EFFECTS OF ABSCISIC ACID AND JASMONIC ACID ON TOLERANCE AGAINST DROUGHT AND SALT STRESS IN APPLE SEEDLINGS (*Malus domestica*)

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ABA	abscisic acid
Abz-E_3M	abscinazole-E3M
JA	jasmonic acid
NaCl	Sodium chloride
PDJ	*n*-propyl dihydrojasmonate
FW	fresh weight
DW	distilled water
DAT	Days after treatment
HPLC	high performance liquid chromatography
GC-MS-SIM	gas chromatography-mass spectrometry-selected ion monitoring
RT-PCR	Reverse transcription-polymerase chain reaction
pH	Power of hydrogen ion
v/v	volume per volume
℃	degree Celsius
SE	standard error
ns	no significant difference
e.g.	for example
et al.	et alia (Latin) and others
kg ha^{-1}	kilogram per hectare

min	minute
mL	milliliter
mg L^{-1}	milligram per liter
L	liter
μmol	micromolar
MdNCED1	*9-cis-epoxycarotenoid deoxygenase 1*
MdCYP707A1	*ABA 8′-hydroxylase 1*
MdAOS1	*Allene oxide synthase 1*
MdJAR	*Jasmonate resistant*
MdJAZ2	*Jasmonate ZIM-domain 2*
MdP5CS	*Δ1-pyrroline-5-carboxylate synthetase*
MdOAT1	*Ornithine aminotransferase 1*
MdPDH	*Proline dehydrogenase*
MdSOS1	*Salt overly sensitive 1*
MdNHX1	*Na^+/H^+exchanger 1*
ROS	reactive oxygen species
MDA	malondialdehyde
DPPH	diphenylpicryl phenylhydrazine
SOD	superoxide
POD	peroxidase
CAT	catalase
APX	peroxidase

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

With human-induced climate change from increased CO_2 and other heat-trapping gases in the atmosphere in the 21st century—the global warming, which is directly related to the increase in surface heating. The extra heat from global warming will result in an increase in actual evaporation, higher evapotranspiration in plants and a continuous shortfall in precipitation, establishing agricultural drought more quickly and with greater intensity (Trenberth et al., 2014). Agricultural drought is the lack of ample moisture required for normal plant growth and development to complete the life cycle. Being the single most devastating environmental stress, agricultural drought can decrease crop growth and productivity, affect mineral uptake and assimilation and limits light harvesting and carbon fixation (Kumawat and Sharma, 2018).

Artificial irrigation water on these arable lands seems to be the most direct and effect solution to solve this problem. Irrigation with saline water, uneven distribution of irrigation water, land clearing, and improper drainage may accumulate salts in the soil layers, and results in another problem—salinity stress (Shrivastava and Kumar, 2015). With a climate predominated by little rainfall and adverse evapotranspiration rates, and soil characteristics that restrain salt leaching, arid irrigated lands are prominent salinity hotspots. Moderate problems are also observed even by using enough quality water or in climate suitable area. The use of fertilizers and other inputs in association with irrigation and insufficient drainage could be another important reason for soil salinity, markedly in cases of intensive agriculture in compacted and limited leaching soils.

Wastewater treatment, industrial or mining operation effluents are often rich in salts, therefore their mismanaged subsurface injection, surface disposal or use for irrigation, can also lead to soil salinity (Rengasamy, 2010). Finally, the use of traditional salt based de-icing agents in excess contributes to the accumulation of salt in the soil and water. It has been estimated that worldwide 20% of total cultivated and 33% of irrigated agricultural lands are afflicted by high salinity. It has been estimated that more than 50% of the arable land would be salinized by the year 2050 (Ladeiro, 2012).

Agriculture drought severely affects plant growth and development with substantial reductions in crop growth rate and biomass accumulation. The main consequences of this stress in crop plants are reduced rate of cell division and expansion, leaf size, stem elongation and root proliferation, and disturbed stomatal oscillations, plant water and nutrient relations with diminished crop productivity, and water use efficiency (M. Farooq et al., 2012). Soil salinity could be another threat for agricultural productivity. Salts in the soil occur as ions and these ions are released from weathering minerals in the soil. They may also be applied through irrigation water or as fertilizers, or sometimes migrate upward in the soil from shallow groundwater. Plants absorb essential nutrients from soil in the form of soluble salts, but salts excessive accumulation strongly suppresses the plant growth. High soil salinity inhibits the growth, development, and productivity of plants through the effects of osmotic stress, ion-toxicity, accumulation of reactive oxygen species (ROS), etc (Ladeiro, 2012). Especially in the hot and dry regions of the world, the soils are frequently saline with low agricultural potential. The inadequate irrigation management in these regions

further leads to secondary salinization of soil and the affected areas continues to increase each year. Soil salinity also affects ecological soil functions. The adverse impact of increased EC on important soil processes such as respiration, residue decomposition, nitrification, and denitrification through the decrease of soil biodiversity and microorganism activity (Rengasamy, 2010). On drying, sodic soils become dense, cloddy and structureless because natural aggregation is destroyed. At the soil surface, dispersed clay can act as adhesive, forming relatively dense crusts that impede seedling rooting and emergence. For all important crops, average yields are only a fraction somewhere between 20% and 50% of record yields, these losses are mostly due to drought and high soil salinity, which seriously impede the agricultural sustainability, and directly threatening human's health and survival (Shrivastava and Kumar, 2015).

Plants are often subjected to periods of soil and atmospheric water deficits during their life cycle as well as, in many areas of the globe, to high soil salinity. Different plant species are highly variable with respect to their optimum environments, and a harsh environmental condition, which is harmful for one plant species, might not be stressful for another. This is also reflected in the multitude of different stress-response mechanisms (Chhabra, 1996). Understanding how plants respond to salt, drought and co-occurring stresses can play a major role in stabilizing crop performance under saline and drought conditions and in the protection of natural vegetation.

Ion uptake and compartmentalization are crucial not only for normal growth but also for growth under saline conditions because the stress disturbs ion homeostasis (Chen et al., 2018). Plants, whether glycophyte or halophyte, cannot tolerate large amounts of salt in the cytoplasm and therefore they either restrict

the excess salts in the vacuole or compartmentalize the ions in different tissues to facilitate their metabolic functions against saline conditions. Halophytes limit sodium uptake into xylem or partition sodium in older tissues that serve as storage compartments that are eventually sacrificed. About one-third of the halophyte species also have salt glands that secrete ions from the leaves (Chen et al., 2018). However, for most horticultural plants, especially fruit trees, are non-halophytes, meaning that their tolerance to salt is relatively low or their growth limit of salinity is very low. It has been shown that salt stress inhibits the growth of apple seedlings (Liu et al., 2006). Both the photosynthesis and nutrients intake were affected by water or salt stress in apple (Avery, 1977).

Inadequate water availability in drought-prone environments affects the growth and productivity of crops by lowering tissue water status and turgor (Blum, 2005). Accumulation of osmotic adjustments such as proline in plant tissues helps lower water potential without decreasing actual water content and protects the enzymes and macro molecules of cells from the damaging effects of ROS. Therefore, effective osmotic adjustment is among the imperative physiological adaptations of plants grown in drought-prone conditions (Muhammad et al., 2012).

Changed dynamics of phytohormones such as ABA and JA and antioxidant defense systems are also major physiological adaptations for plants under abiotic conditions, including drought and salt stress (Wani et al., 2016). Higher ABA accumulation in plants under dehydration signals the leaves to induce stomatal closure and avoid water loss via transpiration. The production of antioxidants and the increase of antioxidant enzymes activity help to douse the produced ROS, minimize detrimental effects of oxidative stress induced by abiotic stress to normalize their

metabolic activities (Alkadi, 2020).

Plants challenged with drought or salinity also undergo many adaptive mechanisms at molecular levels to modulate growth and development. Many stress-inducible genes such as osmoprotectants-related, detoxifying enzymes-related, and transporters-related, as well as many regulatory proteins such as protein kinases and phosphatases synthesis genes functioned within the regulation of transcription factors or phytohormones. (Krasensky and Jonak, 2012).

The purpose of this research is to delve into the different mechanisms that apple seedlings go through when under drought and NaCl stress conditions by monitoring water potential, antioxidant defense and osmotic adjustments. This will be accompanied with an analysis on plant hormones in response to drought and NaCl stress, as well as measuring the activity of the genes involved in the global response to the stress.

1.2 LITERATURE REVIEW

1.2.1 Apple

Apple is an edible fruit produced by an apple tree (*Malus domestica*), belongs to family Rosaceae. And the apple tree is the most widely grown species in the genus *Malus* (Bramlage, 2001). The apple tree originated in Central Asia and where its wild ancestor, *Malus sieversii*, is confirmed and still exist today. Apples have been cultivated more than 3000 years in Asia and Europe and then brought to North America by European colonists. For now, the seven continents except Antarctica all have been cultivated. This fruit has been featured prominently in literature, poetry, art, religion and folklore throughout history. Apples have been symbolic since early time and were often featured in mythology (Janik, 2011). Even for now, many companies and enterprises take apple as the company's name or even brand name, such as Apple Inc., Apple Daily, Apple Film and Apple Advertising Production company.

The apple is a deciduous tree, grows very large if cultivation from seed, even up to nine meter in the wild. Generally, apple cultivars are propagated by grafting onto rootstocks, which control the size of the resulting tree. There are more than 7,000 known cultivars of apples, resulting in a range of desired characteristics (Bramlage, 2001). Different cultivars are bred for various tastes and use, including cooking, eating raw and cider production. Apple fruits production has become increasingly intensified within recent 100 years, with the use of dwarfing rootstocks and cultivation techniques designed to improve

production efficiency. Additionally, the development and application of insecticides, fungicides and herbicides has permitted to produce the high-quality apple fruit in many areas where production was difficult previously. Apple has become one of the most popular fruit, is considered to a major functional food resource (Pollack, 2001). China ranks first in apple cultivation area and production in the world, accounting for almost half of the world's apple production of 86 million tonnes in 2018 year. But France, Italy and United states lead the way in terms of yield per ton/hectare (Zhang et al., 2020).

Japan introduced apple from Europe and America in the Meiji Restoration era and now has developed and selected several good varieties (Oraguzie et al., 2002). In 2014, 'Fuji', 'Tsugaru', 'Ohrin', and 'Jonagold' accounted for 53.5%, 11.2%. 7.4%, and 6.9%, respectively, of the 816,300 tons of apples produced in Japan. Most of the remaining 21% consisted of new or old cultivars (such as 'Sekaiichi', 'Akibae', 'Shinano sweet', etc) developed in Japan, each constituting less than 1% or a few percent of total apple production (Igarashi et al., 2016). Aomori-ken is the biggest county for apple cultivation in japan, produced 445,500 tons in 2018 year, counting for 59% total apple fruits yield in the whole Japan. Relying on the first-class quality and high yields of Aomori apples, this county has become the world's famous Apple tourist resort (Masaki, 2019) (Fig. 1.1).

'Tsugaru', registered in 1973, is an early-maturing species, which is derived from the cross between 'Golden Delicious' and 'Orange Pippin'. The fruit of 'Tsugaru' is juicy, sweet and crispy, has always been the second only to Fuji in crop volume in japan (Igarashi et al., 2016). It is also the representative cultivar of Hokkaido apple cultivation region. Limited by the new cultivar and cultivation

environment, the cultivation area of 'Tsugaru' in Japan is decreasing year by year.

Throughout the life of an apple tree, a variety of stresses, including abiotic stress (such as drought, high salinity and extreme temperature) and biotic stress (such as insects and pathogen attack), affect growth, quality and yield (Liu et al., 2006). Apples cannot grow in tropical region because they require a period of cold (temperatures below 7 ℃) to natural dormancy and flower differentiation normally. Standard cultivars usually need 500-1,000 chill units, while some low chill cultivars require 400-600 h. In areas of adequate winter chilling, cold tolerance is often a concern as is later blooming to avoid spring frosts (Foster, 2016). Global warming causes more erratic climatic conditions, new pathogens, pests and in some areas an increased frequency of extreme weather is offering new challenges to apple producers worldwide. The excess use of fertilizers and other inputs in association with irrigation and insufficient drainage have caused serious soil salinity, affect growth, quality and yield (Shrivastava and Kumar, 2015). Unlike the chilling or drought stress, which effect rapidly, damages clearly, the influences of soil salinity on trees could be inconspicuous but permanent and harmful, especially for fruit trees, aimed at high yield and quality fruits, which roots system are earlier damaged. Once planted, they are generally exposed to salt stress injury over several years (Du Zhongjun, 2001). Thus, the development of techniques for the avoidance or reduction of injury from environmental stress and/or the breeding of stress-tolerant fruit tress is important. In 2010, the apple fruit's genome sequencing was finished, which is benefit for the research on disease control and selective breeding (such as high cold resistance, drought resistance and salt tolerance and special characters variety) in apple production. Apple breeders are assisting by developing high-quality cultivars

through both conventional breeding and genetic engineering (Velasco et al., 2010).

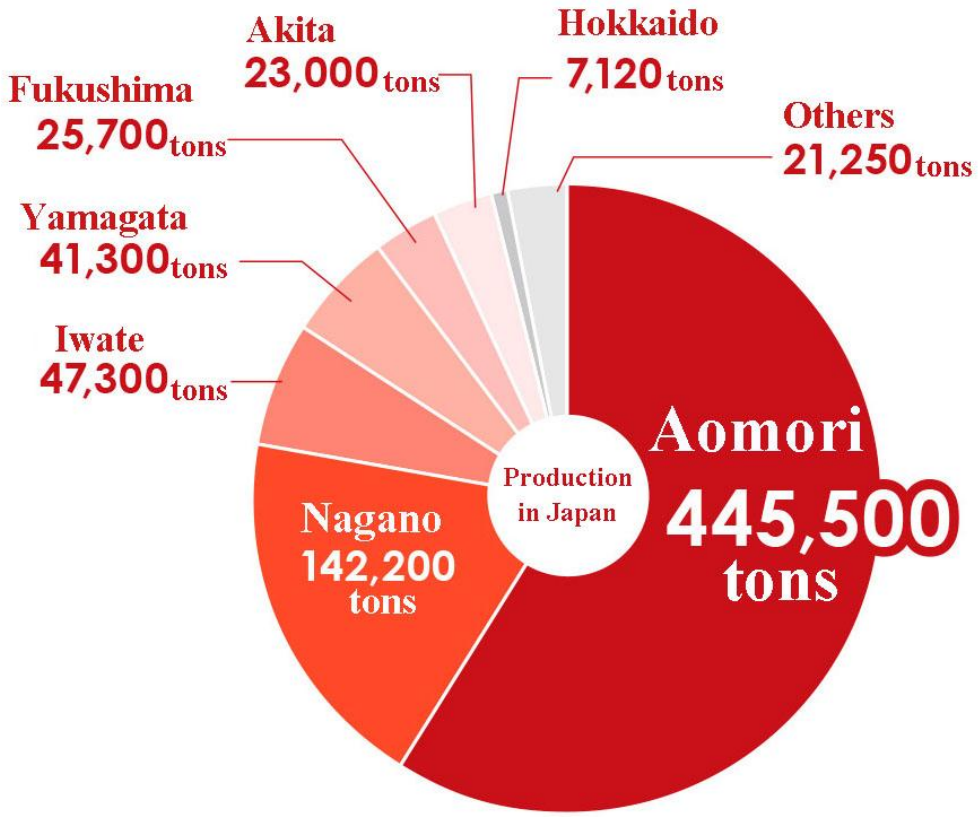

Fig. 1.1 The yields of apple fruits in Japan in 2018 (https://www.aomori-ringo.or.jp/overview/volume/).

1.2.2 Drought stress and salt stress

Drought is a temporary dry period, in contrast to the permanent aridity in arid

areas, it is a recurring extreme climate event over land characterized by below-normal precipitation over a period of months to years (Dai, 2011). Over most parts of the world even in wet and humid regions, drought may occur. Drought is usually divided into three types (1) Meteorological drought; (2) Hydrological drought; (3) Agricultural drought (Dai, 2011). In this study, the agricultural drought (soil drought) was only discussed.

Agricultural drought is the lack of ample moisture required for normal plant growth and development to complete the life cycle. Plants are subjected to the drought conditions when either the water supply to the roots is limited or the loss of water through transpiration is very high (Anjum et al., 2011). Drought stress severely reduces rate of cell division and expansion, limits leaf size and root proliferation, disturbs stomatal oscillations, plant water and nutrient absorption efficiency and as a result affects crop growth rate and biomass accumulation, speeds up plant senescence even death. The severity of the damage caused by the drought is generally driven by various factors including, the rainfall patterns, moisture holding capacity of the soil, and water losses through evapotranspiration. A recent study analyzed the data of studies published from 1980 to 2015 to report up to 21 and 40% yield reductions in wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.), respectively due to drought on a global scale (Daryanto et al., 2016). The plant response to drought stress generally varies from species to species, depending on plant growth stage and other environmental factors. Certain changes in their growth patterns and physiological process will activated positively to cope with the drastic effects of drought stress.

The stress can happen not only independently but also together, in the field, generously plants are routinely subjected to a combination of different abiotic

stresses (Mittler, 2006). Soil salinity is another major environmental constraint to crop production characterized by a high concentration of soluble salts. More than 800 million hectares of land throughout the world are salt affected. This amount accounts for more than 6% of the world's total land area, affecting an estimated 45 million hectares of irrigated land, and is expected to increase due to global climate changes and many unreasonable irrigation practices (Shrivastava and Kumar, 2015).

There are two types of salinity: natural or primary salinity and secondary or human-induced salinity (Parihar et al., 2015). Most of salt affected lands have arisen from natural causes such as parent rock weathering, geological deposits or groundwater flow, from the accumulation of salts over long periods of time in arid and semiarid zones. NaCl is one of the most soluble and abundant salt released in soil. Soils are classified as saline when the EC is more than 4 dS m^{-1} at 25 ℃, which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa (Parihar et al., 2015). The deposition of oceanic salts carried in wind and rain could be another natural factor to increase soil salt concentrations. Each liter rainwater contains 6 to 50 mg NaCl, and the concentrations decreasing along with the distance from the coastline. It has been statisticsed that each 100 millimeter of rainfall per year would deposit 10 kg ha^{-1} NaCl, if 10 mg L^{-1} NaCl in rainwater was contained (Munns and Tester, 2008).

