An ABCC-type transporter endowing glyphosate resistance in plants

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Glyphosate is the most widely used herbicide in world agriculture and for general vegetation control in a wide range of situations. Global and often intensive glyphosate selection of very large weedy plant populations has resulted in widespread glyphosate resistance evolution in populations of many weed species. Here, working with a glyphosate-resistant (GR) *Echinochloa colona* population that evolved in a Western Australia agricultural field, we identified an ATP-binding cassette (ABC) transporter (*EcABCC8*) that is consistently up-regulated in GR plants. When expressed in transgenic rice, this *EcABCC8* transporter endowed glyphosate resistance. Equally, rice, maize, and soybean overexpressing the *EcABCC8* ortholog genes were made resistant to glyphosate. Conversely, CRISPR/Cas9-mediated knockout of the *EcABCC8* ortholog gene *OsABCC8* increased rice susceptibility to glyphosate. Subcellular localization analysis and quantification of glyphosate cellular levels in treated *ABCC8* transgenic rice plants and isolated leaf protoplasts as well as structural modeling support that EcABCC8 is likely a plasma membrane–localized transporter extruding cytoplasmic glyphosate to the apoplast, lowering the cellular glyphosate level. This is a report of a membrane transporter effluxing glyphosate in a GR plant species, and its function is likely conserved in crop plant species.

Echinochloa colona | glyphosate resistance | ABC transporter | glyphosate exporter | plasma membrane

Glyphosate is the world's most widely used herbicide (1, 2), lethal to a wide range of plant species, both annual and perennial. Around one million tons of glyphosate is being used annually for weed control across the world, especially because of high adoption of glyphosate-resistant (GR) transgenic crops. Consequently, glyphosate selection on huge numbers of genetically diverse weedy plant species has resulted in glyphosate resistance evolution, now known in 48 GR weed species worldwide (3).

Glyphosate specifically inhibits the plastidic 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), disrupting the biosynthesis of aromatic amino acids (4). In evolved GR weeds, resistance can be due to specific EPSPS resistance mutations, EPSPS amplification (5, 6), or due to enhanced glyphosate metabolism (7). Glyphosate is foliar absorbed, readily translocated throughout the plant to meristematic regions, and must move across the plasma membrane (PM) and enter plastids to inhibit EPSPS. Importantly, glyphosate resistance can be unrelated to EPSPS but rather due to reduced glyphosate translocation within the plant (5, 8–10). First observed in GR *Lolium rigidum* (11), this reduced glyphosate translocation has often been observed in GR *Lolium spp* (12–14), *Conyza* spp (15–17), *Sorghum halepense* (18, 19), *Amaranthus palmeri* (20, 21), *Digitaria insularis* (22), and *Ambrosia trifida* (23). In some GR weed species, there is biochemical evidence that glyphosate is sequestered in vacuoles, or its cellular uptake is reduced (24–26).

There is speculation that if tonoplast or PM ATP-binding cassette (ABC) transporters could move glyphosate out of the cytoplasm then this could endow glyphosate resistance (8). Transcriptome studies revealed a few ABC transporter genes were constitutively higher expressed or induced by glyphosate treatment in GR versus susceptible (S) plants (27, 28). However, there has been no specific evidence of ABC transporter genes endowing glyphosate resistance. In contrast, in human cancer, anticancer drug resistance is well known to be mediated by ABC transporters, including the multidrug resistance proteins (MRPs) (29). For example, HsMRP1 (also known as HsABCC1) can extrude many anticancer drugs from cancer cells, thereby enabling ongoing cell function and division (30, 31).

Plant genomes encode between 120 to 140 ABC transporters, divided into the ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCI subfamilies (32, 33). Plant ABC transporters function in multiple physiological processes such as transport of hormones, lipids, metals, and secondary metabolites, detoxification of xenobiotics, plant–microbe interactions, etc. (32, 33). Laboratory-generated *Arabidopsis* transporter variants in the ABCB or ABCG family can be resistant to the antibiotic kanamycin (34) or some auxin and/or dinitroaniline herbicides (35, 36) and paraquat (37). The ABCC (also known as MRP) transporter family typically transports organic acids alone, or as glutathione or glucose conjugates, from the cytoplasm into vacuoles. Only some of these

Significance

Glyphosate is the world's dominantly used herbicide to control weedy plant species in a wide range of situations, especially in global field crops of soybean, maize, canola, and cotton with genetically engineered glyphosate resistance. Persistent glyphosate selection has led to worldwide evolution of glyphosate-resistant weeds. Several biochemical and physiological mechanisms have been identified that endow glyphosate resistance. To be toxic to plants, glyphosate must be present in the cytoplasm, and thus mechanisms reducing the cytoplasmic glyphosate to a sublethal level could confer resistance. Here, we provide evidence of a plant ABC transporter (ABCC8) that likely serves as a plasma membrane glyphosate exporter, lowering the cytoplasmic glyphosate level and thereby endowing glyphosate resistance.

AGRICULTURAL SCIENCES

transporters have been functionally characterized (32, 33, 38). Multispecificity has been shown for several plant ABCCs (e.g., AtABCC2 transports phytochelatins, phytochelatin conjugates, glutathione conjugates, glucuronate conjugates, chlorophyll catabolites, and auxin conjugates) (32, 39). AtABCC1, AtABCC2, and OsABCC1 can sequester phytochelatin-conjugated arsenic to vacuoles for detoxification (40, 41). AtABCC5, an ortholog of maize ZmMRP4, can transport phytate to vacuoles for Pi storage and stomatal aperture regulation (42).

Echinochloa colona (awnless barnyardgrass, also known as jungle rice) is a global agricultural C4 weed infesting many warm season crops. *E. colona* is a genetically diverse, resistance-prone weed species, including there being many GR biotypes in various parts of the world (3). Here, we report on our GR *E. colona* population that we have long studied (43). Using GR versus S plants, our leaf disk study found a trend of increased glyphosate efflux and therefore reduced glyphosate cellular content (44). We speculated whether glyphosate resistance in this GR *E. colona* could be due to increased ability of plant ABC transporters to extrude glyphosate from the cytoplasm, additionally to the recently discovered glyphosate metabolism (7). Accordingly, and following transcriptomics indications, we cloned and characterized a plant ABC transporter (ABCC8) from GR *E. colona* and here confirm that heterologous expression of this ABC transporter and overexpression of its orthologs in plants confer glyphosate resistance by reducing the cytoplasmic glyphosate level. This is evidence of a plant ABC transporter conferring field-evolved herbicide resistance.

Results

RNA Sequencing Analysis and Multiple Step Validations Linked Two ABC Transporter Gene Contigs with Glyphosate Resistance in GR *E. colona*. According to the selection criterion of twofold change and $P < 0.05$, initially nine out of 18 differentially expressed candidate contigs with membrane transporter gene annotation in RNA sequencing (RNA-seq) analysis were selected from the GR versus S *E. colona* samples and validated using RT-qPCR (*SI Appendix*, Tables S1 and S2). Five of these (*SI Appendix*, Table S3) were further confirmed using an additional six GR and six S spare samples for RNA-seq and were subjected to several rounds of validation using a series of prephenotyped samples from between and within multiple GR and S populations/lines. This revealed that two ABC transporter contigs, EC_v4.g098055 and EC_v4.g102032 (*SI Appendix*, Table S3), showed consistently and significantly higher expression in all GR versus S comparisons and lower expression in additional S populations (QBG1 and Grossy). Furthermore, as we have shown that the level of glyphosate resistance in the GR line is influenced by temperature (7), expression of the two ABC transporter contigs were tested for response to temperature. Significantly higher expression was recorded under 35/30 °C than 25/20 °C growth temperatures (*SI Appendix*, Table S3). These multiple test results indicated that higher expression of the two ABC transporter contigs, EC_v4.g098055 and EC_v4.g102032, might be associated with glyphosate resistance in this GR *E. colona* population.

