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EFFECTS OF ABIOTIC STRESSES ON SORBITOL AND RIBITOL ACCUMULATION AND SORBITOL BIOSYNTHESIS AND METABOLISM IN TOMATO [Solanum lycopersicum L.]

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EFFECTS OF ABIOTIC STRESSES ON SORBITOL AND RIBITOL ACCUMULATION AND SORBITOL BIOSYNTHESIS AND METABOLISM IN TOMATO [Solanum lycopersicum L.]

Dissertation

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food and Environment at the University of Kentucky

By
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2019

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ABSTRACT OF DISSERTATION

EFFECTS OF ABIOTIC STRESSES ON SORBITOL AND RIBITOL ACCUMULATION AND SORBITOL BIOSYNTHESIS AND METABOLISM IN TOMATO [Solanum lycopersicum L.]

Abiotic stresses are responsible for limiting crop production worldwide. Among diverse abiotic stresses, drought and salinity are the most challenging. Plants under these conditions have diverse strategies for tolerating stress. Osmotic adjustment and osmoprotection occur in plants during salinity and drought stress through accumulation of compatible solutes to a high level without interfering with cellular metabolism. Polyols (sugar alcohols) including sorbitol and ribitol are one such class of compatible solutes. Using plants of wild-type (WT) and three genetically-modified lines of tomato (Solanum lycopersicum cv. ‘Ailsa Craig’), an empty vector line ‘TR22’, and 2 sdh anti-sense lines ‘TR45’, and ‘TR49’ designed to severely limit sorbitol metabolism, the objective of this work was to characterize the sorbitol cycle in tomato in response to abiotic stresses. Sorbitol and ribitol content, as well as the enzymatic activities, protein accumulation, and gene expression patterns of the key sorbitol cycle enzymes ALDOSE-6-PHOSPHATE REDUCTASE (A6PR), ALDOSE REDUCTASE (AR), and SORBITOL DEHYDROGENASE (SDH), were measured in mature leaves in response to drought stress by withholding water and by using polyethylene glycol as a root incubation solution to mimic drought stress, to salt stress by incubating roots in NaCl solution, and to incubation of roots in 100 mM sorbitol and ribitol.

A6PR, not previously reported for tomato, and AR both exhibited increased activity correlated to sorbitol accumulation during the drought osmotic, and salt stresses, with SDH also increasing in WT and TR22 to metabolize sorbitol. The level of sorbitol accumulation was considerably lower than that of the common sugars glucose and fructose so was not enough to have a significant impact on tissue osmotic potential but could provide other important osmoprotective effects. Use of the sdh antisense lines indicated that SDH has the key role in sorbitol metabolism in tomato as well as a likely role in ribitol metabolism. Like sorbitol, ribitol also accumulated significantly more in the antisense lines during the stresses. Expression and/or activity of A6PR, AR, and SDH were also induced by the polyols, although it is not clear if the induction was due to a polyol signal, the osmotic effect of the incubation solution, or both. In addition, a unique post-abiotic stress phenotype was observed in the sdh anti-sense lines. After both drought and salt stresses and during a recovery phase after re-watering, the antisense lines failed to recover. This may have been due to their accumulation of ribitol. The sdh anti-sense lines were uniquely sensitive to
ribitol but not sorbitol, with an apparent foliar and seed germination toxicity to ribitol. The determination that sorbitol, and perhaps ribitol as well, plays a role in abiotic responses in tomato provides a cornerstone for future studies examining how they impact tomato tolerance to abiotic stresses, and if their alteration could improve stress tolerance.

KEYWORDS: drought stress, salt stress, aldose-6-phosphate reductase, aldose reductase, sorbitol dehydrogenase

Afaf Almaghamsi

06/14/2019
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CHAPTER 1.

LITERATURE REVIEW

Plants require water, energy from sunlight, carbon from CO$_2$, mineral nutrients from the soil, and adequate temperatures for growth. In the open field, plants are continuously exposed to a complex set of abiotic stresses which result from insufficient or excessive levels of those required inputs, and their responses to these stresses are equally complex (Cramer et al., 2011). The definition of abiotic stresses, such as drought, salinity and low temperature, are environmental conditions which adversely affect plant growth, development, and yield due to low or excessive levels of water, salt, and temperature, respectively. To some extent, a plant is able to tolerate and/or overcome abiotic stresses by complex and dynamic systems involving a wide range of biochemical and physiological processes to perceive and to cope with the deleterious conditions (Vincent et al., 2007; Ahuja et al., 2010; Cramer et al., 2011; Saidi et al., 2011; Walbot et al., 2011).

There is serious concern about sufficient future global production of food from crop plants because of the increasing world population and global climate changes (Rosegrant and Cline, 2003; Lobell et al., 2008; Hussain et al., 2015). Some of the major abiotic stresses affecting crop plants include drought stress and salinity of irrigation water. This is especially true for arid climates around the world, including Middle Eastern countries like Saudi Arabia, where increasing demands for irrigation from scarce groundwater resources that are commonly saline is resulting in increasing soil salinity and fears of reductions in crop yields (Al Tokhais, 2013; Al Naeem, 2015). Drought affects more than 10% of arable land and causes desertification, while soil salinization has increased rapidly on a global scale leading to decreased average yields for many major crops in the affected regions (Machado and Serralheiro, 2017; Zorb et al., 2019).

Many organisms including higher plants synthesize and accumulate soluble compounds in response to abiotic stresses such as salt and drought stress (Stoop et al., 1996; Nuccio et al., 1999; Loescher and Everard, 2000; Sakamoto and Murata, 2002). These compounds are known as compatible solutes due to their ability to accumulate to a high level without interfering with cellular metabolism. One such class of compatible solutes is
polyols (sugar alcohols) including mannitol and sorbitol (Williamson et al., 2002). Polyols are reduced forms of aldose and ketose sugars; they are common in all living organisms, from bacteria to animals (Loescher, 1987; Da Costa et al., 1998; Noiraud et al., 2001). Polyols are important metabolites, which may function as carbon sources, energy sources and/or osmoprotective solutes in plants (Conde et al., 2015). According to Bieleski (1982), more than 30% of all photosynthetically fixed carbon in some non-Rosaceae species is transported as polyols.

Sorbitol is one of the most widespread polyols in plants. It is a major translocated photoassimilate in the phloem of woody Rosaceae species such as apple (Malus domestica) (Loescher et al., 1982), peach (Prunus persica) (Lo Bianco et al., 2000), and pear (Pyrus pyrifolia N.) (Hwa-Young et al., 2007). As a result there are numerous studies on sorbitol biosynthesis, accumulation, and metabolism in these important crop species. However, there is limited information available about sorbitol biosynthesis and accumulation in plant species in which sorbitol is considered a minor secondary product. Sorbitol has been detected, for example, in tomato (Solanum lycopersicum L.) (Schauer et al., 2005; Tari et al., 2010), Arabidopsis thaliana (Nosarzewski et al., 2012; Aguayo et al., 2013), barley (Hordeum spp.) (Chen et al., 2007), the pulp of grape (Vitis vinifera L.) berries (Conde et al., 2015), maize (Zea mays L.) kernels (Shaw et al., 1984), and soybean (Glycine max L.) seed (Kuo et al., 1990).

Exposure to various abiotic stresses induces the accumulation of unwanted and harmful reactive oxygen species (ROS) in plants as a secondary, oxidative stress. These ROS include hydrogen peroxide, hydroxyl radicals, and superoxide anions. Excessive ROS cause oxidative damage to DNA, proteins, and lipids (Sarvajeet et al., 2010). Plant cells combat the accumulation of ROS by the production of compatible solutes including sorbitol which has an ability to scavenge ROS (Smirnoff and Cumbes, 1989) and improve stress tolerance. Many studies have provided evidence that under various stress conditions osmoprotectants, such as glycine betaines, sugars, polyols and polyamines, upregulate antioxidant enzyme activities to reduce adverse effects of oxidative stress (Ashraf and Foolad, 2007; Kubis, 2008; Koyro et al., 2012). It has also been reported that exogenous application of sorbitol improved plant growth in rice (Oryza sativa L.) (Theerakulpisut and
Gunnula, 2012) by promoting the antioxidant defense system against salinity stress. In yeast (*Saccharomyces cerevisiae*), accumulation of glycerol reduced stress-induced excesses of NADH (Ansell *et al.*, 1997). Downregulation of sorbitol biosynthesis and consequently sorbitol levels in apple also downregulated the expression of many stress response genes suggesting that sorbitol plays a role in abiotic and biotic stress tolerance via modulating the expression of stress response genes either directly or indirectly (Wu *et al.*, 2015).

Polyols can play important roles in preventing water loss from cells and in turgor maintenance (Da Costa and Huang, 2006). In addition to its role as a photoassimilate in Rosaceous tree fruits, sorbitol has been reported to have critical roles in osmotic adjustment during drought stress in apple (Wang and Stutte, 1992) and cherry (*Prunus cerasus* L., *P. avium* × *pseudocerasus*) (Ranney *et al.*, 1991). In plant species that do not commonly produce sorbitol, the accumulation of sorbitol in response to an abiotic stress has also been reported. For example, in *Arabidopsis thaliana*, sorbitol levels increased dramatically during drought stress. The accumulation of sorbitol was 30% greater than in non-stressed plants (Nosarzewski *et al.*, 2012). In tomato, salt stress caused a >2-fold increase in leaf sorbitol content above the level of non-stressed plants (Tari *et al.*, 2010). In addition, sorbitol significantly accumulated in the pulp of grape berries in response to water deficits (Conde *et al.*, 2015). In the sucrose- and sorbitol-translocating medicinal species common plantain (*Plantago major* L.), salt stress increased the sorbitol-to-sucrose ratio in the phloem sap (Nadwodnik *et al.*, 2008; Pommerrrenig *et al.*, 2007). Sorbitol plays an important role in osmotic adjustment in developing maize kernels and in soybean seed (Shaw *et al.*, 1984; Kuo *et al.*, 1990). Since sorbitol is not a major translocated photoassimilate and its level is much lower than fructose and glucose in tomato, its contribution to osmotic adjustment may not be its only function. It may have other roles, no less significant than maintaining the osmotic balance, such as mitigating damage caused by ROS and altering gene transcription as mentioned above, preventing membrane injury, and stabilizing proteins and enzymes (Bohnert and Jensen, 1996; Le and McQueen-Mason, 2006; Ashraf and Foolad, 2007).
In mature apple leaves, sorbitol is synthesized from glucose-6-phosphate using NADPH as a cofactor by the aldo-keto reductase ALDOSE 6-PHOSPHATE REDUCTASE (A6PR, EC 1.1.1.200), also known as SORBITOL 6-PHOSPHATE DEHYDROGENASE (Negm and Loescher, 1981; Sengupta et al., 2015;), followed by hydrolysis of the phosphate group via SORBITOL 6-PHOSPHATE PHOSPHATASE (Zhou et al., 2003) (Figure 1). The regulation of sorbitol synthesis via A6PR has been reported in the leaves of apple and other members of the Rosaceae (Kanayama et al., 1995, 2006; Sakanishi et al., 1998) in which sorbitol is the primary photosynthetic product. Several studies have reported that transgenic apple trees with suppressed A6PR gene expression showed reductions in sorbitol accumulation and a shift to increased sucrose content (Kanamaru et al., 2004; Teo et al., 2006). Stressful environmental conditions like low temperature increased A6PR mRNA levels in peach leaves that contributed to increasing sorbitol content (Deguchi et al., 2002). However, information about the role of A6PR in sorbitol biosynthesis in many plants species including tomato is scarce. A6PR was detected in tomato by immunoblot analysis (Mehta et al., 1991), and Ohta et al. (2005) reported detecting A6PR activity but did not provide data. The introduction of the apple A6PR gene into plant species which do not accumulate the polyol such as tobacco (Nicotiana tabacum L.) and persimmon (Diospyros kaki thunb.) increased sorbitol production and enhanced salt tolerance (Tao et al., 1995; Sheveleva et al., 1998; Gao et al., 2001).

ALDOSE REDUCTASE (AR), also an aldo-keto reductase like A6PR, may also play an important role in polyol biosynthesis as a key enzyme leading to the accumulation of sorbitol (Figure 1) in stress situations, correlated with osmotic balance of the cytoplasm and protection of the function of macromolecules in both animals and plant systems (Jeffery et al., 1983; Bartels and Nelson, 1994; Sengupta et al., 2015). Both A6PR and AR use NADPH as a co-factor in plants, animals and bacteria (Garcia-Perez et al., 1989; Bartels et al., 1991; Colrat et al., 1999; Simpson et al. 2009; Sengupta et al., 2015). AR has been reported in non-Rosaceous plants (which translocate sucrose) such as rice, oat (Avena sativa), barley (during a specific stage of embryogenesis where the embryo acquires
Figure 1. Putative metabolic pathway for sorbitol and ribitol in tomato. Abbreviations: SORBITOL-6-PHOSPHATE PHOSPHATASE (SorPP; EC 3.1.3.50); SORBITOL DEHYDROGENASE (SDH; EC 1.1.1.14); RIBITOL DEHYDROGENASE (RDH; EC 1.1.1.56); ALDOSE REDUCTASE (AR; EC 1.1.1.21); ALDOSE-6-PHOSPHATE REDUCTASE (A6PR; EC 1.1.1.200); nicotinamide adenine dinucleotide (NAD⁺); reduced nicotinamide adenine dinucleotide (NADH); nicotinamide adenine dinucleotide phosphate (NADP⁺); reduced nicotinamide adenine dinucleotide phosphate (NADPH). (KEGG Pathway Database, 2019)
desiccation tolerance), and the *Xerophyta viscosa* Baker where AR was enhanced during exposure to water stress, salt stress and abscisic acid (Li and Foley, 1995; Roncarati et al., 1995; Mundree et al., 2000; Sree et al., 2000). During abiotic stresses, a 20% increase in sorbitol level was associated with increased activity of AR in tomato (Tari et al., 2010). The activity of AR under osmotic stress using polyethylene glycol (PEG), salt stress, and ABA treatment in rice and other cereals increased and was correlated with sorbitol accumulation (Sree et al., 2000). In addition, high expression of AR was associated with accumulation of sorbitol in barley embryos during development (Bartels et al., 1991). AR gene expression increased in bromegrass (*Bromus inermis* Leyss) under low temperature stress and ABA (Stephen and Chen, 1993). AR gene expression was also enhanced under high temperature, drought, heavy metals and UV-B in *Digitalis* spp. and alfalfa (*Medicago sativa* L.) (Gavidia et al., 2002; Hideg et al., 2003). A unique aldo-keto reductase was identified in peach, which showed increased expression along with sorbitol accumulation in response to abiotic stresses (Kanayama et al., 2014).

