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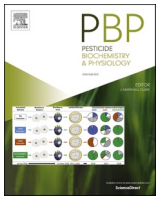

PsbA gene over-expression and enhanced metabolism conferring resistance to atrazine in *Commelina communis*

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ABSTRACT

Commelina communis L. is a troublesome weed in agronomic fields and increasingly threatens the yield security of corn in north-eastern China. Previously, we found that a *C. communis* population (JL-1) has evolved resistance to atrazine. Although the potential genetic and enzymic differences contributing to atrazine resistance in this population have been investigated, the specific molecular mechanisms underlying *C. communis* resistance are still poorly understood. Here, the expression level of the target gene *PsbA* and the non-target-site resistance (NTSR) mechanism for this population were studied. The results showed that the decline in chlorophyll content in JL-1 leaves was less than in the susceptible JS-10 population following atrazine treatment. JL-1 exhibited an enhanced expression of the *PsbA* gene compared with JS-10 of 7.28- and 14.28-fold higher at 0 and 24 h after treatment with atrazine, respectively. The cytochrome P450 monooxygenase (P450) inhibitor piperonyl butoxide (PBO) increased the phytotoxicity of atrazine in both populations of *C. communis*. Seven candidate genes associated with NTSR of Jl-1 were identified through RNA-seq and validated by quantitative real-time PCR, including 5 upregulated genes involved in herbicide metabolism. In addition, the activities of glutathione S-transferases and P450s in JL-1 were increased compared with JS-10. Collectively, *PsbA* gene overexpression and enhanced metabolism are likely to be responsible for JL-1 resistance to atrazine.

1. Introduction

The Asiatic dayflower (*Commelina communis* L.) is the most problematic summer broadleaf weed species across north-eastern China (Yang et al., 2018). It infests agronomic crops such as corn (*Zea mays* L.) and soybeans (*Glycine* max L.), leading to considerable yield losses (Yang et al., 2019). Due to several factors, including rapid growth and limited effective selective herbicides, the distribution area of this weed species is expanding. Atrazine is currently one of the few selective herbicides used to control *C. communis*. This compound belongs to the triazine group of photosystem II (PS II) inhibitors and blocks electron transfer of PS II by competing for binding to the plastoquinone binding site (QB) on target protein D1 (Fuerst and Norman, 1991; Hess, 2000). Recently, owing to extensive atrazine use over a long period of time, some *C. communis* populations have evolved resistance to this herbicide in north-eastern China (Yang et al., 2021).

The resistance mechanism for herbicides is divided into target-site resistance (TSR) and non-target-site resistance (NTSR) (Powles and Yu, 2010). The mutation of target proteins is the most common TSR mechanism in various weeds. *PsbA*, which encodes the D1 protein, is the target gene of atrazine. The serine (Ser)264-glucine (Gly) substitution in *PsbA* gene is known to decrease the efficiency of atrazine in numerous species, including *Kochia scoparia* L. (Kumar et al., 2020), *Poa annua* L. (Svyantek et al., 2016), *Sisymbrium orientale* L. (Dang et al., 2017) and *Amaranthus hybridus* L. (Hirschberg and Mcintosh, 1983). To date, there have been at least eight mutations in the *PsbA* gene, namely leucine (Leu)218-Val, Val219-isoleucine (Ile), alanine (Ala)251-Val, phenylalanine (Phe)255-Ile, Ser264-Gly, Ser264-threonine (Thr), asparaginate (Asn)266-Thr and Phe274-Val, which confer resistance to PSII-inhibiting herbicides in weed species (Mengistu et al., 2000; Powles and Yu, 2010). Additionally, increased target gene and/or protein expression can also be a mechanism of resistance. For example, overexpression of the target gene 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) is a common mechanism for glyphosate resistance (Gaines et al., 2020). Decreased sensitivity to multiple acetyl-CoA carboxylase (ACCase) inhibitors in *Digitaria sanguinalis* L. was also related to an

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increase in *ACCase* expression levels (Laforest et al., 2017). However, enhanced *PsbA* gene expression endowing resistance has not yet been described for PS II inhibitors.

NTSR involves mechanisms that reduced the amount of herbicide reaching the target site, including reduced absorption and translocation, and enhanced herbicide metabolism (Gaines et al., 2020). Increased herbicide detoxification is by far the main NTSR mechanism in weeds. Nevertheless, this mechanism is complex because there are multiple herbicide-degrading genes, such as cytochrome P450 monooxygenases (P450s), glucosyltransferases (GTs), glutathione S-transferases (GSTs) and ATP-binding cassette (ABC) transporters that can be involved (Yuan et al., 2007). In recent years, resistance mediated by increased metabolism has been increasingly detected to atrazine. Previously published studies indicated that metabolic resistance to atrazine in *A. palmeri* and *A. tuberculatus* was associated with enhanced GST activity (Nakka et al., 2017; Vennapusa et al., 2018). For *A. tuberculatus*, elevated expression of one GST belonging to the phi-class was found to be strongly correlated with resistance to atrazine (Evans Jr et al., 2017). Whole-transcriptome sequencing (RNA-seq) technology has been a powerful tool for investigating the NTSR mechanism. It has been used to identify candidate genes involved in metabolic resistance in various weed species (Duhoux et al., 2015; Fang et al., 2019; Wang et al., 2021; Zhao et al., 2017). Eleven contigs that may participate in metabolic resistance to mesosulfuron-methyl were identified in *Beckmannia syzigachne* Steud. using RNA-seq (Wang et al., 2021). In *Alopecurus aequalis* Sobol, a total of 17 contigs were identified as possibly contributing to metabolism-based resistance (Zhao et al., 2017).

