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Physiological basis of salt stress tolerance in rice expressing the antiapoptotic gene *SfIAP*

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Abstract. Programmed cell death-associated genes, especially antiapoptosis-related genes have been reported to confer tolerance to a wide range of biotic and abiotic stresses in dicotyledonous plants such as tobacco (*Nicotiana tabacum* L.) and tomato (*Solanum lycopersicum* L.). This is the first time the antiapoptotic gene *SfIAP* was transformed into a monocotyledonous representative: rice (*Oryza sativa* L.). Transgenic rice strains expressing *SfIAP* were generated by the *Agrobacterium*-mediated transformation method and rice embryogenic calli, and assessed for their ability to confer tolerance to salt stress at both the seedling and reproductive stages using a combination of molecular, agronomical, physiological and biochemical techniques. The results show that plants expressing *SfIAP* have higher salt tolerance levels in comparison to the wild-type and vector controls. By preventing cell death at the onset of salt stress and maintaining the cell membrane's integrity, *SfIAP* transgenic rice plants can retain plant water status, ion homeostasis, photosynthetic efficiency and growth to combat salinity successfully.

Additional keywords: cell life, NaCl, programmed cell death, terminal doxynucleotidyl transferase D-UTP nick end labelling, TUNEL.

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Introduction

Salinity is a general term used to describe the presence of elevated levels of different salts such as sodium chloride, magnesium and calcium sulfates, and bicarbonates in soil and water. Increased salinity levels may result from the rising of water tables to, or close to, the ground water surface (Katerji *et al*. 2003). Salinity can develop naturally, but where human intervention has disturbed natural ecosystems and changed the hydrology of the landscape, the movement of salts into rivers and onto land has been accelerated. This can dramatically affect our natural environment and reduce the viability of our agricultural sector. It has been estimated that more than 800 million ha of land is affected by natural salinity and a further 77 million ha of land has been salinised as a consequence of human activities (Metternicht and Zinck 2003; Eynard *et al*. 2005; Munns and Tester 2008; Shabala and Cuin 2008). Plant production was significantly reduced in salt-affected land, with an estimated 30–50% yield loss for rice (*Oryza sativa* L.), 10–90% for wheat (*Triticum aestivum* L.), 50–70% for cotton (*Gossypium hirsutum* L.) and 30–90% for sugarcane (*Saccharum officinarum* L.). Changes in the global environment due to climate change are predicted to cause further yield losses as a result of soil salinity (Eynard *et al*. 2005).

Programmed cell death (PCD), particularly apoptosis, is a cellular suicide process by which damaged or harmful cells are eliminated from multicellular organisms. Cells undergoing apoptosis have distinct morphological changes, including cell shrinkage, membrane blebbing, chromatin condensation, apoptotic body formation and fragmentation (Gilchrist 1998; Bredesen 2000; Collazo *et al*. 2006). The cell suicide programme is a physiological and genetically controlled process, and is evolutionarily conserved across animal and plant species (Williams and Dickman 2008). Furthermore, it acts as a host plant defence mechanism in response to biotic and abiotic stresses (Del Pozo and Lam 2002; Khurana *et al*. 2005). Emerging evidence indicates that the expression of anti-PCD genes in transgenic plants may be an efficient way of enhancing abiotic stress tolerance in economically important crops. For example, antiapoptotic genes from mammals, nematodes and baculovirus such as *Bcl*-2, *Bcl-xL*, *ced*-9 and *p35* have been transformed into tobacco (*Nicotiana tabacum* L.) and tomato (*Solanum lycopersicum* L.) plants and shown to confer resistance to fungi, tomato spotted wilt virus, and abiotic stresses such as salinity, drought, heat, cold, wounding, UV radiation, aluminium, acifluorfen, sufentrazone, menadione and hydrogen peroxide (Dickman *et al*. 2001; Qiao *et al*. 2002; Li

and Dickman 2004; Xu *et al*. 2004; Shabala *et al*. 2007; Wang *et al*. 2009*a*, 2009*b*).

SfIAP is an IAP (inhibitor of apoptosis) family member, isolated from the insect *Spodoptera frugiperda* (Huang *et al*. 2000). IAP was first identified in baculovirus and has been found to function as a cell death suppressor (Crook *et al*. 1993). *SfIAP* has previously been transformed into tobacco and tomato, and reported to confer tolerance to salinity, heat, fumonisin B1 and resistance to the necrotrophic fungus *Alternaria alternata* (Kabbage *et al*. 2010; Li *et al*. 2010). The ability to confer tolerance to salt stress was attributed to its E3 ubiquitin ligase activity (Kabbage *et al*. 2010). In research conducted by Li *et al*. (2010) and Kabbage *et al*. (2010), transgenic tobacco and tomato plants expressing *SfIAP* were studied at the morphological and molecular levels. However, the biochemical and physiological basis of salt stress tolerance due to the expression of *SfIAP* in a monocotyledonous plant has not been investigated to date. In this study, *SfIAP* from the insect *Spodoptera frugiperda* was introduced to *Oryza sativa* L. Japonica cv. Nipponbare. The enhancement of tolerance to salt stress in transgenic Nipponbare expressing *SfIAP* was confirmed using agronomical assessment. Physiological characteristics from the cell to whole-plant level of transgenic rice expressing *SfIAP* under control and salt stress conditions were investigated.

Materials and methods

Generation of constructs

The binary vector *pCAMBIA1301* was modified to contain the maize (*Zea mays* L.) polyubiquitin-1 (Ubi-1) promoter controlling the expression of the antiapoptotic gene *SfIAP*. The gene was sequenced in its original vector before the transformation to confirm the presence and integrity of the coding sequence, and the promoter–gene and gene–terminator borders.

