Down-regulation of OsSAG12-1 results in enhanced senescence and pathogen-induced cell death in transgenic rice plants

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Senescence is a highly regulated process accompanied by changes in gene expression. While the mRNA levels of most genes decline, the mRNA levels of specific genes (senescence associated genes, SAGs) increase during senescence. *Arabidopsis SAG12* (*AtSAG12*) gene codes for papain-like cysteine protease. The promoter of *AtSAG12* is SA-responsive and reported to be useful to delay senescence by expressing cytokinin biosynthesis gene isopentenyltransferase specifically during senescence in several plants including *Arabidopsis*, lettuce and rice. The physiological role of *AtSAG12* is not known; the homozygous *atsag12* mutant neither fails to develop senescence-associated vacuoles nor shows any morphological phenotype. Through BLAST search using AtSAG12 amino acid sequences as query, we identified a few putative homologues from rice genome (OsSAGs; *Oryza sativa* SAGs). OsSAG12-1 is the closest homologue of AtSAG12 with 64% similar amino acid composition. Expression of *OsSAG12-1* is induced during senescence and pathogen-induced cell death. To evaluate the possible role of *OsSAG12-1* we generated RNAi transgenic lines in Japonica rice cultivar TP309. The transgenic lines developed early senescence at varying levels and showed enhanced cell death when inoculated with bacterial pathogen *Xanthomonas oryzae pv.oryzae.* Our results suggest that OsSAG12-1 is a negative regulator of cell death in rice.

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1. Introduction

Rice is the most important crop in the world that provides staple food nearly half of the world's population and accounts for more than 50% of their daily calorie intake (*www.irri.org*). Supply of rice always remained a concern to meet the hunger of the growing population. Delaying leaf senescence by extending the available time for photosynthesis may be a very effective way to increase crop yield (Liu *et al.* 2010; Zhang *et al.* 2010).

Senescence of the leaves is associated with sequential programmed death of matured cells, tissues, organs or sometimes the entire plant (Lim *et al.* 2007). The senescence in perennial evergreen plants is a continuous process in which the older leaves undergo this programmed death. In the contrast, annual plants like rice undergo a process known as reproductive senescence during which the entire plant undergoes this death process after completion of the reproductive phase of life. In a senescing leaf, many genes that are expressed in green leaves, including those genes involved in photosynthesis, are down-regulated, while a subset of genes, generally referred to as senescence-associated genes (SAGs), are up-regulated. Even though several SAGs are reported for different plants, a few are reported to be strictly associated

Keywords. OsSAG12; rice; RNAi; SAG12; *Xanthomonas oryzae*

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with senescence, such as *SWEET POTATO CYSTEINE PROTEASE 3* (*SPCP3*) from sweet potato (Chen *et al.* 2006), *SENESCENCE ASSOCIATED PROTEIN 15* (*SPA15*) of sweet potato and its homologue from rice (Yap *et al.* 2003), *SAG12* from *Arabidopsis (Otegui et al.* 2005*)*, SAG39 from rice (Liu *et al.* 2010), etc. Through PCR based subtractive hybridization 14 genes from rice have been shown to be associated with dark induced senescence (Lee *et al.* 2001). However, the physiological roles played by most of these SAGs are not known (Lim *et al.* 2007; Park *et al.* 2007; Fischer-Kilbienski *et al.* 2010).

Arabidopsis SAG12 gene (referred as *AtSAG12* hereafter) is the best characterized gene amongst the SAGs reported from plant species (Miller *et al.* 1999; Noh and Amasino 1999a; Otegui *et al.* 2005; Ay *et al.* 2009; Brusslan *et al.* 2012). The plant hormone cytokinin negatively regulates senescence process. Expression of cytokinin biosynthesis gene isopentenyltransferase (ipt) under the *AtSAG12* promoter significantly delayed natural and stress-induced senescence in *Arabidopsis*, wheat and lettuce (McCabe *et al.* 2001; Liu *et al.* 2005; Sykorova *et al.* 2008). Two *AtSAG12* homologues from *Brassica napus*, *BnSAG12-1* and *BnSAG12-2* have been identified by screening the cDNA library (Noh and Amasino, 1999b). These two genes under their native promoters showed senescence-specific induction in transgenic *Arabidopsis*, indicating the presence of evolutionarily conserved expression regulation system for the *SAG12* genes (Noh and Amasino 1999b). Expression of *AtSAG12* is restricted to the senescence-associated vacuoles (SAV) (Otegui *et al.* 2005). In spite of the large volume of research, the physiological role of AtSAG12 is not known. The homozygous *atsag12* mutants show normal development of SAVs and normal senescence (Otegui *et al.* 2005).

AtSAG12 homologue from rice is not yet identified. Expression of SAG39 gene of rice with 55% identity to AtSAG12 has been shown to be associated with senescence (Liu *et al.* 2010). SAG39 promoter-driven expression of *ipt* gene (*pSAG39-ipt*) in the transgenic rice plant shows delayed senescence, supporting the similar expression regulation to that of *AtSAG12* (Liu *et al.* 2010). However, like the *Arabidopsis* counterpart, the physiological role of SAG39 is also unclear. Here we report the identification, expression profile and physiological role of an AtSAG12 homologue from rice. Our data shows that OsSAG12-1 is negative regulator of cell death in contrast to the proposed function of this class of proteases.

