The rice *OsSAG12-2* gene codes for a functional protease that negatively regulates stress-induced cell death

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Senescence is the final stage of plant development. Although expression of most of the genes is suppressed during senescence, a set of genes referred as senescence-associated genes (SAGs) is induced. *Arabidopsis thaliana SAG12 (AtSAG12)* is one such gene that has been mostly studied for its strict association with senescence. *AtSAG12* encodes a papain-like cysteine protease, expressed predominantly in senescence-associated vacuoles. Rice genome contains multiple *AtSAG12* homologues (*OsSAGs*). *OsSAG12-1*, the closest structural homologue of *AtSAG12*, is a negative regulator of developmental and stress-induced cell death. Proteolytic activity has not been established for any *SAG12* homologues *in vitro*. Here, we report that *OsSAG12-2*, the second structural homologue of *AtSAG12* from rice, codes for a functional proteolytic enzyme. The recombinant OsSAG12-2 protein produced in *Escherichia coli* undergoes autolysis to generate a functional protease. The matured OsSAG12-2 protein shows 27% trypsin-equivalent proteolytic activity on azocasein substrate. Dark-induced senescence activates *OsSAG12-2* expression. Down-regulation of *OsSAG12-2* in the transgenic artificial miRNA lines results in enhanced salt- and UV-induced cell death, even though it does not affect cell viability in the stress-free condition. Our results show that *OsSAG12-2* codes for a functional protease that negatively regulates stress-induced cell death in rice.

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1. Introduction

Annual plants like rice and *Arabidopsis* show reproductive senescence, a process in which the entire plant undergoes senescence upon maturity of seeds. Senescence can also be induced by various external and internal factors, such as plant hormones, prolonged darkness and biotic/abiotic stresses (Guo and Gan 2005; Carrion *et al.* 2013). Although most cellular metabolic processes including transcription are reduced during senescence, expression of a set of genes, referred to as *SENESCENCE-ASSOCIATED GENES* (*SAG*s), is induced. Various genes encoding degradative enzymes such as proteases and nucleases, enzymes involved in lipid and carbohydrate metabolism, constitute SAGs (Guo and Gan 2005). Although large numbers of SAGs are reported from different plants, only a few are strictly associated with senescence. *Arabidopsis thaliana SAG12* (*AtSAG12*) is one such gene for which expression is strictly associated with senescence (Miller *et al.* 1999; Otegui *et al.* 2005). Cytokinin negatively regulates senescence. Expression of cytokinin biosynthetic enzyme iospentenyltransferase (*ipt*), under *AtSAG12* promoter, results in a delay in senescence in *Arabidopsis*, lettuce and wheat (McCabe *et al.* 2001; Liu *et al.* 2005; Sykorova *et al.* 2008). However, the physiological role of AtSAG12 is not ascertained. The mutants of *AtSAG12* are not defective in senescence, and show normal growth and development (Otegui *et al.* 2005).

Rice genome contains multiple genes having sequence similarity with *AtSAG12* (Singh *et al.* 2013a). *OsSAG12-1*, the closest homologue of *AtSAG12* from rice, negatively

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regulates senescence- and pathogen-associated cell death. OsSAG12-1 protein shares 47% sequence identity and 64% similarity with AtSAG12. Expression of *OsSAG12-1* is induced during senescence and pathogen inoculation and its down-regulation in transgenic RNAi lines results in developmental and stress-induced cell death (Singh *et al.* 2013a). Down-regulation of *OsSAG12-1* in transgenic rice RNAi lines results in the induction of both developmental and stress-induced cell death. OsSAG12-1 is annotated as 'cysteine proteinase EP-B1 precursor, putative, expressed', in the rice genome database (*http://rice.plantbiology.msu.edu*).

AtSAG12 expression is mostly restricted in the senescence-associated vacuoles (SAVs) (Otegui *et al.* 2005; Carrion *et al.* 2013). *AtSAG12* codes for a putative cysteine protease. Involvement of cysteine proteases in senescing leaves of wheat is established by in-gel protease activity assay (Martinez *et al.* 2007). Inhibition of cysteine proteases using E-64 (*trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane) protease inhibitor reduces proteolytic activity of dark-induced SAVs (Carrion *et al.* 2013). However, to the best of our knowledge, proteolytic activity *in vitro* has not been established for any of the SAG12 homologues. Although *OsSAG12-1* negatively regulate stress-induced senescence (Singh *et al.* 2013a), biochemical function of *OsSAG12-1* is not known. Thus, it is uncertain whether the proteolytic activity of this class of genes is required for negative regulation of senescence. Rice *LOC_Os08g44270* (referred as *OsSAG12-2* hereafter) gene is annotated as 'vignain precursor, putative and expressed' in the rice genome database. To determine the biochemical function of OsSAG12-2, we studied the *in vitro* proteolytic activity of recombinant protein purified from *E. coli*. To investigate the biological role of OsSAG12-2, we generated down-expression RNAi lines. Our results show that *OsSAG12-2* codes for a functional proteolytic enzyme, and down-regulation of this gene exaggerates stress-induced cell death.

