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The Alfalfa Genome

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Physiological, Morphological, Biochemical, and Genetic Responses of Alfalfa to Salinity

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Abstract

Alfalfa is an important forage crop known for its perennial nature, high biomass yield, high nutritive value, and high water demand. Global reduction in the availability of clean water in arid and semiarid regions is forcing farmers to irrigate with low-quality degraded/recycled waters, which are generally higher in salts than freshwater. High salt concentrations in soils not only affect soil properties but also result in reduced plant growth and tissue ion toxicities. Plants employ a wide variety of morphological, anatomical, physiological, biochemical, and molecular mechanisms to cope with salinity stress. The self-incompatibility and polyploidy of alfalfa makes the genetic dissection of these complex mechanisms challenging. Understanding these mechanisms is critical to effectively manipulate underlying genetic determinants needed to develop salt-tolerant alfalfa genotypes. This chapter focuses on the effects of salinity on alfalfa growth and development, mechanisms

of salinity tolerance in plants, and the current status of the knowledge related to salinity tolerance in alfalfa. Recent studies on the effects of salinity on growth, biomass yield, photosynthesis, water and ion relationships, nutritive value, and antioxidant capacity provide a clear picture of different component traits involved in the salinity response of alfalfa. Based on these studies and the molecular information generated on model plants, tremendous progress has been made to fill the major knowledge gaps in different pathways regulating salt stress responses in alfalfa. This knowledge will facilitate the manipulation of genetic components that would allow alfalfa to thrive when irrigated with recycled/highly saline waters and expand alfalfa cultivation to marginal lands.

9.1 Introduction

Alfalfa (*Medicago sativa* L.) is an important forage crop that is grown worldwide due to its perennial nature, wide adaptability, high biomass yield, resistance to multiple cuttings, and tolerance to various biotic and abiotic stresses (Riday and Brummer 2002; Singer et al. 2018). Additionally, alfalfa has high nutritive value, protein content, and palatability to ruminants. It can be used fresh for animal grazing or dehydrated to hay and meal pellets. Alfalfa is the 3rd most valuable field crop (US\$10.8 billion) in the

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United States, behind corn grain (US\$52.9) and soybean (US\$31.2), according to the 2019 data (<https://www.nass.usda.gov/>).

Water is the most crucial resource for agriculture. With the reduced availability of good quality irrigation water, the demand for alternate/degraded waters, which have higher salt concentrations than freshwater, is growing. Salinity is a mounting threat to global agriculture. About one billion hectares of arable land is affected by salinity/sodicity (Shahid et al. 2018), and millions of hectares of land are destroyed each year due to salinity worldwide (Pimentel et al. 2004). It has been predicted that about 50% of the cultivated land may be affected by salinity by 2050 (Bartels and Sunkar 2005).

Earlier studies on alfalfa classify it as moderately sensitive to salinity, based on the electrical conductivity of soil-paste (EC_e) with a salinity threshold of 2.0 dS m^{-1} (Maas and Hoffman 1977). However, recent studies showed that alfalfa could produce high biomass yield with an EC_e of 11.0 dS m^{-1} (equivalent to $EC_{iw} = 24 \text{ dS m}^{-1}$) while maintaining its nutrient composition and antioxidant capacity, and with a slight but significant increase in forage crude protein and quality (Cornacchione and Suarez 2017; Ferreira et al. 2015). These observations propose that alfalfa is moderately tolerant to salinity, and developing new salt-tolerant cultivars may lead to the expansion of alfalfa cultivation to marginal lands, unsuitable for producing staple crops. To realize this goal, the understanding of genetic determinants regulating morphological, physiological, and biochemical traits responsible for salinity tolerance in alfalfa is crucial. Genetics studies in alfalfa indicate considerable variation in salinity tolerance in alfalfa (Cornacchione and Suarez 2017; Sandhu et al. 2017; Yu et al. 2016).

