A Novel Naturally Occurring Class I 5-Enolpyruvylshikimate-3-Phosphate Synthase from Janibacter sp. Confers High Glyphosate Tolerance to Rice

Shu-yuan Yi1,*, Ying Cui2,*, Yan Zhao1, Zi-duo Liu1, Yong-jun Lin2 & Fei Zhou2

As glyphosate is a broad spectrum herbicide extensively used in agriculture worldwide, identification of new aroA genes with high level of glyphosate tolerance is essential for the development and breeding of transgenic glyphosate-tolerant crops. In this study, an aroA gene was cloned from a Janibacter sp. strain isolated from marine sediment (designated as aroAJ. sp). The purified aroAJ. sp. enzyme has a $K_m$ value of 30 $\mu$M for PEP and 83 $\mu$M for S3P, and a significantly higher $K_i$ value for glyphosate (373 $\mu$M) than aroAE.coli. AroAJ. sp is characterized as a novel and naturally occurring class I aroA enzyme with glyphosate tolerance. Furthermore, we show that aroAJ. sp can be used as an effective selectable marker in both japonica and indica rice cultivar. Transgenic rice lines were tested by herbicide bioassay and it was confirmed that they could tolerate up to 3360 g/ha glyphosate, a dosage four-fold that of the recommended agricultural application level. To our knowledge, it is the first report of a naturally occurring novel class I aroA gene which can be efficiently utilized to study and develop transgenic glyphosate-tolerant crops, and can facilitate a more economical and simplified weed control system.

The shikimate pathway is essential for the biosynthesis of aromatic amino acids in plants, fungi and microorganisms1. The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC2.5.1.19), also called aroA enzyme, plays a crucial role in the penultimate step of the shikimate pathway by catalyzing the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl group of shikimate-3-phosphate (S3P) to form 5-enolpyruvylshikimate-3-phosphate (EPSP). Glyphosate is the most widely used broad spectrum herbicide that mimics the carbocation state of PEP and binds EPSPS competitively2–4. The inhibition of EPSPS is a reversible reaction in which glyphosate binds to the binding site of PEP and forms a stable but non-covalent ternary complex with the enzyme and S3P (EPSPS-S3P-glyphosate)5. The ternary complex blocks the formation of EPSP, affecting the growth of the organism. Glyphosate provides an effective, efficient and economical way to control weeds6. As glyphosate is an unselective herbicide, the development of glyphosate-tolerant crops has dramatically changed weed management practices and increased the yield of crops.

Since the mutant aroA gene cloned from Salmonella typhimurium was first reported in 19837, the aroA enzyme (EPSPS) identified as the target of glyphosate resistance has been studied extensively over the past three decades8. Different aroA enzymes from various organisms have been divided into two classes on the basis of their intrinsic glyphosate sensitivities and their substantial sequence variations9. In general, class I aroA enzymes are naturally sensitive to glyphosate and generally found in Escherichia coli, Aeromonas salmoncida, Petunia hybrida and Arabidopsis thaliana. Class II aroA enzymes share less than 30% sequence similarity with class I enzymes, and can retain their activity at a high concentration of glyphosate, which are isolated from Agrobacterium tumefaciens CP4, Pseudomonas sp. strain PG2982, Bacillus subtilis, Ochrobactrum anthropi, Staphylococcus aureus and other bacteria species10–16. In class II enzymes, two conserved regions have been proved to be key regions involved in...
glyphosate tolerance: RXHXE and NXTR (X represents non-conserved amino acids), in both of which the positive charge of the side chain of Arg hinders the binding of glyphosate11.

Several class II enzymes have been used to generate transgenic plants33–35. Among them, only the one from Agrobacterium tumefaciens CP4 has been used for the production of transgenic glyphosate-tolerant crops.

Glyphosate insensitivity can be also achieved in class I aroA enzymes through site-directed mutagenesis33–35 or natural selection26–28. However, most of the mutants show lower affinity for their substrate PEP. Among them, the double mutated aroA (TIPS) has been successfully used to produce the first commercial transgenic glyphosate-tolerant maize (GA21). In recent years, considerable attention has been paid to the exploration of new types of aroA enzymes with commercial feasibility.

In this study, a novel aroA enzyme from a glyphosate-tolerant strain Janibacter sp (designated as aroA<sub>sp</sub>) was isolated from marine sediment and further functionally characterized. Through the sequence analyses and phylogenetic analyses, aroA<sub>sp</sub> is characterized as a new class I aroA enzyme. The K<sub>m</sub> values for PEP and S3P of this enzyme were found to be similar to those of the analog from aroA<sub>E. coli</sub>, but its K<sub>i</sub> value for glyphosate was significantly higher. Finally, the function of aroA<sub>sp</sub> was evaluated in both <i>Japonica</i> and <i>Indica</i> rice cultivar, zhonghua11 (ZH11) and minghui86 (MH86), respectively. The results show that it can be used as an effective marker for direct selection and the generated transgenic rice lines were conferred high glyphosate tolerance (up to 3360 g/ha glyphosate). Taken together, our study indicates that the naturally occurring novel class I aroA<sub>sp</sub> gene has promising potential for the development and breeding of transgenic glyphosate-tolerant crops.

Results

Isolation of gene conferring glyphosate tolerance. With the aim of finding novel aroA genes, the marine sediment sample was enriched with glyphosate and plated on M9 agar plates containing different concentrations of glyphosate. After plate-screening, one strain (named as L42) grew very well at a concentration of 150 mM glyphosate. The L42 strain was gram-positive and coccoid, whose 16S rRNA sequence (1487 bp) displayed the highest similarity (99%) with that of <i>Janibacter</i> sp N2M. Thus, this strain was identified as a <i>Janibacter</i> species. To isolate the gene encoding aroA enzyme from L42 strain, a genomic library was constructed. After screening about 5,000 colonies from the library, one positive colony was found to possess the ability to grow on M9 agar containing 100 mM glyphosate, indicating that the recombinant plasmid in the colony might contain a gene involved in glyphosate tolerance. From this colony the plasmid (named as pZY3) was recovered.

