SILVERLEAF SUNFLOWER (*HELIANTHUS ARGOPHYLLUS*, TORREY AND GREY) IN SUNFLOWER BREEDING: FROM COMPARATIVE MAPPING TO THE GENETICS OF SALT TOLERANCE IN THE SPECIES

by

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(Under the Direction of Roger Boerma and Charles Brummer)

ABSTRACT

Cultivated sunflower (*Helianthus annuus* L.) is the fourth most important oil crop worldwide and its genus *Helianthus* comprises 50 species which are native to North America. Due to a genetic bottleneck during domestication, cultivated sunflower lacks the necessary variability to adapt to changing biotic and abiotic conditions. To overcome this deficiency in the domesticated germplasm, breeders have utilized wild species to expand the genetic variability to be incorporated into elite sunflower breeding populations. *H. argophyllus* is the closest relative of *H. annuus* and has been extensively used in sunflower breeding. We developed a *H. argophyllus* high density genetic linkage map with 1549 EST-SNP markers. Through comparative mapping with a consensus *H. annuus* map sharing 1445 EST-SNP markers we were able to identified 11 colinear chromosomes and four chromosomes rearrangements (two non-reciprocal translocation, one reciprocal translocation and one inversion). In spite of these rearrangements affecting gene-flow between species most of the *H. argophyllus* genome is colinear with *H. annuus* facilitating its introgression and use in sunflower breeding. Since *H. argophyllus* has been reported to be salt tolerant, we also studied the feasibility of using this

species as a source of salt tolerance alleles. We performed QTL analysis in two generations (F2 and BC1S1) of a cross between *H. annuus* and *H. argophyllus* using Bayesian QTL interval mapping to elucidate the complexity of salt tolerance in sunflower. We were able to identify 5 and 10 QTL for the F2 and BC1S1 generations, respectively, responsible for salt tolerance rating, weighted salt tolerance rating, and SPAD value. QTL analysis using two generations of the same cross and QTL comparison with other salt tolerance studies in sunflower allowed us to identify three important genomic regions for salt tolerance. We also discovered that salt tolerance in sunflower is highly complex and epistatic interactions are of greatly importance in the expression of the trait. In addition, we performed QTL analysis of a BC2 Testcross set of families growing under saline and non-saline conditions. This experiment helped us make inferences about the best strategy to be use in the improvement of salt tolerance in sunflower. We found 22 and 26 QTL responsible for nine traits related to productivity and salt tolerance under saline and non-saline conditions, respectively. We found only two QTL in common between both saline treatments which indicated a large genotype by salt condition interaction. The QTL found were also of small effect which it will affect their utility in marker-assisted-selection.

INDEX WORDS: Helianthus annuus, Helianthus argophyllus, Interspecific Hybridization, Comparative Mapping, SNP Markers, QTL Mapping, Salt Tolerance

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DEDICATION

This dissertation is dedicated to my wife Vanesa for walking by my side throughout the rough years of graduate school so far from home. I will be endless grateful for her encouragement, love and patience. None of my accomplishments during these years would be possible without her. To my sons Joaquin and Nicolas, for being the source of the greatest happiness that I have ever experienced in life. To my mother, Gloria for her love, support and resignation of having us far from home. To the memory of my father, Juan Manuel that even is not among us to celebrate this moment… I would like to tell him: EL SACRIFICIO NO FUE EN VANO VIEJO…LO LOGRE!

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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The sunflower genus *Helianthus*, comprising 50 species native to the Americas (Schilling and Heiser, 1981), is an economically and evolutionary important taxon that includes cultivated sunflower (*Helianthus annuus* L.), the fourth oil crop in importance worldwide (Fernández-Martínez et al., 2009). The main use of sunflower seed has been for its oil, but other uses such as snack (confectionary seeds), bird feed, biodiesel production, and potentially biomass are also important. In addition to its role as an important crop, sunflower along with other *Helianthus* species have contributed to science as a model for genetics studies of adaptation and speciation (Lai et al., 2005a; Rieseberg, 1995; Rieseberg et al., 1995a).

Due to a genetic bottleneck during domestication, cultivated sunflower lacks the wide variability necessary to adapt to new arising biotic and abiotic factors restricting productivity. For this reason, sunflower breeders have been looked for variability in wild *Helianthus* species. These species have been donors of favorable alleles for increasing genetic diversity and enhancing agricultural traits in sunflower breeding for many decades (Korell et al., 1996; Quagliaro et al., 2001; Quresh et al., 1993; Seiler, 1992; Tavoljanski et al., 2002; Velasco et al., 2007). In this work we focus our efforts on silverleaf sunflower (*H. argophyllus* Torrey & Gray), the closest relative of cultivated sunflower (Schilling and Heiser, 1981). *H. argophyllus* has been extensively used as a donor of favorable alleles in sunflower improvement (Seiler, 1992; Seiler et al., 2007), and it has been suggested as a source of tolerance to abiotic stresses (Rauf, 2008; Richards, 1992).

For the effective use of *H. argophyllus* in sunflower breeding the knowledge of its genome differences with *H. annuus* it would be of enormous importance. Comparative mapping helps to identify chromosome rearrangements that have occurred during the evolution of plants and animals. These chromosome rearrangements represent a barrier for gene flow between related plant taxa (Barton and Bengtsson, 1986; Rieseberg, 2001; Rieseberg et al., 1995b; Rieseberg et al., 1999) . The study of karyotypic differences helps to explain the common evolutionary history among related taxa (Burke et al., 2004; Hackauf et al., 2009; Jones et al., 2002; Paterson et al., 2009) and it provides clues for the use and introgression of wild germplasm as a source of favorable alleles for crop improvement (Chetelat and Meglic, 2000; Dirlewanger et al., 2004; Foulongne et al., 2003).

In our first experiment we constructed the first high density linkage map of *H. argophyllus* using EST-SNP markers for the purpose of making comparisons with an ultra high-density *H. annuus* linkage map. The objectives of our first study were to: i) identify chromosomal differences between *H. annuus* and *H. argophyllus*, ii) gain a better understanding of the common evolutionary history between both species, and iii) quantify the impact of the chromosomal rearrangements identified in the use of *H. argophyllus* in interspecific crosses with *H. annuus*. These results could be applied to the prediction of introgressed segments from *H. argophyllus* into cultivated sunflower.

Since *H. argophyllus* grows in dry areas, sometimes close to the ocean where salinity is present, it has been suggested that it could be a good source of alleles for salt tolerance (Seiler et al., 2007). Salinity is a major abiotic stress threatening food production in many areas around the world, especially in arid and semi-arid climates. Around 6% of the world's land is affected either by salinity or sodicity, which is a secondary effect of salinity in clay soils

(www.fao.org). The main approach to overcome the problem of salinity for crops under cultivation is the increase in salt tolerance of crops through plant breeding. Cultivated sunflower has been classified as moderately salt sensitive based on water stress index (Katerji et al., 2003). *H. argophyllus* could be a good donor of alleles to improve salt tolerance in sunflower. Salt tolerance, with a few exceptions, is a complex trait involving the function of many genes in most crop species (Ashraf et al., 2009). In sunflower there is just one previous study of the genetics of salt tolerance by means of QTL mapping (Lexer et al., 2003b) in an interspecific cross between *H. annuus* and *H. petiolaris*. Further studies of the genetics of salt tolerance in sunflower are needed to inform the development of improved salt tolerance in cultivated sunflower.

Our objectives in the second experiment in this dissertation were: i) identify QTL responsible for salt tolerance using Bayesian analysis in an interspecific cross between *H. annuus* and *H. argophyllus*, ii) validate the QTL results through QTL comparison between two generations of the same cross, iii) compare our results with previous QTL studies for salt tolerance in sunflower, iv) elucidate the complexity of salt tolerance in sunflower through the identification of important epistatic QTL pairs contributing to salt tolerance, and vi) discuss the implications of our findings for use in sunflower breeding. In order to achieve these objectives we performed QTL analysis for salt tolerance rating and SPAD value in two generations (F_2 and BC2S1) of the same cross between *H. annuus* and *H. argophyllus* growing under high salinity conditions (300 mM of NaCl) in the greenhouse.

Efforts to improve crop productivity under saline conditions through conventional breeding have been conducted in several crops with limited success, mainly due to problems inherent to the nature of salinity in the soil (Flowers, 2004). One of the problems that plant breeders must address when they breed crops for salt tolerance it is the spatial and temporal

heterogeneity of salinity in the field (Cetin and Kirda, 2003). Richards (1983; 1992; 1995) believes that due to the heterogeneity of salinity in soils breeders should concentrate their efforts on selecting for yield in non-saline environments and rather than breeding for salt tolerance. It is expected that genotype by salinity interactions would be present, so the validity Richards' argument would be in doubt. A study of the genotype by salt interaction would provide data to choose the appropriated strategy to breed for salt tolerance in crops.

