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Possible connection between imidacloprid-induced changes in rice gene transcription profiles and susceptibility to the brown plant hopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae)

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ABSTRACT

The chemical pesticide, imidacloprid (IMI) has long-lasting effectiveness against Hemiptera. IMI is commonly used to control the brown planthopper (BPH), *Nilaparvata lugens* Stål (Hemiptera: Delphacidae). Some chemical pesticides, however, can induce the susceptibility of rice to BPH, which has indirectly led to the resurgence of BPH. The mechanism of the chemical induction of the susceptibility of rice to BPH was not previously understood. Here, a 44 K Agilent Rice Expression Microarray was used to identify changes in gene expression that accompany IMI-induced rice susceptibility to BPH. The results showed that 225 genes were differentially expressed, of which 117 were upregulated, and 108 were downregulated. Gene ontology annotation and pathway analysis revealed that differentially expressed genes were mainly classified into the eight functional groups: oxidation reduction, regulation of cellular process, response to stress, electron carrier activity, metabolic process, transport, signal transducer, and organismal development. The genes encoding plant lipid transfer protein, lignin peroxidase, and flavonol-3-O-methyltransferenase may be important responses to the IMI-induced susceptibility of rice to BPH. The reliability of the microarray data was verified by performing quantitative real-time PCR and the data provide valuable information for further study of the molecular mechanism of IMI-induced susceptibility of rice.

1. Introduction

In Southeast Asia and China, the resurgence of brown planthopper is thought to have been caused by the use of chemical pesticides [1,2]. The side effects of pesticides include the destruction of natural enemies [3,4], stimulation of reproduction of male and female adults [5], and pesticide-induced susceptibility of rice to BPH [6].

The brown planthopper (BPH), *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) is a major pest of rice throughout Asia [7]. The BPH damages crops by phloem feeding, nutrient depletion, and by transmitting a number of rice pathogens. In addition, BPH occurrence is related to the resistance or susceptibility of the rice varieties [8]. When living on susceptible rice varieties, where BPH can acquire ample nutrients, the number of eggs laid and survival rates of eggs and nymphs are higher than on resistant varieties. At high rates of BPH feeding plants become completely desiccated, a condition known as "hopperburn". However, when living on resistant varieties, BPH survival and ovipsition rates are significantly lower and BPH population growth is effectively suppressed [9–11]. Control of BPH relies heavily on insecticides, and most chemical classes used extensively against this pest have become compromised by insecticide resistance [12,13].

Neonicotinoids are the fastest-growing class of insecticides in modern crop protection [14]. This insecticide class has gained widespread use against a broad spectrum of sucking and certain chewing pests. Additional benefits of neonicotinoids include relatively low risk for non-target organisms and the environment, high target specificity, and versatility in application methods. This important insecticide class needs careful placement in insect resistance management in order to sustain its efficacy [15]. Imidacloprid (IMI), the first commercialised neonicotinoid insecticide, was introduced for planthopper control in the early 1990s. IMI proved extremely effective against insects that were resistant to compounds used previously. It consequently became the most commonly used insecticide against *N. lugens* over much of Asia. The wide application of IMI against pests has increased the risk of resistance in target insects [16,17]. This increased use has also resulted in various negative effects on crop plants and non-target insects [18,19]. For example, high doses of IMI reduced the length of the active growth stage, the grain weight, and the levels of the rice hormone zeatin

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riboside [20,21]. Insecticide-induced changes in plant quality have even been linked to the resurgence of *N. lugens* in rice [12]. The mechanism of insecticide-induced changes in plant quality is still unclear although this phenomenon has been widely observed.

More specifically, pesticide foliar sprays caused the increased infestation of rice by the BPH, also called pesticide-induced susceptibility [6]. However, the molecular mechanism of this susceptibility is not known. Thus, characterising the effect of IMI on gene expression in rice will provide a better understanding of insecticide-induced susceptibility to BPH.

Gene expression profiling by cDNA microarray is one of the most powerful tools for providing an overview of gene expression under various environmental stresses. Recently, several successful studies have examined plant gene expression profiles in response to pathogens [22,23], abiotic stress [24], and chemicals stress [25]. The Agilent Rice Oligo Microarray is a successful gene expression profile microarray. This chip represents more than 40,000 rice genes and transcripts, which allows for the comprehensive identification of imidacloprid-responsive genes.

The present study examined physiological and gene expression profile changes in the moderately resistant rice variety ZhenDao 2 after exposure to IMI. The objective of this study was to further elucidate the mechanism of insecticide-induced susceptibility to BPH.

2. Materials and methods

2.1. Rice variety and insecticide

The rice (*Oryza sativa* L.) variety, Zhen Dao 2 (japonica rice) was used in the trials. This variety of rice was selected because it is sensitive to pesticide application [26]. Seeds were sown outdoors in a standard rice-growing soil in cement tanks (height 60 cm, width 100 cm, and length 200 cm). When the seedlings reached the six-leaf stage, they were transplanted into 16 cm diameter plastic pots with four hills per pot and three plants per hill. Rice plants at the tillering stage were used in the experiments.

