ENGINEERING SALINITY TOLERANCE IN RICE BY EXOGENOUS EXPRESSION OF CELL DEATH REGULATORS

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Abiotic stress, *Agrobacterium*-mediated transformation, anti-apoptosis, *AtBAG4*, *AtBI*, *AtBAG4 + Hsp70*, biotic stress, callus proliferation, cell death, cell membrane integrity, DAB, D_{50} survival, dry weight, electrolyte leakage, embryogenic callus, glasshouse, growth, *Hsp70*, *Hsp90*, *in vitro*, *in vivo*, leaf damage, NaCl, *OsBAG4*, panicle length, photosynthesis, PCD, programmed cell death, pro-apoptosis, pro-death, pro-survival, *p35*, ROS, RWC, salinity, salinity stress sensitive, salinity stress tolerance, *SfIAP*, survival, transgenic lines, TUNEL, yield components.

Abstract

By 2050, the world population is expected to rise to 9.6 billion; to sustain this increase in population agriculture needs to be significantly more efficient. How do we make crop plants more efficient? A logical starting point is to look at what are the most important crops and the factors that hinder their productivity. Environmental factors result in over 70% of crop losses worldwide. Of these drought and salinity are the most significant causes of crop losses. Rice (*Oryza sativa* L.) is an important staple crop that feeds more than one half of the world's population and is the model system for monocotyledonous plants. Rice is also the most sensitive to salinity stress, among the agronomically important cereals.

Programmed cell death (PCD) or simply the decision of whether a given cell should live or die is essential for all multi-cellular (Metazoan) organisms. Recent studies have shown that PCD pathways are keys to mediating plant responses to a range of abiotic stresses, including salinity. The expression of PCD related (e.g. pro-survival) genes in transgenic plants may be an efficient way of enhancing abiotic stress tolerance in economically important crops. The research for this PhD describes the generation of transgenic rice plants expressing pro-survival genes from a variety of sources and assessment of the physiological and yield factors including photosynthetic rates, membrane integrity, ion homeostasis, growth rates and yield components during salinity stress. The results reveal that the manipulation of PCD pathways using pro-survival genes from diverse origins significantly improves salinity tolerance in rice at the whole plant level.

A total of 139 transgenic rice lines expressing pro-survival genes including *AtBAG4* and *AtBI* from *Arabidopsis thaliana*, *Hsp70* and *Hsp90* from *Citrus tristeza* virus, *OsBAG4* from *Oryza sativa*, *p35* from baculovirus and *SfIAP* from *Spodoptera frugiperda;* and more than 30 lines of transgenic rice expressing the reporter gene (*GUS*) were generated by *Agrobacterium*-mediated transformation. These transgenic rice lines were validated by PCR and RT-PCR. The effects of pro-survival genes on

callus proliferation and the efficiency of *Agrobacterium*-mediated transformation were assessed during the selection on hygromycin supplemented media. The transformation efficiency was significantly improved when embryogenic callus were used for transformation. Transformation conducted on the mix of embryogenic and non-embryogenic callus resulted in low transformation efficiency in rice.

Rapid screening for salinity stress tolerance in transgenic rice expressing pro-survival genes and wild type Nipponbare under *in vivo* conditions concurred with previous studies that leaf damage and D_{50} survival are good indicators for salinity stress tolerance in rice and that young seedlings are more sensitive to salinity stress than older plants. The research also revealed that expression of five pro-survival genes significantly improved salinity stress tolerance in rice, and these were *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP*. Expression of *AtBI, Hsp90* and the combination of *AtBAG4* and *Hsp70* in transgenic rice did not show enhanced tolerance to salinity stress. This might be due to the low level of transgene expression in the host plant.

Glasshouse evaluation of representative transgenic rice lines expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* confirmed that the expression of pro-survival genes enhanced tolerance to salinity stress in rice. Under salinity stress wild type and vector control plants exhibited high levels of reactive oxygen species (ROS), significant cell death, membrane damage, low photosynthetic efficiency, low relative water content, stunted growth and low yield components. In contrast, transgenic rice lines expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* exhibited low levels of ROS, maintained cell membrane integrity and Na^+ homeostasis thereby maintaining photosynthetic efficiency, water content and cell life that lead to maintenance of growth rates, yield components and enhanced tolerance to salinity stress.

This study provided evidence of biochemical and physiological basis of salinity stress tolerance in rice expressing pro-survival genes. It contributed to the knowledge of crop improvement strategies for salinity stress tolerance and the findings of this project can be applied to other crops. In addition, this is the first time a model for salinity-induced cell death switch for salinity stress tolerance in rice has been proposed.

List of Publications and Conferences

Thi My Linh Hoang, Brett Williams, Harjeet Khanna, James Dale and Sagadevan G. Mundree (2014).Physiological basis of salt stress tolerance in rice expressing the antiapoptotic gene *SfIAP*.*Functional plant biology*.http://dx.doi.org/10.1071/FP13308

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Thi My Linh Hoang, Brett Williams, Harjeet Khanna, James Dale and Sagadevan G. Mundree (2012). Engineering tolerance to salinity stress in rice by manipulation of programmed cell death pathways. *Poster in the International Gordon Research conference on "Salt and water stress in plants"*, 24-29 June 2012, Hongkong, China.

Sagadevan G Mundree and **Linh Thi My Hoang (2011).**Engineering tolerance to salinity stress in rice using anti-apoptosis genes. *Proceeding of International Symposium on "100 years of Rice Science and Looking Beyond"***.** Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Volume 1 Pp 48-50.

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List of Abbreviations

ATP = adenosine-5'-triphosphate

BAP = 6-benzylaminopurine

bp = basepair(s)

BRM = bacterial re-suspension media

CaMV = *Cauliflower mosaic* virus

cDNA = complementary DNA

CTAB = cetyl trimethyl ammonium bromide

C-terminal = carboxyl-terminal

DAB = 3,3'-diaminobenzidine

DEPC = diethylpyrocarbonate

dH_2O = distilled water

DMSO = dimethylsulphoxide

DNA = deoxyribonucleic acid

dNTPs = deoxyribonucleoside triphosphates

dsDNA = double-stranded DNA

DTT = 1,4-dithiothreitol

DW = dry weight

2,4 D = 2,4-dichlorophenoxyacetic acid

EDTA = ethylenediaminetetraacetic acid

E. coli = *Escherichia coli*

FAO = Food and Agriculture Organization

GUS = β-glucuronidase

IRRI = International Rice Research Institute

Kbp = kilo basepair(s)

LB = Luria-Bertani

mRNA = messenger RNA

MS = Murashige & Skoog

NAA = α- naphthalene acetic acid

Nos = gene coding nopaline synthase

N-terminal = amino-terminal

OECD = Organisation for Economic Co-operation and Development

PBS = phosphate buffered saline

PCR = polymerase chain reaction

pH = log (proton concentration)

RNA = ribonucleic acid

RNase = ribonuclease

RT-PCR = reverse transcription PCR

RWC = relative water content

SDS = sodium dodecyl sulphate

ssp = subspecies

ssDNA = single-stranded DNA

UNFPA = United Nations Population Fund (previous name: UnitedNations Fund for Population Activities)

USDA = United States Department of Agriculture

TAE = Tris acetate EDTA

TE = Tris-EDTA

TUNEL = terminal dUTP nick end labelling

Tween 20 = polyoxyethylene (20) sorbitan monolaurate

U = unit

Ubi = ubiquitin

UV = untra violet

VC = vector control

WT = wild type

X-gal = 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

X-gluc = 5-bromo-4-chloro-3-indolyl-β-D-glucuronide-cyclohexylamine salt

Units:

cm = centimetre(s)

dS = deciSiemens

°C = degree Celsius

d = day(s)

KDa = kilo Dalton(s)

g = gram(s)

h = hour(s)

l = litre(s)

M = molar

m = meter(s)

MW = molecular weight

min = minutes

mol = mole(s)

rpm = revolutions per minute

s = second(s)

V = volt(s)

vol = volume(s)

v/v = volume per volume

W = watt

w/v = weight per volume

Prefixes:

M = Million

K = kilo

m = milli

μ = micro

n = nano

ρ = pico

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature:

Date: 12/06/2014

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Chapter 1: Introduction

This chapter provides the brief background and context of the research, and its purposes. The significance and scope of this research is mentioned in section 1.4. Section 1.5 describes the three specific objectives of this project and an outline of the remaining chapters of the thesis.

1.1 BACKGROUND

The world population is increasing rapidly, within 50 years from 1950 to 2010 the population was almost double and it reached 7.2 billion people in mid-2013 (UNFPA, 2014). By 2050, the world population has been predicted to reach 9.6 billion people (UNFPA, 2014). To have enough food for the increasing population crop productivity needs to increase by approximately 44 million metric tons annually. This is a challenge because there is very little potential for future expansion of arable lands whereas there are more and more environmental stresses coming (Eckardt 2009; FAO 2009, 2012; Cominelli *et al.* 2013). Of the abiotic stresses that limit world crop production, soil water deficits and salinisation are the two most critical factors (Munns 2011). This project focussed on salinity.

Salinity is a growing problem worldwide. It has been estimated that more than 800 million hectares (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 & Zinck 2003; Eynard *et al.* 2005; Munns & Tester 2008; Shabala & Cuin 2008). Crop yields are significantly reduced in salt-affected land with an estimation of 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.) (Eynard *et al.* 2005). Changes in the global environment due to climate change are predicted to cause further yield losses (Eynard *et al.* 2005).

The primary effects of high salinity on plant productivity are osmotic and ionic stresses. Osmotic stress occurs after the concentration of salts around the roots of the plant increase beyond a threshold tolerance level while ionic stress occurs when the concentration of salt in old leaves reaches a toxic level due to the influx of large

amounts of Na^+ into the plant, resulting in increased Na^+ concentrations in the vacuole and cytoplasm leading to interruption of metabolic processes and cell death (Munns & Tester 2008; Yu *et al.* 2012; Wang *et al.* 2013). To minimize the effect of salinity stress on plants, a number of strategies have been employed. Among these, water and soil management practices have facilitated agricultural production on soils marginalized by salinity but additional gain by these approaches seems problematic. More recently, crop improvement strategies that are based on the use of molecular marker techniques and biotechnology are being used in conjunction with traditional breeding efforts to develop salt tolerant crop species (Ribaut & Hoisington 1998; Arzani 2008; Bhatnagar-Mathur *et al.* 2008). Implementation of biotechnology strategies to achieve this goal requires substantial research effort and the identification of salt tolerance effectors, salt tolerance genes and the regulatory components that control osmotic homeostasis during stress events (Hasegawa *et al.* 2000; Yu *et al.* 2012).

Programmed cell death (PCD) or simply the decision of whether a given cell should live or die is essential for all multicellular (Metazoan) organisms. Under several stimuli, this decision is dependent on: i) the battle between anti-apoptotic (pro-survival) and pro-apoptotic (pro-death) proteins encoded by anti-apoptotic and pro-apoptotic genes (Li & Dickman 2004; Williams & Dickman 2008); ii) the ratio between pro-survival and pro-death proteins as this ratio determines the sensitivity of PCD (Fulda *et al.* 2010); and iii) signal transduction pathways because cell death signals can lead to activation of pro-death proteins while survival signals can reduce the activity of the pro-death proteins and increase the levels of pro-survival proteins (Zha *et al.* 1996; von Freeden-Jeffry *et al.* 1997; Strasser *et al.* 2000). Recently, it has been suggested that PCD pathways are key players in mediating plant responses to abiotic stresses, including salinity. Expression of pro-survival genes in transgenic plants has been shown to enhance biotic and abiotic stress tolerance in model crops. For example, expression of pro-survival genes has been used to improve tolerance to biotic and abiotic stresses in tobacco, tomato and banana(Dickman *et al.* 2001; Doukhanina *et al.* 2006; Paul 2009; Wang *et al.* 2009b; Paul *et al.* 2011).

Rice is an important crop that feeds more than half of the world population and is the model system for monocotyledonous plants that include members of the agronomically important cereals. Asia accounts for 90% of the world's production

and consumption of rice (Khush 2005). In humid and sub-humid Asia, rice is the single most important source of employment and income for rural people, and it provides 50-80% of the calories consumed for more than 3 billion Asians (Hossain & Fischer 1995; Khush 2005). However, rice is very sensitive to salinity stress and is currently listed as the most salt sensitive cereal crop with a threshold of 3 dSm^{-1} for most cultivated varieties (USDA 2013).

Rice is a member of the genus *Oryza* in the Family Poaceae. *Oryza* has many species, of which two – *Oryza sativa* L. and *O. glaberrima* Steud – are cultivated. *Oryza sativa* is most commonly grown in Asia while *O. glaberrima* is native to West and central Africa, where it is grown but it is being substituted by *Oryza sativa* (Linares 2002; OECD 2006; Oka 2012).

Oryza sativa is herbaceous and lives for one year with a growth period varies depending on the cultivar, season of cultivation and cultivation region, as well as the ecological conditions (Dingkuhn & Asch 1999).Three growth stages have been identified during the life cycle of rice including vegetative or seedling (from germination to panicle initiation), reproductive (panicle initiation to flowering) and ripening (flowering to mature grain); the length of each stage varies between cultivars (IRRI 2007). *Oryza sativa* has two subspecies: *indica* and *japonica* (OECD 2006). Identification of the two subspecies can be based on morphological and physiological differences. The japonicas have dark and erect leaves, a reasonable number of tillers, and grains that are more rounded and shorter than those of indica. Japonica are more tolerant of cold and lodging conditions and produce rice that, when cooked, is much stickier than the grains produced by indica. Japonica usually responds moderately to additional nitrogen. Indica, in contrast, are tall with droopy leaves and tiller heavily. Indica rice grains are longer and, when cooked, are drier and less sticky than japonica grains. Indica are most common in tropical regions and respond strongly to additional nitrogen especially in seedlings. The application of fertilizer to indica, however, leads to an increase in leafiness and tillering so that not much advantage in yield is gained (OECD 2006; Oka 2012).

Rice is an ideal model system for research in monocots and cereals as it has: the smallest fully sequenced genome in comparison with other major cereals (Sasaki & Burr 2000); a large-scale analysis of expressed sequence tags (ESTs) (Jantasuriyarat *et al.* 2005); a rich full-length cDNA collection (Kikuchi *et al.* 2003;

Lu *et al.* 2008); a high quality and density molecular map (Matsumoto *et al.* 2005); a gene expression atlas for all growth stages (Fujita *et al.* 2010; Wang *et al.* 2010); a rich mutant library, and more importantly it has an efficient transformation, a tool for modern plant biology that is not available in other cereals such as maize and sorghum (Izawa & Shimamoto 1996). In addition, some earlier disadvantages such as rice having longer life cycle and needing more space to grow during experiments than *Arabidopsis* (the model plants for dicots), have been solved (Ohnishi *et al.* 2011).

1.2 CONTEXT

Previous reports have shown that rice induces programmed cell death pathways upon salinity stress (Li *et al.* 2007; Liu *et al.* 2007; Jiang *et al.* 2008). Manipulation of PCD pathways to increase tolerance against salinity stress shows great potential to increase crop production in salt affected areas (Joseph & Jini 2010). Recently, a report has been released on PCD in rice however it was hindered by an incomplete experimental approach and assessment of plants (Yang *et al.* 2013).The transgenic rice expressing antisense – *OsPDCD5* in the Yang *et al*. study were exposed to salinity stress over a short period (only 3 days). This short period of salt exposure did not provide sufficient evidence of salt tolerance as screening for salinity stress tolerance at the seedling stage in rice requires longer exposure to account for toxicity effects, usually around eleven to sixteen days (Yeo & Flowers 1986; Gregorio *et al.* 1997; Hoang 2002; Lee *et al.* 2003; Platten *et al.* 2013). More importantly, none of the important indicators of salt tolerance at seedling stage which are essential criteria for salt tolerance assessments such as growth rate, percentage survival or standard evaluation score (SES) (Flowers & Yeo 1981; Gregorio *et al.* 1997; Platten *et al.* 2013) or essential evidence of PCD inhibition in transgenic rice upon salinity stress exposure such as absence of TUNEL-positive nuclei or DNA laddering were presented in that study. In this project a selection of key plant and virus derived genes, associated with programmed cell death pathways was investigated. Specifically *AtBAG4* and *AtBI* from *Arabidopsis thaliana, Hsp70*and *Hsp90* from *Citrus tristeza* virus, *OsBAG4* from *Oryza sativa, p35* from baculovirus, *SfIAP* from *Spodoptera frugiperda* and a combination of *AtBAG4+Hsp70,*for generating salinity tolerance in rice; assessment for salinity stress tolerance was conducted at both the seedling and reproductive stages in rice's life cycle.

1.3 PURPOSES

Programmed cell death plays important roles during normal development and during the interaction between plants and environment. Manipulation of PCD pathways using pro-survival genes in tobacco, tomato and banana has highlighted the possibility to enhance tolerance to biotic and abiotic stresses in other economical crops. Pro-survival genes such as CED-9, *AtBAG4*, *p35* and *SfIAP* have been reported to enhance tolerance to salinity stress in tomato and tobacco (Doukhanina *et al.* 2006; Shabala *et al.* 2007; Wang *et al.* 2009b; Kabbage *et al.* 2010; Li *et al.* 2010). However, pro-survival genes confer tolerance to salinity stress in a monocot have not been mentioned. This project therefore aims at manipulation of cell death pathways via the transformation of pro-survival genes from different sources to rice for improvement of salinity tolerance in this important crop.

1.4 SIGNIFICANCE, SCOPE AND DEFINITIONS

This research employed a preferable method (biotechnology-genetic engineering) for crop improvement strategies to address one of the most serious environment effectors that limit crop productivity- salinity. The project provided evidence of biochemical and physiological basis of salinity stress tolerance in rice expressing pro-survival genes. It contributed to the knowledge of crop improvement strategies for salinity stress tolerance and the findings of this project can be applied to other crops. In addition, the research also proposed a model for salinity-induced cell death switch for salinity stress tolerance in rice.

1.5 THESIS OUTLINE

To fulfil the overall aim, three specific objectives were achieved:

1. Generation and characterisation of transgenic rice expressing *AtBAG4*, *AtBAG4+Hsp70*, *AtBI*, *Hsp70*, *Hsp90*, *OsBAG4*, *p35*, *SfIAP* and vector control (vector backbone without gene of interest).
2. High throughput screening for salinity stress tolerance of transgenic rice at seedling stage to select prominent salinity stress tolerant lines.
3. Glasshouse evaluation of representative transgenic rice lines with full assessment on salinity stress tolerance at both seedling and reproductive stages based on agronomical, biochemical and physiological parameters.

The following chapters will provide an up-to-date literature review of the research topic, the general materials and methods used in this project, followed by the results of three specific objectives respectively and a general discussion and conclusion.

Chapter 2: Literature Review

This chapter begins with an overview of salinity as a growing worldwide problem (section 2.1) and the impact of salinity on crop productivity (section 2.2). The response of plants to salinity stress is summarised in section 2.3 and approaches to improving salinity stress tolerance with an emphasis on genetic engineering is presented in section 2.4. Section 2.5 provides an overview of programmed cell death and its roles during development and interaction of organisms with environment. Section 2.6 presents the literature review of programmed cell death in mammals with details of execution and regulation of apoptosis - the most understood form of programmed cell death. The programmed cell death and factors that induce cell death in plants are mentioned in section 2.7. Section 2.8 briefly describes seven pro-survival genes that used in this project and section 2.9 highlights the implications from the literature and develops the conceptual framework for the study.

2.1 SALINITY - A GROWING WORLDWIDE PROBLEM

Salinity is a general term used to describe the presence of elevated levels of different salts such as sodium chloride, magnesium and calcium sulphates 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. Salts in the soil may originate from many sources, particularly rainfall and rock weathering. Although rainwater contains only 6-50mg/Kg of sodium chloride, over time, salt deposited by rain can accumulate in the landscape. Wind also transports salt from the sea or lakes into neighbouring soil, contributing to an increase of the inland salt level. Another source of salinity is human activities such as irrigation and extensive clearing of vegetation (Rengasamy 2006; Munns & Tester 2008; Rengasamy 2010).Salinity hazard is measured as electrical conductivity (EC) of soluble salts in water or soil in deciSiemens per meter (dSm^{-1}) or microSiemens per centimetre (μScm^{-1}).

The primary effect of salinity stress on crop productivity is the inability of the plant to compete with ions in the soil solution for water (physiological drought). While an appropriate concentration of salts is vital for plants, salinity that is beyond the normal range will cause stress or even death to most plants. Soil and irrigation water with electrical conductivity in the range of 0.25 - 0.75 dSm^{-1} is considered to be optimum and 8 -10 dSm^{-1} is considered to be the practical upper limit for agriculture (Corwin & Lesch 2003). Moreover, poor irrigation practices contribute to salinity as often more water is applied than can be used by the crops. In this case, excess water percolates into the water table causing groundwater to rise which brings soluble salts to the soil surface (Ashraf 1994; Qadir *et al.* 2009). This can dramatically affect our natural environment and reduce the viability of our agricultural sector.

Both primary (natural) and secondary (related to human activities) salinization result in salt-affected soils. Two types of salt-affected soils have been classified: saline and sodic soils. The distinguishing features of saline and sodic soils are presented in Table 2.1.

Table 2.1: Distinguishing features of saline and sodic soils (FAO 1988)

Characteristics	Saline soils	Sodic soils
Dominated chemicals	- Dominated by neutral soluble salts consisting of chlorides and sulphates of sodium, calcium and magnesium.	- Appreciable quantities of neutral soluble salts generally absent. Measurable to appreciable quantities of salts capable of alkaline hydrolysis, e.g. Na_2CO_3, present.
	- pH of saturated soil paste is less than 8.2.	- pH of the saturated soil paste is more than 8.2.
	- An electrical conductivity of the saturated soil extract of more than 4 dSm^{-1} at	- An exchangeable sodium percentage (ESP) of 15 or more is the generally accepted limit above which soils are

	25°C is the generally accepted limit above which soils are classed as 'saline'. - There is generally no well-defined relationship between pH of the saturated soil paste and exchangeable sodium percentage (ESP) of the soil or the sodium adsorption ratio (SAR) of the saturation extract. - Although Na is generally the dominant soluble cation, the soil solution also contains appreciable quantities of divalent cations, e.g. Ca and Mg. - Soils may contain significant quantities of sparingly soluble calcium compounds, e.g. gypsum.	classed as 'sodic'. Electrical conductivity of the saturated soil extract is generally less than 4 dSm^{-1} at 25°C but may be more if appreciable quantities of Na_2CO_3 etc. are present. - There is a well defined relationship between pH of the saturated soil paste and the exchangeable sodium percentage (ESP) of the soil or the SAR of the saturation extract for an otherwise similar group of soils such that the pH can serve as an approximate index of soil sodicity (alkali) status. - Sodium is the dominant soluble cation. High pH of the soils results in precipitation of soluble Ca and Mg such that their concentration in the soil solution is very low. - Gypsum is nearly always absent in such soils.
Physical features of the soils	- In the presence of excess neutral soluble salts the clay fraction is flocculated and the soils have a stable	- Excess exchangeable sodium and high pH result in the dispersion of clay and the soils have an unstable structure. - Permeability of soils to water

	structure. - Permeability of soils to water and air and other physical characteristics are generally comparable to normal soils.	and air is restricted. Physical properties of the soils become worse with increasing levels of exchangeable sodium/pH.
Effect on plant growth	- In saline soils plant growth is adversely affected: • chiefly through the effect of excess salts on the osmotic pressure of soil solution resulting in reduced availability of water • through toxicity of specific ions, e.g. Na, Cl, B, etc.	- In sodic soils plant growth is adversely affected: • chiefly through the dispersive effect of excess exchangeable sodium resulting in poor physical properties • through the effect of high soil pH on nutritional imbalances including a deficiency of calcium • through toxicity of specific ions, e.g. Na, CO_3, Mo, etc.
Soil improvement	- Improvement of saline soils essentially requires removal of soluble salts in the root zone through leaching and	- Improvement of sodic soils essentially requires the replacement of sodium in the soil exchange complex by calcium through use of soil amendments and leaching and

	drainage. Application of amendments may generally not be required.	drainage of salts resulting from reaction of amendments with exchangeable sodium.
Geographic distribution	- Saline soils tend to dominate in arid and semi-arid regions.	- Sodic soils tend to dominate in semi-arid and sub-humid regions.
Ground-water quality	- Groundwater in areas dominated by saline soils has generally high electrolyte concentration and a potential salinity hazard.	- Groundwater in areas dominated by sodic soils has generally low to medium electrolyte concentration and some of it may have residual sodicity so has a potential sodicity hazard.

The distribution of soil salinity over the world (Borém *et al.* 2012)is shown in table 2.2.

Table 2.2: Global distribution of soil salinity (saline and sodic soils)

Regions	Total area	Saline soils		Sodic soils	
	Million ha	Million ha	%	Million ha	%
Africa	1,899	39	2.0	34	1.8
Asia, the Pacific, and Australia	3,107	195	6.3	249	8.0
Europe	2,011	7	0.3	73	3.6

Latin America	2,039	61	3.0	51	2.5
Near East	1,802	92	5.1	14	0.8
North America	1,924	5	0.2	15	0.8
Total	**12,781**	**397**	**3.1**	**434**	**3.4**

2.2 SALINITY AND CROP PRODUCTION CONSTRAINTS

Salinity stress has a significant effect on the production of most crops, but the extent of these adverse effects depends on the type and duration of salinity and also upon sensitivity of the crop at different growth stages. For example, previous reports have indicated yield losses in the range of 10 to 90% for wheat, 30 to 50% for rice, 50 to 75% for cotton and 30 to 90% for sugarcane that grew on salt affected areas (Eynard *et al.* 2005). These adverse effects are caused by low osmotic potential of the soil solution resulting in osmotic stress, ionic stress (Munns & Tester 2008), effects on energy balance (Rengasamy 2006), production of reactive oxygen species (Ashraf 2009), inhibition of enzymatic activities, alterations in different metabolic activities of plants, disturbances in solute accumulation and combinations of these factors (Wang & Li 2006).

Osmotic stress occurs after the concentration of salts around the roots of the plant increases beyond the threshold tolerance level (Munns & Tester 2008) (e.g. 3 dSm^{-1} for rice) (USDA 2013). Osmotic stress results in reduced leaf area and in cereals it is reflected in a reduction in the number of tillers (Munns & Tester 2008). Ionic stress starts when the concentration of salt in old leaves reaches toxic levels resulting in the death of those leaves. The rate at which old leaves die becomes higher than the rate of emergence of new leaves leading to a lower photosynthetic capacity and lower growth rate (Munns & Tester 2008). Energy balance is also affected as plants subjected to salinity require more energy to take-up water from saline soil. Rengasamy (2006) reported that in non-saline sandy loam soils, plants are able to take up water until the soil dries to 5% soil-water content but with salinity at the level of 6.4 dSm^{-1} to 10 dSm^{-1}, plants can take up water only up to 14 to 18% soil-water content, respectively.

Reactive oxygen species (ROS) play an important role in many important physiological phenomena such as program cell death (PCD), cell signalling, senescence, gene regulation, and pathogen defence (Gechev *et al.* 2006). Under salinity stress, however, electron transport within the different sub-cellular compartments is reduced resulting in the overproduction of ROS. ROS can cause substantial oxidative damage to bio-molecules such as nucleic acids, proteins and membranes lipids (Ashraf 2009). An overview of how plants overcome these effects is summarized below.

2.3 RESPONSE OF PLANTS TO SALINITY STRESS

Plants may be divided into two groups – halophyte or glycophyte –based on their level of salinity tolerance. Halophytes are plants that tolerate relatively high salt concentrations (400mM NaCl), while glycophyte can tolerate only low concentrations (Maas&Nieman1978). Most crops are glycophyte and their growth is retarded during salinity stress. Among the cereals, rye (*Secale cereale*) is the most tolerant (threshold of 11 dSm^{-1}) and rice is the most sensitive crop plant with a threshold of 3 dSm^{-1} for most cultivated varieties (USDA 2013).

Plant adaptations to salinity stress may be placed into three categories: i) osmotic stress tolerance, ii) Na^+ or Cl^- exclusion, and iii) Na^+ or Cl^-compartmentalization.

Osmotic stress reduces cell expansion in root tips and young leaves, and causes stomata closure. A reduced response to the osmotic stress would result in greater leaf growth and stomata conductance, but the resulting increased leaf area would benefit only plants that have sufficient soil water. Greater leaf area expansion would be productive when a supply of water is ensured such as in irrigated food production systems, but could be undesirable in water-limited systems, which could potentially 'run-dry' before the grain is fully matured. Osmotic stress tolerance is also achieved through overproduction of osmoprotectants such as glycine betaine, proline, mannitol and trehalose.

Exclusion of Na^+ by roots ensures that it does not accumulate to toxic concentrations in leaves. Failure to exclude Na^+ manifests its toxic effect after days or weeks and causes premature death of older leaves. Tolerance of tissue to accumulated Na^+ requires compartmentalization of Na^+ into vacuole to avoid toxic

concentrations within the cytoplasm, especially in mesophyll cells of the leaf. Toxicity occurs with time, after leaf Na^+ increases to high concentrations in the older leaves (Munns & Tester 2008). Plants can defend themselves against ROS by employing a system to scavenge or detoxify these elements (Ashraf 2009).

2.4 APPROACHES TO IMPROVING SALINITY STRESS TOLERANCE - GENETIC MODIFICATION

Conventional breeding approaches to improve tolerance of plants to abiotic stress, especially salinity, have had limited success (Ashraf & Akram 2009). Recently, molecular techniques such as Marker Assisted Selection (MAS) have been employed along with conventional breeding to improve crop tolerance to salinity stress. Quantitative trait loci (QTL) analysis has been attempted in order to understand the genetics of salt tolerance and to deal with component traits in breeding programs. However, due to the lack of precise knowledge of the key genes underlying the QTLs, the introgression of genes involved in stress tolerance through MAS has often resulted in introgression of undesirable agronomic characteristics from the donor parents. Genetic modification has become a powerful tool in plant breeding programs since it allows the introduction of gene(s) controlling traits without affecting the desirable characteristics of an elite genotype (Bhatnagar-Mathur *et al.* 2008).The recent advent of an efficient *Agrobacterium*-mediated transformation method for rice (Hiei *et al.* 1994) displays great potential from the genetic improvement of this plant.

To date, genetic engineering for salinity tolerance in plants has focused on genes that encode compatible organic solutes, antioxidants (detoxification of ROS), ion transport, heat-shock and late embryogenesis abundant proteins (Ashraf *et al.* 2008) (Figure 2.1). Despite some promising reports, the development of a salt-tolerant cultivar by way of transgenics has still awaited more investigation.

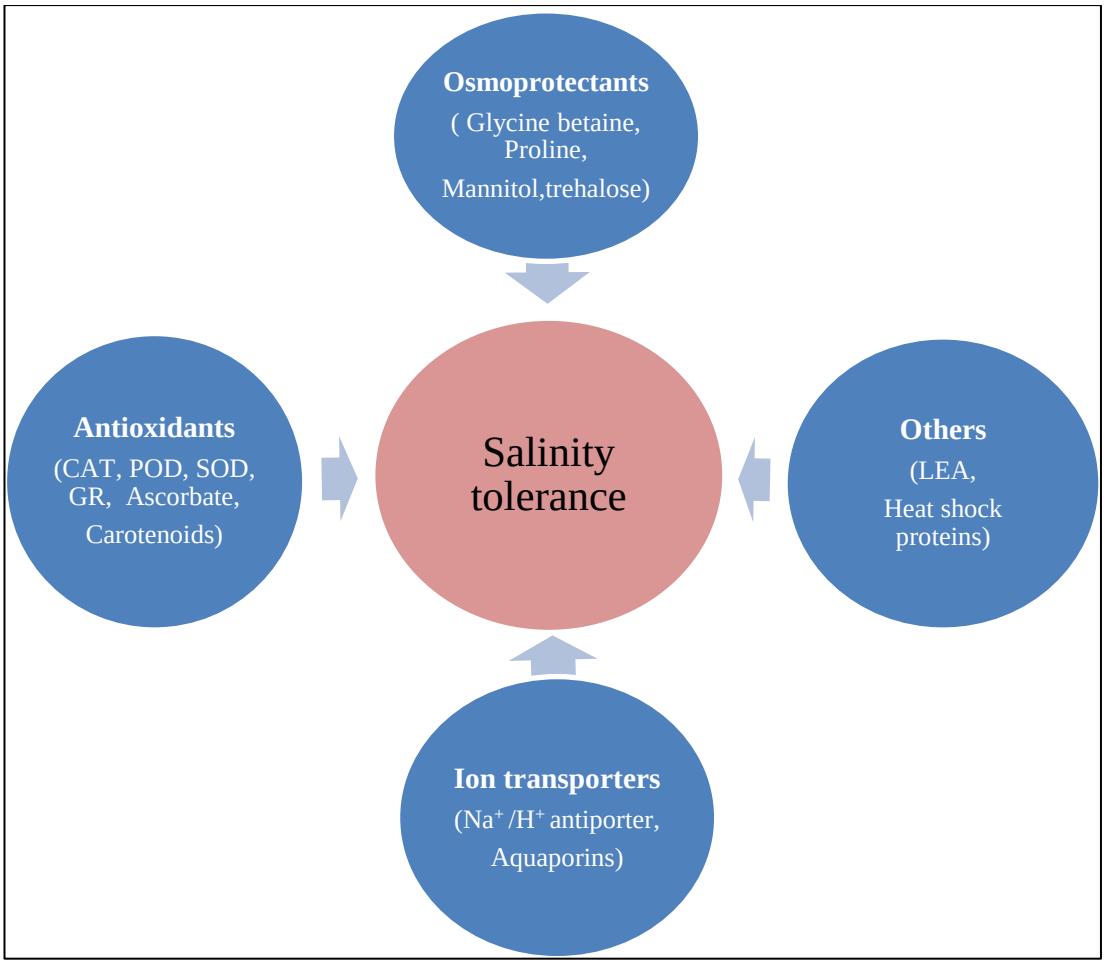


Figure 2.1: Genetic modification approaches to salinity stress tolerance

2.4.1 Compatible organic solutes

Overproduction of different types of compatible organic solutes is the most common plant response to salinity stress (Ashraf & Foolad 2007). However, many crops lack the ability to synthesize the special compatible organic solutes (osmoprotectants) that are naturally accumulated by stress-tolerant organisms. A number of studies have focused on over-expressing osmoprotectants in plants to generate stress-tolerant crops (Bhatnagar-Mathur *et al.* 2008). For example, genes involved in glycine betaine biosynthesis like *BetA* or *CodA* and *COX* for chloline oxidase, have been transformed into *Arabidopsis*, tobacco, rice and cabbage for enhanced tolerance to salinity stress (Hayashi *et al.* 1997; 1998; Sakamoto *et al.* 1998; Holmstrom *et al.* 2000; Huang *et al.* 2000a; Bhattacharya *et al.* 2004).

For proline biosynthesis and accumulation, genes such as Δ^1-pyrroline-5-carboxylate reductase (*TaP5CR*), Δ^1-pyrroline-5-carboxylatesynthetase (*P5CS*) (*P5CS129A*), *DREB* (dehydration responsive element binding protein) and *AtProDH* cDNA encoding proline dehydrogenase (*ProDH*) have been transformed into a variety of plants including*Arabidopsis*, rice, tobacco and wheat for improvement of salinity stress tolerance in those plants (Nanjo *et al.* 1999; Sawahel & Hassan 2002; Su & Wu 2004; Cong *et al.* 2008; Ma *et al.* 2008).

Other genes such as Mannitol 1-phosphate dedydrogenase *(mtlD)* and glucitol-6-phosphate dehydrogenase (*GutD*), L-myo-inositol 1-phosphate synthase, L-2,4-diaminobutyric acid acetyltransferase (*ectA*), L-2,4-diaminobutyric acid transaminase (*ectB*); L-ectoine synthase (*ectC*), Myo-inositol Omethyltransferase (*ImtI*) and trehalose-6-phosphate synthase (*TPS1*) were introduced into a number of plants and there are reports that salt tolerance in those transgenic plants was higher than that of the controls (Thomas *et al.* 1995; Sheveleva *et al.* 1997; Nakayama *et al.* 2000; Abebe *et al.* 2003; Majee *et al.* 2004; Cortina & Culiáñez-Macià 2005; Tang *et al.* 2005).

Although much work has been devoted to genetically engineering plants for the overproduction of various osmoprotectants, there has been little success in attaining the desired protective levels of these osmolytes in plants using these approaches (Ashraf & Foolad 2007).

2.4.2 Expression of ROS scavengers

In order to protect the cell from oxidative injury, plants have evolved a complex antioxidant defence system to scavenge and detoxify ROS (Asada 1999; Bhatnagar-Mathur *et al.* 2008; Ashraf & Akram 2009). This system includes various enzymes such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR); as well as non-enzymatic antioxidant compounds such as arscorbate, α-tocopherol, carotenoids and glutathione (Ashraf 2009). Many studies have shown that over-expression of antioxidant enzymes like DHAR and different types of SOD increase salt tolerance in plants such as *Arabidopsis* (Ushimaru *et al.* 2006), Chinese cabbage (Tseng *et al.* 2007), rice (Tanaka *et al.* 1999), and tobacco (Badawi *et al.* 2004; Wang *et al.* 2004).Other genes encoding glyoxalase pathway enzymes and glutathione S-transferase and catalase have also

been transformed into rice and tobacco, and shown to marginally increase tolerance to salinity stress (Roxas *et al.* 2000; Verma *et al.* 2005; Yadav *et al.* 2005; Al-Taweel *et al.* 2007). However, the protective effects were minimal and were only observed under strictly controlled conditions (Ahmad & Srivastava 2008).

2.4.3 Ion transporters

Enabling plants to re-establish ion homeostasis under stressful environments has been another approach to improving salinity tolerance in plants (Bhatnagar-Mathur *et al.* 2008). Researchers have reported that antiporter genes, when engineered into the plant improve salinity tolerance. For example, the *MsNHX1* gene was over-expressed in *Arabidopsis* leading to an increase in salinity tolerance of transgenic plants (Bao-Yan *et al.* 2008). Transgenic rice over-expressing *AgNHX1* was reported to increase tolerance to salinity stress (Ohta *et al.* 2002). Similarly over-expression of other genes such as *ZmOPR1,EhCaBP,GhNHX1, PgNHX1, AtNHX1* and *OsNHX1* in transgenic *Arabidopsis,* tobacco, wheat, maize and rice also resulted in increase of tolerance to salinity stress (Pandey *et al.* 2002; Fukuda *et al.* 2004; Wu *et al.* 2004; Xue *et al.* 2004; Yin *et al.* 2004; Verma *et al.* 2007; Gu *et al.* 2008).

2.4.4 Late embryogenesis abundant proteins

Late embryogenesis abundant proteins (Vanninia *et al.* 2006) may play protective roles in tolerance of plants to environmental stresses (see review by Chinnusamy *et al.*(2005). Xu *et al.* (1996) reported an increase in tolerance to soil water deficit and salinity stress in transgenic rice over-expressing the *HVA1* gene. Another study also showed some positive effectsof *HVA1* expression on tolerance to salinity stress in transgenic rice (Rohila *et al.* 2002).

Although many studies have been conducted for genetic engineering using genes that code for compatible solutes, antioxidant production, ion transporter and LEA proteins, the improvement in salinity stress tolerance of plant has met with limited success. In more than 35 studies listed above only three studies reported a complete assessment for salinity stress tolerance in transgenic plants (Xue *et al.* 2004; Yin *et al.* 2004; Verma *et al.* 2007).

2.4.5 Programmed cell death genes-potential candidates for salinity stress tolerance

Programmed cell death (PCD) is a physiological and genetically controlled process that is evolutionarily conserved across kingdoms and allows multicellular organisms to eliminate excessive or damaged cells which arise during development and in response to abiotic and biotic stress (Williams & Dickman 2008; Fomicheva *et al.* 2012).

The expression of anti-PCD genes in transgenic plants has been reported to enhance tolerance to biotic and abiotic stresses. For example, anti-apoptotic (pro-survival) genes from mammals, nematodes, insect, baculovirus and plants such as *Bcl*-2, *Bcl-xL*, *ced*-9, *SfIAP*, *p35* and *AtBAG4* have been transformed into tobacco, tomato and banana 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 & Dickman 2004; Xu *et al.* 2004; Doukhanina *et al.* 2006; Shabala *et al.* 2007; Wang *et al.* 2009a; Wang *et al.* 2009b; Kabbage *et al.* 2010; Li *et al.* 2010; Paul *et al.* 2011). Manipulation of PCD pathways may be an efficient approach for improving salinity stress tolerance in economically important crops. Programmed cell death has been studied extensively in animals and the underlying mechanisms in plants are gradually being discovered. The following section will outline: i) Mechanisms of mammalian and plant programmed cell death, ii) PCD during development and in response to abiotic and biotic stress and iii) a brief introduction of the seven genes used in this project.

2.5 PROGRAMMED CELL DEATH

Programmed cell death refers to the death of a cell that is genetically "programmed", often requiring the induction of specific genes to activate the cell death machinery (Williams & Dickman 2008).Alongside cell division and cell migration, PCD enables the organism to strictly control cell numbers and tissue size and to protect itself from unwanted cells that threaten cellular homeostasis (Hengartner 2000). The role of programmed cell death, particularly apoptosis, a physiological form of cell death, during development and interaction between multicellular organisms and the environment has become the subject of many studies since the first time it was reported in the nematode *Caenorhabditis elegans* by Kerr

et al.(1972). Over the past four decades, a number of PCD functions and involvements in development and in interaction of multicellular organisms with environment have been discovered.

2.5.1 PCD – a physiological mechanism for normal development

Regulation of homeostatic balance between cell division and cell death is fundamental for development and well-being of all multicellular organisms (Rudin & Thompson 1997). Genetically regulated mechanisms in multicellular organisms not only determine which cells live but also which cells die (Raff 1992; Chinnaiyan & Dixit 1996). Programmed cell death, apoptosis in particular, is a well regulated physiological form of cell autodestruction that plays an important role in organogenesis, tissue remodelling and normal cellular homeostasis during development of multicellular organisms (Faucheu *et al.* 1995; Zimmermann *et al.* 2001; Rupinder *et al.* 2007; Fuchs & Steller 2011). The sculpturing of shape during developing limb to form foetal fingers and toes together with the resorption of the tadpole tail during metamorphosis into a frog are the two well-known examples of the programmed cell death involvement in normal development (Zuzarte-Luís & Hurlé 2002). Evidence indicates that abnormal regulation of programmed cell death especially apoptosis is associated with a wide range of diseases. Insufficient apoptosis results in excessive cell accumulation causing autoimmunity or cancer; inappropriate cell death can lead to chronic degenerative diseases, heart failure, cerebral ischemia, Alzheimer disease, infertility and immunodeficiency (Kondo 1988; Leijon *et al.* 1994; Edwards 1998; Danial & Korsmeyer 2004; Rami *et al.* 2008; Lukiw & Bazan 2010; Whelan *et al.* 2010).

The roles of programmed cell death during development of animals were thoroughly reviewed in Fuchs & Steller (2011), especially in regulation of sculpting structure and driving morphogenesis, deletion of unwanted or redundant transient functional structures, control cell numbers and elimination of unwanted and potentially dangerous cells (Figure 2.2). In plants, PCD is involved in many stages of development from the embryo to reproduction and aging such as embryogenesis, somatic embryogenesis (Giuliani *et al.* 2002; Suarez *et al.* 2004; Hill *et al.* 2013), sex determination in unisexual species (Dellaporta & Calderon-Urrea 1994; Beers 1997), seed development (Young & Gallie 2000) and senescence (Greenberg 1996; Yen & Yang 1998; Simeonova *et al.* 2000; Yoshida 2003).

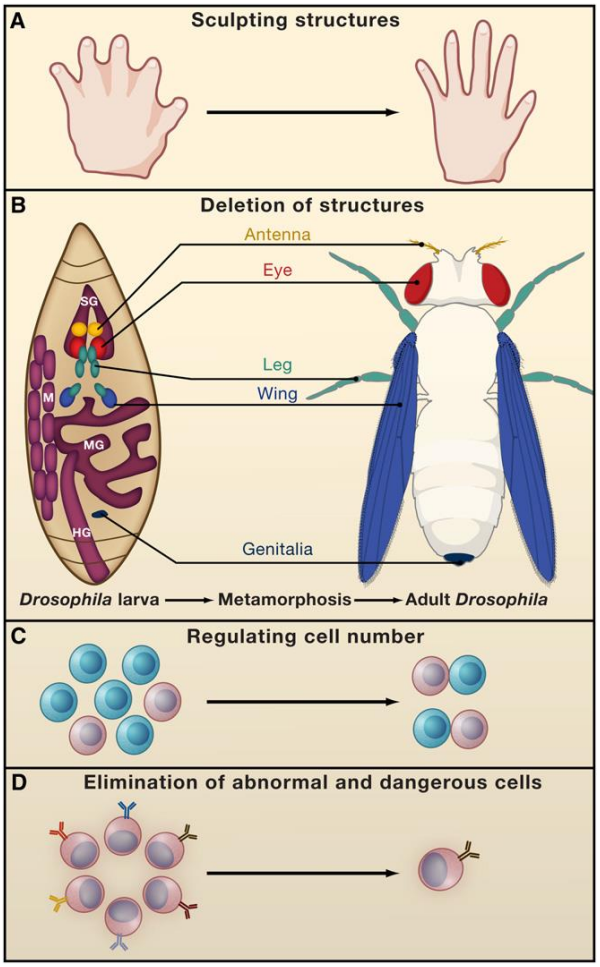


Figure 2.2: Function of PCD during development of multicellular organism (Fuchs & Steller 2011)

2.5.2 PCD – a defence mechanism in interaction between organisms and environment

In addition to development, PCD pathways are also used for adaptation to environmental stresses (Vaux *et al.* 1994; Mittler & Lam 1996; Vaux & Strasser 1996). The Hypersensitive response (HR) of plants to pathogen infection is one example of the programmed cell death functions as a defence mechanism of the organism to pathogen invasion. Plants lack of an immune system which can produce specialized cells, such as T cells in animal systems that can attack, disable and eliminate pathogen, they instead, induce programmed cell death as one of general defence strategies. Hypersensitive response, which occurs at the site of pathogen attack and involves programmed cell death in and around the infection site, is one of the strategies that plants employ to cope with pathogen invasion (Lam *et al.* 2001; Lam 2004). During interaction between the biotrophic pathogen and host plant, programmed cell death in HR help plants to prevent infection as biotrophy by

definition require living cells for growth and colonisation. However, in some instances plants infected by necrotrophic pathogens e.g. *Sclerotinia sclerotiorum*, cell death is disadvantageous for the plant as necrotrophic pathogens require dead or dying cells for nutrients. The role of PCD in plant-pathogen interaction, therefore, depends upon the context and in some circumstances plants involved in the process as a passive participant (Williams & Dickman 2008).

Cell death in response to abiotic stress provides an advantage to plants in some circumstances but not in others. For example programmed cell death during hypoxia-induced aerenchyma formation in root of maize enables the plants to survive and develop in wetlands where there is limited or no oxygen present (Drew *et al.* 2000). However, in response to most of other abiotic stresses such as salinity, drought, heat, cold, wounding, UV radiation, aluminium, acifluorfen, sufentrazone, menadione and hydrogen peroxide, prevention of cell death brings more benefit to the plants than execution of cell death as evidenced in many studies (Dickman *et al.* 2001; Lincoln *et al.* 2002; Qiao *et al.* 2002; Li & Dickman 2004; Xu *et al.* 2004; Doukhanina *et al.* 2006; Shabala *et al.* 2007; Wang *et al.* 2009a; Wang *et al.* 2009b; Kabbage *et al.* 2010; Li *et al.* 2010). Thus manipulation of programmed cell death pathways, particularly through the use of pro-survival genes to improve tolerance to abiotic stress in economically crops is worth to be investigated.

2.5.3 PCD – a conserved mechanism

Programmed cell death particularly apoptosis, the physiological form of PCD, has been studied for more than 40 years and is known to occur in many species across all kingdoms. For example human *Bcl-2* can partially complement *Ced-9* mutant of nematode *Caenorhabditis elegans* even though the two genes have limited sequence homology. The animal-origin anti-apoptotic genes *Ced-9* and *Bcl-2* confer tolerance to a wide range of biotic and abiotic stresses, when being over expressed in transgenic plants, have also been reported (Qiao *et al.* 2002; Chen & Dickman 2004; Shabala *et al.* 2007; Wang *et al.* 2009a; Paul *et al.* 2011).

Since the first evidence that a genetic program existed for physiological (programmed) cell death came from studying development in the nematode *Caenorhabditis elegans* (Kerr *et al.* 1972; Horvitz *et al.* 1982; Ellis & Horvitz 1986; Vaux *et al.* 1988) the study of pathways and regulation of programmed cell death has been carried out on several model systems including *C. elegans*, the vinegar fly

Drosophila melanogaster and the mouse. The conservation of the core apoptotic machinery has been found across vast evolutionary distances from worm to human, however it is somewhat obscure in plants (Williams & Dickman 2008; Fuchs & Steller 2011) as shown in Figure 2.3. This is likely due structural differences between animal and plant cells.

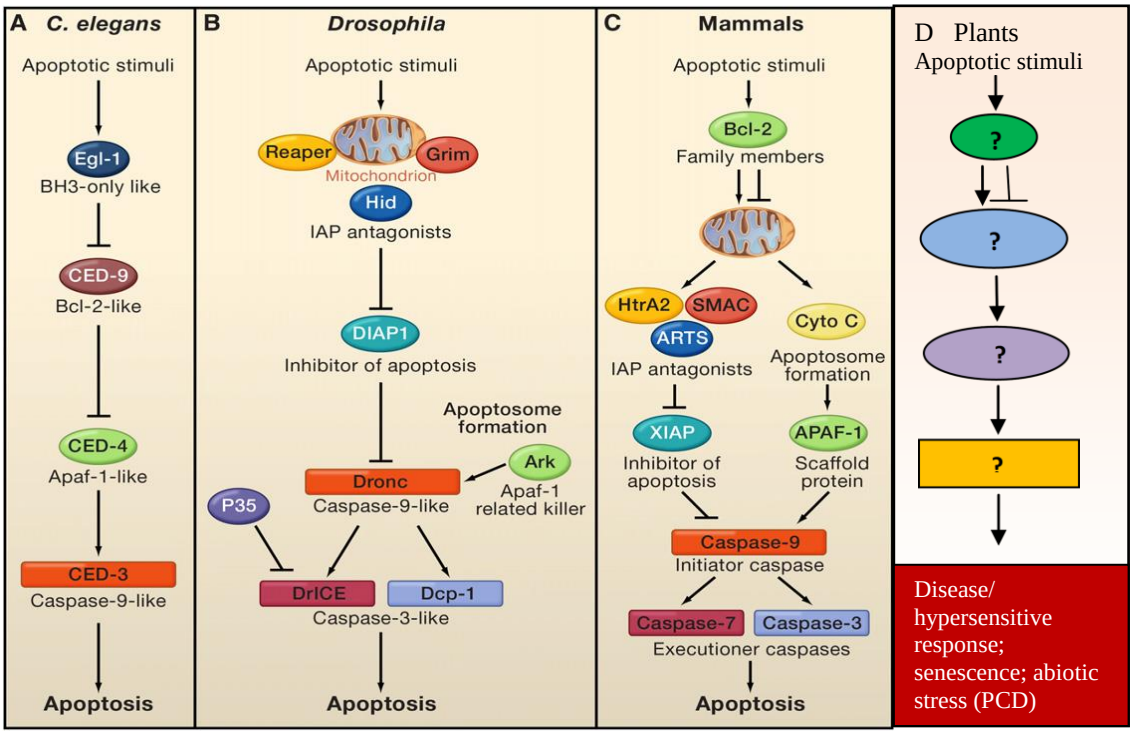


Figure 2.3: Evolutionary conservation of the core apoptotic machinery [(Fuchs & Steller 2011) and (Williams & Dickman 2008)]

As the core apoptotic machinery is conserved across kingdoms, details of a well-studied programmed cell death model would be helpful to establish an understanding of programmed cell death in plants. Of the three well-studied model systems above, mammalian programmed cell death is the most complicated model and it provides up to date knowledge about apoptosis therefore it is selected to be discussed in the following section.