In order to address important questions about breeding for salt tolerance in sunflower, we performed a third experiment. In this experiment a BC2 Test Cross set of families (resembling the type of cultivar grown for sunflower production) was grown under saline (150 mM of NaCl) and non-saline conditions in the greenhouse and morphological traits were measured. The objectives for the third study in this dissertation were: i) identified QTL for morphological traits in an interspecific population between *H. annuus* and *H. argophyllus* growing under saline and non-saline conditions, ii) make comparisons of the QTL found for the morphological traits under different saline conditions, iii) assess the importance of *H. argophyllus* as donor of favorable alleles for salt tolerance to sunflower breeding, and iv) make inferences about the best strategy to be used for the improvement of salt tolerance in sunflower.

LITERATURE REVIEW

Sunflower

The genus Helianthus, comprising 50 species native to the Americas (Schilling and Heiser, 1981), is an economically and evolutionary important taxon that includes cultivated sunflower, one of the main oilseed crops worldwide (*Helianthus annuus* L.). Cultivated sunflower is a diploid species with chromosome number 2n=34, and is an outbreeding species with a self-incompatibility systems preventing self-fertilization. Sunflower seeds are consumed in other forms than for its oil. Significant amounts of seeds, especially large sized seeds, are sold

as roasted or dehulled snacks for human consumption. Smaller sized seeds are used for bird feed and small animal. In recent years, production of biodiesel from sunflower oil has acquired a greater importance due to a larger demand of alternative energy sources (Knapp, personal communication).Besides its important role as crop, sunflower along with several other *Helianthus* species have contributed to science as model for genetics studies of adaptation and speciation (Lai et al., 2005a; Rieseberg, 1995; Rieseberg et al., 1995a; Vekemans).

Interspecific Hybridization in Sunflower Breeding

Due to a bottleneck of gene-flow that occurred during domestication, cultivated sunflower lacks the genetic diversity to adapt to emerging biotic and abiotic stresses. For this reason, wild sunflower species have been used extensively in sunflower breeding as a donor of favorable alleles for increasing genetic diversity and enhancing agricultural traits (Korell et al., 1996; Quagliaro et al., 2001; Quresh et al., 1993; Seiler, 1992; Tavoljanski et al., 2002; Velasco et al., 2007). These diverse species are adapted to a wide range of habitats and possess considerable variability for most economic and agronomic characteristics (Seiler, 1992).

There are 12 wild annual *Helianthus* species with the same haploid chromosome number as cultivated sunflower that can be easily used in interspecific crosses (Heiser et al., 1969). Among these species, silverleaf sunflower (*H. argophyllus* Torrey & Gray), the closest relative of common sunflower (Schilling and Heiser, 1981), has been widely used as donor of disease resistance alleles (Dussle et al., 2004; Heiser Jr, 1951; Miller and Gulya, 1991; Radwan et al., 2004; Seiler et al., 2007; Slabaugh et al., 2003; Wieckhorst et al.), fertility restoration to PET1 cytoplasm (Chepurnaya et al., 2003), and cytoplasmic male sterility (Horn et al., 2002). It has also been suggested that it can be a source of favorable alleles for salt and drought tolerance (Rauf, 2008; Richards, 1992) and insect resistance (Rogers and Thompson, 1980; Rogers et al.,

1987; Sujatha and Lakshminarayana, 2007). It is important to remember that most of *H. argophyllus* introgressions into *H. annuus* have been done by phenotypic selection and location of the introgressed segments are unknown. A few exceptions include the identification of genomic locations of introgressions of *H. argophyllus* segments harboring downy mildew (Plasmopara halstedii (Farl.) Berl. and de Toni) resistance (*R*) genes (Dussle et al., 2004; Slabaugh et al., 2003). These include the introgression line (RHA340), which is resistant to downy mildew races 2, 3, and 4 (Miller and Gulya, 1988), and another introgression line carrying a segment of ARG1575-2 on linkage group 1 developed by phenotypic selection with resistance to races 300, 700, 730, and 770 of downy mildew (Dussle et al., 2004).

A cross between *H. annuus* and *H. argophyllus* produces offspring with reduced pollen viability and chromosomal abnormalities (Quillet et al., 1995), resulting in restrictions for gene introgression. For a more efficient use of *H. argophyllus* in sunflower breeding it is essential to achieve a better understanding of the genomic differences with *H. annuus*. Knowledge of the differences between these species would help in the development of strategies for introgression through marker-assisted-selection.

Comparative Mapping

Comparative mapping narrates the history of chromosome rearrangements that have occurred during the evolution of plants and animals. These chromosome rearrangements represent a barrier for gene flow between related plant taxa (Barton and Bengtsson, 1986; Rieseberg, 2001; Rieseberg et al., 1995b; Rieseberg et al., 1999). The study of such karyotypic differences, as well as the conserved or synteny regions among genomes of dissimilar species, is of special interest for both plant breeders and evolutionary biologists. The understanding of these karyotypic differences helps to explain the common evolutionary history among related taxa

(Burke et al., 2004; Hackauf et al., 2009; Jones et al., 2002; Paterson et al., 2009). It also helps to locate and identify useful genes in crop species by extrapolating information regarding gene order from well studied model species (Choi et al., 2004; Dilbirligi et al., 2006). More pragmatically, it provides guidance for the use and introgression of wild germplasm, potentially a source of favorable alleles for crop improvement (Chetelat and Meglic, 2000; Dirlewanger et al., 2004; Foulongne et al., 2003).

The use of high-density genetic maps to compare common markers among species still remains the main option in comparative mapping for species where the full genome sequence is not available. As a rule, the more DNA markers that are used the more accurate the identification of homologous loci, chromosome rearrangements, and collinear segments becomes. The development of high-throughput DNA marker genotyping technologies have permitted the construction of very dense genetic maps in plants (Anithakumari et al., ; Deulvot et al., ; Eckert et al., 2009; Yan et al., 2009). As a result of these applications, more accurate comparative studies have been conducted (Jermstad et al., 2010 , Shinozuka et al., 2010).

Cytological studies have identified meiotic abnormalities predicting differences in two reciprocal translocations between *H. annuus* and *H. argophyllus* (Chandler et al., 1986; Quillet et al., 1995). A more recent comparative mapping study using DNA markers has reported differences of five non-reciprocal translocations and two inversions between genomes (Heesacker et al., 2009). In this work a *H. argophyllus* map of 299 DNA markers (SSR, INDEL, and SSCP) was constructed and compared against an *H. annuus* reference map using 131 DNA orthologous markers. Besides the seven chromosomal rearrangements the study of Heesacker et al. (2009), showed a complex evolutionary history, including entire chromosome duplications and fusion of chromosome fragments. Despite the importance of this seminal study on *H.*

argophyllus, further research with more dense maps would be of immense interest to better elucidate karyotypic evolution between both species.

The Problem of Salinity

Soil salinity is an important abiotic stress imposing constraints to agricultural productivity in many areas around the world. According with FAO (http://www.fao.org/nr/land/en/), 6% of the world's land is affected either by salinity or sodicity, as a secondary effect of salinity in clay soils. The definition of salinity has been adopted from FAO (www.fao.org). This definition classifies a soil as saline when the electrical conductivity of the saturated extract (ECe) is greater than 4 dS m^{-1} and soils with an ECe of 15 dS m^{-1} or more are strongly saline.

Salinity problems occur due to natural or human-induced processes and result in an excessive accumulation of soluble salts that can suppress plant growth. Natural or primary salinity occurs due to the accumulation of salt in soils over a long period of time, and is caused by two natural processes. The first one is the weathering of the parental material containing soluble salts, especially chlorides of sodium, magnesium, and calcium. The second is the deposition of oceanic salt carried inland by wind and incorporated to soils by rainfall (Peck, 1997; Tanji, 1990). Human-induced or secondary salinity is caused by human activities resulting in changes of the hydrological balance of soils. These changes could be the result of the replacement of natural perennial vegetation by annual crops. In Western Australia the replacement of natural vegetation by farmlands has been the cause of the increase in salinity of more than 1.8 millions of hectares (George et al., 1997). The consequence of this replacement is that annual crops with shallow root systems are not able to use existing water supplies from precipitations. The hydrological balance is then disturbed and the excess of water results in a rise

of the water table mobilizing salts stored in the subsoil to roots zone. Another human activity that could cause an increase in the salinity level of soils is irrigation. Excess irrigation with salty water or even with good quality water in poor drained soils could accumulate of salts in roots zone (Chhabra, 1996; Ghassemi et al., 1995).

With the prediction in population growth from 6.1 billion in mid-2001 to 9.3 billion in 2050 (http://www.unfpa.org/swp/2001/), it is evident the need of more agricultural land area and/or the increase in productivity of the current areas under cultivation in order to increase the food supply. One obvious way to increase food supply is claim for cultivation the areas of the world affected by salinity or cultivate desserts and dryland areas with the use of irrigation. This practice would result in increase soil salinity. Regardless of how we increase the world's food supply we will need to make extra efforts to improve crop performance under saline conditions.

