12-2017

Polyamines Regulate Chilling Response in Ripening Tomato Fruits by De Novo Transcription

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POLYAMINES REGULATE CHILLING RESPONSE IN RIPENING TOMATO FRUITS BY DE NOVO TRANSCRIPTION

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Horticulture

West Lafayette, Indiana

December 2017

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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To my dearest wife (Ruby) and baby (Nael) for being part of my journey to graduation

ACKNOWLEDGMENTS

First and foremost, I would like to express my special appreciation and thanks to my advisor, Prof. Dr. Avtar K. Handa, for accepting me as his MS student and as a part of his wonderful lab in Purdue. He offered so much personal and technical advice, patiently supervising me, and always steering me in the right direction in my studies. I would also like to acknowledge my committee members; Dr. Lori Hoagland, Dr. Gurmukh Johal and Dr. Peter Hirst for their valuable comments and suggestion for my thesis and defense, and for being a significant part of my thesis approval.

I would like to thank all my fellow lab members for their immeasurable assistance throughout my studies, with special thanks goes to two important women namely Dr. Tahira Fatima and Ms. Tatsiana Datsenka (Tanya). I am indebted to Tahira for her time and effort in guiding my thesis writing, editing my thesis draft and suggesting possible improvements; and to Tanya for all the selfless help she provided with the lab works by teaching me the protocols and setting up the instruments and always willing to help whenever I needed. Many thanks also to my fellow graduate students especially Dr. Kexin Wang for assisting me with RNA-seq data analyses, and Ms. Bazgha Zia who was always willing to help especially with the course assignments. I would never have been able to finish my MS study without their helps.

I would also like to thank all Horticulture and Landscape Architecture Department staff and faculties, especially department Head, Dr. Hazel Wetzstein for giving me a good opportunity to be a teaching assistant during my final semester; and Ms. Colleen Flynn for keeping all graduate students organized and always there for us whenever we needed help.

I am gratefully indebted to my employer, Malaysian Agricultural Research and Development Institute (MARDI), which granted me a study leave and provided me an indispensable generous sponsoring. My study in Purdue would not have been possible without the financial support from MARDI. A very special thank you goes to Datuk Abd Shukor Abd Rahman (Former Director General, MARDI), Tengku Ab Malik Tengku Maamun (Former Director, Horticulture Research Center, MARDI), Dr. Pauziah Muda (Former Deputy Director, Postharvest Handling Program, Horticulture Research Center MARDI) and Dr. Razali Mustaffa (Deputy Director, Fruit Program, Horticulture Research

Center MARDI) for approving and supporting my application to study in the USA. You all have made my dream come true. In addition, I would like to thank Human Resource Management Center MARDI, especially the Training and Competency Program for providing unfailing supports during a significant part of my study leave. Special thanks go to Ms. Zainab Yunus, Mr. Nik Mohd Adib Ghazali, Ms. Asiah Mansor and all other officers and staffs who cared about my financial affair and study progress and not to mention my family welfare while we are living in a foreign country. Without their support and financial help, it would not have been possible for me to pursue and to complete this MS study successfully.

Last but not least, I must express my very profound gratitude to my entire family and friends. Most importantly, I am greatly indebted to my loving and supportive wife, Wan Rubitah (Ruby) who was always there with me throughout my stay in Purdue, cheering me up and stood by me through the good times and bad. She encouraged me and gave me warm hugs when I was frustrated. I am also very grateful to our precious baby, Nael, for being such a good baby boy and always cheering me up. He was born during my final year of study and being a motivation for me to finish this study. Special thanks also go to my mother, mother-in-law, father-in-law, my siblings, sibling in-laws and my friends for their prayer, love and sacrifice and their life-long support. They were always supporting me and encouraging me with their best wishes.

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Institution: Purdue University
Degree Received: December 2017
Title: Polyamines Regulate Chilling Response in Ripening Tomato Fruits by De Novo Transcription
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Plants response to various abiotic stress, including chilling injury, by differentially regulating genes responsible for protection against these stresses. Polyamines (PAs); putrescine (Put), spermidine (Spd), and spermine (Spm), are ubiquitous metabolite in all organisms and have been implicated in abiotic stresses in plants. Dr. Handa laboratory has previously developed high Spd/Spm tomato fruits genotypes by expressing yeast S-adenosylmethionine decarboxylase (SAMDC). In present investigation, the role of Spd/Spm in chilling injury was evaluated by using two independent transgenic lines with high Spd/Spm (556 and 579) and isogenic parental Ohio 8245 (WT) genotypes. Fruits at onset of ripening (Breaker, BR) and fully ripe (Breaker+8 days, BR+8) from both transgenic lines and WT were subjected to chilling by storing at 5°C or 8ºC. Results showed that the 556 and 579 fruits exhibit more chilling tolerance phenotype at BR+8 stage but performed poorly at BR stage compared to WT fruit. RNA-seq analyses of transcriptome showed differential expression of a large number of genes in response to chilling treatment of high Spd/Spm fruit compared to WT fruit, which suggesting a significant effect of PAs in chilling response mechanism. PAs are implicated in regulating genes related to redox homeostasis (Thioredoxin family protein), stress signaling pathway (MAPK cascade), various cold responsive genes, protein chaperon (heat shock proteins) and cellular antioxidant system (CAT, SOD and APX). It can be postulated that, being positive charged molecules, PAs bind to DNA, RNA and transcription factors and differentially regulate expression of genes associated with chilling tolerance in ripened fruits that help acclimate fruits to sub optimal chilling temperatures

CHAPTER 1. INTRODUCTION

Temperature is the most important environment factor that influence deterioration rate of fresh produce after harvest. In general, there will be two to three-fold increase in the rate of deterioration with every 10°C increase of temperature at optimum range (Kader, 2002). In many cases, it may take up to several weeks for fruit and vegetables before consumed after harvested. Throughout postharvest period, fruits and vegetables are undergoing many biochemical processes that affect many quality attributes. Optimal storage temperatures for tomato depends on fruit maturity. For mature-green tomato, recommended temperature is 12.5°C at which tomato can be stored up to 14 days without significantly affect the color and sensory properties, and thereafter decay incidences are expected to increase (Suslow and Cantwell, 1997). For light red tomato, the range suggested for refrigerated storage is 10 to 13°C (Suslow and Cantwell, 1997), but tomatoes stored at 10°C have lower aroma and flavor rating than those stored at 13°C (Maul et al., 2000). The fully ripe tomatoes can be stored at far lower temperature such as 7°C, but they withstand this storage for a couple of days before chilling injury incidence set-in. Cherry and grape tomatoes, however, are sometimes stored at sub-optimal temperatures (2-5°C) as they are often packed together with other fresh cut vegetables in retail refrigerator, and could last for 14-18 days (Cantwell et al., 2009).

1.1 Effects of chilling temperature on tomato flavor

Flavor is the first quality attribute in fruits that undergo modifications in cold storage, both in air and controlled atmosphere storage (Kader, 2008). Flavor is a result of combination of aroma and taste sensations (Acree, 1993), which stemmed from complex interactions among sugars, organic acids and more than 400 volatile compounds (Baldwin et al., 2000; Sanz et al., 1997). Tomato flavors are significantly contributed by about 30 aroma volatiles with cis-3-hexenal, hexanal, cis-3-hexenol, β-ionone, β-damascenone, 1-penten-3- one, 3-methylbutanal and 2-isobuthylthiazole being the most important ones (Yilmaz, 2001). Hexanal and 2-isobutylthiazole volatiles are commonly associated with moldy in odor, flavor and after-taste (Krumbein et al., 2004). In general, the lower the storage temperature,

greater is decrease in aroma volatile level, even at the recommended temperature range. Storage of light-red tomato at 13°C would likely increase the negative impact on tomato flavor compared to storage at 20°C (Maul et al., 2000). Kader et al. (1978) reported that temperature below 16°C may impair tomato flavor by lowering the volatile content leading to reduced tomato-like aroma. Comparison of storage at two recommended temperatures, 10°C and 13°C, showed that 10°C resulted in more loss of flavor and an unfavorable sensory quality with off-odor and less freshness than 13°C (Ponce-Valadeza et al., 2016). At 13°C, significant chemical aroma profile was detected as early as on day 6 of storage and as expected, storing tomato at lower temperatures risk chilling injury and adversely affect aroma and flavor. Light red tomatoes stored at 4°C or 5°C (Renard et al., 2013; Maul et al., 2000) were rated significantly lower in tomato flavor and sweetness as opposed to 20°C for the same duration, with about 66% decrease in volatiles concentration at lower temperature. The aroma concentration, however, recovered slightly by returning the fruits to ambient temperature after 6 days at 4°C or 5°C (Renard et al., 2013). Storage at 4-5°C is generally used in domestic refrigeration. Based on a survey, approximately 60% of consumers store tomatoes in the refrigerator at home (Farneti et al., 2012). Consumers need to be made aware of the detrimental effect of too low temperature on tomato flavor and aroma so that flavor and aroma is not lost at the point of tomato supply chain (Zhang et al., 2016). Transcriptome analysis performed using RNA sequencing (RNA-Seq) on tomato fruits stored at 5°C revealed that there was significant loss of flavor volatiles after 8 days of storage and they did not recover even after fruits return to ambient temperature which was associated with reductions in transcripts encoding essential enzymes for volatile biosynthesis. On the other hand, sugar and acid contents did not change under similar storage temperature (Zhang et al., 2016), suggesting that chilling conditions impair aroma not the other determinants of taste of tomato.

1.2 Effects of chilling temperature on tomato nutritional quality

Tomato fruit is arguably one of the important reservoir of carotenoid, particularly lycopene that imparts red color to fruit (Rao and Rao, 2007). Being a very strong antioxidant, it is also a source of health benefits in addition to other metabolite such as vitamin E, ascorbic acid, phenolic compounds and flavonoids (Beecher, 1998). At recommended temperature,

it is reported that lycopene content during ripening increased at 10°C throughout storage period (Khairi et al., 2015), but this increase was more rapid (up to 3-fold) at higher temperature (15-25°C). Storage at 7°C or 5°C on the other hand lowered accumulation of lycopene (Toor and Savage, 2006; Javanmardi and Kubota, 2006 and Farneti et al., 2012). For ascorbic acid, the concentration was higher when being stored at 10°C for up to 14 days, as compared to storage at ambient temperature (Gharezi et al., 2012; Oms-Oliu et al., 2011). Storing tomato fruits at low temperature also could retain ascorbic acid concentration to the extent similar with fruits ripen on vine (Tsaniklidis et al., 2014). Storage for a short period of time such as 10 days, storage at 7°C, 15°C and 25°C resulted in small differences in ascorbic acid content (Toor and Savage, 2006). Taken together, available findings suggest that low temperature storage within the recommended range would not cause only the deleterious effect on tomato for certain duration but would maintain the ascorbic acid level with some increase in the lycopene content.

1.3 Chilling injury

Although storage at optimal temperatures (10-13°C) have enabled extension of tomato postharvest life up to a few weeks, much lower temperature is still required for certain cases in commercial practice such as in mixed shipment of various types of fruits in one container regardless of storage requirement. In addition, the ability to cope with lowest possible temperature would make distant transportation or long-term storage feasible. Nevertheless, storing tomato below the threshold temperature for certain duration would result in some chilling stress. A wide variety of physiological and biochemical changes and cellular dysfunctions takes place in chilling-sensitive species in response to chilling stress (Wang, 1982). These include, but not limited to stimulation of ethylene production and respiratory rate, interference in energy production, decrease in photosynthesis rate, inactivation of enzymes, cell membrane dysfunction, and alteration of cellular structure. Observation in the tomato fruits encountered chilling stress revealed that after 15 days of storage at 2°C, prominent ultrastructural modifications were present as seen by the swelling of mitochondria and plastids which ultimately hardly discernible after 21 days due to degeneration. These phenomena may lead to development of chilling injury symptoms after a prolonged exposure to chilling conditions, characterized by internal discoloration,

surface lesions, water-soaking of the tissue, decay, off-flavor unable to ripen normally (Saltveit and Morris, 1990)

1.4 Mechanisms of chilling injury

Conceptually, chilling injury incidence can be broken down into primary and secondary effects. The primary event takes place when the temperature dips below its threshold for specific duration and alter membranes, whereas the secondary event is time-dependent and adversely affect a multitude of metabolic processes as a result of the primary event and leading to detectable chilling injury symptoms (Orr and Raison, 1990). Cell membranes are considered as a primary site for development of chilling injury (Rui et al., 2010). Under non-chilling temperatures, the cell membranes are in fluid form, but at chilling injury temperature their fluidity is reduced due to increase membrane rigidity. The first contemporary hypothesis which associate the chilling injury and cell membrane was advanced by Lyons (1973) who proposed that lipid phase transition in the cell membranes under chilling conditions may accounts for the primary response during chilling disorder which consequently causing molecular and structural alteration in the lipid matrix. Study by Sharom et al. (1994) on postharvest tomato fruits confirmed this hypothesis and showed that indeed tomato fruits undergo membrane lipid phase transition which was associated with chilling injury development but are more in an indirect way rather than direct effect of the chilling. These authors also reported that phase transition is not reversible when temperature is increased above the chilling threshold and become even more intensified upon returning of fruits to ambient temperature after chilling (Sharom et al., 1994).

This focus on membrane phase transitions has led to several studies examining the relationship between unsaturated membrane lipids and chilling sensitivity. Chilling injury was shown to be induced by the decline of unsaturated fatty acids level and the membrane permeability (Imahori et al., 2008). Lower ratio of unsaturated to saturated fatty acids may indicate an increase in lipid peroxidation under chilling conditions. Genetic engineering approach confirmed that plant chilling tolerance can be enhanced by increasing unsaturated lipids in the cell membranes (Goyal et al., 2016). The lipid peroxidation was found to be responsible to the chilling injury development, which may be achieved by the concerted activities of a membranous lipolytic enzymes such as phospholipase D (PLD) and

lipoxygenase (LOX), with both enzymes are believed to be the major contributor to membrane damage (Wang, 2010).

The unsaturated fatty acids undergo oxidative deterioration with the action of free radical such as reactive oxygen species (ROS) (Slater, 1972), causing lipid peroxidation, membrane damage, and reduced activity of antioxidant systems (Hodges et al.,2004; Singh and Singh, 2012). Chloroplast, mitochondria and peroxisomes are primary sources of ROS in plant cells. ROS also are toxic products of aerobic metabolism that build up within cells during abiotic stress (Huang et al., 2012) and capable of causing oxidative injuries to DNA, lipids, and proteins (Mittler et al., 2004, Torres and Dangl, 2005). ROS and their toxic products accumulate and generate more toxic ROS such as hydroxyl radicals (OH·) if not being removed or detoxified by the enzymatic and non-enzymatic antioxidants (Hodges et al., 2004).

1.5 Chilling stress response mechanisms

Plants develop mechanisms to adapt to low temperature by regulating several types of genes, such as cold-regulated genes (CORs) (Verlues et al., 2005; Lissarre et al., 2010) which includes more than 100 genes participating in chilling tolerance and cold acclimation in plants (Chinnusamy et al., 2004; Fowler and Thomashow, 2002). Many COR genes are regulated by CBF/DREB1 (C-repeat binding factor/ dehydrin responsive element binding protein 1) gene family of transcription factor (Liu et al., 1998) and included CBF1, CBF2 and CBF3 (Hu et al., 2015). They recognize the C-repeat/DRE DNA regulatory elements of COR genes and bind them (Stockinger et al., 1997; Kanaya et al., 1999). The C-repeat binding factor (CBF) cold-responsive pathway is considered the best-known cold tolerance pathway in plants (Van-Buskirk and Thomashow, 2006 and Chinnusamy et al., 2007) and has been widely studied in Arabidopsis to explain cold acclimation and subsequently survival at freezing temperature of Arabidopsis (Caffagni et al., 2014). Tomato which is considered chilling sensitive plants, also has CBF-mediated cold-responsive pathway, but the CBF regulon comprised of smaller and less functionally diverse genes than that of Arabidopsis (Carvallo et al., 2011; Zhang et al., 2004). CBF locus present in tomato consisting three CBF genes arranged in tandem on chromosome 3 (Jaglo et al., 2001; Zhang et al., 2004). Unlike Arabidopsis that has all three cold-inducible CBF genes, in tomato

only CBF1 induced in response to chilling (Zhang et al., 2004). The overexpression of Arabidopsis CBF1 (AtCBF1) in tomato also has been reported to enhance chilling tolerance of transgenic tomato plants but similar effect was not found in freezing tolerance (Hsieh et al., 2002 a,b). Partially in contrast to previous studies, Caffagni et al. (2014) reported that, CBF1 and CBF3 were induced when tomato seedlings were exposed under chilling condition; with stronger induction observed for CBF1. These genes were induced more strongly in chilling tolerant cultivar (cv Albenga) compared to sensitive one (cv San Marzano) suggesting CBF cold-responsive pathway play role in conferring chilling tolerance of tomato seedlings (Caffagni et al., 2014).

In addition to tomato seedlings, similar cold-responsive pathway was also found in harvested tomato fruits. Zhao et al. (2009a) quantified CBF1 expression in tomato fruits in two contrasting cultivars with different chilling tolerance and found that CBF1 was induced within one hour of exposure to chilling storage, with a higher expression in the tolerant than sensitive cultivar. The CBF1 expression was highly correlated (correlation coefficient -0.9176) with chilling injury index suggesting that the expression of this gene would be a good molecular marker in selecting chilling tolerance traits in tomato fruits (Zhao et al., 2009a). In another investigation, Zhao et al., (2009b) reported that two peaks of CBF1 expression were observed one at 2 hours and other at 16 hours after fruit storage at 2°C, with the second peak was coincided with ethylene production. They confirmed that both chilling and endogenous ethylene were essential condition for normal expression of CBF1 particularly in harvested tomato fruits. They further suggested that CBF and ethylene signaling pathway share similar downstream genes such as ATERF4, ATERF5, and ACO2 (Fowler and Thomashow, 2002; Ito et al., 2006; Yu et al., 2001). The increased ethylene production was found to enhance chilling tolerance of several other fruits after harvest (Zhao et al., 2009a). Recently, Zhang et al. (2016) have reported that, CBF transcripts were substantially elevated upon chilling of tomato fruits at 5°C for 8 days but they return to the basal level once being transferred to ambient temperature (Zhang et al., 2016), suggesting that the response system was appropriately deactivated right after stress being removed from the fruit.