2.6 PROGRAMMED CELL DEATH IN MAMMALS

Mammalian programmed cell death can be categorized into three types based on morphological criteria: apoptosis (type I), autophagy (type II) and necrosis (type III) (Kourtis & Tavernarakis 2009; Kroemer *et al.* 2009). Cells undergoing apoptosis have distinct morphological changes including cell shrinkage, membrane blebbing, chromatin condensation, apoptotic body formation and fragmentation, minor

modification of cytoplasmic organelles, and the apoptotic bodies were engulfed by resident phagocytes *in vivo* (Gilchrist 1998; Bredesen 2000; Collazo *et al.* 2006; Kroemer *et al.* 2009). In contrast to apoptosis, autophagic cells (PCD type II) lack chromatin condensation and the engulfment of dying cells is achieved by autophagolysosome within the dying cell. This process is independent of phagocytes. Cells undergoing autophagy usually have massive vacuolization of cytoplasm and accumulation of double-membrane autophagic vacuoles (autophagosome) (Baehrecke 2005; Kourtis & Tavernarakis 2009; Kroemer *et al.* 2009). Type III of programmed cell death in mammals is termed necrosis. Cells undergoing necrosis have cytoplasmic and cytoplasmic organelle swelling, plasma membrane rupture, and moderate chromatin condensation (Kroemer *et al.* 2009). Necrosis is more "accidental" cell death event in comparison with autophagy and apoptosis which are genetically controlled (Williams & Dickman 2008). Other forms of cell death in mammals related to inflammation response during pathogen invasion have also been observed. This includes pyroptosis (or caspase-1-dependent cell death) and necroptosis (or programmed necrosis). Pyroptosis is featured by the rapid rupture of the plasma membrane and release of pro-inflammatory intracellular contents (see review by Bergsbaken *et al.* 2009). Cells undergoing Necroptosis or regulated necrosis have similar cellular morphology to necrosis and secondary necrosis including cell rounding, cytoplasmic swelling, plasma membrane rupture and spilling of the intracellular contents (Berge *et al.* 2010).

Amongst the aforementioned types, apoptosis has been studied the most and the best understood form of PCD in mammals. It is a genetically controlled and highly orchestrated cell death. Basically, the excecution of apoptosis in mammals relies on the activation of caspases (cysteine aspartic acid specific proteases), a family of higly specific cysteine proteases that are ubiquitously expressed as inactive precusors (zymogens) with little or no protease activities (Fuchs & Steller 2011). Caspases can be thought of as central excecutioners of apoptotic pathways because they bring about most of visible changes that characterise apoptotic cell death. For example hallmarks of apoptosis such as DNA fragmentation and membrane blebbing are associated with caspase-3 activities (Hengartner 2000; Zimmermann *et al.* 2001).

2.6.1 Caspases

Caspases are cysteine proteases that use a cysteine residue as a catalytic nucleophile and cleave their target proteins at specific aspartic acid residues (Bredesen 2000; Boatright & Salvesen 2003). Caspases are very selective processing proteases as they usually make one, or rarely two, breaks per target protein. This is due to the fact that caspases specific recognise a tetrapeptide sequence (P4-P3-P2-P1) with an aspartic acid residue at C-terminus (P1 substrate position) and hydrolyse the peptide bond after it (Fomicheva *et al.* 2012). At least 14 caspases have been characterised in mammals (Shi 2002). Members of the caspase family share similarities in amino acid sequences, structure and substrate specificity and have been named "caspase" followed by an Arabic number, this number is assigned based on its date of publication (Alnemri *et al.* 1996; Nicholson & Thornberry 1997; Johnson 2000). The primary sequence of a caspase encodes an N-terminal pro-domain followed by a 20 kDa (p20 or large) and a 10 kDa (p10 or small) subunit. The p20 and p10 subunits associate to form the catalytic core of the enzyme (Shiozaki & Shi 2004).The N-terminal pro-domain and the large subunit is separated by an aspartate cleavage site, and the large and small subunits are separated at an interdomain linker containing one or two aspartate cleavage sites (Zimmermann *et al.* 2001).

The mammalian caspase family can be divided into two subfamilies. The first one is involved in inflammation, where caspases act as pro-cytokine activators and include members caspases-1, -4, -5, -11, -12, -13 and -14. The other subfamily is involved in apoptosis and includescaspase-2, -3, -6, -7, -8, -9 and -10. The apoptotic subfamily can be further categorised into two subgroups: initiator caspases caspase-2, -8, -9 and -10; and executioners or effector caspases caspase-3, -6 and -7 (Zimmermann *et al.* 2001; Shi 2002; Boatright & Salvesen 2003; Fomicheva *et al.* 2012).The initiators and effectors were classified according to the point of entry into the apoptotic pathway and similarity in their structure. Initiator caspases are the first to be activated in a particular death pathway, once activated they specifically cleave and activate the effector caspases (Shi 2002; Boatright & Salvesen 2003). Structurally, initiator caspases have an extended pro-domain with one or two motifs, DED (death effector domain) and CARD (caspases recruitment domain), responsible for the interaction with adaptor molecules; effector caspases on the other hand have a

shorter pro-domain (Fomicheva *et al.* 2012). A schematic diagram of the mammalian caspases is shown in Figure 2.4

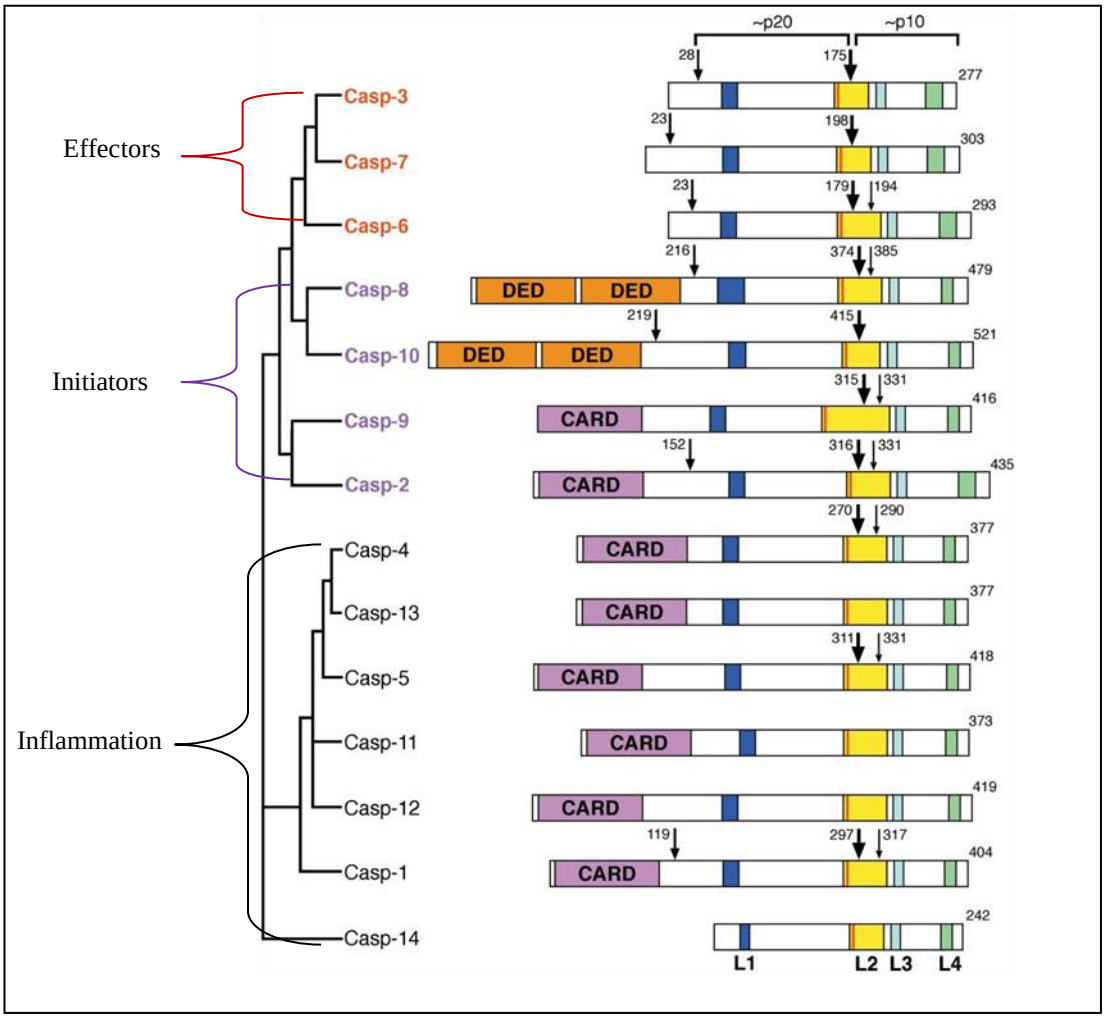


Figure 2.4: Mammalian Caspases family, adaptation from (Shi 2002). The large arrow indicates position of the first activation cleavage. The medium and small arrows represent additional sites of cleavage. The four surface loops (L1-L4) that shape the catalytic groove are indicated. The catalytic residue Cys is shown as a red line at the beginning of loop L2. DED: Death Effector Domain. CARD: Caspase Recruitment Domain. This diagram is scaled according to the lengths of caspases and the location of functional segments.

Since unregulated caspase activity would be lethal for a cell, caspases are synthesized as single-chain zymogens and stored in the cytoplasm as relatively inactive precursors (pro-caspases). Pro-caspases must undergo an activation process during apoptosis to become active caspases (Srinivasula *et al.* 1998; Yang *et al.* 1998; Chen & Wang 2002; Boatright & Salvesen 2003; Shi 2004).

2.6.2 Mechanism of caspases activation

The activation of caspases during apoptosis has been reported to occur through two signalling pathways defined as extrinsic and intrinsic pathways in reference to the origin of the cascade signal. The extrinsic pathway is responsible for elimination of unwanted cells during development, immune system education and immune-system-mediated tumour removal (immunosurveilance). The intrinsic pathway is used to eliminate cells in response to chemotherapeutic drugs, ionizing radiation, mitochondrial damage and certain developmental cues (Boatright & Salvesen 2003). Although each pathway is capable of functioning independently, cross-talk between pathways is common. For example both pathways cooperate to enhance apoptosis through a BH3-only protein member of Bcl-2 pro-apoptotic protein, Bid; and more importantly, both pathways converge, leading to the activation of the effector caspase-3 (Schimmer 2004; Williams & Dickman 2008).

Extrinsic pathway

The extrinsic pathway is associated with a group of trans-membrane proteins, "death receptors", which act as surface sensors for the presence of specific extracellular death signals from ligands of tumor necrosis factor (TNF) family (Fomicheva et al. 2012). Death receptors transmit apoptotic signals initiated by specific death ligands and can activate the caspase cascade within seconds of ligand binding (Vaux & Korsmeyer 1999). To date, eight human death receptors have been identified including Fas (CD95, Apo1), TNFR-1 (p55, CD120a), TRAMP (Apo3, WSL1, DR3, LARD) TNF related apoptosis inducing ligand (TRAIL)-R1 (DR-4), (TRAIL)-R2(DR-5, Apo, killer); DR-6; EDA-R and NGF-R (Chinnaiyan *et al.* 1995; French & Tschopp 2003; Rupinder *et al.* 2007). Briefly, the extrinsic pathway of caspase activation is initiated bythe ligation of the respective ligand (FasL) to the death receptor (Fas) to form microaggregates at the cell surface. This complex allows the adaptor molecule FADD (Fas-associated protein with death domain) to be recruited to it cytosolic tail by a multi-step mechanism. FADD recruits pro-caspase-8 or pro-caspase-10 by protein-protein interaction via homologous death effector domain (DED) to assemble a death-inducing signaling complex (DISC). During DISC assembly pro-caspase-8 orpro-caspase-10 is activated and released to cytoplasm where it cleaves and hence activates downstream caspase, typically caspase-3. The active caspase-3 cleaves several death substrates leading to the well-

known apoptotic hallmarks including nuclear fragmentation, DNA fragmentation, membrane blebbing and other morphological and biochemical changes (Chinnaiyan *et al.* 1995; Algeciras-Schimnich *et al.* 2002; Boatright & Salvesen 2003; Yin *et al.*2006; Portt *et al.* 2011; Fomicheva *et al.* 2012). Steps involved in the extrinsic activation of caspases in mammals is summarised in Figure 2.5

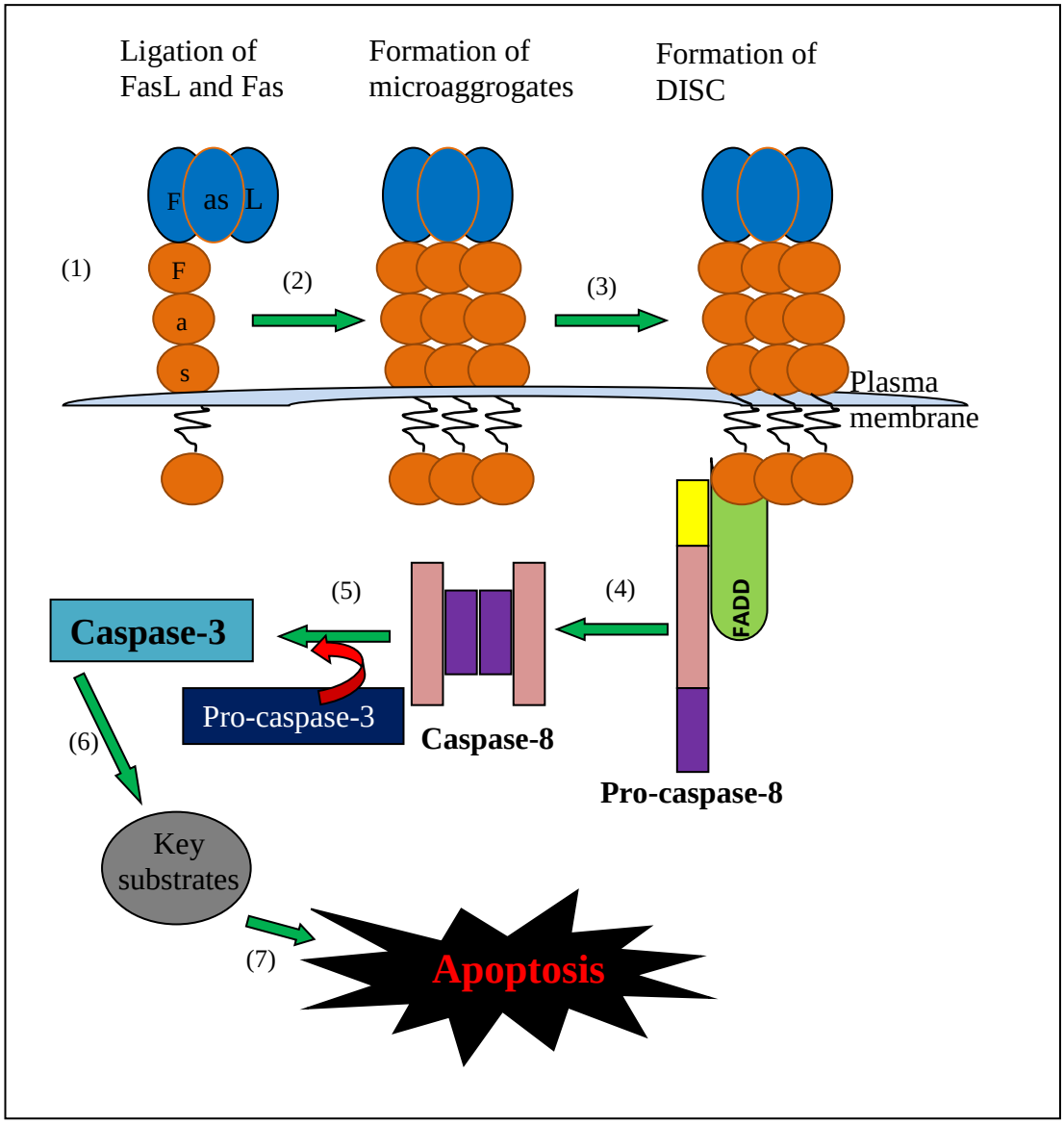


Figure 2.5: Steps involved in the extrinsic activation of caspases in mammals. (1): ligation of FasL and Fas; (2): formation of microaggrogates;(3): formation of DISC; (4): activation of caspase-8; (6): activation of caspase-3 and (7) execution of apoptosis

Intrinsic pathway

The intrinsic pathway involves the participation of mitochondrion as a central organelle therefore it is also termed as mitochondrial pathway. The mitochondrial

pathway is induced by several stimuli such as UV radiation, DNA damage, voltage changes, oxidative stress [hydrogen peroxide (H_2O_2) or nitrogen oxide (NO)] or growth factor withdrawal (starvation), resulting in the dissipation of mitochondrial membrane potential and increased permeability. The permeabilization of the mitochondrial outer membrane leads to the release of apoptogenic molecules and proteins including cytochrome c, certain caspases, endonuclease G, Smac/Diablo and apoptosis inducing factor (AIF) from the inter-membrane space of mitochondrion to cytoplasm, resulting in both caspase-dependent and caspase independent PCD (Brenner & Mak 2009; Paul 2009).

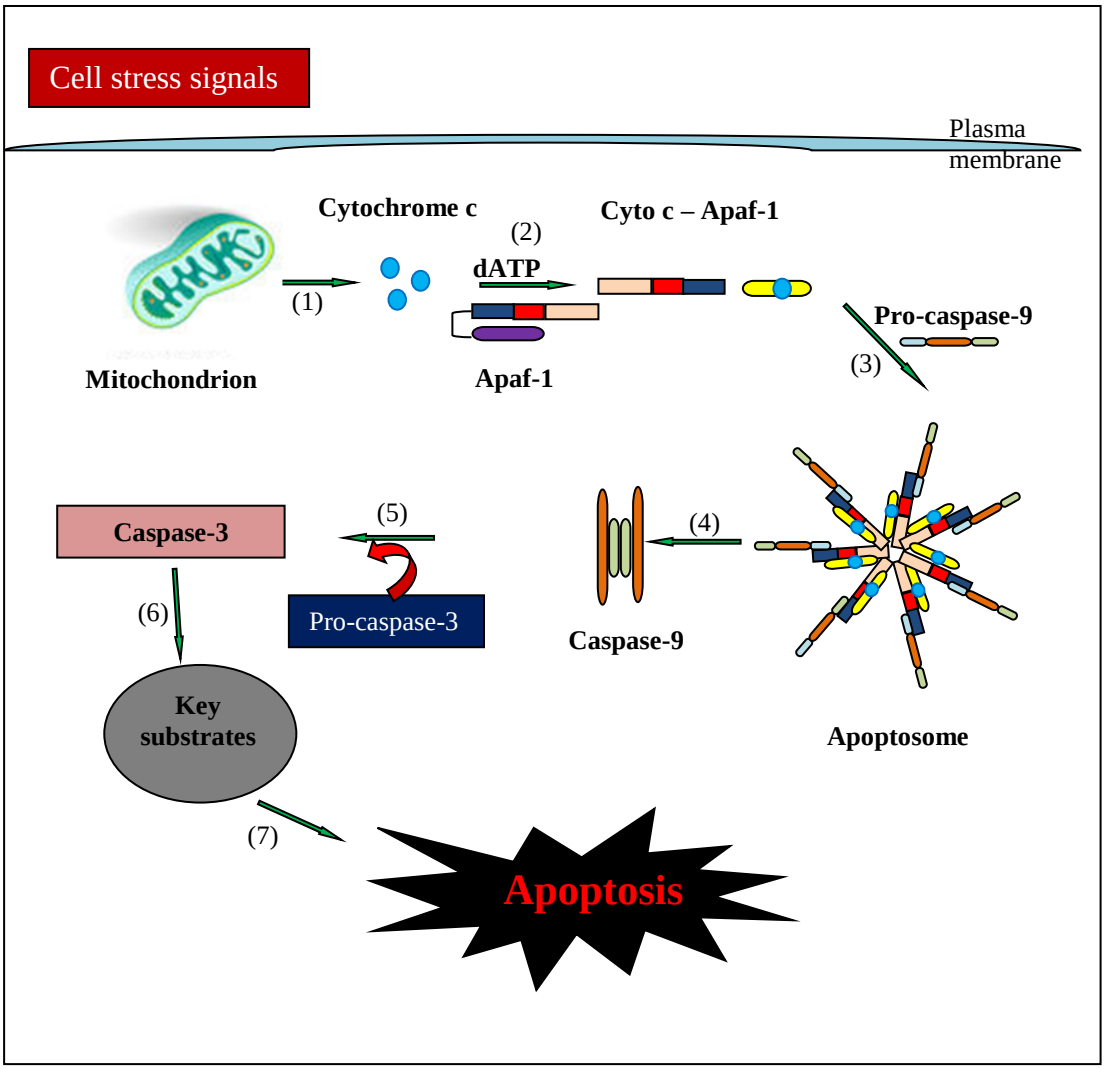


Figure 2.6: Steps involved in the intrinsic activation of caspases in mammals. (1): release of Cytochrome c from mitochondrion. (2): formation of Cytochrome c-Apaf 1 complex; (3): formation of "apoptosome"; (4): activation of caspase-9; (6): activation of caspase-3and (7) execution of apoptosis

The release of cytochrome c into cytosol and the presence of dATP are essential requirements for apoptotic mediated by mitochondria (Liu *et al.* 1996; Goldstein *et al.* 2000; Purring-Koch & McLendon 2000). Upon releasing, cytochrome c binds to Apaf-1 (apoptotic protease activating factor-1, the mammalian homolog of *C. elegans* Ced-4) in the presence of dATP to form an Apaf-1 complex (Zou *et al.* 1997; Hu *et al.* 1999) which then binds to pro-caspase-9 to assemble an oligo-protein complex termed "apoptosome" (Cain *et al.* 2000; Gupta 2001; Acehan *et al.* 2002; Gewies 2003). The apoptosome activates caspase-9 by dimerization (Purring-Koch & McLendon 2000; Pop *et al.* 2006). Active caspase-9 activates downstream caspase, typically caspase-3, resulting in apoptosis. Steps involved in the intrinsic activation of caspases in mammals leading to programmed cell death is shown in Figure 2.6

2.6.3 Regulation of apoptosis

From the previous section it is clear that apoptosis is mainly executed by activation of caspase-mediated signalling cascades. In this section the regulation of apoptosis by regulation of caspase activation via death receptors (extrinsic pathway), mitochondrial-driven PCD pathway (intrinsic pathway) and direct regulation of initiator and effector caspases activation will be discussed.

Regulators of the death receptors (extrinsic pathway)

The regulation of apoptosis-mediated by death receptors can be at multiple levels including regulation of expression of ligands and death receptors and regulation of intracellular regulatory molecules (Chen & Wang 2002). The expression of ligands and death receptor such as Fas-ligand and Fas is regulated by a number of factors (Schattner *et al.* 1995; Wang & Lenardo 1997; Zheng *et al.* 1998). Intracellular regulatory molecule such as FLIP (FLICE inhibitory protein) can negatively regulate apoptosis by competing with pro-caspase-8 during the binding of this zymogen to FADD (Fas associated death domain). FLIP contains DED (death effector domain), when expressed at high level it can bind to FADD using DED motif therefore compete with pro-caspase-8 for recruitment at DICS (death-inducing signalling complex) (Yu & Shi 2008; Bagnoli *et al.* 2010).

Regulators of mitochondrial-driven PCD (intrinsic pathway)

Members of B cell lymphoma 2 (Bcl-2) protein family provides a critical role in regulation of mitochondrial-driven PCD pathway. They either can disrupt or maintain the integrity of mitochondrial membranes thereby promote or prevent the release of apoptogenic proteins such as cytochrome c from inter-mitochondrion membrane space which can activate pro-caspase-9 through assembling of apoptosome leading to apoptosis (Zheng *et al.* 1998; Heiden *et al.* 1999; Chen & Wang 2002; Youle & Strasser 2008; Fuchs & Steller 2011; Martinou & Youle 2011).

Bcl-2 family is characterised by the presence of one or more conserved sequence motifs within α helical segments known as Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4. These BH domains are the only areas of sequence conservation between family members and strongly influence whether the family member is pro or anti-apoptotic (Danial 2007; Williams & Dickman 2008). Many members of Bcl-2 family have a conserved C-terminal transmembrane region (TM) that is responsible for their localization on the outer mitochondrial membrane, endoplasmic reticulum and nuclear envelope to the cytosolic aspect (Strasser *et al.* 2000; Soriano & Scorrano 2010). Bcl-2 proteins family can be divided into two groups: pro-apoptotic and anti-apoptotic depend upon their functions. The pro-apoptotic group consist of two subgroups known as i) multi-domain proteins subgroup, Bax (Bcl-2 antagonist X), Bak (Bcl-2 antagonist killer) and Bok (Bcl-2 related ovarian killer); and ii) BH3 only subgroup containing proteins, Bid, Bad, Bim, Puma, Noxa, Bmf, Hrk, Bik, Spike, Bcl-G, Bcl-Rambo, Hrk/DP5, Bcl-X_s. The anti-apoptotic or pro-survival group consists of Bcl-2, Bcl-X_L, Bcl-B, Bcl-W, A1, Mcl-1 proteins that prevent cell death or promote survival (Chao & Korsmeyer 1998; Strasser *et al.* 2000; Cory & Adams 2002; Mund *et al.* 2003; Danial 2007; Brenner & Mak 2009; Danial *et al.* 2010; Soriano & Scorrano 2010) (Figure 2.7). BH1, BH2 and BH3, the three functionally important Bcl-2 homology regions, are in close spatial proximity and form an elongated hydrophobic groove that may provide biding sites for other Bcl-2 family members (Muchmore *et al.* 1996).

At least four models of how Bcl-2 family members regulate apoptosis have been proposed [see review by (Strasser *et al.* 2000)]. The first model was generated based on the findings that cell death signals can lead to activation of both Ced-4-like proteins and Bcl-2 pro-apoptotic proteins; while survival signals can reduce the

activity of the pro-apoptotic Bcl-2 family members and increase the levels of Bcl-2 anti-apoptotic proteins, as evidenced in Zha *et al.*(1996) and von Freeden-Jeffry *et al.*(1997). Ced-4 and its homologs act as adaptors for pro-caspases and are assumed to trigger their conversion into active tetrameric enzyme complex. Bcl-2 anti-apoptotic proteins and its homologs bind to and block the action of Ced-4-related proteins on caspases activation thereby promoting survival. The Bcl-2 pro-apoptotic proteins promote cell death by preventing binding of Bcl-2 anti-apoptotic proteins to Ced-4 or its homologs (Strasser *et al.* 2000).

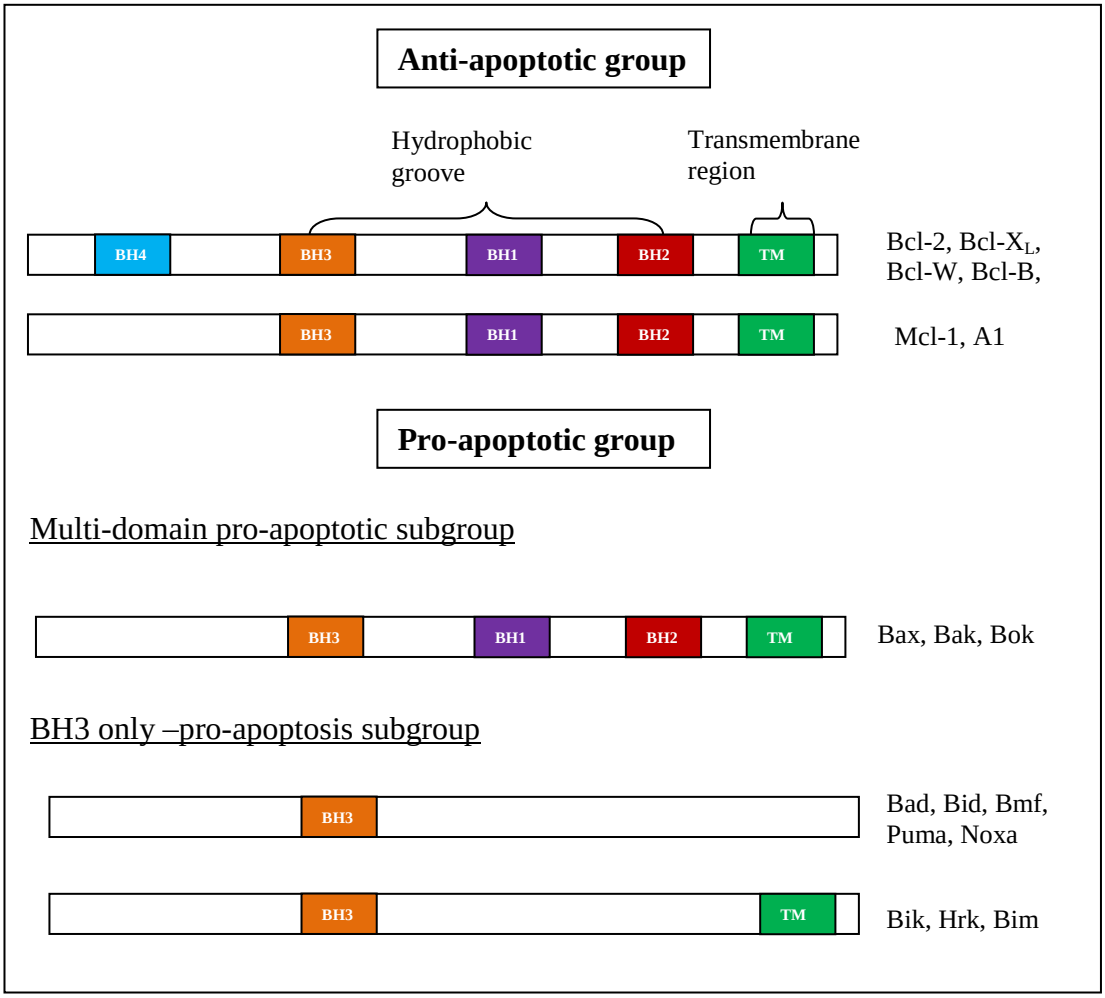


Figure 2.7: Bcl-2 protein family. (BH) Bcl-2 homology domain; (TM): Transmembrane region

The second model suggests that pro-apoptotic and anti-apoptotic proteins in Bcl-2 family function as transmembrane channels that promote or prevent the efflux of molecules that cause caspases activation (Strasser *et al.* 2000). This model was proposed based on the similarities in the three-dimensional structure of Bcl-X_L to pore-forming bacterial toxin (Muchmore *et al.* 1996) and the ability to form ion

channels of Bcl-2, Bcl-X_L and Bax at non-physiological pH in synthetic lipid membranes (Antonsson *et al.* 1997; Minn *et al.* 1997; Schendel *et al.* 1997) together with findings that many Bcl-2 family members have a conserved C-terminal transmembrane region that enables them to localise on nuclear envelope, mitochondrial outer membranes and endoplasmic reticulum towards the cytosolic aspect (Akao *et al.* 1994; Kroemer 1997).

The third model proposed that apoptotic stimuli cause disturbances in mitochondria leading to the release of cytochrome c thereby activating caspase cascades; and Bcl-2 anti-apoptotic proteins function by maintaining the integrity of the mitochondria and prevent releasing of cytochrome c. Cytochrome c is one of the four essential molecules (cytochrome c, Apaf-1, pro-caspase-9 and dATP) that are required for activation of pro-caspase-3 through the intrinsic pathway (Liu *et al.* 1996; Li *et al.* 1997; Zou *et al.* 1997). The release of cytochrome c from the mitochondrial inter-membranes space to the cytosol of dying cells was inhibited by Bcl-2 anti-apoptotic group (Bcl-2 and Bcl-X_L) while Bcl-2 pro-apoptotic group was reported to promote this process (Liu *et al.* 1996; Kluck *et al.* 1997; Yang *et al.* 1997). This model was supported by the finding of Shimizu et al. (1999) that Bcl-2 pro-apoptotic proteins Bax and Bak can accelerate the opening of mitochondrial porin channel called voltage dependent anion channel or VDAC on synthetic liposome, allow cytochrome c to pass through VDAC out of liposome. Bcl-2 anti-apoptotic protein (Bcl-X_L) in contrast can bind directly to VDAC and close the channel consequently preventing the release of cytochrome c [see review by Strasser *et al.*(2000)].

The fourth model was proposed by Strasser *et al.* (2000) in which a large macromolecular structure or lattice may be formed by Bcl-2 family members in membranes. The conformation of individual proteins in this lattice are affected by their neighbours and maybe induced to switch between two stages: anti-apoptotic and pro-apoptotic. When a Bcl-2 pro-apoptotic protein binds to a Bcl-2 anti-apoptotic protein, it induces the switch conformation of the anti-apoptotic protein. The anti-apoptotic protein in its switched state can induce nearby anti-apoptotic proteins to switch their conformation, and entire lattice will become conducive to caspase activation and apoptosis induction.

Although several mechanisms have been proposed to explain the function of Bcl-2 anti-apoptotic and pro-apoptotic members in regulation of apoptosis, there still remain some unclear and unanswered questions that limited understanding of how Bcl-2 regulate cell death (García-Sáez 2012).

Inhibitor of Apoptosis (IAP) protein – a direct regulator of caspases

Although pro-caspases have a low protease activity, this activity is significant; and since pro-caspases are widely expressed in living cells, unregulated caspase activation would be lethal. Therefore cells must have an efficient mechanism to prevent unnecessary caspase activation. Inhibitor of apoptosis (IAP) protein is one of an important family of caspase inhibitors (Fuchs & Steller 2011). The first member of the IAP family was identified by Crook *et al.*(1993) from the baculovirus *Cyndia pomonella* when genomic DNA of this virus was co-transfected into the SF-21 (*Spodoptera frugipera*) insect cell line together with *Autographa californica* nuclear polyhedrosis virus DNA containing a deletion in *p35.* A polypeptide of 31 kDa coded by the 1.6 kb SalI-SstI open reading frame was found to be sufficient to suppress cell death and rescue the wild type infection. Since then several IAPs have been characterized including Cp-iap from *Cyndiapomonella* granulosis virus, Op-iap from *Orgyia pseudotsugata* nuclear polyhedrosis virus, Ac-iap from *Autographa californica* multiply-embedded nuclear polyhedrosis virus (AcMNPV) (Crook *et al.* 1993; Birnbaum *et al.* 1994; Clem & Miller 1994), NAIP, c-IAP1, c-IAP2, XIAP (also known as hILP or MIHA) from human (Rothe *et al.* 1995; Roy *et al.* 1995; Deveraux *et al.* 1997), DIAP1, DIAP2 from Drosophila (Hay *et al.* 1995) and SfIAP from *Spodoptera frugiperda* (Huang et al. 2000b).

IAP family members are characterized by the presence of one to three baculoviral IAP repeat (BIR) domains, a region of approximately 70 amino acids. In some IAP members BIR domains allow them to bind to and inhibit initiator and effector caspases as well as downstream proteases thereby preventing apoptosis (Deveraux & Reed 1999; Vaux & Silke 2005). For example XIAP has been reported to inhibit cytochrome c-induced caspase activation especially the cytochrome c-induced cleavage of pro-caspase-3. It also inhibited caspase-8-induced destruction of nuclei and interfered with the activation of pro-caspase-3 by caspase-8. In addition, XIAP can inhibit the proteolytic activities of caspase-3 and caspase-7 and can directly bind to these proteases *in vitro*. XIAP also inhibits the activation of pro-

caspase-9 in the granulose cell. All these inhibitions lead to prevention of apoptosis and have been attributed to the function of BIR and RING (really interested new gene) domains within IAP. RING domain can function as E3 ubiquitin ligase and may play a role in apoptosis regulation via the proteasome (Deveraux *et al.* 1997; Vaux & Silke 2005; Cheng *et al.* 2008; Kabbage *et al.* 2010). Unlike FLIP or Bcl-2 anti-apoptotic proteins which can only regulate death receptor or mitochondrial-driven PCD pathways respectively, IAPs are unique in that they are capable of inhibiting both extrinsic and intrinsic pathways due to their inhibition of caspase cleavage at the initial phase of the cascade (Straszewski-Chavez *et al.* 2004).

The activity of IAP family members is regulated by IAP antagonists, a protein family whose members can bind to the BIR domain of IAP and inactivate the anti-apoptotic function. In Drosophila the anti-apoptotic activity of DIAP1 has been reported to be blocked by *reaper*, *hid* and *grim* encoded proteins (Goyal *et al.* 2000). In mammalian systems the three well-known IAP antagonists are Smac (second mitochondria-derived activator of caspases), Diablo (Direct IAP binding protein with low pI) and HtrA2/Omi identified by Du *et al.*(2000), Verhagen *et al.*(2000) and Suzuki *et al.*(2001) respectively. Smac/Diablo is synthesized as precursor product which has a mitochondrial import sequence at its N-terminus that is removed upon import. In healthy cells, Smac/Diablo localizes in mitochondria and is released to the cytosol upon cellular stress to promote apoptosis by preventing IAP inhibition of caspases. Smac/Diablo has been reported to interact with all mammalian IAPs examined and can also bind OpIAP (Verhagen & Vaux 2002). HtrA2/Omi is also released from mitochondria upon cellular stress. It inhibits IAP function by direct binding to IAPin similar way to Smac (Suzuki *et al.* 2001).

In summary, mammalian programmed cell death is complex and it is regulated by many factors. A detailed apoptotic pathway and its regulation has been summarised in Portt *et al*. (2011) (Figure 2.8)

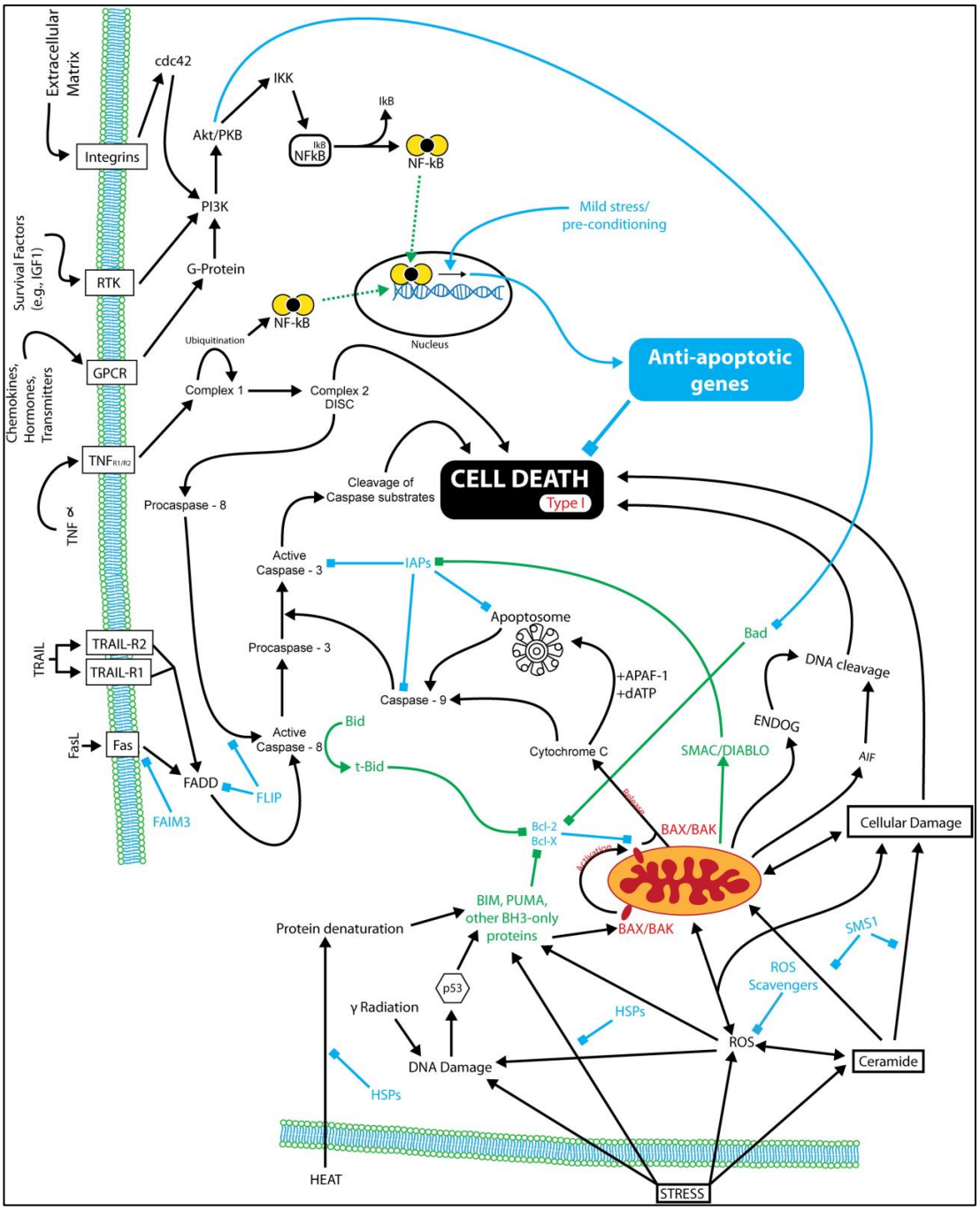


Figure 2.8: Schematic representation of programmed cell death type I (apoptosis) pathways and regulation (Portt *et al.* 2011)

2.7 PROGRAMMED CELL DEATH IN PLANTS

2.7.1 Function of PCD in plant development and interaction with environment

Most of the functions of PCD (apoptosis and autophagy) that were witnessed in other multicellular organisms such as in animal are also observed in plants. For example, the involvement of PCD in tissue remodelling has been reported in leaf

shape remodelling of lance plant (Gunawardena *et al.* 2004). PCD functions in deletion of temporary functional structures that no longer required for the plant development such as suspensor and aleurone layer cells (Pennell & Lamb 1997; Bozhkov *et al.* 2005). The death of aleurone layer cells during seed germination in cereals is an example of the function of PCD in removing a no longer required structure in plant during development. Aleurone is the outer surrounding layer of endosperm, a store of nutrients materials, in mature seeds. The cereal endosperm contains two specialized tissues: the starchy endosperm and the aleurone layer. Starchy endosperm cells die upon the completion of grain filling and the dead starchy endosperm is the reserved source of carbon and nitrogen that is used for embryo growth during seed germination. Nutrients required for the growth of the embryo during seed germination are initially obtained from the store in the embryo and subsequently from mobilization of the materials stored in the endosperm. The hydrolytic process of materials stored in endosperm required hydrolytic enzymes which are synthesised in aleurone cells (Kuo *et al.* 1996; Wang *et al.* 1996b; Fath *et al.* 2000). However, aleurone layer cells are not required for young plants and are therefore programmed to die after contributing their hydrolytic enzymes usually a few days after seed germination. (Wang *et al.* 1996b; Bethke *et al.* 1999; Fath *et al.* 2000; Fath *et al.* 2002).

PCD also plays a key role in the specialization of cells including the development of xylem tracheary elements (Fukuda *et al.* 1998; Groover & Jones 1999)or cell death in root cap cells which protect the root meristem (Wang *et al.* 1996a). Additionally, PCD plays a role in the redistribution of nutrients, for example cell death during senescence recycles nutrients from older to younger organs (Greenberg 1996; Yen & Yang 1998; Simeonova *et al.* 2000; Yoshida 2003). PCD has been found to occur throughout the plant life cycle in many sites of the plants (Pennell & Lamb 1997) (Figure 2.9).

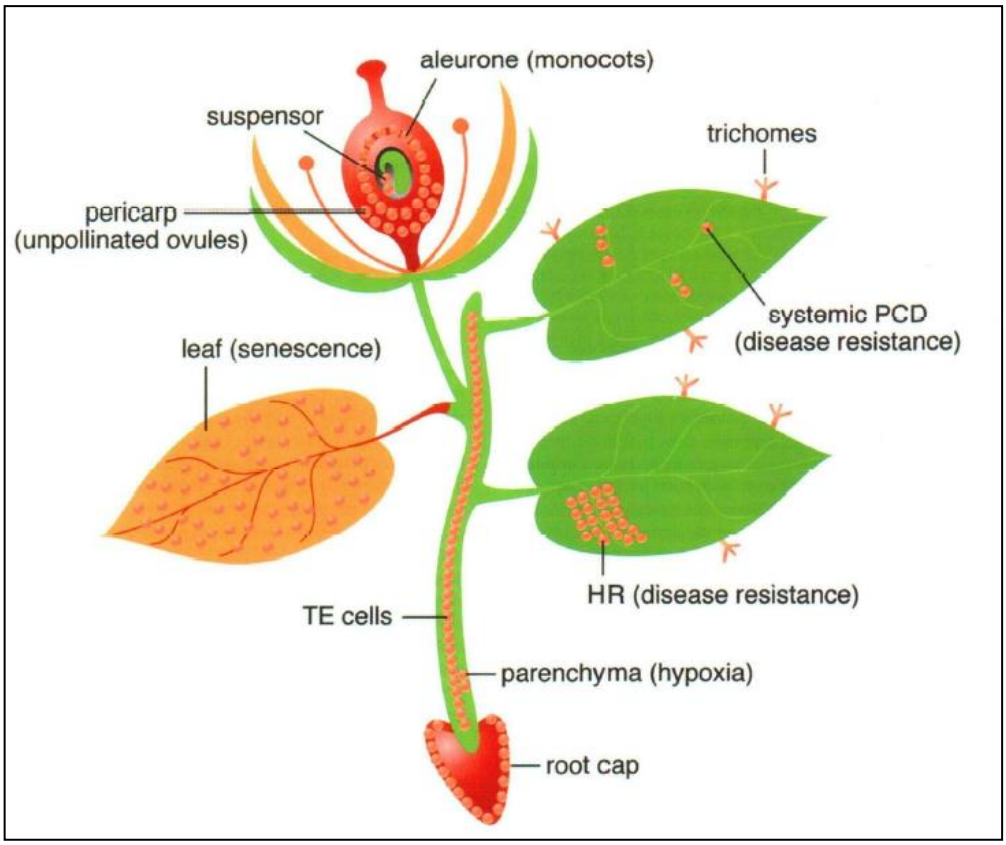


Figure 2.9: Sites of cell death in vascular plants (Pennell & Lamb 1997) www.plantcell.org "Copyright American Society of Plant Biologists."

In terms of defence, PCD is induced as general defence strategies in plants to compensate for the absence of immune system as well as the inability to move to escape environmental challenges during pathogens invasion. Hypersensitive response is a typical example of programmed cell death function as a defence mechanism during pathogens attack. HR not only prevents reproduction of the pathogen but also kills adjacent uninfected cells to create a "barrier of death" separating the pathogen from healthy tissues (Dangl & Jones 2001; Lam *et al.* 2001; Lam 2004). Hypoxic conditions in maize triggered cell death in the cortex of the roots and stem to form aerenchyma which facilitates an efficient transportation of oxygen from aerial organs to waterlogged stem bases and roots is another example of PCD function to enable plants to cope with unfavourable environmental conditions (Pennell & Lamb 1997; Drew *et al.* 2000).

Pennell and Lamb (1997) have summarised the function of PCD during plants development and interaction with environment in Figure 2.10, in which examples of PCD removal of cells that serve a temporary function, are unnecessary or unwanted,

or give rise to specialised tissues; removal of cells during sculpturing of plant body and leaf lobbing and deletion of cells during plant interactions with pathogens were included.

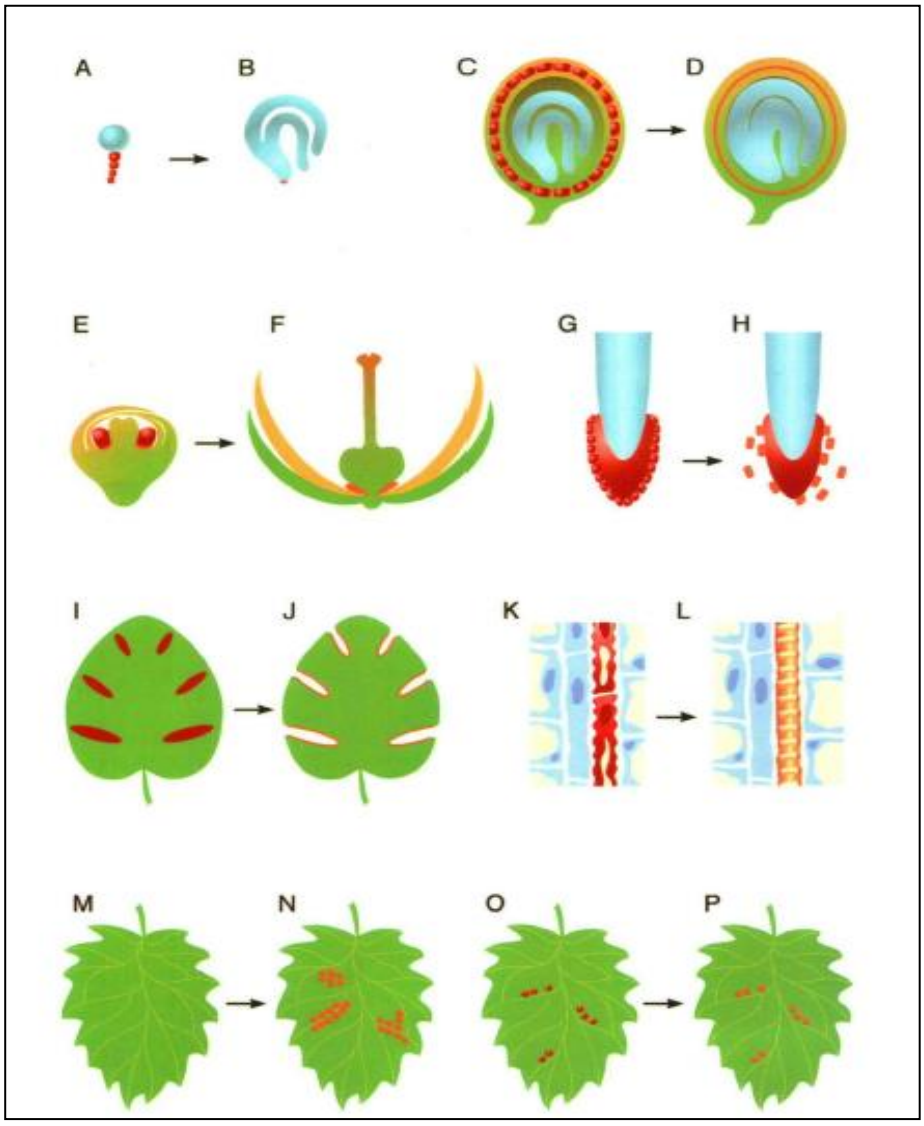


Figure 2.10: Function of PCD in plants (Pennell & Lamb 1997) www.plantcell.org "Copyright American Society of Plant Biologists." Note: the red regions represent cells that have been targeted for PCD and the orange regions represent cells that have died by PCD.Deletion of temporary functional cells: (A & B) suspensor cells in embryos;(C & D) aleurone cells in seeds. Deletion of unnecessary or unwanted cells: (E & F) stamen primordia cells in unisexual flowers, (G & H) root cap cells, (I & J) deletion of cells during sculpturing of plant body and leaf lobbing; deletion of cells during specialization: (K & L) TE cells (specialized death cells to form columns for water transportation in vascular plant); and deletion of cells during plant interactions with pathogens: (M & N) cells in an HR; (O & P) cells in uninfected leaves in response to HR-derived signals.

