Expression Analysis of Rice Polygalacturonase cDNA Responding to Brown Planthopper [*Nilaparvata lugens* (Stål)]

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ABSTRACT

A cDNA (*OsKPG*) encoding polygalacturonase (PG) from rice (*Oryza sativa* cv. KDML105) was cloned and sequenced. The cDNA full length was 1,103 bp and carried 277 deduced amino acids with 4 highly conserved domains among PG family. The expression of *OsKPG* was investigated under brown planthopper [*Nilaparvata lugens* (Stål)] attack. The transcription levels of *OsKPG* under different phytohormone applications including ethylene, abscisic acid (ABA), 6-benzyladenine (6-BA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were also analyzed in leaves. Semi-quantitative RT-PCR revealed that the expression of *OsKPG* was high upon the brown planthopper attack, or under the application of ethylene or 6-BA treatments. This is the first evidence demonstrating that *OsKPG* may play a role in response against insect herbivore infestation through ethylene and cytokinin signaling pathways in rice.

Keywords: polygalacturonase; *Oryza sativa* cv. KDML105; insect herbivore; ethylene; cytokinin

INTRODUCTION

Plants and insects have interacted for more than 350 million years, leading plants to develop an elegant defense system that has the ability to recognize foreign molecules, signals from damaged cells, and activate the plants immune response against the herbivores (War *et al.*, 2012). The signaling pathways that enable plants to mount defenses against insect herbivores are known to be complex. Some defenses are constitutive while others are inducible. Constitutive defenses are present and offer continuous protection against herbivores such as furanocoumarin, saponin and cardenolide (Wittstock and Gershenzon, 2002). Some intracellular signals in wounded tissues induced by herbivores are jasmonic acid (JA); salicylic acid (SA) and ethylene (Fürstenberg-Hägg *et al*., 2013).

Ethylene is known as a major signal in plants that can induce defense-related genes, leading to produced defense-related proteins. For example, ethylene can induce chitinases accumulation in rice (Rakwal *et al*., 2004). The activity of peroxidase in barley is increased by ethylene application as well as infestation by 2 aphids; *Schizaphis graminum* (biotype C) and *Rhopalophum padi*. It has been suggested that ethylene is involved in the oxidative responses of barley plants induced by infestation (Argandoña *et al*., 2001). Ethylene can also regulate the cysteine proteinase (*mir-1*) expression in maize (*Zea mays* L.) genotype Mp708, leading to defense against insect herbivore attack (Harfouche *et al.*, 2006).

Polygalacturonases (PGs) have been known to play a major role in many processes of plants such as seed germination (Sitrit *et al*., 1999), cell elongation and flower development (Xiao *et al*., 2014); senescence and abscission of leaves (Lee *et al*., 2001) and fruit ripening (Gayathri and Nair, 2015). Evidence has shown that plant endogenous PG induced by mechanical damage could degrade pectin in plant cell walls to release oligogalacturonides (OGAs) (Orozco-Cardenas and Ryan, 1999). The OGAs have been known to act as elicitor molecules that trigger a variety of plant responses against pathogens and insects (Shibuya and Minami, 2001). These responses included the accumulation of phytoalexins (Davis *et al*., 1986), glucanase, and chitinase (Davis and Hahlbrock, 1987; Broekaert and Pneumas, 1988), the production of reactive oxygen species (ROS) (Galletti *et al*., 2008) and nitric oxide (Rasul *et al*., 2012). This evidence suggests that PG may be a key molecule to activate defense responses during herbivore and pathogen attacks (Bergey *et al*., 1999). In addition, PG is one of several enzymes induced by ethylene. For examples, the expression of PG gene in cucumber fruit (*CUPG1*) is induced by water stress (water loss after harvesting)

and exogenous ethylene, but not by the application of abscisic acid (ABA) (Kubo *et al*., 2000); PG gene isolated from papaya (*cpPG*), which controls the process of pulp softening during papaya ripening, is strongly induced during ripening and highly ethylene-dependent (Fabi *et al*., 2006). Moreover, the PG in ethylene-stimulated abscission of tomato pedicel shows an abundant accumulation in the cortical and vascular tissues in the abscission zone at 8 hours after ethylene treatment (Qi *et al*., 2014).