9.2 Effects of Salinity on Alfalfa

9.2.1 Growth

Salinity typically induces osmotic stress (first phase) and ionic stress (second phase) that directly impact biochemical, physiological, and

molecular plant responses. Osmotic stress limits plants' ability to uptake water from the soil, while the ionic stress results in the build-up of toxic levels of salt ions. These, together, lead to stunted plant growth and development (Munns and Tester 2008). In alfalfa, ionic toxicity is primarily caused by Na^+ and, to some extent, by Cl^- . For plant species sensitive to salinity, the salt concentration does not reach a level to cause osmotic stress (Sandhu and Kaundal 2018). In those cases, ionic stress is the key to suppressing plant growth. In alfalfa, it has been shown that some genotypes have a high salt tolerance index (STI) (performance under salt/performance under control conditions) for biomass yield resulting in almost no reduction when irrigated with saline water of high electrical conductivity ($EC_{iw} = 17 \text{ dS m}^{-1}$) (Sandhu et al. 2017). Hence, for alfalfa, both the osmotic and ionic stresses are critical during salinity stress.

Roots are initially exposed to the high salinity of water or soil. Then, the stress signal is distributed throughout the plant. Leaves are ultimately affected, leading to several modifications under stress. Plants employ a wide variety of morphological, anatomical, physiological, molecular, and biochemical mechanisms to cope with salinity stress. Alfalfa responds to salinity stress by reducing young growth, leaf size and by accelerating the senescence of older leaves (Cornacchione and Suarez 2015). On the other hand, roots are more tolerant to salinity compared to shoots and suffer a smaller reduction in biomass than shoots. In alfalfa, biomass yield and forage quality are important traits that must be maintained in salinity-tolerant cultivars.

9.2.2 Shoot Biomass

Biomass yield is a complex trait regulated by several genes in a tetraploid species, such as alfalfa. On top of that, various biotic (e.g., insect or fungal attack) or abiotic stresses (e.g., drought, heat, salinity) stresses make it harder to dissect the genetic networks underlying this trait. High salinity of irrigation water or soil is known to reduce alfalfa shoot biomass, and that has been

reported by several authors cited in a recent review (Al-Farsi et al. 2020b). Most researchers who evaluated several genotypes agree that there is a significant variability in alfalfa response to salinity (Al-Farsi et al. 2020a; Cornacchione and Suarez 2017; Sandhu et al. 2017). Researchers at the US Salinity Laboratory (USDA-ARS) in California evaluated 12 genotypes (G01-G12) under greenhouse conditions with EC_{iw} of 2.0 (control) and 17.0 $dS\ m^{-1}$ (saline treatment) for 18 months (Sandhu et al. 2017). In this long-term study, biomass was determined based on the average of 11 harvests. Salinity impact on plant growth was genotype-dependent, and the maximum reduction recorded under salinity was 61% for G06, while G03 and G10 had no reduction in biomass. Interestingly, G02 had a significant reduction in biomass at 17.0 $dS\ m^{-1}$ compared to control, but its biomass under salinity was slightly higher than G03 and G10. Thus, under salinity, G02, G03, and G10, respectively, were the most productive in shoot biomass (Sandhu et al. 2017). The genotypes G03 and G10 also had the highest STI. The performance of the best genotypes was consistent with their mother plants, with shoot biomass inversely correlated with their shoot accumulation of both Na and Cl (Sandhu et al. 2017). These authors also reported that the reduction in biomass under salinity was primarily attributed to the number of shoots per plant rather than plant height. Hence, high branching ability under salinity may be an important morphological trait to be considered while breeding for salinity tolerance in alfalfa.

When evaluating a perennial forage such as alfalfa, it is important to stress that long-term experiments of at least one year, preferably two, should be conducted to allow plants to establish and adjust to salinity. Also, one should use the same number of cuts used in the region where the genotype is cultivated. Although greenhouses in semiarid and arid areas usually get hot and dry during summer months, outdoor studies should be done to confirm that plants will perform as expected under extreme heat and evapotranspiration during summer months.