2. Materials and methods

2.1 *RNAi vector construction and transformation*

The siRNA target area was amplified from TP309 genomic DNA with primers (OsSAG12-1 F1-TTC AAG GAC AAC GTG AGG TAC A and OsSAG12-1 R1-GTA GAA CTG GAA GGA CTG GTC G. The amplicon was cloned into the pTZ57R/T vector (T/A cloning vector, Fermentas, USA). The orientation of the insert was checked with appropriate enzymes. From the two oppositely oriented clones *Bam*HI-*Kpn*I and *Spe*I-*Sac*I fragments were cloned into pTCK303 vector (Wang *et al.* 2004). The Japonica rice cultivar TP309 (*Oryza sativa* L. ssp. *japonica* cv.Taipei 309) was used in the study. Transformation of embryogenic calli was carried out by using *Agrobacterium*-mediated gene transfer technique as described previously (Nandi *et al.* 2000). T0 transgenic plants were regenerated in the presence of hygromycin 50 mg/L. After regeneration the plants were grown for 3 weeks on liquid MS medium supplemented with 50 mg/L hygromycin to confirm the selections. The regenerated plantlets were transferred to soil, covered with plastic dome and grown inside the tissue culture room for 1 week before transferring to the glass house.

2.2 *Trypan blue staining*

Freshly harvested leaves were boiled with lactophenol-trypan blue solution (10 mL lactic acid, 10 mL glycerol, 10 mL molten phenol, 10 mg trypan blue dissolved in 10 mL water) for 1 min in the microwave oven. The leaves were destained for overnight with lactophenol solution without trypan blue and finally placed in 50% ethanol before viewing under light microscope (Swain *et al.* 2011).

2.3 *RNA isolation and RT-PCR*

Total RNA was isolated from the leaves of *OsSAG12-1* RNAi lines and wild-type plants using trizol as described earlier for *Arabidopsis* (Nandi *et al.* 2004). 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 presence of 2.5 mM EDTA. The RNA was converted into cDNA using MMLV reverse transcriptase (Fermentas, USA), oligo-dT primer 2.5mM $MgCl_2$, 2μg RNA and RNase inhibitor at 42°C for 1 h. The PCR followed by reverse transcription was carried out by the same gene-specific primers that were used for generating RNAi target. RT-PCR for rice ubiquitin was followed as control (Primers used Rice Ubq F1-CCA GGA CAA GAT GAT CTG CC and Rice Ubq R1-AAG AAG CTG AAG CAT CCA GC).

2.4 *Southern blot analysis*

Genomic DNA was extracted from leaves of transgenic and control plants using the cetyltrimethylammonium bromide (CTAB) method (Maguire *et al.* 1991). A total of 10 μg of

genomic DNA digested with *Bam*HI was resolved by electrophoresis on 0.8% agarose gel. Transfer of DNA, hybridization with radioactive GUS gene probe and detection of the band was carried out as per standard protocols (Sambrook and Russel 2001).

2.5 *Xanthomonas oryzae pv.oryzae (Xoo) inoculation*

Xoo culture was grown in 10 mL volume bacto™ protease peptone-sugar (PS) media at 28°C by rotating shaker at 220 rpm for 2 days. The culture was centrifuged at room temperature and pellet was resuspended in the sterile water. Leaves were inoculated by clipping with a scissor dipped in the bacterial solution (Subramoni and Sonti 2005). The inoculated plants were covered with polybags for maintaining 70–80% moisture for 3 days. Symptoms were analyzed at 10 days post inoculation.

2.6 *Subcellular localization*

GFP coding sequences (CDS) was amplified from pEGFP-N1vector (Clonetech, USA) using specific primers (EGFP-EcoRI-GAA TTC ATG GTG AGC AAG GGC and EGFP-SacI-GAG CTC TTA CTT GTA CAG CTC), cloned in pBSK+ vector (pBSK-GFP). *OsSAG12-1* full-length CDS was amplified from TP309 wild-type using primer set OsSAG12-1-Kpn1-GGT ACC ATG GGG AGG GTT ATT and OsSAG12-1-EcoRI-GAA TTC CTG GGT TTC CTT GGC. The amplicon was cloned in TA and then transferred to pBSK-GFP clone, which generated pBSK-OsSAG12-1-GFP. The OsSAG12-1-GFP fragment was released as *Kpn*I and *Sac*I fragments and cloned into pTCK303 vector under maize ubiquitin promoter to generate final construct. For vector control only GFP was cloned directly, under maize ubiquitin promoter of pTCK303 within *Bam*HI and *Sac*I sites. All the recombinant vectors Ubi:GFP (vector control) and Ubi:OsSAG12-1-EGFP were transferred into *Agrobacterium* strain C58 using the standard cloning techniques. Healthy and fresh onion scales (1–1.5×1 cm) were placed on a 9 cm plate and their inner surfaces were immersed into 6 mL resuspension *Agrobacterium* solution (OD600=1–1.5) consisting of 5% (g/v) sucrose, 100 mg acetosyringone/L and 0.02% (v/v) Silwet-77 for 6–12 h at 28°C. Then the onion scales were transferred to a Petri dish containing about 25 mL 1/2 MS (Murashige and Skoog salts, 30 g sucrose/L and 0.7% (g/v) agar, pH 5.7) and co-cultivated with *Agrobacterium* for 1–2 days. The scales were rinsed with water, and the onion epidermal cell layers were peeled and directly transferred to glass slides. Fluorescence images were screened using a confocal laser microscope LSM 5 PASCAL (Carl Zeiss) equipped with 20× lenses and analysed by LSM 5 Image Browser software.

