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ROLE OF OsWAK124, A RICE WALL-ASSOCIATED KINASE, IN RESPONSE TO ENVIRONMENTAL HEAVY METAL STRESSES

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Abstract

Members of the *Arabidopsis* cell wall-associated kinase (WAK) family play important roles in both development and stress responses. There are about one hundred and twenty five*OsWAKs* annotated in the rice genome but their functions in rice growth and development are largely unknown. In this paper, we reported a functional role of the OsWAK124 (Os12g0266200) in rice heavy metal responses. Confocal GFP experiments located OsWAK124 in the cell wall and analyses of *OsWAK124 promoter*::*GUS* transgenic lines suggested that *OsWAK124* promoter is primarily active at the meristematic tissues under normal growth condition. Under stress conditions, however, *OsWAK124* promoter activity is induced in non-meristematic tissues, such as leaf, stem and root, and the activity in the meristematic tissues is further enhanced. Various transgenic rice lines carrying either RNA interference (RNAi) or overexpression constructs were generated. Transgenic lines were tested for their responses to various stress conditions including salicylic acid, NaCl, $AlCl_3$, $CuSO_4$ and $CdSO_4$. Our analyses showed that rice seedlings overexpressing *OsWAK124* are more resistant to the three tested heavy metals (Al, Cu, and Cd), which suggested that OsWAK124, like some *Arabidopsis* WAK members, plays a role in environmental heavy metal stress responses.

Key words: Wall-associated kinase, Heavy metal stress, Rice, Transgenic.

Introduction

In plants, the cell wall serves as a physical barrier between cell and outside environment. Meanwhile, this physical barrier allows cells respond to various outside stimuli very efficiently to achieve continuous communications between a plant cell and its environment. There are certain biomolecules physically located at the cell wall as bridge signals between a cell and its outside environment. Receptor-like kinases (RLK) are thought to act as such bridging signal molecules (Chae *et al*., 2009). There are more than 600 and 1100 *RLKs* in *Arabidopsis* and rice genomes, respectively (Shiu *et al.*, 2004). *RLKs* can be divided into 43 subfamilies according to their sequence divergence. The *WALL-ASSOCIATED KINASE* (*WAK*) and *WAK-like* (*WAKL*) gene family belong to the *RLK* super gene family. *AtWAK1-5* were found in a 30 kb cluster in *Arabidopsis* chromosome 1 (He *et al.,* 1999) and 22 additional *AtWAKLs* were identified in the *Arabidopsis* genome and found to be distributed in all five chromosomes (Verica *et al.*, 2002). Most of the AtWAK/AtWAKLs have three domains: a less conserved extracellular domain with EGF, a transmembrane domain, and a highly conserved Serine/Threonine protein kinase domain. AtWAK/AtWAKLs associate with the cell wall very tightly and can only be released by boiling plant cellhomogenate in 4% SDS and 50 mM dithiothreitol or by treating with pectinase (Wagner & Kohorn, 2001), suggesting AtWAK/AtWAKL may be covalently cross-linked with cell wall matrix. Based on their biochemical and structural properties, AtWAKs are thought to be excellent candidates for signal transduction between the cytoplasm and the cell wall.

AtWAK/AtWAKLs were shown to play important functions in plant development, biotic and abiotic resistance. The expressions of *AtWAK* genes are both environmentally and developmentally regulated (Verica *et al.*, 2003). *AtWAK1* and *AtWAK2* expressions were both detected at organ junctions, in shoot and root apical meristems, and in expanding leaves and sepals (Wagner & Kohorn, 2001). Plants transformed with *AtWAK2* antisense construct under the control of the dexamethasone-inducing promoter have small rosette leaves when induced. These results showed that AtWAK2 were required for leaf cell expansion (Wagner & Kohorn, 2001).Through analyzing inducible *AtWAK4* antisense *Arabidopsis* lines, AtWAK4 was found to be essential in regulating cell elongation and lateral root development (Lally *et al.*, 2001).*AtWAK1*'s expression can be induced by pathogen attack, and PR (pathogenesis-related) gene expressions are down regulated in *AtWAK1* antisense transgenic lines. These *AtWAK1* down regulation transgenic lines are more labile than wild type under pathogen attack (He *et al.*, 1998). At WAKL22/RFO1 is a novel type of dominant disease-resistant protein that has broad spectrum to *Fusarium* races (Diener & Ausubel, 2005). *Magnaporthe oryzae* can significantly induce the *OsWAK1* transcripts, and constitutive overexpression of *OsWAK1* transgenic lines confer resistant to the compatible *Magnaporthe oryzae* race (Li *et al.*, 2009). The mRNA and protein level of *AtWAK1* can be induced quickly upon aluminum treatment, *AtWAK1* overexpressing transgenic lines showed more aluminum resistant than wildtype (Sivaguru *et al.*, 2003). *WAKL4* expressions were identified to confer plants more resistant to Na^+, K^+, Cu^{2+}, Zn^{2+} and Ni^{2+} by using a T-DNA inserted at *AtWAKL4* promoter -40bp mutant, WAKL4 can influence the zinc transporter genes expression, and then the Zn^{2+} accumulation in shoots (Hou *et al.*, 2005).

