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# Leaf proteome analysis provides insights into the molecular mechanisms of bentazon detoxification in rice

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ABSTRACT

Bentazon is a widely used herbicide that selectively removes broad-leaf weeds by competing with plastoquinone for the binding site in the D1 protein and interrupting the PET (photosynthetic electron transfer) chain. However, monocotyledonous plants, such as rice, show strong resistance to bentazon due to *CYP81A6* induction, which results in herbicide detoxification. Here, we confirmed that rice was sensitive to bentazon treatment during the initial exposure period, in which bentazon rapidly inhibited photosynthesis efficiency and electron transfer, based on results of chlorophyll fluorescence analysis. In order to gain a comprehensive, pathway-oriented, mechanistic understanding of the effects directly induced by bentazon, we employed 2D-DIGE (two-dimensional difference gel electrophoresis) to analyze the leaf proteome after 8 h of bentazon treatment coupled with individual protein identification by MALDI-TOF (Matrix assisted laser desorption/ionization-time of flight) MS/MS. Proteomic analyses revealed that bentazon induced the relative upregulation or downregulation of 30 and 71 proteins (by 1.5-fold or more, $p < 0.05$), respectively. The pathways involved include photosynthesis processes, carbohydrate metabolism, antioxidant systems, and DNA stabilization and protein folding. Protein analysis data revealed that bentazon primarily suppressed photosynthesis processes, and showed inhibitory effects on carbohydrate metabolism and ATP synthesis, whereas several stress response proteins were induced in response to bentazon. Importantly, we identified a 519 kD protein containing two histidine kinase-like ATPase domains and a C3HC4 RING type zinc finger domain which may function as a transcript factor to drive expression of detoxification genes such as *CYP81A6*, leading to bentazon tolerance. This study identifies, for the first time, a candidate transcription factor that could up-regulate *CYP81A6* expression, and provides a foundation for further research to advance our knowledge of mechanisms of bentazon resistance in rice.



## 1. Introduction

Herbicides are the dominant method of crop weed control in most broad-area grain cropping systems, and their use has contributed significantly to improved crop productivity and sustainability of agricultural systems in many parts of the world. However, herbicide-resistant weeds present an increasing challenge to this practice, and have become a major threat to the sustainability and profitability of cropping systems. The two primary mechanisms of herbicide resistance in weeds are resistance conferred by mutations in genes that encode proteins containing target sites of the herbicide (target-site resistance), and resistance as a result of mutations outside of target sites (non-target-site resistance) [1]. Most herbicides are designed to target specific enzymes or proteins, thus target-site resistance develops from either genetic diversity or mutations in target sites. Mutations that produce structural differences at the protein level can prevent binding of a single herbicide or a group of related herbicides, such as ACCase (acetyl-CoA carboxylase), the target of DM (diclofop methyl), in rice [2]. DM selectively inhibits the activity of ACCase in monocotyledonous plants, but lacks efficacy in dicotyledonous plants, due to structural changes in ACCase not present in monocotyledonous plants.

In contrast to target-site resistance, non-target-site herbicide resistance involves multi-herbicide resistance proteins, including but not limited to P450s (cytochrome P450 monooxygenase), glutathione *S*-transferase, glycosyltransferase, and ABC transporters [3]. Non-target-site resistance depends on sophisticated detoxification mechanisms against toxic chemicals, in which the oxidation phase reaction is carried out by P450s. Reactions mediated by P450s produce oxygenated compounds with increased reactivity or solubility, thus setting the stage for subsequent detoxification reactions [4]. The function of P450s in

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non-target herbicide resistance has been established through the correlation of P450 enzyme activity with herbicide resistance [5,6].

Bentazon [3(1-isopropyl)-(1*H*)-2,1,3-benzothiadiazine-4(3*H*)-one 2,2-dioxide] is a widely used herbicide that selectively removes broadleaf weeds by competing with plastoquinone (QB) for the binding site in the D1 protein and interrupting the PET (photosynthetic electron transfer) chain [7]. Rice develops resistance to this herbicide due to the ability of bentazon to induce expression of the P450 gene *CYP81A6*. Sensitivity of CYP81A6 mutants to bentazon further confirmed that CYP81A6 is a key mediator of bentazon resistance [8,9]. It has also been shown that unknown protein factor(s) can rapidly induce *CYP81A6* transcription within 2 h of bentazon exposure [10]. *CYP81A6* is therefore used as a selective mrker in modern crop breeding [11–13].

The majority of previous studies related to herbicide–plant interactions on their specific metabolism and detoxification pathways in plants focused on effects at the physiological and biochemical levels. However, little is known about the impact of herbicides on transcription or translation, particularly on transcriptomic or proteomic scales. In this study, we employed a 2-D DIGE proteomic approach to evaluate changes in expression patterns after bentazon treatment. DIGE is a powerful tool for separating complex mixtures of proteins by charge and size (electrophoresis), and different sample types are detected by different fluorescent. To the best of our knowledge, this is the first study to identify protein that may play important roles in upregulation of *CYP81A6* transcription, as well as to clarify the molecular mechanisms by which these pathways participate in *CYP81A6* induction and bentazon detoxification in rice.