3. Results and discussion

3.1 *Identification of OsSAG12-1*

Approximately 60 genes from rice genome were picked up by tBLASTn search when AtSAG12 amino acid sequence was used as the query. To identify the homologue we gave the priority to sequence similarity and query coverage. Table 1 shows the list of 10 closest homologues from rice genome with the query coverage ranging from 56.65% to 94.22%. The rice gene *LOC_Os01g67980* annotated as cysteine protease EP-B1 precursor, putative, and expressed showed highest similarity (referred as *OsSAG12-1* hereafter), was selected for further study. This homologue from rice showed 47% identity and 64% similarity with *AtSAG12-1* peptide (figure 1A). The PROSITE pattern search and PFAM analyses (*http://myhits.isb-sib.ch/cgi-bin/motif_scan*) identified two conserved regions in both the homologues. The larger C-terminal conserved domain comprising approximate 210 aa belongs to the peptidase family (pfam:fs_peptidase_C1), whereas, the smaller

Table 1. Homologues of AtSAG12 as obtained by using tBLASTn search from the rice genome

Accession No.	Description	E-value	Query coverage
1. LOC_Os01g67980	cysteine proteinase EP-B 1 precursor, putative, expressed	2.6e−79	94.22%
2. LOC_Os07g01800	cysteine proteinase EP-B 1 precursor, putative, expressed	3.4e−79	90.46%
3. LOC_Os08g44270	vignain precursor, putative, expressed	4.6e−81	87.86%
4. LOC_Os04g57490	cysteine protease, putative, expressed	1.1e−77	87.86%
5. LOC_Os01g73980	cysteine proteinase 2 precursor, putative, expressed	6.8e−80	69.08%
6. LOC_Os05g01810	cysteine proteinase 2 precursor, putative, expressed	2.5e−80	69.08%
7. LOC_Os01g42790	cysteine proteinase 2 precursor, putative, expressed	4.2e−50	65.32%
8. LOC_Os03g54130	cysteine protease 1 precursor, putative, expressed	2.8e−85	56.65%
9. LOC_Os12g17540	vignain precursor, putative, expressed	3.9e−96	56.65%
10. LOC_Os04g13140	vignain precursor, putative, expressed	1.4e−96	56.65%

The 10 closest homologues have been arranged in descending order of query coverage.

(A)

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At 18    FCFSITLS-RPLDNELIMQKRHIEWMTKHGRVYADVKEENNRYVVFKNNVERIEHLNSIP  76
          C +I    R L+++  +   +  W   H  V     E++ R+  FK+NV  I H ++
Os 25    LCAAIPFDERDLESDEALWDLYERWQEHH-HVPRHHGEKHRRFGAFKDNVRYI-HEHNKR  82

At 77    AGRTFKLAVNQFADLTNDEFRSMYTGFKGVSALSSQSQTKMSP-FRYQNVSSGALPVSVD  135
          GR ++L +N+F D+  +EFR+ + G                P F Y+ V    LP +VD
Os 83    GGRGYRLRLNRFGDMGREEFRATFAGSHANDLRRDGLAAPPLPGFMYEGVRD--LPRAVD  140

At 136.  WRKKGAVTPIKNQGSCGCCWAFSAVAAIEGATQIKKGKLISLSEQQLVDCDTND-FGCEG  194
         WR+KGAVT +K+QG CG CWAFS V ++EG   I+ G+L+SLSEQ+L+DCDT D  GC+G
Os 141   WRRKGAVTGVKDQGKCGSCWAFSTVVSVEGINAIRTGRLVSLSEQELIDCDTADNSGCQG  200

At 195   GLMDTAFEHIKATGGLTTESNYPYKGEDATCNS-KKTNPKATSITGYEDVPVNDEQALMK  253
         GLM+ AFE+IK +GG+TTES YPY+  + TC++ +        I G+++VP N E AL K
Os 201   GLMENAFEYIKHSGGITTESAYPYRAANGTCDAVRARRAPLVVIDGHQNVPANSEAALAK  260

At 254   AVAHQPVSVGIEGGGFDFQFYSSGVFTGECTTYLDHAVTAIGYGESTNGSKYWIIKNSWG  313
         AVA+QPVSV I+ G   FQFYS GVF G+C T LDH V  +GYGE+ +G++YWI+KNSWG
Os  261  AVANQPVSVAIDAGDQSFQFYSDGVFAGDCGTDLDHGVAVVGYGETNDGTEYWIVKNSWG  320

At 314   TKWGESGYMRIQKDVKDKQGLCGLAMKASYPT  345
         T WGE GY+R+Q+D     GLCG+AM+ASYP
Os 321   TAWGEGGYIRMQRDSGYDGGLCGIAMEASYPV  352
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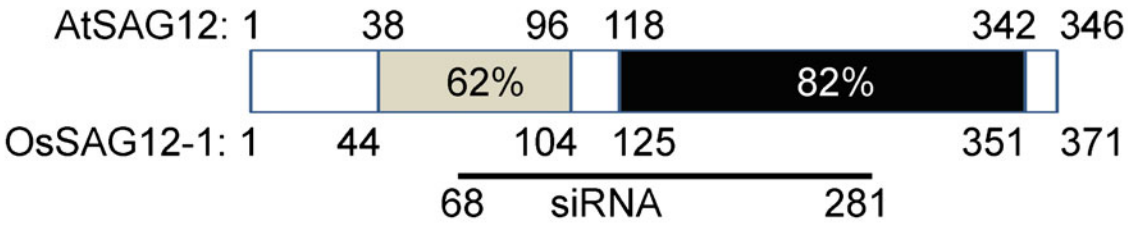
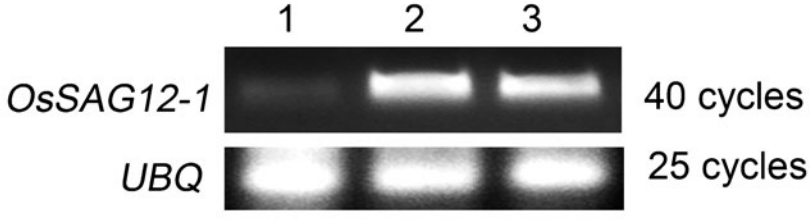
(B)



