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Expression of a bacterial *aroA* gene confers tolerance to glyphosate in tobacco plants

Feng LIU, Yueping CAO*
Department of Plant Science, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, P. R. China

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Abstract: Glyphosate is a widely used herbicide that inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-encoding *aroA* gene in the shikimate pathway. The discovery and cloning of the *aroA* gene with high resistance is central to breeding a transgenic glyphosate-resistant plant. A novel $aroA_{Pantoea}$ gene from *Pantoea* G-1 was previously isolated and cloned. The $aroA_{Pantoea}$ enzyme was defined as a new class I EPSPS with glyphosate resistance. The $aroA_{Pantoea}$ gene was introduced into tobacco through *Agrobacterium*-mediated transformation. The transgenic tobacco plants were confirmed by PCR, RT-PCR, and Southern blot. The analysis of glyphosate resistance also showed that the transgenic tobacco plants could survive at 15 mM glyphosate; the glyphosate resistance level of the transgenic plants is higher than the agricultural application level recommended by most manufacturers. Overall, this study shows that $aroA_{Pantoea}$ can be used as a candidate gene for the development of genetically modified crops.

Key words: *aroA*, glyphosate, *Pantoea*, tobacco, transformation

1. Introduction

As a broad-spectrum herbicide, glyphosate is used to kill most kinds of weeds (Steinrücken and Amrhein, 1980). Glyphosate blocks plant growth by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), which is a critical enzyme in the shikimate pathway (Schönbrunn et al., 2001) that is important for the synthesis of aromatic amino acids and a number of secondary metabolites in plants and microorganisms (Amrhein et al., 1980). EPSPS catalyzes shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (Baerson et al., 2002), respectively. Glyphosate and PEP are analogous; therefore, glyphosate inhibits EPSPS synthesis and causes plant death (Duke and Powles, 2008). Because of this nonselective property, glyphosate also kills food crops. Today, much attention is paid to finding glyphosate-tolerant genes for genetically modified crops.

Since the transgenic tobacco overexpressing mutant *aroA* gene was first reported (Stalker et al., 1985), many *aroA* genes have been identified and cloned over the past three decades (Rogers et al., 1983; Sost and Amrhein, 1990; Zhou et al., 1995). EPSPS proteins have been divided into two major types: class I and class II (He et al., 2001). Class I EPSPS is found in all plants and microorganisms, including *Convolvulus arvensis*, *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae* (Rogers et al., 1983; Sost et al., 1990; Wang et al., 2003; Funke et al., 2009; Huang et al., 2014; Tian et al., 2014) and is sensitive to glyphosate. Class II enzymes are found in *Pseudomonas stutzeri* A1501, *Agrobacterium tumefaciens* strain CP4, *Halothermothrix orenii* H168, and *Bacillus cereus* (Li et al., 2009; Tian et al., 2012, 2013). Class II *aroA* genes have been used to enhance glyphosate tolerance in transgenic plants (Cao et al., 2012; Yi et al., 2016). A novel *aroA* gene cloned from *Pseudomonas putida* 4G-1 has also been defined as neither class I nor class II (Sun et al., 2005). Until now, only the *aroA* gene from *Agrobacterium tumefaciens* strain CP4 has been commercialized successfully (Zhou et al., 1995).

In our previous study, an *aroA* gene from a glyphosate-tolerant strain, *Pantoea* G-1 (designated as $aroA_{Pantoea}$), was isolated from heavily contaminated soil and functionally characterized (Liu and Cao, 2015). Its glyphosate resistance level has not been systematically studied in transgenic plants. In the current study, we transplant the $aroA_{Pantoea}$ gene into tobacco and assess the glyphosate tolerance of transgenic tobacco plants in order to demonstrate that the glyphosate resistance of transgenic tobacco can reach up to 15 mM glyphosate (1.23 kg a.e. ha^{-1}), the glyphosate resistance level of the transgenic plants being higher than the agricultural application level recommended by most manufacturers (Guo et al., 2015). Furthermore, transgenic tobacco plants were used to evaluate the potential of $aroA_{Pantoea}$ in developing glyphosate-resistant crops.