Although the functions of PCD in plants and animals are similar; and some typical morphological features of PCD in animals such as cell shrinkage, DNA

cleavage, DNA fragmentation were also observed during plant PCD, the question about the similarity of molecular mechanisms involved in PCD between the two kingdoms are still open (Fomicheva *et al.* 2012). Plant cells display several unique features as compared with their animal counterparts that include the presence of chloroplast, vacuoles and totipotency. Additionally, unlike animal cells, plant cells are held together by rigid cell walls which prevent active phagocytosis; plants also lack "true" caspases. Taken together, it has been suggested that plants do not exhibit "classical" apoptosis (van Doorn 2011). Van Doorn (2011) therefore proposed a classification of plants PCD in which 2 categories of PCD were described: vacuolar plant cell death and necrotic plant cell death. There are many cases of plant PCD however, not falling within either of the proposed categories, classification of plant PCD therefore should base on other criteria such as molecular mechanisms and basic components of PCD apparatus rather than morphology alone (Fomicheva *et al.* 2012). Other authors, Reape *et al.*(2008), described three different modes of programmed cell death in plants including apoptotic-like PCD (AL-PCD), autophagy and necrosis. Reape *et al.* (2008) also proposed an apoptotic-like regulation of PCD in plants in which mitochondrial membrane permeabilisation plays a central role via the forming of permeability transition pore (PTP), which is induced by the changes in phosphate and/or ATP level, build-up of Ca^{2+} and ROS production following cellular stress (Reape & McCabe 2010).

Despite intense search, caspases, which are the most characteristic proteases and known to have essential functions in initiation and execution of apoptosis in animal cells, have not been found in plants (Vartapetian *et al.* 2011; Domínguez & Cejudo 2012). However a numbers of caspase-like proteases in plants have been identified including metacaspases (Uren *et al.* 2000), vacuolar processing enzymes (VPE) (Hatsugai *et al.* 2004) and subtilisin-like proteases (saspases and phytapases) (Chichkova *et al.* 2004; Coffeen & Wolpert 2004; Chichkova *et al.* 2010).

2.7.2 Metacaspases

Metacaspases are cysteine-dependent proteases that were identified in protozoa, fungi and plants by Uren *et al.*(2000) using bioinformatics and functional screen-based approaches. Metacaspases are categorised into type I and type II. Type I metacaspases have an N-terminal pro-domain containing a zinc-finger motif followed by a proline-rich repeat motif. Type II metacaspases lack such a pro-

domain but possess a linker region between putative large (p20) and small (p10) subunits (Uren *et al.* 2000; Vercammen *et al.* 2004; Vercammen *et al.* 2007). While protozoa and fungi have only type I metacaspases, plants have both types of metacaspases though type I are more prevalent than type II (Tsiatsiani *et al.* 2011). Metacaspases are similar to caspases in the way that they contain a caspase-specific catalytic dyad of histidine and cysteine and a caspase-like secondary structure (Carmona-gutierrez *et al.* 2010). However metacaspases and caspases have different cleavage specificity, metacaspases hydrolysed proteins at the site after a basic residues usually arginine or lysine (Vercammen *et al.* 2004; Watanabe & Lam 2005) whereas caspases hydrolysed target proteins at the site after aspartate (acid residues) (Thornberry *et al.* 1992; Thornberry *et al.* 1997). This difference together with the phylogenetic analysis for PCD-associated proteins, which showed that caspases and metacaspases form distant groups within clan CD peptidases (Koonin & Aravind 2002), leads to the suggestion that metacaspases should be regrouped to a separate family in the clan CD cysteine peptidases (Vercammen *et al.* 2007). Emerging evidence however indicates that metacaspases are caspases (He *et al.* 2008; Bozhkov *et al.* 2009; Coll *et al.* 2010; Tsiatsiani *et al.* 2011). Metacaspases and caspases fulfil the criteria of homology including common cellular program, PCD and common substrates (at least in part) and represent variants of the same enzyme that has varied in revolution. Metacaspases exist virtually in all eukaryotic organisms lacking caspases, and the presence of caspases excludes that of metacaspases therefore caspases and their aspartate specificity may constitute a secondary development in just one branch of eukaryotes that includes higher animals; caspases evolved from metacaspases rather than just falling from the sky (Carmona-gutierrez et al. 2010). This means metacaspases and caspases come from a common ancestor region and evolutionary linked, therefore PCD pathways are conserved across the plants and animal kingdoms.

2.7.3 Vacuolar processing enzymes

One of the distinct features between animal and plant cells is the presence of vacuoles in plant cells. Vacuoles contain copious amounts of hydrolytic enzymes such as proteases, nucleases and lipases; and a large amount of various proteins such as defence proteins and storage proteins required for seeds germination and subsequent growth (Hara-Nishimura & Hatsugai 2011). In mature plant cells,

vacuoles can take up the majority of the cell volume (Rojo *et al.* 2003; Lam 2005). During nutrient deficiency, vacuoles play a major role in the recycling of cellular contents through pathways such as autophagy (Hanaoka *et al.* 2002). Vacuolar processing enzyme (VPE) was originally discovered as a novel cysteine protease which is capable of converting several pro-proteins precursors into their respective mature forms, and responsible for maturation of seed storage proteins (Hara-Nishimura *et al.* 1991). VPE was also found to be responsible for the maturation or activation of several vacuolar proteins (He *et al.* 2008; Bozhkov *et al.* 2009; Zhang *et al.* 2010). The finding that VPE has caspase-1 activity and is essential for virus-induced hypersensitive cell death, even though it is structurally unrelated to caspases, was reported by Hatsugai *et al.*(2004). This finding provides the link between the plant legumain and caspase-like functions (Lam 2005). VPE is considered to have capase-like activity and is an executor of plant cell death (Hara-Nishimura *et al.* 2005) including cell death in response to pathogen infection (defence strategies) and during normal development (Yamada *et al.* 2005; Hatsugai *et al.* 2006; Hatsugai & Hara-Nishimura 2010).

In response to pathogen invasion plants induce two classes of vacuole-mediated PCD: i) destructive death initiates with vacuolar membrane collapse and release of vacuolar hydrolytic enzymes into the cytosol resulting in rapid and direct cell death, ii) non-destructive occurs via the fusion of the vacuole and plasma membranes which allows passage of defence proteins into extracellular space where bacteria pathogens proliferate. Interestingly, both ways involve caspase-like activity enzymes: destructive death involves VPE that has caspase-1 activity and non-destructive death uses the proteasome subunit PBA1 (Yamada *et al.* 2005) which has caspase-3 activity (Hatsugai & Hara-Nishimura 2010).

2.7.4 Subtilisin – like proteases – saspases and phytaspase

Other plant proteases which have caspase-like activity but are structurally unrelated to caspases include the saspases which were identified during victorin-induced PCD in oat (*Avena sativa)*. Victorin is a host specific toxin caused by the necrotrophic fungus *Cochliobolus victoriae* in victoria blight of *Avena sativa*. Saspases are proteases which exhibit caspase-like specificity but contain an active-site Ser residue. The two saspases, purified from *A. Sativa* contain a similar amino acid sequence to subtilisin-like proteases from plants and are likely involved in a

PCD-induced signalling cascade as processive proteases though no direct evidence for this involvement has been identified(Coffeen & Wolpert 2004). More recently, phytaspase (plant aspartate-specific protease) a PCD-related subtilisin-like protease has been identified in tobacco and rice (Chichkova *et al.* 2010). In terms of substrate specificity, phytaspases are more similar to animal caspase-6 and to an unidentified protease involved in PCD during embryogenesis in Norway spruce than saspases. Phytaspase is synthesised as a pro-enzyme which is autocatalytically processed to become the mature enzyme and localised in extraculluar (apoplastic) fluid before PCD. Under PCD conditions mature phytaspase is relocated to intracellular. Overexpression and silencing of the phytaspase gene illustrated that phytaspase is essential for PCD-related responses to tobacco mosaic virus and abiotic stresses including oxidative and osmotic stresses (Chichkova *et al.* 2010). Phytaspases and caspases are very different in structure, one is a subtilisin-like serine protease (phytaspase) and other is cysteine-dependent protease (caspase) but they share a number of important characters including D cleavage specificity and being involved in the implementation of PCD pathways. They are both synthesised as pro-enzymes in cells but the way in which they are processed to become mature enzyme is not similar. Caspases activation is regulated by a number of regulators within the cell while phytaspase is physically separated from its intracellular targets (Chichkova *et al.* 2012). A "backspace" model has been proposed for phytaspase behaviour in which phytaspase is constitutively processed and the mature phytaspase is secreted into apoplast in healthy plant cells. Upon PCD induction by several death stimuli, phytaspase is quickly relocalised to the cytoplasm where it gains access to intracellular targets for PCD execution (Chichkova *et al.* 2010; Chichkova *et al.* 2012). However, the mechanisms by which phytaspase was rapidly shifted to cytosol is remained unknown and how mature phytaspase presents in extracellular fluid without any detrimental effect to cells needs further investigation (Chichkova *et al.* 2012). Currently, the relationship between phytaspase and saspases is not completely clear. Both of them are subtilises and share many common features, phytaspase however has higher hydrolytic stringency than saspase and even higher than caspase-3; and it has a known protein target (VirD2 protein of *Agrobacterium tumefaciens*) whereas targeted protein of saspases remains unknown [see review by Vartapetian *et al*. (2011)].

Although plant caspase-like proteases have been identified, their target proteins and the way in which they are activated, regulated and participate in plant PCD pathways awaits further investigation.

2.7.5 Plant PCD regulators

Similar to the case of true caspase, attempts to identify plant homologues of mammalian core regulators of apoptosis using informatics tools at the primary sequence level such as BLAST or FASTA have failed. A search for functional similarity based on prediction from structural similarity has been conducted with assumption that distantly related proteins may have limited overall (undetectable) sequence homology but key features such as helical structure, hydrophodicity, water accessible surfaces, electrostatic potential, fold and catalytic sites may be conserved; and functional predictions can be made independently of the primary sequence (Doukhanina *et al.* 2006; Williams & Dickman 2008). Using this approach, a family of *Bcl-2* associated gene product (BAG) proteins of *Arabidopsis* has been identified by profile-sequence (PFAM) and profile-profile (FFAS) algorithms (Doukhanina *et al.* 2006). The BAG family has been identified in yeast and animals, and is believed to function through a complex interaction with signalling molecules and molecular chaperones; under stress conditions, the BAG proteins recruit molecular chaperones to target proteins and modulate their functions by altering protein conformation (Sondermann *et al.* 2001; Takayama & Reed 2001). The search of the *Arabidopsis thaliana* genome sequence resulted in recognition of seven homologues of the BAG proteins family with limited sequence but high structural similarity to their human counterparts and contained putative Hsp70 biding sites (Doukhanina *et al.* 2006). Of the seven homologues of BAG family in *Arabidopsis thaliana,* four with domain organization similar to animal BAGs including AtBAG1-4 which are predicted to localize in cytosol, and three (AtBAG5-7) contain a calmodulin-binding motif near the BAG domain. This is a novel feature associated with plant BAG family and possibly reflecting differences between animal and plant PCD (Kabbage & Dickman 2008). AtBAGs have been speculated to bind Hsp70 in a manner similar to their animal counterparts this is at least the case of AtBAG4. AtBAG4 conferred tolerance to a wide range of abiotic stress in transgenic tobacco. AtBAG6 may have a role in basal resistance by limiting disease development in *Botrytis cinerea.* The functional differences between AtBAG4 and AtBAG6 lead to a hypothesis that the BAG family

has developed specialized roles for cell regulation (Kabbage & Dickman 2008). Similarly to their mammalian counterparts, the proposed function of plant BAG proteins is to coordinate signals for cell growth and to induce cell survival or cell death pathways in response to stress (Doukhanina *et al.* 2006). *Arabidopsis* BAG family members are localised to a variety of subcellular organelles for a range of cellular functions including the important function in PCD pathways and cytoprotection (Williams *et al.* 2010).

Despite limited understanding of the molecular mechanisms driving programmed cell death in plants, it is undoubted that PCD occurs in plants during development and during the interaction between plants, the environment and pathogen challenge.

2.7.6 Salinity induced PCD

Salinity stress-induced PCD has been reported in many plants including barley (Hatsugai *et al.* 2006), *Arabidopsis* (Huh *et al.* 2002), rice (Li *et al.* 2007; Liu *et al.* 2007; Jiang *et al.* 2008), tobacco (Doukhanina *et al.* 2006; Shabala *et al.* 2007) and tomato (Li *et al.* 2010). Under salinity stress, PCD was induced in response to ion disequilibrium (Huh *et al.* 2002). In addition, ROS levels were reported to increase in plants resulting in significant cellular damage during salinity stress (Borsani et al. 2005; Zhu et al. 2007). ROS can play a dual role acting as both toxic compounds and secondary messengers in signal transduction pathways in a variety of scenarios (Miller et al. 2008; Miller et al. 2010). PCD was shown to be triggered by ROS signals that originate from different organelles such as chloroplast and mitochondria (Foyer & Noctor 2005; Rhoads et al. 2006). Recently, Joseph and Jini (2010)provided an in-depth review of salinity-induced PCD in plants. The paper focused on the roles of the mitochondria, ROS, mechanisms for ion specific signaling, and the induction of nuclear and DNA degradation in plants during salt-induced PCD pathways. The review described that the induction of PCD in plants following exposure to excessive salinity appears after increased ROS and dissipation of mitochondrial membrane potential resulting in increased permeability.

Being sessile, plants are particularly vulnerable to aberrant environmental conditions including saline soils and water deficit. To mitigate salinity stress, plants implement a range of strategies, however, if these mechanisms are unable to cope with the increased stress imposed by excess salinity the plant will implement

selective PCD as a last ditch effort to survive (Hara-Nishimura *et al.* 1991; Greenberg 1996). Paradoxically, studies have shown that inhibition of PCD during stress promotes survival (Shabala *et al.* 2007; Wang *et al.* 2009b; Li *et al.* 2010). These findings suggest that manipulation of programmed cell death pathways, particularly prevention of PCD may help to enhance tolerance to salinity stress in crops. In the next section we discuss the various PCD genes used in this project for the generation of salinity tolerant rice.

2.8 PRO-SURVIVAL GENES USED IN THIS PROJECT

The below pro-survival genes have been selected for this study because: i) they associated with regulation of PCD induced by abiotic stress; and ii) they are key plant and virus derived pro-survival genes.

2.8.1 AtBAG4

The *Arabidopsis thaliana* genome contains seven homologs of the BAG family, including four with a domain organization similar to animal BAGs. BAG-like genes appear to be widely distributed in plants and several expressed sequence tags (ESTs) of putative BAG genes from different plants have been shown to have 42 to 79% similarity to the BAG domain of *AtBAG4* (Kabbage & Dickman 2008). These ESTs were derived from plants exposed to both biotic and abiotic stresses as well as ESTs associated with tissue-specific expression. Transgenic tobacco lines over-expressing *AtBAG4* have enhanced tolerance to several abiotic stress stimuli through inhibition of programmed cell death (Doukhanina *et al.* 2006).

All BAG family members contain a highly conserved region known as the *BAG* domain, a key characteristic of the BAG domain is its ability to bind to *Hsp70/Hsp90* proteins to facilitate their chaperone activity. It has been speculated that BAGs recruit Hsp70 and Hsp90 (p61) proteins and induce conformational changes that prevent receptor signalling in the absence of a ligand. The broad-spectrum activity of *Hsp70s* and *Hsp90s* requires the recruitment of co-chaperones and other chaperone systems. BAG protein may coordinate signals for cell growth and induce survival pathways and/or inhibit PCD pathways in response to stress (Doukhanina *et al.* 2006).

2.8.2 AtBI

BAX inhibitor-1 (BI-1) is an pro-survival protein that is evolutionarily conserved and predicted to be a transmembrane protein that localizes predominantly to the endoplasmic reticulum (ER) (Chae *et al.* 2003; Chae *et al.* 2004; Watanabe & Lam 2004; Lee *et al.* 2007). Plant BI-1 mRNA can be detected in various tissue types and its expression level is enhanced during senescence and under several types of biotic and abiotic stresses (Kawai-Yamada *et al.* 2004; Watanabe & Lam 2006; Kotsafti *et al.* 2010). Over-expression of *BI-1* was shown to suppress BAX, pathogen, or abiotic stress-induced cell death in a variety of cells from yeast, plant, and mammalian origins (Sanchez *et al.* 2000; Kawai-Yamada *et al.* 2001; Matsumura *et al.* 2003; Watanabe & Lam 2009). These observations support the hypothesis that BI-1may have a conserved function in diverse organisms (Watanabe & Lam 2008). Recent genetic analysis of *Arabidopsis BI-1* (*AtBI1*) demonstrated that *AtBI1* is not required for normal plant growth and development, but plays a protective role against both phytotoxin- and heat stress-induced PCD (Watanabe & Lam 2006). Expression of *AtBI1* is up-regulated under several stress conditions induced by bacterial and fungal pathogens, ozone, norflurazon, or salicylic acid (Sanchez *et al.* 2000; Kawai-Yamada *et al.* 2004). Plant *BI-1* is thus likely to play an important role as a survival factor under multiple stress conditions including *Fusarium* infection (Watanabe & Lam 2008).

2.8.3 Hsp70 and *Hsp90*

Heat-shock proteins are powerful chaperones that are expressed in response to a variety of physiological and environmental stresses. Heat shock proteins have different functions depending on their location in intracellular or extracellular. Hsp70 and Hsp90 have anti-apoptotic function, they can interact with different programmed cell death regulator proteins thereby block the apoptosis process at distinct key points (Joly *et al.* 2010). The broad-spectrum activity of *Hsp70s/Hsp90* requires the recruitment of co-chaperones and other chaperone systems (Brodsky & Bracher 2007).

2.8.4 OsBAG4

OsBAG4 was identified from *Oryza sativa* using the same methods that were used for *Arabidopsis* i.e. via profile-sequence (PFAM) and profile-profile (FFAS)

algorithms. Using this approach, six BAG family members, phylogenetically grouped into two groups, were identified in *Oryza sativa*. Group 1 includes four members OsBAG1-OsBAG4 which, similarly to their animal counterparts, contain ubiquitin-like domain at N-terminus domain and group 2 consists of two members OsBAG5 and OsBAG6 which have calmodulin-binding motifs like AtBAG5 and AtBAG6. The proposed function of OsBAG family members is to involve in cell elongation, differentiation, response to tresses and cell death process (Rana *et al.* 2012).

2.8.5 *p35*

The *p35* gene was first identified by sequencing analysis of a region of the *Autographa californica* multicapsid nucleopolyhedrovirus genomeadjacent to the insertion of a transposable element in 1987, but the function of the *p35* gene product (p35 protein) was unknown until four years later (Clem *et al.* 1991; Clem 2001). To date the role of *p35*, have been extensively studied in a range of organisms (Clem *et al.* 1991; Clem & Miller 1994; Bump *et al.* 1995; Xue & Horvitz 1995; Bertin *et al.* 1996). Expression of *p35* is known to inhibit PCD across broad evolutionary boundaries including mammals (Viswanath *et al.* 2000), insects (Davidson & Steller 1998), nematodes (Xue & Horvitz 1995) and plants(Lincoln *et al.* 2002; Freitas *et al.* 2007; Wang *et al.* 2009b). Research conducted by Clem et al.(Clem *et al.* 1991; Clem & Miller 1994) showed that expression of *p35* in SF-21 (*Spodoptera frugipera*) insect cell line is sufficient to suppress apoptosis triggered by AcMNPV infection and actinomycin D. The Baculovirus p35 protein has been attributed to prevent the programmed cell death in *Caenorhabditis elegans* by acting as a competitive inhibitor of cysteine proteases (Xue & Horvitz 1995). The p35 protein was also suggested to act as an inhibitor of human interleukin-1β converting enzyme (ICE) (caspase-1) by preventing the autoproteolytic activation of ICE from its precursor form and blocking ICE-induced apoptosis (Bump *et al.* 1995). The expression of a baculovirus anti-apoptotic gene *p35* in transgenic tomato, tobacco and passion fruit plants suppressed the cell death caused by a fungal toxin (AAL-toxin) and the infection of certain bacterial or fungal pathogens and enhance tolerance to a range of abiotic and biotic stresses (Lincoln *et al.* 2002; Freitas *et al.* 2007; Wang *et al.* 2009b).

2.8.6 *SfIAP*

SfIAP is an IAP (inhibitor of apoptosis) family member, isolated from the insect *Spodoptera frugiperda* (Huang et al. 2000b). *SfIAP* has previously been transformed into tobacco and tomato and reported to confer tolerance to salinity, heat, fumonisin B1 and resistance to necrotrophic fungus *Alternaria alternate* (Kabbage *et al.* 2010; Li *et al.* 2010). All aspects of plant's life are controlled by the regulated synthesis of new polypeptides and the precise degradation of pre-existing proteins (proteolysis). Ubiquitin/26S proteasome is arguably the dominant proteolytic system in plants (Smalle & Vierstra 2004). Proteolysis via Ubiquitin/26S proteasome pathway requires sequential enzyme activities including a ubiquitin activating enzyme (E1) which forms a thioester bond with the C terminus of ubiquitin in a present of ATP and then transfers the activated ubiquitin to a ubiquitin conjungating enzyme (E2), E2 then transfers ubiquitin directly to a ubiquitin-ligating ezyme (E3) which transfer ubiquitin to the targeted substrate (Smalle & Vierstra 2004; Kabbage *et al.* 2010).The ability to confer tolerance to salinity stress of *SfIAP* was attributed to its E3 ubiquitin ligase activity (Kabbage *et al.* 2010).

2.9 SUMMARY AND IMPLICATION

In summary, programmed cell death play important roles during normal development and during the interaction between plants and environment. Depending on the context, PCD maybe benefit or be harmful to the plants as discussed in previous sections. In some circumstances plants involved in PCD as a passive participant. Manipulation of PCD pathways using pro-survival genes in tobacco, tomato and banana has highlighted the possibility to enhance tolerance to biotic and abiotic stresses in other economical crops. Therefore pro-survival genes from different sources were transformed into rice and accessed for their ability to confer tolerance to salinity stress. The overview of this project with essential steps to achieve the three specific objectives is shown in Figure 2.11

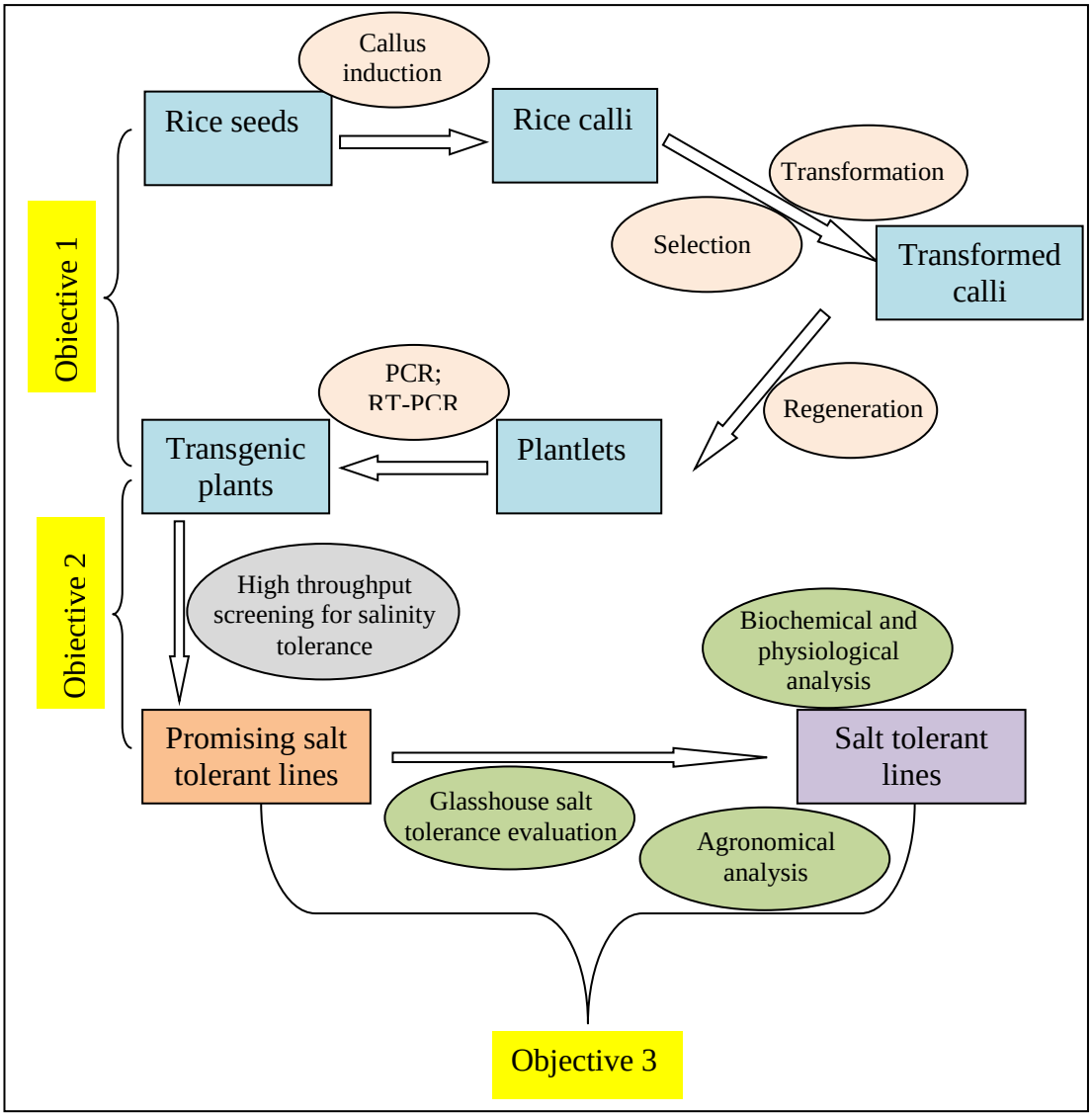


Figure 2.11: Project overview

The following chapters will present the materials and methods used in this project, followed by the results of three specific objectives respectively and a general discussion and conclusion of overall project.

Chapter 3: General Materials and Methods

This chapter describes the general materials used for this project including Nipponbare rice seeds, pro-survival genes, vector backbone and source of specialized reagents in section 3.1. Section 3.2 presents general methods for generation and characterization of transgenic rice as well as methodology for salinity tolerance screening.

3.1 GENERAL MATERIALS

3.1.1 Nipponbare rice seeds

Rice seeds (*Oryza sativa* L. spp. Japonica cv. Nipponbare) were kindly provided by Yanco Agricultural Institute, NSW, Australia.

3.1.2 Pro-survival genes

List of pro-survival genes used in this project is shown in Table 3.1. Of the seven genes used, *SfIAP*, *p35* and *AtBAG4* were kindly provided by Professor Martin Dickman, Texas University, USA. Other pro-survival genes used in this project were kindly provided by the banana research team from the Centre for Tropical Crops and Biocommodities, Queensland University of Technology (QUT).

Table 3.1: List of pro-survival genes and combination used in this study

Genes	Sources
AtBAG4, AtBI	*Arabidopsis thaliana*
AtBAG4 + Hsp70	A combination *of AtBAG4* and *Hsp70*
Hsp70,Hsp90 (p61)	*Citrus tristeza* virus
OsBAG4	*Oryza sativa*
p35	Baculovirus
SfIAP	*Spodoptera frugiperda*

3.1.3 Plant expression vector

The pro-survival genes in Table 3.1 together with maize (*Zea mays* L.) polyubiquitin-1 (Ubi-1) promoter were cloned into the plant binary vector pCAMBIA 1301 (Figure 3.1).

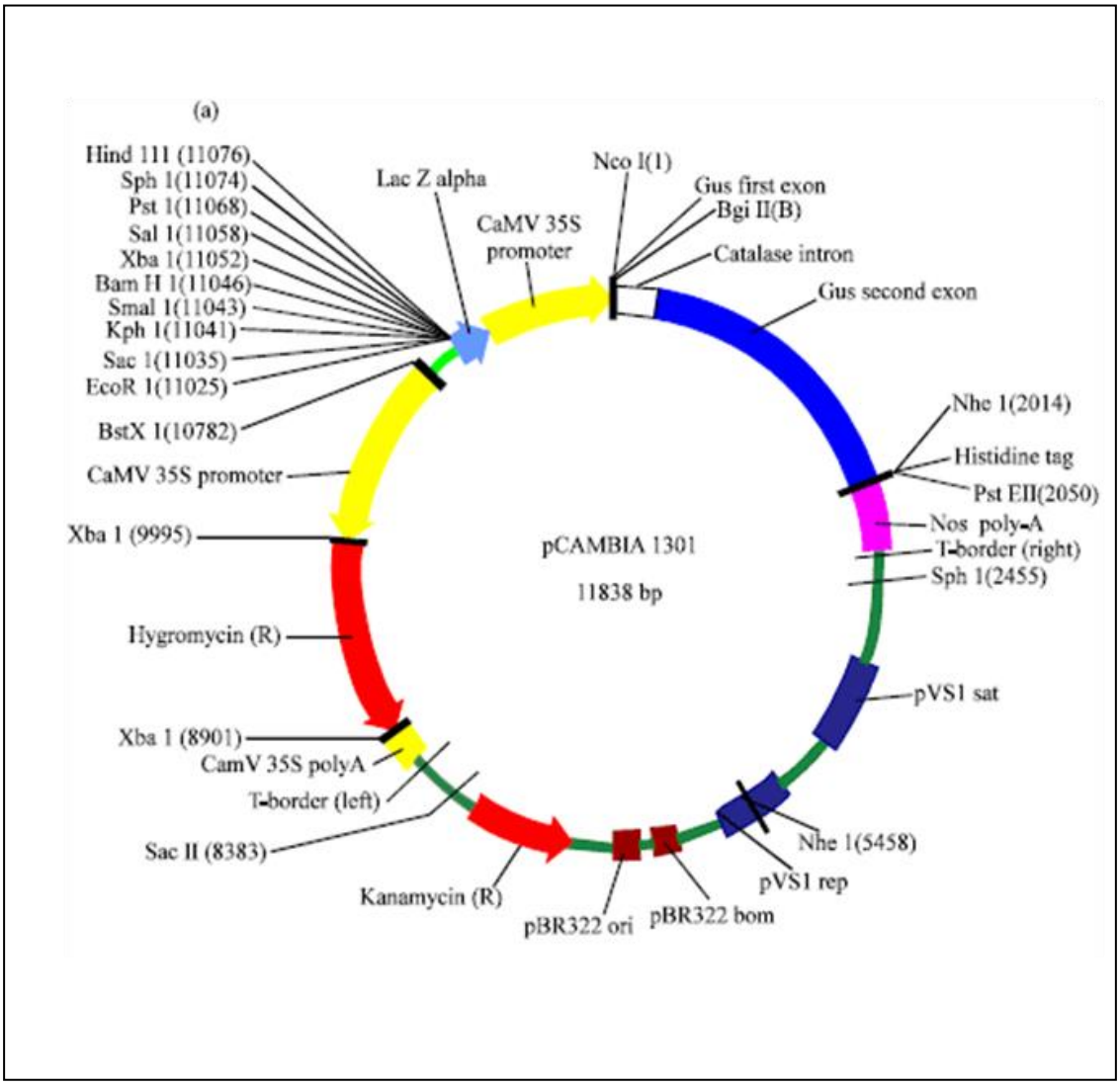


Figure 3.1: Plasmid map of the binary plant expression vector pCAMBIA1301. The T-DNA of this base vector has hygromycin gene driven by CaMV35S promoter cloned in it and this was used as the plant selection marker.

3.1.4 Sources of specialised reagents

All general reagents were sourced from scientific supply companies including Crown Scientific (Aus), Invitrogen (USA), Promega (USA), Qiagen (Netherlands), Roche Diagnostics (Switzerland) and Sigma Aldrich (USA).

3.1.5 Media for tissue culture and transformation

Refer to appendices.

3.1.6 Solution for plant nucleic acid extraction

CTAB buffer: 2% CTAB (cetyltrimethylammonium bromide), 2 M NaCl, 25 mM EDTA pH 8, 100 mM Tris-HCl, 2% polyvinylpyrrolidone (PVP 40)

EDTA: Ethylene diamine tetra-acetic acid, pH 8

$CHCl_3$:IAA: chloroform: isoamyl alcohol (24:1)

3.1.7 Solution for gel electrophoresis

Agarose gel loading dye (6X): 0.25% (w/v) bromophenol blue, 50% TE and 50% glycerol

TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA

TAE buffer: 10 mM Tris-acetate, 0.5 mM EDTA, pH 7.8

3.1.8 Solutions for the detection of cell death

PBS: 137 mM NaCl, 10 mM phosphate (10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4), 2.7 mM KCl, pH 7.4

Fixation: 4% paraformaldehyde, 0.1% PBS, pH 7.2

Permeability: 0.1% Triton X100, 0.1% tri sodium citrate, pH 6

Staining solution: TUNEL reactions mix (Roche Diagnostics - Switzerland).

Propidium Iodide: 0.5 mg mL^{-1}

3.2 GENERAL METHODS

3.2.1 Generation of transgenic rice

Induction of rice callus

Embryogenic callus (scutellum-derived callus) was induced from mature seeds of rice (*Oryza sativa* L. spp Japonica cv. Nipponbare) using 2N6 callus induction media (Appendix 1). Dehusked Nipponbare seeds were rinsed three times with deionised water, surface-sterilised in 70% (v/v) ethanol for one minute followed by vigorous shaking in a solution of 80% commercial bleach (4.0% hypochlorite w/v) containing one drop of Tween-20 for 30 min. Seeds were then rinsed 4–5 times in

sterilised distilled water and blotted on sterile Whatman® filter paper before being placed onto 90 x 14 mm petri dishes containing 25 mL of solidified 2N6 callus induction media (Appendix 1). All steps following sterilisation of the seeds were carried out in a laminar flow cabinet. Seeds contained in petri dishes were kept in the dark at 27°C for 3 weeks. Pieces of proliferating embryogenic callus were selected and pre-cultured on fresh 2N6 media four days prior to transformation.

Agrobacterium transformation

A 200µL aliquot of *Agrobacterium tumefaciens* strain AGL1 from a glycerol stock, was added to 10 ml of YM media (Appendix 1) supplemented with 25 mgL^{-1} rifampicin and 100 mgL^{-1} kanamycin in a 15 mL falcon tube. The tube was then inoculated for three days at 28°C with 200 rpm shaking speed. After three days, 5 mL of the culture was transferred into a 50 mL falcon tube containing 20 ml of LB media (Appendix1) supplemented with 25 mgL^{-1} rifampicin and 100 mgL^{-1} kanamycin. An 1 mL aliquot of each culture was assessed by spectrophotometer to determine optical density at a wavelength of 600 nm (OD_{600}). Following confirmation of optical density the *Agrobacterium* culture was centrifuged at 5000 rpm for 10 min, the supernatant was decanted and the resulting pellet was resuspended in bacterial re-suspension media (BRM, Appendix 1) in a 50 mL Falcon tube supplemented with 100µM acetosyringone to an OD_{600} of 0.7. The culture was gently agitated on a rotary shaker at 70 rpm at room temperature for 5 h to facilitate T-DNA induction in BRM.

Four day pre-cultured embryogenic calli were transferred to a 50 mL Falcon tube and 10 mL of preheated (45°C) 2N6 media was added. The mixture was incubated for 5 min at 45°C in a water bath. Following incubation, the 2N6 media was removed and replaced with 10 mL of induced *Agrobacterium* culture. Sterile Pluronic F68 solution was added into the mixture at a final concentration of 0.02%. The mixture was then shaken for 5 min at 70 rpm before centrifugation at 2000 rpm for 10 min. After incubating for 30 min at room temperature, *Agrobacterium*-exposed calli were blotted on sterile Whatman® filter paper and transferred to 90 x 14 mm petri dishes containing 25 mL of co-cultivation media (CCM, Appendix 1) supplemented with 100µM acetosyringone. The cultures were co-cultivated in the dark for 3 days at 27°C. After three days, transformed calli were washed four times in 30 mL 2N6 liquid media containing 200 mgl^{-1} timentin to eliminate excess

bacteria. For the final washing, the callus was placed on a rotary shaker at 70 rpm for 10 min before blotting on sterile Whatman® filter paper. Dry calli were transferred onto petri dish containing 25 mL of 2N6 selection media (Appendix 1) supplemented with 200 mgL^{-1} timentin and 25 mgL^{-1} hygromycin.

Hygromycin selection

Transformed calli were selected using 25 mgl^{-1} hygromycin for the first two weeks after transformation. To kill residual *Agrobacterium,* 200 mgL^{-1} timentin was also added to the media. The transformed calli were transferred onto fresh 2N6 selection media containing 50 mgL^{-1} hygromycin and 200 mgL^{-1} timentin every 14 days. Cultures were incubated in the dark at 27°C and monitored for proliferation of resistant callus.

Callus proliferation

After seven weeks on hygromycin selective media, proliferation of transformed calli was recorded by measuring callus clump diameter. Six levels of callus proliferation were determined based on the diameter of a callus clump. Proliferation level 1 (PL1) indicates that callus clump had a diameter between 3-6 mm; 7-8 mm; 9-11 mm; 12-14 mm; 15-17 mm and 18 mm or above were recorded as proliferation level 2 (PL2),level 3(PL3), level 4 (PL4), level 5 (PL5) and level 6 (PL6) respectively.

Plant Regeneration

After seven weeks on selection media, individual proliferating antibiotic resistant callus clumps (cream white in colour) was transferred to 100 x 20 mm petri dishes containing 30 mL of regeneration media (Appendix 1) supplemented with 200 mgL^{-1} timentin and 25 mgL^{-1} hygromycin. Cultures on the regeneration media were incubated at 27°C in the dark for the first 7 days and then exposed to day/night cycle of 16 h/8 h at 25°C for shoot formation. After three weeks on regeneration media, several green points and little plantlets were observed. All green regenerating calli were transferred onto fresh regeneration media in 100 x 20 mm petri dishes supplemented with 200 mgL^{-1} timentin and 25 mgL^{-1} hygromycin. The cultures were incubated at 25°C using 16 hours photoperiod.

Rooting

Shoots with at least three well-formed leaves were transferred to 120 x 60 mm culture vessels containing 50 mL of rooting media (Appendix1) supplemented with 200 mgL^{-1} timentin and 25 mgL^{-1} hygromycin. Culture vessels were incubated in tissue culture growth room at 25°C with a 16 hour photoperiod and monitored for plant growth, elongation and root development.

3.2.2 Molecular characterization of transgenic plants

Nucleic acid purification from plant tissue

Total gDNA

Genomic DNA was extracted from leaf tissues of putative transgenic lines using either the DNA Easy kit (Qiagen - Netherland) flowing the manufacturer's instruction or the cetyltrimethylammonium bromide method (CTAB) (Stewart & Via 1993).This method is based on the use of cetyltrimethylammonium bromide, a positively-charged detergent that, in the presence of high concentrations of salt, interacts with the negatively charged DNA to form a soluble complex (Sambrook 2001).

Approximately 100 mg of frozen rice leaves in a 2 mL Eppendorf containing a bead was frozen by immersing in liquid nitrogen and homogenised using TissueLyser (Qiagen - Netherland) at maximum frequency (30) for 30 sec and the tube was immersed back in liquid nitrogen. A 700 μL aliquot of pre-warmed CTAB buffer was added to the powdered sample and the sample was returned to the TissueLyser to homogenise at maximum frequency (30) for 1 min.

Following homogenization the tube was incubated at 65°C in a water bath for 30 min, with occasional inversion to mix the contents. Proteins, pigments, and other contaminating organic compounds were extracted by adding 700 μL chloroform: isoamyl alcohol (24:1) to the slurry. The resulting mixture was gently inverted 8-10 times to maximize DNA yield and the microtube was centrifuged at 14,000 rpm for 5 min at room temperature. The resulting aqueous supernatant (approximately 500 μL, containing the DNA) was carefully transferred to a fresh 1.5 mL microtube.

The DNA in the supernatant was precipitated with two thirds the volume of cold isopropanol. The tube contents were gently mixed, incubated at room temperature for 10 min and centrifuged at 14,000 rpm for 10 min at room

temperature. A small white pellet was observed at the bottom of the tube. The isopropanol was decanted and replaced with 1 mL chilled 70% (v/v) ethanol and the sample was centrifuged at 14,000 rpm for 5 min at room temperature. The ethanol was decanted and the DNA pellet was allowed to dry at room temperature in a fume hood (30 min) or under vacuum for 15 min before resuspending in 100 μL sterile DNase, RNase free water overnight at 4°C.

Total RNA

RNA was extracted from leaf tissue of transgenic lines, wild type Nipponbare and vector-control plants using TRI Reagent® (Sigma - USA). Approximately 100 mg of leaf tissue was snap frozen with liquid nitrogen in a 2 mL Eppendorf or Cryovial tubes containing a sterile bead. Samples were homogenised using TissueLyser (Qiagen - Netherland) at maximum frequency (30) for 30 sec and returned to liquid nitrogen. Following grinding 1 mL of TRI Reagent® was added to the powdered sample and the sample was returned to the TissueLyser to homogenise at maximum frequency (30) for 1 min. The mixture was incubated at room temperature for 5 min.

To remove proteins and other organic contaminants 200 μL of chloroform was added to each sample and the mixture was shaken vigorously for 15 sec, followed by 10 min incubation at room temperature. The tube was centrifuged at 14,000 rpm for 15 min at 4°C. A 350 μL of the resulting aqueous supernatant, containing the RNA, was carefully transferred to a fresh 1.5 mL microtube. To precipitate the RNA, 500 μL of cold isopropanol was added in the supernatant. The tube contents were gently mixed and the sample was incubated at room temperature for 10 min. RNA was precipitated by centrifugation at 14,000 rpm for 10 min at 4°C. The isopropanol was decanted and replaced with 1 mL chilled 70% (v/v) ethanol and then centrifuged at 9,000 rpm for 5 min at 4°C. The ethanol was then decanted, and the RNA pellet was dried under a vacuum for 15 min before resuspending it in 50 μL sterile DNase, RNase free water. The tubes were incubated at 58°C for 15 min to assist the resuspension of RNA. Isolated RNA was used immediately or stored at - 80°C for later use.

Quantification of nucleic acid

The quantity and quality of extracted gDNA and RNA were estimated using a NanoDrop2000 spectrophotometer (Thermo Scientific - USA). Absorbance was measured at wavelengths of 230, 260 and 280 nm. The purity of the gDNA and total RNA was assessed using the ratio of absorbance at 260 and 280 nm and 260 and 230 nm.

Amplification of nucleic acid

Polymerase chain reaction (PCR)

Each reaction was performed in a final volume of 20 µl containing 10 µL of 2X GoTaq green (Taq DNA polymerase, 400 µM dATP,400 µM dGTP,400 µM dCTP,400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes (Promega - USA), 0.5 µL of each 10 µM forward and reverse primers, 100 ng of genomic DNA and DNase, RNase free water up to 20 µL. PCR was carried out either in a Peltier Thermal Cycler – 200 (MJ research - USA) or in a Peltier Thermal Cycler (Gradient Cycler - Bio-RAD - USA).

PCR profile: initial denature at 94°C for 2 min followed by 30 cycles of denaturing at 94°C for 30 sec, annealing at 54-57°C (depending on primers), extension at 72°C for 1 min. Final extension was at 72°C for 2 min.

Reverse Transcription Polymerase chain reaction (RT-PCR)

SuperScript® III first strand synthesis system (Invitrogen - USA) was used to carry out RT-PCR reaction following the manufacturer's instruction and using gene specific primers. Briefly, cDNA was synthesised using RNA mix and cDNA synthesis mix. RNA mix was prepared using 1 µL of 50 nM Oligo $(dT)_2$, 1 µL of 10 nM mix dNTPs, 2 µg RNA (previously treated with DNase to destroy any DNA residue) and DNase, RNase free water to a final volume of 10 µL. The mixture was incubated at 65°C for 5 min and then quenched on ice for 2 min. cDNA synthesis mix (prepared freshly during the incubation of RNA mixture) containing 2 µL of 10 X RT buffer, 4 µL of 25 mM $MgCl_2$, 2 µL of 0.1 M DTT, 1 µL of RNase OUT (40 U/µL) and 1 µL of SuperScript ™ III RT (200 U/µL) was added to the chilled RNA mix. The mixture was spun briefly and incubated at 50°C for 50 min followed by incubation at 85°C for 5 min following by chilling on ice. A 1 µL of aliquot RNase H (supplied with the kit) was added to the mixture and the mixture was incubated at

37°C for 20 min. RT-PCR was set up using 2 X GoTaq green (Taq DNA polymerase, 400 μM dATP,400 μM dGTP,400 μM dCTP,400 μM dTTP, 3 mM $MgCl_2$, yellow and blue dyes (Promega - USA), 0.5 μL of each 10 μM forward and reverse gene specific primers, 2 μL of cDNA and DNase, RNase free water up to 20 μL. RT-PCR was carried out either in a Peltier Thermal Cycler – 200 (MJ research - USA) or in a Peltier Thermal Cycler (Gradient Cycler - Bio-RAD - USA). RT-PCR profile: initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 54-57°C (depending on primers), extension at 72°C for 1 min. Final extension was at 72°C for 2 min.

Gel electrophoresis

Agarose gels of 1.5% (w/v) were prepared using agarose (Roche diagnostics - USA) dissolved in 1 X TAE buffer containing 0.5 X SYBR Safe DNA gel stain (Invitrogen - USA). Each gel was cast and run in an EasyCast Mini Gel System. The gels were loaded with 15 μL of each PCR product combined with 3 μL of bromophenol blue loading dye 6 X. A molecular weight marker (Marker X – Roche Diagnostics - Switzerland) was added to one lane to determine the approximate sizes of the PCR products. Electrophoresis was carried out at 120 V and in 1 X TAE buffer, for 30 min. The gel was viewed and photographed using a Syngene Geldoc system (G-box and GenSnap version 6.07) (Syngene - UK).

3.2.3 Glasshouse acclimation

Wild type, vector-control and transgenic rice plants were acclimatized in the glasshouse for salinity tolerance screening assays. Rooted plants were taken out of tissue culture jars. Agar sticking to the roots was removed by washing under tap water. Plants were then transferred to a 40 mm diameter plastic garden pot filled with commercial potting mix (Searles, Sunshine coast, Qld, Australia). Pots were placed in a container filled with tap water to one third of the height of the pot. The container was covered with clear plastic bag and placed in a glasshouse at 28°C, day/night cycle of 16 h/ 8 h for 1 week to allow plants to acclimatise. During the acclimation, the plants were sprayed with tap water twice per day. After 1 week, the bag was removed.

3.2.4 Salinity tolerance screening assay

The experiments were conducted from August to December 2012 at the QUT glasshouse at Carseldine, Brisbane, Australia with temperature adjusted at 28ºC/21ºC day/night and no supplemental light was used. Rice tissue culture plants were acclimatised using 40 mm plastic pots and premium potting mix (Searles) for 7 days. Pots were placed in a container fill with tap water. The plants were grown for another 7 days then Aquasol fertilizer (Yates, Padstow, NSW, Australia) containing nitrogen, phosphorus, potassium and trace elements (N:P:K:23:3.95:14) was added. One week later the salinity stress experiment at seedling stage was started; water in the container was drained out and 100 mM NaCl in tap water was filled in until a level of 1 cm above potting mix level was achieved. At this stage the plants had 3 fully expanded leaves and the fourth leaf was just emerged. The water level was maintained daily at 1 cm above soil level by adding tap water (not salt water) into the container. The salinity stress experiment at reproductive stage was carried out basically followed 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 fertilizer (Osmocote pots, planter & indoor, Scotts, Bella Vista, NSW, Australia, with a NPK ratio of 13.8:3.2:9.9) at 14 days post acclimation. The pots were placed in a container filled with tap water supplemented with fertilizer (Aquasol) to 1 cm above the compost level. The plants were grown for another 16 days and the water was drain out of the container. NaCl solution (100 mM NaCl in tap water) or tap water (control) was added to a level of 1 cm above the potting mix level. The water level was maintained daily to the 1 cm above potting mix level by adding tap water into containers to compensate the water lost due to transpiration and evaporation. It is worth to note that the concentration of sodium in tap water is very low, it is approximately 16 to 100 mg L^{-1} (approximately 0.7 mM to 4.4 mM) (Queensland Urban Utilities 2009).

3.2.5 Measurement of morphological parameters

Morphological data including the shoot height, number of leaves and number of tillers in the plants under salinity stress (100 mM NaCl) and non-stressed conditions (control) were recorded at day 0 and day 13 of the salinity stress tolerance screening at seedling stage. On day 13 plants were carefully removed from the pot,

soil was washed off with salt solution (100 mM NaCl in tap water for plants from 100 mM NaCl treatment) or tap water for the control condition (0 mM NaCl) and the fresh weight was recorded using a digital balance. Samples were then dried at 70°C in a vacuum oven until constant weight was reached and this weight was recorded as dry weight. Shoot growth was calculated as shoot height at day 13 minus shoot height at day 0. For salt tolerance screening at the reproductive stage data were recorded on number of tillers per plant, number of panicles per plant and number of spikelets per panicle at harvest.