2. Materials and methods

2.1 *Materials*

The vector pTCK303 was a kind gift from Kang Chong, Institute of Botany, Chinese Academy of Science, Beijing. Plant growth materials were obtained from local market. Chemicals and DNA modifying enzymes were procured from Sigma-Aldrich and NEB respectively unless mentioned otherwise. Oligonucleotide primers used for the study are listed in the supplementary table 1.

2.2 *Bacterial expression, purification, proteolytic assay and Western blot analysis of OsSAG12-2*

OsSAG12-2 coding DNA sequence (CDS) was cloned in pET28a (+) using *Nde*I and *Xho*I restriction enzymes and transformed into *E. coli* strain BL21 (DE3) cells. Protein expression was induced with 0.5 mM IPTG. Proteins were resolved in 12% SDS-PAGE, and transferred to PVDF membrane for western blot analysis. His-tagged protein was detected by using primary anti-His antibody (Millipore, USA, Cat# 05-531) after 1:5000 dilutions and infrared (IR) dye-conjugated secondary anti-rabbit antibody (LICOR, USA Cat# P/N925-32211). The blot was scanned at 800 nm channel for secondary antibody and 700 nm for ladder under IR-image scanner (LICOR, USA model Odyssey). Protein purification was carried out using Ni^{2+} Agarose column (Qiagen, Hilden Germany). The proteolytic assay was carried out as described earlier (Nandi *et al.* 1999). In brief, required amount of enzyme was taken in 225 μL mixture having final concentrations of 1 mM of $CaCl_2$, 10 mM Tris-HCl (pH 8.0). To this, an equal volume of azocasein (2.5%, Sigma Cat # A2765) was added and incubated at 37°C for 30 min before snap chilling on ice. The reaction was stopped by adding trichloroacetic acid (TCA) at 5% final concentration. The mixtures were centrifuged and supernatants were mixed with equal volume of 0.5 N NaOH before taking absorbance at 428 nm.

2.3 *Generation of transgenic RNAi lines*

The amiRNA construct was generated as described earlier (Warthmann et al. 2008). In brief, three PCRs were performed with specific sets of primers on pNW55 as the template, yielding fragments of 256, 87 and 259 bp lengths. The resulting fragments were gel purified and then fused by one PCR with the two flanking primers G-4368 and G-4369. The fusion product of 554 bp was cloned into pTCK303 RNAi vector under maize ubiquitin promoter between *Bam*HI-*Sac*I restriction sites (Wang *et al.* 2004).

The Japonica rice (*Oryza sativa* L. ssp. *japonica*) cultivar TP309 was used for the study. Transformation of embryogenic calli was carried out using *Agrobacterium*-mediated gene transfer technique (Nandi *et al.* 2000; Singh *et al.* 2016). T0 transgenic plants were regenerated in the presence of hygromycin (50 mg/L). After regeneration, the plants were grown for 3 weeks in liquid MS medium supplemented with 50 mg/L hygromycin to confirm the selections. The regenerated plantlets were transferred to soil, covered with a plastic dome and grown inside the tissue culture room for one week before transferring to the glass house.

