

Chapter 5

Manipulating Programmed Cell Death Pathways for Enhancing Salinity Tolerance in Crops



Ahmad Arzani

Abstract One of the key challenges for researchers is to obtain a deeper understanding of the strategies and mechanisms of plant adaptation to environmental stress that help overcome the limitations associated with climate change and loss of biodiversity. In this context, tolerance to salinity stress is one of the main abiotic factors constraining the plant growth, and production is of special importance. Programmed cell death (PCD) plays a protective role against biotic and abiotic stresses. PCD might play an important role in the maintenance of normal tissue homeostasis, regulation of cell metabolism, and remodeling of tissues after injury and infection as well as the elimination of damaged cells. Salinity stress induces an alteration in chloroplasts, mitochondria, cytoplasm, plasma membrane (PM), endoplasmic reticulum (ER), Golgi apparatus, vesicle formation and trafficking, and vacuoles formation which may result in PCD in plants. The overexpression of pro-survival genes including anti-apoptotic genes and those involved in suppression of apoptosis genes in the transgenic plants to enhance abiotic stress tolerance has been the subject of a number of investigations, particularly in the context of salinity tolerance. Therefore, the development of transformed plants for resistance to apoptosis could be an effective approach to improving salinity tolerance, while the use of complementary techniques like RNA-interfering (RNAi)-mediated gene knock-downs has been shown to be an interesting and appealing alternative. The objective of this review is to summarize the current state of knowledge on improving salinity tolerance in crop plants through manipulation of PCD pathways.

Keywords Abiotic stress · Molecular networks · Salt · Transformation · Vacuolar processing enzyme

A. Arzani (✉)

Department of Agronomy and Plant Breeding, College of Agriculture, Isfahan University of Technology, Isfahan, Iran

e-mail: a_arzani@cc.iut.ac.ir

Abbreviations

AIF	Apoptosis-inducing factor
AL-PCD	Apoptotic-like PCD
ASPP	Apoptosis-stimulating proteins of p53
ACD	Autophagic cell death
BAG	Bcl-2-associated athanogene
Bak	BCL-2 antagonist/killer-1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma2
Bcl-xl	BCL-2-like 1
Ca ²⁺	Calcium ion
ER	Endoplasmic reticulum
FB1	Fumonisin B1
GORK	Guard cell outward-rectifying K ⁺ channel
H ₂ O ₂	Hydrogen peroxide
HR	Hypersensitivity
IAP	Inhibitor of apoptosis
K ⁺	Potassium ion
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia-1
Na ⁺	Sodium ion
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
PM	Plasma membrane
RNAi	RNA interfering
ROS	Reactive oxygen species
PCD	Programmed cell death
PLC	Phospholipase C
SKOR	Outward-rectifying K ⁺ channel
VPE	Vacuolar processing enzyme

5.1 Introduction

Abiotic stress threatens staple crop production, coupled with the expanding world population necessitate not only efficient breeding strategies for developing abiotic stress tolerance crop plants but also the extension of plant production into the marginal regions including saline soil and water (Arzani and Ashraf 2016). Soil or water salinity is one of the key abiotic stresses that cause plant growth and yield reduction worldwide (Arzani 2008). Abiotic stress can be sensed and appropriate responses triggered implicating changes in growth, development, and metabolism (Conde et al. 2011). Plants' response to salinity stress represents the sum of numerous parallel-distributed processes that act to alleviate hyperosmolarity and reestablish ionic homeostatic conditions in cells (Arzani and Ashraf 2016). Programmed cell

death (PCD) is also among the evolved plant strategies to overcome these adverse conditions. PCD operates during growth and development as well as in response to various hostile environmental conditions. In this way the removal of damaged and superfluous cells can be facilitated; thus, cellular differentiation and homeostasis are supported in plants (De Pinto et al. 2012).

Therefore, PCD plays not only a protective role against abiotic and biotic stresses but also a major role in plant development. PCD is a highly coordinated process with series of steps involving specific nucleases and proteases and results in the selective elimination of the cells. In animals, autophagy, apoptosis, and programmed necrosis are the three major PCD forms, clearly characterized by their morphological features (Bialik et al. 2010; Ouyang et al. 2012). Autophagy is usually defined by the accumulation of autophagic vacuoles. Chromatin condensation, the formation of apoptotic bodies and nuclear fragmentation by the caspases as the executioners of apoptosis are the hallmarks of apoptosis. A more passive form of PCD is necrosis, which is distinguished by the presence of PM rupture and cytoplasmic swelling.

Autophagic cell death (ACD) is one of the characterized types of PCD. Autophagy process is initiated with the generation of double membrane-bound autophagosomes, encompassing cytoplasmic organelles and macromolecules, headed for recycling (Huett et al. 2010). There is increasing recognition that autophagic cells commit suicide to prevent excessive stress by undertaking cell death, which discriminates from programmed necrosis and apoptosis (Bialik et al. 2010). Nonetheless, autophagy regulates an enormous number of physiological and pathological functions such as cell differentiation, infections, starvation, cell survival, and death (Liu et al. 2010; Michaeli et al. 2016). The role of autophagy in cell death has been reviewed by Minina et al. (2014). In addition, recent advances in plant autophagy regarding mechanisms of selective autophagy, regulation of autophagy, and role of autophagy in recycling and availability of nutrients have recently been reviewed by Michaeli et al. (2016).

“Apoptosis,” as the second form of PCD, comes from a Greek root word that has been used to refer to “dropping off” the leaves or petals from a tree (Kerr et al. 1972). Given the definition roots, apoptosis is likely the most frequent type of PCD, while the biological impact of other non-apoptotic types may also be a driving force of the PCD especially in plants. Apoptosis is characterized by morphological alterations of nucleus and cytoplasm including cell shrinkage, pyknosis (DNA condensation), and karyorrhexis (nuclear fragmentation) as well as biochemical changes such as internucleosomal cleavage of DNA, a number of intracellular substrate cleavages by specific proteolysis, and phosphatidylserine externalization (Ouyang et al. 2012).

Programmed necrosis as the third type of PCD contributes to cell swelling, cell lysis, and organelle dysfunction (Wu et al. 2012). Therefore, PCD may have a role in the maintenance of tissue homeostasis, regulation of cell metabolism, and remodeling of tissues after injury and elimination of damaged cells (Wynn et al. 2013). In contrast to the wealth of knowledge regarding the molecular mechanisms of PCD, in plants the molecular networks regulating PCD are still in their infancy, and

information on this topic is scarce. This is in spite of the abundance and the importance of PCD throughout plant life span occurring as a conspicuous part of development (dPCD) as well as a response to abiotic and biotic stresses (ePCD) (Lam 2004; Huysmans et al. 2017). Although plants react differently to various abiotic stresses, the initial recognizing and induction of reactive oxygen species (ROS) generation are a common set of response to abiotic factors in all plant species (Sewelam et al. 2016). Nonetheless, production of ROS is a crucial factor in plant stress response and is also associated to in signaling of PCD (Chen et al. 2009a; Kumar et al. 2016).