Contrary to primary salinity, secondary salinity is introduced by human interventions; mainly irrigation with saline water or other ill-suited irrigation practices often coupled with poor drainage conditions (Parihar et al., 2015). In arid coastal areas, irrigation with highly saline water such as seawater or contaminated groundwater is the primarily reason for constant or increasing

salt accumulation in the upper soil layers, thus soil salinity in these regions is the major factor limiting crop production and land development (Daliakopoulos et al., 2016). Inland, the excess application of chemistry fertilizer, broad irrigation or/and plastic mulching and some others improper drainage might the major causes for secondary salinity. In arid regions, poorly drained soils, also allow for too much evaporation leading to salt residuals on the soil surface (Daliakopoulos et al., 2016). More than one third of the world's irrigated land is affected by soil salinization and this condition poses a threat for food security and environmental conservation (Singh, 2015). The yield reductions in various crops due to salinization have been reported by various researchers around the world. Maas and Hoffman (1977) concluded that the agricultural crops yield decreases linearly with increase in salinity beyond the threshold level. A linear decrease in both grain and dry matter yields of corn and alffalfa with an increase in salt levels was reported by Hanks et al (1978). Not just for the yield, the quality of vegetables, fruits and flowers were affected and lowered when roots exposed salinization soil long-term (Shannon and Grieve, 1998).

Whether the drought stress or salinity conditions have direct and indirect effects on plant growth and yield. The damage to plant growth and yield is much serious when these processes occur simultaneously. Knowledge of them is important because they represent suitable targets for genetic suppression to improve plants salt or/and drought stress tolerance.

1.2.3 Physiological response to drought stress and salt stress

Plants undergo several morphological and biochemical adaptations at subcellular,

cellular, and organ level to survive under drought. Drought escape, dehydration avoidance, and dehydration tolerance are three important adaptive mechanisms types of plants exposed to drought (Farooq et al., 2012).

Drought escape is the ability for the plant to complete its life cycle to undergo dormancy before the onset of drought season. Many desert plants possess this ability, which exhibit extremely short life cycles and produce seeds during short rainy seasons in order to ensure them from extinction. Drought escape is an advantageous strategy, and flowering may be critical as flowering time is a major trait of crop adaptation in areas where the growing season is restricted by terminal drought (Shavrukov et al., 2017).

Drought avoidance is the ability for the plant to sustain high plant water status under drought by catching more water from the soil or by minimizing water loss through transpiration. Roots are the only organ capable of extracting water from the soil profile, thus more rooting depth, root proliferation, and root length density will ensure with fetch more water from soil, which are considered important drought avoidance traits (Matsui and Singh, 2003). Drought stress inhibited root growth in general, even in tolerant genotypes, but the effect was more prominent on over-ground parts, which enhanced the root-shoot dry weight ratio. Many xerophytes have small leaves as their adaptation to survive in harsh environments. As earlier mentioned, a small leaf area is advantageous to restricted water use and evaporation. Therefore, less small stomata, smaller leaf area, and vertical leaf orientation are among the major drought avoidance traits to minimize transpiration to save water under drought conditions (Matsui and Singh, 2003).

Dehydration-tolerant is the ability for the plant to maintain metabolic activities

at low tissue water potential. Accumulation of organic and inorganic solutes under drought and/or salinity, which help lower water potential without decreasing actual water contents, and these solutes do not pose any detrimental effects on membranes, enzymes, and other macromolecules, even at higher concentration, which are called compatible solutes or osmotic adjustment substances (Sanders and Arndt, 2012). Limited water supply under drought promotes oxidative stress with overproduction of ROS. These ROS are highly reactive and deteriorate normal plant metabolism through oxidative damage to lipids, protein, and other macromolecules in the absence of any protective mechanism. And these excess ROS could be eliminated to maintain the normal physiological metabolism through their own antioxidant defense system with enzymatic and non-enzymatic components (Simova-Stoilova, 2008). Plant growth and development is regulated by certain growth substances produced internally called phytohormones. Generally, under stress conditions, the concentration of growth retardants increases at the expense of growth promoters to regulate plant water budget. For instance, higher ABA accumulation in roots under drought condition signals the leaves to induce stomatal closure and avoid water loss via transpiration (Liu et al., 2005). ABA and JA induced leaf senescence in drought-stressed plants contributes to nutrient remobilization thus allowing the rest of the plant to benefit from the nutrients accumulated during the lifespan of the leaf (Jibran, 2013). Dehydration-tolerant plants usually own higher osmotic adjustment function, stronger antioxidant defense system, and more effective phytohormones regulation pathway. This ability not just cope with the drought stress, is also suitable for the salt stress or other biotic stress such as pathogen or insect infection.

Similar with the drought stress, salt stress can also disrupt the water relations

between plants and soil. High salt in soil interferes with plant growth as it leads to physiological drought and ion toxicity (Chen et al., 2018). Physiological drought refers to the drought caused by plants' inability absorption of water from the soil for some physiological reasons. For example, the excessive soil solution concentration, extreme soil temperature (too low or high) and the serious hypoxia in the soil and so on, can destroy the normal physiological process of plant roots absorb water and cause water shortage. Its relation to different types of drought is not unambiguous, even if there is a meteorological drought or agriculture drought, it does not mean necessarily physiological drought. Accordingly, the stage of physiological drought depends on plant types, and the ontogenesis stage of plants (Muhammad, 2012). Whether the agriculture drought or physiological drought, the adverse effects on plants should be the same: inhabiting the growth, decreasing the yields and quality even causing death.

Toxicity occurs as a result of uptake and accumulation of certain toxic ions from the irrigation water within a crop itself. These toxic constituents include mainly sodium, chloride and sulphate. Soil salinity imposes ion toxicity, osmotic stress, nutrient deficiency and oxidative stress on plants, and thus limit water uptake from soil (Shrivastava and Kumar, 2015).

Mechanisms in plants for salt tolerance including two main types: minimizing the intake of salt into the plant and minimizing the concentration of salt in the cytoplasm. According to salt tolerance ability, Greenway and Munns (1980) defined halophytes as plants that can grow and complete their life cycle in soil normally with a salt content of over 0.33 MPa (70 mM L^{-1} salt concentrations); defined glycophytes or non-halophytes as plants which cannot grow and complete their life cycles

properly in such conditions. Halophytes have both types of mechanisms, they can exclude the Na^+ effectively, also can compartmentalize these inevitably uptake ions in vacuoles, which strategies allows them growing for long periods of time in salinity soil. Some glycophytes also exclude the salt well, but they cannot compartmentalize the excess Na^+ in tissues as effectively as do halophytes (Flowers, 1977). Most glycophytes cannot exclude the salt well and are easily to be toxic exposed salinity soil. Lots of fruit trees including apple tree are glycophytes and sensitive to high salt areas. Marginal and tipburn of fruit tree leaves are usually strong indicators of Na^+ and Cl^- toxicity, and these injuries may be the dominant factors in reducing fruit crop yields and quality (DE LIMA, 2018).

To mount an effective response to cope with salt stress, plants have developed the ability to sense both the hyperosmotic component and the Na^+ ion component under the salt stress (Deinlein et al., 2014). Three main categories are classified here: firstly, osmotic tolerance, including the accumulation of various osmotic adjustment substances such as proline, betaine, soluble proteins and sugars, which are regulated by long distance signals that reduce shoot growth and is triggered before shoot Na^+ accumulation; secondly, ion exclusion, where Na^+ and Cl^- transport processes in roots reduce the accumulation of toxic concentrations of Na^+ and Cl^- within leaves; finally, tissue tolerance, where high Na^+ concentrations are transport into old leaves and compartmentalized in the vacuole at the cellular and intracellular level (Tester and Davenport, 2003).

For the growth response to osmotic effect caused by salt stress, plants produce a suite of reactions identical to those of water stress caused by drought. That implies that any improvement in drought resistance would make a plant more adapted to saline soil.

However, the processes that adapt a plant specifically to saline soil also involve the regulation of the uptake and compartmentation of salt, to delay the time as much as possible when it accumulates to toxic levels in leaves that are actively photosynthesizing. Breeding or genetic engineering of plants better adapted to saline soil should focus on these processes. Therefore, understanding the mechanisms operating at a whole plant level has implications for screening techniques to distinguish plants that are tolerant of salinity as distinct from soil drying.

1.2.4 Water relations between plants and soil

Water is the most substance in plants, and it cannot replace by anything else, accounted for 60% to 90% of the total plant weight. It is majorly absorbed by the root systems from soil (Fig. 1.2). A good relationship between plant and soil is the important factor for high yields and quality (Kramer and Boyer, 1995). Without water, the transport of substances, biofilm assembly and metabolism in plant cells cannot process normally, thus retards the growth of plants severely, and even leads to death.

Water potential is the potential energy of water per unit volume relative to pure water in reference conditions, expressed in potential energy per unit volume and very often is represented by the Greek letter ψ. Plants water potential quantifies the tendency of water to move from soil to plants due to osmosis, gravity, mechanical pressure and matrix effects such as capillary action. The level of water potential in plants reflects the relationship between water supply and demand, that is, the degree of water stress (McCutchan and Shackel, 1992). Under normal conditions, plant water potential is lower than soil water potential, which guarantees the properly absorb water and soluble fertilizers from the soil for growth. When exposed drought conditions, soil water potential decreases even lower the plant water potential, roots cannot take up the

required water smoothly and consequently, the growth will be affected. It has been studied that the soil water potential up to -3.0 MPa will cause the permanent wilting for xerophytes, -1.5 MPa for medium plants and over -1.5 MPa for wet plants (Frank et al., 1973).

One of the first effects observed after dehydration is stomatal closure, even before detection of any leaf water deficit to reduce transpiration of water. At low plant water status, the most important being osmotic adjustment and protection of the membrane system. The osmotic adjustment of plants can allow the maintenance of root or shoot growth under drought conditions by synthesizing the contents of various osmotic protective substances, the water potential can be reduced to a state where soil moisture can be reabsorbed (Sanders and Arndt, 2012). Structural integrity of cellular membranes ensures that the plant maintains high osmotic pressure, avoids the exosmosis of cell fluid and maintains the normal operation of multiple cell functions, which is also important for survival under severe dry periods, or in situations where random droughts occur.

Early responses to drought and salt stress have been considered mostly identical. Drought and salinity share a physiological water deficit that attains almost all plant organs. Osmotic balance is also essential for plants growing in saline medium. Failure of this balance results in loss of turgidity, cell dehydration and ultimately, death of cells (Shrivastava and Kumar, 2015). An increase of salt in the root medium can lead to a decrease in leaf water potential and, hence, may affect many plant processes. It has been shown that the water potential in cucumber (*Cucumis sativa*.L) decreases linearly with increasing salinity levels (Khan, 2013).At very low soil water potentials, this condition interferes with the plant's ability to extract water from the soil and maintain

turgor. However, under prolonged salt stress plants respond in addition to dehydration to hyper-ionic and hyper-osmotic stress, too.

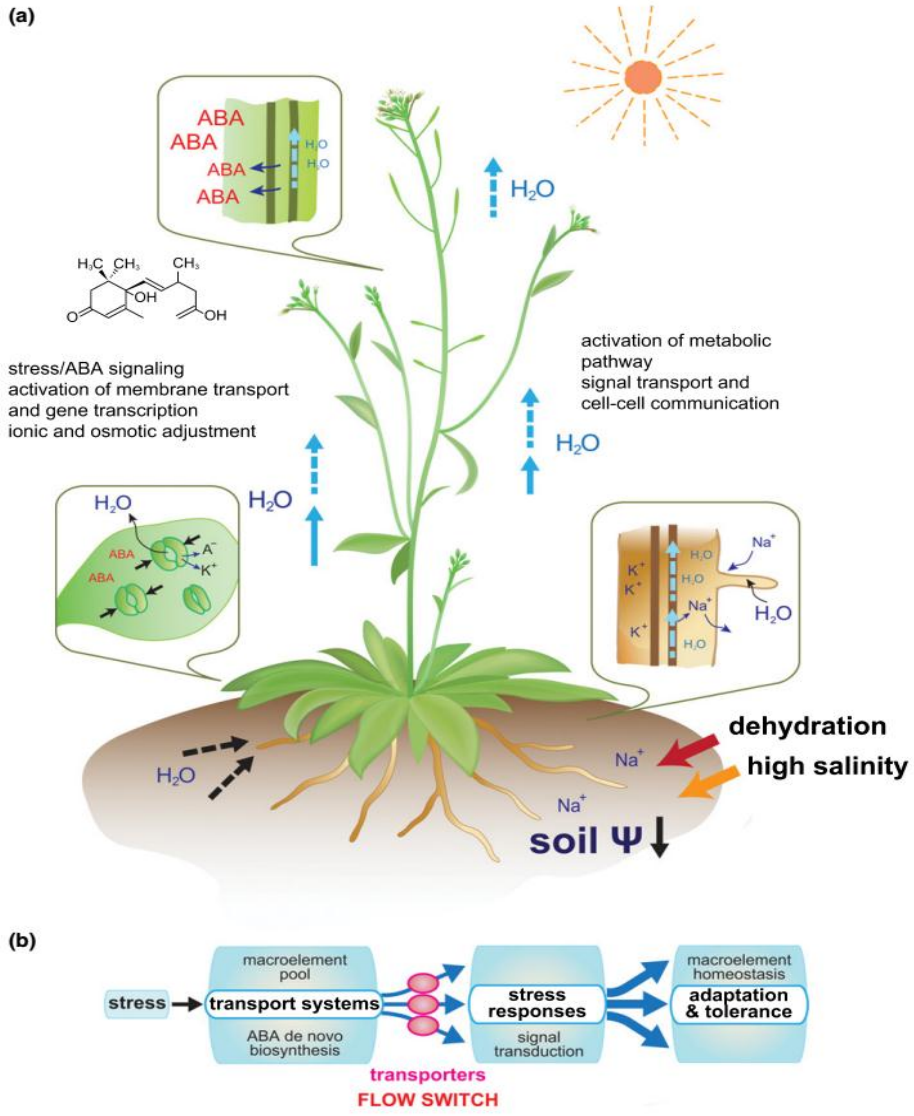

Fig. 1.2 Plant transport system and responses to water deficit and high salinity stresses (Osakabe et al., 2014).

1.2.5 Ion toxicity in salt stress

Under salinity, plants response follows a biphasic model, with current metabolic data indicating an early similarity with drought, whereas in the long-term plants are

responding to ion toxicity (Shani and Ben-Gal, 2005). In plants, roots take up ions dissolved in water from soil to the whole plant tissues. The vascular tissues play the main roles in this long-distance transport of these ions between organs and tissues (Osakabe et al., 2014).

Ion toxicity occurs as a result of excessively uptake and accumulation of certain toxic ions from soil within a crop itself. These toxic constituents include mainly Na^+, Cl^- and SO_4^{2-}. They can reduce crop productivity and eventually cause crop failures (Shani and Ben-Gal, 2005). Ion toxicity induced by the salinity is the result of replacement of K^+ by Na^+ in biochemical reactions, and Na^+ and Cl^- induced conformational changes in proteins. K^+ is necessary for osmoregulation and protein synthesis, maintaining cell turgor and stimulating photosynthesis (Wang et al., 2013). The deficiency of K^+ initially leads to chlorosis and then necrosis.

Continued transport of salt into transpiring leaves eventually results in very high Na^+ and Cl^- concentrations. Once the salts load exceeds the ability of the cells to compartmentalize salts in the vacuole, salts then would rapidly build up in the cytoplasm and inhibit enzyme activity. Alternatively, they might build up in the cell walls and dehydrate the cell. In addition, the salt taken up by plant concentrates priority in the old leaves, which lost chlorophyll more rapidly and died earlier.

Na^+ increment inside plants also had toxic effects on seed germination, mainly by affecting the plant water relations or through displacement of Ca^{2+} by Na^+ from critical cell wall binding sites, which could disrupt cell wall synthesis and hence inhibit plant growth (Wu and Wang, 2012). Not all plants are equally affected but most crops and woody perennial plants are sensitive. Apple trees are considered to a salt-sensitive species in which accumulation of potentially toxic concentrations of Na^+ and Cl^- in

leaves is dependent on the capability of the root system to exclude ions (Smolik et al., 2004).

1.2.6 Salt exclusion

Salt exclusion functions to reduce the concentrations at which salt accumulates in upper ground organs. Some plants can secrete salt from the plant body to the outside through salt glands (as in *Limonium bicolor*) or into salt bladders for temporary storage and then the salt will scatter from salt bladders when it encounters strong winds or other external stimuli (Smolik et al., 2004). Partial salt excluders plant such as reed (*Phragmites communis* L.), excluding most of the Na^+ and Cl^- into the soil solution or by accumulating much more salt in the vacuoles of parenchyma tissues and parenchyma of roots and xylem than in the shoot to avoid injury of photosynthetic organs (Matsushita and Matoh, 1991). Most salt-exclusion halophytes only transport 2% of the Na^+ or Cl^- absorbed by the roots to the shoots and the remaining salt is excluded into the soil solution (Fig. 1.3). Another strategy is compartmentalizing excessive salt ions into the vacuole, which reduces the water potential of the plant and helps it to absorb water from the saline soil and, on the other hand, this reduces the ions content in cytoplasm and avoids damage to enzymes and biological substances in the cytoplasm.

Most woody perennial plants have the same salt tolerance mechanism as salt-exclusion plants even though these woody plants have a significantly lower salt tolerance than salt-exclusion plants. The net accumulation of Na^+ in cells is due to the balance between the influx and efflux of Na^+. A reduction in the Na^+ accumulation in plant cells can be caused by either a decrease in influx or an increase in efflux. The SOS (salt overly sensitive) pathway plays essential roles in the Na^+ efflux, within this

signaling pathway, SOS3, SOS2, and SOS1 three proteins are involved (Liu and Zhu, 1998). The SOS1 is a Na^+/H^+ antiporter located on the plasma membrane, functioned for efflux of Na^+ from the cytoplasm under salt stress and it can be activated by the complex composed of SOS3 and SOS2. Additionally, the SOS pathway also plays roles on blocking the Na^+ influx into the root cells by Ca^{2+} sensitive pathway.