Rice transformation and molecular characterisation of transgenic plants

Embryogenic *Oryza sativa* L. Japonica cv. Nipponbare calli were initiated, maintained and transformed as described by Khanna and Raina (1999). Molecular characterisation of transgenic plants including PCR and reverse transcription-PCR (RT-PCR) were carried out using gene specific primers for *SfIAP* and hygromycin specific primers for the vector control. Briefly, DNA was extracted from 100 mg fresh rice leaf tissue using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). PCR analysis was carried out in a 20-μL reaction mixture containing 10 μL of 2× GoTaq green (Promega, Madison, WI, USA), 0.5 μL of each 10 μM forward and reverse primers, 100 ng of genomic DNA and DNase, and RNase-free water up to 20 μL. PCR was conducted in a Peltier Thermal Cycler (Gradient Cycler, Bio-RAD, CA, USA) using the PCR profile as 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min and a final extension at 72°C for 2 min.

RNA was extracted from 50 mg of leaf tissue using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions and was treated with RNase-free DNase (Promega). RT-PCR was carried out using the SuperScript III first-strand synthesis system (Invitrogen-Life Technologies Australia Pty Ltd, Musgrave, Vic., Australia) using gene-specific primers following the manufacturer's instructions.

Salt stress experiments at seedling and reproductive stages

The experiments were conducted from August to December 2012 at the Queensland University of Technology glasshouse at Carseldine, Brisbane, Australia, with temperature adjusted at 28°C : 21°C day : night and no supplemental light was used. Rice tissue culture plants were acclimatised using 40-mm plastic pots and premium potting mix (Searles, Sunshine coast, Qld, Australia) for 7 days. Pots were placed in a container filled with tap water. The plants were grown for another 7 days then Aquasol fertiliser (Yates, Padstow, NSW, Australia) containing nitrogen, phosphorus, potassium and trace elements (N : P : K: 23 : 3.95 : 14) was added. One week later, the salt stress experiment at the seedling stage was started: water in the container was drained out and 100 mM NaCl in tap water was poured in until a level of 1 cm above the potting mix level was achieved. At this stage, the plants had three fully expanded leaves and the fourth leaf had just emerged. The water level was maintained daily at 1 cm above the soil level by adding tap water (not salt water) into the container. The salt stress experiment at the reproductive stage was carried out basically following the methods described in Moradi and Ismail (2007) using 30-day-old well acclimatised rice plants. Briefly, the acclimatised plants in 40-mm plastic pots were transplanted into 140-mm plastic pots containing premium potting mix (Searles) supplemented with Osmocote fertiliser (Osmocote Pots, Planters and Indoor, Scotts, Bella Vista, NSW, Australia) with a NPK ratio of 13.8 : 3.2 : 9.9) at 14 days after acclimation. The pots were placed in a container filled with tap water supplemented with fertiliser (Aquasol) to 1 cm above the compost level. The plants were grown for another 16 days and the water was drained out of the container. NaCl solution (100 mM NaCl in tap water) or tap water (control) was added to reach a level 1 cm above the potting mix. The water level was maintained daily to 1 cm above the potting mix level by adding tap water into containers to compensate for the water lost due to transpiration and evaporation. It is worth to note that the concentration of sodium in tap water is very low: ~16 to 100 mg L^{-1} (~0.7–4.4 mM) (Queensland Urban Utilities 2009).

An initial screening for salt stress tolerance was conducted on 10 independent RT-PCR positive transgenic rice lines expressing *SfIAP*, vector controls (VC) and wild-type (WT) Nipponbare plants in a glasshouse at the seedling stage. Morphological data such as shoot growth, third leaf damage and survival rate were captured and analysed statistically. The best independent transgenic line expressing *SfIAP* was selected for further study.

The investigation of tolerance to salt stress at the seedling stage was conducted in a glasshouse at a temperature of 28°C : 21°C day : night. Ten plants of selected transgenic lines expressing *SfIAP*, VC and WT controls were acclimatised for 7 days. Plants were treated with 100 mM NaCl in tap water 14 days after acclimation. Shoot height was measured at Day 0 and Day 13 following NaCl stress; shoot DW was determined after 13 days of NaCl treatment. The level of salt tolerance of

transgenic lines and controls at the reproductive stage was determined based on yield components, including the length of panicles and the number of spikelets per panicle. Ten replicates of transgenic lines and the appropriate controls were subjected to 100 mM NaCl stress for 30 days after acclimation. Assessment of relative yield was performed at harvest.

Terminal deoxynucleotidyl tranferase D*-UTP nick end labelling assay*

The terminal deoxynucleotidyl tranferase D-UTP nick end labelling (TUNEL) assay was carried out using an *in situ* cell death detection kit, Fluorescein (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia) following the manufacturer's instructions. Briefly, root tip fragments (~1 cm) were cut from plants, washed three times with fresh 1× PBS and fixed in 4% paraformaldehyde solution at 4°C for 1 h. Following fixation, root tips were washed twice with fresh PBS, immersed in fresh permeability solution (0.1% triton × 100 and 0.1% sodium citrate) and microwaved at 700 W for 1 min. Samples were immediately cooled down using fresh PBS, followed by two more PBS washes. A 50-µL aliquot of TUNEL reaction mix was added to the root tips in a 1.5-mL Eppendorf tube. As a negative control, 50-µL aliquots of TUNEL labelling solution without the enzyme were also included. Samples were incubated at 37°C for 1 h under high humidity. After incubating, the samples were washed twice with fresh PBS and counterstained with a 0.5 mg mL^{-1} propidium iodide (Sigma-Aldrich Pty Ltd, Sydney, NSW, Australia) in the dark at room temperature for 15 min. Stained root tips were washed twice with fresh PBS and squash-mounted onto slides and examined under an A1 Confocal Microscope (Nikon, Japan).

Relative water content determination

The relative water content (RWC) of plant leaves was examined using the method described by Lafitte (2002). First, ~10 cm of leaf was cut off from the middle part of the youngest fully expanded leaf, weighed (FW) and placed in a 15-mL Falcon tube. The tube was kept on ice until it was filled with distilled water and kept in the dark at 4°C overnight. The next morning, the leaf was blotted dry with a tissue towel for 30 s and weighed (turgid weight). The samples were then dried in a vacuum oven at 70°C for 3 days and weighed for DW. The RWC was calculated as shown in Eqn 1:

$$RWC = \frac{(FW - DW) \times 100}{(TW - DW)}, \quad (1)$$

where *TW* is the turgid weight.

