020) Codon optimization with deep learning to enhance protein expression. Sci Rep 10(1):17617. https://doi.org/10.1038/s41598-020-74091-z

Funke T, Han H, Healy-Fried ML, Fischer M, Schönbrunn E (2006) Molecular basis for the herbicide resistance of Roundup Ready crops. Proc Natl Acad Sci 103(35):13010–13015. https://doi.org/10.1073/pnas.0603638103

Ge X, d'Avignon DA, Ackerman JJ (2012) Vacuolar glyphosate-sequestration correlates with glyphosate resistance in ryegrass (Lolium spp.) from Australia, South America, and Europe: a 31P-NMR investigation. J Agric Food Chem 60(5):1243–1250. https://doi.org/10.1021/jf204089c

Gomes MP, Smedbol E, Chalifour A, Hénault-Ethier L, Labrecque M, Lepage L, Lucotte M, & Juneau P (2014) Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid: an overview. J Exp Bot 65:4691–4703. https://doi.org/10.1093/jxb/eru269

Geerts A, Roels F (1981) Quantitation of catalase activity by microspectrophotometry after diaminobenzidine staining. Histochemistry 72(3):357–367. https://doi.org/10.1007/BF00501778

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48(12):909–930. https://doi.org/10.1016/j.plaphy.2010.08.016

Gillen SL, Waldron JA, Bushell M (2021) Codon optimality in cancer. Oncogene 40(45):6309–6320. https://doi.org/10.1038/s41388-021-02022-x

Herrmann KM, Weaver LM (1999) The shikimate pathway. Annu Rev Plant Physiol 50(1):473–503. https://doi.org/10.1146/annurev.arplant.50.1.473

Hoagland RE (1980) Effects of glyphosate on metabolism of phenolic compounds: VI. Effects of glyphosine and glyphosate metabolites on phenylalanine ammonia-lyase activity, growth, and protein, chlorophyll, and anthocyanin levels in soybean (Glycine max) seedlings. Weed Sci 28(4):393–400. https://doi.org/10.1017/S0043174500055545

Hu Z, Weijian L, Yali F, Huiquan L (2018) Gibberellic acid enhances postharvest toon sprout tolerance to chilling stress by increasing the antioxidant capacity during the short-term cold storage. Sci Hortic 237:184–191. https://doi.org/10.1016/j.scienta.2018.04.018

Jalal A, Oliveira Junior JC, Ribeiro JS, Fernandes GC, Mariano GG, Trindade VDR, Reis ARD (2021) Hormesis in plants:

physiological and biochemical responses. Ecotoxicol Environ Saf 207:111225. https://doi.org/10.1016/j.ecoenv.2020.111225

Jiang W, Ye Q, Wu Z, Zhang Q, Wang L, Liu J, Hu X, Guo D, Wang X, Zhang Z, He H, Hu L (2023) Analysis of CAT gene family and functional identification of OsCAT3 in rice. Genes 14(1):138. https://doi.org/10.3390/genes14010138

Less H, Angelovici R, Tzin V, et al (2010) Principal transcriptional regulation and genome-wide system interactions of the Asp-family and aromatic amino acid networks of amino acid metabolism in plants. Amino Acids 39, 1023–1028. https://doi.org/10.1007/s00726-010-0566-7

Less H, Angelovici R, Tzin V et al (2010) Principal transcriptional regulation and genome-wide system interactions of the Asp-family and aromatic amino acid networks of amino acid metabolism in plants. Amino Acids 39(4):1023–1028. https://doi.org/10.1007/s00726-010-0566-7

Li WY, Lu P, Xie H, Li GQ, Wang JX, Guo DY, Liang XY (2019) Effects of glyphosate on soybean metabolism in strains bred for glyphosate-resistance. Physiol Mol Biol Plants 25(2):523–532. https://doi.org/10.1007/s12298-018-0597-1