1.6 Polyamines

Polyamines (PAs) are low-molecular weight, aliphatic nitrogen organic cations, that are ubiquitous in all living organisms. It has long been established that they implicated in multiple of fundamental processes in the plant, such as cell division and elongation, morphogenesis, seed germination, flowering and senescence (Bais and Ravishankar 2002; Kusano et al., 2007). There are three main PAs in plants namely diamines putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm), which carry different number of positive charges in the physiological pH of the cell. In some plants, thermospermine (tSpm) is also present in addition to or in place of Spm (Kusano et al., 2014). The three major PAs are present in different level among plant species, with Spd and Put being relatively abundant compared to Spm (Takahashi and Tong, 2015). Spd and Spm have been suggested as the positive regulators whereas Put is a negative regulator of amino acid metabolism in the cell (Handa and Mattoo, 2010). PAs are found in free molecular bases, or often covalently conjugated to small molecules like phenolic acids (soluble conjugated forms) and covalently bound to macromolecules like nucleic acids proteins (insoluble bound forms) (Groppa and Benavides, 2008; Kasukabe et al. 2004)

PAs cellular homeostasis is maintained through the regulation of its biosynthesis and catabolism (Bitrian et al., 2012; Kusano et al., 2014). Briefly, Put is either synthesized by decarboxylation of arginine, catalyzed by arginine decarboxylase or from ornithine catalyzed by ornithine decarboxylase. Spd are synthesized from Put, which require addition of aminopropyl group decarboxylated S-adenosylmethionine (dcSAM), catalyzed by spermidine synthase. Decarboxylation of S-adenosylmethionine (SAM) by SAMdecarboxylase provides the required dcSAM for this reaction. Spm is synthesized from the reaction of Spd with SAM catalyzed by spermine synthase. PAs catabolism is exerted through diamine oxidase (DAO) and polyamine oxidase (PAO) (Gill and Tuteja, 2010).

1.7 Polyamines in chilling stress response in tomato

PAs have been reported to participate in myriad of plant response including to various abiotic stresses such drought, chilling, salinity, heavy metal toxicity, high temperature,

flooding and osmotic stress. Their roles in enhancing plant tolerance have been shown by either exogenous application of PAs compounds or by the development of transgenic plants overexpressing genes involved in PAs biosynthesis (Alca´zar et al., 2010; Pal et al., 2015; Rangan et al., 2014; Alcázar et al., 2006, 2010, 2011; Kusano et al., 2007; Alet et al., 2011). Nevertheless, effects of PAs differ among their concentrations and forms, plant species and stress levels. Despite positive correlation between elevated PAs content and plant stress tolerance, there are a few reports demonstrated the adverse effect of higher polyamines level on plants (Pal et al., 2015).

Global approaches in quest of correlations between transcript and/or metabolites with chilling stress very often recognized a significant function of PAs biosynthetic pathway in the plant response against the stress (Alcazar et al., 2011). Considerable evidence of PAs role in conferring the chilling tolerance is reported by a higher increase in endogenous PAs levels in chilling tolerant plants compared to chilling sensitive plants upon encountering stress (Lee, 1997). Their role in inducing such tolerance has also been implicated by exogenous application of PAs or PAs-biosynthetic inhibitors or also by transgenic plants overexpressing PAs biosynthetic genes (Zhang et al., 2015). Different PAs compounds (Put, Spd and Spm) were found to exert different effects on different plant species (Handa and Mattoo, 2010).

1.7.1 Putrescine

Considerable evidences point to correlate putrescine (Put) levels with chilling tolerance, especially in Arabidopsis. Arginine decarboxylase (ADC) is considered the key enzyme responsible for accumulation of putrescine under stress conditions, with ADC1 and ADC2 genes encode two isoforms of the gene (Alcazar et al., 2006). Both ADC1 and ADC2 were found to be induced after exposure to chilling temperature (Urano et al., 2003; Hummel et al., 2004; Alcazar et al., 2006) and during cold acclimation before being exposed to freezing temperature (Alet et al., 2011). Moreover, even a small decrease of the ambient temperature (20ºC to 17ºC) may increase expression of ADC1 and ADC2 (Usadel et al., 2008). An essential *cis*-acting element, C-repeat (CRT) or dehydration-responsive element (DRE), is found in ADC1 promoter (Hummel et al., 2004; Yamaguchi and Shinozaki, 1994). CRT/DRE is commonly present in the promoter regions of many cold-responsive

genes (Zhang et al., 2013a). It has been suggested to mediate the early and transient ADC1 upregulation under cold stress (Hummel et al., 2004; Yamaguchi and Shinozaki, 1994). Put is considered responsible for chilling tolerance in other crops such as rice (Lee et al, 1997), where ADC activity and Put levels increased after exposure to chilling temperature in a chilling-tolerant rice cultivar while there was only a slight Put increase in a chilling-sensitive cultivar (Alcazar et al., 2011).

1.7.2 Spermine and spermidine

SAM decarboxylase 1 (SAMDC1), SAM decarboxylase 2 (SAMDC2) and spermine synthase (SPMS) have been reported to be induced after exposure to chilling (Urano et al., 2003; Usadel et al., 2008). Analysis of the promoter region of SAMDC1 and 2 revealed the presence of Dehydration Response Element (DRE) and Low Temperature Responsive Element (LTREs). DRE with a core sequence CCGAC and LRTE with a core GCCGAC motif contained in the promoter region of cold responsive genes (Basu et al, 2014). SAMDC is considered a primary and rate limiting enzyme in the synthesis of Spd and Spm (Mehta et al., 2002; Urano et al., 2003). The relationship of Spd with chilling tolerance has been shown in several types of plants such as in cucumber seedlings, whereby the **c**hilling-tolerant cultivar showed a marked increase of free Spd in leaves, during chilling as well as upon rewarming, whereas no increase of Spd and any other PAs in chilling-sensitive cultivar was obtained (Zhang et al., 2009). In zucchini, treatment with exogenous Spd before storage increased the endogenous levels of Spd by 2-fold and significantly reduced chilling injury (Wang, 1993). In rice seedling, exogenous Spd enhanced chilling tolerance even after rewarming that induced activities of antioxidant enzymes and promote normal physiological function of chloroplasts (Yan-hua et al., 2015). Exogenous Spm was also capable of improving chilling tolerance in maize seedlings that could be associated mainly to stabilizing membrane and enhancing antioxidants production (Saeidnejad et al., 2012). Spd has been implicated in the inhibition of chilling-induced activation of microsomal NADPH oxidases, a family of enzymes that generate reactive oxygen species (ROS) (Shen at al., 2000).

1.7.3 PAs act in direct binding and protective actions

It has been suggested that PAs can exert their effects on plant stress directly by simply acting as protective compound (Pal et al., 2015). Owing to their cationic feature at physiological pH, PAs have been proposed to bind to nucleic acids, phospholipids, proteins and other anionic cellular compounds (Slocum et al., 1984), catalyzed by transglutaminase enzyme (Rangan et al., 2014). Such binding of PAs to the phospholipid head groups of cell membrane (Hura et al., 2015; Pal et al., 2015) has been suggested to stabilize membranes under stress conditions (Roberts et al., 1986). Moreover, they can reduce membrane damage from lipoxygenase activity (Lester et al., 2002). In addition, PAs also may directly function as free radical scavengers (Bors et al. 1989). Of the three main PAs, Spm which has four amino groups was found to be more powerful scavenger than Spd which has only three; indirectly suggesting effect of amino groups in free radical inactivation (Besford et al.,1993). Khan et al. (1992) and Ha et al. (1998) have proposed that Spm and Spd were capable of quenching chemically generated singlet oxygen from damaging DNA.

Kim et al. (2002) reported that chilling tolerance in tomato leaves was induced following exogenous application of Put possibly through cell membrane stabilization as indicated by lower electrolyte leakage percentage. The exogenous Put application on tomato seedling also reduced malondialdehyde content (MDA) (Song et al., 2014). Both electrolyte leakage and MDA levels are indirect measurement of cell membrane damage, with the former is measured based on deterioration of membrane permeability, whereas the latter is measured based on membrane lipid peroxidation (Fahmy et al., 2015). On the other hand, inhibition of Put biosynthesis by difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), increased membrane damage (Kim et al., 2002).

In order to mitigate the damage caused by stress-induced ROS accumulation, cells generate antioxidant enzymes to detoxify ROS (Gill and Tuteja, 2010), which including Superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and other enzymes of ascorbate-glutathione pathway (Jaleel et al., 2007). Of the antioxidant enzymes, SOD, CAT and POD play significant role in antioxidant system. SOD function in dismutation of superoxide (•O2-) to hydrogen peroxide (H_2O_2) and O2, whereas CAT and POD scavenge H_2O_2 (Egert and Tevini, 2002). Application of exogenous Put on

tomato seedling effectively induced SOD, POD and CAT activities under chilling stress condition, while in contrast, application of D-arginine (D-Arg), a Put biosynthesis inhibitor diminished the activities (Jiang et al., 2002). Apart from Put, exogenous application of Spd on tomato seedling was also reported to enhance chilling tolerance by inducing gene expressions of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), and their corresponding enzymes (Diao et al., 2016).

Recently, Goyal et al. (2016) have come up with a new finding that PAs may have capability of inducing and stabilizing Pathogenesis-Related Protein 1b1protein (PR1b1) in tomato under chilling stress. Polyamines were postulated to mediate sustained accumulation of PR1b1 protein in transgenic tomato fruits overexpressing high Spd and Spm, suggesting a possible defense response mechanism related to Cold Stress-Induced Disease Resistance (SIDR) phenomenon, which confer chilling tolerance. It has been suggested that Spd and Spm modulate protein synthesis by inducing structural changes in RNA structure (Igarashi and Kashiwagi, 2000) consequently stabilizing proteins from degradation (Gugliucci and Menini, 2003).

1.7.4 PAs act as signaling molecules and involved in crosstalk with other pathways

Beyond acting as a protective compound, a large body of evidence show that PAs also participate in stress signaling and interconnected with other metabolic routes and hormonal cross-talk in mediating plant stress responses and tolerance (Anwar et al., 2014; Palet al., 2015; Alcazar et al., 2010). Apart from being a potential ROS scavenger, PAs also being a source of ROS by generating H_20_2 (Pal et al., 2015). A massive increase in endogenous PAs beyond threshold may promote their catabolism catalyzed by PAO, generating H_20_2 in apoplast and peroxisomes (Liu et al., 2015; Wimalasekera et al., 2011). It has been known that H_20_2 acts as a signal molecule in both abiotic and abiotic at low cytosolic concentration (Cona et al., 2006) which in turn may trigger induction of antioxidant enzymes (Moschou et al., 2008; Zhang et al., 2015). PAs may promote induction of antioxidant enzymes via regulation of their expression as demonstrated by higher transcript levels of antioxidant enzyme-encoding genes detected following exogenous PAs treatment or in the transgenic plants overexpressing PAs biosynthetic genes under stress condition

(Tanou et al., 2014; Zhang et al., 2015). In addition to H_20_2, PAs catabolism also generated nitric oxide (NO) catalyzed by diamine oxidases (DAO) and polyamine oxidase (PAO) (Wimalasekara et al, 2011; Yamasaki and Cohen, 2006). NO is a highly diffusible gaseous molecule which mediate various physiological functions and defense responses against abiotic and biotic stresses in plants (Wilamalsekara et al., 2011). H_20_2 and NO may act either as two independent signaling molecules, or may interrelated with each other or with other stress mediators in PAs-induced plant response to stress (Tun et al., 2006). NO also acts as an intermediate signaling molecule in ethylene, auxin, cytokinin and abscisic acid signaling (Pal et al., 2015).

One of the most recent study in tomato shows that under chilling stress, Spd and Spm induced accumulation of NO in tomato seedlings resulted in enhanced chilling tolerance, whereas Put had no clear effect on NO accumulation (Diao et al., 2017). This is consistent with a previous finding by Diao et al. (2016). Moreover, Spd and Spm treatment, but not Put treatment, cause substantial elevated level of H_2O_2, suggesting that Spd and Spm induced NO generation in tomato seedlings via an H_2O_2-dependent mechanism, through the NR and NOS-like pathways. PAs-induced elevation of H_2O_2 in cell could be attributed to Put, Spd, or Spm catabolism (Wimalasekera et al., 2011). It has been demonstrated that H_2O_2 can acts as a signal in the signal transduction network of abiotic stresses at low concentration (Tanou et al., 2009; Jiang et al., 2012), while it may cause cell injury and death at excessive concentration (Quan et al., 2008).

PAs also may be implicated in ABA-mediated stress response in tomato. In Put-treated tomato seedlings, a substantial increase of leaf ABA content was observed under stress condition, while the effect was significantly reduced by D-arginine (D-Arg), under similar condition. In parallel to this, the expression of 9-cis-epoxy-carotenoid dioxygenase (LeNCED1) a key ABA biosynthesis regulator in tomato, was greatly induced in the Put-treated tomato plants compared to that in the control, suggesting possible action of Put in inducing ABA generation thereby conferring chilling tolerance (Diao et al., 2017). This is in disagreement with earlier finding by Kim et al. (2002) who reported that both Put and ABA act independently in enhancing chilling tolerance of tomato leaves with ABA (Kim et al., 2002).

Goyal et al. (2016) have found that sustained accumulation of PR1b1 protein in high-Spd/Spm transgenic tomato fruits after recovering from chilling was positively correlated with salicylic acid (SA) level suggesting a crosstalk between PAs and SA in response to chilling stress. SA plays an important role in thermogenesis and in the activation of certain plant defense responses such as PR gene expression (Gaffney et al., 1993; Klessig and Malamy, 1994). PR1b1 accumulation did not correlate with other signaling compound such as methyl jasmonate and ethylene (Goyal et al., 2016). Similar relationship between PAs and SA was also reported in other crops such as in maize leaves (Nemeth et al., 2002). The exogenous SA treatment caused a substantial increase in Put and Spd content under chilling stress; for instance, in peach fruits, Cao et al. (2010) reported that the combination of SA and heat treatment increased the PAs concentrations and effectively alleviated the chilling injury. One of derivative of SAs, methyl salicylate (MeSA) was found to enhance chilling tolerance of cherry tomato possibly by elevating PAs and NO level through arginine catabolism (Zhang et al., 2011). Arginine is a precursor for biosynthesis of PAs and NO. In this study, MeSA treatment induced gene expression and activities of ADC, ODC and NOS, resulting in elevated levels of both PAs and NO. Zhang et al. (2013) have reported that arginine catabolism can be activated by heat treatment, which in turn accumulate higher PAs under chilling stress and thereby alleviate chilling injury in tomato.

1.8 Hypothesis and Objectives

As has been reviewed above, there is a large body of evidence demonstrating the role of PAs in the protective mechanism of plants against chilling stress. Nevertheless, the information regarding mechanism of PAs in tomato chilling tolerance are still scarce and elusive. In addition to that, while most of the studies have emphasized on the whole plants or seedlings response under chilling conditions at field, little is known about the postharvest chilling response of tomato fruits in association with PAs. It has been reported by Serrano et al. (1996) that several pre-storage treatments which aimed at alleviating chilling injury such as temperature pre-conditioning and controlled atmosphere have resulted in increase in PAs levels and subsequent chilling injury protection in some fruits

but no report on tomato fruits. Only in recent years, Zhang et al. (2011) reported that exogenous methyl salicylate (MeSA) pre-storage treatment on tomato fruits induced higher accumulation of PAs along with nitric oxide that in turn indirectly involved in chilling injury protection mechanism. Similar relationship of PAs and chilling tolerance in tomato fruits was found by Zhang et al. (2013), but through application of heat treatment prior to chilling storage. To the best of my knowledge, there is no report of direct application of exogenous PAs on tomato fruits before postharvest storage and chilling tolerance of fruits. However, recently Goyal et al. (2016) came up with an interesting finding that overexpression of PAs biosynthesis genes in transgenic tomato resulted in sustained accumulation of pathogenesis related protein 1b1 (PR1b1) which possibly one of the defense response mechanism against chilling stress in tomato fruits. Likewise, in the present study we utilized transgenic tomato fruits overexpressing SAMDC under an induceable promoter resulting in higher levels of Spd and Spm to gain insight into function of PAs in tomato fruits response to postharvest chilling storage. I have determined the effect of chilling on two stages of fruit ripening using a concerted approach involving physiology, biochemistry and transcriptome by RNA-seq analyses. This has allowed me to address complex biological systems underlying the PAs functions and the fruit chilling response. Postharvest chilling response of tomato have been reported by Zhang et al. (2016) using RNAseq analyses to understand the loss of flavor after chilling of ripened fruits. Cruz Mendevil et al. (2015a,b) have reported effects of pre-heat treatment on chilling tolerance of tomato fruits. However, little is known about the roles of PAs in this subject matter. Hypothesis, general and specific objectives of my investigations are as follows:

1.8.1 General objective

To gain insight of the physiological and molecular characteristics of PAs action in tomato fruits during storage at chilling temperatures

1.8.2 Specific objectives

- To study effects of PAs on phenotypic changes in response to chilling in tomato fruits
- To investigate effect of PAs on transcriptional changes in tomato fruit in response to chilling

1.8.3 Hypothesis

Endogenous increase in PAs will enhance chilling tolerance leading to improved storage of tomato at chilling temperatures

CHAPTER 2. MATERIALS AND METHODS

2.1 Plant materials and growth condition

Two transgenic tomato lines (556HO and 579HO) expressing yeast S-adenosylmethionine decarboxylase (ySAMdc) and accumulate spermidine and spermine in ripened tomato fruits (Mehta et al., 2002) were compared with parental Ohio 8245, a wild type (WT), as control throughout the study. Ohio 8245 is the parental line used for developing the 556 and 579 transgenic lines. The transgenic lines are homozygous for ySAMdc gene driven by fruit/ethylene-specific promoter E8 and has been described by Mehta et al. (2002). Transgenic and WT plants were grown in similar condition and time under glasshouse (photoperiod: 16 hours day/ 8 hours night; temperature: 23°C day/18°C night). Growing mix media with high porosity (52Mix, Conard Fafard Inc., MA USA) were used for cultivation. Tomato fruits were initially tagged at a breaker stage, characterized as "a definite break in color from green to tannish-yellow, pink or red on not more than 10 percent of the surface" (Figure 1a) in accordance to United States Department of Agriculture Grade Standards for Fresh Tomatoes. Fruit samples were then collected on either 0 day (BR) or 8 days (BR+8) after the breaker stage depending on the experimental design. BR+8 is characterized by fully ripe stage fruits with the whole fruit surface turns red, but not overripe (Figure 1b). Samples were immediately subjected to chilling treatment after harvest.