In animals, ICE-/CED-like family proteases, named caspases, play a central role in PCD such as apoptosis and pyroptosis (Green 2011). In spite of the absence of caspases (abbreviation of cysteiny aspartate-specific proteases) in plants, the meta-caspases were postulated as the functional caspase homologs in plants (Bonneau et al. 2008). In recent years, significant knowledge has been gained in these areas including the characterization of two PCD types: vacuolar PCD and necrotic PCD. The apoptotic cells can be eliminated in the animal using macrophages, whereas in plant lytic vacuoles progressively engulf and digest the cytoplasmic content during vacuolar cell death. On the other hand, necrosis is an alternative form of cell death which is triggered by severe stress and characterized by mitochondrial dysfunction, premature rupture of the plasma membrane, and organized cell disassembly. Vacuolar processing enzyme (VPE) is a plant cysteine proteinase that is mediator driving the execution of various PCD and is considered as a counterpart of animal caspase 1 (Hatsugai et al. 2015).

Climate change and biodiversity loss create new challenges for developing dynamic strategies of plant adaptation to the changing environment. Stress-induced PCD markedly influences plant growth and yield, and it is an important threat to agriculture production (Mittler and Blumwald 2010). The applied and basic research on stress-induced PCD and stress responses, with the eventual goal of manipulating them for practical use, are incredibly challenging areas that attract the growing interest. Therefore, research on PCD-induced abiotic stress and stress responses in plants has strengthened significantly during the past years, and thereby understanding of regulatory mechanisms and knowledge of the immunity role will undoubtedly help to reach the eventual goal to lessen yield losses (Petrov et al. 2015; Wang et al. 2015). The objective of this review is to summarize the current state of knowledge on improving salinity tolerance in crop plants through manipulation of PCD pathways.

5.2 PCD in Response to Abiotic Stress

Plants tolerate the adverse environmental conditions by employing various adaptation mechanisms including toxin exclusion and dramatic amelioration of susceptibility (hypersensitivity) where the abiotic stress is extreme. The monitored level of applied heat stress-induced PCD in plant cells, where heat shock could be responsible for the cell death morphology, is reported in *Arabidopsis* (Hogg et al. 2011),

tobacco (Vacca et al. 2004), soybean (Zuppini et al. 2006), maize (Wang et al. 2015), and lace plant (Dauphinee et al. 2014). PCD in plant species has been induced by low or high temperature in tobacco (Koukalova et al. 1997), cucumber (Balk et al. 1999), *Arabidopsis thaliana* (Swidzinski et al. 2002), and maize (Wang et al. 2015).

Plant symptoms illustrating either undesirable or desirable response to salinity stress can be visually rated in the field. Nevertheless, a reduction in growth which is manifested by leaf burn and necrotic lesions on the leaves is a well-known indicator of exposure of plants to salinity (Tanou et al. 2009). It is suggested that leaf necrosis could be caused by the failure of the cells to avert the accumulation of Na^+ ions into the cytoplasm (Greenway and Munns 1980); in other words, leaf necrosis may be a symptom of the breakdown of ionic regulation (Subbarao and Johansen 1994). In barley, Patterson et al. (2009) compared two barley cultivars (Sahara and Clipper) exposed to 100 mM NaCl treatment and observed that “Sahara” cultivar had significantly less leaf necrosis and higher leaf Na^+ concentrations than “Clipper,” concluding that “Sahara” has a higher tolerance to accumulated Na^+ . However, despite a general consensus attributing leaf necrosis to an undesirable reaction in plant salinity stress, it is probably most disputable, and it could also be considered as the fundamental lack of knowledge about the reaction at the cellular level and entirely limited to macroscopic observations. Nevertheless, pathogen-induced HR cell death is one of the most efficient plant defense strategies, whereas pathogen-secreted toxin-induced cell death is a necrotrophic pathogen tactic for infection. Interestingly, although distinct mechanisms may regulate toxin-induced cell death and pathogen-induced cell death, both were mediated by the same VPE (Kuroyanagi et al. 2005).

Leaf margin, leaf tip burn, and leaf necrosis are among the plant responses to drought stress which can be found at the late vegetative stage. It was suggested that drought-induced leaf necrosis can be illustrated by the lack of anthocyanin pigmentation (Rosenow et al. 1983). Therefore, leaf necrosis is considered distinctly different from that of the disease symptom, where leaf necrosis is known as desirable plant reaction of host resistance named as “hypersensitivity (HR).” HR is a plant-specific PCD which is essential for defense response to restrict the spread of pathogens. Apoptosis is generally regarded as a critical physiological cell death program required for the tissue homeostasis as well as an active suicidal response to various pathological or physiological stimuli in the mammalian organism (Kabbage et al. 2017). Among the several cell death pathways that have been postulated, apoptotic-like PCD (AL-PCD) seems to be an interesting operational mode in plants leading to a corpse morphology that is similar to the apoptotic morphology perceived in animal cells (Reape and McCabe 2008). It is now established that AL-PCD is an essential cellular process in plants that have a crucial role in the developmental, stress-induced, and senescence processes as well as in response to pathogen infection (Lam et al. 1999). Apart from the developmental and biotic stimuli, it has been shown that AL-PCD is induced by abiotic stresses such as high-fluence UV radiation and heat stress (Foyer and Noctor 2005; Doyle et al. 2010).

Caspases are either involved or not involved in PCD. Accordingly, PCDs can be categorized into two groups, caspase-independent and caspase-dependent PCD

(Kroemer and Martin 2005). Apoptosis is entirely contingent upon caspase activation and thus caspase-dependent PCD represents typical apoptosis. Caspase-independent mechanism of cell death comprises paraptosis, autophagy, necrosis-like PCD, apoptosis-like PCD, and mitotic cataclysm. The non-caspase PCD was found to be associated with caspase-independent elimination, including the use of mitochondrial protein apoptosis-inducing factor (AIF) (Cande et al. 2002; Kroemer and Martin 2005; Zanna et al. 2005). Analysis of the *Arabidopsis* genome indicated the incidence of five close homologs of AIF which detected monodehydroascorbate reductases (MDARs) (Lisenbee et al. 2005) while AIF initially characterized in mammalian mitochondrial DNA (Susin et al. 1999). Of AL-PCD regulation especially relevant to plant cells is the affirming dual target sites of MDAR that is to both chloroplasts and mitochondria.