WAKs were identified broadly existed in higher plants (He *et al.*, 1996). Kaur *et al.* (2013) cloned *HvWAK1* from barley genome, and it was found only expressed in root and involved in root elongation under normal and stress conditions. A *LeWAK* was cloned from tomato cDNA, and its expression rapidly increased in

tomato roots and cultured cells when challenged by *Orobanche ramosa* (Lejeune *et al.*, 2006). 4 *TaWAKs* and 2 *TaWAKLs* genes were isolated from wheat genome, and their expression patterns were studied by qRT-PCR (Liu *et al*., 2006).

There are one hundred and twenty five *OsWAK* genes that were annotated from rice genomes through reiterative database search and manual reannotation (Zhang *et al.*, 2005), but their functions are largely unknown. Rice is the staple food for more than half of the world population, and rice will face various biotic and abiotic stresses during their three to four months growth period. WAKs were shown not only have important role in plant development (Lally *et al.*, 2001), but also in increasing plant resistance to biotic and abiotic stresses from previous research (Diener & Ausubel, 2005; Hou *et al.*, 2005). So to understand the functions of OsWAKs, especially in biotic and abiotic stresses resistance, and their possible usage in plant molecular breeding, will help the rice production stable under various stresses. Here transgenic lines of *OsWAK124* promoter::*GUS*, *OsWAK 124* down and up regulation were constructed, and the subcellular location, expression pattern and functions of *OsWAK124* in heavy metal responses were studied by using these transgenic lines thereby.

Materials and Methods

Plant materials and growth conditions: Rice wild type Zhonghua 11 (*Oryza sativa* 'Nipponbare') and transgenic lines were germinated and grown under natural conditions in Kimura B solution about one month, then planted into pots with paddy soil under natural conditions until harvest (Zhang *et al.*, 2007). Seeds harvested from independent transgenic lines were selected in Kimura B with 50 μg/mL hygromycin B (Roche, Germany), the positive lines were chosen for further studies.

Generation of *OsWAK124* transgenic rice lines: The full length of *OsWAK124* fragment was amplified with specific primers (forward: 5'-GAAGGATCCAAGGCTA TTGTCTG -3'; reverse: 5'-TTCGAGCTCAAATAGACA TTTCTCTT -3'. The introduced restriction site was underlined), a 413bp RNAi fragment by primers (forward:5'-GGTGCTGGAGTGGGCCGTGGCGTCTGT -3'; reverse: 5'-ACACCAAAGCAAATCCAACCC GCACC-3'), a 2.7 kb *OsWAK124* promoter fragment by primers (forward: 5'-AGGAAGCTTGGGACTCGTAA CATAAAC-3'; reverse:5'-ATCAGC GGATCCAGACAA TAGCCTTG-3') by using Easy-A high fidelity DNA polymerase (Stratagene, USA). From these amplified fragments, the transformation vectors pCAMBIA1301-Ubi-*OsWAK124*::*GFP*, pCAMBIA1301-Ubi-*OsWAK124*-RNAi and pCAMBIA-1300G-*OsWAK124 P*::*GUS* were constructed by using molecular cloning technologies. The respective transgenic rice lines were obtained by *Agrobacterium tumefaciens*-mediated transformation of Zhonghua 11 embryonic callus (Xu *et al.*, 2009).

T-DNA insertion confirmation and *OsWAK124* mRNA level analysis: Genomic DNAs of wildtype Zhonghua 11 and hygromycin resistant transgenic lines were extracted by CTAB method (Murray & Thompson, 1980). These transgenic lines were further confirmed by PCR amplification of *HPT* (*HYGROMYCIN PHOSPHATE TRANSFERASE*) gene (forward: 5'-CTGAACTCACCG CGACGTCTGTC-3'; reverse: 5'-TAGCGCGTCTGCT GCTCCATACA-3'). pCAMBIA-1300G-*OsWAK124 P*::*GUS* transformed lines were also further confirmed by PCR amplification of *GUS* gene (forward:5'- CTGTGGG CATTCAGTCTGGATCG-3'; reverse: 5'-GTTACCGCC AACGCGCAATATGC -3'). Total RNAs of wildtype Zhonghua 11 and selected transgenic lines were extracted by Trizol (Invitrogen, USA) method. After trace DNA removed by DNase I (Takara, China), 1μg RNA was reverse-transcripted by ReverTra Ace (Toyobo, China) with Oligo dT_{20} primer. Semi-quantitative RT-PCR was used to analyze the expression level of *OsWAK124* by using its specific primers (forward: 5'-GTGGCAAT CCCTACACCAAAGA-3'; reverse: 5'-CAAGAAGACG AGAGAAAGGACC -3'). The house-keeping gene *OsACTIN1* was chosen as template loading control (forward: 5'-GACATTCAGCGTTCCAGCCATGTAT-3'; reverse: 5'-TGGAGCTTCCATGCCGATGAGAGAA-3').