2.2 Experimental design

Fruits were harvested at breaker (BR) or Breaker+8 days (BR+8) to determine the effect of chilling injury at initial and fully ripe stages of fruit. All fruits were randomly divided into three replicates and being subjected to chilling treatments described as follows:

For effect of chilling treatment on BR fruits, they were stored in the dark at 8 ±1°C under 80–90% relativity humidity (RH) for up to 5 weeks. Samples were withdrawn from storage every week and subsequently transferred to ambient temperature (20 $\pm$ 2°C) for up to 5 days for rewarming or ripening. Evaluations were done only after rewarming period. For effect of chilling treatment on BR+8 fruits, they were stored in the dark at 5±1 °C for up

to 2 weeks. Samples were withdrawn at the end of storage period and subsequently transferred to ambient temperature (20 ± 2°C) for up to 5 days for rewarming. Evaluations were performed either immediately after chilling or after rewarming period.

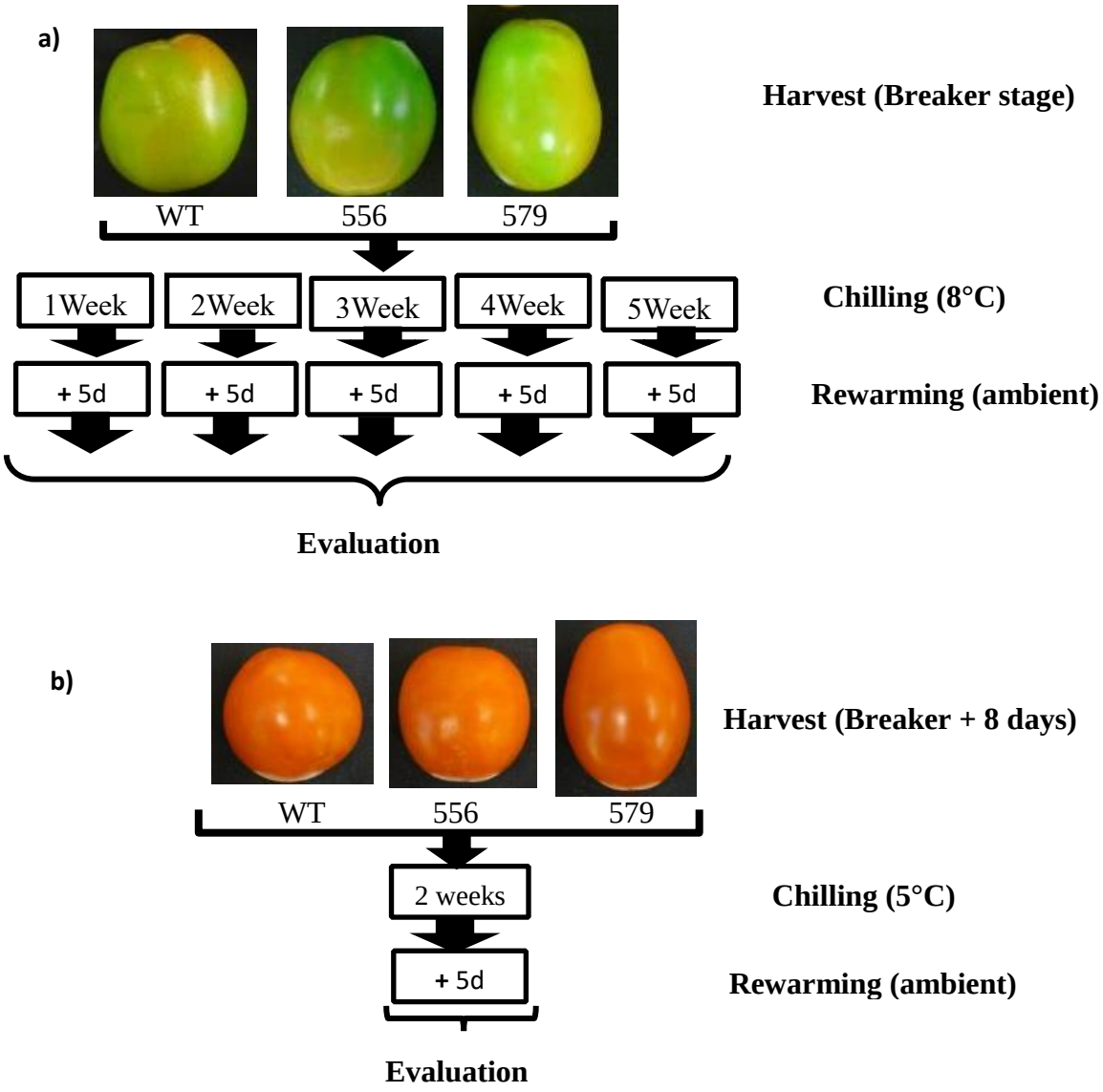


Figure 1: Experimental design to study effects of chilling treatment on fruits harvested at (a) BR stage and (b) BR+8 stage

2.3 Evaluation of phenotypic changes of fruits in response to chilling

2.3.1 Physical changes

Chilling injury symptom evaluation: Chilling injury (CI) of fruits was evaluated at 5 days of rewarming period at 20 $\pm$ 2°C after being exposed to chilling treatment at different duration. CI symptom typically will not become pronounced until a few days after the injury happen. The severity of the symptoms was assessed visually using a following scale [0 = no symptom; 1 = symptom covering <25% of the fruit surface; 2 = symptom covering <50%, but >25% of surface; 3 = symptom covering <75%, but >50% of surface, and 4 = symptom covering >75% of surface]. CI symptoms vary between maturity stages and severity. These include abnormal color, delay or no ripening and abnormal texture (soft and watery fruits). CI index was calculated by using the following formula: {P [(CI symptom severity) X (Number of fruit at the CI severity)]/(Total number of fruits evaluated) X 4} X 100 and expressed as CI %.

Fruit color evaluation: Fruit skin color was determined by using reflectance colorimeter (model CR-400, Minolta, Japan). Measurements were taken at three points (shoulder, equator and base) of the fruit surface of individual fruit. Color was expressed in coordinate hue angle (Hº) which describes the relative amounts of redness and yellowness where 0°/360° is defined for red/ magenta, 90° for yellow, 180° for green and 270° for blue color (McGuire, 1992; Voss, 1992). This value was automatically determined by instrument according to the following equation: hue = tan -1 (b*/a*)2; where b* and a* are two color indices on the hue circle which respectively defines yellowness or blueness; and redness or greenness (McGuire 1992; Voss 1992).

2.3.2 Chemical changes

Quantification of polyamines (PAs) content: Extraction and dansylation of PAs in tomato fruits were carried out as described by Anwar et al. (2014) with some modifications. Instead of freshly ground samples, lyophilized samples were used in this present study along with modification on sample weight. Briefly, 20mg lyophilized tomato pericarp powder were homogenized in 800 µl of 5% cold perchloric acid (PCA) and then immediately incubated on ice for 1 hour. Homogenate was then centrifuged at 20,000g for

30 min at 4^{o}C. 100 µl supernatant was used to quantify free PAs, whereas another 200 µl was used to quantify conjugated PAs. The remaining pellet from the same centrifuged homogenate was washed twice with 5% PCA and re-suspended in 800 µl of 5% cold PCA to quantify bound PAs. Samples for conjugated and bound PAs, as well as the PAs standard (Put, Spd and Spm), were hydrolyzed with an equal volume of 12N HCl at 110°C for 18 hours. As an internal control, 400 µl of 1,7-heptanediamine was added to all samples except the PAs standards. 200 µl saturated sodium carbonate was added to all samples. Dansylation process was done by adding dansyl chloride followed by dark incubation at 60°C for 1 hour. 100 ul proline was added to terminate the dansylation, followed by light incubation for 30 min at the same temperature. After incubation, dansylated PAs were extracted in 500 ul toluene and were air dried. The dried samples were dissolved in 200 ul acetonitrile and then diluted. The extracted PAs were quantified using HPLC (Waters 2695 Separation Module equipped with Waters 2475 Multi Lambda fluorescence detector).

Quantification of ethylene production: The ethylene production in tomato fruit was measured after subsequent rewarming, by using gas chromatography (GC) (Shimadzu GC-8A), equipped with a flame ionization detector. Individual fruit were weighed and placed in an airtight jar (volume 470mL) at 20^{o}C that was capped for 2 hours to accumulate any emitted gas. Subsequently, 1 ml gas samples were withdrawn from the headspace of jar by inserting a syringe through a fitted septum to be directly injected into the GC. The ethylene production rate of tomato fruit was expressed as µl/kg/hr, by using the following calculation:

[jar volume (ml) - fruit weight (g)] / [fruit weight (kg) x time in jar (hour)] x 1000 x C2H4 reading taken from GC (ppm)

Determination of Malondialdehyde content: Malondialdehyde (MDA) was measured only after rewarming period. Tomato pericarp tissue (0.25g) was ground using liquid nitrogen and homogenized in 1ml 0.1 % (w:v) Trichloroacetic (TCA) solution. After centrifugation at 12 000 g for 15 min., 1 ml aliquot of the supernatant was mixed with 0.5 % (w:v) Thiobarbituric (TBA) in 20 % TCA. The mixture was heated to 100°C for 30 min and quickly cooled in an ice bath to stop the reaction. The mixture was briefly vortexed and

centrifuged at 10,000g for 10 min. 1ml supernatant was collected into a cuvette and measured using spectrophotometer (Shimadzu UV-1800) at 532, 600 and 450nm wavelength. The amount of MDA–TBA complex (red pigment) was calculated according to the formula: 6.45×(A532 −A600)−0.56×A450 and expressed as μmol/g, where one unit was defined as 1 μmol MDA per g of pericarp tissue.

2.4 Evaluation of transcriptional changes of fruits in response to chilling

2.4.1 RNA isolation and cDNA synthesis

Total RNA was extracted by using the phenol/ lithium chloride extraction method, as reported by Eggermont et al. (1996) with some modification to the amounts of reagents and samples used. Briefly, pericarp tissue of six tomato fruits were pooled for each biological replicate, and were ground to fine powder with liquid nitrogen and were mixed with 1.5 ml RNA extraction buffer in a 2 ml Eppendorf tube. RNA extraction buffer is a combination of 1:1 (v/v) RNA buffer and Phenol Chloroform Isoamyl Alcohol (PCI). Samples were vortexed and incubated for five minutes at room temperature followed by centrifugation for 10 minutes at 13,000 rpm. The top phase was carefully transferred into a fresh Eppendorf tube and was mixed well with 1 ml PCI. They were centrifuged again for five minutes at 13,000 rpm and the top phase was mixed well with 1.2 ml Chloroform Isoamyl alcohol (CI), followed by another centrifugation. The top phase was mixed well with 250 µl of 8M Lithium Chloride and was incubated at -20ºC overnight. The incubated samples were centrifuged at 4ºC for 15 minutes at 13,000 rpm and the supernatant were removed carefully with the pellet visible on the bottom. One ml of ice cold 80% ethanol was added, followed by centrifugation for two minutes at 13,000 rpm. Ethanol was removed as much as possible after centrifugation and the pellets were air-dried. Forty µl of RNAse-free water were added to dissolve the pellets for further processes including RNA quality check, purification, quantification as well as cDNA synthesis. RNA quality was checked by electrophoresis running on ethidium-bromide-stained 1% standard agarose gel. RNA was further purified by RNA Clean & ConcentratorTM-25 (Zymo Research; Catalog No: R1017). RNA concentration was measured by using NanoDrop 2000 UV-Vis

Spectrophotometer (Thermo Scientific). First strand cDNA was synthesized using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad; Catalog No: 1708840) as a reagent and the reaction mix was incubated in a thermal cycler (PTC-200 Peltier) using the following reaction protocol; Priming for 5min at 25^{o}C, Reverse transcription for 30 min at 42^{o}C and RT inactivation for 5 min at 85^{o}C. The cDNA quality was checked by using PCR Master Mix (Promega; Catalog No. M7502) reagent with tomato elongation factor one gene (EF1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes as housekeeping genes and subjected to PCR reaction with a thermal cycler (T100 BIO-RAD) in a 20-µl reaction mixture under the following thermal cycle conditions: an initial 94ºC for 2 min; 25 cycles of 94ºC for 20 s, 60ºC for 20 s, and 72ºC for 30 s and a final 72ºC for 5 min. Ethidium bromide-stained PCR products were visualized after electrophoresis in 1.5% agarose gels.

2.4.2 Gene expression analysis using RT-PCR and qRT-PCR

cDNA products were synthesized as described in 2.4.1 and were diluted 10-fold prior to qRT-PCR. Gene-specific primers (as listed in Table 1) were designed by using primers 3 software. GoTaq® qPCR Master Mix (Promega, Catalog No. A6001/2) was used as reagent system for quantitative PCR. StepOnePlusTM Real-Time PCR System (Applied Biosystems, USA) was used with program sequence as follows: 95°C for 10 minutes (holding stage); 95°C for 15 seconds and 60°C for 60 seconds (cycling stage: 40 cycles); 95°C for 15 seconds and 60°C for 60 seconds (melt curve stage). To determine relative expression level of transcript, the threshold constant (Ct) value was normalized to the GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) gene as a housekeeping gene and using WT fruits as a calibrator. 2-ΔΔCT formula was used for calculation and data were expressed as “fold change”.

Table 1 Tomato genes and the primer sequence combinations used in qRT-PCR analysis

Gene/symbol/description	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
Solyc01g111300.2 (Cold shock protein-1 (AHRD V1)	AGGCTCAAGGGAACTGTCAAGT	ATACTCAACATCCTCACCTTCAGCTA
Solyc03g121090.2 (Cold induced protein-like)	GTACAGAAAACAAAAGAAAATAAGGAGAGT	TCAAGGGCAAACAATATATCCAA
Solyc03g026280.2 (CRT binding factor 2)	GCTGCCTATCCCTGCTTCCT	GCCTCCTCATCCACGAAGTC
Solyc04g058170.1 (Calmodulin-like protein)	TGATGGCCTTGTGTGTCTTG	TTTGCCTTTGTGATAGCATCAT
Solyc02g078360.2 (Thioredoxin family protein)	ATCGAAGGCGGAGCTTGATA	GAAAATGGGCATGTGGGAAA

2.4.3 Differential expression analysis using high-throughput sequencing of cDNA (RNA-seq)

Triplicate biological replicates were used for RNA-seq analyses. RNA samples from tomato pericarp were prepared as described in 2.4.1 and were sent to Purdue genomic center for RNA-seq analysis using Illumina technology. Briefly, quality control checks on raw high throughput sequence data was done by using FASTQC program. All reads in quality score > 30 derived from FASTQC result, were aligned to *Solanum lycopersicum* genome using TopHat version 2.1.1 with default parameters. The number of reads that map to a gene were calculated by using HTSeq with "htseq-count -s no" option, whereby it counts the reads number regardless of the strand. The annotation information was downloaded from Sol Genomics Network (SGN, http://solgenomics.net), which store genome maps and sequences data for species in Solanaceous family including tomato. The tomato genome information used was "ITAG2.4\underline{\hspace{0.5em}}release" version. The result was reported in Reads Per Kilobase of transcript per Million mapped

reads (RPKM) to normalize for sequencing depth and gene length according to following equation: RPKM = (NR * 1,000 * 1,000,000) / (GL * TN). (NR=number of reads mapped to a gene sequence; GL=Length of the gene sequence; TL=Total number of mapped reads of a sample; 1,000,000=to account for per million scaling factor; 1,000=to account for the kilobase of gene RPKM values).

2.5 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the statistical software SAS 9.4 for Windows. Significant effects were determined at the 5% level and 1% level which represent significant difference and highly significant difference respectively indicated by * and ** symbols respectively. Means separation was performed using Duncan Multiple Range Test, whereby mean bars in the graphs with the same letters are not significant different. Each bar in the graphs represents the mean with standard error of three biological replication.

CHAPTER 3. RESULTS

3.1 Transgenic fruits with high PAs did not show chilling tolerance phenotype at BR stage

Previously in an initial study, the fruits were subjected to 5°C to induce chilling stress. All fruits showed noticeable chilling injury symptom at the same severity starting from week 2 of storage (data not shown). As such, for the present study, the temperature was raised to 8°C in order to distinguish the symptom severity more clearly. As the optimal storage temperature for tomato is 12°C (Wang, 2010), storage at 8°C is still considered sub-optimal and could induce chilling stress. Evaluation for phenotypic aspects as well as other parameters were not carried out until fruits being kept at ambient temperature for 5 days upon removal from storage. This is due to the facts that chilling stress symptoms may not be observable until the fruits are removed from chilling condition to ambient temperature (Wang, 2010).

Chilling stress symptoms started being detected upon removal at 3 weeks of storage followed by 5 days rewarming (3W+5d), and was only appeared on line 579 (Figure 2). Tomatoes are chilling-sensitive at temperature below 10°C if held for longer than 2 weeks (Suslow and Cantwell, 2000). The symptoms can be characterized as incomplete development of red skin color with slight shriveling at the circular area of calyx scar. Chilling injury symptoms may vary such as internal discoloration, surface lesions, water-soaking of the tissue, decay, off-flavor unable to ripen normally (Saltveit and Morris, 1990). The symptoms on 579 fruits were more pronounced after longer exposure to chilling temperature whereby severe symptoms were observed in the end of experiment; 5 weeks of storage + 5 days rewarming (5W+5d). At this point, the fruits were not able to ripen with only slightly red color developed on the skin surface. Comparing between line 556 and WT, there was no obvious difference between these two lines in terms of physical appearance until in the end of experiment (5W+5d). Both lines were able to completely ripen with full red color developed on the skin surface without any visible symptom of chilling injury. The difference in color development on the skin surface of the three samples was further confirmed with reflectance colorimeter (Figure 3). The skin color of fruits was expressed in hue angle value which describes the relative amounts of redness and yellowness whereby

0°/360° is defined for red/magenta, 90° for yellow, 180° for green and 270° for blue color (McGuire 1992; Voss 1992). In short, generally lower value indicates color more to red. Regardless of storage period, there was significant difference in hue angle value among the samples which was consistent with visual observation. Line 579 fruits recorded the highest hue angle value throughout the experiment which indicate that they were least red compared to both line 556 and 579. There was no significant difference between line 556 and WT at every point of evaluation, suggesting that fruits from both lines could ripen to similar extent.