Sequence Analysis of the Two Candidate ABC Transporter Genes in GR and S *E. colona* Revealed No Amino Acid Substitutions. Full coding sequences of the two ABC transporter contigs were cloned from GR and S *E. colona* lines. The coding sequence of the contig EC_v4.g098055 was 4,299 base pairs (bp), sharing 90% amino acid sequence identity to *Panicum hallii* ABCC8 (XP_025810778.1), 87% to *Zea mays* MRP1 (NP_001105942.2), 77% to *Oryza sativa* ABCC8 (KAF0901840.1), and 71% to *Glycine max* ABCC8 (XP_014633115.1). The coding sequence of the contig EC_v4.g102032 was 4,527 bp, sharing 88% sequence identity to *P. hallii* ABCC10 (XP_025806285.1) and 87% to *Dichanthelium oligosanthes* ABCC10 (OEL24435.1). Amino acid sequences of

contigs EC_v4.g098055 and EC_v4.g102032 only share 39% identity. The phylogenetic tree analysis indicates that contig EC_v4.g098055 has a close evolutionary relationship with ABCC8 and contig EC_v4.g102032 with ABCC10, from various plant species (*SI Appendix*, Fig. S1 *A and B*). Based on this, the two ABC transporter genes are named as *EcABCC8* and *EcABCC10* (National Center for Biotechnology Information [NCBI] accession nos.: MT249005, MT249006), respectively.

Alignment of the *EcABCC8* coding sequences between GR and S *E. colona* samples showed only one single nucleotide polymorphism (SNP), and this did not cause any amino acid change. No SNPs were found in *EcABCC10* in GR and S *E. colona* sequences. In addition, *EcABCC8* and *EcABCC10* sequences from the two supplementary S populations (QBG1 and Crossy) were also cloned and compared with the S. Three silent SNPs in *EcABCC10* and four in *EcABCC8* were found. These results are indicative that glyphosate resistance could be due to overexpression but not mutation of the two ABC transporter genes in this GR *E. colona* population.

Heterologous Expression of *EcABCC8* and Overexpression of Its Orthologs in Planta Confer Glyphosate Resistance.
Heterologous expression of *EcABCC8* in rice. The two ABC transporter genes (*EcABCC8* and *EcABCC10*) were used to transform rice for functional characterization in comparison to rice expressing the *GFP* gene. Treatment of *GFP* and *EcABCC10*-OE seedlings (24 from four T_1 lines) with glyphosate at the field rate of 540 g · ha⁻¹ caused 100% mortality (*SI Appendix*, Fig. S2), and therefore, no further analysis on *EcABCC10*-OE lines was conducted. Conversely, 30 T_1 rice seedlings, from five *EcABCC8*-OE lines, survived this glyphosate treatment. Segregation revealed a resistance versus susceptibility ratio of 22:8, indicating single gene 3:1 inheritance (Fig. 1*A*). There was no resistance to the primary glyphosate metabolite aminomethyl phosphonic acid (AMPA) (50 mM) or to a widely used alternative herbicide glufosinate (250 g · ha⁻¹), relative to the GFP control (*SI Appendix*, Fig. S3). Subsequently, 20 T_2 seedlings from four *EcABCC8*-OE lines were treated at the same glyphosate field rate (540 g · ha⁻¹), and all survived (Fig. 1*B*). Quantification of glyphosate dose response in one homozygous T_2 *EcABCC8*-OE line (Fig. 1*C*) revealed a GR_{50} value (herbicide dose causing 50% growth reduction) of $1,847 \pm 282$ in comparison to 84 ± 12 g · ha⁻¹ for the GFP control line, giving 22-fold ($P = 0.005$) glyphosate resistance. These results clearly established that heterologous expression of the ABC transporter gene *EcABCC8* in rice conferred glyphosate resistance.
Homologous expression of EcABCC8 orthologs in rice, maize, and soybean. *OsABCC8* (LOC_Os06g36650), *ZmABCC8* (Zm00001d046226), and *GmABCC8* (Glyma.07G011600.1) are orthologous genes of *EcABCC8* in rice, maize, and soybean, sharing 77, 87, and 71% identity in protein sequence to EcABCC8, respectively. Four T_1 lines each from rice, maize, and soybean (with six to seven seedlings per line) overexpressing the respective ortholog gene were tested for glyphosate resistance. In each of these three crop species, overexpression of the *ABCC8* orthologs endowed resistance at the glyphosate field rate (540 g · ha⁻¹), relative to their respective GFP or wild-type (WT) controls (Fig. 2). The ratio of surviving to dead plants was 22:6, 19:5, and 21:7 in T_1 rice, maize, and soybean, respectively, close to single gene 3:1 inheritance. One T_1 line each from *OsABCC8*-OE rice, *GmABCC8*-OE maize, and *ZmABCC8*-OE soybean was used for quantifying glyphosate resistance levels. GR_{50} values from the glyphosate dose responses (*SI Appendix*, Fig. S4) are $1,330 \pm 222$ versus 93 ± 18 g · ha⁻¹ for *OsABCC8*-OE versus *GFP* plants, 568 ± 83 versus 41 ± 1.5 g · ha⁻¹ for *GmABCC8*-OE versus GmWT, and $5,860 \pm 140$ versus 337 ± 42 g · ha⁻¹ for *ZmABCC8*-OE versus ZmWT. Based on the GR_{50} ratio, a level of glyphosate resistance of 14-, 13.8- and 17-fold was obtained for *OsABCC8*-OE ($P = 0.005$), *GmABCC8*-OE ($P = 0.006$), and *ZmABCC8*-OE ($P = 0.003$)
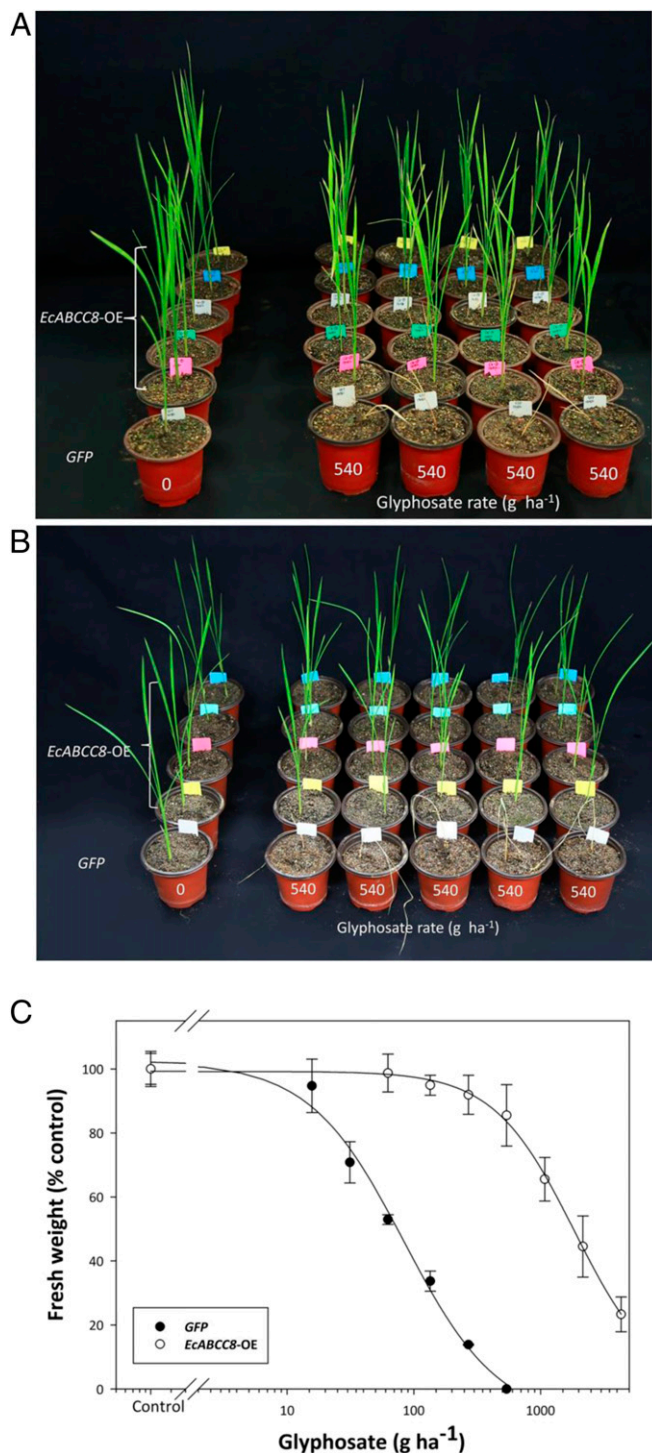
2 of 11 | PNAS
https://doi.org/10.1073/pnas.2100136118