* Correspondence: yuepingcao@sjtu.edu.cn

2. Materials and methods

2.1. Construction of the plant expression vector

The construction of the plant expression vector was performed according to a previous report (Xu et al., 2010). To ensure that the *aroA* gene would be localized to the plant chloroplast, the DNA fragment that encoded the chloroplast transit peptide of *Arabidopsis* (TSP) was inserted into the front of the *aroA* gene (Della-Cioppa et al., 1986). The final construction, D35S:TSP:aroA:Nos, was introduced into *A. tumefaciens* GV3101 through electroporation. The recombinant plasmid was transformed into the tobacco plants using *Agrobacterium*-mediated transformation.

2.2. Tobacco transformation

Construction of the PHB vector was used for the tobacco (*Nicotiana tabacum* cv. Xanthi) transformation according to a previous report (Yan et al., 2011). To begin, 50 mg L^{-1} hygromycin was used as the selected marker. The transgenic tobacco plants were confirmed through PCR amplification of the *aroA* gene. The PCR fragments were amplified using specific primers (5-ATGCAGGACTCCCTGACTTTACAG-3;5-TCAGGCGCTCTGGCTGATTTTTGCCA-3), resulting in 1287 bp of amplified PCR product ($aroA_{Pantoea\ sp.}$). The PCR products were analyzed using 1% agarose gels and electrophoresis.

2.3. RT-PCR and qRT-PCR

To analyze tissue-specificity expression of the $aroA_{Pantoea}$, transgenic tobacco leaves, stems and roots of 30-day-old seedlings were used respectively in the greenhouse. The total RNA extraction from the tobacco tissues was conducted using an RNA prep pure kit for plants (Tiangen Biotech). The first-strand cDNA synthesis was performed using M-MLV reverse transcriptase (Takara). For RT-PCR, 2 µL of cDNA was used as a template for 30 amplification cycles using PCR. The fragment products were analyzed using 1% agarose gels and electrophoresis.

qRT-PCR analysis was used to observe the $aroA_{Pantoea}$ gene expression according to a previous report (Liu et al., 2015; Yıldırım, 2017). Specific primers (5-CGGCAATGACAATCGCTACC-3 and 5-AAAATCGTGCCCCTCTTCCA-3) were used to amplify the $aroA_{Pantoea}$ gene. A ubiquitin gene (accession number: U66264.1) was used as the housekeeping gene. The transgenic tobacco plants were sprayed with 15 mM glyphosate at the six-leaf stage and the leaves were harvested at 12, 24, 36, and 48 h after glyphosate treatment and used for gene expression analysis. The expression level of the $aroA_{Pantoea}$ gene was calculated using the $2^{-\Delta\Delta t}$ method. Each sample was biologically repeated three times.

2.4. Determining the level of chlorophyll content

The chlorophyll content was tested according to a previous report (Guo et al., 2015; Sucu et al., 2017). Forty-day-old plants of wild-type tobacco and transgenic tobacco were sprayed with 15 mM glyphosate. The SPAD values of the leaves from the different tobacco plants were calculated using a SPAD-502 Plus chlorophyll measuring instrument at different times (0, 12, 24, 36, 48, 60, and 72 h) after glyphosate treatments. This meter measures absorption at 650 and 940 nm wavelengths in order to determine the chlorophyll levels (Gao et al., 2014; Yıldırım and Uylas, 2016). At least six replications were confirmed for each treatment.

2.5. Determining the level of shikimic acid

The level of shikimic acid was calculated according to a previous report (Gao et al., 2014). First, 40-day-old plants were sprayed with 15 mM glyphosate, and approximately 0.2 g of young tobacco leaves was taken at different times (0, 12, 24, 36, 48, 60, and 72 h) and put into an ice-bath mortar for continuous grinding. Then 1.0 mL of HCl was added and the mixture was centrifuged at 12,000 rpm for 10 min (4 °C). Next, 2 mL of 0.1% periodic acid was added to 200 µL of supernatant for 3 h. Then 2 mL of NaOH (0.1 mol/L) and 1.5 mL of glycine (0.1 mol/L) were added to the mixture. Finally, the absorbance of the solution was measured at 380 nm using a spectrophotometer. The shikimate content was measured using a shikimate standard curve.