In plants, the role of mitochondrial proteins triggering cell death is still in its infancy and debatable (Reape and McCabe 2010). However, a pivotal role of the mitochondrion in plant PCD has also been implicated in plant responses to salinity stress (Yao et al. 2004; Lin et al. 2006; Chen et al. 2009b; Wang et al. 2010; Monetti et al. 2014; Hamed-laouti et al. 2016). ROS produced from the electron transport chain in mitochondrion causes dysfunction of mitochondrial lipids and proteins (Yao et al. 2004) leading to the opening of a nonspecific pore in the inner mitochondrial membrane, also called the permeability transition pore (PTP) and release of “caspase-like” proteins (Yao et al. 2004; Reape and McCabe 2010; Sirisha et al. 2014). The dysfunction of mitochondria has been proposed as a prerequisite for the establishment of NaCl-induced PCD in several plant species comprising both glycophyte (*A. thaliana*, rice, tobacco) and halophyte (*Cakile maritima*, *Thellungiella halophila*) (Lin et al. 2006; Chen et al. 2009b; Wang et al. 2010; Monetti et al. 2014; Hamed-laouti et al. 2016). There are considerable evidence and speculation that interaction between ROS and antioxidants would supply a boundary for the environmental metabolic signals mediating activation of the acclimation of the cells to stress or alternatively induction of PCD (Foyer and Noctor 2005).

A dual biological role for ROS might be attributed to the leaf senescence including regulation of the expression of senescence-associated genes and elevation of the program of cell death by direct oxidizing target macromolecules. Interestingly, taking in account the chloroplasts is one of the sources of ROS production in plants (Doyle et al. 2010) would help to resolve the question as to what extent the PCD reaction is responsive to the environmental stimuli in the plant kingdom. In addition, photoreduction of oxygen and energy transfer from triplet excited chlorophyll to oxygen, respectively, are responsible for generating superoxide radicals ($O_2^{\cdot-}$) and singlet oxygen (1O_2) in chloroplasts (Kim et al. 2012). The PCD is induced with increasing singlet oxygen (1O_2) concentration in chloroplasts, but the output of $1O_2$ -mediated chloroplast leakage and liberate of chloroplastic proteins to the cytosol on the $1O_2$ -mediated collapse of cells needs to be elucidated.

PCD, a genetically controlled cell response, has evolved under selective pressure and thus should be advantageous to the plant. Despite the recent progress in the understanding expression of the ROS-responsive genes which induced in response to abiotic and biotic stress, many challenges remain, particularly with regard to the

beneficial effects of the ROS-dependent genes influencing PCD on plant growth and resistance to both abiotic and biotic stresses. Hence, it appears possible that induction of the ROS-dependent PCD pathway in plants can be part of physiological changes that normally occur during an acclimation response to enhance stress resistance.

PCD has been perceived traditionally as a vital protective mechanism for disease resistance in plants. Today, it appears that PCD plays a fundamental role in the regulation of much more diverse cellular functions, such as in response to biotic and abiotic stress as well as developmental processes (see a recent review by Huysmans et al. 2017). It should be acknowledged that since the dissection of the PCD at the whole-plant level is difficult, most of the attempts have been made at in vitro cell assays. Here a new scenario for the biological roles of PCD at the whole-plant level to facilitate the possible explanation contributing to the induction of PCD in response to abiotic stress is presented. The results of assessment of abiotic stress tolerance in the C_4 model plant, *Setaria viridis* (L.) Beauv. accessions originated from diverse geographical areas of the world, a portion of which has been published elsewhere (Saha et al. 2016), suggested to us that PCD might have occurred in response to salinity stress (Saha et al. unpublished data). Interestingly, only one accession showed leaf necrosis after 4 weeks of treatment at 300 mM NaCl concentration (Fig. 5.1) and astonishingly ranked as one of the most salinity-tolerant genotypes. Further in vitro positron emission tomography (PET) study showed a clear difference in Na^{22} uptake and transport in this accession compared to a sensitive accession (Ariño-Estrada et al. 2017). However, observations at the cellular level are more pertinent for assessing the possible role of PCD in salinity tolerance than



Fig. 5.1 Leaf necrosis resulted from 300 mM NaCl treatment for 4 weeks in one of the *Setaria viridis* (L.) Beauv. accessions

those at the whole-plant level. It is also important to note that leaf necrosis was observed at the reproductive growth stage, while the PET imaging has been conducted at the seedling stage. The inconsistency with the previously characterized leaf necrosis, as being regarded typical for the sensitive plant to salinity stress, can be explained by developmental stage differences in high levels of NaCl accumulation occurred in the leaf cells at either the reproduction (present study) or the vegetative/seedling stage (previous studies). In addition, a complex combination of differential expression of genes encoding the photosynthetic enzymes and anatomical characterization was functionally essential for evolving more effective photosynthetic mechanism in the C_4 plants. C_4 plants exhibit higher adaptation to tropical regions than C_3 plants and assumed to have an evolutionary adaptation in hot areas of the world in response to diminishing ambient CO_2 concentration (Sage 2004). Therefore, this finding inspires us to look for an alternative interpretation. Further work on the macroscopic, microscopic, and molecular aspects of the salt-tolerant leaf necrosis structure is underway to test the hypothesis that leaf necrosis might be a favorable plant response (i.e., HR) to salinity.

As illustrated in Fig. 5.2, plant cells undergoing PCD exhibit the following features: condensation of the cytoplasm and the nucleus, the retraction of the plasma membrane from the cell wall, loss of membrane integrity, DNA laddering, release of cytochrome c from mitochondria, increase in activity of the proteases of caspase-1-like and caspase-3-like, and alterations in the K^+ efflux and ion homeostasis (Wang et al. 2010; Poor et al. 2013; Reape and McCabe 2013; Reape et al. 2015).

5.3 PCD in Response to Salinity Stress

Although to date no report on HR-like response has been documented for plant salinity tolerance, several researchers have investigated PCD at the cellular level. The influences of NaCl stress primarily on chloroplasts, mitochondria, cytoplasm, plasma membrane (PM), endoplasmic reticulum (ER), Golgi apparatus, vesicle formation and trafficking, and vacuoles have been investigated in plants. The degradation of the inner chloroplast membrane due to the NaCl-induced stress on the ultrastructure of plant leaves has been reported by Hernandez et al. (1995). The disintegration of organellar membranes (particularly the degradation of thylakoid membrane of chloroplast) in cells was found to be one of the major effects of salinity stress (Mitsuya et al. 2000). Salinity stress caused swelling of thylakoid as one of the main alterations of chloroplast ultrastructure in barley (Zahra et al. 2014) and rice (Yamane et al. 2012). The wrinkled effects of salinity on chloroplast ultrastructure have been observed at 100 mM NaCl treatment in tomato (Khavari-Nejad and Mostofi 1998) and at 200 mM NaCl treatment potato (Fidalgo et al. 2004) cells under in vitro conditions. The chloroplasts can play a similarly important role as do the mitochondria in triggering PCD, by regulating ROS signaling. The chloroplasts generate more ROS in a less efficient photosynthesis caused by salinity stress, and hence the ROS leads to cell death (Doyle et al. 2010; Kim et al. 2012; Aken and Breusegem 2015; Reape et al. 2015).