Pan et al.
An ABCC-type transporter endowing glyphosate resistance in plants

Fig. 1. Heterologous expression of *EcABCC8* in rice endows glyphosate resistance. Growth of T_1 (*A*) (five lines) and T_2 (*B*) (four lines) transgenic rice seedlings expressing *EcABCC8* (*EcABCC8*-OE) or *GFP* control, 3 wk after glyphosate treatment. Only glyphosate surviving T_1 seedlings from *EcABCC8*-OE lines were shown in *A*, and the ratio of surviving to dead plants was 22:8. (*C*) Glyphosate dose response of *EcABCC8*-OE and the *GFP* control. The three- to four-leaf stage seedlings were foliar sprayed with glyphosate, and results were assessed 3 wk after treatment. Data points are means ± SE (*n* = 3).

The apparently weaker effect of GmABCC8 overexpression on soybean growth at the glyphosate field rate (540 g · ha⁻¹) (Fig. 2*C*) is likely due to intrinsically higher sensitivity of soybean to glyphosate and relatively lower sequence homology of *GmABCC8*
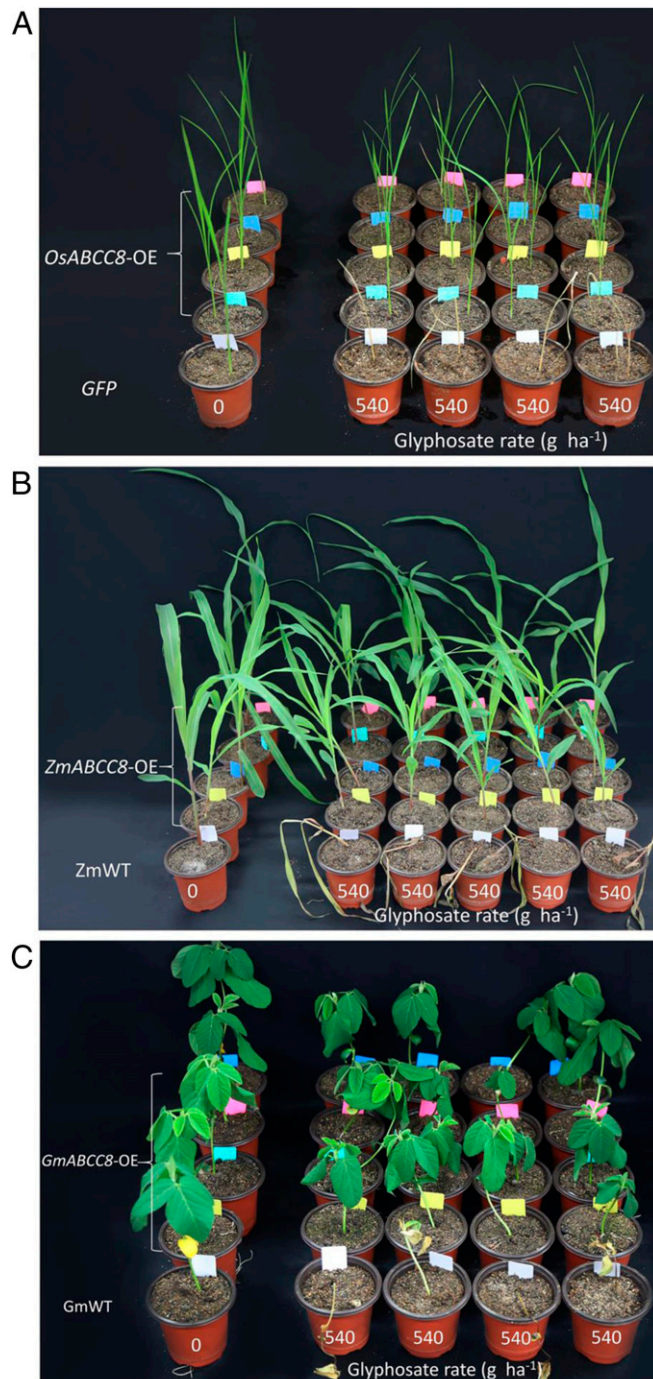

Fig. 2. Overexpression of *EcABCC8* ortholog genes in crop plants confers glyphosate resistance. Growth response to glyphosate of T_1 plants overexpressing *OsABCC8* (*OsABCC8*-OE) in rice (*A*), *ZmABCC8* (*ZmABCC8*-OE) in maize (*B*), and *GmABCC8* (*GmABCC8*-OE) in soybean (*C*), relative to *GFP* or untransformed WT controls. Plants at the four- to six-leaf stage were foliar sprayed with a field-relevant glyphosate rate (540 g · ha⁻¹), and photos were taken 3 wk after treatment. Note only glyphosate surviving T_1 seedlings from *ABCC8* overexpressing lines are shown. The ratio of surviving to dead plants was 22:6, 19:5, and 21:7 for *OsABCC8*-OE rice, *ZmABCC8*-OE maize, and *GmABCC8*-OE soybean, respectively, close to single gene 3:1 inheritance.

line, respectively. These results suggest that the ABCC8 transporter EcABCC8 and its orthologs have conserved function in plant species.

Pan et al.
An ABCC-type transporter endowing glyphosate resistance in plants

PNAS | **3 of 11**
https://doi.org/10.1073/pnas.2100136118

(71%) to *EcABCC8* compared to *OsABCC8* (77%) and *ZmABCC8* (87%).

CRISPR/Cas9 Knockout of *OsABCC8* Increases Glyphosate Susceptibility in Rice. To further confirm the function of the ABC transporter ABCC8, we generated *OsABCC8* nonfunctional, knockout (KO) rice mutants. Among seven T_1 nonfunctional KO mutants, four of them had a nucleotide insertion (allele1, *SI Appendix,* Fig. S5) and three had a nucleotide deletion in the *OsABCC8* gene (allele2, *SI Appendix,* Fig. S5), leading to a frame shift with premature transcription termination. The *OsABCC8* gene was sequenced in 12 to 18 randomly chosen T_2 plants derived from each of the seven T_1 nonfunctional KO lines, and as expected, these T_2 lines all had identical deletion (*osabcc8-1*) or insertion (*osabcc8-2*) variants in *OsABCC8*.

Glyphosate susceptibility of these two T_2 nonfunctional KO variants was tested in comparison to the WT line. As expected, WT seedlings were S to glyphosate, with growth reduction (in shoot fresh weight) by $17 \pm 3.3\%$ at glyphosate as low as $26 \text{ g} \cdot \text{ha}^{-1}$ (Fig. 3*A*) and marked reduction ($48 \pm 5.3\%$ in shoot fresh weight) at $105 \text{ g} \cdot \text{ha}^{-1}$ (Fig. 3*B*). However, the two *OsABCC8*-KO variants were even more glyphosate S than the WT, suffering $39 \pm 4.2\%$ and $42 \pm 7.2\%$ growth reduction, respectively, at $26 \text{ g} \cdot \text{ha}^{-1}$ and 100% mortality at $105 \text{ g} \cdot \text{ha}^{-1}$ (Fig. 3 *A* and *B*).

The GR_{50} value estimated from glyphosate dose response was $86 \pm 9.6 \text{ g} \cdot \text{ha}^{-1}$ for WT and 24 ± 1.7 and $22 \pm 2.1 \text{ g} \cdot \text{ha}^{-1}$ for the two nonfunctional *OsABCC8*-KO variants, respectively, giving up to 3.9-fold ($P < 0.033$) increased glyphosate susceptibility (Fig. 3*C*). From these results, it is clear that KO of the *OsABCC8* gene increases glyphosate susceptibility. In contrast, as shown in Fig. 2*A*, overexpression of the *OsABCC8* gene in rice confers glyphosate resistance.