Despite much information on PG, reports about the relationship between phytohormones and PG in the herbivore-defense mechanism are rare. This study is the first report to show that *OsKPG* is involved in herbivore attack, probably, under ethylene and cytokinin signaling in rice.

MATERIALS AND METHODS

Plant material

Seeds of rice (*Oryza sativa* cv. KDML105) were submerged in water for 2 days and germinated in a moisture chamber. After 5 days, the seedlings were transferred into plastic pots (9 cm in diameter) containing soil and grown in a greenhouse under natural light conditions. The rice seedlings at 10 day old were used for *OsKPG* cloning and expression analysis under insect attack. The brown planthopper [*Nilaparvata lugens* (Stål)] (BPH) used for infestation were collected from rice fields in Phayao province, Thailand and fed on tillers of a susceptible rice variety "Taichung Native 1" (TN1) for mass propagation. The 40 day seedlings were chosen for expression analysis under phytohormone treatments.

Cloning of a full-length *OsKPG* cDNA from rice

First-strand cDNA was synthesized using Superscript III Reverse transcriptase (Invitrogen, USA) following the manufacturer's instructions. The first-strand cDNA was used as template for PCR amplification. The partial cDNA sequence encoding the OsKPG protein was obtained using degenerate forward primer which was designed to correspond to the highly conserved N terminal region of the PG proteins. The forward primer, OsKPGFD, was designed to match a sequence coding for domain I of PG family. A poly-T oligonucleotide, oligo $(dT)_{18}$ was used as a reverse primer. The amplification conditions were an initial denaturation at 94ºC for 2 min, followed by 35 cycles at 94ºC for 45 s, annealing at 55ºC for 30 s, extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min. The DNA fragments were ligated into the pTZ57R vector (Fermentas, Germany). DNA sequencing was performed using the dideoxynucleotide chain termination method (Sanger *et al*., 1977) using an automated sequencer (ABI). The amino acid sequence similarity was analyzed using the BLAST program (Altschul *et al*., 1990). The 5' terminal of the *OsKPG* cDNA sequences were obtained using 5' Full Core Set (Takara, Japan) following the manufacturer's instructions. The primers used for partial cDNA cloning and 5' RACE PCR are shown in Table 1.

Sequence analysis

The complete sequence of *OsKPG* cDNA was translated into peptide sequence. The peptide sequence was aligned with 11 PG protein sequences from 10 plant species [*Oryza sativa japonica* (XP_015623329), *Oryza sativa indica* (EAY87758), *Setaria italic* (XP_004954128), *Brachypodium distachyon* (XP_003570492), *Zea mays* (NP_001140630), *Triticum urartu* (EMS61824.1), *Sorghum bicolor* (XP_002452925), *Phoenix dactylifera* (XP_008784338), *Ananas comosus* (OAY70358), *Aegilops tauschii* (EMT11866) and *Lycopersicon esculentum* (Bergey *et al.*, 1999)] using Clustal W (Goujon *et al*., 2010) and the initial phylogenetic tree was then bootstrapped 1,000 times using the UPGMA method, which was included in the MEGA5 software package (Tamura *et al*., 2011).

Table 1 The sequences of PCR primer sets used for partial cDNA cloning and 5′ RACE PCR

Primers	Sequences
OsKPGFD	5′-GCNCCNAAYACNGAYGGNATHCCN-3′
Oligo $(dT)_{18}$	5′-TTTTTTTTTTTTTTTTTT- 3′
OsKPG_deFD	5′-GCNCCNAAYACNGAYGGNATH- 3′
OsKPGRT-p	5′-ATCGTGGATGTAGCC-Ⓟ- 3′
OsKPG_A1	5′-CACAATGTTACGAGATGGGC- 3′
OsKPG_A2	5′-ACCGCTATGGCATCATCACC- 3′
OsKPG_S1	5′-CGTTCATTGGTCAGTGCTGG- 3′
OsKPGS2	5′-GCAGTGAGATGTCTGGTGGG- 3′
OsKPG_3'FD	5′-CAAGACCTCCTCTTTTGCAGC- 3′
OsKPG_3'RV	5′-ATGAGAATGGGAATGCCACCC- 3′