Sequence analysis of aroA<sub>sp</sub> gene. DNA sequencing analysis revealed that the pZY3 plasmid contained a 1,299 bp DNA fragment that consisted of a complete open reading frame (ORF1) and encoded 432 amino acids with an estimated molecular weight of 45.1 kDa. The overall GC content of the ORF1 was 73.6%. The deduced amino acid sequence was then used to search for homologous sequences through the BLAST program, and the protein was found to share the highest homology (67% amino acid identity) with aroA enzyme from <i>Janibacter</i> sp HTC22649 (GenBank: EAP999471), indicating that the ORF1 from L42 strain encoded an aroA enzyme. Thus, the aroA gene from the <i>Janibacter</i> sp was designated as aroA<sub>sp</sub>.

Multiple alignments of amino acids showed that aroA<sub>sp</sub> shared only 26–35% amino acid identity with most class I and class II aroA enzymes. Several key residues involved in S3P and PEP binding were well conserved in aroA<sub>sp</sub>, including S24, K25, S26, R127, S173, Q175, R348, R389, S202, R348 and R389, which are indicated in two classes of the aroA enzymes in Fig. 1. The highly conserved region containing residues XLGNAGTAXRXL (Fig. 1a, Frame 1) has been demonstrated to be critical for the substrate PEP binding in aroA enzymes26. It was indeed present in aroA<sub>sp</sub> and showed higher sequence identity with that in class I aroA enzymes than that in class II analogs. In addition, two other regions RXHTE (Fig. 1a, Frame 2) and NPTR (Fig. 1a, Frame 3), which are well conserved in the class II aroA enzymes, were completely absent in aroA<sub>sp</sub>. Further phylogenetic analysis indicated that aroA<sub>sp</sub> belonged to class I aroA but showed little sequence similarity with other class I aroA enzymes (Fig. 1b). Taken together, these results suggest that aroA<sub>sp</sub> is a new member of class I aroA enzymes based on its amino acid sequence.

Glyphosate insensitivity assay of aroA<sub>sp</sub> in <i>E. coli</i>. <i>E. coli</i> AB2829, which is deficient in aroA, can only grow in minimal medium when foreign aroA complements the deficiency. Plasmids pGEX-6p-1-aroA<sub>sp</sub> and pGEX-6p-1-aroA<sub>E. coli</sub> were obtained by cloning aroA<sub>sp</sub> and aroA<sub>E. coli</sub> into GST carrier vector pGEX-6p-1. Then, the function of the isolated aroA<sub>sp</sub> gene in <i>E. coli</i> was investigated by comparing the growth characteristics of <i>E. coli</i> AB2829 harboring either plasmid pGEX-6p-1-aroA<sub>sp</sub> or pGEX-6p-1-aroA<sub>E. coli</sub> at glyphosate concentrations of 0, 50 and 100 mM. All the strains grew well in M9 medium without glyphosate, and the growth of each strain was about 55% and 40%, which was 2.7 and 2-fold higher than that of the strain containing aroA<sub>E. coli</sub>. However, bacterial cells containing aroA<sub>sp</sub> showed better growth in the presence of 50 mM and 100 mM glyphosate, with the growth decreasing to 30% (Fig. 2b), and the cells were severely inhibited in the presence of 100 mM of glyphosate with the growth of only 20% (Fig. 2c). In contrast, cells carrying aroA<sub>sp</sub> grew well under all the conditions with 50 or 100 mM of glyphosate. In medium containing 50 mM and 100 mM glyphosate, the growth of this strain was about 55% and 40%, which was 2.7 and 2-fold higher than that of the strain containing aroA<sub>E. coli</sub> (Fig. 2d). These results indicate that aroA<sub>sp</sub> can functionally complement the deficiency of aroA in <i>E. coli</i> AB2829 and definitely carries a high glyphosate-tolerant capability, whereas the aroA<sub>E. coli</sub> gene derived from bacterium strain DH5α does not have such capability.

Expression and purification of aroA<sub>sp</sub> enzyme. After induction by IPTG, soluble expression of the aroA<sub>sp</sub>-GST fusion was performed in bacterium strain BL21, and the induced fusion protein, which comprised an aroA<sub>sp</sub> (47 kDa) and a GST tag (26 kDa), showed an expected molecular mass of 73 kDa on SDS-PAGE (Fig. S2a). Affinity-purified aroA<sub>sp</sub>-GST fusion was cleaved by 3C protease, resulting in a 47 kDa aroA<sub>sp</sub> protein.
with high purity as indicated by a single band on SDS-PAGE. Soluble protein was successfully produced in BL21 under the conditions used in this study, suggesting a proper folding and conformation for its functionality. GST carrier within the aroA<sub>J. sp</sub>-GST fusion may play a role for this soluble expression. At the same time, the aroA<sub>E. coli</sub>-GST fusion was expressed in parallel and run on the same SDS-PAGE as a control, and aroA<sub>E. coli</sub> of a similar size was also obtained after cleavage of the fusion (data not shown). The concentration of the purified aroA<sub>J. sp</sub> and aroA<sub>E. coli</sub> enzyme was 0.3 mg/ml and 1.5 mg/ml, respectively.

**Kinetic properties of purified aroA<sub>J. sp</sub> enzyme.** As shown in Fig. S2b and S2c, aroA<sub>J. sp</sub> enzyme had the highest activity at pH 8.0 and 40 °C, and maintained about 40% activity over a pH range from 6 to 10 and 30% activity over a temperature range from 10 to 60 °C, implying high stability and applicability of this enzyme under different conditions.