Regulation of the *EcABCC8* Expression in *E. colona* May Involve DNA Methylation. In order to investigate possible mechanisms regulating *EcABCC8* expression in *E. colona*, the 1, 990 bp promoter sequences of the *EcABCC8* were obtained from five plants of each GR and S populations. Sequence alignment showed only three SNPs in the promoter region comparing the GR and S samples (*SI Appendix,* Fig. S6). Global DNA methylation analysis identified three differentially methylated regions in the *EcABCC8* gene between the GR and S samples (*SI Appendix,* Fig. S7). Compared to S, the level of methylation in the GR *EcABCC8* gene was lower in two promoter regions at the CHH context (0.25 versus 0.39 and 0.54) and higher in one exon region under the CG contexts (0.55 versus 0.36) (*SI Appendix,* Fig. S7). These results indicate that epigenetic mechanisms may be involved in *EcABCC8* expression regulation, that is, a lower level of methylation in the promoter region is likely related to a higher level of *EcABCC8* expression in GR versus S plants.

Tissue Expression, Subcellular Location, and Function of ABCC8.
***ABCC8* is expressed in both above- and below-ground plant tissue.** The RT-qPCR results demonstrated that the *EcABCC8* gene expresses in leaf, stem, and root tissues of GR and S *E. colona* plants, with up to 10-fold higher expression in GR than in S (*SI Appendix,* Fig. S8). *EcABCC8* expression in leaf and stem of both GR and S plants was up to threefold higher than in the root (*SI Appendix,* Fig. S8).
***EcABCC8* is likely localized to the PM.** Rice and *Arabidopsis* protoplast transit expression systems were used to determine the subcellular localization of EcABCC8. Multiphoton confocal laser scanning microscopy examination of transformed rice protoplasts showed that protoplasts expressing *35S:GFP* alone had strong fluorescent signals in cytoplasm around the periphery of the large vacuole in the cytoplasm (Fig. 4*A*). When expressed together, green and red fluorescent signals respectively from the *35S:EcABCC8-GFP* and the PM marker *35S:SCAMP1-mRFP* completely merged to the
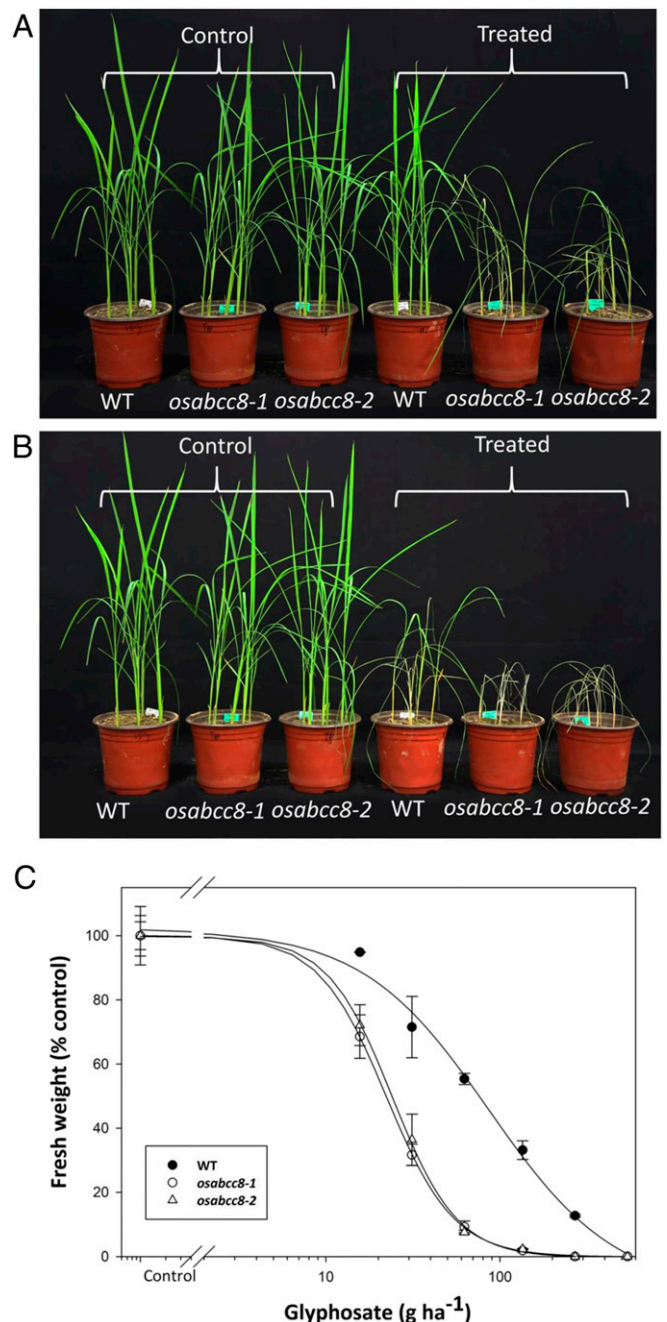

Fig. 3. KO of the *EcABCC8* ortholog gene in rice increases susceptibility to glyphosate. Growth response of the *osabcc8-1* and *osabcc8-2* KO mutants versus WT rice seedlings to glyphosate treatment at (*A*) 26 g · ha^{-1} and (*B*) 105 g · ha^{-1}. (*C*) Glyphosate dose response of the two KO versus WT lines. Plants at the three- to four-leaf stage were foliar treated with glyphosate, and results were assessed 3 wk after treatment. Data points are means ± SE (*n* = 3).

PM (Fig. 4*A*). However, this was not the case for the coexpressed tonoplast marker *35S:AtTPK3-mRFP*, which had red fluorescence outlining the large central vacuole, close to but clearly distinguishable from the green fluorescence of the *35S:EcABCC8-GFP* (Fig. 4*B*). In addition, line intensity scan analysis also revealed complete overlapping of fluorescence distribution of EcABCC8 with the PM marker (Fig. 4 *C, Upper*) and separation from the tonoplast marker (Fig. 4 *C, Lower*), supporting that EcABCC8 is more likely a PM- than a tonoplast-located transporter. Similar
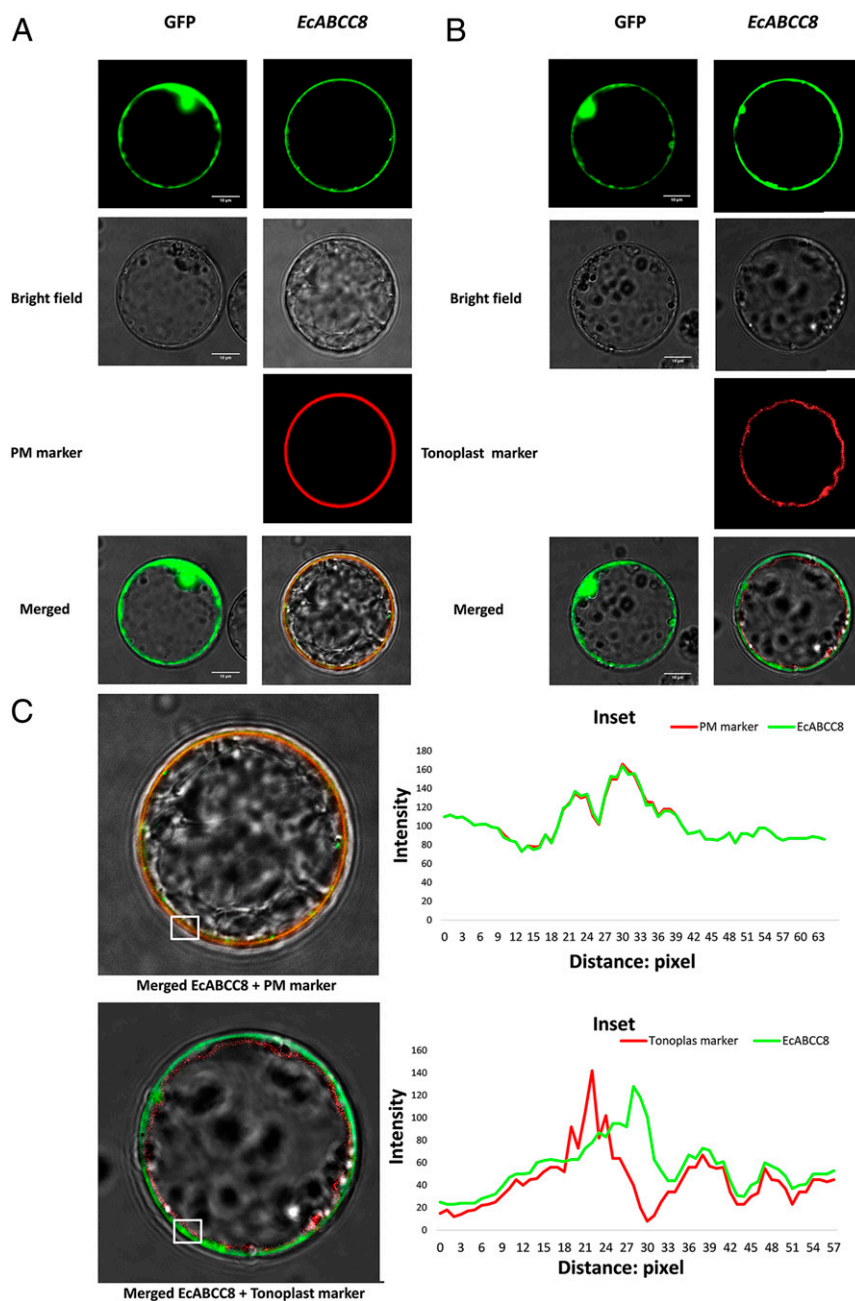
4 of 11 | PNAS
https://doi.org/10.1073/pnas.2100136118