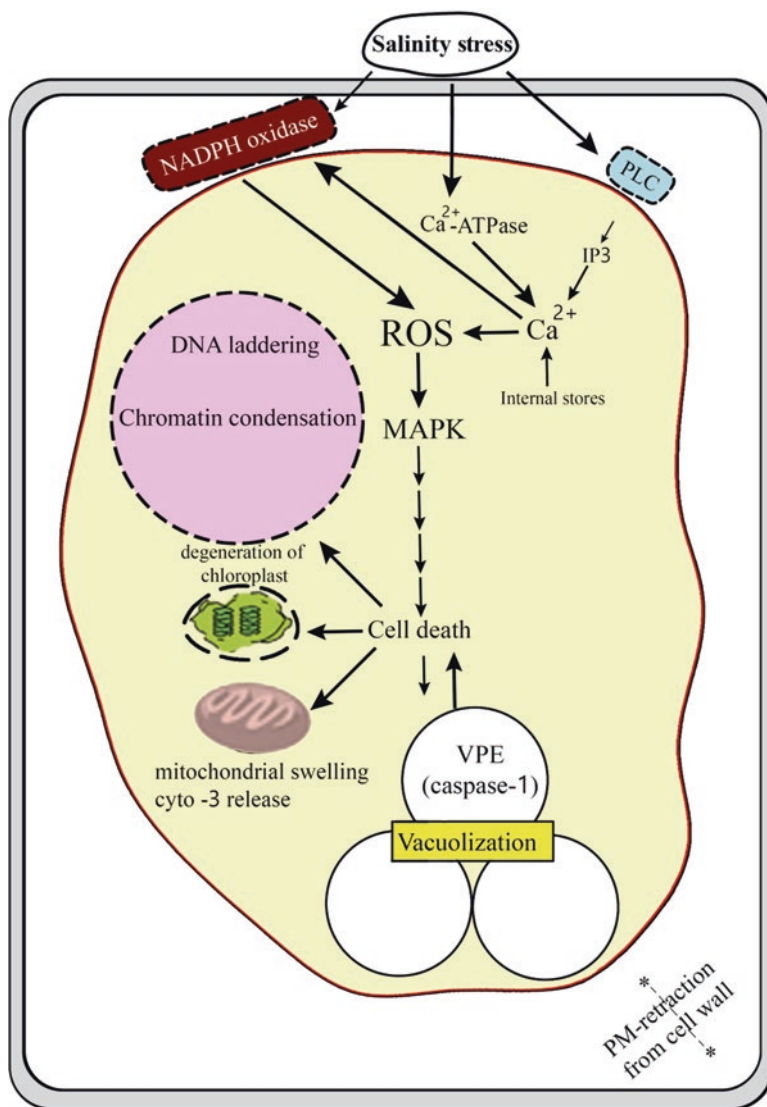


Fig. 5.2 Overview of salinity stress-induced programmed cell death (PCD) in plants. Salinity stress causes the following changes in the plant cell: (1) phospholipase C (PLC), a plasma membrane (PM) enzyme, liberates IP₃ from membrane phospholipid, which results in release of Ca²⁺ from internal stores; (2) an increased Ca²⁺-ATPase gene expression leads to increase in this membrane-bound enzyme, which provides energy to drive the cellular Ca²⁺ pump. An increase Ca²⁺ in cytosol triggers PM-bound NADPH oxidase activity, which produces superoxide in the apoplast. This reactive oxygen species (ROS) transmits death signals through mitogen-activated protein kinases (MAPK) signaling pathway. In addition, death signals can be emitted by vacuolar processing enzyme (VPE) releasing from vacuole. The ROS levels could also be increased by a sense of release of either cytochrome c (Cyt c) or cytochrome f (Cyt f) from mitochondrion. Signals are transmitted to nucleus and ultimately cell execution proteins is synthesized, which results in PCD

A profound downgrading in cytoplasmic streaming was observed at 100 mM NaCl treatment. In plants, cytoplasmic streaming is a marked attribute of cell compartment, in which vesicles and organelles transport along the strands of cytoplasm containing actin filaments. Cytoplasmic streaming indirectly explores some features of the metabolic function in the cell (Mansour and Salama 2004; Shimmen and Yokota 2004; Pieuchot et al. 2015). It has been postulated that salinity stress causes an increase in cytoplasmic Ca^{2+} , which may detain cytoplasmic streaming through the support of internal Ca^{2+} stores (Knight 2000). Calcium does not solely play a key role in signaling function but might also trigger PCD and mediate death-specific enzymes in both animal and plant cells (Boursiac et al. 2010). In plants, Zhu et al. (2010) used the RNA-interfering (RNAi) silencing of the Ca^{2+} pump *NbCA1* and showed that endomembrane Ca^{2+} pump operates in mediating the kinetics of a PCD pathway triggered by the pathogen. As shown in Fig. 5.2, a plasma membrane enzyme, phospholipase C (PLC), liberates IP3 from membrane phospholipid. The release of Ca^{2+} from internal stores is in turn mediated by IP3. It is hence suggested that the amplification of the stress signals during stress through enhancing the level of the stress-induced Ca^{2+} signal could be mediated by a stress-induced PLC gene (Hirayama et al. 1995). Likewise, cytosolic Ca^{2+} was increased by salinity stress in tobacco cells after a few minutes of treatment, and membrane potential of mitochondria was diminished before the occurrence of PCD (Lin et al. 2005). Pretreatment of protoplasts with Ca^{2+} chelators such as EGTA or LaCl_3 delayed salinity stress-induced PCD through the increase in cytosolic Ca^{2+} implying an essential role for Ca^{2+} in the triggering of PCD in plants (Lin et al. 2005; Li et al. 2007a).