2.6. Southern blot analysis

Selected PCR-positive T_0 transgenic tobacco plants were further analyzed by Southern hybridization for the integration of the $aroA_{Pantoea}$. Tobacco genomic DNA was extracted from the leaves of the putative transgenic plants and wild-type plants using the CTAB method. Genomic DNA (100 µg) was entirely digested overnight at 37 °C using *EcoR* I. The digestion products were separated on 1.0% agarose gel through electrophoresis and transferred to a Hybond N^+ nylon membrane. The PCR fragment of the *aroA* gene was amplified using primers (5-AGCGTTTCCAGCCAGTTC-3, 5-CTCCCATCTTCTCCAGCAC-3) and was labeled using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). The hybridization and detection steps were performed according to the manufacturer's instructions.

2.7. Analysis of glyphosate resistance in transgenic tobacco

To detect the glyphosate tolerance of T_0 plants, the tobacco transformants were grown in MS culture medium for 3 weeks with 16 h of light and 8 h of dark. Seedlings were transferred to soil and grown in the greenhouse for 1 month. Five- to-six-leaf-stage transgenic and wild-type tobacco plants were sprayed with 15 mM glyphosate and the results were observed after 2 weeks.

2.8. Seed germination assays

The tobacco seeds (transgenic and wild-type) were disinfected with 15% NaClO for 15 min and then rinsed at least three times with sterile water. The T_1 sterilized

transgenic line NT-1 was grown on MS medium containing glyphosate (0, 300, 500, and 1000 μM) in petri dishes in a controlled environment chamber at 25 °C with a 16/8-h day/night cycle. A photograph was taken after 2 weeks of growth and the root length was measured.

2.9. Statistical analysis

Student's t-test was used for analysis of significant differences with SAS 9.2 at a level of $P < 0.01$. All of the figures were created using Origin 8.0 software.

3. Results

3.1. Construction of plant expression vector

A plant expression vector containing the *aroA* gene was constructed in order to test the glyphosate resistance of the $aroA_{Pantoea}$ gene (GenBank: ARH59536.1) in the transgenic T_0 plants. In the vector, the signal peptide sequence of *Arabidopsis* and the *aroA* coding sequence were cloned into the T-DNA box of the PHB vector (Figure 1).

3.2. Obtaining transgenic tobacco with an $aroA_{Pantoea}$ gene

The plant expression vector, PHB-TSP-$aroA_{Pantoea}$, was transferred into tobacco leaf disks via an *Agrobacterium*-mediated transformation. The tobacco callus medium was first screened for a 50-mg L^{-1} hygromycin selection, which is a marker of the PHB vector. Twenty hygromycin-resistant plants were regenerated during this genetic transformation process. A molecular analysis was executed with PCR in order to determine the integration of the $aroA_{Pantoea}$ gene into the T_0 generation. Only nine hygromycin-resistant plants showed amplification of the expected 1287-bp fragment of $aroA_{Pantoea}$, while no amplification was observed in the nontransgenic plants (Figure 2).

3.3. Expression analysis of the gene by RT-PCR and qRT-PCR

The leaves of four transgenic tobacco lines showed transcription levels of the $aroA_{Pantoea}$ gene using RT-PCR. Transgenic lines were expressed at the transcriptional level while the wild-type did not have any specific bands (Figure 3a). The transcription expression level in different tissues (leaf, stem, and root) was also tested. The highest expression level of the $aroA_{Pantoea}$ gene was detected in the leaves, while a relatively low transcription expression level was found in the stem and roots (Figure 3b).

qRT-PCR was used to analyze the target gene expression in the leaves of wild-type and transgenic tobacco plants at various times after glyphosate application. The transcription expression level of the $aroA_{Pantoea}$ gene increased threefold after 36 h (Figure 4). Consequently, the expression of the $aroA_{Pantoea}$ gene increased at the transcription level after glyphosate application.

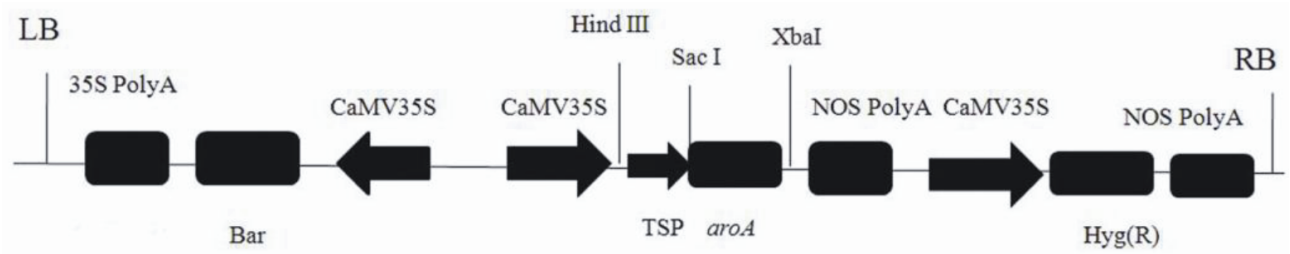

Figure 1. The *aroA* expression vector for tobacco transformation.