Pan et al.
An ABCC-type transporter endowing glyphosate resistance in plants

Fig. 4. Subcellular location of EcABCC8. (*A*) Colocalization of the EcABCC8 and the PM marker and (*B*) lack of colocalization of the EcABCC8 and the tonoplast marker in rice protoplasts. (*C*) Line scan analysis showing overlapping of fluorescence distribution of EcABCC8 (green) and the PM maker (red) (*Upper*) and separation of EcABCC8 (green) and the tonoplast marker (red) (*Lower*) in areas of interest (boxed). (Scale bars, 10 μm.)

results were also obtained with the *Arabidopsis* protoplasts (*SI Appendix*, Fig. S9).

In addition, subcellular location of the *EcABCC8* ortholog *GmABCC8* was also probed using the same markers and is also likely localized to the PM (*SI Appendix*, Fig. S10).

ABCC8 enhances glyphosate efflux, reducing glyphosate cellular level in rice leaf discs. To avoid complication by any other glyphosate resistance mechanisms in the GR *E. colona* populations (e.g., AKR-mediated glyphosate metabolism, see ref. 7), glyphosate efflux and net content were examined in 1 mm leaf discs of *EcABCC8*-OE versus *GFP* transgenic rice seedlings, in contrast to the ortholog gene KO (*osabcc8-1* versus WT) rice seedlings.

Glyphosate efflux from leaf discs of rice seedlings was rapid over the first 30 min and then slowed (Fig. 5*A*). The rate of

glyphosate efflux to the apoplast (external solution) was nearly twofold faster in *EcABCC8*-OE leaf discs (b = 0.15 ± 0.02 [μg · g^{-1} fresh weight (FW) min^{-1}]) than in *GFP* control (b = 0.08 ± 0.02), leading to significantly ($P = 0.016$) lower glyphosate content in *EcABCC8*-OE than in the *GFP* samples (Fig. 5*A*). In contrast, glyphosate efflux rate was twofold slower in leaf discs of the nonfunctional *osabcc8-1* (b = 0.04 ± 0.01) than in the WT control (b = 0.08 ± 0.01) (Fig. 5*B*), resulting in significantly ($P = 0.026$) higher glyphosate content in *osabcc8-1* than in the WT samples (Fig. 5*C*). These results are consistent with the hypothesis that the *EcABCC8* codes for a PM transporter that can move glyphosate out of the cytoplasm into the apoplast, therefore reducing the glyphosate level in the cytoplasm.
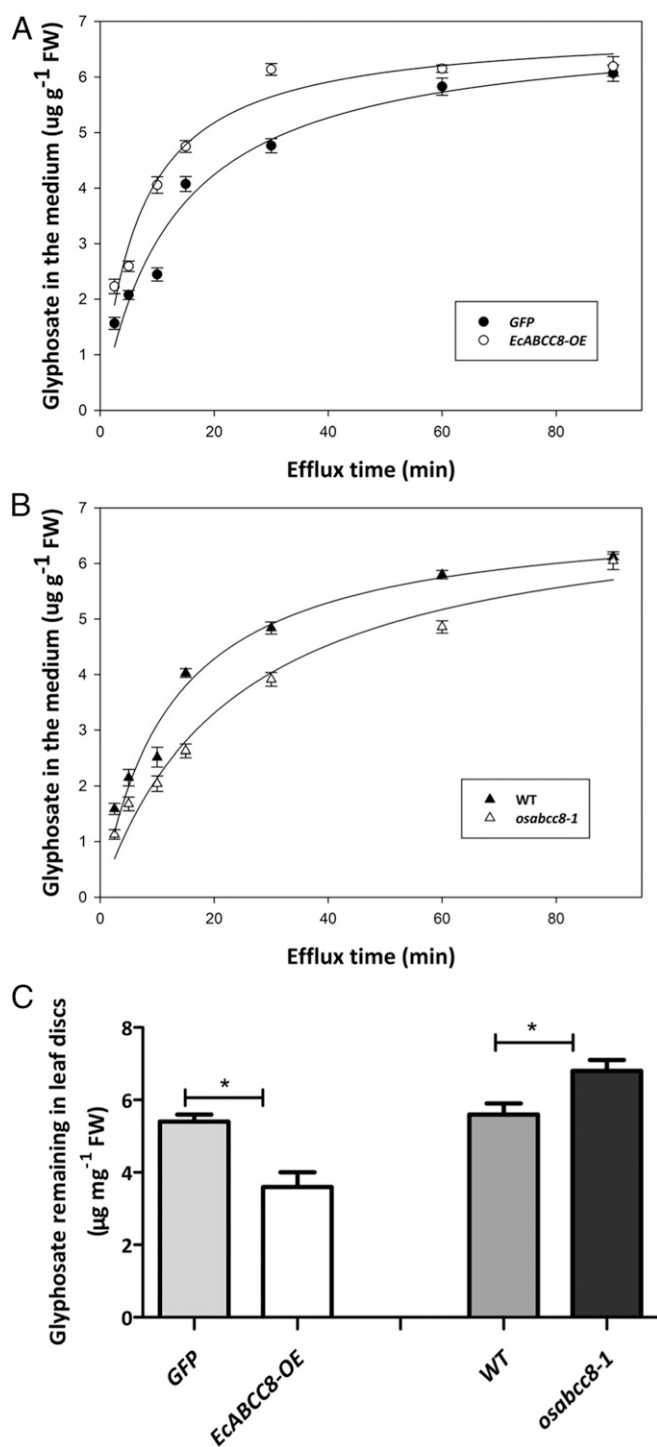
Pan et al.
An ABCC-type transporter endowing glyphosate resistance in plants

PNAS | 5 of 11
https://doi.org/10.1073/pnas.2100136118

Fig. 5. Glyphosate efflux from leaf discs to the external solution. Glyphosate efflux from leaf discs of rice seedlings (*A*) expressing *EcABCC8* (*EcABCC8*-OE) versus *GFP* control and (*B*) *osabcc8-1* KO mutant versus WT. (*C*) Glyphosate content in leaf discs after efflux. Data points are means ± SE (*n* = 3). Significance of difference by the Student's *t* test is indicated by *$P < 0.05$. The experiments were repeated with similar results.