Rice infestation procedure

The 10 day old seedlings were thinned to 10 plants per pot. All pots were transferred into the 50 x 40 x 50 (W x L x H) cm cages covered with a nylon net (32 holes/cm^2). To provide suitable humidity for insect survival, the experiment was conducted at a temperature of 28ºC to 30ºC and kept relatively high humidity at 70% to 80%. The rice seedlings were infested with 2nd – 3rd instar of brown planthopper at a density of 8 to 10 per seedling. The seedling without insect infestation was used as a control. Five seedlings were harvested after 5, 10 and 15 days of herbivore infestation and stored at -80ºC.

Phytohormone treatment

The fourth leaf blades from 40 day old rice plants were chopped to 2 to 3 cm. The chopped leaf blades were incubated in 3 mM MES buffer, pH 5.8, supplemented with various phytohormones including 1 mM ethephon, 100 μM of abscisic acid (ABA), 100 μM 6-benzyladenine (6-BA) and 100 μM 2,4-dichlorophenoxyacetic acid (2,4-D) to examine the effect of these phytohormones (Pitakrattananukool *et al*., 2012). Three leaves from each experiment were collected at 0, 6, 12 and 24 hours after incubation. All experiments were performed under continuous lighting.

Semi-quantitative reverse transcriptase PCR reaction and analysis

Total RNA was extracted from the rice tissues using Trizol reagent (Invitrogen, USA) and first-strand cDNA was synthesized. Twenty ng of first-strand cDNA was used as the PCR template. The amplified PCR product (237 bp) was obtained using *OsKPG* specific primers (Table 2). The RT-PCR reaction was performed by initiating for template denaturation for 2 min at 94ºC, followed by 28 cycles of denaturation for 30 s at 94ºC, annealing for 30 s at 62ºC and extension for 30 s at 72ºC. Rice *β-Actin* transcripts were used as the internal standards. The primers used for RT-PCR are shown in Table 2. Agarose electrophoresis was performed to visualize the PCR products. To monitor the relative level of gene expression, the intensities of the band were analyzed using Scion Image software (Scion, Frederick, MD). The level of *OsKPG* expression was presented as the ratio of expression of *OsKPG* to *β-Actin*.

Table 2 The sequences of PCR primer sets used for RT-PCR

Primers	Sequences
PGRT-FD	5′-TCACGGCGAGGTTTGAGAATAAAGACTGCCAT-3′
PGRT-RV	5′-ACTGCTGCCATGAGCACGGACTGGCACCCGGACA-3′
βActin-FD	5′ - CAAGGCCAATCGTGAGAAG - 3′
βActin-RV	5′ - AGCAATGCCAGGGAACATA - 3′

RESULTS

Cloning of full-length *OsKPG* cDNA from rice (KDML105)

The partial sequence (705 bp) of *PG* cDNA from rice (*Oryza sativa* cv. KDML105) was cloned. Then the full length of *PG* cDNA was amplified using 5′ RACE PCR technique. The 1,103 bp full-length cDNA was cloned into pTZ57R and was analyzed. This sequence contained a 26 bp 5′ noncoding region, 243 bp 3′ noncoding region and translated into 277 amino acids. The four conserved domains found in higher plant and fungi PG sequences were observed in OsKPG sequence (domain I-IV). Domain I (NTD) analyzed as substrate-binding region. Domain II (G/QDD) had a carboxylate group in the three aspartic acids that may be a component of the catalytic site. Domain III (G/SHG) contained the histidine residue that is thought to participate to the catalytic reaction. Domain IV (RIK) was thought to be as a substrate-binding region (Bussink *et al*., 1991; Rao *et al*., 1996; Palanivelu, 2006). Sequence analysis is shown in Figure 1.

