

Original Research Article

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## Expression of Defense Responsive Gene in Rice after the Infection of *Rhizoctonia solani*

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### ABSTRACT

Rice sheath blight, caused by *Rhizoctonia solani* is one of the major diseases of rice. The pathogen infects rice plants directly through stomata or using lobate appressoria and hyphal masses called infection cushions. Initially the mycelium looked thread like structure, milky white in colour than mycelium converted mature Sclerotia. After the screening of different rice genotypes using 0 to 9 scaling (SES-IRRI), according to Leaf detach method, Pusa Basmati-1 was found highly susceptible variety and Swarna *sub-1* was susceptible one. Among wild rice, *O. australiensis* was observed resistant species, while *Oryza rufipogon* was found moderately resistant species. Approximately the same results were found in humidified chamber condition. During molecular analysis, In control condition, 8A1 gene/primer was not expressed in Pusa Basmati-1 and Swarna *sub-1*, it was expressed in stressed condition in both the cultivar with the product size 155 bp. In both wild rice namely *O. rufipogon* and *O. australiensis*, 8A1 gene was expressed in control as well as stressed condition with the product size 155 bp. While 4C<sub>7</sub> gene/primer was expressed continuously in control and stressed condition in susceptible rice cultivars; Pusa Basmati-1, Swarna *sub-1* and wild rice accessions- *O. rufipogon* and *O. australiensis* with the product size 162 bp.

#### Keywords

Sheath blight,  
SES, 8 A1, 4C<sub>7</sub>,  
*R. solani*.

#### Article Info

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### Introduction

Rice sheath blight, caused by the fungal pathogen *Rhizoctonia solani* Kuhn [Sexual stage: *Thanetophorus cucumeris* (Frank) Donk] is one of the major production constraints in rice-growing countries of the world. Currently, the best method for controlling sheath blight is applying commercial fungicides. Breeding for sheath blight resistant rice plants has not been very

successful mainly because of lack of resistant donors in the cultivated varieties of rice (Bonmann *et al.*, 1992).

Rice sheath blight is a necrotrophic disease. Consequently, very little is known about the host–fungus interaction at the molecular level. As a first step to define the defense-related gene expression profile, we first observed the

infection process of the pathogen and the corresponding expression pattern of the pathogenesis-related genes, *PR1b* and *PBZ1* (Midoh and Iwata, 1996). The most common resistance reaction in plants is the hypersensitive response (HR), which localizes the pathogen at the infection site through cell death. The local responses at the point of infection also trigger a subsequent non-specific resistance that occurs throughout the entire plant; this phenomenon is called systemic acquired resistance (SAR). Typical SAR results in an increase of endogenous salicylic acid (SA) and transcriptional activation of defense-related genes including pathogenesis-related genes *PR1*, *PR2* and *PR5* etc. It finally leads to an enhanced resistance to a broad spectrum of pathogens throughout the plant (Ryals *et al.*, 1996; Durrant and Dong, 2004). The HR typically occurs in the diseases caused by biotrophic pathogens, in which defence signaling is initiated by R protein–Avr protein interactions, and many of these pathways require SA (Denby *et al.*, 2004).

There is no resistance in cultivated rice varieties, however; some level of resistance to sheath blight (ShB) was reported in Jasmine, Teqing, NDR 2030, and Giri (Sinha *et al.*, 2004). The resistance reaction of different wild rice accessions can be transferred to adopted genetic background of cultivated rice (Prasad and Eizenga, 2008; Nelson *et al.*, 2011). Different rice accessions including 15 *Oryza* species belonging to *O. australiensis*, *O. minuta* and *O. officinalis* were found resistant/moderately resistant to ShB and sheath rot and transferred in cultivated rice for ShB resistance (Lakshmanan, 1991; Amante-Bordeos *et al.*, 1992; Ritu and Goel, 2007).

