# Cloning and Expression Analysis of the Rice OCPI2 Gene Postprint

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## Abstract

To investigate the function of protease inhibitor genes in rice defense against herbivorous insect damage, this study used the rice variety 'Zhonghua 11' as material, cloned the coding sequence of the rice serine protease inhibitor gene OCPI2, analyzed its sequence characteristics and constructed a phylogenetic tree using bioinformatics software, and simultaneously employed real-time quantitative PCR technology to explore the expression patterns of this gene under herbivorous insect feeding and plant hormone induction. The results showed that: (1) The full-length coding sequence of the rice OCPI2 gene was 219 bp, encoding 72 amino acids, with a predicted protein molecular weight of 7.72 kDa and a theoretical isoelectric point of 5.21, lacking a signal peptide and transmembrane structure. (2) The OCPI2 protein was closely related to the homologous protein from Triticum urartu (EMS61613.1). (3) The OCPI2 gene contained one potato_{inhibit} conserved domain and belonged to the serine protease inhibitor family. (4) Feeding by Chilo suppressalis and Nilaparvata lugens, mechanical wounding, and methyl salicylate treatment all induced OCPI2 gene expression, while methyl jasmonate treatment consistently suppressed OCPI2 expression. These findings suggest that the OCPI2 gene may be involved in rice induced defense responses against herbivorous insects, providing a theoretical basis for in-depth investigation of OCPI2 function in rice anti-insect defense responses.

## Full Text

## Cloning and Expression Analysis of the OCPI2 Gene in Rice

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**Abstract**

To investigate the function of protease inhibitor genes in rice defense against herbivorous insects, this study cloned the coding sequence of the rice serine protease inhibitor gene *OCPI2* from the rice variety 'Zhonghua 11'. Bioinformatics software was employed to analyze its sequence characteristics and construct a phylogenetic tree, while real-time quantitative PCR was used to examine the expression patterns of this gene under herbivorous insect feeding and plant hormone treatments. The results revealed: (1) The coding region of rice *OCPI2* is 219 bp in length, encoding a protein of 72 amino acids with a predicted molecular weight of 7.72 kDa and a theoretical isoelectric point of 5.21, lacking both a signal peptide and transmembrane structures. (2) The OCPI2 protein shows close phylogenetic relationship with its homolog in *Triticum urartu* (EMS61613.1). (3) OCPI2 contains a potato_{inhibit} conserved domain and belongs to the serine protease inhibitor family. (4) Feeding by *Chilo suppressalis* and *Nilaparvata lugens*, mechanical wounding, and methyl salicylate treatment all induced *OCPI2* expression, whereas methyl jasmonate treatment consistently suppressed its expression. These findings suggest that *OCPI2* may participate in the induced defense response of rice against herbivorous insects, providing a theoretical foundation for further investigation of its function in rice anti-herbivore defense.

**Keywords:** rice, protease inhibitor, OCPI2, gene cloning, induced expression pattern

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## Introduction

Protease inhibitors (PIs) are widely distributed in animals, plants, and microorganisms as small polypeptides or proteins with molecular weights ranging from 5–25 kDa. They exist as dimers or tetramers in organisms and play crucial roles in regulating proteolytic activity and various biological processes related to metabolism and cellular physiology (Habib & Fazili, 2007). In plants, protease inhibitors serve as storage proteins and endogenous protein regulators, participating not only in physiological processes such as seed germination and dormancy but also in regulating plant resistance to herbivorous insects (Hörger & Van der Hoorn, 2013; Singh et al., 2020; Xie et al., 2021). For instance, some plant protease inhibitors induce programmed cell death by regulating insect endogenous protease activity, while others enter the insect digestive tract and bind to digestive enzymes, inhibiting their activity and thereby interfering

with nutrient absorption and insect growth and development (Meekins et al., 2017). Additionally, certain protease inhibitors can enter the insect hemolymph and disrupt the immune system (Rahbé et al., 2003). Therefore, identifying plant protease inhibitor genes, elucidating their physiological functions, and applying them in pest control are of great significance for developing sustainable agriculture.