Subcellular localization of OsWAK124: By using the roots of transgenic rice plants transformed by pCAMBIA1301-Ubi-*OsWAK124*::*GFP*, the subcellular localization of OsWAK124 was detected by confocal microscope (Leica, Germany) system with the GFP fluorescence (Hou *et al.*, 2005).

***OsWAK124* expression pattern:** By using *OsWAK124 P*::*GUS* transgenic rice lines, the expression patterns of *OsWAK124* were analyzed under normal and stress conditions, respectively (Hou *et al.*, 2005).

Phenotype of *OsWAK124* transgenic lines under normal and stress conditions: The wildtype Zhonghua 11 and transgenic lines were planted in the same conditions as mentioned above. The young seedlings of Zhonghua 11 and transgenic lines with three to five leaves were treated by different concentration of salicylic acid (SA), NaCl, $CuSO_4$, $AlCl_3$and $CdSO_4$ (Guangzhou reagents, China), respectively, in Kimura B (Zhang *et al.*, 2007). After treated by above abiotic and simulated biotic stresses, their growth phenotypes were recorded.

Results

Transgenic rice lines of *OsWAK124* were constructed: By using specific *OsWAK124* primers, respective *OsWAK124* fragments were amplified. Through regular molecular cloning technologies, transformation vectors pCAMBIA1301-Ubi-*OsWAK124*::*GFP*, pCAMBIA1301-Ubi-*OsWAK124*-RNAi and pCAMBIA-1300G-*OsWAK 124 P*::*GUS* were constructed successfully, which were sequentially confirmed by restriction enzyme digestion and sequencing.

Transgenic lines were generated by transforming *OsWAK124* via *Agrobacterium tumefaciens*-mediated transformation procedure with three constructs: pCAMBIA 1301-Ubi-*OsWAK124*::*GFP*, pCAMBIA 1301-Ubi-*OsWAK124*-RNAi (Fig. 1), and pCAMBIA-1300 G-*OsWAK124 P-GUS* (Fig. 2). The obtained transgenic lines

were further confirmed by hygromycin resistance selection and PCR verifications. Expression levels of *OsWAK124* were analyzed by semi-quantitative RT-PCR. Our results showed that *OsWAK124* expression can be upregulated in the *OsWAK124* overexpression transgenic rice lines and down regulated in the *OsWAK124* RNAi transgenic rice lines, respectively (Fig. 3). The *OsWAK124* overexpression line NO 2 and *OsWAK124* RNAi line NO 6 were selected for further study.

***OsWAK124* is localized at the cell wall:** The GFP signal of *OsWAK124*::*GFP* overexpression transgenic line's roots was detected by confocal to indicate OsWAK124 subcellular localization. As shown in Figure 4A, the GFP signals showed that the OsWAK124 was localized at the cell boundary. To further test whether OsWAK124 was localized at the cell wall, or at the plasma membrane, roots were treated with 5 mol/L NaCl solutions to induce plasmolysis. As shown in Figure 4B, GFP signal was detected at the cell wall area suggesting that OsWAK124 is localized at the cell wall, not at the plasma membrane.

***OsWAK124* expression can be increased by stress conditions:** The expression pattern of gene could reveal its function to some extent. By using *OsWAK124 P*::*GUS* transgenic lines, the expression pattern of *OsWAK124* was studied. Under the natural conditions, only the shoot-root transition zone was obviously stained with color, while other parts including leaf, stem, root and root hairs, were all not stained (Fig. 5). These results mean that *OsWAK124* primarily expresses at the shoot-root transition zone under normal condition.

In order to investigate the expression patterns of *OsWAK124* under various stresses conditions, young seedlings of *OsWAK124P*::*GUS* transgenic line were cultivated in Kimura B with 20 μmol L^{-1} SA, 200 mmol L^{-1} NaCl and 75 nmol L^{-1} $CdSO_4$ for 3 days, respectively, then various parts of the treated seedlings were stained in GUS solution. The expression of *OsWAK124* is strongly increased under SA, NaCl and $CdSO_4$ treatment at the shoot-root transition zone. And furthermore the GUS staining of leaves, roots and lateral roots were obviously induced under SA, NaCl and $CdSO_4$ stresses, while there were almost no GUS staining under natural condition (Fig. 6). These results indicated that OsWAK124 is involved in response to these environment stresses, and thus consistent with results that *OsWAK124* expression can enhance plants resistance to various stresses conditions.

The expression level of *OsWAK124* is correlated to its resistance to stress: The *OsWAK124* overexpression line NO 2 and RNAi transgenic line NO 6 and wildtype Zhonghua 11 were germinated and then cultivated in Kimura B for 15 days. These plants with consistent development situation were selected to cultivate in Kimura B only (Fig. 7A) or Kimura B with 100 mmol L^{-1} NaCl (Fig. 7B), 200 μmol $L^{-1}AlCl_3$ (Fig. 7C), 75 nmol $L^{-1}CdSO_4$ (Fig. 7D) and 20 μmol $L^{-1}CuSO_4$ (Fig. 7E) for 7 days, respectively. Although the *OsWAK124* overexpression and RNAi transgenic lines and wildtype Zhonghua 11 did not show too much difference under normal growth condition (Fig. 7A), the *OsWAK124* overexpression line NO 2 grew obviously better than *OsWAK124* RNAi transgenic line NO 6 treated by NaCl, $AlCl_3$,$CdSO_4$ and $CuSO_4$ (Fig. 7B, 7C, 7D, 7E). These results indicated that *OsWAK124* overexpression endowed rice plant more resistant to salt and heavy metal abiotic stresses.