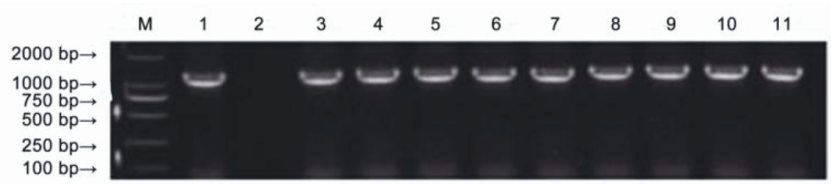

Figure 2. The glyphosate-tolerant transgenic plants were confirmed by PCR analysis. M: 2000-bp DNA marker; 1: PHB-TSP-$aroA_{Pantoea}$ plasmid as the positive control; 2: nontransgenic tobacco as the negative control; 3–11: transgenic plants.

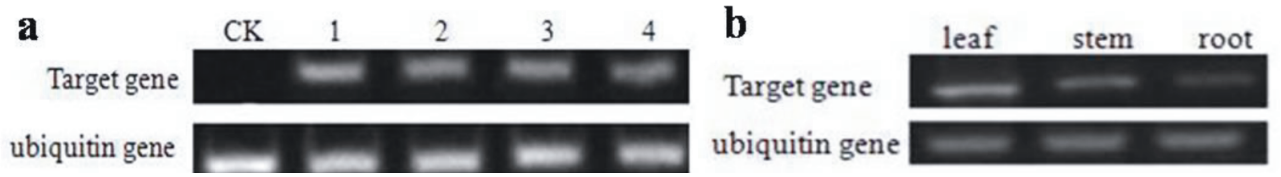

Figure 3. Expression analysis of *aroA* by semiquantitative RT-PCR. a) Expression analysis of four transgenic lines. CK: Nontransgenic plant; 1–4: four putative transgenic lines. b) mRNA level of the *aroA* in different tissues of transgenic lines.

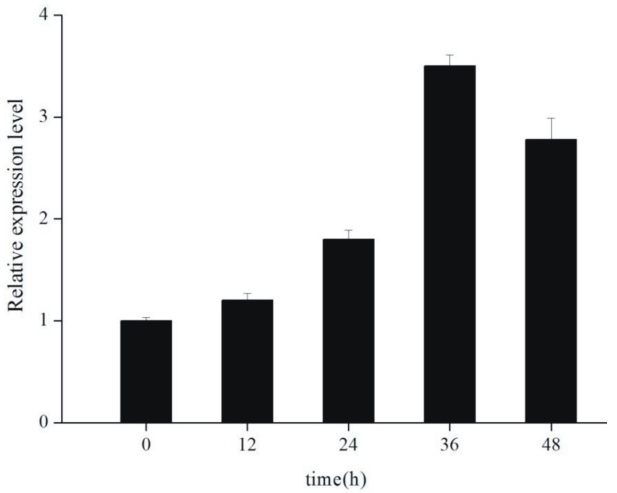

Figure 4. mRNA level of the *aroA* after glyphosate treatment. Quantitative real-time polymerase chain reaction (qRT-PCR) was used for detecting expression level. Error bars are SE (n = 3).

3.4. Chlorophyll content and shikimic acid assay

There were no significant differences in chlorophyll content between the leaves of the wild-type and transgenic plants before glyphosate treatment. The chlorophyll content decreased significantly 72 h after glyphosate treatment in the wild plants. However, the transgenic tobacco showed no significant alteration in chlorophyll content at any time (Figure 5a).

The accumulation of shikimic acid is often used as a biomarker for glyphosate effects. The results demonstrated that the amount of shikimic acid in the wild tobacco significantly increased after glyphosate treatment. The content of shikimic acid in the transgenic tobacco was much lower than that of the wild plants (Figure 5b). This showed that the growth and plant development of the transgenic tobacco that overexpresses $aroA_{Pantoea}$ was normal after glyphosate treatment.