(C)

(D)



◀ **Figure 1.** OsSAG12-1 homology with AtSAG12 and expression profile. (**A**) Two-way BLAST output of AtSAG12 (At) and OsSAG12-1 (Os) amino acids. (**B**) The proteinase inhibitor I29/cathepsin propeptide and the C1 peptidase domains are shown in grey and black boxes respectively and comparatively less similar areas are shown by white boxes. The percent similarity within the domains between *Arabidopsis* and rice homologues are shown inside the boxes and corresponding positions in their peptides are shown above and below the boxes. The line below denotes the area selected for generating RNAi transgenic lines. (**C**) Photograph of three different leaves from WT plants that have been used to determine *OsSAG12-1* expression. The numbers 1, 2 and 3 below the leaves indicate the increasing order of age. (**D**) Reverse-transcription PCR for detecting expression of *OsSAG12-1* and *Ubiquitin* (UBQ) mRNA. The lane number of the gel corresponds to the leaf number of the photo in panel (**C**).

N-terminal region encompassing N-terminal 68 amino acids belongs to a Proteinase inhibitor I29, cathepsin propeptide (pfam_ls:Inhibitor_I29) (figure 1B). These two domains from rice and *Arabidopsis* share 82% and 62% similar amino acid compositions respectively (figure 1B). Similar to the expression profile of its counterpart in *Arabidopsis*, the *OsSAG12-1* expression is extremely low in the early vegetative phase and in the green leaves and rapidly increases during senescence (figure 1C and D).

3.2 *Generation of OsSAG12-1 RNAi transgenic lines*

For generating siRNA lines, a 639 bp region from rice genomic DNA was selected that is specific for OsSAG12 peptide coding region spanning from 68 to 281 aa position (figure 1B). The region was amplified by PCR using rice genomic DNA as template as the *OsSAG12-1* ORF was not interrupted with intron and cloned two times in opposite orientation in the rice RNAi vector pTCK303 (Wang *et al.* 2004) as shown in figure 2A. This vector construct when transformed into rice would generate a transcript from ubiquitin promoter having duplex stem formed by the oppositely oriented part of *OsSAG12-1* ORF and hair-pin structure from the rice intronic region suitable for generating siRNA molecules against the endogenous *OsSAG12-1* transcript in the host (Wang *et al.* 2004).

*OsSAG12-1*RNAi transgenic rice plants were generated by transforming embryogenic calli obtained from TP309 seeds using EHA105 strain of *Agrobacterium* (supplementary figure 1). The transformed calli were selected two rounds and regenerated in the presence of hygromycin. The regenerated plants were further screened in the liquid medium NB6 medium supplementd with 50 mg/L hygromycin for 4 weeks before transferring to soil. Integration of transgene was confirmed in the first three plants by Southern blot that were