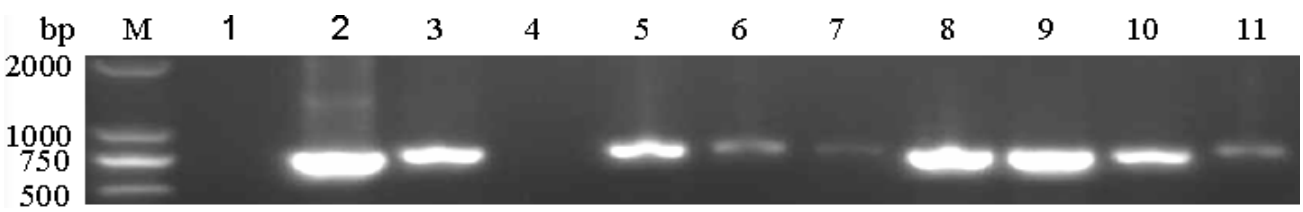

Fig. 1. The confirmation of *OsWAK124* overexpression and RNAi transgenic lines by PCR amplification of *HPT* gene
M: DL2000 marker; 1: WT (negative control); 2: Construct (positive control); 3-7: *OsWAK124* overexpression transgenic lines; 8-11: *OsWAK124* RNAi transgenic lines

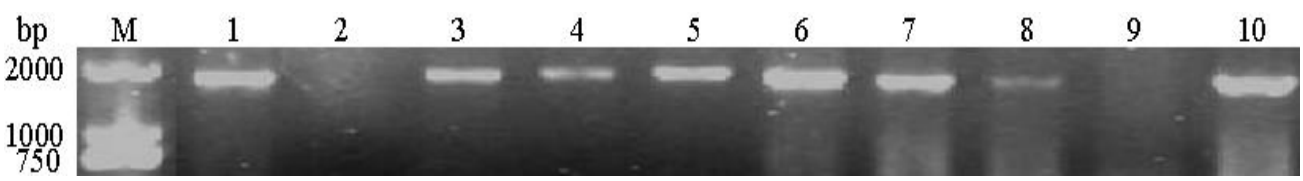

Fig. 2. The confirmation of *OsWAK124*::*GUS* transgenic lines by PCR amplification of *GUS* gene.
M: DL2000 marker; 1: Construct (Positive control); 2: WT (Negative control): 3-10: *OsWAK124 GUS* transgenic lines

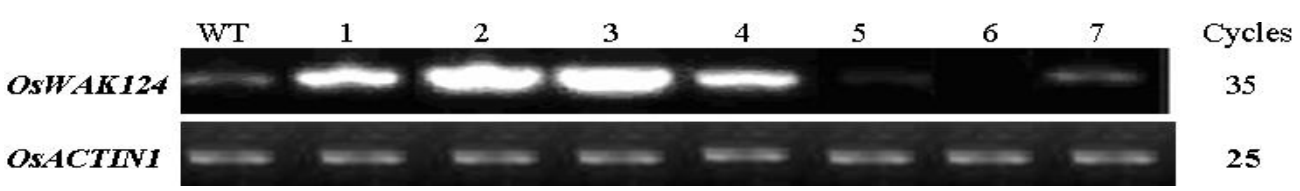

Fig. 3. *OsWAK124* expression level of *OsWAK124* overexpression and RNAi transgenic lines detected by semi-quantitative RT-PCR.
WT ; 1-4: *OsWAK124* overexpression transgenic lines; 5-7: *OsWAK124* RNAi transgenic lines

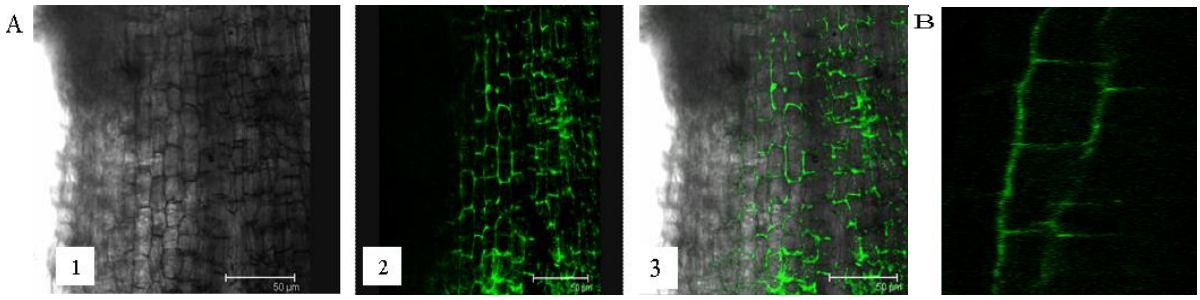

Fig. 4. The subcellular localization of OsWAK124::GFP detected by Confocal.
A. GFP signal under natural condition; B. GFP signal under plasmolysis
1. Bright field; 2. GFP signal; 3. The merged result of picture 1 and 2