The proteins encoded by aroA<sub>J. sp</sub> and aroA<sub>E. coli</sub> were purified separately with a GST system and used for enzymatic activity assay. Due to the structural difference from class II enzymes, class I aroA enzymes are known to be naturally glyphosate-sensitive. The of PEP and the <i>K<sub>i</sub></i> value for glyphosate of the purified aroA<sub>E. coli</sub> enzyme were determined to be 60 and 0.91 μM in this experiment, respectively, and the corresponding <i>K<sub>i</sub></i>/<i>K<sub>m</sub></i> ratio was 0.015. Kinetic constants of the aroA<sub>J. sp</sub> enzyme were significantly different from those of the aroA<sub>E. coli</sub> enzyme (Table 1, Fig. 3). In the presence of KCl, the <i>K<sub>m</sub></i> values of the aroA<sub>J. sp</sub> enzyme for PEP and S3P were 30 and 83 μM,
respectively, which were lower than those of aroA\textsubscript{E. coli} indicating a high substrate affinity of the aroA\textsubscript{J. sp} enzyme. The IC\textsubscript{50} and the \(K_i\) values for glyphosate of the aroA\textsubscript{J. sp} enzyme were 3.6 mM and 373 \(\mu\)M, respectively, and the IC\textsubscript{50} value was 90-fold that of the aroA\textsubscript{E. coli} enzyme. The \(K_i/K_m\) value of aroA\textsubscript{J. sp} enzyme was calculated to be 12.4, which is 800-fold that of aroA\textsubscript{E. coli}, suggesting that the aroA\textsubscript{J. sp} enzyme has a higher level of glyphosate tolerance than the aroA\textsubscript{E. coli} counterpart.

Table 1. Kinetic properties of aroA\textsubscript{J. sp} and aroA\textsubscript{E. coli}\textsuperscript{a}. The results are the averages of two sets of experiments conducted in triplicate. \textsuperscript{b}The PEP or S3P concentration was set at 0.05, 0.067, 0.1, 0.2, 0.5, and 1 mM, while the concentration of the other one was fixed at 1.0 mM. \textsuperscript{c}Competitive inhibition by glyphosate with respect to PEP was demonstrated by lines converging on the x axes of Lineweaver-Burk plots. The PEP concentration was set at 0.067, 0.1, 0.2, and 0.5 mM, respectively, while the glyphosate concentration was 0, 5, 10, 20, and 50 \(\mu\)M in determining the inhibition of aroA\textsubscript{E. coli}; and the glyphosate concentration was 0, 0.1, 0.5, 1, 2 and 5 \(\mu\)M in determining the inhibition of aroA\textsubscript{J. sp}; S3P concentration was fixed at 1 mM. \textsuperscript{d}The glyphosate concentration causing 50\% inhibition of enzyme activity, which was determined by fitting the data to the equation: \(V = V_{\text{min}} + (V_{\text{max}} - V_{\text{min}})/(1 + ([I]/IC_{\text{50}})^n)\), and \(V\) was determined at 1 mM PEP and 1 mM S3P with the glyphosate concentration ranging from 0.0001 mM to 100 mM.

| Enzyme         | Sp act (nKat/mg) | \(K_i\) (PEP)\textsuperscript{b} (\(\mu\)M) | \(K_i\) (S3P)\textsuperscript{b} (\(\mu\)M) | \(K_i\) (\(\mu\)M) | IC\textsubscript{50}\textsuperscript{d} (mM) | \(K_i/K_m\)(PEP) |
|----------------|------------------|--------------------------------------------|--------------------------------------------|-------------------|------------------------------------------|------------------|
| AroA\textsubscript{J. sp} | 27.9 ± 3         | 30 ± 4                                     | 83 ± 11                                    | 373 ± 50          | 3.6 ± 0.2                                | 12.4             |
| AroA\textsubscript{E. coli} | 15.6 ± 4         | 60 ± 7                                     | 115 ± 10                                   | 0.9 ± 0.09        | 0.04 ± 0.01                              | 0.015            |

**Figure 2.** Growth curve of E. coli AB2829 harboring either pGEX-6p-1-aroA\textsubscript{J. sp} or pGEX-6p-1-aroA\textsubscript{E. coli} in liquid M9 minimal medium supplemented with glyphosate at concentrations of 0 mM (a) 50 mM (b) and 100 mM (c). (d) Bar charts of growth analysis of the strains. The results presented are the averages of two sets of experiments done in triplicate.

**Nuclear transformation directly using aroA\textsubscript{J. sp} as selectable marker.** Since the aroA\textsubscript{J. sp} showed high glyphosate-tolerant capability in E.coli and had a high \(K_i/K_m\) value, it was tested in a japonica rice variety zhonghui11 (ZH11) to explore the possibility of employing aroA\textsubscript{J. sp} as a selectable marker in crops. To ensure efficient expression of aroA\textsubscript{J. sp} maize Ubi promoter was used, because it conferred a higher level of foreign gene expression than Act1 and CaMV 35S promoters in monocot study\textsuperscript{29}. Since aroA was located in the chloroplast in plants, a chloroplast transit peptide from Arabidopsis thaliana (CTP) was added to the N-terminus of aroA\textsubscript{J. sp} to target the protein into the chloroplasts in rice. The resulted pU130-aroA\textsubscript{J. sp} (Fig. 4a) was transformed into ZH11.
From 3 independent transformation experiments, glyphosate-resistant calli were selected on medium containing 200 mg/L glyphosate. Resistant calli appeared after 5 weeks on average and could be transferred to the differentiation medium after one more week, resulting in a callus formation rate of around 50% (Table 2).

16, 8 and 13 resistant lines were obtained out of 84, 67 and 75 resistant calli, respectively. All T₀ lines were confirmed by PCR (data not shown) and Southern blot analysis (Fig. 4b–d). The results tentatively suggest that direct selection for glyphosate tolerance using \( \text{aroA}_{J.\ sp} \) as selectable marker can readily produce transgenic rice lines with a high transgenic positive rate.