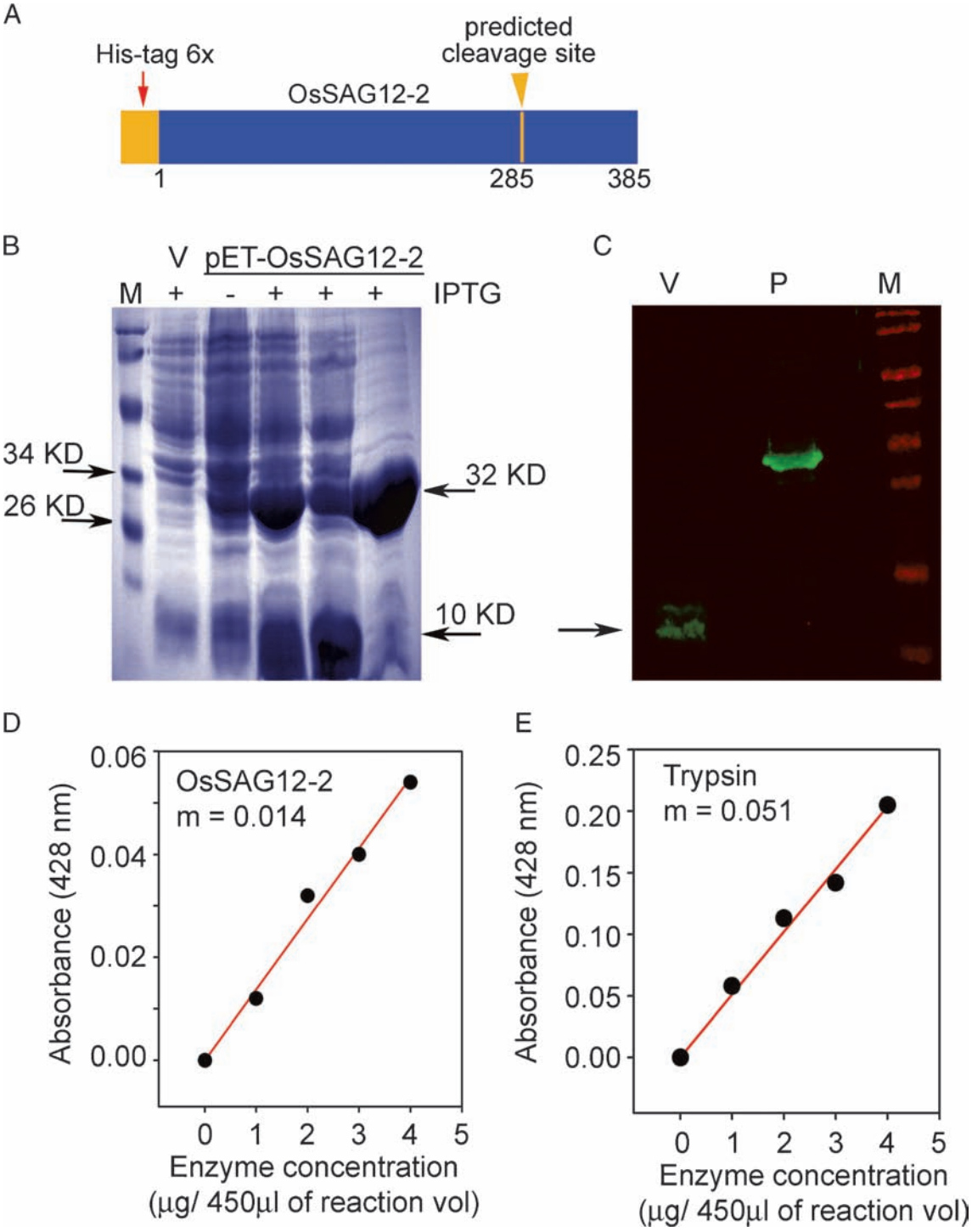

Figure 1. Expression of OsSAG12-2 protein in *E.coli* and Proteolytic activity analysis. (**A**) Sketch of recombinant His-OsSAG12-2 protein. The numbers indicate amino acid positions. Putative cysteine protease cleavage site and 6X 'His-tag' positions are indicated with arrows. (**B**) Coomassie brilliant blue stained polyacryl amide gel showing proteins from empty vector or pET-OsSAG12-2-expressing BL21(DE3) *E.coli* cells. Expression was induced at 37°C with 0.5 mM IPTG for 3 h. M, protein marker; V, only vector; + and –, either induction with IPTG or not; Total, total protein; S, supernatant; P, pellet. (**C**) Confirmation of OsSAG12 protein by Western blot; V-pET28a(+) vector, P-purified protein, M-pre-stained protein ladder. (**D**) Enzymatic kinetics graph for trypsin. (**E**) Enzymatic kinetics graph for OsSAG12-2.