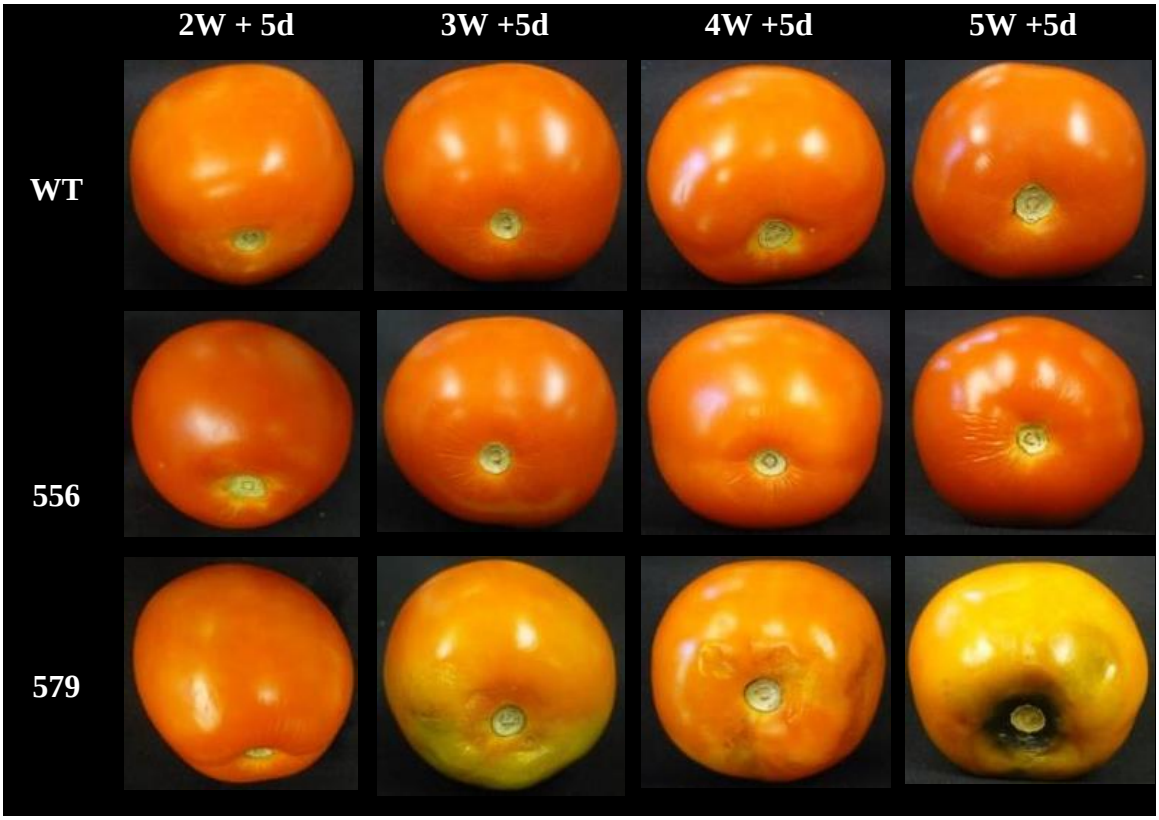


Figure 2: Physical condition of fruits (BR stage) after being stored at 8ºC and subsequently held at ambient temperature for 5 days. (556 and 579 = transgenic; WT=wild type. 2W–5W=Week 2 to Week 5 of storage; 5d= 5 days rewarming).

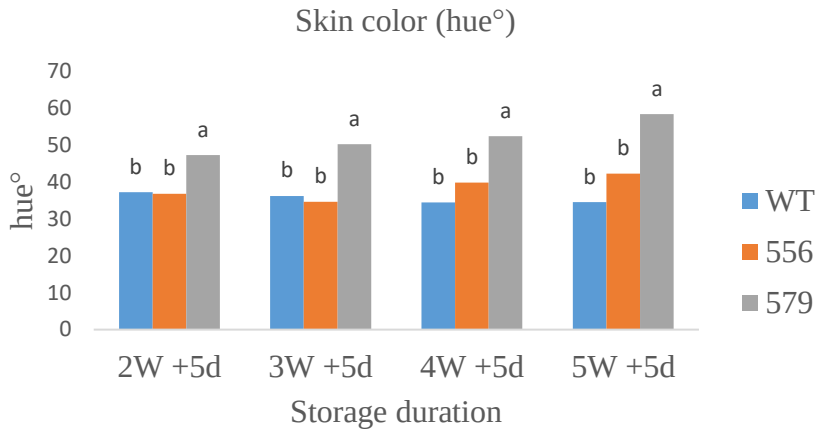


Figure 3: Hue^{o} value of fruit skin (BR stage) after being stored at 8^{o}C and subsequently held at ambient temperature for 5 days. Generally, lower hue^{o} value indicates color more to red. (556 and 579 = transgenic; WT=wild type. 2W–5W = Week 2 to Week 5 of storage; 5d= 5 days rewarming). Mean bars with the same letter at each storage duration point are not significantly different.

To confirm the visual chilling injury symptom assessment, malondialdehyde (MDA) content was measured in all the fruit samples. MDA content which is the final product of lipid peroxidation has been used as a direct indicator of loss of cell membrane structural integrity (Liu et al., 2013). Lipid peroxidation which results from ROS accumulation is considered as the first evidence of chilling stress in plants (Aghdam, 2013). In this present study, thiobarbituric acid reactive substances assay (TBARS) was used to determine MDA content in fruits. Thiobarbituric acid (TBA) reacts with MDA to yield a fluorescent 2:1 TBA/MDA adduct with absorbance at 532nm (Du and Bramlage, 1992). Consistent with the chilling injury development, the 579 line fruits showed the significant higher ($p \leq 0.05$) accumulation of MDA content throughout storage period (Figure 4), suggesting severe cell membrane degradation associated with chilling injury in this line. On the other hand, both 556 and WT fruits did not differ ($p \leq 0.05$) in MDA content, consistent with their physical condition (Figure 2).

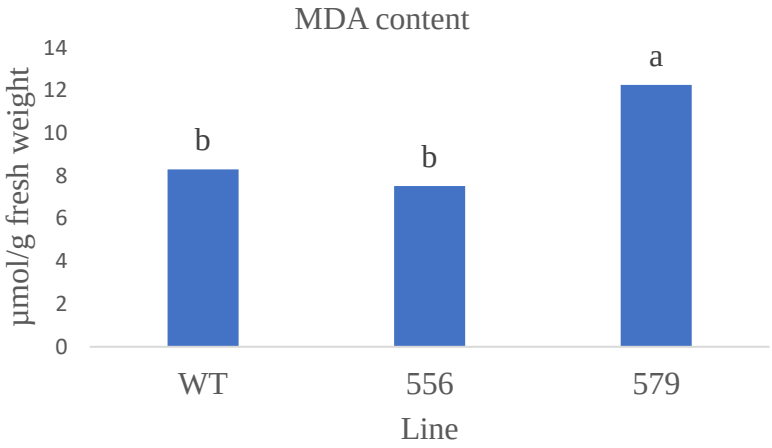


Figure 4: Malondialdehyde content of fruits (BR stage) (µmol/g fresh weight pericarp) after being stored at 8ºC for 5 weeks and subsequently held at ambient temperature for 5 days.

3.2 PAs protect ethylene biosynthesis machinery in BR stage fruits

There was a significant difference ($p \leq 0.05$) in ethylene production among all lines when measured after each week of storage and being ripen for another 5 days at ambient (Figure 5). In the WT fruits ethylene production ($p \leq 0.05$) become detectable in 3W+5d fruits which declined thereafter. On the other hand, line 579 and 556 produced similar high levels of ethylene in 2W+5d fruits, which declined significantly with longer exposure to low temperature in 556 but not in 579 fruits. However, at all storage conditions examined, the rates of ethylene production were higher in transgenic fruits than WT fruits (Figure 5). Interestingly, the ethylene production pattern was negatively correlated with ripening ability of fruits after storage, as shown by both visual color development (Figure 2) as well as hueº value (Figure 3). On the other hand, it showed a consistency with chilling injury symptom, wherein the 579 line which had the most severe symptom produced the highest ethylene. This unlikely trend of ethylene, ripening and chilling injury symptom might shed some light on chilling stress status of fruits under sub-optimal storage temperature for fruits harvested at BR stage. Apart from that, it also likely to be attributed to protection of ethylene biosynthesis machinery by PAs, possibly through stabilization of genes regulating the ethylene biosynthesis under adverse condition, hence continuously generated ethylene. The nexus of PAs and ethylene has been suggested, considering the facts that biosynthetic pathways for both compounds use S-Adenosyl methionine (SAM) as the substrate (Fluhr

and Maatoo, 1996). A potential competition between ethylene and PAs have been suggested for their biosynthesis (Anwar et al., 2014). Nambeesan et al. (2010) have reported that expression of yeast spermidine synthase (ySpdSyn) also increase ethylene production in the harvested tomato.

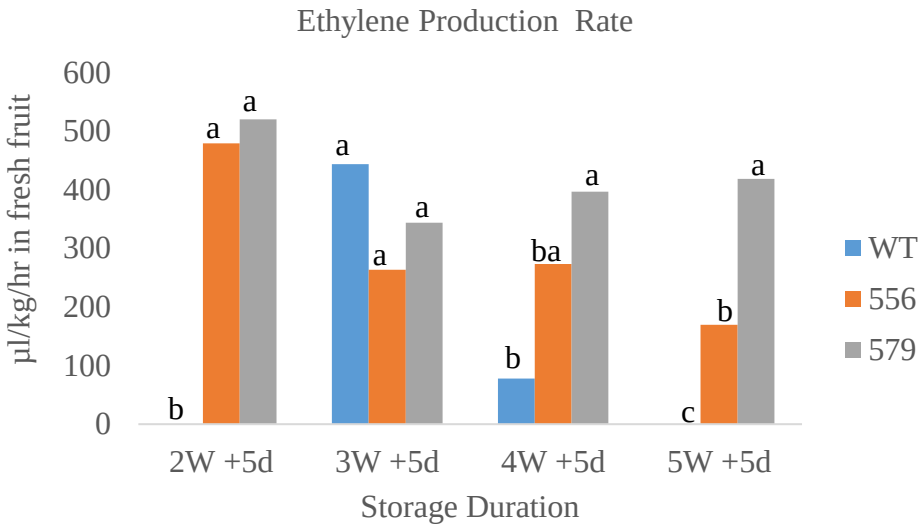


Figure 5: Ethylene production (µl/kg/hr) in fruits (BR stage) after being stored at 8ºC and subsequently held at ambient temperature for 5 days. (556 and 579 = transgenic; WT=wild type. 2W–5W = Week 2 to Week 5 of storage; 5d= 5 days rewarming). Mean bars with the same letters in each storage duration point are not significantly different.

3.3 Transgenic fruits at BR stage accumulate higher spermidine level under chilling stress

As expected, spermidine (Spd) level was significantly higher ($p \leq 0.05$) in both transgenic lines and remained higher throughout storage period without any significant difference between each other (Figure 6). For spermine (Spm), there was no significant difference ($p \leq 0.05$) among the lines except at week 5, whereby 579 recorded relatively higher Spm level compared to WT as well as 556. Interestingly, utrescine (Put) which was relatively lower in both transgenic lines had a tremendous increasing trend in WT line after 4 weeks and 5 weeks of storage. Seemingly negative correlation of Put and chilling injury symptom in WT might suggest a possible function of this form of PAs in chilling response in tomato harvested at BR stage. The role of Put in alleviating chilling injury has been demonstrated

by Song et al. (2014) and Zhaou et al. (2016) in tomato plants. Comparing different fractions of PAs, generally conjugate fractions were consistent with their free PAs counterpart in throughout storage. On the other hand, the bound fractions showed slight difference among all lines, except for at 4W+5d time point when WT interestingly showed higher level in three types of PAs, and at W5+5d time point when 579 showed higher level of Put, Spd and Spm.

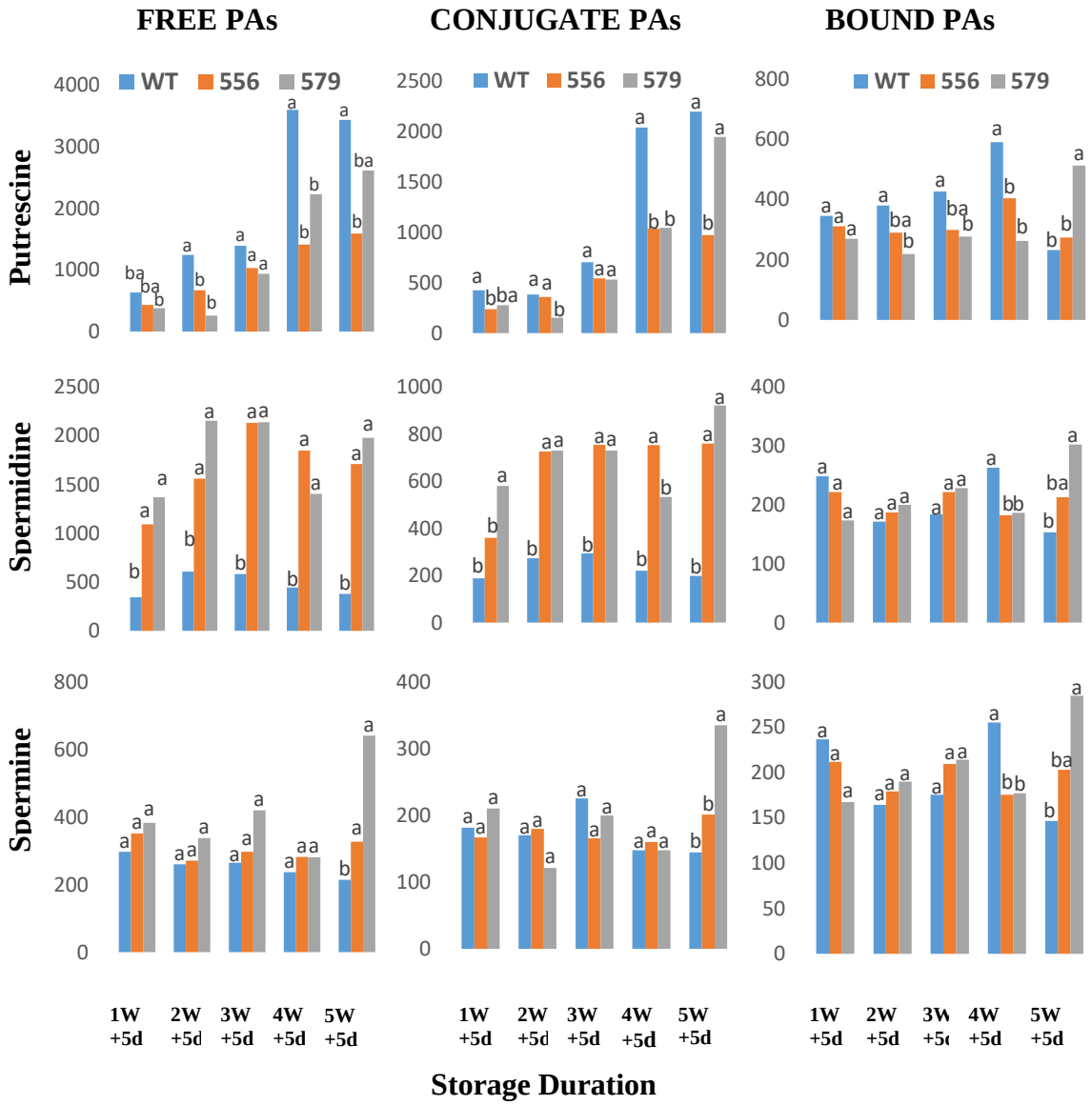


Figure 6: Free, conjugated and bound Put, Spd and Spm levels in fruit pericarp (ηmol/gDW) harvested at BR stage, stored at 8ºC for up to 5 weeks and subsequently held at ambient

temperature for 5 days. (WT=wild type; 556 and 579 = transgenic. 1W–5W = Week 1 to Week 5 of storage; 5d= 5 days of rewarming). Mean bars with the same letters in each storage duration point are not significantly different

3.4 Polyamines protect ripened (BR+8) tomato fruits from chilling injury

The response of more advance ripening stage fruits to chilling was investigated by keeping BR+8 fruits at 5°C for up to only 2 weeks with additional 5 days of rewarming (2W+5d), since from a preliminary observation, it was found that 2 weeks duration was sufficient to detect chilling injury development. Similar to what was observed in BR stage fruits, additional 5 days rewarming period was needed for symptom to appear. Interestingly, chilling treatment on BR+8 fruits has resulted in opposite effects compared to effect on BR stage fruits. Most severe chilling injury symptom was observed in WT, whereby the symptom can be characterized by severe water-soaked-like appearance in WT fruits pericarp. The transgenic 556 and 579 fruits on the other hand presented more chilling tolerance phenotype judged by no visible symptom in the former whereas only slight shriveling on pericarp of the latter (Figure 7).

Figure 7: Physical condition of fruits (BR+8) after being stored for 2 weeks at 5°C and subsequently rewarmed at ambient temperature for 5 days (556 and 579 = transgenic; WT=wild type)

Consistent with severity of chilling injury symptoms, malondialdehyde (MDA) content measured after 2 weeks of storage followed by subsequent rewarming for 5 days was significantly higher ($p \leq 0.05$) in WT compare to transgenic fruits from both lines (Figure 8). These results imply lower lipid peroxidation and cell damage in transgenic vs WT fruits consistent with observed phenotype (Figure 7) (Hodges et al., 1999). In general, relatively higher MDA content was recorded in BR+8 fruits compared to that of BR fruits (Figure 4) likely due to excessive reactive oxygen species (ROS) production when ripening in progress (Fry et al., 2001; Cheng et al., 2008) which increase consequent lipid peroxidation.

