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# Rice SAPs are responsive to multiple biotic stresses and overexpression of OsSAP1, an A20/AN1 zinc-finger protein, enhances the basal resistance against pathogen infection in tobacco

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ABSTRACT

Eukaryotic A20/AN1 zinc-finger proteins (ZFPs) play an important role in the regulation of immune and stress response. After elucidation of the role of first such protein, OsSAP1, in abiotic stress tolerance, 18 rice stress associated protein (*SAP*) genes have been shown to be regulated by multiple abiotic stresses. In the present study, expression pattern of all the 18 *OsSAP* genes have been analysed in response to different biotic stress simulators, in order to get insights into their possible involvement in biotic stress tolerance. Our results showed the upregulation of *OsSAP1* and *OsSAP11* by all biotic stress simulator treatments. Furthermore, the functional role of *OsSAP1* in plant defence responses has been explored through overexpression in transgenic plants. Constitutive expression of *OsSAP1* in transgenic tobacco resulted into enhanced disease resistance against virulent bacterial pathogen, together with the upregulation of known defence-related genes. Present investigation suggests that rice *SAPs* are responsive to multiple biotic stresses and *OsSAP1* plays a key role in basal resistance against pathogen infection. This strongly supports the involvement of rice SAPs in cross-talk between biotic and abiotic stress signalling pathways, which makes them ideal candidate to design strategies for protecting crop plants against multiple stresses.



## 1. Introduction

Zinc-finger proteins (ZFPs) are known to play various important roles in diverse organisms. Human A20 protein comprises of seven zinc-fingers at N-terminal and an ovarian tumour (OTU) domain at C-terminal end [1]. In some cases A20 domain is also accompanied by another zinc-finger, AN1, and these A20/AN1 proteins are associated with immune and stress responses [2]. The most studied human A20 protein inhibits NF-κB activation, a transcription factor that plays a critical role in immune regulation and inflammatory responses in humans [3]. These A20/AN1 proteins regulate immune response by influencing the ubiquitination status of target proteins in NF-κB pathway via de-ubiquitination and E3 ubiquitin ligase activities [4]. In plants, the first A20/AN1 ZFP, *Oryza sativa* Stress Associated Protein1 (OsSAP1) was identified in rice. *OsSAP1* transcript was shown to be induced by multiple stresses, namely cold, desiccation, salt, submergence, heavy metals, ABA and wounding [5]. Further, 18 and 14 genes were identified from rice and Arabidopsis genome, respectively, encoding A20/AN1 zinc-finger containing SAPs. The quantitative real-time PCR based expression analyses of the rice *SAP* gene family revealed that all the genes were inducible by abiotic stress treatments [6]. Recently, abiotic stress-inducible *SAPs* have also been identified from other plant species like maize, banana, *Medicago* and *Aleuropus*. Overexpression of *SAPs* from rice and other plants has been shown to confer abiotic stress tolerance in transgenic plants and also protect

*Abbreviations:* CA, cholic acid; DAB, 3,3′-diaminobenzidine; $H_2$DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HR, hypersensitive response; JA, jasmonic acid; NBT, nitro-blue tetrazolium; PA, picolinic acid; PR, pathogenesis-related; ROS, reactive oxygen species; SA, salicylic acid; SAP, stress associated protein; ZFP, zinc-finger protein.

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crop-yield loss [5,7–14]. These studies have suggested that SAPs may act as ubiquitin ligase, redox sensor and regulator of gene expression during stress in plants [15].

Plants respond to a variety of microbial pathogens and insect herbivores by activation of a battery of defense responses. The pathogen-induced defence responses depend on the production of molecules that are recognized by corresponding resistance or *R*-genes of plants [16]. This perception of pathogen infection leads to a cascade of signal transduction that involves several events like protein phosphorylation, ion fluxes and reactive oxygen species (ROS) production for hypersensitive response (HR) resulting in cell death [17]. *R* gene-mediated responses are also associated with salicylic acid (SA) production that leads to the induction of pathogenesis-related (PR) proteins for resistance against pathogens [18]. Jasmonic acid (JA) and ethylene also control plant defence mechanisms against necrotrophic pathogens and herbivorous insects [19].

It has now been established that innate immunity responses in plants and animals share many common conserved elements [20]. The involvement of human A20/AN1 ZFPs like A20, ZNF216 and Rabex-5 in innate immune responses suggests a potential role for SAPs in plant defence signalling against pathogens [2,3,21]. Additionally, rice and banana *SAPs* were reported to be induced by wounding; a common feature of insect infestation [5,14]. Furthermore, overexpression of a few plant ZFPs has also been shown to confer disease resistance in tobacco against virulent pathogens [22,23].