**Glyphosate tolerance of the transgenic indica rice.** As \( \text{aroA}_{J.\ sp} \) could be used as direct selectable marker in japonica rice, we further applied it in a conventional indica rice variety, minghui86 (MH86). After 8 weeks of selection followed by the differentiation of calli, 28 putative transgenic plants were generated (named as P1–P28). The transformation efficiency for MH86 was about 5% based on the numbers of the total transformants and the calli used for transformation. PCR analysis showed that a specific fragment of 815 bp was amplified from 22 of the 28 T₀ transgenic lines, indicating an integration of \( \text{aroA}_{J.\ sp} \) gene into the rice genome (Fig. 5a). Those PCR positive lines were then named as PP1–PP22, and chosen for RT-PCR analysis (Fig. 5b). The result demonstrated a proper transcription of the integrated \( \text{aroA}_{J.\ sp} \) gene in 20 rice transformants (marked as ME1–20). The copy number of T-DNA integration in these T₀ transgenic lines (ME1–20) was determined by Southern blotting (Fig. 5c). T₂ progenies from four lines (ME1, ME2, ME6 and ME7) were further tested by Southern blotting. The

---

**Figure 3.** (a) The V-S curve of \( \text{aroA}_{J.\ sp} \) and \( \text{aroA}_{E.\ coli} \) assayed at fixed S3P and various PEP concentrations. (b) The Lineweaver-Burk plots \( \text{aroA}_{J.\ sp} \) and \( \text{aroA}_{E.\ coli} \) assayed at fixed S3P and various PEP concentrations. (c) The V-S curve of \( \text{aroA}_{J.\ sp} \) and \( \text{aroA}_{E.\ coli} \) assayed at fixed PEP and various S3P concentrations. (d) The Lineweaver-Burk plots \( \text{aroA}_{J.\ sp} \) and \( \text{aroA}_{E.\ coli} \) assayed at fixed PEP and various S3P concentrations. (e) The \( K_i \) values of \( \text{aroA}_{E.\ coli} \) determined by lines converging on the x-axis of Lineweaver-Burk plots. (f) The \( K_i \) values of \( \text{aroA}_{J.\ sp} \) determined by lines converging on the x-axis of Lineweaver-Burk plots.
result confirmed that in these lines, the transgene was integrated into the genome as a single copy and was stably inherited (Fig. 5d). In order to further assay the tolerant capability for crop breeding, the ME1 line and ME2 line were chosen for further characterization, because their T-DNA insertions were mapped to the intergenic region (data not shown). The data collected from the field experiment for agronomic performance showed the homozygous T2 transgenic rice lines (ME1-P and ME2-P) had no significant differences from their corresponding negative transgenic lines (ME1-N and ME2-N) (Table 3).

Spray with glyphosate resulted in a strong inhibition of growth and the ultimate death of the non-transgenic rice plants (210 g/ha), but did not influence the normal growth of the homozygous T2 transgenic rice plants at 3360 g/ha glyphosate (Fig. 5e).

In addition, T4 generation seedlings of ME1 line were transplanted into a weedy field without weed control. Only 7 days after spray with 840 g/ha glyphosate, the weeds were dead, while the transgenic rice plants reached tillering stage, and grew normally 15 days after spray (Fig. 5f), suggesting the applicable potential of aroA in weed control.

**Discussion**

In modern agricultural system, herbicides have greatly contributed to the reliable global food production as they can easily remove weeds. Among these chemicals, glyphosate is the most widely used one due to its broad-spectrum herbicidal activity and minimal human and environment toxicity. Since the glyphosate use pattern commenced in 1996, the planting area of transgenic herbicide-tolerant crops accounts for more than 80% of the total planting area of transgenic crops in 2014 (ISAAA Annual Report: 2014 http://www.isaaa.org/resources/publications/annualreport/2014/pdf/ISAAA-Aannual_Report-2014.pdf). In the last three decades, a number of promising enzymes were identified. However, among those enzymes, only two aroA variants have been utilized for developing commercial glyphosate-tolerant crops: CP4, a naturally occurring class II type, and TIPS, a mutant class I type. Therefore, identification of more novel glyphosate-insensitive aroA genes which can be used to generate glyphosate-tolerant crops with commercial feasibility will bring great positive impact on global food security by facilitating a more economical and simplified weed control system.

Mutagenesis is one method to improve the glyphosate tolerance of aroA enzymes, but when the tolerance for glyphosate is increased, the binding affinity for PEP and catalytic efficiency may be decreased at the same time, as glyphosate and PEP bind to the same site. To date, TIPS is the only class I enzyme which is essentially insensitive to glyphosate but maintains high affinity for PEP. Thus, the exploration of new types of aroA enzymes with both intrinsic glyphosate tolerance and high affinity with PEP is very necessary.
Microbial biodiversity provides opportunities to extract genes and proteins with unique properties for industrial and environmental applications. As a result, in this study, a *Janibacter* sp strain that could grow at a 150 mM concentration of glyphosate was isolated from marine sediment, and the *aroA* gene from this strain was cloned and named as *aroA* _sp_. Phylogenetic analysis and conserved domain analysis revealed that *aroA* _sp_ is characterized as a novel and naturally occurring class I *aroA* enzyme but with glyphosate tolerance.