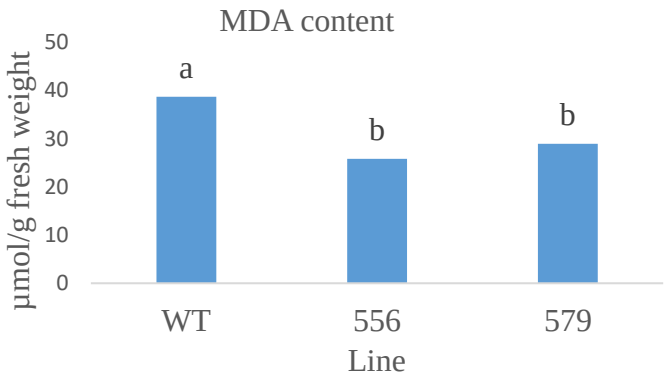


Figure 8: Malondialdehyde content of BR+8 fruits (µmol/g fresh weight of pericarp) after being stored at 5ºC for 2 weeks and subsequently held at ambient temperature for 5 days. Mean bars with the same letters are not significantly different.

3.5 Ripened transgenic fruits accumulate higher spermidine and spermine under chilling stress

To determine whether putative chilling tolerance in 556 fruits was attributed to high accumulation of PAs, their quantification was performed on fruits after 2 weeks of storage at 5°C. For free fraction, there was significant difference ($p \leq 0.05$) only in spermidine (Spd) and spermine (Spm), whereas no significant difference in putrescine (Put) (Figure 9). Both 556 and 579 fruits were equally high ($p \leq 0.05$) in Spd, whereas for Spm, 556 fruits recorded

significantly ($p \leq 0.05$) higher level. WT fruits on the other hand had the lowest level of both Spd and Spm. For conjugated and bound fractions, there were no significant difference in any forms of PAs. The seemingly correlation between higher Spd and Spm level in both transgenic fruits with their lower chilling injury incidence, suggest a possible role of these forms of PAs in chilling tolerance mechanism.

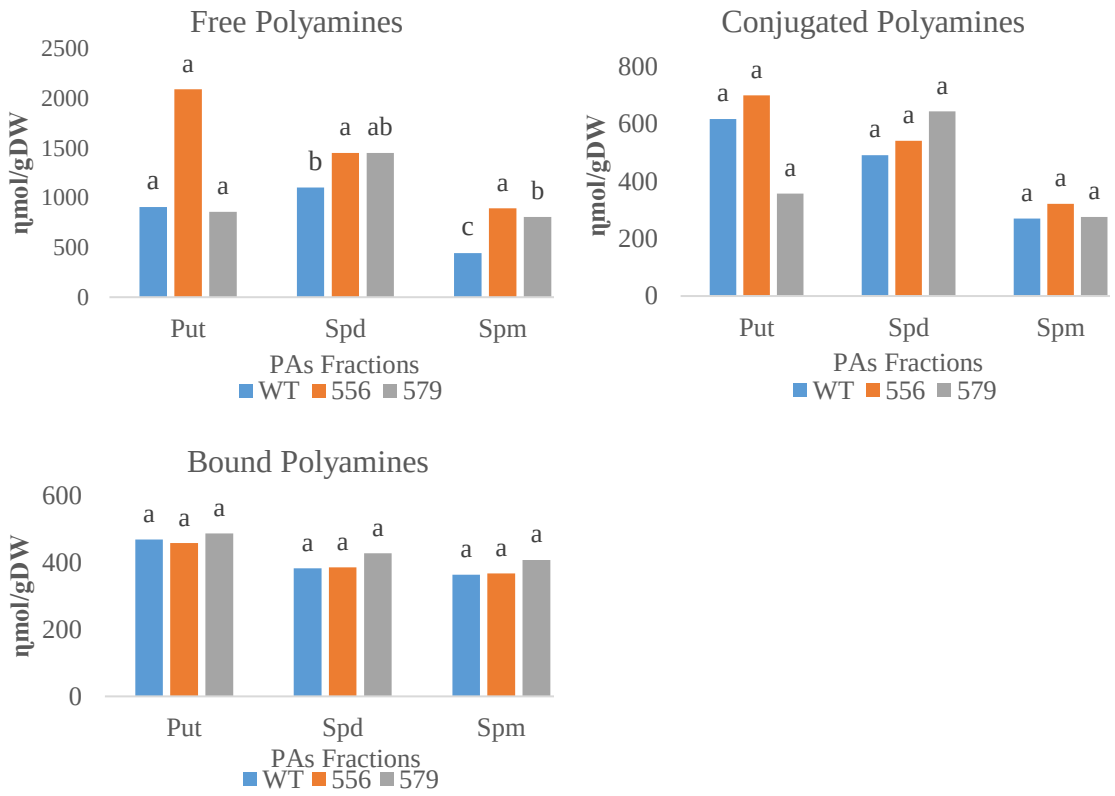
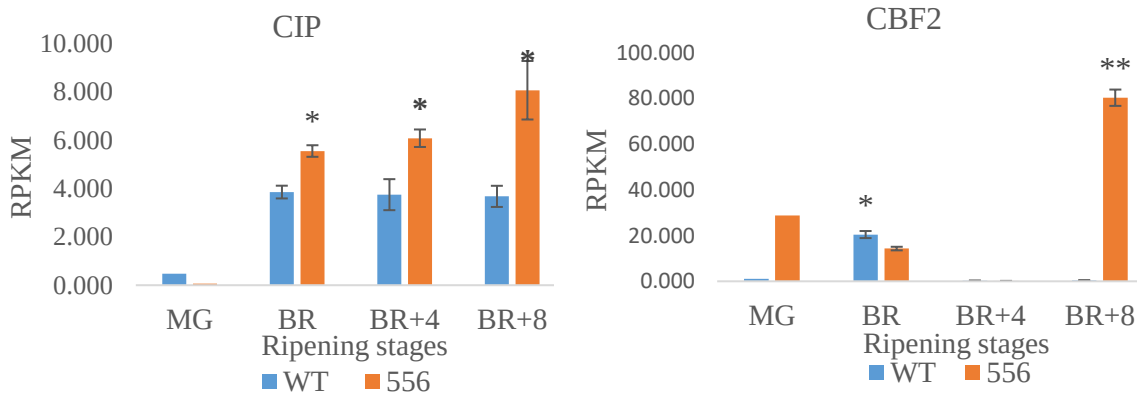


Figure 9: Free, conjugated and bound Put, Spd and Spm levels in fruits pericarp (ηmol/gDW) harvested at BR+8 stage, stored at 5ºC for 2 weeks and subsequently held at ambient temperature for 5 days. (WT=wild type; 556 and 579 = transgenic). Mean bars with the same letters in PAs fraction point are not significantly different.

3.6 High PAs enhance transcript level of genes associated with chilling tolerance during ripening in the absence of chilling stress

A preliminary screening of RNA-seq data was performed to find out the presence of genes associated with chilling tolerance in ripening fruits in the absence of chilling stress. Gene expression level was determined in fruits harvested at different ripening stages namely mature Green (MG), breaker (BR), breaker+4 (BR+4) and breaker+8 (BR+8). Nevertheless, this data only involves comparison between WT and 556 fruits due to unavailability of 579 fruit samples at that time. Search was performed by using The Sol Genomics Network (SGN; http://solgenomics.net/) and yielded 20 genes associated with chilling tolerance. Of these, only 14 genes were significantly upregulated ($p \leq 0.05$) at BR stage, whereby 10 genes were upregulated at BR+8 stage in 556 fruits. Upregulation of these particular genes in 556 fruits is likely associated with the accumulation of Spd and Spm as these polyamines increase during the ripening of 556 fruits (Mehta et al., 2002). Out of the 10 genes, expression patterns of five genes that exhibited RPKM >5 at at least at one stage of ripening are shown in Figure 10. The efficacy of RNA-seq for these genes was evaluated by qRT-PCR analysis (Figure 11). The genes selected included those encoding cold induced protein (CIP), CRT binding factor 2 (CBF2), calmodulin-like protein (CLP), thioredoxin family protein (TRX) and cold shock protein (CSP).



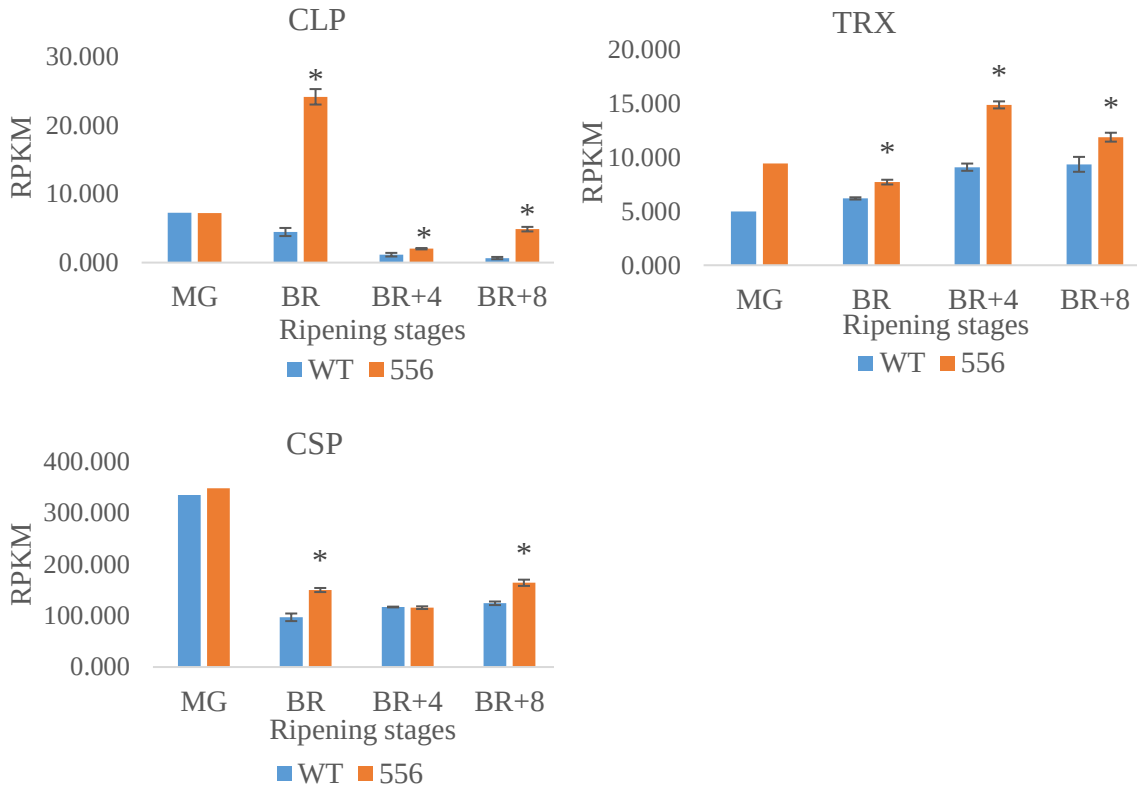
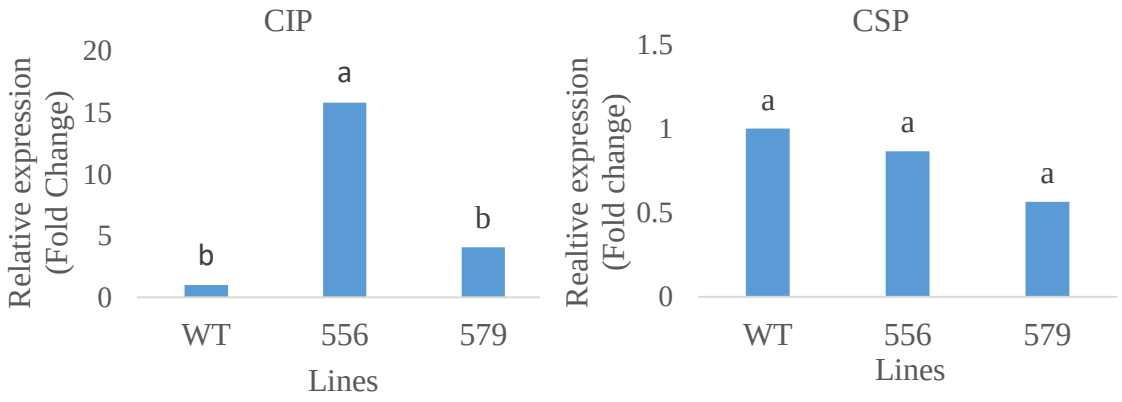


Figure 10: List of genes associated with chilling tolerance which were highly upregulated at fully ripe stage. Error bars indicate standard error with three biological replicates. Statistical difference between lines at each ripening stage point is denoted by * and ** symbols which indicate significant ($P < 0.05$) and highly significant ($P < 0.01$) respectively.

3.7 qRT-PCR shows higher expression of genes associated with chilling tolerance in ripened tomato fruits

Various mechanisms are employed by plants in their efforts to cope with chilling stress, including but not limited to expression of cold-responsive genes (Knight and Knight, 2012). These genes possibly involved in mediating physiological and biochemical changes required for plants' adaptation under adverse chilling condition (Thomashow, 1998). The effects of chilling stress on expression of genes associated with chilling tolerance in ripened fruits (BR+8) was investigated by using qRT-PCR. Five genes were selected from the above mentioned preliminary RNA-seq result (Figure 10). Gene expression in high-PAs

556 and 579 fruits was relatively compared with WT as control. Unlike physical evaluation and MDA, gene expression analysis was performed on fruits right after 2 weeks of storage without additional 5 days of rewarming since we aimed at investigating the response of fruits to chilling stress without any possible interference by chilling injury symptom development which typically occur during rewarming period. Of the total five genes investigated, three of them were significantly ($p \leq 0.05$) upregulated in 556 fruits. These included genes encoding for cold induced protein-like (CIP), CRT binding factor 2 (CBF2) and thioredoxin family protein (TRX) (Figure 11). There was no significant difference ($p \leq 0.05$) in genes encoding for cold shock protein-1 (CSP) among the lines. On the other hand, transcripts levels of calmodulin-like protein (CLP) were significantly higher in WT fruits. Uniquely higher ($p \leq 0.05$) expression of CIP and CBF2 in 556 compared to WT and 579 after 2 weeks of low temperature storage could be associated with chilling tolerance response, since it was consistent with the phenotypic changes, in which 556 fruits were appeared to be the most tolerant (Figure 7). However, since this qRT-PCR analysis was only performed on five candidate genes, these data were not representative enough to support this speculation. To obtain more comprehensive analysis, RNA-seq was performed for chilled and un-chilled fruits to delve into regulation of physiological and biochemical network of chilling response.



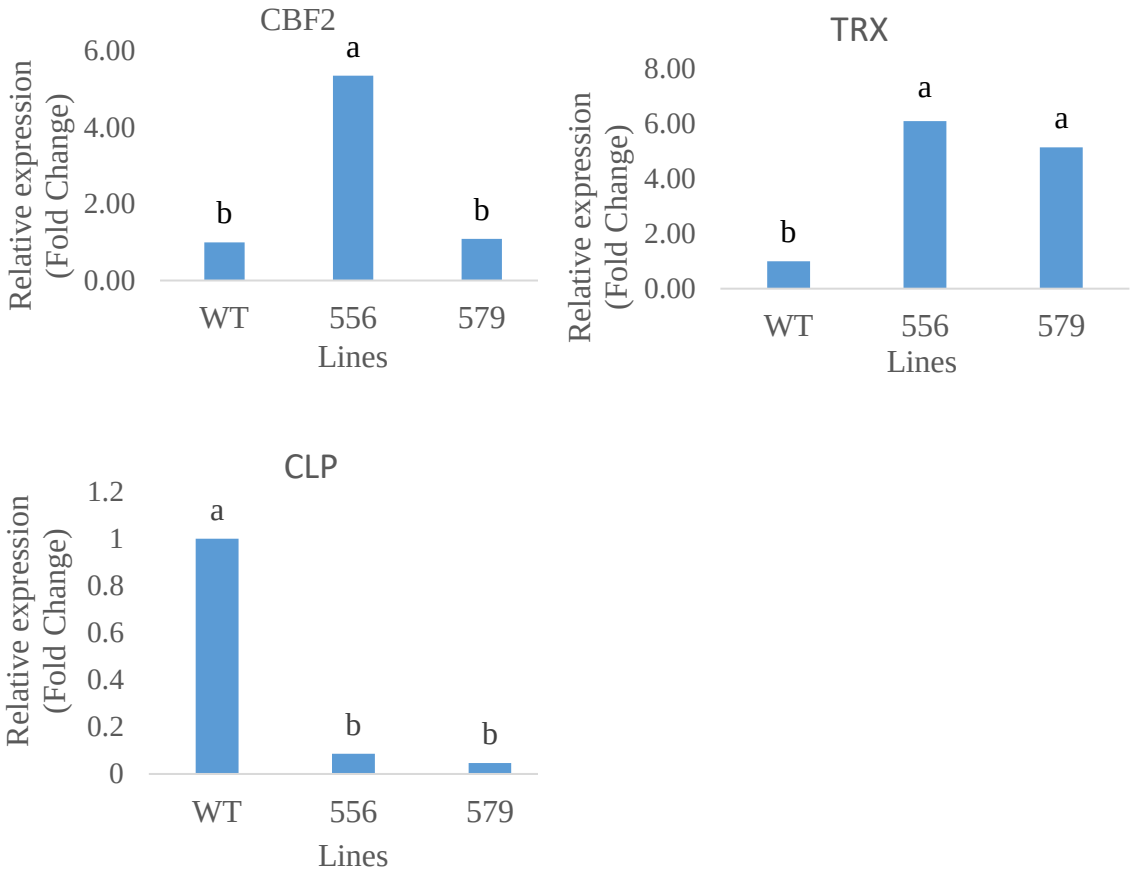


Figure 11: qRT-PCR analysis of five different genes associated with chilling tolerance in fruits harvested at BR+8 stage and stored for 2 weeks at 5°C. The transcripts expression levels were determined relative to a calibrator represented by WT line which was subjected to similar condition and time. Mean bars with same letters are not significant different ($p < 0.05$).