ABCC8 reduces glyphosate accumulation in rice leaf protoplasts. To mimic the in vivo situation, rice seedlings of each *EcABCC8*-OE versus *GFP* and *OsABCC8*-KO versus WT were glyphosate treated at a low rate (68 g · ha^{-1}), and then leaf protoplasts were isolated, and glyphosate content was quantified. By 2 and 6 h after foliar glyphosate treatment, the glyphosate content in leaf

protoplasts was up to 4.2-fold less ($P \leq 0.016$) in *EcABCC8*-OE than in *GFP* lines (*SI Appendix*, Fig. S11*A*) and up to 1.9-fold higher ($P \leq 0.024$) in *OsABCC8*-KO than in WT lines (*SI Appendix*, Fig. S11*A*).

To avoid complication by the in vivo approach, leaf protoplasts were also isolated from each line and then treated in vitro with 60 μM glyphosate and followed by glyphosate quantification. Although the absolute glyphosate content is understandably different, relative glyphosate levels in ECABCC8 versus GFP and the nonfunctional KO versus WT was found to be similar, between the two treatment approaches. By 1 and 2 h after glyphosate treatment, the protoplast glyphosate level was up to 3.1-fold less ($P \leq 0.032$) in *EcABCC8*-OE than in *GFP* lines and up to 2.2-fold higher ($P \leq 0.028$) in *OsABCC8*-KO than in WT lines (*SI Appendix*, Fig. S11*B*).

Measurement of time-dependent glyphosate accumulation in isolated rice protoplasts of GFP revealed that the glyphosate level rapidly increased in the first 10 min (initial uptake) following treatment and remained unchanged for 10 min and then slowly increased until 100 min (Fig. 6*A*). This is similar to glyphosate uptake in broad bean protoplasts when measured for a shorter timespan at glyphosate concentration of 0.1 mM (45). However, glyphosate protoplast accumulation in *EcABCC8*-OE displayed a distinct pattern with clear departure from that of GFP 20 min after treatment because of ongoing decrease in glyphosate levels over time (Fig. 6*A*). This result also implies that after rapid glyphosate uptake in the early phase, there then was glyphosate extrusion by the EcABCC8 transporter evident in the later slow uptake phase (after 20 min in our experimental conditions).

As a control, time-dependent accumulation of the glyphosate metabolite AMPA was also measured in isolated rice protoplasts of *EcABCC8*-OE and GFP lines. However, no difference in AMPA levels between the two lines was observed throughout the experiment period (Fig. 6*B*). This is expected, as *EcABCC8*-OE plants are not resistant to AMPA (*SI Appendix*, Fig. S3).

Thus, consistent results from the in vivo and in vitro treatment situations in contrasting *EcABCC8*-OE and *OsABCC8*-KO lines confirmed that the ABC transporter ABCC8 reduces glyphosate accumulation in the cytoplasm.

3D Reconstruction Reveals Structural Interactions of the *EcABCC8* and Glyphosate. The plant ABC transporter EcABCC8 is a mono-subunit protein containing three transmembrane domains (TMDs) and two intracellular nucleotide-binding domains (NBDs) (Fig. 7*A*). The overall spatial organization of EcABCC8 is very similar to its mammalian homolog MRP1 (46, 47). The characteristic TMD0 domain is located in space closely to TMD1 (Fig. 7*B*) in both "open" and close" (inward facing and outward facing) conformations.

Results of blind docking of the glyphosate molecule into EcABCC8 and the molecular dynamics (MD) investigations reveal the binding mechanism which is typical for ABCC8/MRP1 proteins and consists of ligand interactions in the substrate interaction area (cargo site) of these exporters. The glyphosate molecule primarily interacts with EcABCC8 in an inward-facing conformation. The correspondent binding site consists of residues Arg355, Tyr526, Glu566, Glu1097, Ser1136, Val1139, and Phe1140 (Fig. 7*C*). A transfer of glyphosate from the intracellular space between the NBD into the primary binding cargo site results in a reduction of total energy by 2,100 kJ · mol^{-1}, establishing a favorable condition for this process to occur. In the predicted binding site, glyphosate directly forms several favorable interactions, particularly the salt bridge with Arg355, two attractive charges with Arg355 and Glu1097, two conventional hydrogen bonds with Arg355 and Glu566, and one carbon hydrogen bond with Ser1136 (Fig. 7*D*). The free interaction energy between the glyphosate and EcABCC8 calculated from the MD ensemble is −97 kJ · mol^{-1}.
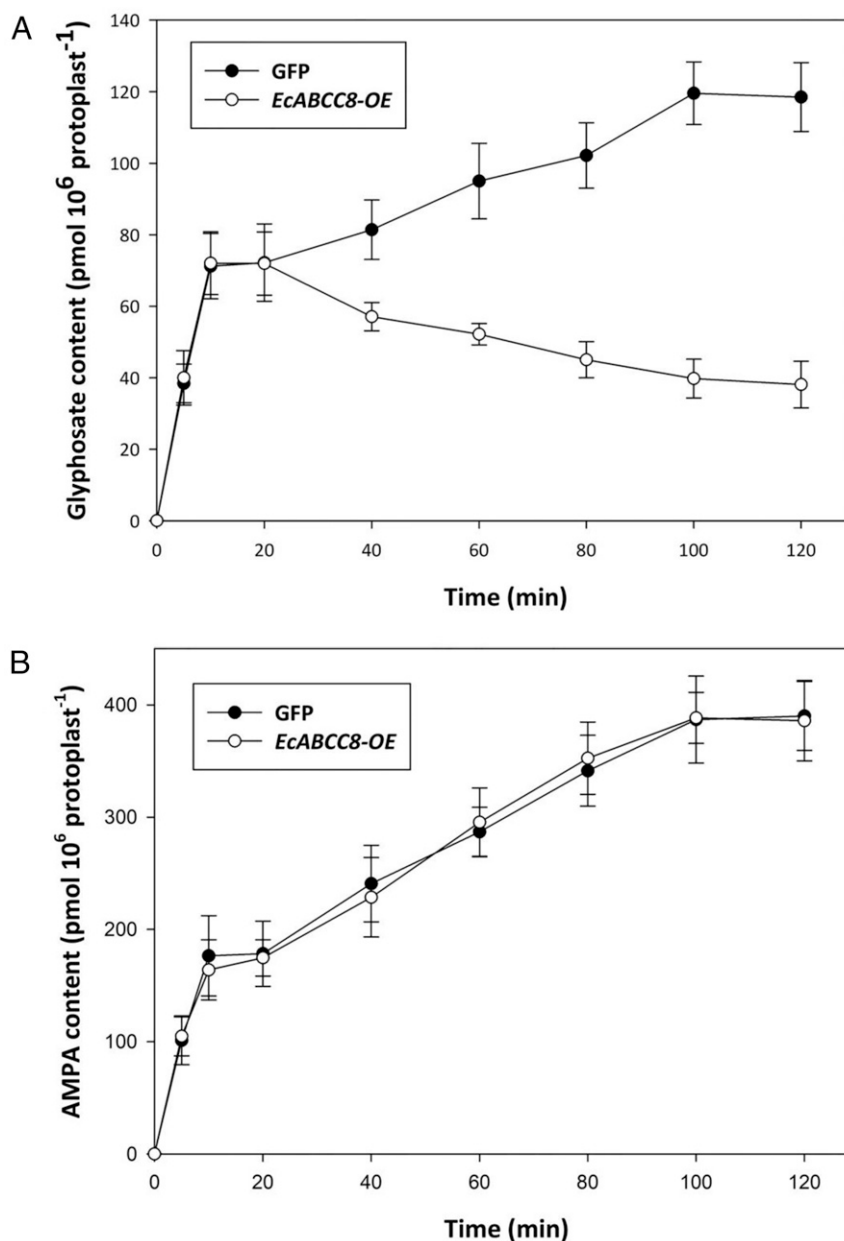
Fig. 6. Glyphosate and AMPA levels in rice protoplasts. Time-dependent glyphosate (*A*) and AMPA (*B*) accumulation in rice protoplasts of *EcABCC8*-OE versus *GFP*. Glyphosate or AMPA was present at 60 μM. Data points are means ± SE (*n* = 3). The experiment was repeated with similar results.