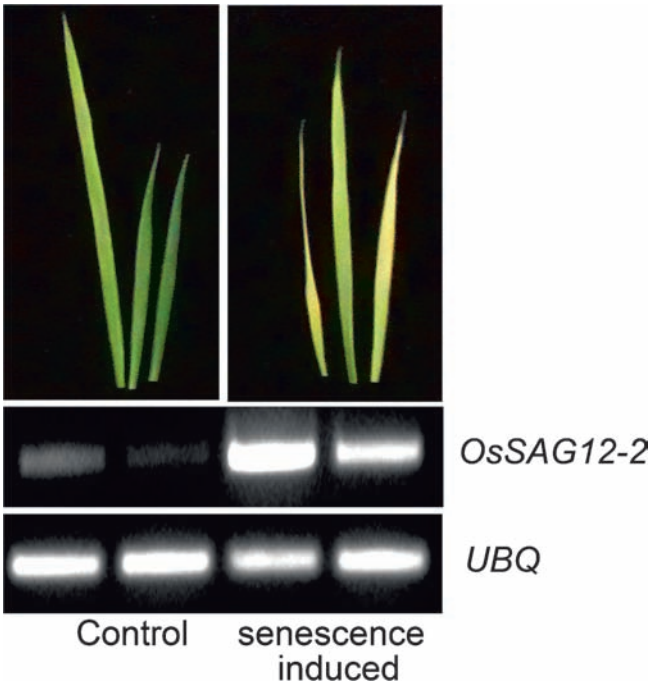

Figure 2. Senescence-induced expression of *OsSAG12-2* gene. Senescence was induced in 3-weeks-old rice plants (upper panel) for 5 days and accumulation of *OsSAG12-2* transcript was determined by reverse-transcription PCR (lower panel).

2.4 *RNA isolation and RT-PCR*

Total RNA was isolated from the leaves of OsSAG12-2 RNAi lines and wild-type (WT) plants using trizol reagent (Chomczynski and Sachhi 1987). The extracted RNA samples were treated with RNase-free DNase I (Fermentas, USA) for 1 h at 37°C and then heat-inactivated at 72°C for 10 min in the presence of 2.5 mM EDTA. The RNA was converted into cDNA using MMLV reverse transcriptase (Fermentas, USA), oligo-dT primer 2.5 mM $MgCl_2$, 2 µg RNA and RNase inhibitor at 42°C for one hour (Singh *et al.* 2013b). The PCR followed by reverse transcription was carried out by the gene specific primers (supplementary table 1).

2.5 *Salt treatment and UV treatment*

Leaves of soil-grown 60-days-old plants were cut and floated in NaCl solutions of different concentration in 6-well transparent plates. For control sets, the leaves were floated in the water. The plates were covered and incubated at 23°C under 12 h/12 h light/dark cycle. Plates were photographed every day. UV treatment and associated cell death measurement were carried out as described previously (Bhattacharjee *et al.* 2015).

3. Results and discussion

3.1 *OsSAG12-2 codes for a functional proteolytic enzyme*

The objective of this study was to investigate whether *SAG12*-like genes from rice code for functional proteases. Previously we showed that down-regulation of *OsSAG12-1* results in developmental cell death and heightened stress-induced cell death (Singh *et al.* 2013a). However, we could

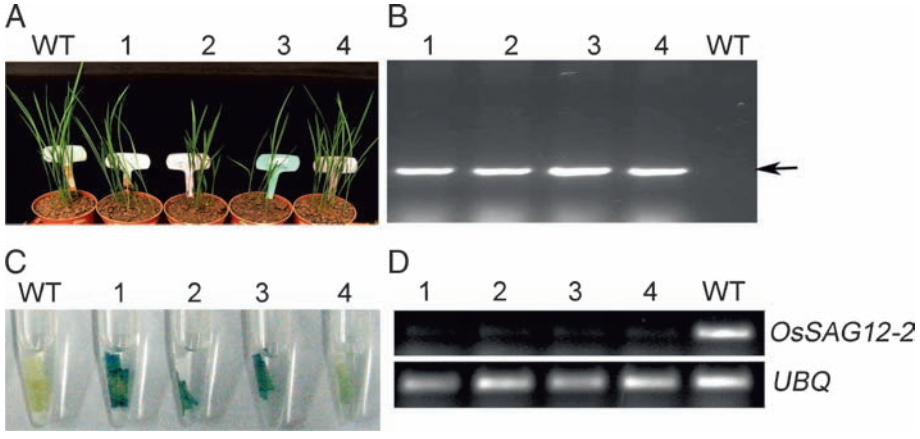

Figure 3. Confirmation of *OsSAG12-2* amiRNA transgenic lines. (**A**) Growth phenotypes of untransformed WT and four different *OsSAG12-2* amiRNA lines. Plants were germinated on soil and grown for 15 days under normal conditions before taking photograph. (**B**) PCR detection of hygromycin resistance marker *hptII* gene, in transgenic lines. Arrow indicates the *hptII*-specific band, (**C**) GUS-expression in the leaves of transgenic plants. (**D**) mRNA abundance of *OsSAG12-2* in WT and transgenic plants after 5 days of dark incubation.