The function of Ca^{2+} -ATPases is amended substantially in response to abiotic stress in plants. Ca^{2+} -ATPase membrane-bound enzyme hydrolyzes ATP to supply energy to run the cellular Ca^{2+} pump. The transcript levels of genes encoding a putative ER Ca^{2+} -ATPase have been increased by salinity stress in tobacco cells (Perez-Prat et al. 1992) and in tomato (Wimmers et al. 1992). In tobacco, Perez-Prat et al. (1992) observed an increased Ca^{2+} -ATPase gene expression in both adapted and unadapted cells cultured at 428 mM NaCl, while the levels of transcripts were much higher in adapted cells than in unadapted cells. Likewise, an enhanced Ca^{2+} -ATPase transcript has been observed in plants treated with 50 mM NaCl for 24 h in tomato (Wimmers et al. 1992). In addition, it was suggested that the regulation of expression of Ca^{2+} -ATPase gene could be regulated by the RNAi such as 22 nt miR4376 in tomato (Wang et al. 2011). It has been demonstrated that Ca^{2+} -ATPase comprises five functional domains which are named based on their function or position. Hence, they include P-domain (the catalytic core), A-domain (actuator domain), N-domain (nucleotide-binding domain), as well as S- and T-domains (membrane-embedded domains). The ATP hydrolysis is performed by the cytoplasmic domains (A, P, and N), whereas the T- and S-domains play role in the ion transport, together with conformational changes through tertiary contacts and linkers (Palmgren and Nissen 2011).

It is argued that the burden of PCD categorizations should be put on the tonoplast disruption relating to cytoplasmic clearing since the vacuole elaborates on various plant PCD types including HR, differentiation of tracheary elements,

senescence of various plant tissues, and so on (van Doorn 2011). Accordingly, the only terminology of two classes of PCD comprising necrosis and vacuolar cell death was suggested by van Doorn (2011). Vacuolar cell death is caused by a progression of an autophagy-associated phenomenon and the release of hydrolases from ruptured vacuoles (Bagniewska-Zadworna and Arasimowicz-Jelonek 2016). In addition, vacuole disintegration and tonoplast disruption are extremely rapid and irreversible processes and represent an unequivocal step in a cell headed for death in plant roots (Bagniewska-Zadworna and Arasimowicz-Jelonek 2016). In *Physcomitrella patens*, it was shown that knockout of vacuolar ACA pump (*PCA1*) gene could lead to higher sensitivity to salinity stress, because of diminished level of NaCl-triggered Ca^{2+} in the cytosol (Qudeimat et al. 2008). Furthermore, the rapid enlargement of vacuolar volume has been observed under in vitro salinity conditions in mangrove [*Bruguiera sexangula* (Lour.) Poir.] cells and barley (*Hordeum vulgare* L. cv. Doriru) root meristematic cells (Mimura et al. 2003). Paradoxically, this phenomenon was not confirmed in pea (*Pisum sativum* L.) (Mimura et al. 2003). The accumulation of Na^+ ions in the central vacuole causes enhanced vacuolar volumes and is considered as one of the strategies employed by the cell in response to salinity stress.

The detrimental effects of ROS on plant tissues are being increasingly recognized, but the biochemical mechanism linking the ROS production and PCD is poorly known. The main enzymes responsible for superoxide anion generation are cell wall-associated peroxidases and apoplastic plasma membrane-bound NADPH oxidases which are regulated by various environmental and developmental stimuli (Gechev et al. 2006; Sagi and Fluhr 2006). Salinity stress causes an increase in Ca^{2+} in the cytosol and triggers PM-bound NADPH oxidase activity, which produces superoxide in the apoplast (Monetti et al. 2014). Nevertheless, ROS are involved in signaling pathways and mediating PCD activation (Chen et al. 2009a; Mittler 2017) as they influence the activity of mitogen-activated protein kinase (MAPK), which is able to induce several nuclear transcription factors (Fig. 5.2). The overaccumulation of ROS in the cells causes oxidative cellular damage and cell death through reacting with different cellular components. In addition, it is now appreciated that ROS involves in triggering a programmed or physiological pathway for cell death that were not previously thought to be associated straightforwardly with executing cells through oxidation (Mittler 2017). In general, the quantity of ROS accumulation can activate opposing pathways leading to either survival or PCD. A supportive data was obtained by *Chlamydomonas reinhardtii* subjected to hydrogen peroxide (H_2O_2) treatment. The programmed cell suicide event is shown to be triggered by enhanced level of H_2O_2 which resulted in caspase-3-like protein recruitment, DNA laddering, and increased cleavage of PARP (a poly-(ADP)-ribose polymerase-like enzyme) (Vavilala et al. 2015). In general, a variety of genes, transcription factors, and signaling molecules associated with the inducible expression of genes mediating salinity-induced PCD. Certainly, this series includes some of those appointed to control ROS accumulation, release of Cyt c and Ca^{2+} , and mitochondrial permeability transition (Lin et al. 2005, 2006; Li et al. 2007b; Chen et al. 2009a; Monetti et al. 2014; Biswas and Mano 2015; Bahieldin et al. 2016; Pan et al. 2016).

PM and its proteins involved in a wide spectrum of cellular processes including signal perception-transduction and cellular homeostasis, which are regulated by various developmental and environmental stimuli (Mansour 2014; Mansour et al. 2015). Salinity stress-induced PCD has also associated with the retraction of the PM from the cell wall, most likely due to raising the osmotic pressure leading to plasmolysis (Dauphinee et al. 2014; Zhang et al. 2016). Salinity stress is also known to cause membrane disturbance resulting in the loss of membrane integrity, which allows intracellular components to leak out of the cells. NaCl-induced K^+ efflux is believed to be responsible for the effect of salinity on the loss of membrane integrity and non-specific membrane damage in a number of species (Shabala et al. 2006; Cuin et al. 2008; Demidchik et al. 2010, 2014).

The cell membrane is the first living tissue that perceives signals of abiotic stresses including salinity and because of important role and abundance of lipids, which is one of the most sensitive ROS targets (Mansour 2014; Mansour et al. 2015). Salinity stress may cause electrolyte leakage as it is one of the integral parts of the plant's response to stress. Demidchik et al. (2014) suggested that the main consequence of electrolyte leakage is stress-induced K^+ release which outwardly rectifying K^+ channels activated by ROS are responsible for this in plant cells. The K^+ loss results from ion channel-mediated K^+ efflux can induce PCD (Demidchik et al. 2010, 2014). The phenomenon of ROS generation, leading also to PCD, is not an independent process but may largely be influenced by the K^+ loss in conditions of stress-induced electrolyte leakage. In plant cells, highly selective outward-rectifying potassium channel (SKOR), guard cell outward-rectifying K^+ channel (GORK), and annexins catalyzing K^+ efflux can be activated by ROS ($\bullet OH$ and H_2O_2). In addition, under salinity and oxidative stress, PCD could be induced by GORK-mediated K^+ efflux (Demidchik et al. 2014).

