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(71) Applicant: **THE CHINESE UNIVERSITY OF HONG KONG** [CN/CN]; Room 226 Pi Ch'iu Building, Shatin, Nt Hong Kong (CN).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SUN, Samuel, Sai Ming** [US/CN]; 10a, Staff Residence Block 16, The Chinese University Of Hong Kong, Shatin, N.t., Hong Kong (CN). **LAM, Hon-ming** [CN/CN]; Rm 702, Daisy Li Hall, New Asia College, The Chinese University Of Hong Kong, Shatin, Nt., Hong Kong (CN).

(74) Agents: **MURASHIGE, Kate, H.** et al.; Morrison & Foerster Llp, 12531 High Bluff Drive, Suite 100, San Diego, CA 92130-2040 (US).

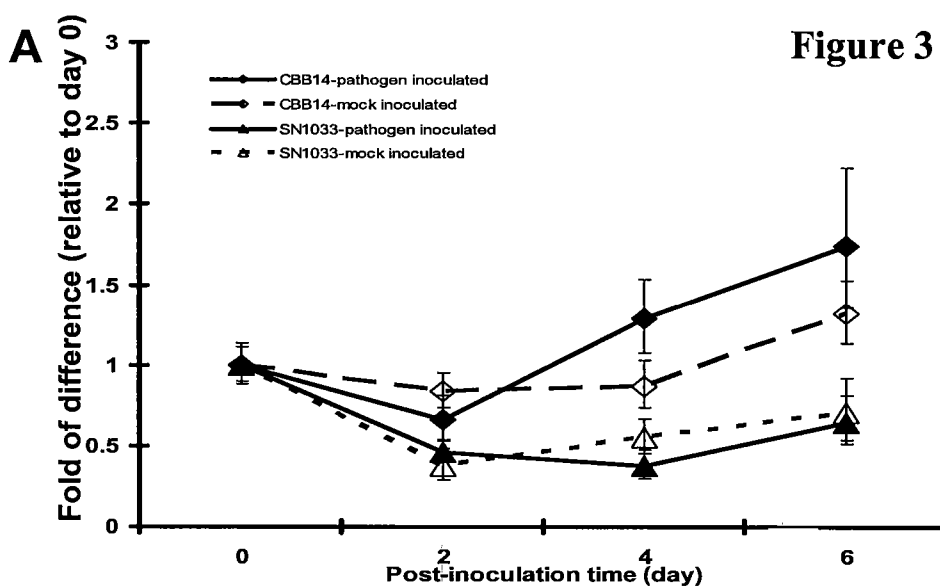
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(54) Title: METHOD TO IMPROVE PLANT RESISTANCE TO INFECTIONS



(57) Abstract: The disclosed invention relates to expression systems that effect production of a protein in plants that confers resistance to trauma. The expression systems are used to modify plants to improve their resistance to infections and wounding.

METHOD TO IMPROVE PLANT RESISTANCE TO INFECTIONS

Related Application

This application claims benefit of U.S. Serial No. 60/947,365 filed 29 June 2007 and of U.S. Serial No. 60/947,590 filed 2 July 2007. The contents of these applications are
5 incorporated herein by reference in their entirety.

Reference to Sequence Listing Submitted Via EFS-WEB

The entire content of the following electronic submission of the sequence listing via the USPTO EFS-WEB server, as authorized and set forth in MPEP § 1730 II.B.2(a)(C), is incorporated herein by reference in its entirety for all purposes. The sequence listing is
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Technical Field

The invention relates to proteins that improve the resistance of plants to infections, including infections by pathogen and wounding. The invention also concerns methods to improve the resistance of plants to infections by effecting expression of the genes encoding
15 these proteins.

Background Art

Preformed and induced defense mechanisms provide a wide spectrum of resistance toward numerous pathogens encountered by the plant host. Pathogen specific defense responses are usually initiated by the recognition of a pathogen avirulent (Avr) protein by
20 the corresponding resistance (R) protein of the host. Ultimately, the plant host will produce a series of defense molecules (including pathogenesis-related proteins) to restrict or kill the pathogens. The processes between the initiation of resistance and the production of resistance proteins involve a complex signal transduction network which is yet to be fully elucidated.

25 In *Arabidopsis thaliana*, many important hubs of the defense signaling network have been identified by molecular genetic approaches, including EDS1 (Enhanced Disease

Susceptibility 1), NPR1 (Non-Expresser of PR Genes 1) and NDR1 (Non Race-Specific Disease Resistance 1). Using similar tactics and together with biochemical studies, the involvement of phytohormone signals in defense responses has been corroborated in *A. thaliana*, especially the roles of salicylic acid (SA), and the other phytohormones such as jasmonic acid (JA) and ethylene (ET).

Many known signaling strategies are employed in plant defense responses. For instance, some R proteins are receptor kinases while other protein kinases also play significant roles. Biochemical signals such as calcium flux and oxidative burst are also important. Furthermore, there are several reports on the participation of other signaling components such as G-proteins and RING (Really Interesting New Gene) zinc finger proteins.

RING zinc finger proteins are a group of diverse proteins with highly conserved zinc binding domains. Based on the type of cysteine (C) and histidine (H) residue combination, the RING zinc finger domain can be classified into canonical and modified RING zinc fingers. The canonical RING zinc finger can be further grouped into two subclasses: HC subclass (consensus: C-X₂-C-X₉₋₃₉-C-X₁₋₃-H-X₂₋₃-C-X₂-C-X₄₋₄₈-C-X₂-C) (SEQ ID NO:1) and H2 subclass (consensus: C-X₂-C-X₉₋₃₉-C-X₁₋₃-H-X₂₋₃-H-X₂-C-X₄₋₄₈-C-X₂-C) (SEQ ID NO:2) (Stone, S. L., *et al.*, *Plant Physiology* (2005) 137:13-30). Modified RING zinc fingers include RING-C2, RING-v, RING-D, RING-S/T and RING-G.

Many members of the RING zinc finger protein family (including both HC and H2 subclasses) are E3 ubiquitin ligases. Different subclasses of the RING zinc finger domain determine specificity toward different E2 ubiquitin conjugating enzymes. Other RING zinc finger proteins can bind to nucleic acids or interact with other protein targets. Besides the ubiquitin mediated degradation pathway, RING zinc finger proteins also play important roles in organelle transport and transcription/translation regulations.

In rice, more than 30 resistance loci (*Xa* loci) against the pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) have been identified and 6 *Xa* genes were cloned mainly by map-based cloning approaches. Several pathogenesis-related (*PR*) genes have been reported to contribute directly to the resistance mechanism. However, only a few key components of the signal transduction pathway from the onset of R protein-Avr protein interaction to the actual resistance development have been studied. To obtain new signal transduction components related to *Xoo* resistance in rice, cDNA clones differentially expressed in rice lines harboring *Xa* loci were searched for.

The present inventors have cloned and characterized a novel RING zinc finger protein gene (*OsRHC1*) from rice. *OsRHC1* is differentially expressed under wounding in near isogenic lines containing the *Xa14* or *Xa23* resistance loci, but not in the corresponding susceptible recurrent parents. Ectopic expression of *OsRHC1* in transgenic *A. thaliana* enhances its resistance toward bacterial pathogens and such protective function depends on the action of the 26S proteasome.

Disclosure of the Invention

A variety of genes encoding infection resistance proteins is known in plants, and various transgenic plants modified to produce them have been used in attempts to confer resistance to infections. However, these resistance proteins appear to have a limited spectrum of activity with respect to the types of pathogens that they will successfully recognize. Many cause negative side effects (such as programmed cell death) as well. The present invention provides materials that can be used to confer resistance to infections on a wide variety of plants, without apparent negative side effects. The invention provides recombinant materials for the production of a protein designated OsRHC1 which is a RING zinc finger protein that confers resistance to infections of a broad spectrum of pathogens. Because the protein of the invention which is derived from a monocot (rice) is also effective in dicots (*Arabidopsis*) it is applicable to a broad spectrum of plants as well.

In one aspect, the invention is directed to expression systems that produce the OsRHC1 protein and proteins closely related thereto that are RING zinc finger proteins and are able to improve resistance of plants to infections. Transgenic plants modified with the expression systems of the invention have enhanced ability to resist infections either from pathogenic organisms or by wounding.

Thus, in another aspect, the invention is directed to plant cells or plants that have been modified to contain an expression system that produces this RING zinc finger protein. The plants may either be heterologous from the origin of OsRHC1 or may be rice plants modified to overexpress this protein.

In still another aspect, the protein produced by this expression system may be used to conduct screening assays to identify compounds or combinations of compounds that modulate resistance to infections in plants.

The invention also relates to antibodies that are immunospecific for the OsRHC1 protein. These antibodies are useful for detecting and purifying this protein.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence-encoding region of the *OsRHC1* gene and the amino acid sequence of the OsRHC1 protein (SEQ ID NOS:42-43).

Figure 2A shows the full-length amino acid sequence of OsRHC1 (SEQ ID NO:43) aligned to seven annotated proteins (SEQ ID NOS:44-50) exhibiting high degree of similarity. Figure 2B shows membrane bound and soluble protein fractions extracted from CBB23 and JG30 followed by Western blot analysis using anti-OsRHC1 antibodies.

Figures 3A and 3B are graphs showing expression of *OsRHC1* in bacterial blight resistant lines CBB14 and CBB23 (carrying the *Xa14* locus and *Xa23* locus, respectively) and their susceptible recurrent parents (SN1033 and JG30, respectively).

Figure 4A is a graph showing wounding-induced expression of *OsRHC1* by real-time PCR. Figure 4B shows a Western blot of the corresponding protein.

Figures 5A-C show pathogen inoculation tests of transgenic *A. thaliana* expressing *OsRHC1*. The expression of the transgene *OsRHC1* in the transgenic lines was confirmed by Northern blot analysis in Figure 5A. The disease symptoms were visible as shown in Figure 5B and the rosette leaves (not at the site of infection) were harvested to estimate the titer of pathogens shown in Figure 5C.