Effect of Salinity on Plants

Salinity inhibits plant growth through two mechanisms. First, it reduces the ability of the plant to take up water resulting in reduced in growth. Osmotic balance is essential for plants growing in salinity soils. If such balance is not reached, the result is loss in turgidity, increase cell dehydration, and finally cell death (Ashraf, 2004). Osmotic adjustments in plants under salt stress can be achieved by accumulation of high concentrations of inorganic or organic solutes, or both, within cells. The attempt to adjust the osmotic balance within cells leads to the second mechanism of growth inhibition. Accumulation of salt related ions, mainly Na^+ and Cl^-, within the cell produce damage that reduces plant growth, a process referred to the specific ion effect of salinity (Greenway and Munns, 1980). With high accumulation of ions over a long period of time the leaves will start to died (mainly old leaves). This leaf death is essential for the survival of the plant. If new leaves are produce at a rate greater than the death of old leaves, there is a

greater opportunity of survival and reaching the reproductive stage (Munns, 2002). The two mechanisms of growth inhibition give rise to a two-phase growth response to salinity over time. The first phase of growth reduction is rapid and is due to the salt concentration outside of roots. The second phase of growth reductions takes time to develop, and result from internal damage due to salt accumulation within cells (Munns, 2002).

One of the first and most rapid effects of salinity on plants is the decrease in stomatal aperture due to perturbed water relations (Fricke et al., 2004). This effect is caused by a decrease in osmotic potential in the roots zone (Hatami et al., ; Vysotskaya et al.). Photosynthesis rate will be affected by stomatal closure as well as some other non-stomatal effects on bioenergetic processes (Melesse and Caesar, 2008; Sudhir and Murthy, 2004). Chlorophyll content is increased under moderate salinity but high salinity concentrations decrease chlorophyll content in a short time period (Santos, 2004). The reduction in photosynthetic rate leads to formation of Reactive Oxygen Species (ROS) inducing oxidative stress (Sairam et al., 2005; Vaidyanathan et al., 2003). The excess of these citotoxic compounds can destroy normal metabolism through oxidative damage of lipids, proteins, and nucleic acids (McCord, 2000).

High ionic salt concentration produce visible symptoms such as necrosis and leaf tip burn due to Na^+ and Cl^- ions (Wahome et al., 2001). The increase in ions concentration within cells may disturb membrane integrity and cellular functions through the inhibition of enzymes. NaCl uptake by the plant also competes with the uptake of other ion nutrients such as K^+, N, P and $Ca2^+$ causing nutritional deficiency symptoms (Grattan and Grieve, 1998).

Mechanism of Salt tolerance in Plants

Plants will survive under salinity conditions if they are able to continue taking up water and exclude a large proportion of the salt from the soil solution. The mechanism of salinity

tolerance can be classified in three categories; tolerance to osmotic stress, Na^+ exclusion, and tissue tolerance (Munns and Tester, 2008b). Relative importance of osmotic tolerance still remains unknown for most species due to the relative difficulty of quantifying this parameter. There should be an association between osmotic tolerance and tissue tolerance of Na^+ because genotypes that can tolerate high Na^+ concentrations in leaves should also be tolerant to osmotic stress due to their high osmotic adjustment (Munns and Tester, 2008b).

. The site of the plant where ion salt toxicity is expressed most obviously in the leaf blade, where Na^+ it is transported by the transpiration stream (Munns, 2002). Most Na^+ transported from roots (soil solution) to the shoots remains and is accumulated in leaves. The processes determining the accumulation of Na^+ in the shoots will be those that control the entrance (influx) or exit (efflux) of Na^+ from the root xylem. Roots must exclude most of the Na^+ and Cl^- in the soil solution. Na^+ inters into cells through K^+ pathways due to the fact that the hydrated ionic radii of Na^+ and K^+ are similar and difficult to differentiate (Blumwald, 2000). Cells use high and low affinity channels for uptake K^+ and Na^+ can get into cells through these K^+ transporters. Three classes of low affinity K^+ channels have been identified. Two of these are involved in the influx of Na^+ into the cells via K^+ outward rectifying channels (KORCs) (Maathuis and Sanders, 1995; Wegner and Raschke, 1994) and voltage independent cation channels (VIC) (Amtmann et al., 1997; White, 1999). Regarding the high affinity channels, there are two families involved in K^+ transport into the cell that will determine the Na+/K+ ratio into cells.. The K+ uptake transporter-high affinity K^+ transporters (KUP-HAK), (Quintero and Blatt, 1997; Santa-Maria et al., 1997) and the high affinity K^+ transporters (HKT) (Schachtman and Schroeder, 1994; Wang et al., 1998).

Mutations in any of the genes encoding constitutive proteins of these channels could modify the uptake of K^+ and Na^+ interfering in the Na^+/K^+ relationship conferring more or less susceptibility to salinity through Na^+ exclusion from cells. Another cell strategy for salt tolerance is the regulation of Na^+ concentration in the cell cytosol through extrusion of Na+ mediated plasma membrane H^+-ATPase (Sussman, 1994).

Plants cannot tolerate high salt concentrations in the cytoplasm, and to survive, they either need to restrict the excess of salts, as previously described, or compartmentalize the ions in different tissues not affecting essential enzymatic activity. One way to avoid the noxious effects of Na^+ in vital tissues is partitioning Na^+ in older tissues as storage compartments that will be eventually sacrificed (Parida and Das, 2005). Na^+ can also be stored in the vacuoles of the cells avoiding high concentrations of the ion in the cytosol where it would be more toxic (Munns and Tester, 2008b). The increase of Na^+ in the vacuoles will require an increase of the osmotic pressure of the cytosol to maintain the cellular volume. This can be achieve by an increase of compatible solutes (Parida and Das, 2005) such as, proline, glycine betaine sugars, and polyols (Ashraf, 2004; Ashraf and Harris, 2004; Juan et al., 2005).

Breeding for Salt Tolerance in Crops

Efforts to improve crop salt tolerance using conventional breeding have been conducted in several species with some success. Some promising results to improve salinity tolerance in alfalfa through recurrent selection have been accomplished (Al-Khatib et al., 1992). In this study researchers used shoot length of alfalfa seedlings growing in saline media as a selection criterion for salt tolerance. The selected individuals produced more shoot fresh and dry weight than the unselected individuals. Furthermore, Johnson et al., (1992) suggested that direct selection for biomass yield in alfalfa growing under saline conditions could improve forage yield at low and

moderated salinity levels. In white clover Rogers et al., (1997) selected individuals growing under saline culture with low Cl^- accumulation in shoots. They found that within the cultivar Haifa, the individuals with the less concentration of Cl^- in the shoots also produced the largest amount of dry matter. Selection for shoot growth in maize seedlings growing under saline conditions identified individuals with high dry weight production (Ashrai and McNelly, 2006). These results seemed to justify improving maize populations under salt conditions through recurrent selection.

Saranga et al., (1992) exploited inter-specific variation for salt tolerance in a cross between *Lycopersicum esculentum* and *L. pennellii* by selection of BC1 plants and BC1S1 families for fruit yield and dry matter under saline conditions. They reported high gains from selection for yield in this population. Although considerable progress has been made for improving crops for salt tolerance through conventional breeding, the progress is not satisfactory (Flowers, 2004). The main reasons for this relatively "lack" of success in using conventional breeding for salt tolerance includes the high genetic complexity and low magnitude of genetic variation of the trait along with: i) it is time consuming and labor intensive, ii) unsiderable genes could be transferred along with salt tolerant genes (when interspecific crosses are involved), and iii) reproductive barriers restrict transfer of favorable alleles from inter-specific and intergeneric sources (Ashraf et al., 2008).

Salt tolerance is a complex trait involving the function of many genes (Ashraf et al., 2009) and the screening and selection of salt tolerant individuals is influenced by environmental factors (Ashraf et al., 2008). Due to the proposed problems with conventional breeding for salt tolerance, it has been suggested that a best alternative for salt tolerance improvement would be the identification and use of molecular markers associated with salt tolerance loci (quantitative

trait loci or QTL). In this approach molecular markers which are unaffected by the environment areemployed for selection in the breeding programs (Flowers, 2004). Rice has been the target of intensive research on molecular breeding for salt tolerance. In rice, there are several studies reporting QTL for survival days of seedling under salinity (Lin et al., 2004), seedlings traits related to salt tolerance (Haq et al., 2008; Lee et al., 2007; Prasad et al., 2000), physiological traits determining salt tolerance (Ammar et al., 2009; Koyama et al., 2001), and for salt tolerance of seedlings based on visual score (Lee et al., 2006). Maybe one of the most successful findings is a major QTL (Saltol), explaining 80% of phenotypic variation, responsible for maintaining the Na^+/K^+ homeostasis within the cell in seedlings under saline conditions. This QTL is located on chromosome 1 and was found in a cross between the cultivar IR9 and a salt tolerance landrace (Bonilla et al., 2002). Assessment of rice genotypes using microsatellite markers associated with Saltol QTL was done, validating the effects and usefulness of the QTL in rice breeding (Mohammadi-Nejad et al., 2008). Furthermore, near isogenic lines (NILs) have been developed isolating Saltol and other minor QTL for salt tolerance from the rice landrace in different elite backgrounds (de Ocampo et al., 2008). The availability of these NILs will allow the pyramidization of multiple QTL to increase the level of salinity tolerance in rice elite germplasm. It is estimated that the use of Saltol as well as several other salt tolerant QTL (Gautam et al., 2009), will have huge economic advantages over conventional breeding (Alpuerto et al., 2009).