Therefore, to investigate the role of SAPs in defence response, expression pattern of all the 18 rice *SAP* genes was analysed in response to wounding, pathogen elicitors and defence signal molecules using quantitative real-time PCR. Majority of the *SAPs* showed responsiveness towards a variety of biotic stress treatments. One gene, *OsSAP1*, which was found responsive to almost all the stress treatments, was tested for function. We analysed its role in biotic stress tolerance through overexpression in transgenic tobacco plants. *OsSAP1* overexpressing transgenics showed enhanced basal resistance in tobacco leaves against virulent bacterial pathogen, *Pseudomonas syringae* pv. tabaci that was linked with constitutive elevated expression of known defence-related genes. Our data demonstrate for the first time that *OsSAP1* is involved in modulation of defence responses against biotic stress, in addition to its earlier proven roles in abiotic stress response.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

For quantitative real-time PCR analysis, rice seeds (*O. sativa* subsp. *indica* var Pusa Basmati 1) were surface sterilized with 0.1% mercuric chloride and few drops of detergent for 10 min. After sterilization, seeds were washed at least five times with sterile water and kept soaked overnight in dark. The following day, seeds were evenly spread on a 5–6 cm thick cotton bed, soaked in sterile water. The seeds were allowed to germinate in culture room maintained at 28 °C with a 16/8 h light/dark cycle and light intensity at 200 μmol $m^{-2}$ $s^{-1}$.

Transgenic tobacco plants expressing *OsSAP1* constitutively (*Nicotiana tabacum* cv. xanthii) were raised earlier in our lab [5]. These were grown for four generations and homozygous lines for *OsSAP1* were used in this study. Tobacco plants were grown in the phytotron maintained at 25 ± 2 °C with a 16 h photoperiod and 75 ± 5% relative humidity under 200–250 μmol $m^{-2}$ $s^{-1}$ light intensity.

### 2.2. Wounding and chemical treatments

Nine-day-old seedlings were used for wounding, alpha-picolinic acid (PA), cholic acid (CA), SA, JA and hydrogen peroxide ($H_2O_2$) treatments. The mid portion of the leaf was incised with a scalpel blade for wounding. Chemical treatments were applied by foliar spraying of PA (24.3 mM), CA (20 μM), SA (1 mM) and JA (4.7 mM). Except for PA which was made in fresh water, all the above mentioned chemicals were made fresh in 4 mM potassium phosphate buffer, pH 6.0. Hydrogen peroxide was applied at the concentration of 20 mM by immersing the roots of the seedlings in $H_2O_2$ solution. All the chemicals were purchased from Sigma–Aldrich, USA.

### 2.3. Quantitative real-time PCR analysis

Expression pattern of all the 18 *OsSAP* genes have been analysed in response to different biotic stress simulators by measuring relative transcripts levels through real-time PCR as described earlier [6]. Wounding and chemical treatments were given to nine-day-old rice seedlings as mentioned above. The samples were harvested at different time points ranging from 5 min to 6 h and 5 min to 24 h after wounding and chemical treatments, respectively. Controls were treated with buffer for all the time points. All experiments were repeated at least twice.

For expression analysis of known biotic stress responsive genes in wild-type and *OsSAP1* transgenic tobacco plants, tobacco homologs of Arabidopsis genes/ESTs involved in plant-pathogen interactions were identified from public database (http://www.genome.jp/kegg/pathway/ath/ath04626.html). Samples were harvested from eight-week-old wild-type and *OsSAP1* transgenic tobacco plants in unstressed condition (0 h), or after 2 h, 6 h and 8 h post-infection (hpi) with virulent (*P. syringae* pv. tabaci) and avirulent (*P. syringae* pv. tomato DC3000) strains. The data are represented as mean value of three biological replicates for each sample. For both experiments, cluster analysis was performed using MultiExperiment Viewer (http://www.tm4.org/mev.html). Details of primer sequences used for real-time PCR analysis are given in Supplementary Tables S1 and S2.

### 2.4. Disease resistance assays

Eight-week-old wild-type and *OsSAP1* transgenic tobacco plants were used for disease resistance assays against *P. syringae* pv. tomato DC3000 (Pst DC3000, avirulent) and *P. syringae* pv. tabaci (Ps tabaci, virulent). The bacterial culture of late log phase ($OD_{600}$ = 0.6–1) was harvested and re-suspended in 10 mM $MgCl_2$. The inoculum density was adjusted to $OD_{600}$ = 0.001–0.01 (~$10^5$–$10^7$ cfu $ml^{-1}$) by serial dilutions in 10 mM $MgCl_2$. Bacterial suspensions were syringe-infiltrated abaxially into intervenal areas of fully expanded tobacco leaves. Symptom development was monitored daily and photographs were taken at 1, 3 and 5 days post-infection (dpi). These experiments were repeated at least three times.

For the determination of bacterial growth *in planta*, tobacco leaves were inoculated as described above using a 100 μl inoculum at the density of ~$10^3$ cfu $ml^{-1}$. Infected leaves were harvested at different time points (1, 3 and 5 dpi) and surface-sterilized with 70% ethanol for 1 min, rinsed with sterile water and blot-dried. Leaf discs of 0.5 $cm^{-2}$ were excised from infected area and homogenized in 10 mM $MgCl_2$. The bacterial population in tobacco leaves was determined by a serial dilution of homogenate, plated onto King's B or LB medium containing appropriate antibiotic. Colonies were counted after incubating the plates at 28 °C for 48 and 96 h for *Pst* DC3000 and *Ps* tabaci, respectively. Experiments were repeated at least twice.



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