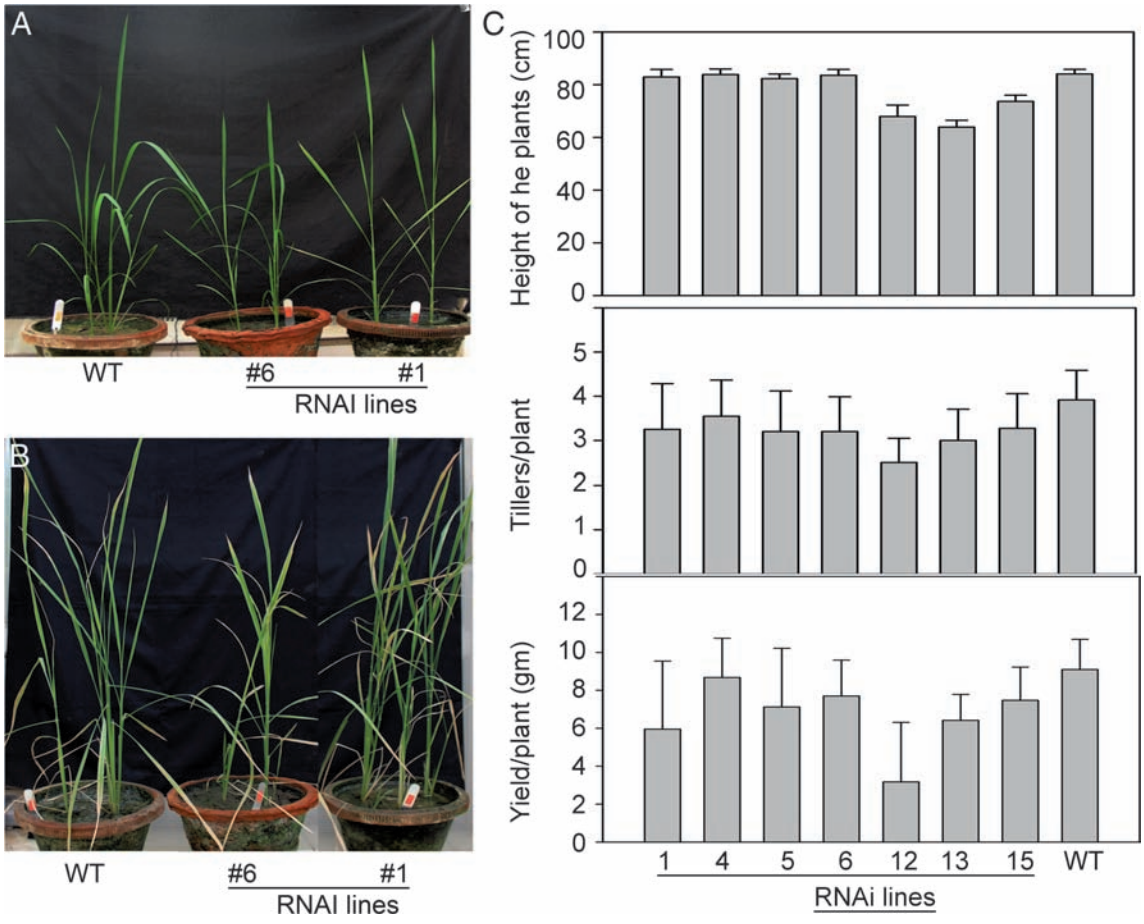

Figure 4. Morphological phenotypes of RNAi lines. (**A**) Phenotype of 60-days-old soil grown transgenic plants. (**B**) Phenotype of 120-days-old soil grown transgenic plants. (**C**) Growth parameters of WT and transgenic plants. Plant height was recorded from 90-days-old greenhouse-grown plants. Number of tillers and seed yield were counted at maturity.

not purify the recombinant OsSAG12-1 protein expressed in *E.coli.* We observed that the expression of the second closest homologue, *OsSAG12-2* was induced upon senescence (described later). The amino acid sequence of OsSAG12-2 was 51% identical and 65% similar to that of AtSAG12 (supplementary figure 1), and 61% identical and 72% similar to OsSAG12-1 sequence (supplementary figure 2). To investigate whether *OsSAG12-2* codes for a functional protease, we produced recombinant protein in *E.coli. OsSAG12-2* was cloned in IPTG-inducible vector pET28a(+) to generate an N-terminal 'His-tag'-fused full-length OsSAG12-2 protein. The predicted size of the His-OsSAG12-2 full-length peptide was 42 kDa (figure 1A). However, in the transformed *E.coli* cell-lysate we observed two IPTG-induced bands of approximately 32 and 10 kDa molecular weight proteins (figure 1B). Whereas the 32 kDa fragment was present in both soluble and precipitated fractions, the 10 kDa fragment was mostly present in the soluble fraction. It was intuitive that the His-OsSAG12-2 peptide was cleaved into two fragments in the *E.coli.*, resulting in two bands. The scan of OsSAG12-2 polypeptide for the possible proteolytic site by cysteine protease (MEROPS peptidases database, *http://merops.sanger.ac.uk*) identified one possible cleavage site at the C-terminal end of the protein (figure 1A). After the predicted cleavage, only the larger product (~32 kDa) would retain the 'His-tag' but not the smaller one (~10 kDa). Western blot analysis of the recombinant proteins using anti-'His-tag' antibody supported the predicted cleavage (figure 1C). The digestive enzymes of the animal gut system such as trypsin and chymotrypsin undergo proteolytic modifications before becoming fully functional (Pace and Barrett 1984). Thus, we postulated that *OsSAG12-2* codes for a functional protease which undergo an autoproteolysis to produce two smaller fragments. However, whether the