9.2.3 Photosynthesis

The efficiency of photosynthesis determines growth rate under stress conditions. The effect of salinity stress on photosynthesis in alfalfa is dependent on multiple factors like genotype, salinity level, developmental stage, and growth conditions. Plants irrigated with high-salinity water develop smaller leaves, darker in color due to the higher chlorophyll concentration (Sandhu et al. 2017). A greenhouse study was conducted with 15 commercial alfalfa genotypes from Argentina and the USA, with waters dominated by sulfate or chloride and salinities ranging from 0.85 to 24.0 $dS\ m^{-1}$ (Cornacchione et al. 2018). This study revealed no difference between sulfate- or chloride-dominated waters for photosynthetic rate (Pn), stomatal conductance (gs), or transpiration rate (Tr). However, increased salinity significantly reduced leaf area (Cornacchione et al. 2018). Also, a highly positive correlation was found between the specific leaf weight and chlorophyll ($R^2 = 0.9653$), suggesting that, under high salinity, the leaves became thicker (with a thicker palisade layer that could have contained more chloroplasts per leaf unit area). Still, in this study, Pn increased with salinity while leaf area was reduced, suggesting that this reduction in Pn with salinity could be either “the cause or the effect” of the decreased biomass per plant.

9.2.4 Mineral Imbalance

Mineral balance in plant cells plays a critical role in plant growth and development. High concentrations of salt ions, including Na^+ and Cl^- , in irrigation water or soil, are known to affect the mineral balance of other nutrients necessary for plant growth and development, such as the previously reported Na^+ antagonism to K^+ and Ca^{2+} and Cl^- antagonism to NO_3^- uptake (Maas and Grattan 1999). Most alfalfa salinity studies show increased tissue concentrations of Na and decreased concentrations of K and Ca under

salinity (Cornacchione and Suarez 2015; Ferreira et al. 2015; Cornacchione and Suarez 2017; Sandhu et al. 2017). K is an important nutrient and an important cell osmoticum, whereas Ca is critical for plasma membrane and cell wall stability. Although salinity may lead to reduced accumulation of some mineral nutrients, alfalfa irrigation with saline waters of $EC_{iw} = 24 \text{ dS m}^{-1}$ and mimicking the waters of the California Central Valley, and of ground-water-fed wells of the islands of Lanzarote and Fuerteventura, Spain ($EC_{iw} = 10 \text{ dS m}^{-1}$), resulted in darker green shoots, which had a significantly higher N%, crude protein, and forage quality (Ferreira et al. 2015; Díaz et al. 2018). The salinity experiment performed in California lasted for 300 days, with plants being cut monthly with shoot mineral analysis and forage values performed at 84 days after sowing (DAS) and at 299 DAS (Ferreira et al. 2015). EC_{iw} ranged from 3.1 dS m^{-1} (control) to 30 dS m^{-1} . Although concentrations of N, P, Mg, and S increased significantly at $EC_{iw} = 18 \text{ dS m}^{-1}$, those of K and Ca decreased (Ferreira et al. 2015). Interestingly, these authors also reported that Zn, Mo, and Mn increased significantly in shoots with salinity. The plants likely absorbed more micronutrients to boost their defenses against the salinity stress. None of the plants, even at 24 dS m^{-1} showed visual symptoms of mineral deficiency. For the salinity experiment performed in Spain, the relative biomass reduced by 50% under EC_{iw} of 5 dS m^{-1} , but forage crude protein increased, slightly increasing the relative forage value (RFV) and metabolizable energy (ME) (Díaz et al. 2018). Shoot mineral concentrations for N, Mg, S, and Na increased with salinity, while K and Ca decreased. The interplay of salt ions among themselves and with minerals is complex, and care should be taken to ensure that the plant has enough macro and micronutrients to sustain its growth and biomass production.

9.2.5 Forage Nutritional Value and Antioxidants

It is widely accepted that alfalfa is the most important forage crop used as a high protein source for dairy and beef cattle, sheep, horses, and birds, among other livestock (Radović et al. 2009). Besides protein, alfalfa silage contains higher concentrations of minerals compared to other cereals (Khorasani et al. 1997). Also, according to these authors, cows absorb more potassium (89%) from alfalfa silage than from cereal silages (74–83%). However, alfalfa demands a high volume of water in arid and semiarid lands to produce high forage biomass from several cuts a year. Among approximately 270 million hectares of irrigated land worldwide, close to 40% is located in arid/semiarid zones (Smedema and Shiati 2002). This irrigation, even if done with low-salinity water, is linked to increased salinity of irrigated lands. Although alfalfa is more tolerant to salinity than previously reported and can produce a reasonable amount of biomass under salinities of irrigation water up to 24 dS m^{-1} (Cornacchione and Suarez 2015, 2017; Ferreira et al. 2015), there are very few reports on the effect of salinity on mineral composition, forage parameters, and antioxidant capacity under salinity. We would like to emphasize that we are not using the term “nutritional value” because that implies evaluating the forage in livestock and their feed conversion into weight gain and milk production. These studies are more challenging to perform as they involve evaluating the forage through livestock parameters, and we found none with alfalfa in the literature. Thus, we cite the works that have been done evaluating mineral composition and/or antioxidant capacity of alfalfa biomass under salinity. Field-cultivated alfalfa in the San Joaquin Valley was reported to produce over 20 t/acre when irrigated with freshwater, but its