Figures 6A and 6B show expression of defense marker genes in transgenic *Arabidopsis thaliana* without (A) and with (B) *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) inoculation.

Figures 7A-D are graphs showing expression of defense marker genes (*PR1* (A), *PR2* (B), *PDF1.2* (C) and *Thi2.1* (D)) when treated with MG132 (a 26S proteasome inhibitor).

Figures 8A and 8B show the results of pathogen inoculation test of *OsRHC1* transgenic *A. thaliana* in the *npr1-3* background. Figure 8A shows the expression of the *OsRHC1* gene and Figure 8B shows the expression of four defense marker genes.

Figure 9 shows the results of PCR screening of the *OsRHC1* transgene in transgenic rice lines.

Figure 10A shows the expression of *OsRHC1* by real-time PCR and Figure 10B shows production of the corresponding protein in transgenic rice lines.

Figures 11A-C show expression of defense marker genes *PR1* (A), *PBZ1* (B) and *GRCWP* (C) in *OsRHC1* transgenic rice lines.

Figure 12 shows the results of autoubiquitination assay conducted on the RING-HC-C-terminal portion of OsRHC1.

Figure 13 shows the DNA sequence (SEQ ID NO:51) and deduced amino acid sequence (SEQ ID NO:52) of a binding partner for OsRHC1.

Modes of Carrying Out the Invention

A protein designated rice RING-HC subclass protein-1 (OsRHC1) is a 409-amino acid protein overexpressed in rice in response to pathogen or wound-induced infections. This protein and its variants, which share at least 90%, preferably 95%, more preferably 98% or 99% sequence identity over the entire length of this 409-amino acid sequence (shown in Figure 1) are able to confer resistance to the negative effects of infection to a wide variety of plants when said plants are modified to produce these proteins (collectively referred to as OsRHC1 proteins). The present invention provides expression systems that can be used to modify a wide variety of plants, both monocots and dicots, to enhance their ability to resist infections. The generic capability of such expression systems to confer resistance is confirmed in the examples hereinbelow which demonstrate that the protein, which has its origin in the monocot, rice, is able to confer these properties on the dicot *A. thaliana*.

The techniques for constructing expression vectors operable in plants, for modifying plant cells, for regenerating plant cells into intact plants and recombinant manipulation of plants in general are by this time well known. A summary of such techniques is found, for example, in U.S. patent 7,109,033 which is incorporated herein by reference for its disclosure of these techniques.

As noted in this patent, promoters useful in plant expression may be constitutive, inducible and/or tissue-specific. Transformation techniques include use of *Agrobacterium*, lipofection, electroporation, and the like. Techniques for regeneration of plants from transformed plant cells are also well established.

Accordingly, once the nucleotide sequence encoding the OsRHC1 protein is available, methods of preparing transgenic plants that produce these proteins are well within the ordinary skill of the art. The nucleotide sequence natively producing this protein has been deposited in GenBank with Accession No. EF584506 and synthetic alternatives having variations in codon usage are possible.

Thus, according to the invention, a suitable expression system is constructed for operability in plants wherein the nucleotide sequence encoding the proteins of the invention is operably linked to suitable control sequences operable in plants. This expression system is used to modify plant cells or plants so that the protein is produced either ubiquitously in

plant tissues or in specialized desired locations in the plant, depending on the choice of control system and method of transformation. The resulting plants, whether monocots or dicots, are then permitted to produce the protein in response to pathogen or wound-induced infection so as to enhance their ability to resist damage caused by these infectious events.

5 As shown below, OsRHC1 is an E3 ubiquitin ligase which enhances the destruction of unwanted proteins by directing them to the proteasome. This property is shared in common with other RING proteins, and represents one aspect of its protective function. This protein is the first E3 that harbors transmembrane domains at the N-terminal region and RING-HC at the C-terminal cytoplasmic tail that has been found to be involved in plant
10 disease resistance.

 In addition, the protein itself, produced in sufficient quantity and isolated and purified to a suitable extent (at least 50% pure by weight, preferably 75% pure, more preferably 90% or 95% pure) can be used as a screening tool. Compounds or combinations of compounds that are able to bind the protein are candidates for modulating the ability of
15 plants to resist infection. Compounds or combinations of compounds that, by binding the protein, are able to agonize its activity will enhance the infection-resisting capability of plants that are able to produce this protein.

 Thus, the binding assay may be used as a preliminary screen. As it has been shown that OsRHC1 is an E3 ubiquitin ligase, the assay for ubiquitin ligase activity described
20 below in Example 9, or a similar assay for such activity is used to demonstrate the agonist activity of a candidate compound. Thus, a suitable candidate will enhance the ability of OsRHC1 to effect ubiquitination.

 Also useful for purifying the proteins of the invention and for detecting them are antibodies that are specifically immunoreactive with said proteins. The term "antibodies" is
25 understood to mean complete antibodies, polyclonal or monoclonal, as well as the immunospecific fragments thereof such as Fab fragments, as well as recombinantly produced forms such as single-chain F_v antibodies. Thus, the term "antibodies" refers both to any form of the antibody and to any portion thereof that retains its immunospecific characteristics. Such antibodies can be used, for example, on affinity columns, etc., for
30 purification.

 In the examples below, the nucleotide sequence encoding the OsRHC1 protein shown in Figure 1 has been retrieved from rice and deposited. Further, it was demonstrated that the OsRHC1 expression, both at an mRNA level and at a protein level, could be

induced in a line of rice that exhibits resistance in response to a pathogen and in response to wounding.

Transgenic *A. thaliana* plants were obtained using an expression construct for the OsRHC1 protein, and these transgenic plants were shown to have enhanced expression of four defense marker genes, both under regular growth conditions and when salicylic acid or jasmonic acid was added. The *OsRHC1* transgenic *A. thaliana* also showed constitutive expression of the OsRHC1-encoding DNA and was protected by this expression when challenged with a *Pseudomonas*. Similarly, overexpression of this DNA in rice resulted in expression of several defense marker genes.

The following examples are offered to illustrate but not to limit the invention.

Example 1

Identification and Cloning of OsRHC1-Encoding cDNA

One partial cDNA clone was obtained via suppression subtractive hybridization techniques with the PCR-select cDNA subtraction kit (Clontech 637401), using total RNA extracted from six to eight-week-old CBB14 which is bacterial blight resistant (tester) and SN1033 the susceptible parent of CBB14 (driver) rice lines collected four days after pathogen (*Xoo* race LN44) inoculation. Inoculation was performed by clipping method described in Zhang, Q., *et al.*, *Acta Agr. Sin.* (1996) 22:135-141. Using the DNA sequence information of this partial clone, 5'-Rapid-Amplification of cDNA Ends (5'-RACE) experiment and subsequent PCR amplifications using specific primers were performed. Gene specific primers 5'-TTCTCCATGTTTCGGTAAACCTTTC-3' (SEQ ID NO:3), 5'-TAAAGTTGTGAT TGAGACTACATGG-3' (SEQ ID NO:4) and 5'-ACATTGCACAACCAACATGTAC-3' (SEQ ID NO:5) were employed in the 5'RACE reactions. To amplify the full length coding region, PCR using the primer pair 5'-CCTCACTTTTGTCTCCAC-3' (SEQ ID NO:6) and 5'-CGACATTGCACAACCAAC-3' (SEQ ID NO:7) were performed. All clones were stored in the plasmid vector pBluescript® KSII(+) (Stratagene) and propagated in the *E. coli* strain DH5α.

The resulting cDNA clone (GenBank accession number EF584506) encodes an intact open reading frame of 409 amino acid residues (Fig. 1). EF584506 is 99% identical to a directly deposited rice cDNA clone (accession number: NM_001057564). The corresponding gene in the rice genome appears to be a single copy gene located on chromosome 3. BlastP search showed that the protein encoded by our clone exhibits 99%

identity to a rice clone annotated as a zinc finger family protein (accession number: ABF98464), but missing 64 amino acid residues at the N-terminus. Further analysis using the conserved domain database (CDD) revealed that the predicted protein harbors a RING zinc finger domain. The pattern of the conserved cysteine and histidine residues in the RING zinc finger domain exhibited a signature for the RING-HC subclass. The clone was designated as *OsRHC1* accordingly.

The predicted amino acid sequence of the *OsRHC1* protein was compared with two RING zinc finger proteins, EL5 (RING-H2 subclass) and XB3 (RING-HC subclass) from rice that are involved in disease resistance. No significant homology was found except at the RING zinc finger domain (data not shown). The RING zinc finger domain of *OsRHC1* is located at the C-terminus (Fig. 2A) while such domain in EL5 and XB3 is located in the middle portion or close to the C-terminus of the protein, respectively. Prediction by the TopPred and the iPSORT programs suggested that *OsRHC1* may possess multiple transmembrane domains (Fig. 2A) while EL5 only has one and XB3 does not possess any transmembrane region with high certainty (data not shown).

BlastP analysis revealed that *OsRHC1* shares high amino acid sequence homology to seven other annotated proteins deposited in GenBank from various plant species (Fig. 2A). These proteins exhibit greater than 50% identity (spanning full length) to *OsRHC1*, with multiple transmembrane domains at the N terminal half, and a RING-HC domain at the C-terminus. The consensus of the RING-HC domain for this group of proteins is Cys-X₂-Cys-X₁₁-Cys-X-His-X₃-Cys-X₂-Cys-X₆-Cys-X₂-Cys (SEQ ID NO:8). There is apparently no published information on the functions of these homologues.