**SORBITOL DEHYDROGENASE (SDH, EC 1.1.1.14)** is the key enzyme in sorbitol metabolism, catalyzing the oxidation of sorbitol to fructose using NAD\(^+\) as a cofactor (Figure 1), or the opposite reaction, reduction of fructose to sorbitol using NADH (Loescher, 1987). SDH can oxidize several other polyols such as ribitol and xylitol (Aguayo et al., 2015). SDH activity has been identified in Rosaceae species like plum (*Prunus salicina*) (Guo et al., 2012) and apple (Yamada et al., 1998; Park et al., 2002; Nosarzewski et al., 2004; Wang et al., 2009). SDH activity has also been identified in nonsorbitol translocating species including maize (Poaceae family), soybean (Fabaceae family), tomato (Solanaceae family) and *Arabidopsis thaliana* (Brassicaceae family) (Doehlert, 1987; Kuo et al., 1990; Ohta et al., 2005; Nosarzewski et al., 2012; Aguayo et al., 2013). The importance of SDH in sorbitol metabolism in apple was demonstrated by transcript analysis of *SDH* cDNA (Yamada et al., 1998, 1999; Park et al., 2002), where SDH expression was detected in all apple tissues. SDH also plays an important role in sorbitol metabolism during drought stress in peach (Lo Bianco et al., 2000). In *Arabidopsis thaliana* leaves, SDH activity was detected prior to stress, while drought stress increased the level 2-fold more than control levels (Nosarzewski et al., 2012).
Ribitol (C₅H₁₂O₅) is a sugar alcohol not currently considered as having an important function in most plant species. According to Kegg’s plant metabolic pathway database (www.genome.jp/kegg/pathway), ribitol is a product of riboflavin breakdown and is converted to D-ribulose by RIBITOL DEHYDROGENASE (RDH) (Figure 1). Ribitol conversion to ribulose by NAD⁺-dependent RDH has been reported for bacteria (Adachi et al., 2001) but not for plants. Riboflavin may be photoxidised to ribitol and lumichrome in etiolated seedlings (Treadwell and Metzler, 1972). Green plants may also be capable of enzymatic hydrolysis of riboflavin to ribitol and lumichrome (Kumar and Vaidyanathan, 1964). Ribulose can be phosphorylated by RIBULOKINASE to ribulose-5-phosphate, and two molecules of ribulose-5-phosphate plus one molecule of guanosine triphosphate (GTP) make one molecule of riboflavin (Fischer et al., 2006). SDH was reported to play a role in ribitol metabolism in Arabidopsis (Nosarzewski et al., 2012). A 10-fold increase in ribitol content in Arabidopsis knockout mutants lacking functional SDH during drought stress was observed, with little change in ribitol content of wild type plants in the same conditions (Nosarzewski et al., 2012). Sorbitol and ribitol were oxidized by recombinant Arabidopsis SDH (Aguayo et al., 2013, 2015). Also, sheep (Ovis aries) liver SDH catalyzed the interconversion of ribitol to D-ribulose (Lindstad et al., 1998), and tomato SDH was capable of metabolizing ribitol as well (Ohta et al., 2005) at lower rates than sorbitol, as was also observed with Arabidopsis SDH (Nosarzewski et al., 2012). Thus, Nosarzewski et al. (2012) suggested that SDH may be the functional RDH in plants, the first step in cycling ribitol back to riboflavin.

Nosarzewski et al. (2012) also reported that the Arabidopsis sdh knockout mutants exhibited a unique post-drought stress phenotype, dying after re-watering while wild type plants resumed growth. The knockout mutants were also uniquely sensitive to ribitol, which was inhibitory and/or toxic to germinating seed, while sorbitol had no effect. Thus, it was hypothesized that the excessive accumulation of ribitol in the Arabidopsis knockouts was responsible for the plant death. This response has not been observed in any other species to date.

Sugars may modulate gene expression in plants, enhancing or repressing expression of some genes, while some may be minimally affected (Koch, 1996; Iido et al., 2004). SDH
protein level and activity in apple fruit tissue was induced by sorbitol treatment while treatment with the SDH product fructose decreased it (Archbold, 1999; Iida et al., 2004). On the other hand, a reduction of the sorbitol supply to girdled apple fruit (Archbold, 1999; Beruter et al., 1997) or by suppression of A6PR expression in source leaves (Zhou et al., 2006), reduced SDH activity. These findings supported the hypothesis that sorbitol (and other polyols) may function as a signal molecule in the utilization of soluble carbohydrates. It is unknown if A6PR and AR expression and/or activities are induced in a similar manner.

Tomato is one of the most widely planted crops in the world (FAO, 2017). As a result, it is grown in diverse climates and production systems and is exposed to numerous abiotic stresses which can detrimentally impact yield. With reduced water supplies or irrigation frequency, tomato yields decline (Kirda et al., 2004; Nangare et al., 2016). Sorbitol accumulation during abiotic stress in tomato was observed by Tari et al. (2010), but accumulation of ribitol has not been reported in tomato as a response to abiotic stresses. Ohta et al. (2005) created tomato plants with an SDH anti-sense construct, significantly reducing SDH activity, thus altering sorbitol metabolism. Given the possible role(s) for sorbitol in tomato stress responses, the antisense SDH plants provide a unique opportunity to compare tomato sorbitol accumulation, biosynthesis, and metabolism, and ribitol accumulation, during abiotic stresses similar to prior studies of sdh knockout mutants of Arabidopsis (Nosarzewski et al., 2012).

Objectives

The first objective of this work was to characterize the sorbitol cycle in tomato by quantifying sorbitol and ribitol content as well as the enzymatic activities, protein accumulation, and gene expression patterns of the key sorbitol cycle enzymes AR, A6PR, and SDH in mature leaves of wild type, empty vector, and sdh anti-sense tomato plants in response to abiotic stresses, including drought stress by withholding water, using PEG as an osmotic root zone solution to mimic drought stress, salt stress by watering with NaCl solution, and root incubation in polyols (sorbitol and ribitol). The second objective was to determine if SDH is the functional RDH in plants as proposed by Nosarzewski et al. (2012) by measuring ribitol content of (sdh) anti-sense tomato plants. The third objective was to determine if there were phenotypic differences between the sdh antisense tomato plants and
wild type and control plants under drought stress and after re-watering. Based on the studies cited above, the sdh antisense plants should exhibit an increased accumulation of sorbitol, higher activities of A6PR and/or AR, and a lower activity of SDH than WT plants during abiotic stress. If SDH is critical to ribitol metabolism in tomato, ribitol should accumulate along with sorbitol in the antisense plants. Finally, if an excess ribitol level is toxic and sdh tomato plants behave similar to sdh Arabidopsis mutants, then tomato sdh antisense plants should exhibit a phenotype different from the control groups following severe drought and re-watering, with growth slowly resuming or plant death upon relief from the abiotic stress.
CHAPTER 2.

Effects of Water Deficit and Osmotic Stress on Sorbitol and Ribitol Accumulation and Sorbitol Biosynthesis and Metabolism in Tomato [Solanum lycopersicum L.]

2.1 Introduction

Tomato is one of the most widely planted crops in the world (FAO, 2017). As a result, it is grown in diverse climates and production systems and is exposed to numerous abiotic stresses which can detrimentally impact yield. With reduced water availability or irrigation frequency creating moderate drought stress, tomato yields decline (Kirda et al., 2004; Nangare et al., 2016). Many organisms including higher plants synthesize and accumulate soluble compounds in response to abiotic stresses such as salt and drought stress (Stoop et al., 1996; Nuccio et al., 1999; Loesch and Everard, 2000; Sakamoto and Murata, 2002). These compounds are known as compatible solutes, due to their ability to accumulate to a high level without interfering with cellular metabolism. One such class of compatible solutes is polyols (Williamson et al., 2002). Polyols, often called sugar alcohols, are reduced forms of aldose and ketose sugars (Bieleski, 1982; Noiraud et al., 2001). They are common in many organisms including bacteria, yeast, marine algae, higher plants, and animals (Da Costa et al., 1998). The most frequently found polyols in plants are sorbitol, mannitol, xylitol, and myo-inositol (Bieleski, 1982) which can act as compatible solutes during periods of abiotic stress and help maintain osmotic balance and provide osmoprotection within plant cells (Mechri et al., 2015). Plants under water stress generate secondary stresses called oxidative stresses which are caused by an excessive concentration of reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radicals, and superoxide anions. Excessive ROS cause oxidative damage to DNA, proteins, and lipids (Williamson et al., 2002; Gill et al., 2010). Plants cells have complex defense mechanisms to combat the accumulation of ROS, including the accumulation of compatible solutes such as polyols which have the osmoprotective ability to scavenge ROS (Smirnoff and Cumbes, 1989).

Sorbitol is unique in that it is also the main translocated photoassimilate in important crop species in the Rosaceae family like apple (Malus domestica Borkh.), pear (Pyrus spp.), and peach (Prunus persica) (Zimmermann et al., 1975; Wallaart, 1980; Moing
et al., 1992; Nadwodnik et al., 2008). Sorbitol levels may rise dramatically in some species during water stress conditions. Among the Rosaceae, sorbitol has been reported to have critical roles in osmotic adjustment during drought stress, such as in apple (Wang and Stutte, 1992) and cherry (Prunus cerasus L., P. avium x pseudocerasus) (Ranney et al., 1991). In non-Rosaceae plant species, where sorbitol is a minor secondary compound, drought stress has also increased sorbitol accumulation. Water stress enhanced the accumulation of sorbitol to more than 30% above non-stressed levels in Arabidopsis thaliana plants (Nosarzewski et al., 2012), and also increased it in barley (Hordeum spp.) (Chen et al., 2007), and in the pulp of grape (Vitis vinifera L.) berries (Conde et al., 2015). Although water stress-induced sorbitol accumulation in tomato (Solanum lycopersicum L.) has not been reported, salt stress caused a >2-fold increase in leaf sorbitol content above the level of non-stressed plants (Tari et al., 2010).

Sorbitol can be synthesized from glucose-6-phosphate by ALDOSE-6-PHOSPHATE REDUCTASE (A6PR, EC 1.1.1.200) (Deguchi et al., 2002; Figueroa and Iglesias, 2010; Borisa et al., 2017), also called SORBITOL-6-PHOSPHATE DEHYDROGENASE, which reduces glucose-6-phosphate to sorbitol-6-phosphate (Hirai, 1981; Loescher, 1987). Then, the phosphate group is hydrolyzed by SORBITOL-6-PHOSPHATE PHOSPHATASE catalyzing the last step in sorbitol biosynthesis (Zhou et al., 2003). Sorbitol can also be synthesized from glucose by ALDOSE REDUCTASE (AR, EC 1.1.1.21) (Tari et al., 2010). Both enzymes use NADPH as a cofactor and are considered key enzymes of the polyol pathway leading to the accumulation of sorbitol in plants (Sree et al., 2000; Tari et al., 2010).

A6PR has been purified and characterized from many species, including apple, loquat (Eriobotrya japonica), cherry (Prunus spp.), and pear (Pyrus pyrifolia N.) (Hirai, 1981; Negm and Loescher, 1981; Kanayama, 1993; Esteban et al., 2002; Hwa-Young et al., 2007). It is considered to play the primary role in sorbitol biosynthesis in apple, pear and other members of the Rosaceae family. Extensive molecular analysis of the promoter region of the A6PR gene in apple showed that the gene responds to various abiotic stresses including low temperature and salt stress (Kanayama et al., 2006; Liang et al., 2012).

In plants, there is a growing body of information about AR and its possible role in stress responses (Sengupta et al., 2015). AR was reported to reduce a range of substrates in
addition to aldoses, including aliphatic and aromatic aldehydes, to their corresponding alcohols (Negm, 1986). AR protein was observed as a drought-responsive protein in barley embryos and showed sequence similarities to human AR (Bartels et al., 1991). AR accumulated during a specific stage of embryogenesis in barley, when embryos need drought tolerance (Bartels et al., 1991). Accumulation of AR protein in bromegrass (*Bromus inermis* Lyess) was noted under low temperatures and desiccation stress (Stephen and Chen, 1993). AR activity in rice (*Oryza sativa* L.) significantly increased over 40% in shoots exposed to polyethylene glycol solution-induced osmotic stress (Sree et al., 2000). According to Tari et al. (2010), AR increased in tomato leaves under salt stress and that was followed by a 20% increase in sorbitol content. A6PR was not studied as a possible source of sorbitol in these studies.

Sorbitol is metabolized to fructose by the enzyme SORBITOL DEHYDROGENASE (SDH), and has been reported from Rosaceae species (apple, peach, pear, cherry) as well as Arabidopsis, maize (*Zea mays* L.), strawberry (*Fragaria x ananassa* Duch.), and tomato (Nosarzewski et al., 2004, 2012; Ohta et al., 2005; de Sousa et al., 2008; Sutsawat et al., 2008). SDH also plays an important role during drought stress in peach (Lo Bianco et al., 2000) as well as in Arabidopsis leaves where its level increased 2-fold more than control levels during drought stress (Nosarzewski et al., 2012), lowering sorbitol content after stress was relieved. SDH has also been reported to play a role in ribitol metabolism in Arabidopsis (Nosarzewski et al., 2012). A 10-fold increase in ribitol content in *Arabidopsis thaliana* knockout mutants lacking functional SDH during non-stress and stress conditions was observed, with little change in WT plants in the same conditions. A unique phenotype was also reported for the knockout mutants; they died upon re-watering after drought stress, and seed germination was inhibited by ribitol but not sorbitol, suggesting that ribitol in excess may be toxic. Sorbitol and ribitol were oxidized by recombinant *Arabidopsis thaliana* SDH (Aguayo et al., 2013; Aguayo et al., 2015). Thus, SDH may be the functional RDH in plants. The ribitol content of most plant species has not been reported.