### **Materials and Method**

The present investigation was carried out at the Net house and laboratory of Department

of Plant Molecular Biology and Genetic Engineering, Narendra Deva University of Agriculture and Technology, Kumarganj, Faizabad (U.P.) India. During the Kharif season of 2013-14 and 2014-15. The experiment was laid out in a complete randomized design (CRD) and randomized complete block design (RCBD) in pots (Net house) condition with three replications. The details of materials used, experimental procedures followed and protocols are given below:

### **Plant Materials**

Seeds of rice and wild rice were collected/procured from NBPGR, Pusa, New Delhi and C.R.R.I Cuttack, Orissa (M.P) and Department of Genetics and Plant Breeding, N.D. University of Agriculture and Technology, Kumarganj, Faizabad India.

### ***R. solani* isolates**

A multinucleate compatible, highly virulent strain of *R. solani*, D-14 belonging to AG1-IA anastomosis group was obtained from the Rice Pathology Laboratory, G.B. Pant University of Agriculture and Technology, Pantnagar, U.K. India. This isolate was grown on Potato dextrose Agar at 28 + 1° C for 6 days and used as the inoculation purpose.

### **Screening of resistance by detached leaf methods**

Five week old leaves of different hosts and non hosts were cut in the size of 9 cm long and kept in plastic-dishes. Inoculation was made aseptically at the centre of a leaf by placing a 5 mm mycelia agar disc from a four days old culture of *R. solani* grown on PDA plates as described by Galindo *et al.*, (1982). After inoculation, proper moist condition was maintained inside the plastic Petri-dishes using three fold what man filter paper

saturated with sterile double distilled water. There were three leaves per Petri-dishes, replicated three times for each of the hosts. 72 Hrs. after inoculation, the severity of leaf infection was rated following 0 to 9 scales (Jia *et al.*, 2002), scale with 0 having no lesion to 9 having 90 to 100 % of the leaf surface covered with lesions.

### **Humidified chamber condition**

Inoculation procedure for infection in cultivated varieties of rice and wild rice by *R. solani* isolates was performed according to Park *et al.*, (2008). Immature sclerotia developed on 4-6 days Old mycelia of *R. solani* strain D-14 was grown on Potato Dextrose Agar (PDA) medium. An immature sclerotium of *R. solani* was placed underneath the leaf sheath with 10 ml of sterilized water. Inoculated and non-inoculated plants were placed in humidified chamber condition at  $28 \pm 1^\circ \text{C}$  for 24 hrs. Scoring of disease was carried out on 0-9 rating scale (Standard Evaluation System of IRRI) as described below:

- 0= absolutely free from infection
- 1= lesions limited to lower 20% of plant height
- 3= 20-30% disease
- 5= 31-45% disease
- 7= 46-65% disease
- 9= more than 65% disease.

### **Inoculation of different hosts for RNA isolation**

Inoculation of the different hosts plants for the total RNA isolation purpose were done in the humid chamber condition. The non inoculated control plant was also served as negative control condition which placed in the humidified chamber. Leaves were harvested from the non inoculated (control) and inoculated plants after 72 hours. of

inoculation. The harvested leaves were briefly rinsed with in sterile distilled water, dried with paper towel, put immediately in mortar pestle and crushed in the presence of liquid nitrogen and stored in the deep freezer ( $-80^\circ\text{C}$ ).

### **Isolation of total RNA**

Collected the fresh leaves of rice in ice container from infected and (control) non infected plants and ground 100 mg leaves sample in liquid nitrogen in fine powder with the help of mortar and pestle. Transferred the powder into autoclaved Eppendorf tubes and added 1 ml trizol buffer solution mix the content by shaking. Incubate the tube 2-3 minutes at room temperature ( $20-30^\circ\text{C}$ ). Centrifuged the sample at 9000 rpm for 10 minute at  $4^\circ\text{C}$  and transferred the supernatant into a new sterile centrifuge tube and discard the pellet. 200  $\mu\text{l}$  (0.2ml) of chloroform was added into the tube and Mixed vigorously and stored the tube for 2-3 min at room temperature. Centrifuged and transferred the upper aqueous layer to a fresh tube. The RNA was precipitated by adding 0.5 volume of isopropanol and 0.5 volume of RNA ppt. solution. Stored the tubes for 10 minutes at room temperature and recovered the RNA by centrifugation at 9000 rpm for 10 minute at  $4^\circ\text{C}$ .