Example 2

Demonstration That *OsRHC1* Is Membrane Bound

To verify that the *OsRHC1* is membrane bound as depicted by bioinformatics tools, membrane-bound and soluble proteins were separated using a fractionation protocol (modified from Jiang and Rogers, *J. Cell Biol.* (1998) 143:1183-1199). For Western blot analysis, the proteins were electrophoretically separated on a polyacrylamide gel (4% stacking; 10% resolving) before transferred to an activated PVDF membrane (pre-treated in absolute methanol for 20 minutes followed by protein transfer buffer for 15 minutes) using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad 170-3949). The blocking and detection steps were performed according to the manufacturer's manual (Western Breeze™ Immunodetection Kit, InvitroGen WB7106). Primary antibodies

(polyclonal) targeting the OsRHC1 protein was raised by a commercial service (InvitroGen, Custom antibody) via injecting a synthetic peptide ('N'-CGYPPEVVRKMPKRD-'C') (SEQ ID NO:9) into rabbits and antibodies were purified using affinity column before use. Anti-rabbit secondary antibody conjugated to an alkaline phosphatase (provided in Western Breeze™ Immunodetection Kit, InvitroGen WB7106) was used to recognize the primary antibodies. Western blot analysis confirmed that the OsRHC1 protein was tightly associated to membranes (Fig. 2B).

Example 3

OsRHC1 is Wound-Inducible in the Rice Lines CBB14 and CBB23

To study the expression pattern of *OsRHC1*, real-time PCR analyses was performed using reverse-transcribed RNA samples from two near isogenic pairs (CBB14 containing *Xa14* and its susceptible recurrent parent SN1033; CBB23 a resistant line containing *Xa23* and its susceptible recurrent parent JG30). Rice lines were grown on regular field soil in a green house (temperature 24-28°C; RH 70-80 %; under natural light). Inoculation of the *Xoo* races LN44 and P6 was performed by clipping method described in Zhang, Q., *et al.*, *supra* (1996). Mock inoculation and wounding treatment followed the same procedure except that the pathogen was replaced by water. For the time-course experiments, samples were collected at 0, 2, 4, 6 days at around the same time of the day (between 8-10 am). Day 0 sample was collected before treatment.

For evaluating expression of *OsRHC1* via real-time PCR, total RNA was extracted by the phenol extraction method of Ausubel, *et al.*, Current Protocols in Molecular Biology (1995) J. Wiley & Sons, New York. The cDNA samples were generated by reverse transcription (18-mer oligo-dT; SUPERScript™ II RNaseH (InvitroGen 18064-071)) of DNase I (InvitroGen 18068-015)-treated RNA samples.

Real-time PCR amplification of cDNA was conducted using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in 96-wells PCR plate with dome cap. Reaction was carried out in a 20 µl reaction volume containing 10 µl SYBR Green PCR Master Mix (Applied Biosystems 4309155) with 0.3 µM each of the forward and reverse primers. *OsRHC1* primers for real-time PCR were 5'-AAAGAAGAGCAAGCCCCGG TTAT-3' (SEQ ID NO:10) and 5'-GCCTCCATACCTCTTCTGCAA-3' (SEQ ID NO:11). All reactions were set independently for at least four times and at least three sets of consistent data were used for analysis. The expression level of actin (*O. sativa OsAc1D*; accession number: X15865) with the primer set

5'-CTTCATAGGAATGGAAGCTGCGGGTA-3' (SEQ ID NO:12) and 5'-GACCACCTTGATCTTCATG CTGCTA-3' (SEQ ID NO:13) was used to normalize the results. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen, *Methods* (2001) 25:402-408.

5 To validate the reliability data, amplification efficiencies between the target genes and the housekeeping genes of all the real-time PCR reactions were compared, and dissociation curves of all PCR products were examined to ensure the quality of PCR. At least two independent batches of plant samples were used and gene expression patterns were consistently observed. All PCR products were sequenced at least one time to verify that the
10 right targets were being quantified.

When an incompatible *Xoo* strain (LN44 for *Xa14* and P6 for *Xa23*) was inoculated, the rice lines containing *Xa14* or *Xa23* exhibited an induction of *OsRHC1* gene expression while the susceptible recurrent parents were non-responsive as shown in Figures 3A and 3B, respectively. However, such induction was also observed in mock inoculated samples
15 which had been wounded, suggesting that *OsRHC1* could be wounding-inducible. The amplitude of induction was much stronger in the case of CBB23 which harbors the *Xa23* locus that confers broad spectrum resistance.

The effect of wounding on *OsRHC1* expression in CBB23 line and its susceptible recurrent parent JG30 was further analyzed. Both RNA and protein samples were collected
20 after wounding by leaf clipping. CBB23 and JG30 rice lines (eight-week-old plants) were wounded by clipping. Day 0 leaf samples were collected before wounding. Leaf tissues about 6-8 mm away from the wounding site were collected at 2, 4, and 6 days after clipping. Total RNA and membrane bound protein samples were prepared in parallel. Real-time PCR experiments were performed as described above. Western blot analysis was performed
25 using the anti-OsRHC1 antibody as described in Example 2.

The induction peak of *OsRHC1* gene expression appeared on Day 4 after treatment in CBB23 (Fig. 4A). Western blot analysis of membrane-bound proteins showed that the production of the OsRHC1 protein in CBB23 was greatly enhanced on Day 6 (Fig. 4B), after the induction of gene expression on Day 4. The response in JG30 was not obvious
30 when compared to CBB23, indicating that the presence of the *Xa23* locus may play a role in the wounding induction of *OsRHC1*.

Example 4

Production of Transgenic *Arabidopsis* Lines

To test whether OsRHC1 could mediate resistance in dicots, an *A. thaliana* was modified to produce OsRHC1 protein and challenged with *Pseudomonas syringae* pv
5 *tomato* DC3000. *OsRHC1* cDNA was inserted into a binary vector and the transgene expression was driven by the Cauliflower Mosaic Virus 35S promoter. *Agrobacterium*-mediated transformation of the wildtype Col-0 *A. thaliana* line was performed using a vacuum infiltration method (Bechtold, N., *et al.*, *Methods Mol. Biol.* (1998) 82:259-266). Transgenic plants with single insertion locus were screened by kanamycin resistance
10 phenotype (encoded by the selection marker gene in the binary vector) of offspring. A 3:1 (resistant:sensitive) ratio verified by Chi-Square test in the T1 generation suggested a single insertion event.

Only positive transformants containing a single insertion locus were propagated to obtain homozygous lines for further experiments. The transgene expression in three
15 independent homozygous transgenic lines was examined by Northern blot analysis. As shown in Figure 5A, three transformed lines, B-1-4, G-1-5, and H-2-9, showed high levels of production of mRNA. However, the Col-0 line, the wildtype, showed no production of the mRNA.

A. thaliana was grown in a growth chamber (temperature 22-24°C; RH 70-80%;
20 light intensity 80-120 μ E of a 16 h light-8 h dark cycle). The preparation of the *Pst* DC3000 culture, inoculation (by a dipping method), and subsequent titering were performed as previously described (modified from Kim, H. S., *et al.*, *Plant Cell* (2002) 14:1469-1482; Uknes, S., *et al.*, *Plant Cell* (1992) 4:645-656). Six-week-old seedlings were challenged with *Pst* DC3000 in a concentration of 10^8 colony forming unit/ml in 10 mM MgSO_4
25 supplemented with 0.02% (v/v) Silwet L-77 (Pieterse, C.M. J., *et al.*, *Plant Cell* (1998) 10:1571-1580; Ton, J., *et al.*, *Mol. Plant-Microbe Interact.* (2002) 15:27-34).

Pst DC3000 inoculation caused severe yellowing and necrosis in infected Col-0 and transgenic plants transformed with the empty vector V7, while the disease symptoms were much reduced in all *OsRHC1* transgenic lines as shown in Figure 5B.

30 The titers of pathogen inside the rosette leaves were consistent with the observed phenotypes (Fig. 5C). Furthermore, the H-2-9 line that exhibited the highest level of transgene expression also gave the lowest pathogen titer (comparing Figs. 5A and 5C).

Example 5Expression of OsRHC1 Enhances the Expression of Defense Marker Genes in Transgenic *Arabidopsis thaliana*

The expression of four defense marker genes, *PR1*, *PR2*, *PDF1.2* and *Thi2.1*, was
 5 tested in transgenic *A. thaliana*. In *A. thaliana*, these genes are indicators of defense pathways mediated by different phytohormones including SA, JA, and ET.

Leaf tissues of six-week-old *A. thaliana* transgenic lines (B-1-4, G-1-5 and H-2-9) expressing *OsRHC1* and the untransformed wild-type (Col-0) were harvested to prepare total RNA, followed by real-time PCR as described in Example 3. The primers used are as
 10 follows:

PR1: 5'-TCAAGATAGCCCACAAGATTATC-3' (SEQ ID NO:14) and
 5'-CTTCTCGTTCACATAATTCCCAC-3' (SEQ ID NO:15);

PR2: 5'-ACCACCACTGATACGTCTCCTC-3' (SEQ ID NO:16) and
 5'-AACTTCATACTTAGACTGTCGATC-3' (SEQ ID NO:17);

15 *PDF1.2*: 5'-CCCTTATCTTCGCTGCTCTTGT-3' (SEQ ID NO:18) and
 5'-CCCTGACCATGTCCCACTTG-3' (SEQ ID NO:19);

Thi2.1: 5'-AGCACTGCAAGTTAGGGTGTGA-3' (SEQ ID NO:20) and
 5'-ACATTGTTCCGACGCTCCAT-3' (SEQ ID NO:21).

The tubulin (*A. thaliana* β -tubulin 4, accession number: M21415) with the primer set
 20 5'-GAAGGTGCTGAGTTGATTG-3' (SEQ ID NO:22) and
 5'-GGACTTGACGTTGTTTGG-3' (SEQ ID NO:23) was used to normalize the results.

The expressions of *PR1* (solid), *PR2* (open), *PDF1.2* (hatched), and *Thi2.1* (dotted) in each transgenic line as shown in Figure 6A were compared to those of Col-0 (expression level set to 1).

25 In six-week-old seedlings under regular growth conditions, all four defense marker genes exhibited enhanced expression when compared to the wild type Col-0 (Fig. 6A). The fold of induction was particularly higher for the *PR1* and the *PDF1.2* genes which are mediated by two different signaling pathways. Among three independent transgenic lines tested, the H-2-9 line that showed highest expression of the transgene and best protection in
 30 the pathogen inoculation experiment also gave the highest fold of induction of *PR1* and *PDF1.2* (comparing Figs. 5 and 6A).