Ohta et al. (2005) reported that the *SDH* gene exists in the tomato genome as a single copy and created sorbitol dehydrogenase (*sdh*) antisense tomato plants. The transformed plants had SDH activity approximately 50% lower than that of the wild type average. In
addition to the unknown role that sorbitol and SDH may play during abiotic stress in tomato, accumulation of ribitol has not been reported in tomato as a response. The objective of the present study was to characterize the sorbitol cycle in tomato by quantifying sorbitol content as well as the enzymatic activities of the key sorbitol cycle enzymes, AR, A6PR, and SDH, in mature leaves of wild type (WT), empty vector, and of sorbitol dehydrogenase (sdh) antisense tomato lines in response to drought stress by withholding water and by using PEG as an osmotic incubation solution to mimic drought stress, to determine if ribitol accumulated in response to drought stress, and to determine if the sdh antisense plants have a unique phenotype after drought stress and re-watering.

2.2. Materials and Methods

2.2.1 Plant material

Plants of wild-type (WT) and three genetically modified lines of tomato (Solanum lycopersicum cv. ‘Ailsa Craig’), an empty vector line ‘TR22’, and 2 sdh antisense lines ‘TR45’ and ‘TR49’ (a gift from Dr. Yoshinori Kanayama, Tohoku University, Japan), were used. The sdh anti-sense lines were selected in preliminary assays as ones with a WT level of activity (TR22) and the lowest SDH activity (TR45, TR49) (Appendix 1, Figure A.1). One seed of each was germinated and grown in each container (15 cm diameter, 14 cm depth) in PROMIX growing media (Premier Horticulture Inc., Quakertown, PA) in a growth room under fluorescent and incandescent lights (37 µmol·m²·s⁻¹) on shelves at 21 ± 2 °C, or under natural lighting (180 µmol·m²·s⁻¹) in a greenhouse at 22 ± 4 °C (location indicated for each experiment below). The seed/plants were watered to runoff every 3 d and received periodic fertilization and pest management treatment as needed. For studies with rooted plants, stem pieces with 2-4 leaves were excised from stock plants of each genotype on the same day and rooted in water for up to 14 d, until adventitious roots were abundant.

2.2.2 Drought stress by withholding water

Twelve replicate containers with 4 plants each, a WT, TR22, TR45, and TR49 plant, were grown in the greenhouse and watered to runoff every 3 d for 2 weeks prior to withholding water. To impose drought stress, six replicate containers of plants with 2-4
leaves each were subjected to drought stress by withholding water until incipient wilting was observed after about 10 d. Six control containers continued to be watered regularly. On day 10, plants in three control and three drought–stressed containers were sampled. For assessing post-stress recovery, three drought-stressed containers were re-watered to runoff. Plants from three re-watered and 3 control containers were sampled after 24 h in one experiment or 72 h in a separate experiment. On the sampling day, leaves from each plant in the replicate containers were collected, immediately frozen in liquid nitrogen, and stored at -80 °C for subsequent analyses.

2.2.3 Drought stress using polyethylene glycol (PEG)

Three replicate rooted cuttings with 2-4 leaves per plant of each line, WT, TR22, TR45 and TR49, were grown in the growth room for 7 d in water or in 25 mM PEG 6000 solutions (-0.15 MPa) in 50 mL tubes, chosen through preliminary studies which allowed stress to develop slowly with no visible injury. Treated and control plants were sampled after 7 d. Leaves from each plant in the replicate tubes were collected, immediately frozen in liquid N₂, and stored at -80 °C for subsequent analyses.

2.2.4 Tissue analyses

2.2.4.1 Sorbitol, ribitol, fructose, and glucose quantification

Following procedures of Nosarzewski et al. (2012) for measurement of sorbitol, ribitol, fructose, and glucose content of leaf tissues, frozen tissues were lyophilized, 0.1 g of dry tissue was pulverised in a mortar and pestle, and 2-deoxy-D-glucose was added as an internal standard. The tissue was extracted with 1 mL of 80 % ethanol 3 times. Each time the sample solution was heated for 30 min at 80 °C in a closed Eppendorf vial, centrifuged at 2000 x g for 10 min, and the supernatant pipetted off. The supernatants were combined, evaporated to dryness under N₂, and the dried residues re-dissolved in 1 mL Millipore-purified water. A 300 µL aliquot of each sample was dried under N₂, mixed with 100 µL hydroxylamine solution (50 mg hydroxylamine dissolved in 1 mL pyridine), and heated in a sealed glass vial for 30 min at 80 °C. After cooling the samples, 100 µL of N,O(trimethylsilyl)trifluoroacetamide (BSTFA) (Thermo Fisher Scientific, Waltham, MA) was added, and the solution heated at 80 °C for 10 min. The samples were then ready for analysis. Ribitol, sorbitol, fructose and glucose were determined using a HewlettPackard
5890 II gas chromatograph (Agilent, Santa Clara, CA), equipped with a 60 m X 0.32 mm DB-5 column with a 1 µm film thickness (J & W Scientific, Folsom, CA) and a flame ionization detector. The operating conditions were 210 to 270 °C at 2.5 °C per min, then held at 270 °C for 20 min. Injector and detector temperatures were held at 270 °C. Helium was used as the carrier gas at a linear flow rate of 30 cm·s⁻¹. Based on peak areas of each compound at the same retention times as the respective standards, quantitative values were derived from the areas relative to the area of the 2-deoxy-D-glucose internal standard, and tissue concentrations were calculated.

2.2.4.2 SORBITOL DEHYDROGENASE activity assays

The SDH enzyme was assayed as described by Nosarzewski et al. (2004). Frozen leaf tissue (~0.4 g) was ground in 3 mL of 0.5 M Tris-HCl (pH 7), containing 0.2% (w/v) ascorbic acid, 0.1% (v/v) Triton X100 (Sigma, St. Louis, MO) and 1% (w/v) polyvinylpolypyrrolidone (PVPP). After centrifugation at 20000 x g for 20 min, the supernatant was desalted on a Sephadex G-50 column (Sigma). The SDH activity assay mixture contained 0.45 mL of desalted extract, 0.8 mL of 30 mM Tris-HCl (pH 9.6), 1 mM nicotinamide adenine dinucleotide (NAD⁺), and 235 mM sorbitol. The enzyme activity was followed by the reduction of NAD⁺ at 340 nm. All assays were run at 21 °C. The protein content of the Sephadex-purified extracts was determined spectrophotometrically at 595 nm using the Bradford Assay (Bradford, 1976). Enzyme activity was calculated as nmol NADH per mg protein per min.

2.2.4.3 ALDOSE REDUCTASE activity assays

AR (EC 1.1.1.21) was extracted from frozen tissue by reported methods (Sree et al., 2000; Tari et al., 2010). One g of frozen tissue was ground in liquid N₂ and extracted with 10 mL of extraction buffer containing 20 mM potassium phosphate buffer at pH 7.0, 5 mM β-mercaptoethanol and 0.5 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 20000 x g for 30 min, the supernatant was desalted on a Sephadex G-50 column. The purified extract was used for enzyme activity. The AR activity was determined in a total volume of 1 mL. The reaction mixture contained 0.05 mL of 20 mM D, L-glyceraldehyde, 0.05 mL of 2 mM NADPH and 0.025 mL of enzyme extract in 0.875 mL
of 0.135 M potassium phosphate buffer (pH 7.4). The enzyme activity was followed by the oxidation of NADPH at 340 nm. All assays were run at 21 °C. The protein content of the Sephadex-purified extracts was determined spectrophotometrically at 595 nm using the Bradford Assay (Bradford, 1976). Enzyme activity was calculated as nmol NADPH per mg protein per min.

2.2.4.4 ALDOSE-6-PHOSPHATE REDUCTASE activity assays

A6PR (EC 1.1.1.200) activity was extracted and assayed by methods described by (Lo Bianco et al., 2000). Frozen leaf tissue (1 g) was ground with a pre-cooled mortar and pestle in 10 mL of extract buffer (0.2 M HEPES-NaOH at pH 7.5, containing 10 mM dithiothreitol (DTT), 3 mM Mg-acetate, 6% (v/v) glycerol, 0.1% (v/v) Tween 20 and 1% (w/v) PVPP) which was added during grinding. The supernatant was filtered and centrifuged at 20000 x g for 20 min and desalted using Sephadex G-25 (medium) columns. A6PR was assayed using 0.05 mL of desalted extract, 0.1 M Tris buffer (pH 9), 0.11 mM NADPH, and 50 mM glucose-6-phosphate in 1 mL final volume. The enzyme activity was followed by the oxidation of NADPH at 340 nm. All assays were run at 21 °C. The protein content of the Sephadex-purified extracts was determined spectrophotometrically at 595 nm using the Bradford Assay (Bradford, 1976). Enzyme activity was calculated as nmol NADPH per mg protein per min.

2.2.4.5 Real-time PCR analysis of SDH and A6PR expression

To determine the expression of SDH and A6PR genes, procedures based on Nosarzewski et al. (2012) were used. Total RNA was isolated from frozen leaf tissues using an RNeasy plant Kit (Qiagen, Germantown, MD) and DNase treated (RNase-Free DNase set, Qiagen, Germantown, MD). The concentration of RNA isolated from the above tissue was measured using a Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000 and 2000c). The first strand cDNA for real-time PCR analysis was synthesized with oligo(dT)20 primer using 2 μg of total RNA in a final volume of 20 μL, containing 1 μL 50 μM oligo and 1 μL 10 mM dNTP. The total RNA mix was heated at 65°C for 5 min and then subjected
to a reverse transcription reaction by adding the following components: 4 μL 5× RT Buffer, 1 μL 100 mM DTT and 1 μL SUPERSCRIPT III reverse transcriptase (Invitrogen, Germany) at 50-55°C for 10 min. The reaction was inactivated at 80°C for 10 min and treated with RNase cocktail (Ambion; 37°C, 20 min).

Real-time PCR was performed with Bio-Rad iQ5 on 1 μL of first-strand cDNA, using iQ™ SYBR® Green Supermix, SDH and A6PR gene-specific primers, and TIP41 like – protein (TARGET OF RAPAMYCIN (TOR) PROTEIN KINASE SIGNALING PATHWAY REGULATOR-LIKE 41) primers which was selected as the endogenous control for control of the quantity of total RNA present in the sample (Expósito-Rodríguez et al., 2008). SDH, A6PR and control TIP41 like – protein gene-specific intron spanning primers were used. Nine replicate real-time PCR runs were performed with each sample of each gene. The RT-qPCR was performed under the following conditions: an initial denaturation step for 20 sec at 95 °C, followed by 40 cycles of amplification with 5 sec of denaturation at 95 °C, 30 sec of annealing and extension at 60 °C. The melt curve was obtained by heating the amplicon with 95 °C for 15 sec followed by +0.3°C increments from 60°C for 1 min to 95 °C for 15 sec. Relative Gene Expression was calculated by the 2(-Delta Delta C(T)) Method (Livak and Schmittgen, 2001).

The sequences of primer pairs used were:

SDH 5’- GGATGAAGGCTGTCGGTATTT -3’ and 5’-GCACATTCATGCCCAATCAC -3’,
A6PR 5’- AGACTACTTGGCACGGAATG -3’ and 5’- GCAATCTCGGGTGAGAAAGA -3’,
and TIP41 5’-CACGGTTTGGAGATCGAGTG-3’and 5’- CCATCTCCGGAAGTGAGTT -3’.

2.2.4.6 Western blot analysis

SDH protein in tomato leaves was detected using apple SDH purified antibody according to the protocol described in Nosarzewski et al. (2004). A6PR protein in tomato leaves was detected by A6PR apple antibody (a gift from Dr. Yoshinori Kanayama, Tohoku University, Japan) and a goat anti-mouse secondary antibody using the Bio-Rad Opti-4CN Detection kit according to protocol (Laemmli, 1970; Richardson et al., 2008; Emilie et al., 2011). Western blots were obtained after transferring protein from 12.5% SDS-PAGE gels,
run with 10 μg of apple and tomato protein per lane, to nitrocellulose membranes and were treated with a wash buffer (0.05% w/v Tween 20, 0.1% w/v BSA in TBS). Blots were incubated with blocking buffer (ImmunoPure ABC Phosphatase Staining Kit, Thermo Fisher Scientific, Waltham, MA) for 1 h and later incubated with primary SDH antibody (Nosarzewski et al., 2004) or primary apple A6PR antibody. Blots were washed in the same manner and exposed to the secondary antibody according to the ImmunoPure ABC Phosphatase Staining Kit and the Bio-Rad Opti-4CN Detection Kit (Hercules, CA) manufacturer protocols for detecting SDH and A6PR, respectively. Blots were also stained with Coomassie Blue Stain (SimplyBlue™ SafeStain, Thermo Fisher Scientific) to visualize equal protein loading.

2.2.4.7 Detection of SDH, A6PR and AR DNA in tomato leaf tissue

AR, SDH and A6PR gene presence were each detected in tomato leaves by PCR using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and the following AR, SDH and A6PR specific primers: AR: forward (5’-acattaaagtgtttctatggga-a-3’), reverse (5’-tcggaaaccaaaataaatgt-t-3’); SDH: forward (5’-tggegtgaagtcagatggg-3’), reverse (5’-cacttagttggttgctgc -3’); and, A6PR: forward (5’-ttgtgaatcagacatggc-3’), reverse (5’-ttcgagttcaccaaaagac-3’). The gene specific primers were designed to include intron sequences in amplified fragments. At an annealing temperature of 47 °C for 35 PCR cycles, amplicon sizes obtained during the PCR reaction were AR 400 bp, SDH 543 bp, and A6PR 411 bp, and were visualized on 1% agarose gel ethidium bromide.