Li X, Jia J, Zhao P, Guo X, Chen S, Qi D, Cheng L, Liu G (2020) LcMYB4, an unknown function transcription factor gene from sheepgrass, as a positive regulator of chilling and freezing tolerance in transgenic Arabidopsis. BMC Plant Biol 20(1):238. https://doi.org/10.1186/s12870-020-02427-y

López JL, Lozano MJ, Fabre ML, Lagares A (2020) Codon usage optimization in the prokaryotic tree of life: how synonymous codons are differentially selected in sequence domains with different expression levels and degrees of conservation. Mbio 11(4):e00766-20. https://doi.org/10.1128/mBio.00766-20

Lynch JH, Qian Y, Guo L et al (2020) Modulation of auxin formation by the cytosolic phenylalanine biosynthetic pathway. Nat Chem Biol 16:850-856. https://doi.org/10.1038/s41589-020-0519-8

Maeda H, Dudareva N (2012) The shikimate pathway and aromatic amino acid biosynthesis in plants. Annu Rev Plant Biol 63:73–105. https://doi.org/10.1146/annurev-arplant-042811-105439

Mahajan G, Matloob A, Walsh M, & Chauhan BS (2018) Germination Ecology of Two Australian Populations of African turnipweed (Sisymbrium thellungii). Weed Sci 66(6):752–757. https://doi.org/10.1017/wsc.2018.55

Mao H, Zhao W, Yang X, Sheng L, Zhu S (2023) Recruitment and metabolomics between Canna indica and rhizosphere bacteria under Cr stress. Front Microbiol 14:1187982. https://doi.org/10.3389/fmicb.2023.1187982

Noctor G (2006) Metabolic signalling in defence and stress: the central roles of soluble redox couples. Plant Cell Environ 29(3):409–425. https://doi.org/10.1111/j.1365-3040.2005.01476.x

Okumu MN, Vorster BJ, Reinhardt CF (2019) Growth-stage and temperature influence glyphosate resistance in Conyza bonariensis (L.) Cronquist. S Afr J Bot 121:248–256. https://doi.org/10.1016/j.sajb.2018.10.034

Ouyang C, Liu W, Chen S et al (2021) The naturally evolved EPSPS from goosegrass confers high glyphosate resistance to rice. Front Plant Sci 12:756116. https://doi.org/10.3389/fpls.2021.756116

Ozturk L, Yazici A, Eker S, Gokmen O, Romheld V, Cakmak I (2008) Glyphosate inhibition of ferric reductase activity in irondeficient sunflower roots. New Phytol 177(4):899–906. https://doi.org/10.1111/j.1469-8137.2007.02340.x

Pandey P, Irulappan V, Bagavathiannan M, Senthil-Kumar M (2017) Impact of combined abiotic and biotic stresses on plant growth and avenues for crop improvement by exploiting physio-morphological traits. Front Plant Sci 8:537. https://doi.org/10.3389/fpls.2017.00537

Parthasarathy A, Cross PJ, Dobson RCJ, Adams LE, Savka MA, Hudson AO (2018) A three-ring circus: metabolism of the three proteogenic aromatic amino acids and their role in the health of plants and animals. Front Mol Biosci 5:29. https://doi.org/10.3389/fmolb.2018.00029

Sedigheh HG, Mortazavian M, Norouzian D, Atyabi M, Akbarzadeh A, Hasanpoor K, Ghorbani M (2011) Oxidative stress and leaf senescence. BMC Res Notes 4:477. https://doi.org/10.1186/1756-0500-4-477

Shafiq S, Akram NA, Ashraf M, García-Caparrós P, Ali OM, Latef AAHA (2021) Influence of glycine betaine (natural and synthetic) on growth, metabolism and yield production of drought-stressed maize (Zea mays L.) Plants. Plants 10(11):2540. https://doi.org/10.3390/plants10112540

Shaner DL et al (2005) Glyphosate-resistant crops: history, status and future. Pest Manag Sci 61(3):219–224. https://doi.org/10.1002/ps.1008