yield dropped to a bit over 16 t/acre when the alfalfa cultivar (Salado/801S) was irrigated with drainage water of 4.7–6.9 dS m⁻¹ (Suyama et al. 2007). These authors reported that alfalfa irrigated for five years with these drainage waters had 4–10 mg kg⁻¹ of Se, highly present as selenite in the soil used for the experiment. The maximum limit of Se tolerated by most ruminants in forage is 2.0 mg kg⁻¹ (NRC 2000).

Although mineral nutrients may be added to an animal diet through other feed sources, it is crucial to evaluate the mineral composition of alfalfa cultivated with water of elevated salinity. Four commercial alfalfa cultivars grown in sand tanks for ten months and irrigated with water salinities ranging from EC_{iw} = 3.0 (control) to 24.0 dS m⁻¹ maintained sufficient concentrations of mineral nutrients for their growth and biomass accumulation (Ferreira et al. 2015). This study on the “potential nutritional value” of alfalfa reported that crude protein significantly increased at 18 dS m⁻¹ in both harvest dates, while relative forage value (RFV) increased significantly for EC_{iw} = 18 dS m⁻¹ at 84 DAS but remained constant at 299 DAS (Ferreira et al. 2015). However, after three years of field growth, “Salado/801S” alfalfa irrigated with either freshwater (EC_{iw} = 4.7 dS m⁻¹) or drainage water (EC_{iw} = 6.9 dS m⁻¹) had similar shoot concentrations of K, Ca, Mg, and P, and twice as much NO₃ nitrogen when irrigated with 6.9 dS m⁻¹ drainage water (Suyama et al. 2007).

9.3 Mechanisms of Salinity Tolerance in Plants

Nonselective cation channels (NSSCs) are the primary route through which Na⁺ enters roots. The mechanisms by which plants sense salt upon exposure are not well understood. Recently, an extracellular salt sensor, MONOCATION-INDUCED [Ca²⁺]I INCREASES 1 (MOCA1), has been identified, which senses Na⁺ and some other monovalent cations (Jiang et al. 2019). MOCA1 is responsible for the production of glycosyl inositol phosphorylceramide (GIPC) sphingolipids at the plasma membrane. Upon

binding with monovalent cations, GIPCs can interact with the Ca²⁺ channel, increasing the intracellular cytosolic Ca²⁺ concentration. An increase of cytosolic Ca²⁺ level activates the Salt Overly Sensitive (SOS) pathway (Zhu 2000). SOS3 is a calcium-binding protein (calcineurin B-like protein 4) that senses increased calcium concentration in the cytosol of plant cells in response to salt stress. Binding of SOS3 to Ca²⁺ promotes interaction between SOS3 and SOS2 (calcium-induced protein kinase 24), and this process activates SOS2 protein kinase. Subsequently, active SOS2–SOS3 complex phosphorylates SOS1 at Ser¹⁰⁴⁴ position that activates SOS1 by relieving from an autoinhibitory state of SOS1 (Quintero et al. 2011). Active SOS1 extrudes Na⁺ from cytosol to outside. The SOS pathway is conserved in several plant species (Munns and Tester 2008; Zhao et al. 2020).