Phylogenetic analysis of the OsKPG

The deduced amino acid sequence of OsKPG was analyzed by alignment among members of PG sequences from 10 plant species using Clustal W and phylogenetic tree was constructed by UPGMA method. Comparative analysis revealed the OsKPG displayed high similarity to PGs members, *Oryza sativa japonica* (XP_015623329.1) and *indica* (EAY87758.1) at 99%, and to other monocot plant species such as *Setaria italic* (XP_004954128.1), *Brachypodium distachyon* (XP_003570492.1), *Zea mays* (NP_001140630.1), *Triticum urartu* (EMS61824.1) and *Sorghum bicolor* (XP_002452925.1) at 89, 88, 87, 86 and 86 % respectively. OsKPG showed low homology at 28.4% identity to PG from dicot specifically one induced by wounding in *Lycopersicon esculentum* (Bergey *et al.*, 1999) (Figure 2).

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ATACAAGACCTCCTCTTTTGCAGCTGATGGTCCAAGGACATTATTGTTGCAAATATAA
                          M  W  S  K  D  I  I  V  A  N  I  T
CATTGAAGAATTCACCTTTCTGGCACTTCCACCCATATGATTGCACGAATATAACTGTTT
  L  K  N  S  P  F  W  H  F  H  P  Y  D  C  T  N  I  T  V  S
CTAATGTTACTATCTTAGCTCCTATTTCTAGTGCTCCAAACACAGATGGCATAGATCCAG
  N  V  T  I  L  A  P  I  S  S  A  P  N  T  D  G  I  D  P  D
                                   I
ATTCTTGTCAGGATGTGCTTATTGAGAATTGCTACATTTCAGTTGGTGATGATGCCATAG
  S  C  Q  D  V  L  I  E  N  C  Y  I  S  V  G  D  D  A  I  A
                                            II
CGGTAAAGAGTGGGTGGGATCAATATGGGATTGCATATGGGCGCCCATCTCGTAACATTG
  V  K  S  G  W  D  Q  Y  G  I  A  Y  G  R  P  S  R  N  I  V
TGATACGCAATGTAATGGCTCGTTCATTGGTCAGTGCTGGAATTTCAATTGGCAGTGAGA
  I  R  N  V  M  A  R  S  L  V  S  A  G  I  S  I  G  S  E  M
                                               III
TGTCTGGTGGGATTGCAAATGTTACAGTGGAGGATGTCCGCATTTGGGAGTCACGGCGAG
  S  G  G  I  A  N  V  T  V  E  D  V  R  I  W  E  S  R  R  G
GTTTGAGAATAAAGACTGCCATAGGAAGAGGGGGCTACATCCGCGATATCTCCTATCGCA
  L  R  I  K  T  A  I  G  R  G  G  Y  I  H  D  I  S  Y  R  N
     IV
ACATAACCTTCGACAATGTCCGTGCTGGTATTGTGATAAAGGTTGACTACAATGAGCACG
  I  T  F  D  N  V  R  A  G  I  V  I  K  V  D  Y  N  E  H  A
CTGATGATGGGTATGACCGGGATGCCTTTCCAGACATCACAAACATATCATTCAAGGAAA
  D  D  G  Y  D  R  D  A  F  P  D  I  T  N  I  S  F  K  E  I
TACATGGGCGAGGTGTCCGGGTGCCAGTCCGTGCTCATGGCAGCAGTGACATTCCCATCA
  H  G  R  G  V  R  V  P  V  R  A  H  G  S  S  D  I  P  I  K
AGGACATCAGCTTTCAGGACATGTCTATCGGCATCAGCTACAAGAAGAAACATATTTTCC
  D  I  S  F  Q  G  M  S  I  G  I  S  Y  K  K  K  H  I  F  Q
AGTGTTCCTTCATTGAGGGGCGTGTCATCGGGTCAGTGTTTCCAAAACCATGCGAGAATT
  C  S  F  I  E  G  R  V  I  G  S  V  F  P  K  P  C  E  N  L
TGGATCTCTACAATGAGCAAGGGCAGCTTGTTAAGCGTGCAGCAATGGTAAACAGCACGG
  D  L  Y  N  E  Q  G  Q  L  V  K  R  A  A  M  V  N  S  T  E
AAGTTGATTATGACATATGATGATATAGGTGGCTAAGGCTAACAGCAGAACATGGGTGGC
  V  D  Y  D  I  stop
ATTCCCATCCTCATTTTGTTCTTTTAGTCTTTCTGTGTTTTTTGAGATTAGAACTGTAT
ATACATAGGAGATTCACCTCTTCATAGGTTTGAATGCAGTGGCTAATTGTATCTCTCATT
TCATTTGTCCACCAGCATTTTGTTCTTGCTGCCAAACATGTAATACAACCTTGCAATCTT
AGAAATACACTTGGCCATTCACC
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Figure 1 Nucleotide sequence and deduced amino acid sequence of *OsKPG*. Bold letters indicate start and stop codons, while the 5′ and 3′ UTR are indicated in italics. The predicted amino acid sequence is shown below the nucleotide sequence in single-letter code. The four functional domains of PG are underlined and Roman numerals indicate the substrate-binding domains (I, IV) and catalytic domains (II, III).