Plants trigger salt tolerance responses can also by compartmentation, exclusion, and secretion of Na^+/H^+ ions into vacuoles, a unique structural feature of plant cells, play a major role in pH regulation, K^+ and Na^+ homeostasis of cells in higher plants (Ahanger and Agarwal, 2017). Na^+/H^+ exchangers (NHXs) are intracellular membrane proteins that localized in the vacuole and involved in sequestration of Na^+ into the vacuole under stressful conditions (Yamaguchi et al., 2013). These vacuolar antiporters regulate the exchange of H^+/Na^+ across tonoplast to reduce the toxicity of Na^+ concentration in the cytoplasm.

Under salt stress, due to the functions of SOS pathway and NHXs proteins, excess Na^+ in the cytoplasm is either discharged into the extracellular domain of the external solution or compartmentalized into the vacuole, provide the effective mechanism for reconstruction of Na^+/K^+ homeostasis under salt stress.

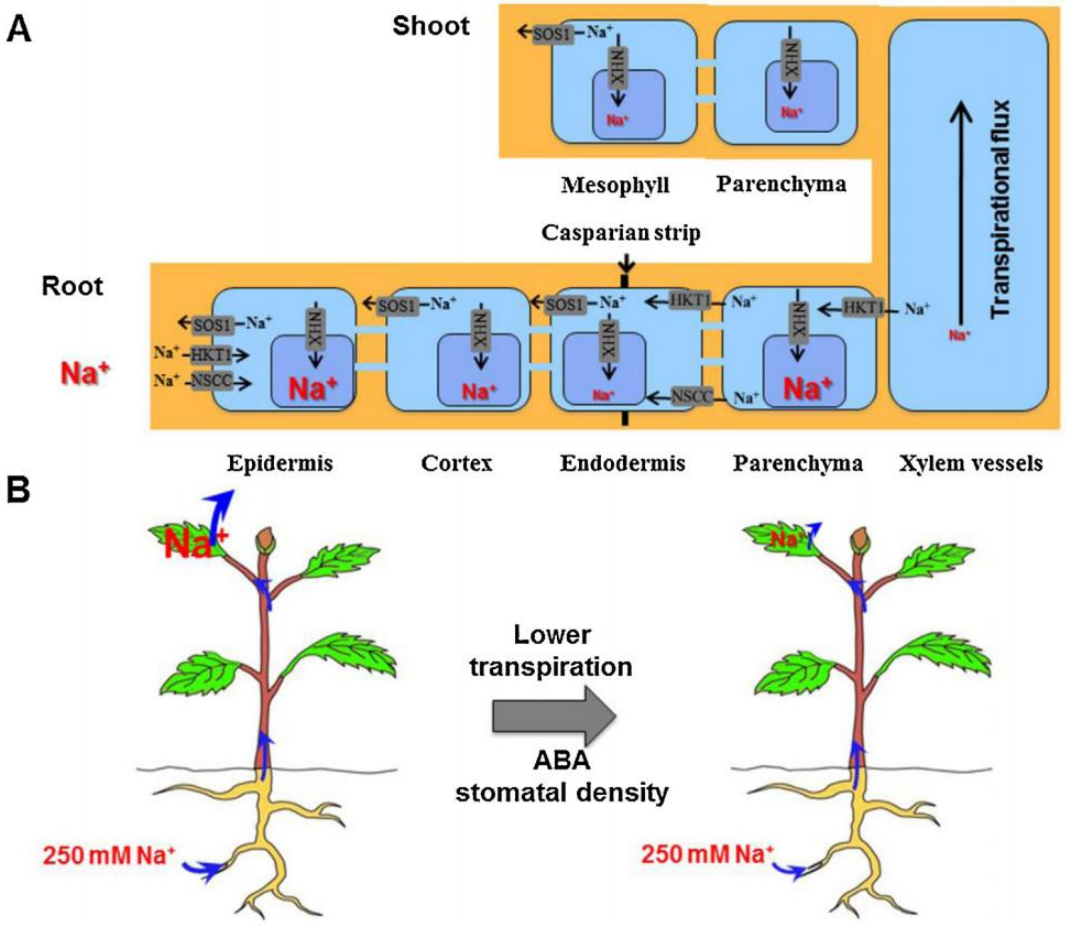

Fig. 1.3 Na^+ uptake from soil, transport and exclusion from root xylem vessels and compartmentalization in plants (Hasegawa and Paul, 2013).

1.2.7 Oxidative stress and membrane damage

Exposure of plants to adversity conditions such as drought and salt stress can lead to osmotic stress and ion toxicity incipiently, which in turn leads to the generation of

excess ROS such as O_2^-, $^1O_2$, H_2O_2 and OH (Simova-Stoilova et al., 2008). These ROS are highly reactive and interact with a wide range of molecules to deteriorate normal plant metabolism through oxidative damage to lipids, protein, and other macromolecules in the absence of any protective mechanism.

Membrane injury is the result of lipid peroxidation, which is related to an enhanced production of highly toxic ROS induced by salt stress (Simova-Stoilova et al., 2008). The content of MDA, a product of lipid peroxidation, is generally an indicator of free radical damage to cell membranes causing severe oxidative stress (Liu et al., 2020). Multiple studies have confirmed that adversity induce oxidative stress and cause the increase of MDA and H_2O_2. The concentrations of MDA were positively correlated with the degree of stress and increased with the severity of stress.

Biological membranes have important physiological functions, including the stabilization of the intracellular environment, regulation or selection the substances in and/or out of the cell and being the site of multiple biochemical reactions (Sten-Knudsen, 2002). Membrane injury affects the permeability of the plasma membrane, accelerates the substances outward infiltration from cell. Electrolyte leakage accompanies plant response to stresses, such as salinity, drought or pathogen attack, is a hallmark of stress response in intact plant cells. This index is widely used as a test for the stress-induced injury of plant cell and 'a measure' of plant stress tolerance. It can be detected almost instantaneously after the application of a stress factor and lasts from a few minutes to several hours, which is mainly caused by the efflux of K^+ and some counterions (Cl^-, HPO_4^{2-}, NO_3^-, $citrate^{3-}$, $malate^{2-}$) that move to balance the efflux of positively charged potassium ions (Ahanger and Agarwal, 2017). The stress-induced electrolyte leakage is usually accompanied by accumulation of

ROS and often results in programmed cell death, finally leads to photosynthetic declination, leaf yellowing and organ shedding.

Plants douse the ROS through their antioxidant defense system. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), accumulate in higher plants under stress conditions to avoid oxidative damages (Ahmad et al., 2016). Initially, SOD catalyzes the conversion of O_2^- to H_2O_2 that is further reduced to H_2O by CAT or APX by using ascorbate as an electron donor. Various studies have also shown that genetically engineered plants containing higher levels of ROS scavenging enzymes, such as SOD, APX, and POX, have improved tolerance to abiotic stresses such as drought and salinity (Ahmad et al., 2016; Reddy et al., 2015). Additionally, improved activities of CAT, APX, and SOD by ABA or JA application decreased H_2O_2 and MDA concentrations in drought-prone tomato seedlings (Muhammad et al., 2012).

Except the antioxidant enzymes, antioxidant such as polyphenolic compounds on scavenging free radical ability has been confirmed due to the presence of hydroxyl substituents and their aromatic structure. Diphenylpicrylhydrazyl (DPPH) is a free radical, stable at room temperature, which produces a violet solution in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncolored ethanol solutions. Exogenous ABA application enhanced the DPPH scavenging free radical ability level in apple seedlings and enhanced the salinity tolerance (Sales et al., 2017). The use of DPPH provides an easy and rapid way to evaluate stress tolerance variety according to the antioxidants level of extracting solutions.

The ability of increasing production of antioxidant compounds and enzyme activities in response to oxidative stress has been an effective method to enhance the

salt or drought tolerance.

1.2.8 Osmolytes in drought and salt stress response

One of the key strategies of plant adaptation to adverse environmental conditions, including excessive salinity, is a stress-induced regulation of the qualitative composition and quantitative content of law-molecular-weight organic osmolytes with the functions of chemical chaperones, antioxidants, and signaling molecules. These compatible solutes include soluble sugars, sugar alcohols, proline, glycine betaine, organic acids etc., (Shrivastava and Kumar, 2015). These solutes provide protection to plants from stress by contributing to cellular osmotic adjustment, ROS detoxification, protection of membrane integrity and enzymes/protein stabilization.

The phenomenon of proline accumulation is known to occur under water deficit, salinity, low temperature, heavy metal exposure and UV radiations, etc (Shrivastava and Kumar, 2015). Apart from acting as osmolyte for osmotic adjustment, proline contributes to stabilizing membranes and proteins structures, scavenging free radicals and buffering cellular redox potential under stress conditions. It may also act as protein compatible hydrotrope, alleviating cytoplasmic acidosis and maintaining appropriate $NADP^+$/NADPH ratios compatible with metabolism (Hayat et al., 2012). In many plant species, proline accumulation under salt stress has been correlated with stress severity, and its concentration has been shown to be generally higher in salt tolerant than in salt sensitive plants (Kumar, 2003).

In plants, proline is synthesized by two pathways including glutamate pathway and ornithine pathway (Fig.1.4). The glutamate pathway accounts for major proline accumulation during osmotic stress. In this pathway, the proline is synthesized from glutamatic acid via intermediate Δ'-pyrroline-5-carboxylate (P5C). The reaction is

being catalyzed by Δ'-pyrroline-5-carboxylate synthetase (P5CS) and Δ'-pyrroline-5-carboxylate reductase (P5CR) (Hayat et al., 2012). Proline catabolism occurs in mitochondria by means of the chronological action of proline dehydrogenase (PDH) or proline oxidase producing P5C from proline and P5C dehydrogenase (P5CDH) converts P5C to glutamate. PDH transcription is activated by rehydration and proline but repressed by dehydration, thus preventing proline degradation during abiotic stress. In another pathway, proline can be synthesized from ornithine, which is transaminated to P5C by ornithine-δ-aminotransferase. It has been suggested that ornithine pathway is important during seedling development and in some plants for stress-induced proline accumulation. Accumulation of proline has been suggested the self-protective mechanisms of plants in response to adversity stimuli. Numerous studies have reported proline as an antioxidant suggesting its role as ROS scavenger and singlet oxygen quencher.

Exogenous proline application reduces ROS levels and prevents lipid peroxidation in rice exposed to salinity (Deivanai et al., 2011). It is reported that proline, applied exogenously at a low concentration, ameliorated the adverse effects of salinity in plants. in contrast, higher proline concentrations did not prove beneficial (Hayat et al., 2012). Higher levels of exogenous proline feedback inhibition of P5CS enzymes, blocked the biosynthetic portion of this cycle and thereby resulted in toxic effects and poor plant growth.

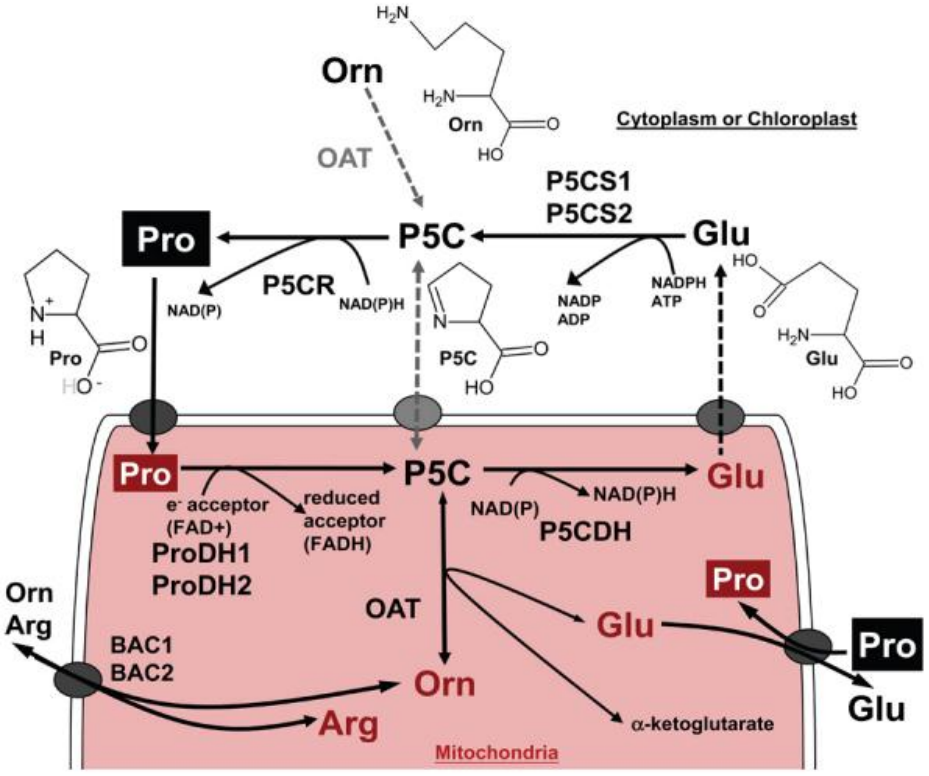

Fig.1.4 The pathways of proline biosynthesis and metabolism (Hayat et al., 2012).

1.2.9 Hormonal response to drought and salt stress

The similarity between drought and salt stress also applies to hormonal responses, apart from oxidative stress and membrane damage. Plant hormones as watchdog of physiological response, such as ABA, ethylene, salicylic acid, jasmonic acid (JA) and

brassinosteroids, play key roles in integrating plants growth and develop. Among these, ABA is defined as a stress hormone because of its rapid accumulation in response to biotic and abiotic stresses and its mediation of many stress responses that help plant survival over the stresses (Zhang et al., 2006) (Fig. 1.5). Under osmotic conditions such as high salinity and drought, ABA is known to stimulate short-term responses like closure of stomata, resulting in maintenance of water balance and longer-term growth responses through regulation of stress-responsive genes. And nitric oxide accumulation in guard cells might be necessary for ABA-induced stomatal closure, which placing nitric oxide as a new component of the ABA signaling transduction pathway during adaptive plant responses to drought and salt stress (Garcıa-Mata and Lamattina, 2002).

ABA regulates the expression of multiple stress-responsive genes by binding the ABA-responsive elements ABREs within these gene promoters. It has been validated that induction of AREB1/ABF2, AREB2/ABF4 and ABF3 by dehydration, high salinity and ABA treatment and enhanced plants drought tolerance. Other transcription factors from the MYC, MYB and NAC protein families are also known to function in an ABA-dependent manner (Zhang et al., 2006).

Exogenous ABA application can induce the endogenous ABA accumulation and trigger a series of reactions. It was shown that ABA application reduced the Na^+ concentration or its translocation to shoots in rice, increased in the K^+/Na^+ ratio, enhanced salinity tolerance; or increased proline accumulation in the shoot of the bean under water deficit which also correlated with enhanced drought tolerance; or induced the expression of antioxidant genes and enhanced the capacity of antioxidant defense systems, both the enzymatic and non-enzymatic constituents has been documented (Zhang et al., 2006). Thus, it is evident that ABA employs a sophisticated process for mediating plant

defense responses against abiotic stresses.

Progression of the response at stress stimulus is associated with a change in endogenous ABA levels, which are controlled by a precise balance between biosynthesis and catabolism rates of the hormone (Waśkiewicz et al., 2013). Accumulation of ABA can be accomplished by the increase of biosynthesis and decrease of degradation metabolism, functioned with 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzyme and ABA 8'-hydroxylation (CYP707A) (Kondo et al., 2012). ABA mainly produced in leaf vascular tissues in response to water deficit stress and transported to guard cells by specific ATP-dependent transporters where it induced stomatal closure and that appeared to be no requirement for root-shoot delivery of ABA (Osakabe et al., 2014). It has been reported that ABA concentrations can increase up to 30-fold during drought; and other types of stresses, such as salinity and cold, also cause ABA biosynthesis and accumulation (Acharya and Assmann, 2009).

The sensibility and rapidity of ABA accumulations ensure to activate several stress responses when subjected to abiotic stresses such as drought and salt. Nonetheless, it should be rapidly degraded and deactivated once the stress is relieved to avoid part inhibition of plant growth and functions (Zhang et al., 2006). Modulation of ABA levels in tissues and cells is critical for balancing defense and growth processes when plants experience non-optimal environments.

Many studies showed that JA plays an important role in abiotic stress tolerance, and considerable interests have focused on JA due to its ability to induce a protective effect on plants under stress. Under salt stress, JA recovered salt inhibition on dry mass production in rice and diminished the inhibitory effect of NaCl on the rate of $^{14}CO_2$ fixation and protein content (Kang et al., 2005). Similar with ABA, Zhang et al. (2006) clearly defined

that JA will be increased rapidly in response to salinity, whereas indole-3-acetic acid and salicylic acid are declined. Additionally, de Ollas et al. (2013) found that JA transient accumulation was needed for ABA response to salt tolerance in citrus (*Citrus reticulata Blanco*). The high levels of JA accumulation in plants after salt treatments may change the balance of endogenous ABA, which might be an effective protection against high salinity.