The movement of ions from the cytoplasm to the vacuole is critical in keeping ion concentration low in the cytoplasm. Tonoplast-localized Na⁺/H⁺ exchangers (NHX), including NHX1 and NHX2, mediate sequestration of Na⁺ in the vacuole leading to enhanced salinity tolerance (Apse et al. 1999). It has been suggested that both NHX1 and NHX2 also play roles in K⁺ homeostasis as they have equal affinity for both Na⁺ and K⁺ ions (Maathuis et al. 2014). These findings suggest that NHX1 and NHX2 would import Na⁺ into the vacuole when the cytoplasmic concentration of Na⁺ is higher than K⁺. Na⁺/K⁺ homeostasis plays an important role in salinity tolerance in plants. Multiple potassium channels and transporters have been implicated in K⁺ homeostasis during salinity stress, including AKT1, GORK, TPK1, HAK5, CHX17, NHX5, and NHX6 (van Zelm et al. 2020).

High-affinity potassium transporters (HKTs) play vital roles in ion transport during salinity stress in plants (Horie et al. 2009). Substitution of a single amino acid in the second pore-loop region determines affinity for Na⁺ or K⁺. These transporters belong to two subclasses: HKT1 has an affinity for Na⁺, and HKT2 has an affinity for K⁺. In Arabidopsis, HKT1 has been shown to inhibit root-to-shoot Na⁺ transport but promotes Na⁺ transport from shoot-to-root and provides

tolerance to salt stress by keeping Na^+ content low in leaves (Kaundal et al. 2019).

There are many additional signaling pathways and elements that contribute to salinity tolerance like osmolytes, ROS signaling, hormone signaling, IP3 signaling, and sugar signaling (Park et al. 2016; van Zelm et al. 2020). Import of Na^+ also increases production of reactive oxygen species (ROS) and 3', 5'-cyclic guanosine monophosphate (cGMP) levels. Ca^{2+} , cGMP, and ROS serve as important secondary messengers during salinity stress. cGMP inhibits the import of Na^+ , reduces K^+ efflux, and increases Ca^{2+} import. Increase of Ca^{2+} promotes ROS production mediated by RbohD, RbohF, and RbohJ. Ca^{2+} and ROS form a positive feedback loop in the signaling pathway (van Zelm et al. 2020).

9.4 Progress Toward Understanding Salinity Tolerance in Alfalfa

Several genes have been identified that play critical roles in salinity tolerance in alfalfa, indicating that salinity stress tolerance is a multigenic trait (Bhattarai et al. 2020). Here, we discuss the importance of different component traits of the salinity tolerance mechanisms in alfalfa.

9.4.1 Transporters

Several transporter proteins are critical during salinity stress. The salt overly sensitive (SOS) pathway that consists of three important proteins (SOS1, SOS2, and SOS3) plays an important role in the removal of Na^+ from the cytoplasm in response to salinity stress (Zhu 2000). *MsSOS1*, *MsSOS2*, and *MsSOS3* were upregulated in response to salt stress in salt-tolerant genotypes (Sandhu et al. 2017). Coexpression of Arabidopsis SOS pathway genes in alfalfa has been shown to enhance salt tolerance in alfalfa by enhancing plant growth, lowering Na^+ accumulation, increasing K^+ accumulation

in the leaves, increasing proline content, and enhancing activities of antioxidant enzymes (Wang et al. 2019). Additionally, the SOS core pathway in alfalfa was shown to be activated in response to ionic stress (Luo et al. 2019b). These findings indicate that the SOS pathway is also conserved in alfalfa.

Upregulation of *NHX1*, *HKT1*, and *AKT1* was observed in salt-tolerant genotypes of alfalfa, suggesting that these genes play a vital role in Na^+/K^+ homeostasis in alfalfa (Sandhu et al. 2017). The complementation of *NHX* genes from various plant species including *SeNHX1* (*Salicornia europaea NHX1*), *SsNHX1* (*Suaeda salsa NHX1*), and *TaNHX2* (*Triticum aestivum NHX2*) have been shown to provide tolerance to salt stress in alfalfa (Hrbáčková et al. 2020; Zhang et al. 2012). Arabidopsis vacuolar proton-pumping pyrophosphatase (H^+ -PPase) gene *AVPI* functions with vacuolar H^+ -ATPase (V-ATPases) to establish a difference in electrochemical potential for H^+ across the tonoplast, which helps in the sequestration of Na^+ into the vacuole (Gaxiola et al. 2001; Schilling et al. 2017). Constitutive expression of *AVPI* protects various plants from salinity stress and improves growth and yield. Overexpression of Arabidopsis *AVPI* in alfalfa enhanced salinity tolerance and membrane integrity in response to 200 mM NaCl (Bao et al. 2009) and resulted in the accumulation of more cations (Na^+ , K^+ and Ca^{2+}) than wild-type plants in response to salinity stress (Schilling et al. 2017).