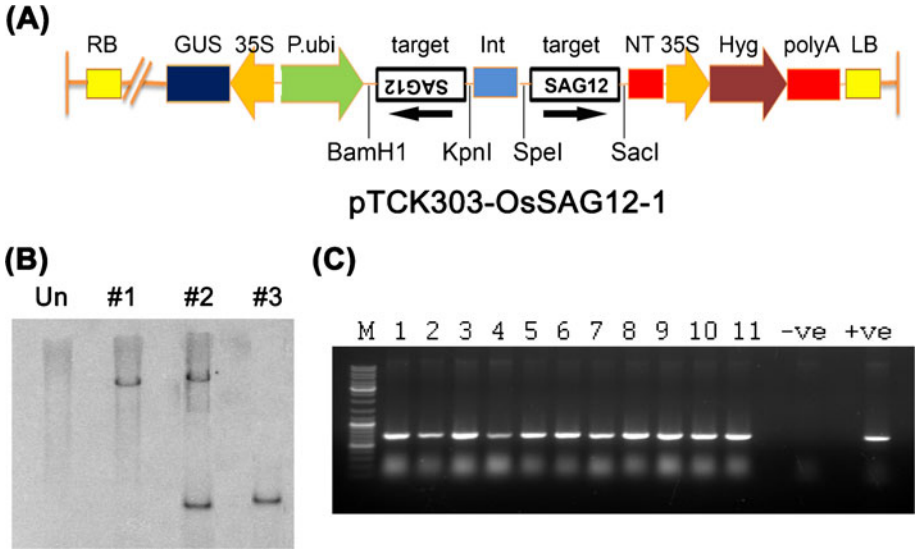
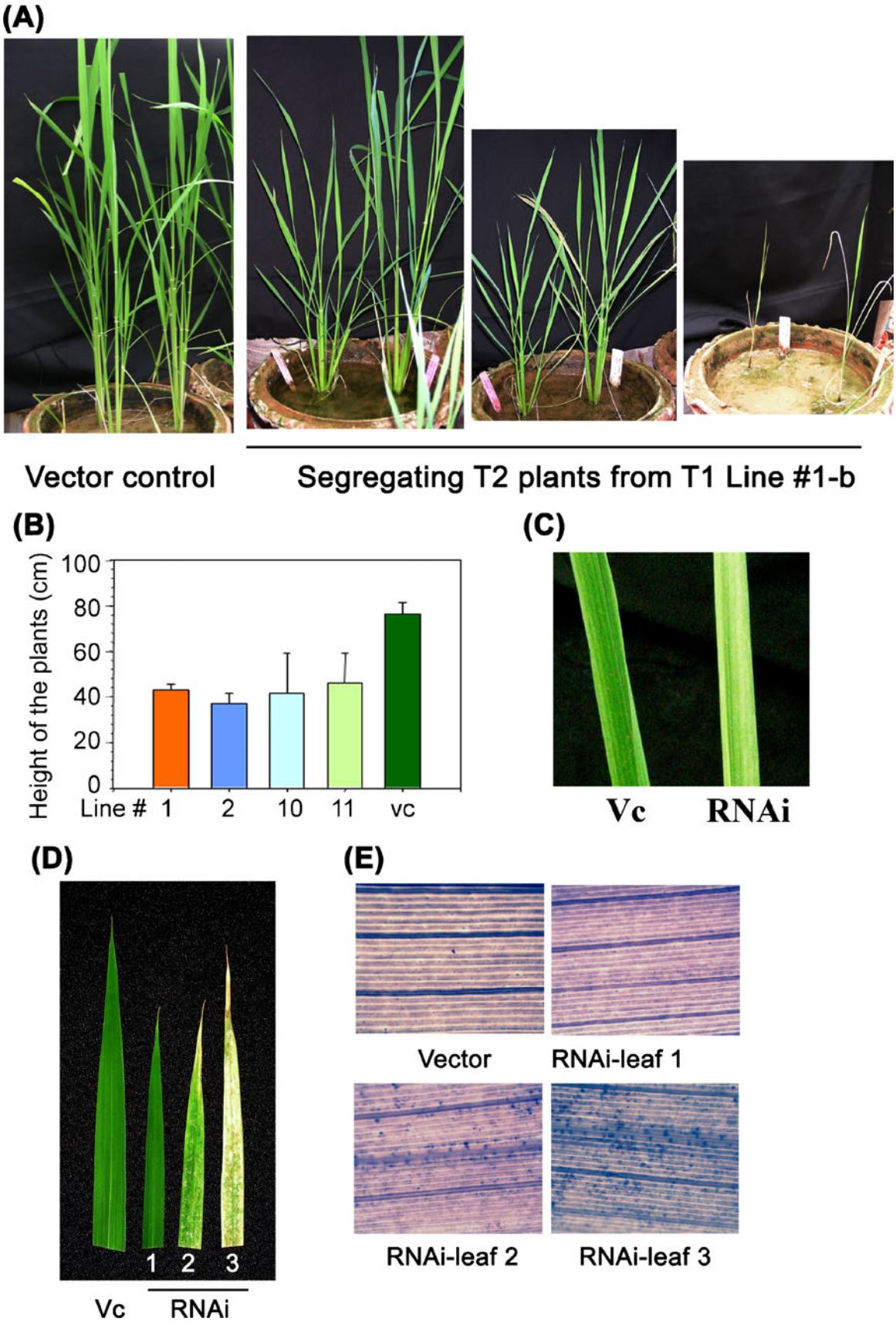


Figure 2. RNAi vector and confirmation of transgenic plant generation. (**A**) Map of pTCK303-OsSAG12-1 RNAi vector. RB – right border, GUS – glucuronidase, P.ubi – maize ubiquitin promoter, target – RNAi target, int- rice intron, NT – NOS terminator, 35S – CaMV 35S promoter, Hyg – *hpt* gene coding for hygromycin resistance, poly A – terminator, LB – left border. The restriction enzymes used for cloning the construct and the direction of RNAi target cloning are mentioned below the figure. (**B**) Southern hybridization to detect stable integration in T0 transgenic lines. The numbers indicate transgenic line numbers. Un – untransformed plant. The genomic DNA was digested with *Bam*HI and probed with radio-labelled GUS coding DNA. (**C**) Confirmation of transgenic plants by PCR for the presence of *hpt* gene. In (**B**) and (**C**) DNA was isolated from the leaves of the T0 lines after transferring to the soil.


(A)
Vector control
Segregating T2 plants from T1 Line #1-b
(B)
Height of the plants (cm)
100
80
60
40
20
0
Line # 1 2 10 11 vc
(C)
Vc RNAi
(D)
1 2 3
Vc RNAi
(E)
Vector
RNAi-leaf 1
RNAi-leaf 2
RNAi-leaf 3

transferred to the soil (figure 2B). All the T0 plants were tested for the presence of the *hygromycin phosphotransferase* (*hpt*) gene that confers hygromycin resistance in the transgenic plants by PCR (figure 2C). The seeds were collected from several independent T0 plants and the next generation was analysed .