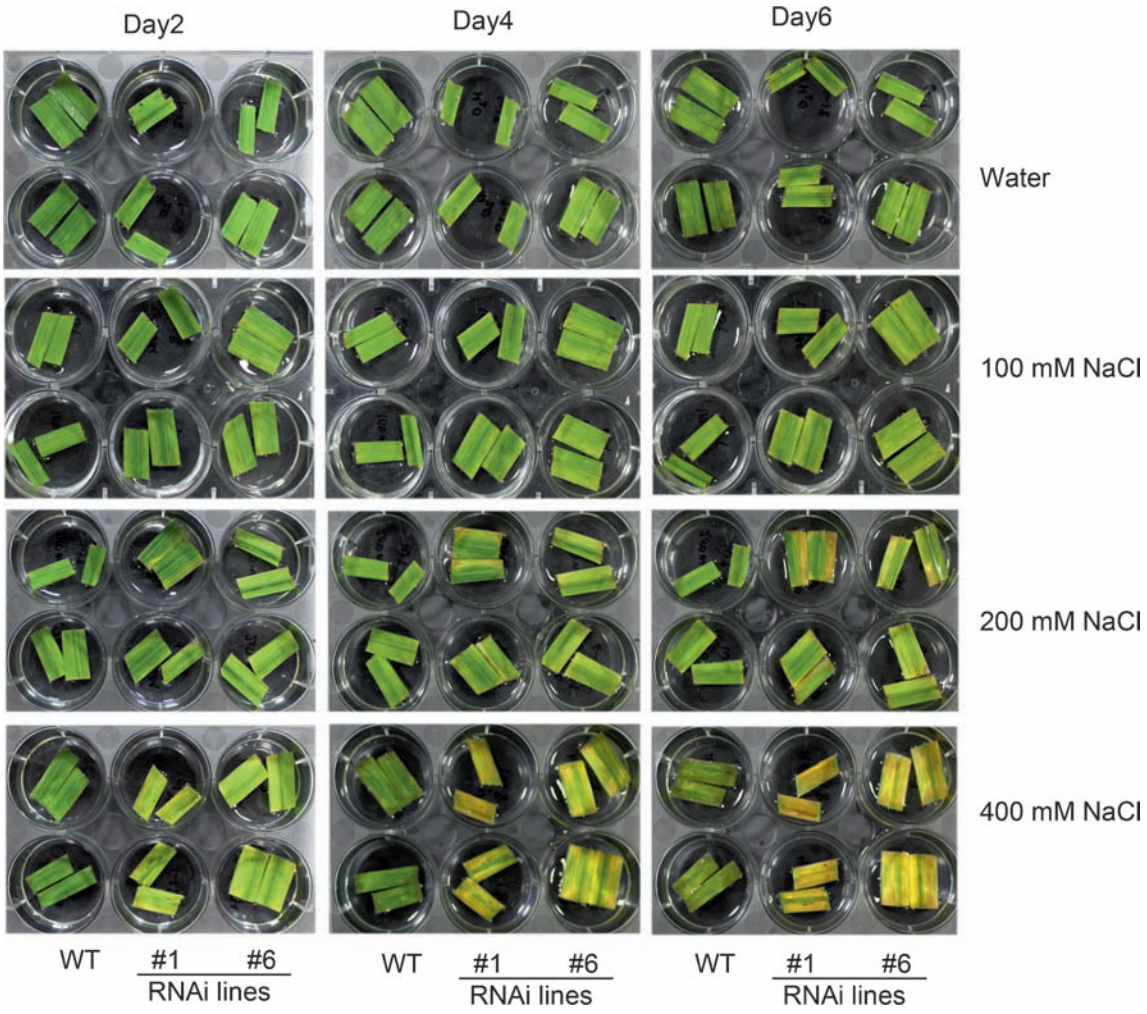

Figure 5. Effect of NaCl treatment on WT and RNAi plants. Cell death phenotype in *OsSAG12-2* RNAi and WT leaves after treatment with 0 (only water), 100, 200 and 400 mM NaCl treatment. All pictures were taken at same magnification.

proteolytic cleavage of OsSAG12-2 was an autolysis event like several digestive proteases, or an *E.coli* protease assisted event, remains to be asserted.