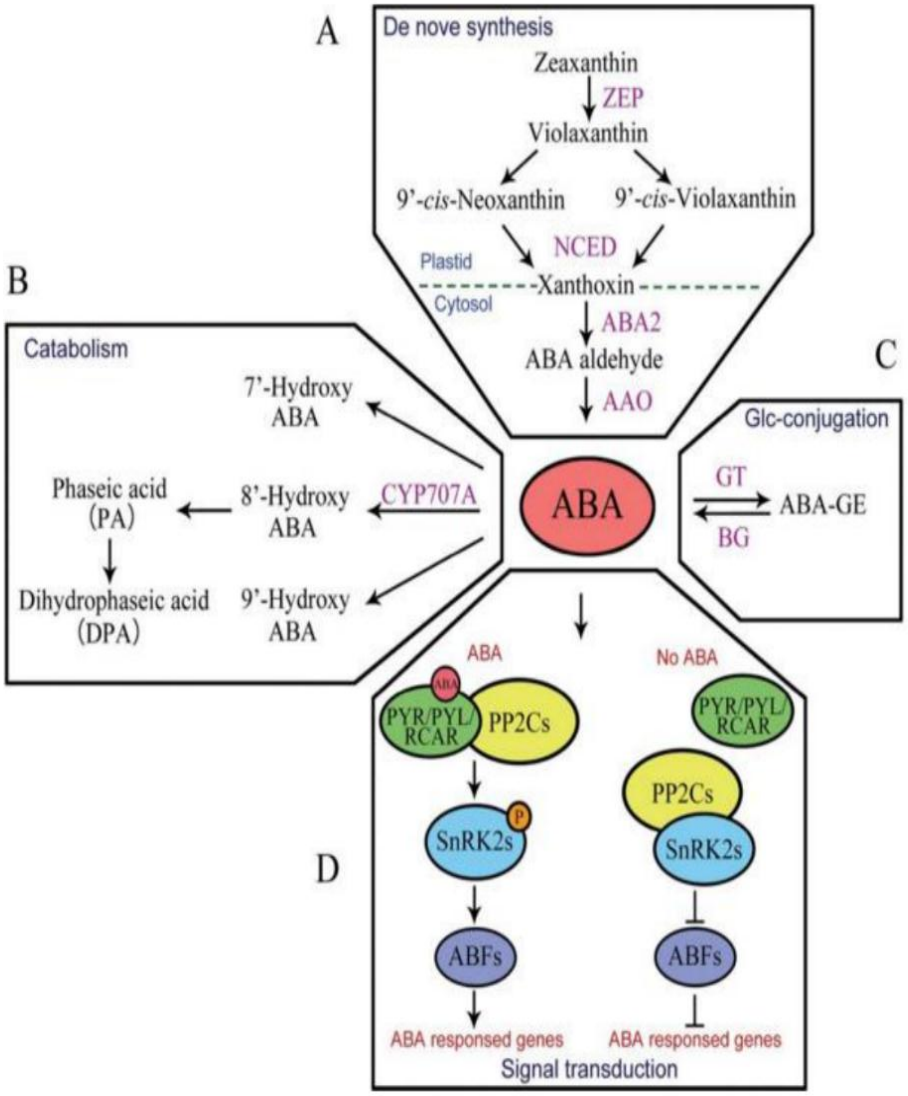

Fig. 1.5 ABA metabolism, signal transduction, and reactivation pathways. (A) ABA biosynthesis pathways, (B) ABA catabolic pathway, (C) ABA-glucose conjugation, (D) ABA signal transduction (Waśkiewicz et al., 2013).

The biosynthesis of JA in plants derives from α-linolenic acid (18:3), with the allene oxide synthase (AOS) being one of the key enzymes (Fig. 1.6). The JA signaling mechanism against abiotic stress involves bioactive form of JA by Jasmonate-Resistant (JAR), which conjugates JA and isoleucine to jasmonoyl-L-isoleucine (JA-Ile), and ultimately activates the downstream responses via degradation of jasmonate ZIM-domain (JAZ) repressor proteins (Ruan et al., 2019).

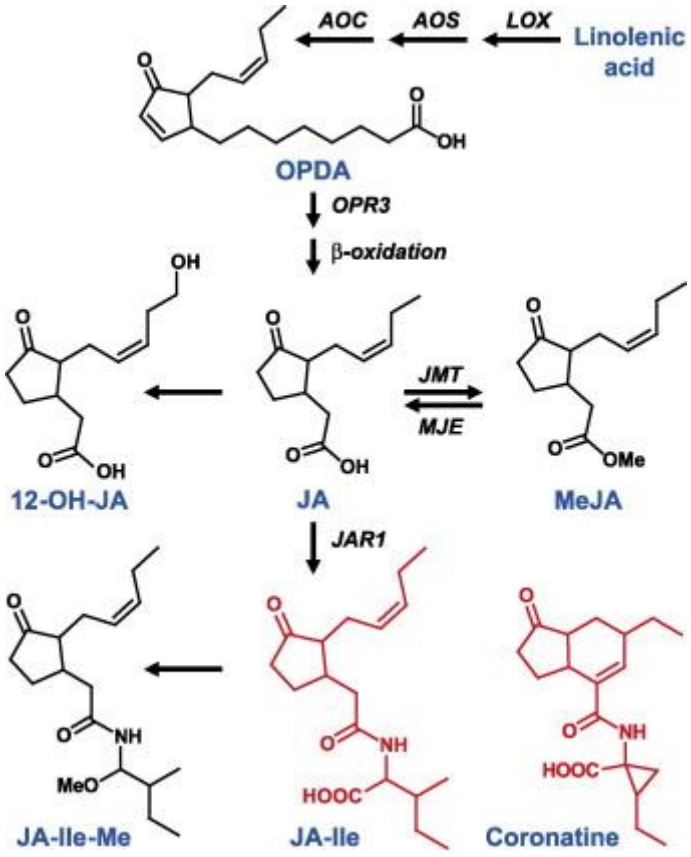

Fig. 1.6 The biosynthesis of JA in plants (Ruan et al., 2019).

In recent years, plant growth regulators were considered a new way to improve plant stress tolerance, alleviate stress damage, and ensure the quality and yield of plants (Rostami and Azhdarpoor, 2019). JA is naturally occurring plant growth regulators, regulating the morphological, physiological and biochemical processes in plants. Previous studies have documented that foliar application of JA could modulate plant physiological responses towards abiotic stress tolerance (Kang et al., 2005) (Fig. 1.7). Exogenous JA is effective in protecting plant from salt-induced oxidative damage because it enhances the activity of antioxidant enzymes (Ruan et al., 2019). A synthetic analog of JA, *n*-propyl dihydrojasmonate (PDJ), has been widely used in Japan for the promotion of apple coloration (Kondo et al., 2000). PDJ could inhibit *Botrytis cinerea* pathogen infection in apples (Suktawee et al., 2019). PDJ treatment also improved chilling tolerance in apple fruits through induction of scavenging activity against reactive oxygen (Kondo, 2009). Therefore, PDJ application may mitigate biotic and abiotic stresses including salinity.

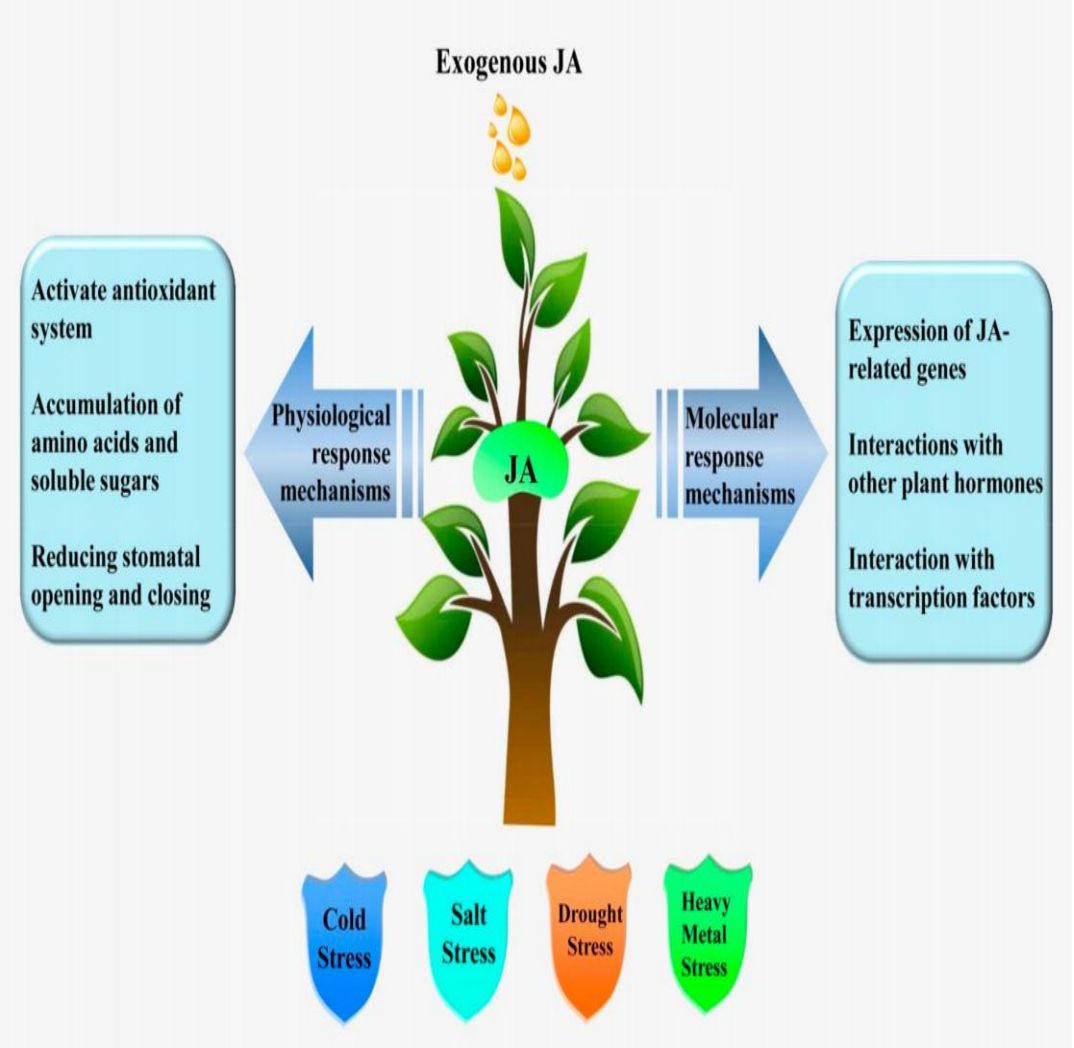

Fig. 1.7 The role of jasmonic acid (JA) in plant response to abiotic stress (Wang et al., 2020).

CHAPTER 2

DROUGHT TOLERANCE INDUCED BY A COMBINATION OF ABSCISIC ACID AND ABSCINAZOLE IN APPLE SEEDLINGS

2.1 INTRODUCTION

Drought stress triggers the accumulation of ROS, which, in-turn affects redox homeostasis and results in oxidative stress as shown by a heightening of lipid peroxidation (Simova et al., 2008). The closure of stomata is a spontaneous physiological reaction that enables a reduction of evapotranspiration losses under drought stress (Li et al., 2017). Upon the perception of drought stress, plants initiate the accumulation of proline and soluble sugars for protection from damage during such conditions (Shrivastava and Kumar, 2015). Multiple antioxidant enzymes, including SOD, CAT, and POD, are induced under drought stress to scavenge the excess ROS (Ahmad et al., 2016).

ABA is a plant hormone that accumulates under various stresses including drought. High concentrations of ABA can quickly induce stomatal closure to minimize transpiration losses. It can also mitigate stress damage by promoting the synthesis of multiple osmotic protective substances, and by activating multiple defense response systems (Zhang et al., 2006). Previous research has shown that the improvement of antioxidase activity under abiotic stress is regulated by endogenous ABA (Guajardo, 2016). Thus, high concentrations of endogenous ABA could be beneficial in protecting plants against drought. It has been reported that pretreatment with ABA increases the endogenous ABA concentrations in wheat seedlings and enhances drought tolerance (Bano et al., 2012).

Our previous research has shown that using Abz-E2B, an inhibitor of ABA 8'-hydroxylase (the key enzyme in ABA metabolism), prevented drought damage by effectively increasing endogenous ABA accumulation in apple seedlings (Kondo et al.,

2012). Similarly, Abz-E3M was an even more practical and effective inhibitor, increasing the ABA concentration in both rice and maize (Takeuchi et al., 2016). However, the effect of exogenous ABA combined with Abz-E3M has not been studied on plants are water stressed. Therefore, in this study, ABA and a combination of ABA and Abz-E3M were applied to apple seedlings to study their mitigation effects against drought.

2.2 MATERIALS AND METHODS

2.2.1 Plant materials and treatments

Ninety-day-old (*Malus domestica*) 'Tsugaru' apple seedlings were used in the study. Germinated seeds were sown into plastic trays (26 × 52 × 6 mm, 72 holes) containing moist vermiculite and turfy soil with a volume ratio of 1:2. About 0.5 -1.0 L of water was applied to the bottom of each tray every morning to keep the soil moist. Commercial Hyponex® solution (Hyponex Japan Co., Osaka, Japan) was added as liquid fertilizer at 14-day intervals. All seedlings were grown in a greenhouse covered with polyvinyl film at Chiba University (35.78 °N, 139.90 °E). The greenhouse environments were monitored and regulated. The average daily temperature was 25-30 ℃ during the day and 15-20 ℃ at night, under natural light [with an average daily photosynthetic photon flux density (PPFD) of 500 μmol m^{-2} s^{-1}], and with an average relative humidity of 45-65% during the day and 65-75% at night. Drought was imposed by with-holding water and nutrient solution from the growing trays. Uniform seedlings were selected and then divided into three groups of 140 seedlings. Three treatments were performed under drought conditions as follows: (a.) application of 100 μM ABA; (b.) application of 100 μM ABA +100 μM Abz-E3M; (c.) application of distilled water (ddH_2O) (control group). The leaves were sprayed with ABA, ABA plus Abz-E3M or ddH_2O solutions containing a surfactant (0.5% of Approach BI® (Maruwa Biochemical Co., Tokyo, Japan)), and allowed to dry naturally. Mature, fresh leaves were collected at day-0 (before chemical and drought treatment), day-2, and day-4 after the start of the drought

treatment. They were frozen with liquid nitrogen and then kept at -80 ℃ until analysis.

2.2.2 Measurement of the water potential of apple seedling leaves

Leaf water potential measurements were performed at 4:00 am (before dawn) each time, to obtain stable and optimal data, as described by Sales et al. (2017). Samples (three replicates) were transferred to a sample cup and their water potential was immediately measured using a WP4-T water potential meter (Decagon Devices Inc.; Pullman, WA, USA).

2.2.3 Measurement of lipid peroxidation

The amount of malonaldehyde (MDA) produced in the leaves was measured to assess the level of lipid peroxidation. MDA concentrations were determined using the spectrophotometric method described by Heath and Packer (1968). The OD_{450}, OD_{532}, and OD_{600} were recorded with a UV-VIS spectrophotometer (2J1-0010, HITACHI, Japan) and three replications were performed per test group.

2.2.4 Measurement of proline concentrations

The proline concentrations were measured using the method described by Sarker et al. (2005). Three replications per test group (0.5 g fresh weight per sample) were each homogenized with 10 mL of 3% sulfosalisylic acid (w/v) and centrifuged at 10,000 rpm for 15 min. The absorbance of the supernatant was measured at 520 nm using a spectrophotometer (HITACHI, Japan U-2910).

2.2.5 DPPH antioxidant assay

The antioxidant DPPH activity test was performed according to a modified version of the method described by Sales et al. (2017). Fresh samples of 0.5 g (three

replications) were mixed with 25 mL of methanol and homogenized. The samples were measured with a spectrophotometer at 515 nm. The results were compared with the Trolox standard curve at 25-800 μM.

2.2.6 Antioxidative enzyme activity assay

The enzymatic activities of SOD, POD, CAT, and APX in the leaves were determined using the methods described by He et al. (2014). A 1 g sample of leaf tissue was homogenized in a 50 mL tube with 15 mL of phosphate buffer (50 mM, pH 7.8) containing 2% (w/v) polyvinylpyrrolidone and 0.5 mM EDTA in three replications. After centrifuging at 4 ℃ for 15 min at 15000 rpm, the supernatant was used to determine enzymatic activity. The activity of SOD was measured using the nitroblue tetrazolium reduction method described by He et al. (2014). Each unit of SOD activity was calculated using the amount of enzyme required for 50% inhibition. The activity of POD was determined based on the methyl catechol method of oxidation using H_2O_2. The change of OD_{560} was recorded for 3 min at 60-s intervals using a spectrophotometer. The CAT levels were determined by reading the decrease in absorption for 180 s at 240 nm. The activity of APX was assayed by documenting the decrease in absorbance at 290 nm of ascorbate within 1 min.

2.2.7 Quantitative analysis of endogenous ABA

The ABA methods of extraction and analysis were the same as described by Sales et al. (2017). Fresh samples (1 g) were homogenized with 20 mL extraction solution comprised of 80% (v/v) methanol, 0.1% L (+) ascorbic acid (Kanto Chemical Co., Tokyo, Japan), and 0.1% butylated hydroxytoluene (BHT;

2,[6]-di-tert-butyl-p-cresol; Sigma-Aldrich Co., St. Louis, MO, USA), using 0.2 μg ABA-d_6 as an internal standard. The solution was purified using high-performance liquid chromatography (HPLC; flow rate, 1.5 mL min^{-1}; detection at 254 nm) that was equipped with an ODS-Mightysil RP-18 column (250 × 4.6 mm i.d.). The solution was methylated using ethereal diazomethane and finally analyzed using gas chromatography-mass spectrometry for ion monitoring (GC-MS-SIM; model QP5000; Shimadzu, Kyoto, Japan).

2.2.8 Expressions of the *MdNCED1* gene

Total RNA was extracted using the cetyltrimethylammonium bromide (CTAB) method of Kondo et al. (2012) and cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix and gDNA Remover (Toyobo Co., Ltd., Osaka, Japan). Quantitative real-time PCR analysis was performed. The *ubiquitin* gene was used as a reference gene, for which the primer sequence was designed as follows:

forward: 5′-TCGCTGGAAAGCAGCTCGAAGA-3′,

reverse: 5′-GCTTTCCGGCAAAGATCAGACG-3′.

The *MdNCED1* gene primer was referenced to Sales et al. (2017), and used as follows:

forward: 5′-GTATCACGTCCAAATCACTGAACC-3′,

reverse: 5′- ATTTGAGGTATGGCTTCTGAACG-3′.

The results were analyzed using the $2^{-\Delta\Delta CT}$ method (Yang et al., 2012) and three replicates were performed for each set of treatments.

2.2.9 Statistical analysis

Data are presented as means ± SE, subjected to analysis of variance procedures,

and separated by the Tukey-Kramer test at $P \leq 0.05$ using the SPSS statistical analysis software.