## 2. Materials and methods

### 2.1. Plant materials

Indica rice seeds (Jiazhe B) were germinated on filter paper at 30 °C in the dark for 2 d. The germinated seeds were transferred to a net floating on liquid culture medium for 3 weeks. The seedlings were then incubated in a controlled-environment growth chamber at 28 ± 0.5 °C with a light:dark cycle of 12 h:12 h, with light provided by cool-white fluorescent bulbs (≈54 $\mu Em^{-2} s^{-1}$). After one month, 5-leaf rice seedlings approximately 20 cm in height were treated with 500 mg/l bentazon. At this concentration, the rice grew normally without morphologic changes and effectively induced *CYP81A6* transcription to an extent that changes in protein expression could be detected following bentazon exposure [10].

### 2.2. Photosynthesis ratio measurements and RNA extraction for real-time PCR (qRT-PCR)

To investigate the time kinetics of bentazon effects on photosynthesis, four parameters were measured after bentazon treatment using a portable chlorophyll fluorometer (MINI-PAM, Walz, Germany): $F_v/F_m$ (maximum quantum yield), ΦII (quantum yield of photochemistry in PSII), ETR (electron transport rate), and qP (photochemical quenching). In order to study the effects of the bentazon on transcription of PET chain and carbon fixation-related genes, samples were collected every hour after herbicide treatment, for a total of 10 h. Leaf tissues were ground in liquid nitrogen, and total RNA was extracted using 1 ml RNAiso reagent according to the manufacturer's instructions (TaKaRa Company, China). After DNase treatment, cDNA was reverse transcribed from 500 ng RNA. qRT-PCR was performed in 10 μl reactions, with the following cycling parameters: 94 °C for 2 min; 40 cycles of 94 °C denaturation for 30 s and extension at 60 °C for 1 min. The relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method; qRT-PCR primers are listed in Table 1. The housekeeping gene *25S* rDNA was used as an internal standard; four replicates per treatment were performed.

### 2.3. Leaf proteome preparation

After 8 h of bentazon treatment, plant leaves were collected for protein extraction protein using the modified method of Carpentier et al. [14]. Approximately 3 g of fresh weight leaf tissue was ground to a fine powder in liquid nitrogen and incubated at −20 °C overnight with 10% w/v TCA (trichloroacetic acid) in pre-cooled acetone. After centrifugation at 16,000 *g* for 20 min, the supernatant was discarded, and the pellets were vortexed, mixed and washed with 25 ml cold acetone four times in order to eliminate residual TCA and pigment. Pellets were dried under vacuum to volatilize the acetone until the pellets were reduced to powder. The resultant powder was suspended in 4 ml lysis buffer (40% urea, 15% thiourea, 4% CHAPS, 0.4% Tris base, and 1× protease inhibitor) and incubated for 12 h to extract cellular protein. Three biological replicates were prepared.

### 2.4. DIGE used in 2-D electrophoresis by Ettan system

Tissue residue was removed after centrifugation, and the supernatant (total soluble protein solution) was collected for clean-up using a 2-D clean kit (GE healthcare, USA) according to the manufacturer's instructions. Protein concentration was determined using the 2-D Quant

**Table 1**
Sequences of primer pairs used in real-time PCR.

| Gene name | Primers | GeneID |
|---|---|---|
| Plastocyanin | Forward: 5′-TTCCTCTGCCGCCGTCACCA-3′<br>Reverse: 5′-AACACCAGCACGCCGCCATT-3′ | Os06g0101600 |
| Ferredoxin | Forward: 5′-GTGCGGTTCGGATAAAGTCA-3′<br>Reverse: 5′-GCCAAACAATCGCTAACAGAGT-3′ | Os03g0659200 |
| Ferredoxin/$NADP^+$ reductase | Forward: 5′-GGGGAGAACCCGAAGAAGCC-3′<br>Reverse: 5′-CCACGGAATGGAGCAACACC-3′ | Os01g0919100 |
| ATPase β chain | Forward: 5′-TCCTTATTAGGGAGAATGCC-3′<br>Reverse: 5′-TTGCGATAATGTCCTGAAGT-3′ | Os12g0207500 |
| RuBisCO large subunit | Forward: 5′-ATGTTCGGATGCTCTGACGC-3′<br>Reverse: 5′-CACCTGCAAATGAAGATGATGC-3′ | Os02g0152400 |
| Phosphoglycerate kinase | Forward: 5′-CTTCAACTGAGGGAGTGACCAAA-3′<br>Reverse: 5′-AGGTCCGTTCCAAATGATAGTGTT-3′ | Os02g0169300 |
| Phosphoribulokinase | Forward: 5′-GAAGCCAGGAAACCAGAT-3′<br>Reverse: 5′-TGATACCTCATGCCCAAA-3′ | Os02g0698000 |
| Putative TF | Forward: 5′-GTACAGGCAGTCCACGATAT-3′<br>Reverse: 5′-CGAGTAGAGCCACTTGAGAT-3′ | B9FDA1 |
| 25S rDNA | Forward: 5′-AAGGCCGAAGAGGAGAAAGGT-3′<br>Reverse: 5′-CGTCCCTTAGGATCGGCTTAC-3′ | AK119809 |