The mitochondrion has recently acquired renewed attention in toxicology because of its crucial role in signaling and mediating cell death in certain cell types. It was proposed that mitochondria can also be associated in signaling pathways relevant to PCD induction, which is the mitochondrial release of cytochrome c and Ca^{2+} into the cytosol where they trigger cell death caspases (Lin et al. 2005, 2006; Reape et al. 2015). In plants, the permeability of the mitochondrial membrane increases due to mitochondrial generated ROS which releases apoptotic mediators such as cytochrome c (see Fig. 5.2; Tiwari et al. 2002). Salinity stress caused partially or fully inactivation of the photosynthetic reaction centers which results in the downgraded conversion of light energy into chemical energy (Turan and Tripathy 2015), leading to increased ROS formation (Ambastha et al. 2017). In rice, a recent study of the ultrastructure of seedling leaves has proposed the involvement of chloroplasts in PCD induced by salinity stress (Ambastha et al. 2017). Salinity stress also reported inducing cell death in isolated protoplasts of tobacco (Lin et al. 2006) and rice (Ambastha et al. 2017).

Salinity stress may cause substantial amendments in the Golgi bodies and disordered vesicle formation and trafficking in plant cells. In *Arabidopsis thaliana*, high NaCl levels promoted vesicle formation, which may imply elevated levels of macro-

autophagy, plausibly to recycle degenerated intracellular elements (Liu et al. 2009). The ultrastructural alterations were observed in not only mitochondria but also Golgi bodies, which eventually resulted in autophagy in a halophyte plant, *Thellungiella halophila* (Wang et al. 2010).

5.4 Types of NaCl-Induced Cell Death in Plants

The first approach taken by plant cells for apoptosis-like PCD through DNA fragmentation does not include the NaCl-induced osmotic stress because osmotic stress cannot be accounted for the activation of endonucleases. DNA laddering results in PCD were only found in cells with NaCl and KCl treatment and not in sorbitol-treated cells, indicating that ionic component is to be associated with the PCD (Affenzeller et al. 2009; Vavilala et al. 2016). Hence, the effects of Na⁺ ion toxicity have attracted much greater interest as an apt target for dissecting the PCD and the salinity tolerance mechanisms than the osmotic effects (Arzani and Ashraf 2016). Potassium serves as a macronutrient with important roles in a variety of physiological processes in plants, including nucleic acid and protein synthesis. The second approach taken for NaCl-induced PCD is related to ion homeostasis disturbance that results from an excessive amount of Na⁺ and a K⁺ deficit in the cytosol. It is postulated that reduction of cytosol K⁺/Na⁺ ratio in the cells would be an essential component in triggering PCD (Joseph and Jini 2010). Under saline conditions, the influx of Na⁺ through plasma membrane by the nonselective cation channels (NSCC) causes plasma membrane depolarization which leads to K⁺ leakage from the cell through depolarization-activated potassium outward-rectifying channels (KORs) (Shabala 2009; Demidchik 2014; Kim et al. 2014). K⁺ deficit results from the release of K⁺ from the cytoplasm, which in turn may trigger the effectors of PCD, cysteine proteases (Shabala 2009; Demidchik et al. 2010). The final way taken for the PCD is associated with NaCl-induced oxidative stress, generating ROS, which causes PCD through the deleterious effects to nucleic acids, proteins, lipids, and enzymes, as well as increased peroxidation of membrane lipids and membrane leakage. The enhanced ROS and reduced mitochondrial membrane potential were observed in protoplasts of *Nicotiana tabacum* treated with salinity stress. Similarly, increase in cytosolic Ca²⁺ was found a few minutes after salinity treatment, and decreased membrane potential of mitochondria was also noticed before the occurrence of PCD in tobacco BY2 cells (Monetti et al. 2014). In *Thellungiella halophila*, salinity stress-induced PCD through caspase-like proteases under in vitro conditions was observed. Cells undergoing PCD exhibited attributes such as DNA laddering, retraction of plasma membrane from the cell wall, Cyt c release, and increase in caspase-3-like protease activity (Wang et al. 2010). The cells subjected to in vitro salt stress (500 mM NaCl) showed PCD symptoms such as DNA laddering, nuclear condensation, reduced cell viability, and positive TUNEL in wheat (Rezaei et al. 2013).

5.5 Engineering PCD Pathway to Enhance Salinity Tolerance

Transgenic plants regenerated from the cells transformed with recombinant DNA are becoming increasingly pervasive and will approach ubiquity in research laboratories. The production of transgenic plants has become commonplace and has been employed as a routine tool for the introduction of a foreign or related gene to an agronomically important crop variety and for elucidating mechanisms of gene expression. Transgenic plants expressing novel salinity tolerance genes can be employed to improve crop performance under saline conditions (Arzani and Ashraf 2016). The prosperous development of transgenic plants with the desired trait, such as salinity tolerance, relies on object identification of the genes that are key players in governing that trait. Although overexpression of the majority of salinity tolerance genes being in model plants such as tobacco or *Arabidopsis* plants, the list of candidate genes mainly associated with Na⁺ exclusion in the transgenic plants from both *Arabidopsis* and field crops has been compiled by Arzani and Ashraf (2016). The various strategies to engineer PCD pathways that enhance salinity tolerance are as follows:

5.5.1 Manipulation of Anti-PCD Genes

The development of transformed plants for resistance to apoptosis could be an effective approach to improving salinity tolerance. It has been revealed that the generation of transgenic plants expressing anti-PCD genes led to enhancing biotic and abiotic tolerance. The family of apoptosis-stimulating proteins of p53 (ASPP) with iASPP, as the most evolutionary conserved member (Sullivan and Lu 2007), is one of the most promising candidates for use as anti-apoptotic factors. The ASSP family members bind to key player proteins regulating cell growth (APCL, PP1) and apoptosis (p53, p63, p73, Bcl-2, and RelA/p65) and most likely regulate the apoptotic function of p53, p63, and p73 (Sullivan and Lu 2007). The iASPP proteins only inhibit the apoptotic function of P53 (including p63 and p73) and do not impact the cell-cycle arrest activity of p53.

The expression of different apoptotic Bcl-2 genes can be activated by p53 as a transcription factor (Levine and Oren 2009).

In mammals, the family of Bcl-2 (B-cell lymphoma2) proteins, localized in the outer mitochondrial membrane, is a key regulator of mitochondrial outer membrane permeabilization (MOMP) and subsequent apoptosis. Bcl-2 proteins comprise both anti-apoptotic member (Bcl-2, Bcl-XL, and Mcl-1) proteins and the pro-apoptotic (Bax, Bak, and Bad) members (Le Pen et al. 2016). They exert influence on balancing the mitochondrial membrane potential. Although the members of Bcl-2 family, caspases, and the members of the inhibitor of apoptosis (IAP) family are important regulators of apoptosis in animals, conservation cycle does not evidently occur in plants. However, plant PCD and animal apoptosis have many common morphological resemblances. The expression of anti-apoptotic (pro-survival) genes has generally

been investigated in the model or crop plants using animal and plant target genes. For example, tomato plants were transformed with animal anti-apoptotic *Bcl-xL* and *Ced-9* genes and led to retarded cell death or lack of cucumber mosaic virus symptoms (Xu et al. 2004). Tomato and tobacco plants expressing *SfIAP* gene from an insect (*Spodoptera frugiperda*) preclude cell death caused by the necrotrophic fungus *Alternaria alternata*, salinity, heat, and fungal toxin fumonisin B1 (FB1) treatment (Li et al. 2010). Likewise, expression of *SfIAP* gene has enhanced salinity tolerance in rice (Hoang et al. 2014).