Plant protease inhibitors are classified into several types, including serine protease inhibitors, cysteine protease inhibitors, aspartic protease inhibitors, and metalloproteinase inhibitors (Laskowski et al., 1980). Among these, serine and cysteine protease inhibitors are the most widely applied. Serine protease inhibitors represent the most abundant class of protease inhibitors in plants (Rustgi et al., 2018). Although structurally complex, they are functionally conserved and can inhibit various serine proteases while regulating plant immune responses to enhance resistance against insects (Clemente et al., 2019; Benbow et al., 2019; Ferreira et al., 2023). For example, Losvik et al. (2017) found that overexpressing the serine protease inhibitor gene *Barley chymotrypsin inhibitor 2c* (*CI2c*) in barley (*Hordeum vulgare*) significantly enhanced resistance to the green peach aphid (*Myzus persicae*). Further studies revealed that CI2c reduced aphid fecundity by inhibiting digestive enzyme activity and interfering with nutrient absorption and growth. Cysteine protease inhibitors, on the other hand, affect insect development by hydrolyzing cysteine proteases and show particular efficacy against coleopteran insects. Cingel et al. (2017) transformed potato (*Solanum tuberosum*) with cysteine protease inhibitor genes *oryzacystatin-I* and *oryzacystatin-II* (*OC-I* and *OC-II*) to generate transgenic potatoes expressing both proteins. Bioassays demonstrated that *Leptinotarsa decemlineata* larvae fed on these transgenic potatoes exhibited slower development, reduced body weight, and increased mortality.

Rice (*Oryza sativa*) is one of the world's most important food crops, and its stable production is crucial for global food security. However, rice is frequently damaged by various pests during growth, leading to reduced yield and quality (Lou et al., 2013). Currently, rice pest control in China relies primarily on chemical insecticides, which not only pose risks to human health but also cause environmental pollution, excessive pesticide residues in agricultural products, and increased pest resistance (Mao et al., 2019). Therefore, developing green control strategies is essential for integrated rice pest management. Identifying insect resistance genes and breeding resistant rice varieties represent key priorities, with the discovery and utilization of plant protease inhibitor genes being an important research direction. For example, serine protease inhibitors play vital roles in rice defense against *Chilo suppressalis*, *Cnaphalocrocis medinalis*, and the blast fungus *Magnaporthe oryzae* (Zhang et al., 2020; Liu et al., 2021; Zhang et al., 2022). Transgenic rice overexpressing the trypsin inhibitor gene *AvrPiz-t interacting protein 4* (*APIP4*) showed enhanced resistance to both *C. suppressalis* and *M. oryzae* (Zhang et al., 2020; Liu et al., 2021). Additionally, introducing exogenous protease inhibitor genes can enhance plant resistance to pests and diseases. The cowpea trypsin inhibitor gene (*CpTI*) is a broad-spectrum

insect resistance gene, and its introduction into rice confers strong resistance to multiple rice pests (Xu et al., 1996). Transgenic rice expressing the barley trypsin inhibitor *CMe* gene showed significantly increased resistance to the rice weevil (*Sitophilus oryzae*), as the expressed trypsin inhibitor reduced weevil gut trypsin activity, thereby inhibiting its growth and development (Alfonso-Rubí et al., 2003). Thus, discovering novel protease inhibitor genes is valuable for green pest control in rice.

Previous transcriptome sequencing results indicated that both *C. suppressalis* and brown planthopper (*Nilaparvata lugens*) damage induced upregulation of the rice protease inhibitor gene *Oryza sativa chymotrypsin protease inhibitor 2* (*OCPI2*), suggesting its important role in rice defense against these pests (Liu et al., 2016; Liu et al., 2021). Using the rice variety 'Zhonghua 11' ('ZH11') as material, this study cloned the complete coding sequence of *OCPI2*, performed bioinformatics analysis, and investigated its induced expression characteristics using real-time quantitative PCR. The study addresses three main questions: (1) whether the *OCPI2* coding sequence is consistent across different rice varieties; (2) the physicochemical properties, structural features, family classification, and interspecies homology and evolutionary relationships of the encoded protein; and (3) the expression patterns of *OCPI2* following damage by different insects (*C. suppressalis* and *N. lugens*), mechanical wounding, and treatment with different plant hormones (methyl salicylate and methyl jasmonate), aiming to establish a foundation for further functional analysis of *OCPI2* in rice insect resistance.