3.8 Large number of common genes are up and down regulated in two independent transgenic lines

Transcriptomic sequencing of tomato fruits was performed after they were exposed to chilling storage for 24 hours. The shorter cold treatment was used for this analyses in order to avoid the interference by genes or proteins involved in more advanced stages of the chilling stress, which are more related to chilling injury symptoms (Sanchez-Bel et al., 2012; Cruz-Mendívil et al., 2015a,b). Genes differentially expressed in two transgenic lines compared to WT fruits after chilling stress were determined as described in the Materials

and Methods chapter. Cut-off criteria with a minimum 2-fold change in abundance between high-PAs transgenic fruits and WT fruit after 24 hours of chilling was used to define differentially up and down regulated genes (DEGs) as shown in Venn diagram (Figure 12a and b). A total of 6523 and 5707 genes were differentially expressed in 556 and 579, respectively, compared to WT fruits after 24 hours of chilling. Among them 5018 and 4352 were up regulated in 556 and 579 fruits, respectively, and 1505 and 1355 were down regulated in 556 and 579 genotypes respectively, compared to WT genotype (Figure 12). A large number of DEGs were common between the two transgenic lines. This represented 3824 up and 1080 down regulated DEGs (Figure 12). These common genes in both transgenic lines are of interest in the present study, considering that both lines are genetically similar, and that the common genes might suggest connection between high PAs and chilling tolerance phenotype.

In addition to common genes between line 556 and 579, another intriguing comparison is the differential expression between line 556 and WT under two conditions; before and after chilling stress (Figure 13). In 556, 2902 DEGs upregulated without chilling and increased to 3824 after chilling at 5ºC. Similarly, higher number of genes were downregulated after chilling (1505) compared to before chilling (590) (Figure 13). On a different note, 499 upregulated DEGs and 85 downregulated DEGs were common in 556 between with and without chilling respectively. The upregulated and downregulated genes in this analysis can be considered as chilling-induced and chilling-repressed respectively. Considering that there was an increase of both upregulated and downregulated DEGs in 556 fruits after being subjected to chilling at 5ºC, it can be presumed that some genes were induced and some were repressed following chilling, which also might be linked with high endogenous PAs in the fruits.

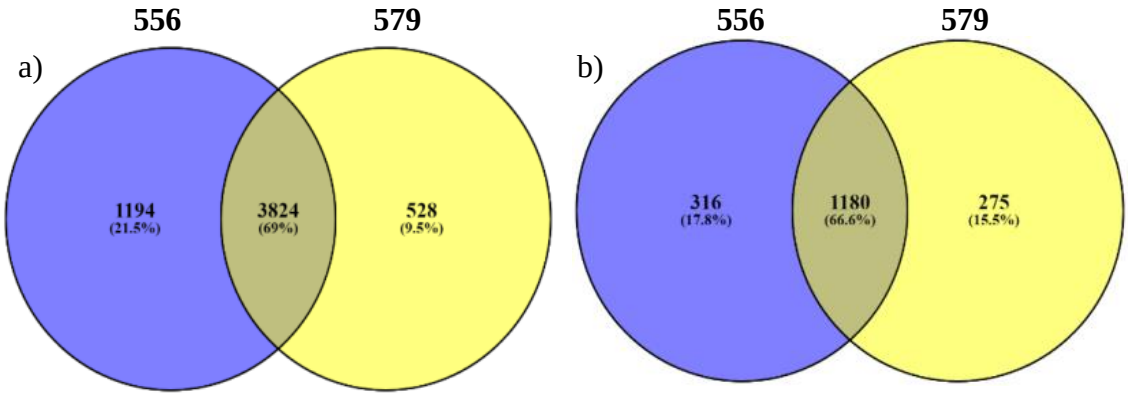


Figure 12: Venn diagrams showing DEGs of 556 and 579 fruits compared to those from WT after chilling. (a) upregulated DEGs after chilling and (b) downregulated DEGs after chilling (24 hours at 5ºC).

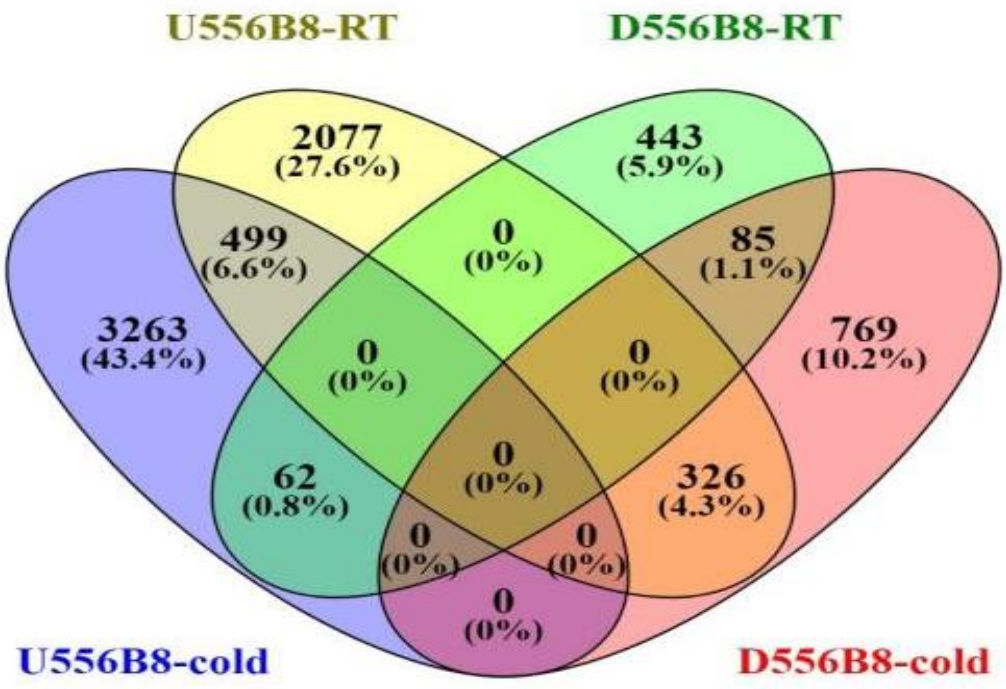


Figure 13: Venn diagrams showing DEGs of 556 fruits compared to those from WT either before chilling (immediately after harvest) and after chilling (storage at 5ºC for 24 hours). U556B8-RT=Upregulated DEGs before chilling; D556B8-RT=Downregulated DEGs before chilling; U556B8-cold=Upregulated DEGs after chilling; D556B8-cold= Downregulated DEGs after chilling.

3.9 Pathway Analysis of Differentially Expressed Genes

In order to gain more insight into gene functions of above described upregulated and downregulated DEGs, the Gene List Analysis for gene ontology (GO analysis) was performed by using PANTHER (Protein Annotation Through Evolutionary Relationships) Classification System for two groups of data after chilling as follow: common elements in upregulated 556 and 579 (Figure 12a) and common elements in downregulated 556 and 579 (Figure 12b). The data on common DEGs of 556 and 579 upon chilling was used because it might shed more light on relationship between PAs and chilling tolerance. The data sets were broken down to PANTHER GO-Slim Biological Process, Cellular Component, and Slim Molecular Function. For the most part, both groups of data had similar distribution of ontology whereby among the assigned molecular function terms (Figure 14a and 15a), majority DEGs were related to catalytic activity followed by binding. For biological processes terms (Figure 14b and 15b), majority DEGs were related to metabolic process followed by cellular process. For cellular component terms (Figure 14c and 15c), majority of DEGs were related to organelle and cell part. Since the focus of this present study was to examine the chilling stress response, the biological process terms of the upregulated common DEGs of line 556 and 579 after chilling was mined down to reveal the response of stimulus fraction (Figure 16). In this fraction, 74 out of 150 genes related to stress response were found. Further mining revealed the list of stress response related DEGs as in Table 2. Among the genes listed down were those from family of mitogen-activated protein kinase (MAPK), thioredoxin, heat shock proteins, peroxidase and autophagy, which have been long associated with a diverse plants abiotic stress response (Sinha et al., 2011; Wang et al., 2017; S Al-Whaibi, 2011 and Kapoor et al., 2015). The upregulation of these genes after chilling might suggest their possible role in chilling tolerance mechanism.

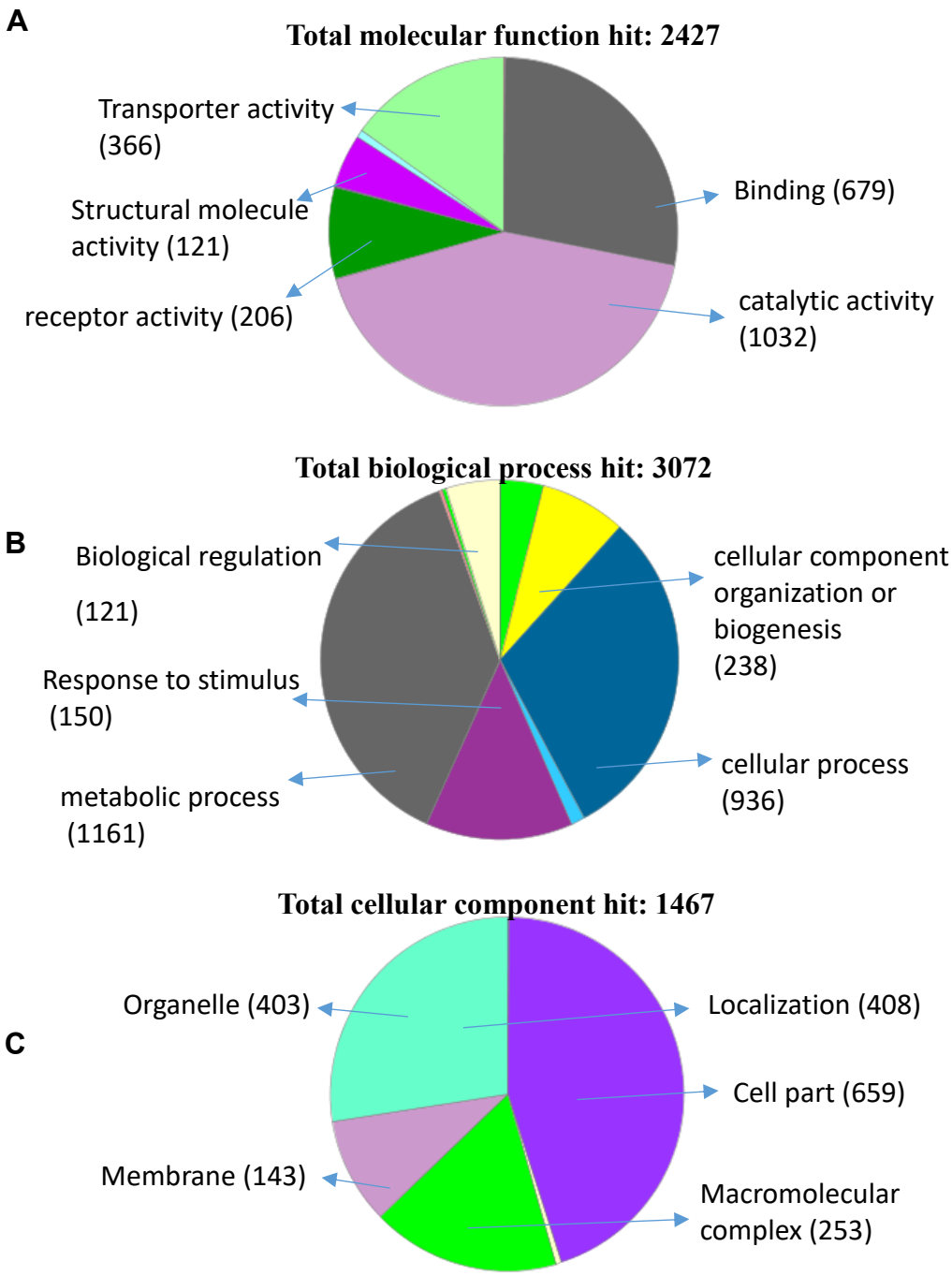


Figure 14: Gene Ontology (GO) terms assigned to the UPREGULATED common DEGs of line 556 and 579 after chilling. A) Molecular function, B) Biological process, C) Cellular component. Total number of DEGs are 3465. Number of genes for each fraction is in bracket

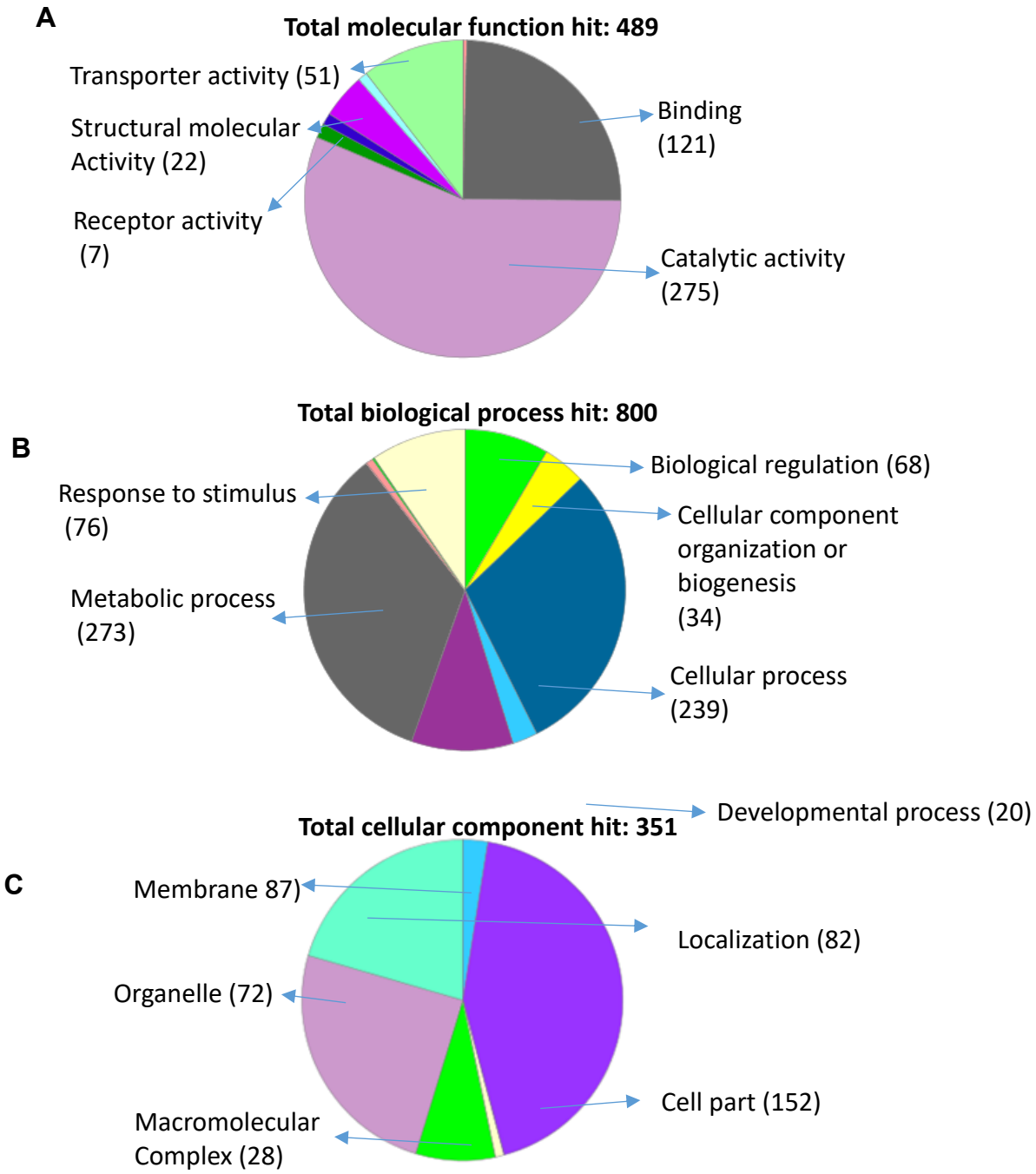


Figure 15: Gene Ontology (GO) terms assigned to the DOWNREGULATED common DEGs of line 556 and 579 after chilling. A) Molecular function, B) Biological process, C) Cellular component. Total number of DEGs are 1042. Number of genes for each fraction is in bracket.