2.2.5 Differentiation between A6PR and AR

2.2.5.1 Sequence alignment analysis

Using protein sequences of AR (EC 1.1.1.21) and A6PR (EC 1.1.1.200) enzymes in tomato from the NCBI database (https://www.ncbi.nlm.nih.gov/gene/), sequences were aligned and compared using the NCBI alignment program (Global Alignment).
2.2.5.2 AR inhibitor

To distinguish between AR and A6PR activities separately, the potent AR-specific inhibitor sorbinil was used in assays of AR and A6PR. Sorbinil was added to the assay mixtures described above to a final concentration of 1 mM (Bohren et al., 2000; Song et al., 2017). Activities of each enzyme with and without added sorbinil were compared.

2.2.6. A6PR, AR and SDH gene promoter region motifs

Promoter regions of A6PR and AR genes in tomato were analyzed to ascertain the presence of abiotic stress transcription factor binding sites (motifs) using Genomatix software (Intrexon Bioinformatics Germany GmbH, Germany).

2.2.7 Statistical analysis

Two-way (Genotype by Treatment (GxT)) analysis of variance (ANOVA) was performed using Sigmaplot 13 (Systat Software, San Jose, CA, USA). Differences were considered significant if $P \leq 0.05$. Because the GxT interaction was significant in most analyses (Appendix 1, Tables 1..1-1..3), GxT means were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. All data presented are means ± SE. RT-PCR results were statistically analyzed by using t-test and two-way ANOVA at $P \leq 0.05$.

2.3 Results

2.3.1 Carbohydrate content and enzyme activities during drought stress by withholding water

Sorbitol was detected in all genotypes in control conditions, and drought stress resulted in accumulation of sorbitol in all genotypes (Fig. 2.1A). Sorbitol content under control conditions in the antisense lines was greater than in WT and TR22. During drought stress, sorbitol concentration increased significantly in all genotypes but was higher in TR22, TR45 and TR49 than in WT. The concentration of sorbitol one day after re-watering declined in WT and TR22 compared to its level during drought stress, while in TR45 and TR49 there was no change in sorbitol content from the levels during drought stress.
Figure 2. 1. Leaf sorbitol (A), ribitol (B), fructose (C) and glucose (D) concentrations after 10 d of withholding water and then re-watering for 24 h. The experiment was performed in the greenhouse. WT= wild type, TR22=empty vector, and TR45 and TR49=sdh antisense plants. NC= control, DS= drought stress, RW= re-watering. Means (n=3) were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. 
Ribitol was also detected in all genotypes under control conditions, and, like sorbitol, its level in TR49 and TR45 was significantly higher than in WT and TR22 (Fig. 2.1B). Ribitol content was 10-fold lower than sorbitol content, however. Drought stress significantly increased ribitol accumulation in all genotypes, but its level in TR45 and TR49 was 2-3 times higher than in WT and TR22. One day after re-watering, ribitol content decreased in TR22 and TR49 but there was no change in WT or TR45. Fructose accumulated significantly in TR22 and TR45 during drought stress, but not in WT and TR49 (Fig. 2.1C). Glucose increased significantly in WT and TR22 under drought treatment but did not change in the \textit{sdh} antisense lines TR45 and TR49 (Fig. 2.1D). Twenty-four hours after re-watering, glucose declined in WT and TR22, but did not change in the antisense lines. In control and drought-stressed conditions overall, leaf fructose and glucose contents were substantially greater than sorbitol or ribitol content.

SDH activity under well-watered conditions was detectable in all genotypes but its level in WT was 3-fold or more than in the other genotypes (Fig. 2.2A). SDH activity was detected in the antisense lines, albeit very low. Drought stress significantly increased SDH activity in WT and TR22, while its activity in TR45 and TR49 did not change. One day after re-watering, SDH activity was significantly less in WT and TR22 compared to its activity during drought stress, but TR22 activity was still greater than the pre-stress level. SDH activity in TR45 and TR49 one day after re-watering did not change. In well-watered conditions, A6PR and AR activities were detected in all genotypes but their levels in TR45 were higher than in the other genotypes (Fig. 2.2B,C). A6PR and AR activity under drought stress significantly increased in WT, TR45 and TR49 but not TR22.

A unique phenotype after drought stress and re-watering was observed for the antisense lines (Figure 2.3). Although the WT and empty vector genotypes recovered and resumed growing when re-watered, TR49 did not recover and senesced and TR45 slowly recovered after 2 weeks of re-watering, but never reached full recovery or resumed normal growth.

In the drought stress study with re-watering after 3 d, sorbitol was detected in all genotypes in pre-stress conditions, but the level in \textit{sdh} antisense plants (TR45, TR49) was significantly higher than in WT and TR22 (Fig. 2.4A). Drought stress caused a significant
Figure 2. 2. SDH (A), A6PR (B), and AR (C) activities after 10 d of withholding water and then re-watering for 24 h. The experiment was performed in the greenhouse. WT=wild type, TR22= empty vector, and TR45 and TR49=sdh antisense plants. NC=control, DS= drought stress, RW= re-watering. Means (n=3) were separated by Fisher’s Least Significant Difference at \( P \leq 0.05 \).
Figure 2. 3 Unique drought stress phenotype. 1) Well-watered plants; 2) at 17 days of drought stress; 3) at 1 day after re-watering; 4) at 2 days after re-watering. WT= wild type, TR22= empty vector, and TR45 and TR49= sdh antisense plants.
Figure 2.4. Leaf sorbitol (A) and ribitol (B) content after withholding water for 10 d and then re-watering for 72 h in a greenhouse. WT= wild type, TR22= empty vector, and TR45 and TR49= sdh antisense plants. NC= control, DS= drought stress, RW= re-watering. Means (n=3) were separated by Fisher’s Least Significant Difference at P≤0.05.
increase in TR49 and TR45 sorbitol content, but not the other genotypes. Three days after re-watering, leaf sorbitol content of stressed TR45 and TR49 plants was significantly lower than when sampled during stress and the amount of sorbitol in all genotypes was identical. Ribitol content after 10 d of withholding water increased significantly in TR45 and TR49 (Fig. 2.4B), but there was no change in WT and TR22. Three days after re-watering, ribitol content in TR45 and TR49 had significantly declined.

SDH activity under pre-stress conditions was very low and the same in all genotypes (Fig. 2.5A). Drought stress induced SDH activity in WT and TR22 but not in TR45 or TR49. WT and TR22 SDH activities 3 d after re-watering was reduced to the level of well-watered controls. A6PR activity after 10 d of withholding water increased significantly in TR22, TR45, and TR49. The highest level was reached by TR49 which was 66% higher than other genotypes. A6PR activity 3 d after re-watering was reduced to control levels in all genotypes (Fig. 2.5B). AR activity was detectable in all genotypes under control conditions (Fig. 2.5C). Drought stress induced a significant increase in activity of AR in all genotypes, and 3 d after re-watering AR activities were similar to or less than control plants.

2.3.2 Osmotic stress using incubation in PEG

Although sorbitol was detectable in all genotypes prior to osmotic stress (Fig. 2.6A), the content in WT and TR22 was significantly lower than its level in TR45 and TR49. A one-week incubation in 25 mM PEG contributed to a significant increase in sorbitol content in TR45 and TR49 but not in WT and TR22. The sorbitol level was approximately 7 times greater in PEG-treated sdh antisense plants than in PEG-treated WT plants.

Ribitol was also detected in all genotypes prior to osmotic stress at low levels, less than 1 µmol/g DW (Fig. 2.6B). PEG incubation led to a significant increase in ribitol content in sdh antisense plants, 7- to 9-fold more than the pre-stress content, respectively. Ribitol content of WT and TR22 plants did not change with PEG treatment. Under well-watered conditions the activity of SDH in all genotypes was low (Fig. 2.7A). After incubation in 25 mM PEG, SDH activity increased in WT and TR22 more than 3-fold.
Figure 2. 5. SDH (A), A6PR (B), and AR (C) activities after withholding water for 10 d and then re-watering for 72 h in a greenhouse. WT=wild type, TR22=empty vector, and TR45 and TR49=sdh antisense plants. NC=control, DS= drought stress, RW=re-watering. Means (n=3) were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. 
Figure 2. 6. Leaf sorbitol (A) and ribitol (B) concentrations after one-week incubation in 25 mM PEG. WT=wild type, TR22= empty vector, and TR45 and TR49=sdh antisense plants. NC=Control. Means (n=3) were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. 
Figure 2.7. Leaf SDH (A), A6PR (B), and AR (C) activities after 1-week incubation in 25 mM PEG solution. WT=wild type, TR22=empty vector, and TR45 and TR49=sdh antisense plants. NC=Control. Means (n=3) were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. 
compared to its pre-treatment level. SDH activity in TR45 and TR49 after incubation in 25 mM PEG did not change. A6PR activity when well-watered was detectable in WT, TR22, TR45, and TR49, all at almost the same level (Fig. 2.7B). Incubation in PEG caused a significant increase in A6PR activity in all genotypes. The maximum increase of A6PR activity occurred in the antisense plants TR45 and TR49, which were 2-3 times higher than in WT and TR22 plants. AR activity under well-watered conditions in all genotypes was very low without any significant differences among genotypes (Fig. 2.7C), but incubation in 25 mM PEG caused a significant elevation in AR in only the sdh antisense plants TR45 and TR49. The increase in sorbitol content was correlated with the increase in A6PR and AR activities in tomato leaves across all genotypes and treatments (Fig. 2.8).

2.3.3 Expression patterns of A6PR and SDH

Real-time PCR was employed to determine relative expression levels of A6PR and SDH in all genotypes (Fig. 2.9). A6PR transcripts were expressed in the 4 genotypes, but the relative expression of A6PR in the sdh anti-sense plants TR45 and TR49 was significantly higher than WT and empty vector (TR22) plants in stress conditions by 50% or more (Fig. 2.9A, B). Conversely, SDH expression in WT and TR22 was significantly higher than in sdh antisense plants under both conditions (Fig. 2.9C, D).

2.3.4 Identification of A6PR and SDH protein in tomato leaves

The Western blots showed A6PR protein in all genotypes at 34.7kDa (Fig. 2.10.1). TR45 and TR49 appeared to have more A6PR protein accumulation than WT and TR22 under stress conditions. Western blot analyses also revealed an accumulation of SDH protein in WT and TR22 under stress conditions (Fig. 2.10.2). In contrast, SDH protein in sdh antisense plants TR45 and TR49 was not detectable.

2.3.5 Detection of SDH, A6PR and AR DNA from tomato leaf tissue

AR, SDH and A6PR gene presence was detected in WT tomato leaves by PCR using AR, SDH and A6PR specific primers. AR (401 bp), SDH (543 bp) and A6PR (411 bp)
Figure 2.8. Linear regression of A6PR (A,C) and AR (B,D) activities with sorbitol content across all genotypes in control conditions and after 1-week incubation in 25 mM PEG solution (A,B) and after withholding water for 10 days (C,D).
Figure 2.9. Relative expression of *A6PR* (A,B) and *SDH* (C,D) in control (A,C) and drought stress (B,D) conditions, respectively. Control = NC, and drought stress = DS. Means (n=9) were separated by t-test at $P \leq 0.05$. 
Figure 2. Western blots of A6PR protein (1A) and SDH protein (2A) during stress conditions in wild type (WT), empty vector (TR22), and sdh antisense tomato plants (TR45, TR49), protein level loading detection by Coomassie Blue stain (1B,2B), (3) AR, SDH and A6PR genes in wild type tomato leaves were detected using gene specific primers by PCR.
amplicons were obtained during the PCR reaction and were visualized on 1% agarose gel with ethidium bromide (Fig. 2.10.3).

2.3.6 Differentiation between A6PR and AR

2.3.6.1 Protein Sequence Alignment analysis

Protein sequence alignment analysis of A6PR versus AR from tomato indicated the similarity between A6PR and AR was only 35% (Fig. 2.11A), while the identities between tomato A6PR and apple A6PR were 71% (Fig. 2.11B).

2.3.6.2 AR inhibitor

The AR-specific inhibitor sorbinil reduced AR activity by 90% but did not affect the activity of A6PR (Fig. 2.12).

2.3.7 Analysis of promoter region motifs for A6PR, AR and SDH genes

Analysis of the tomato A6PR and AR gene promoter regions showed the presence of the ABA response element AREB-1 and a salt tolerance zinc finger (ZAT10) motif (Fig. 2.13A, B; 2.14A, B). Analysis of the tomato SDH gene promoter showed many motifs such as dehydration responsive element binding factors, plant specific NAC transcription factors, MYB-like proteins, and HD-Zip I proteins (Fig. 2.15A-E), which generally play a crucial role in plant growth, development, and adaption to the environment as responses related to abiotic stress.
Figure 2. 11. Protein sequence alignment analysis of A6PR versus AR in tomato (A) and A6PR in tomato versus A6PR in apple (B). Query=A6PR, Sbjct=AR (A), Query=A6PR in tomato, Sbjct=A6PR in apple.

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  - MA
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  - L SG+K+P +GLG WR + +D + AI + GYRH D AA+

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**Sbjct 59:** YQVQDEVHGKIAAHITG--VERSTFTITLKMDLSDRPMKTLNLQELQLYLDF 117
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**Query 105:** LVHPFWATKHTGQVTTTATASLGDEIGVLDDLSTLEDTHWGMENLSLGLVRSIGISNYDI 164
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- L +++ P+V Q+E HP +++ E +++ C+K+ I VTA+++PLG V

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- I + R A W +

**Sbjct 282:** QACDIDPDQMRLDDGEELFVNKSDGPKYSVADIWDHEI 319

Figure 2. A6PR and AR activities with AR-specific inhibitor sorbinil (+I) and without (−I). Different letters indicate a significant difference due to inhibitor within enzyme by t-test at $P<0.05$. 