Proteolytic activity assay was carried out using the 32 kDa fragment of the His-OsSAG12-2 fusion Ni^{2+}-agarose column purified protein. Purified protein was capable of cleaving the substrate azocasein substrate *in vitro* (figure 1D). To compare the proteolytic activity of purified protein with commercially available trypsin, both the proteins were used at an increasing concentration and substrate levels were kept in abundance. For determining the rate of reaction, we have taken only the ranges of enzyme concentration that produce a first order kinetics for both trypsin and OsSAG12-2 (figure 1D and E). The slope of the linear regression lines (denoted by m, in the plots, figure 1D and E) was taken as enzyme activity with respect to the quantity of protein. At 0 to 4 µg of protein concentrations, trypsin activity was 0.051 A_{428} per µg of protein, whereas for OsSAG12-2 fragment it was 0.014 A_{428} per µg of protein. Thus, OsSAG12-2 possesses approximately 27% of trypsin equivalent activity on azocasein substrate.

3.2 *Expression of OsSAG12-2 is enhanced in dark-induced senescing leaves*

To investigate, whether the expression of *OsSAG12-2* is associated with senescence, we monitored its mRNA accumulation after inducing senescence in rice by dark treatment. WT TP309 rice plants were grown under normal condition for 3 weeks, after which plants were dark-treated for another 5 days. The experimental control plants were kept under light with similar growth conditions. Expression of *OsSAG12-2* was monitored by reverse-transcription PCR.

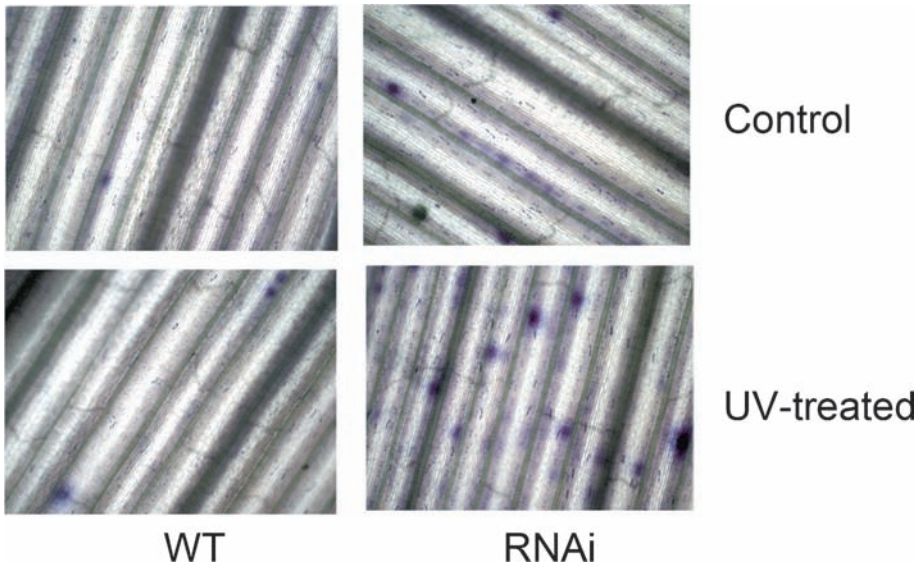

Figure 6. UV-induced cell death in WT and RNAi lines. Plants were treated with UV in laminar air-flow chamber for 5 h and transferred to growth room. After 24 h of treatment, samples were harvested for trypan blue staining.

As shown in figure 2, *OsSAG12-2* expression is highly induced in the dark-treated senescing leaves compared to the control leaves. The results were similar to that was reported earlier for *OsSAG12-1* gene (Singh *et al.* 2013a).