3.2.6 Relative water content

Relative water content plant leaves was examined using the method described by Lafitte (2002). Approximately 10 cm of leaf was cut off from the middle part of the youngest fully expanded leaf, weigh (fresh weight – 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 dark at 4ºC overnight. The next morning, the leaf was blotted dry with tissue towel for 30 s and weigh (turgid weight – TW). The samples were then dried in a vacuum oven at 70ºC for 3 days and weigh for Dry weight (DW). The relative water content was calculated as RWC= (FW-DW) *100/(TW-DW)

3.2.7 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 seedling stage the third leaf was used to measure net photosynthesis at day 0, 3, 7, 10 and 13 after salinity 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 vapor pressure deficit based on leaf temperature was recorded around 1.05 – 1.4 kPa. Relative humidity in the sample cell was maintained at 57% ± 3%. Measurement started from 9 am – 12 pm.

3.2.8 Electrolyte leakage measurement

Electrolyte leakage from leaves at the seedling and reproductive stages was measured using a CM 100-2 conductivity meter (Reid & Associates CC, South Africa) following manufacturer's instruction. Briefly, leaf tissue was excised from plants, placed in a plastic bag and immediately put on ice. Leaf was washed twice with deionised water and blotted dry with towel paper. Approximately 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 two min over 60 min periods. Samples were removed, dried in an oven at 70ºC overnight for measurement of dry weight. Electrolyte leakage was calculated as the slope of electrolyte leakage over time and normalized by dry weight.

3.2.9 Sodium and potassium measurement

The amount of Na^+ and K^+ in leaf of rice plants in salt tolerance screening experiments at both the seedling and reproductive stages were determined using an Atomic Absorption Spectrophotometer (Shimadzu A-7000, Shimadzu Scientific Instrument, Sydney, NSW, Australia). Leaf samples for sodium and potassium analysis were undertaken on the same leaf that was used for photosynthesis measurements at both seedling and reproductive stage (third leaf and flag leaf respectively).Samples were prepared as described in Dionisio-Sese & Tobita (2000). Fifteen milligrams of dried leaf was cut into 0.5 cm long and immersed in 30 mL 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 121ºC. Samples were cool down at room temperature and filtered using the Whatman filter paper No40 (ashless).

3.2.10 TUNEL assay

The 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 tips fragments (approximately 1 cm) were cut from plants, washed three times with fresh 1 X Phosphate Buffer Saline (PBS) and fixed in 4% paraformaldehyde solution at 4ºC for 1 h. Following fixation, root tips were washed twice with fresh PBS, immersed in fresh permeability solution (0.1% triton X 100 and 0.1% sodium citrate) and microwaved at 700 W for 1 min. Samples were immediately cool down using fresh PBS, followed by two more PBS washes. A 50 µL 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 was also included. Samples were incubated at 37ºC 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,

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 a Nikon A1 Confocal Microscope.

3.2.11 Histochemical GUS assay

Rice calli (transformed with vector control pCAMBIA1301) that survived after 7 weeks on hygromycin selection were histochemically assayed for *GUS* expression by addition of 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) (Progen Biosciences) and overnight incubation at 37°C as described by Jefferson *et al.* (1987).

3.2.12 Data analysis

All experiments in this study were conducted using a randomized completed block designed (Montgomery, 2005; Hinkelmann, 2007). Graphs and standard errors were prepared in Excel. Where applicable data were analysed using one-way ANOVA and Tukey's HSD tests (Minitab Version 16).

Chapter 4: Generation and characterisation of transgenic rice plants expressing pro-survival genes

Chapter 4 begins with an introduction to the first specific objective of the project: generation and characterisation of transgenic rice plants expressing pro-survival genes. Methodology used to achieve this objective is briefly described in section 4.2. The results section (4.3) reports on the generation of transgenic rice plants from mature rice seeds through callus induction, *Agrobacterium*-mediated transformation, hygromycin selection, regeneration of putative transgenic plants and finally the characterisation of those plants. A discussion on the results and findings of this specific objective is presented in section 4.4.

4.1 INTRODUCTION

The world's population is expected to rise above 9 billion by 2050. To feed this increasing population, food production is required to increase by 120-170% i.e. cereal production needs to increase by approximately 44 million metric tons annually. The demand for increasing food production, under very little potential for future expansion of arable lands and growing environmental stress conditions, is a challenge (Eckardt 2009; FAO 2009, 2012; Cominelli *et al.* 2013; United States Census Bureau 2013). The use of modern biotechnology, molecular breeding techniques and genetic engineering of crop species can significantly contribute to overcoming these challenges (Eckardt 2009). Genetic engineering allows for the introduction of genes controlling traits without affecting the desirable characteristics of an elite genotype and has become a powerful tool for the generation of "improved" future crops. Genetic modification allows the generation of elite crops which can survive in adverse environmental conditions (Bhatnagar-Mathur *et al.* 2008; Agarwal *et al.* 2013). A number of techniques have been developed for gene transfer to plant cells, including protoplast-mediated techniques using polyethylene glycol and/or electroporation, microprojectile bombardment and *Agrobacterium*-mediated transformation. Of all these techniques, *Agrobacterium*-mediated transformation is the most efficient and reliable system (Cho *et al.* 1998).

Of the abiotic stresses that limit world crop production, soil water deficits and salinisation are the two most critical factors (Munns 2011). Previously, attempts have focussed on developing drought and salinity tolerant crops using different approaches (Singh *et al.* 2010a; Singh *et al.* 2010b; Reguera *et al.* 2012; Cominelli *et al.* 2013). Recently, pro-survival (anti-apoptotic) genes have been reported to play important roles in mediating plant adaption to environmental stresses. For example, overexpression of *AtBAG4*, a *Bcl-2* associated anthanogene (BAG) from *Arabidopsis thaliana* in tobacco enhanced tolerance to several abiotic stress stimuli including salinity stress (Doukhanina *et al.* 2006). Expression of *AtBI1*,a BAX inhibitor-1from *Arabidopsis thaliana*, was found up-regulated under several stress conditions induced by bacterial and fungal pathogens, ozone, norflurazon, or salicylic acid (Sanchez *et al.* 2000; Kawai-Yamada *et al.* 2004).Over-expression of *AtBI-1* was shown to suppress BAX, pathogen, or abiotic stress-induced cell death in a variety of cells from yeast, plant, and mammalian origins (Sanchez *et al.* 2000; Kawai-Yamada *et al.* 2001; Matsumura *et al.* 2003; Watanabe & Lam 2009). Plant *BI-1* is thus likely to play an important role as a survival factor under multiple stress conditions (Watanabe & Lam 2008). The expression of a baculovirus pro-survival gene (*p35)* in transgenic tomato, tobacco and passion fruit plants suppressed cell death caused by the fungal toxin (AAL- toxin- the sphinganine analog mycotoxin caused by *Alternariaalternate* f. sp. *lycopersici*) and the infection of bacterial or fungal pathogens and enhanced tolerance to a range of abiotic and biotic stresses (Lincoln *et al.* 2002; Freitas *et al.* 2007; Wang *et al.* 2009). *SfIAP*, a member of the IAP (inhibitor of apoptosis) family of anti-apoptosis proteins isolated from the insect *Spodoptera frugiperda* has also 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). Salinity stress induces PCD in rice, (Li *et al.* 2007; Liu *et al.* 2007; Jiang *et al.* 2008), therefore the manipulation of PCD pathways to prevent death upon exposure to salinity stress shows great potential to increase crop production in salt affected areas (Joseph & Jini 2010).

In light of these seminal studies, the aim of the research described in this chapter was to explore a range of pro-survival genes, with different proposed functions in PCD pathways, for generating salinity stress tolerant transgenic rice.

The specific objectives of this chapter were to:

1. Transform Nipponbare rice embryogenic calli with *AtBAG4*, *AtBI*, *AtBAG4+Hsp70*, *Hsp70*, *Hsp90*,*OsBAG4*, *p35* and *SfIAP* genes
2. Evaluate the potential effect of pro-survival genes on calli proliferation during the selection period
3. Select transformants and regenerate transformed lines
4. Characterise transformed lines using PCR and RT-PCR

4.2 MATERIALS AND METHODS

4.2.1 Transformation constructs

The modified binary vector pCAMBIA1301 containing the maize polyubiquitin-1 (Ubi-1) promoter controlling the expression of the pro-survival genes *AtBAG4*, *AtBI*, *AtBAG4+Hsp70*, *Hsp70*, *Hsp90*,*OsBAG4*, *p35* and *SfIAP* was kindly provided by Banana research team, Centre for Tropical Crops and Biocommodities (CTCB), Queensland University of Technology (QUT). The genes were sequenced in their original vector prior to transformation to confirm the presence and integrity of the coding sequence and the promoter-gene and gene-terminator borders. A schematic representative of a gene construct is shown in Figure 4.1.

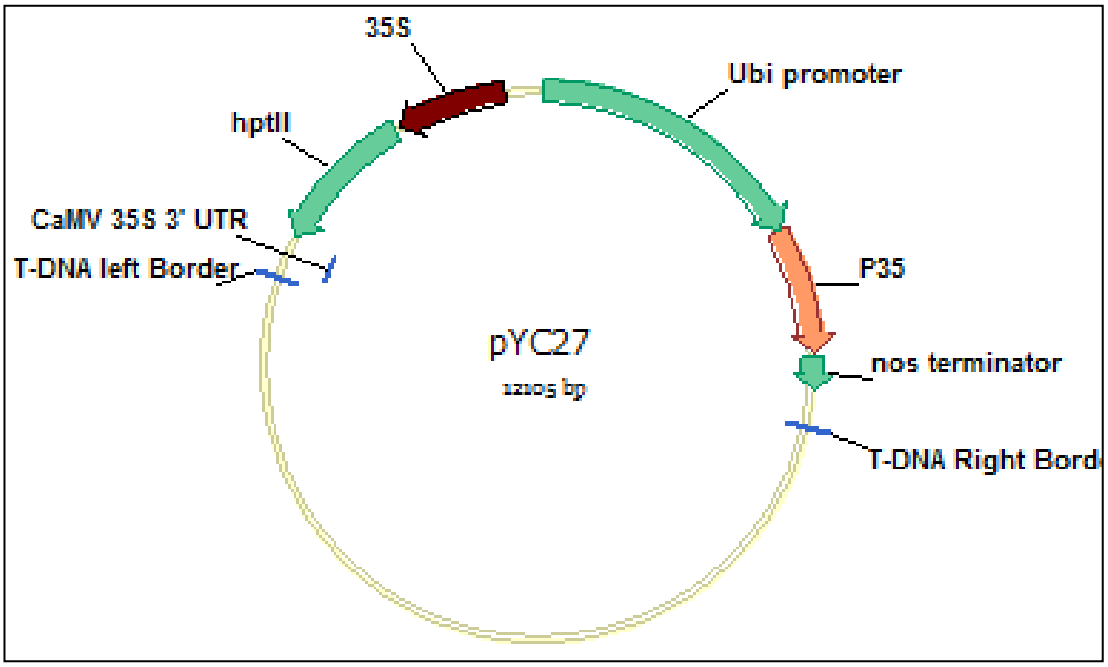


Figure 4.1: Schematic representations of the gene construct (pYC27, *p35* gene)

4.2.2 Rice transformation

Embryogenic callus was induced from scutellum of mature seeds of rice *Oryza sativa* L. spp. Japonica cv. Nipponbare using 2N6 callus induction media as described in chapter 3 (section 3.2.1). Binary vectors harbouring the genes of interest and a vector control (VC = pCAMBIA1301) were transferred to *Agrobacterium* strain AGL1 (see chapter 3 section 3.2.1 for detail). Four day-old pre-cultured rice scutellum-derived calli were transformed with the genes of interest by *Agrobacterium*-mediated transformation using recombinant Agrobacterium cultures with an optical density at wavelength of 600 nm (OD_{600}) of 0.7 together (see chapter 3 section 3.2.1 for details). Due to the fact that the transformation of 9 constructs (8 pro-survival genes and VC) and wild type Nipponbare (WT) (negative control transformation) cannot be achieved within 1 transformation (1 day); and monitoring of calli after transformation is also impossible for all genes at once, the transformation experiments were conducted in four sets. i) Set 1 *p35*, *SfIAP* and (VC); ii) set 2 *AtBAG4*, *AtBAG4+Hsp70* and VC; iii) set 3 *Hsp70* and *Hsp90* and VC; and iv) set 4 *OsBAG4*, *AtBI* and VC. Transformation set 2 & 3 were conducted on the same day using the same batch of scutellum-derived calli. Both shared the VC transformation with 2 replicates were transformed one with transformation set 2 and one was with transformation set 3. The reason for separating into 2 sets was to ensure similar conditions (including duration of transformation experiment) for transformation in all 4 sets. Wild type Nipponbare (WT) was included in each transformation as a control. Three replicates of at least 10 or 5 petri plates each containing 10 calli clumps were generated for each pro-survival gene construct and VC respectively (total at least 30 plates containing 300 calli clumps for each pro-survival gene construct and 15 plates containing 150 calli clumps for VC in each transformation set). The transformed calli were selected on half hygromycin selection media (2N6 selection media supplemented with 25 mgL^{-1} hygromycin) for 2 weeks then were sub-cultured to full hygromycin selection media (2N6 selection media containing 50 mgL^{-1} hygromycin) for 7 weeks with 3 subculturing intervals. Dead calli clumps, recognised by their dry black colour and no sign of proliferation were discarded during sub-culture. Proliferating calli were sub-cultured to new full hygromycin (50 mgL^{-1}) selection media every 2 weeks.

4.2.3 Callus proliferation

In order to evaluate the effect of different pro-survival genes on the proliferation of rice calli during selection, levels of calli clump proliferation were measured. After seven weeks of full hygromycin selection, the diameter of calli clump was recorded. Based on the diameter of a calli clump, six levels of callus proliferation were determined. Proliferation level 1 (PL1) denoted calli clumps with a diameter between 3-6 mm; PL2: 7-9 mm; PL3: 9-11 mm; PL4: 12-14; PL5: 15-17 mm; PL6:18 mm or above.

4.2.4 Plant Regeneration and rooting

After nine weeks on selection media containing hygromycin, individual proliferating antibiotic resistant calli clumps (cream-white in colour) were transferred to regeneration media supplemented with 200 mgL^{-1} timentin and 25 mgL^{-1} hygromycin for shoot generation. Shoots with at least three well-formed leaves were transferred to 120 x 60mm culture vessels containing 50 ml of rooting media supplemented with 200 mgL^{-1} Timentin and 25 mgL^{-1} hygromycin for rooting (see chapter 3section3.2.1 for details). The healthiest, well rooted plant from each calli clump was selected as a transformed independent event. Transformed events were confirmed transgenics by PCR; and expression of pro-survival gene in rice plants were confirmed by RT-PCR.

4.2.5 PCR confirmation of transgenic plant

For details of sample collection, DNA extraction, RNA extraction, PCR, RT-PCR and agarose gel electrophoresis please see chapter 3 section 3.2.2. The primers used for PCR are outlined in table 4.1

Table 4.1: List of primers for PCR

Gene	Sequences	Tm	PCR product length (bp)
AtBAG4	5' TGTGGATTTTTTTAGCCCTGCCTTC 3' 5' CAAAAAGCGGCGGAAGCAGAGTC 3'	65 66	220
AtBI	5' TGTGGATTTTTTTAGCCCTGCCTTC 3' 5' TGGATCCCCTCAGTTTCTCCTTTTCTTCTT 3'	65 65	880

AtBAG4 + Hsp70	5' TGTGGATTTTTTTAGCCCTGCCTTC 3' 5' ACCAAAGTCTAAACCCAGAAGCACC 3'	65 66	110
	5' TGTGGATTTTTTTAGCCCTGCCTTC 3' 5' CAAAAAGCGGCGGAAGCAGAGTC 3'	65 66	220
SfIAP	5' TGTGGATTTTTTTAGCCCTGCCTTC 3' 5' CAAGCGATCCGCTCTTGAATAGC 3'	64 65	220
Hsp70	5' TGTGGATTTTTTTAGCCCTGCCTTC 3' 5' ACCAAAGTCTAAACCCAGAAGCACC 3'	65 66	110
Hsp90	5' TGTGGATTTTTTTAGCCCTGCCTTC 3' 5' AAGGATCCTTAGGAAGTATTGTATGACCTG 3'	65 66	1600
OsBAG4	5' TGTGGATTTTTTTAGCCCTGCCTTC 3' 5' TAGGATCCGAGGCTAGTCGAATTGCTCCCA 3'	65 67	850
p35	5' TAGAGTTTACTACAGAATCGAGCTGG 3' 5' GTAAAACGACGGCCAGT 3'	65 55	450
Vector control (Hygromycin)	5' AGC TGC GCC GAT GGT TTC TAC AA 3' 5' ATC GCC TCG CTC CAG TCA ATG 3'	62 59	700
VirC	5'GCCTTAAAATCATTTGTAGCGACTTCG 3' 5'TCATCGCTAGCTCAAACCTGCTTTCTG 3'	62 62	738

4.3 RESULTS

4.3.1 Generation of transgenic rice plants

Callus induction, transformation and selection

Embryogenic calli derived from scutellum of mature seeds were reported to be a good material for high efficient transformation in rice (Hiei *et al.* 1994), therefore we induced callus from scutellum of Nipponbare mature seeds for generation transgenic rice expressing pro-survival genes. A total of 3,000 mature seeds were used to generate scutellum-derived embryogenic calli for four sets of transformation. The callus induction protocol used in this project worked well with no contamination and the rate of callus formation was very high (more than 90% - data not shown). Within 4 weeks, embryogenic calli which are ready for transformation were obtained from mature rice seeds using this protocol. The process of callus induction from mature seeds is shown in Figure 4.2.

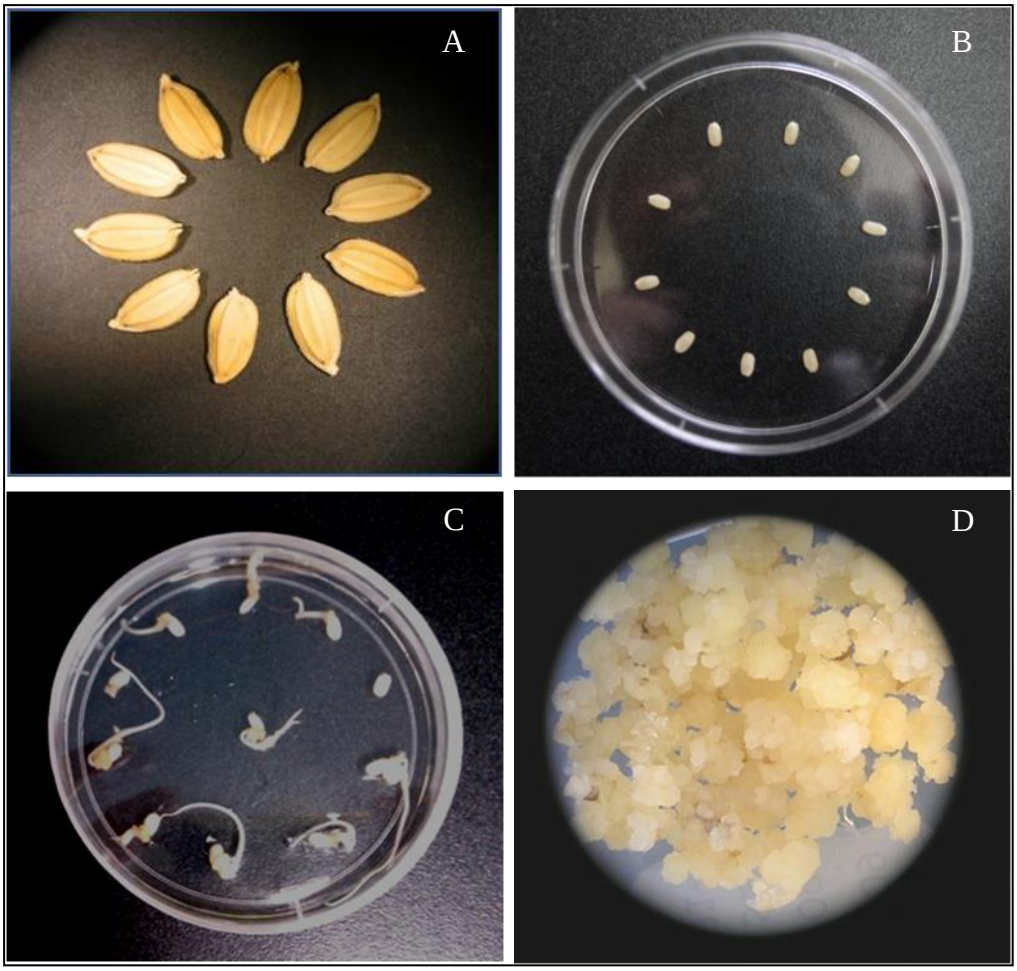


Figure 4.2: Induction of embryogenic callus from Nipponbare mature seeds. (A): Nipponbare seeds; (B): Dehusked Nipponbare seeds on callus induction media; (C):Callus derived from seeds; (D) Callus ready for transformation.

A total of 2,850 transformed callus clumps were obtained from the four sets of transformation. To select putative transgenic callus, transformed callus were selected by addition of the antibiotic hygromycin (50 mgL^{-1}) to the media. The efficacy of selection between untransformed and transformed callus following seven weeks on hygromycin selection media is shown in Figure 4.3. Using hygromycin selection, untransformed calli die after 7 weeks whereas potential transformed calli continue proliferating.

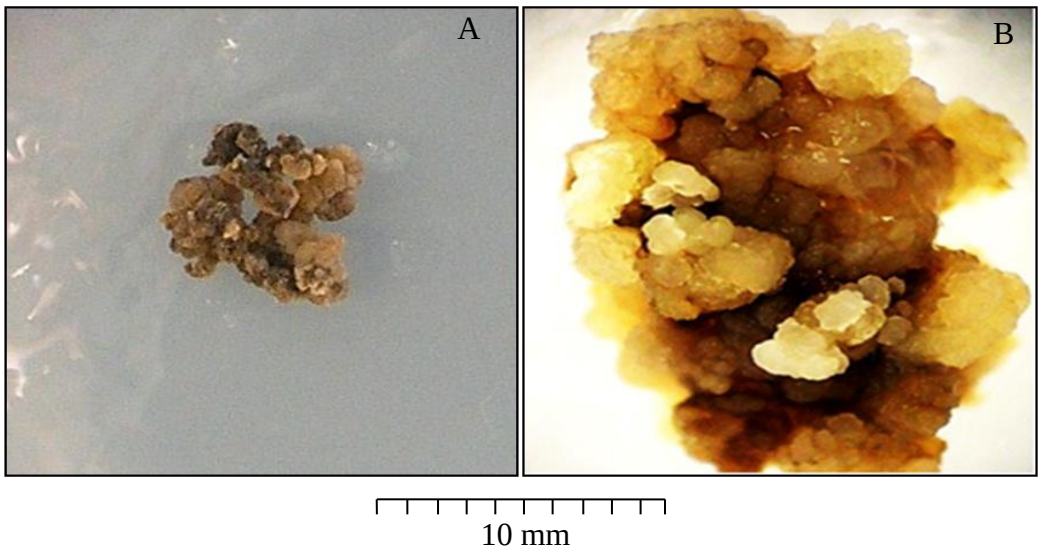


Figure 4.3: Representative of untransformed and transformed rice calli. (A): Wild type Nipponbare calli after 7 weeks on fullhygromycin selection media (untransformed); (B): Transformed Nipponbare calli after 7 weeks onfull hygromycin selection media.

Histochemical GUS assay of 10 random VC transformed callus pieces (from 10 different calli clumps) after 7 weeks on full hygromycin selection media showed expression and activity of the *GUS* transgene (Figure 4.4). No sign of residual *Agrobacterium* contamination was observed by eye in and around the calli clumps during selection.

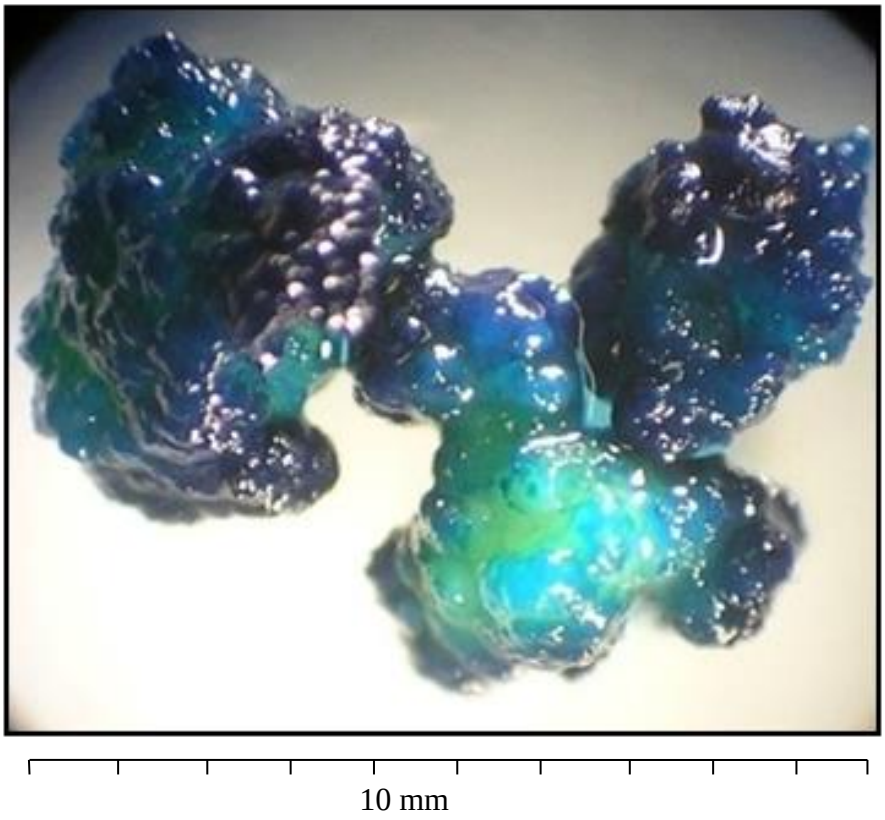


Figure 4.4: Histochemical staining of VC (*GUS*) transformed rice calli after 7 weeks on full hygromycin selection media

Expression of pro-survival genes leads to increased calli proliferation but not transformation rate

The percentage of calli clumps transformed with *GUS* (vector control-VC) and pro-survival genes that survived and proliferated after 7 weeks on full hygromycin selection media was shown in Figure 4.5. More than 90% of the wild type (WT) control embryogenic calli (untransformed) were dead after 7 weeks on full hygromycin selection media, while this number was less than 20% on average for both VC and pro-survival transformed embryogenic calli. There was no noticeable difference in the survival rate between the VC and pro-survival genes in each set of transformation. This result suggest that expression of *AtBAG4, AtBI, AtBAG4+Hsp70, Hsp70, Hsp90,OsBAG4, p35* and *SfIAP* does not affect transformation efficiency at the callus stage; and that this is a suitable time to transfer the survived calli to regeneration media.

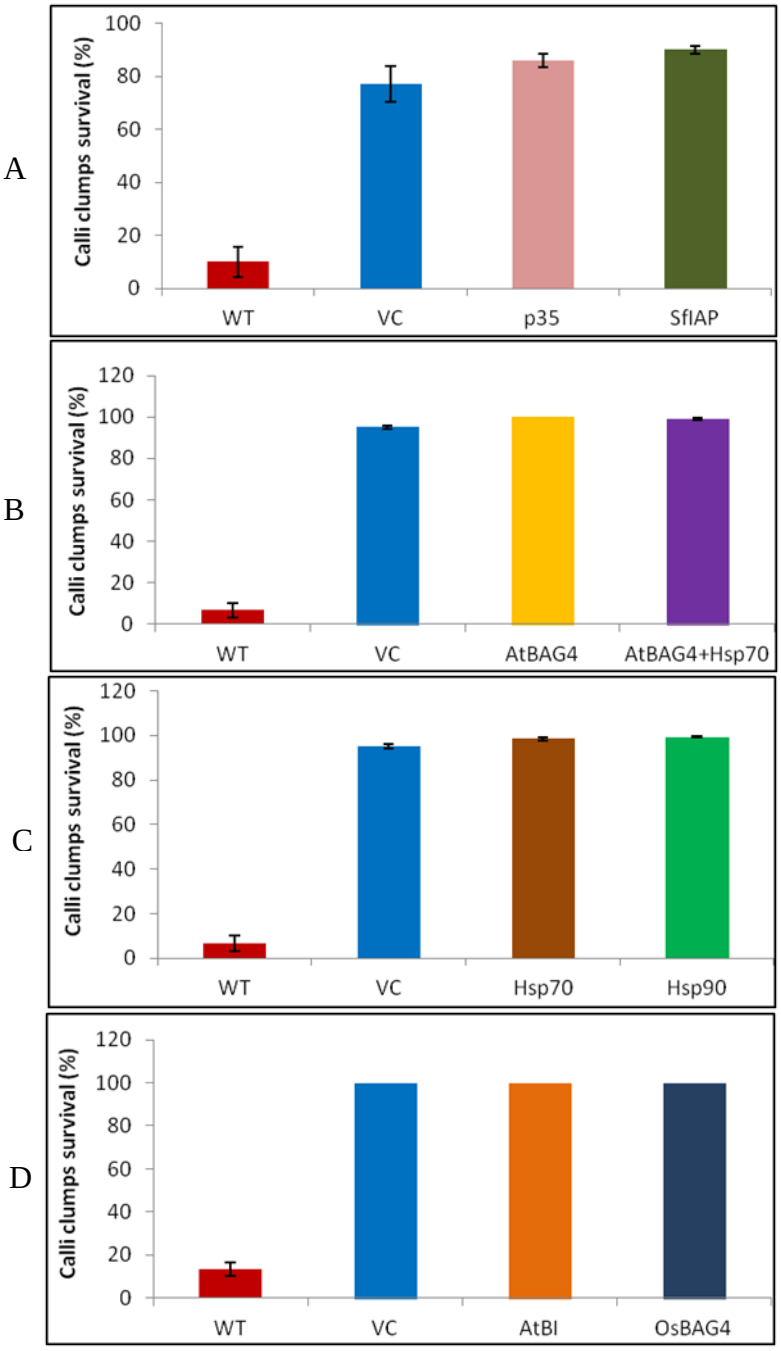


Figure 4.5: Percentage of calli survival after 7 weeks on full hygromycin selection media. A: transformation set 1; B: transformation set 2; C: transformation set 3 and D: transformation set 4. Data are mean and standard error (n = at least 30).

During the 7 weeks selection period we noted that the levels of calli proliferation were different between calli transformed with VC and pro-survival genes and between calli of individual transformation experiments. Although growth conditions for rice calli were optimal, some stress still occurs i.e. nutrients deficiency. The genes used in this study promote survival by preventing stress-

induced cell death allow continuous proliferation of calli. The levels of calli proliferation therefore, were investigated. Based on the diameter of calli clump and the health of calli pieces (nodular and cream colour), six levels of calli proliferation were recorded. An example of the calli proliferation levels is shown in Figure 4.6.

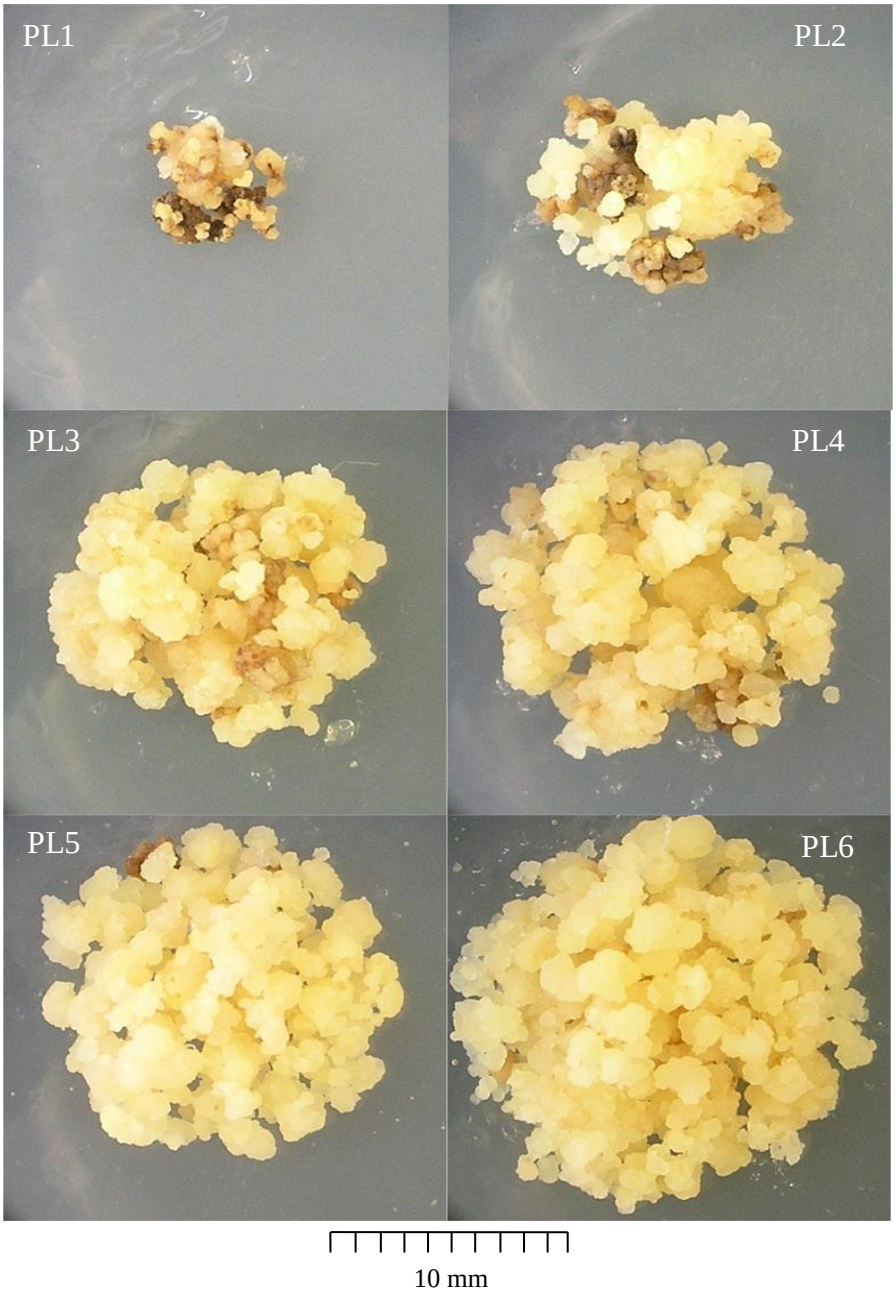


Figure 4.6: Examples of Proliferation Level of Nipponbare calli. PL1: callus diameter's 3-6 mm; PL2: 7-9 mm; PL3: 9-11 mm; PL4: 12-14; PL5: 15-17 mm; PL6:18 mm or above.

The proliferation profile of embryogenic calli transformed with different genes after 7 weeks on full hygromycin selection media is shown in Figure 4.7. No remarkable differences were observed in the proliferation profile of calli transformed with the VC and *p35*. Calli transformed with *SfIAP*, however, had a different proliferation profile in comparison with that of the VC and *p35* with approximately 4.5 % of calli clumps proliferated at a high level (levels 5, 6), while no calli clumps transformed with VC proliferated to these levels (Figure 4.7A). Proliferation profiles of calli transformed with *AtBAG4* and *AtBAG4+Hsp70* were not different but they were different to calli transformed with the VC. Most calli transformed with *AtBAG4* and *AtBAG4+Hsp70* proliferated at PL1 (approximately 60%) while calli transformed with VC had highest proliferation at PL2 (around 45%) followed by PL1 and PL3 (approximately 20% each) (Figure 4.7B). Calli transformed with *Hsp70* and *Hsp90* proliferated at different rates in PL1 and PL2 but were similar to each other in other levels of proliferation (PL3, 4, 5, 6). Their proliferation profiles were different to that of calli transformed with VC (Figure 4.7C). There was no difference in proliferation profiles between calli transformed with *AtBI*, *OsBAG4* and VC (Figure 4.7D).

Although there was variation in proliferation levels of calli transformed with different genes, a common trend observed from overall proliferation profiles of all transformation sets is that most of the calli clumps proliferated at PL1 and PL2 (more than 65% for each gene in all transformation sets). Of the nine genes and combination tested, *SfIAP* appeared to induce the highest level of calli proliferation (PL 5, 6) calli. *AtBAG4*, *OsBAG4*, *Hsp70* and VC did not produce any calli clumps up to PL6.

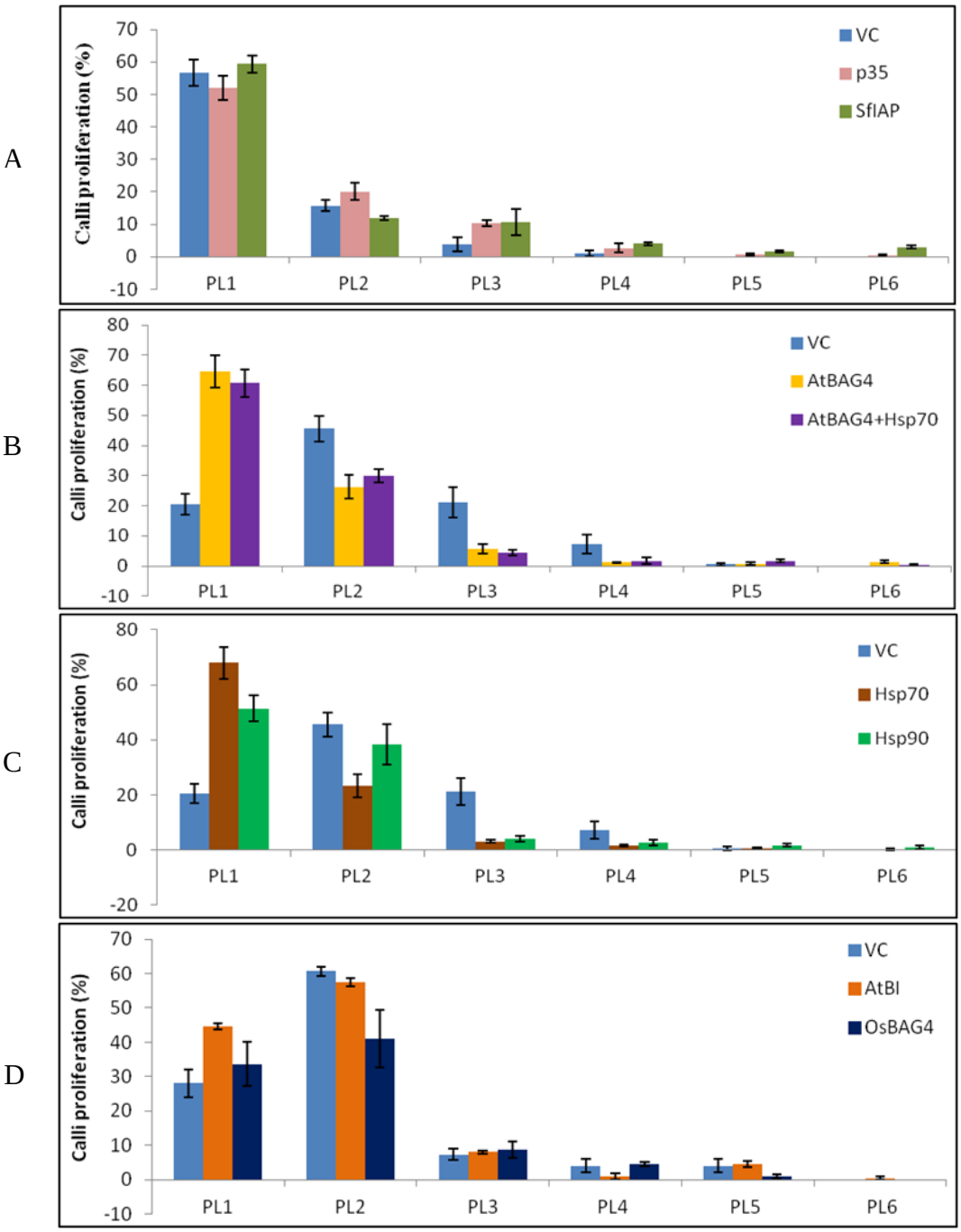


Figure 4.7: Calli proliferation profile after 7 weeks on full hygromycin selection. A: transformation set 1; B: transformation set 2; C: transformation set 3 and D: transformation set 4. Data are mean and standard error (n = at least 50).

Effect of pro-survival genes on rice plant regeneration

To regenerate transgenic plants, proliferating calli were transferred to regeneration media after 9 weeks on hygromycin selection media (2 weeks on half selection and 7 weeks on full selection media) (Figure 4.8).

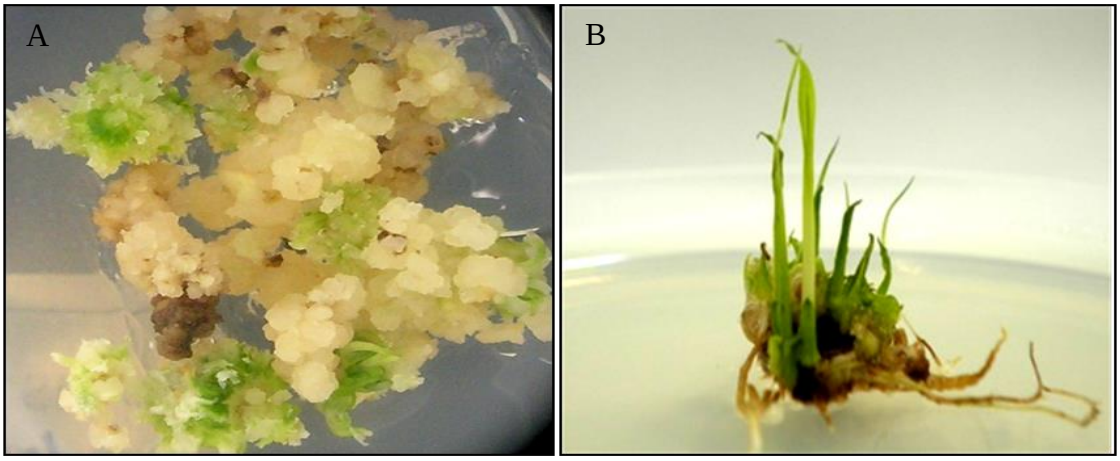


Figure 4.8: Regeneration of transformed rice calli. A: transformed embryogenic calli after 3 weeks on regeneration media. B: plantlet regenerated from calli transformed with *p35* after 6 weeks on regeneration media.

The percentage regeneration of putatively transformed calli was recorded after 6 weeks on regeneration media (Figure 4.9). No differences were observed in the regeneration percentage of calli transformed with VC and *p35* in transformation set 1. However, the regeneration ability of embryogenic calli transformed with *SfIAP* was definitely lower (Figure 4.9A) with only 4.33% of putatively *SfIAP* transformed calli regenerated compared to 10.95% and 11.39% in calli transformed with the VC and *p35* generated, respectively. No noticeable differences were observed in regeneration of calli transformed with *AtBAG4*, *AtBAG4+Hsp70* or *AtBI* and *OsBAG4* (transformation sets 2 & 4) when compared to the VC (Figure 4.9B, D). The percentage regeneration of calli transformed with *Hsp90* was higher than that of VC and *Hsp70* in the transformation set 3. However, it was only around 1.47 fold higher (Figure 4.9C). A possible explanation for the differences in regeneration levels of calli transformed with different pro-survival genes is that responses of these genes to different developmental cues (i.e. phytohormone and the change of growth conditions from dark for calli culture to light for plant regeneration) may be different leading to differences in regeneration rate.

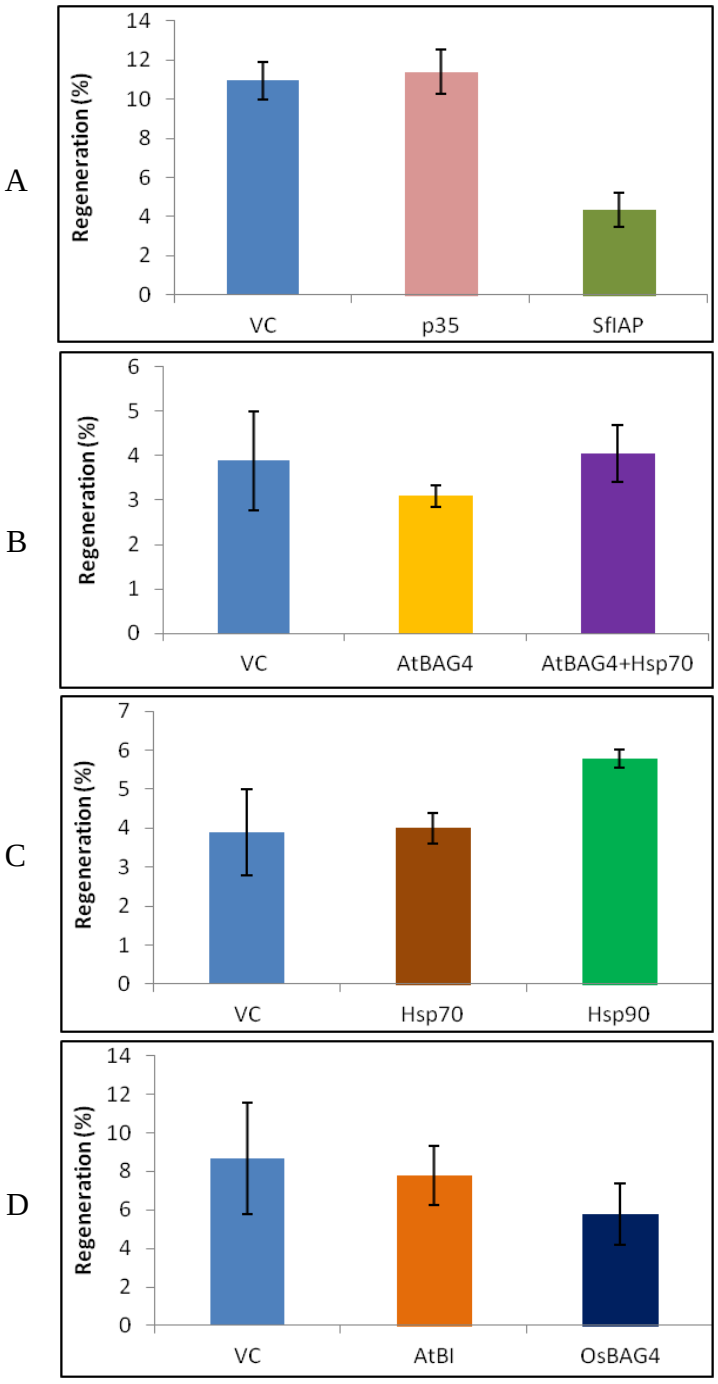


Figure 4.9: Regeneration of embryogenic calli transformed with different pro-survival genes and VC. A: transformation set 1; B: transformation set 2; C: transformation set 3 and D: transformation set 4. Data are mean and standard error (n = at least 50).

4.3.2 Confirmation of transgenic event and gene expression

To confirm that the plants contained stably integrated copies of the transgenes, genomic DNA was extracted from leaves and was used in a PCR with gene specific primers. Additionally, to avoid the possibility of recording a false PCR positive due

to the presence of residual *Agrobacterium*, any transgene plantlets which have gene specific-PCR positive were screened further using a second PCR with VirC primers to detect the presence of *VirC* gene. *VirC* gene is located outside of the T-DNA borders of the binary vectors and therefore should not be transferred to the plant.

RT-PCR was used to confirm the expression of transgene in transgenic plants. Total RNA was extracted from leaves of transgenic plants, treated with DNase to remove any DNA contamination. PCRs with *hpt* (hygromycin phosphotransferase) primers and gene specific primers were conducted to detect DNA residue in RNA samples. DNA free RNA was use as template for cDNA synthesis.

PCR and RT-PCR confirmation of transformation set 1

Rice embryogenic calli transformed with the *p35* pro-survival gene regenerated 36 putatively transformed lines which were characterized by PCR using primers as listed in Table 3.1. Of the 36 lines tested, 32 lines were PCR positive with gene specific primers (Appendix 2). A PCR using *VirC* primers was conducted to detect *Agrobacterium* residue in all PCR positive lines. Result showed that no *Agrobacterium* residue was present in these transgenic lines (Appendix 2). Expression of *p35* gene in the transgenic lines was examined using RT-PCR. Results showed that *p35* was expressed in 29 out of 32 transgenic lines tested (Appendix 2). The transformation efficiency, calculated from the resulted PCR positive transgenic lines on total number of calli clumps, was around 10.67% ± 1.45% (Table 4.2).

Table 4.2: Summary of PCR and RT-PCR confirmation of transformation set 1

Gene	# lines tested	PCR positive (# lines)	VirC-PCR positive(# lines)	RT-PCR positive(# lines)	TE (%)
p35	36	32	0	29	10.67 ± 1.45
SfIAP	12	11	1	10	3.33 ± 0.88
GUS	21	21	0		10 ± 0.95

Note: TE = Transformation efficiency (calculated on # PCR positive/Total # callus clumps used for transformation)

In total 12 putatively transformed lines were generated from the *SfIAP* transformation experiment. PCR with gene specific primers showed that except for line 5 which is negative (non-transgenic), all other lines were PCR positive (Figure 4.10A). The remaining lines were therefore checked with VirC-PCR to detect the presence of residual *Agrobacterium*. Out of 12 lines tested, 1 line (line 12) was found positive with VirC-PCR (Figure 4.10B). This result indicates that *Agrobacterium* remains in this line despite the inclusion of Timentin in selection media; and PCR positive for transgene in this line maybe caused by the bacterial presentation. RT-PCR conducted on 10 transgenic lines indicated that *SfIAP* is expressing in all *SfIAP* transgenic lines (Figure 4.10C). The transformation efficiency of *SfIAP* is around 3.33% ± 0.88% (Table 4.2).

For PCR confirmation of VC, hygromycin primers were used (Table 4.1). All 21 putative transformed lines were found positive with PCR (Appendix 2). PCR with *Vir*C specific primers showed that no *Agrobacterium* residue was present in putatively transformed lines tested. The transformation efficiency for VC is around 10% ± 0.95% (Table 4.2).