Table 3. Comparison of agronomic traits between transgenic homozygous lines and transgenic-negative lines under field conditions*. ME1-P and ME2-P represent homozygous transgenic lines of ME1 and ME2, while ME1-N and ME2-N are their corresponding negative transgenic lines. *The parameters were given as means (± standard deviation) for data collected from 10 plants in triplicate for each plant type. *Means significantly different from the control (P < 0.05).

| Line | Plant height (cm) | Panicle per plant | Panicle length (cm) | Seed-set rate | Weight per 1,000 grains (g) | Yield/plant (g) |
|------|------------------|-------------------|---------------------|--------------|-----------------------------|-----------------|
| ME1-P | 110.50 ± 3.36 | 12.30 ± 2.00 | 25.75 ± 0.39 | 0.73 ± 0.02 | 23.36 ± 1.13 | 32.11 ± 2.70 |
| ME1-N | 112.00 ± 1.30 | 12.70 ± 0.44 | 27.33 ± 1.33 | 0.70 ± 0.03 | 22.76 ± 1.10 | 32.20 ± 1.74 |
| ME2-P | 107.05 ± 3.20 | 12.73 ± 1.76 | 24.80 ± 0.44* | 0.71 ± 0.06 | 23.86 ± 1.13 | 30.27 ± 9.22 |
| ME2-N | 112.87 ± 2.66 | 14.63 ± 1.14 | 25.94 ± 0.40 | 0.74 ± 0.01 | 24.29 ± 1.01 | 38.72 ± 2.54 |

*Means significantly different from the control (P < 0.05).
**Methods**

**Materials and Mediums.** Marine sediment was sampled at site IR-CTDS(16°59.9412′N 124°58.2958′E) on the south west Indian Ridge. The glyphosate tolerant bacteria *J. sp* was isolated from the marine sediment. Bacterial strains, *Escherichia coli* DH5α, *E. coli* aroA mutant strain AB2829, *E. coli* BL21 (DE3) and the zhonghua11 *japonica* rice variety were stored in our laboratory. The pUC118 vector was purchased from Takara (Japan), and the vector pGEX-6p-1 was kept in our laboratory. Vector pCAMBIA1300 was a gift from the Center of Application of Molecular Biology to International Agriculture, Australia. All enzymes used for restriction digestion and ligations were purchased from Takara (Japan). S3P was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PEP and glyphosate were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade. The medium used for isolating glyphosate tolerant strain was minimal medium M9 (6.8 g/L NaHPO4, 3.0 g/L K2HPO4, 1.0 g/L NH4Cl, 0.5 g/L NaCl and 0.12 g/L MgSO4) prepared in artificial sea water (distilled water with 0.5 g/L MgCl2, 0.5 g/L MgSO4, 0.5 g/L CaCl2, 0.55 g/L KCl, 0.16 g/L NaHCO3, 0.08 g/L KBr, 34 mg/L SrCl2, 22 mg/L H3BO3, 4 mg/L Na2SiO3, 2.4 mg/L NaF), and the minimal medium used for the
glyphosate tolerance test in *E. coli* was M9 (6.8 g/L Na₂HPO₄, 3.0 g/L K₂HPO₄, 1.0 g/L NH₄Cl, 0.5 g/L NaCl and 0.12 g/L MgSO₄) supplemented with 0.4% (w/v) glucose as a carbon source plus the appropriate antibiotics.

**Isolation of glyphosate-tolerant strain.** The marine sediment was used for enrichment with glyphosate for 7 d, and then the enriched culture was diluted with distilled water and plated on M9 agar plates containing 60 mM glyphosate. After 48 h of incubation at 28 °C, the colonies were further screened at 100 and 150 mM glyphosate concentrations. One strain numbered L42 grew well in the presence of 150 mM glyphosate and was chosen for further study. The 16S rRNA sequence of L42 strain was used for species identification.

**Isolation of gene associated with glyphosate tolerance.** A genomic library of the L42 strain was constructed. The chromosomal DNA was extracted from the strain and partially digested with *Sal* III to produce 4–9 kb fragments. These DNA fragments were inserted into the pUC118 vector. The ligation mixture was incubated at 4 °C overnight and transformed into *E. coli* DH5α. The transformants containing the recombinant pUC118 were transferred onto M9 agar plates containing 20 mM glyphosate and then the plates were incubated at 37 °C for 48 h. One colony exhibiting glyphosate tolerance was isolated from the plates containing a recombinant plasmid (pZY3) with an insert of approximately 3.5 kb.

**Sequence analysis.** The inserted fragment from the plasmid pZY3 was sequenced by the Genescript Company (Nanjing, China), and the nucleotide sequences were analyzed using the Softberry Gene Finding Tool (http://linux1.softberry.com/berry). Sequence alignments were performed using the BLAST program (http://blast.ncbi.nlm.gov/blast) combined with the ClustalW program software. The phylogenetic tree of aroA gene was constructed by MEGA5.

**Construction of prokaryotic expression vectors pGEX-6p-1-aroA*<sub>E. coli</sub>* and pGEX-6p-1-aroA*<sub>J. sp</sub>*.** The oligonucleotide primer sequences were as follows: primer 1 (5′-CGCGGATCCATGACCGATCTGCTGATTGGCATGC-3′) (the BamHI site is underlined); primer 2 (5′-CGCGAATTCTTCCGCGCCAGCTCGCTCG-3′) (the EcoRI site is underlined), which was designed according to the sequence derived from the insert of plasmid pZY3 and supplied by the Genescript; primer 3 (5′-CGCGGATCCATGAAATCACTGGCTGATGCTGTCG-3′); and primer 4 (5′-CGCGAATTCTCAGGCTGCCTGGCTAATCCGCGCCAGCTCG-3′), which was specific for amplifying *aroA*<sub>E. coli</sub> gene and was designed based on the sequence available in GenBank (GenBank: X00557). The *aroA*<sub>E. coli</sub> and *aroA*<sub>J. sp</sub> genes were amplified, using the recombinant plasmid pZY3 and the genomic DNA from *E. coli* as the template, respectively. The *FastPfu* DNA polymerase (Transgen, Beijing, China) was used for all the reactions under the following conditions: 30 cycles of 94 ºC for 20 sec, 55 ºC for 20 sec, and 72 ºC for 1 min. After PCR, the amplified products were gel-purified, digested with *BamHI* and *EcoRI*, and ligated into the pGEX-6p-1 vector.