Si-Sun C, Seung-Yeul S, Sun-Ok P, Han-Na L, Ji-soo S, Ji-yeon K, Ji-Hoon P, Sangyong K, Joung LS, Gie-Taek C, Eung-Soo K (2019) Cell factory design and culture process optimization for dehydroshikimate biosynthesis in Escherichia coli. Front Bioeng Biotechnol 2019:7–241. https://doi.org/10.3389/fbioe.2019.00241

Sørensen MA, Pedersen S (1991) Absolute in vivo translation rates of individual codons in Escherichia coli. The two glutamic acid codons GAA and GAG are translated with a threefold difference in rate. J Mol Biol 222(2):265–280. https://doi.org/10.1016/0022-2836(91)90211-n

Suh H, Hepburn AG, Kriz AL, Widholm JM (1993) Structure of the amplified 5-enolpyruvylshikimate-3-phosphate synthase gene in glyphosate-resistant carrot cells. Plant Mol Biol 22:195–205. https://doi.org/10.1007/BF00014928

Tzin V, Galili G (2010) New insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. Mol Plant 3(6):956–972. https://doi.org/10.1093/mp/ssq048

Vázquez-García JG, Alcántara-de la Cruz R, Palma-Bautista C, Rojano-Delgado AM, Cruz-Hipólito HE, Torra J, Barro F, De Prado R (2020) Accumulation of target gene mutations confers multiple resistance to ALS, ACCase, and EPSPS inhibitors in lolium species in chile. Front Plant Sci 11:553948. https://doi.org/10.3389/fpls.2020.553948

Vennapusa AR, Agarwal S, Rao Hm H, Aarthy T, Babitha KC, Thulasiram HV, Kulkarni MJ, Melmaiee K, Sudhakar C, Udayakumar M, Vemanna SR (2022) Stacking herbicide detoxification and resistant genes improves glyphosate tolerance and reduces phytotoxicity in tobacco (Nicotiana tabacum L.) and rice (Oryza sativa L.). Plant Physiol Biochem 189:126–138. https://doi.org/10.1016/j.plaphy.2022.08.025

Vivancos PD et al (2011) Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. Plant Physiol 157:256–268. https://doi.org/10.1104/pp.111.181024

Wang C, Chen N, Liu J et al (2022) Overexpression of ZmSAG39 in maize accelerates leaf senescence in Arabidopsis thaliana. Plant Growth Regul 98:451–463. https://doi.org/10.1007/s10725-022-00874-1

Wiersma AT, Gaines TA, Preston C, Hamilton JP, Giacomini D, Robin Buell C et al (2015) Gene amplification of 5-enol-pyruvylshikimate-3-phosphate synthase in glyphosate-resistant Kochia scoparia. Planta 241(2):463–474. https://doi.org/10.1007/s00425-014-2197-9

Wu S, Chen W, Lu S, Zhang H, Yin L (2022) Metabolic engineering of shikimic acid biosynthesis pathway for the production of shikimic acid and its branched products in microorganisms: advances and prospects. Molecules 27:4779. https://doi.org/10.3390/molecules27154779

You J, Hu H, Xiong L (2012) An ornithine δ-aminotransferase gene OsOAT confers drought and oxidative stress tolerance in rice.

Plant Sci 197:59–69. https://doi.org/10.1016/j.plantsci.2012.09.002

Yuan J, Zhong S, Long Y, Guo J, Yu Y, Liu J (2022) Shikimate kinase plays important roles in anthocyanin synthesis in petunia. Int J Mol Sci 23(24):15964. https://doi.org/10.3390/ijms232415964

Zhang X, Henriques R, Lin SS, Niu QW, Chua NH (2006) Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat Protoc 1(2):641–646. https://doi.org/10.1038/nprot.2006.97

Zhao HC, Zhao H, Wang BC, Wang JB (2005) Effect of local stress induction on resistance-related enzymes in cucumber seeding. Colloids Surf B 43(1):37–42. https://doi.org/10.1016/j.colsurfb.2005.01.017