### **Analysis of transcript levels by reverse transcriptase-mediated PCR**

To analyze the expression patterns of these genes during the interaction with *R. solani*, RT-PCR analysis was conducted. Total RNA was reverse-transcribed into first-strand cDNA with oligo-dT primer according to kit protocol (Genei two step MMLV-RT-PCR kit) and the cDNA was used as a template for PCR to analyze the selected genes. The primers for the PCR were designed based on the sequences of the genes (Table 1). Two

primers namely 8A<sub>1</sub> and 4C<sub>7</sub> were amplified using the control and stressed cDNA template. Following condition was standardized for the amplification of genes

with different primers which was performed by using thermal cycler (PTC-100, Bio-Rad, USA).

Stage	Step	Temp (°C)	Duration	No. of cycle
1	Initial denaturation	94	4 min.	1
2	Denaturation	94	1 min.	} 35
3	Annealing (vary with temp)	55	1.5 min.	
4	Extension	72	2 min.	
5	Total extension	94	10 min.	} 1
6	Storage	4		

## Results and Discussion

### *R. solani* isolates

A multinucleate compatible, highly virulent strain of *R. solani*, D-14 belonging to AG1-IA anastomosis group was colonized into black brown (Figure 1a). Initially the mycelium looked thread like structure, milky white in colour than mycelium converted mature Sclerotia.

### Screening of resistance by detached leaf methods/ Humidified chamber condition

According to Leaf detach method, using 0 to 9 scaling, Pusa Basmati-1 was found highly susceptible variety and Swarna sub-1 was found susceptible one. Among wild rice, *O. australiensis* was observed resistant species. While *Oryza rufipogon* was found moderately resistant species. Approximately the same results were found in humidified chamber condition (Table 2, Figures 1 and 2).

### Analysis of transcript levels by reverse transcriptase-mediated PCR

Total RNA was isolated using trizol method (Fig. 3) and reverse-transcribed into first-strand cDNA with oligo-dT primer and the cDNA was used as a template for PCR to

analyze the selected genes. In present experiment, In control condition, 8A<sub>1</sub> gene/primer was not expressed in Pusa Basmati-1 and Swarna sub-1, it was expressed in stressed condition in both the varieties with the product size 155 bp. While 8A<sub>1</sub> gene/primer was expressed in both wild rice namely *O. rufipogon* and *O. australiensis* in both control as well as stressed condition with the product size 155 bp (Fig. 4).

In control condition, 4C<sub>7</sub> gene/primer was expressed continuously in both control and stressed condition in both susceptible rice varieties; Pusa Basmati-1 and Swarna sub-1 with the product size 162 bp. 4C<sub>7</sub> gene/primer was expressed in both wild species namely *O. rufipogon* and *O. australiensis* in both condition control as well as stressed condition with the product size 162 bp (Fig. 4). However, the roles of ethylene insensitive- 3-like protein (4C7), translationally controlled tumor protein (9B12) and oxygen-evolving enhancer protein 2 (8B2) in response to pathogens are unknown.

Rice sheath blight disease is an important fungal disease of rice, which has become a major constraint to rice production during the last two decades (Kobayashi *et al.*, 1997). A highly virulent strain of *R. solani*, D-14 belonging to AG1-IA anastomosis group was

colonized into black brown. Initially the mycelium looked thread like structure, milky white in colour than mycelium converted mature Sclerotia. Sunder *et al.*, (2003) had also reported that colony colour ranged from brown, light brown, dark brown and yellowish brown. The results are also supported by Hoa (1994), reported that sclerotial colour ranged from brown, light/dark brown, black brown, chocolate brown, salmon and dark salmon.

