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Enhanced detoxification and degradation of herbicide atrazine by a group of *O*-methyltransferases in rice

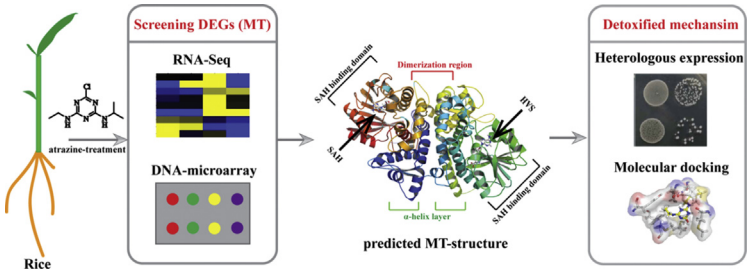
Yi Chen Lu [a, e, 1], Fang Luo [a, 1], Zhong Ji Pu [c], Shuang Zhang [d], Meng Tian Huang [a], Hong Yang [a, b, *]

[a] Jiangsu Key Laboratory of Pesticide Science, College of Sciences, Nanjing Agricultural University, Nanjing, 210095, China
[b] State & Local Joint Engineering Research Center of Green Pesticide Invention and Application, Nanjing Agricultural University, Nanjing, 210095, China
[c] School of Life Science and Biotechnology, Dalian University of Technology, Dalian, 116024, China
[d] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, 214122, China
[e] College of Food Science and Light Industry, Nanjing Tech University, Nanjing, 211800, China

HIGHLIGHTS

- Many ATR-responsive *O*-methyltransferases were identified in rice.
- Yeast with MTs accumulated less ATR and more degraded products.
- UPLC-TOF-MS^2 characterized a *O*-methylated-modified metabolites.
- *O*-MT and ATR-metabolites interaction was analyzed by molecular-docking.

GRAPHICAL ABSTRACT



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ABSTRACT

Atrazine (ATR) as a toxic herbicide has become one of the seriously environmental contaminants worldwide due to its long-term intensive use in crop production. This study identified novel methyltransferases (MTs) involved in detoxification and degradation of ATR residues in rice plants. From a subset of MTs differentially expressed in ATR-exposed rice, forty-four *O*-methyltransferase genes were investigated. Total activities were significantly enhanced by ATR in rice tissues. To prove detoxifying capacity of the MTs in rice plants, two rice *O*-MTs (LOC_Os04g09604 and LOC_Os11g15040) were selected and transformed into yeast cells (*Pichia pastoris* X-33). The positive transformants accumulated less ATR and showed less toxicity. Using UPLC-TOF-MS/MS, ATR-degraded products in rice and yeast cells were characterized. A novel *O*-methylated-modified metabolite (atraton) and six other ATR-derivatives were detected. The topological interaction between LOC_Os04g09604 enzyme and its substrate was specially analyzed by homology modeling programs, which was well confirmed by the molecular docking analysis. The significance of the study is to provide a better understanding of mechanisms for the specific detoxification and degradation of ATR residues in rice growing in environmentally relevant ATR-contaminated soils and may hold a potential engineering perspective for generating ATR-resistant rice that helps to minimize ATR residues in crops.

1. Introduction

Atrazine (ATR) is a triazine herbicide predominantly used for controlling annual broadleaf weeds in crop fields (Villanueva et al.,

* Corresponding author. Weigang No.1, Chemistry Building, College of Sciences, Nanjing Agricultural University, Nanjing 210095, China.
E-mail address: hongyang@njau.edu.cn (H. Yang).
[1] The authors made equal contribution to the study.

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2005; Arora et al., 2014). Due to its low-cost and high-effectiveness, ATR has been used in more than 80 countries for 40 years (Zhang et al., 2014a). Intensive use of ATR has led to the widespread resides in ecosystem, making it as a seriously environmental problem. According to the reports, the concentration of ATR was detected at 42 μg L^{-1} in surface waters, 102 μg L^{-1} in river basins of agricultural areas, and 108 μg L^{-1} in the rivers of North America (USEPA, 2002; Powell et al., 2011). ATR residues at 1.0–42.5 μg kg^{-1} in a field lysimeter soil after 22 years were still detected (Jablonowski et al., 2009). Environmental accumulation of ATR residues risks crop production and negatively affects human health through the food chain (USEPA, 2002; Zhang et al., 2014a; Powell et al., 2011).

To cope with the environmental challenges, plants have developed diverse protective strategies for decaying toxic compounds. For example, glutathione *S*-transferases (GSTs) and cytochrome P450 monooxygenases (P450s)-mediated catabolic processes have been implicated in the mechanism for degradation of organic toxicants (Siminszky, 2006; Lu et al., 2015; Tan et al., 2015). Importantly, involvement of methyltransferases (MTs; EC 2.1.x.y) has been emerging as a potential mechanism for plant response to environmental toxicants (Feng et al., 2016; Lu et al., 2016). MTs are a group of metabolic enzymes that modify structures and functions of various natural and artificial chemicals; they catalyze the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to an acceptor substrate, generating group-methyl derivatives (Roje, 2006). Based on the group of substrates they target, MTs can be subdivided into three major categories including *C*-methyltransferase, *N*-methyltransferases and *O*-methyltransferases. Recently, MTs were reported to methylate atoms of oxygen from a variety of secondary metabolites, implying the active involvement of many critical responses such as chemical defense, signal transduction and inactivation of reactive hydroxyl groups in environmental stimuli (Ibrahim et al., 1998). Research on human lung showed a detoxified pathway for PAH *O*-quinones through *O*-methylation of PAH-catechols by the catechol-*O*-methyltransferase (*O*-MT) (Zhang et al., 2011). Furthermore, *O*-MTs in fungus detoxified toxic phenolic compounds and facilitated degradation of lignin (Pham and Kim, 2016). In high plants, *O*-methylation of secondary metabolites such as phenylpropanoids, flavonoids, alkaloids, sterols or thiols were also reported (Chiron et al., 2000). But so far, data on biological functions of MTs are only limited to the natural compounds, little is known about their role in catabolizing toxicants in crops.