2.3 RESULTS

2.3.1 Effects of exogenous ABA and Abz on water potential and MDA concentrations in apple seedlings under drought stress

The water potential was the lowest in the control group at 4 day after drought treatment (DAT) (Fig. 2.1A). In contrast, that of the ABA+Abz group was higher than that in the other treatments. Drought stress increased the MDA concentrations, but this effect was noticeably weakened by the ABA+Abz treatment (Fig. 2.1B).

2.3.2 Effects of exogenous ABA and Abz on proline concentrations in apple seedlings under drought stress

The proline concentration was highest in the control group at 4 DAT, but in the ABA+Abz group, it was the lowest at 4 DAT, with a decrease of 45.92% as compared to the control group (Fig. 2.2).

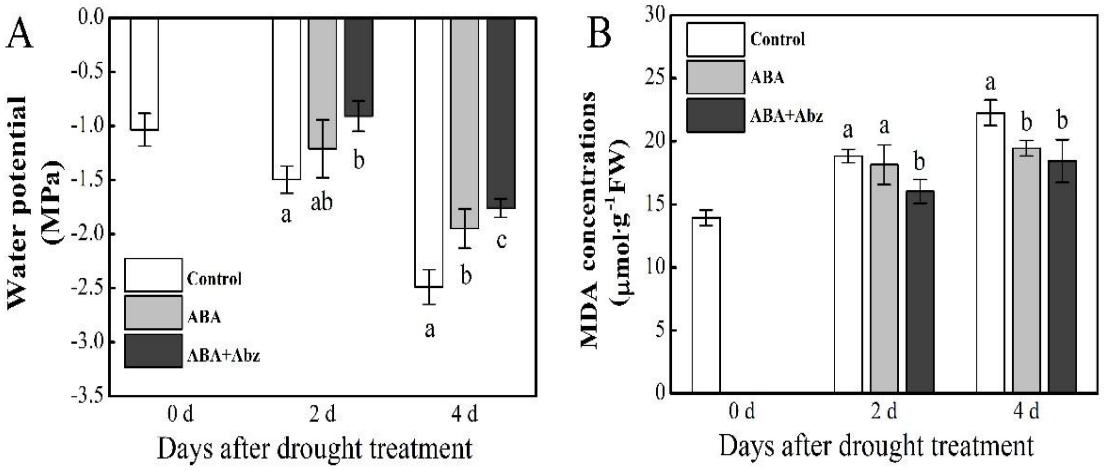

Fig. 2.1 Changes of water potential (A) and MDA concentrations (B) in apple leaves under drought stress. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey– Kramer test at $P \leq 0.05$.

2.3.3 Effects of exogenous ABA and Abz on antioxidant and enzyme activity in apple seedlings under drought stress

The DPPH radical scavenging activity in the ABA+Abz group was significantly higher than in other groups at 2 DAT (Fig. 2.3). At 4 DAT, the ABA+Abz and ABA treatments were higher than in the control group. Similarily to the DPPH activity, the activity of the antioxidant enzymes SOD, POD and APX in the control group increased at 2 DAT but decreased significantly at 4 DAT (Fig. 2.4A, B, D). In contrast, the activity of the ABA+Abz or ABA groups was not reduced at 4 DAT in SOD, CAT or APX. In fact, the activity CAT increased at 4 DAT under drought conditions (Fig. 2.4C).

2.3.4 Effects of exogenous ABA and Abz on endogenous ABA concentrations and *MdNCED1* gene expression in apple seedlings under drought stress

Endogenous ABA concentrations in the control group at 2 DAT were nearly triple the levels found on day-0 (Fig. 2.5A). Concentrations in the ABA+Abz treatment further increased by 2.32- and 4.60-fold at 2 and 4 DAT, respectively, in comparison to the control group. Although the concentration of endogenous ABA in the ABA treatment increased dramatically at 2 DAT, no significant difference between the ABA treated and control treatment were found at 4 DAT. The expression of the *MdNCED1* gene in all three groups was induced at 2 DAT and increased further at 4 DAT (over 25-fold), with maximum expression in the control group (Fig. 2.5B).

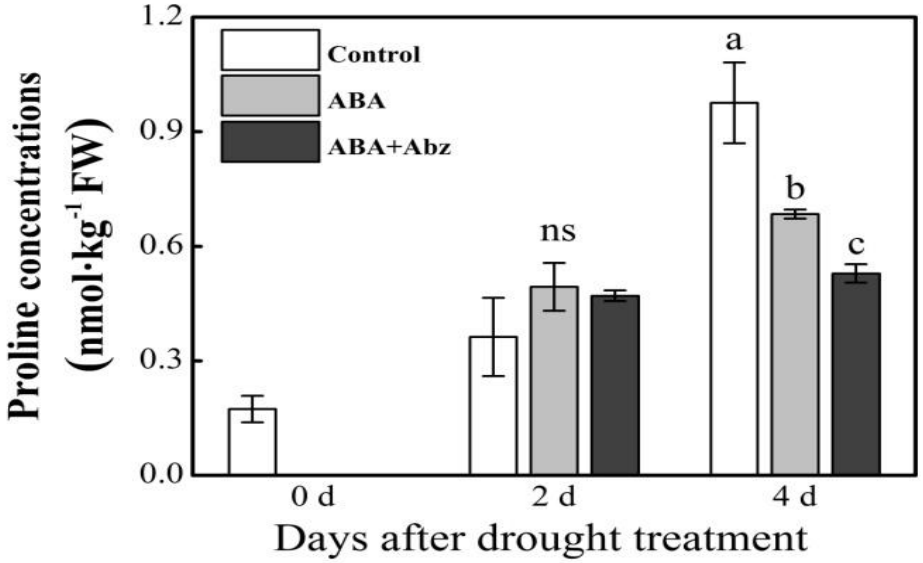

Fig. 2.2 Proline concentrations in apple leaves under drought stress conditions. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey–Kramer test at $P \leq 0.05$.

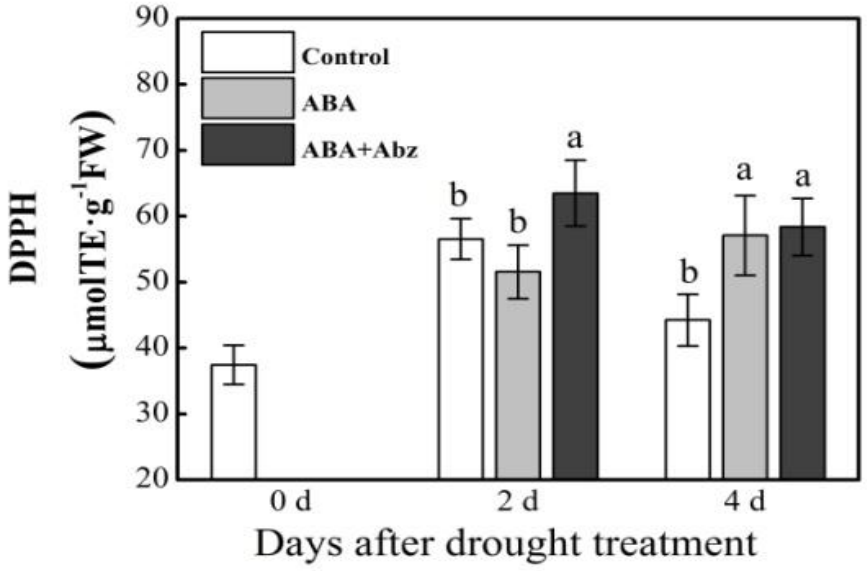

Fig. 2.3 DPPH radical scavenging abilities in apple leaves under drought stress conditions. Data are the mean ±SE of three replications. Different letters indicate significant differences by Tukey–Kramer test at $P \leq 0.05$.

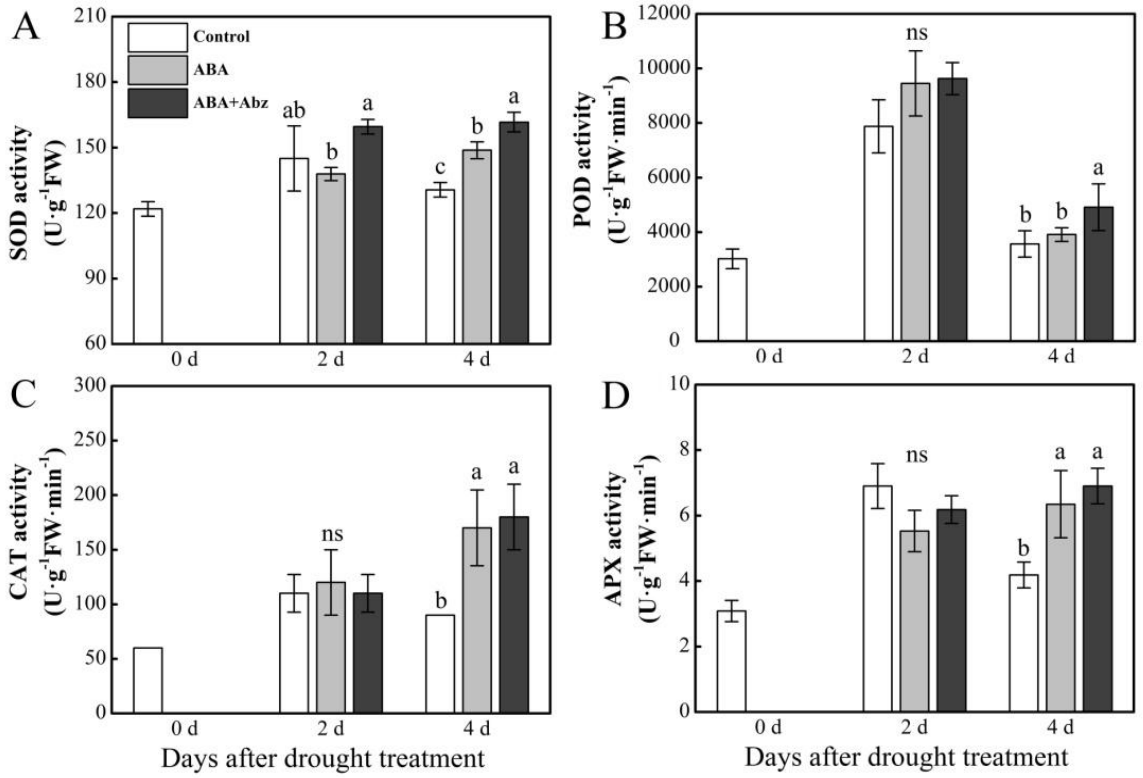

Fig. 2.4 Antioxidant enzyme activities of SOD (A), POD (B), CAT (C) and APX (D) in apple leaves under drought stress. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey–Kramer test at $P \leq 0.05$.

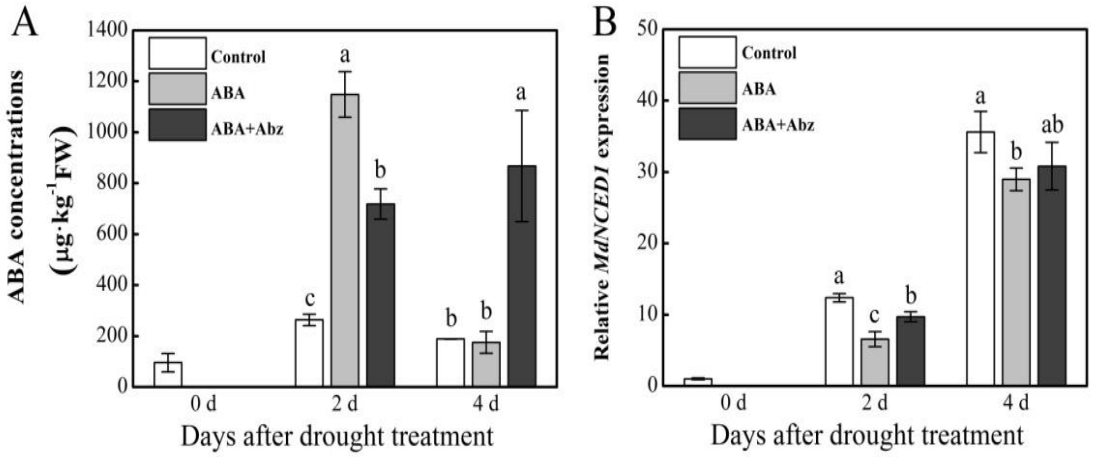

Fig. 2.5 Endogenous ABA concentrations (A) and the expressions of the *MdNCED1* (B) gene in apple leaves. The experimental values were plotted compared with the control (*Ubiquitin*) values. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey–Kramer test at $P \leq 0.05$.

2.4 DISCUSSION

Sruamsiri (1986) found that in strawberries, there is a water potential threshold of -1.7 MPa for wilting and -2.5 MPa for irreversible drought effects. Our experiment showed similar results, with water potentials of -1.46 and -2.49 MPa at 2 and 4 DAT. It was observed that ABA and ABA+Abz treatments maintained higher water potentials in the leaves as compared to the control group and that the ABA+Abz treatment maintained the highest values. It was deduced that ABA induces stomatal closure rapidly to reduce water loss. ABA and ABA+Abz treatments quickly increased the endogenous ABA concentrations at 2 DAT, and the ABA+Abz group maintained higher ABA concentrations, providing a greater tolerance against drought stress.

MDA, which is a product of lipid peroxidation, reflects the degree of cell membrane damage by oxidative stress (Aroca et al., 2012). In this study, seedlings subjected to drought showed an elevated level of MDA, indicating that there was severe oxidative damage being caused by the drought conditions. Proline manifested similar results, as judged from the increased concentrations. It is regarded as a membrane stabilizer and an essential molecule for plant tolerance and recovery from environmental stresses (Zhong et al., 2020). The ABA and ABA+Abz treatments inhibited the elevations of both MDA and proline concentrations, implying that there was less injury to the seedlings under the imposed drought conditions by

maintaining the stability and functionality of the cell membranes. A lower MDA concentration was also observed in maize and wheat seedlings with ABA application under adverse conditions (de Souza et al., 2014). Proline concentrations generally increase with increases in stress levels (Murmu et al., 2017). In our study, the proline concentrations decreased in the ABA and ABA+Abz treatments, which may reflect lower stress damage under drought conditions. ABA may prevent lipid peroxidation by inhibiting excess ROS produced under drought stress.

ROS, such as superoxide radicals (O_2^-) and H_2O_2, directly and indirectly damage enzymes, biological membranes, and cellular components, causing severe oxidative damage in plants. SOD is a critical antioxidant that converts O_2^- into O_2 and H_2O_2. Although ROS can play a key role as a signaling molecule, its excess accumulation can be toxic, and should be eliminated for the proper functioning of CAT, POD and APX (Yang et al., 2015). In our study, the activities of SOD, CAT, and APX in the ABA and ABA+Abz treated leaves increased after the application of drought conditions, but only at 4 DAT. POD activity at 4 DAT in the ABA treatment was not different from the control. Thus it is likely that the combined activities of CAT, APX and SOD played a critically protective role in scavenging the O_2^- and H_2O_2 during the stress conditions. Simultaneously, radical scavenging abilities were also shown by DPPH at 4 DAT. Those results, therefore, indicate that both ABA and ABA+Abz were able to enhance antioxidant activities in apple seedlings under drought conditions.

In this study, the endogenous ABA concentrations increased rapidly at 2 DAT in both the ABA and ABA+Abz treatments but the increase was not sustained and

reduced markedly at 4 DAT in the ABA treatment. Abz-E3M is the inhibitor of ABA 8'-hydroxylase, which inhibits ABA catabolism, and thus is likely the reason that a higher ABA concentration was maintained at 4 DAT. Though the expression of the *MdNCED1* gene also increased at 2 and 4 DAT, it was lower than in the control group. It was deduced that ABA and ABA+Abz treatments did not need the increase of *MdNCED1* expression because the stomata were closed quickly (Sales et al., 2018), the high endogenous ABA concentrations inhibited its synthesis.

2.5 CONCLUSION

Both the ABA and ABA+Abz treatment mitigated the stress damage caused by drought conditions. Application of a combination of ABA and Abz inhibited the degradation of endogenous ABA, maintained endogenous ABA concentrations, stabilized leaf water potential and increased antioxidant activities, resulting in an induced tolerance to drought stress.

CHAPTER 3

SALT STRESS IN APPLE SEEDLING WAS MITIGATED BY *N*-PROPYL DIHYDROJASMONATE, A SYNTHETIC ANALOG OF JASMONIC ACID

3.1 INTRODUCTION

Sodium chloride in soil causes ion toxicity, ROS formation, membrane dysfunction, oxidative damage and multiple metabolic activity attenuation (Annunziata et al., 2017). Exposure to unfavorable abiotic environment leads plants to developed strategies to respond appropriately, and these include alteration of phytohormone such as JA and ABA and amino acid such as proline levels. JA can reinforce stress resistance in plants exposed to sodium chloride by enhancing antioxidant enzymes activities and triggering stress response genes expression (Ruan et al., 2019). Pedranzani et al. (2003) showed that salt tolerant tomato (*Solanum lycopersicum*) showed higher levels of JA concentrations and *AOS* gene expressions. The JA signaling mechanism against abiotic stress involves activation of JA by *JAR* gene, which conjugates JA and isoleucine to jasmonoyl-L-isoleucine (JA-Ile), and ultimately activates the downstream responses via degradation of jasmonate ZIM-domain (JAZ) repressor proteins (Ruan et al., 2019).

Plants accumulate ABA through the functions of two key enzymes, 9-*cis*-epoxycarotenoid deoxygenase (NCED) and ABA 8′-hydroxylase (CYP707A) (Waśkiewicz et al., 2013). Over-expression of *OsNCED5* gene enhanced salt tolerance by controlling ABA biosynthesis and genes related to abiotic stress in rice (*Oryza sativa* L.) (Huang et al. 2019). Sales et al. (2017) found that inhibition of CYP707A1 attenuated the catabolism of ABA and increased endogenous ABA concentrations under NaCl stress in apple seedlings, and decreased Na^+ intake and mitigated salinity damage in apple seedling. Generally, crosstalk exists between ABA and JA with regards to their signaling behavior

(Yang et al., 2019). de Ollas et al. (2013) found that JA transient accumulation was needed for ABA response to salt tolerance in citrus (*Citrus reticulata Blanco*).