In the past, IRRI developed the standard evaluation system for rice sheath blight disease based on relative lesion length, which is the average vertical height of the uppermost lesion expressed as a percentage of the average plant height (IRRI. 2002). According to Leaf detach method, using 0 to 9 scaling, Pusa Basmati-1 was found highly susceptible variety and Swarna *sub-1* was found susceptible one. Among wild rice, *O. australiensis* was observed resistant species. While *Oryza rufipogon* was found Moderately

Resistant species. Approximately the same results were found in humidified chamber condition (Table 2, Figures 1 and 2). The similar results were found by Shamim *et al.*, 2014, they screened 52 rice cultivar in humidified chamber condition, leaf detach condition and field condition against sheath blight and observed that Kalanamak, Pusa Basmati, Swarna, IR-64 were found the susceptible cultivar while *O. australiensis* and *O. grandiglumis* were found the resistance. Prasad *et al.*, (2008), reported that the detached-leaf inoculation method separated cvs. Jasmine 85 and TeQing, moderately resistant checks, from Lemont and Cocodrie, highly susceptible checks, based on disease reaction (lesion length). Eizenga *et al.*, (2002), used the toothpick method/ humidified chamber method to evaluate disease intensity based on the leaf lesion length of both the leaf blade and sheath summed together. Data from the first three leaves were useful in interpreting disease response as reported earlier.

**Table.1** The primers for the PCR

Gene/ primer	Forward primer	Reverse primer	Annealing temperature (°C)
4C7	5-CCAATTCGAGCCGTCAT-3	5-CAGCAGCAGTTCTTCATCC-3	55
8A1	5-GTGGTCCAATAGAGTGATGC-3	5-AGAGACATACCAATTGCAGG-3	55

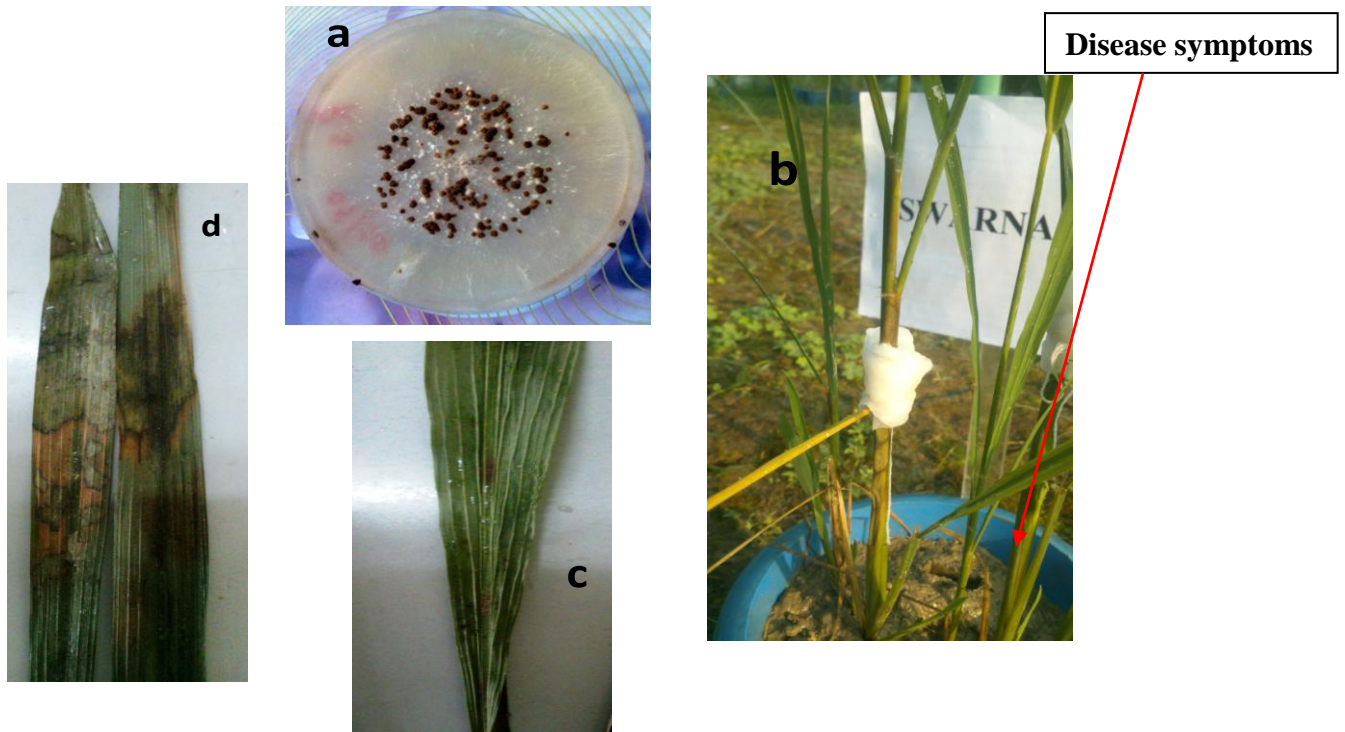
**Table.2** Screening of resistance by detached leaf methods/ Humidified chamber condition