**Cell growth in the presence of glyphosate.** Strain *E. coli* AB2829<sup>11</sup> was transformed with pGEX-6p-aroA*<sub>J. sp</sub>* plasmid and the transformants were cultured in 50 mL LB medium containing 100 µg/mL ampicillin at 37 °C until the OD<sub>600</sub> values reached 0.1. The culture was collected by centrifugation and washed twice using liquid M9 minimal medium. Bacteria in the culture were then grown with shaking at 37 °C in liquid M9 minimal medium supplemented with glyphosate at the concentrations of 0, 50 and 100 mM. The OD<sub>600</sub> values of the cultures were determined at approximately 5-h intervals to record the growth rates of the strains till 50 h. OD<sub>600</sub> increments were calculated as (OD<sub>a</sub>-OD<sub>b</sub>)/OD<sub>0</sub>, in which ODa and ODb represent the OD<sub>600</sub> value before and after growing for 50 h, and OD<sub>0</sub> is the final OD<sub>600</sub> value without glyphosate. The results presented are the averages of two sets of experiments done in triplicate. The strain AB2829 harboring pGEX-6p-aroA*<sub>J. sp</sub>* was used as the control.

**Expression and purification of aroA*<sub>J. sp</sub>* and aroA*<sub>E. coli</sub>* enzymes.** *E. coli* BL21 (DE3) was transformed separately with pGEX-6p-1-aroA*<sub>J. sp</sub>* and pGEX-6p-1-aroA*<sub>E. coli</sub>* plasmids, and cultivated in LB medium containing 100 µg/mL ampicillin at 37 °C until the OD<sub>600</sub> values reached 0.6–1.0. IPTG (0.5 mM) was then added, and the cultures were further incubated at 18 °C for 8 h. The cells were harvested and resuspended in 50 mL Hepes buffer (50 mM Hepes, 100 mM KCl, and 2 mM dithiothreitol, pH 7.0). After treatment by high pressure crushing, the cells were centrifuged at 10,000 g for 40 min at 4 °C, and the supernatant was loaded onto a Glutathione-S-Transferase (GST) agarose at 4 °C. The glutathione-S-transferase (GST)-tagged aroA enzymes were purified using a GST Fusion System (GE Healthcare Sweden). The GST tag was removed by digestion with a 3C protease solution (10 U/µL, PreScission; Pharmacia). The molecular mass of the protein was determined by a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the enzyme concentration was measured by a Bradford Protein assay kit (Sangon, China).

**Enzyme assay.** AroA enzyme activity was determined by measuring the release of inorganic phosphate using the malachite green dye assay method<sup>11</sup>. The reaction (final volume, 50 µL) was performed at 28 °C in 50 mM Hepes buffer (pH 7.0), 1 mM S3P, 1 mM PEP, and 0.4 µg of the purified enzyme. After incubation for 4 min, 0.8 mL malachite green (0.045%) /ammonium molybdate (4.2%) colorimetric solution was added; 1 min later, the reaction was terminated by adding 0.1 mL 34% (w/v) sodium citrate solution. After 30 min of incubation at room temperature, the absorbance of the samples was measured at 660 nm; the same reaction solution without S3P was used as the blank control. The *Kₘ* values for substrates, *Kᵥ* value and *IC₅₀* value for glyphosate were calculated as described by Tian et al.<sup>12</sup>
Generation of transgenic rice. To evaluate the potential application of the isolated araoA sp gene in developing glyphosate tolerant crops, a japonica rice variety, zhonghua11 (ZH11) and an indica rice variety, minghui86 (MH86), were selected as the plant materials for genetic transformation. The chloroplastic targeting signal peptide (CTP) coding sequence is from Arabidopsis thaliana and contains 120 nucleotides (Genebank: AAB72287.1). The nucleotide sequence of the araoA sp gene and CTP were codon-optimized according to the codon bias in rice and was chemically synthesized to produce ctp-araoA sp fusion gene (Takara, Japan). The hpt expression cassette in the binary vector pCAMBIA1300 (provided by the Center for the Application of Molecular Biology in International Agriculture, Australia) was removed by using Xhol and EcoRI restriction sites, resulting in pU130 transformation vector. Maize Ubi promoter and ctp-araoA sp fusion gene were subcloned into pU130 using multiple clone sites, and pU130-araoA sp (Ubi-1: araoA sp: 35S polyA) was obtained. The pU130-araoA sp was introduced into A. tumefaciens EHA105 by electroporation, and by following the callus culture and transformation procedures as described by Hiei et al. the resistant calli were obtained from the selection medium containing 200 mg/L glyphosate.

Molecular analysis of transgenic rice. To confirm the correct integration of the araoA sp gene into the rice genome, the genome DNA from young leaves of rice plants was extracted by CTAB method and used as the template for PCR to amplify an 815 bp fragment of araoA sp with Primer 5 (5′-TTCTCTTAAGCGAAAAACCCC-3′) and Primer 6 (5′-AGGAGGGGCGACGAGACTG-3′). Expression of araoA sp gene in T0 transgenic rice was analyzed by RT-PCR. Total RNA from rice plants was extracted using the Trizol reagent (Transgen, China) according to the manufacturer’s instructions. For the first strand cDNA synthesis, 1 μg of genomic DNA was used with M-MLV reverse transcriptase (Invitrogen, USA) after DNase I digestion (Invitrogen, USA). The endogenous actin gene was amplified with the primers actin-F (5′-GCCACACTGCTCCGATCTAT-3′) and actin-R (5′-GGGACACCTCTGATCTCTCAT-3′) as an internal control. The ctp-araoA sp fragment was amplified with primer 7 (5′-GGCTCTATATAGCCGTCGGGTTCC-3′) and primer 8 (5′-GGCTCTATATAGCCGTCGGGTTCC-3′), resulting in a 1.3 kb amplified DNA product. For Southern blot analysis, 10 μg of genomic DNA digested with HindIII or SacI was electrophoresed on 0.8% agarose gel and transferred onto a Hybond nylon membrane (GE Healthcare UK Limited). The araoA sp probe was DIG labeled using Primer 5 (5′-TTCTCTTAAGCGAAAAACCCC-3′) and Primer 6 (5′-AGGAGGGGCGACGAGACTG-3′). Hybridization and detection steps were performed according to the manufacturer’s instructions (Roche, Mannheim, Germany).