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## Materials and Methods

**1.1 Plant Materials** This study used two rice varieties: 'ZH11' for experimental treatments and 'Taichung Native 1' ('TN1') for brown planthopper rearing. Plump rice seeds were soaked in water and placed in a 37°C incubator. After germination, seeds were wrapped in moist towels and returned to the 37°C incubator until roots and shoots emerged, then transplanted into sterilized nutrient soil. Plants were grown under controlled conditions (temperature: 30 ± 2°C, photoperiod: 16 h light/8 h dark, relative humidity: 75 ± 10%) for 40 days before selecting uniformly vigorous plants for experiments.

**1.2 Insect Materials** *Chilo suppressalis* and *Nilaparvata lugens* were used in this study. *C. suppressalis* larvae were reared on wild rice stems until pupation, and adults were fed 20% honey solution. Rearing conditions were: temperature 28 ± 2°C, photoperiod 16L:8D, relative humidity 75 ± 10%. Brown planthoppers were maintained on 40-day-old 'TN1' rice seedlings under conditions of 26 ± 2°C, 16L:8D photoperiod, and 60 ± 10% relative humidity.

**1.3 Experimental Treatments**

**1.3.1 *C. suppressalis* Infestation Treatment** Transparent plastic cylinders (diameter: 1.5 cm, height: 7 cm) were placed around the base of rice stems, sealed with sponge at both ends, and kept in the growth chamber for 3 days to eliminate mechanical damage effects from the cylinders. Three-instar *C. suppressalis* larvae, starved for 2 h, were introduced onto rice stems inside the cylinders, and their boring behavior was monitored. Timing began when larvae had bored halfway into the stem. At different time points after infestation (0, 1, 2, 4, 8, 12, 24, 48 h), the damaged stem sections were excised, immediately frozen in liquid nitrogen, and stored at -80°C. Control samples were taken from 'ZH11' stems fitted with cylinders but without insects, with three biological replicates per treatment.

**1.3.2 Brown Planthopper Infestation Treatment** Rice plants were fitted with transparent plastic cylinders at the base three days before infestation (see 1.3.1). Fifteen fourth-instar brown planthopper nymphs were introduced into each cylinder, and timing commenced immediately. At various time points after infestation (0, 1, 2, 4, 8, 12, 24, 48 h), damaged stem sections were harvested, snap-frozen in liquid nitrogen, and stored at -80°C. Control samples were taken from 'ZH11' stems with cylinders but no planthoppers, with three biological replicates per treatment.

**1.3.3 Mechanical Wounding Treatment** Rice stems were wounded by gently piercing both sides of the basal 2–3 cm region 100 times with an insect pin (tip diameter: 0.32 mm). At different time points after wounding (0, 1, 2, 4, 8, 12, 24, 48 h), the wounded stem sections were collected, immediately frozen in liquid nitrogen, and stored at -80°C. Control samples were taken from unwounded 'ZH11' stems under identical conditions, with three biological replicates per treatment.

**1.3.4 Plant Hormone Treatment** Methyl salicylate and methyl jasmonate (both from Sigma-Aldrich, USA) were dissolved in 50 $\text{mmol} \cdot \text{L}^{-1}$ phosphate buffer (pH 8.0) to final concentrations of 70 $\text{g} \cdot \text{mL}^{-1}$ and 100 $\text{g} \cdot \text{mL}^{-1}$, respectively, with 0.01% Tween-20 added before spraying. Two milliliters of either solution were evenly sprayed onto 40-day-old 'ZH11' rice plants. At 0, 1, 2, 4, 8, 12, 24, and 48 h after treatment, stem sections were harvested, snap-frozen in liquid nitrogen, and stored at -80°C. Control plants were sprayed with 2 mL of 50 $\text{mmol} \cdot \text{L}^{-1}$ phosphate buffer (pH 8.0) under the same conditions, with three biological replicates per treatment.