9.4.2 Osmoregulators

Salinity stress induces osmotic stress that leads to loss of turgor pressure, which in turn causes changes in membrane structure, leading to membrane leakage (Zhu 2002). To maintain turgor pressure in the cell during osmotic stress, plants synthesize compatible organic solutes (or osmolytes) and inorganic solutes for osmotic adjustment (Zhu 2002). Common solutes synthesized by plants include soluble sugars (fructose and sucrose), complex sugars (raffinose, trehalose, and fructans), polyols (pinitol,

mannitol, glycerol, arabinitol, and sorbitol), charged metabolites (betaines, prolines, aspartate, glutamate, glycine, putrescine, choline, malate, and oxalate), specific proteins, and ions (mainly K^+). Some osmolytes also serve as osmoprotectants, antioxidants, and as signaling molecules (Park et al. 2016). Overexpression of *Glycine soja* S-locus LecRLK gene *GsSRK* in alfalfa enhanced salinity tolerance by controlling osmotic regulation, ion homeostasis, and ROS scavenging (Song et al. 2019; Sun et al. 2018). Salt-tolerant alfalfa genotypes are known to accumulate more proline than their corresponding sensitive cultivars (Torabi and Halim 2010). *P5CS1*, a gene required for proline biosynthesis, was upregulated in salt-tolerant alfalfa cultivars compared to salt-sensitive ones (Sandhu et al. 2017). Further, by employing a biotechnological approach, it was shown that coexpression of Arabidopsis *SOS1*, *SOS2*, and *SOS3* in alfalfa enhances proline biosynthesis and salinity tolerance (Wang et al. 2019). Additionally, increased soluble sugar levels have been linked with salinity tolerance in alfalfa (Rahman et al. 2015). A positive association has been shown between higher levels of accumulation of pinitol and sucrose in leaves and starch in roots of salt-tolerant genotype of alfalfa in response to salt stress (Bertrand et al. 2015). Alfalfa plants also synthesize osmoprotectant Pro betaine during salinity stress (Trinchant et al. 2004).

9.4.3 ROS Production, Oxidative Stress, and Antioxidant System

Excess production of ROS in plants in response to salinity stress is a secondary effect due to various factors, including water deficit induced stomatal closure, increase of leaf and plant temperature, CO_2 deficit, and photosynthesis inhibition (Gill and Tuteja 2010). Salinity stress induces accumulation of H_2O_2 (hydrogen peroxide), O_2^- (superoxide radicals), and free radicals in cellular regions, including mitochondria, chloroplast, and

apoplastic space. Overproduction of ROS during salinity stress (and in response to other stresses) severely affects the balance between oxidants and antioxidants (Gill and Tuteja 2010). Salinity stress-induced oxidative stress has an additive negative effect on different crop species, including alfalfa.

Plants produce antioxidants to remove ROS that in turn helps to maintain balance between oxidants/antioxidants. Plants produce both non-enzymatic antioxidants (ascorbic acid, glutathione, vitamin E, phenolics, etc.) and enzymatic antioxidants (catalase, peroxidase, superoxide dismutase, etc.) (Ashraf 2009). These facts indicate that, in addition to non-enzymatic antioxidants, the expression status of detoxifying enzymes may determine salinity tolerance of different cultivars of alfalfa. In comparison to control, salt-treated alfalfa seedlings show higher activities for antioxidative enzymes catalase (CAT) and peroxidase (POD); and higher accumulation of antioxidant reduced glutathione (Luo et al. 2019a). A recent study comprising proteomics and metabolomics studies revealed that in response to salinity stress in alfalfa, ROS scavenging systems including CAT, POD, and proteins associated with glutathione metabolism were upregulated, which indicated that the antioxidant system played critical roles in protecting alfalfa in response to salinity stress (Li et al. 2020). Coexpression of Arabidopsis SOS pathway genes in alfalfa has been shown to enhance activities of antioxidant enzymes, CAT, POD, and superoxide dismutase (SOD), and led to enhanced salt tolerance (Wang et al. 2019).