Glyphosate tolerance assay of transgenic rice. Glyphosate-tolerance of transgenic rice plants was assayed by herbicide spraying. The T0 and T1 transgenic rice plants containing araoA sp gene were planted in the weed controlled field and sprayed with either different amounts of glyphosate or 1% (vol/vol) solution of the herbicide Roundup which contains 41% isopropylamine salt of glyphosate (Monsanto, Malaysia). In addition, T1 generation seedlings of ME1 line were also transplanted into a weedy field without weed control to investigate the potential to be used in no-till system. In the weedy field, there were mainly Gramineae weeds (Echinochloa crusgalli (L.) Beauv), Cyperaceae weeds (Cyperus difformis L.) and Cyperus fuscus L., Pontederiaceae weeds (Monochoria vaginalis (Burm.f) Presl ex Kunth) and Scrophulariaceae weeds (Lindernia procumbens (Krock.) Borbas) in this research.

References
1. Bentley, R. The shikimate pathway-a metabolic tree with many branches. Crit. Rev. Biochem. Mol. Biol. 25, 307–384 (1990).
2. Boocock, M. R. & Coggins, J. R. Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate. FEBS Lett. 154, 127–133 (1983).
3. Steinrücken, H. C. & Amrhein, N. 5-Enolpyruvylshikimate-3-phosphate synthase of Klebsiella pneumoniae 2. Inhibition by glyphosate [N-(phosphonomethyl)glycine]. Eur. J. Biochem. 143, 351–357 (1984).
4. Priestman, M. A. et al. Interaction of phosphate analogues of the tetrahedral reaction intermediate with 5-enolpyruvylshikimate-3-phosphate synthase in atomic detail. Biochemistry. 44, 3241–3248 (2005).
5. Schönbrunn, E. et al. Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate-3-phosphate synthase in atomic detail. Proc. Natl. Acad. Sci. USA 98, 1376–1380 (2001).
6. Green, J. M. Current state of herbicides in herbicide-resistant crops. Pest Manag. Sci. 70, 1351–1357 (2014).
7. Comai, L. & Sen, I. C. & Stalker, D. M. An altered araoA gene product confers resistance to the herbicide glyphosate. Science 221, 370–371 (1983).
8. Pollegioni, L., Schönbrunn, E. & Siehl, D. Molecular basis of glyphosate resistance—different approaches through protein engineering. FEMS J. 278, 2753–2766 (2011).
9. Funke, T., Han, H., Healy-Fried, M. L., Fischer, M. & Schönbrunn, E. Molecular basis for the herbicide resistance of Roundup Ready crops. Proc. Natl. Acad. Sci. USA. 103, 13016–13015 (2006).
10. Priestman, M. A., Funke, T., Singh, I. M., Crupper, S. S. & Schönbrunn, E. 5-Enolpyruvylshikimate-3-phosphate synthase from Staphylococcus aureus is insensitive to glyphosate. FEBS Lett. 579, 728–32 (2005).
11. Dill, G. M., Cafajec, C. A. & Padgette, S. R. Glyphosate-resistant crops: adoption, use and future considerations. Pest Manag. Sci. 64, 326–331 (2008).
12. Tian, Y. S. et al. Isolation from Ochrobactrum antropi of a novel class II 5-enolpyruvylshikimate-3-phosphate synthase with high tolerance to glyphosate. Appl. Environ. Microb. 76, 6001–6005 (2010).
13. Tian, Y. S. et al. Functional characterization of Class II 5-enolpyruvylshikimate-3-phosphate synthase from Halotolerant thermotolerans H168 in Escherichia coli and transgenic Arabidopsis. Appl. Environ. Microb. 93, 241–250 (2012).
14. Tian, Y. S. et al. Complementary screening, identification and application of a novel class II 5-enolpyruvylshikimate-3-phosphate synthase from Bacillus cereus. World J. Microb. Biot. 29, 549–557 (2013).
15. Wang, L. et al. Characterization of a class II 5-enolpyruvylshikimate-3-phosphate synthase with high tolerance to glyphosate from Sinorhizobium fredii. World J. Microb. Biot. 30, 2967–2973 (2014).
16. Zhang, Y. et al. Characterization and site-directed mutagenesis of a novel class II 5-enolpyruvylshikimate-3-phosphate synthase from the deep-sea bacterium Alaminovox sp. L27. Enzyme Microb. Tech. 63, 64–70 (2014).
17. Li, L. et al. A novel RPMXR motif among class II 5-enolpyruvylshikimate-3-phosphate synthases is required for enzymatic activity and glyphosate resistance. J. Biotechnol. 144, 330–336 (2009).
18. Ye, G. N. et al. Plastid-expressed 5-enolpyruvylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. Plant J. 25, 261–270 (2001).
19. Kahrizi, D., Salmanian, A. H., Afshari, A., Moieni, A. & Mousavi, A. Simultaneous substitution of Gly96 to Ala and Ala183 to Thr in 5-enolpyruvylshikimate-3-phosphate synthase gene of E. coli (k12) and transformation of rapeseed (Brassica napus L.) in order to make tolerance to glyphosate. *Plant Cell Rep*. **26**, 95–104 (2007).
20. Yan, H. Q. *et al.* Novel AraA from *Pseudomonas putida* confers tobacco plant with high tolerance to glyphosate. *PLoS One.* **6**, e19732 (2011).
21. Zhao, T., Lin, C. Y. & Shen, Z. C. Development of transgenic glyphosate-resistant rice with G6 gene encoding 5-enolpyruvylshikimate-3-phosphate synthase. *Agr. Sci. China.* **10**, 1307–1312 (2011).
22. Chhapanekar, S. *et al.* Transgenic rice expressing a codon-modified synthetic CP4-EPSPS confers tolerance to broad-spectrum herbicide, glyphosate. *Plant Cell Rep.* **34**, 721–731 (2015).