3.3 *OsSAG12-1 RNAi lines develop early senescence*

The T1 seeds from *OsSAG12-1* RNAi plants and from empty vector transformed lines were germinated on soil. Since the T0 plants were hemizygous, the segregation of the transgene was followed by GUS assay (supplementary figure 2). Only the GUS-positive plants were retained and grown. To our surprise we observed that T1 plants from most of the T0 lines showed varied degree of early senescence phenotypes. Almost 15% of the plants died before transition to reproductive stage, similar to that shown in figure 3A (the plants in the right most pot). The other plants from T1 lines eventually gave rise to seeds. Interestingly, the T2 generation of most of these T1 lines showed varied degree of early death phenotype as mentioned earlier for T1 generation. The figure 3A shows differently growing T2 plants obtained from a single T1 plant. All RNAi lines showed reduced overall growth including height compared to the vector transformed plants (figure 3B). Figure 3B shows the average and standard deviation of heights of the T1 progeny plants obtained from 4 independent T0 RNAi lines and vector-transformed plants at 60 days after sowing. Other than those extreme phenotypic plants that died early, most of the remaining plants also showed accelerated senescence phenotype especially in their flag leaves. The flag leaves from RNAi plants showed comparatively early senescence compared to vector-transformed plants (figure 3C). Flag leaves are the primary source of nutrient mobilization during the grain filling stage of rice and showed faster senescence compared to the other leaves (Biswas and Choudhuri, 1980). Majority of the other leaves also showed a range of early senescence phenotypes. While the tips of most of the leaves from vector-transformed plants were green after 60 days, the RNAi plants showed varied degree of senescence (figure 3D). This is in contrast to the flag leaves that showed early senescence throughout the leaf blade. To determine if the senescing phenotypes of leaves were associated with the cell death, we stained the leaves with trypan blue, a dye that specifically binds with dead cells. Figure 3E shows the trypan blue staining from the cut end of the same leaves shown in figure 3D. The results indicate that indeed there is significant enhancement in the number of dead cells in the RNAi plants compared to the vector-transformed plants and the senescing phenotype is in consistence with the cell death phenotype. Thus the normal function of OsSAG12-1 appears to negatively regulate the senescence process.

3.4 *The apparent healthy leaves of OsSAG12-1 RNAi plants show enhanced cell death when inoculated with Xanthomonas oryzae*

As described in the previous section, the *OsSAG2-1* RNAi lines showed range of cell death phenotypes. A large number of leaves from these RNAi plants, as shown for leaf-1 from RNAi line (figure 3D and E), did not show any obvious cell death and senescing phenotype. We wanted to test if these apparent healthy leaves would show enhanced cell death induced by a pathogen like *Xanthomonas oryzae* pv *oryzae* (Xoo). Xoo is known to induce cell death in rice (Park *et al.* 2008; Aparna *et al.* 2009). The vegetative healthy leaves from the 40-day-old transgenic plants were inoculated with Xoo and cell death followed. The leaves from RNAi plants showed significantly enhanced symptoms than the vector-transformed plants (figure 4A). The extent of cell death phenotype from four different transgenic lines and vector-transformed plants are shown in the figure 4B. All the RNAi lines showed much enhanced cell death compared to the control plants. We tested if there was any correlation of *OsSAG12-1* RNA suppression of cell death phenotype. The total RNA from the inoculated vector-transformed RNAi plants and as well as un-inoculated vector-transformed plants were extracted and accumulation of *OsSAG12-1* RNA was monitored after reverse-transcription PCR. As expected we could detect amplification in the inoculated vector transformed plant but not from the un-inoculated leaves after 40 cycles of PCR (figure 4C). The inoculated leaves from the RNAi T1 line #10, #11 and #2 showed reduced accumulation of the *OsSAG12-1* transcript (figure 4C). The level of this transcript accumulation was inversely correlated with cell death phenotype (figure 4A). The results clearly demonstrate that even though these leaves do not produce developmental cell death, the RNAi lines having reduced expression of *OsSAG12-1* exaggerated the cell death induced

◀ **Figure 3.** Phenotypes of OsSAG12-1 RNAi plants. (**A**) Morphological phenotype of 60-day-old soil-grown T2 plants obtained from vector transformed and RNAi plants obtained from a single T1 line #1-b that was apparently healthy in T1 generation. (**B**) Average heights of T1 progeny plants from four different T0 transgenic lines after 60 days of growth. Each bar represents mean and standard deviation of 8 T1 progeny plants. (**C**) Photograph of flag leaves from vector transformed and RNAi lines at the same age during early grain filling stage. (**D**) Photograph of tip of leaves showing ranges of senescence phenotype. The numbers 1, 2 and 3 are assigned to link the cell death phenotype in the plants as shown in the next panel. (**E**) Trypan blue staining of the same leaves as shown in (**D**) near the cut end.