In our previous study, a *C. communis* population (JL-1) from Jilin Province, China, exhibited a low level of resistance to atrazine compared with the sensitive JS-10 population, with the resistance index value of 2.9. To investigate its resistance mechanism, the *PsbA* gene sequences of the two populations were compared, and the results showed that there was no amino acid mutation known to confer atrazine resistance in these two populations (Yang et al., 2021). Based on these findings, it is speculated that JL-1 resistance to atrazine is related to elevated *PsbA* gene expression levels or alterations in non-target-site factors. To explore this speculation, this study was performed to (1) determine the effect of atrazine on the chlorophyll levels in JL-1 and JS-10; (2) compare the expression levels of the target gene *PsbA* between JL-1 and JS-10; (3) estimate the influence of the P450 inhibitor piperonyl butoxide (PBO) on resistance to atrazine; (4) explore critical factors implicated in JL-1 non-target-site resistance to atrazine by RNA-seq; and (5) evaluate the activities of enzymes associated with JL-1 resistance.

2. Materials and methods

2.1. Chemicals

50% atrazine suspension concentrate (SC) was purchased from Shandong Binnong Technology Co., Ltd. (Shandong, China).

2.2. Plant materials and chlorophyll content assay

Resistant (JL-1) and susceptible (JS-10) *C. communis* populations were used in this study. The seed information for the two populations, methods to break seed dormancy and seedling growth conditions were described in our previous study (Yang et al., 2021). When the *C. communis* plants reached the three- to four-leaf stage, all the seedlings were treated with atrazine at a concentration of 1275 g a.i. ha^{-1} (labelled field rate) using an ASS-3 Walking Spray Tower (450 L ha^{-1}; National Engineering Research Center for Information Technology, China). Their leaves were cut at 0, 1, 3, 7, 14 and 21 days after treatment and then used to extract chlorophyll. The method used to extract chlorophyll was the same as that described by Yu et al. (2020). The Chl (a + b) ($C_{(a+b)}$) content was calculated based on $C_{(a+b)} = 6.63OD_{665} + 18.08OD_{649}$. The experiment was conducted with three independent replicates.

2.3. Quantitative real-time PCR (qPCR) analysis of PsbA

Atrazine application was performed as described above with distilled water spray used as a control. Leaf samples for the control (JL_CK and JS_CK) and treatment (JL_T and JS_T) groups were collected at 24 h after treatment. Each sample contained the newest emerging leaf and the second fully expanded leaf from each of five individuals. The experiment was performed with three biological replications. Leaves were stored at −80 °C after being rapidly frozen with liquid nitrogen. The total RNA from each sample was extracted according to the protocol for the RNAprep Pure Plan Kit (Tiangen Biotech Beijing Co., Ltd., China). The RNA quality was assessed using 1% agarose gel electrophoresis. The RNA quantity and integrity were evaluated with an Agilent 2100 (Agilent, CA). One thousand nanograms of RNA was used for cDNA synthesis based on the manufacturer's instructions for All in One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech, China). qPCR was performed with an ABI 7500 Fast Sequencer (Applied Biosystems, USA) as described by Yu et al. (2021). To normalize the expression level of the target gene, the reference gene was selected from ubiquitin (*UBQ*), capsine phosphatase (*CAP*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) with BestKeeper software. The relative expression level of the *PsbA* gene was calculated using the $2^{-\Delta\Delta CT}$ method. The specific primers for the target genes were designed with Oligo software and are listed in Table 1. Each sample was analysed using three technical replicates.

2.4. Effects of P450 inhibitor PBO on sensitivity to atrazine

JL-1 and JS-10*C. communis* seedlings at three to four-leaf stages were treated with PBO, atrazine or PBO plus atrazine. Atrazine was applied at doses of 79.69, 159.38, 318.75, 637.5 and 1275 g a.i. ha^{-1} following the method described earlier. Seedlings treated with distilled water were controls. PBO (500 g a.i. ha^{-1}) was sprayed 1 h before atrazine application. At 21 DAT, the dry weight of aboveground plant tissue was taken and expressed as a percent of the control group. Five pots were included in each treatment, and each pot contained five plants. The experiment was independently replicated three times.

2.5. cDNA library construction and sequencing

RNA samples for *PsbA* gene expression analysis were also used for RNA-Seq. The cDNA library was constructed in accordance with a previously reported method (Yu et al., 2020) and then sequenced with the Illumina HiSeqTM 4000 platform (Illumina, San Diego, CA).