Electrolyte leakage measurement

Electrolyte leakage from leaves at the seedling and reproductive stages was measured using a CM 100–2 conductivity meter (Reid and Associates CC, Durban, South Africa) following the manufacturer's instructions. Briefly, leaf tissue was excised from plants, placed in a plastic bag and immediately put on ice. Each leaf was washed twice with deionised water and blotted dry with a paper towel. Next, ~5 cm of the middle part of the leaf was cut into 0.5 cm pieces, rinsed with deionised water and loaded into wells of the CM 100–2 conductivity meter containing 1.25 mL of deionised water. Measurement was carried out every 2 min over a 60-min period. Samples were removed and dried in an oven at 70°C overnight for measurement of DW. Electrolyte leakage was calculated as the slope of electrolyte leakage over time and normalised by DW.

Gas exchange measurements

Net photosynthesis (*A*) was measured using an Infra Red Gas Analyser LI-6400 XT (John Morris Scientific, Chatswood, NSW, Australia). For measurement at the seedling stage, the third leaf was used to measure *A* at Day 0, 3, 7, 10 and 13 after the salt stress treatment. Reproductive stage measurement was taken on the flag leaf after 30 days of NaCl exposure. The in-chamber quantum sensor (ParIn_µm) was set at 800 µmol m^{-2} s^{-1} and the vapour pressure deficit based on leaf temperature was recorded to be ~1.05–1.4 kPa. Relative humidity in the sample cell was maintained at 57% ± 3%. Measurement took place over 0900 hours to 1200 hours.

Sodium and potassium measurement

The amount of Na^+ and K^+ in the leaves of rice plants in the salt tolerance screening experiments at both the seedling and reproductive stages were determined using an atomic absorption spectrophotometer (Shimadzu A-7000, Shimadzu Scientific Instruments, Sydney, NSW, Australia). Leaf samples for sodium and potassium analysis were undertaken on the same leaf that was used for photosynthesis measurements at both the seedling and reproductive stages (the third leaf and the flag leaf respectively). Samples were prepared as described in Dionisio-Sese and Tobita (2000). Fifteen mg of dried leaf was cut into 0.5-cm pieces and immersed in 30 mL of deionised water in a 50-mL Falcon tube. The mixture was boiled in a water bath for 1 h followed by 20 min autoclaving at 121°C. Samples were cooled down at room temperature and filtered using Whatman filter paper No. 40 (ashless) (Thomas Scientific, NJ, USA).

Statistic analysis

All experiments in this study were conducted using a randomised completed block design, and data were analysed using one-way ANOVA and Tukey's Honestly Significant Difference (HSD) tests (Minitab ver. 16, Sydney, NSW, Australia).

Results

Generation and molecular characterisation of transgenic rice plants expressing SfIAP

To elucidate the physiological role of *SfIAP* in tolerance to salt stress in rice, the coding region of *SfIAP* was placed under the control of maize ubiquitin promoter (Fig. 1*a*). The expression cassettes and a vector control (the vector backbone without the gene of interest) were introduced into *Oryza sativa* L. japonica cv. Nipponbare by *Agrobacterium*- mediated transformation of Nipponbare embryogenic calli. Eleven PCR-confirmed independent transgenic lines were generated (Fig. 1*b*). The expression of *SfIAP* in the independent transgenic lines was examined using RT-PCR (Fig. 1*c*). Under normal tissue culture growth conditions, no significant difference in morphology was observed between transgenic plants expressing *SfIAP* and the controls (VC and WT).

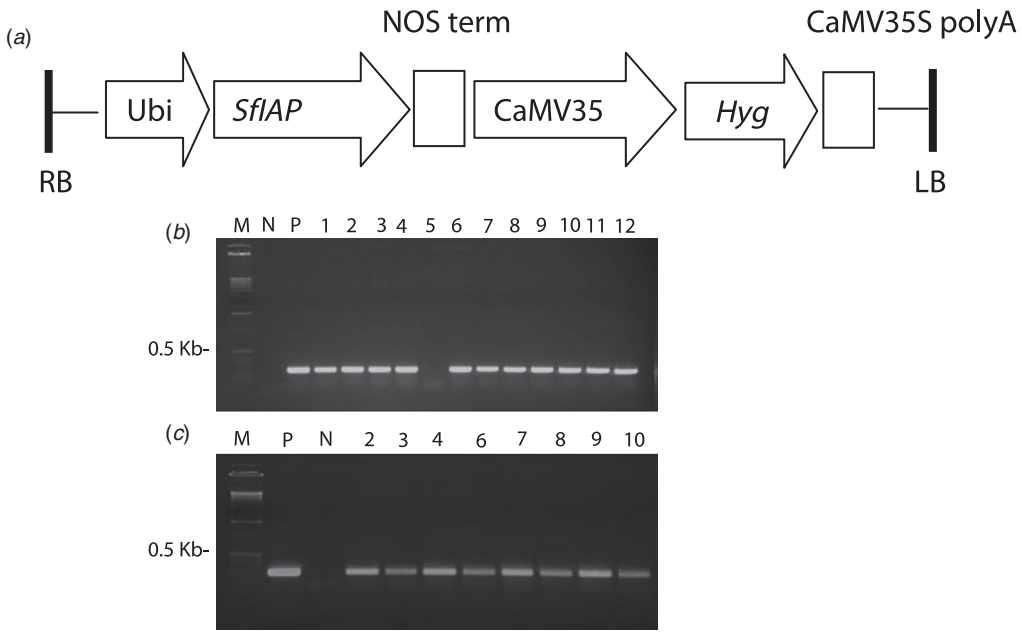

Fig. 1. Schematic diagram of gene constructs and molecular analysis of transgenic rice lines expressing *SfIAP*. (*a*) Schematic diagram of *SfIAP* overexpression gene constructs. LB, left border; RB, right border. (*b*) PCR confirmation of transgenic lines; M, marker; N, negative control; P, positive control (plasmid DNA); 1–12, independent *SfIAP* trangenic lines 1–12. (*c*) Reverse transcription-PCR analysis on transgenic plants expressing *SfIAP*.