3.5. Glyphosate tolerance in transgenic tobacco plants and Southern blot analysis

In our study, 2 weeks after spraying 15 mM glyphosate, the leaves of the wild-type tobacco turned yellow, leading to death, whereas the transgenic tobacco continued to grow well after treatment (Figure 6). The results also showed that the transgenic tobacco overexpressing $aroA_{Pantoea}$ had more glyphosate resistance than the wild-type.

First, the transgenic tobacco plants were confirmed by PCR and RT-PCR. Second, we identified three transgenic tobacco plants through Southern blot hybridization in these transgenic lines. As shown in Figure 7, the results confirmed that the $aroA_{Pantoea}$ gene was stably integrated into the tobacco genome. The copy number of the $aroA_{Pantoea}$ gene in the transgenic NT-1 line was a single copy. The T_1 progeny from the NT-1 line showed a 3:1 segregation ratio for resistance to glyphosate (data not shown), which suggests one expressed copy of $aroA_{Pantoea}$.

3.6. Glyphosate tolerance in T_1 seedlings

To investigate the genetic stability of T_1 transgenic plants, we observed the glyphosate tolerance of transgenic tobacco in solid MS medium with different concentrations of glyphosate. The results also showed that the transgenic NT-1 line grew well in 1000 µM glyphosate, whereas the wild-type did not germinate at 300 µM (Figure 8a). The root length was measured, and the results showed that the NT-1 line that overexpressed $aroA_{Pantoea}$ had a significantly

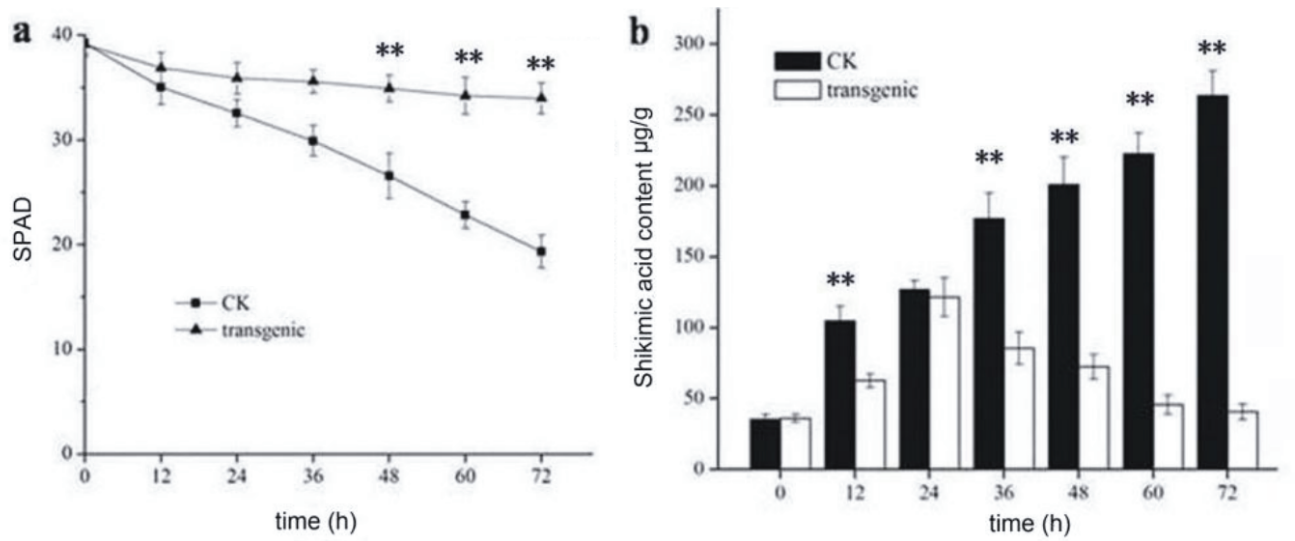

Figure 5. Physiological content in in transgenic and nontransgenic plants. a) Chlorophyll contents. b) Contents of the shikimic acid. CK: Nontransgenic plant. Data represent means ± SE from three replicates. Statistical significance was determined by Student's t-test (**, $P < 0.01$). Asterisks indicate the difference between CK and transgenic plants of $aroA_{Pantoea}$.