2.6. De novo transcriptome assembly and gene function annotation

Strict quality control was performed on raw reads (Yu et al., 2021). All the clean reads were used for de novo transcriptome assembly with Trinity software, and the longest transcript of each locus was considered a “unigene” for further analysis (Grabherr et al., 2011). Subsequently, the resulting unigenes were aligned and annotated with the NCBI non-redundant protein sequences (Nr) database, NCBI non-redundant nucleotide sequence (Nt) database, Kyoto Encyclopedia of Genes and Genomes Ortholog (KO) database, manually annotated and reviewed protein sequence (SwissProt) database, protein family (Pfam) database and clusters of orthologous groups of proteins (KOG) database using the BLAST program. The gene ontology (GO) annotation and functional classification for the unigenes were performed with Blast2GO and WEGO software, respectively. Furthermore, the unigenes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway based on the results of KOG database annotation.

Table 1
Sequences of the primers used in this study.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
UBQ	AGAAGACCTACACCAAGCCCA	CAGCCCCGCACTCCCCATTAG
GAPDH	GTCTTTCCGTGTTCCTACTGTGG	CTTTCAACTTCCCCTCAGATGCT
CAP	ACAACTCATAAATCTGCTCCTCCG	ACCGATGCTCCAATAACCCTT
PsbA	AGGTGTGGCTGGTGTATTCGG	TGCGGACTCGTTTTCTGTGGT
c144893_g1	CGGTGAGGGACAGAAGGCATC	GCCAGTAAGAGCCACAGCCAAGAA
c148186_g1	TTAATTGGTAGGAGCAGATTGAGGT	CCTTTTTCAGTGGCGACAACAT
c146029_g1	CAACCAGCACTCTCCCAAAAT	ACTGCCAATGCCTCGTCAA
c183753_g1	GCTAGGTCCGTTCCAGTTCCC	CCCTCTATGCCCCTTCTCCGT
c165541_g2	GATAGGCCCGGTCTTCTCGCA	GCACACGAACACCACAGACTC
c154372_g2	AATACCTTCCATCCTCCACCA	GCGACCGATTCCTCAACACTCA
c149751_g1	CCTCATAGTCCTTTTTATTGCCCTT	TCTTGGGGGGAAGTTTGGGAA

2.7. Differentially expressed gene (DEG) analysis

Clean reads for the samples were mapped back onto the reference database that was assembled in the above description with Bowtie 2 v.2.2.3. The gene expression level of the unigenes was estimated with the union model in HTSeq software and described as fragments per kilobase of exon model per million mapped reads (FPKM). Differential expression analysis was implemented with DESeq software (Anders and Huber, 2010). Genes with q value (adjusted P value) < 0.05 were considered DEGs. Expression differences were compared between the treatment and control groups for the JL-1 (JL-1_T vs. JL-1_CK) and JS-10 (JS-10_T vs. JS-10_CK) populations. In addition, expression differences were also compared between the JL-1 and JS-10 populations for the treatment (JL-1_T vs. JS-10_T) and control (JL-1_CK vs. JS-10_CK) groups.

2.8. Candidate resistance gene selection and qPCR validation

Candidate NTSR genes were strictly selected based on the following standards: (1) DEGs involved in herbicide metabolism, oxidation, and plant stress physiology; (2) DEGs that were significantly differentially expressed between the JL-1 and JS-10 populations in the control or treatment group with |log2 (fold change)| > 3, or DEGs that had significantly different expression levels between the treatment and control groups in the JL-1 population (|log2 (fold change)| > 3). The validation of these candidate resistance genes with qPCR was performed as described above. RNA samples used for RNA-Seq were also used to assess the relative expression level of the candidate genes. The qPCR primer sequences are shown in Table 1.

2.9. GST and P450 activity tests

The cultivation of *C. communis* seedlings and atrazine spray were the same as above. The aboveground leaf tissues were collected at 0, 1, 3, 5, 7, 10 and 14 days after treatment (DAT) and then stored at −80 °C. Leaves from all the samples were frozen in liquid nitrogen, followed by grinding into fine powder. To 100 μg of powdered sample 900 μL of precooled phosphate buffer (PSB, 0.05 mol L^{-1}, pH 7.8) was added before being centrifuged at 4 °C and 12,000 ×g for 10 min. The supernatant was used for GST and P450 activity tests. The GST and P450 activities were determined following the manufacturers' protocols for GST and P450 Enzyme-Linked Immunosorbent Assay Kits (Kenuodi Biotechnology Co., Ltd., China), respectively. The experiment was independently repeated three times with three technical replications.

2.10. Statistical analysis

All the data are presented as means with standard error (SE). The chlorophyll contents were subjected to a non-liner regression analysis using SigmaPlot software (Systat Software, USA). In the *PsbA* gene expression level assay and qPCR validation of the candidate genes experiments, two-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test were performed to assess the significant differences ($P < 0.05$) among different treatments via SPSS software (IBM Crop, USA). The percentage of dry weight relative to the control was fit to a log-logistic non-linear regression model with SigmaPlot software, and the model was as follows (Seefeldt et al., 1995):

$$Y = C + (D - C) \Big/ \left[1 + (X/X_0)^b\right].$$

3. Results

3.1. Chlorophyll contents

The Chl(a + b) contents significantly and negatively responded to atrazine treatment in both JL-1 and JS-10 ($P < 0.05$). The inhibitory effect of atrazine on Chl(a + b) accumulation was greater in JS-10 than in JL-1. It was estimated that 11.02 and 5.27 days were required for JL-1 and JS-10 to inhibit Chl(a + b) content by 50%, respectively. At 21 DAT, the Chl(a + b) content in JL-1 was 0.95 mg g^{-1}, 10.33 times higher than that in JS-10 (Fig. 1).