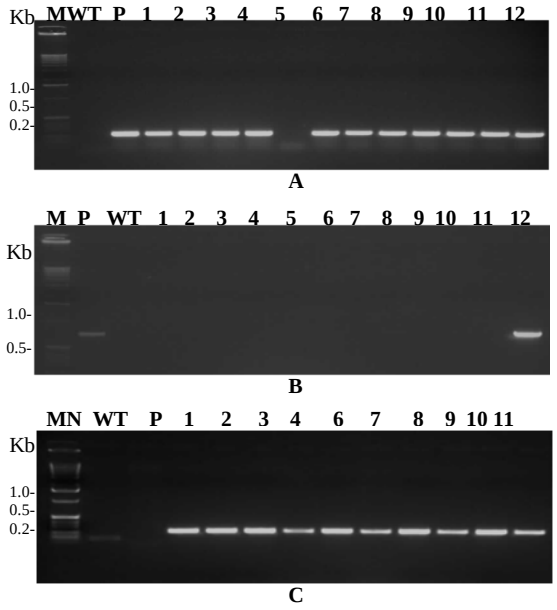


Figure 4.10: Molecular characterisation of *SfIAP* transformed rice plants. A: PCR with gene specific primers; B: PCR with VirC primers; C: RT-PCR. M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA or *Agrobacterium* extraction); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120 V.

PCR and RT-PCR confirmation of transformation set 2&3

Table 4.3: Summary of PCR and RT-PCR confirmation of transformation set 2 & 3

Gene	# lines tested	PCR positive (# lines)	VirC-PCR positive(# lines)	RT-PCR positive(# lines)	TE (%)
AtBAG4	10	9	0	9	3 ± 0.23
AtBAG4+Hsp70	18	16	0	15	3.1± 0.32
Hsp70	15	15	0	15	4 ± 0.38
Hsp90	19	18	0	18	5.77 ± 0.22
GUS	8	8	0		3.9 ±1.1

The confirmation of transgenic lines (PCR) and expression (RT-PCR) of *AtBAG4, AtBAG4+Hsp70, Hsp70, Hsp90* and VC is summarised in Table 4.3. As shown, none of the transgenic lines were positive with VirC-PCR indicating that no *Agrobacterium* contamination was present in putatively transformed lines tested. All putative transformed *Hsp70* plants tested were positive with both PCR and RT-PCR (Appendix 2). PCR for *AtBAG4+ Hsp70* transgenic plants were conducted using 2 pairs of specific primers, one for *AtBAG4*and other for *Hsp70*. Of the 18 putatively *AtBAG4+ Hsp70* transformed lines, 16 lines and 17 lines had PCR positive results with *AtBAG4* and *Hsp70* gene specific primers respectively (Figure 4.11 A, B, C, D). There was one line that had PCR negative with both *AtBAG4* and *Hsp70* gene specific primers (line number 2) and one line that had PCR negative for *AtBAG4* but positive for *Hsp70* (line number 14). Of all *AtBAG4* and *Hsp90* transformed plants lines tested each has one line negative with gene specific-PCR, the remaining lines were both PCR and RT-PCR positive (Appendix 2, Table 4.3). All VC transformed lines in transformation set 2 & 3 tested were PCR positive. The transformation efficiency of pro-survival genes and the VC in transformation set 2 & 3 are similar but significantly lower than in transformation set 1.

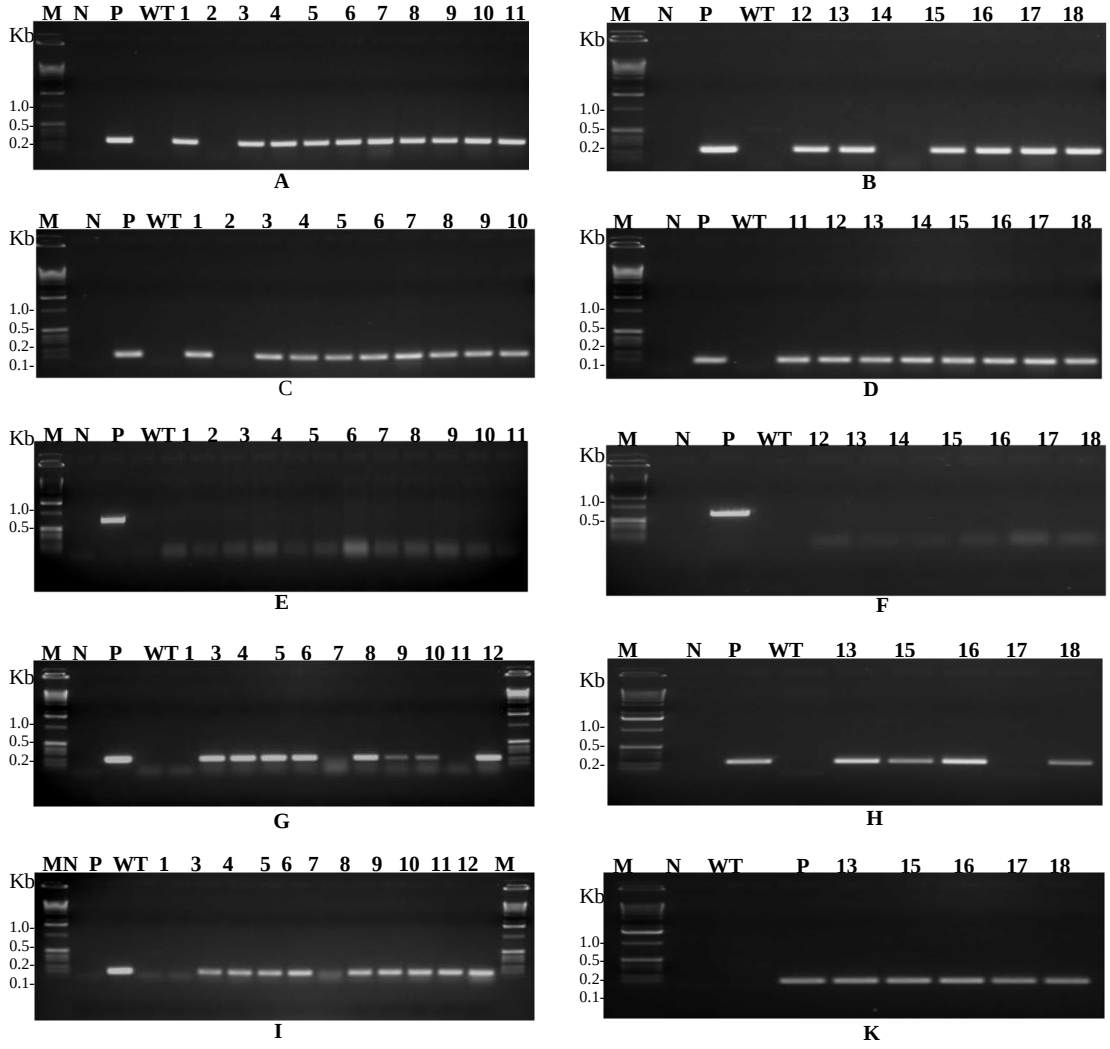


Figure 4.11: Molecular characterisation of *AtBAG4+ Hsp70* transformed rice plants. A and B: PCR with *AtBAG4* gene specific primers; C and D:PCR with*Hsp70* gene specific primers; E and F: PCR with VirC primers; G and H: RT-PCRwith*AtBAG4* gene specific primers; I and K:RT-PCRwith*Hsp70* gene specific primers. M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA or *Agrobacterium* extraction); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120V.

PCR and RT-PCR confirmation of transformation set 4

The transformation set 4 includes *AtBI OsBAG4* and VC. PCR with gene specific primers on putatively *AtBI* and *OsBAG4* transformed plants showed that each has one line negative with PCR (non transgenics) all other transformed lines tested were PCR positive. None of the PCR positive lines had contaminating *Agrobacterium*. RT-PCR of the PCR positive lines showed that 24 out of 26 transgenic lines of the *AtBI* were expressing (Appendix 2, Table 4.4) and 19 of 20

OsBAG4 lines were expressing the transgenes (Figure 4.12).All 11 putative transformed lines of the VC were positive for *hpt* (Appendix 2 and Table 4.4). The transformation efficiency in this transformation set is higher than in transformation sets 2 & 3 but lower than that in transformation set 1.

Table 4.4: Summary of PCR and RT-PCR confirmation of transformation set 4

Gene	# lines tested	PCR positive (# lines)	VirC-PCR positive(# lines)	RT-PCR positive(# lines)	TE (%)
AtBI	27	26	0	25	7.77 ± 1.55
OsBAG4	21	20	0	19	5.77 ± 1.6
GUS	11	11	0		8.67 ± 2.9

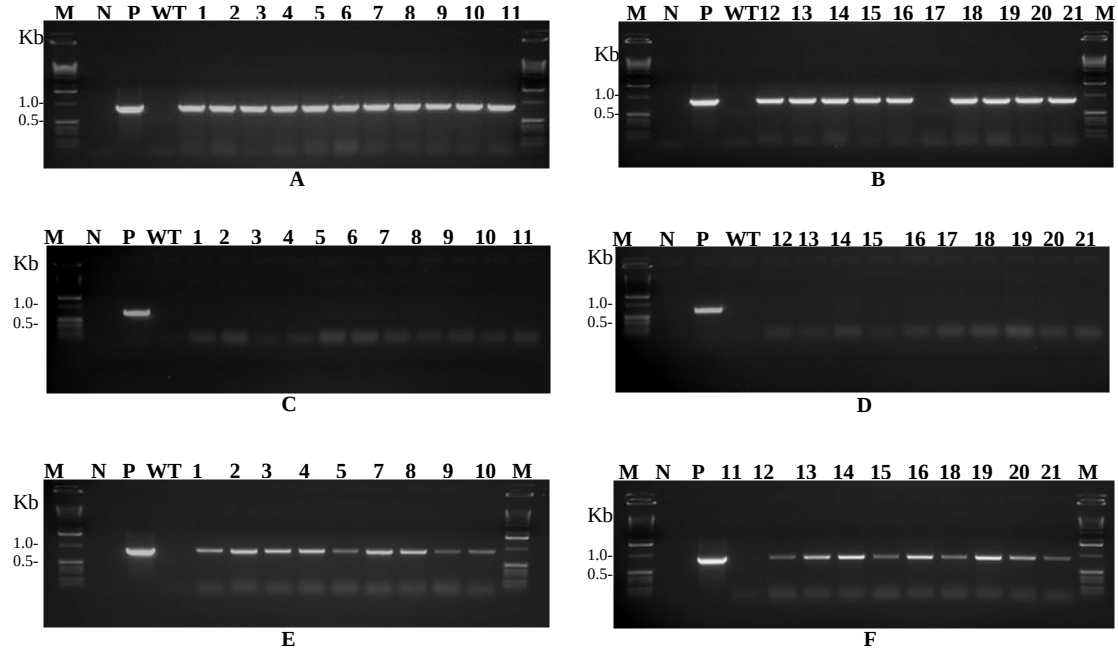


Figure 4.12: Molecular characterisation of *OsBAG4*transformed rice plants. A and B: PCR with*OsBAG4* gene specific primers; C and D: PCR with VirC primers; E and F: RT-PCRwith*OsBAG4* gene specific primers. M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA or *Agrobacterium* extraction); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120V.

4.4 DISCUSSION

Agrobacterium-mediated transformation has been used to genetically engineer rice since 1990 (Raineri *et al.* 1990). However, the efficiency of transformation remained fairly low until 1994 when Hiei *et al.* (1994) developed an efficient protocol for *Agrobacterium*-mediated transformation using scutellum-derived calli from mature rice seeds. Since then, *Agrobacterium*-mediated transformation protocols have been developed for javanica rice (Dong *et al.* 1996), indica rice (Rachid *et al.* 1996; Khanna & Raina 1999; Sahoo *et al.* 2011), elite japonica rice (Nishimura *et al.* 2006) and New rice for Africa (NERICA) (Ishizaki & Kumashiro 2008).This chapter describes i) the generation of transgenic rice via *Agrobacterium*-mediated transformation using scutellum-derived calli from mature Nipponbare seeds and a selective range of pro-survival genes including *AtBAG4, AtBI, AtBAG4+Hsp70, Hsp70, Hsp90,OsBAG4, p35* and *SfIAP;* ii) characterisation of the transformed rice plants using PCR and RT-PCR techniques.

The transformation efficiency of the reporter gene (*GUS*) in set 1 (10% ± 0.95%) and set 4 (8.67% ± 2.9%) of transformation experiments was significantly higher (almost 2.5 folds) than that of sets 2 & 3 (3.9% ± 1.1%) despite similar experimental conditions. The efficiency of *Agrobacterium*-mediated transformation in rice is affected by several key factors including i) the addition of phenolic compounds to the bacterial culture and/or to co-cultivation media to induce expression of *vir* genes on the Ti plasmid; ii) the pH of co-cultivation media and the temperature during co-cultivation; iii) the active cell division and type of tissue, iv) composition of media and v) bacterial strain and vector (Hiei *et al.* 1994; Hiei *et al.* 1997).

Phenolic compounds such as acetosyringone (4-acetyl-2,6-dimethoxyphenol) and α_-hydroxyacetosyringone (4-(2-hydroxy-acetyl)-2,6-dimethoxyphenol) exudates from wounded tobacco cells can activate *vir* genes on Ti plasmid (Stachel *et al.* 1985). *Vir* genes are induced during *Agrobacterium* - plant cell contact and their products direct the transfer of T-DNA into the plant nucleus where it is integrated into the genome (Sorensen & Hansen 2011). Monocotyledonous plants, particularly grasses, do not produce or produce insufficient amount of these phenolic compounds to serve as a signal for inducing expression of *vir* genes (Suriya-arunroj *et al.* 2004). Therefore addition of acetosyringone is key for the successful transformation of rice

(Hiei *et al.* 1994). Other research also reported that the addition of acetosyringone into co-cultivated media increases *Agrobacterium*-mediated transformation efficiency in monocots such as wheat (He *et al.* 2010; Rashid *et al.* 2010) and rice (Tripathi *et al.* 2010). In this study, the same amount of freshly prepared acetosyringone (100 μM) was added to co-cultivation media in all four sets of transformation. Therefore the difference in transformation efficiency was not caused by this factor. Other factors that affect the expression of *vir* genes such as pH of co-cultivated media and temperature during the cultivation (Alt-Moerbe *et al.* 1988; Alt-Mörbe *et al.* 1989) were maintained at optimum levels and were identical for the 4 sets of transformations. Composition of media and stage of callus development as well as *Agrobacterium* strain and vectors were also identical for all transformation sets.

Taken together, the above factors did not contribute to a significant drop in the transformation efficiency of the *GUS* gene in sets 2 & 3 in comparison with that of sets 1 & 4. The only possibility leading to this difference is the quality of the scutellum-derived calli used in these transformation sets. Although the procedure to induce callus from scutellum of mature rice seeds and the experimental conditions were similar in the four sets, calli derived from scutellum can be embryogenic or non-embryogenic. The use of non-embryogenic calli for transformation would affect the regeneration capacity of transformed calli (Sahoo *et al.* 2011). Perhaps, non-embryogenic calli were missed during selection to the pre-subculture for transformation in sets 2 & 3 as the regeneration rate of other genes in these sets was also low in comparison to others in different transformation sets (Figure 3.9). This reason is quite feasible as no significant difference in proliferations capacity of *GUS* transformed calli was observed between the four transformation sets in this study (Figures 3.5, 3.7), whereas the regeneration capacity of *GUS* transformed calli in transformation set 2 & 3 were significantly lower than that of sets 1 & 4 leading to the significantly lower transformation efficiency of these two sets. This observation is consistent with a study by Sahoo *et al.*(2011) where the scrutiny of embryogenic calli from non-embryogenic calli at the point of subculturing for transformation (4 days prior to the transformation) was suggested to be essential for high transformation efficiency in rice.

Anti-apoptotic (pro-survival) genes such as Bcl-xL and CED-9 have been reported to enhance the efficiency of *Agrobacterium*-mediated transformation in Banana and sugarcane by inhibiting *Agrobacterium*-induced PCD and enhancing recovery of transformed embryos (Khanna *et al.* 2007). Such enhancement of *Agrobacterium*-mediated transformation efficiency was not observed in rice using *AtBAG4, AtBAG4+Hsp70, AtBI, Hsp70, Hsp90, OsBAG4, p35* and *SfIAP* at both calli and regeneration stages. Despite some high proliferation levels of calli transformed with *SfIAP* in transformation set 1, expression of pro-survival genes did not affect the transformation efficiency as the regeneration rate of *SfIAP* transformed calli was not enhanced (Figures 4.7, 4.9). *Agrobacterium* is a pathogen and as such upon contact the plant cell induces a hypersensitive response (HR). The hypersensitive response is one of the earliest and most universal responses of plants to stress. Moreover, recent studies have indicated that HR cell death is programmed. Pro-survival genes may suppress the HR to result in higher survival rate thereby increase the transformation efficiency. However I did not observe this phenomenon in this study. There are four possible reasons that may have contributed to my observations: i) CED9 and Bcl-xL (which function at the mitochondrial levels in animals) may affect different pathways in plants compared to the genes being tested. Though the ultimate result may be the same (inhibition of cell death), there is an enormous amount of information that is missing as far as the events leading to this response. For example, *SfIAP* and *OpIAP* provide cytoprotection to different abiotic and biotic stresses, though they are supposed to be analogous biochemically. ii) I used larger callus clumps compared to the embryogenic cell suspension used in by Khanna *et al.* 2007. It is more difficult to determine cells vitality after 3-7 days of transformation than it was with embryos. iii) The age of callus at the time of record in this study was also different with that in the study by Khanna *et al*. (2007). Due to the difficulty in distinguishing between dead and living calli in the first week of transformation level of calli proliferation was recorded after seven weeks on full hygromycin selection media (at the end of callus selection stage). iv) Transformation efficiency was based on number of putatively independent transgenic lines (each calli clumps contains several callus pieces but only the single healthiest plant from each callus clump was selected for an independent transformed event) over total numbers of callus pieces used for transformation, not number of transgenic plants per 100 embryos". Taken together, I may observe the enhancement of pro-survival genes on

the efficiency of *Agrobacterium*-mediated transformation in rice if I use similar experimental conditions described in the study by Khanna *et al.* (2007).

In summary, a total of 139 transgenic rice expressing *AtBAG4*, *AtBAG4+Hsp70*, *AtBI*, *Hsp70*, *Hsp90*, *OsBAG4*, *p35* and *SfIAP* and more than 30 VC transgenic lines were generated. These lines will be tested for salinity stress tolerance in the next chapter.

Chapter 5: High throughput screening of transgenic rice for enhanced salinity tolerance

In the previous chapter I used *Agrobacterium*-mediated transformation to generate a total of 139 transgenic rice lines expressing pro-survival genes *AtBAG4, AtBAG4+Hsp70, AtBI, Hsp70, Hsp90, OsBAG4, p35* and *SfIAP* and more than 30 transgenic lines harbouring a vector control (VC). In order to select salinity tolerant transgenic lines for further study, a rapid salinity tolerance screening assay was developed and conducted on 10 transgenic rice lines for each pro-survival gene. This chapter describes the development and results of the rapid screen of *AtBAG4, AtBAG4+Hsp70, AtBI, Hsp70, Hsp90, OsBAG4, p35* and *SfIAP* transgenic rice through assessment of two key indicators: i) leaf damage and ii) survival of plants exposed to 100 mM NaCl at seedling stage.

5.1 INTRODUCTION

Salinity tolerance is the ability of plants to grow and complete their life cycle on a substrate that contains high concentrations of soluble salts (Parida & Das 2005). Screening for salinity tolerance in rice has been successfully conducted under controlled conditions (Flowers & Yeo 1981; Chowdhury *et al.* 1995; Lutts *et al.* 1996; Gregorio *et al.* 1997; Lee *et al.* 2003; Moradi & Ismail 2007).Typically, salinity tolerance of transgenic plants is screened using either *in vitro* or controlled *in vivo* (greenhouse) conditions (Ashraf & Akram 2009). For example transgenic rice expressing *HVA1* (Xu *et al.* 1996), *CodA* (Sakamoto *et al.* 1998), *p5cs* (Su & Wu 2004), *ZFP252* (Xu *et al.* 2008) were examined for salinity tolerance in controlled environments.

One important agronomic character that cannot be omitted when studying the salinity tolerance of plants is that of survival. Flowers and Yeo (1981), when studying the effect of different concentrations of NaCl on the survival of the moderately salinity sensitive rice cultivar IR28 and the salinity tolerant IR 2153-26-3-5-2, declared that the D_{50} (the time taken for 50% of the individuals die) was a useful index of salinity tolerance. They found that the D_{50} varied from cultivar to

cultivar and the percentage survival of the salinity tolerant cultivars is greater than that of the salinity sensitive cultivars. The use of survival as a good index for salinity tolerance has also been confirmed by other studies (Flowers & Yeo 1989; Gazitt *et al.* 1998).

According to Noble and Rogers (1992), leaf damage is one of the most commonly used criteria (besides yield, survival and plant height) for identifying salinity tolerance. This is due to its ease of measurement and also because of its necessity for estimating the yield of many crop plants exposed to salinity. Leaves play an important role in the determination of yield as they are the main photosynthetic organs. Gregorio *et al.*(1997) developed an effective protocol for the rapid screening of salinity stress tolerance in rice which is based on scoring of leaf injury and growth of rice plant under salinity stress in controlled conditions (glasshouse).

Concern has been expressed about the validity of results from studies carried out under controlled environments rather than true field conditions (Richards 1983; Shannon & Noble 1990; Daniells *et al.* 2001). However, Houshman *et al.*(2005), demonstrated that the response of *in vitro* selected genotypes of wheat was comparable with that of field selected genotypes for salinity stress tolerance. Based on the results from genotypes selected *in vitro*, greenhouse and field conditions, the authors concluded that *in vitro* screening methods are suitable for determining salinity tolerance and suggested that *in vitro* testing for salinity tolerance should be conducted prior to field screening.

In the context of the above, the aim of this chapter was to develop a rapid screen and assess the salinity tolerance of transgenic rice expressing pro-survival genes to: i) determine which pro-survival gene conferred tolerance to salinity stress in rice and ii) select best putative transgenic lines for further study. To fulfil these aims two specific objectives were set out:

1. To develop *in vitro* and *in vivo* salinity stress tolerance screening assays
2. To use the developed assays to screen for salinity stress tolerance in transgenic rice expressing *AtBAG4, AtBAG4+Hsp70, AtBI, Hsp70, Hsp90, OsBAG4, p35* and *SfIAP*.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

WT Nipponbare and ten transgenic rice lines expressing each of *AtBAG4,AtBAG4+Hsp70, AtBI, Hsp70, Hsp90, OsBAG4, p35* and *SfIAP* were randomly selected from RT-PCR positive lines (chapter 4).

5.2.2 *In vitro* salinity stress tolerance assay

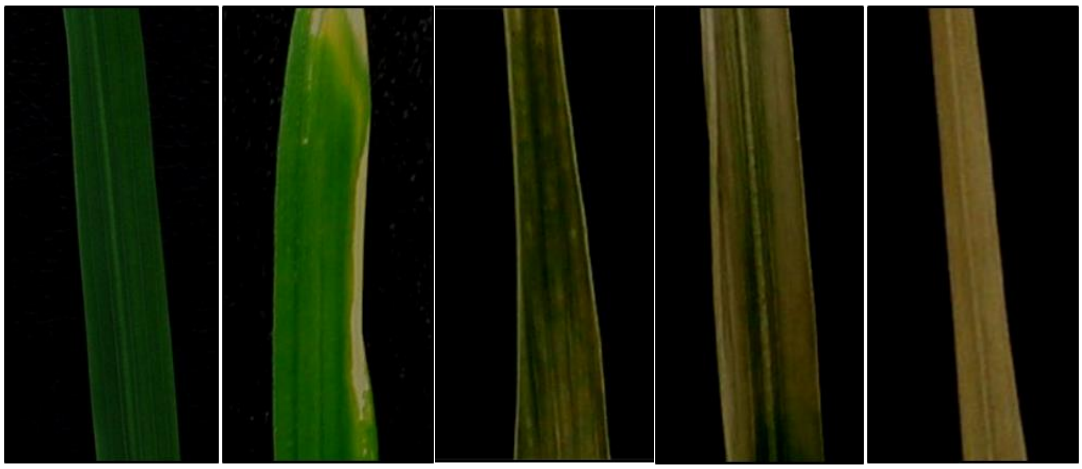
For *in vitro* evaluation of salinity stress tolerance, WT Nipponbare plantlets (four leaf stage) were subjected to liquid MS media (Appendix 1) supplemented with 50 mM and 100 mM NaCl. Cultures were placed in a growth room at 25°C, 16h/8h day/night cycle. Data on leaf damage and plant survivalwere recorded weekly over a four-week period.

5.2.3 *In vivo* salinity stress tolerance assay

Screening for salinity stress tolerance on WT Nipponbare and transgenic lines expressing pro-survival genes was conducted in a growth chamber (Thermoline) at a temperature of 28ºC/21ºC, photoperiod of 16 h, light intensity of 350 µmol photons $m^{-2}s^{-1}$ and 70% relative humidity. Ten plants of each selected transgenic line expressing *AtBAG4, AtBAG4+Hsp70, AtBI, Hsp70, Hsp90, OsBAG4, p35* and *SfIAP*, as well as WT controls were acclimatised for seven days (see chapter 3 for details). For development of the salinity stress tolerance assay, 10 WT Nipponbare (four leaf stage) plants were subjected to 50 mM and 100 mM NaCl; for salinity stress tolerance screening on transgenic rice, 10 transgenic rice plants (four leaf stage) of each selected line were treated with 100 mM NaCl in tap water 14 days post-acclimation (see chapter 3 for details). Leaf damage was measured at day 13 post salinity stress and survival was recorded at day 21 after the salinity stress.

5.2.4 Assessment of total leaf damage

As shown in Figure 5.1, leaf damage was recorded as one of five levels 0%, 25%, 50%, 75% and 100%: i) 0% indicates a healthy leaf with no injury; ii) 25% indicates that necrosis was confined to the leaf margins; iii) 50% indicates a chlorotic leaf; iv) 75% indicates necrosis in most of the leaf and v) 100% indicates dry, dead leaf. A plant was recorded dead when all leaves were at level 5.



0% 25% 50% 75% 100%

Figure 5.1: Examples of five levels of leaf damage assessment.

5.3 RESULTS

5.3.1 Development of an in vitro salinity stress tolerance screen using WT Nipponbare

It is beneficial to assess plants *in vitro* prior to their transfer to soil as glasshouse trials are both time-consuming and expensive (Rai *et al.* 2011). In light of this an *in vitro* salinity stress tolerance screening assay was developed. As evident in Figure 5.2A, percentage leaf damage of WT Nipponbare exposed to different concentrations of NaCl over a 28 day period increased with increasing NaCl concentrations and duration of treatment. Leaves were partly damaged i.e. necrosis was confined to the leaf margins at all concentrations tested after 7 days of NaCl treatment (level 1). As the duration of the treatment increased to 21 days, approximately 50% of WT Nipponbare leaves at 100 mM NaCl treatment were necrotic (level 4). At this concentration of NaCl, entire leaves were affected. Following 28 days of NaCl treatment, approximately 68% and 82% of leaves were drying out, exhibiting severe chlorosis, or ceasing to grow in the treatments with 50 mM and 100 mM NaCl respectively.

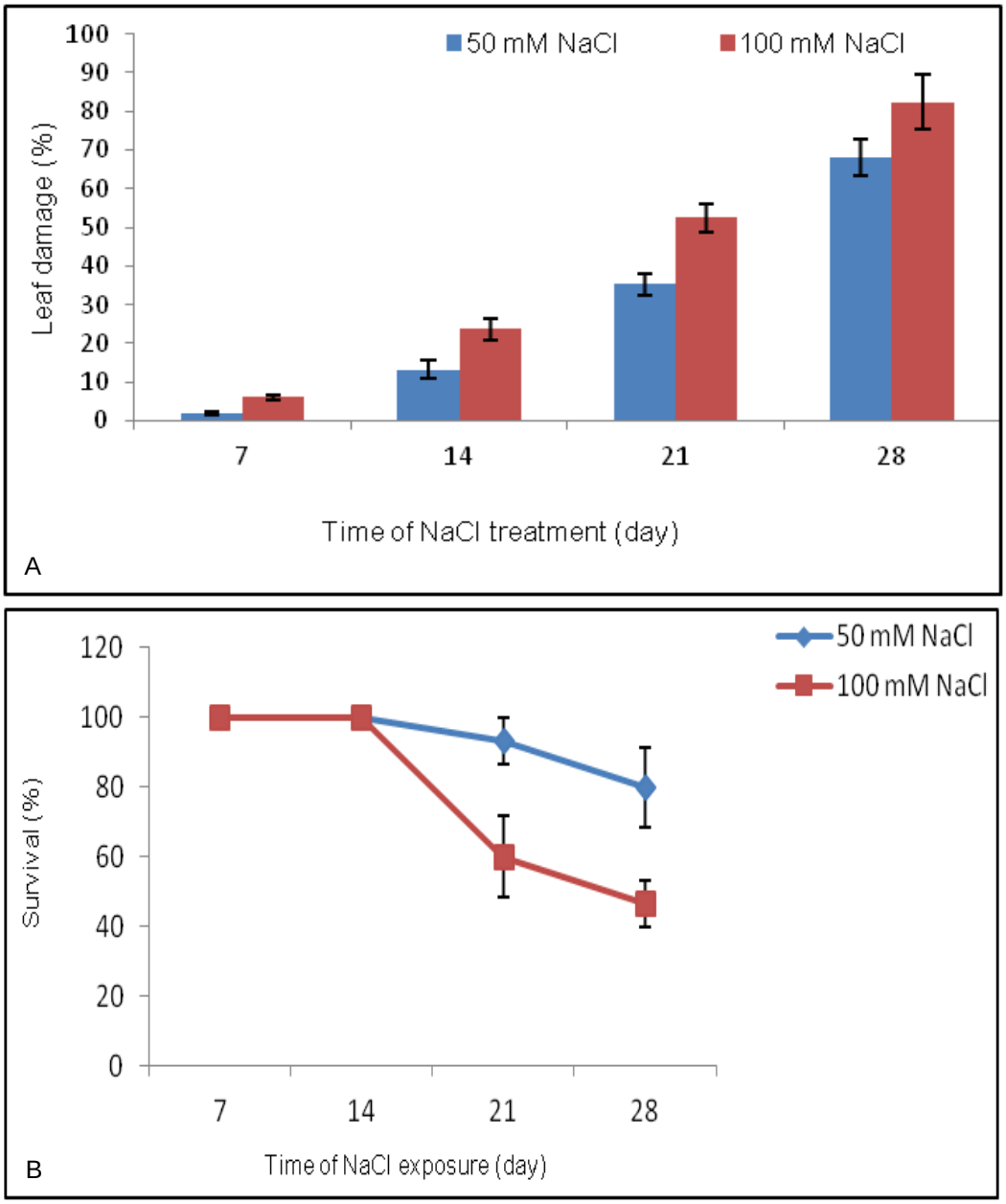


Figure 5.2: Salinity stress assays of WT Nipponbare under *in vitro* conditions. (A): Leaf damage and (B): Survival of WT plants exposed to different NaCl concentrations. Data are mean and standard error of three replicates.

Similar to leaf damage, survival of WT Nipponbare decreased with increasing NaCl concentrations and duration of treatment. Following 14 days of NaCl exposure, all shoots of WT Nipponbare in both levels of NaCl treatments were able to survive and grow, though some leaves turned yellow. As the treatment time increased to 21 days, approximately 6.67% and 40% of explants died in 50 mM and 100 mM NaCl treatments, respectively. The percentage survival of WT Nipponbare was remarkably

reduced following28 days of exposure to 100 mM NaCl, where only 46.67% of explants survived (Figure 5.2B); therefore D_{50} of WT under 100 mM NaCl stress is 28 days. When sub-cultured onto fresh saline media, the survival rate declined further with all plantlets dead at 42 days and 70 days of NaCl exposure in 100 mM and 50 mM NaCl treatments, respectively (data not shown). These results confirm that WT Nipponbare is a salinity sensitive cultivar and that its survival and leaves were seriously affected by salinity levels beyond threshold level [salinity threshold level of rice is approximately 3 dSm^{-1} (USDA 2013), while 50 mM and 100 mM NaCl equal to approximately 5-6 dSm^{-1} and 11-12 dSm^{-1} respectively].

5.3.2 WT Nipponbare exhibit higher leaf damage and lower survival rate during salinity stress under in vivo (growth chamber) compared to in vitro conditions.

Percentage leaf damage of chamber grown WT Nipponbare plants exposed to different concentrations of NaCl was monitored over four-week growth period in a growth chamber (*in vivo* condition) (Figure 5.3A). Similar to *in vitro* salinity stress, the percentage leaf damage of WT Nipponbare under *in vivo* salinity stress condition increased with increasing NaCl concentration and exposure time. However the effect of salinity stress was more pronounced on rice plants under *in vivo* than *in vitro* conditions. After 14 days of NaCl treatment *in vivo*, more than 58% of the leaves were damaged in 100 mM NaCl treatment while this number was only around 24% under the equivalent concentration of NaCl under *in vitro* conditions. Percentage leaf damage at 100 mM NaCl treatment after 21 days of NaCl exposure *in vivo* was as high as that recorded under *in vitro* conditions for 28 days (Figures 5.2A, 5.3A).

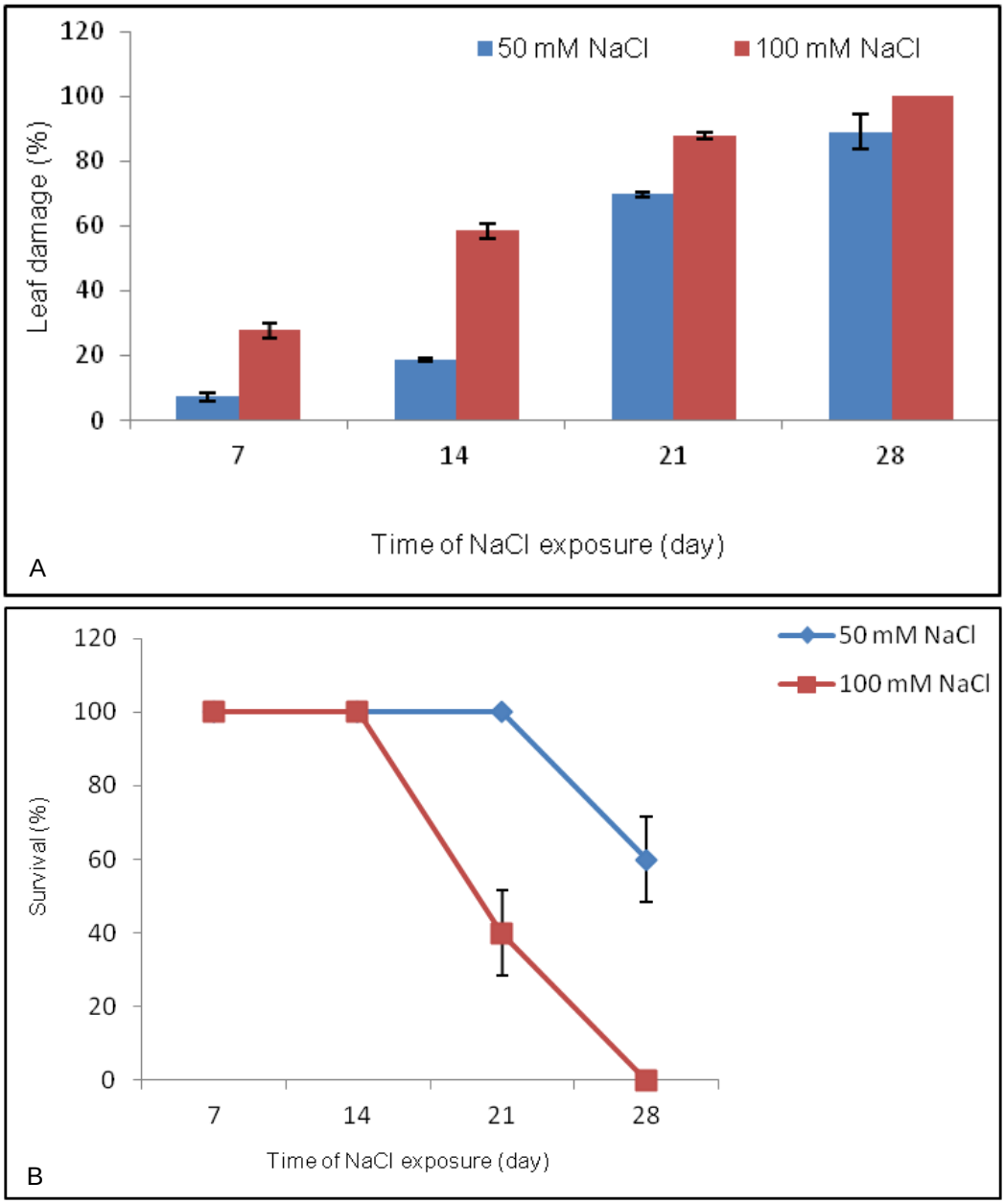


Figure 5.3: Salinity stress assays on WT Nipponbare under *in vivo* conditions (growth chamber). (A): Leaf damage and (B): Survival of WT plants exposure to different NaCl concentrations. Data are mean and standard error of three replicates.

As shown in Figure 5.3B, treatment with 100 mM NaCl*in vivo* reduced the survival rate of WT Nipponbare plants markedly following 21 days of exposure (approximately 40% plants survived). This effect was as significant as that of WT Nipponbare plants after 28 days exposure to 100 mM NaCl under *in vitro* condition. After 28 days of NaCl exposure *in vivo*, survival of WT Nipponbare plants was substantially decreased and 100% and 40% of plants die upon treatments with 100

mM and 50 mM NaCl, respectively while these numbers were recorded around 20% and 53.33% in 100 mM and 50 mM NaCl treatments in *in vitro* , respectively. This result together with the result from leaf damage suggest that salinity stress under *in vitro* conditions causes less damage to rice plants compared to *in vivo* conditions i.e. WT Nipponbare plants were more salinity stress tolerant under *in vitro* than *in vivo* conditions.

Considering the results from salinity stress tolerance screening under *in vitro* and *in vivo* conditions we used 100 mM NaCl and the *in vivo* screening method for screening salinity stress tolerance in transgenic rice plants expressing *AtBAG4, AtBAG4+Hsp70, AtBI, Hsp70, Hsp90, OsBAG4, p35* and *SfIAP*.

5.3.3 Expression of the survival genes AtBAG4, Hsp70, OsBAG4, p35 and SfIAP significantly improves salinity stress tolerance in rice

The previous section (5.3.2) demonstrated that approximately 60% of leaves of WT plants were damaged after 14 days exposed to 100 mM NaCl; the D_{50} survival was 21 days post-salinity stress. Therefore in this experiment we recorded leaf damage and survival rate at 13 (D_{50} leaf damage) and 21 days after salinity stress treatment. The percentage of total leaf damage observed was noticeable lower in most of the transgenic rice lines expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* tested than WT under salinity stress conditions (Figure 5.4A, Appendix 3A). Approximately 80%-100 % damage in leaves (i.e. almost all leaves necrosis, dry and death) was recorded in WT Nipponbare plants after 13 days exposure to 100 mM NaCl while only one line of *AtBAG4*, three lines of *OsBAG4*, two lines of *p35* and four lines of *SfIAP* show damage in leaves to this level. None of the ten transgenic rice lines expressing *Hsp70* tested showed an equal level of leaf damage to the WT (Table 5.1).

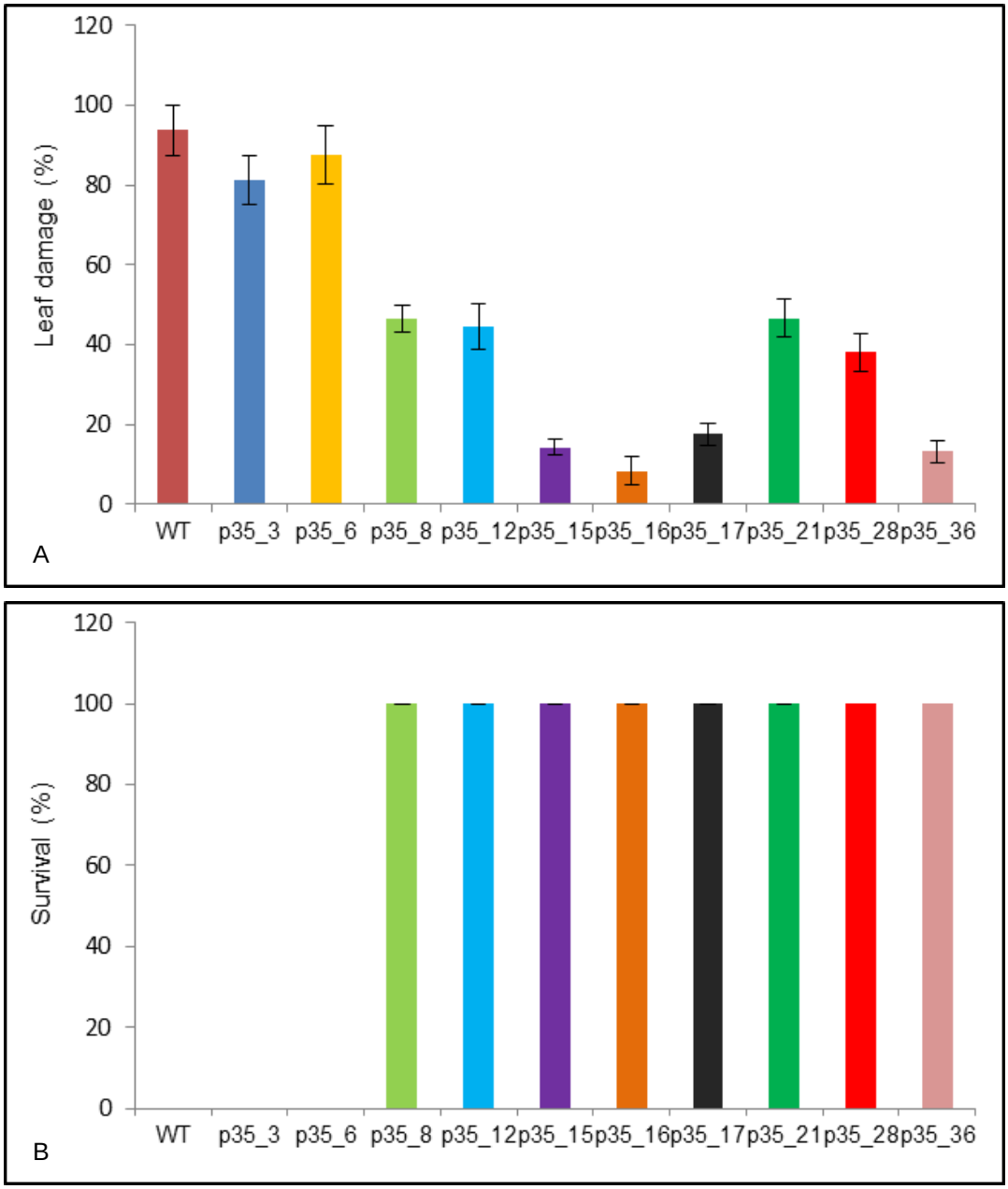


Figure 5.4: Representative of salinity tolerance screening on transgenic plants in *in vivo* conditions (growth chamber-Thermoline). (A): Leaf damage and (B): Survival of WT and *p35* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.

The survival rate of transgenic rice lines expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* after 21 days exposure to 100 mM NaCl was significantly higher than that of WT Nipponbare plants (Figure 5.4B, Appendix 3A). Eight of the ten lines tested on *Hsp70* and *p35* transgenic rice survived (100%) while all WT plants were dead following 21 days of the NaCl treatment. Approximately 60% of the *AtBAG4*

and *SfIAP* transgenic lines tested showed higher survival rate than WT (Table 5.1). Survival rate was also higher in four *OsBAG4* transgenic rice lines compared to WT, other lines did not show a noticeable difference (Appendix 3).These results suggest that over-expression of *OsBAG4* might improve salinity stress tolerance in rice however further studies are required to confirm this observation. Taken together, the results of leaf damage and survival suggest that over expression of pro-survival genes *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* can improve salinity stress tolerance in rice.

Table 5.1: Expression of pro-survival genes improve salinity tolerance in rice

Gene	**# lines tested**	**Leaf damage**		**Survival rate**	
		Less damaged than WT	**ND to WT**	**Higher than WT**	**ND to WT**
AtBAG4	9	8	1	6	3
Hsp70	10	10	0	8	2
OsBAG4	9	6	3	4	5
p35	10	8	2	8	2
SfIAP	10	6	4	6	4

Note: ND stands for No Difference

Due to the variation in number of leaves and shoot height of one acclimatised *OsBAG4* transgenic line, only nine lines were used for this salinity stress tolerance screening experiment.

Representatives of putative salinity tolerant and salinity sensitive lines are shown in Figure 5.5. Under salinity stress putative tolerant lines maintained growth rate and continue to produce new leaves whereas WT and salinity sensitive lines had serious leaf damage and dead following prolonged exposure to NaCl.

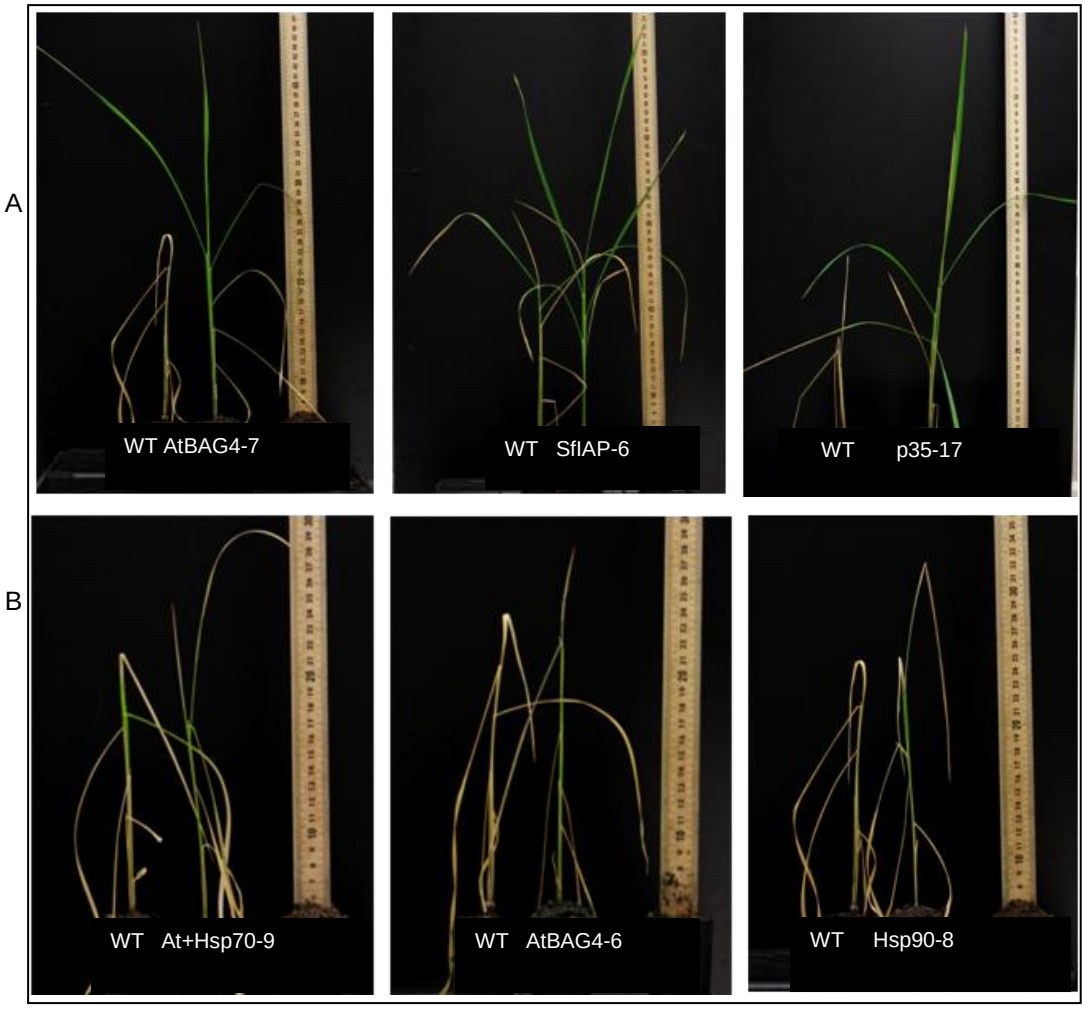


Figure 5.5: Representatives of putative salinity tolerant transgenic rice lines (A) and salinity sensitive transgenic rice lines (B)

5.3.4 Expression of AtBAG4+Hsp70 and Hsp90 did not improve salinity tolerance in rice

Only one line of the *Hsp90* plants showed lower leaf damage than WT, the other nine transgenic lines showed no difference to WT (Figure 5.6A). Leaf damage was also found to be similar between five lines of *AtBAG4+ Hsp70* and WT. Three lines out of the ten *AtBAG4+ Hsp70* transgenic lines tested showed higher damage in leaves (almost 100% damage) and two lines exhibited less leaf damage than WT following 13 days of exposure to 100 mM NaCl (Appendix 3).

Consistent with leaf damage, the survival rate of *Hsp90* and *AtBAG4+ Hsp70* transgenic lines was the same as that of WT or even lower. Of the ten *Hsp90* and *AtBAG4+ Hsp70* transgenic lines tested, only one *Hsp90* line had a higher survival rate, the other nine lines showed no noticeable difference (Figure 5.6B). Approximately 60% of the *AtBAG4+ Hsp70* transgenic lines tested were not able to

survive after 21 days of NaCl exposure; three lines were able to survive but at levels equivalent to the WT. One line showed a higher survival rate than WT (Appendix 3). These results indicate that transgenic rice lines expressing *Hsp90* and *AtBAG4+ Hsp70* are not tolerant to salinity stress.