**1.4 Total RNA Extraction and cDNA Synthesis** Rice stem tissues were ground in liquid nitrogen, and 100 mg samples were used for total RNA extraction following the RNAiso protocol. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). One microgram of total RNA was used as template for cDNA synthesis using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time)

(TaKaRa) according to the manufacturer's instructions. The 20 L reaction was performed in two steps: first, genomic DNA elimination; second, reverse transcription. The first-step reaction contained 1 g total RNA, 2 L 5× gDNA Eraser Buffer, and 1 L gDNA Eraser, brought to 10 L with RNase-free $dH_2O$, and incubated at 42°C for 2 min. The second-step reaction combined the first-step product (10 L) with 1 L PrimeScript RT Enzyme Mix I, 1 L RT Primer Mix, and 4 L 5× PrimeScript Buffer 2 (for Real Time), brought to 20 L with RNase-free $dH_2O$, and incubated at 37°C for 15 min followed by 85°C for 50 s. All procedures were performed in a clean bench using RNase-free consumables, with reaction mixtures prepared on ice. Synthesized cDNA was stored at -20°C for subsequent experiments.

**1.5 Gene Cloning and Sequence Analysis of Rice *OCPI2*** Based on the *OCPI2* gene (*LOC_{Os01g42860}*) sequence from the Rice Genome Annotation Project (RGAP) for 'Nipponbare' (*Oryza sativa* spp. *japonica*), primers flanking the complete CDS were designed using Primer 5 software (primer sequences listed in ) and synthesized by Shanghai Sangon Biotech. PCR amplification was performed using cDNA from 'ZH11' stems damaged by *C. suppressalis* larvae for 24 h as template. Amplified products were electrophoresed on 1% agarose gel, gel-purified, ligated into pMD-18T vector (TaKaRa), and transformed into competent *E. coli*. Transformants were plated on ampicillin-containing medium overnight, and single colonies were selected for colony PCR verification. Positive clones were sequenced by Wuhan Hetaiqing Biotechnology.

Sequencing results were aligned with the 'Nipponbare' *OCPI2* sequence using Jalview software. Physicochemical properties including molecular weight and theoretical isoelectric point were predicted using the online ExPASy tool (https://web.expasy.org/cgi-bin/protparam/protparam). Signal peptide prediction was performed using SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/), transmembrane structure prediction using TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), and conserved domain analysis using the NCBI Conserved Domain Service (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

**1.6 Homologous Sequence Alignment and Phylogenetic Analysis** Homologous amino acid sequences from other Poaceae species were retrieved using the NCBI Blastp tool. Sequence alignment and phylogenetic tree construction were performed using Jalview and MEGA 11 software, respectively. The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method with 1,000 bootstrap replicates.

**1.7 Real-Time Quantitative PCR** Expression of *OCPI2* under different treatments was analyzed using a CFX96 Real-Time PCR System (Bio-Rad, USA). Gene-specific primers for *OCPI2* were designed using Primer 5 software (sequences in ) and synthesized by Sangon Biotech (Shanghai). Reactions were performed in 10 L volumes containing 5 L 2X M5 HiPer SYBR Premix Es Taq (Mei5 Biotechnology), 2 L cDNA template, 0.4 L primer mix, and 2.6 L

dd$H_2O$. The amplification program consisted of initial denaturation at 95°C for 60 s, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. The rice *18S ribosomal RNA* gene (*Os18s*, AK059783) served as an internal reference, and relative expression levels were calculated using the $2^{-}\Delta\Delta$Ct method.

**1.8 Data Processing and Analysis** Student's t-test was used to analyze *OCPI2* expression levels in response to pest infestation, mechanical wounding, and hormone treatments. All statistical analyses were performed using SPSS 22.0 software.

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## Results

**2.1 Cloning and Analysis of Rice *OCPI2*** Using cDNA from 'ZH11' stems damaged by *C. suppressalis* larvae for 24 h as template, PCR amplification with *OCPI2*-specific primers yielded a band of 300–400 bp on agarose gel ([Figure 1: see original paper]). The purified PCR product was ligated into pMD18-T vector and transformed into *E. coli* DH5$\alpha$ competent cells. Sequencing of positive clones revealed a 342 bp amplification product containing the complete 219 bp *OCPI2* CDS. Alignment with the 'Nipponbare' *OCPI2* sequence (*LOC_{Os01g42860}*) from RGAP showed 100% identity between 'ZH11' and 'Nipponbare' ([Figure 2: see original paper]).