3.2. PsbA gene expression level

UBQ was determined as the best reference gene and was used to normalize the expression of the genes studied in this research (Table S1). Atrazine application had no significant effect on the expression of *PsbA* in both the JS-10 and JL-1 populations. However, there were significant differences ($P < 0.01$) in *PsbA* expression between JL-1 and JS-10 populations (Table 2). In comparison with JS-10, JL-1 exhibited a higher expression level of the *PsbA* gene both before and after treatment with

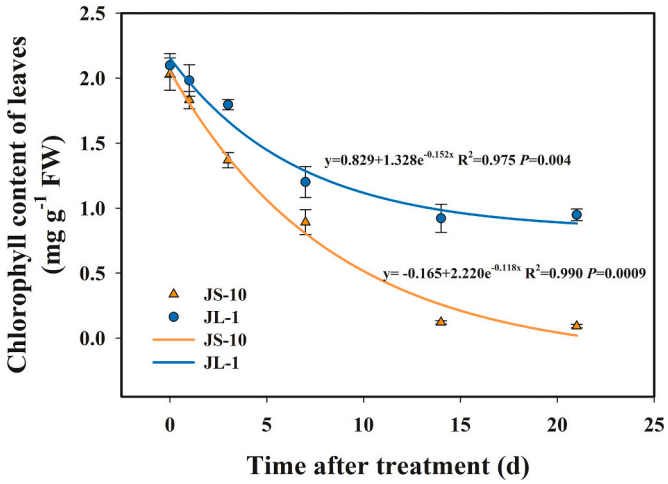

Fig. 1. Chlorophyll content in two *Commelina communis* L. populations (JL-1 and JS-10) following treatment with atrazine.

Table 2
Two-way ANOVA of the relative expression level of *PsbA* and candidate genes from JL-1 and JS-10 populations in response to atrazine.

Gene	Factor	*df*	F value	*p* value[a]
PsbA	Population	1	39.593	<0.001**
	Atrazine treatment	1	2.261	0.171
	Population×atrazine treatment	1	1.243	0.297
c144893_g1	Population	1	26.183	0.001**
	Atrazine treatment	1	39.397	<0.001**
	Population×atrazine treatment	1	28.789	0.001**
c148186_g1	Population	1	33.837	<0.001**
	Atrazine treatment	1	3.575	0.095
	Population×atrazine treatment	1	3.538	0.097
c146029_g1	Population	1	11.258	0.011*
	Atrazine treatment	1	9.702	0.014*
	Population×atrazine treatment	1	0.814	0.393
c183753_g1	Population	1	32.444	<0.001**
	Atrazine treatment	1	0.065	0.805
	Population×atrazine treatment	1	0.074	0.792
c165541_g2	Population	1	5.022	0.055
	Atrazine treatment	1	0.147	0.711
	Population×atrazine treatment	1	3.447	0.100
c154372_g2	Population	1	96.860	<0.001**
	Atrazine treatment	1	20.286	0.002**
	Population×atrazine treatment	1	28.470	0.001**
c149751_g1	Population	1	211.004	<0.001**
	Atrazine treatment	1	544.438	<0.001**
	Population×atrazine treatment	1	97.729	<0.001**

[a] * represents significant difference at 0.05 probability level; ** represents significant difference at 0.01 probability level.

atrazine (Fig. 2). Enhanced *PsbA* gene expression was detected in the resistant JL-1 population compared with the sensitive JS-10 population.

3.3. Effect of PBO on C. communis resistance to atrazine

To determine whether differences in atrazine metabolism led to resistance, the sensitivities of the JL-1 and JS-10 populations to atrazine plus the P450s inhibitor PBO were evaluated. PBO alone had no effect on plant growth in either the JL-1 or JS-10 populations. However, PBO increased atrazine toxicity to JL-1 and JS-10. Without PBO application, the GR_{50} (herbicide dose leads to 50% growth inhibition) value for JL-1 was estimated at 392 g a.i. ha^{-1}. This was reduced by 52% when the plants were treated with atrazine plus PBO (188 g a.i. ha^{-1}). Similarly, the GR_{50} value for JS-10 declined from 141 g a.i. ha^{-1} to 78 g a.i. ha^{-1} (Fig. 3).

3.4. RNA sequencing and de novo assembly, gene annotation, and functional classification

A total of 322,288,295 raw reads and 313,368,633 clean reads were obtained from the 12 samples, with the raw reads per sample ranging from 26,323,616 to 26,667,945 and the clean reads ranging from

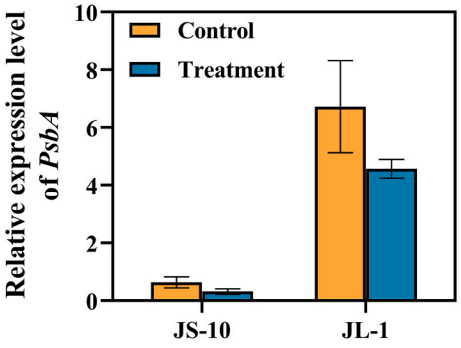

Fig. 2. Relative expression of the *PsbA* gene in untreated and atrazine treated plants of the JL-1 and JS-10 populations.