When the plants were subjected to the challenge of *Pst* DC3000, the levels of *PR1* and *PR2* transcripts in Col-0 increased (data not shown) but the expression levels of these genes were even higher in transgenic lines (Fig. 6B). While the level of *Thi2.1* in Col-0 did

not alter significantly by the pathogen inoculation (data not shown), its expression was elevated in the transgenic lines (Fig. 6B). The expression of *PDF1.2*, on the other hand, was repressed by *Pst* DC3000 inoculation in both Col-0 and transgenic lines (Fig. 6B).

To show the relationship between the function of OsRHC1 and ubiquitin-mediated protein degradation, the effects of MG132 (a 26S proteasome inhibitor) on the expression of defense marker genes were studied in the transgenic lines. Four-week-old seedlings were subjected to MG132 treatment. The 26S proteasome inhibitor (MG132) was applied to the transgenic *A. thaliana* using a protocol modified from previous reports (Abas, L., *et al.*, *Nature Cell Biol.* (2006) 8:249-256; Dong, C. H., *et al.*, *Proc. Nat'l Acad. Sci. USA* (2006) 103:8281-8286; Guo, H., *et al.*, *Cell* (2003) 115:667-677; Oñate-Sánchez, L., *et al.*, *Plant Physiol.* (2002) 128:1313-1322). In brief, 50 mg/L MG132 dissolved in 1% (v/v) DMSO supplemented with 0.01% (v/v) Silwet L-77 were poured onto MS square plates to cover the roots but not the aerial tissues of the seedlings. Mock treatment was performed with the same procedures except that no MG132 was added. After four hours, the seedlings were harvested for RNA extraction followed by real-time PCR.

Expression of the transgene was not affected by such treatment (data not shown). In Col-0, no significant effects of MG132 on the expression of defense marker genes were observed. On the other hand, the induction effects of overexpressing *OsRHC1* on the four defense marker genes were diminished under MG132 treatment, as shown in Figures 7A-7D for *PR1*, *PR2*, *PDF1.2*, and *Thi2.1*, respectively, where open bars represent mock treatment and solid bars represent MG132 treatment

In summary, it appears that the ability of the OsRHC1 protein to enhance the expression of the defense marker genes was, in all cases, inhibited by an inhibitor of the 26S proteasome. It thus appears that the ability of the invention protein to enhance expression of the defense marker genes may be dependent on the 26S proteasomal activity.

Example 6

The Protective Function of the *OsRHC1* Clone in Transgenic *Arabidopsis thaliana* is Dependent on the Function of NPR1

The function of OsRHC1 in relation to a known hub in the defense signaling network was positioned using the model plant system. NPR1 which mediates both SA and JA/ET signals and plays a central role in defense signaling in *A. thaliana*. *OsRHC1* was transformed as described above into the *npr1-3* *A. thaliana* mutant that is depleted of NPR1. Independent transformants with a single insertion locus were selected. At the time of

inoculation, the expression of transgene (under the control of the Cauliflower Mosaic Virus 35S promoter) in individual lines was examined with real-time PCR as described in Example 3. The steady-state level of *OsRHC1* in an *npr1-3* background was found to be comparable to that in the transgenic lines with a Col-0 background (data not shown).

5 Eight-week-old transgenic lines (D-2, E-1, F-1, G-1 and G-2) expressing *OsRHC1*, the untransformed *npr1-3* mutant, and the wild-type Col-0 were challenged by *Pst* DC3000 and the subsequent estimation of pathogen titer was obtained as shown in Figure 8A. Expression of defense marker genes was determined as described in Example 5. The expressions of *PR1* (solid), *PR2* (open), *PDF1.2* (hatched), and *Thi2.1* (dotted) in each line
10 was compared to those of Col-0 (expression level set to 1), as shown in Figure 8B. No significant increase in the expression of four selected defense marker genes was found in any of these transgenic lines.

 When the *npr1-3* transgenic lines were subjected to the challenge of *Pst* DC3000, no protection effects could be observed in the transgenic lines. Both the disease symptom
15 development (data not shown) and pathogen titer of these transgenic lines resembled that of the untransformed *npr1-3* mutant. Thus, protection appears to require NPR1.

Example 7

Construction of *OsRHC1* Transgenic Rice

 The nucleotide sequence of Figure 1 encoding *OsRHC1* was subcloned into a double
20 T-DNA binary vector, pSB130 (from Dr. Liu Qiaoquan and Prof. Samuel Sun at the Chinese University of Hong Kong). The vector pSB130 carries two T-DNA. One T-DNA harbors the hygromycin resistance gene (selectable marker) and the other possesses a multiple cloning site downstream from a maize ubiquitin promoter for cloning of target genes. The recombinant construct was transformed into the *Agrobacterium EHA105* for rice
25 transformation, and transgenic rice lines were constructed.

 Figure 9 shows PCR screening of the *OsRHC1* transgenes in T2 transgenic rice lines (parent: Aichi Asahi). The forward and reverse primers for PCR are from the maize ubiquitin promoter and the *OsRHC1* coding region respectively as follows:

 Forward primer: 5'-CTGATGCATATACATGATGG-3' (SEQ ID NO:24);

30 Reverse primer: 5'-ACATTGCACAACCAACATGTAC-3' (SEQ ID NO:25).

 A total of five *OsRHC1* transgenic rice lines were obtained.

Example 8Over-Expression of *OsRHC1* and Defense Marker Genes in Rice

The expression of the *OsRHC1* and three rice defense marker genes (*PR1*, glycine rich cell wall protein encoding gene (*GRCWP*) and *PBZ1*) was studied via real-time PCR as described in Example 3. *PR1* is a well known PR protein. Glycine rich cell wall protein (encoded by *GRCWP*) is a structural protein commonly found in strengthened cell wall to hinder pathogen attacks. *PBZ1* is induced by probenazole (PBZ), N-cyanomethyl-2-chloro-isonicotinamide (compounds known to induce disease resistance) as well as the fungal blast pathogen *M. grisea*. *PBZ1* is induced faster by incompatible strains of *M. grisea* than compatible strains. *PR1* and *PBZ1* are induced by over-expression of *NHL*, a key signaling component in rice defense response.

The RNA was extracted from 8-week-old plants of the transgenic rice lines (at the T3 generation) carrying a single insertion of *OsRHC1* and their wild type parent (Aichi Asahi). The primers used in real-time PCR are as follows:

O. sativa OsRHC1 forward primer: 5'-AAAGAAGAGCAAGCCCGGTTAT-3' (SEQ ID NO:26);

O. sativa OsRHC1 reverse primer: 5'-GCCTCCATACCTCTTCTGCAA-3' (SEQ ID NO:27);

O. sativa PR1 (BF889437) forward primer: 5'-CGGACAGAGGCCTTACTAAGTTATTT-3' (SEQ ID NO:28);

O. sativa PR1 (BF889437) reverse primer: 5'-GACCTGTTTACATTTTCACGTCTTTATT-3' (SEQ ID NO:29);

O. sativa GRCWP (BF889438) forward primer: 5'-GAGGCAACGGACACCACTAAG-3' (SEQ ID NO:30);

O. sativa GRCWP (BF889438) reverse primer: 5'-TGTAAGCAGAGAGAGAGGCTCATT-3' (SEQ ID NO:31);

O. sativa PBZ1 (D38170) forward primer: 5'-AAGCTCAAGTCACACTCGAC-3' (SEQ ID NO:32);

O. sativa PBZ1 (D38170) reverse primer: 5'-GATGTCCTTCTCCTTCTCC-3' (SEQ ID NO:33).

For normalization, the actin primers are:

O. sativa OsAct1D (X15865) forward primer: 5'-CTTCATAGGAATGGAAGCTGCGGGTA-3' (SEQ ID NO:34);

O. sativa OsAc1D (X15865) reverse primer:

5'-GACCACCTTGATCTTCATGCTGCTA-3' (SEQ ID NO:35).

Figure 10A shows the over-expression of *OsRHC1* in the transgenic rice lines as measured by real-time PCR. Western blot analysis conducted as described in Example 2, gives the results shown in Figure 10B. Transformants generally exhibited higher protein content than wildtype.

Figure 11 shows an induction effect by overexpressing *OsRHC1* on the expression of the three rice defense marker genes. In general, the degree of induction of the three defense marker genes is positively correlated with the level of *OsRHC1* expression. For instance, the two transgenic lines R8 and R12 which exhibited higher level of *OsRHC1* also induced the expression of the three defense marker genes to a larger extent (comparing Figs. 10 and 11).

Example 9

OsRHC1 is an E3 Ubiquitin Ligase

This example demonstrates that OsRHC1 is capable of autoubiquitination, a property common to ubiquitin E3 ligases.

A partial fragment of OsRHC1 (OsRHC1p) lacking transmembrane domain located at the N-terminus was prepared. Only the RING-HC domain at the C-terminus is included as the presence of the transmembrane domains makes extraction from *E. coli* cells difficult.

The appropriate C-terminal portion of the encoding sequence was amplified with primer set HMOL5743 (5'-CCGGAATTCGTTGTTCTACTATTACGAAATGG-3') (SEQ ID NO:36) and HMOL2625 (5'-CAGGTCGACGTTAAACATCATATACGGGCATG-3') (SEQ ID NO:37) flanking the C-terminal half containing the RING-HC domain. The PCR reaction was run with the following cycle profile: 94°C 5 min; 30 cycles of 94°C 30s, 55°C 30s and 72°C 1 min; followed with 72°C extension for 5 min. The amplified product was subcloned into pGex-4T-1 vector with *EcoRI* and *XhoI* restriction sites so as to be fused with GST coding region in frame. The fusion protein was then expressed in DE3 cell with 1.5 mM IPTG induction at 30°C for 2 hours during growth phase. GST-OsRHC1p protein was extracted by lysing the bacterial cells with 1 mg/ml lysozyme at room temperature for 1 hour, followed by 5 freeze/thaw cycles with liquid nitrogen and warm water bath. The extracted protein was purified with GST SpinTrap Purification Module (GH Healthcare).