ProtParam analysis of the deduced amino acid sequence revealed that *OCPI2* encodes a 72-amino-acid protein with a predicted molecular weight of 7.72 kDa and theoretical isoelectric point of 5.21. SignalP 5.0 prediction indicated no signal peptide ([Figure 3: see original paper]A), suggesting OCPI2 is a non-secreted protein. TMHMM Server 2.0 analysis revealed no transmembrane domains ([Figure 3: see original paper]B). Conserved domain analysis using the NCBI tool identified a potato_{inhibit} domain ([Figure 3: see original paper]C), and database searches confirmed that OCPI2 belongs to the serine protease inhibitor family (Volpicella et al., 2011; Wang et al., 2023).

**2.2 Homology and Phylogenetic Analysis of OCPI2** To investigate the evolutionary relationships of OCPI2, eight Poaceae CPI proteins were selected for homology analysis and phylogenetic tree construction: *Panicum virgatum* (XP_{039836276}.1), *Lolium rigidum* (XP_{047070641}.1), *Triticum dicoccoides* (XP_{037471069}.1), *Triticum urartu* (EMS61613.1), *Oryza glaberrima* (XP_{052139272}.1), *Lolium perenne* (XP_{051224251}.1), *Zea mays* (NP_{001232814}.2), and *Miscanthus lutarioriparius* (CAD6334936.1). All nine species contained a potato_{inhibit} conserved domain, and CPI proteins showed high homology across species ([Figure 4: see original paper]A). Phylogenetic analysis indicated that OCPI2 is most closely related to homologs from *T. urartu*, *L. rigidum*, *T. dicoccoides*, and *L. perenne*, while more distantly related to those from *Z. mays*, *O. glaberrima*, *P. virgatum*, and *M. lutarioriparius* ([Figure 4: see original paper]B).

**2.3 Expression Analysis of *OCPI2* Under Insect Herbivory** To examine the effects of herbivorous insect damage on *OCPI2* expression, real-time quantitative PCR was used to analyze induction by *C. suppressalis* and *N. lugens* feeding. During 24 h of feeding by a single third-instar *C. suppressalis* larva, *OCPI2* expression showed two peaks at 2 h and 8 h, both significantly different from the control (15-fold and 6-fold induction, respectively). When damaged by 15 fourth-instar brown planthopper nymphs, *OCPI2* expression increased rapidly within 1 h, reaching a peak 6.7-fold higher than the control, then declined to normal levels by 8 h. Continued infestation produced a subsequent fluctuating pattern of increased then decreased expression. Except at 8 h, *OCPI2* expression at all other time points showed significant differences from the control ([Figure 5: see original paper]B). These results demonstrate that both *C. suppressalis* and *N. lugens* feeding induce *OCPI2* expression, albeit with different temporal patterns, suggesting that *OCPI2* may be involved in rice resistance to both pests.

**2.4 Expression Analysis of *OCPI2* Under Mechanical Wounding** Mechanical wounding significantly increased *OCPI2* expression within 1 h, reaching 29.1-fold higher than the control. Expression began to decline after 2 h and returned to normal levels by 4 h, followed by minor fluctuations of increase and decrease ([Figure 6: see original paper]).

**2.5 Expression Analysis of *OCPI2* Under Exogenous Plant Hormone Treatments** Methyl salicylate treatment rapidly induced *OCPI2* expression within 1 h (12.6-fold higher than control), which returned to normal levels by 2 h with no further significant differences. In contrast, methyl jasmonate treatment downregulated *OCPI2* expression at 1 h, showed extremely significant suppression at 2 h, and maintained downregulation until 12 h when expression recovered to control levels. However, expression decreased significantly again at 24 h and remained below normal at 48 h ([Figure 7: see original paper]B). These results indicate that methyl salicylate induces *OCPI2* expression, whereas methyl jasmonate suppresses it.