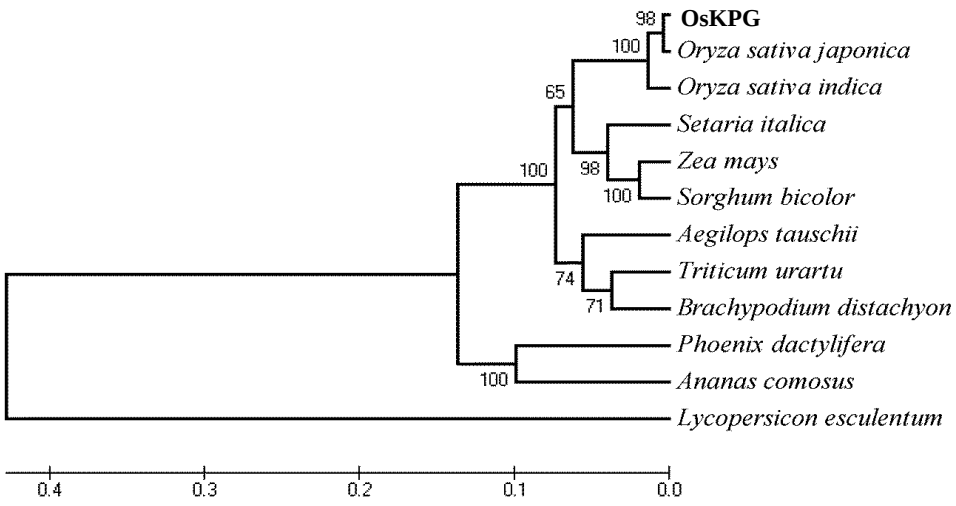

Figure 2 The phylogenetic analysis of the relationship among OsKPG amino acid sequence and amino acid sequences of PG from 10 different plant species. The numbers at an internal node show the bootstrapping value.

Expression profile of the *OsKPG* under brown planthopper infestation

To examine *OsKPG* expression under insect infestation, 2nd - 3rd instar of *Nilaparvata lugens* (Stål) was introduced to 10 day old rice seedlings in the cage, as mentioned in material and methods, for 15 days. Then, the seedlings were collected to evaluate the *OsKPG* mRNA intensities (Figure 3A). RT-PCR revealed that the *OsKPG* expression in rice seedlings attacked with brown planthopper had increased in day 10 and 15 by 1.3 and 1.5 fold respectively when compared to the seedlings without insect attack (Figure 3B).

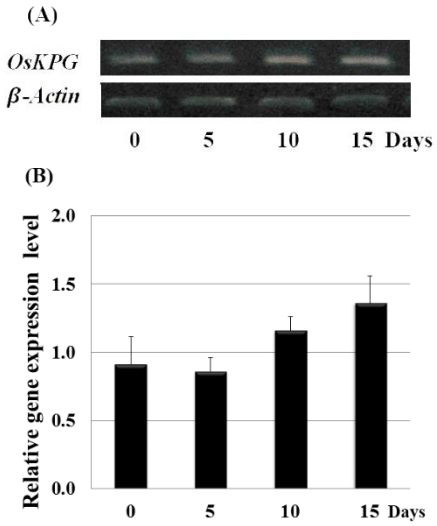

Figure 3 The expression of *OsKPG* under herbivore infestation. The expression of *OsKPG* after infestation with brown planthopper for 5, 10 and 15 days (A). The relative expression of *OsKPG* under herbivore infestation, presented as the ratio of expression of *OsKPG/β-Actin* (B). *β-Actin* was used as the internal control.