A6PR-I  A6PR+I  AR-I  AR+I

nmol NADPH min$^{-1}$ mg$^{-1}$ protein
Figure 2. Tomato A6PR gene promoter region motif analysis by Genomatix. A) Promoter sequences of the A6PR gene with some transcription factor motifs for an ABA response element and a salt tolerance zinc finger element indicated at the 157-173 bp and 359-381 bp positions of the analyzed sequence, respectively. (B) ABA response element sequence logo (C) salt tolerance zinc finger sequence logo. Sequence logo is a graphical representation consisting of stacks of symbols, one stack for each position in the sequence. The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each nucleic acid at that position. (Schneider et al., 1990; Crooks et al., 2004).
Figure 2. Tomato AR gene promoter region motif analysis by Genomatix. (A) 1 and 2 represent the promoter sequences of the AR gene with 4 transcription factor motifs for ABA response elements starting at 938, 939, 916 and 872 bp and one GA- and ABA-responsive zinc finger-like factor starting at 18 bp position of the analyzed sequences. (B1, 2, 3, 4) ABA response elements sequence logos. (C) GA-and ABA-responsive zinc finger like factor sequence logo.
A) Tomato *SDH* gene promoter region

1- Dehydration responsive element binding factors

2- NAC transcription factors
3- MYB-like proteins
4- HD-ZIP class III protein ATHB9

Figure 2. 15. (A) Tomato *SDH* gene promoter region motif analysis by Geomatix showing the promoter sequences of *SDH* gene with several transcription factor motifs: 1) a dehydration responsive element binding factor starting at 10 bp, 2) a plant specific NAC transcription factor sequence starting at 66 bp, 3) a MYB-like protein starting at 85 bp, and 4) an HD-ZIP class III protein starting at 257 bp. Of analyzed sequence B) Dehydration responsive element binding factor sequence logo. C) Plant specific NAC transcription factor sequence logo. D) MYB-like proteins sequence logo. E) HD-ZIP class III protein sequence logo.
2.4 Discussion

The results clearly showed the accumulation of sorbitol in tomato leaf tissue in all genotypes when subjected to drought stress by withholding water and by using PEG to mimic drought stress (Figs. 2.1A, 2.4A, 2.6A). These results confirmed that sorbitol plays a role in the drought stress response in tomato. These results are consistent with similar observations in Arabidopsis, apple, peach, and barley (Bohrent et al., 1995; Wang et al., 1996; Lo Bianco et al., 2000; Šircelj et al., 2007; Chen et al., 2007; Nosarzewski et al., 2012) where sorbitol levels increased under abiotic stress conditions. In particular, TR45 and TR49 sdh antisense plants lacking significant SDH activity accumulated higher levels of sorbitol than WT and TR22 similar to sdh knockout mutants of Arabidopsis (Nosarzewski et al., 2012).

The sorbitol biosynthetic pathway has not yet been fully characterised in *Solanum lycopersicum*, so the source of the accumulated sorbitol was not clear. Tari et al. (2010) suggested that sorbitol accumulation in tomato was due to the activity of AR but they did not study A6PR. Using gene specific primers for PCR analysis (Fig.2.10.3), both AR and A6PR genes were identified in tomato leaf tissue. Increased AR and A6PR activities under drought stress were correlated with the accumulation of sorbitol, so both or either alone could be the source of the sorbitol accumulation (Fig. 2.8). Both are considered as possible biosynthetic sources of sorbitol in other plant species (Hirai, 1981; Bartels et al., 1991; Sree et al., 2000; Zhou et al., 2003). The present results showed: 1) activity of A6PR from tomato leaves, also reported by (Ohta et al., 2005) but without data; 2) that A6PR activity was enhanced by drought stress in all of the genotypes but its activity remained high in sdh antisense plants after re-watering (Figs. 2.2B, 2.5B, 2.7B); 3) the expression of A6PR by using RT-qPCR was induced by stress (Fig. 2.9A.B), and; 4) the presence of A6PR protein in tomato leaves was detected by immunoblot analysis as previously shown by Mehta et al. (1991). The sdh antisense plants TR45 and TR49 had the highest level of A6PR protein (Fig. 2.10.1). These facts support the presence of A6PR in tomato which likely played an important role in sorbitol biosynthesis and accumulation.

According to protein sequence analyses, the similarity between A6PR and AR in tomato is only 35%, while the identity between tomato A6PR and apple A6PR is 71% (Fig.2.11). Since the similarity between A6PR and AR protein is very low, this suggests
that they are two different enzymes with different substrate specificities even though they are both members of the aldo-keto oxidoreductase superfamily. Barley AR was partially purified and characterized as having maximum activity in the 70% ammonium sulfate fraction with glyceraldehyde as substrate (Bartels et al., 1991). However, A6PR from rice showed high specificity towards G6P (Yadav and Prasad, 2014). We confirmed that A6PR and AR are two different enzymes by using the AR-specific inhibitor sorbinil, with the inhibitor reducing AR activity by 90% but not affecting the activity of A6PR (Fig. 2.12).

Plants have large numbers of transcription factors (TFs). These TFs are classified by their DNA-binding domains (Stracke et al., 2001). The TFs influence the transcription of genes and because of that they are involved in regulating various biological functions such as stress signaling, seed maturation, pathogen defense and flower development (Jakoby et al., 2002). Abiotic stresses such as drought and salt stresses have been reported to increase ABA-transcriptional activators that regulate stress-related gene expression in both Arabidopsis and tomato. In tomato, the bZIP transcription factor SIAREB was involved in responses to water deficit and high salinity stresses (Hsieh et al., 2010). Another TF family reported to increase in response to multiple abiotic stresses are zinc finger TFs, which were induced by osmotic stresses such as salt and drought in both Arabidopsis and tomato plants mainly by maintaining photosynthesis and increasing polyamine biosynthesis (Hichri et al., 2014).

Analysis of the tomato A6PR and AR gene promoter regions showed the presence of an ABA response element (AREB-1) which is upregulated by ABA and water stress (Fig. 2.13, 2.14). Additionally, a salt tolerance zinc finger (ZAT10) motif was detected (Fig. 2.13) in A6PR. Both of these motifs were also found in the apple A6PR promoter region (Kanayama et al., 2006; Liang et al., 2012). Analysis of the tomato SDH gene promoter showed many motifs that could be responsive to transcription factors such as plant specific NAC transcription factors, MYB-like proteins, HD-Zip I proteins and a dehydration responsive element binding factor (Fig. 2.15). These could play crucial roles in adaption to the environment as responses related to abiotic stress (Li et al., 1995; Hichri et al., 2014; Lia et al., 2015; Hu et al., 2017; Filichkin et al., 2018).
The sorbitol level in TR45 and TR49 sdh antisense plants under control conditions was at a higher level than WT and TR22 as was observed with Arabidopsis sdh knockout mutants (Nosarzewski et al., 2012). Sorbitol content of WT and TR22 declined during stress recovery after re-watering for 24 h, but there was no change in its levels in TR45 or TR49 (Fig. 2.1A) as also observed in the Arabidopsis sdh knockout mutants (Nosarzewski et al., 2012). Re-watering for 3 d resulted in a reduced sorbitol content in sdh antisense TR45 and TR49 (Fig. 2.4A) which is in contrast to that with the Arabidopsis sdh knockout mutants (Nosarzewski et al., 2012) where the level of sorbitol remained at their drought stress level. This may be due to the presence of a very low yet detectable level of SDH activity in the sdh antisense tomato plants in the present work whereas no activity was detected in the Arabidopsis sdh knockout mutants.

SDH is the key enzyme in sorbitol metabolism, which catalyzes the oxidation of sorbitol to fructose in higher plants (Loescher, 1987), and it has been well-characterized in the Rosaceae family of fruit trees, where sorbitol is the primary translocatable photosynthate. Expressed sequence tags of SDH-like sequences have been found in many non-Rosaceae species which do not synthesize sorbitol as a primary photosynthetic product (Ohta et al., 2005). The SDH gene exists in the tomato genome as a single copy (Ohta et al., 2005). The results clearly confirmed the existence of SDH in tomato by its activity (Figs. 2.2A, 2.5A, 2.7 A), from DNA purification, from its transcript by using RT-qPCR (Fig. 2.9CD), and by the identification of SDH protein in tomato leaves using immunoblots (Fig. 2.10.2). The greatest increase in SDH transcript levels and activity occurred as a result of drought stress, and that was correlated with an increase in leaf sorbitol concentration in WT and TR22, followed by decreased SDH activity 1 and 3 d after re-watering as sorbitol levels in WT and TR22 decreased. SDH activity and SDH transcript levels were low during stress and after stress recovery in sdh antisense lines TR45 and TR49, implying that SDH functions as a sorbitol–metabolizing enzyme where it reduced the sorbitol content during stress recovery even at a low level of activity.

The results also showed the presence of ribitol in tomato leaf tissue (Figs. 2.2B, 2.4B, 2.6B), based on a retention time during GC analyses that matched a ribitol standard. Leaf ribitol content increased when subjected to drought stress by withholding water and
by using PEG in TR45 and TR49 but not in WT and TR22, which is consistent with the observation in Arabidopsis (Nosarzewski et al., 2012) but which has not previously been shown in tomato. The results of this study provided evidence for the potential importance of SDH in ribitol metabolism in a second plant species in addition to Arabidopsis. Ribitol as a possible SDH substrate was suggested from SDH assays with tomato SDH (Ohta et al., 2005) and with drought-stressed sdh knockout mutants in Arabidopsis (Nosarzewski et al., 2012). In the present results, ribitol levels significantly decreased after re-watering (1 and 3 d) reaching the level found in control plants. These results contrast with what was reported in Arabidopsis (Nosarzewski et al., 2012) where the levels of ribitol in sdh plants after re-watering were similar to their level during stress, but are similar to the decline in sorbitol content in the sdh antisense tomato, also likely due to the very low level of SDH activity in the antisense plants. In total, the present results support the suggestion that SDH is also a RIBITOL DEHYDROGENASE in tomato as was suggested with Arabidopsis (Nosarzewski et al., 2012).

The leaf content of sorbitol and ribitol were considerably lower than the other major monosaccharides in tomato leaves, fructose and glucose (Fig. 2.1C.D). Thus, the poyols would have a negligible effect on osmotic adjustment in the leaves. However, they could have antioxidant effects as ROS scavengers and osmoprotectants of DNA, RNA, and cell membranes (Smirnoff and Cumbes, 1989; Beligh et al., 2002; Williamson et al., 2002; Gill et al., 2010). Furthermore, the presence of ribitol implies that riboflavin catabolism occurred during drought stress and that SDH functions to recycle the ribitol component back to a form available for riboflavin biosynthesis. To our knowledge, this is the first study to report on: 1) the presence and activity of A6PR in tomato; and, 2) the presence and stress-related accumulation of ribitol in tomato.

The changes in the sorbitol cycle enzymes and the sorbitol and ribitol content of the antisense genotypes was correlated with a unique phenotype (Fig. 2.3) previously only seen in drought stressed sdh knockout mutants of Arabidopsis (Nosarzewski et al., 2012). Upon re-watering after drought stress, the antisense genotypes failed to recover and resume growth in contrast to the wild type and empty vector genotypes. The Arabidopsis knockouts
were uniquely sensitive to ribitol which may have been toxic, implying the same sensitivity and outcome were occurring in the antisense tomato to create the unique phenotype.

2.5 Conclusion

Drought is the most critical abiotic factor adversely affecting plant growth and limiting crop production. Many mechanisms have been developed by plants to resist drought. This study was performed to determine the ability of tomato plants to biosynthesize and accumulate sorbitol, and to determine if ribitol has any involvement in the stress response. The expression and activities of the key sorbitol cycle enzymes A6PR, not previously reported for tomato, AR, and SDH in tomato changed in response to drought stress and to PEG-induced osmotic stress, which led to sorbitol and ribitol accumulation. This finding suggests an important role for SDH in sorbitol metabolism, and in ribitol catabolism as well.
CHAPTER 3.

Effects of Salt Stress and Incubation in Sorbitol and Ribitol on Sorbitol and Ribitol Accumulation and Sorbitol Biosynthesis and Metabolism in Tomato [Solanum lycopersicum L.]

3.1 Introduction

Salt stress is one of the most critical limiting factors of plant growth and productivity. In some regions of the world, especially arid Middle Eastern countries like Saudi Arabia, increasing demands for irrigation from scarce groundwater resources that are commonly saline is resulting in increasing soil salinity and fears of reductions in crop yields (Al Tokhais, 2013; Al Naeem, 2015). Soil salinity in agricultural soils means the soil has high concentrations of soluble salts, and the high salt is directly toxic and also creates a water deficit or osmotic stress because of the decreased osmotic potential in the soil solution, affecting water and mineral nutrient uptake (Boyer, 1982; Allakhverdiev et al., 2000). Such conditions have adverse effects on plant growth, yield and quality of a wide variety of irrigated vegetable crops including tomato (Solanum lycopersicum L.) (Malash et al., 2008; Machado and Serralheiro, 2017).

Plants have several mechanisms to exclude salt and/or tolerate its presence. High concentrations of Na\(^+\) and Cl\(^-\) (salt) ions negatively affect the absorption of beneficial ions such as K\(^+\) and Ca\(^{2+}\). Excess Na\(^+\) competes with K\(^+\) thereby preventing its uptake which inhibits plant growth and productivity. Keeping in balance cellular K\(^+\) level and the K\(^+\)/Na\(^+\) ratio is the most important factor for salt tolerance, and plants have a number of strategies for restricting Na\(^+\) movement to young meristematic tissues and allowing greater movement of K\(^+\) or keeping it in the more physiologically active tissues (Chakraborty et al., 2018). Tomato cultivars tolerant to salt stress showed a high level of K\(^+\), Ca\(^{2+}\), proline and high-antioxidant enzyme activities, with a lower Na\(^+\) level, than salt-susceptible cultivars (Gharsallah et al., 2016). The Na\(^+\) not stored in the vacuoles of tomato followed the transpirational water flux, leading to higher Na\(^+\) accumulation in mature leaves (Maggio et al., 2007). Plants cells under salt stress produce ionic stress signals, leading to the expression of numbers of genes which in turn produce or activate proteins that prepare plants for salt tolerance (Marco et al. 2015; Zhang et al., 2018). Hence, excess salt is either transported to the vacuole or isolated in older tissues which eventually are sacrificed,
thereby protecting the more actively-growing parts of the plant from salinity stress (Reddy et al., 1992; Zhu, 2003).