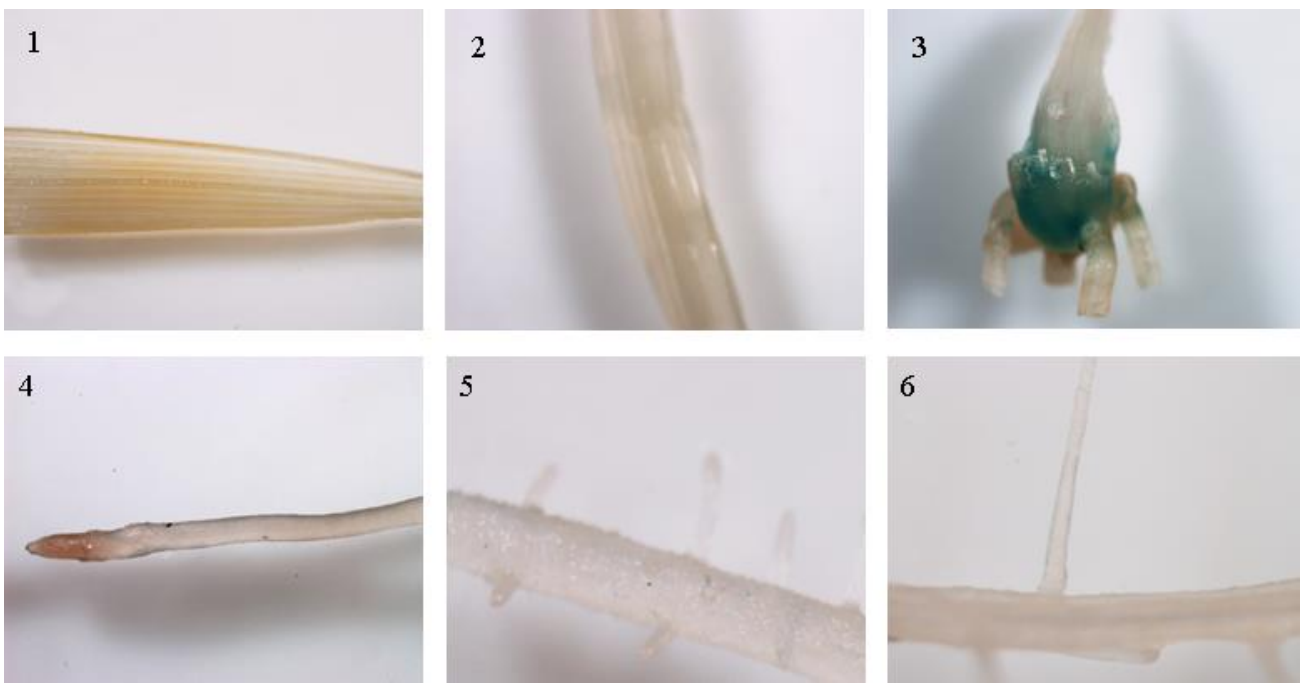

Fig. 5. The expression pattern of *OsWAK124* under normal condition
1. Leaf; 2. Shoot; 3. Shoot-root transition zone; 4. Root meristem and elongation zone; 5. Root maturation zone; 6. Root hair

Salicylic acid (SA) is thought to be an important signal molecule that mediates plant cellular signal transduction triggered by pathogen infection, so exogenous SA can mimick pathogen infection to induce pathogen resistance response in plants (He *et al.*, 1998). After culturing in Kimura B for 15 days, the seedlings of Zhonghua 11, *OsWAK124* overexpression line NO 2 and RNAi transgenic line NO 6 with consistent development situation were selected to cultivate in Kimura B with 5 μg mL^{-1}SA, and sprayed with 10 μg mL^{-1} SA added with 0.05% Tween-20 as surfactant to enhance its effect. After 7 days treatment, *OsWAK124* overexpression transgenic line NO 2 was the strongest, while *OsWAK124* RNAi transgenic line NO 6 was the weakest, and the colour of its leaves was partly changed yellowish (Fig. 7F). This experiment showed that overexpression of *OsWAK124* increased resistance of rice seedlings to pathogen attacks.

Discussion

WAKs and WAKLs are good candidates for signaling molecules across the plasma membrane of plant cells (Kohorn, 2000). So far functional studies of this gene family was mainly focused on *Arabidopsis*. Although there are one hundred and twenty five *OsWAKs* annotated in rice genomes (Zhang *et al.*, 2005), their functions are largely unknown. Here *OsWAK124* was chosen to study. The sequences of *OsWAK124* （LOC Os12g16540.1） was used as query to do blast in Michigen State University rice genome database (http://rice.plantbiology.msu.edu/). The full length of *OsWAK124* is 2521 bp, ORF 1287 bp, encoding 429 amino acids. It has 3 exons and 2 introns, and there are typical *WAK* gene domains, such as GUB-WAK-bind and 2 EGF domains (Fig. 8A). The 3 exons and 2 introns structure of *OsWAK124* gene is same as *AtWAKs*' (Verica & He, 2002). *OsWAK124* belongs to *OsWAK-RLP* subfamily. There are 13 members in this subfamily. The only difference is that this subfamily does not have transmembrane and intracellular Ser/Thr kinase (STK) domains (Fig. 8B).