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## Discussion

Protease inhibitors are important defense proteins in plants that are rapidly synthesized upon herbivore attack to protect against insect feeding (Volpicella et al., 2011). Genetic engineering of protease inhibitor genes into crops has proven effective for developing insect-resistant transgenic varieties and controlling field pests. Rice, a crucial food crop, suffers damage from multiple pests including *C. suppressalis* and *N. lugens*. Therefore, studying rice protease inhibitor genes and their anti-herbivore mechanisms is essential for rice production.

This study successfully cloned the complete *OCPI2* cDNA sequence from rice. Sequence analysis revealed a 219 bp coding region encoding 72 amino acids

with a potato_{inhibit} domain, confirming its membership in the serine protease inhibitor family. Numerous studies have shown that plant serine protease inhibitors regulate anti-herbivore responses by modulating serine protease activity. For example, soybean Bowman-Birk inhibitor (sBBI) delays development of the melon fruit fly (*Bactrocera cucurbitae*) (Kaur et al., 2017), while *Adenanthera pavonina* trypsin inhibitor (ApTI) increases mortality in soybean looper (*Anticarsia gemmatalis*) larvae (Meriño et al., 2020). These findings suggest that OCPI2 plays an important role in rice anti-herbivore defense.

Induced expression analysis revealed that *OCPI2* is upregulated by feeding from both *C. suppressalis* and *N. lugens*, as well as by mechanical wounding. The response pattern to mechanical wounding resembled that of the piercing-sucking insect *N. lugens*, though the wounding response was more intense. Our mechanical wounding treatment using insect pins created wounds similar to those caused by brown planthopper feeding but more severe and concentrated, eliciting a stronger *OCPI2* response. Similarly, Singh et al. (2009) reported mechanical wounding induction of *OCPI2* and additionally found induction by abscisic acid, low temperature, and salt stress. However, studies of other rice protease inhibitor genes showed different patterns: *OsLTPL164* and *OsLTPL151* were induced by *C. suppressalis* feeding within 3 h but only after 6 h by mechanical wounding (He et al., 2018). Although the maximum response intensity induced by the chewing insect *C. suppressalis* was higher than that by brown planthopper, the response speed and duration were less rapid and persistent. Comparable results were reported in poplar, where different herbivore species induced distinct expression patterns of Kunitz-type trypsin inhibitor genes (Eberl et al., 2021). These findings demonstrate that different herbivores induce distinct expression patterns of protease inhibitor genes, and that insect feeding and mechanical wounding elicit clearly different *OCPI2* responses. Insect feeding causes not only mechanical damage but also introduces saliva containing elicitors or effectors that trigger specific plant defense responses (Jones et al., 2022; Liu et al., 2023).

Jasmonic acid (JA) and salicylic acid (SA) are key endogenous hormones that regulate plant defense against various herbivores (Erb & Reymond, 2019; Liu et al., 2021). The JA pathway positively regulates rice resistance to both *C. suppressalis* and *N. lugens* (Xu et al., 2021), while the SA pathway also participates in rice resistance to brown planthopper (Guo et al., 2018). Previous reports showed that exogenous JA induces synthesis of trypsin inhibitor genes and accumulation of protease inhibitors in rice (Farmer et al., 1992). However, our results demonstrate that methyl salicylate rapidly induces *OCPI2* expression, whereas methyl jasmonate consistently suppresses it, indicating antagonistic regulation by JA and SA. This is consistent with known antagonistic interactions between JA and SA signaling pathways, where each suppresses defense responses mediated by the other (De et al., 2014). Nevertheless, some studies have reported synergistic regulation of herbivore resistance by JA and SA pathways (Liu et al., 2021), suggesting that both antagonistic and synergistic interactions between these hormones may be important for effective defense

against herbivores. Whether other plant hormones regulate *OCPI2* remains to be investigated.

In summary, this study successfully cloned the complete coding sequence of the rice serine protease inhibitor gene *OCPI2*, analyzed its physicochemical properties and evolutionary relationships using bioinformatics approaches, and examined its transcriptional responses to herbivore feeding, mechanical wounding, and hormone treatments. Our results demonstrate that *OCPI2* participates in rice induced defense against insect herbivores, though its specific mechanisms require further investigation.

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*Note: Figure translations are in progress. See original paper for figures.*