Increasing carbohydrate levels are very important for abiotic stress tolerance. The effect of salt stress on increased accumulation of glucose in tomato leaves has been reported (Ghanem et al., 2009). According to Shaba et al. (2010), salinity increased the soluble sugars in leaf and root tissues of tomato. Soluble sugars increased cell membrane tolerance by reducing entry of Na$^+$ and Cl$^-$ ions (Prado et al., 2000). Many organisms including vascular plants also accumulate unique non-toxic solutes, osmoprotectants, and/or compatible solutes in response to salt and drought stress (Stoop et al., 1996; Nuccio et al., 1999; Loescher et al., 2000; Sakamoto et al., 2002) as a mechanism to elevate osmotic pressure and maintain turgor and the gradient for water uptake (Rhodes et al., 1994). One such class of compatible solutes is polyols (or sugar alcohols) which are the reduced form of aldose and ketose sugars. They are enzymatically-produced plant compounds which contribute to abiotic stress tolerance by acting as compatible solutes during abiotic stresses (Chen et al., 2002; Aguayo et al., 2015). Polyols are the dominant forms of translocated carbohydrates in some species, and they are often translocated in association with sugars such as sucrose or raffinose (Williamson et al., 2002). It has been suggested that polyols, because of their water-like hydroxyl groups, might imitate the structure of water and maintain an artificial sphere of hydration around macromolecules to provide protection (Stoop et al., 1996).

Plants under abiotic stress will generate secondary oxidative stresses which are caused by excessive accumulation of reactive oxygen species (ROS) that can cause oxidative damage to DNA, proteins, and lipids (Sarvajeet et al., 2010). Plants cells have complex defense mechanisms to combat the accumulation of ROS; one of the most important mechanisms is the accumulation of compatible solutes including polyols like sorbitol which have an ability to scavenge ROS (Smirnoff and Cumbes, 1989). Sorbitol is a main photosynthetic product and the primary translocated carbohydrate in many members of the Rosaceae family, including apple (Malus x domestica Borkh.) (Loescher et al., 1982), Prunus species such as peach (Prunus persica)(Nii, 1997), and pear (Pyrus pyrifolia) (Hwa-Young et al., 2007). The accumulation of sorbitol in apple leaves (Wang et al. 1996;
Šircelj et al., 2007) and in Malus hupehensis (Meng et al., 2008) has been reported to support osmotic adjustment during salt and drought stress, respectively. Little information is available about sorbitol biosynthesis and accumulation in other plant species where sorbitol is not the main photosynthetic product. For example, sorbitol accumulation has been detected in Arabidopsis thaliana (Nosarzewski et al., 2012), barley (Hordeum vulgare) (Chen et al., 2007), and common plantain (Plantago major) (Breins, et al., 1983; Pommerrenig et al., 2007) in response to drought and/or salt stress. In tomato, sorbitol accumulated in mature leaves of drought- and salt-stressed tomato plants (Chapter 2; Schauer et al., 2005; Tari et al., 2010).

Sorbitol-6-phosphate may be formed from glucose-6-phosphate via the action of ALDOSE-6-PHOSPHATE REDUCTASE (A6PR), also known as SORBITOL-6-PHOSPHATE DEHYDROGENASE (Hirai 1981; Negm et al., 1981; Kanayama et al., 1992). Then, the phosphate group is hydrolyzed by SORBITOL-6-PHOSPHATE PHOSPHATASE catalyzing the last step in sorbitol biosynthesis (Zhou et al., 2003). The expression of the A6PR gene was enhanced by high-salinity stresses in apple (Kanayama et al., 2006). Transforming with the A6PR gene from apple into tobacco (Nicotiana tabacum L.) and Japanese persimmon (Diospyros kaki Thunb. cv Jiro) increased sorbitol production and enhanced salt tolerance (Tao et al., 1995; Sheveleva et al., 1998; Gao et al., 2001). Drought stress significantly increased A6PR activity in tomato leaf tissue (Chapter 2).

Another enzyme capable of sorbitol biosynthesis from glucose is ALDOSE REDUCTASE (AR), which also belongs to the superfamily of aldo-keto reductases (Bartels et al., 1991) and which catalyses the NADPH-dependent reduction of various aldehydes and reactive carbonyl metabolites (Hideg et al., 2003). The activity of AR in rice and other cereals increased during water and salt stress suggesting a role for sugar alcohol accumulation and sorbitol biosynthesis (Sree et al., 2000), but polyols were not quantified. During salt stress, a 20% increase in sorbitol levels was correlated with increased activity of AR in tomato (Tari et al., 2010). We also observed that drought stress induced the activity of AR in tomato (Chapter 2).
Sorbitol is metabolized to fructose by the enzyme SORBITOL DEHYDROGENASE (SDH). SDH can oxidize several other polyols such as ribitol and xylitol (Aguayo et al., 2015). SDH activity has been identified in many members of the Rosaceae family, such as apple (Malus × domestica) (Yamada et al., 1998; Nosarzewski et al., 2004), peach (Prunus persica), and Japanese pear (Pyrus serotina) (Oura et al., 2000). SDH activity has also been reported in non-sorbitol translocating species including soybean (Kuo et al., 1990), maize (Doehlert, 1987), tomato (Ohta et al., 2005), and Arabidopsis thaliana (Nosarzewski et al., 2012; Aguayo et al., 2013). In Arabidopsis thaliana leaves, SDH activity was detected prior to drought stress, and after re-watering its level increased 2-fold more than control levels (Nosarzewski et al., 2012). Our results highlighted a similar increase in SDH of tomato in response to drought stress (Chapter 2). As drought stress increased tomato leaf sorbitol content, SDH activity significantly increased. SDH activity has not been assessed in relation to salt stress in any non-Rosaceae species.

Sugars may modulate gene expression in plants, enhancing or repressing expression of some genes, while some may be minimally affected (Koch, 1996; Iido et al., 2004). SDH protein level and activity in apple fruit tissue was induced by sorbitol treatment while treatment with the SDH product fructose decreased it (Archbold, 1999; Iida et al., 2004). On the other hand, the reduction of sorbitol supply to girdled apple fruit (Beruter et al., 1997; Archbold, 1999) or by suppression of A6PR expression in source leaves (Zhou et al., 2006), reduced SDH activity. These findings supported the hypothesis that sorbitol (and other polyols) may function as a signal molecule in the utilization of soluble carbohydrates. It is unknown if A6PR and AR expression and/or activities are induced in a similar manner.

Ribitol is a five-carbon sugar alcohol not considered to have a crucial functional role in most plant species. A 10-fold increase in ribitol content in Arabidopsis thaliana knockout mutants lacking functional SDH during non-stress and stress conditions was observed, with little change in wild type (WT) plants in the same conditions (Nosarzewski et al., 2012). Sorbitol, ribitol and xylitol were oxidized by recombinant Arabidopsis thaliana SORBITOL DEHYDROGENASE (Aguayo et al., 2013, 2015). In drought-stressed tomato, ribitol content increased, but the level in sdh antisense plants was much higher than in WT plants (Chapter 2).
In addition to the unknown role that sorbitol may play in tomato during drought stress, ribitol accumulated in tomato as a response to drought stress and a unique phenotype in \textit{sdh} antisense plants was observed (Chapter 2). As with \textit{sdh} knockout mutants in Arabidopsis (Nosarzewski et al., 2012), the \textit{sdh} antisense tomato plants failed to recover and resume growth after re-watering at the end of a period of drought stress.

The objective of the present study was to determine sorbitol and ribitol contents, as well as the enzymatic activities of the key sorbitol cycle enzymes AR, A6PR, and SDH, of mature leaves of WT and of \textit{sdh} anti-sense tomato in response to salt stress. In addition, we investigated if sorbitol and ribitol content influenced the expression of \textit{SDH}, \textit{A6PR} and \textit{AR} and the accumulation of polyols (sorbitol under ribitol treatment and ribitol under sorbitol treatment), and if a unique phenotype of \textit{sdh} antisense plants also occurred during or after salt stress.

3.2 Materials and Methods

3.2.1 Plant material

Plants of wild-type (WT) tomato (\textit{Solanum lycopersicum} cv. ‘Ailsa Craig’), and three genetically-modified lines, an empty vector line ‘TR22’, and 2 \textit{sdh} anti-sense lines ‘TR45’ and ‘TR49’ (gifts from Dr. Yoshinori Kanayama, Tohoku University, Japan), were used. One seed of each was germinated and grown in containers (15 cm diameter, 14 cm depth) in PROMIX growing media (Premier Horticulture Inc., Quakertown, PA) in a growth room under fluorescent and incandescent lights (37 \textmu mol·m⁻²·s⁻¹) on shelves at 21 ± 2 °C, or under natural lighting (180 \textmu mol·m⁻²·s⁻¹) in a greenhouse at 22 ± 4 °C. The seed/plants were watered to runoff every 3 d and received periodic fertilization and pest management treatment as needed. For studies with rooted plants, stem pieces with 2-4 leaves were excised from stock plants of each genotype on the same day and rooted in water for up to 14 d, until adventitious roots were abundant.

3.2.2 Salt stress by using NaCl

At 6 weeks from the beginning of germination, treatments were started. To impose salt stress, six replicate pots of plants with 2-4 leaves each were subjected to salt stress by
watering with 50 mM NaCl every three days. The NaCl concentration was chosen through preliminary studies with a range of NaCl concentrations to identify one (50 mM) which allowed stress to develop slowly without evident injury, and with incipient wilting observed after about 14 d. Six replicate control pots continued to be watered regularly. On day 14, leaves from each plant in the six control and six salt–stressed pots were collected, immediately frozen in liquid N₂, and stored at -80°C for analysis of polyols, fructose, glucose, SDH, A6PR, and AR expression and activity.

3.2.3 Incubation in sorbitol and ribitol solutions

Six replicate rooted cuttings of WT, TR22, and TR45 and TR49 were grown in the growth room in 50 mL tubes for 5 d. Three replicate rooted cuttings of each line were kept in water and the other three of each line were held in 100 mM sorbitol or ribitol. Treated and control plants were sampled after 5 d. Leaves from each replicate plant were collected, immediately frozen in liquid N₂, and stored at -80°C for analysis of polyols and SDH, A6PR, and AR expression and activity.

3.2.4 Tissue analyses

3.2.4.1 Polyols, fructose, and glucose quantification

Following the procedures of Nosarzewski et al. (2012) for measurement of sorbitol, ribitol, fructose, and glucose content of leaf tissues, frozen tissues were lyophilized, 0.1 g was pulverised in a mortar and pestle, and 2-deoxy-D-glucose was added as an internal standard. The tissue was extracted with 1 mL of 80 % ethanol 3 times. Each time the sample solution was heated for 30 min at 80 °C in a closed Eppendorf vial, centrifuged at 2000 x g for 10 min, and the supernatant pipetted off. The supernatants were combined, evaporated to dryness under N₂, and the dried residues re-dissolved in 1 mL Millipore-purified water. A 300 µL aliquot of each sample was dried under N₂, mixed with 100 µL hydroxylamine solution (50 mg hydroxylamine dissolved in 1 mL pyridine), and heated in a sealed glass vial for 30 min at 80 °C. After cooling the samples, 100 µL of N,O(trimethylsilyl)trifluoroacetamide (BSTFA) (Thermo Fisher Scientific, Waltham, MA) was added, and the solution heated at 80 °C for 10 min. The samples were then ready for GC analysis. Ribitol, sorbitol, fructose and glucose were determined using a HewlettPackard 5890 II gas chromatograph (Agilent, Santa Clara, CA), equipped with a 60
m X 0.32 mm DB-5 column with a 1 µm film thickness (J & W Scientific, Folsom, CA) and a flame ionization detector. The operating conditions were 210 to 270 °C at 2.5 °C per min, then held at 270 °C for 20 min. Injector and detector temperatures were held at 270 °C. Helium was used as the carrier gas at a linear flow rate of 30 cm·s⁻¹. Based on peak areas of each compound at the same retention times as the respective standards, quantitative values were derived from the areas relative to the area of the 2-deoxy-D-glucose internal standard, and tissue concentrations were calculated.

3.2.4.2 SDH activity assays

The SDH enzyme was assayed as described by Nosarzewski et al. (2004). Frozen leaf tissue (~0.4 g) was ground in 3 mL of 0.5 M Tris-HCl (pH 7), containing 0.2% (w/v) ascorbic acid, 0.1% (v/v) Triton X100 (Sigma, St. Louis, MO) and 1% (w/v) polyvinylpolypyrrolidone (PVPP). After centrifugation at 20000 x g for 20 min, the supernatant was desalted on a Sephadex G-50 column (Sigma). The SDH activity assay mixture contained 0.45 mL of desalted extract, 0.8 mL of 30 mM Tris-HCl (pH 9.6), 1 mM nicotinamide adenine dinucleotide (NAD⁺), and 235 mM sorbitol. The enzyme activity was followed by the reduction of NAD⁺ at 340 nm. All assays were run at 21 °C. The protein content of the Sephadex-purified extracts was determined spectrophotometrically at 595 nm using the Bradford Assay (Bradford, 1976). Enzyme activity was calculated as nmol NADH per mg protein per min.

3.2.4.3 AR activity assays

AR was extracted from frozen tissue by the methods of Tari et al. (2010) and Sree et al. (2000). One g of frozen tissue was ground in liquid N₂ and extracted with 10 mL of extraction buffer containing 20 mM potassium phosphate buffer, pH 7.0, 5 mM βmercaptoethanol and 0.5 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 20000 x g for 30 min, the supernatant was desalted on a Sephadex G-50 column (Sigma). The purified extract was used for enzyme activity. The AR activity was determined in a total volume of 1 mL. The reaction mixture contained 0.05 mL of 20 mM D, L-glyceraldehyde, 0.05 mL of 2 mM NADPH and 0.025 mL of enzyme extract in 0.875 mL of 0.135 M potassium phosphate buffer (pH 7.4). The enzyme activity was followed
by the oxidation of NADPH at 340 nm. All assays were run at 21 °C. The protein content of the Sephadex-purified extracts was determined spectrophotometrically at 595 nm using the Bradford Assay (Bradford, 1976). Enzyme activity was calculated as nmol NADPH per mg protein per min.