The family of Bcl-2-associated athanogene (BAG) proteins is conserved in the eukaryotic organisms. The anti-cell death activity of BAG has been described through constitutive overexpression of *AtBAG4* in rice (Hoang et al. 2015). All BAG proteins share a common signature motif at the C terminus (BD domain), which directly mediates binding to the Hsp70/Hsc70 heat shock proteins (see the recent review by Kabbage et al. 2017). The ubiquitous 70 kDa Hsp70 family proteins play a crucial role, as molecular chaperones in mediating the refolding of denatured proteins and the folding of newly synthesized proteins. Therefore, Hsp70 proteins can assist anti-apoptotic Bcl-2 proteins through protein-protein interaction at marked essential points to suppress apoptosis pathways (Joly et al. 2010). Overexpression of *Hsp70* derived from *Citrus tristeza* virus in rice conferred tolerance to salinity stress (Hoang et al. 2015). In rice, transgenic plants overexpressing *Bcl-2* gene significantly alleviated PCD symptoms through reduction of NaCl-induced K⁺ efflux and inhibition of the expression of VPEs (Kim et al. 2014).

5.5.2 Overexpression of Inhibitor of Apoptosis (IAP) Genes

Although plant genomes do not contain IAPs, tolerance to cell death induced by stress has been detected in the ectopic expression of viral and animal IAPs in plants. In tobacco, transgenic plants overexpressing the baculovirus *Orgyia pseudotsugata* nuclear polyhedrosis virus IAP (OpIAP) protein were resistant to tomato-spotted wilt virus and the necrotrophic fungi *Cercospora nicotianae* and *Sclerotinia sclerotiorum* (Dickman et al. 2001).

5.5.3 Interfering RNA (RNAi)-Induced Apoptosis Gene Silencing

Alternatively, small interfering RNA (siRNA)-induced transcriptional gene silencing system can be used to knockdown or knockout the expression of apoptotic genes. Long noncoding (lncRNA) and microRNAs miRNA are the two foremost subtypes of regulatory noncoding RNA (ncRNAs). They comprehensively regulate the interrelated steps and mediate the regulated cell death including apoptosis and necrosis through their interaction as well as in association with assorted

intracellular components (Su et al. 2016). Cytoplasmic mRNAs can be silenced by miRNAs through either promoting translation repression, expediting mRNA decapping, or triggering an endonuclease cleavage (Bagga et al. 2005; Wu et al. 2006; Pasquinelli 2012; Nam et al. 2014). As such, the alternative cleavage and polyadenylation mechanisms that produce varied 3'-UTR isoforms influence the efficiency of miRNA targeting, while the translation inhibition is dependent on the CCR4-NOT complex and the miRNA-induced silencing complex (miRISC), which causes the recruitment of eIF4A2 and locked on the mRNA region between the start codon and the pre-initiation complex (Nam et al. 2014). In humans, loss of microRNA-mediated repression of *Bcl2* gene expression, in many instances, causes chronic lymphocytic leukemia (CLL) (Anderson et al. 2016). RNAi-mediated silencing of *P69B* a substrate of two matrix metalloproteinases (SI2/3-MMP) from tomato and located upstream of *SI2/3-MMP* in tomato transgenic plants led to reduced expression of the cell death marker genes *tpoxC1*, *hsr203j*, and *Hin1* (Zimmermann et al. 2016). VPEs are cysteine proteinases that function as key moderators of stress-induced PCD in plants. Suppression of *OsVPE3* gene in the transgenic lines of rice led to improved salinity tolerance (Lu et al. 2016). Transgenic rice plants overexpressing Bcl-2 resulted in inhibition of salt-induced PCD through a significant reduction of the transient increase in the cytosolic Ca^{2+} , suppression of *OsVPE2* and *OsVPE3*, expression, and inhibited K^+ efflux across the plasma membrane (Kim et al. 2014). In *Arabidopsis*, inhibition of FB1-induced cell death was observed using loss of function mutation in all four VPE (α VPE, β VPE, γ VPE, and δ VPE) genes (Kuroyanagi et al. 2005). In *Nicotiana benthamiana*, silencing VPE_{1a} and VPE_{1b} diminished sensitivity to cell death caused by the elicitor of bacterial hairpin but did not affect cell death caused by ethylene-inducing peptide1 (Nep1), the fungal necrosis, and the elicitor of oomycete boehmerin (Zhang et al. 2010). Therefore, although VPE_{1a} and VPE_{1b} may involve in elicitor-triggered immunity, they execute cell death in a context-specific manner.

5.5.4 Repression of ROS-Induced PCD

The signaling and biological roles of ROS (e.g., $\cdot\text{O}_2^-$, H_2O_2 , $\cdot\text{OH}$, $^1\text{O}_2$) in higher-order eukaryotic cells are still controversial and are unclear. Paradoxically, it is conceivable that both the stimulatory and inhibitory capacities of ROS can be related to its conspicuous biological properties, which comprise half-life, chemical reactivity, and lipid solubility (D'Autreaux and Toledano 2007). ROS, on the one hand, appear to act as signaling molecules that mediate intercellular pathways controlling cell growth, differentiation, inflammation, survival, and immunity when available at a moderate levels (D'Autreaux and Toledano 2007; Foyer and Noctor 2016; Gilroy et al. 2016; Mittler 2017). On the other hand, the excessive generation of ROS results in oxidative damage to essential biological molecules such as DNA, RNA, membranes (lipid peroxidation), and proteins, which causes the demolition of cellular integrity through amending their functionality. During normal homeostasis,