3.3 *OsSAG12-2 RNAi lines show almost normal growth and development under stress-free condition*

To investigate the physiological role of *OsSAG12-2*, we generated transgenic lines under-expressing the gene, through artificial microRNA (amiRNA) technology. The amiRNA construct was generated as described earlier (Warthmann et al. 2008). The vector contains hygromycin resistance marker for selection of transgenic plants. We tested T1 progeny of four independent T0 amiRNA lines (figure 3A). The presence of hygromycin resistance gene in T1 plants was confirmed by RT-PCR (figure 3B) and by the expression of GUS reporter gene (figure 3C). To examine the effect of amiRNA expression in the suppression of *OsSAG12-2*, we determined the abundance of the *OsSAG12-2* transcript in the transgenic plants after dark incubation for 5 days. The untransformed WT plants were used as control. All the amiRNA plants accumulated highly reduced level of *OsSAG12-2* transcript, compared to the untransformed plants (figure 3D), which confirmed efficient suppression of *OsSA12-2* in the transgenic lines. All the transgenic lines were morphologically almost similar to untransformed plants (figure 3A).

Phenotypes of *OsSAG12-2* plants (figure 3A) was in sharp contrast to the *OsSAG12-1* RNAi lines, of which a fraction of plants grew slow and developed spontaneous cell death and early senescence symptoms (Singh *et al.* 2013a). To evaluate the effect of *OsSAG12-2* suppression, we grew T3 population of seven independent transgenic lines and followed the entire growth period (figure 4). Although most of the lines grew normally like WT plants, a few showed reduced growth (figure 4A and B). To quantitatively determine the growth pattern, we measured plant height, the number of tillers per plant and the total seed yield per plant. The plant height recorded at 90 days of growth showed that majority of the transgenic lines had no effect of *OsSAG12-2* suppression, whereas others showed modest suppression of growth (figure 4C). A similar effect was also observed for the number of tillers per plant and the seed yield per plant (figure 4C). The negligible growth-defects in most of the RNAi plants suggest that *OsSAG12-2* does not influence the growth and development of plants under stress-free condition. It is possible that the plants that showed reduced growth might have experienced some kind of stress, as the RNAi lines showed exaggerated stress response compared to the WT plants (discussed in the following section).

3.4 *Down-regulation of OsSAG12-2 enhances salt- and UV-induced cell death*

To investigate whether stress-induce cell death is aggravated in the RNAi lines, we studied the effect of salt and UV treatment on the RNAi plants. Leaves from WT and two of the *OsSAG12-2* RNAi lines were floated on water or salt solutions (100, 200 and 400 mM NaCl) and visible

cell death phenotype was observed over 6 days. Both the transgenic lines developed a higher level of cell death symptoms compared to the untransformed plants when floated in salt solutions (figure 5). Exaggeration of salt-induced cell death was dose dependent. As the concentration of salt was increased the difference between WT and RNAi lines became more obvious. The results suggest that *OsSAG12-2* negatively regulates stress-induced cell death in rice. The results were further confirmed by inducing cell death with UV light. UV-treated RNAi leaves showed a much higher level of cell death compared to WT plants (figure 6).

Proteolytic activity is directly associated with the process of senescence, especially for breaking down of cellular constituents (Lim *et al.* 2007; Roberts *et al.* 2012; Carrion *et al.* 2013). In spite of a large number of reports about the induction of several proteolytic enzyme coding genes during senescence, their role in the process of execution or regulation of cell death largely remain obscure. Application of cysteine protease inhibitor AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride] or DFP (diisopropyl-fluorophosphate) delays senescence in iris flower (Pak and van Doorn 2005). Similarly, application of cysteine protease inhibitor E-64 inhibits the formation of senescence-associated vacuoles (SAV) which are important for degradation of chloroplastic proteins, and delays dark-induced senescence in tobacco leaves (Carrion *et al.* 2013). AtSAG12 also expresses in SAVs. However, *atsag12* mutants are neither defective in SAV formation, nor in senescence (Otegui *et al.* 2005). In contrary, down-regulation of rice homologues, *OsSAG12-1* promotes developmental and stress induced senescence (Singh *et al.* 2013a). OsSAG12-2 which is structurally close to both AtSAG12 and OsSAG12-1 also support a similar, negative regulatory role for stress- induced senescence. Thus, it is possible that rice SAG12 homologues are recruited to control excessive cell death. Finding cellular targets of OsSAG12 genes will improve our understanding about this complex process of programmed cell death.

4. Conclusion

OsSAG12-2 is structurally related to the earlier reported senescence-associated genes OsSAG12-1 and AtSAG12. *AtSAG12* is the most characterized senescence-associated gene, in terms of expression pattern, and utility in controlling senescence of plants. The physiological role of AtSAG12 is not known; however, it is believed to aid cell death process by its proteolytic activity. In contrast, rice homologues OsSAG12-1 and OsSAG12-2 function as negative regulators of cell death. *OsSAG12-2* codes for a functional proteolytic enzyme. This proteolytic activity may be required to control the activity of such proteins that influence stress-induced cell death.

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