Various antioxidant enzymes such as SOD, CAT and POD have been induced against salt stress (Ahmad et al., 2016). As a free radical scavenger, proline is synthesized by the enzyme Δ1-pyrroline-5-carboxylate synthatase (P5CS) and ornithine-delta-aminotransferase (OAT) and degraded through oxidation by proline dehydrogenase (PDH) (Cao et al., 2020). It has been shown that proline accumulation under salt stress in plants tissues is attributed to the high expression of *P5CS* gene and decreased expression of *PDH* gene (Cao et al., 2020). This resulted in protection against NaCl-induced ROS and lipid peroxidation through the increase of antioxidant enzyme activities (Reddy et al., 2015). ABA induced proline production in salt stressed plants by activating *P5CS* gene expression (Guan et al., 2019). ABA regulates intracellular Na^+ dynamic balance via inducing the expression of *salt overly sensitive1* (*AtSOS1*) and *Na^+/H^+ exchanger 1* (*AtNHX1*) genes in *Arabidopsis thaliana*. Both genes encode the plasma memberane Na^+/H^+ transpoter and Na^+/H^+ exchanger protein, functioning as Na^+ efflux carrier from the cell and Na^+ compartmentalization in vacuolar (Yamaguchi et al., 2013).

A synthetic analog of JA, *n*-propyl dihydrojasmonate (PDJ), has been widely used in Japan for the promotion of apple coloration (Kondo et al., 2000). PDJ could inhibit *Botrytis cinerea* pathogen infection in apples (Suktawee et al., 2019). PDJ treatment also improved chilling tolerance in apple fruits through induction of scavenging activity against reactive oxygen (Kondo, 2009). Therefore, PDJ application may mitigate biotic and abiotic stresses including salinity.

In the present study, the endogenous JA, ABA and proline concentrations, SOD, POD, CAT and APX antioxidants activities and Na^+, K^+ concentrations, as well as their respective gene expressions were examined to clarify whether PDJ can mitigate NaCl stress in apple seedlings or not.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials and treatment

Ninety-day-old 'Tsugaru' apple seedlings [*Malus domestica*] were used. The germinated apple seeds were sown in plastic trays (26 × 52 × 6 cm) with a capacity of 72 seeds per tray with a spacing of 4 × 4 cm, containing moist vermiculite as a substrate. The trays were set under the greenhouse for growth and a half liter of water was added in the bottom of each tray every day to keep it moist, and 0.2% (v/v) Hyponex® solution was applied as a nutritent (Hyponex Japan Co., Osaka, Japan). The seedlings were grown under natural light conditions with average daytime temperature range in the green house was 25 to 30 ℃, and 15 to 20 ℃ at night.

Treatment consisted of an application of 0.8 mM of PDJ solution containing 0.5% Approach BI® (Maruwa Biochemical Co., Tokyo, Japan) on both sides of the leaves. After 24 h, 100 mM sodium chloride was applied to seedlings by dipping into 1 L NaCl solutions per tray. The apple seedlings were then divided into four groups as follows: (1) a group without any treatment (Untreated control); (2) a group treated with 0.8 mM of PDJ solution, but without NaCl ($PDJ^{+}NaCl^{-}$); (3) a group treated with 100 mM NaCl solution, without PDJ application ($PDJ^{-}NaCl^{+}$); (4) a group administered with a combination of 100 mM NaCl and PDJ ($PDJ^{+}NaCl^{+}$). The concentrations of PDJ and NaCl were based on a previous study by Sales et al. (2017). Two hundred forty (240) seedlings were sampled from each group, divided into 3 replications and healthy fresh leaves were collected at 1, 4, 7 and 10 days

after the beginning of NaCl treatment, frozen with liquid nitrogen, and kept at −80 °C until further analysis.

3.2.2 Determination of ion leakage and lipid peroxidation

Ion leakage was determined according to Jambunathan (2010). The fourth leaf from the tip of each seedling (six replications per each treatment) was sampled and ion leakage was determined using the electrical conductivity meter (LAQUAtwin-pH-22, HORIBA, Japan). The relative ion leakage was expressed as the percentage of conductivity before and after boiling of leaves in respective aqueous extracts. The amount of malondialdehyde (MDA) was measured according to Heath and Packer (1968). MDA was determined spectrophotometrically at OD_{450}, OD_{532} and OD_{600} using the UV-VIS spectrophotometer (2J1-0010, HITACHI, Japan).

3.2.3 Measurement of hydrogen peroxide (H_2O_2) and proline concentrations, and antioxidative enzymes activity

The concentrations of H_2O_2 in fresh leaves (0.5 g, three replications) were measured according to the method of Shi et al. (2005). The absorbance was at 390 nm and the final concentration was calculated according to standard curve derived from different concentrations of H_2O_2. The proline concentrations were assayed based on the method of Sales et al. (2017). The fresh leaf tissues (0.5 g, three replications) were homogenized with 10 mL sulfosalisylic acid 3% (w/v) then centrifuged at 15000 g for 10 min. After reaction with ninhydrin solution, the compound was measured at 520 nm by a spectrophotometer (U-2910; Hitachi, Tokyo, Japan). A standard curve was obtained using L-proline. For antioxidant

enzyme analysis, the leaf tissues (three replications of 0.5 g) were homogenized with 15 mL phosphate buffer (50 mM, pH 7.8) containing 0.5 mM EDTA and 2% (w/v) polyvinylpyrrolidone and centrifuged at 15000 g for 15 min. The antioxidant enzyme activities of SOD, POD, CAT and ascorbate peroxidase (APX) were measured according to the method of He et al. (2014).

3.2.4 Determination of chlorophyll (Chl), Na^+ and K^+ concentrations

After extraction of chlorophyll in the leaves using N, N-Dimethylformamide, it was measured at absorbance 663 and 645 nm, by the spectrophotometer (U-2910; Hitachi, Tokyo, Japan). The Chl a, b and total Chl concentrations were calculated according to Sestak et al. (1971). The Na^+ and K^+ concentrations in the leaves were determined according to Sales et al. (2017). The leaf samples (0.5 g dry weight, three replications) were mixed with 15 mL nitric acid, then converted to ashes at 450 ℃ and finally ground into powder. The residues were dissolved in 0.1 N hydrochloric acid and filtered. The solutions were analyzed by atomic absorption spectrophotometry (ICPE-9000; ICP-AES Multitype ICP Emission Spectrometer, Shimadzu, Japan).

3.2.5 Analysis of JA and ABA

JA in leaves was analyzed according to Suktawee et al. (2019). Leaf samples (1 g FW, three replications) were homogenized in 10 mL saturated NaCl solution, 20 mL diethyl ether containing 0.005% butylated hydroxytoluene (BHT) and 500 μL of 1 M citric acid and 100 μL [(±)-2-(2,3-$^2H_2$) JA (100 mg L^{-1})] as an internal standard. The final extract was dissolved with methanol and analyzed using gas chromatography/mass spectroscopy-selective ion monitoring (GC-MS -SIM) (QP

5000; Shimadzu, 25 m × 0.25 mm I.D. column) as described by Kondo et al. (2009). The ABA was extracted using the method described by Sales et al. (2017). Leaf samples (1 g FW, three replications) were homogenized with 20 mL cold 80% (v/v) methanol with 100 mg L^{-1} BHT butylated hydroxytoluene and 0.5 g polyvinylpolypyrrolidone as a stabilizer with 0.2 μg ABA-d_6 as an internal standard. The extract was purified with high performance liquid chromatography (HPLC; flow rate, 1.5 min^{-1}; detection at 254 nm; ODS-Mightysil RP-18 column, 250 mm × 4.6 mm i.d.) and finally measured with gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM; model QP5000; Shimadzu, Kyoto, Japan).

3.2.6 Total RNA isolation, cDNA synthesis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted using the cetyltrimethylammonium bromide as reported by Kondo et al. (2012). The first-strand cDNA was synthesized from 1 μg of total RNA according to the instruction manual of ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). And qRT-PCR was performed on a StepOnePlus™ system (Applied Biosystems, USA) using the THUNDERBIRD™ SYBR® qPCR Mix (Toyobo, Osaka, Japan). The primers except for *MdUbiquitin-11* gene were designed using Beacon Designer 7 (Premier Biosoft International) software (Table 1). The *MdUbiquitin-11* gene was used as an internal control gene. The results were calculated using the $2^{-\Delta\Delta CT}$ method (Yu et al., 2007). Three biological replicates were performed for each set of treatment.

3.2.7 Statistical analysis

All data were statistically analyzed with SPSS statistics software (IBM SPSS statistics 20, IBM Corp., Armonk, NY) and means separated using Duncan's multiple range test at the $P \leq 0.05$ level.

Table. 3.1 Primers used for real-time RT-PCR

Gene	**Forward/reverse primer (5′–3′)**	**Accession No.**
MdNCED1	(F) TACTACTACTACTACTACTACA (R) GAAGGTTGTTGATAGGAA	XM_009356579
MdCYP707A1	(F) ACCGAGGAGATTACCATA (R) CTTCAATGCCAGGAACTA	XM_008358695
MdAOS1	(F) GAACCTGTTATCCACTCT (R) GGAAGACTCGTAGAAGAA	XM_008366758.3
MdJAR	(F) CTGCCTTCAATCAACATT (R) AATCAACAACTTCCAACTT	XM_008377840.2
MDJAZ2	(F) ATTCTTGTCTTCCGTCTCT (R) ACCTTGCCTATCGTGATT	NM_001328912.1
MdP5CS	(F) TTATCGGACTTGTGAATA (R) ACAGGTATCTTAGTTGAC	XM_008389606.3
MdOAT1	(F) CGAGTATCTAACAAGCAT (R) ACACGAGACAATAATAGC	XM_017327930.2
MdPDH	(F) AAGCCAATGTGCCTCTAT (R) AGGAGTAGGTCAAGTAATCG	XM_008359589.3
MdSOS1	(F) ACCTGCTGACTGGCCCT (R) ATGTTCCAGTAAGCAGCTTGG	XM_008391743.1
MdNHX1	(F) GGTGTTCGGATTGATGAC (R) AGTGGTTGGTTCTGATGA	NM_001328858.1
Ubiquitin11	(F) TCGCTGGAAAGCAGCTCGAAGA (R) GCTTTCCGGCAAAGATCAGACG	XM_008360582.2

3.3 RESULTS

3.3.1 Chlorophyll, MDA and H_2O_2 concentrations, and ion leakage

At 10 days after treatment (DAT), the concentrations of Chl a, b and total Chl were lowest in the PDJ^-NaCl^+ group compared to the other groups (Fig. 3.2a). The chlorophyll a/b (Chl a/b) ratio was highest in the PDJ^-NaCl^+ group, followed by the PDJ^+NaCl^+ and PDJ^+NaCl^- groups and the untreated control group was lowest.

The ion leakage, and MDA and H_2O_2 concentrations in leaves exhibited a similar tendency. These increased in the PDJ^-NaCl^+ and PDJ^+NaCl^+ groups through NaCl treatment (Fig. 3.2b,c,d). PDJ^+NaCl^+ group showed lower concentrations compared to PDJ^-NaCl^+ group at 7 and 10 DAT. Ion leakage and MDA concentrations at 7 and 10 DAT were not significantly different between PDJ^+NaCl^- group and the untreated control. PDJ^+NaCl^- group increased H_2O_2 concentrations compared to the untreated control at 1 and 4 DAT.

3.3.2 Endogenous JA concentrations and expressions of JA-related genes

The endogenous JA concentrations in the PDJ^-NaCl^+ and PDJ^+NaCl^+ groups were significantly higher from 4 to 10 DAT compared to the untreated control (Fig. 3.3a). JA in the PDJ^+NaCl^+ group was 1.8 folds compared to that in the PDJ^-NaCl^+ group at 1 DAT. JA concentrations in the PDJ^+NaCl^- group increased at 1 and 4 DAT. Both of *MdAOS1* and *MdJAR* genes were up-regulated by NaCl treatment compared with the untreated control group at 4, 7 and 10 DAT (Fig. 3.3b,c). Compared to the PDJ^-NaCl^+ group, the *MdAOS1* and *MdJAR* gene expressions in the PDJ^+NaCl^+ group presented higher level at 7 and 10 DAT respectively. On the

other hand, the expressions of *MdJAZ2* gene in the $PDJ^{-}NaCl^{+}$ group were significantly lower than those in the untreated control (Fig. 3.3d). In the $PDJ^{+}NaCl^{+}$ group, the expression of *MdJAZ2* gene were lower than that in the $PDJ^{-}NaCl^{+}$ group at 1 and 4 DAT.

Fig. 3.1 Morphological observation of 'Tsugaru' apple seedlings at 10 days after the treatments of NaCl and PDJ.

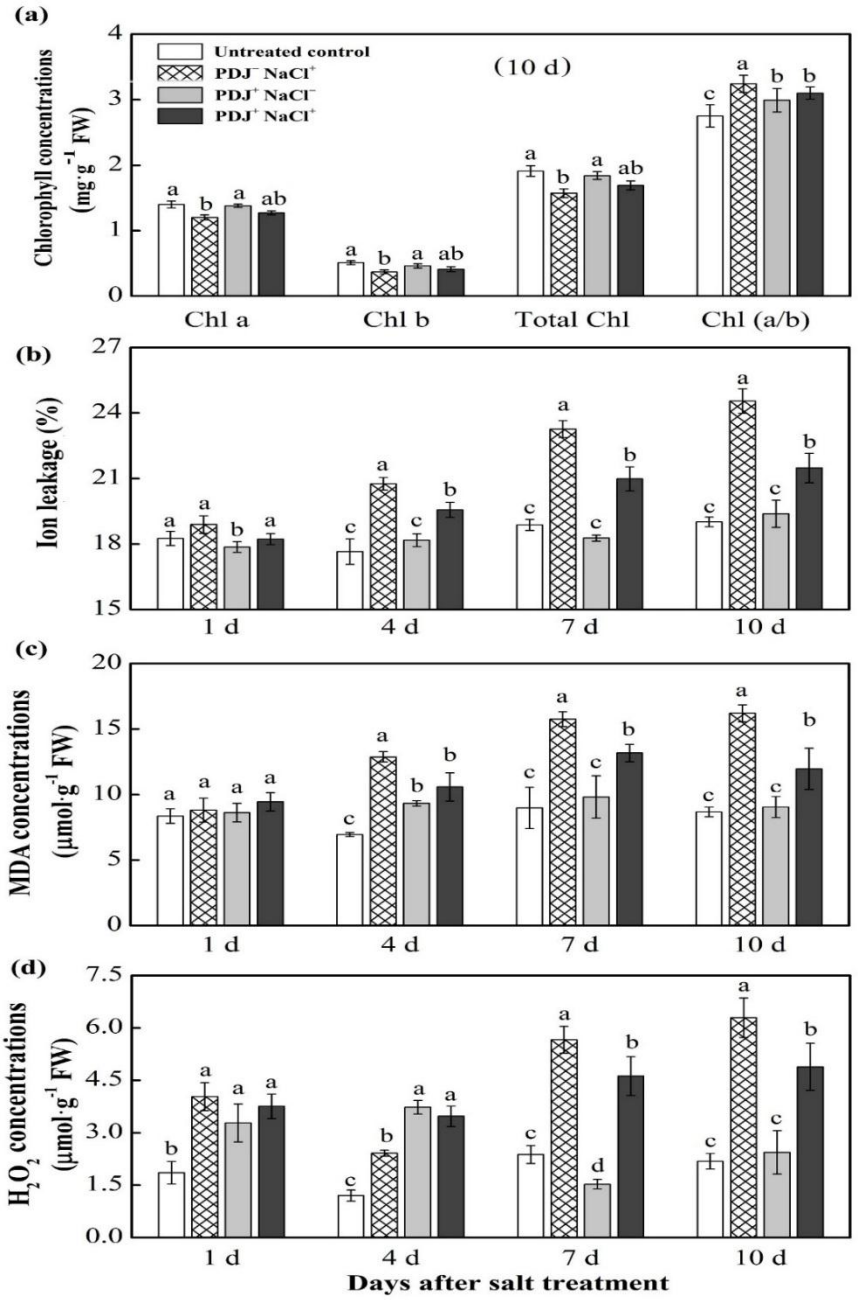

Fig. 3.2 Changes of chlorophyll (a) ion leakage (b), MDA (c) and H_2O_2 (d) concentrations in apple leaves under different treatments. Chlorophyll concentrations were revealed only at 10 DAT in this study. The data represent the average value ± SE of six replications in ion leakage and of three replications in another indexes. Different letters indicate significant differences by Duncan's multiple range test at $P \leq 0.05$.