Abundant work has been done with excellent results in wheat. Ogbonnaya et al., (2008) found that a major QTL for Na^+ exclusion in shoots. While in contrast, Ma et al., (2007) found 47 QTL responsible for salt tolerance in several related traits during germination and the seedling stage located on almost all chromosomes. Munns et al., (2000) discovered a landrace wheat line

with low accumulation of Na^+ in the shoots. Later, Munns et al., (2003) concluded that the trait is dominated by two dominant genes. One QTL for low Na+ concentration in the leaf blade, named Nax1, accounted for 38% of the phenotypic variation and was mapped on chromosome 2A. This QTL was validated in genetically diverse backgrounds (Munns et al., 2003). The presence of second locus for Na^+ exclusion was confirmed through the observation of lines without Nax1 that possess low Na^+ accumulation and was named Nax2 (Lindsay et al., 2004). Both loci, Nax1 and Nax2 were physiologically characterized and their effects as Na+ excluders were confirmed. The development of molecular markers associated with Nax1 allowed its utilization for selection of low Na^+ concentration in leaves of progenies in the durum wheat breeding program at CIRO Australia (Munns et al., 2006).

Even though QTL for salinity tolerance have been discovered in several crops, most of them have small to moderate effects. This has made them difficult to use in a marker assisted selection program across a wide range of germplasm. As mentioned above, there are a few successful examples in molecular breeding for salt tolerance. Most of the information of QTL for salt tolerance found in crops has not been translated to practical applications through marker assisted selection.

Salt Tolerance in Sunflower

Sunflower has been classified as moderately salt sensitive based on water stress index (Katerji et al., 2003). Salinity delays sunflower germination and emergence due to the absorption of Na+ and Cl- through the hypocotyl (Katerji et al., 1994). The deleterious effects due to salinity, are associated with the inability of the plant to break down lipids and supply the embryonic tissues with soluble sugars (Ashraf et al., 2003). Salt also affects leaf expansion and biomass accumulation in seedlings (Delgado and Sánchez-Raya, 1999; Rawson and Munns,

1984). In addition, salt also results in an increase in oleic acid, decrease in linoleic acid, and a decrease in oil yield (Flagella et al., 2004). Loss of achene yield was also observed due to reduction in the number of seeds per head, while the percentage of seed oil was unaffected (Francois, 1996). Salt-tolerance in sunflower seems to be related to exclusion of Na^+ in the leaf lamina and to maintenance of almost uniform concentrations of this ion in leaves of all ages (Ashraf and O Leary, 1995). Accumulation of antioxidants under saline conditions was also linked with salt tolerance (Rios-Gonzalez et al., 2002). Furthermore, poliamides and other compatible solutes were found in higher concentrations in salt tolerant sunflower cultivars (Mutlu and Bozcuk, 2005). Genetic studies on salt tolerance, measured as dry shoot weight, showed heritabilities of 0.45 and 0.72 for two populations differing in salt tolerance (Ashraf et al., 1995). There is also significant variation in salt tolerance within the species and a the tolerance does not vary during the plant's stage of development (Ashraf and Tufail, 1995). These studies indicate the potential for selection during the initial vegetative growth stage.

Limited genetic studies of salt tolerance in sunflower have been reported. Lexer et al., (2003b), using QTL mapping, studied salt tolerance from an evolutionary point of view. They studied how, from two parental salt sensitive species (*H. annuus* and *H. petiolaris*), the salt tolerant species *H. paradoxus* could arise. They found that both species contributed favorable alleles for a rapid ecological divergence of the hybrid neospecies. Extending the previous results Lexer et al., (2004) found that Ca-dependent salt tolerant genes in wild sunflowers play and adaptative role and that transgressive segregation explained the origin of adaptative genetic variation in sunflower. These results focused on the evolutionary aspects of salt tolerance in sunflower but, it could be also useful to elucidate the genetics of this trait within the species. In

addition, the QTL information found could be used for the introgression of favorable salt tolerant alleles into elite sunflower breeding populations.

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CHAPTER 2

HIGH-RESOLUTION COMPARATIVE MAPPING BETWEEN COMMON SUNFLOWER (*HELIANTHUS ANNUUS* L.) AND SILVERLEAF SUNFLOWER (*HELIANTHUS ARGOPHYLLUS* TORREY AND GREY)[1]

[1] Rey, J.I., J.E. Bowers, J.M. Burke, E.R. Boerma and S.J. Knapp to be submitted to *Theoretical and Applied Genetics*

ABSTRACT

Comparative mapping has been shown to be an important tool in the study of the genome differences between dissimilar species. Through the study of genome differences we can describe the evolutionary history between species and as well as determine the implications for gene introgression from one species to the other. Silverleaf sunflower (*Helianthus argophyllus* Torrey and Grey) is the closest relative of common sunflower (*Helianthus annuus* L.) and has been extensively used in sunflower breeding. Knowledge of the genome differences between these two species would be useful to elucidate the evolutionary relationship between these to *Helianthus* species and to establish an efficient strategy for the introgression of *H. argophyllus* into *H. annuus*. We constructed a high-density EST-SNP linkage map of *H. argophyllus* and compared it to a consensus *H. annuus* map to examine the genome differences between these two species. Through the comparison of 1445 EST-SNP markers common to both maps we found the presence of 11 colinear chromosomes and four chromosome rearrangements between both species (two non-reciprocal translocations, one reciprocal translocation and one inversion). These four chromosome rearrangements are smaller than previously estimated in other studies. Karyotypic evolution was estimated to be 1.1 to 2.7 chromosomal rearrangements/MYA and is slower than previous estimates in sunflower, but still relatively fast compare with the evolution in other genera. In spite of the presence of four chromosome rearrangements, both genomes are mostly colinear and should allow the the introgression of *H. argophyllus* into *H. annuus*.

INTRODUCTION

Comparative mapping narrates the history of chromosome rearrangements that have occurred during the evolution of plants and animals. These chromosome rearrangements represent a barrier for gene flow between related plant taxa (Barton and Bengtsson, 1986; Rieseberg, 2001; Rieseberg et al., 1995b; Rieseberg et al., 1999). Then, the study of such karyotypic differences, as well as the conserved or synteny regions among genomes of dissimilar species is of special interest for both, plant breeders and evolutionary biologists. The understanding of these karyotypic differences helps to explain the common evolutionary history among related taxa (Burke et al., 2004; Hackauf et al., 2009; Jones et al., 2002; Paterson et al., 2009). It also helps to locate and identify useful genes in crop species extrapolating information regarding gene order from well studied model species (Choi et al., 2004; Dilbirligi et al., 2006). And, more pragmatically, it gives clues for the use and introgression of wild germplasm, potentially a source of favorable alleles for crop improvement (Chetelat and Meglic, 2000; Dirlewanger et al., 2004; Foulongne et al., 2003).

The use of high-density genetic maps to compare common markers among species still remains the main option in comparative mapping for species where the full genome sequence is still unknown. As a rule, the more DNA markers are used, the more accurate the identification of homologous loci, chromosome rearrangements, and collinear segments becomes. The development of high-throughput DNA markers genotyping technologies have permitted the construction of very dense genetic maps in plants (Anithakumari et al., 2010; Deuvot et al., 2010 ; Eckert et al., 2009; Yan et al., 2010) (Deulvot et al., ; Yan et al.). As a result of these applications, more accurate comparative studies have started to arise (Jermstad et al., ; Shinozuka et al.).

The genus Helianthus, comprising 50 species native to the Americas (Schilling and Heiser, 1981), is an economically and evolutionary important taxon that includes common sunflower, one of the main oilseed crops worldwide (*Helianthus annuus* L.). Besides its important role as crop, sunflower along with several other Helianthus species have contributed to science as model for genetics studies of adaptation and speciation (Lai et al., 2005a; Rieseberg, 1995; Rieseberg et al., 1995a; Vekemans). Moreover, wild sunflower species have been used widely in sunflower breeding as donor of favorable alleles for increasing genetic diversity and enhancing agricultural traits (Korell et al., 1996; Quagliaro et al., 2001; Quresh et al., 1993; Seiler, 1992; Tavoljanski et al., 2002; Velasco et al., 2007).