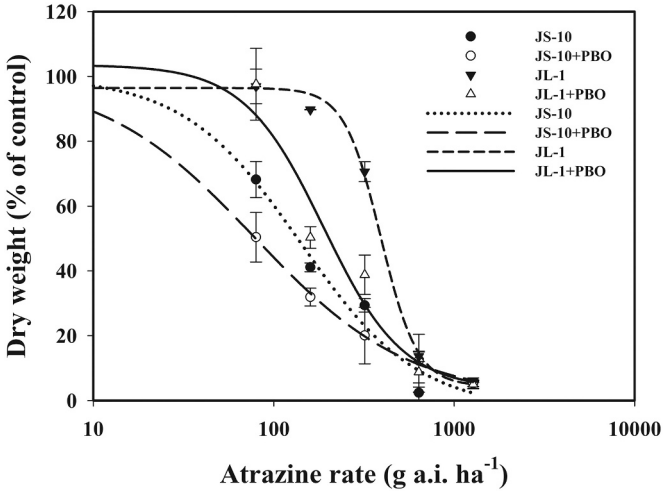

Fig. 3. Dose-response to atrazine of JL-1 and JS-10 populations with or without the P450 inhibitor piperonyl butoxide (PBO) at 21 days after treatment.

25,521,180 to 28,946,778 (Table S2). The clean reads were assembled into 299,471 transcripts, with an N_{50} of 880 bp. The detailed distribution of transcript lengths is shown in Table 3. Among all the transcripts, 226,676 unigenes were obtained, and their length averaged 340 bp. The lengths of the unigenes ranged from 201 bp to 16,508 bp, with an N_{50} of 807 bp (Table 3).

A total of 32% of the total unigenes (72,727) were successfully annotated in at least one of the seven databases, including 5410 unigenes annotated in all the databases (Table S3). A total of 12,269 unigenes were mapped to the GO database, and they were clustered into 29 functional subgroups, including 15 subgroups for the "biological process" group, 9 subgroups for the "cellular component" group and 5 subgroups for the "molecular function" group (Fig. S1). In total, 36,235 unigenes were successfully clustered into 26 groups in the KOG database, and most unigenes (10715) were enriched in the "general function prediction only" subgroup, followed by the "posttranslational modification, protein turnover, chaperones" subgroup and the "signal transduction mechanism" subgroup (Fig. S2). In the KEGG analysis, the unigenes successfully annotated with the KOG database were classified into five groups, namely "cellular processes", "environmental information processing", "genetic information processing", "metabolism" and "organismal system". "Translation" belonging to the "genetic information processing" group was the predominantly enriched pathway (Fig. S3).

3.5. DEGs analysis

In the atrazine treatment group, 1716 genes were significantly different in expression between the JL-1 and JS-10 populations, of which 622 DEGs were upregulated and 1054 DEGs were downregulated. By contrast, 1572 DEGs were noted in the control group, with 750 upregulated genes and 822 downregulated genes. In addition, 48 DEGs were detected in the resistant JL-1 population after atrazine treatment, including 35 upregulated DEGs and 13 downregulated genes, whereas 957 DEGs were identified in the sensitive JS-10 population (Fig. 4A). A total of 706 DEGs in the control group (JL-1_CK vs. JS-10_CK) were also noted in the atrazine treatment group (JL-1_T vs. JS-10_T). JL-1 and JS-10 shared 9 genes that displayed different expression levels after atrazine treatment (Fig. 4B).

3.6. Candidate genes involved in NTSR selection and qPCR validation

Considering the selection standards of candidate resistance genes as described above, a total of 7 DEGs were putative NTSR genes (Table 4).

Table 3
Summary statistics for the *Commelina communis* L. transcriptome assembly.

Types	Number (N)						Maximum length (bp)	Minimum length (bp)	Median length (bp)	N_{50} (bp)
	<300 bp	300–500 bp	500–1000 bp	1000–2000 bp	>2000 bp	Total				
Transcript	112,376	75,597	63,351	36,539	11,608	299,471	16,508	201	374	880
Unigene	95,366	57,992	41,886	23,035	8397	226,676	16,508	201	340	807