SfIAP *confers tolerance to salt stress in rice*

More than 60% of the transgenic lines tested exhibited greater growth rates, a higher survival percentage and less leaf damage in comparison to the WT and VC plants. The remaining 40% were not significantly different to the WT and VC plants (data not shown). Consistent with the initial experiment, the expression of *SfIAP* in transgenic rice enhanced tolerance to salt stress at both the seedling and reproductive stages. Shoot growth and DW, two indicators of salt tolerance, were significantly greater in transgenic rice expressing *SfIAP* than in the WT and VC plants after 13 days of exposure to 100 mM NaCl at the seedling stage (Fig. 2*a*, *b*). Yield components, including panicle length and number of spikelets per panicle, were significantly higher in transgenic rice expressing *SfIAP* than in the control plants (Fig. 2*c*, *d*). Under nonstress conditions, there was no significant difference in shoot growth, DW and yield components between transgenic rice expressing *SfIAP*, and the WT and VC plants (Fig. 2).

Constitutive expression of SfIAP *reduces cell damage and death caused by salt stress in rice*

To investigate the effect of *SfIAP* expression on cell damage and death caused by salt stress, a TUNEL assay was conducted on the root tips of rice plants exposed to 100 mM NaCl after 36 h. The results show that no cell death or only a small amount of cell death (less than 5%) was detected in transgenic rice plants expressing *SfIAP*, whereas WT Nipponbare and VC plants underwent noticeable cell death (Fig. 3*a*, *b*). As shown in Fig. 3*c*, the growth of control plants (WT and VC) was seriously affected by 100 mM NaCl after 13 days of exposure, but it was much less pronounced in *SfIAP* transgenic plants. Note that under normal growth conditions, no cell death was observed in controls or transgenic rice plants (data not shown).

SfIAP *transgenic rice demonstrates better water retention and cell membrane integrity than the controls under salt stress*

In order to understand the physiological mechanisms of enhanced salt tolerance in transgenic rice expressing *SfIAP* under salt stress conditions, the water status of transgenic plants and the controls in control condition and in the 100 mM NaCl treatment at both the seedling and reproductive stages were investigated. The results showed that under control condition, the RWC of *SfIAP* transgenic rice was not different from that of WT and VC rice plants at both the seedling and reproductive stages (Fig. 4*b*, *d*). However, under the 100 mM NaCl treatment at the seedling stage, the water retention of WT and VC plants was significantly lower compared to transgenic plants expressing *SfIAP* (Fig. 4*b*). The RWC of *SfIAP* transgenic rice plants at the reproductive stage under the salt stress treatment was noticeably higher than that of the control plants (Fig. 4*d*). To further elucidate the physiological mechanisms of enhanced salt tolerance in *SfIAP* transgenic plants, the cell membrane integrity of *SfIAP* transgenic and control plants was analysed by measuring leaf cell electrolyte leakage under control and salt stress conditions. The relative electrolyte leakage of the leaf cells was calculated as the slope of electrolyte leakage against time in 1 h increments; the greater the gradient of the slope, the more electrolytes leaked out of the cells. Under control conditions, at both the seedling and reproductive stages, the relative electrolyte leakage of transgenic plants and the control plants was not significantly different (Fig. 4*a*, *c*). However, under the 100 mM NaCl

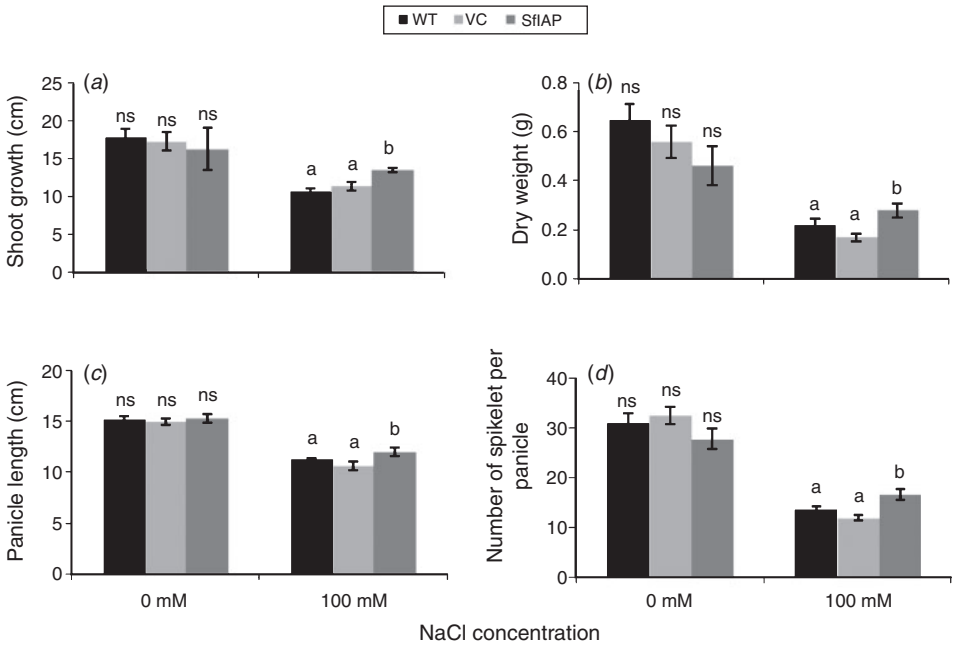

Fig. 2. Growth and yield components of transgenic rice expressing *SfIAP* and control plants under normal and NaCl stress conditions. (*a*) Shoot growth and (*b*) DW of rice after 13 days of exposure to 0 or 100 mM NaCl at the seedling stage. (*c*) Panicle length and (*d*) number of spikelets per panicle of rice exposed to 0 or 100 mM NaCl at the reproductive stage. Data are means ± s.e.,($n \geq 3$). ns, no significant difference; WT, wild-type; VC, vector controls. Bars followed by the same letter represent no significant difference by Tukey's Honestly Significant Difference (HSD) test at 95% confidence intervals. Statistics compared mean values among individuals in each treatment.

treatment, the relative electrolyte leakage was dramatically increased in WT and VC plants. Electrolyte leakage was also observed in *SfIAP* transgenic rice plants but at significantly lower level compared with the controls. This result indicated that *SfIAP* transgenic plants exhibited less cell membrane damage than control plants without the gene of interest.