Our confocal results indicated that OsWAK124 is located at the cell wall (Fig. 4); this is exactly like to its suggested name. AtWAK and AtWAKLs were identified by confocal and biochemical method that they were located at plant cell wall (He *et al.*, 1996; Wagner & Kohorn, 2001; Hou *et al.*, 2005).

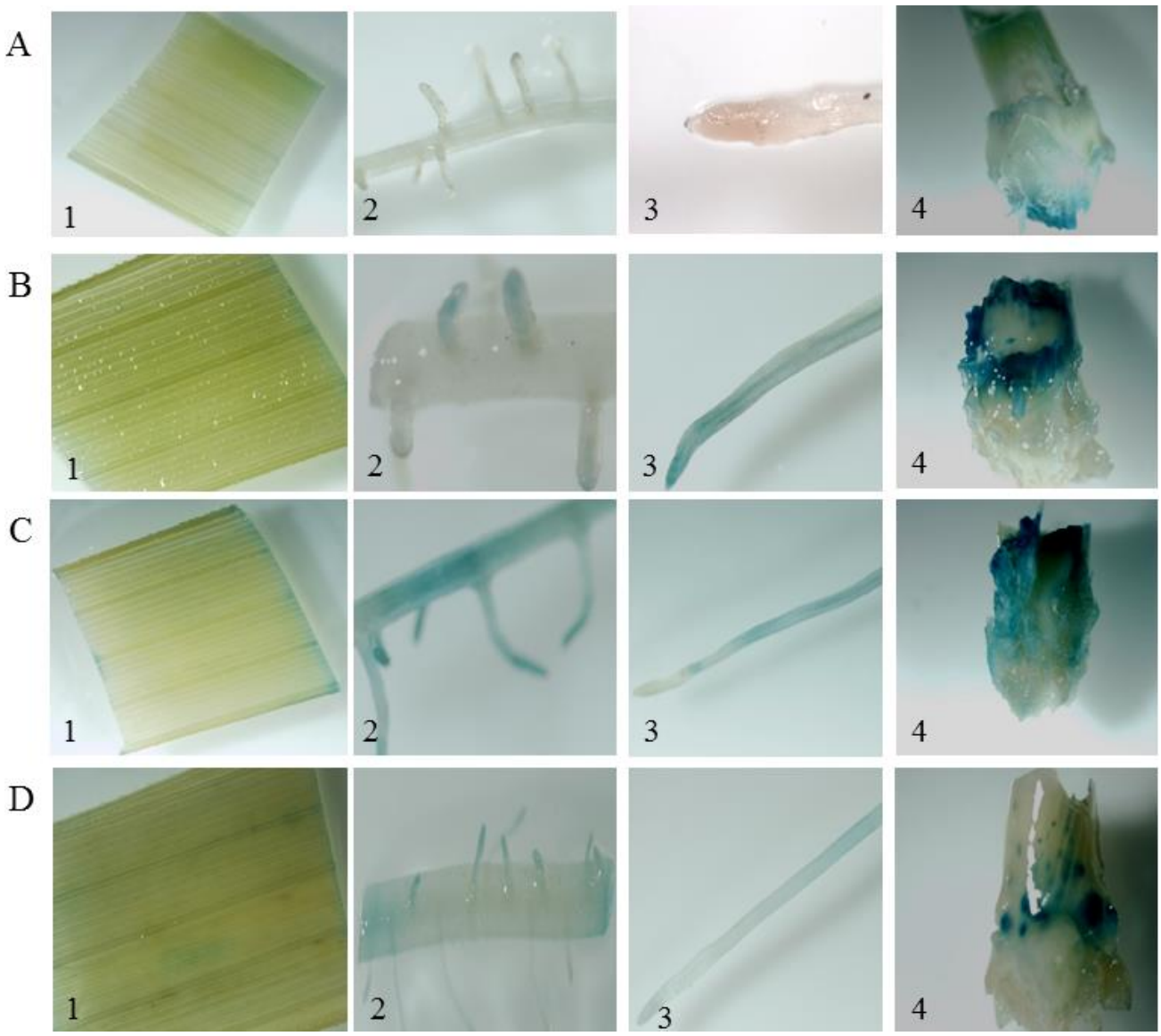

Fig. 6. The GUS staining of *OsWAK124P*::*GUS* transgenic line under different culturing conditions
A. *OsWAK124P*::*GUS* transgenic line cultured in Kimura B; B. *OsWAK124P*::*GUS* transgenic line cultured in Kimura B with 75 nmol/ L $CdSO_4$; C. *OsWAK124P*::*GUS* transgenic line cultured in Kimura B with 100 mmol/ L NaCl; D. *OsWAK124P*::*GUS* transgenic line cultured in Kimura B with 20 μmol/ L SA