The *in vitro* ubiquitination assay was performed in ubiquitination buffer (40 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 mM ATP, 2 mM dithiothreitol, 300 ng/μl ubiquitin,

25 μ M MG132, 5 μ l wheat germ extract (to provide E1 and E2 enzymes) (Promega)) plus either 400 ng GST-OsRHC1p or GST only protein. As negative control, the same reaction buffer without the addition of ATP and ubiquitin was used (modified as described by Bazirgan, O. A., *et al.*, *J. Biol. Chem.* (2006) 281:38989-39001; Matsuda, N., *et al.*, *J. Cell. Sci.* (2001) 114:1949-1957). The reaction mixtures were kept at room temperature for 2 hours, then subjected to 10% SDS-PAGE gel electrophoresis, and followed by Western blot analysis with anti-OsRHC1 specific antibody. (Fig. 12).

Autoubiquitination of GST-OsRHC1 was observed in the reaction including ATP and ubiquitin (+ve), but not in the reaction without ATP and ubiquitin (-ve).

10 These results demonstrate that, like other E3 ligases, OsRHC1 undergoes autoubiquitination.

Example 10

Identification of an OsRHC1 Binding Partner

A protein encoded by a clone deposited in GenBank Accession No. ABA98865.1 was identified as a binding partner. This was ascertained using a yeast two hybrid protocol and verified by co-precipitation. The protein encoded by this deposited clone is expressed in *Oryza sativa* (Japonica Cultivar-Group) but it has no identified function. However, since it interacts with OsRHC1, it is presumed to modulate plant defense responses.

The yeast two hybrid protocol employed a commercial kit, the BD Matchmaker™ library construction and screening kit (Clontech K1516-1). *OsRHC1* was first amplified with the oligos HMOL2624 (5'-CCGAATTCATGCCAGCCCCTTCGCTTC-3') (SEQ ID NO:38) and HMOL2625 (5'-CAGGTCGACGTAAACATCATATACGGGCATG-3') (SEQ ID NO:39), digested with the *EcoRI* and *SaII*, subcloned into pGBKT7 in reading frame and transformed into yeast strain Y187. Proteins were extracted from the yeast clones transformed with pGBKT7-OsRHC1 and the control pGBKT7. Western blot analysis with anti-c-Myc epitope tag antibody confirms the presence of DNA binding domain fused OsRHC1 proteins.

Samples of RNA from several rice lines (each containing one of the following *R* genes: *Xa2*, *Xa12*, *Xa14*, *Pita*, *Pib*, and *Pik*) inoculated with the corresponding incompatible pathogens (T2 for *Xa2*; P1 for *Xa12*; LN44 for *Xa14*; Ken54-04 for *Pita*, *Pib* and *Pik*) for 4 days were used as starting materials to construct an AD domain fusion yeast library in the yeast strain AH109 according to the manufacturer's manual. Two rounds of library screening were performed by mating between pGBKT7-*OsRHC1* transformed Y187 and the

AH109 yeast library. Yeast diploid mating products were selected on SD minus Trp, Leu and His (SD/-3) agar plates and incubated at 30°C for 4 days. Only colonies grown to 2-3 mm diameter were further streaked onto SD minus Trp, Leu, His and Ade (SD/-4) agar plates. Selected clones were tested by colony-lift filter assay for *lacZ* reporter gene activity
5 (Yeast Protocols Handbook, Clontech PT3024-1). The partial clone that encoded expressed protein (accession number: ABA98865.1) (labeled as HML1797) produced a positive result. Retransformation of pGBKT7-OsRHC1 and pGADT7-HML1797 into AH109 confirmed this was not due to mutation.

To verify the result of yeast-2-hybrid experiments, co-immunoprecipitation assays
10 were conducted. The full-length coding region of ABA98865.1 was amplified with primers HMOL5311 (5'-AACCCGGGATGGCCGTGGGGTCAGAG-3') (SEQ ID NO:40) and HMOL5312 (5'-TTCCCGGGTCAAAATAAAAACAAATAAAAAAACAC-3') (SEQ ID NO:41), digested with *Sma*I and subcloned into *Sma*I linearized pGADT7-Rec vector to generate a fusion protein with an in-frame HA tag (HA-ABA98865.1); this was designated
15 HML1846. This construct was transcribed and translated *in vitro* by RiboMAX RiboMAX™ large scale RNA production systems-T7 (Promega), wheat germ extract (Promega) and Transcend™ biotin-lysyl-tRNA system (Promega) in combination, respectively.

Total protein was extracted from a rice line overexpressing *OsRHC1* (modified from
20 Boyes, D. C., *et al.*, *Proc. Natl. Acad. Sci. USA* (1998) 95:15849-15854; Greve, K., *et al.*, *Biochem. J.* (2003) 371:97-108. Samples from rice containing 100 µg protein were mixed with 40 µl HA tag fused protein above in a co-immunoprecipitation buffer containing 50 mM Tris/HCl (pH 7.5), 250 mM NaCl, 2 mM MgCl₂, 0.5 mM CaCl₂, 10% (v/v) glycerol, 1.5% (v/v) Triton® X-100, 1 mM PMSF, 2 mg/L leupeptin (modified from Boyes, *et al.*,
25 1998, *supra*; Greve, *et al.*, 2003, *supra*), using the BD Matchmaker™ Co-IP Kit (Clontech 630449). Anti-HA epitope tagged antibody was employed for pulling down the protein complexes. Protein signal was detected by anti-OsRHC1 antibody.

Western blot showed that OsRHC1 was pulled down by HA tag fused ABA98865.1, but no protein was detected on Western blot when the rice protein extract was treated with
30 unrelated protein fused with HA tag.

Claims

1. A recombinant expression system that comprises a nucleotide sequence encoding a protein that has the amino acid sequence shown in Figure 1 or a variant thereof that is at least 95% identical to said amino acid sequence and that confers on plants
5 resistance to trauma, wherein the nucleotide sequence is operatively linked to control systems that effect expression in plant cells.
2. The expression system of claim 1, wherein said protein has the amino acid sequence shown in Figure 1.
3. A plant or plant cell modified to contain the expression system of claim 1.
- 10 4. A method to confer an enhanced ability to resist infections or wounding on a plant, which method comprises modifying said plant to contain the expression system of claim 1.
5. A method to prepare a protein that has the amino acid sequence shown in Figure 1 or variants thereof that are at least 95% identical to said amino acid sequence and
15 that confers on plants resistance to trauma, which method comprises culturing cells that comprise the expression system of claim 1 under conditions wherein said protein is produced and recovering the protein from the culture.
6. A protein prepared by the method of claim 5.
7. A protein that has the amino acid sequence shown in Figure 1 or a variant
20 thereof that is at least 95% identical to said sequence and that confers on plants resistance to trauma.
8. A method to identify a compound or combination of compounds that modulate the ability of plants to resist infections or wounding, which method comprises
contacting the protein of claim 6 or 7 as a test protein with a candidate compound or
25 candidate combination of compounds and

determining the ability of said compound or combination of compounds to bind said protein, whereby a compound or combination of compounds that bind said protein are candidates for modulating the ability of plants to resist infection.

9. A method to identify a compound or combination of compounds that
5 modulate the ability of plants to resist infections or wounding, which method comprises
contacting the protein of claim 6 or 7 as a test protein with a candidate compound or
candidate combination of compounds and
determining the ability of said compound or combination of compounds enhance or
diminish autoubiquitination of said protein.
10. Antibodies specifically immunoreactive with the protein of claim 6 or 7.

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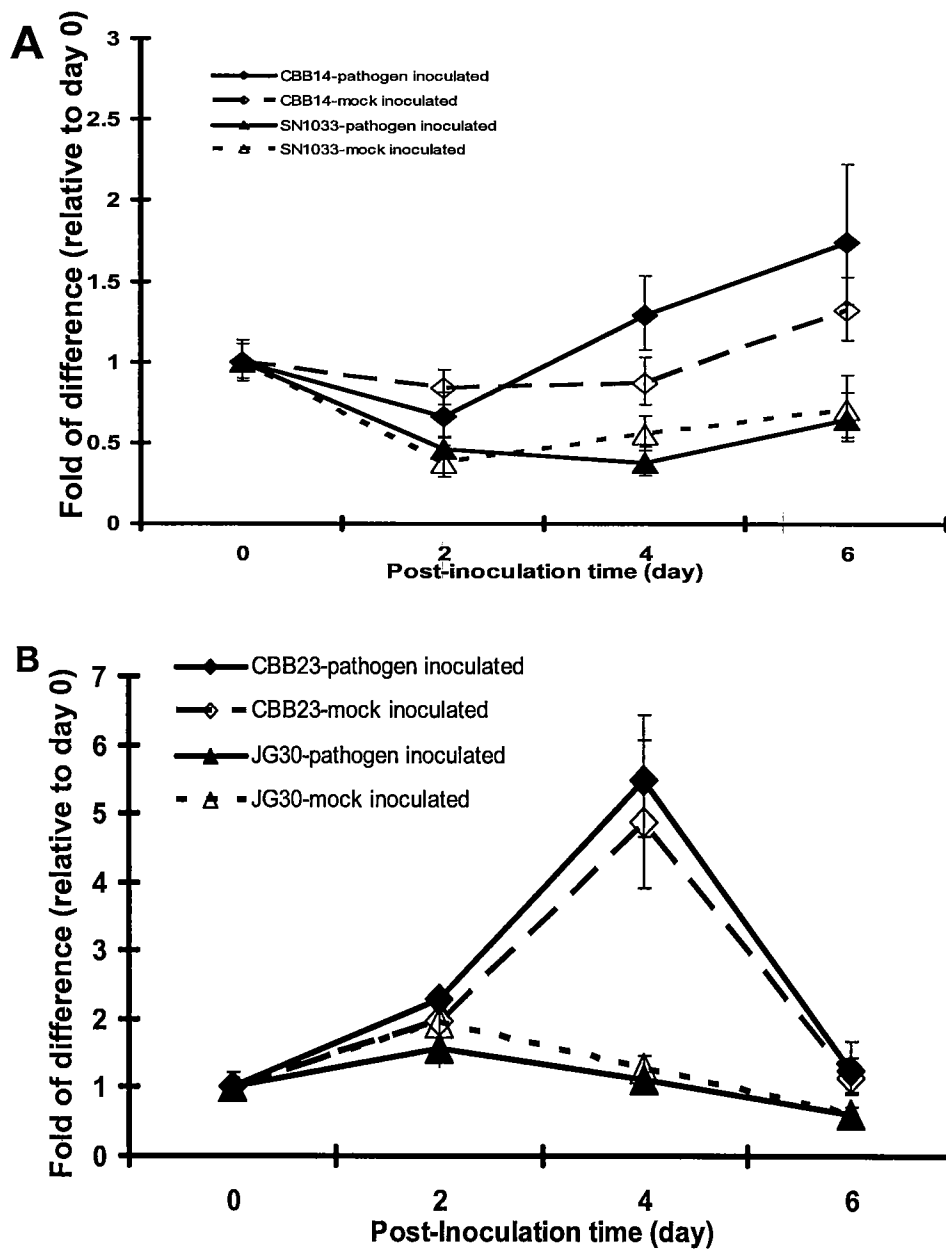
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Figure 1