SfIAP *transgenic rice plants accumulate less* Na^+ *and more* K^+*, and maintain a lower* $Na^+:K^+$ *ratio than the control plants under salt stress condition*

It is well documented that under salt stress condition, salt-tolerant rice cultivars accumulate less Na^+ in leaves and shoots compared with salt-sensitive rice cultivars (Dionisio-Sese and Tobita 1998, 2000; Lee *et al*. 2003; Moradi and Ismail 2007; Ghosh *et al*. 2011). In this study, we investigated the sodium content in the leaves of transgenic rice and controls under control and salt stress conditions. As shown in Fig. 5*a*, *d*, the Na^+ concentration in leaves of rice grown under control conditions was not significantly different between the transgenic and the control plants. In contrast, under 100 mM NaCl stress at the seedling stage, the concentration of Na^+ in leaves of WT Nipponbare and VC plants increased dramatically: a ~20-fold increase in comparison with that under control conditions (from 0.044 mmol g^1 and 0.062 mmol g^{-1} DW under control conditions to 1.25 mmol g^{-1} and 1.17 mmol g^{-1} DW under the 100 mM NaCl treatment, respectively). Transgenic plants expressing *SfIAP* showed only a fivefold increase in Na^+ concentration (from 0.064 mmol g^{-1} DW to 0.333 mmol g^{-1} DW). The *SfIAP* plants maintained a ~3.5-fold lower Na^+ concentration in comparison with WT and VC plants under salt stress conditions. At the reproductive stage, the salt stress treatment resulted in a twofold increase in sodium concentration in the leaves of WT and VC plants compared with *SfIAP* transgenic plants (1.911 mmol g^{-1}, 1.914 mmol $g^{-1}$and 0.944 mmol g^{-1} DW, respectively). In addition to maintaining a lower level of Na^+ in leaves under salt stress, transgenic rice plants expressing *SfIAP* also maintained a sevenfold higher K^+ concentration in leaves compared with WT and VC plants (Fig. 5*b*, *e*). Under the 100 mM NaCl treatment, WT and VC plants exhibited a significant decrease in K^+ concentration in leaves compared with those under control conditions (from 0.407 mmol $g^{-1}$and 0.404 mmol g^{-1} DW in control conditions to 0.172 mmol g^{-1} and 0.165 mmol g^{-1} DW under the 100 mM NaCl treatment at the seedling stage), whereas *SfIAP* transgenic plants had only a slightly decrease in leaf K^+ concentration (from 0.432 mmol g^{-1} DW to 0.382 mmol g^{-1} DW). More importantly, *SfIAP* transgenic plants exhibited a significantly lower ratio between Na^+ and K^+ compared with the controls (Fig. 5*c*, *f*). Interestingly, no significant difference in leaf Na^+ and K^+ concentrations or leaf $Na^+:K^+$ ratio were observed between transgenic plants and the controls under control conditions, except for the $Na^+:K^+$ ratio at the seedling stage. This indicates that under salt stress conditions, *SfIAP* can maintain cell life and allows normal control of ion transport to continue, thereby maintaining Na^+ homeostasis.