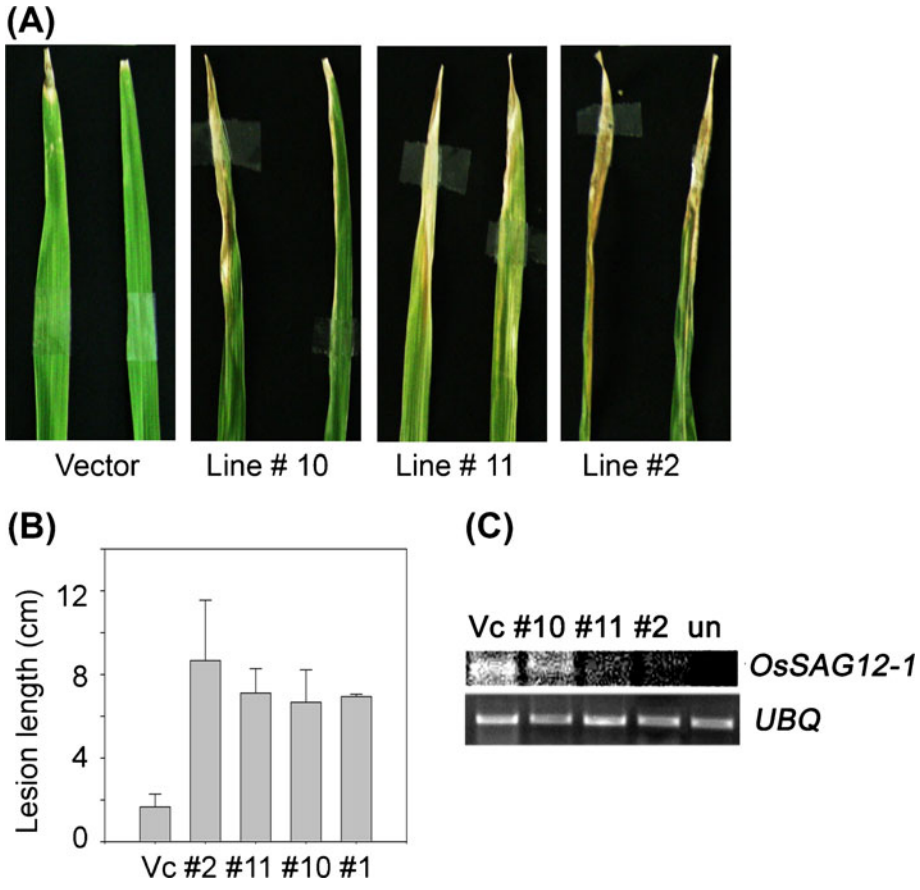


Figure 4. Xoo-induced cell death is enhanced in RNAi lines. (**A**) Healthy leaves from 40-days-old soil-grown plants were cut inoculated with Xoo at 10^8 CFU/mL. Photograph was taken at 10 days post inoculation. (**B**) Average and standard deviations of lesion length from the leaves of vector control (Vc) and RNAi lines after 10 days post inoculation with Xoo (N=5). (**C**) RT-PCR to determine relative abundance of OsAG12-1 mRNA from the vector control (Vc), indicated transgenic lines and un-infected vector transformed plant (Un).

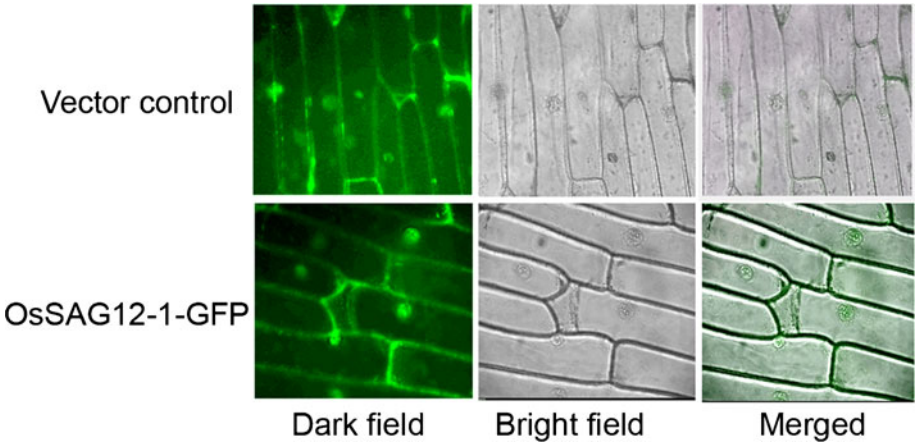


Figure 5. Subcellular localization of OsSAG12-1-GFP. OsSAG12-1-GFP construct was transformed into onion peel by *Agrobacterium* mediated gene transfer. The bright field, dark field images were taken at 3 days post inoculation by a confocal microscope.

by pathogens. In other words the normal function of the *OsSAG12-1* product is likely to minimize the cell death phenotype.

3.5 *OsSAG12-1-GFP localization is not restricted to vacuolar compartments*

AtSAG12 has been shown to be localized with senescence-associated vacuoles (Otegui *et al.* 2005). To determine if the rice homologue also shows similar pattern to sub-cellular localization, we generated OsSAG12-1-GFP construct and followed its localization after transforming onion-peel cells. The recipient vector providing Ubi:GFP served as vector control. As expected the vector-control-transformed cells showed fluorescence throughout the cells (figure 5). However, contrary to our expectation, we observed dispersed fluorescence in the Ubi:OsSAG12-1-GFP tissues. Even though further experiments are required with sub-cellular compartment specific dyes to determine its localization, this initial experiment indicated that OsSAG12-1-GFP is not restricted to the vacuolar compartment as observed in *Arabidopsis*.

4. Conclusion

Our present study provides new insight into the possible physiological role of cysteine-proteases that up-regulate during senescence. It is hypothesized that largely these proteases are responsible for degradation of proteins in the senescing tissue into smaller fragments or into amino acids for recycling/transporting into the developing tissues. Our data indicate that there are proteases that function to negatively regulate the senescence/cell death, essentially to ensure that process of cell death goes slow or at optimum pace matching physiological requirement by the plant. One possible functional mechanism of this kind of cysteine protease could be that they have specific targets having pro-cell-death function such as other enzymes that take part in degradation process. Alternatively, the OsSAG12-1 gene product may be involved in controlling the expression of other SAGs that are required for cell death.