2 / 12

**Figure 2B****Figure 3**

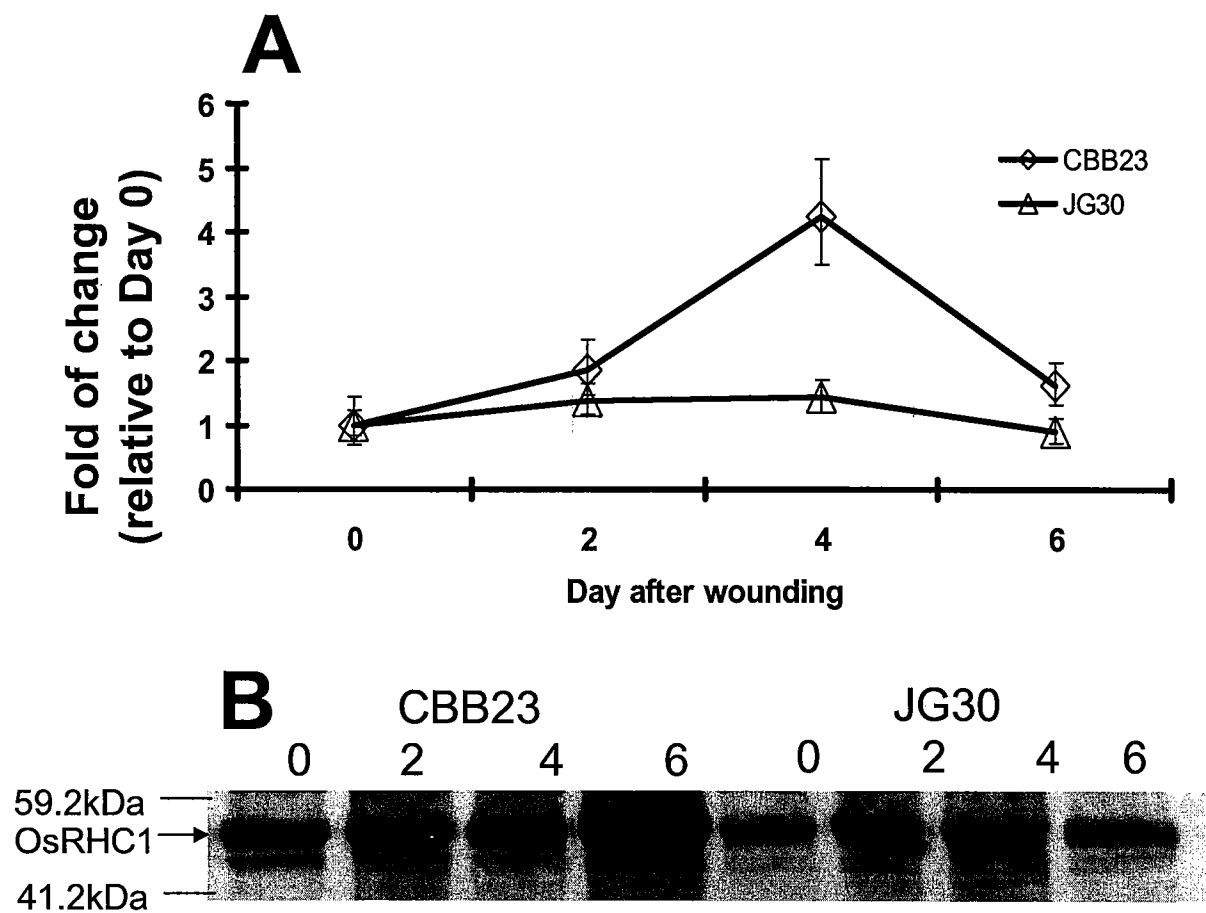


Figure 4

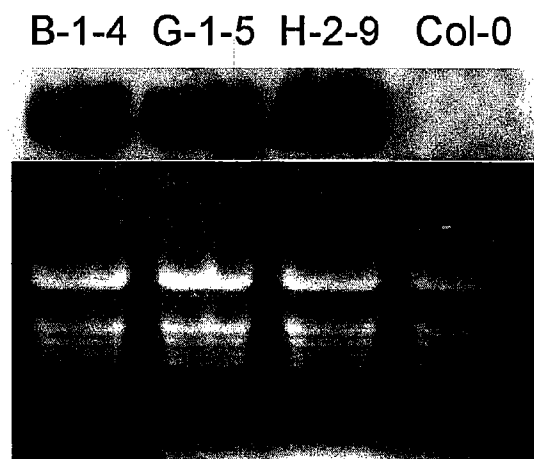


Figure 5A

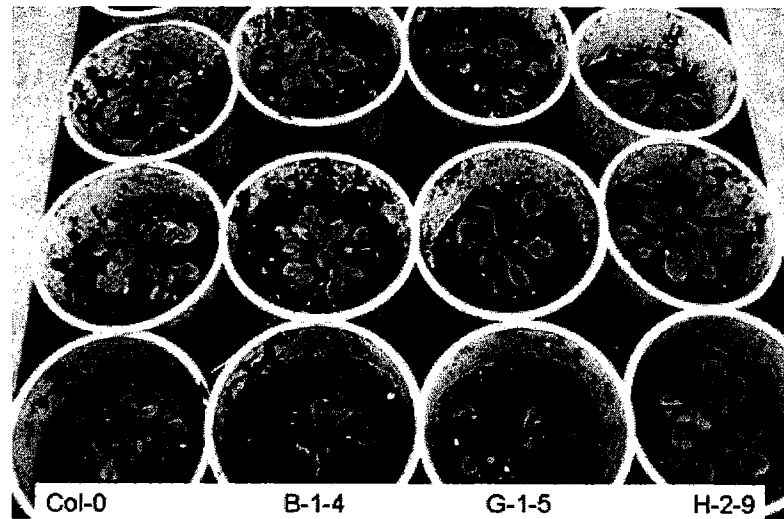


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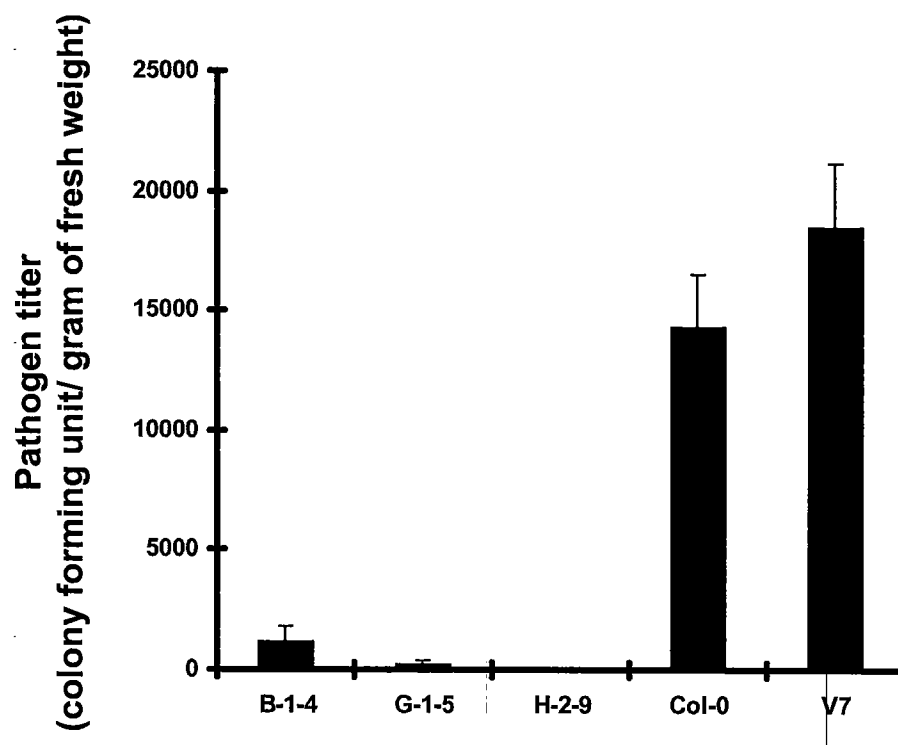