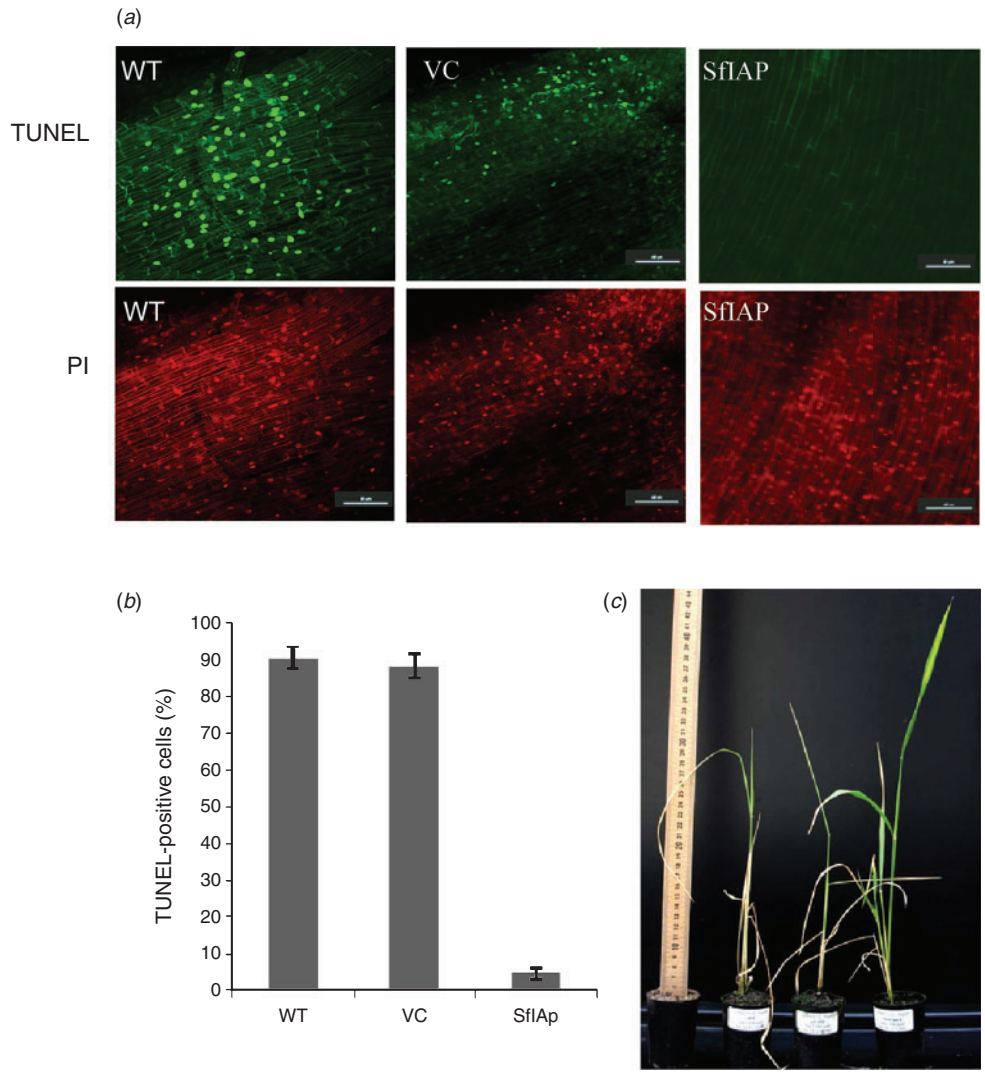

Fig. 3. *SfIAP* reduces cell death and damage in transgenic rice plants under salt stress. (*a*) Terminal deoxynucleotidyl tranferase D-UTP nick end labelling (TUNEL) assays on rice root tips after 36 h of exposure to 100 mM NaCl. (*b*) Percentage of TUNEL-positive cells. Data are means and s.e. ($n = 3$). (*c*) Wild-type (WT), vector control (VC) and *SfIAP* transgenic rice plants after 13 days of exposure to 100 mM NaCl. Scale bars are 50 µm.

SfIAP *transgenic rice plants exhibit greater photosynthetic efficiency than control plants under salt stress conditions*

Photosynthesis is a fundamental physiological process that provides a source of energy for plants to grow and cope with environmental stresses. To investigate the possibility of *SfIAP* enhancing salt tolerance in rice by maintaining photosynthetic efficiency under salt stress, we examined and compared the *A* of transgenic plants expressing *SfIAP* constitutively with WT and VC rice plants under control and salt stress conditions at both the seedling and reproductive stages. *A* was measured at 0, 3, 7, 10 and 13 days after 100 mM NaCl was added to the media in the seedling salt stress experiment. As evident in Fig. 6*a*, under control growth conditions, no significant difference was observed in *A* between *SfIAP* transgenic plants and the controls over 13 days of the experiment. The trend of variation in *A* was also the same for all plants tested. However, when exposed to 100 mM NaCl stress, the transgenic plants expressing *SfIAP* behaved differently from the control plants (Fig. 6*b*). Significant differences were observed between the transgenic plants expressing *SfIAP* and the control plants after 7 days of salt exposure. *A* in all transgenic and control plants decreased at Day 10 in both the control and salt stress treatments. This was probably due to the natural physiological senescence of the leaf at a specific age (we used the third leaf for photosynthesis measurements). However, *A* in control plants under salt stress conditions decreased more rapidly than the transgenic plants expressing *SfIAP*. After 13 days of exposure to 100 mM NaCl, the *A* of WT and VC plants was significantly lower than that of the *SfIAP* transgenic plants; the *A* of *SfIAP* transgenic plants was not significantly different from that of control plants under control conditions. This result indicated that transgenic rice expressing *SfIAP* can maintain photosynthetic efficiency to ensure the energy source for plants to grow under salt stress conditions. The *A* of transgenic rice plants and that of the controls under control and salt stress at

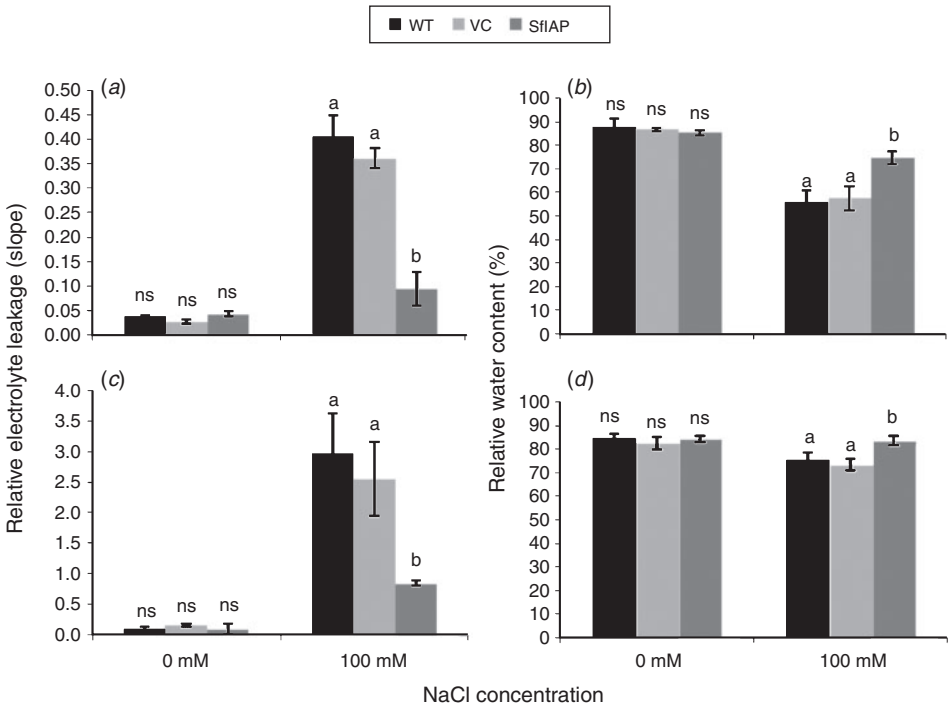

Fig. 4. (*a*, *c*) Electrolyte leakage and (*b*, *d*) water status in transgenic and wild-type rice plants under normal and salt stress conditions. (*a*, *b*) seedling stage; (*c*, *d*) reproductive stage. Data are means ± s.e. ($n \geq 3$). ns, no significant difference; WT, wild-type; VC, vector control. Bars followed by the same letter represent no significant difference by Tukey's Honestly Significant Difference (HSD) test at 95% confidence intervals. Statistics compared mean values among individuals in each treatment.

the reproductive stage was not significantly different (Fig. 6*c*). This is probably due to the status of the leaf that we used to measure photosynthesis. We used the flag leaf of the main culm for *A* measurement. At the time of measurement, the flag leaf was not damaged in all plants tested, although other leaves from the NaCl-treated plants were dramatically damaged in control plants.

Discussion

Previous studies have shown that expression of antiapoptosis genes can facilitate enhanced stress tolerance levels in plants. Therefore, the first aim of this study was to generate transgenic rice plants expressing *SfIAP* and assess their tolerance levels to conditions of high salinity. Phenotypic growth parameters and molecular approaches have both demonstrated that plants expressing *SfIAP* have higher salt tolerance levels via improved growth and the absence of salinity-induced cell death.