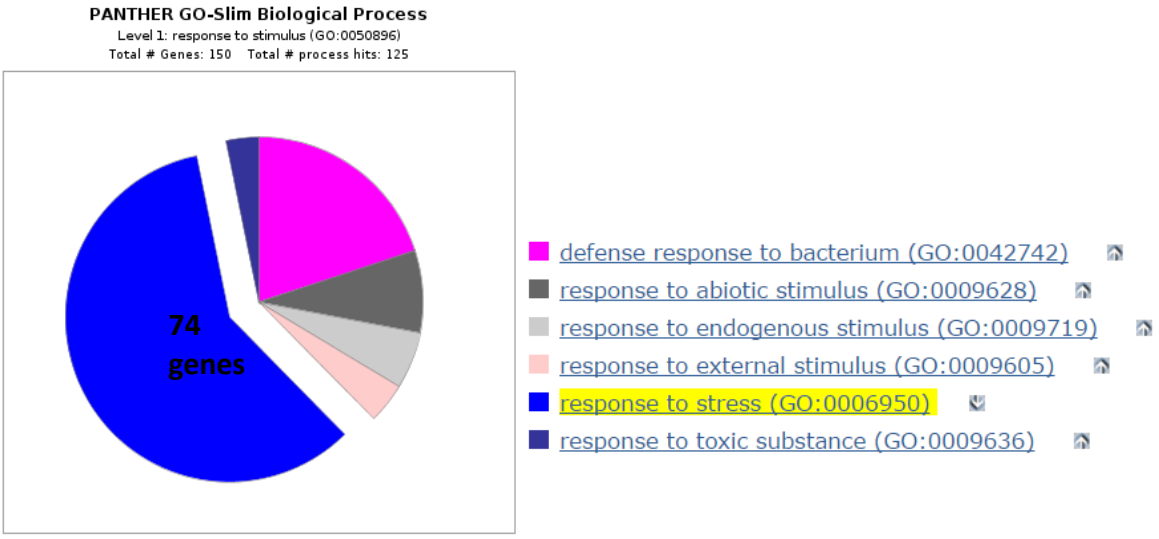


Figure 16: The GO level 1 Biological Process of the response to stimulus was further mined down to the reveal the percentage of molecules involved in stress response in upregulated common DEGs of line 556 and 579 after chilling

Table 2 List of upregulated common DEGs of line 556 and 579 after chilling which are related to stress response as derived from GO level 1 Biological Process of the Response to Stimulus

	Gene name/ symbol	**Family/Protein class**	**PANTHER Protein Class**
1	Solyc08g006010.2/ Uncharacterized protein	Autophagy-related protein 18a (pthr11227:sf33)/	
2	Uncharacterized protein Solyc03g121780.1	ATMRK1-RELATED (PTHR23257:SF691)/ non-receptor serine/threonine protein kinase	non-receptor serine/threonine protein kinase
			non-receptor tyrosine protein kinase
3	Uncharacterized protein Solyc05g056300.2	Thioredoxin f1, chloroplastic-related (pthr10438:sf312)	oxidoreductase

Table 2 continued

4	Uncharacterized protein Solyc04g014480.2	15.7 kda heat shock protein, peroxisomal (pthr11527:sf178)	chaperone
5	Uncharacterized protein Solyc02g090330.2	Autophagy-related protein 101 (pthr13292:sf1)	-
6	Uncharacterized protein Solyc08g078720.2	23.5 kda heat shock protein, mitochondrial-related (pthr11527:sf223)	chaperone
7	Uncharacterized protein Solyc01g108070.2	Protein deglycase dj-1 (pthr43444:sf1)	RNA binding protein
			cysteine protease
			transcription factor
8	Uncharacterized protein Solyc06g065650.2	Protein tify 8 (pthr33077:sf30)	
9	Uncharacterized protein Solyc07g006760.2	Mitogen-activated protein kinase kinase kinase (pthr23257:sf650)	non-receptor serine/threonine protein kinase
			non-receptor tyrosine protein kinase
10	Uncharacterized protein Solyc08g008550.2	Subfamily not named (pthr24361:sf449)	-
11	Uncharacterized protein Solyc02g078140.2	Protein kinase 6-like protein (pthr23257:sf635)	non-receptor serine/threonine protein kinase
			non-receptor tyrosine protein kinase
12	Uncharacterized protein Solyc09g082540.2	Carboxylate clamp-tetratricopeptide repeat protein (pthr22904:sf408)	chaperone
13	Uncharacterized protein Solyc05g012130.2	Non-specific serine/threonine protein kinase (pthr24361:sf576)	-
14	Uncharacterized protein CTR3	Serine/threonine-protein kinase ctr1 (pthr23257:sf677)	non-receptor serine/threonine protein kinase

Table 2 continued

			non-receptor tyrosine protein kinase
15	Uncharacterized protein Solyc04g080470.2	Dna glycosylase superfamily protein (pthr43003:sf5)	DNA glycosylase
16	Uncharacterized protein Solyc04g074240.2	Endoplasmic reticulum resident protein 44 (pthr18929:sf141)	
17	Uncharacterized protein Solyc06g048510.2	WD repeat domain phosphoinositide-interacting protein 3 (pthr11227:sf36)	-
18	Uncharacterized protein Solyc03g118070.2	Sucrose nonfermenting 4-like protein (pthr13780:sf75)	kinase modulator
19	Uncharacterized protein Solyc03g025890.1	DNA mismatch repair protein msh3 (pthr11361:sf119)	DNA binding protein
20	Uncharacterized protein Solyc01g108020.2	Thioredoxin m3, chloroplastic (pthr10438:sf314)	oxidoreductase
21	Peroxidase Solyc02g064970.2	Peroxidase 25 (pthr31235:sf130)	-
22	Uncharacterized protein Solyc05g055780.2	DNA repair protein reca homolog 3, mitochondrial-related (pthr22942:sf51)	DNA strand-pairing protein
			hydrolase
23	Uncharacterized protein Solyc05g042040.2	Gh01724p (pthr23333:sf27)	
24	Uncharacterized protein MAPKKKe	Cell division control protein 15 (pthr24361:sf434)	
25	Uncharacterized protein Solyc09g074980.2	Mismatch repair endonuclease pms2 (pthr10073:sf51)	DNA binding protein
26	Uncharacterized protein Solyc07g018340.2	Muts protein homolog 4 (pthr11361:sf113)	DNA binding protein

Table 2 continued

27	Uncharacterized protein Solyc11g006980.1	Germin-like protein subfamily 1 member 3-related (pthr31238:sf67)	-
28	Uncharacterized protein Solyc06g065790.1	Mitogen activated protein kinase kinase kinase-like protein-related (pthr24361:sf544)	-
29	Uncharacterized protein Solyc01g102810.2	Protein stichel-related (pthr11669:sf38)	DNA-directed DNA polymerase nucleotidyltransferase
30	Uncharacterized protein Solyc07g062060.2	Methionine-r-sulfoxide reductase b1 (pthr10173:sf44)	reductase
31	Uncharacterized protein Solyc07g065990.1	Oleosin 20.3 kda-related (pthr33203:sf8)	-
32	Uncharacterized protein Solyc03g117530.1	Hus1-like, isoform a (pthr12900:sf6)	-
33	Ammonium transporter Solyc03g033300.1	Subfamily not named (pthr43029:sf2)	transporter
34	Uncharacterized protein Solyc04g016530.2	Dna-(apurinic or apyrimidinic site) lyase (pthr22748:sf14)	-
35	Peroxidase Solyc03g080150.2	Subfamily not named (pthr31235:sf115)	-
36	Uncharacterized protein Solyc10g006520.2	Subfamily not named (pthr43601:sf1)	oxidoreductase
37	MLO-like protein Solyc10g044510.1	Mlo-like protein 4 (pthr31942:sf45)	-
38	Uncharacterized protein Solyc08g067250.2	Subfamily not named (pthr23240:sf20)	-

Table 2 continued

39	Uncharacterized protein Solyc01g010700.2	Snf1-related protein kinase regulatory subunit gamma-1-like (pthr13780:sf83)	kinase modulator
40	Uncharacterized protein Solyc04g078250.2	Metal transporter nramp2-related (pthr11706:sf52)	cation transporter
41	Uncharacterized protein Solyc10g009050.2	F3h9.11 protein (pthr12956:sf48)	hydrolase
42	Uncharacterized protein Solyc05g007430.2	Subfamily not named (pthr12956:sf42)	hydrolase
43	Serine/threonine-protein phosphatase Solyc01g009280.2	Subfamily not named (pthr11668:sf349)	calcium-binding protein protein phosphatase
44	Uncharacterized protein Solyc08g074610.1	Mediator of rna polymerase ii transcription subunit 34 (pthr13710:sf107)	DNA helicase
45	Uncharacterized protein Solyc11g073220.1	Dna repair protein rad51 homolog 4 (pthr22942:sf56)	DNA strand-pairing protein hydrolase
46	Uncharacterized protein Solyc02g094100.2	Fidgetin-like protein 1 (pthr23074:sf119)	non-motor microtubule binding protein
47	Uncharacterized protein Solyc06g071450.2	General transcription factor iih subunit 3 (pthr12831:sf1)	nucleic acid binding transcription factor
48	Uncharacterized protein Solyc07g042010.2	ATP-dependent dna helicase q-like 3 (pthr13710:sf112)	DNA helicase
49	Uncharacterized protein Solyc01g079750.2	Mitogen-activated protein kinase kinase kinase 19 (pthr24361:sf485)	-
50	Uncharacterized protein Solyc02g031910.2	Protein kinase superfamily protein with octicosapeptide/phox/bem1p domain (pthr23257:sf584)	non-receptor serine/threonine protein kinase non-receptor tyrosine protein kinase

Table 2 continued

51	Uncharacterized protein Solyc01g111920.2	F-box protein skip23 (pthr13710:sf119)	DNA helicase
52	Uncharacterized protein Solyc08g008240.2	Serine/threonine/tyrosine kinase-related (pthr23257:sf602)	non-receptor serine/threonine protein kinase
			non-receptor tyrosine protein kinase
53	Uncharacterized protein Solyc01g006210.2	Non-structural maintenance of chromosomes element 1 homolog (pthr20973:sf1)	-
54	Uncharacterized protein Solyc12g019740.1	Subfamily not named (pthr43601:sf3)	oxidoreductase
55	Uncharacterized protein Solyc09g059440.1	Cell division control protein 45 homolog (pthr10507:sf1)	replication origin binding protein
56	Tau2 Solyc11g012710.1	Snf1-related protein kinase regulatory subunit beta-2 (pthr10343:sf78)	kinase modulator
57	Uncharacterized protein Solyc04g055110.2	Arm-repeat/tetratricopeptide repeat-like protein (pthr22904:sf400)	chaperone
58	Uncharacterized protein Solyc05g050950.2	DNA repair protein rad51 homolog 3 (pthr22942:sf53)	DNA strand-pairing protein
			hydrolase
59	Uncharacterized protein Solyc03g080010.2	DNA mismatch repair protein muts, type 2 (pthr11361:sf107)	-
60	Heat shock cognate protein 80 HSC80	Heat shock protein 90-2-related (pthr11528:sf69)	Hsp90 family chaperone
61	Uncharacterized protein Solyc10g080560.1	DNA-3-methyladenine glycosylase (pthr10429:sf1)	DNA glycosylase
			damaged DNA-binding protein
			glycosidase
62	Uncharacterized protein Solyc07g065330.2	Germin-like protein subfamily 3 member 4 (pthr31238:sf52)	-

Table 2 continued

63	Uncharacterized protein Solyc09g065630.2	Subfamily not named (pthr33077:sf21)	-
64	Uncharacterized protein Solyc08g076610.2	Cell cycle checkpoint protein rad1 (pthr10870:sf1)	exodeoxyribonuclease
			hydrolase
65	Uncharacterized protein Solyc05g014690.1	Werner syndrome atp-dependent helicase (pthr13710:sf105)	DNA helicase
66	Uncharacterized protein Solyc01g008250.2	Thioredoxin x, chloroplastic (pthr10438:sf316)	oxidoreductase
67	Uncharacterized protein Solyc01g099280.2	Sucrose nonfermenting 4-like protein (pthr13780:sf75)	kinase modulator
68	Uncharacterized protein Solyc12g099250.1	Subfamily not named (pthr23257:sf668)	non-receptor serine/threonine protein kinase
			non-receptor tyrosine protein kinase
69	Uncharacterized protein Solyc01g020220.1	Replication factor c subunit 3 (pthr11669:sf40)	DNA-directed DNA polymerase
			nucleotidyltransferase
70	Uncharacterized protein Solyc05g050960.2	DNA repair protein rad51 homolog 3 (pthr22942:sf53)	DNA strand-pairing protein
			hydrolase
71	Uncharacterized protein Solyc01g096960.2	Protein restricted tev movement 2 (pthr43670:sf7)	chaperone
72	Uncharacterized protein Solyc06g082190.2	Protein kinase family protein (pthr23257:sf615)	non-receptor serine/threonine protein kinase
			non-receptor tyrosine protein kinase
73	Uncharacterized protein Solyc06g065910.2	Carboxylate clamp-tetratricopeptide repeat protein-related (pthr22904:sf423)	chaperone

Table 2 continued

74	Uncharacterized protein Solyc01g080410.2	Peptide methionine sulfoxide reductase 2 (pthr10173:sf46)	reductase

3.10 Polyamines regulate expression of genes associated with chilling tolerance as early response towards chilling stress in both transgenic fruits

20 genes associated with chilling were extracted from RNA-seq data to see their status after being exposed to chilling only for 24 hours. This is to compare with previous data before and after chilling for 2 weeks at 5ºC (Figure 10 and 11). This would shed some light on PAs effects in inducing early response towards chilling stress. Out of four genes that show significant difference ($p \leq 0.05$), only three of them recorded significantly higher ($p \leq 0.05$) transcripts in both 556 and 579 fruits, namely Cold acclimation protein (CAP), Zinc finger CCCH domain-containing protein (ZFN), and Thioredoxin family protein (TRX) (Figure 17). CAP was found to be associated with the development of chilling tolerance in cereals and Arabidopsis (Breton et al., 2003). The zinc finger proteins are a super family of proteins implicated in some activities that regulate resistance mechanism for a number of biotic and abiotic stresses (Gupta et al, 2012), by binding with nucleic acids for their function whereby CCCH represent cysteine and histidine components (Gupta et al., 2012). Thioredoxins (Trxs) are a multigenic family of proteins in plants that play a critical role in redox balance regulation through thiol-disulfide exchange reactions and influences plant development and stress responses (Kapoor et al., 2015). Common transcription level between those two transgenic lines could be associated with effects of high PAs on chilling tolerance. On the other hand, CBF2 was significantly highest ($p \leq 0.05$) in 556 fruits only. Unlike evaluation at 2 weeks of storage, more cold-responsive genes showed common transcription level between 556 and 579 transgenic fruits, implying that PAs pose more effects of chilling tolerance at early stage of stress (24 hours of chilling), rather than after extended period (2 weeks of chilling). In contrast, higher gene expression was uniquely found in 556 fruits at 2 weeks stress point.

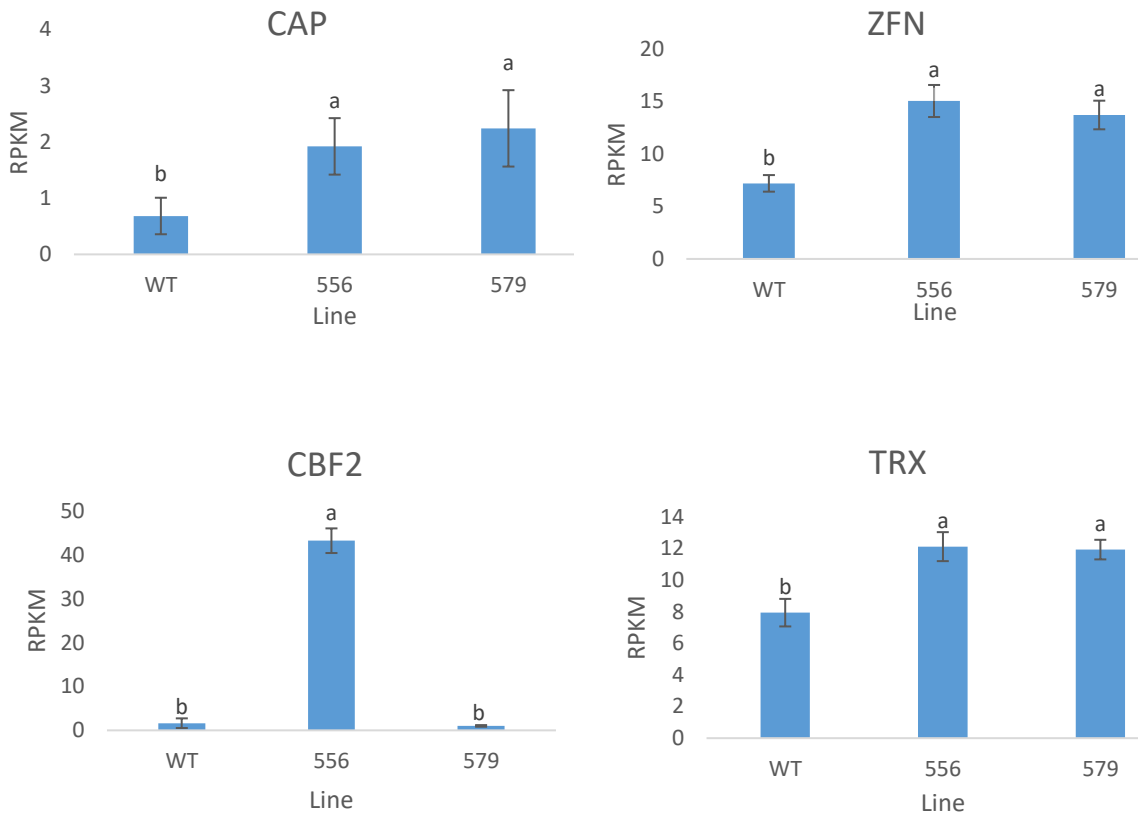


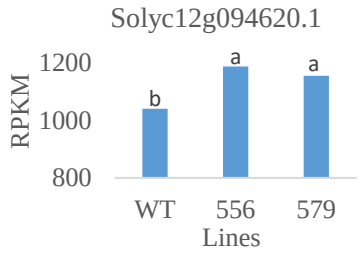
Figure 17: Expression level of genes associated with chilling tolerance after 24 hours of storage at 5ºC, derived from RNA-seq data. Mean bars with same letters are not significant different ($p \leq 0.05$).