Figure 6. The transgenic plants (T_0) were sprayed with glyphosate at 15 mM after 2 weeks. CK: Nontransgenic plant; 1–3: transgenic plants.

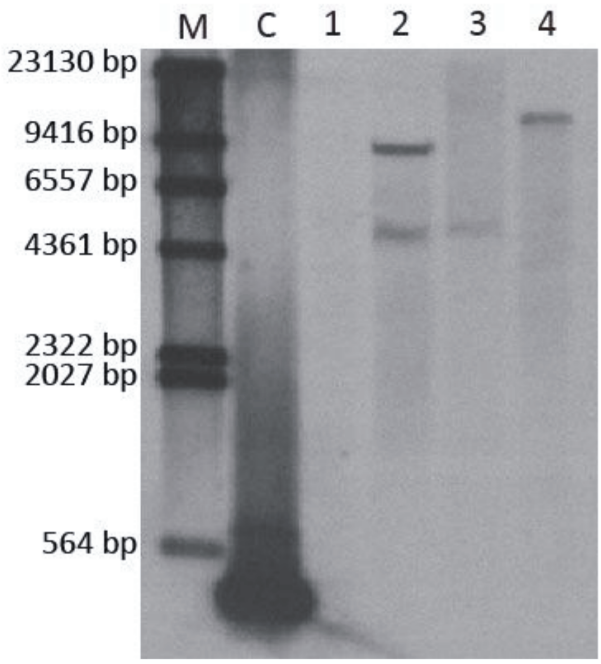

Figure 7. Southern blot analysis of T_0 transgenic tobacco. M: DNA marker; C: probe as the positive control; 1: nontransgenic plant as the negative control; 2–4, transgenic NT-3, NT-2, and NT-1, respectively.

longer root length than the wild-type. It is apparent that the NT-1 line had stronger glyphosate tolerance than the wild-type (Figures 8b and 8c), and that the $aroA_{Pantoea}$ gene could be a stable inheritance for glyphosate resistance.

4. Discussion

In our previous study, a new *aroA* gene that belongs to the class I EPSPS with high glyphosate tolerance (Liu and Cao, 2015) was obtained from *Pantoea* G-1 in glyphosate-polluted soil. A class I *aroA* gene can be naturally glyphosate-resistant, and some *aroA* genes have been assessed in *Proteus mirabilis* and *Janibacter* sp. (Tian et al., 2014; Yi et al., 2016). Although *Pantoea* sp. widely exists in soil, there were no previous reports on glyphosate resistance.

Two pathways for the improvement of glyphosate tolerance have been recently identified. One involves the mutation of amino acids in active *aroA* sites, which has been studied by many scholars (Sost et al., 1990; He et al., 2001; Liang et al., 2008). The second pathway is using the *aroA* in class II EPSPS to indirectly induce glyphosate tolerance (Fitzgibbon and Braymer, 1990; Tian et al., 2010; Cao et al., 2012). Since the 1980s, only the *CP4-EPSPS* gene has been commercially used for many years (Guo et al., 2015).

Glyphosate effectively inhibits *aroA* by blocking the synthesis of aromatic amino acids, thus causing plant death. The transcription level of the *aroA* gene in different plant tissues is different. The transcription level showed that the *aroA* gene is expressed extensively in roots, stems, and leaves, being most highly expressed in the leaf. This expression model is consistent with the *aroA* expression observed in *Convolvulus arvensis* L. and *Camptotheca acuminate* (Gong et al., 2006; Huang et al., 2014). Glyphosate treatment could induce a significant increase of *aroA* gene expression level. The expression of the *aroA* gene could be induced by spraying glyphosate at a significantly upregulated level. In this experiment, the peak induction was 36 h after spraying glyphosate, which was 3-fold higher than the control group's expression level. In field bindweed, the expression level of EPSPS was also responsive to glyphosate (Huang et al., 2014). These results show that the *aroA* gene plays an important role in the response to glyphosate.