### 3.2.4.4 A6PR activity assays

Frozen leaf tissue (1 g) was ground in a pre-cooled mortar and pestle in 10 mL of extract buffer (0.2M HEPES-NaOH, pH 7.5, containing 10 mM dithiothreitol (DTT), 3 mM Mg-acetate, and 6% (v/v) glycerol; 0.1% (v/v) Tween 20; and 1% (w/v) PVPP), which was added during grinding. The supernatant was filtered and centrifuged at 20000 x g for 20 min and desalted using Sephadex G-25 (medium) columns. A6PR was assayed using 0.05 mL of desalted extract, 0.1 M Tris buffer (pH 9), 0.11 mM NADPH, and 50 mM glucose-6-phosphate in 1 mL final volume (Lo Bianco et al., 2000). The enzyme activity was followed by the oxidation of NADPH at 340 nm. All assays were run at 21 °C. The protein content of the Sephadex-purified extracts was determined spectrophotometrically at 595 nm using the Bradford Assay (Bradford, 1976). Enzyme activity was calculated as nmol NADPH per mg protein per min.

### 3.2.4.5 Real-time PCR analysis of SDH and A6PR expression

To determine the expression of SDH and A6PR genes, procedures based on Nosarzewski et al. (2012) were used. Total RNA was isolated from frozen leaf tissues using an RNeasy plant Kit (Qiagen, Germantown, MD). The concentration of RNA isolated from the above tissue was measured using a Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000 and 2000c). The first strand cDNA for real-time PCR analysis was synthesized with oligo(dT)20 primer using 2 μg of total RNA in a final volume of 20 μL, containing 1 μL 50 μM oligo and 1 μL 10 mM dNTP. The total RNA mix was heated at 65°C for 5 min and then subjected to a reverse transcription reaction by adding the following components: 4 μL 5× RT Buffer, 1 μL 100 mM DTT and 1 μL SUPERSCRIPT III reverse transcriptase (Invitrogen, Germany) at 50-55°C for 10 min. The reaction was inactivated at 80°C for 10 min and treated with RNase cocktail (Ambion; 37°C, 20 min).
Real-time PCR was performed with Bio-Rad iQ5 on 1 μL of first-strand cDNA, using iQTM SYBR® Green Supermix, SDH and A6PR gene-specific primers, and and TIP41 like – protein (TARGET OF RAPAMYCIN (TOR) PROTEIN KINASE SIGNALING PATHWAY REGULATOR-LIKE 41) primers which was selected as the endogenous control for normalisation (a critical factor providing a necessary control for error associated with sample preparation) of the quantity of total RNA present in the sample (Expósito-Rodríguez et al., 2008). Nine replicate realtime PCR runs were performed with each sample of each gene. Relative Gene Expression was calculated by the 2(-Delta Delta C(T)) Method (Livak and Schmittgen, 2001).

The sequences of primer pairs used were:

**SDH** 5’- GGATGAAGGCTGTCGGTATTT -3’ and 5’-GCACATTCTATGCCCAATC -3’,

**A6PR** 5’- AGACTACTTGCGACGGAATG -3’ and 5’- GCAATCTCGGTGAGAAAGA -3’, and

**TIP41** 5’-CACGGTTGGAGATCGAGTG-3’ and 5’- CCATCTCCGGCAAGTGAGTT -3’.

3.2.5 Statistical analysis

Two-way (Genotype by Treatment (GxT)) analysis of variance (ANOVA) was performed using Sigmaplot 13 (Systat Software, San Jose, CA, USA). Differences were considered significant if $P \leq 0.05$. Because the GxT interaction was significant in most analyses (Appendix 1, Tables 1..4-1.6), GxT means were separated by Fisher’s Least Significant Difference at $P\leq0.05$. All data presented are means ± SE. RT-PCR results were statistically analyzed by using t-test and two-way ANOVA at $P \leq 0.05$.

3.3 Results

3.3.1 Salt stress

3.3.1.1 Polyol, glucose, and fructose content of tomato leaves and enzymatic activities after two weeks of salt stress by irrigation with 50 mM NaCl solution

Sorbitol was detectable in all genotypes under control conditions (Fig. 3.1A). A 2 week irrigation with NaCl solution contributed to a significant increase in sorbitol content
in all genotypes, but in sdh antisense plants the sorbitol level was more than 2-fold greater than in WT and TR22. Ribitol was detected at a very low concentration in all genotypes under control conditions (Fig. 3.1B), but irrigation with the NaCl solution also led to a clear increase in its level in all genotypes. In sdh antisense plants, the accumulation of ribitol was approximately 1.5- to 2-fold higher than its level in WT and TR22. Fructose under salt stress accumulated significantly only in WT and TR49 (Fig. 3.1C). Glucose increased significantly in WT and TR22 under salt treatment but was not changed in sdh antisense TR45 and TR49 lines (Fig. 3.1D). The glucose and fructose levels in stressed plants were several folds greater than the levels of sorbitol and ribitol.

SDH activity under control conditions in all genotypes was low (Fig. 3.2A). After NaCl treatment SDH activity increased in WT and TR22 more than 5 times its level in control conditions. SDH activity in the sdh antisense genotypes TR45 and TR49 after treatment did not change. A6PR and AR activities in control conditions were detectable in all genotypes at similar levels, respectively (Fig. 3.2 B,C). NaCl stress significantly increased A6PR activity in all genotypes. The greater increase in A6PR activity was in sdh antisense lines TR45 and TR49. Salt stress induced AR activity in all genotypes except WT (Fig. 3.2B,C). Salt stress resulted in a unique phenotype in the antisense lines, failing to recover after thorough rinsing of the media in water and re-watering without salt for several days (Fig. 3.3) Activities of both A6PR and AR across genotypes and treatments were correlated with the increase in sorbitol content (Fig. 3.4).

3.3.1.2 Expression patterns of A6PR and SDH under salt stress

Real-time PCR was employed to determine relative expression levels of A6PR and SDH in all genotypes. A6PR transcripts were expressed in the four genotypes under control conditions (Fig. 3.5A), but relative expression was 66% greater in the sdh anti-sense plants TR45 and TR49 than WT and TR22 plants during salt stress (Fig. 3.5B). In contrast, the
Figure 3.1. Leaf sorbitol (A), ribitol (B), fructose (C) and glucose (D) concentrations after 14 d of irrigation with 50 mM NaCl solution. WT= wild type, TR22= empty vector, and TR45 and TR49 = sdh anti-sense plants. NC= control, SS= salt stress. Means (n=3) were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. 
Figure 3.2. Leaf SDH (A), A6PR (B), AR (C) activities after 14 days of irrigation with 50 mM NaCl solution. WT = wild type, TR22 = control vector, and TR45 and TR49 = *sdh* antisense plants. NC = control, SS = salt stress. Means (n=3) were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. 

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Figure 3. 3. Unique salt stress phenotype. 1) Well-watered control plants, 2) plants after two weeks of watering with 50 mM NaCl, 3) one week after rinsing the media with five container volumes of Millipore water and then re-watering. TR45 and TR49 never recovered but WT and TR 22 achieved full recovery. WT=wild type, TR22=empty vector, and TR45 and TR49 = sdh anti-sense plants.
Figure 3.4. Linear regression of A6PR (A) and AR (B) activities with sorbitol content across all genotypes after 14 days of irrigation with water or 50 mM NaCl solution.
Figure 3. 5. Relative expression of A6PR and SDH genes in tomato leaves from control (A, C) and salt stress (B, D) conditions, respectively. WT= wild type, TR22= empty vector, and TR45 and TR49 = sdh anti-sense plants. NC= control, SS= salt stress. Means (n=9) were separated by t-test at P≤0.05.
relative expression of SDH in WT and TR22 was significantly higher than in sdh antisense plants under both normal (Fig. 3.5C) and stress conditions (Fig. 3.5D).

### 3.3.2 Irrigation with 100 mM ribitol solution

All genotypes responded to ribitol irrigation by increasing leaf sorbitol levels significantly (Fig. 3.6A). Leaf ribitol content was similar in WT, TR45, and TR49, but was significantly greater in TR22 (Fig. 3.6B). Seed germination after irrigation with ribitol presented no problem to WT and TR22, but the anti-sense lines presented a unique phenotype, failing to germinate (Fig. 3.6C). Irrigation with equal concentrations of mannitol or xylitol had no effect on germination of any line. WT SDH activity was significantly higher than the other genotypes in control conditions (Fig. 3.7A). Incubation in 100 mM ribitol for one week enhanced SDH activity in WT and TR22 but did not change it in sdh antisense lines TR45 and TR49. Incubation in ribitol significantly increased A6PR activity in all genotypes, with the greatest increase relative to control conditions in sdh antisense plants TR45 and TR49 (Fig. 3.7B). Incubation in 100 mM ribitol also increased AR activity in WT, TR45, and TR49 but not TR22 plants. (Fig. 3.7C).

### 3.3.3 Irrigation with 100 mM sorbitol solution

All genotypes responded to incubation in 100 mM sorbitol solution by increasing sorbitol levels significantly (Fig. 3.8A). Treatment with sorbitol led to increased ribitol content in TR45 and TR49 but there was no effect on ribitol content in WT and TR22 (Fig. 3.8B). In addition, individual leaves of the all genotypes incubated in the ribitol solution indicated that the all lines were sensitive to ribitol with signs of toxicity, but incubation with sorbitol did not elicit the same response (Fig. 3.8C). Seeds of the all genotypes were germinated and grown for 7 days in the greenhouse then transferred to a dark room, with 100 mM sorbitol supply for one week that effected negatively the sdh anti-sense seedlings caused their death without any effect on control group (Fig. 3.8D).

Incubation in 100 mM sorbitol for one week significantly enhanced SDH activity in WT and TR22, while SDH activity did not change in sdh antisense lines TR45 and TR49
Figure 3. Leaf sorbitol (A) and ribitol (B) concentrations after 5 days of incubation of rooted plants in 100 mM ribitol solution. C) Seed germination after watering with 200 mM ribitol, mannitol, or xylitol. WT= wild type, TR22 = empty vector, and TR45 and TR49 = *sdh* anti-sense plants. NC= control, T= treatment with ribitol. Means (n=3) were separated by Fisher’s Least Significant Difference at *P*≤0.05.
Figure 3.7. Leaf SDH (A), A6PR (B), and AR (C) after 5 days of incubation of rooted plants in 100 mM ribitol solution. WT = wild type, TR22 = empty vector, and TR45 and TR49 = sdh anti-sense plants. NC = control, T = treatment with ribitol. Means (n=3) were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. 
Figure 3. 8. Leaf sorbitol (A) and ribitol (B) concentrations after 5 days of incubation of rooted plants in 100 mM sorbitol solution. Means (n=3) were separated by Fisher’s Least Significant Difference at P≤0.05. C) Incubation of individual leaves in 100 mM ribitol or 100 mM sorbitol after 2 days. WT=wild type, TR22=empty vector, and TR45 and TR49 = sdh anti-sense plants. NC=control, T= treatment with sorbitol.
A6PR activity in control conditions was detectable at similar levels in WT, TR22, TR45, and TR49 plants. Incubation in sorbitol significantly increased A6PR activity in all genotypes, but the greatest increase was in sdh antisense lines TR45 and TR49 (Fig. 3.9B). AR activity did not differ among genotypes or treatments (Fig. 3.9C).

3.4 Discussion

In the present work the accumulation of sorbitol and ribitol, and biosynthesis and metabolism of sorbitol, in leaf tissue of four tomato genotypes, two of which had anti-sense sdh to reduce SDH activity, were tested when subjected to salt stress. These results confirmed accumulation of sorbitol in the salt stress response of tomato (Fig. 3.1A), consistent with what Tari et al. (2010) reported, and were parallel to the response to drought stress (Chapter 2). A similar response to salt stress in Plantago spp. occurred (Ahmad et al., 1979; Lambers et al., 1981; Konigshofer, 1983; Smeeckens and Tienderen, 2001), where the increase of sorbitol content doubled due to salt stress (Pommerrenig et al., 2007), as well as in barley genotypes (Chen et al., 2007). The sdh antisense tomato lines accumulated higher levels of sorbitol than WT and TR22, analagous to what was found for SDH knockout mutants of Arabidopsis (Nosarzewski et al., 2012) and to the same sdh anti-sense tomato lines (Chapter 2), both in response to drought stress. The accumulation of polyols (e.g., sorbitol, mannitol) as efficient osmolytes improves tolerance to both salinity and drought stress (Everard et al., 1994; Stoop and Pharr, 1994; Hu et al., 2005; Rejskova et al., 2007). The results indicated that sorbitol accumulation is a component of salt stress responses in tomato.

Salt stress induced fructose accumulation in 2 genotypes, WT and TR49, and glucose content in WT and TR22 (Fig. 3.1C.D). The effect of short-term treatments (10 days) by a high salt level (150 mm NaCl) on the accumulation of carbohydrates has been reported for tomato leaves (Ghanem et al., 2009). According to others (Khavarinejad and Mostofi, 1998; Shaba et al., 2010), salinity increased the soluble sugars in leaf and root tissues of tomato, but the starch content was not affected. An increase in general carbohydrate content as a response to salt stress has been reported for many plant species;
Figure 3.9. Leaf SDH (A), A6PR (B), and AR (C) activities after 5 days of incubation of rooted plants in 100 mM sorbitol solution. WT= wild type, TR22= empty vector, and TR45 and TR49 = sdh anti-sense plants. NC= control, T= treatment with sorbitol. Means were separated by Fisher’s Least Significant Difference at $P \leq 0.05$. 