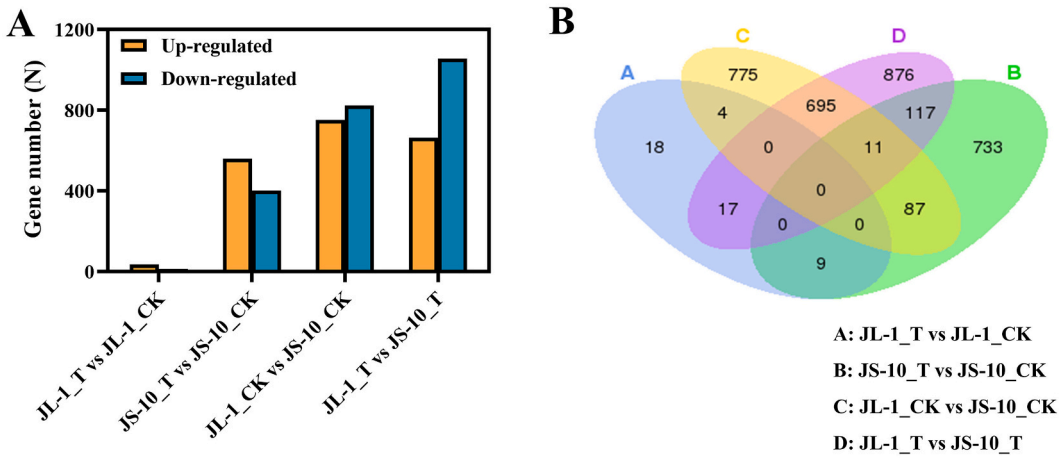

Fig. 4. Differentially expressed genes (DEGs) of *Commelina communis* L. in response to atrazine. (A) The number of DEGs in different comparison groups. (B) Venn diagram of the four DEG comparison groups. JL-1_CK and JL-1_T represent JL-1 population without and with atrazine treatment, respectively; JS-10_CK and JS-10_T represent JS-10 population without and with atrazine treatment, respectively.

Table 4
The candidate differentially expressed genes related to non-target-site resistance of *Commelina communis* L. to atrazine.

Gene ID	Function annotation	Log_2 (Fold change)			
		JL-1_CK vs JS-10_CK	JL-1_T vs JS-10_T	JL-1_T vs JL-1_CK	JS-10_T vs JS-10_CK
c144893_g1	Glutathione S-transferase U1	0.32	10.14	8.52	−1.31
c148186_g1	Glutathione S-transferase U17	3.35	4.02	−1.36	−2.05
c146029_g1	Cytochrome P450 monooxygenases 71A9	4.90	3.11	−1.55	0.22
c183753_g1	Glucosyltransferases 73C6	8.18	6.85	−0.69	0.64
c165541_g2	Glucosyltransferases 71A16	−2.97	3.90	4.02	−2.85
c154372_g2	Peroxidase	4.75	4.84	−0.15	−0.27
c149751_g1	Peroxisomal sarcosine oxidase	0.30	3.50	−0.69	−3.93

These candidate genes were primarily divided into three groups. One group included five candidate genes related to herbicide metabolism. Among them, two genes (c144893_g1 and c148186_g1) belong to the GST family, one gene (c146029_g1) was annotated to the P450 family, and two genes (c183753_g1 and c165541_g2) encoded GTs. Another group contained one candidate gene (c154372_g2) encoding peroxidase (POD), which is associated with antioxidative stress responses. The last group was implicated in plant stress defense. One candidate gene (c149751_g1) annotated to the peroxisomal sarcosine oxidase (PSO) family was obtained.

To verify the RNA-Seq results of candidate genes, their expression levels in response to atrazine were evaluated with qPCR. The expression of the selected candidate genes assayed by qPCR were similar to their abundance in RNA-Seq. Atrazine markedly induced the expression of *GSTU1* in JL-1, with no obvious effect in JS-10. In the control group, there was no significant difference in *GSTU1* expression between JL-l and JS-10; nevertheless, the expression level for JL-1 was 9.2-fold higher than that of JS-10 after atrazine treatment (Fig. 5A). There was a significant effect of population on the expressions of *GSTU17*, *CYP71A9* and *GT73C6* ($P < 0.05$) (Table 2). Their expression levels of JL-1 were increased compared with JS-10 in both the control and treatment groups (Fig. 5B-D). For example, JL-1 exhibited 729-fold and 208-fold higher expression of *GT73C6* than JS-10 in the control and treatment groups, respectively (Fig. 5D). In view of *CYP71A9*, atrazine treatment significantly influenced its expression ($P < 0.05$) (Table 2). The expression level of *CYP71A9* was inhibited by atrazine in both JL-1 and JS-10 populations (Fig. 5C). *GT71A16* expression was not obviously affected by population and atrazine treatment (Table 2). However, population and atrazine treatment had extremely significant effects on the expression of *POD* and *PSO* ($P < 0.01$) (Table 2). Regardless of whether atrazine was applied, less *POD* expression was detected in JS-10 in contrast with JL-1 ($P < 0.05$). In JL-1, *POD* expression in the control group was significantly higher than that in the atrazine treatment group ($P < 0.05$), whereas this phenomenon was not observed in JS-10 (Fig. 5F). After atrazine treatment, *PSO* expression was inhibited by 91% and 85% in JS-10 and JL-1 compared with the control group, respectively. In addition, its expression levels in JL-1 were higher than those in JS-10 regardless of atrazine application ($P < 0.05$) (Fig. 5G). Collectively, the candidate NTSR genes showed higher expression levels in the resistant JL population than in the susceptible JS-10 population, except for *GT71A16* (Fig. 5).