Silverleaf sunflower (*H. argophyllus* Torrey & Gray), the closest relative of common sunflower (Schilling and Heiser, 1981), has been widely used as donor of disease resistance alleles (Dussle et al., 2004; Heiser Jr, 1951; Miller and Gulya, 1991; Radwan et al., 2004; Seiler et al., 2007; Slabaugh et al., 2003; Wieckhorst et al.), fertility restoration to PET1 cytoplasm (Chepurnaya et al., 2003), and cytoplasmic male sterility (Horn et al., 2002). It has been also suggested that it can be a source of favorable alleles for salt and drought tolerance (Rauf, 2008; Richards, 1992) and insect resistance (Rogers and Thompson, 1980; Rogers et al., 1987; Sujatha and Lakshminarayana, 2007). It is important to understand that most of *H. argophyllus* introgressions into *H. annuus* have been done by phenotypic selection and location of the introgressed segments are unknown. A few exceptions include the identification of genomic locations of introgressions of *H. argophyllus* segments harboring downy mildew [Plasmopara halstedii (Farl.) Berl. and de Toni] resistance (*R*) genes (Dussle et al., 2004; Slabaugh et al., 2003). These include the introgression line (RHA340), which is resistant to downy mildew races 2, 3, and 4 (Miller and Gulya, 1988), and, another introgression line carrying a segment of

ARG1575-2 on linkage group 1 developed by phenotypic selection with resistance to races 300, 700, 730, and 770 of downy mildew (Dussle et al., 2004).

The cross between *H. annuus* and *H. argophyllus* produces offspring with reduced pollen viability and chromosomal abnormalities (Quillet et al., 1995), resulting in restrictions for gene introgression. For a more efficient use of *H. argophyllus* in sunflower breeding it is essential to achieve a better knowledge of the genomic differences with *H. annuus*. Knowledge of the differences between these species would help in the development of strategies for introgression through marker-assisted-selection. Primary cytological studies have identified meiotic abnormalities predicting differences in two reciprocal translocations between *H. annuus* and *H. argophyllus* (Chandler et al., 1986; Quillet et al., 1995). More recent comparative mapping studies using DNA markers found differences of five non-reciprocal translocations and two inversions between genomes (Heesacker et al., 2009). In this work a *H. argophyllus* map of 299 DNA markers (SSR, INDEL, and SSCP) was constructed and compared against an *H. annuus* reference map using 131 DNA orthologous markers. Besides, seven chromosomal rearrangements, the study of Heesacker et al. (2009), showed a complex evolutionary history, including entire chromosome duplications and fusion of chromosome fragments. Despite the importance of this seminal study on *H. argophyllus*, further studies with more dense maps would be of immense interest to better elucidate karyotypic evolution between both species. More abundant markers such as single nucleotide polymorphisms markers (SNPs) together with high-throughput genotyping technologies are critical tools to achieve the goal of more precise studies in comparative mapping.

In this paper we construct the first high-density linkage map of *H. argophyllus* using EST-SNPs markers coupled with the high-throughput genotyping Illumina Infinium assay. We

compared the *H. argophyllus* map with an ultra-dense consensus *H. annuus* linkage map and obtained precise, clear, and high-definition results. In our work, we redefine Heesaker et al. (2009) findings and present a more accurate picture of karyotypic evolution of *H. annuus* and *H. argophyllus*. This work contributes to evolutionary biology by providing information to better understand the evolutionary history between these two Helianthus species. And, also provides plant breeders accurate information for a more efficient use of *H. argophyllus* in sunflower breeding.

MATERIAL AND METHODS

Mapping population

An interspecific hybrid testcross mapping population was developed by crossing a single male sterile *H. annuus* inbred line NMS373, with a single male fertile plant randomly selected from an intraspecific population created by crossing two single plants from two different ecotypes of *H. argophyllus*, accessions ARG1820 and ARG1834 provided by the Germplasm information Network, Iowa (GRIN). The intraspecific cross was made with the purpose of increasing the number of heterozygous loci segregating in the second cross with the inbred line. Leaf samples were collected from 94 four week-old F_1 plants growing in the greenhouse. DNA was isolated from frozen tissue using a modified CTAB method (Murray and Thompson, 1980) and DNA concentrations were evaluated using the Quant-iT dsDNA BR kit (Invitrogen) measuring the Pico green fluorescence on a BioTek Synergy HT Microplate Spectophotometer (BioTek Instruments, Winooski, VT). DNA concentrations for each sample were adjusted to 50 ng/µL for SNP genotyping.

Marker genotyping and genetic mapping

Multiplexed genotyping was carried out using Illumina's Infinium™ assay according to the manufacturer's protocol. A custom 9480 EST-SNP marker array was used. The assay involved the generation of hundreds of templates with specific target and address sequences using allele-specific extension followed by ligation and amplification with universal primers. Fluorescent products are hybridized to precoded beads on an array matrix from which the signal intensities are subsequently determined using Illumina's BeadArray Reader. Signal intensities were quantified and matched to specific alleles using GenomeStudio software (Illumina). The software assigns three clusters, corresponding to the segregating genotypes, on a graph based on the fluorescence obtained. The homozygous and heterozygous clusters were checked visually and manual re-clustering was made as needed, determined by the expected allele transmission of markers in the population.

The *H. argophyllus* map was generated from a population derived from an intraspecific *H. argophyllus* hybrid (ARG1820 x ARG1834) crossed to a nuclear male-sterility *H. annuus* inbred line NMS373. The mapping population generated in this way allows the segregation of the heterozygous *H. argophyllus* hybrid in the homozygous inbred line background, or a one-way pseudo-testcross mapping strategy (Burke et al., 2004; Grattapaglia and Sederoff, 1994). To detect linkage in the repulsion phase, the data was duplicated and inverted and then added to the original data. The map was generated using a combination of colormapping (Kiss et al., 1998) and the program MapDisto v. 1.7 (Lorieux, 2007). Genotype markers were grouped using colormapping and the order of the markers was determined using the commands Order and Ripple of MapDisto using a LOD=3. The Kosambi function (Kosambi, 1944) was used to convert the recombination fractions to centimorgans (cM). The deviation from the expected Mendelian ratio (1:1) for each locus was determined by segregation X^2 tests.

Sunflower linkage group naming conventions and identification of chromosomal rearrangements

The *H. argophyllus* and *H. annuus* maps were compared through a dot-plot constructed from orthologous SNP markers mapped in both species. In *H. annuus*, the prefix ANN was used together with the linkage group number according with the standard public linkage groups nomenclature. The *H. argophyllus* linkage groups were identified with the prefix ARG followed by a number or a combination of numbers depending if the *H. argophyllus* linkage groups were or not colinear with *H. annuus* groups. If both linkage groups were collinear the same number was use following both suffixes (i.e. ARG1 is colinear with ANN1). When the *H. argophyllus* linkage groups were formed by a fusion of two *H. annuus* fragments, these groups were identify using group numbers from the fussed *H. annuus* groups, i.e. ARG6/15 is product of the fusion of chromosomes ANN6 and ANN15. Inversions were identified using the suffix INV following the proper linkage group number in *H. argophyllus*.

RESULTS

Linkage genetics maps

The *H. argophyllus* map is composed of 1549 loci distributed across 17 linkage groups that collectively span 1321 cM representing a density of 0.85 cM/locus (Table 1). This represents an increase of 1250 loci from the previous *H. argophyllus* map constructed by Heesacker et. al. (2009). Also, our map represents a reduction in the number of linkage groups from 21 to 17. This is important given that 17 is the haploid chromosome number in sunflower. Map length for our *H. argophyllus* maps was approximately the same that in the previous work, being just 49 cM shorter. The largest gap between markers is 34 cM for linkage group 10 and with all other linkage groups gaps were smaller than 15 cM (Table 1). From the 9480 EST-SNPs markers screened in the Infinium assay, 2269 were appropriate for intraspecific mapping (heterozygous

for *H. argophyllus* and homozygous for *H. annuus*) and we were able to map 1549 markers representing 68% of these "mappable" markers. The consensus *H. annuus* map used to make comparisons with the *H. argophyllus* map was assembly joining three linkage maps from different *H. annuus* populations. This map is composed of 9998 DNA markers spanning 1310 cM across 17 linkage groups (Bowers et. al. 2011, unpublished data).