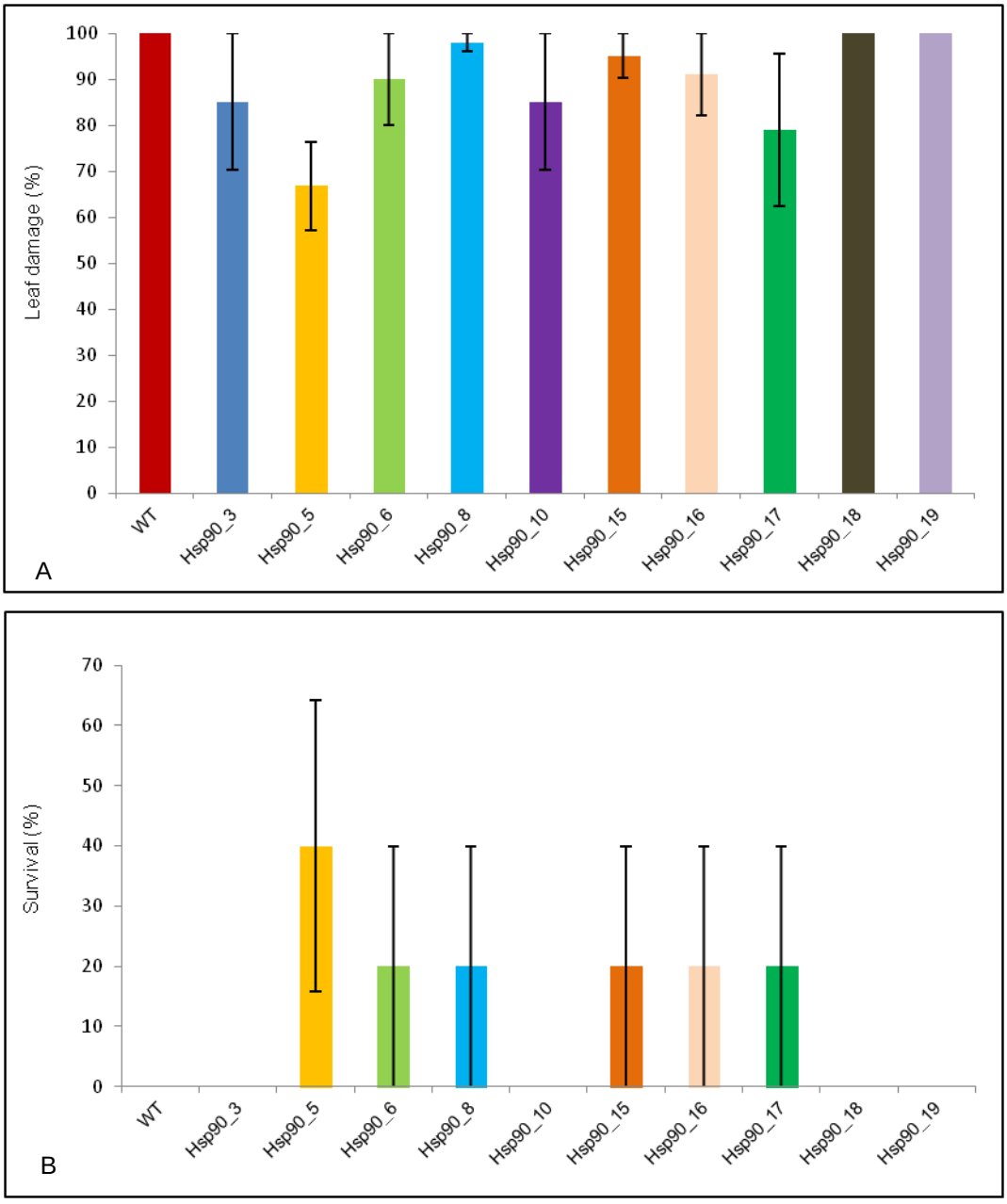


Figure 5.6: Salinity tolerance screening of *Hsp90* transgenic plants in controlled conditions (growth chamber-Thermoline). (A): Leaf damage and (B): Survival of WT and *Hsp90* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.

5.3.5 AtBI transgenic rice plants showed no improvement in tolerance to salinity stress in comparison to WT

The Thermoline that was used for salinity stress tolerance screening of WT in the salinity stress assays under *in vivo* conditions (section 5.3.2) and on *AtBAG4, Hsp70, OsBAG4* and *AtBAG4 +Hsp70* transgenic rice malfunctioned, therefore an alternative Thermoline cabinet was used for the assessment of the *AtBI* plants. Although all growth conditions were identical to the previous experiments, the plants including WT grew faster; by 14 days after acclimation the fifth leaf was emerged. No sign of death was seen at day 13 of the salinity stress screening experiment I therefore measured growth rate and number of tillers for salinity stress tolerance assessment instead of survival. Salinity stress at the beginning of the tillering period will affect the number of tillers per plant. More salinity tolerant rice cultivars will maintain the tillering ability while salinity sensitive cultivars will reduce this ability. Total leaf damage of transgenic rice plants expressing *AtBI* in eight out of ten lines tested were not different from that of WT (Figure 5.7A). *AtBI* transgenic line 8 showed less damage in leaves and *AtBI* transgenic line 26 showed more damage in leaves in comparison to WT. Shoot growth of six out of ten *AtBI* transgenic lines tested was equal to that of WT, and that of the other four lines was remarkably decreased in comparison to WT (Figure 5.7B). Consistent with leaf damage and growth rate, tillering ability of *AtBI* plants was not different to WT. Taken together, these results suggest that expression of *AtBI* might not improve tolerance to salinity stress in rice.

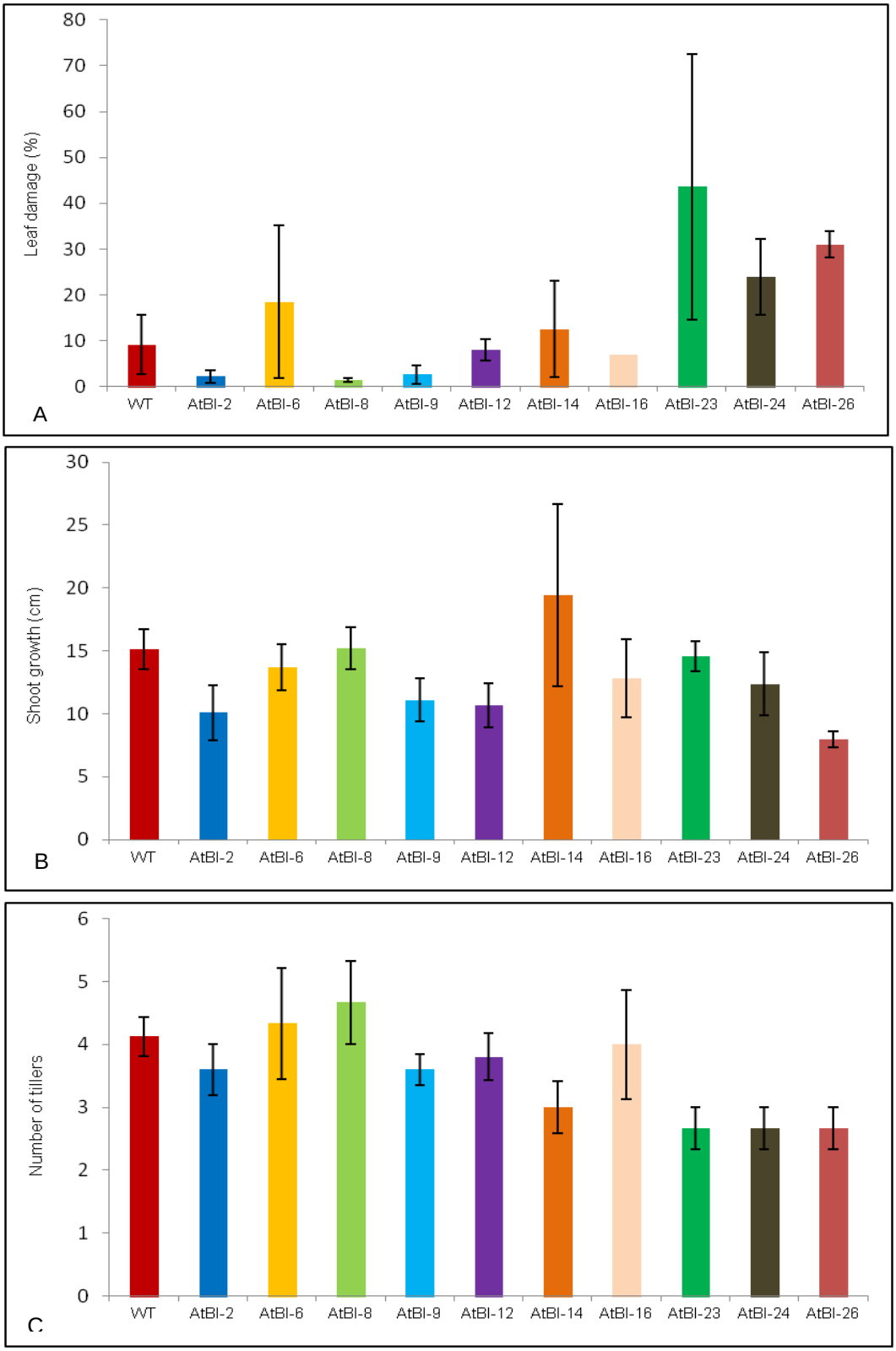


Figure 5.7: Salinity tolerance screening of *AtBI* transgenic plants in controlled conditions (growth chamber-Thermoline). (A): Leaf damage; (B): shoot growth and (C): number of tillers of WT and *AtBI* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.

5.4 DISCUSSION

Previous studies have suggested that percentage leaf damage and D_{50} survival of rice exposed to salinity stress are good indicators for salinity tolerance. Under salinity stress, more salinity tolerant cultivars have less damage in leaves, higher survival rate and longer D_{50} survival than salinity sensitive cultivars (Flowers & Yeo 1981; Noble & Rogers 1992; Hoang 2002). In this study, it was observed that the percentage leaf damage, survival rate and D_{50} survival of WT Nipponbare during salinity stress under *in vitro* conditions were less pronounced than that under *in vivo* conditions (sections 5.3.1 and 5.3.2). In other words, plants were more tolerant to salinity stress under *in vitro* than in glasshouse conditions. This is perhaps due to the large supply of sucrose in the tissue culture medium which may have altered the "source-sink" relationship of shoots, and shoots may have been preferentially utilising sucrose in the media for growth rather than through photosynthesis (La Rosa *et al.* 1984; Hoang 2002). Therefore screening for salinity tolerance is better conducted under *in vivo* than *in vitro* conditions.

Flower and Yeo (1981) reported that young rice seedlings were more sensitive to salinity stress than older seedlings. In this study it was observed that *AtBI* transgenic plants and WT Nipponbare which had five leaves at the beginning of the salinity stress experiment showed less damage to leaves and could prolong the D_{50} survival while other transgenic plants and WT Nipponbare that were exposed to salinity stress at the four leaf stage showed more pronounced effects of leaf damage and lower survival rates. One possible explanation for this observation is that older plants with more leaves will have greater photosynthetic capacity thereby providing the necessary energy for the plants to continue growing and overcome salinity stress. Furthermore, more leaves means more cells and the concentration of Na^+ accumulation could be less in each cell therefore the ion toxicity effect is less pronounced and thereby leads to better growth in older and bigger seedlings. Perhaps due to this reason both WT and *AtBI* transgenic plants showed less leaf damage and more tillers after 13 days of exposure to 100 mM NaCl in comparison to WT in the previous experiments. Although same age tissue culture materials were used for acclimation and experimental conditions were similar in all experiments, the variation still occurred between experiments as it was with *AtBI*. I therefore suggest that for tissue culture derived materials, the onset of salinity stress tolerance

screening at seedling stage or rapid screening for salinity stress tolerance should rely on developmental state through the number of leaves rather than time after acclimation. The point at which the plant has three fully expanded leaves and the fourth leaf start emerging is ideal for initiation of salinity stress tolerance screening at seedling stage.

This research correlated with previous research and showed that *AtBAG4, p35* and *SfIAP* consistently confer tolerance to salinity stress in transgenic plants (Doukhanina *et al.* 2006; Wang *et al.* 2009; Kabbage *et al.* 2010; Li *et al.* 2010). In addition, transgenic rice plants expressing *OsBAG4* and *Hsp70* were also found to be more tolerant to salinity stress than WT Nipponbare. However *AtBAG4+Hsp70*, *AtBI* and *Hsp90* did not show enhanced tolerance to salinity stress in transgenic rice. This is perhaps not because of the genes as at least one line out of ten lines tested in these genes exhibited more tolerance to salinity stress than WT Nipponbare (see sections5.3.4; 5.3.5 and 5.3.7 for details). It might also not because of gene silencing as mRNA was detected through RT-PCR (Chapter 4). It might be due to other reasons such as the level of gene expression. The *AtBAG4+Hsp70*, *AtBI* and *Hsp90* might be expressed at low level in transgenic rice thus insufficient to confer tolerance to salinity stress. Although not performed in this project due to time constraints, quantitative real-time PCR, assessment of copy number by Southern blot and level of protein expression by Western blot would indicate whether expression level played a significant role for salinity tolerance within the *AtBAG4+Hsp70*, *AtBI* and *Hsp90* transgenic lines. Another possibility of low protection level to salinity stress in transgenic rice expressing combination of *AtBAG4* and *Hsp70* is that these two genes may compete each other when expressing in the transgenic rice. Both are co-chaperon and they may function similarly in conferring salt tolerance leading to competition and reduced activities

Transgene expression levels are influenced by a number of factors such as the location of the transgene and the number of transgenes per integration site (Stam *et al.* 1997). T-DNA can be integrated at different chromosomal locations. If inserted into a transcriptionally active region in euchromatin, the expression may be affected by the regulatory factors of nearby host genes (Koncz *et al.* 1989; Kertbundit *et al.* 1991). If they are inserted in or near repetitive DNA or heterochromatin, they might be inactivated (Pröls & Meyer 1992; Stam *et al.* 1997; Cheng *et al.* 2001). Two or

more T-DNAs can be inserted at the same site of a chromosome. These T-DNAs can be arranged as a direct repeat “head to tail” or as an inverted repeat “head to head” or “tail to tail”. Transgenes that are organised as inverted repeat usually show low expression level (Jones *et al.* 1987; Stam *et al.* 1997). The copy number of transgenes at a given locus might affect gene expression level, however this is still controversial as some studies suggest that high copy number of transgenes leads to low gene expression and gene silencing [see review by Kooter *et al.*(1999)], other studies report no relationship between high transgene copy low level transgenes expression (Hadi *et al.* 2012). I am aware of possible reasons that may lead to no difference in tolerance to salinity stress of *AtBAG4+Hsp70*, *AtBI* and *Hsp90* transgenic rice and WT; however I did not conduct any further studies to elucidate these possibilities due to: i) time limitation of PhD candidature and ii) I focussed on further evaluation of genes that confer tolerance to salinity stress in rice (*AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP*).

In summary rapid screening for salinity stress tolerance of transgenic rice expressing *AtBAG4*, *AtBAG4+Hsp70*, *AtBI*, *Hsp70*, *Hsp90*, *OsBAG4*, *p35* and *SfIAP* show that plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* are more tolerant to salinity stress than WT Nipponbare. Some lines of *AtBAG4+Hsp70*, *AtBI*, and *Hsp90* showed greater tolerance to salinity stress than WT Nipponbare however most of the transgenic rice lines expressing these genes were demonstrated equivalent tolerance levels to the WT controls. Therefore, *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* transgenic rice were selected for further study of whether expression of pro-survival genes can confer enhanced salinity tolerance in a glasshouse.

Chapter 6: Glasshouse evaluation of representative transgenic rice lines expressing pro-survival genes

In the previous chapter, I reported that the expression of pro-survival genes *AtBAG4*, *Hsp70, OsBAG4*, *p35* and *SfIAP* resulted in the enhancement of tolerance to salinity stress in transgenic rice. In this chapter, I demonstrated that pro-survival genes inhibited salinity-induced programmed cell death (PCD) in transgenic plants, and the enhanced tolerance to salinity stress was evaluated by assessing the agronomical, physiological and biochemical parameters. The results section (6.3) in this chapter is presented following the format of my paper published in Functional Plant Biology journal.

6.1 INTRODUCTION

Rice has been reported to be relatively tolerant to salinity stress during germination, active tillering and towards maturity but sensitive during early seedling and reproductive stages (Heenan *et al.* 1988; Zeng *et al.* 2001). There was a weak link between tolerance to salinity stress at seedling and reproductive stages in some genotypes within the species *Oryza saltiva* L. (Zeng *et al.* 2002). Therefore, it is imperative that the screening for salinity stress tolerance in rice should be conducted at both seedling and reproductive stages (Wankhade *et al.* 2013).

Agronomical parameters such as growth, dry weight, number of tillers, yield components such as number of panicles per plant, panicle length, number of spikelets per panicle and physiological parameters including relative water content, electrolyte leakage, net photosynthesis, Na^+, K^+ accumulation and the $Na+/K^+$ ratio were reported to be significantly different between salinity sensitive and salinity tolerant plant cultivars under salinity stress (Schachtman & Munns 1992; Lutts *et al.* 1996; Dionisio-Sese & Tobita 2000; Khatkar & Kuhad 2000; Çiçek & Çakirlar 2002; Zeng*et al.* 2002; Lee *et al.* 2003; Moradi & Ismail 2007; Cha-um *et al.* 2009b; Vysotskaya *et al.* 2010).

Electrolyte leakage has been widely used to determine the damage to cell membranes during water deficit, freezing, oxidative and salinity stresses (Stachel *et al.* 1985; Dionisio-Sese & Tobita 1998; Bajji *et al.* 2002; Verslues *et al.* 2006; Ishida *et al.* 2007; He *et al.* 2010; Rashid *et al.* 2010; Tripathi *et al.* 2010). During salinity stress the amount of electrolyte leakage from leaves increased in salinity sensitive rice cultivars but not in salinity tolerant rice cultivars (Dionisio-Sese & Tobita 1998; Cha-um *et al.* 2009b).

The influx of large amounts of Na^+ into the plant, when exposed to salinity stress, increases the Na^+ content, which results in deleterious effects by competing with K^+ in enzyme activation and protein biosynthesis (Shabala & Cuin 2008; Wang *et al.* 2013). One of the mechanisms that plants employ to tolerate salinity stress is to exclude Na^+ uptake by roots thereby maintaining low concentrations of Na^+ in the leaves. A failure in Na^+ exclusion manifests its toxic effect after days or weeks and causes premature death of older leaves(Munns & Tester 2008). Na^+ concentration in the leaves of rice is correlated to the level of salinity stress tolerance in both Indica and Japonica varieties (Moradi & Ismail 2007; Platten *et al.* 2013)

Photosynthesis is a fundamental physiological process that provides energy for plants to grow as well as arsenal to facilitate plant adaptation to environmental and biotic stresses. Physiological response studies in salinity tolerant and salinity sensitive rice cultivars have shown that during salinity stress, the net photosynthesis and relative water content were maintained in salinity tolerant but not in salinity sensitive rice cultivars (Dionisio-Sese & Tobita 2000; Moradi & Ismail 2007; Cha-Um *et al.* 2009a).

The TUNEL assay (Terminal deoxynucleotidyl tranferase d UTP Nick End Labelling) is a commonly used and established method for detecting two characteristic features of apoptosis, DNA fragmentation and the formation of apoptotic bodies, which occur from the coalescence of specifically cleaved DNA. This assay relies on the presence of nicks in the DNA (resulting from DNA fragmentation during apoptosis) which can be identified by terminal deoxynucleotidyl tranferase, an enzyme that will catalyze an addition of dUTPs that are secondarily labelled with a marker (fluorescein) (Roche Applied Science – *In situ* cell death detection kit, fluorescein, 11684795910 instruction manual). This technique has been used to detect apoptotic-like hallmarks in plants during exposure

to biotic and abiotic stresses such as in studies by Li & Dickman (2009), Liu *et al. (2007),* Li *et al.* (2010), Paul *et al*. (2011).

In light of these developments, research in this chapter focused on investigating whether expression of *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* can inhibit salinity-induced PCD and enhanced tolerance to salinity stress in transgenic rice at the two most sensitive periods of their life cycle: the seedling and reproductive stages.

The two specific objectives of the research in this chapter were:

1. To determine whether expression of pro-survival genes in representative salt tolerant transgenic lines can inhibit salinity-induced PCD.
2. To evaluate and compare the level of salinity stress tolerance between the representative salt tolerant pro-survival transgenic lines, VC and WT Nipponbare at both the seedling and reproductive stages.

6.2 MATERIALS AND METHODS

6.2.1 Plant materials

The four representative lines for each of the five pro-survival genes included AtBAG4-3, AtBAG4-5, AtBAG4-7, AtBAG4-10, Hsp70-1, Hsp70-3, Hsp70-11, Hsp70-13, OsBAG4-7, OsBAG4-15, OsBAG4-19, OsBAG4-20,p35-15, p35-16, p35-17, p35-36 and SfIAP-4, SfIAP-6, SfIAP-7, SfIAP-10. These were selected from the results of rapid screening for salinity stress tolerance in chapter 5 and were used for further experimentation. WT and VC plants were used as controls. The plants were acclimatised at QUT's plant house in Carseldine, Brisbane in 40 mm plastic pots containing potting mix as described in chapter 3.

6.2.2 Salinity stress at seedling and reproductive stage

Salinity stress experiments were conducted from August to December 2012 at the QUT glasshouse at Carseldine, Brisbane, Australia with temperature adjusted at 28ºC/21ºC day/night under ambient light conditions. Details of acclimation of tissue cultured rice plants and the set up for salinity tolerance screening at seedling and reproductive stages were described in chapter 3.

Ten plants of each selected transgenic line expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP*, VC and WT controls were acclimatised for seven days.

Plants were treated with 100 mM NaCl in tap water 14 days post-acclimation. Shoot height was measured at day 0 and day 13 following NaCl stress, shoot dry weight was determined after 13 days of NaCl treatment.

The level of salinity tolerance of transgenic lines and controls at the reproductive stage was determined based on yield components including the number of panicles per plant and the number of spikelets per panicle. Ten replicates of transgenic lines and the appropriate controls were subjected to 100 mM NaCl stress 30 days post-acclimation. This time point is coincident with the panicle initiation (PI) stage (beginning of reproductive stage) of glasshouse growing Nipponbare plants developed from tissue culture plantlets. The assessment of relative yield was performed at harvest.

6.2.3 Measurement of physiological parameters

Physiological parameters were examined on the representative salinity tolerant line for each of the five selected genes including AtBAG4-7, Hsp70-11, OsBAG4-20, p35-16 and SfIAP4. For convenience, the five selected lines are renamed as AtBAG4, Hsp70, OsBAG4, p35 and SfIAP in this chapter.

Except for photosynthetic efficiency that was measured at day 0 (before NaCl was add in) and 3, 7, 10 and 13 following salinity stress, all other physiological parameters including leaf relative water content, electrolyte leakage, Na^+ and K^+ were determined at day 13 following exposure to NaCl at seedling stage. The third leaf was used for photosynthetic efficiency, electrolyte leakage, and Na^+, K^+ measurements. The youngest fully expanded leaf was used for relative water content analysis.

For screening of salinity tolerance at the reproductive stage, all physiological data was recorded at 30 days after salinity stress. The flag leaf of the main culm was used for photosynthetic efficiency, electrolyte leakage, and Na^+, K^+ measurements. Relative water content was measured using flag leaf of other culms. See chapter 2 for details of each measurement.

6.2.4 TUNEL assays

Root tips of WT, VC and transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* respectively, following exposure to 0 mM and 100 mM NaCl for 36 h were examined for apoptotic-like hallmarks using TUNEL assays. In this study, the

TUNEL assay was carried out using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche) following the manufacturer's instructions. See chapter 2 for details of these assays. Numbers of cell death were recorded as percentage on three replicates

6.2.5 Detection of H_2O_2

In Situ H_2O_2 production in rice leaves exposed to 100 mM NaCl for 30 h was detected by 3,3'-Diaminobenzidine (DAB) Enhanced Liquid Substrate System for Immuno-histochemistry solution, D3939 (Sigma, Saint Louis, Missouri, USA) following the manufacturer's instruction. Briefly, pieces of youngest fully expanded leaf (approximately 1 cm long) were excised from rice plants and immediately immerged into 0.5 ml of mixed D3939 solutions for 60 min at room temperature. DAB solution was decanted and 1 ml of Ethanol: Acetic acid (3:1 v/v) was added to the samples for chlorophyll destaining overnight.

6.3 RESULTS

6.3.1 Expression of pro-survival genes suppress ROS in transgenic rice during exposure to salinity stress

ROS levels were reported to increase in plant cells during salinity stress (Borsani *et al.* 2005; Zhu *et al.* 2007; Chawla *et al.* 2013). To elucidate whether the expression of pro-survival genes in rice coincides with reduced ROS production caused by salinity stress, *in situ*H_2O_2 production was detected in leaves of rice after 30 h exposure to 100 mM NaCl. As shown in Figure 6.1 more H_2O_2production was observed in WT and VC leaves while lower levels of H_20_2 were detected in the leaves of transgenic rice expressing pro-survival gene. This result indicates that the expression of pro-survival genes can suppress the level of H_2O_2 production in rice during salinity stress.

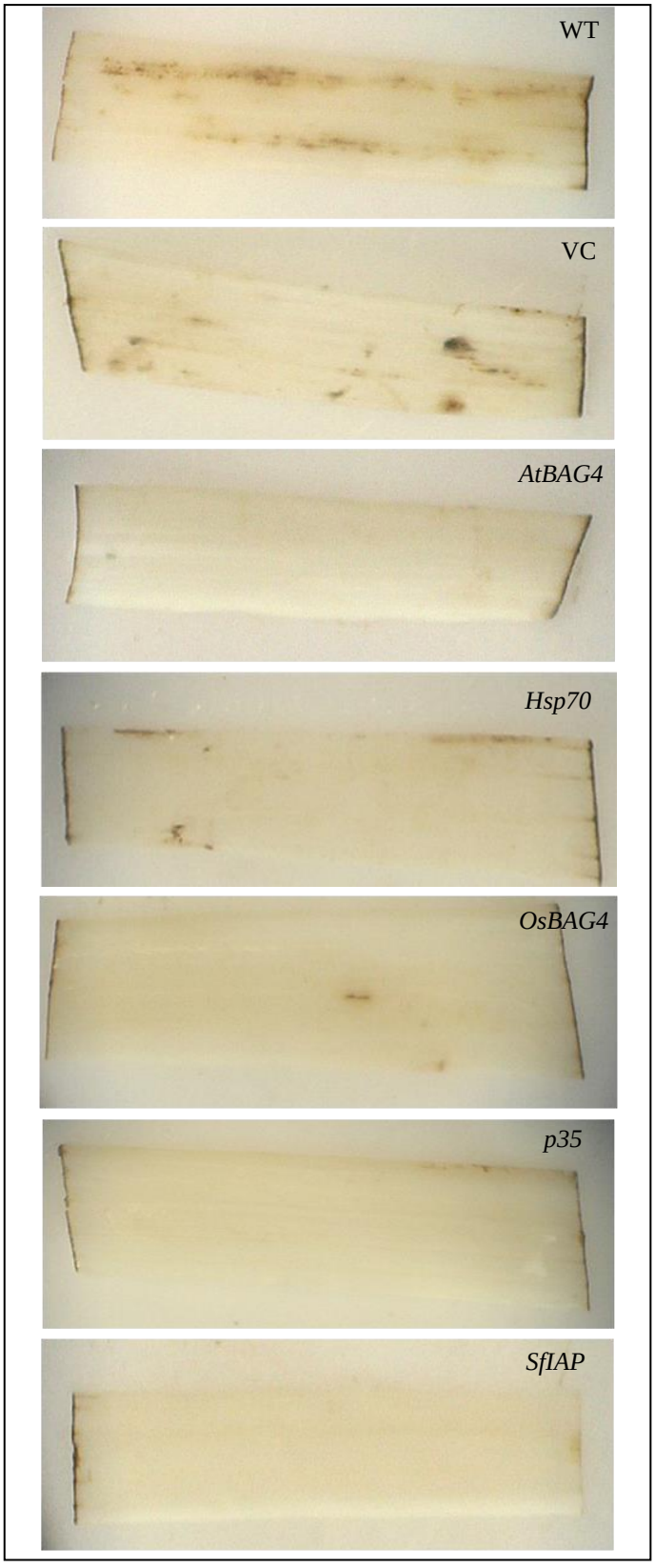


Figure 6.1: Expression of pro-survival gene suppresses ROS (H_2O_2) level in leaf of rice exposed to 100 mM NaCl. DAB (3,3'-Diaminobenzidine) staining was conducted 30 h after salinity stress.

6.3.2 Pro-survival genes inhibit salinity stress–induced PCD in rice

To investigate whether expression of pro-survival genes in rice inhibits salinity-induced PCD, TUNEL assays were conducted on root tips of rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP*, VC and WT Nipponbare after 36h exposure to 100 mM NaCl. The data showed no cell death or small amounts of cell death in transgenic rice plants expressing pro-survival genes; however in the WT Nipponbare and VC plants significantly greater cell death was observed (Figure 6.2). Approximately 90% of cells were TUNEL-positive in both WT and VC while these numbers were less than 5% in transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP*. These results indicate that expression of pro-survival genes in transgenic rice suppressed PCD caused by salinity stress. To further assess salinity tolerance-mediated by the five pro-survival genes, agronomical and physiological assessments were conducted on transgenic rice and controls exposed to 0 (non-stressed conditions) and 100 mM NaCl at seedling and reproductive stages.

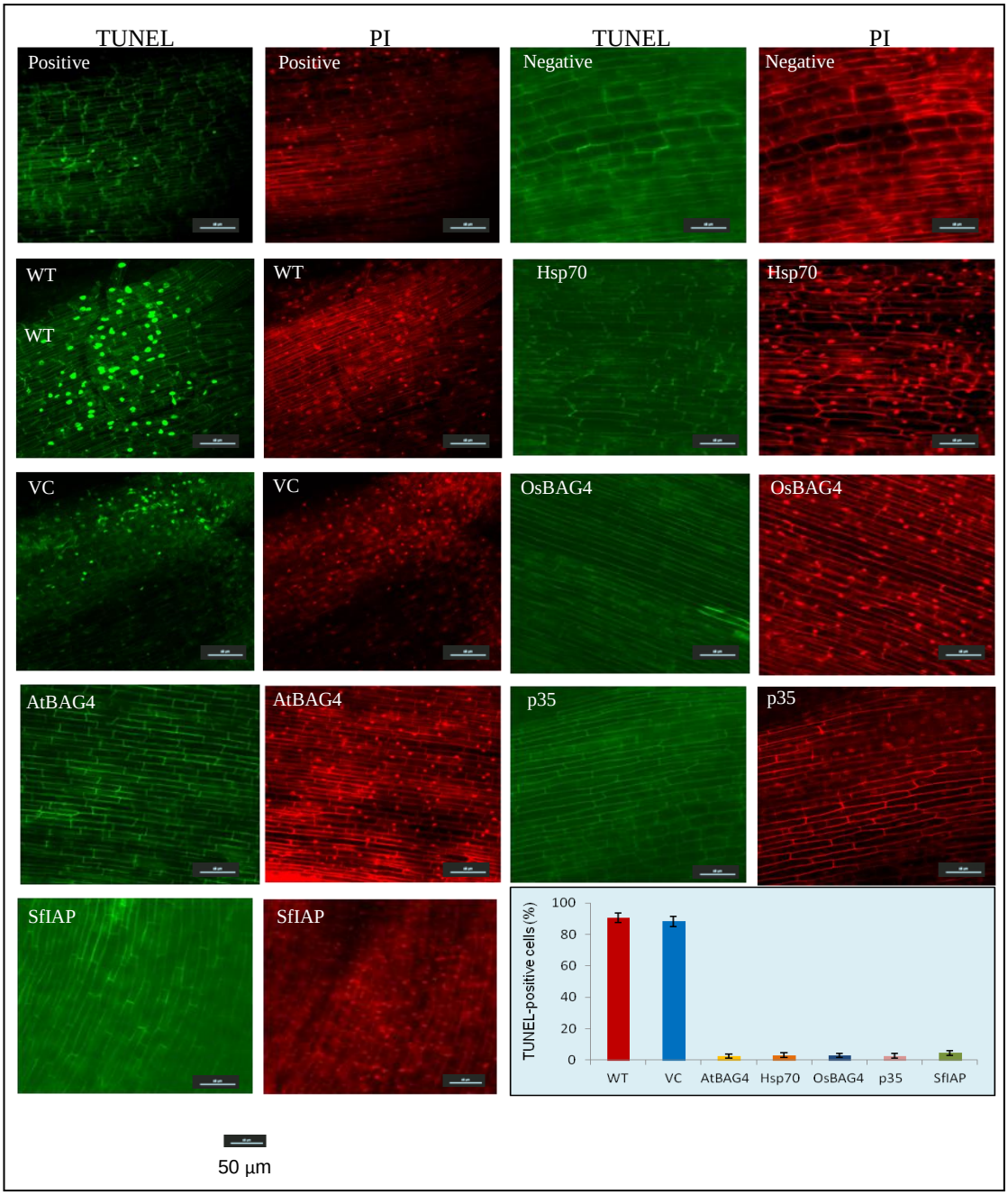


Figure 6.2: Expression of *AtBAG4*, *Hsp70OsBAG4*, *p35*and *SfIAP* suppress salinity-induced PCD in rice. WT, VC, *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* transgenic plants were subjected to 100 mM NaCl at the seedling stage, TUNEL assays and propidium iodide counter-staining were carried out at 36h after salinity stress. Positive control samples for TUNEL assay was root tips treated with DNase. Nucleic acid in TUNEL positive cells are selectively stained and fluoresces green, indicating the presence of apoptotic-like bodies, whereas all nucleic acid is counter-stained with propidium iodide and fluoresces red. Magnifications as indicated.

6.3.3 Transgenic rice expressing pro-survival genes exhibit greater shoot growth in comparison to WT and VC plants under salinity stress

The effects of NaCl on leaves and on whole plants of WT, VC and transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35*, *SfIAP* is shown in Figure 6.2. There were no noticeable differences in the leaves and overall plant appearances between the WT, VC and the transgenic lines under control conditions (0 mM NaCl) (Figure 6.3A). However under 100 mM NaCl treatment, WT and VC plants exhibited serious damage to leaves and the entire plant. In general, leaves of WT and VC plants exhibited chlorosis, wilting and drying from the tip to the base of the leaf in the first 7 days following exposure to 100 mM NaCl. However, the leaves of transgenic plants expressing pro-survival genes remained green and looked healthy. After 13 days exposure to 100 mM NaCl almost all leaves of WT and VC get necrosis and die (Figure 6.3B).

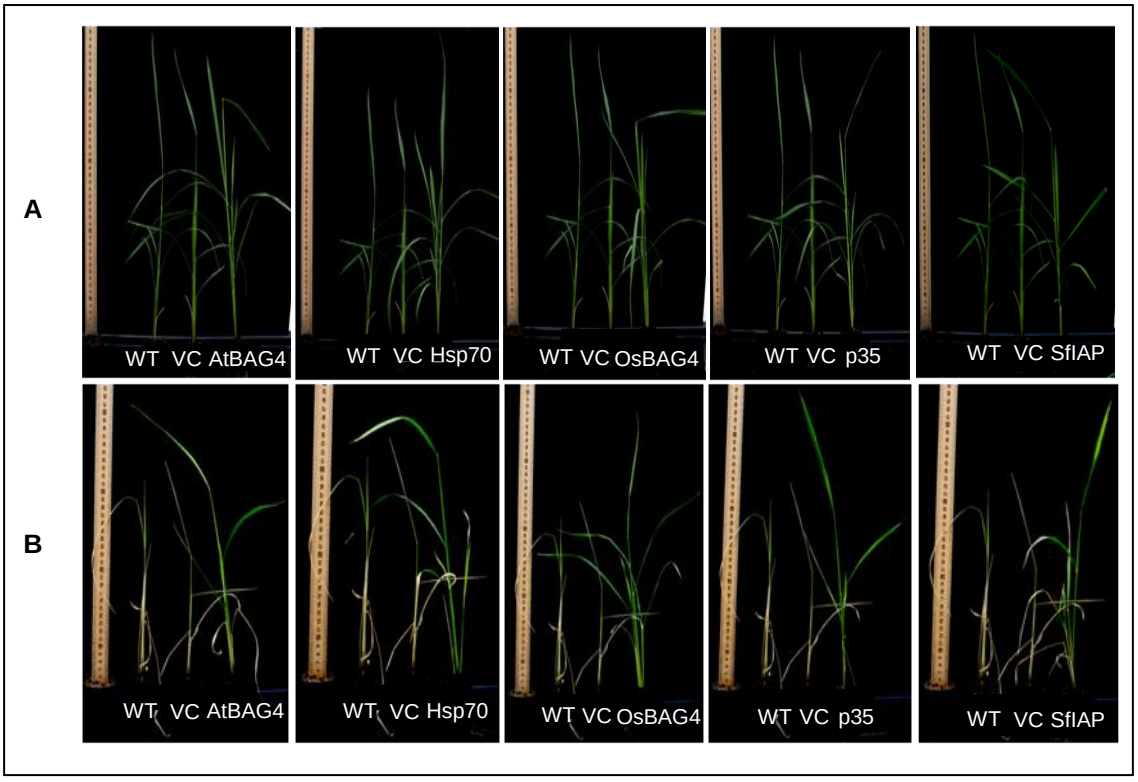


Figure 6.3: Morphology of WT, VC and transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* after 13 days under (A) control (0 mM NaCl) and (B) salinity stress conditions (100 mM NaCl)

Under control conditions at seedling stage, no significant differences (One-way ANOVA: DF = 6; F = 0.79; P = 0.588) between the transgenic lines expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35*, *SfIAP*, the WT and VC plants were observed (Figure

6.4A). Conversely, data on shoot growth over a period of 13 days exposure to 100 mM NaCl of WT, VC showed a significant reduction (One-way ANOVA: DF = 6; F = 34.43; P = 0.000) in comparison to that of the transgenics (Figure 6.4B). Shoot growth of transgenic rice expressing *AtBAG4*, *Hsp70* and *OsBAG4* were similar; *p35* transgenic rice exhibited a slightly lower growth of shoot in comparison to *AtBAG4*, *Hsp70* and *OsBAG4* transgenic plants however the difference in shoot growth was not statistically significant. On the other hand, the shoot growth of *SfIAP* transgenic plants was the lowest of the transgenic lines tested these were however significantly higher than that of the VC and the WT plants. This result indicates that, transgenic rice plants expressing *AtBAG4*, *Hsp70*, *OsBAG4 p35* and *SfIAP* genes can maintain better growth under salinity stress than the non transgenic and the vector control plants.

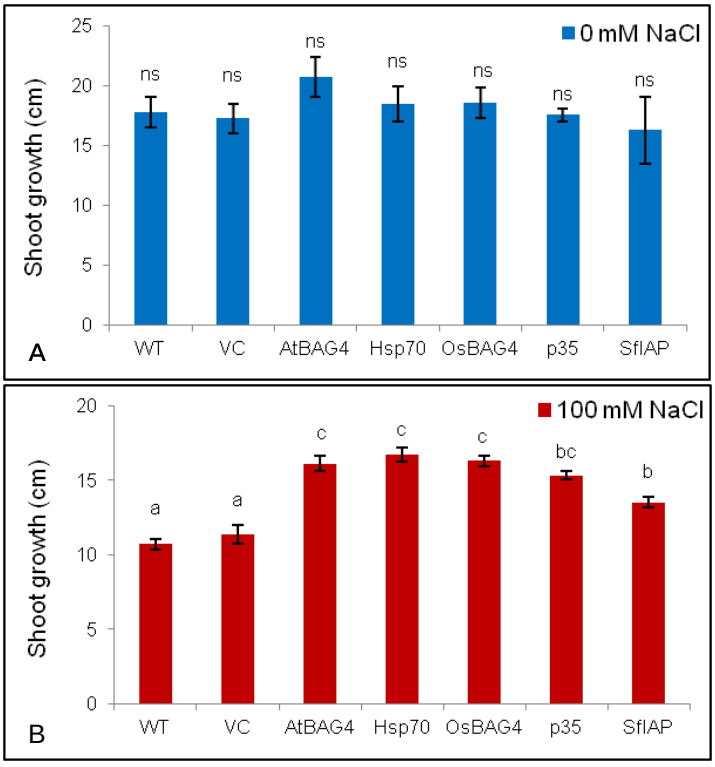


Figure 6.4: Relative shoot growth of WT, VC and transgenic plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* grown at 0 mM (A) and exposed to 100 mM NaCl (B) at the seedling stage. Data represent the mean and standard error of three replicates. ns indicates no statistically significant difference. Bars that share a common letter are not significantly different by Tukey HSD test at 95% confidence intervals.

6.3.4 Transgenic rice expressing pro-survival genes produce more tillers than WT and VC under salinity stress.

Under glasshouse conditions, Nipponbare WT, VC and transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* in control condition (0 mM NaCl) started tillering 23-25 days after acclimation. The salinity stress tolerance at seedling stage started at 14 days after acclimation and lasted for 13 days (i.e. total 27 days post-acclimation) therefore overlapping the early tillering stage. Due to this fact, an investigation on the effect of salinity stress on tillering ability of the transgenic rice and the controls was conducted. The number of tillers produced by the transgenic plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* were recorded after 13 days of 0 mM and 100 mM NaCl exposure and compared to that of the WT and VC plants.

No significant difference (One-way ANOVA: DF = 6; F = 0.58; P = 0.741) in number of tillers per plant was observed between the transgenic rice plants and the controls under normal growth conditions at both the seedling and reproductive stages (Figure 6.5A, C). However, 100 mM NaCl treatment at seedling stage caused a significant reduction (One-way ANOVA: DF = 6; F = 15.60; P = 0.000) in the number of tillers in WT and VC plants in comparison to those from *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* transgenic plants (Figure 6.5B).The number of tillers varied between transgenic rice lines with the highest observed in *Hsp70* transgenic plants and the lowest recorded in *SfIAP* transgenic plants. No statistically significant difference was noticed in the number of tillers between *OsBAG4*, *AtBAG4*, *p35* and *SfIAP* plants. Under salinity stress at the reproductive stage, *AtBAG4* and *Hsp70* transgenic plants showed significantly higher number of tillers per plant in comparison with the VC and WT controls plants (Figure 6.5D).The number of tillers per plant in *OsBAG4*, *p35* and *SfIAP* transgenic plants were slightly higher than that of the controls however it was not statistically significant. This result indicates that salinity stress effects on the tillering in rice plants is more pronounced at seedling stage (i.e. prior to tillering stage) than at the reproductive stage (i.e. nearly end of tillering stage). In addition, the expression of pro-survival genes in rice resulted in the maintenance of tillering under salinity stress conditions even though different pro-survival has different effects on tillering of transgenic rice.

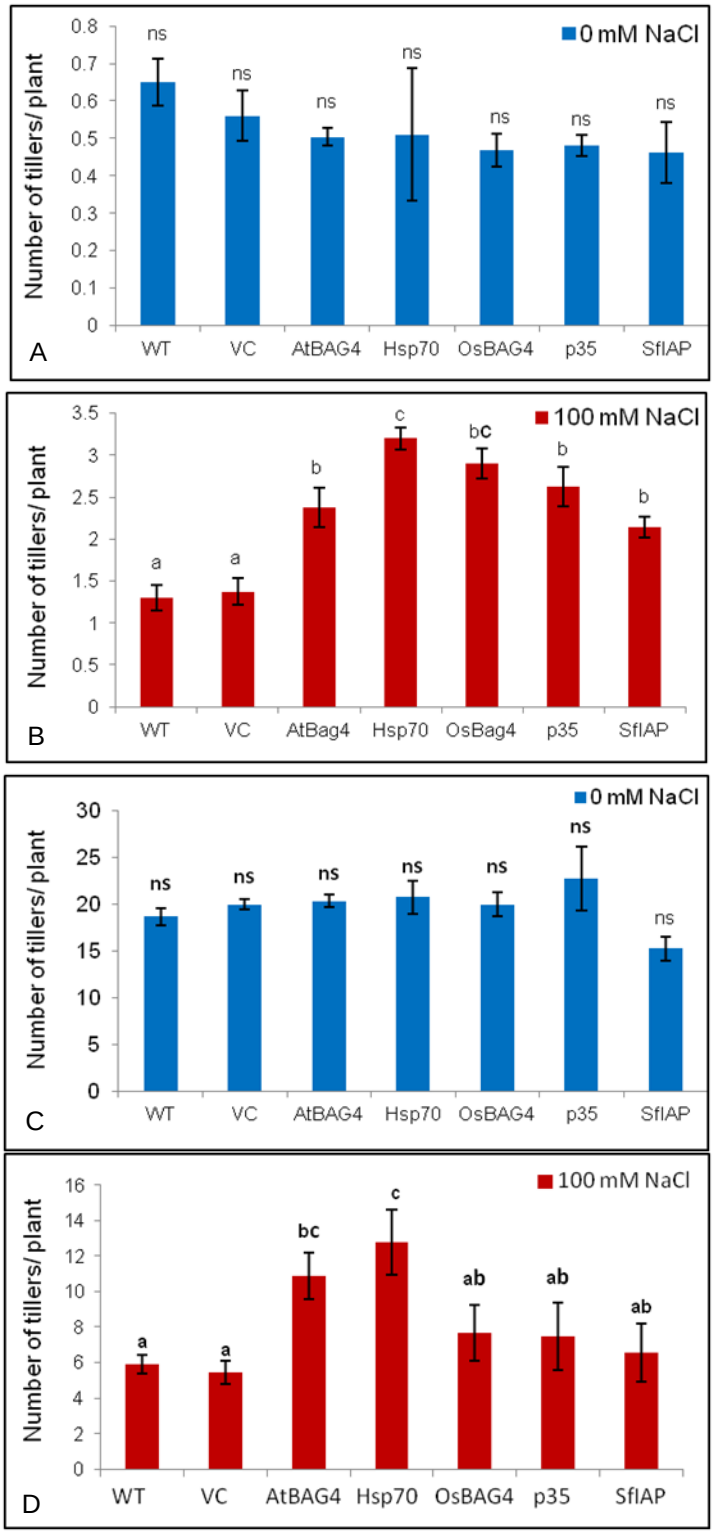


Figure 6.5: Number of tillers of WT, VC and transgenic plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* grown at 0 mM and exposed to 100 mM NaCl (A & B) at the seedling stage; (C & D) at the reproductive stage. Data represent the mean and standard error of three replicates. ns indicates no statistically significant difference. Bars that share a common letter are not significantly different by Tukey HSD test at 95% confidence intervals.

6.3.5 Expression of pro-survival genes result in significant increase in dry weight under salinity stress

To further elucidate the basis of salinity stress tolerance in transgenic rice expressing pro-survival genes, dry weight was recorded on WT, VC and transgenic rice plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* after 13 days exposure to 100 mM NaCl at the seedling stage; the result is shown in Figure 6.6. Under non stress conditions, no significant difference (One-way ANOVA: DF = 6; F = 0.87; P = 0.534) was observed in dry weight between transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* genes and the controls (Figure 6.6A). However, in 100 mM NaCl treatment, the dry weight of transgenic rice expressing pro-survival genes was significantly higher than that of the WT and VC (One-way ANOVA: DF = 6; F = 9.54; P = 0.000). Interestingly, there was no noticeable difference in dry weight between the transgenic lines expressing each of the five pro-survival genes tested (Figure 6.6B). This result suggests that transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* genes maintained growth during salinity stress and produced more cells while WT and VC failed to do so.

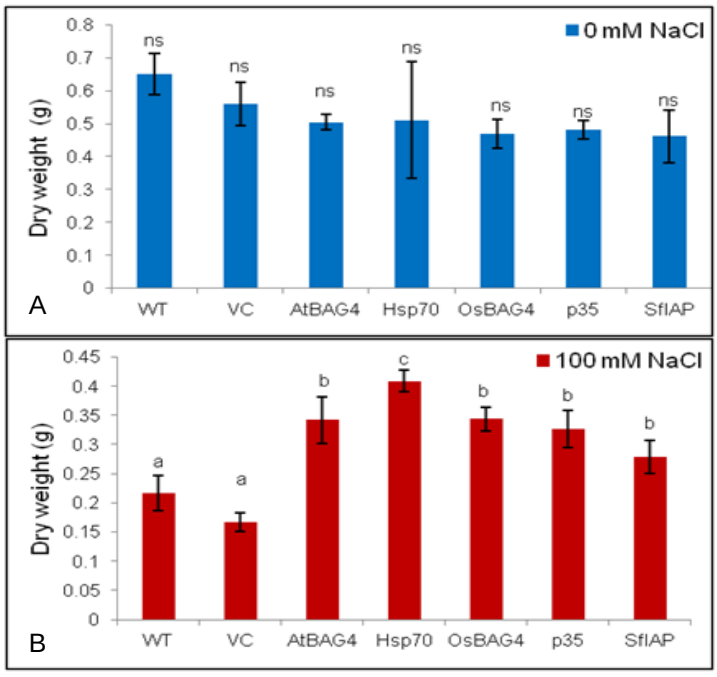


Figure 6.6: Dry weight of WT, VC and transgenic plants expressing *AtBAG4, Hsp70,OsBAG4, p35* and *SfIAP* grown at 0 mM (A) and exposed to 100 mM NaCl (B) at the seedling stage. Data represent the mean and standard error of three replicates. ns indicates no statistically significant difference. Bars that share a common letter are not significantly different by Tukey HSD test at 95% confidence intervals.

6.3.6 Transgenic rice plants expressing pro-survival genes exhibit higher yield components than the WT and VC plants

Yield components including number of panicles per plant and number of spikelets per panicle were not significantly different between WT, VC and transgenic plants expressing *AtBAG4, Hsp70,OsBAG4, p35* and *SfIAP* under non-stressed conditions (One-way ANOVA: DF = 6; F = 1.38 and1.90; P = 0.271 and 0.079, respectively). The number of panicles per plant varied between transgenic plants expressing pro-survival genes under salinity stress. *Hsp70* transgenic plants had the highest number of panicles per plant, followed by *AtBAG4* transgenic plants. Number of panicles per plant in *AtBAG4* and *Hsp70* transgenic plants were significantly higher (One-way ANOVA: DF = 6; F = 2.57; P = 0.022) than that in WT and VC plants under salinity stress conditions (Figure 6.7B). Although *OsBAG4, p35*and *SfIAP* transgenic plants showed a slightly higher number of panicles per plant, these numbers were not significant in comparison to WT and VC (Figure 6.7B). Similarly, under salinity stress number of spikelets per panicle in transgenic plants expressing pro-survival genes was significantly higher (One-way ANOVA: DF = 6; F = 10.19 P = 0.000) than that in WT and VC plants (Figure 6.7D). These results suggest that transgenic rice plants expressing pro-survival genes showed no negative effects under non-stressed conditions and responded well to salinity stress by maintaining higher yield components.