High salinity levels stress plants in two ways: water deficit and ion toxicity. Salinity-induced water deficit is due to the high osmotic pressure in the soil solution, which makes it harder for the roots to draw water from the soil; ion toxicity is caused by high concentrations of salts, particularly Na^+ within the cells, which can compete with K^+ for enzyme activation and protein biosynthesis (Munns and Tester 2008; Shabala and Cuin 2008; Wang *et al*. 2013). The maintenance of cell membrane integrity and stability under water stress is an important component of tolerance against water deficit in plants.

The cell membrane is the first site of signal perception of biotic stress as well as a primary defence against many abiotic stresses, including salinity (Ghosh *et al*. 2011). Cell membrane damage can be measured by monitoring electrolyte leakage from the cells (Bajji *et al*. 2002). In this study, electrolyte leakage from leaves of WT and VC Nipponbare rice undergoing salt stress was significantly higher than that observed from leaves of transgenic rice expressing *SfIAP* at both the seedling and reproductive stages (Fig. 4*a*, *c*). These results indicate that *SfIAP* leaf cells maintain higher cell membrane integrity than control plants under salt stress and correlate with previous studies which showed a significant difference between the electrolyte leakage levels of salt-sensitive and salt-tolerant rice cultivars during salt stress (Dionisio-Sese and Tobita 1998; Cha-um *et al*. 2009*b*).

Plants that withstand exposure to high salinity environments restrict the uptake of Na^+ from the soil and the influx into their cells. Increased Na^+ levels are toxic to cells because Na^+ has similar physicochemical properties to K^+, so it can compete with K^+ for major binding sites in key metabolic processes such as enzymatic reactions, ribosome functions and proteins biosynthesis in the cytoplasm leading to disturbances in the metabolism. As K^+ is responsible for the activation of more than 50 enzymes in cytoplasm, the disruption to the metabolism is severe (Shabala and Cuin 2008; Marschner 2011; Wang *et al*. 2013). One of the mechanisms that plants employ to adapt to salt stress, especially ion toxicity, is to exclude Na^+ from the roots and subsequently maintain low concentrations

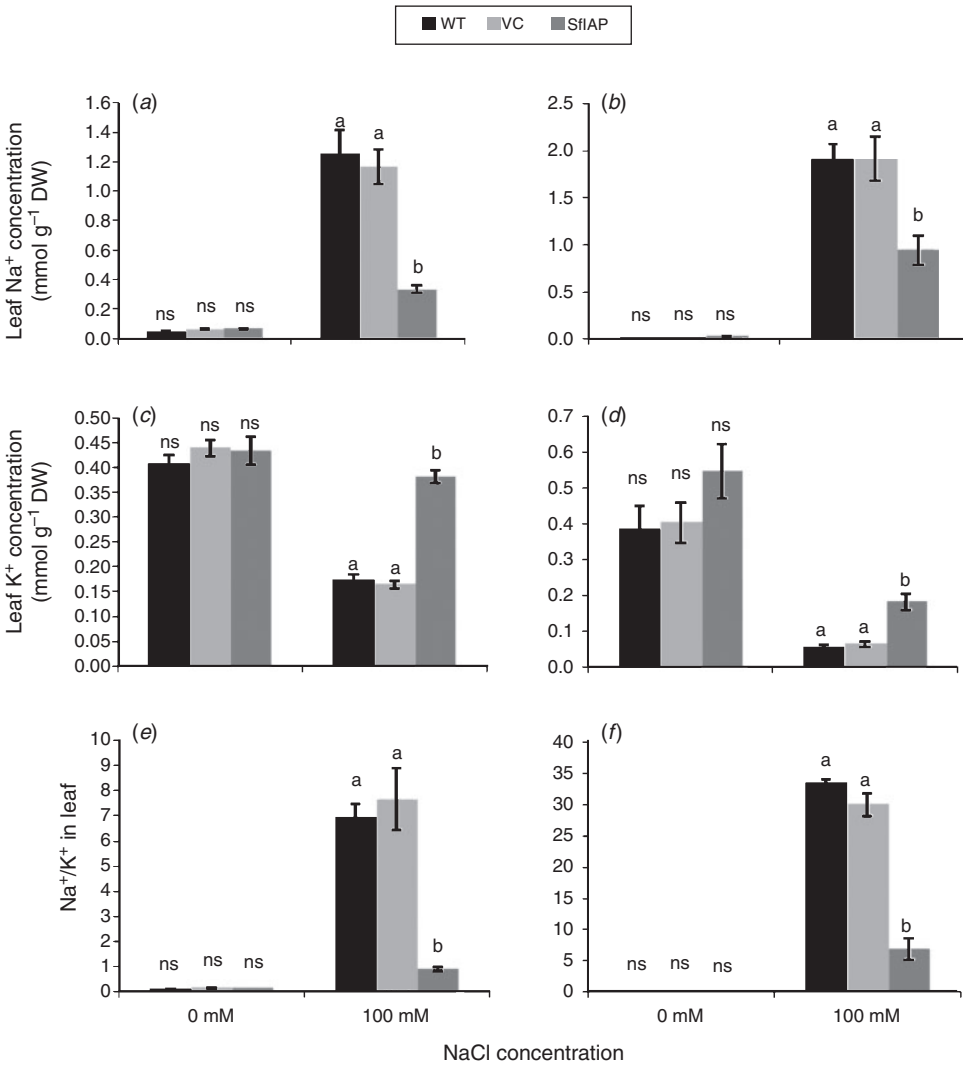

Fig. 5. (*a*, *d*) Sodium and (*b*, *e*) potassium concentrations and (*c*, *f*) sodium : potassium ratios in leaves of rice exposed to NaCl at seedling and reproductive stages. (*a*, *b*, *c*) Seedling stage; (*d*, *e*, *f*) reproductive stage. Data are means ± s.e.,($n \geq$t 3). ns, no significant difference. Bars followed by the same letter represent no significant difference by Tukey's Honestly Significant Difference (HSD) test at 95% confidence intervals. Statistics compared mean values among individuals in each treatment. Note: The value of Na^+ : K+ in leaf of wild-type (WT), vector control (VC) and *SfIAP* plants at 0 mM NaCl in (*f*) are 0.072 ± 0.007, 0.061 ± 0.007 and 0.059 ± 0.008 respectively.