Rice (*Oryza sativa*) is a staple crop that nourishes nearly half the world's population. Since maize/rice, rice/wheat or rice/rapeseed cropping cycles are a popular cultivation modality in most areas, particularly in China and Asia, farmers have to face the problem that ATR residue in soils is most likely accumulated in rice. There are reports indicating that ATR at field concentrations affects physiological metabolisms and cereal production (Arora et al., 2008, 2014; Su and Zhu, 2006; Zhang et al., 2014b). Our recent studies with rice and alfalfa show that ATR could be biochemically transformed into many derivatives and conjugates (Zhang et al., 2014b; Lu et al., 2016), suggesting that ATR can be degraded or detoxified in crops. However, the mechanism underlying the precise ATR degradation and detoxification inside crops are largely unknown. Also, none of the MTs responding to organic toxicants such as herbicide ATR have been characterized in plants. To explore the capability of ATR degradation and detoxification in rice growing in ATR-contaminated soils, we performed a global search for MTs in response to ATR and then functionally characterized two representative ATR-induced *O*-MTs by multiple approaches. Thus, the objective of the study to: (1) screen and identify global *O*-MTs genes in response to ATR in rice and (2) characterize the function of some *O*-MTs genes to figure out the mechanism responsible for ATR degradation and detoxification by *O*-MTs in rice plants.

2. Materials and methods

Plant culture and treatment. Atrazine (with a purity of 99.6%) was obtained from Institute of Pesticide Science, Academy of Agricultural Sciences in Jiangsu, Nanjing, China. Other chemicals for experiments were analytical grade. Seeds of rice (*Oryza sativa,* Japonica) were soaked with water in darkness at 30 °C on a culture dish for 24 h. After germination, seedlings were transferred to a black plastic pot (20 seedlings per pot) containing half-strength nutrient solution (Lu et al., 2016), and grew in a growth chamber (30°C/25 °C light/dark; 300 μmol m^{-2} s^{-1} photosynthetic photon fluxdensity 14 h). After 6 d, seedlings were treated with 0.4 mg L^{-1} ATR for 2, 4, 6 and 8 d, respectively. This concentration used in this study was based on the previous biological response and realistic environmental contamination as well (Ghosh and Philip, 2006; Zhang et al., 2014a). Treatment solutions were renewed every two days.

Bioinformatics analysis of MTs. The amino acid sequences were processed using CLUSTALW (Version 2.0). The neighbor joining (NJ) method was used to construct phylogenic trees with Molecular Evolutionary Genetic Analysis (MEGA) Version 5.1. Protein domains were predicted with the database Pfam (http://pfam.janelia.org/). The *cis*-acting regulatory DNA elements of MTs in the promoter regions were predicted and analyzed with the PlantCare website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Chromosomal location was conducted with the Oryzabase website (http://www.shigen.nig.ac.jp/rice/oryzabaseV4/). The *Arabidopsis thaliana* amino acid sequences were retrieved from the website Tair (http://www.arabidopsis.org/).

Cloning, amplification and ectopic expression of two *O*-MT genes in yeast. Two *O*-MTs genes (LOC_Os04g09604 and LOC_Os11 15040) were cloned and amplified by successive RT-PCR. The condition for PCR amplification was 98 °C for 30 s, 98 °C for 10 s, 60 °C for 20 s and 72 °C for 60 s for each cycle (total 34 cycles), followed by additional 10 min at 72 °C to ensure complete extension. The primers were summarized in Table S1.

Sequences of LOC_Os04g09604 and LOC_Os11g15040 were digested with EcoR I and Xba I and inserted between the EcoR I/Xba I site of the modified pPICZαC vector. The recombinant plasmid pPIC-MTs was linearized with Pme I and inserted into the yeast (*Pichia pastoris* strain X-33) cells by electroporation (Wu and Letchworth, 2004). The cells were plated on YDP medium with zeocin and incubated at 30 °C for 2 d. DNA of the cells was extracted. The inserted MTs genes were confirmed by PCR with the primers listed in Table S1.

For recombinant protein generation, the positive colonies were cultivated in 50 mL of BMGY medium (2% peptone, 1% yeast extract, 1.34% YNB, 0.4 μg mL^{-1} biotin and 1% glycerol) at 30 °C shaking at 200 rpm for 48 h until the OD_{600} reached 2.0. The cells were harvested by centrifuging at 3000 × *g* for 5 min at room temperature. To induce expression, the cellular pellet was resuspended in an equal volume of BMMY (2% peptone, 1% yeast extract, 1.34% YNB, 0.4 μg mL^{-1} biotin, 1% glycerol and 1% methanol). To maintain induction, methanol was added to the culture to a final concentration of 1% every 24 h.

Assay of transformed yeast cell resistance to atrazine. The *P. pastoris* strain X-33 transformants (transfected with linearized pPICZαC or pPICZαC-MT) were grown in YPD medium. After induction with 1% methanol for 24 h, the yeast cells were diluted to 10^{-3} cell μL^{-1} and 0.5–10 μL of cells was transferred to the fresh YPD medium supplemented with 0, 0.4, 2.0 and 10 mg L^{-1} ATR. For ATR feeding experiments, transformants were added in YPD media