Treating plants with glyphosate affected EPSPS activity by reducing chlorophyll content and causing the accumulation of shikimic acid (Powell et al., 1992; Guo et al., 2015). Spraying glyphosate can cause the accumulation of shikimic acid content in glyphosate-sensitive plants, but tolerant plants lack shikimic acid accumulation

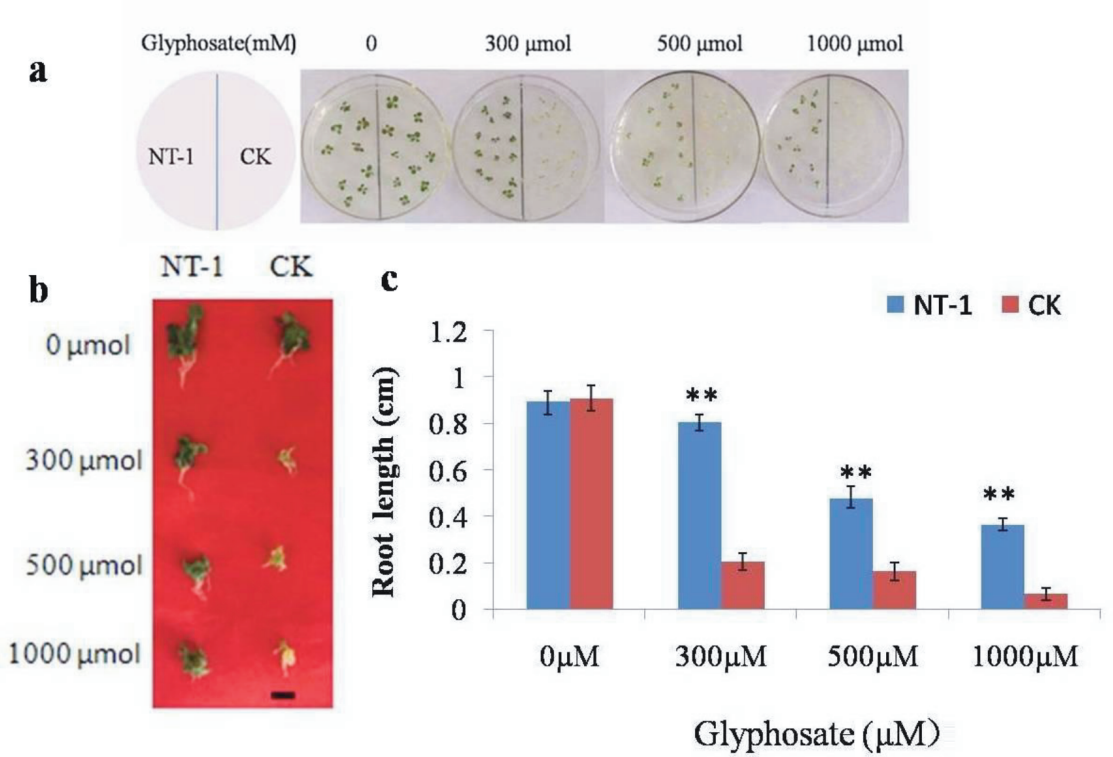

Figure 8. a) Comparative germination of transgenic seeds on MS medium containing various glyphosate concentrations (0, 300, 500, 1000 µM) in petri dishes. b) The comparative image of root length between NT-1 and CK on MS medium containing various glyphosate concentrations (0, 300, 500, and 1000 µM) in petri dishes. c) The root length of the tobacco plants. Asterisk means significant difference at $P < 0.01$, bar = 0.5 cm.

(Gao et al., 2014). Shikimic acid content can be used as a marker of glyphosate resistance (Binarová et al., 1994). Low shikimate accumulation in transgenic plants revealed higher glyphosate tolerance compared to the wild-type plants. When spraying with 15 mM glyphosate, the transgenic tobacco plants with the new *aroA* gene were morphologically normal. In addition, the glyphosate resistance level of the transgenic tobacco is higher than the agricultural application level recommended by most manufacturers. We also compared our results with previous studies, which showed that the glyphosate tolerance level of transgenic *A. thaliana* tobacco is 10 mM and 5 mM (Peng et al., 2012; Han et al., 2014), respectively. The 15 mM glyphosate tolerance of transgenic tobacco is obviously higher.

In summary, we assessed the glyphosate resistance of the $aroA_{Pantoea}$ gene in transgenic tobacco plants and confirmed that $aroA_{Pantoea}$ shows high tolerance to glyphosate, indicating that the novel *aroA* gene would likely enhance glyphosate tolerance in engineered crops.

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