IMI (10% WP, Yangnon Chemical Co., Ltd., Yangzhou, Jiangsu, China) was used in the trials. This insecticide was selected because its foliar spray significantly affects the physiology and biochemistry of rice plants [18]. Two concentrations, 30 and 60 ppm, were designed based on previous studies [20,21].

2.2. Imidacloprid treatment to rice

Foliar sprays were applied with 30 and 60 ppm IMI using a Jacto sprayer (Maquinas Agricolas Jacto S.A., Brazil) equipped with a cone nozzle (1 mm diameter orifice, pressure 45 psi, flow rate 300 ml/min). Control plants at the same stage of growth were sprayed with a mixture of adjuvants and tap water, similar in composition to the mixture containing the insecticide but lacking the active component. Each treatment and control was replicated three times, excluding the damage test following BPH infestation described in the following section. The level of malondialdehyde (MDA) and oxalic acid in the rice leaves and sheaths were measured 3, 6, and 9 days after IMI foliar sprays (3, 6, and 9 DAF). The rice sheaths at 6 DAF were used for the gene expression analysis.

2.3. Assessment of rice damage by BPH following IMI treatment

To examine whether IMI increased the susceptibility of rice to BPH infestation, resistance tests were conducted in 16 cm diameter plastic pots and arranged in a randomised complete block design. A single six-leaf seedling was transplanted to a plastic pot. Five days after transplantation, foliar sprays were conducted with the same concentrations of IMI and methods described above. Control plants were sprayed with a mixture of adjuvants and tap water, similar in composition to the mixture containing the insecticide but lacking its active component. Each treatment and control was replicated thirty times (30 pots). Thirty third-instar nymphs were released onto a single plant at 7 DAF, rather than immediately, so that there would be a lower residue concentration of imidacloprid and lower residue lethal efficacy to the third instar nymphs. Insect mortality was checked at 48 h after the release of nymphs, and dead nymphs (if any) were replaced with live nymphs of the same age to maintain a given density. The potted rice was then placed inside a cylindrical (25×25 cm) cage made of clear plastic film in order to prevent the nymphs from escaping. When BPH adults emerged (about 10 days after the release), the scale of damage to the rice plant was assessed according to a 9-level evaluation method, modified from the screening method used to determine the resistance of rice varietals [6]. The symptom description of a nine-scale injury rating was as follows:

Scale 1: slight injuries, few yellow pitches on leaf sheaths; scale 3: leaf sheaths slightly yellow; scale 5: leaf sheaths clearly yellow, reducing tillering; scale 7: leaf sheaths severely yellow, plant dwarfing and severely reduced tillering; scale 9: general withering [27].

$$\text{Injury index} = \frac{\sum_{i=1,3,5,7,9} (\text{the number of plant with i injury scale} \times \text{i})}{\text{Total number ofrice plants} \times (\text{the maximal injury scale})}$$

2.4. Quantification of IMI-induced biochemical changes

MDA levels, oxalic acid levels, and photosynthetic capacity are important indicators of resistance to BPH [28–30]. We measured MDA, oxalic acid, leaf chlorophyll, and the photosynthetic rate of the rice plants to examine the susceptibility of rice plants to BPH after exposure to IMI.

MDA content was measured using the thiobarbituric acid (TBA) method [31]. Leaf blades were ground in liquid nitrogen using a mortar and pestle, and 15 ml 0.1% (w/v) trichloroacetic acid (TCA) was added to 0.5 g frozen powder. This mixture was centrifuged at 4000 rpm for 15 min, and 4 ml 0.5% thiobarbituric acid (TBA) was added to 1 mL of the supernatant (extracted solution). The supernatant with TBA was then bathed in boiling water for 30 min after evenly shaking. Next, the mixture was centrifuged at 4000 rpm for 10 min after rapidly cooling on ice. The absorbance of the supernatant at 532, 450, and 600 nm was detected using a UV755 B spectrometer (Shanghai Precision Instrument Co., Ltd. Shanghai, China). The value for non-specific absorption at 600 nm was subtracted, and a standard curve of sucrose (from 2.5 to 10 mol/mL) was used to rectify the results from the interference of soluble sugars in samples. The MDA concentration was calculated using the absorption coefficient of 155 mM^{-1} cm^{-1}.

The photosynthetic rate of rice leaves was measured using a Li-6400 Portable Photosynthesis System (Li-COR Company, USA). Measurements were taken between 10:30 A.M. and 12:00 A.M. on sunny and calm days under relatively constant light intensity. Three rice leaves were tested per hill. The photosynthetic rate of control leaves was measured immediately after measuring the rate of treated leaves to minimise errors due to changes in light intensity.

Leaf chlorophyll content was determined using a chlorophyll meter (SPAD-502, Minolta, Japan). Three flag leaves for both IMI-treated and mock-treated plants were measured at 3 and 6 DAF, respectively. Three measurements at random positions in the centre of the leaf were taken for each plant, and the average of the three measurements was used for analysis. Ten leaves from each