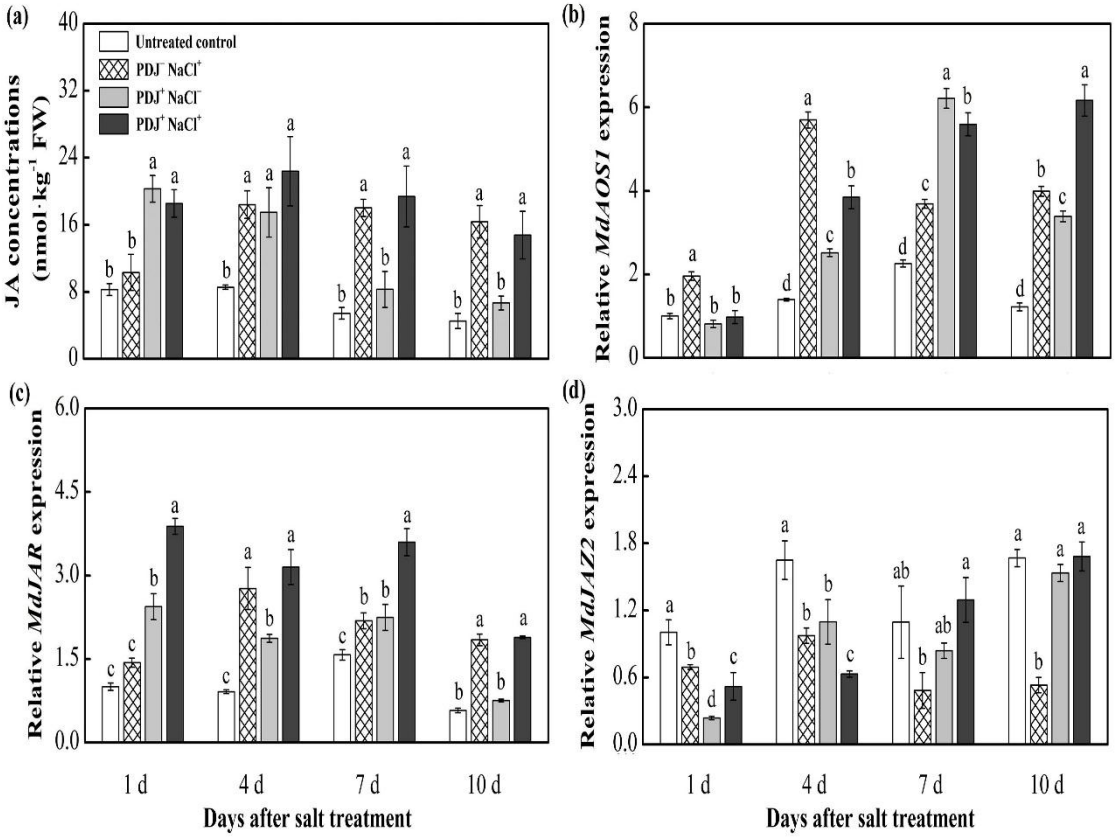

Fig. 3.3 Endogenous JA concentrations (a) and gene expressions of *MdAOS1* (b), *MdJAR1* (c) and *MdJAZ2* (d) in apple leaves under different treatments. The data represent the average value ± SE (n = 3). The experimental values are plotted compared with the control (*Ubiquitin gene*) values. Different letters indicate significant differences by Duncan's multiple range test at $P \leq 0.05$.

3.3.3 Endogenous ABA concentrations and expressions of ABA-related genes

NaCl treatment ($PDJ^{+}NaCl^{+}$ and $PDJ^{-}NaCl^{+}$) significantly increased ABA concentrations compared to other treatments at 4, 7 and 10 DAT (Fig. 3.4a). The ABA concentrations in $PDJ^{+}NaCl^{+}$ treatment were higher than those in $PDJ^{-}NaCl^{+}$ at 1 DAT but lower at 7 and 10 DAT. The ABA concentrations in $PDJ^{+}NaCl^{-}$ group were increased at 1 DAT but significantly declined at 7 and 10 DAT, compared to the $PDJ^{-}NaCl^{+}$ group. The *MdNCED1* gene expressions in both $PDJ^{+}NaCl^{+}$ and $PDJ^{-}NaCl^{+}$ groups were up-regulated at 7 DAT (Fig. 3.4b). The *MdNCED1* gene expressions in $PDJ^{-}NaCl^{+}$ group were highest compared to other treatments at 10 DAT. The expression of *MdCYP707A1* gene in $PDJ^{+}NaCl^{-}$ and $PDJ^{+}NaCl^{+}$ groups showed similar tendency with *MdNCED1* gene (Fig. 3.4c).

3.3.4 The antioxidant enzyme activities

The SOD, CAT and POD enzyme activities in the $PDJ^{-}NaCl^{+}$ group increased at 4 DAT but declined significantly at 10 DAT, compared to the untreated control group (Fig. 3.5a,b,c). Foliar application of PDJ before NaCl treatment ($PDJ^{+}NaCl^{+}$ group) increased antioxidant enzymes activities of SOD, CAT, POD and APX compared to those of the $PDJ^{-}NaCl^{+}$ group at 7 DAT (Fig. 3.5a,b,c,d).

3.3.5 Proline concentrations and expressions of proline-related genes

The proline concentrations in the $PDJ^{-}NaCl^{+}$ group significantly increased at 4, 7 and 10 DAT and followed by $PDJ^{+}NaCl^{+}$ group (Fig. 3.6a). In general, the expressions of both *MdP5CS* and *MdOAT1* genes were significantly upregulated in $PDJ^{+}NaCl^{+}$ and $PDJ^{-}NaCl^{+}$ groups compared to untreated group at 4 to 10 DAT, (Fig. 3.6b, c). In contrast, the *MdPDH* gene expression was downregulated in the

PDJ^{-}NaCl^{+} group (Fig. 3.6d).

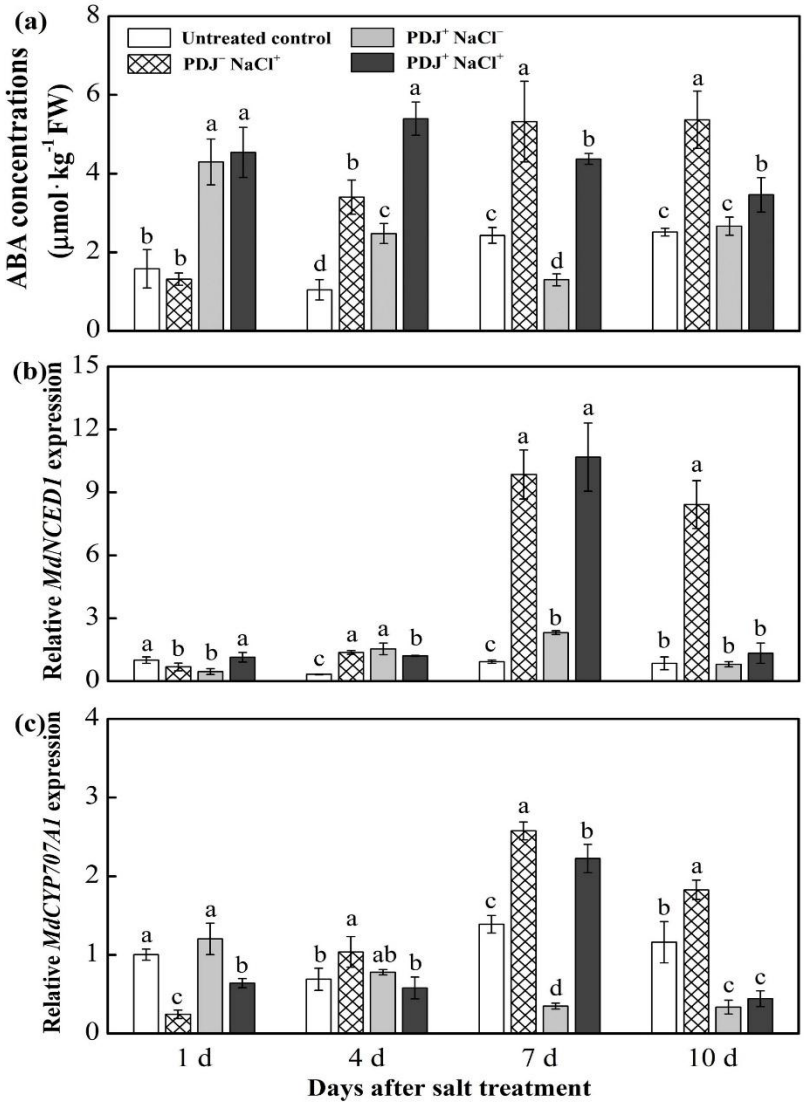

Fig. 3.4 Endogenous ABA concentrations (a) and gene expressions of *MdNCED1* (b) and *MdCYP707A1* (c) in apple leaves under different treatments. The data represent the average value ± SE (n = 3). Different letters indicate significant differences by Duncan's multiple range test at $P \leq 0.05$.

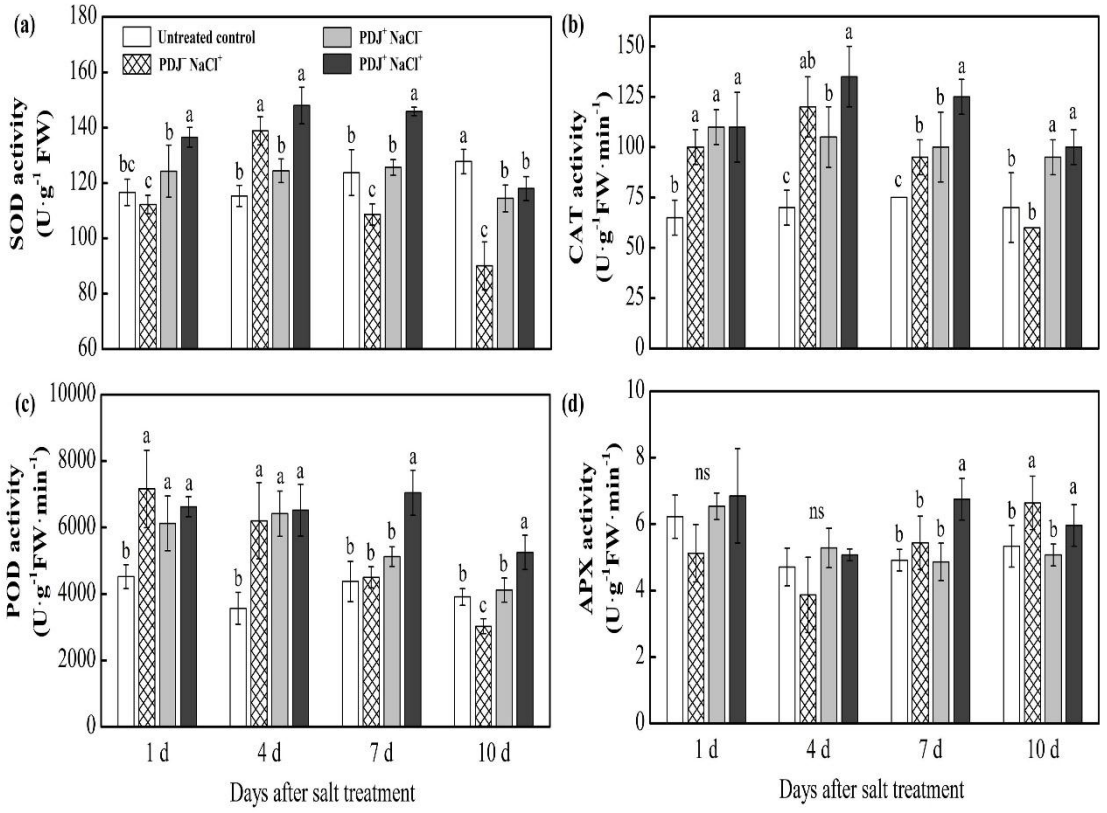

Fig. 3.5 Antioxidant enzymes of SOD (a), CAT (b), POD (c) and APX (d) activity in apple leaves under different treatments. The data represent the average value ± SE (n = 3). Different letters indicate significant differences by Duncan's multiple range test at $P \leq 0.05$.

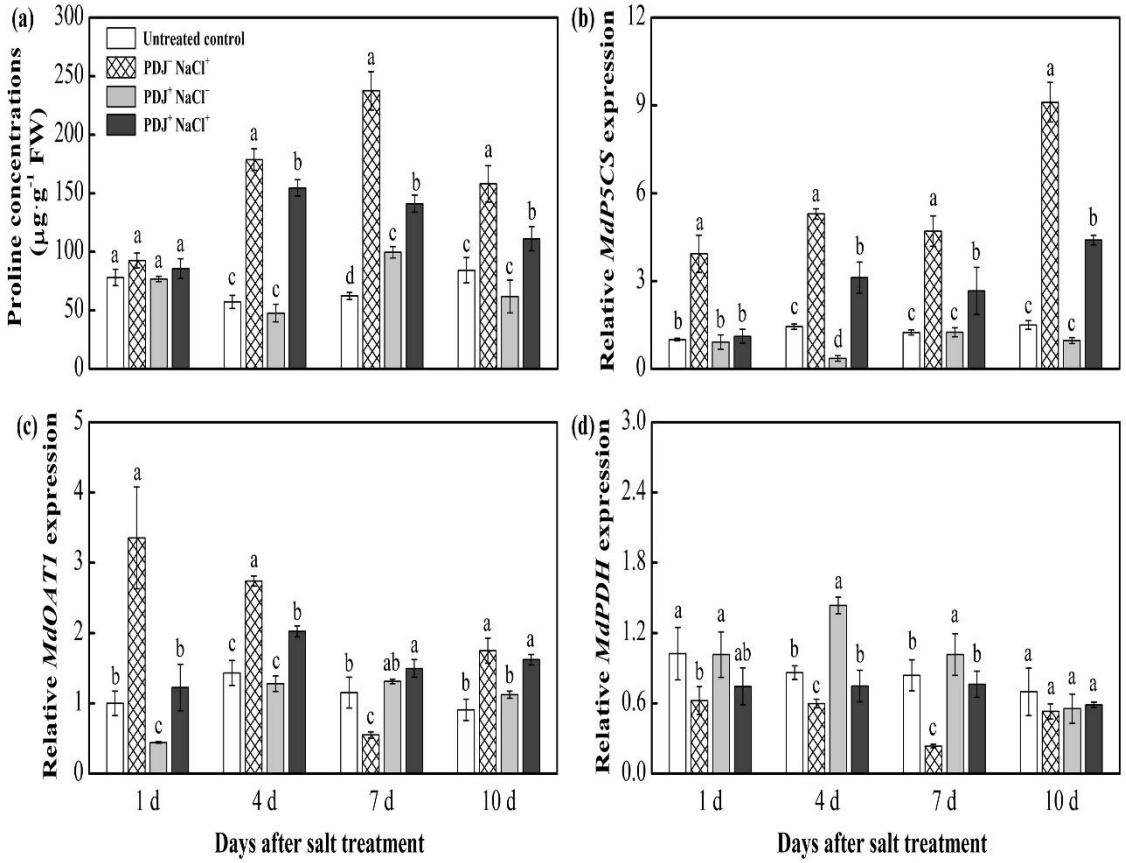

Fig. 3.6 Proline concentrations (a) and gene expressions of *MdP5CS* (b), *MdOAT1* (c) and *MdPDH* (d) in apple leaves under different treatments. The data represent the average value ± SE (n = 3). Different letters indicate significant differences by Duncan's multiple range test at $P \leq 0.05$.

3.3.6 The Na^+ and K^+ concentrations and expressions of ion transport-related genes

The Na^+ concentrations in the PDJ^-NaCl^+ and PDJ^+NaCl^+ groups increased by 6.8 and 2.5 folds respectively than those in the untreated control group at 10 DAT. (Fig. 3.7a). PDJ^+NaCl^- treatment did not influence the Na^+ concentrations compared to the untreated control, but enhanced the K^+ levels at 7 and 10 DAT. The K^+ concentrations in the PDJ^+NaCl^+ group was highest, followed by the PDJ^+NaCl^- and PDJ^-NaCl^+ groups, and the untreated control group was lowest at 10 DAT. The highest K^+/Na^+ ratio was the PDJ^+NaCl^- group and the lowest was the PDJ^-NaCl^+ group. Both the expression of *MdSOS1* and *MdNHX1* genes in the PDJ^-NaCl^+ group increased at 4 DAT (Fig. 3.7b, c). The expression of *MdSOS1* and *MdNHX1* genes in PDJ^+NaCl^+ group was increased significantly compared to the other treatments at 7 and 10 DAT.

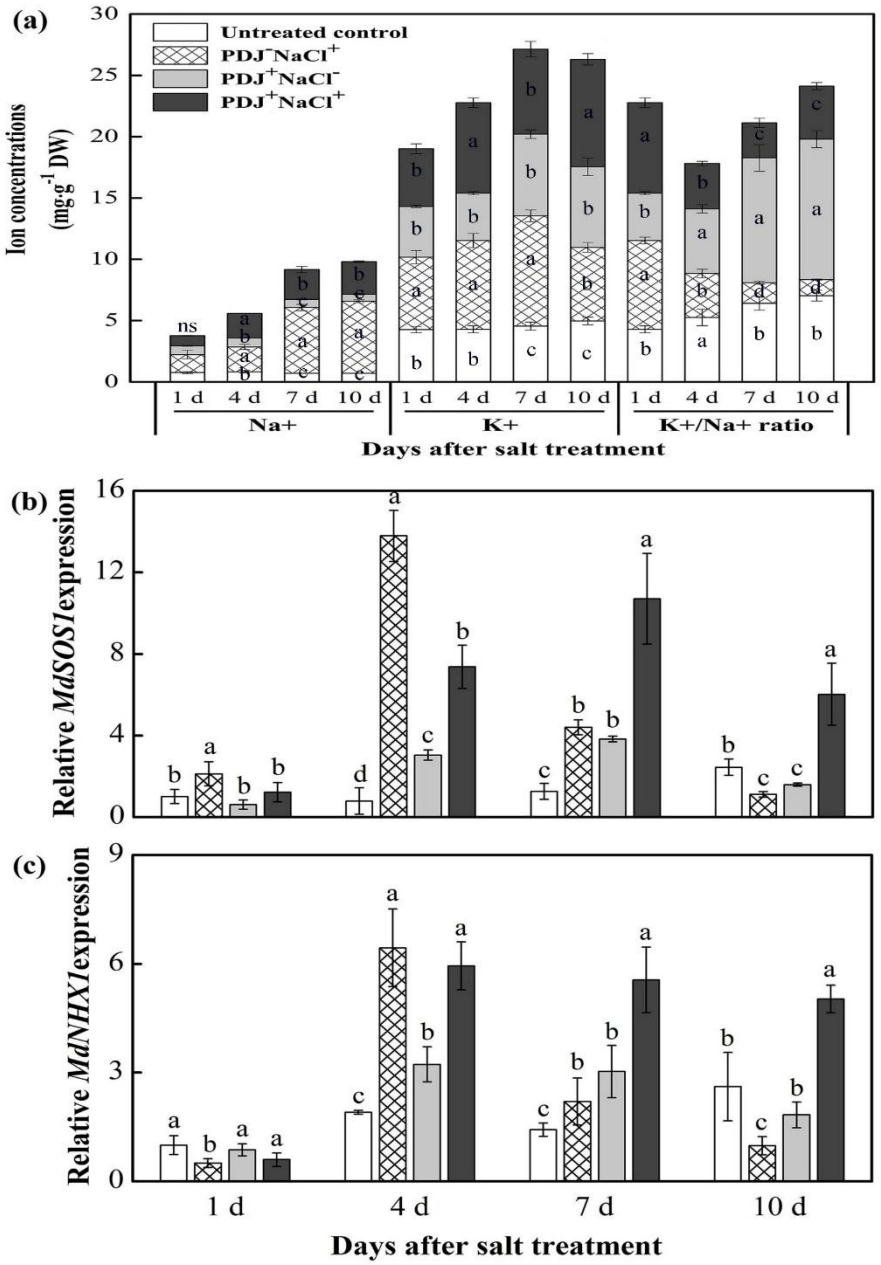

Fig. 3.7 Na^+ and K^+ concentrations (a) and *MdSOS1* (b) and *MdNHX1* (c) gene expressions in apple leaves under different treatments. The data represent the average value ± SE (n = 3). Different letters indicate significant differences by Duncan's multiple range test at $P \leq 0.05$.