Macrosynteny

Throughout the comparison of 1445 orthologous EST-SNPs markers between *H. argophyllus* and *H. annuus* maps we could infer the presence of 12 colinear linkage groups (LG01, LG02, LG03, LG04, LG05, LG08, LG09, LG11, LG13, LG14, LG15 and LG17). The rest of *H. annuus* linkage groups show different kinds of rearrangements in *H. argophyllus* (two non-reciprocal translocation, one reciprocal translocation and one inversion). Graphical representation by means of a dot-plot of the colinear and rearrangements segments can be seen in Figure 1. From observation of Figure 1 we can describe 22 colinear segments resulting from three translocations (two non-reciprocal and one reciprocal) and one inversion. The *H. argophyllus* chromosome ARG6/15 seems to arise from the fusion of chromosome ANN6 and ANN15 (Figure 1). One duplicated segment of chromosome ANN4 seems to have inserted in chromosome ANN7 forming chromosome ARG4/7. The translocated segment spans 6 cM in *H. annuus* and 21 cM in *H. argophyllus*. One segment of chromosome ANN10 broke and fused inverted in the same chromosome forming ARG10-INV. In the inverted fragment we can see that recombination is reduced in *H. argophyllus* in one of the ends. Another particularity that we can observe in this *H. argophyllus* linkage group is the relatively large segment (35 cM) without marker coverage. Both chromosomes, ANN12 and ANN16, split into two segments each and then fused reciprocally to form chromosomes ARG12/16 and ARG 16/12. Reduction in

recombination frequency in *H. argophyllus* is observed across linkage groups and is perceive as a curvature in the dot-plot for linkage groups ARG1, ARG2, ARG4, and ARG5.

DISCUSSION

Chromosomal rearrangements

Regarding chromosome rearrangements differences between *H. annuus* and *H. argophyllus,* we found less chromosomal rearrangements than previously reported by Heesaker et al. (2009). We identified the same translocation in chromosome ARG6/15, but unlike in the previous study this chromosome was not duplicated in our study. It has been reported that this translocation is also presented in *H. petiolaris* (Burke et al., 2004). Since the divergence between *H. petiolaris* and *H. annus* is earlier than to the divergence between *H. annuus* and *H. argophyllus*, our finding supports the conjecture of Heesacker et al. (2009) that chromosomes ANN6 and ANN15 arose from the breakage of an ancestral chromosome 6/15. We found neither the duplications nor the inversion in this chromosome reported in the previous work (Heesacker et al., 2009).

The translocation forming chromosome ARG4/7 was missing in the previous work by Heesacker et al. (2009) as well as the segmental duplication of chromosome ANN4 involved in the translocation. They found a translocation involving ANN13 instead of ANN4 joined with ANN7. They also did not report the reciprocal translocation of chromosome ARG12/16 instead; they found this translocation as non-reciprocal. Another rearrangement reported here and not in Hessacker et al. (2009) is the inversion in chromosome ARG10. This inversion was supported by eight markers spanning 10 cM and is inserted 3 cM downstream of chromosome ARG10. This implies the need of two breaks and two fusions to form the inversion. The ability to detect chromosomal rearrangements via comparative mapping is directly proportional to the density of

markers. It follows that large scale rearrangements are easily detectable while those involving small chromosomal segments could go undetected. Our study represents a 11-fold increase, when compared with a previous study in number of DNA orthologous markers between species used in comparative mapping. This fact should explain most of the differences found between both studies. Heesaker et al. (2009) reported some putative inversions and duplications supported by a few markers that we could not find. These differences could be due to mapping errors or/and higher marker density in our study.

Heterogeneous recombination rate across *H. argophyllus* genome

Syntenic relationships between *H. annuus* and *H. argophyllus* were very colinear showing a high level of conserved gene order between both genomes and only disrupted for a few major rearrangements. For some of the colinear linkage groups such as ARG1, ARG2, ARG4 and ARG5 we observed a reduction in recombination frequency in some regions in *H. argophyllus* compared to the *H. annuus* linkage groups. This phenomenon is common in plants and can vary among species, within a species, and between different areas within a genome, chromosome, or even a small region in the genome (Nachman, 2002). Factors affecting recombination frequencies could be attributed to genetics and environmental causes (Esch and Horn, 2008).

One plausible explanation for our observation are the differences that could exist between species in heterochromatin and euchromatin patterns across genome regions (Kim et al., 2005; Wang et al., 2006). For example, heterochromatin comprises 50% of the sorghum genome, with a 43-fold suppression of recombination on average in heterochromatic versus euchromatic regions (Kim et al., 2005). In a different way, tomato heterochromatin has 75% of its genome with a 1000-fold suppression of recombination in heterochromatic regions (Wang et al., 2006).

The mechanisms underlying the heterocromatin suppression of recombination are still unclear, but, it is suggested that it is related to epigenetic modifications (Yan et al., 2005).

Reduction in recombination could be also associated with some chromosome features such as centromeres, pericentromeric heterochromatin, and telomeres (Kunzel et al., 2000). Nonrandom patterns of DNA markers distributions similar to the ones shown in Figure 2 for chromosomes ARG1, ARG5, ARG6, ARG4/7 and ARG8 could provide insight into the location of important chromosome features such as centromeric and telomeric regions. The same type of pattern was observed by Bowers et al.(2003) in sorghum and was associated with centromeric regions. They utilized probes for sorghum repetitive sequences homologous to pHind22 and Cen38 to the sorghum BAC libraries to co-hybridize the probes with RFLPs previously mapped. They found that some of these probes were associated with markers mapped to marker-dense regions in 8 out of the 10 linkage groups in sorghum. Earlier studies from Tanskley et al (1992) comparing molecular with physical maps, associated high density clustering of markers with centromeric, pericentromeric, and telomeric regions in tomato. More evidences for these non-random distributions of markers can be observed from the comparison between both species maps from dot-plot in Figure 1. For linkage groups ARG5 and ANN5 there was a tendency of markers to cluster in a small region in one of the species while duplicated loci are spread across all the linkage groups for the other species (dotted lines in Fig.1). This pattern was also observed in other cereal species (Bowers et al., 2003) and could be the result of the accumulation of duplicated genes in pericentromeric regions.

Reduction in recombination will have practical implications in genetic analysis. For genetic mapping in areas where recombination rate is low and linkage disequilibrium is high, markers at considerable distance from the causative polymorphism of a gene will be in linkage

disequilibrium with it. On the contrary, high resolution genetic mapping will be difficult in these regions and much larger progeny population size will be needed to recover the crossovers necessary for fine mapping.

Karyotypic evolution

H. argophyllus diverged from *H. annuus* 0.74 to 1.67 MYA at a rate of karyotypic evolution estimated in the range of 2.2 to 3.2 chromosomal rearrangements/MYA (Strasburg and Rieseberg as cited in Heesacker et al. (2009)). With the four chromosomal rearrangements (three translocations and one inversion) found in this study the rate of karyotypic evolution it is estimated between 1.1 and 2.7 chromosomal rearrangements/MYA. The estimated rate of karyotypic evolution in our study is slower than in previous studies for these two species (Heesacker et al., 2009) and between other Helianthus species (Burke et al., 2004). When we compare our results with those from other animal and plant species (Lagercrantz, 1998), the rate of evolution in *Helianthus* still is relatively rapid.

Comparison of linkage maps reveals that chromosomal evolution between *H. argophyllus* and *H. annuus* since the divergence from a common ancestor has been primarily through chromosome translocations. This observation could be extended to some of the other members of the genus Helianthus. Supporting this statement are the results of Burke et al. (2004), who found that 8 out of 11 chromosome rearrangements responsible for the differences between *H. petiolaris* and *H. annuus* were translocations. On the contrary, results on other plant families have shown that inversions are primarily involved in the divergence of some species as it is in the case of the Solanaceae family (Doganlar et al., 2002). It has been proposed that inversions are more frequent than translocations in wild populations because chromosomal interchanges usually have negative effects on an organism's fertility (Burnham, 1962).

If heterozygous translocations are detrimental for an organism's fertility, one can speculate why karyotypic evolution in *Helianthus* has been mainly through translocations? One explanation could be that the fixation of this type of rearrangements could be facilitated by gene redundancy due to the ancient polyploidization in *Helianthus* (Barker et al., 2008; Sossey-Alaoui et al., 1998). Sterility due to translocation is less frequent if genome redundancy is present (Rieseberg, 2001). Chromosome duplication reduces the initial underdominance of chromosomal translocation facilitating their establishment. The rapid rate of karyotypic evolution could also explain why translocation-type of rearrangements could be favored. While chromosomal rearrangements do not have effect on fitness when homozygous, they are deleterious when heterozygous (White, 1973). The fixation of such rearrangements requires genetic drift to overcome the selection against heterozygotes. The population dynamics for Helianthus species are favorable for the fixation of these types of rearrangements through genetic drift in small populations (Harrison et al., 2000). Then, the fixation of translocations and the rapid karyotypic evolution in Helianthus would be explained by population dynamics in the genus.