As a result of EcABCC8 transition from the inward-facing (intracellular space) to outward-facing (extracellular space) conformation, the rearrangement of glyphosate and amino acid microenvironment occurs, and glyphosate appears to interact with several other residues located closely to the primary binding site. These include residues Glu566, Arg569, Phe570, Phe986, Phe1140, and Phe1144 (Fig. 7*E*), involving five electrostatic interactions and one conventional H-bond (Fig. 7*F*). Some of these residues correspond to residues in the bovine MRP1 substrate binding site (e.g., residues Phe1140 and Arg1143 of EcABCC8 correspond to Trp1245 and Arg1248 of MRP1). One of the results of the conformational change is the appearance of the two unfavorable contacts between the positively charged nitrogen and the phosphorus of glyphosate and the amino group of Arg1143. The presence of such a contact may cause the subsequent release of the glyphosate molecule from EcABCC8 into the extracellular

space. The free energy of glyphosate/EcABCC8 interaction after EcABCC8 switching to the outward-facing conformation decreased by 138 kJ · mol^{-1}, indicating this stage as a rate-limiting step for low molecular weight compound transport.

Release of the glyphosate molecule from the intracellular space of the EcABCC8 transporter into the extracellular space is accompanied by a decrease of total energy of the investigated system (including the ligand, protein, membrane, and water–salt solution) by 3,500 kJ · mol^{-1}. This proves the high probability of glyphosate release despite strong binding of glyphosate to EcABCC8.

Discussion

Here, in our well-characterized GR *E. colona* population (43), we present several lines of evidence demonstrating that GR plants overexpress a PM-located ABC transporter (EcABCC8) that functions as a cytoplasmic glyphosate exporter. EcABCC8 is
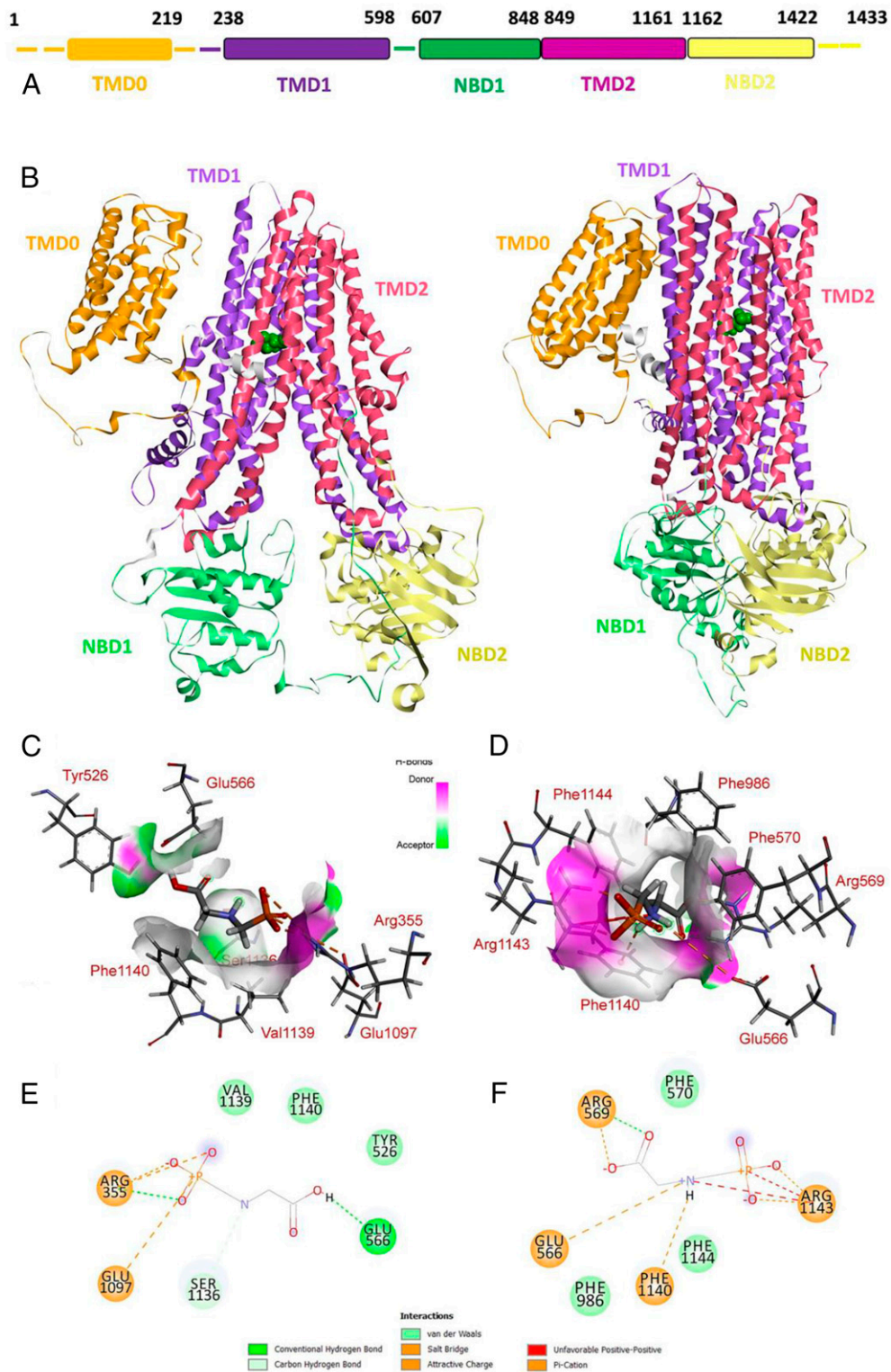
Fig. 7. 3D reconstruction showing structural interactions of the *EcABCC8* and glyphosate. (*A*) Schematic view of the domain structure of EcABCC8. (*B*) General view of EcABCC8 in the inward-facing (*Left*) and outward-facing (*Right*) conformations with glyphosate (green Van der Waals representation) bound into the cargo-binding site. Structural domains in EcABCC8 composition are highlighted by different colors. Spatial structure of the contact interface between the glyphosate and EcABCC8 in the cargo-binding site in (*C*) the inward-facing and (*D*) the outward-facing conformations. Protein contact surface is colored by H-bond donor/acceptor distribution, binding site amino acids represented by sticks, and intermolecular contacts indicated by dotted lines. 2D diagram of molecular interactions between the glyphosate and EcABCC8 in the cargo-binding site in (*E*) in the inward-facing and (*F*) the outward-facing conformations.

able to move glyphosate from the cytoplasm to the extracellular space (apoplast). In this manner, the cellular glyphosate level is lowered, therefore conferring glyphosate resistance. This is evidence of an ABC transporter endowing plants with herbicide resistance.

Using RNA-seq and RT-qPCR validation, we show that higher *EcABCC8* transcript levels are consistently associated with glyphosate resistance in multiple GR versus multiple S *E. colona* lines/populations and under different temperature scenarios (*SI Appendix*, Table S3). Among others (e.g., transcription activation), the higher expression of *EcABCC8* may involve epigenetic regulation, as a lower CHH methylation level in the promoter regions and a higher CG methylation level in the *EcABCC8* gene body was observed in GR versus S sequences (*SI Appendix*, Fig. S7). Generally, the lower the methylation level, the higher the expression level (48, 49). However, in the gene body (exon) region, higher methylation (CG sites) levels could result in higher gene expression (50–52).

In human medicine, it is known that human ABCC-type transporters (e.g., HsABCC1 or HsMRP1) are PM located and are able to pump certain chemotherapeutic drugs and other xenobiotics out of cancer cells, thereby conferring resistance to anticancer drugs (30). We propose that in plants, EcABCC8 serves (serendipitously) as a PM-embedded glyphosate exporter. Indeed, colocalization of the EcABCC8 (and the ortholog GmABCC8) with the PM marker (Fig. 4A and *SI Appendix*, Fig. S10) and lack of colocalization with the known tonoplast marker in rice and *Arabidopsis* leaf protoplasts (Fig. 4 B and C and *SI Appendix*, Fig. S9) supports this.