Figure 5C

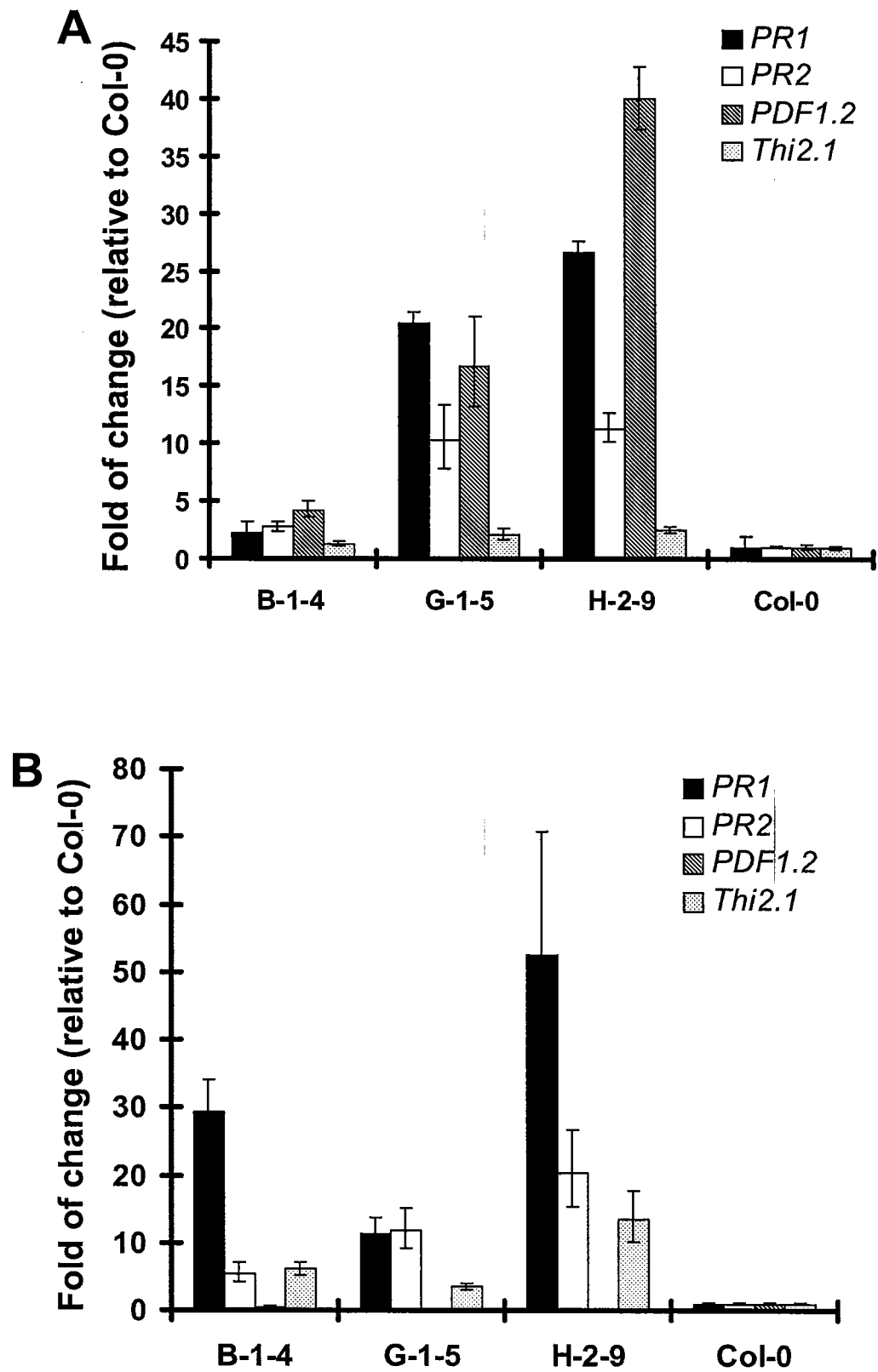
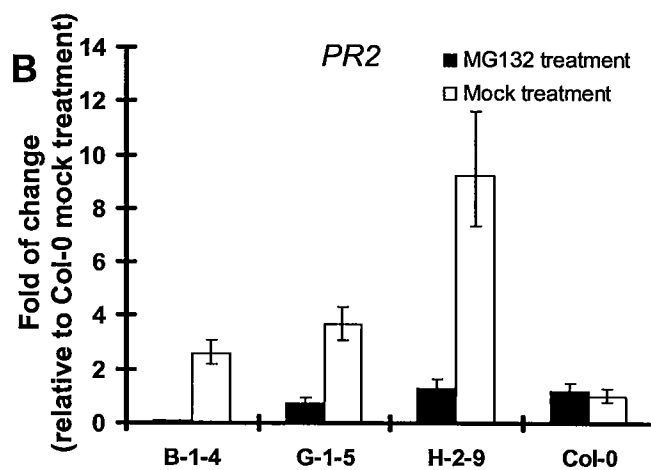
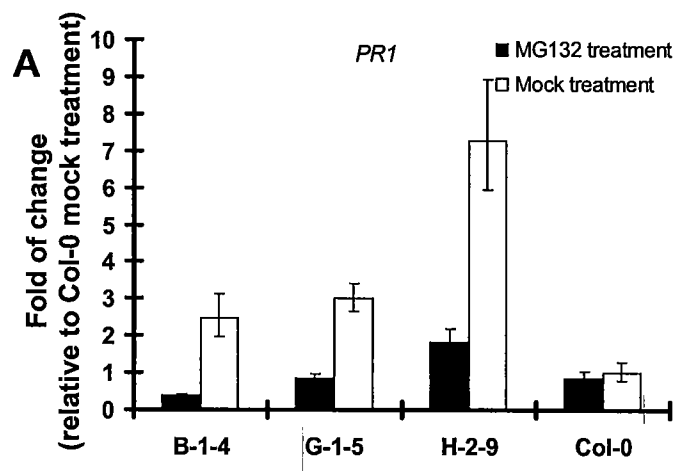
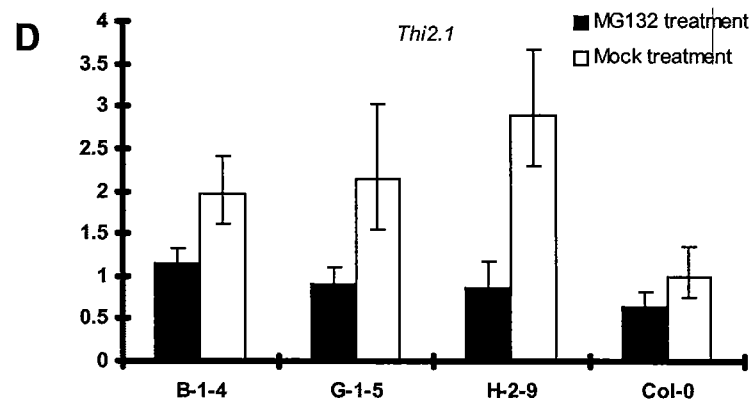
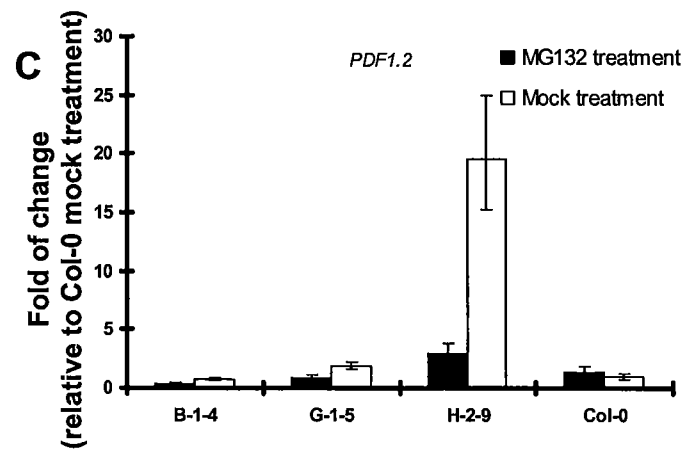