H. argophyllus in sunflower breeding

As previously commented, *H. argophyllus* has been used extensively as donor of favorable alleles in sunflower breeding. The use of *H. argophyllus* has been achived without detailed knowledge of the genomic differences between *Helianthus* species. The results of the macrosynteny analysis of this study complement and improved the results and conclusion from Hessacker et al. (2009). We generated useful information that can be used in the development of valuable tools such as introgression libraries (ILs) (Falke et al., 2008; Salvi et al., ; Zamir, 2001) to better exploit *H. argophyllus* as donor of alleles for complex traits. Currently an IL of *H. argophyllus* in *H. annus* background is being developed with the help of the information

generated in this study (Knapp personal communication). It is thought that ILs will help to dissect complex traits and identify and isolate favorable alleles from *H. argophyllus* will facilitate its final incorporation to *H. annus* assisting the manipulation of *H. argophyllus* alleles within a breeding program through marker assisted selection. Despite the chromosome rearrangements found this study, that will complicate the development of IL, most of the *H. argophyllus* genome is colinear with *H. annuus*. This fact makes those colinear regions easily targeted for the IL development. In addition, reduction in recombination rates in certain regions of the genome targeted for introgression will generate breeding challenges. The reduction in recombination will favor "linkage drag", in which large portions of DNA around the targeted *H. argophyllus* allele are inherited together with the favorable allele. This creates a problem since the breeder may also incorporate deleterious alleles together with the favorable allele into the elite background. The information generated here will be of practical applications since identified the regions with reduced recombination. Taken into consideration this information, breeders can adjust population size in order to obtain recombinant for these regions.

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TABLES AND FIGURES

Table 2.1. Length, number of markers and gaps between markers per linkage group in the *Helianthus argophyllus* linkage map.

LG	Length cM	Loci No	Largest gap
1	151	89	7
2	68	53	11
3	65	112	4
4	55	61	7
5	64	186	9
6	109	75	7
7	76	76	8
8	58	84	11
9	76	111	9
10	70	40	34
11	96	141	8
12	85	128	8
13	77	80	9
14	77	97	6
15	50	59	7
16	52	70	14
17	92	87	6
Total	1321	1549	

Figure 2.1. Dot-plot of common EST-SNP markers mapped in *Helianthus annuus* (y-axis) and *Helianthus argophyllus* (x-axis). The two intersected lines across linkage groups ANN5 and ARG5 point to the accumulation of duplicated genes around the putatively pericentromeric region.

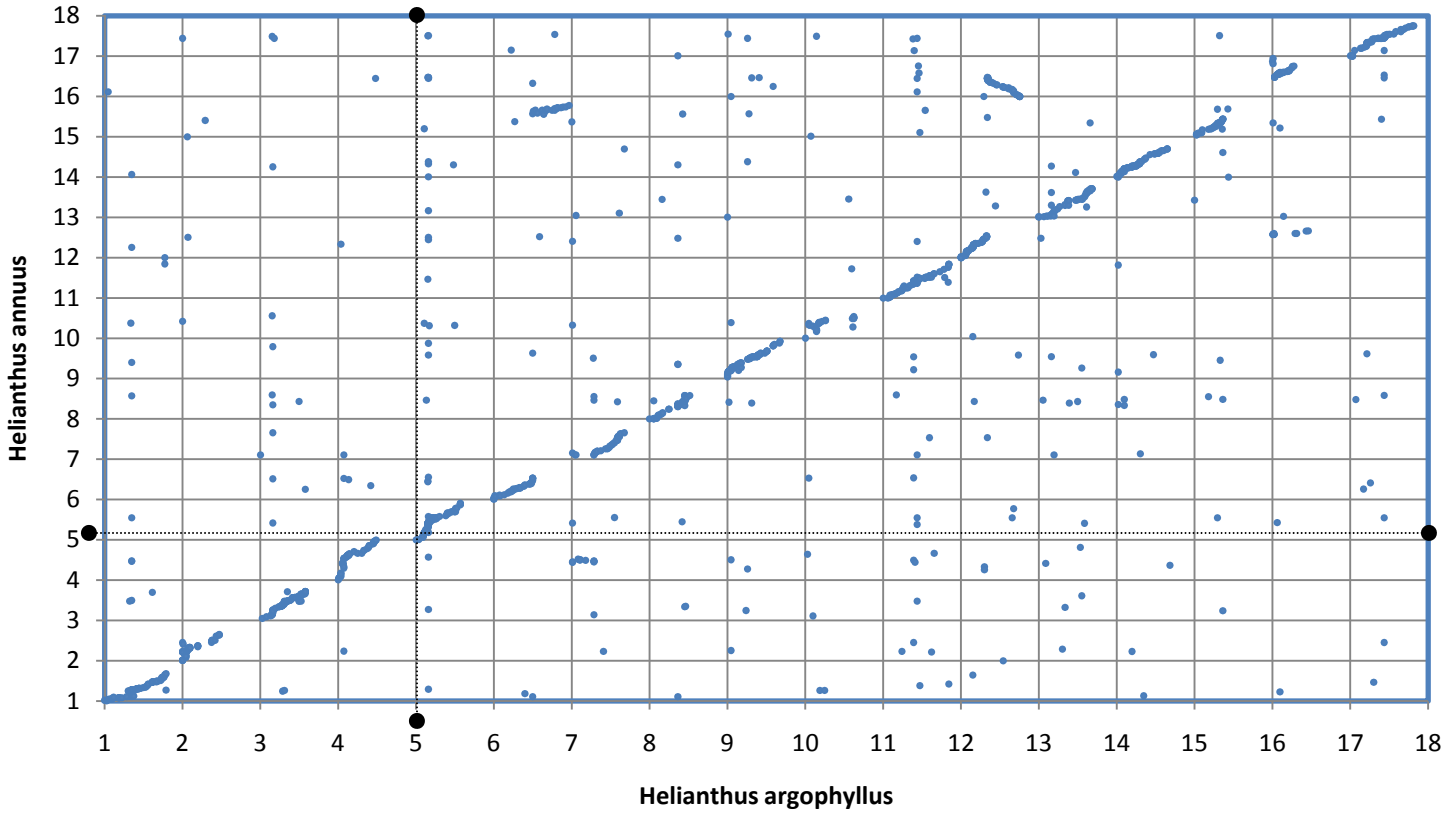
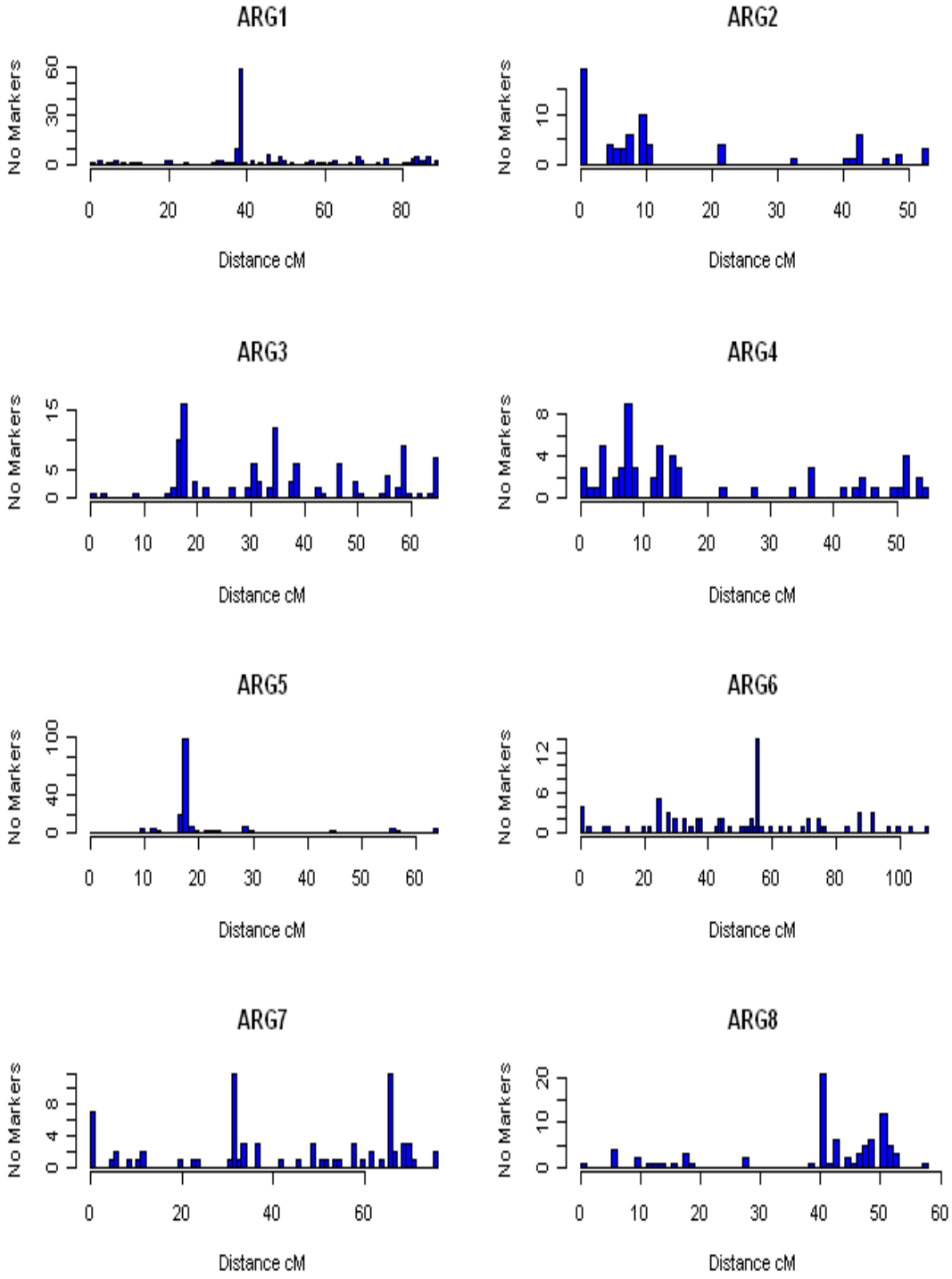
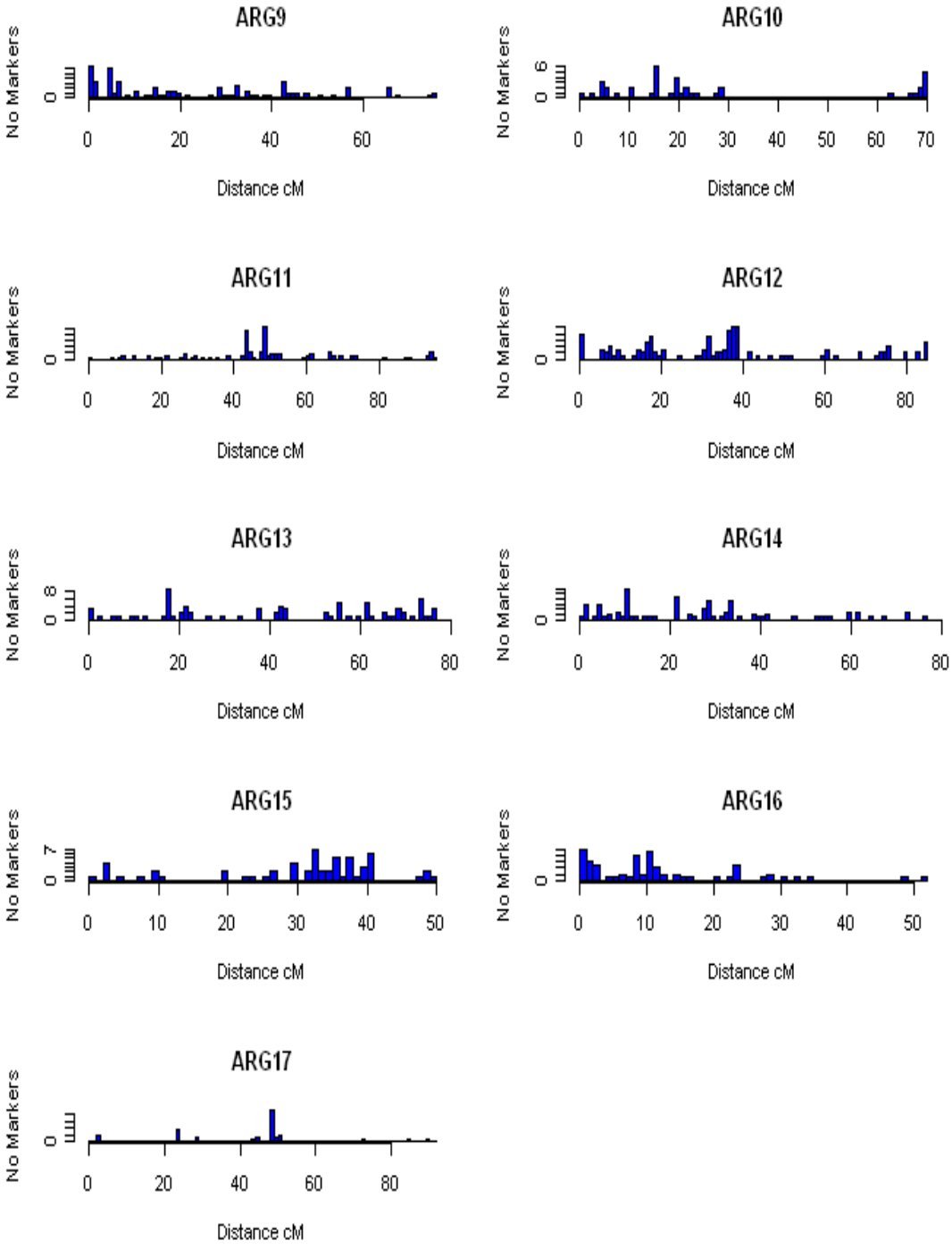