Under natural condition, *OsWAK124* primarily expresses at the shoot-root transition zone, while no expression at other parts, according to GUS staining results (Fig. 6). However, when the *OsWAK124P*::*GUS* transgenic seedlings cultured in Kimura B with $CdSO_4$, NaCl and SA, respectively, *OsWAK124* expression in leaves, roots and lateral roots was obviously induced. These results are consistent with that the overexpression of *OsWAK124* gives rice more resistant to biotic and abiotic stresses (Fig. 7). The promoter region of *OsWAK124* was analyzed by PLACE software (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo *et al.*, 1999) , various biotic and abiotic responsive motifs and their repeat times found in *OsWAK124* promoter are as follows: CURECORECR (3), MYCCO NSENSUSAT (10), OSE2ROOTNODULE (4), GT1CON SENSUS (6), GT1GMSCAM4(1), P1BS(1), CCAA TBOX1 (2), DPBFCOREDCDC3 (1), ASF1MOTIF CAMV(1), SURECOREATSULTR11 (5), CBFHV (3), ABRE LATERD1 (2), ACGTATERD1 (5), BIHD1OS (3), WBOXATNPR1 (5), MYBCORE (5), MYCATRD22 (1), MYB2CONSENSUSAT (2), MYB1AT(1), MYBPLANT (1), BOXLCOREDCPAL (1), CRTDREHVCBF2 (3), MYB2AT (1). These bioinformatics analysis verified that *OsWAK124P*::*GUS* responds to biotic and abiotic stresses.

In order to investigate the functions of *OsWAK124*, the up and down regulation of *OsWAK124* expression transgenic lines were constructed through transformation of Zhonghua 11 embryonic callus by overexpression and RNAi transformation vectors. The selected overexpression, RNAi transgenic lines and wildtype Zhonghua 11 seedlings were tested by 100 mmol L^{-1} NaCl, 200 μmol L^{-1} $AlCl_3$, 75 nmol L^{-1} $CdSO_4$ and 20 μmol L^{-1} $CuSO_4$ for 7 days, respectively. Results showed that the higher the expression of *OsWAK124* is, the more resistanceto salt and heavy metal by the plant. These results are consistent with heavy metal resistant of AtWAK1 (Sivaguru *et al.*, 2003) and AtWAKL4 (Hou *et al.*, 2005).