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the soluble sugar of barley increased with NaCl treatment, and salt stress increased soluble sugars in rice (El-Tayeb, 2005; Amirjani, 2011). More carbohydrate in the cell increased membrane tolerance and selectivity for ion entry for Na$^+$ and Cl$^-$ (Prado et al., 2000).

The sorbitol biosynthetic pathway had not yet been fully characterised in *Solanum lycopersicum* prior to the present study, so the source of the accumulated sorbitol needed to be established. The results demonstrated that AR activity increased in tomato leaf tissue in response to salt stress (Fig. 3.2C), as reported by Tari et al. (2010). However, gene expression (Fig. 3.5A.B) and activity of A6PR (Fig. 3.2B) increased as well, which could also be the source of the sorbitol. A6PR activity has also been shown to increase in tomato during wounding stress (Kanayama et al., 2006; Mehta et al., 1991) and in response to drought (Chapter 2). Regression analyses indicated that activities of both enzymes were positively correlated to increasing sorbitol content (Fig. 3.4). Thus, both enzymes may be responsible for sorbitol production in tomato leaves during salt stress.

SDH is the key enzyme in sorbitol metabolism, catalyzing the oxidation of sorbitol to fructose in higher plants (Loescher, 1987). SDH-like sequences have been found in many non-Rosacease species which do not synthesize sorbitol as a primary photosynthetic product (Jia et al., 2015), such as tomato (Ohta et al., 2005) in which a single-copy of the SDH gene was found. The results clearly confirmed the increased presence of transcript by using RT-qPCR (Fig. 3.5 C.D), and of SDH activity (Fig. 3.2A) in response to salt stress in WT and TR22. In contrast, SDH activity and transcript levels were very low during normal and stress conditions in *sdh* antisense plants (TR45, TR49). However, the sorbitol content in *sdh* antisense plants was higher than in the control genotypes (WT, TR22), likely due to high activity of A6PR and/or AR as well as the lack of sorbitol catabolism in the plants.

The results provided additional confirmation that leaf ribitol content increased when subjected to salt stress in all tomato genotypes (Fig. 3.1), but more accumulated in the *sdh* antisense lines, which is consistent with the observation in Arabidopsis (Nosarzewski et al., 2012). In total, the present results support the hypothesis that SDH is also a ribitol dehydrogenase in tomato as was suggested with Arabidopsis (Nosarzewski et al., 2012).
To study the regulation of gene expression of SDH, A6PR, and AR by polyols, rooted plants of all genotypes were incubated in 100 mM sorbitol or and 100 mM ribitol. Osmotic agents such as sorbitol have been applied in in vitro systems in several studies to simulate water deficiency (Wang et al. 1999; Gopal and Iwama 2007). These osmotic agents are usually chosen because they reduce the osmotic potential without being toxic to the plants. To date, testing the effect of incubation with polyols on the activities of SDH, A6PR and AR in tomato has not been reported. The data clearly showed that SDH (WT, TR22 only) and A6PR activity increased in response to sorbitol and ribitol but that AR activities increased in response to ribitol but not sorbitol treatment (Figs. 3.7C.B, 3.9C.B). Increased SDH activity in response to sorbitol has been shown for apple fruit and shoot tips (Archbold, 1999; Zhou et al., 2006), Japanese pear fruit (Iida et al., 2004) and in Arabidopsis leaves (Nosarzewski et al., 2012). Thus, SDH expression and enzyme activity in tomato may also be regulated by sorbitol and ribitol availability. Similarly, mannitol, another polyol in some higher plant species, has also been reported to stimulate mannitol dehydrogenase activity in celery cell culture (Pharr et al., 1995). Treating plants with ribitol increased accumulation of sorbitol in all genotypes, possibly caused by the perception of the high level of ribitol as a stress signal that elevated sorbitol biosynthesis in all genotypes (Sajan et al., 2002).

Sorbitol content of the control lines WT and TR22 was lower than in the sdh antisense lines TR45 and TR49 under salt stress conditions (Fig. 3.1), but incubation with ribitol increased the level of sorbitol equally in all genotypes including WT and TR22 (Fig. 3.6A). This may be due to two reasons: 1) incubation with ribitol was perceived as a stress signal that interacted with stress response pathways (Sajan et al. 2002) and increased sorbitol biosynthesis by enhancing A6PR and AR activities (Fig. 3.7), and 2) ribitol, which is a SDH substrate, interfered with sorbitol metabolism, even in WT and TR22 plants in which SDH is active. The former reason is more likely because the sdh antisense plants lacking SDH activity also showed increased sorbitol content (Fig. 3.6). On the other hand, sorbitol incubation of the sdh antisense lines TR45 and TR49 resulted in accumulation of a high level of ribitol but the control lines WT and TR22 did not exhibit this response (Fig. 3.8). Plants receiving a high level of sorbitol had elevated SDH activity in WT and TR22 (Fig. 3.9), and the increase may have been sufficient for metabolizing ribitol. However,
ribitol content of $sdh$ antisense lines (TR45 and TR49) increased because of the deficiency in SDH activity.

A unique salt-sensitive phenotype was observed in the antisense lines, unable to recover after rinsing the media of salt and then re-watering (Fig. 3.3), unable to germinate in the presence of ribitol but not xylitol (Fig. 3.6), and foliar toxicity to ribitol but not sorbitol (Fig. 3.8). In contrast, the WT and empty vector genotypes did not show similar responses. A unique phenotype of the $sdh$ anti-sense lines was also observed in response to drought stress and recovery after re-watering (Chapter 2), and in $sdh$ knockout mutants of Arabidopsis (Nosarzewski et al., 2012). Furthermore, the unique phenotype may be a response to the accumulation of ribitol, as reported for Arabidopsis. This was most evident with leaves of the $sdh$ antisense lines incubated with ribitol but not sorbitol; the WT and empty vector lines showed no such response (Fig. 3.8). Inability to remove or metabolize excess ribitol in the antisense lines was very possibly responsible for the unique phenotype.

### 3.5 Conclusions

Salt stress is the most serious threat to agriculture in many parts of this world. Tolerance to salt comes through changes in the molecular and physiological mechanisms that allow plants to adapt to salt stress. This work provided information on physiological, biochemical, and molecular bases of one aspect of salt tolerance of tomato, by the sorbitol cycle and the key sorbitol cycle enzymes A6PR, not previously reported for tomato, AR, and SDH, in tomato plants in response to salt stress, and in response to incubation in sorbitol and ribitol. These results indicated that sorbitol accumulation has a role in the salt stress responses of tomato, and that ribitol accumulation may also occur, the latter perhaps as a result of riboflavin degradation under stress conditions. Expression and/or activity of A6PR, AR, and SDH were sensitive to salt stress and were induced by the polyols. Sorbitol was produced by either or both A6PR and AR and metabolized by SDH. Ribitol was likely metabolized by SDH, as the lack of SDH activity in the antisense lines was correlated to greater ribitol accumulation. The results support the suggestion that SORBITOL DEHYDROGENASE is also a RIBITOL DEHYDROGENASE in tomato (Nosarzewski et al., 2012).
CHAPTER 4

4.1 Summary and Conclusions

Abiotic stresses, such as drought and salt, are major environmental factors that adversely affect plant growth, limit crop production and threaten food security. Improving stress resistance of crop plants is a critical factor for agricultural productivity and environmental sustainability. The objective of this work was to characterize the sorbitol cycle in tomato by quantifying sorbitol content as well as the enzymatic activities, protein accumulation, and gene expression patterns of the key sorbitol cycle enzymes AR, A6PR, and SDH in mature leaves of wild type and of sdh anti-sense tomato plants in response to drought, salt, and osmotic stresses. The results showed that sorbitol accumulated under drought, osmotic and salt stress which was correlated with higher activities of the sorbitol cycle enzymes. The level of sorbitol accumulation was considerably lower than that of the common sugars glucose and fructose so was not enough to have a significant impact on tissue osmotic potential but could provide other important osmoprotective effects. A6PR and AR should both be considered as possible biosynthetic sources of sorbitol in tomato, as their activities significantly increased in response to the stresses. A6PR expression and activity in tomato was characterized for the first time in this work. Use of the sdh antisense lines indicated that SDH has the key role in sorbitol metabolism in tomato as well as a role in ribitol metabolism, not previously known (Figure 4.1). Like sorbitol, ribitol also accumulated significantly more in the antisense lines during the stresses. Expression and/or activity of A6PR, AR, and SDH were also induced by the polyols, although it is not clear if the induction was due to a polyol signal, the osmotic effect of the incubation solution, or both. In addition a unique post-abiotic stress phenotype was observed in the sdh anti-sense lines. After both drought and salt stresses and during a recovery phase after re-watering, the antisense lines failed to recover. This may have been due to their accumulation of ribitol as they were uniquely sensitive to ribitol but not sorbitol, with an apparent toxicity to ribitol. The determination that sorbitol, and perhaps ribitol as well, plays a role in abiotic responses in tomato provides a cornerstone for future studies examining how they impact tomato tolerance to abiotic stresses, and if their alteration could improve stress tolerance.
Figure 4.1. Putative metabolic pathway of sorbitol and ribitol and in tomato. Abbreviations: SORBITOL-6-PHOSPHATE PHOSPHATASE (SorPP; EC 3.1.3.50); SORBITOL DEHYDROGENASE (SDH; EC 1.1.1.14); RIBITOL DEHYDROGENASE (RDH; EC 1.1.1.56); ALDOSE REDUCTASE (AR; EC 1.1.1.21); ALDOSE-6-PHOSPHATE REDUCTASE (A6PR; EC 1.1.1.200); nicotinamide adenine dinucleotide (NAD$^+$); reduced nicotinamide adenine dinucleotide (NADH); nicotinamide adenine dinucleotide phosphate (NADP$^+$); reduced nicotinamide adenine dinucleotide phosphate (NADPH). (KEGG Pathway Database, 2019)
The present work has left a number of important questions unanswered and raised some new questions. These include:

1) Are both AR and A6PR responsible for sorbitol production?

2) Is riboflavin the source of ribitol, and is ribitol re-cycled back to riboflavin?

3) The final step in sorbitol production from A6PR is removal of the phosphate group for sorbitol-6-phosphate, so is there such phosphatase activity in tomato?

To address 1) above, sorbinil (AR-specific inhibitor) could be fed to rooted cuttings of the 4 genotypes, which would be subjected to PEG-induced osmotic and or NaCl salt stress, and then the sorbitol content in all genotypes could be determined to see if losing AR activity affected sorbitol levels. Also, antisense, or clustered regularly interspaced short palindromic repeats (CRISPR)-edited, lines of AR and A6PR could be created to reduce or eliminate activity of either enzyme in WT and in the $sdh$ antisense genotypes, which could then be stressed to determine the effect on sorbitol levels.

To address 2) above, radiolabeled riboflavin and ribitol could be fed to rooted cuttings of the 4 genotypes, which would then be stressed. Extracts of the tissues could be passed through a high performance liquid chromatography system to separate fractions with radioactivity, which could then be subjected to mass spectrometry to identify the radiolabeled products.

To address 3) above, sorbitol-6-phosphate phosphatase activity in tomato could be assayed. If activity was found, the protein could be purified, sequenced, and the sorbitol-6-phosphate phosphatase gene could be sought in the tomato genome.
Appendix 1

*P* values for main effects of genotype (G) and treatment (T) and their interaction (GxT) from the analyses of variance (ANOVA) results for the drought and osmotic stress experiments.

Abbreviations: sorbitol (S), ribitol (R), fructose (F), glucose (G), sorbitol dehydrogenase (SDH), aldose-6-phosphate reductase (A6PR), aldose reductase (AR).

Table 1. 1. Water withheld 10 days, re-watered for 1 day.

<table>
<thead>
<tr>
<th>Measured traits</th>
<th>Effects</th>
<th>S</th>
<th>R</th>
<th>F</th>
<th>G</th>
<th>SDH</th>
<th>A6PR</th>
<th>AR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
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<td>T</td>
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<td>GxT</td>
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<td>0.007</td>
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<td>0.001</td>
</tr>
</tbody>
</table>

Table 1. 2. Water withheld 10 days, re-watered for 3 days.

<table>
<thead>
<tr>
<th>Measured traits</th>
<th>Effects</th>
<th>S</th>
<th>R</th>
<th>SDH</th>
<th>A6PR</th>
<th>AR</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td></td>
<td>GxT</td>
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<td>0.001</td>
<td>0.001</td>
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<td>0.049</td>
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</table>

Table 1. 3. Osmotic stress by polyethylene glycol (PEG) Treatment

<table>
<thead>
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<th>Measured traits</th>
<th>Effects</th>
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<th>R</th>
<th>SDH</th>
<th>A6PR</th>
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<td></td>
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<tr>
<td></td>
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Table 1. 4. 14-day irrigation with 50 mM NaCl

<table>
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<th>Effects</th>
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<th>R</th>
<th>G</th>
<th>F</th>
<th>SDH</th>
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<th>AR</th>
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<tbody>
<tr>
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<td>0.001</td>
<td>0.002</td>
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<tr>
<td></td>
<td>GxT</td>
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<td>0.001</td>
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<td>0.255</td>
<td>0.001</td>
<td>0.127</td>
<td>0.207</td>
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</tbody>
</table>
Table 1.5. Incubation with 100 mM ribitol

<table>
<thead>
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<th>SDH</th>
<th>A6PR</th>
<th>AR</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
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<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>GxT</td>
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Table 1.6. Incubation with 100 mM sorbitol

<table>
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<th>SDH</th>
<th>A6PR</th>
<th>AR</th>
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<tr>
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<td>0.003</td>
<td>0.001</td>
<td>0.001</td>
<td>0.111</td>
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</tbody>
</table>
Figure A. 1. Preliminary screening of the $sdh$ anti-sense tomato lines to determine which have the lowest SDH activity compared with the wild type (WT). Each data point is the mean of 3 replications. The $sdh$ antisense lines were 30, 45, 49, 51, 52, 89, and 92. The empty vector line was 22.
References


glucose transporter translocation/glucose transport through proline-rich tyrosine kinase-2, the extracellular signal-regulated kinase pathway and phospholipase D. Biochem. J. 362:665–674.


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