Rice varieties / sps.	Leaf detach method			Humidified chamber condition		
	0-9 scaling	Disease %	Categories	0-9 scaling	Disease %	Categories
Pusa Basmati-1	9	>90%	HS	9	>75%	HS
Swarna sub-1	7	< 65%	S	7	< 85%	HS
<i>Oryza rufipogon</i>	5	45%	MR	5	40%	MR
<i>O. australiensis</i>	1	< 20%	R	1	< 20%	R

**Note:** HS- Highly susceptible; S- Susceptible; MR- Moderately Resistant  
R- Resistant



**Fig.1** Disease symptoms of sheath blight, a- *R. solani* culture plate, b- inoculated or infected rice plant, c- leaf of *O. australiensis*, d- leaf of Swarna sub-1



**Fig.2** Disease symptoms of sheath blight on (a) Pusa Basmati-1 and (b) *O. australiensis* under leaf detach method condition

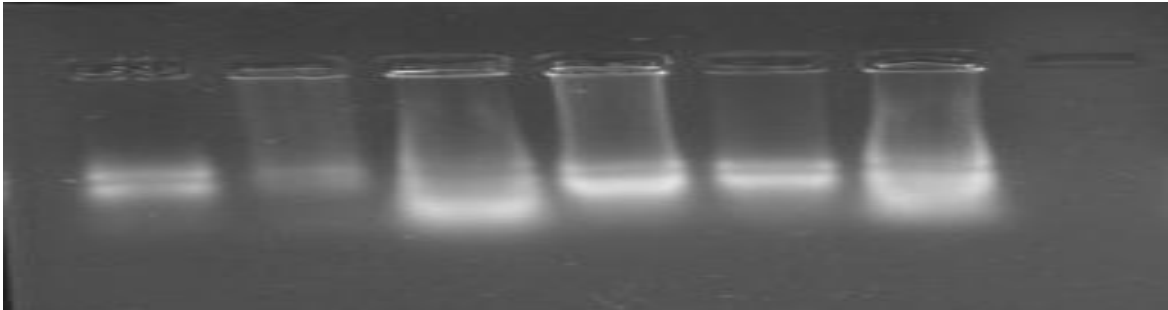
Disease symptoms



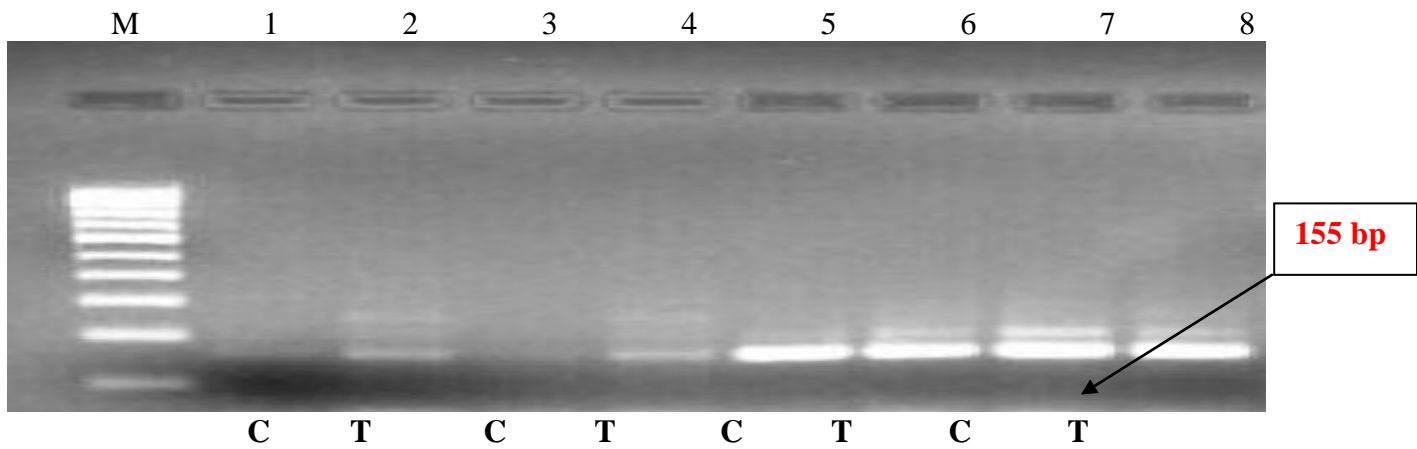
(a)

(b)

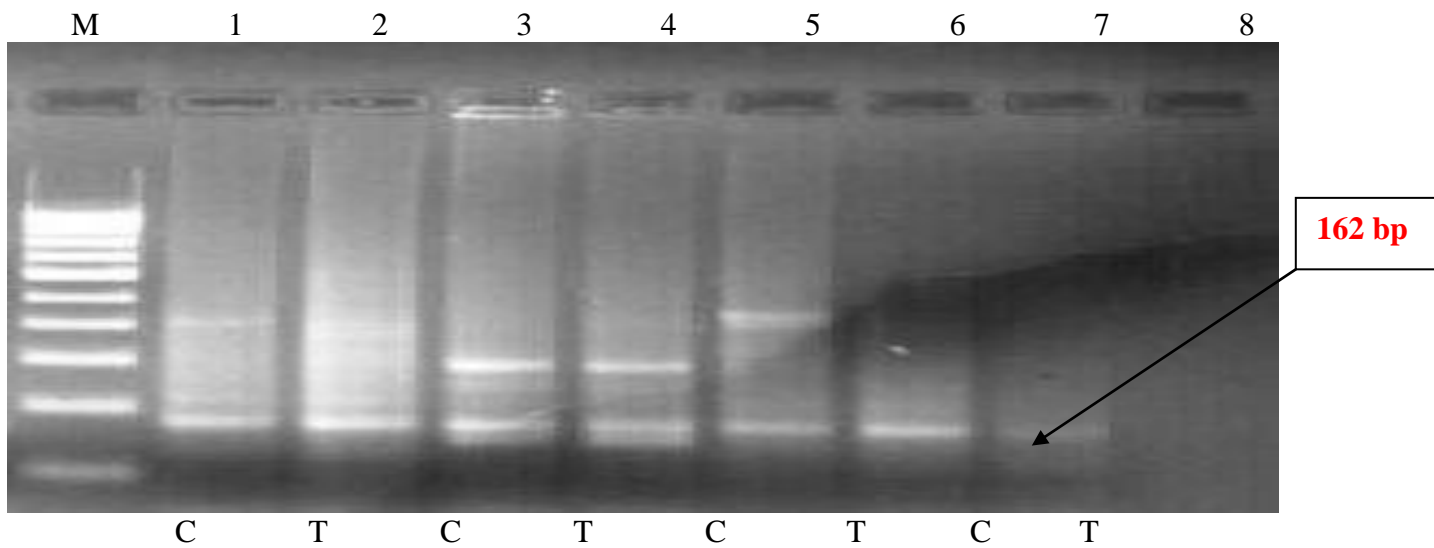
**Fig.3** RNA Gel Electrophoresis of Different rice cultivar at 1.2 % Agarose