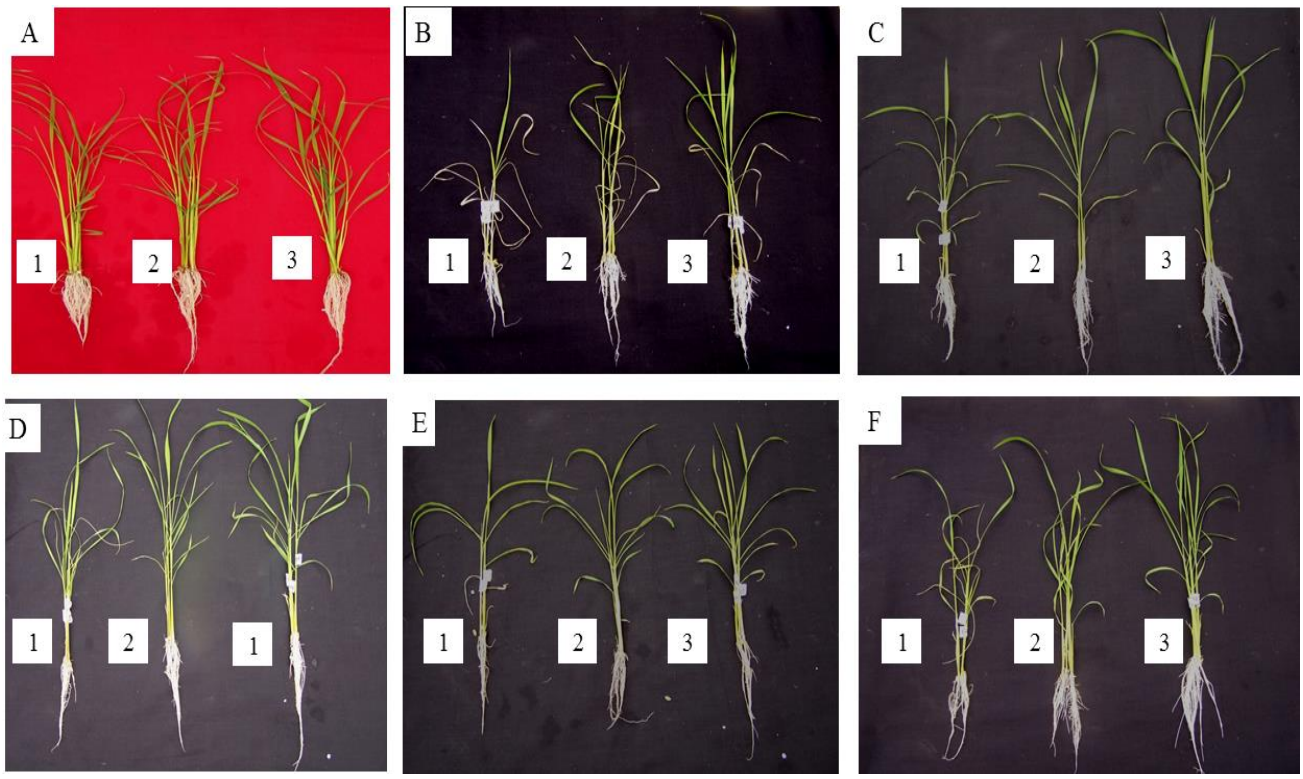

Fig. 7. The phenotypes of *OsWAK124* overexpression line NO2, RNAi transgenic line NO 6 and wildtype Zhonghua 11 under salt and heavy metal treatments.
A. Kimura B 22 days; B. Kimura B 15 days, and Kimura B with 100 mmol/L NaCl 7 days; C. Kimura B 15 days, and Kimura B with 200 μmol/L $AlCl_3$ 7 days; D. Kimura B 15 days, and Kimura B with 75nmol/L $CdSO_4$7 days; E. Kimura B 15 days, and Kimura B with 20 μmol/L $CuSO_4$7 days; F. Kimura B 15 days, and Kimura B with 50 μmol L^{-1} SA 7 days
1. *OsWAK124* RNAi transgenic line NO 6; 2. Zhonghua 11 wildtype; 3. *OsWAK124* over-expression transgenic line NO 2

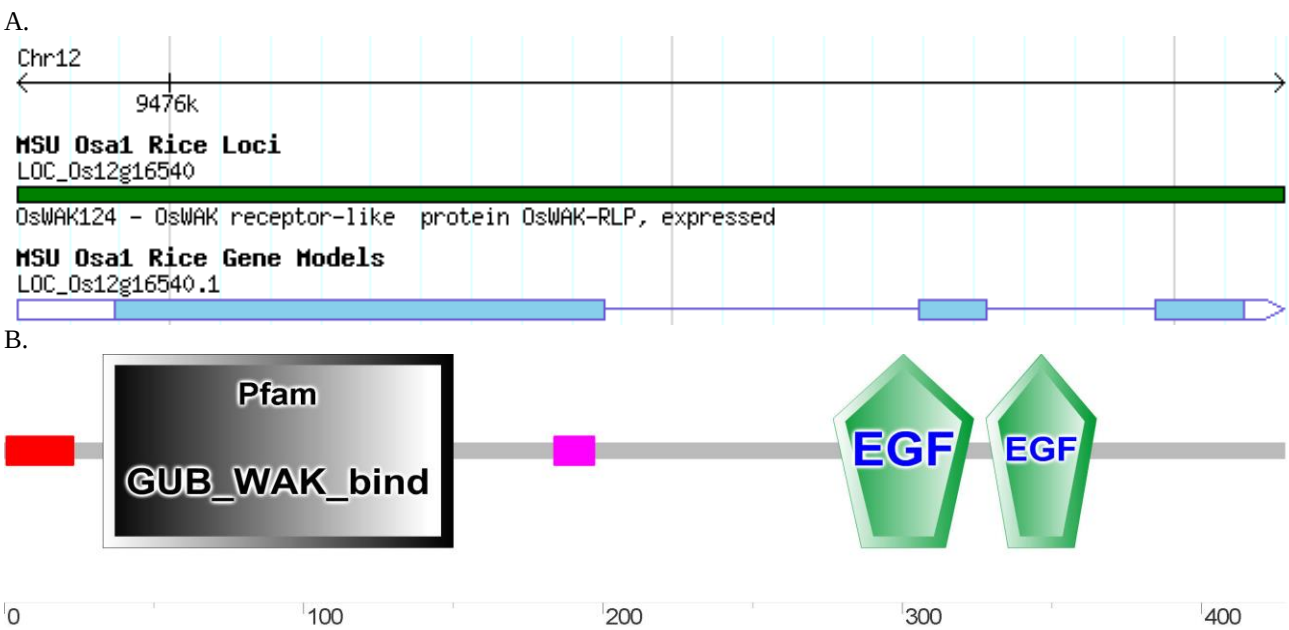

Fig. 8. The gene and protein structure of *OsWAK124*.
A. The exon-intron structure of *OsWAK124* gene; B. The functional domains of OsWAK124

SA can mediate the plant resistant to biotic stress, and mimick biotic stress to plant. AtWAK1 (He *et al.*, 1998), AtWAKL22/RFO1 (Diener & Ausubel, 2005) and OsWAK1 (Li *et al.*, 2009) etc, were reported to play a role in biotic stress resistance. Lejeune *et al.* (2006) reported a putative tomato wall-associated kinase involved in the early steps of tomato-*Orobanche ramosa* interaction. Our preliminary results here also showed that the expression of *OsWAK124* give the plants more resistance to SA treatment, indicates more resistant to biotic stress.

In summary, OsWAK124 is located at plant cell wall. Under normal condition, it primarily expresses at the

shoot-root transition zone. However its expression can be induced in roots and leaves in biotic and abiotic conditions. The overexpression of *OsWAK124* will endow the plant more resistant to salt, heavy metal and SA-mediated stresses.

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