3.7. Activities of GSTs and P450s

The response of GST activity to atrazine was more obvious in JL-1 than in JS-10, and its activity sharply increased at the beginning and then gradually decreased before 10 DAT. During the entire experimental period, the GST activities of JL-1 were elevated compared with JS-10. At 1 DAT, its activity in JL-1 was 9.30 U g^{-1}, 2.8 times higher than that of

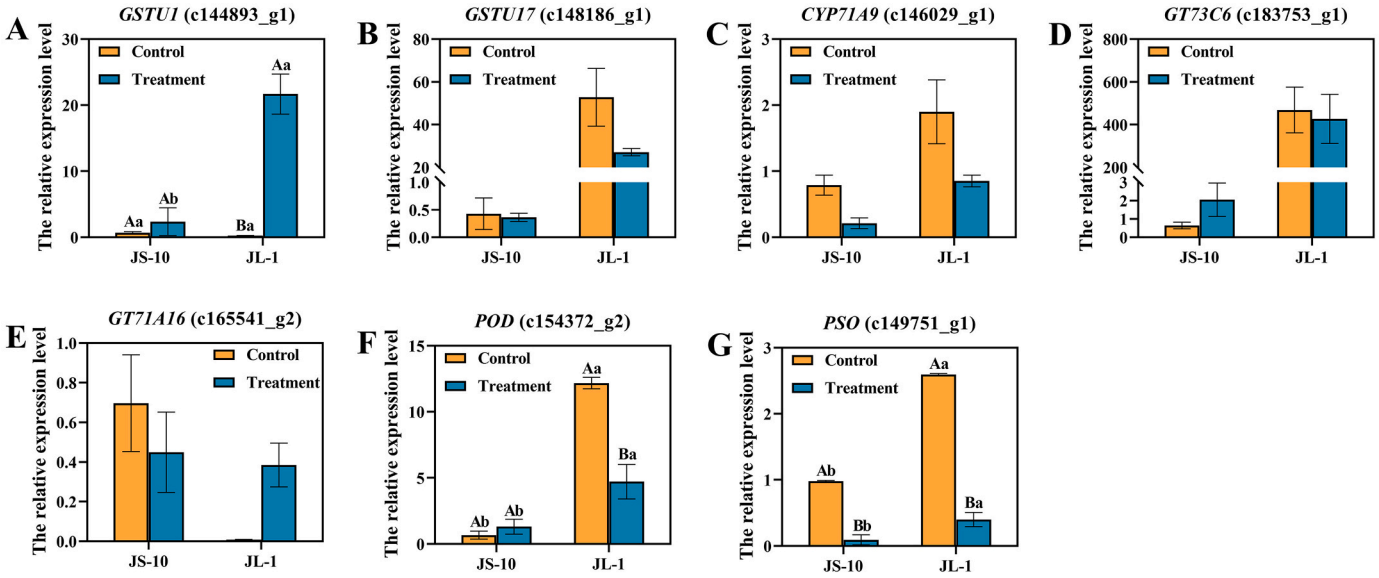

Fig. 5. qPCR validation of the seven candidate genes involved in non-target-site resistance of JL-1 to atrazine. Different lowercase letters represent significant differences between JL-1 and JS-10 populations under the same atrazine treatment at $P < 0.05$. Different uppercase letters represent significant difference between control and treatment groups for the same population at $P < 0.05$.

JS-10 (Fig. 6A). In addition, enhanced P450s activity was also detected in JL-1 population. In JL-1, the P450 activity was 0.046 U g^{-1} at 0 DAT, which was 2.7-fold higher than it was in JS-10 (0.018 U g^{-1}). The P450 activity of JS-10 peaked at 5 DAT and then gradually decreased with time, whereas in JL-1, its activities steadily declined in response to atrazine ($P < 0.05$) (Fig. 6B). In total, the GST and P450 activities were higher in the resistant JL-1 population than in the sensitive JS-10 population at all time points.

4. Discussion

The resistant JL-1 population used in this research was collected from a corn field in Jilin Province where atrazine had been used for over 35 years. In this study, more chlorophyll destruction by atrazine was observed in the sensitive JS-10 population compared with the resistant JL-1 population, strongly suggesting JL-1 has evolved resistance to atrazine possibly through NTSR mechanisms (Yang et al., 2021).

Currently, target-site mutations and target enzyme overproduction are the main reasons for TSR. For JL-1, no mutation at amino acid positions known to confer resistance to atrazine was detected in the target gene coding sequence (Yang et al., 2021). However, JL-1 displayed enhanced expression of *PsbA* compared with the susceptible JS-10 population, suggesting that target gene overexpression was one of the factors contributing to JL-1 resistance to atrazine. Target gene overexpression resulting in weed resistance is commonly reported with the non-selective herbicide glyphosate (Chen et al., 2015; Gaines et al., 2010; Malone et al., 2016; Salas et al., 2012). Recently, Laforest et al. (2017) found that *D. sanguinalis* resistance to ACCase inhibitors had target gene duplication. Target gene overexpression in resistant weed species was likely related to increased gene copy number. Glyphosate-resistant *A. palmeri* contained more copies of the *EPSPS* gene than the susceptible population, and its target gene expression level was positively correlated with the relative copy number (Gaines et al., 2010). However, the reasons for *PsbA* gene overexpression in JL-1 are unknown.