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References

Aparna G, Chatterjee A, Sonti RV and Sankaranarayanan R 2009 A cell wall-degrading esterase of Xanthomonas oryzae requires a unique substrate recognition module for pathogenesis on rice. *Plant Cell* **21** 1860–1873

Ay N, Irmler K, Fischer A, Uhlemann R, Reuter G and Humbeck K 2009 Epigenetic programming via histone methylation at WRKY53 controls leaf senescence in *Arabidopsis* thaliana. *Plant J.* **58** 333–346

Biswas AK and Choudhuri MA 1980 Mechanism of monocarpic senescence in rice. *Plant Physiol.* **65** 340–345

Brusslan JA, Rus Alvarez-Canterbury AM, Nair NU, Rice JC, Hitchler MJ and Pellegrini M 2012 Genome-wide evaluation of histone methylation changes associated with leaf senescence in *Arabidopsis*. *PLoS One* **7** e33151

Chen HJ, Huang DJ, Hou WC, Liu JS and Lin YH 2006 Molecular cloning and characterization of a granulin-containing cysteine protease SPCP3 from sweet potato (Ipomoea batatas) senescent leaves. *J. Plant Physiol.* **163** 863–876

Fischer-Kilbienski I, Miao Y, Roitsch T, Zschiesche W, Humbeck K and Krupinska K 2010 Nuclear targeted AtS40 modulates senescence associated gene expression in *Arabidopsis* thaliana during natural development and in darkness. *Plant Mol. Biol.* **73** 379–390

Lee RH, Wang CH, Huang LT and Chen SC 2001 Leaf senescence in rice plants: cloning and characterization of senescence up-regulated genes. *J. Exp. Bot.* **52** 1117–1121

Lim PO, Kim HJ and Nam HG 2007 Leaf senescence. *Annu. Rev. Plant Biol.* **58** 115–136

Liu F, Vantoai T, Moy LP, Bock G, Linford LD and Quackenbush J 2005 Global transcription profiling reveals comprehensive insights into hypoxic response in *Arabidopsis*. *Plant Physiol.* **137** 1115–1129

Liu L, Zhou Y, Szczerba MW, Li X and Lin Y 2010 Identification and application of a rice senescence-associated promoter. *Plant Physiol.* **153** 1239–1249

Maguire TL, Collins GC and Sedgley M 1991 A modified CTAB DNA extraction procedure for plants belonging to the family proteaceae. *Plant Mol. Biol. Rep.* **12** 106–109

McCabe MS, Garratt LC, Schepers F, Jordi WJ, Stoopen GM, Davelaar E, van Rhijn JH, Power JB and Davey MR 2001 Effects of P(SAG12)-IPT gene expression on development and senescence in transgenic lettuce. *Plant Physiol.* **127** 505–516

Miller JD, Arteca RN and Pell EJ 1999 Senescence-associated gene expression during ozone-induced leaf senescence in *Arabidopsis*. *Plant Physiol.* **120** 1015–1024

Nandi A, Welti R and Shah J 2004 The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene *SUPPRESSSOR OF FATTY ACID DESATURASE DEFICIENCY1* is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell* **16** 465–477

Nandi AK, Kushalappa K, Prasad K and Vijayraghavan U 2000 A conserved function for *Arabidopsis* SUPERMAN in regulating floral-whorl cell proliferation in rice, a monocotyledonous plant. *Curr. Biol.* **10** 215–218

Noh YS and Amasino RM 1999a Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Mol. Biol.* **41** 181–194

Noh YS and Amasino RM 1999b Regulation of developmental senescence is conserved between *Arabidopsis* and *Brassica napus*. *Plant Mol. Biol.* **41** 195–206

Otegui MS, Noh YS, Martinez DE, Vila Petroff MG, Staehelin LA, Amasino RM and Guiamet JJ 2005 Senescence-associated vacuoles with intense proteolytic activity develop in leaves of *Arabidopsis* and soybean. *Plant J.* **41** 831–844

Park CJ, Peng Y, Chen X, Dardick C, Ruan D, Bart R, Canlas PE and Ronald PC 2008 Rice XB15, a protein phosphatase 2C, negatively regulates cell death and XA21-mediated innate immunity. *PLoS Biol.* **6** e231

Park SY, Yu JW, Park JS, Li J, Yoo SC, Lee NY, Lee SK, Jeong SW, *et al.* 2007 The senescence-induced staygreen protein regulates chlorophyll degradation. *Plant Cell* **19** 1649–1664

Sambrook J and Russel DW 2001 *Molecular cloning: A laboratory manual* 3rd edition (Cold Spring Harbor Laboratory press)

Subramoni S and Sonti RV 2005 Growth deficiency of a Xanthomonas oryzae pv. oryzae fur mutant in rice leaves is rescued by ascorbic acid supplementation. *Mol. Plant Microbe Interact.* **18** 644–651

Swain S, Roy S, Shah J, Van Wees S, Pieterse CM and Nandi AK 2011 *Arabidopsis thaliana cdd1* mutant uncouples the constitutive activation of salicylic acid signalling from growth defects. *Mol. Plant Pathol.* **12** 855–865

Sykorova B, Kuresova G, Daskalova S, Trckova M, Hoyerova K, Raimanova I, Motyka V, Travnickova A, Elliott MC and Kaminek M 2008 Senescence-induced ectopic expression of the A tumefaciens ipt gene in wheat delays leaf senescence, increases cytokinin content, nitrate influx and nitrate reductase activity, but does not affect grain yield. *J. Exp. Bot.* **59** 377–387

Wang H, Chen C, Xu Y, Jiang R, Han Y, Xu Z and Chong K 2004 A Practical Vector for Efficient Knockdown of Gene Expression in Rice (*Oryza sativa* L). *Plant Mol. Biol. Report.* **22** 409–417

Yap MN, Lee RH, Huang YJ, Liao CJ and Chen SC 2003 Molecular characterization of a novel senescence-associated gene SPA15 induced during leaf senescence in sweet potato. *Plant Mol. Biol.* **51** 471–481

Zhang P, Wang WQ, Zhang GL, Kaminek M, Dobrev P, Xu J and Gruissem W 2010 Senescence-inducible expression of isopentenyl transferase extends leaf life, increases drought stress resistance and alters cytokinin metabolism in cassava. *J. Integr. Plant Biol.* **52** 653–669

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