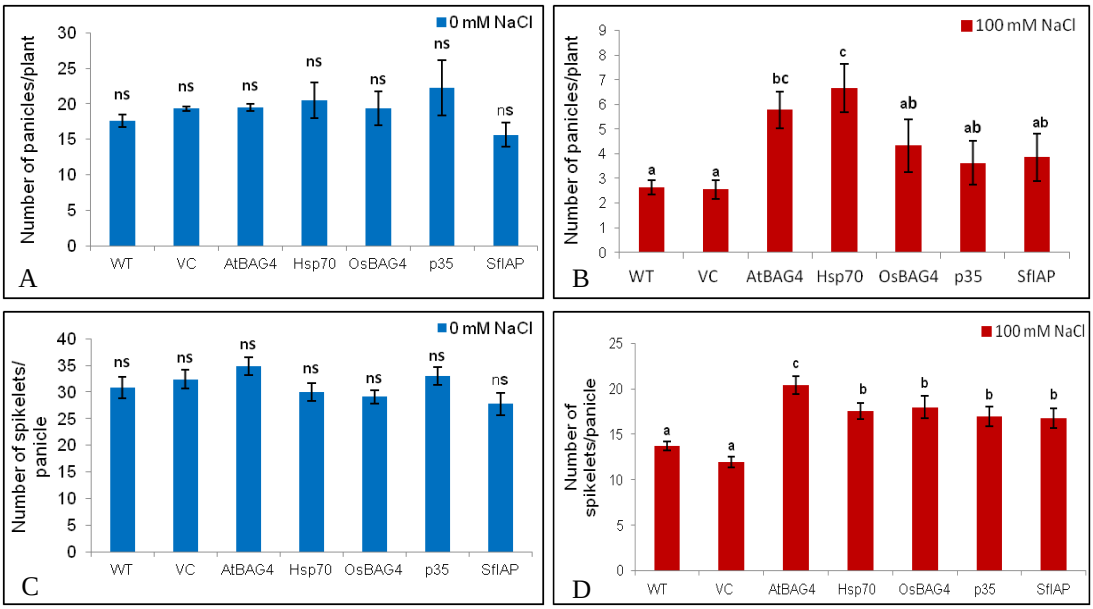


Figure 6.7:Yield components of WT, VC and transgenic rice plants expressing *AtBAG4*, *Hsp70*,*OsBAG4*, *p35* and *SfIAP* grown at 0 mM and exposed to 100 mM NaCl at the reproductive stage.(A & B) indicate number of panicles per plant; (C & D) indicate number of spikelets per panicle. Data represent mean and standard error of three replicates. ns indicates no statistically significant difference. Bars that share a common letter are not significantly different by Fisher's method at 95% confidence intervals.

6.3.7 Expression of pro-survival genes improve water retention and cell membrane integrity of rice during salinity stress

In order to understand the physiological mechanisms of enhanced salinity tolerance in transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP*, the water status of transgenic plants and the WT and VC controls under non-stressed (0 mM NaCl) and salinity stress conditions at both the seedling and reproductive stages were investigated. The results showed that under non-stressed conditions, the relative water content of *AtBAG4*, *Hsp70*,*OsBAG4*, *p35* and *SfIAP* transgenic rice was not different from that of WT and VC plants at both the seedling and reproductive stages (Figure 6.8A, C). However, at 100 mM NaCl treatment at the seedling stage the relative water content in WT and VC plants was lower when compared to transgenic plants expressing *AtBAG4*, *Hsp70*,*OsBAG4*, *p35* and *SfIAP* (Figure 6.8B). Relative water content of *AtBAG4*, *OsBAG4*, *p35* and *SfIAP* transgenic rice plants at reproductive stage salinity tress treatment was noticeably higher than that of the WT

and VC plants (Figure 6.8D). However no significant difference was found in *Hsp70* transgenic plants and the WT and VC (Figure 6.8D).

To elucidate further the physiological mechanisms of enhanced salt tolerance in transgenic plants, cell membrane integrity of *AtBAG4, Hsp70,OsBAG4, p35* and *SfIAP* transgenics and control plants were analysed by measuring leaf cell electrolyte leakage under non-stressed and salinity stress conditions. Although the electrolyte leakage varied between transgenic rice expressing *AtBAG4, Hsp70,OsBAG4, p35, SfIAP*, the WT and VC plants under non-stressed conditions at both seedling and reproductive stage, the values were not high (Figure 6.9A, C). Under 100 mM NaCl stress at the seedling stage however, the electrolyte leakage of WT and VC were almost 10 and 13 folds higher than that at non-stressed conditions. The difference in electrolyte leakage of WT and VC between non-stressed and salinity stress conditions at reproductive stage were recorded as 26.5 and 15.2 folds higher respectively. These figures for *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic plants were approximately 7.6, 11.3, 4.1, 5.1 and 8.8 respectively (Figure 6.9C, D). These results suggest that transgenic plants exhibited less cell membrane damage than control plants.

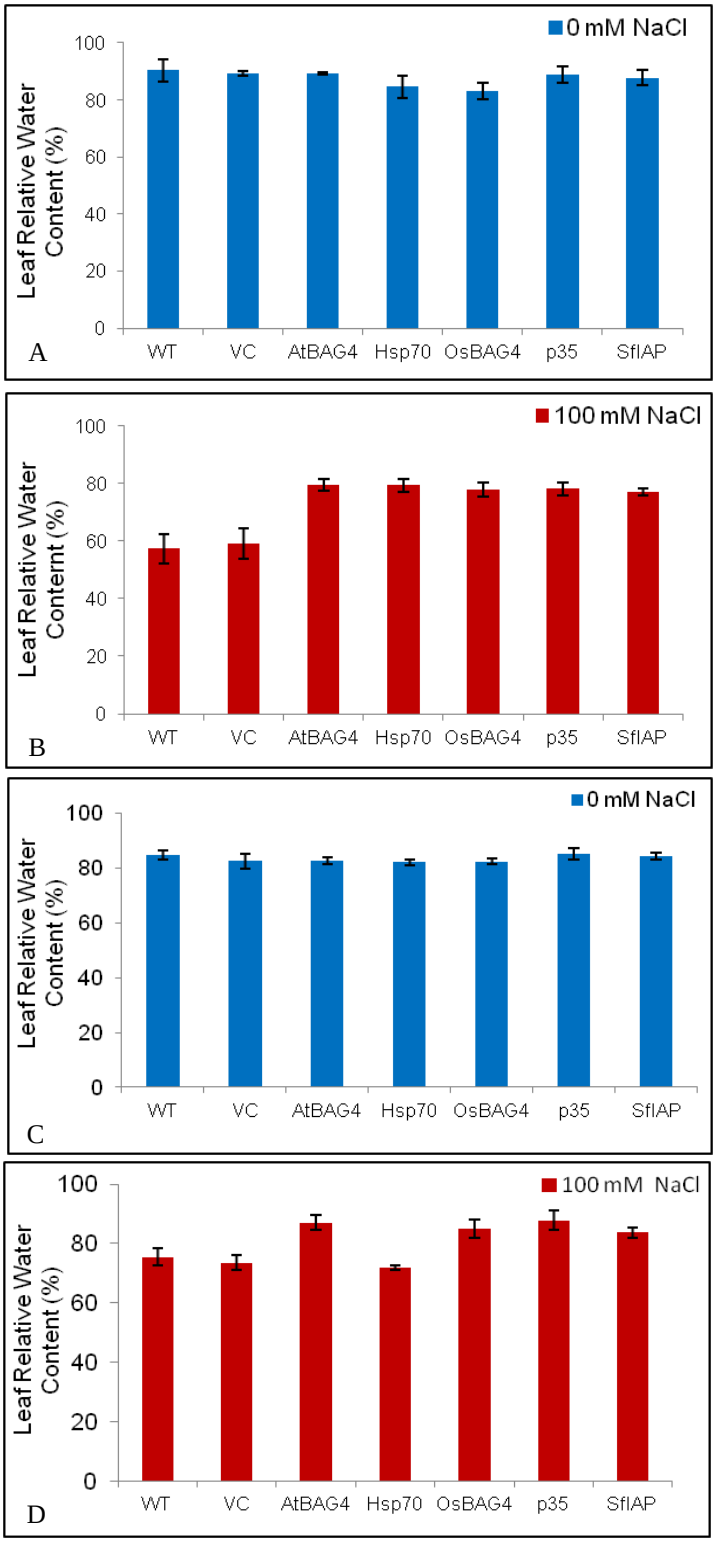


Figure 6.8: Relative water content of WT, VC and transgenic plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* grown at 0 mM and exposed to 100 mM NaCl at the seedling (A & B) and reproductive stages (C & D). Data represent the mean and standard error of three replicates.

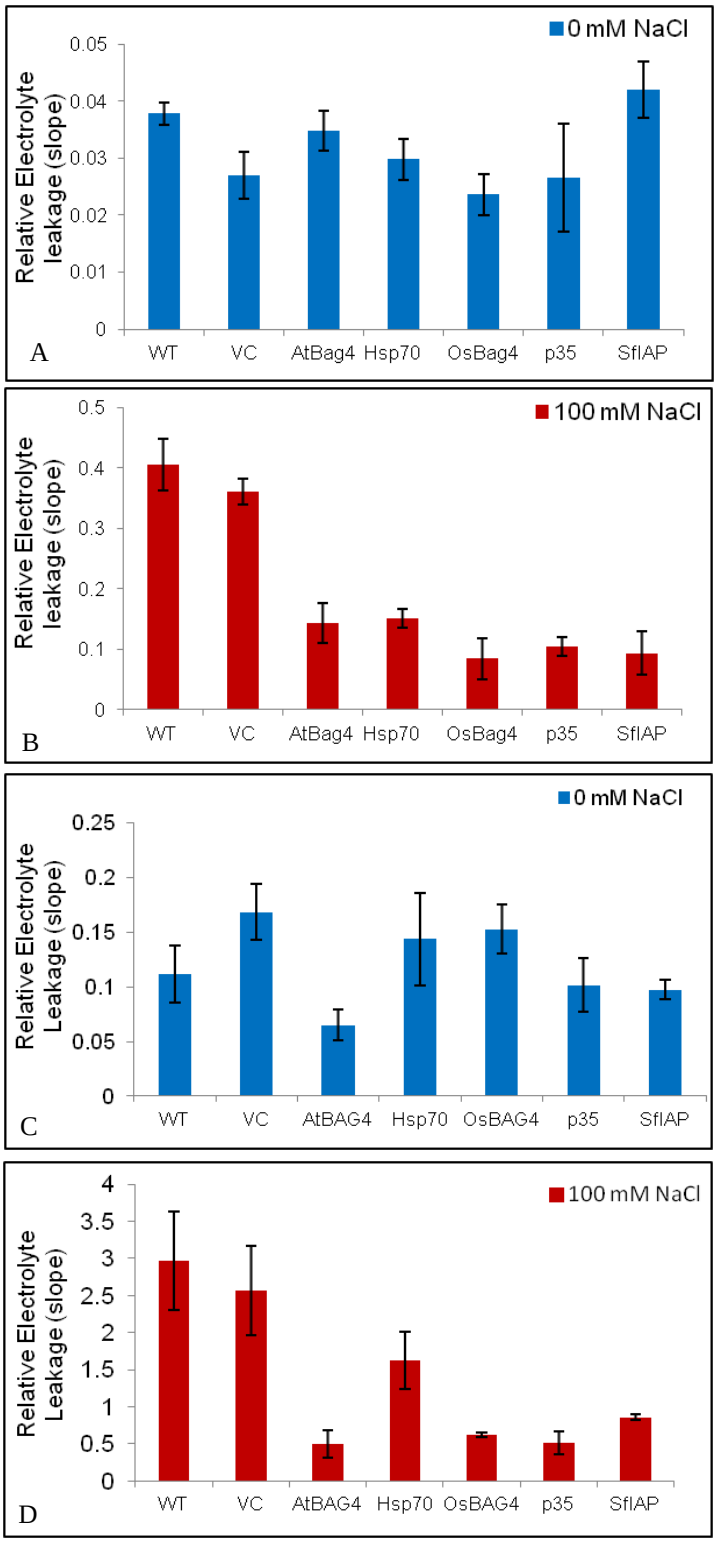


Figure 6.9: Electrolyte leakage of WT, VC and transgenic rice plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* grown at 0 mM and exposed to 100 mM NaCl at the seedling (A & B) and reproductive stages (C & D). Data represent the mean and standard error of three replicates.

6.3.8 Transgenic rice plants accumulate less Na^+, more K^+ and maintain lower Na^+/K^+ ratio than the WT and VC plants under salinity stress

It is well documented that during salinity stress salinity tolerant rice cultivars accumulate less Na^+ in leaves and shoots compared to salinity sensitive rice cultivars (Dionisio-Sese & Tobita 1998, 2000; Lee *et al.* 2003; Moradi & Ismail 2007; Ghosh *et al.* 2011). In this study we investigated the Na^+ concentration in leaves of transgenic rice expressing pro-survival genes and controls under non stressed and salinity stress conditions. As shown in Figure 6.10A, the Na^+ concentration in leaves under non stressed conditions was not significantly different between the transgenic plants expressing *AtBAG4, Hsp70, p35, SfIAP* and the control plants. Although the Na^+ concentration in leaves of *OsBAG4* transgenic plants under non stress condition was significantly higher than that of *AtBAG4, Hsp70, p35* and *SfIAP* transgenic plants as well as WT and VC but this amount is low (approximately 0.128 mmol g^{-1}DW). In contrast, under 100 mM NaCl stress at seedling stage the concentration of Na^+ in leaves of WT Nipponbare and VC plants had dramatically increased, approximately 20 fold in comparison to that under non stressed conditions. Transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* showed only 2.4, 2.7, 2.3, 4.4 and 5.1 fold increase in Na^+ concentration respectively. The *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* plants maintained an approximately 7.75, 6.69, 8.07, 4.24 and 3.6fold lower Na^+ concentration in comparison with WT and VC in salinity stress conditions respectively. At the reproductive stage the salinity stress treatment resulted in a 1.67, 1.29, 6.76, 1.95 and 2.05 fold increase in Na^+ concentration in leaves of WT and VC plants when compared to *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic plants. No significant difference was found in leaf Na^+ concentration between transgenic rice plants expressing different pro-survival genes during salinity stress at seedling stage (Figure 6.10B). In salinity stress treatment at the reproductive stage, *Hsp70* transgenic plants accumulate the highest amount of Na^+ in leaf and *OsBAG4* plants have the lowest leaf Na^+ concentration; leaf Na^+ concentrations of *AtBAG4, p35* and *SfIAP* plants were in the middle of the range (Figure 6.10 D).

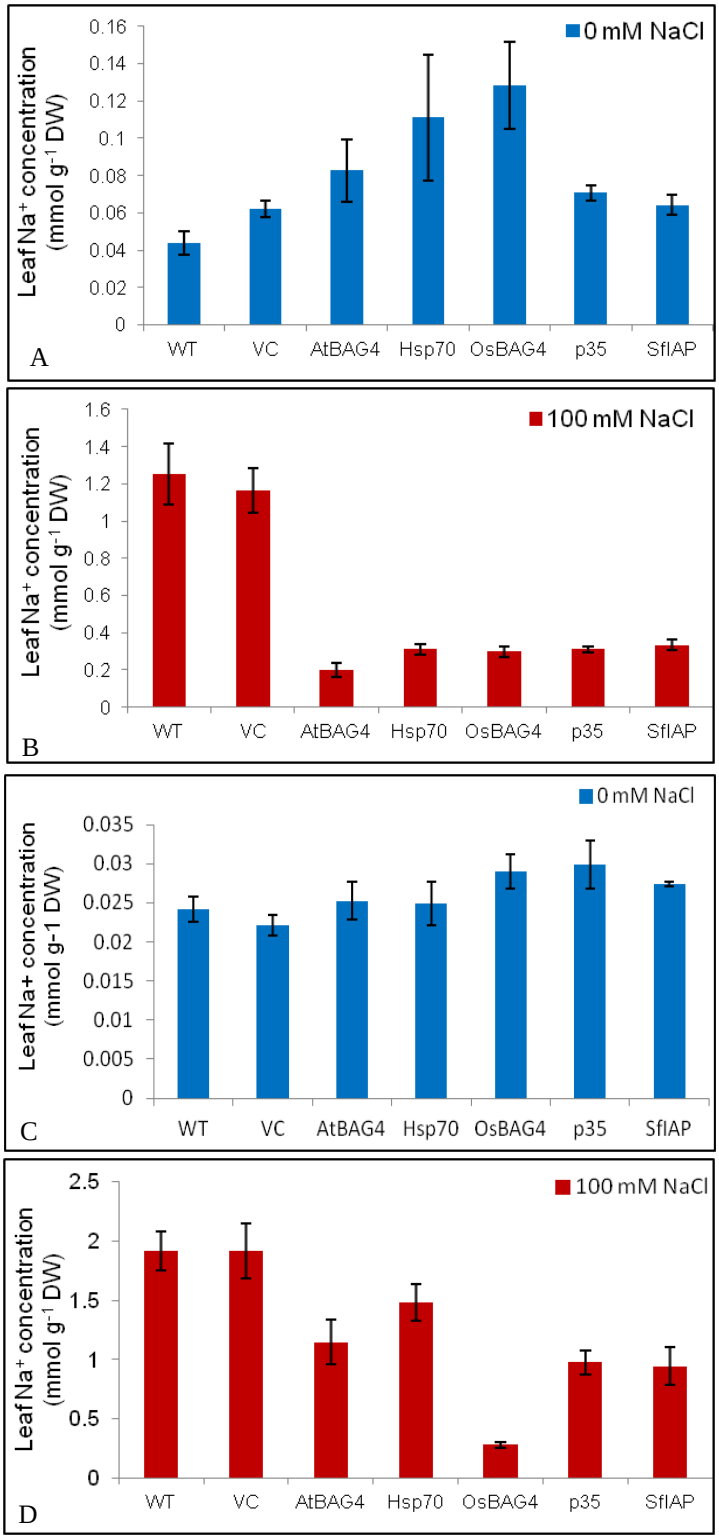


Figure 6.10: Na^+ concentration in leaf of WT, VC and transgenic plants expressing *AtBAG4*, *Hsp70*,*OsBAG4*, *p35* and *SfIAP* grown at 0 mM and exposed to 100 mM NaCl at the seedling (A & B) and reproductive stages (C & D). Data represent mean and standard error of three replicates.

In addition to maintaining a lower level of Na^+ in leaves under salinity stress, transgenic rice plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* also

maintained approximately 2 fold higher K^+ concentrations when compared to WT and VC (Figure 6.11B,D). WT and VC, in 100 mM NaCl treatment at the seedling stage, exhibited a significant decrease in K^+ concentration in leaves compared to that in non-stressed conditions whereas *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* transgenic plants had only slightly decrease in leaf K^+ concentration. At the reproductive stage NaCl, WT and VC plants showed a significant reduction in leaf K^+ concentrations while transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* maintained approximately 5.76, 7.07, 6.94, 9.57 and 3.19 fold higher in leaf K^+ concentrations compared to that in WT and VC, respectively.

More importantly, *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* transgenic plants exhibited a significantly lower ratio between Na^+ and K^+ compared to the controls (Figure 6.12B, D). Interestingly, no remarkably difference in leaf Na^+, K^+ concentrations or leaf Na^+/K^+ ratio was observed between transgenic plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* under salinity stress treatment at the seedling stage. Although there was a variation in either leaf Na^+, K^+ concentrations or leaf Na^+/K^+ ratio between transgenic plants in NaCl treatment at the reproductive stage, all transgenic plants tested maintained Na^+ homeostasis in comparison to WT and VC plants. Perhaps transgenic plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* maintain Na^+ homeostasis by promote the cell life and allow normal control of ion transport to continue under salinity stress conditions.

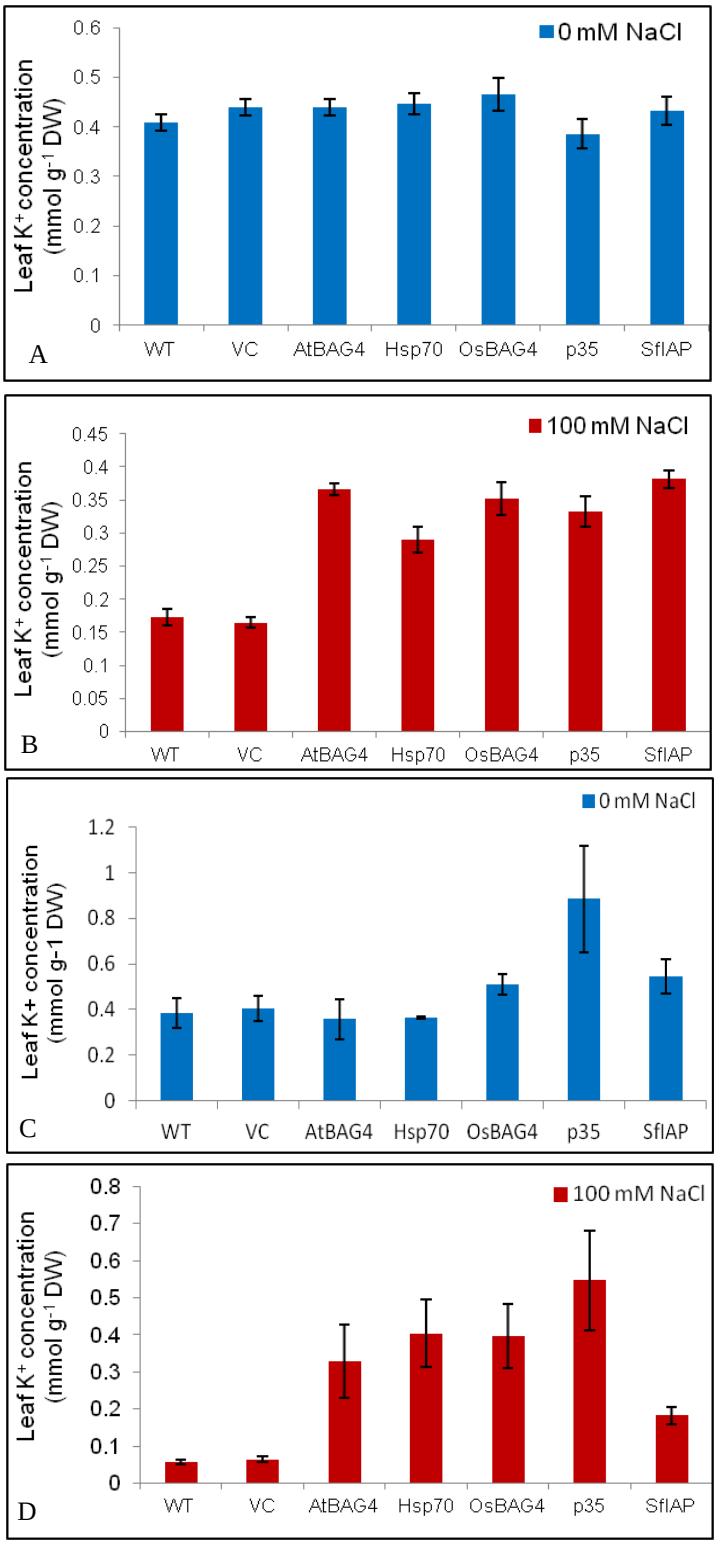


Figure 6.11: K^+ concentration in leaf of WT, VC and transgenic plants expressing *AtBAG4*, *Hsp70*,*OsBAG4*, *p35* and *SfIAP* grown at 0 mM and exposed to 100 mM NaCl at the seedling (A & B) and reproductive stages (C & D). Data represent the mean and standard error of three replicates.

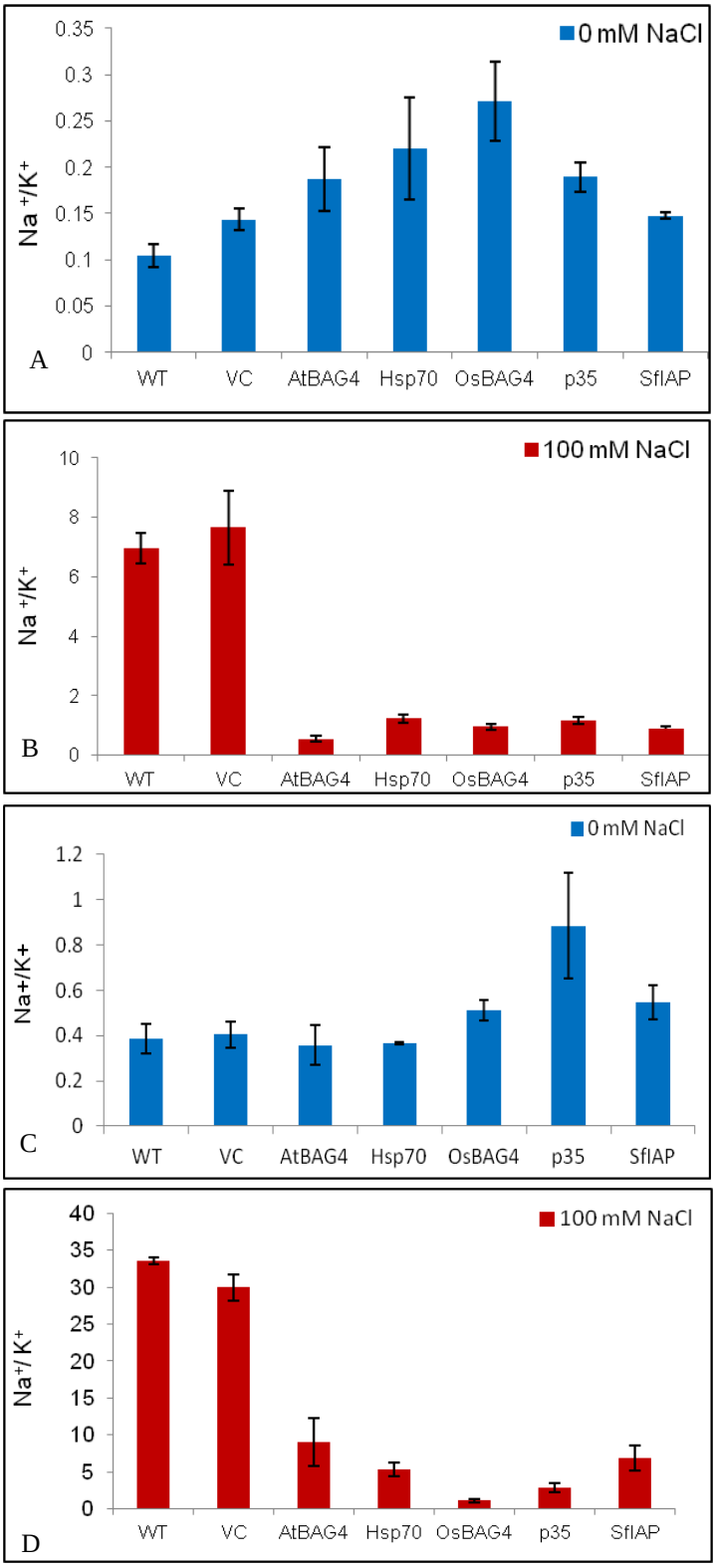


Figure 6.12: Na^+/K^+ ratio in leaf of WT, VC and transgenic plants expressing *AtBAG4*, *Hsp70*,*OsBAG4*, *p35* and *SfIAP* grown at 0 mM and exposed to 100 mM NaCl at the seedling (A & B) and reproductive stages (C & D). Data represent the mean and standard error of three replicates.

6.3.9 Plants expressing pro-survival genes maintain photosynthetic efficiency during salinity stress

Photosynthesis is a fundamental physiological process that provides a source of energy for plants to grow and cope with environmental stresses. To investigate whether expression of *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* respectively, enhanced salt tolerance in rice by maintaining photosynthetic efficiency under salinity stress, the net photosynthesis of transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* to WT and VC rice plants under non-stressed and salinity stress conditions at both the seedling and reproductive stages were examined. Net photosynthesis was measured at 0, 3, 7, 10 and 13 days after 100 mM NaCl being added to the media in seedling salt stress experiment. As evident in Figure 6.13A, under non-stressed conditions, no significant difference was observed in net photosynthesis between *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic plants and the controls over 13 days of the experiment. The trend of net photosynthesis variation was also the same for all plants tested. However, when exposed to 100 mM NaCl stress the transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* differed from the control plants (Figure 6.13B). Differences were observed between the transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* and the control plants after 7 days of salinity stress. Net photosynthesis of all transgenic and control plants decreased at day 10 in both non stressed and salinity stress treatments. This was likely due to the natural physiological senescence of the leaf at a specific age (I use the third leaf for photosynthesis measurement). However, net photosynthesis in control plants under salinity stress condition decreased more rapidly than the transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP*. After 13 days of exposure to 100 mM NaCl net photosynthesis of WT and VC plants were significantly lower than that of the *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic plants; and the net photosynthesis of *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic plants were not different from that of control plants under control condition. These results indicate that transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* can maintain photosynthetic efficiency under salinity stress. The net photosynthesis of transgenic rice plants and the controls under non stressed and salinity stress at the reproductive stage was not significantly different except for *AtBAG4* plants which had a higher net photosynthesis in comparison to WT, VC *Hsp70, OsBAG4, p35* and *SfIAP* (Figure 6.13C, D). 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 net photosynthesis measurement. At the time of measurement the flag leaf was not damaged in all plants tested while other leaves from the NaCl treated plants were dramatically damaged in control plants.

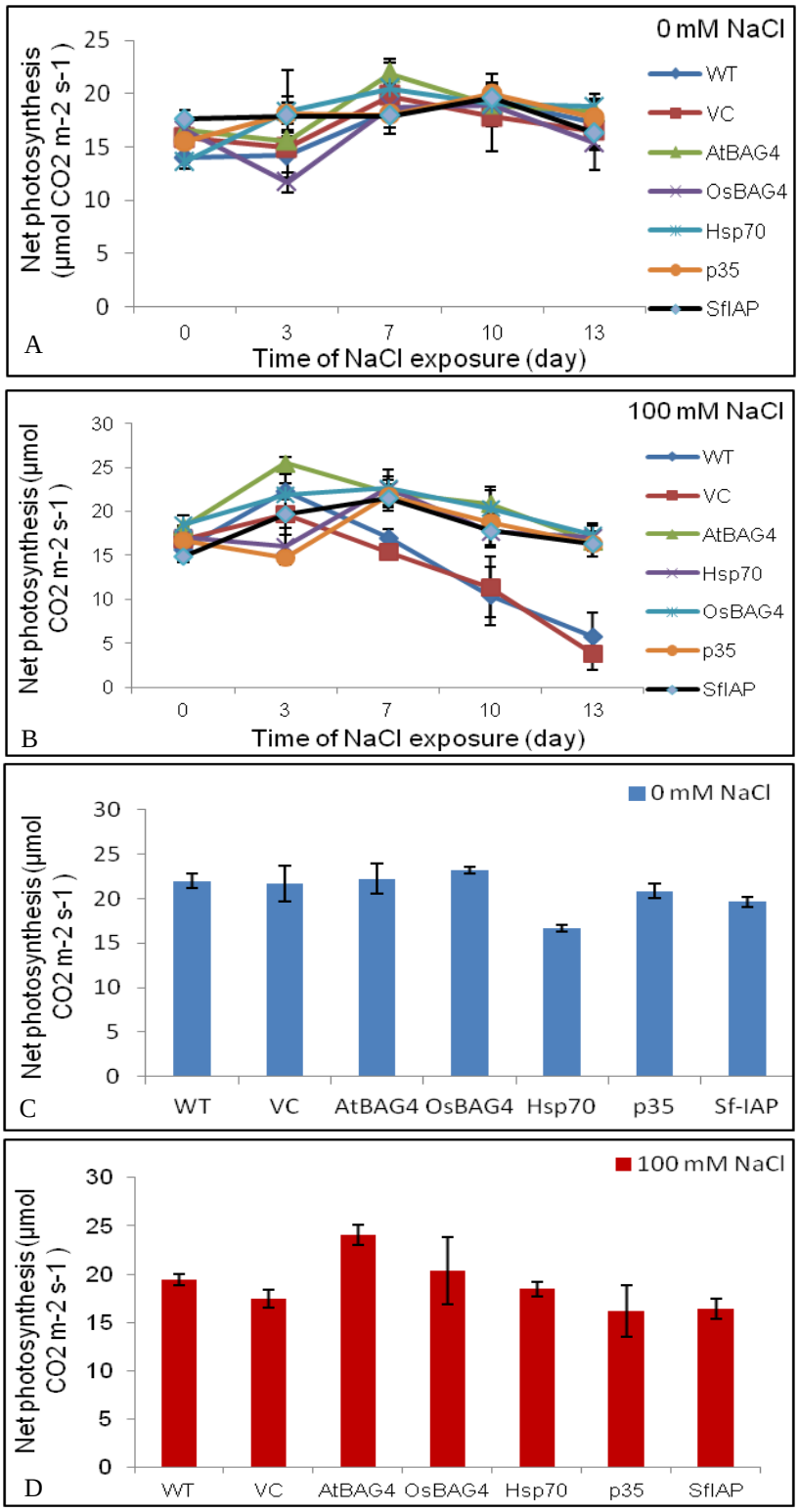


Figure 6.13: Net photosynthesis of WT, VC and transgenic plants expressing *AtBAG4*, *Hsp70*, *OsBAG4*,*p35* and *SfIAP* grown at 0 mM and exposed to 100 mM NaCl (A & B) at the seedling and (C & D) at the reproductive stages.

6.4 DISCUSSION

Previous reports have shown that rice induces PCD pathways when exposed to salinity stress (Li *et al.* 2007; Liu *et al.* 2007; Jiang *et al.* 2008); and salinity stress causes an increase of intracellular ROS levels that can be harmful to cells (Borsani *et al.* 2005; Sairam *et al.* 2005). Therefore the first objective of this chapter was to determine whether expression of pro-survival genes in the representative salinity tolerant transgenic lines coincided with reduced salinity-induced ROS levels. Results obtained in this chapter suggest that expression of pro-survival genes *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* is associated with reduces ROS production in rice during salinity stress (Figure 6.1) and can also prevent PCD caused by salinity stress (Figure 6.2). Among the four types of ROS ($O^{\bullet}_2$, $OH^{\bullet}$, $NO^{\bullet}$ and H_2O_2), H_2O_2 is a relative long-life molecule (1 ms) and it can diffuse some distance cross-linking cell wall structural proteins and more importantly H_2O_2 itself can stimulate further ROS accumulation and function as a local trigger of PCD (Levine *et al.* 1994; Dat *et al.* 2000). H_2O_2 can originate from photosynthesis, photorespiration, respiration and from many other cellular processes. It is a potent inhibitor of photosynthesis as it can inhibit CO_2 fixation up to 50% (Foyer & Shigeoka 2011). The suppression of H_2O_2 in transgenic plants expressing pro-survival genes is perhaps one of the most important steps to protect cells from oxidative damage and enable the plants to maintain photosynthetic efficiency which in turn provides energy for plants to grow and survive under salinity stress conditions. The possible role of the pro-survival genes in suppressing ROS levels during salinity stress will be discussed in the "General discussion" chapter.

Shoot growth in glycophytes is significantly reduced when salinity reaches a threshold level. For most plants the threshold level of salinity is around 40 mM NaCl or less for sensitive plants such as *Arabidopsis* and rice (Munns & Tester 2008). The rate of shoot growth reduces significantly during osmotic stress especially in shoot dry matter or in leaf area (reduction in number of tillers in cereals) over time (Munns & Tester 2008). The results in shoot growth, dry weight and number of tillers of WT, VC and transgenic plants expressing pro-survival genes *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* are consistent with this report. It was observed that relative shoot growth of transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* over 13 days of exposure to 100 mM NaCl was significantly higher than that of the WT

and VC plants (Figure 6.4B).Salinity has been reported to cause a reduction in growth rate by reducing the ability of plants to take up water (Munns 2002). The results in leaf water retention indicate that transgenic rice expressing pro-survival genes maintained significantly higher water content than the WT and VC during salinity stress exposure i.e. water uptake was maintained in *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic plants under salinity stress (Figure 6.8B). The maintenance of water uptake in transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* enabled the plants to continue growing as indicated by the maintenance of high tiller numbers per plant (Figure 6.5) and greater dry weight (Figure 6.6) in transgenic rice but not in WT and VC plants. Previous reports have shown that salinity tolerant rice cultivars exhibited greater growth and biomass (dry weight) and higher relative water content than salinity sensitive plants under salinity stress (Lee *et al.* 2003; Suriya-arunroj *et al.* 2004; Cha-um *et al.* 2009b; Awala *et al.* 2010). The maintenance of growth rates would have led to the maintenance of yield components in transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* when compared to the WT and VC (Figure 6.7). This result agrees with previous reports that salinity stress at the reproductive stage significantly reduced yield components especially the number of tillers per plant and number of spikelets per panicle in salinity sensitive rice cultivars (Zeng *et al.* 2001; Zeng *et al.* 2003). Transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* can maintain growth rates possibly due to their ability to suppress ROS and prevent cell death lead to maintenance of photosynthetic efficiency and subsequently growth rate and yield components.

The maintenance of cell membrane integrity and stability under water stress is an important component of tolerance against water deficit (caused by drought and salinity stress) in plants. Cell membrane damage can be measured by monitoring electrolyte leakage from the cells (Bajji *et al.* 2002). The results from section 6.3.7, indicate that *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* leaf cells maintain higher cell membrane integrity than controls plants under salinity stress, correlates with previous studies which showed a significant difference between electrolyte leakage levels of salinity sensitive and salinity tolerant rice cultivars during salinity stress (Dionisio-Sese & Tobita 1998; Cha-um *et al.* 2009b). 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 and it is one of the most vulnerable targets for ROS due to the predominance of lipids (Ghosh *et al.* 2011).The ability to maintain cell membrane integrity in transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* perhaps due to the ability to suppress H_2O_2 in cells during salinity stress of these plants.

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^+, 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 disturbance in metabolism. As K^+ is responsible for the activation of more than 50 enzymes in the cytoplasm, the disruption to the metabolism is severe (Shabala & Cuin 2008; Marschner 2011; Wang *et al.* 2013). One of the mechanisms that plants employ to adapt to salinity stress, especially ion toxicity is to exclude Na^+ from the roots and subsequently maintain low concentrations of sodium ions in leaves. Failure to exclude Na^+ from the cell manifests in toxic effects days or even weeks after exposure and causes premature death of older leaves (Munns & Tester 2008). The *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* plants consistently maintained low leaf Na^+ concentrations during NaCl treatment at the seedling and reproductive stages (approximately 44.4, 69.1, 66.2, 69.4 and 74 mM and 254.5, 329.2, 62.8, 216.9 and 210 mM in leaf water basis, respectively) whereas dramatic increases were observed in the Na^+ levels in the WT Nipponbare and VC (approximately 278 mM and 259 mM in leaf water basis at seedling stage and 425 mM each in leaf water basis at reproductive stage, respectively). The ability to accumulate less Na^+, more K^+ and low Na^+/K^+ ratio of transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* might be related to the maintenance of cell membrane integrity as cell membranes regulate what can get in and out of a cell. Almost all ion channels are located on membrane. Cell membrane integrity is a key factor that can ensure balanced uptake of ions and nutrients for the cell to grow.

Many studies have suggested that cytosolic K^+ is related to the PCD process as it can affect caspases and caspases-like activities in animal and plants, respectively. Low cytosolic K^+ content in animal tissue correlates with high activities of caspases; and the activation of K^+ efflux, the main cause of cytosolic K^+ content decrease, in

plant cells leads to PCD hydrolase activation (Hughes Jr & Cidlowski 1999; Shabala 2009; Demidchik *et al.* 2010). In this study I found that WT and VC exhibited a significant loss of K^+ during exposure to 100 mM NaCl at both the seedling and reproductive stages (to approximately 38.4 mM and 36.6 mM in leaf water basis at seedling stage and 12.7 mM and 14.3 mM in leaf water basis at reproductive stage, respectively) while *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic plants showed only slight and moderate decreases in leaf K^+ concentration during salinity stress at the seedling and reproductive stages (to approximately 81.3, 64.3, 78.1, 73.8 and 84.8 mM and 73.1, 89.7, 88.1, 121.5 and 40.6 mM in leaf water basis), respectively. This result provides an explanation of PCD inhibition in transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* under salinity stress conditions. Noted that cytosolic K^+ concentration of an animal shrunken cell (cell undergo apoptosis) is around 35 mM – 50 mM (Barbiero *et al.* 1995; Hughes Jr & Cidlowski 1998). The low leaf K^+ concentration of *SfIAP* transgenic plants at the reproductive stage (40.6 mM) was probably associated with PCD during senescence of the leaf at that specific age of development (end of reproductive stage to beginning of ripening stage) whereas very low K^+ concentrations in the WT and VC (12.7 mM and 14.3 mM in 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 leaf senescence but also salinity 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 *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* exhibited a significantly lower Na^+/K^+ ratio in comparison to the WT and VC during salinity stress. Importantly, the lower Na^+ and higher K^+ levels correlated with higher net photosynthetic rates in the *AtBAG4, Hsp70, OsBAG4,p35* and *SfIAP* plants thus providing an energy source and vital ammunition for the plants to develop and cope with the challenges imposed by salinity stress. Consistent with these data previous reports have shown that under salinity stress, salt tolerant rice cultivars exhibit higher net photosynthesis than salt sensitive rice cultivars (Moradi & Ismail 2007; Cha-Um *et al.* 2009a).

The ability to suppress ROS production (Figure 6.1), maintain cell life (Figure 6.2) and cell membrane integrity (Figure 6.9) in transgenic rice expressing *AtBAG4,*

Hsp70, OsBAG4, p35 and *SfIAP* is probably the most important physiological basis that assists the plants to enhance their tolerance to salinity stress at both the seedling and reproductive stages. By reducing ROS levels, preventing cell death at the onset of salinity stress and maintenance of cell membrane integrity, *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic rice plants can retain plant water status, ion homeostasis, photosynthetic efficiency and growth to successfully combat salinity.

In summary this chapter details the investigation of expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* in rice to improve salinity stress tolerance. In contrast to the WT and VC, the *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* plants maintained growth and photosynthetic rates, relative water content, 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 plant cell-death pathways during abiotic stress responses and highlight the potential of using pro-survival genes for the generation of stress tolerant plants.

Chapter 7: General Discussion

This chapter provides a general discussion on the whole project in section 7.1. Section 7.2 describes the significance of the project and suggestions for further study.

7.1 GENERAL DISCUSSION

The decision of whether a given cell should live or die is essential for the well-being of all multi-cellular organisms. Under several stimuli, this decision depends on the result of a battle between anti-apoptotic (pro-survival) and pro-apoptotic proteins or signals (Li & Dickman 2004; Williams & Dickman 2008). For example, cell death signals can lead to activation of both Ced-4-like proteins and Bcl-2 pro-apoptotic proteins; while survival signals can reduce the activity of the pro-apoptotic Bcl-2 family members and increase the levels of Bcl-2 anti-apoptotic proteins (Zha *et al.* 1996; von Freeden-Jeffry *et al.* 1997). Bcl-2 anti-apoptotic proteins and their homologs bind to and block the action of Ced-4-related proteins and caspase activation thereby promoting survival. The Bcl-2 pro-apoptotic proteins promote cell death by preventing binding of Bcl-2 anti-apoptotic proteins to Ced-4 or its homologs (Strasser *et al.* 2000). The ratio of anti-apoptotic (pro-survival) versus pro-apoptotic (pro-death) proteins also regulates PCD sensitivity (Fulda *et al.* 2010). Manipulation of programmed cell death (PCD) pathways has been reported to confer tolerance to a wide range of biotic and abiotic stresses including salinity (Dickman *et al.* 2001; Lincoln *et al.* 2002; Awada *et al.* 2003; Chen & Dickman 2004; Li & Dickman 2004; Doukhanina *et al.* 2006; Shabala *et al.* 2007; Wang *et al.* 2009; Li *et al.* 2010; Paul *et al.* 2011). Due to its broad applicability, the manipulation of PCD pathways to inhibit PCD caused by salinity stress might be a useful approach to increase crop productivity in salt affected areas (Joseph & Jini 2010).

In this project, transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* were found to possess many characteristics that have been reported in salinity tolerant rice cultivars when subjected to salinity stress. These features include, but are not limited to, maintenance of growth and yield components, cellular membrane integrity, high photosynthetic efficiency and low ROS and Na^+ accumulation. In

contrast, the WT and VC rice plants were salinity sensitive and displayed stunted growth, increased cell membrane damage, low photosynthetic efficiency, high cytosolic Na^+ and finally death or yield losses. The only difference between the transgenics and WT and VC plants is that the transgenics were expressing pro-survival genes. The improvement of salt tolerance in the transgenics can be grouped into one of two categories, i) homeostasis of cellular and molecular networks, and ii) improved metabolic and physiological status.

7.1.1 Transgenic rice expressing pro-survival genes maintain homeostasis of cellular and molecular networks

In plants, ROS are versatile molecules playing dual roles as both toxic compounds and signal transduction molecules that mediate responses to environmental stresses, pathogen infection, developmental stimuli and even PCD (Miller *et al*. 2008; Miller *et al*. 2010). The onset of PCD pathways is triggered by increased ROS levels, among other signals, that originate from a variety of organelles including the chloroplast and mitochondria (Foyer & Noctor, 2005; Rhoads *et al*. 2006). During salinity stress, ROS levels have been reported to increase causing significant injury and eventual death (Borsani *et al.* 2005; Zhu *et al.* 2007; Chawla *et al.* 2013). Consistent with these reports, the level of ROS in leaves of WT, VC and transgenic rice expressing *AtBAG4*, *Hsp70*, *OsBAG4*, *p35* and *SfIAP* increased during salinity stress. Importantly, however, despite showing increased levels, the amount of ROS observed in the transgenics was significantly lower than in the WT and VC controls (Chapter 6, section 6.3.1). Expression of *p35* has been reported to inhibit H_2O_2-induced PCD in insect cells by directly sequestering ROS. The antioxidant function of p35 has been attributed to the presence of metal-binding sites in the proteins that could enhance its antioxidant property and/or its three-dimensional structure contains some amino acids that confer electro-dynamically stable configuration conducive to ROS-trapping. The antioxidant role of p35 was also supported by the chemical radio-protectors formed by six cysteine residues in its sequence which can react with certain ROS in a constant rate (Sah *et al.* 1999). Consistent with this report, the results in this PhD study show that expression of *p35* in rice can suppress ROS generated from salinity stress thereby inhibiting cell death cause by salinity.

The exact mechanistic details of how *Hsp70, AtBAG4* and *OsBAG4* genes suppress cellular ROS levels remain unknown, though we can speculate on a few plausible scenarios. i) constitutive expression of *Hsp70, AtBAG4* and *OsBAG4* genes might reduce ROS levels indirectly by facilitating the function of cellular processes which if not working efficiently promote the generation of ROS and ii) maintenance of ion homeostasis.

If left unchecked, copious ROS production can denature proteins. To mitigate the denaturation of proteins, cells employ a complex network of molecular chaperones and foldases which promote efficient and correct folding of cellular proteins (Hartl *et al.* 2011). Members of the highly conserved heat shock protein family are chaperones that play a key role within the promotion of correct protein folding and proteostasis control (Hartl *et al.* 2011). A definitive feature of the BAG family of proteins is their ability to bind and facilitate the function of HSPs (Doukhanina *et al.* 2006; Williams *et al.* 2010). The expression of *Hsp70, AtBAG4* and *OsBAG4* may assist in the folding of proteins and prevention of protein denaturation in high ROS environments, thus maintaining efficiency of cellular processes and mitigating the production of ROS and plant damage. Port *et.al.*(2011) proposed a schematic representation of the processes involved in inducing stress-mediated cell death and its inhibition by key anti-apoptotic proteins. In that scheme stress induced an unknown substrate that mediated activation of: BH3 only BcL-2 proteins, mitochondria (or other ROS producing system such as NADPH oxidase) and sphingomyelinase. This activation led to the action of at least three pro-apoptotic messengers including active Bax, increasing ROS and sphingolipid ceramide thereby causing cell death. Heat shock proteins (HSPs) were proposed to function as anti-apoptotic proteins by blocking that unknown substrate thereby preventing the generation of active Bax, increasing ROS and sphingolipid ceramide. In plants the BH3 only BcL-2 proteins and sphingomyelinase have not yet been identified therefore the inhibition of cell death mediated by pro-survival proteins might be most likely the prevention of increasing ROS.

Accordingly, homeostasis of cellular ROS levels promotes maintenance of cellular membrane integrity. Previous studies have shown that ROS-induced cell death can result from oxidative processes such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA, RNA damage (Mittler 2002).The cell

membrane is the first site of signal perception as well as the primary defence against abiotic stresses including salinity and it is one of the most vulnerable targets for ROS due to the predominance of lipids (Ghosh et al., 2011).

Water retention was maintained in transgenic rice plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* but not in WT and VC plants during salinity stress. This is understandable as the transgenic rice plants expressing the pro-survival genes have higher number of viable cells than the WT and VC plants (see chapter 6 section 6.3.2 for details) therefore they have higher water retention as water is one of the major factors to sustain cell life. When the central vacuole absorbs water in plant cells, it extends the content of the cell within the cell walls, causing the plant to stiffen to provide turgor pressure and also results in cell growth. If there is not enough water in the vacuole the plant will collapse and wilt (Yablonski 2005).

Transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* accumulate low Na^+, high K^+ and maintain low Na^+/K^+ ratios during salinity stress. This is probably a result of the maintenance of cell membrane integrity in transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* during salinity stress. As discussed earlier in chapter 6 high Na^+ levels are toxic to cells because Na^+ has similar physicochemical properties to K^+, 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 disturbance in metabolism (Shabala & Cuin 2008; Marschner 2011; Wang *et al.* 2013). In addition Na^+ can displace Ca^+ from plasma membranes inducing K^+ leaks out of the cytoplasm across the plasma membrane (Cramer *et al.* 1985). This results in a decrease in cytosolic K^+ concentration and effects the Na^+/K^+ ratio hence leads to a disturbance of metabolism. Under typical physiological condition, the influx of Na^+ into plant cells is through the H^+-ATPase channel which is responsible for general transport of ions and nutrients through the plasma membrane; plants maintain a low cytosolic Na^+/K^+ ratio as it is necessary for providing favourable conditions for continued physiological and metabolic activity. During salinity stress increased extracellular Na^+ concentrations create a large electrochemical gradient that favours the passive transport of Na^+ into the cell through K^+ transporters result in high cytosolic Na^+ concentration (Blumwald 2000). To maintain low cytosolic Na^+ concentrations, plant cells need to extrude Na^+ of the cell or compartmentalize Na^+ into vacuoles. The

main mechanism for Na^+ extrusion in plant cells is mediated by the plasma membrane H^+-ATPase (Sussman 1994). As the cell membrane in WT and VC plants was damaged it could not use this strategy to pump Na^+ out of the cell hence the Na^+ concentration was recorded at high levels in leaf cells of those plants. On the contrary, transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* can maintain cell membrane integrity and therefore could use the H^+-ATPase to extrude Na^+ thus maintaining a low concentration of Na^+ in cytoplasm. The high maintenance of low cytosolic Na^+ concentrations facilitates a high concentration of K^+ therefore ensuring a low Na^+/K^+ ratio that could offer an optimal cellular environment for enzymes function thus supporting metabolism. As also discussed in chapter 6 the high cytosolic K^+ concentration in transgenic rice plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* enables the plants to inhibit PCD. Cytosolic K^+ have been suggested to be related to the PCD process as it can affect caspases and caspases-like activities in animal and plants, respectively. Low cytosolic K^+ content in animal tissue correlates with high caspase activity; and the activation of K^+ efflux, the main cause of cytosolic K^+ content decrease, in plant cells leads to PCD hydrolase activation (Hughes Jr & Cidlowski 1999; Shabala 2009; Demidchik *et al.* 2010). The maintenance of high K^+ concentrations in cells of transgenic plants expressing pro-survival genes correlated with less cell death in those plants as evident in chapter 6 section 6.3.2.