It is well established that certain enzymes including CYP450s, GSTs, glucosyl and other transferases, and aryl acylamidase are involved in herbicide metabolism-based resistance (Gaines et al., 2020). To determine whether differences in herbicide metabolism were responsible for atrazine resistance in JL-1, the effect of the P450 inhibitor PBO on atrazine resistance was assayed. PBO increased the phytotoxicity to atrazine in both JL-1 and JS-10, implying that P450s could participate in the degradation of atrazine. However, the increase in sensitivity to atrazine in the resistant population was greater than that in the sensitive population, suggesting metabolism-based resistance in JL-1. In the RNA-Seq and qPCR validation experiments, a total of two *GST* genes, one

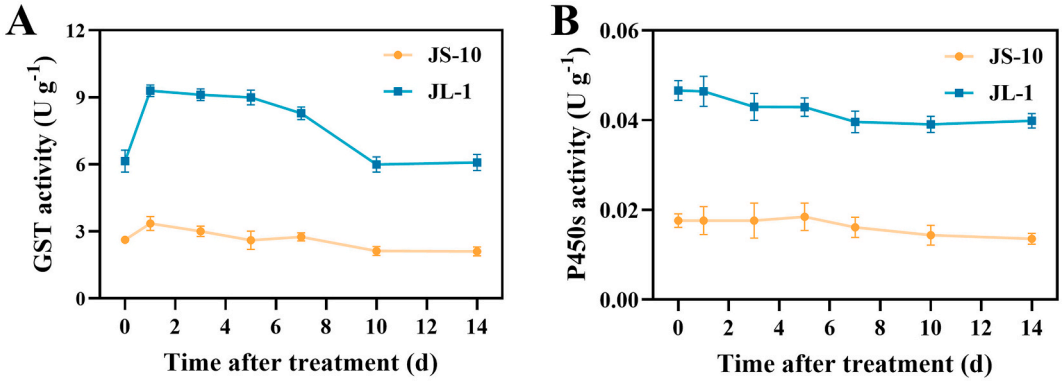

Fig. 6. The activities of glutathione S-transferases (A) and cytochrome P450 monooxygenases (B) in response to atrazine in the JL-1 and JS-10 populations.

CYP450 gene and one *GT* genes, were significantly upregulated in the resistant population compared with the sensitive population. Additionally, the GST and P450 activities in the resistant population were higher than those in the sensitive population. Given the above results, enhanced metabolism may be another reason for *C. communis* resistance to atrazine in JL-1. Increased GST and P450 activities in JL-1 might resulted from the enhanced expression of some *GST* and *CYP450* genes observed in the RNA-seq and qPCR results.

GSTs participate in herbicide detoxification by conjugating GSH to herbicides, leading to herbicide nontoxicity. They play a critical role in the NTSR to herbicide. Previous studies indicated that GSTs were associated with NTSR to atrazine in *A. palmeri* (Nakka et al., 2017) and *A. tuberculatus* (Evans Jr et al., 2017; Vennapusa et al., 2018). In our study, two GST genes (*GSTU1* and *GSTU17*) were identified as candidate genes related to the metabolism-mediated resistance in JL-1. A previous study suggested that *BsGSTU6* was correlated with resistance to fenoxaprop-P-ethyl and mesosulfuron-methyl in *B. syzigachne* (Wang et al., 2020). P450s participate in herbicide metabolism by converting herbicide molecules to more hydrophilic metabolites. A total of 24 *CYP450* genes were validated to be involved in the metabolism of PS II inhibitors (Dimaano and Iwakami, 2021). In the present study, *CYP71A9* upregulation was identified in JL-1. *GmCYP71A10* in *G.* max was verified to catalyse the degradation of the phenylurea class of PS II inhibitors in vitro and in vivo (Siminszky et al., 1999), and it could confer linuron resistance in transgenic *Nicotiana tabacum* L. (Siminszky et al., 2000). *EgP450* in *Elaeis guineensis* Jacq., which belongs to the *CYP71A* subfamily, was also implicated in the detoxification of the PS II inhibitor phenylurea (Phongdara et al., 2012). However, whether *CcCYP71A9* in *C. communis* can metabolize the PS II inhibitor atrazine requires further study. GTs are another critical family of enzymes associated with the degradation of herbicides. Two GTs isolated from tolerant *G. max* could reportedly glycosylate the primary major metabolism of bentazone during the detoxification of the PS II inhibitor (Leah et al., 1992). In comparison with the sensitive JS-10, increased expression of *GT73C6* occurred in the resistant JL-1 population. However, the specific roles of *GT73C6* in metabolism-based resistance of JL-1 need more study.

Beyond herbicide metabolism-related genes, one *POD* gene was distinctly upregulated in the resistant JL-1 population in contrast with the susceptible JS-10 population, which was coincident with our previous finding in which the POD activity of JL-1 was significantly higher than that of JS-10 (Yang et al., 2021). POD is one of the key enzymes involved in defending against oxidative stress. The role of increased POD activity of JL-1in resistance to atrazine requires further study.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2022.105260.

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