endogenous ROS production mainly takes place in the Ero1-PDI oxidative folding system in ER, the electron transport chain in the mitochondrion, and the membrane-bound NADPH oxidase (NOX) complex (Sevier and Kaiser 2008). Considering PCD can be attained by enhanced ROS accumulation and abiotic conditions like salinity stress, genetic programming of cellular metabolism in plants, repressing salinity stress-induced PCD, would lead to an equal relative increase in yield under saline conditions (Xu et al. 2004; Mittler and Blumwald 2010; Hoang et al. 2015). Constitutive overexpression of maize *ABP9* (ABRE-binding protein 9) gene in transgenic *Arabidopsis* plants downregulated cellular ROS content induced by stress and ABA and diminishes cell death (Zhang et al. 2011). Interestingly, aside from the key roles of SOS_1 and SOS_2 in salinity tolerance (see the recent review by Arzani and Ashraf 2016) under salinity stress conditions, they influence the expression of other genes involving in the ROS scavenging activity. Verslues et al. (2007) reported the physical interaction between SOS_2 and $NDPK_2$ (H_2O_2 signaling protein) with CATs. Expression of a baculovirus anti-apoptotic protein, p35, has been observed to suppress PCD induced by H_2O_2 in insect cells through clearly sequestering ROS. It was speculated that the ROS contents can be regulated by either *p35* gene directly or *Hsp70* and *AtBAG4* genes indirectly. In tobacco, transgenic plants expressing p35 (gene from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV)) enhanced abiotic stress tolerance including salinity, which was associated with the capacity to scavenge ROS by p35 (Wang et al. 2009). As a final overview, Table 5.1 summarizes the reported candidate genes involving in PCD pathway and while overexpressed in the transgenic plants to enhance salinity tolerance.

5.6 Concluding Remark

The molecular mechanisms of salinity-induced PCD via autophagy cell death (ACD) remain to be elucidated by studying the autophagic vacuolization of the cytoplasm and the dynamics of the vacuole in various plant species. Apoptosis and anti-apoptosis phenomena occur as a consequence of the successive development of genetic alterations in multiple genes and epigenetic changes that regulate activities of apoptotic caspases responsible for the execution of various PCD. Therefore, another area of research which illuminates these phenomena is that which explores DNA modifications and dynamic histones related to crucial alterations of genome expression during the PCD. Hence, studies to elucidate the common and innovation features existing between abiotic-induced PCD and pathogen-induced PCD will assist in understanding the physicochemical details of apoptotic-like PCD which needs for selectively manipulating target cell in each of the two conditions.

Nevertheless, improving salinity tolerance through manipulation of PCD pathways in crop plants could be attained by:

1. Upregulation/overexpression of anti-apoptosis genes and downregulation or suppression of pro-apoptosis genes which are functionally indispensable and structurally conserved throughout the plant and animal kingdoms. For instance, the

Table 5.1 Candidate genes involving in programmed cell death (PCD) pathway overexpressed^a in the transgenic plants to enhance salinity tolerance

Gene	Function	Origin species	Transgenic plant species	Effects on PCD/mechanism	References
<i>Ced-9</i> homolog of <i>Bcl-2</i>	Anti-apoptotic	<i>Caenorhabditis elegans</i>	<i>Nicotiana benthamiana</i>	Enhanced tolerance to salinity and oxidative stress by altering H ⁺ and K ⁺ flux hypothetically by K ⁺ -permeable channels (KOR and NSCC)	Shabala et al. (2007)
<i>SOS1</i> salt overly sensitive 1	Efflux of Na ⁺ from cells	<i>Homo sapiens</i>	<i>Arabidopsis thaliana</i>	<i>Arabidopsis sos1</i> mutants displayed higher PCD symptoms, showing salinity-induced PCD is regulated by ion disequilibrium	Huh et al. (2002)
<i>Bcl-2</i> B-cell lymphoma	Anti-apoptotic	<i>Homo sapiens</i>	<i>Oryza sativa</i>	Suppressed K ⁺ efflux across the PM by blocking NSCCs, reduction of cytoplasmic Ca ²⁺ , and inhibited the expression of <i>OsVPE2</i> and <i>OsVPE3</i> , leading to the alleviated salinity-induced PCD	Deng et al. (2011)
<i>Bcl-2</i>	Anti-apoptotic	<i>Homo sapiens</i>	<i>Oryza sativa</i>	Alleviated PCD symptoms through reduction of NaCl-induced K ⁺ efflux, inhibition of the expression of VPEs	Kim et al. (2014)
<i>Hsp70</i> heat shock protein	Assist anti-apoptotic <i>Bcl-2</i>	<i>Citrus tristeza virus</i>	<i>Oryza sativa</i>	Enhanced salinity tolerance via alleviation of PCD	Hoang et al. (2015)
<i>IAP</i>	Inhibitor of apoptosis	<i>Spodoptera frugiperda</i> (SfIAP)	<i>Oryza sativa</i>	Enhanced salinity tolerance via precluding PCD	Hoang et al. (2014)
<i>BAG</i>	<i>Bcl-2</i> -associated athanogene	<i>A. thaliana</i> (AtBAG4)	<i>Oryza sativa</i>	Enhanced salinity tolerance via precluding PCD	Hoang et al. (2015)
<i>VPE3</i> RNAi suppressed	Vacuolar processing enzymes (VPEs)	<i>Oryza sativa</i> (<i>OsVPE3</i>)	<i>Oryza sativa</i>	Enhanced salinity tolerance with downregulated <i>OsVPE3</i>	Lu et al. (2016)

Gene	Function	Origin species	Transgenic plant species	Effects on PCD/mechanism	References
<i>p35</i>	Anti-apoptotic	Baculovirus (<i>Autographa californica</i> multiple nucleopolyhedrovirus (AcMNPV))	<i>Nicotiana tabacum</i>	Enhanced salinity tolerance with ability to scavenge ROS	Wang et al. (2009)
<i>p35</i>	Anti-apoptotic	Same as above	<i>Oryza sativa</i>	Enhanced salinity tolerance with ability to scavenge ROS	Hoang et al. (2015)
<i>MKK4</i>	A MAPK kinase	(<i>GlmMCK5</i>)	<i>Nicotiana benthamiana</i>	Reduced salinity tolerance via increase in H ₂ O ₂ -induced HR-like PCD	Zhang et al. (2012)
<i>HSPR</i>	Heat shock protein related	<i>A. thaliana</i> (<i>AtHSPR</i>)	<i>A. thaliana</i>	Protect cells from death upon salinity stress	Yang et al. (2015)
<i>OsSRP-LRS</i> RNAi suppressed; <i>AtSerp1</i> homolog	Serine protease inhibitors (serpins)	<i>Oryza sativa</i>	<i>Oryza sativa</i>	Negatively regulates stress-induced cell death	Bhattacharjee et al. (2015)

^aUnless otherwise stated

protein members of the Bcl-2 family comprised both pro-apoptosis and anti-apoptosis genes that regulate the release of cytochrome c and other apoptotic alterations in the mitochondrion.

2. The repression of the plant caspase-like enzymes including VPEs, metacaspases, and phytaspases also called subtilisin-like proteases (subtilases) are alternative candidates for “silencing” or “downregulation” by emerging genetic and epigenetic tools.

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