Although glyphosate uptake into plant cells can be via both active (at low concentrations) and passive (at high concentrations) mechanisms (8), once glyphosate enters the cytoplasm (pH around seven) it disassociates as anions and cannot freely efflux (25, 26). Therefore, cellular glyphosate efflux must be a transporter-mediated active process. Given that EcABCC8 is a PM-embedded glyphosate exporter, the *EcABCC8-OE* plants will extrude a higher amount of glyphosate to the apoplast, lowering the glyphosate level in the cytoplasm. This was confirmed by quantification of glyphosate efflux in leaf discs and glyphosate content in protoplasts of *EcABCC8-OE* in contrast with the ortholog KO rice plants (Figs. 5 and 6 and *SI Appendix*, Fig. S11). Especially, time-dependent glyphosate accumulation in protoplasts of *EcABCC8-OE* showed a clear opposite trend to that of GFP (decrease versus increase) after the initial uptake of glyphosate (Fig. 6A). We are aware that overexpression of transporters may lead to a mislocalization of membrane proteins (42). However, this functional analysis data on glyphosate cellular distribution, together with ABCC8 subcellular location, strongly suggest a PM localization of EcABCC8 and therefore provide evidence that EcABCC8 and its orthologs confer glyphosate resistance by serving to lower the intracellular glyphosate level.

Cellular elimination of glyphosate may not be easily detectable at the whole plant level. Indeed, no significant difference in ^{14}C-glyphosate uptake and translocation was observed in the GR versus S plants in our previous study (53). This is likely because 1) the ^{14}C-glyphosate foliar uptake assay measures the total amount of glyphosate within the leaf tissue (e.g., in apoplast and symplast), and the herbicide in the apoplast portion may not be easily removed with gentle leaf wash, and 2) glyphosate translocation in the GR population was confounded by the recently discovered glyphosate metabolism (7).

Theoretically, the PM-based extruding mechanism increases glyphosate sequestration to the apoplast, resulting in more glyphosate available for upward movement with the transpiration stream and hence accumulation in leaf tips and edges. Indeed, in glyphosate-treated *EcABCC8*-OE rice seedlings, localized leaf tip damage is evident as compared to extended damage in the whole leaf in *GFP* control seedlings (*SI Appendix*, Fig. S12).

Therefore, it is anticipated that GR plants with more glyphosate translocation to the leaf apical area relative to the S counterpart (e.g., in *Lolium spp*) (12, 14) may likely use the transporter mechanism similar to EcABCC8.

The plant ABCC (MRP) transporters studied so far are largely tonoplast located, sequestering organic anions and xenobiotics mostly as conjugates into vacuoles (33, 38). It is interesting to know whether the exported glyphosate by EcABCC8 is conjugated or not. Our data measure glyphosate that remained in the protoplast, not exported to the apoplast (except for the leaf disc efflux data), and hence cannot address this question. However, glyphosate is water soluble and is unlikely to form glutathione (GSH) and other conjugates. Our structural modeling also supports the view that glyphosate can directly bind to the inward-facing EcABCC8 from the intracellular space (inside the PM, cytoplasm) and is released from the outward-facing transporter to the extracellular space (outside the PM, apoplast) with favorable interaction energy changes (Fig. 7).

As glyphosate is obviously a serendipitous (nonplant) substrate of the ABCC8, the endogenous functions of ABCC8 in planta (e.g., its physiological substrates and whether it can transport GSH or others) remain to be revealed. In addition, ABC transporters usually rely on energization by ATP. However, our experimental system does not answer the question of whether EcABCC8-mediated glyphosate efflux is indeed ATP driven or occurs by facilitated diffusion through the pore of EcABCC8, or if, alternatively, EcABCC8 is a regulator of a so far unknown glyphosate exporter. Indeed, the mammalian sulfonylurea receptor is a non-ATP–dependent ABCC/MRP type transporter regulating K channel (54). Further experiments using PM vesicles or electron cryomicroscopy structures of free EcABCC8 and in complex with glyphosate will better address these above questions.

As ABC transporters are known to transport diverse substrates (55), we examined whether EcABCC8 could export the glyphosate metabolite AMPA. However, *EcABCC8*-OE plants were found not to be resistant to AMPA (*SI Appendix*, Fig. S3) and did not reduce the cellular AMPA levels (Fig. 6B). Our modeling shows that by structural analogy to glyphosate, AMPA can reach the cargo site of ECABCC8. However, AMPA being smaller than glyphosate has fewer stabilizing contacts with amino acids in the binding site. The AMPA-E**cABCC**8 complex is therefore functionally unstable (as evidenced by the small value of free interaction energy of -29 kJ · mol^{-1} as compared to -97 kJ · mol^{-1} for glyphosate). In addition, we also found that *EcABCC8*-OE plants remain S to the herbicide glufosinate (*SI Appendix*, Fig. S3). Likewise, the GR *E. colona* is glufosinate S (43).

Glyphosate resistance in this studied population of *E. colona* involves multiple resistance mechanisms. These include target site EPSPS Pro106Thr mutation (56), non-target site AKR-catalyzed glyphosate metabolism to AMPA (7), and ABC transporter-mediated glyphosate extrusion to the extracellular space (this study). Given that temperature had a positive effect on glyphosate resistance in this population (7), and *EcAKR/ABCC8* expression responds to temperature, non-target site resistance via AKR and EcABCC8 gene overexpression likely plays a more important role than the EPSPS mutation whose effect may be diluted by multiple S alleles in polypoid species (57) such as *E. colona* (58).

Identification and characterization of the ABC transporter EcABCC8 will facilitate discovery of the transporters involved in glyphosate resistance in other plant species. In addition, plant species with varying levels of glyphosate tolerance (or uptake) may be also in part ascribable to the abundance of ABCC8 orthologs, as was demonstrated in the current study that up-regulation of *ABCC8* provides glyphosate resistance in different plant species (Fig. 2).

Identification of ABCC8-associated cell types will help understand its roles in specific cellular processes, as demonstrated for AtABCC5 in guard cell signaling and phytate storage (42).

Pan et al.
An ABCC-type transporter endowing glyphosate resistance in plants

PNAS | 9 of 11
https://doi.org/10.1073/pnas.2100136118

Given that the ABCC8 expresses in both above- and below-ground tissues (*SI Appendix*, Fig. S8), it is tempting to speculate implication of this ABC transporter in other plant cellular processes beyond xenobiotic detoxification, such as root exudation of plant metabolites for soil nutrient acquisition and plant–soil microbe interactions (59) and allelopathy (60).

In summary, here we show in a GR *E. colona* population that overexpression of the ABCC8 transporter endows resistance to glyphosate. This finding that an ABC transporter can endow herbicide resistance will catalyze studies to investigate ABCC8-like and other membrane bound transporters for their capacity to endow plant species with resistance to glyphosate or other herbicides/xenobiotics by moving toxic compounds out of the cytosol.

Materials and Methods

Detailed information on plant material, RNA-seq data analysis and selection of candidate transporter contigs, rice genetic transformation with the two ABC transporter genes *EcABCC8* and *EcABCC10*, homologous overexpression of *EcABCC8* orthologs in other crop plants, rice *OsABCC8* gene KO by CRISPR/Cas9 gene editing, global DNA methylation analysis for *E. colona*, subcellular localization of ABCC8, glyphosate efflux and content in leaf discs of transgenic rice seedlings, glyphosate quantification in leaf protoplasts of transgenic rice plants, and structural reconstruction of *EcABCC8* variant are described in *SI Appendix, Materials and Methods*.

Data Availability. The *EcABCC8* and *EcABCC10* complementary DNA sequences data have been deposited in the GenBank database (access nos. MT249005 and MT249006). All data used in the study are included in the paper and *SI Appendix*. All protocols are described in *SI Appendix, Materials and Methods* or in the references therein. Plant materials are available upon request by qualified researchers to the corresponding author.

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Pan et al.
An ABCC-type transporter endowing glyphosate resistance in plants

PNAS | 11 of 11
https://doi.org/10.1073/pnas.2100136118