23. He, M., Yang, Z. Y., Nie, Y. F., Wang, I. & Xu, P. A new type of class I bacterial 5-enolpyruvylshikimate-3-phosphate synthase mutants with enhanced tolerance to glyphosate. *Biochi. Biophys. Acta.* **1568**, 1–6 (2001).
24. Cao, M. *et al.* Engineering higher yield and herbicide resistance in rice by mediated multiple gene transformation. *Crop Sci.* **44**, 2206–2213 (2004).
25. Funke, T. *et al.* Structural basis of glyphosate resistance resulting from the double mutation Thr297*Ile and Pro313*Ser in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. *J. Biol.Chem.* **284**, 9854–9860 (2009).
26. Sun, Y. C. *et al.* Novel AraA with high tolerance to glyphosate, encoded by a gene of *Pseudomonas putida* 4G-1 isolated from an extremely polluted environment in China. *Appl. Environ. Microb.* **71**, 4771–4776 (2005).
27. Cao, G. *et al.* A novel 5-enolpyruvylshikimate-3-phosphate synthase shows high glyphosate tolerance in *Escherichia coli* and tobacco plants. *PLoS One.* **7**, e38718 (2012).
28. Peng, R. H. *et al.* A novel 5-enolpyruvylshikimate-3-phosphate synthase from *Rahnella aquatilis* with significantly reduced glyphosate sensitivity. *PLoS One.* **7**, e39579 (2012).
29. Schledzewski, K. & Mendel, R. R. Quantitative transient gene expression: comparison of the promoters for maize polyubiquitin1, rice act1n1, maize-derived *Emu* and CaMV 35S in cells of barley, maize and tobacco. *Transgenic Res.* **3**, 249–255 (1994).
30. Alibhai, M. F. *et al.* 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Escherichia coli*, maize (Zea mays L.) and tobacco (Nicotiana tabaccum L.). *Plant Mol. Biol.* **9**, 153–164 (2002).
31. Shrawat, A. K. & Loerz, H. Agrobacterium-mediated transformation of cereals: a promising approach crossing barriers. *Plant Biotechnol. J.* **10**, 153–164 (2012).
32. Chandrasekhar, K. *et al.* Development of transgenic rice harbouring mutated rice 5-enolpyruvylshikimate-3-phosphate synthase (Os-mEPSPS) and *Allium sativum* leaf agglutinin (ASAL) genes conferring tolerance to herbicides and sap-sucking insects. *Plant Mol. Biol. Rep.* **32**, 575–603 (2014).
33. Wang, J. X., Zhao, E. Y. & Xu, P. Use of *aroA-M1* as a selectable marker for *Brassica napus* transformation. *Crop Sci.* **46**, 706–711 (2006).
34. Imai, S. *et al.* Identification of a glyphosate-resistant mutant of rice 5-enolpyruvylshikimate-3-phosphate synthase using a directed evolution strategy. *Plant physiol.* **140**, 184–195 (2006).
35. Chen, J., Zhou, M. & Zhang, X. *et al.* Identification of a mutation in the 5-enolpyruvylshikimate-3-phosphate synthase gene encoding for resistance to glyphosate in *Oryza sativa*. *Nucleic Acids Res.* **34**, 2710–2717 (2006).
36. Tamura, K. *et al.* MEGAS: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739 (2011).
37. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. & Candia, O. A. An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* **100**, 95–97 (1979).
38. Tian, Y. S. *et al.* Improvement of glyphosate resistance through concurrent mutations in three amino acids of the *Ochrobactrum* 5-enolpyruvylshikimate-3-phosphate synthase. *Appl. Environ. Microb.* **77**, 8409–8414 (2011).
39. Shrawat, A. K. & Loerz, H. Agrobacterium-mediated transformation of cereals: a promising approach crossing barriers. *Plant Biotechnol. J.* **1**, 575–603 (2004).
40. Norrander, J., Kempe, T. & Messing, J. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene.* **26**, 101–106 (1983).
41. Chen, R. *et al.* Multiple sequence alignment with the clustal series of programs. *Nucleic Acids Res.* **31**, 3497–3500 (2003).
42. Tamura, K. *et al.* MEGAS: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739 (2011).
43. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. & Candia, O. A. An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* **100**, 95–97 (1979).
44. Hei, Y., Ohta, S., Komari, T. & Kumashiro, T. Efficient transformation of rice (*Oryza sativa*) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282 (1994).
45. Murray, M. G. & Thompson, W. F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325 (1980).

**Acknowledgements**

This work was supported by grants from the National Natural Science Foundation of China (No. 31270162), the National Program of Transgenic Variety Development of China (2011ZX08001-001), and 863 Program of China (No. 2012AA10A304).

**Author Contributions**

Y.L., Z.L. and F.Z. conceived the experiments. S.Y., Y.C. and Y.Z. conducted the experiments. S.Y., Y.C. and F.Z. analyzed the results. S.Y., Y.C. and F.Z. wrote the paper. Y.L., Y.C. and E.Z. revised the paper. All authors reviewed the manuscript.

**Additional Information**

Competing financial interests: The authors declare no competing financial interests.

**How to cite this article**: Yi, S. *et al.* A Novel Naturally Occurring Class I 5-enolpyruvylshikimate-3-Phosphate Synthase from *Janibacter* sp. Confers High Glyphosate Tolerance to Rice. *Sci. Rep.* **6**, 19104; doi: 10.1038/srep19104 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/