Salt stress-induced lipid peroxidation is mediated by ROS. The level of MDA indicates the status of general peroxidation. An increase in ion leakage, malondialdehyde (MDA) level, and H_2O_2 has been observed in response to salt stress in alfalfa (Luo et al. 2019a). Under salinity, a salt-tolerant alfalfa accumulated less H_2O_2 and a lower level of MDA in comparison to the susceptible variety due to activation of the antioxidant enzymes or H_2O_2 scavengers (Rahman et al. 2015).

9.4.4 Phytohormones

Multiple phytohormones are known to play roles in response to salinity stress, including abscisic acid (ABA), auxin, ethylene, gibberellins (GA), brassinosteroids (BRs), jasmonic acid (JA), and melatonin (Kaundal et al. 2021; van Zelm et al. 2020). The role of ethylene in salinity tolerance is dependent on species. For example, ethylene is linked to salinity tolerance in *Arabidopsis* by retaining K^+ in roots and shoots (Yang et al. 2013). In contrast, it appears that ethylene plays a negative regulatory role in salinity tolerance in rice seedlings (Tao et al. 2015). In alfalfa, ethylene provides salinity tolerance during seed germination and seedling growth under saline stress which is dependent on putative ethylene receptor *MsETR2* (Wang et al. 2020). The abscisic acid signaling pathway has been implicated as a positive regulator of salinity tolerance in alfalfa (Li et al. 2020). Exogenous application of melatonin in alfalfa improves antioxidant capacity, reduces Na^+ accumulation that in turn enhances salinity tolerance (Cen et al. 2020).

9.4.5 Transcription Factors

Several alfalfa transcription factors have been shown to play important roles during salinity stress, which include MYB, WRKY, ethylene response factor (ERF), and *Alfin1*. Constitutive expression of alfalfa *MYB2L* in *Arabidopsis* enhanced salt tolerance ability by regulating proline biosynthesis (Song et al. 2019). Overexpression of a salinity stress-inducible transcription factor, *MsMYB4*, provided tolerance against salinity stress in *Arabidopsis* in an ABA-dependent manner (Dong et al. 2018). However, some MYB transcription factors also have been implicated in the negative regulation of salinity tolerance (Kim et al. 2013).

Differential expression of several WRKY transcription factors has been reported in response to salt stress (Mao et al. 2020). Overexpression of alfalfa *WRKY11* in soybean enhanced salinity tolerance at the seedling stage,

suggesting that *MsWRKY11* is a positive regulator of salinity stress (Wang et al. 2018).

ERFs play critical roles in providing salinity tolerance in various plants (Chen et al. 2012a). It has been shown that the expression of alfalfa *ERF8* in tobacco plants enhanced tolerance to salinity stress (Chen et al. 2012a). Overexpression of alfalfa *ERF11* that is induced in response to NaCl and phytohormones including ethylene, enhanced salinity tolerance in *Arabidopsis*, suggesting that *MsERF11* may be critical during salinity stress response (Chen et al. 2012b).

Overexpression of *Alfin1*, a putative transcription factor in alfalfa, regulates the expression of *MsPRP2* and provides salinity tolerance (Winicov and Bastola 1999).

9.4.6 Role of DNA Methylation and Histone Methylation in Salinity Tolerance

The addition of a methyl group to cytosine residues of the DNA molecule is known as DNA methylation, which is a biological process that is catalyzed by a group of methyltransferase enzymes. Environmental stresses could change DNA methylation/demethylation status in plants, modulating gene expression status of various genes (Zhang et al. 2018). Salinity stress-induced DNA methylation has been shown in *Arabidopsis* (Jiang et al. 2014). In alfalfa seedlings, salinity stress-induced slight increase of DNA methylation has been shown in response to 20 dS m^{-1} NaCl treatment (Al-Lawati et al. 2016). The use of 5-azacytidine, a DNA demethylation agent, inhibits the growth of salt-treated alfalfa seedling, suggesting that DNA demethylation decreases salinity tolerance in alfalfa seedlings (Al-Lawati et al. 2016). A recent study indicated that activation of the *MsMYB4* transcription factor is linked to elevated levels of histone H3K4 trimethylation and H3K9 acetylation in specific regions of the promoter sequence (Dong et al. 2020). Although more detailed investigations are warranted, these findings suggest that DNA methylation and histone methylation play regulatory roles in the salinity tolerance of alfalfa.