3.11 Polyamines regulate antioxidant related genes in both high PAs transgenic fruits

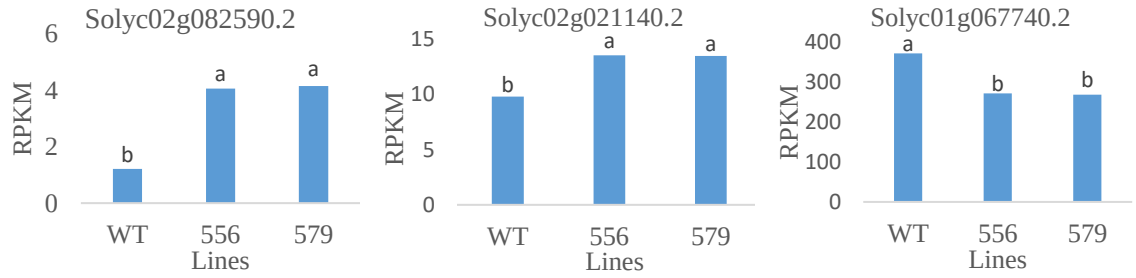
Plant cells generate several antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) in order to reduce oxidative damage by ROS accumulation upon stress (Gill and Tuteja, 2010). ROS include free radicals such as superoxide anion (O2•−), hydroxyl radical (•OH), as well as nonradical molecules like hydrogen peroxide (H_2O_2), singlet oxygen (1O2) (Sharma et al., 2012). In general, SOD detoxifies O2- by forming H202, whereas CAT scavenge H_2O_2 (Jaleel et al., 2009). There were large number of genes were found to be related to each of the enzymes, but only genes with significant ($p<0.05$) expression among three lines based on RPKM value were presented (Figure 18). For catalase, only one transcript

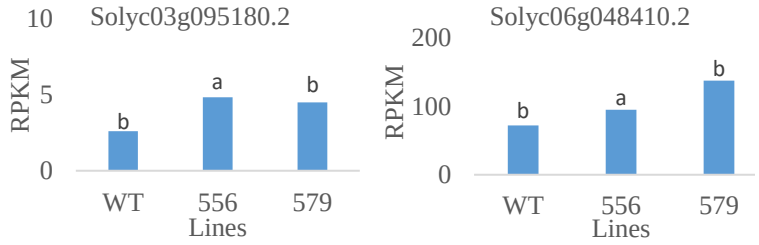
(Solyc12g094620.1) showed a significant expression level whereby the highest was line 556. For SOD, five genes were significantly different among the lines. Line 556 recorded the significantly highest expression in four of them with slight difference with 579, whereas WT had the lowest expression in all but Solyc01g067740.2. For APX, out of three significant transcripts, both 556 and 579 lines were highest in all but Solyc06g005160.2. These results showed that line 556 generally had relatively higher expression of genes related to antioxidant enzymes that could partly implicated in mitigating chilling stress.

a) CAT



b) SOD





c) APX

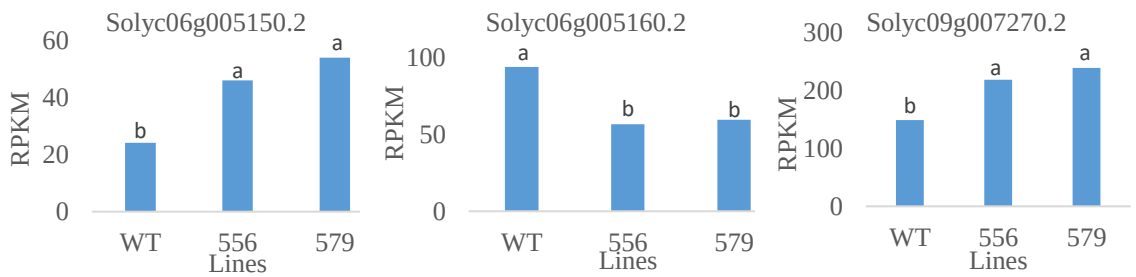


Figure 18: List of genes encoding antioxidant enzymes, derived from RNA-seq data; a) catalase, b) superoxide dismutase and c) ascorbate peroxidase. Mean bars with same letters are not significantly different ($p \leq 0.05$).

CHAPTER 4. DISCUSSION

4.1 High-PAs tomato responded differently to chilling temperature at two ripening stages

Ripening stage is an important determinant of chilling injury susceptibility in tomato (Whitaker, 1994). Tomato at advanced ripening stage are generally more chilling-tolerance than mature green fruits and can be stored at lower temperature (Saltveit, 2005). In this present study, it was observed that tomato with different level of endogenous Spd/Spm, respond differently to chilling stress when harvested at different ripening stages. Interestingly, high-PAs transgenic fruits (line 556 and 579), showed more tolerance to chilling stress when harvested at full ripe stage (BR+8) (Figure 7) compared to those harvested at initial ripe stage (BR) (Figure 2), in comparison with Wild Type (WT) fruits at comparable ripening stages. When stored at 8ºC, fruits from one of the high-PAs transgenic line (579) showed unfavorable phenotype that it was not able to completely ripen after rewarming. Interestingly, in contrast to the failure to ripen, fruits from the line 579 cumulatively produced the highest ($p \leq 0.05$) ethylene production among the lines examined (Figure 5). The relationship between ethylene and PAs have been long proposed considering the facts that both compounds are sharing S-adenosyl-L-methionine (SAM) as the common precursor in their biosynthesis (Anwar et al., 2014). It has been previously shown that 579HO fruits exhibit higher ethylene production during ripening as compared to the WT and 556AZ fruit (Mehta et al., 2002; Mattoo et al., 2006). The rates of ethylene production from fruits of both transgenic lines were highest ($p \leq 0.05$) after the 5 days of rewarming period regardless of storage duration suggesting that inability of 579 fruits to ripe normally was not due to the lack of ethylene biosynthesis but likely, at partly, due to the interruption of conversion of chloroplast to chromoplast (Molina 1976). Another hypothesis could be that PAs protect the ethylene biosynthesis possibly through regulating genes of the ethylene biosynthesis under stress condition. PAs ability in stabilizing molecules involved in gene expression has been reported by Shen et al. (2016) as a PAs transporter LHR1/PUT3 was associated with stabilizing mRNAs of several heat stress responsive genes under an extreme high temperature condition.

Changes in PAs level as an adaptive response towards various abiotic stresses have been evidenced in various investigation and may involve significant increase of one type of PAs, or in some cases may involves all three main PAs; Spd, Spm and Put (Yang et al, 2007; Liu et al., 2015). Interestingly in the present investigation, fruit ripening seemingly does affect differential accumulation three types of PAs under chilling stress, as different types of PAs elevated at different ripening stage under chilling condition (Figure 6 and 9). This was probably due to the fact that PAs accumulation might be influenced by physiological status of the tissues/organs along with stress conditions (Liu et al., 2015). For initial ripe (BR) stage fruits, both transgenic lines retained higher levels ($p \leq 0.05$) of free and conjugated Spd compared to WT parental fruits after 5 weeks of storage at 8ºC followed by 5 days of rewarming at ambient (Figure 6). These results show that the transgenic fruits retained ability to accumulate higher Spd regardless of stress compared to WT fruit and that expression of transgene SAMDC under the control of *SlE8* ripening specific promoter was not affected by stress condition. It also worth noting that there was significant increase of Put throughout the chilling period in all lines for BR stage, whereby WT fruits showed much higher increase after rewarming (Figure 6). Although more experimental work is needed, this finding points out to a possible role of Put in triggering chilling tolerance in fruits at initial ripe stage. Roles of Put in mitigating chilling stress of tomato have been documented by Jiang et al. (2012) in tomato seedling. Likewise, for full ripe stage (BR+8), both Spd and Spm exhibited higher levels ($p \leq 0.05$) in transgenic fruits, after 2 weeks of chilling treatment at 5ºC (Figure 9), and coincided with chilling tolerance phenotype. Based on this result, it is reasonable to speculate that Spd and Spm play more important roles in alleviating chilling stress since they contain more primary amino groups (−NH2), than Put, rendering more efficient protective functions (Liu et al., 2015). The high-PAs transgenic fruits showed more tolerance at full ripe stage suggesting that some mechanisms in ripening process is implicated along with PAs to confer such tolerance. It also shows that a PAs dynamics exists under stress condition. However, a causal relationship between ripening stage, PAs and protection against chilling stress is remained to be established.

4.2 PAs regulate expression of chilling stress-associated genes in full ripe tomato fruits

Regulation of genes implicated in stress response is a pivotal mechanism for plants to cope with adverse conditions (Shen et al., 2016). Positive charged molecules such as PAs has potential to serve as gene regulators through the electrostatic interaction with DNA, RNA, chromatin and transcription factors and modulate promoter elements and protein factors that play roles at the transcription level (Igarashi and Kashiwagi, 2010). With respect to differentially expressed genes (DEG) in response to chilling stress, a widespread up-regulation and down-regulation of gene expression was observed in high PAs transgenic fruits after 24 hours (Figure 12). Although a significant number of genes were up or down regulated only in either 556 or 579 fruits after chilling stress, much larger number of genes were commonly up or down regulated in the two independent transgenic lines (Figure 12). The genes that were differentially regulated only in one and not in the other transgenic line likely represent those which are not affected by high PAs levels but are results of growth environments, or perhaps there are other modifications that work along with PAs in one and not in the other transgenic line. In this sense, the genes that are affected by chilling stress in both transgenic lines are of great interest as they are likely regulated by altered PAs levels in both transgenic compared to WT fruits. The common transcriptional level of both transgenic lines was mostly observed after 24 hours of chilling, suggesting that PAs play significant roles in the early stress responses. Previous transcriptomic studies suggest that many genes upregulated at the early point of chilling conditions play a pivotal role in alleviating chilling stress (Gibson et al., 1994), and provide insight of first molecular response to chilling rather than those implicated in chilling injury symptoms development (Cruz-Mendívil et al., 2015).

In a recent study, Cruz-Mendívil et al. (2015a,b) has reported that after 24 hours of storage at 5ºC, tomato exhibited some adaptive transcriptional responses, which could be associated with chilling tolerance. These included, the up-regulation of genes associated with regulation of transcription (C2H2-type zinger protein and AP2/EREBP and), protein folding (peptidyl-prolyl cis–trans isomerases and chaperonins) and pathogen resistance (NBS, TIR, and LRR families) (Cruz-Mendívil et al., 2015a,b). In the present study, DEGs related to chilling tolerance were classified according to molecular function, cellular component, and biological process categories. Molecular function gene ontology

evaluation of up and down regulated DEG in high PAs fruits compared to WT fruits after chilling treatment predominately represented proteins with various catalytic activities or ability to bind to various macromolecules (Figure 14a and 15a), suggesting PAs play roles by altering metabolome (Liu et al., 2015). Genes encoding transporter activity were other major category of DEGs in response to high PAs. The evaluation of DEGs based on cellular compartment revealed profound effect of chilling on cell parts, organelle, macromolecular and membrane related genes for both up and down regulated DEGs (Figure 14b and 15b). Based on biological process classification, majority of up and down regaled genes represented metabolic and cellular process (Figure 14c and 15c). Another significant subset in biological process classification is response to stimulus (Figure 16), where it revealed the stress response by high-PAs transgenic fruits. There were 74 common upregulated DEGs between 556 and 579 related to stress responses (Figure 16 and Table 2). Among established genes related to stress found in the dataset were gene family of heat shock protein (HSPs), mitogen-activated protein kinase (MAPK) and thioredoxin family protein (TRX). At least three genes related to heat shock protein were upregulated (Table 2). HSPs are ubiquitous proteins found in plant and animal cells, originally described in association with heat shock (Ritossa, 1962) but are now found to be induced by a diverse form of stresses, including chilling (Boston et al., 1996; Lindquist and Craig, 1988; Vierling, 1991). HSPs may have role in stabilizing proteins or in assisting protein folding under adverse condition such as oxidative damage (Park and Seo 2015; Sun et al., 2002). Their presence which is typically undetectable under normal growth conditions but rapidly accumulate under stress conditions (Sabehat et al. 1998; Ding et al. 2001). This rapid response has been implicated in protection against the stress that trigger their accumulation and also against any other subsequent stress conditions encountered by plant (Sevillano et al. 2010). Transcription of HSPs is activated by heat stress transcription factors (Hsfs) family whose bind to the heat shock elements in the HSPs promoters (Kotak et al., 2007). Recently Re et al. (2017) suggested that HSPs may be directly involved in the chilling stress protection in the Micro-tom tomato fruits (chilling tolerant cultivar) in relative to Minitomato (chilling sensitive), and confirmed that HSPs transcript accumulation was exclusively due to chilling response not because of the postharvest fruit ripening

Plants have acquired complex mechanisms to sense and transmit signal in coping with environmental stress. The mitogen-activated protein kinase (MAPK) cascade has emerged as a common signal transduction pathway that connects external stimuli to cellular stress response in plants (Yu et al., 2015). MAPK signaling cascade chiefly consists of three kinases namely MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK) (Wang et al., 2015), which function as sequential signal transducers that transfer information from cellular environment to transcriptional and metabolic response centers through phosphorylation (Wang et al., 2015). In tomato, 16 MPK genes have been identified which are classified into four distinct groups (A-D) based on structure and sequence similarity (Kong et al. 2012). Yu et al. (2015) found that transgenic tomato overexpressing SlMPK7, a novel tomato MAPK gene, resulted in more stress-responsive gene expression along with less ROS, higher antioxidant activity, leading to enhanced chilling stress tolerance compared with the wild-type plants. Interestingly, SlMPK7 expression was not only induced by low temperature but also by signal molecules such as spermidine, salicylic acid, abscisic acid and H_2O_2. Zhou et al. (2013) looked at the relationship of MAPK and ethylene biosynthesis and found that upon chilling and ethephon treatment, tomato MAPK4 (LeMAPK4) expression was increased, thus suggesting the participation of cold-induced ethylene biosynthesis in chilling response of tomato fruit. In other words, MAPK cascade involved in regulating ethylene biosynthesis in chilling (Zhao et al., 2013), which could make ripening process possible even under stress condition. Response of MAPK pathways with Spd and ethylene as shown in previous studies suggest the involvement of SAMDC in this signal transduction system, considering the facts that high Spd/Spm fruits continue to generate high ethylene even under chilling stress in the present finding (Figure 5).

Expression level of 20 cold-responsive genes was investigated after different exposure time of chilling; 2 weeks (Figure 11) and 24 hours (Figure 17), by using qRT-PCR and RNA-seq data respectively. Even though evaluations were made at different time using different tools, the results give insight on when the stress protection system is activated and inactivated. Two weeks and 24-hours time in this study, respectively, represent late and early stress point after chilling. In general, there was only one gene that were significantly ($p \leq 0.05$) expressed in both transgenic lines at both time points namely

thioredoxin family protein (TRX). This suggests that genes encoding TRX might be induced as early as 24 hours of chilling and may continue until 2 weeks. TRX often associated with redox homeostasis and regulating the redox status of target proteins through thiol-disulfide exchange reactions (Wu et al., 2016). They may respond to oxidative stress through several mechanisms such as, repairing the damaged oxidative proteins, activating antioxidant enzymes activity and acting as regulators of scavenging mechanisms or of signaling pathways in the antioxidant network (Wu et al., 2016; Cheng et al., 2014). Comparing between tomato stored at chilling temperature and those stored at room temperature, Vega-Garcia et al. (2010) demonstrated that thioredoxin perodixase protein was significantly accumulated along with glycine-rich RNA-binding protein upon storage at chilling condition. Recently Hu et al. (2015) demonstrated that genetically engineered tomato plants expressing AtGRXS17, a gene of glutaredoxins (GRXs) enhanced chilling tolerance by a crucial function in coordination of signaling and scavenging of ROS. GRXs are small ubiquitous oxidoreductases of the TRX family (Hu et al., 2015). In addition to TRX, genes encoding for cold acclimation protein (CAP) and zinc finger CCCH domain-containing protein (ZFN), also were significantly upregulated in both transgenic lines, implying an effect of PAs in regulating these genes. Nevertheless, both latter genes were only activated at the early point of chilling (24 hours).

Another intriguing gene in this present study is CRT binding factor 2 (CBF2) which is one of three CBFs genes that have been characterized in tomato (Zhang et al., 2016). Nevertheless, in the present investigation, CBF2 was significantly expressed only in 556 fruits and not in both transgenic fruits. This might partly rule out significant role of PAs in regulating this gene, and that CBF pathway is in chilling tolerance mechanism in relation to high endogenous PAs. In contrast, previous investigations reported that as transcription factors, CBFs play a pivotal role in the chilling tolerance mechanisms of some plants by regulating downstream cold-responsive genes to trigger tolerance (Zhou et al., 2009b). In a study by Zhang et al. (2016), transcripts of CBF1, CBF2 and CBF3 were remarkably increased during chilling but returned to un-chilled level after rewarming period. In the present study, significant higher expression level of CBF2 was also observed in line 556 during ripening particularly at BR+8 (Figure 10). High expression of CBF2 during ripening in the absence of chilling could be associated with high production of ethylene that might

induce transient expression of this gene. According to Zhou et al. (2009b), exogenous application of ethephon at an effective concentration could induce tomato CBF1 expression at room temperature, enhance the expression under chilling stress, and decrease the chilling injury of tomato fruits, whereas treatment with 1-Methylcyclopropene (1-MCP), a competitive inhibitor of ethylene, remarkably decreased the CBF1 expression level Zhou et al. (2009b). Ethylene production factor during ripening might also apply to CBF2 expression in this study as well.

Chilling stress resulted in inevitable accumulation of oxygen species (ROS), which consequently lead to chilling injury (Hu et al., 2015). ROS cause oxidative damage to various cellular components such as membrane lipids and structural proteins. To scavenge harmful excess ROS, plants have evolved antioxidant enzyme system including catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD). In the present study, we found that transcript levels of the antioxidant enzymes increased in all line as a normal response to chilling. However, both transgenic lines showed relatively higher ($p \leq 0.05$) expression level in most of these genes suggesting higher antioxidant activity in order to protect cellular membranes and organelles from damaging effects of ROS. Reflecting to this result, both transgenic fruits also had higher cell membrane integrity compared to WT fruits at BR+8 stage, indicated by lowest ($p \leq 0.05$) MDA content (Figure 8). Song et al. (2014) reported that exogenous PAs in particular Put might enhance chilling tolerance of tomato seedlings by enhancing antioxidant enzyme activities, including SOD, POD, and CAT. Edreva et al. (2007) deduced that PAs may eliminate ROS by acting as direct scavengers or binding to the antioxidant enzymes molecules, and they suggested that conjugated PAs are more efficient as scavengers compared to free PAs. However, the present study did not find any significant difference in the levels of conjugated PAs in BR+8 suggesting a limited role if any for the conjugated PAs. The free PAs are the most significant form in this study indicated by tremendous changes in both BR and BR+8 fruits.

CHAPTER 5. CONCLUSION

My results showed that both transgenic lines exhibited higher tolerance phenotype during chilling at full ripe stage (BR+8), than at the onset of ripening BR stage. I provide evidence for differential function for various PAs in chilled tomato fruits, with limited association with conjugated and bound PAs. The transcriptome analyses with and without the chilling stress showed that a large number of genes regaling various processes were altered in response to high PAs and chilling treatment. Among significant effects by the high PAs were upregulation of genes related to redox homeostasis (Thioredoxin family protein), stress signaling pathway (MAPK cascade), various cold responsive genes, protein chaperon (heat shock proteins) and cellular antioxidant system (CAT, SOD and APX). Based on my results I hypothesize that positively charged PAs molecules bind to DNA, RNA and transcription factors, which in turn regulate gene expression of several stress genes under chilling conditions. MAPK cascade likely serve as a signal transduction pathway by transmitting the stress response from the cell membrane into cell nucleus, which in turn activating TRX family genes that activates antioxidant enzymes system in order to regulate ROS homeostasis. Alternatively, various cold responsive genes may encode a diverse array of proteins, including enzymes involved in respiration and metabolism of carbohydrates, lipids, phenylpropanoids and protein chaperones heat shock proteins. Together these results suggest that chilling tolerance is a complex process and it is likely to involve multiple compounds or genes. The present investigation offers a window into PAs role in chilling stress protection and future research would evaluate the crosstalk of PAs with other compounds

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