Expression profile of the *OsKPG* under Phytohormone treatments

The *OsKPG* transcription responded to 4 phytohormones, i.e. ethylene, ABA, 6-BA and 2,4-D was determined in rice leaves. The RT-PCR reactions revealed that the expression of *OsKPG* responded to ethylene and 6-BA showing a 2.0 and 4.0 fold upregulation in rice leaves exposed to 1 mM of ethephon and 100 µM of 6-BA compared with untreated leaves at the beginning of experiment, respectively (Figure 4).

DISCUSSION

Polygalacturonase (*PGs*) was known as a super-family gene for pectin dehydration. There were many *PGs* isolated from numerous organisms. In the case of plants, plants utilize PGs in many processes such as the growth and development processes as well as defending mechanism (Bergey *et al*., 1999). In this work *PG* cDNA from rice *O. sativa* (KDML105) named *OsKPG* was isolated and analyzed. Comparisons of deduced amino acid sequences of full-length *OsKPG* cDNA and 11 plant PG members revealed that *OsKPG* displayed the highest identity (99%) to *O. sativa japonica* and *indica* groups and 98 – 86% identity to PG from other monocot plant species: *S. italic*, *B. distachyon*, *Z. mays*, *T. urartu* and *S. bicolor*. The four functional conserved domains (domain I-IV) of all PG members were also present, suggesting that *OsKPG* belonged to the PG family (Figure 2).

The expression of *OsKPG* in rice seedlings was analyzed after being infested by brown planthopper (Figure 3). RT-PCR revealed that the expression level of *OsKPG* increased up to 1.5 fold than that of intact seedling. Bergey *et al*. (1999) supported this with evidence that the activity of PG was induced in tomato leaves under wounding and herbivore attacks. This led to an increase in endogenous oligogalacturonide elicitor that may be involved in the local and systemic activation of defense responses against herbivores. Therefore, this evidence indicated that *OsKPG* was one of the defense response genes against herbivores in rice leaves.

Meanwhile, the expression of *OsKPG* under phytohormone treatments was investigated. The results revealed that *OsKPG* was upregulated in rice leaves exposed to ethylene and 6-BA to the factor of 2 and 4 respectively. It was not responsive to ABA and 2,4-D application (Figure 4). So far, ethylene and jasmonic acid were well known as the regulators of herbivore and pathogen responsive genes. Ethylene is a main mediator of the signal transduction pathway leading to defense against insect herbivores in maize and other plants (Harfouche *et al*., 2006; von Dahl and Baldwin, 2007; Louis *et al*., 2015). There have been reports that the expression of ethylene biosynthesis gene (*OsACS*) in rice strongly increases after rice is wounded or infested by brown planthopper (Lu *et al*., 2014). The stability of ethylene biosynthesis proteins was under regulation of the increase of cytokinins (Vogel et al., 1998). Moreover, there have been reports that both natural and synthetic cytokinin applications in cotton leaves resulted in increased ethylene production (Suttle, 1986) and some cytokinins showed response to wounding and insect oral secretion applications. These cytokinins were *isopentenyl adenine* (IP), *isopentenyl*

adenosine (IPR), cis-zeatin riboside (cZR), trans-zeatin riboside O-glucoside (tZROG), cis-zeatin riboside O-glucoside (cZROG) and trans-zeatin N7- glucoside (tZ7G). When insect attacked plant cell, herbivore-associated molecular patterns (HAMPs) presented in insect saliva were deposited to the plant cell. These compounds included glucose oxidase, alkaline phosphatase and other proteinaceous elicitors which activated plant defenses through a complex signaling network (Cheng *et al*., 2013). This suggests cytokinin is an integral component of wounding and HAMPs triggered responses in many plant species (Schäfer *et al*., 2015).