9.4.7 Future Prospects of Genetically-Modified Salt-Tolerant Alfalfa

Approximately 450 million tons of alfalfa are produced worldwide on 30 million acres (12,140,570 ha), with the leading producers being the USA (30%), Europe (25%), and Argentina (23%) (Barros et al. 2019). In 2017, the international trade of alfalfa hay reached 8.3 million metric tons with a total value of US\$ 2.3 billion (2nd Alfalfa World Congress 2018). This international congress emphasized the growing need for abiotic and biotic stress resistance breeding and the use of low-quality waters for irrigating forage crops to save freshwater for human consumption. Alfalfa was the first forage crop to be genetically modified for a low concentration of lignin to improve animal digestibility, commercialized with the trade name HarvXtra™ (Barros et al. 2019). A genetically modified glyphosate-tolerant alfalfa was deregulated in the US in 2010 and, a stacked-trait alfalfa with reduced lignin and glyphosate tolerance became available in 2015. This genetically modified alfalfa accounts for ~15% of the alfalfa currently cultivated in the US and is expected to reach 50% in 10 years when Canada and Mexico are expected to deregulate GM alfalfa cultivation and import (Fernandez-Cornejo et al. 2016; Barros et al. 2019). Following on the successes of these previously desired traits incorporated into alfalfa, other of great importance should follow. Some of the desired traits would allow the crop to thrive under reduced irrigation (a must for a high-water demanding crop), irrigation with recycled and with highly saline waters (unfit for most crops), and the production of condensed tannins. Of course, not all those traits may be economically feasible, and the most important ones would have to be chosen, such as traits for drought and salinity tolerance.

9.5 Concluding Remarks

Alfalfa is considered moderately tolerant to salinity; however, salinity tolerance varies among alfalfa genotypes. Upon exposure to high salinity, alfalfa is affected by both osmotic and ionic stresses. As a forage crop, biomass yield and quality are crucial traits for alfalfa. Multiple genotypes have been identified that have a high STI for biomass yield. Recent findings suggest that branching ability under salinity may be an important trait to be considered while breeding for salinity tolerance in alfalfa (Sandhu et al. 2017; Kaundal et al. 2021).

Gene expression-, biochemical-, and physiological analyses indicate that various salt-stress signaling pathways and mechanisms known in model systems are also conserved in alfalfa. Various component traits of salinity tolerance mechanisms have been identified in alfalfa and several candidate genes underlying those traits have been recognized (Sandhu et al. 2017). Progress has been made to develop salt-tolerant transgenic alfalfa plants by expressing many key genes (native/foreign) known to play key roles in salinity tolerance in model systems (e.g., *Arabidopsis*). Different researchers reported tolerance levels of transgenic alfalfa at different stages of development, like during germination, at seedling stage, or at matured stage. Additionally, it is necessary to examine the salinity tolerance ability of transgenic alfalfa in real environmental conditions and over multiple generations.

The genetic dissection of salt tolerance mechanisms is challenging in alfalfa because of its self-incompatible and polyploid nature. Nevertheless, several dozen markers have been identified that are associated with salt stress tolerance by employing GWAS and other approaches that used hundreds of accessions or several advanced breeding populations of alfalfa (Yu et al. 2016; Liu and Yu 2017; Liu et al. 2019; Medina et al. 2020). Validation of various identified markers will

facilitate the use of marker-assisted selection in alfalfa breeding programs.

Besides known genes, hundreds of differentially expressed genes have been identified by the transcriptomics approach in response to salinity stress in salt-tolerant alfalfa cultivars (Kaundal et al. 2021; Lei et al. 2018; Postnikova et al. 2013). Similarly, hundreds of differentially abundant proteins have been identified in salt-tolerant cultivars of alfalfa by a comparative proteomic approach (Rahman et al. 2015; Dong et al. 2018). Future functional genomics studies will expedite the characterization of the most significant genes identified in transcriptomic/proteomic studies to identify their biological roles during salinity stress. Selected candidate genes can then be employed to develop salt-tolerant alfalfa cultivars by traditional breeding and/or genetic engineering.

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