Figure 6



Figures 7A and B



Figures 7C and D

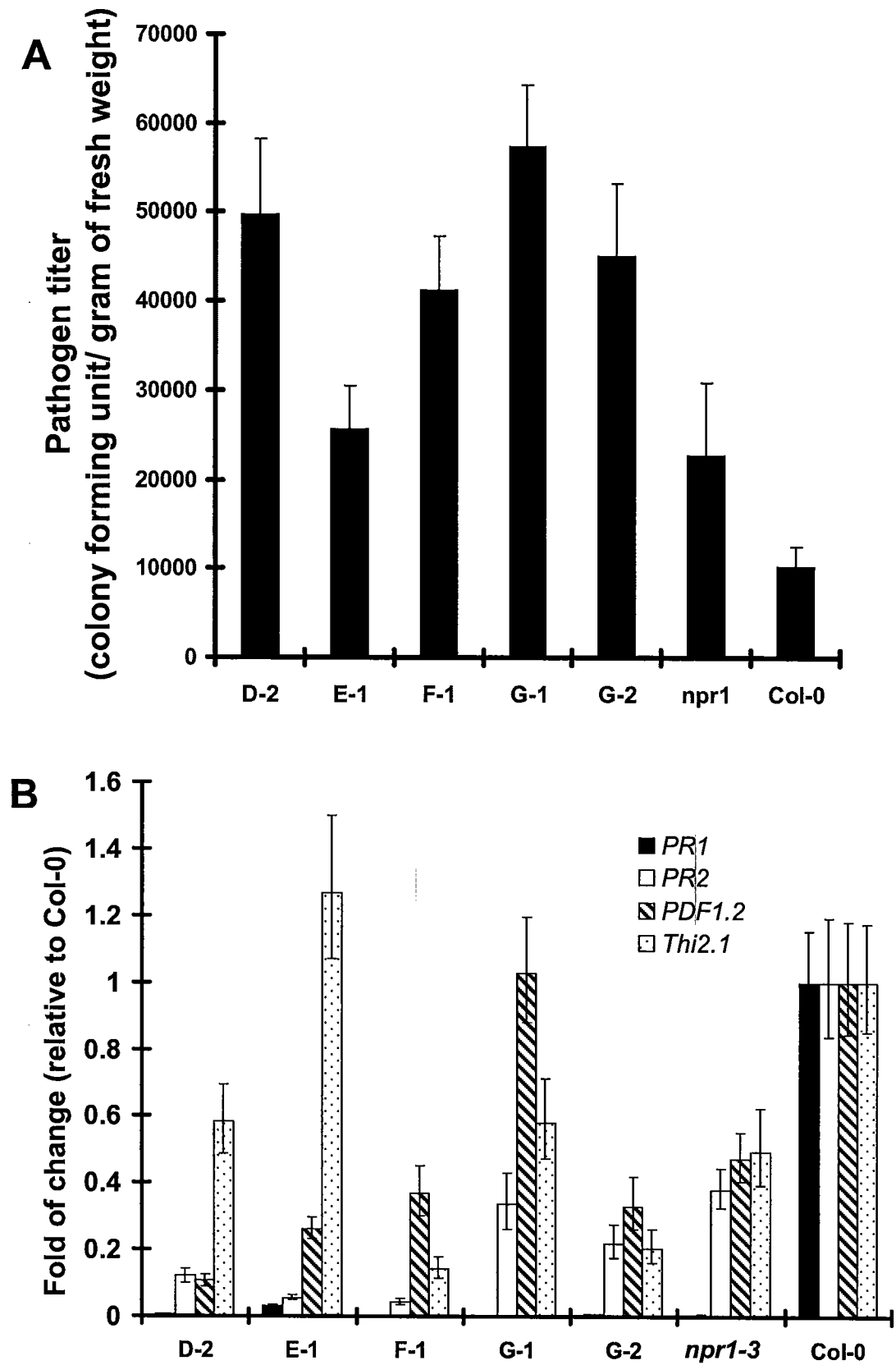


Figure 8

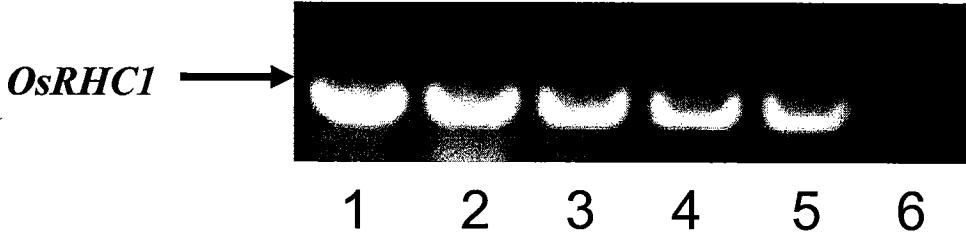


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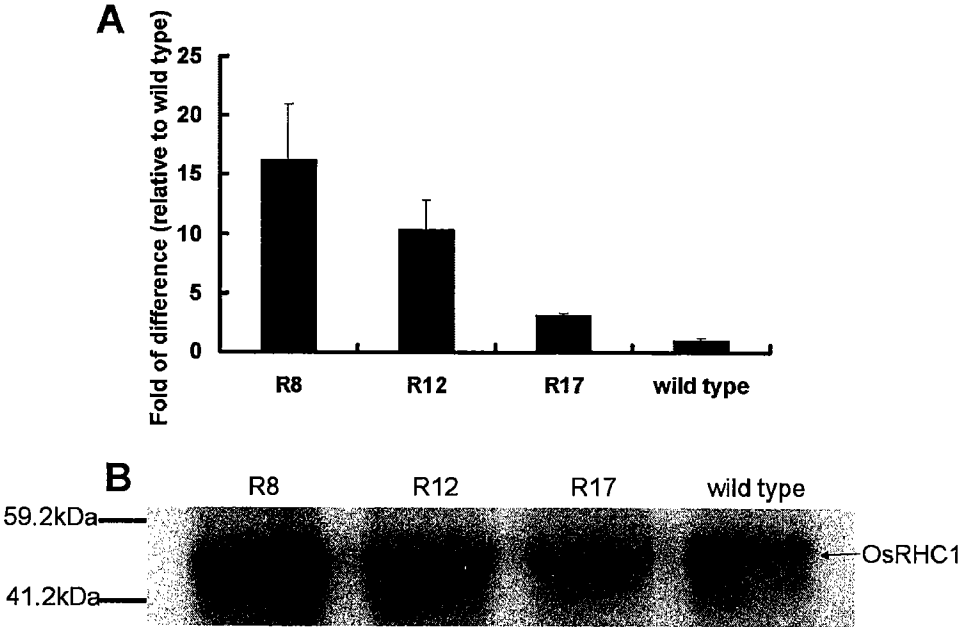


Figure 10

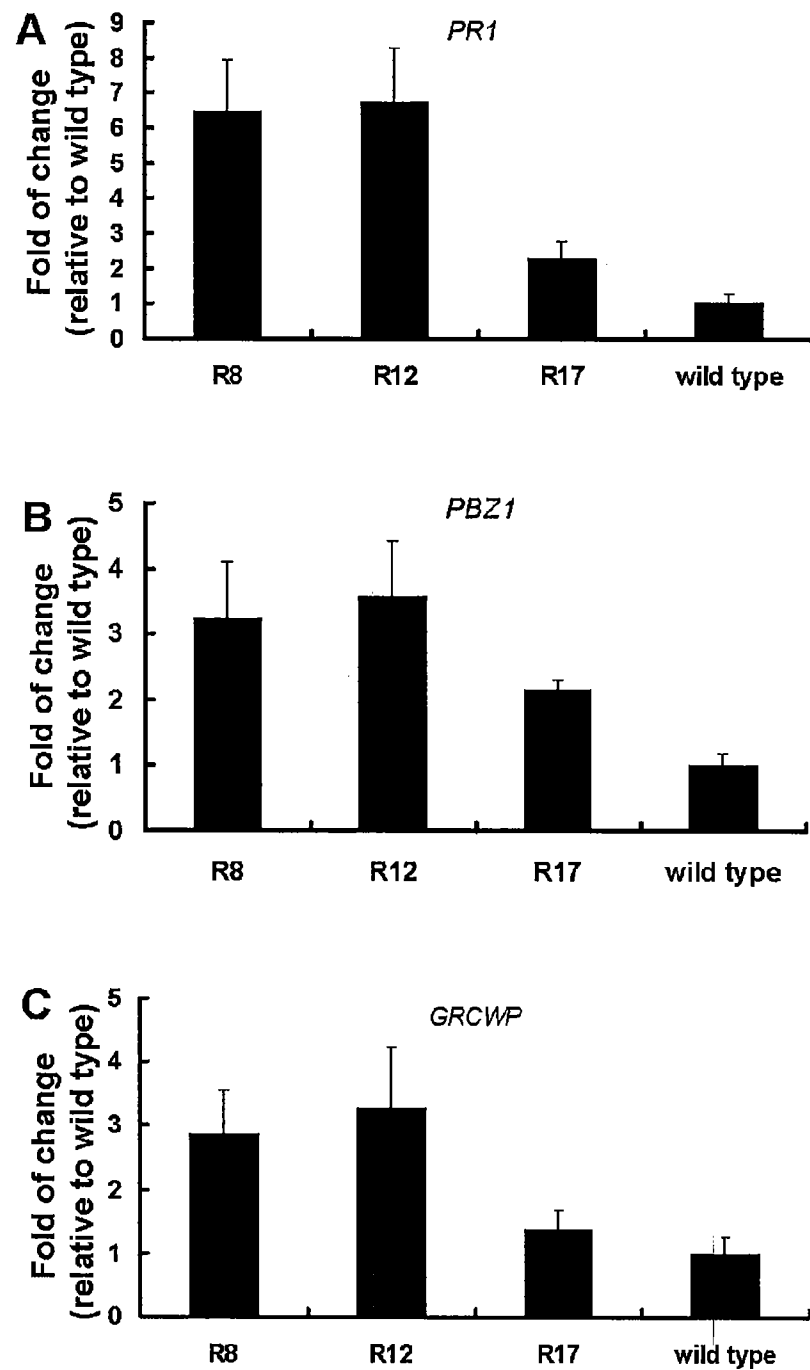


Figure 11

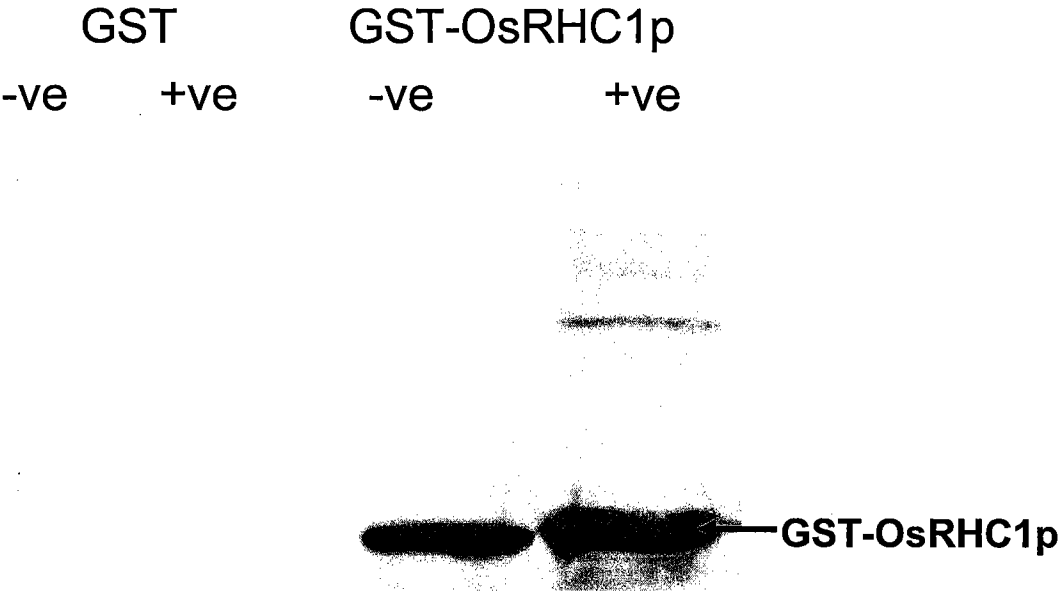


Figure 12

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Figure 13