Our results clearly demonstrated that the expression of rice *OsKPG* responded to the attack of brown planthopper and to the exposure of ethylene or cytokinin treatment. Hereby, we propose that *OsKPG* may play a role in defense response against insect herbivores in rice under ethylene and cytokinin signal transduction.

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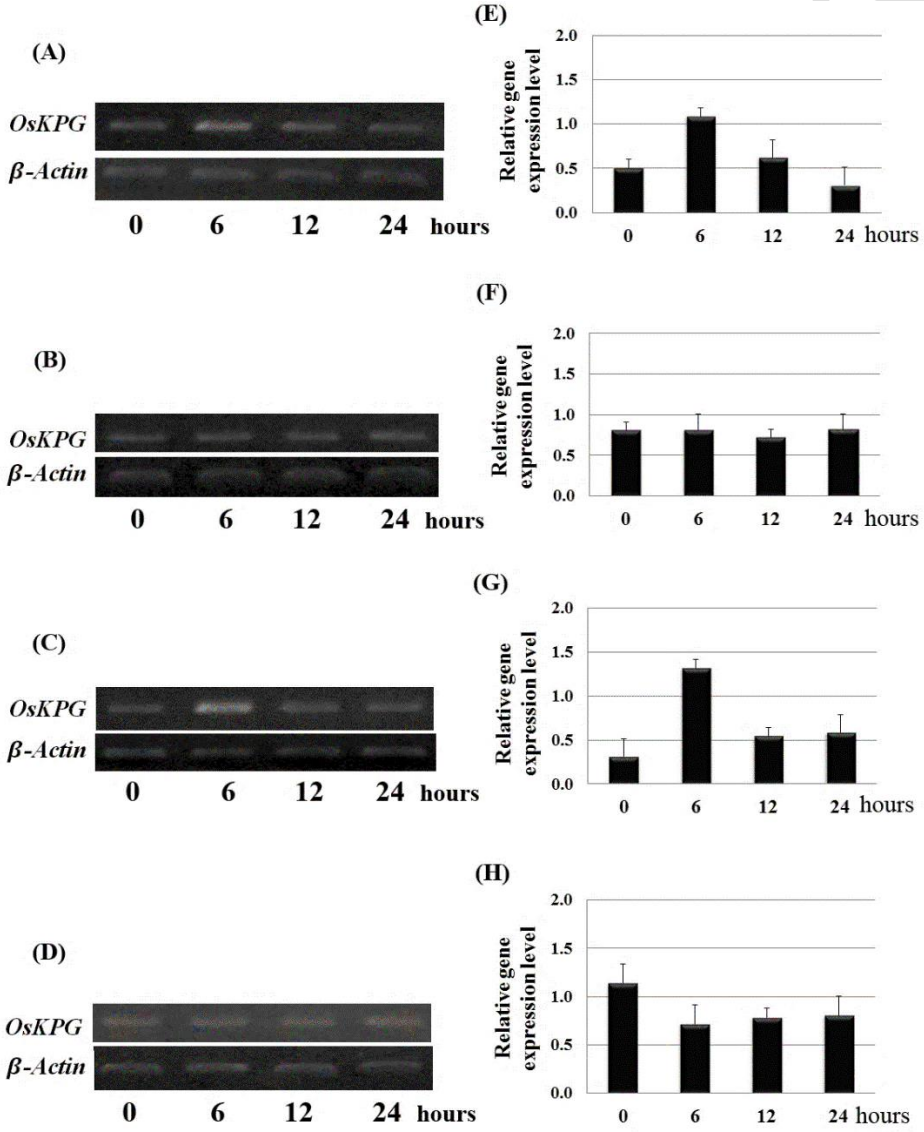

Figure 4 The expression of *OsKPG* under phytohormone treatments. The expression of *OsKPG* in rice leaves incubated in 3 mM MES buffer pH 5.8, supplemented with ethephon (A) and ABA (B), 6-BA (C) and 2,4D (D) for 0, 6, 12, and 24 hours. The relative expression level of *OsKPG* in those rice tissues is presented as the ratio of expression of *OsKPG* /β-Actin (E-H). *β-Actin* was used as an internal control.

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