**Fig.4** PCR with 8 A1 primer M- Marker(100bp), 1 & 2-Pusa Basmati-1, 3 & 4- Swarna sub-1, 5 & 6- *O. rufipogon*, 7& 8- *O. australiensis*



**Fig.5** PCR with 4 C7 primer, M- Marker(100bp), 1 & 2-Pusa Basmati-1, 3 & 4- Swarna sub-1, 5 & 6- *O. rufipogon*, 7& 8- *O. australiensis*. C- Control, T- Treated



During molecular analysis, 8A1 gene/primer was not expressed in Pusa Basmati-1 and Swarna sub-1 in control condition, it was expressed in stressed condition in both the varieties with the product size 155 bp. While 8A1 gene/primer was expressed in both wild rice namely *O. rofipogon* and *O. australiensis* in all condition with the product size 155 bp. The similar results were found by Zhao *et al.*, 2008 for both gene 8 A1 and 4C7. According to the gel analysis of PCR product of their experiment, 8A1 was not expressed initially, it was expressed after 24 hrs of infection. While 4C7 expressed in control as well as treated condition. Further characterization of 8 A1 gene may shed light on the relationship between the ubiquitination pathway and the defense response in plants. It is interesting to note that 8A1 encodes a putative ubiquitin-conjugating enzyme E2. The ubiquitin /proteasome pathway is the major selective protein degradation system in eukaryotes. Growing evidence indicates that this pathway plays an important role in plant defense responses (Dreher and Callis, 2007).

In control condition, 4C<sub>7</sub> gene/primer was expressed continuously in both control and stressed condition in both rice varieties; Pusa Basmati-1 and Swarna *sub-1* with the product size 162 bp. 4C<sub>7</sub> gene/primer was expressed in both wild rice namely *O. rofipogon* and *O. australiensis* in both condition as well as stressed condition with the product size 162 bp. However, the roles of ethylene insensitive- 3-like protein (4C<sub>7</sub>), translationally controlled tumor protein (9B12) and oxygen-evolving enhancer protein 2 (8B2) in response to pathogens are unknown.

In conclusion, rice sheath blight caused by *R.solani* is one of the most common diseases in rice. Pusa Basmati-1 and Swarna sub-1 observed the susceptible varieties after screening in Humidified chamber condition

and leaf detach condition using the SES-IRRI protocol. Among wild rice, *O. australiensis* was observed resistant species, while *Oryza rufipogon* was found moderately resistant sps. During molecular analysis, 8A1 gene/primer was not expressed in control condition in Pusa Basmati-1 and Swarna *sub-1*, while 8A1 gene was expressed in control as well as stressed condition in both wild rice accessions *O. rofipogon* and *O. australiensis*. Another 4C<sub>7</sub> gene was expressed continuously in control and stressed condition in all rice cultivar. So 8A1 gene acts as a defense responsive gene which is induced in stressed condition.

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