3.4 DISCUSSION

In this study, the decrease of chlorophyll concentrations was observed in $PDJ^{-}NaCl^{+}$ group and this symptom was ameliorated by PDJ application prior to NaCl treatment ($PDJ^{+}NaCl^{+}$). Rezai et al. (2013) found that MeJA application inhibited the degradation of chlorophyll in pepper (*Capsicum annuum* L.) under NaCl stress. During the process of chlorophyll degradation, Chl b is first converted to Chl a, then degraded by chlorophyllase (Fang et al., 1998). This process may explain the increased ratio of Chl a/b and the decrease of total chlorophyll concentrations in NaCl treated leaves. Membrane lipids are considered significantly vulnerable to the salinity, and NaCl-induced stress generates ROS, disrupting lipid membrane integrity and ion homeostasis (Reddy et al., 2015). This study revealed that MDA, the final product of lipid peroxidation, and ion leakage rate was significantly inhibited in the $PDJ^{+}NaCl^{+}$ plants. Qiu et al. (2014) reported that exogenous JA treatment ameliorated NaCl injury in wheat (*Triticum aestivum* L.) by decreasing the concentration of MDA, H_2O_2 and ion leakage rate. Meanwhile, at 7 DAT, the APX enzyme activity in the $PDJ^{+}NaCl^{+}$ group was higher than that of the $PDJ^{-}NaCl^{+}$ group. These results suggested that the $PDJ^{+}NaCl^{+}$ treatment retarded the MDA production and ion leakage. Therefore, PDJ may mitigate membrane injury caused by NaCl stress.

It has been shown that ABA induced stomatal closure and up-regulated the expression of stress-related genes to increase salt tolerance (Waśkiewicz et al., 2013). Our results showed that the ABA concentrations were increased in the

$PDJ^{+}NaCl^{+}$ group, as well as up-regulation of *MdNCED1* gene and down-regulation of *MdCYP707A1* gene. Huang et al. (2019) reported that *OsNCED5* transgenic plants presented stronger salt tolerance than wild type plants owing to higher endogenous ABA concentrations and higher expression of genes related to abiotic stress. The JA concentrations in this study continually accumulated in the $PDJ^{+}NaCl^{+}$ group, as well as the *MdAOS1* gene expression. Pedranzani et al. (2003) also showed that salt tolerant tomato showed higher levels of JA concentrations and *AOS* gene expressions. It has been known that the accumulation of endogenous JA could activate the expression of *JAR* gene and enhance the conversion of JA to JA-Ile, resulting in the induction of the proteolysis of JAZ (Ruan et al. 2019). The higher expression of *MdJAZ2* gene in the $PDJ^{+}NaCl^{+}$ group implied the higher accumulation of JA-Ile, whose function is on activating JA signaling pathways directly. Liu et al. (2019) reported that overexpression of *PnJAZ1* gene confers salinity tolerance in moss (*Pohlia nutans*) by activating ABA signaling pathway. de Ollas et al. (2013) showed that both JA and ABA accumulated in salt-stressed plants. Thus, it is reasonable to presume that the application of PDJ initiated JA and ABA response and hence modulating multiple defense systems to overcome the adverse impacts caused by salt.

H_2O_2 is an important signaling molecules involved in many metabolic processes, and its excessive generation, frequently triggers oxidative stress and further induced lipid peroxidation and MDA accumulation (Liu et al., 2020). In this study, the high concentrations of H_2O_2 and MDA in $PDJ^{-}NaCl^{+}$ group suggested an occurrence of oxidative stress. In contrast, the lower level of that in the $PDJ^{+}NaCl^{+}$

group implies a mitigation effect of PDJ application on oxidative injury caused to NaCl stress. This result may be associated with the higher activities of SOD, POD and CAT enzyme activities in the $PDJ^{+}NaCl^{+}$ group. Shu et al. (2016) reported that the accumulation of endogenous ABA mediated H_2O_2 generation, which consequently induced the antioxidant defense systems and enhanced cucumber (*Cucumis sativus* L.) salt tolerance. The increase of these enzyme activities was essential for enhancing salt tolerance in wheat (*Triticum aestivum* L.) and grape (*Vitis vinifera* L.) (Qiu et al., 2014; Haider et al., 2019). The results of this study agreed with a previous report by Kondo et al. (2009), who found that exogenous application of PDJ enhanced the fruits chilling tolerance by increasing radical scavenging ability. Additionally, Shan and Yang (2017) also found that exogenous JA induced other antioxidant metabolites such as ascorbic acid and glutathione synthesis with neutralized toxic effects of NaCl stress. Thus, the reduction of H_2O_2 and enhancement of SOD, POD and CAT enzyme activities in the $PDJ^{+}NaCl^{+}$ group enhanced higher radical scavenging activity to minimize the oxidative injury caused by NaCl stress.

The accumulation of proline plays an essential role on stabilizing membrane structure, and enhancing cell osmotic potential. Guan et al. (2019) found that proline production in salt stressed plants was ameliorated by ABA through activation of *P5CS* gene expression. In this study, proline concentrations were increased by NaCl treatments. In addition, the expressions of proline synthesis genes *MdP5CS* and *MdOAT1* were significantly increased in both $PDJ^{-}NaCl^{+}$ and $PDJ^{+}NaCl^{+}$ groups, with a reversed expression trend of *MdPDH* gene, which encodes the proline

oxidase involved in degradation of proline. Cao et al. (2020) found that proline accumulation was attributed to the decrease in expression of *OsPDH* and increase of *OsP5CS* gene, which are regulated by ABA-dependent signals under NaCl stress. This result may support with the result of this study which NaCl treatment induced proline and ABA accumulation. The lower proline concentrations in $PDJ^{+}NaCl^{+}$ group compared to the $PDJ^{-}NaCl^{+}$ group may imply less injury to the seedlings caused by NaCl stress.

It has been shown that plants increase Na^{+} uptake and reduce K^{+} uptake under salt stress (Garciadeblás et al., 2003). K^{+} ions maintain electrolyte and osmotic balance in the cells of organisms (Zhang et al., 2018). In this study, the Na^{+} ion uptake in the $PDJ^{-}NaCl^{+}$ group was increased significantly, while K^{+} ion decreased under NaCl treatment. High Na^{+} concentrations in plants retard the uptake of K^{+} ion and speed up its efflux, resulting in the increased ion leakage and membrane lipid peroxidation in the leaves, as well as occurance of oxidative stress (Zhang et al., 2018). Generally, lower Na^{+} concentrations and higher K^{+}/Na^{+} ratio was observed in the $PDJ^{+}NaCl^{+}$ group compared with $PDJ^{-}NaCl^{+}$ group. This result may show that PDJ regulates the absorption of Na^{+} in plant tissues. Na^{+} dynamics in a plant cell is mainly regulated by *SOS1* and *NHX1* genes, which functioned as Na^{+} efflux carrier from cell and Na^{+} compartmentalization in vacuolar organelle (Yamaguchi et al. 2013). The up-regulation of the *MdSOS1* and *MdNHX1* genes in the $PDJ^{+}NaCl^{+}$ group was concomitant with reduced Na^{+} concentration. And this process was most likely regulated by endogenous ABA (Osakabe et al., 2014).

3.5 CONCLUSION

PDJ application prior to NaCl treatment could mitigate the NaCl stress by inducing the rapid accumulation of endogenous JA and ABA, enhanced antioxidant enzymes activities, regulated proline concentrations, reduced Na^+ uptake and retarded the increase of MDA and ion leakage (Fig. 3.8).

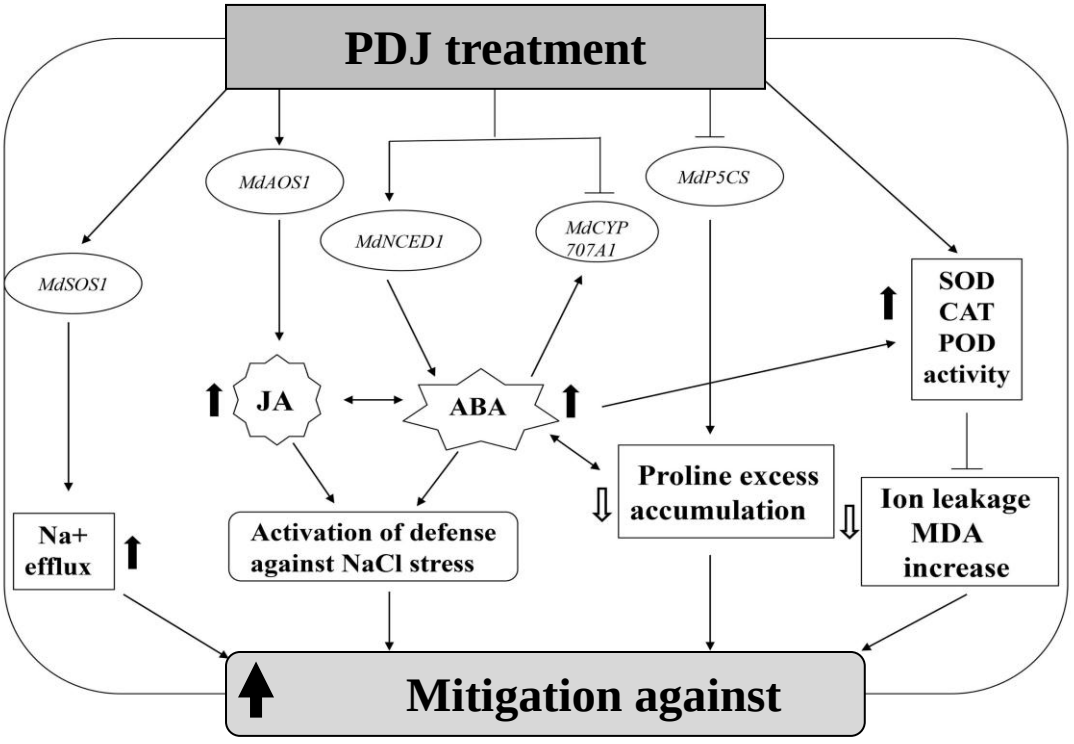

Fig. 3.8 Schematic diagram of the regulation of NaCl tolerance in apple seedings by PDJ treatment. Dark arrows indicate increases, and blank arrows indicated decreases.

4. GENERAL DISCUSSION

Water resources shortage caused by global warming not only affected humanity activity, also increase the rate of drying, establishing agricultural drought more quickly and with greater intensity. Without enough water, the transport of substances, biofilm assembly and metabolism in plant cells cannot process normally, thus retards the growth of plants severely, and even leads to death. One of the first effects observed after dehydration is stomatal closure, even before detection of any leaf water deficit to reduce transpiration of water to maintain the lower water potential (Sanders, 2012). This reaction can be detected within minutes.

The water potential up to -3.0 MPa will cause the permanent wilting for xerophytes, -1.5 MPa for medium plants and over -1.5 MPa for wet plants (Frank et al., 1973). In the present research, drought stress caused very low water potential on apple seedlings, implied the severity damage occurred, proofed by the high MDA concentrations, too.

Accumulation of organic and inorganic solutes under drought and/or salinity, which help lower water potential without decreasing actual water contents, and these solutes do not pose any detrimental effects on membranes. Proline is considered to act as an osmolyte, a ROS scavenger, and a molecular chaperone stabilizing the structure of proteins, thereby protecting cells from damage caused by drought or high salinity stress by accumulating in tissues (Krasensky and Jonak, 2012). The high levels of proline under drought reflects the success of the plant on its intent to overcome stress, but it can also reflect negatively, indicating an oversaturated

system. Because the proline in ABA or ABA+ABz treated groups was lower than that in the drought treated group, implicated a less damages on seedlings leaves sprayed with ABA or ABA+ABz prior to drought treatment.

Water deficit triggers the production of ABA in roots quickly, which is transported to the shoots causing stomatal closure. Leaf cells can also synthesize ABA and translocated around the plant to regulate the activity of guard cells (Osakabe et al., 2014). In this study, the endogenous ABA concentrations increased rapidly at 2 DAT in both the ABA and ABA+Abz treatments, which showed positive responses in the management of water intake and prevent cell oxidation damage. High concentrations of ABA can quickly induce stomatal closure to minimize transpiration losses. It can also mitigate stress damage by promoting the synthesis of multiple osmotic protective substances, and by activating multiple defense response systems. Pretreatment with ABA increased the endogenous ABA concentrations at early stages, stabilized leaf water potential and increased antioxidant activities. But the increase of ABA was not sustained and reduced markedly at 4 DAT in the ABA treatment, compared to ABA+Abz treatment. It has demonstrated that ABA production triggered rapidly to avoid inhibition of plant growth and functioning, and it also rapidly degraded and deactivated (Amjad et al., 2014). Abz-E3M is the inhibitor of ABA 8'-hydroxylase, which inhibits ABA catabolism, and thus is likely the reason that a higher ABA concentration was maintained at 4 DAT. Both the ABA and ABA+Abz treatment mitigated the stress damage caused by drought conditions, with the ABA+Abz treatment possesses a better effect.

Although NaCl stress can also induce stomatal closure, possess the same the

regulatory metabolism with water deficit, but the purpose could be different. Stomata is the most important organ for water transpiration and carbon dioxide gain. Na^+ is taken up from saline soil by roots and transported to the aerial parts of plants through the transpiration stream (Osakabe et al. 2014). Stomatal closure induced by NaCl stress limits the water transpiration, which blocks the power of root cells Na^+ uptake from saline soils. As excess Na^+ is toxic to plants, and reducing Na^+ uptake to be the key, as well as the most efficient approach, to control Na^+ accumulation in crop plants and hence the closure of stomata being one of the first effects observed after NaCl stress.

Exposure to salinity environment leads plants to developed strategies to respond appropriately, and these include alteration of phytohormone such as JA and ABA. Sales et al. (2017) found that inhibition of *CYP707A1* attenuated the catabolism of ABA and increased endogenous ABA concentrations under NaCl stress in apple seedlings, and decreased Na^+ intake and mitigated salinity damage in apple seedling. JA can reinforce stress resistance in plants exposed to sodium chloride by enhancing antioxidant enzymes activities and triggering stress response genes expression (Ruan et al., 2019). And crosstalk exists between ABA and JA with regards to their signaling behavior under stress conditions. PDJ, as one of the synthetic analog of JA, could inhibit *Botrytis cinerea* pathogen infection and improve chilling tolerance in apple fruits. In this study, again proved its effective role on mitigating stress damage.

Excess Na^+ and Cl^- might damage membrane permeability and dehydrate the cell. In addition, these ions taken up by plant concentrates priority in the old leaves, which lost chlorophyll more rapidly and died earlier. In this study, the chlorophyll was affected, as well as the high MDA and H_2O_2 level. Pretreatment with PDJ mitigated the leaf damages by improving oxidation resistance and maintaining

membrane stability and reducing Na^+ uptake.

Whether the application of ABA under drought or spraying PDJ before NaCl stress on apple seedlings, the overall result is similar within the two. Both rely on high levels of ABA at early stages to overcome adversity, as well as managing other pathways that are important in the recovery. What is more, it has been reported that the JA transient accumulation was needed for ABA response to salt tolerance, which was consistent with this study.

As a final remark, it can be concluded that in both experiments the ideal strategy to successfully overcome stress conditions is not only to focus on an efficient mechanism via plant regulators, but also that time is a very important factor in a successful response. By having high levels of ABA at an early stage has been seen that even salt sensitive plant like apple are able to create a strategy that will successfully overcome the stress by managing hormones synthesis, improving oxidation resistance and the aid of osmolytes.

5. SUMMARY

The effects of ABA and PDJ on drought stress and NaCl stress were examined in apple seedlings (*Malus domestica*). The water potential, ion leakage, MDA and proline concentrations, SOD, CAT, APX, and POD activity, Na^+ level, endogenous ABA and JA concentrations, and expressions of the ABA, JA and proline-related genes expression were analyzed. Drought conditions reduced water potential (Mpa) in the leaves but increased proline accumulation, ABA concentrations, and *MdNCED1* expression. The activities of SOD, CAT, APX, and POD in ABA+Abz or ABA treated leaves increased at 4 DAT after the imposition of drought conditions. The ABA+Abz maintained water potential and reduced proline concentration. ABA+Abz had a stronger effect than the application of ABA alone against drought. NaCl stress significantly decreased chlorophyll concentrations, SOD, POD and CAT activities, but increased the ion leakage, MDA, proline and Na^+ concentrations, as well as the H_2O_2, endogenous JA and ABA concentrations in the leaves. Foliar application of PDJ before NaCl treatment (PDJ^+NaCl^+) decreased Na^+ concentrations, ion leakage, MDA and proline accumulation, and H_2O_2 generation, but increased K^+ concentrations, antioxidant capacity and chlorophyll concentrations in the leaves. In PDJ^+NaCl^- group, although the JA and ABA concentrations, and *MdAOS1*, *MdJAR* and *MdNCED1* gene expressions were increased, *MdJAZ2* and *MdCYP707A1* gene expressions were decreased. In addition, the PDJ^+NaCl^+ group showed higher *MdPDH*, *MdSOS1* and *MdNHX1* gene expressions and lower *MdP5CS* gene expressions than PDJ^-NaCl^+ group at 7 and 10 days after treatment. The present total data suggested that ABA or PDJ application could induce rapidly endogenous JA and ABA accumulation under stress conditions,

enhance SOD, POD and CAT activities, which might be a good approach to enhance plant tolerance and allow the plant to recover.

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