of sodium ions in the leaves. Failure to exclude Na^+ from the cell manifests in toxic effects days or even weeks after exposure and causes the premature death of older leaves (Munns and Tester 2008). The *SfIAP* plants consistently maintained low leaf Na^+ concentrations during NaCl treatment at the seedling and reproductive stages (~74 mM and 210 mM on a leaf water basis, respectively), whereas dramatic increases were observed in the Na^+ levels in the WT Nipponbare and VC (~278 mM and 259 mM respectively on a leaf water basis at the seedling stage, and 425 mM each on a leaf water basis at the reproductive stage). Many studies have suggested that cytosolic K^+ is related to the PCD process, as it can affect caspases and caspases-like activities in animals and plants, respectively. Low cytosolic K^+ content in animal tissue correlates with high caspases activity; the activation of K^+ efflux, the main cause of a decrease in cytosolic K^+ content, in plant cells leads to PCD hydrolase activation (Hughes and Cidlowski 1999; Shabala 2009; Demidchik *et al*. 2010). In this study, we found that WT and VC plants exhibited a significant lost of K^+ during exposure to 100 mM NaCl at both seedling and reproductive stages (to ~38.4 mM and 36.6 mM in leaf water basis at the seedling stage, and to 12.7 mM and 14.3 mM on a leaf water basis at the reproductive stage, respectively), whereas *SfIAP* transgenic plants showed only slight and moderate decreases in leaf K^+ concentration during salt stress at the seedling and reproductive stages (to ~84.8 mM and 40.6 mM on a leaf water basis, respectively). This result provides an explanation of PCD inhibition in transgenic rice expressing *SfIAP* under salt stress conditions. Note that cytosolic K^+ concentration of a shrunken animal cell (a cell that has undergone apoptosis) is ~35–50 mM (Barbiero *et al*. 1995; Hughes and Cidlowski 1998). The low leaf

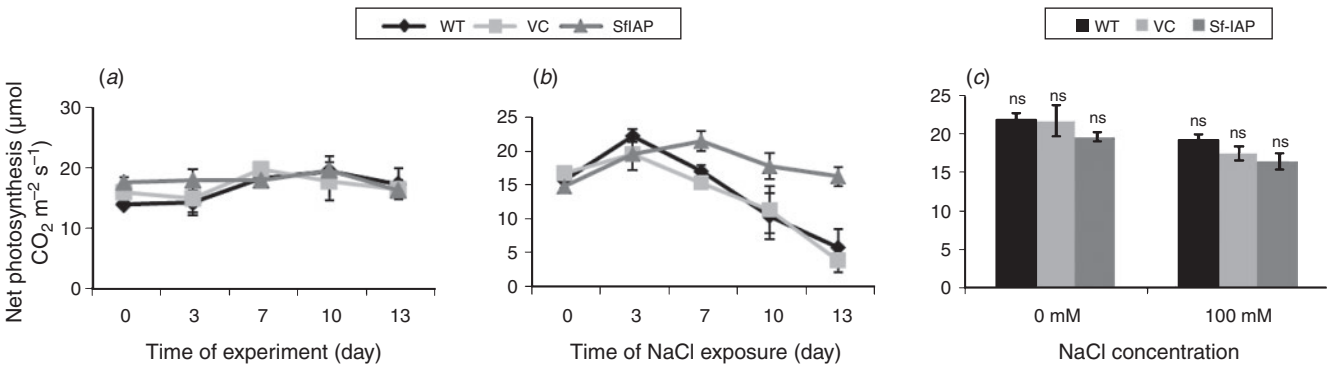

Fig. 6. Net photosynthesis of transgenic rice expressing *SfIAP* and the control plants under (*a*, *c*) normal and (*b*, *c*) salt stress conditions. (*a*, *b*) Seedling stage; (*c*) reproductive stage. Data are means ± s.e. (*n* = at least 3). ns, no significant difference; WT, wild-type; VC, vector control. Bars followed by the same letter represent no significant difference by Tukey's Honestly Significant Difference (HSD) test at 95% confidence intervals. Statistics compared mean values between individuals in each treatment.

K^+ concentration of *SfIAP* transgenic plants at the reproductive stage (40.6 mM) was probably associated with PCD during the natural physiological senescence of the leaf at a specific age (the end of reproductive stage to the beginning of ripening stage), whereas very low K^+ concentrations in the WT and VC plants (12.7 mM and 14.3 mM on a leaf water basis, respectively) suggest that PCD was more pronounced in these plants and that PCD in these plants may be not only due to the natural physiological senescence of leaves but also salt stress. Previous studies have shown that the maintenance of a low Na^+ : K^+ ratio provides favourable conditions for continued physiological and metabolic activity (Yu *et al*. 2012). Transgenic rice plants expressing *SfIAP* exhibited a significantly lower Na^+ : K^+ ratio in comparison to the WT and VC plants during salt stress. Importantly, the lower Na^+ levels correlated with higher rates of *A* in the *SfIAP* plants, thus providing an energy source and vital ammunition for the plants to develop and cope with the challenges imposed by salt stress. Consistent with these data, previous reports have shown that under salt stress, salt-tolerant rice cultivars exhibited higher *A* than salt-sensitive rice cultivars (Moradi and Ismail 2007; Cha-Um *et al*. 2009*a*).

In summary, this paper details the investigation of expressing *SfIAP* in rice to improve salt tolerance. In contrast to the WT and VC controls, the *SfIAP* plants maintained growth and photosynthetic rates, RWC, cell viability, membrane integrity and overall plant health. These findings are consistent with previous data that linked the inhibition of PCD, including the absence of TUNEL-positive nuclei, with improved abiotic stress tolerance. Taken together, these results further demonstrate the importance of PCD pathways during abiotic stress responses and highlight the potential of using antiapoptosis approaches for the generation of stress-tolerant plants.

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