Accumulation or aggregation of malfolded proteins severely compromises metabolic efficiency and is a hallmark of many stresses and key inducer of stress pathways which if overwhelmed can trigger PCD. SfIAP has an E3 ubiquitin function i.e. it can serve as a ubiquitin-ligating enzyme which can regulate PCD via proteasome-mediated degradation (Kabbage *et al.* 2010). E3 enzymes play a critical role in determining the specificity of the ubiquitin/26S proteasome process. They are responsible for substrate recognition and promoting the polyubiquitination of the substrate (Wilkinson 2000). Transgenic rice expressing *SfIAP* might have used this function to degrade aggregated proteins that are harmful to cells thereby mitigating cellular damage during salinity stress.

7.1.2 Transgenic plants expressing pro-survival genes are more physiologically active than their WT and VC counterparts during salinity stress

AtBAG4, Hsp70, OsBAG4, p35 and *SfIAP* transgenic rice plants maintain photosynthetic efficiency under salinity stress. Several studies have suggested that a low cytosolic Na^+/K^+ ratio is required to maintain a favorable ionic homeostasis in plant cells under salinity stress condition (Maathuis & Amtmann 1999; Cuin *et al.* 2003) and a low Na^+/K^+ ratio improves photosynthesis and overall plant growth (Rodrigues *et al.* 2013). The maintenance of photosynthetic capacity in Barley and wheat under salinity stress was associated with low Na^+, high K^+ and a low Na^+/K^+ ratio in cytoplasm (James *et al.* 2006). Consistent with these reports, transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* maintained low cytosolic Na^+, high K^+ and a low Na^+/K^+ ratio which promoted high photosynthetic capacity. Maintaining high photosynthetic efficiency provides essential energy/additional artillery for transgenic plants to cope with salinity stress as energy is required for many cellular processes to sustain growth; energy is also required for pumping the Na^+ out of cells and supports reduced levels of ROS. It is evident that reduced photosynthetic rates increases the formation of ROS (Apel & Hirt 2004; Foyer & Noctor 2005; Foyer & Shigeoka 2011).

Transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* maintain growth rate (shoot growth, dry weight, number of tillers) and yield components (number of panicles per plant and number of spikelets per panicle) during salinity stress. This is probably also a result of the maintenance of high cytosolic K^+ in transgenic rice plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP*. It is well known that salinity causes two types of stress on plants: i) osmotic stress which affects plant growth immediately and is caused by excess salt outside the roots; and ii) ionic stress which develops over time and is due to a combination of ion accumulation in the shoot and an inability to tolerate the ions that have accumulated (Munns 2002; Munns *et al.* 2006; Munns & Tester 2008). In low salt environments plant cells can take up water and nutrients from the soil solution to support higher osmotic pressures compared to that of soil solution. However, in high salt environments, the osmotic pressure of the soil exceeds that of plant cells (osmotic stress) and reduces the ability of plants cells to take up soil water and minerals (Kader & Lindberg 2010). In response to osmotic stress, shoot growth rate decreases immediately (Munns & Tester 2008). High cytosolic K^+ in transgenic plants

expressing pro-survival genes helped the plants to adjust osmotic stress and maintain high growth rates because one of the important cellular roles of K^+ is to contribute to adjustment of osmotic pressure hence maintain cell turgor (Maathuis & Amtmann 1999). The maintenance of growth rate leads to higher yield components in transgenic rice expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* in comparison to WT and VC which had very low cytosolic K^+ under salinity stress condition. Another factor that causes reduced growth rates in high salt environments is inadequate photosynthesis due to limited carbon dioxide uptake as a consequence of stomatal closure (Zhu 2001). *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* transgenic rice plants maintained high net photosynthesis which provided ample energy for their growth and development.

A salinity-induced cell death stress tolerance scheme in rice is proposed (Figure 7.1). In this scheme, the master switch of the cell life/death decision during salinity stress is the "balance of the pro-death and pro-survival signals" of the system. Expression of pro-survival genes coincided with reduced pro-death signals such as ROS levels which in turn supported the maintenance of cell membrane integrity and Na^+ homeostasis. This maintenance promoted sustained photosynthetic efficiency which in turn provided energy for growth. Well-maintained growth further dilutes Na^+ concentration which helps maintain Na^+ homeostasis leading to the increased membrane integrity, relative water content, net photosynthesis and finally growth and yield. Hence the transgenic rice was able to minimise the toxicity caused by the accumulation of sodium ions and water deficit under salinity stress.

In conclusion both agronomic and physiological parameters suggested that transgenic plants expressing *AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP* were more tolerant to salinity stress than the WT and VC. This confirms that manipulation of PCD pathways can improve salinity stress tolerance in rice.

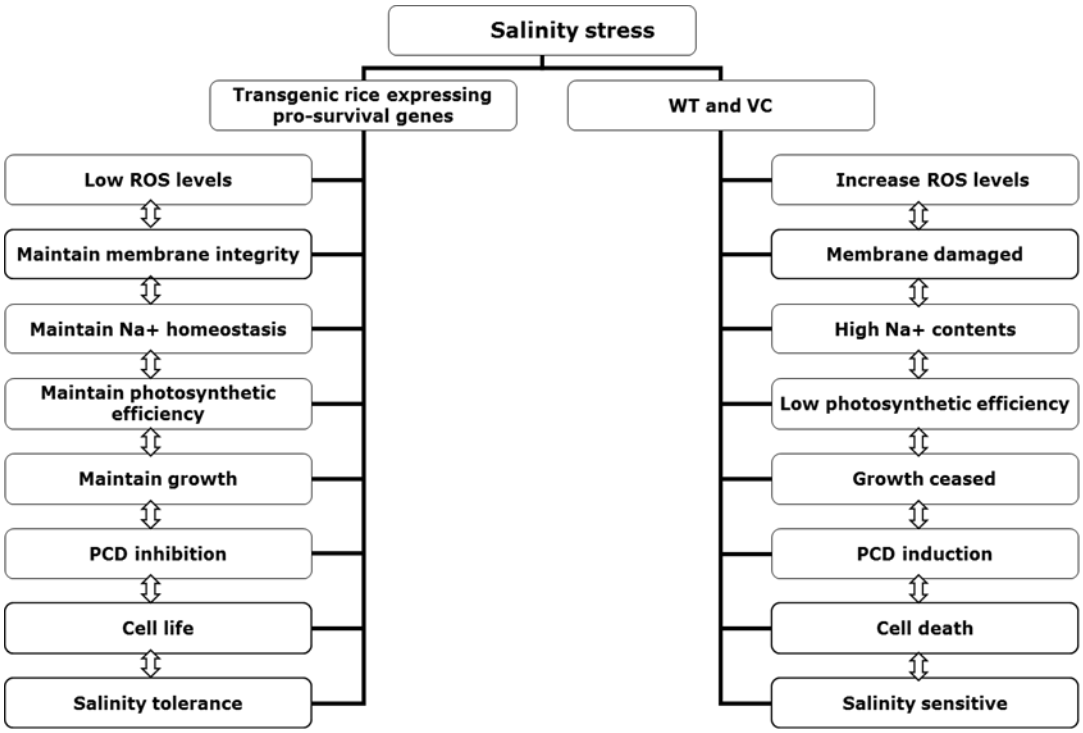


Figure 7.1: Schematic proposing salinity-induced cell death switch for salinity stress tolerance in rice

7.2 CONCLUDING REMARKS

In the next fifty years there will be a massive challenge to sustain an ever-increasing global population. Between the years 1980 – 2000 global population boomed from 4.4 billion through to 6.1 billion, however, food production increased by 50 %. By 2050 this problem will be exacerbated with world population predicted to reach 9.6 billion. In order to sustain this increased population, global food within the next 50 years will have to match that which occurred in the last 10,000 years combined. Salinisation is one of the most devastating forms of land degradation and a major threat to future crop security. Rice is an important staple crop that feeds more than one half of the world's population. Of the agronomically important cereals, rice is the most sensitive to salinity stress.

The research presented in this thesis directly addressed the aforementioned concerns and was focused on the improvement of salinity tolerance in rice via the manipulation of PCD pathways. Previously, the manipulation of cell death pathways has been used to enhance tolerance to biotic and abiotic stresses in dicots plants, both models and agronomically important crops. In this thesis I show that manipulation of

PCD pathways can also be applied to monocots, namely rice, illustrating the broad scoping applications for PCD technology. As such, the knowledge gained from this project is applicable for other crops, both mono- and dicotyledonous. Importantly, this project also provided mechanistic evidence for the biochemical and physiological basis of salinity tolerance in transgenic rice expressing pro-survival genes resulting in the presentation of a model for a salinity-induced cell death switch for salinity stress tolerance in rice.

Five pro-survival genes (*AtBAG4, Hsp70, OsBAG4, p35* and *SfIAP*) that show enhanced tolerance to salinity stress in rice have been shown to perform specific biochemical roles in mammals. For example, members of BAG protein family have been speculated to recruit Hsp70 and Hsp90 (p61) proteins and induce protein conformational changes that prevent receptor signalling in the absence of a ligand. Hsp70 can interact with different programmed cell death regulator proteins thereby block the apoptosis process at distinct key points. *SfIAP* and *p35* are known inhibitors of caspases thereby regulating PCD in mammals via suppression of caspases mediated-signalling pathways.

Although we do not have direct evidence of biochemical roles of these genes in regulation of PCD induced by salinity stress in rice, the biochemical and physiological results in this study suggests that these genes may have a similar function as speculated in mammals and in dicotyledonous plants. *AtBAG4, OsBAG4* and *Hsp70* may inhibit salinity-induced PCD in rice via their chaperon functions. *SfIAP* may function as an E3 ubiquitin and *p35* acted as an antioxidant as were reported elsewhere in the literature. Importantly, expression of these genes leads to more tolerance to salinity stress in rice by switching on the physiological response scheme. It is speculated that the key switch in the responses to salinity stress in rice is the maintenance of ROS levels thereby affecting the signals that could trigger PCD. Although the five pro-survival genes may function differently in regulating of salinity-induced PCD in rice, they converge at one point that regulates cellular metabolism.

In closing, further research should investigate the possibility of the three genes and combination that did not show enhanced salinity tolerance in this study. The further research may focus on quantitative real-time PCR and assessment of copy number by Southern blot to elucidate whether the expression level played a

significant role in salinity tolerance in the *AtBAG4+Hsp70*, *AtBI* and *Hsp90* transgenic lines. The five pro-survival genes that conferred tolerance to salinity stress should be further investigated with other promoters such as salinity stress inducible and tissue specific promoters.

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Appendices

Appendix 1
Media and Solutions

MS Stock Solution 1: Macronutrients (10x concentrated)

Ingredients	Quantity (g)
NH_4N0_3	16.5
KNO3	19
MgS04.7H2O	3.76
KH2PO4	1.7
CaCl2.2H2O	4.5

MS Stock Solution 2: Micronutrients (100x concentrated)

Ingredients	Quantity (mg)
KI	830
H3BO3	620
MnSO4 4H2O	1,320
ZnSO4.7H2O	860
Na2MoO4.2H2O	250
CuSO4.5H2O	25
CoCl2.2H2O	25

MS Stock Solution 3: Vitamins (100x concentrated)

Ingredients	Quantity (mg)
Nicotinic Acid	50
Pyridoxin-Hcl	50
Myo-Inozitol	10,000
Thimine-Hcl	50
Glycine	100

MS Stock Solution 4: Fe-EDTA (100x concentrated)

Ingredients	Quantity (mg)
Na2EDTA.2H2O	3,725
FeSO4.7H2O	2,785

N6 Stock Solution 1: Macronutrients (20x concentrated)

Ingredients	Quantity (g)
KNO3	56,6
(NH4) 2SO4	9,26
MgSo4.7H2O	3,7
KH2PO4	8
CaCl2.2H2O	3,32

N6 Stock Solution 2: Micronutrients (100x concentrated)

Ingredients	Quantity (mg)
KI	80
H3BO3	160
MnSO4 4H2O	440
ZnSO4.7H2O	150
Na2MoO4.2H2O	25

N6 Stock Solution 3: Vitamins (100x concentrated)

Ingredients	Quantity (mg)
Nicotinic Acid	50
Pyridoxin-Hcl	50
Myo-Inozitol	10,000
Thimine-Hcl	50
Glycine	100

N6 Stock Solution 4: Fe-EDTA (100x concentrated)

Ingredients	**Quantity (mg)**
Na2EDTA.2H2O	3,725
FeSO4.7H2O	2,785

MS media

Ingredients	**Quantity for 1 L**
MS Stock Solution 1	100 ml
MS Stock Solution 2	10 ml
MS Stock Solution 3	1 ml
MS Stock Solution 4	10 ml
Sucrose	30 g
pH	5.85
Phytagen	3.5g
Autoclave	121^0C, 15 min

2N6 media

Ingredients	**Quantity for 1 L**
N6 Stock Solution 1	50 ml
N6 Stock Solution 2	10 ml
N6 Stock Solution 3	1 ml
N6 Stock Solution 4	10 ml
Sucrose	30 g
L-proline	0.5g
L-Glutamin	0.5g
Casein Enzymatic Hydrosylate	1 g
2,4 D	2 mg
pH	5.85
Phytagen	3.5g
Autoclave	121^0C, 15 min

YM media

Ingredients	**Quantity for 1 L**
Mannitol	10 g
Yeast extract	0.4 g
KH_2PO_4	0.1 g
K_2HPO4	0.4 g
NaCl	0.1 g
$MgSO_4.7\ H_2O$	0.2 g
pH	6.8
Autoclave	121^0C, 15 min

LB media

Ingredients	**Quantity for 1 L**
Trypton	10 g
Yeast extract	5 g
KH_2PO_4	5 g
pH	7.5
Autoclave	121^0C, 15 min

BRM media

Ingredients	**Quantity for 1 L**
MS Stock Solution 1	10 ml
MS Stock Solution 2	1 ml
MS Stock Solution 3	1 ml
MS Stock Solution 4	1 ml
Cystein	0.4 g
Thiamin	10 g
Glucose	36
Sucrose	68.4 g
pH	5.2
Filter sterile	
Before use add 100 µM acetosynringone (freshly prepared and filter sterile)	

CCM media

Ingredients	**Quantity for 1 L**
MS Stock Solution 1	10 ml
MS Stock Solution 2	1 ml
MS Stock Solution 3	1 ml
MS Stock Solution 4	1 ml
Cystein	0.4 g
Glutamin	0.1 g
Malt extract	0.1 g
Biotin	1 mg
PVP	10 g
Ascorbic acid	10 g
Proline	0.3 g
2.4 D	0.5 mg
Kinetin	0.5 mg
NAA	2.5 mg
Maltose	30 g
Glucose	10 g
Sucrose	30 g
pH	5.3
Agar	7.5 g
Autoclave	121^0C, 15 min
After autoclaved add 100 µM acetosynringone (freshly prepared and filter sterile)	

2N6 selection media

Ingredients	**Quantity for 1 L**
N6 Stock Solution 1	50 ml
N6 Stock Solution 2	10 ml
N6 Stock Solution 3	1 ml
N6 Stock Solution 4	10 ml
Sucrose	30 g
Casein Enzymatic Hydrosylate	1 g
2,4 D	2 mg
pH	5.8
Agar	8 g
Autoclave	121^0C, 15 min

Timetin (add in after autoclaving)	200 mg
Hygromycin	50 mg

2N6 regeneration media

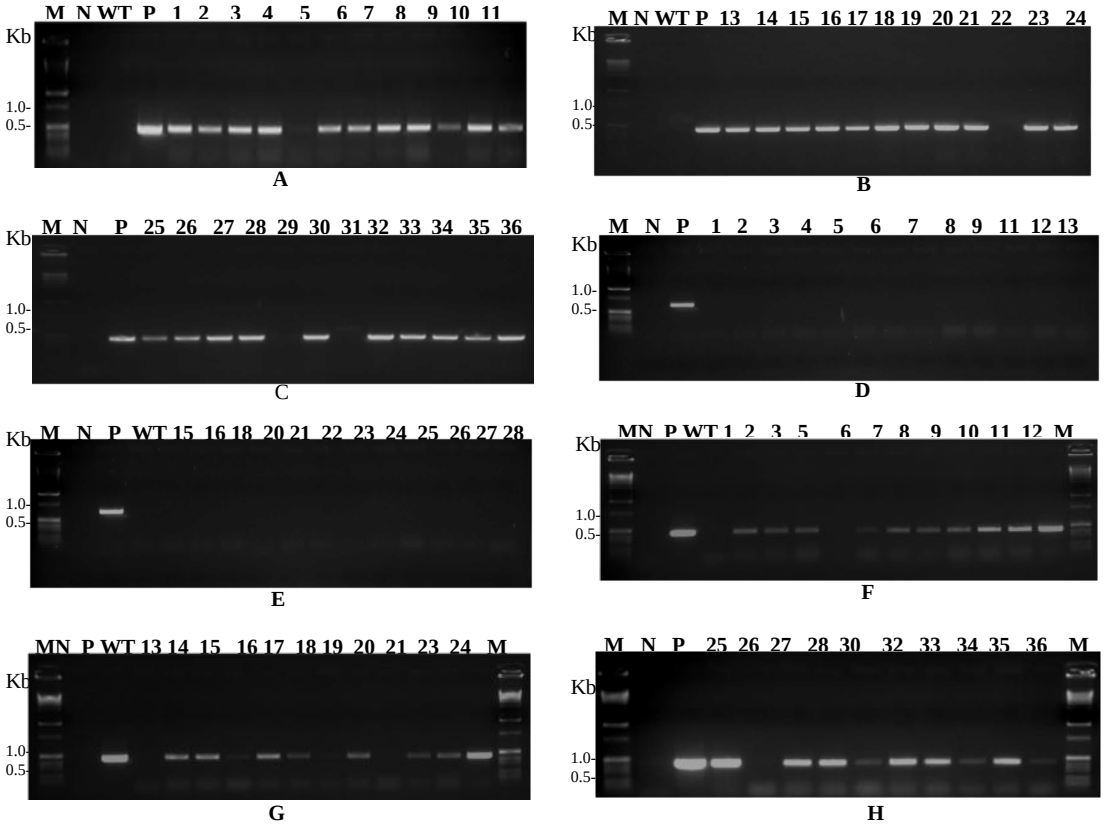
Ingredients	**Quantity for 1 L**
N6 Stock Solution 1	50 ml
N6 Stock Solution 2	10 ml
N6 Stock Solution 3	1 ml
N6 Stock Solution 4	10 ml
Sucrose	30 g
L-proline	0.5g
L-Glutamin	0.5g
Casein Enzymatic Hydrosylate	0.3 g
NAA	0.5 mg
BAP	3 mg
pH	5.8
Agar	8 g
Autoclave	121^0C, 15 min
Timetin (add in after autoclaving)	200 mg
Hygromycin	25 mg

Rooting media

Ingredients	**Quantity for 1 L**
MS Stock Solution 1	50 ml
MS Stock Solution 2	5 ml
MS Stock Solution 3	1 ml
MS Stock Solution 4	10 ml
Sucrose	10 g
IAA	0.2 mg
pH	5.8
Agar	8 g
Autoclave	121^0C, 15 min
Timetin (add in after autoclaving)	200 mg
Hygromycin	25 mg

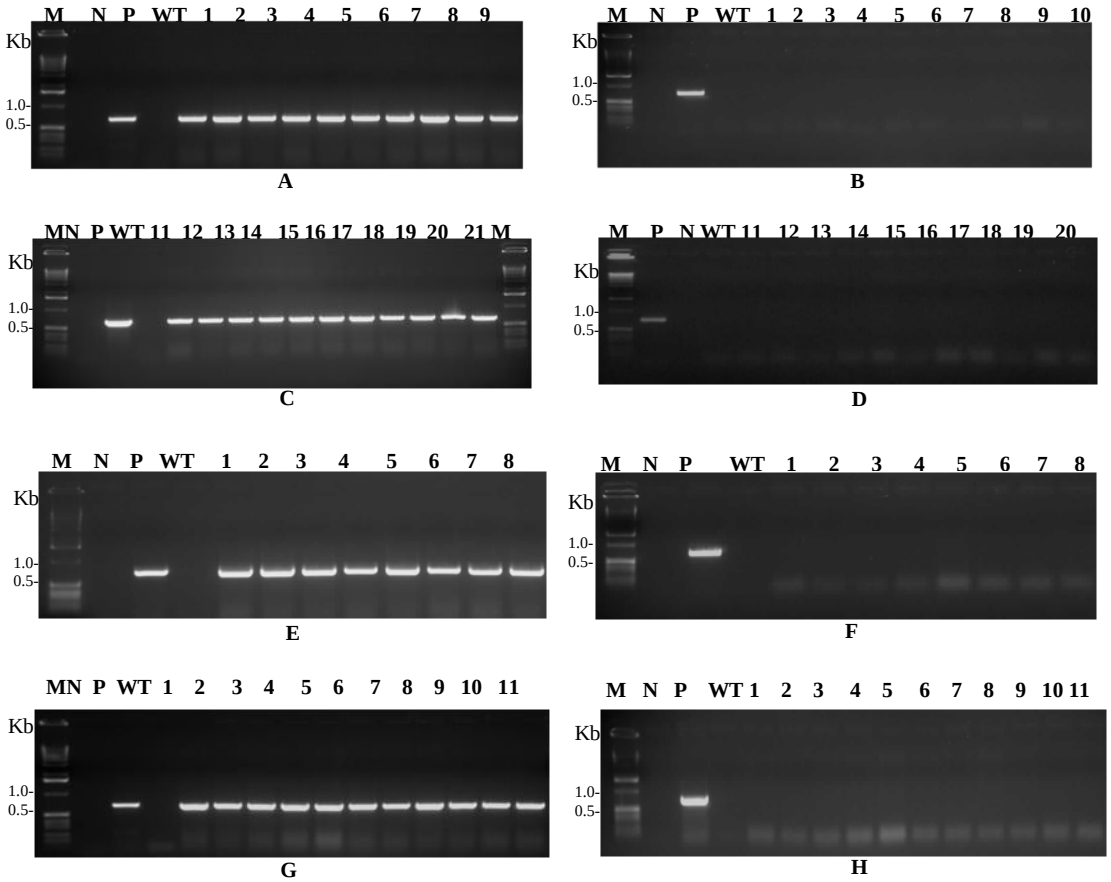
Appendix 2
Additional figures for chapter 4

Molecular characterisation of p35 putative transgenic rice



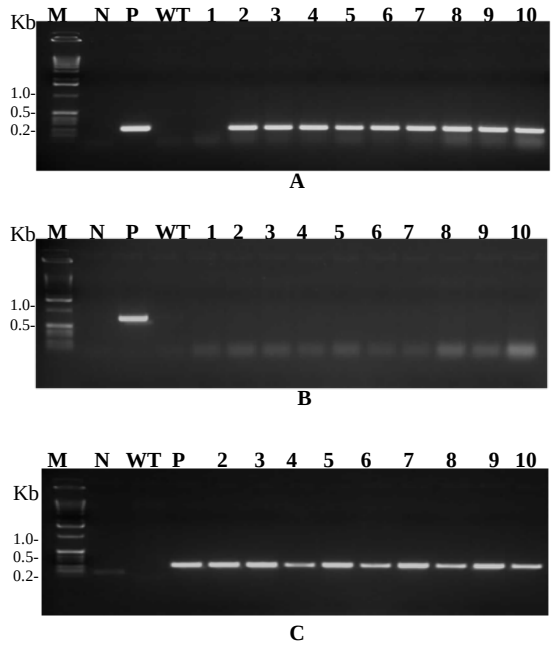
Appendix 2 - Figure 1: Molecular characterisation of p35 transformed rice plants. A, B and C: PCR with gene specific primers; D, E: PCR with VirC primers: RT-PCR. M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120 V.

Molecular characterisation of VC putative transgenic rice



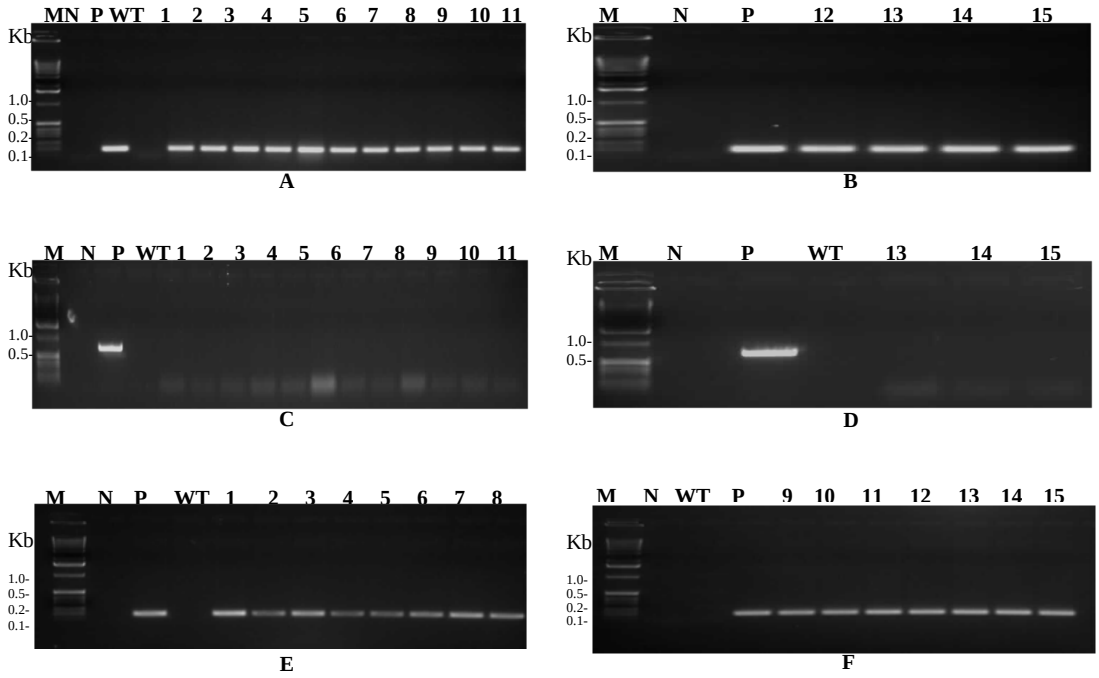
Appendix 2 - Figure 2: Molecular characterisation of vector control transformed rice plants. A, C, E, G: PCR with Hygromycin resistant gene specific primers; B, D, F, H: PCR with VirC primers; M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA or *Agrobacterium* extraction); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120 V.

Molecular characterisation of *AtBAG4* putative transgenic rice



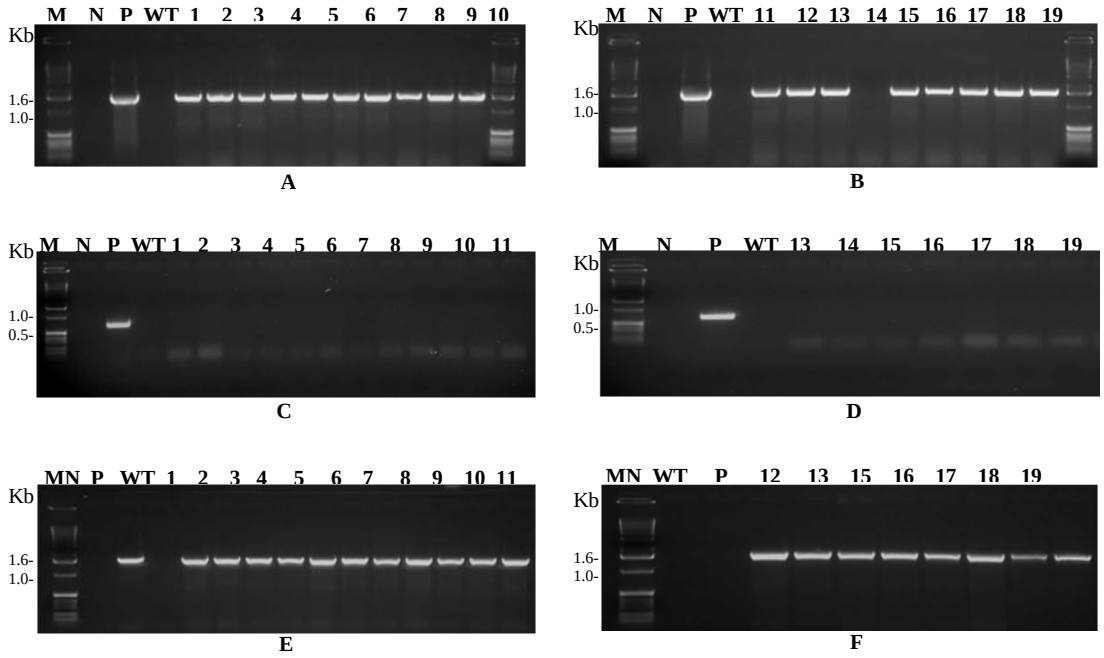
Appendix 2 - Figure 3: Molecular characterisation of *AtBAG4* transformed rice plants. A: PCR with gene specific primers; B: PCR with VirC primers; C: RT-PCR. M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA or *Agrobacterium* extraction); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120 V.

Molecular characterisation of *Hsp70* putative transgenic rice



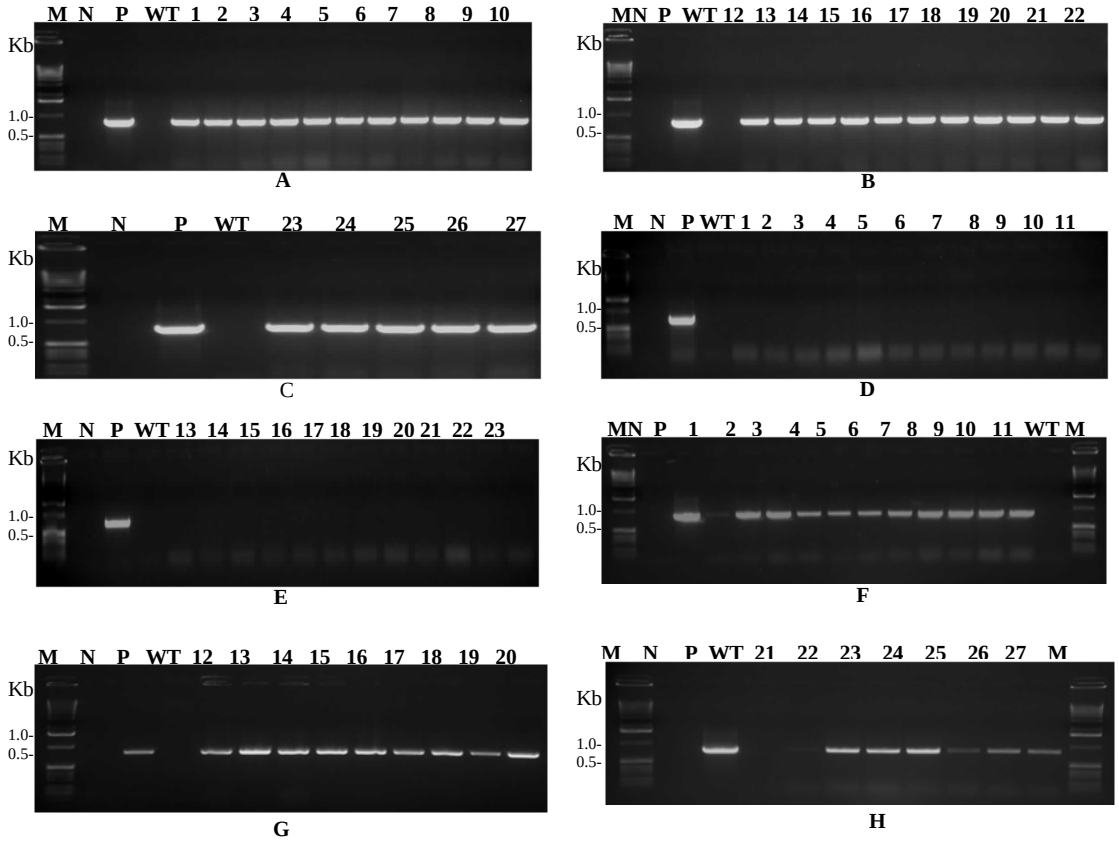
Appendix 2 - Figure 4: Molecular characterisation of *Hsp70*transformed rice plants. A and B: PCR with*Hsp70* gene specific primers; C and D: PCR with VirC primers; E and F: RT-PCRwith*Hsp70* gene specific primers. M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA or *Agrobacterium* extraction); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120 V.

Molecular characterisation of *Hsp90* putative transgenic rice



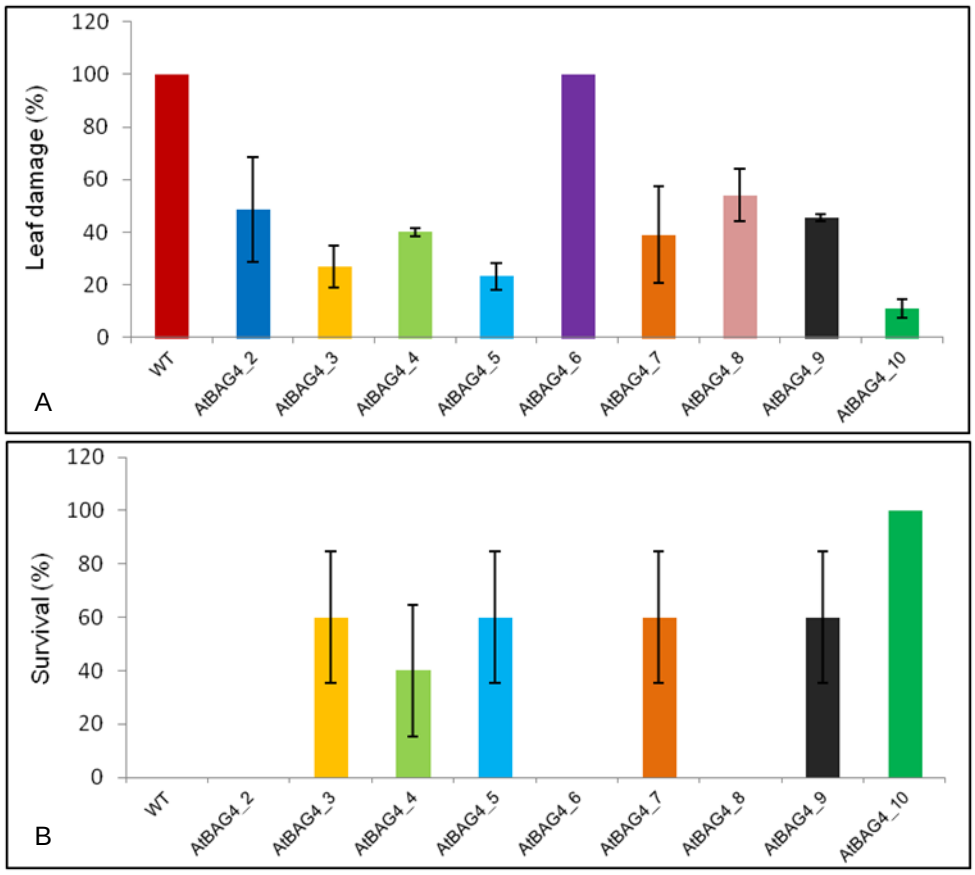
Appendix 2 - Figure 5: Molecular characterisation of *Hsp90* transformed rice plants. A and B: PCR with *Hsp90* gene specific primers; C and D: PCR with *VirC* primers; E and F: RT-PCR with *Hsp90* gene specific primers. M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA or Agrobacterium extraction); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120 V.

Molecular characterisation of *AtBI* putative transgenic rice

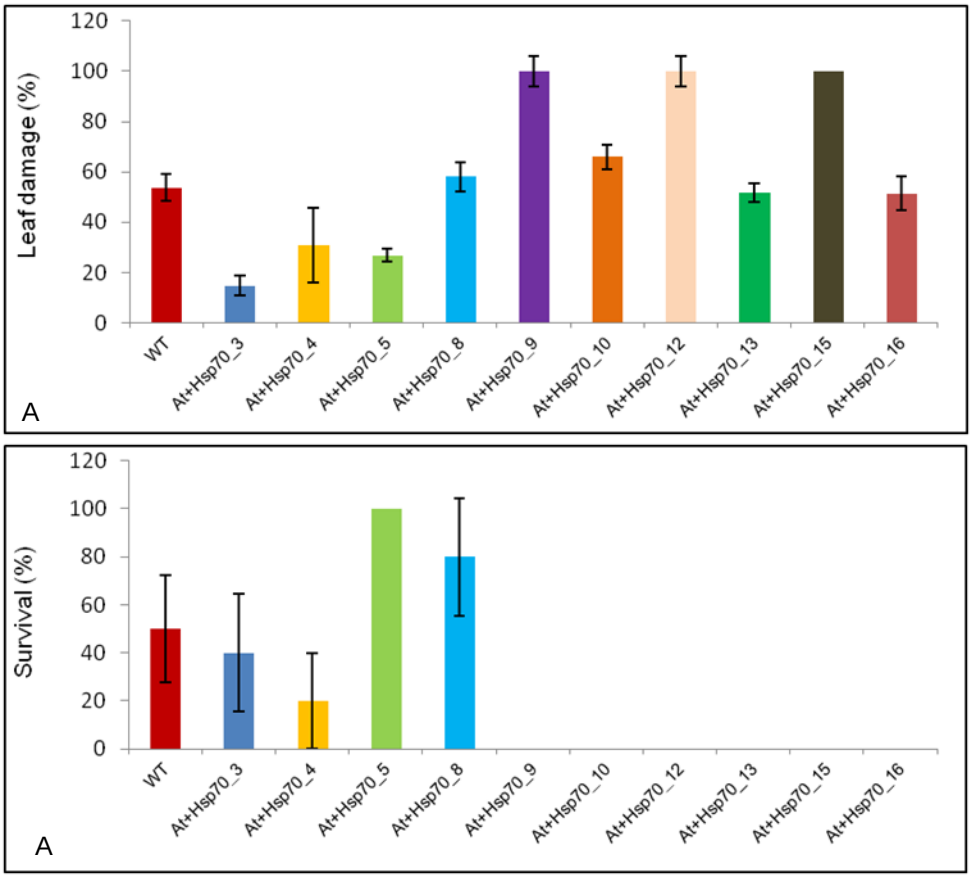


Appendix 2 - Figure 6: Molecular characterisation of *AtBI* transformed rice plants. A, B and C: PCR with *AtBI* gene specific primers; D and E: PCR with VirC primers; F, G and H: RT-PCR with *AtBI* gene specific primers. M= Marker X; N= PCR negative control; WT= wild type Nipponbare, non-transgenic negative control; P= positive control (plasmid DNA or *Agrobacterium* extraction); numbered lanes = transgenic rice lines numbers; Gel = 1.5% Agarose in TAE, run for 35 min at 120 V.

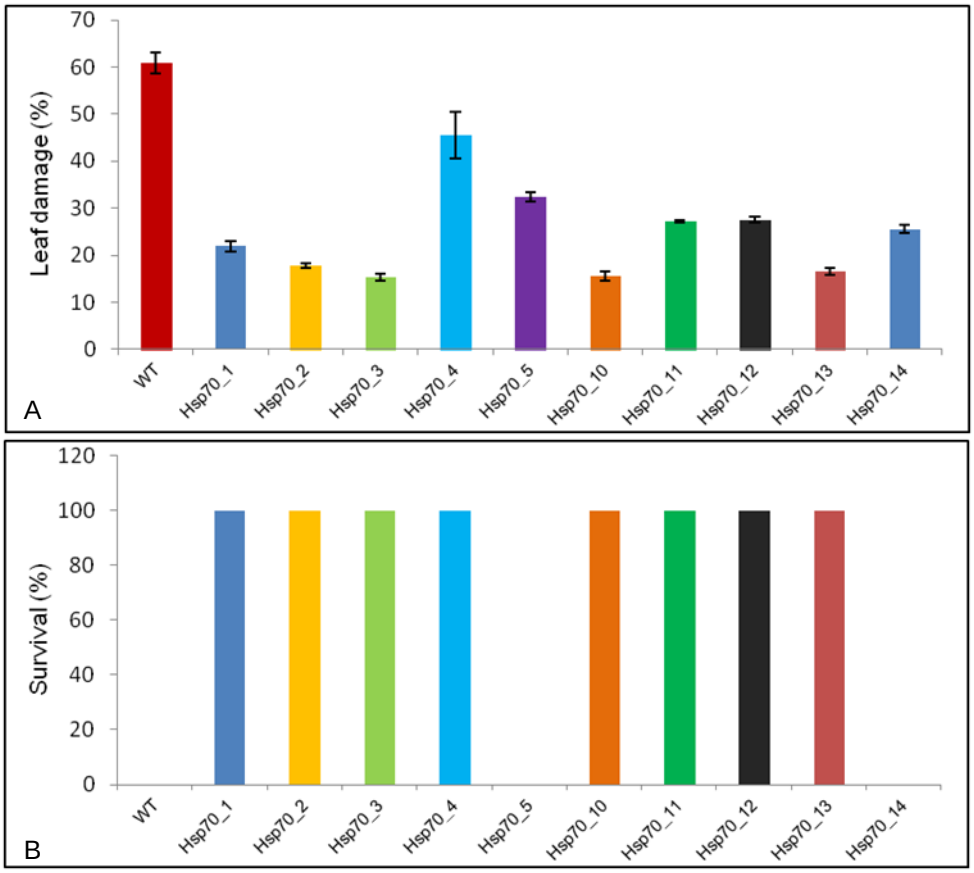
Appendix 3
Additional figures for chapter 5



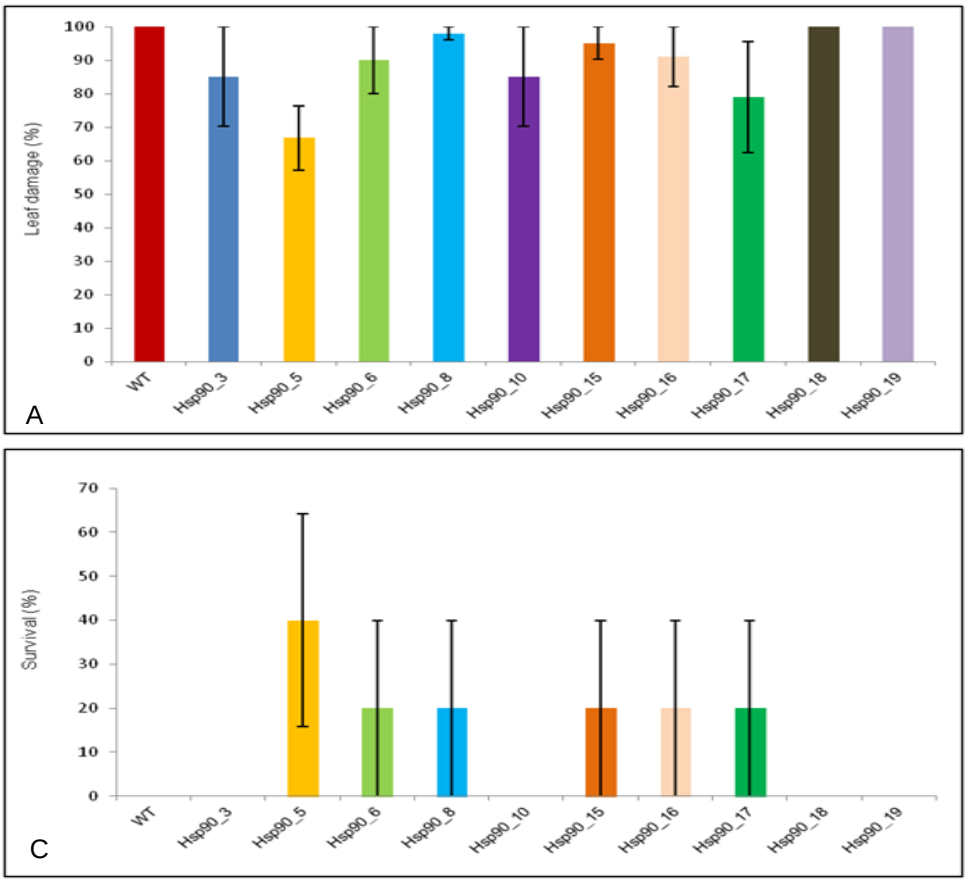
Appendix 3 – Figure 1: Salinity tolerance screening on *AtBAG4* transgenic plants in *in vivo* conditions (growth chamber-Thermoline). (A): Leaf damage and (B): Survival of WT and *AtBAG4* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.



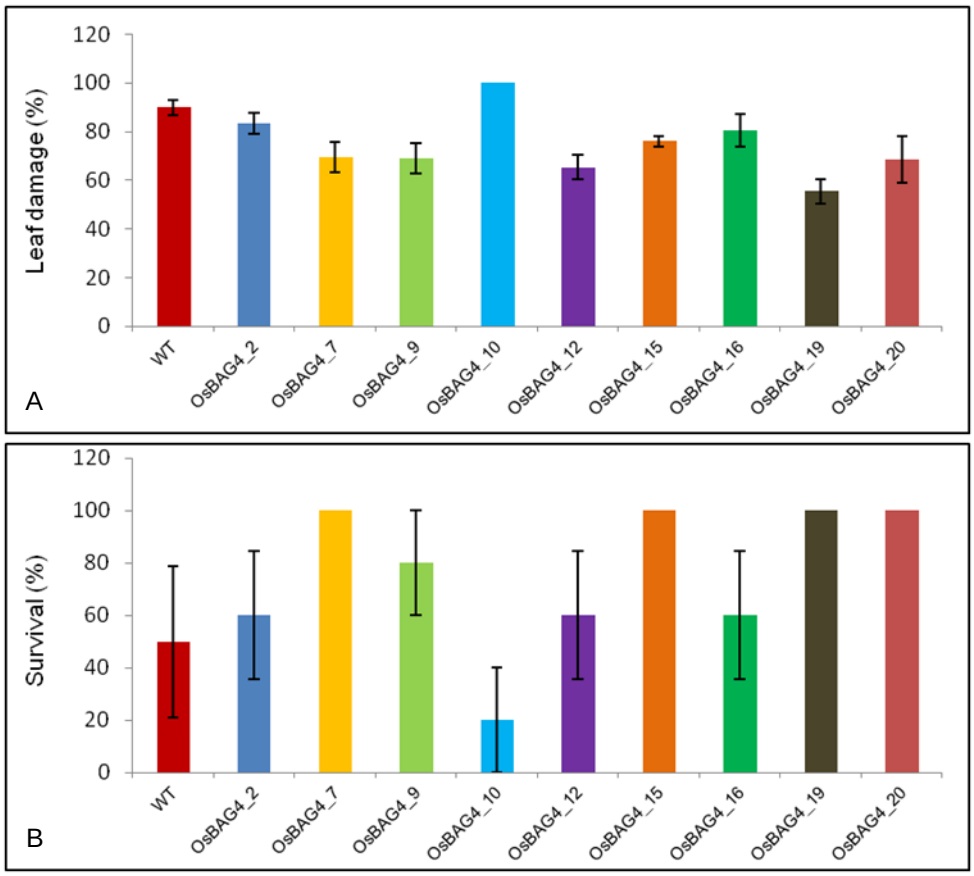
Appendix 3 – Figure 2: Salinity tolerance screening on *AtBAG4+ Hsp70* transgenic plants in *in vivo* conditions (growth chamber-Thermoline). (A): Leaf damage and (B): Survival of WT and *AtBAG4 + Hsp70* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.



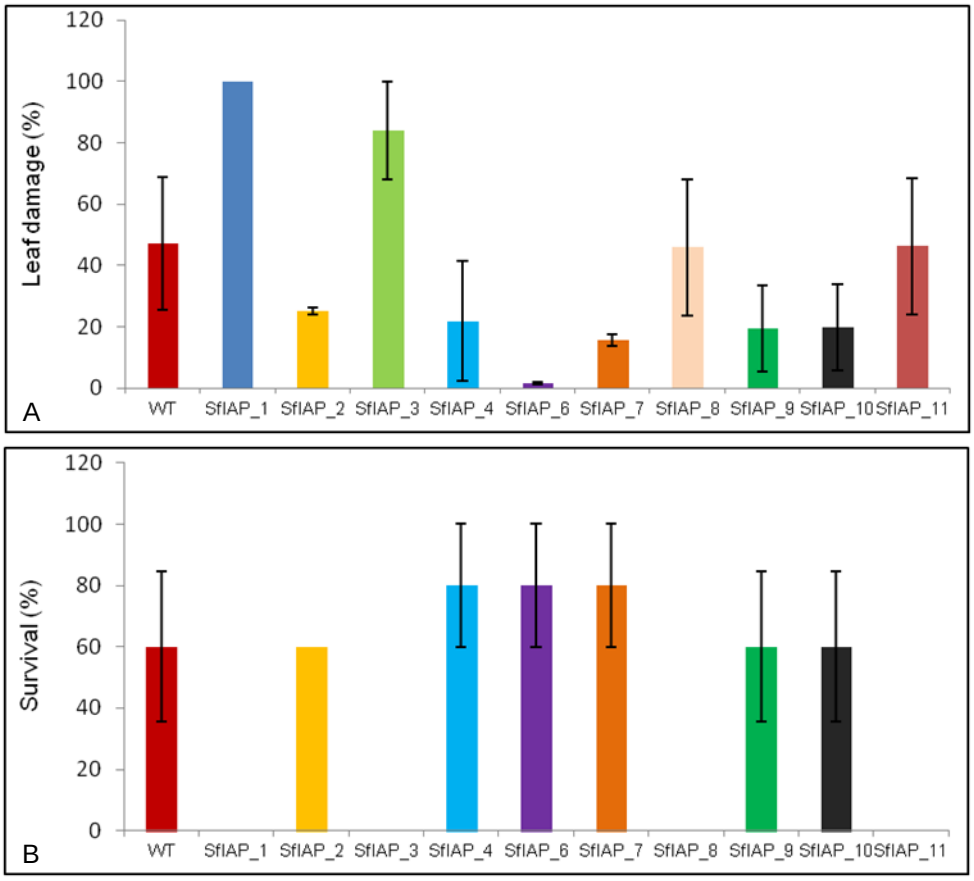
Appendix 3 – Figure 3: Salinity tolerance screening on *Hsp70* transgenic plants in *in vivo* conditions (growth chamber-Thermoline). (A): Leaf damage and (B): Survival of WT and *Hsp70* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.



Appendix 3 – Figure 4: Salinity tolerance screening on *Hsp90* transgenic plants in controlled conditions (growth chamber-Thermoline). (A): Leaf damage and (B): Survival of WT and *Hsp90* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.



Appendix 3 – Figure 5: Salinity tolerance screening on *OsBAG4* transgenic plants in *in vivo* conditions (growth chamber-Thermoline). (A): Leaf damage and (B): Survival of WT and *OsBAG4* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.



Appendix 3 – Figure 6: Salinity tolerance screening on *SfIAP* transgenic plants in *in vivo* conditions (growth chamber-Thermoline). (A): Leaf damage and (B): Survival of WT and *SfIAP* transgenic lines exposed to 100 mM NaCl. Data are mean and standard error of three replicates.