Figure 2.2. Distribution of EST-SNP markers along the *Helianthus argophyllus* linkage map for 1 cM interval




ARG9
No Markers
0
0
20
40
60
Distance cM
ARG10
No Markers
0
6
0
10
20
30
40
50
60
70
Distance cM
ARG11
No Markers
0
0
20
40
60
80
Distance cM
ARG12
No Markers
0
0
20
40
60
80
Distance cM
ARG13
No Markers
0
8
0
20
40
60
80
Distance cM
ARG14
No Markers
0
0
20
40
60
80
Distance cM
ARG15
No Markers
0
7
0
10
20
30
40
50
Distance cM
ARG16
No Markers
0
0
10
20
30
40
50
Distance cM
ARG17
No Markers
0
0
20
40
60
80
Distance cM

CHAPTER 3

GENETIC COMPLEXITY OF SALT TOLERANCE IN AN INTERSPECIFIC CROSS BETWEEN CULTIVATED SUNFLOWER (*HELIANTHUS ANNUUS* L.) AND SILVERLEAF SUNFLOWER (*HELIANTHUS ARGOPHYLLUS* TORREY AND GREY) REVEALED THROUGH BAYESIAN ANALYSIS[1]

[1] Rey, J.I., J.M. Burke, E.R. Boerma and S.J. Knapp to be submitted to *Plant Breeding*

ABSTRACT

Salinity is an important abiotic stress that affects crop productivity in many agricultural areas around the world. Development of salt tolerant cultivars is an effective approach to minimize the lost in productivity in saline soils. Cultivated sunflower (*Helianthus annuus* L.) has been classified as moderately salt sensitive. Silverleaf sunflower (*Helianthus argophyllus*), the closest relative of sunflower, has been suggested as a source of favorable alleles for salt tolerance. With few exceptions, salt tolerance has been shown to be a complex trait in most crop species. In order to elucidate the complexity of salt tolerance in sunflower we used Bayesian interval mapping to detect main-effect and epistatic-effect QTL for salt tolerance rating and SPAD value, an estimate of chlorophyll content, associated with salt tolerance in an intermated-F2 population from an interspecific cross between *H. annuus* and *H. argophyllus*. To validate QTL results from mapping in the F2 population, we used a set of BC2S1 families of the same cross for comparisons with previous results of QTL mapping for salt tolerance in sunflower in a cross between *H. annuus* and *H. petiolaris*. We found important QTL on chromosomes (chr) 1, 3, and 6 that were partially validated through the comparison between generations of the same cross and comparison with previous studies. *H. argophyllus* contributed the favorable alleles on chr 1 and chr 6 and *H. annuus* the one in chr 3. Phenotypic variance explained by these QTL varied between 6 and 10%. We also found important epistatic pairs, especially between QTL on chromosomes 3 and 6 explaining a significant proportion of the phenotypic variance. Our findings showed that salt tolerance in sunflower is highly complex trait and that epistasis plays a key role conferring tolerance. This information should be taken into consideration when applying QTL results to improve salt tolerance through marker-assisted-selection in sunflower.

INTRODUCTION

Soil salinity is a major abiotic stress imposing constraints to agricultural productivity in many areas around the world, with 6% of the world's land affected either by salinity or sodicity a secondary effect of salinity in clay soils (www.fao.org). Almost 20% of the irrigated and 2.1% of the dryland agricultural land is affected by salt (Yamaguchi and Blumwald, 2005). This estimation does not take into account the land that is salt affected and cannot be cultivated due to high salinity levels.

With the prediction in population growth of 50% by 2050 from 6.1 billion in mid-2001 to 9.3 billion (www.unfpa.org/swp/2001/), it is evident of the need for more land area and/or the increase in productivity of the current areas under cultivation in order to increase the food supply. One obvious way to increase food supply is the cultivation the areas of the world affected by salinity or cultivate dryland areas with supplemental irrigation, a practice that could increase soil salinity. Either solution will require the improvement the salt tolerance of crops grown under salinity conditions.

Salt tolerance is a complex trait involving the function of many genes (Ashraf et al., 2009), and the screening and selection of salt tolerant individuals in conventional breeding is highly influenced by environmental factors (Ashraf et al., 2008). Due to the large environmental effect, conventional breeding approaches for salt tolerance may not the best alternative. It is suggested that one of the best methods for salt tolerance improvement will be the identification and use of molecular markers associated with salt tolerance loci/genes (quantitative trait loci or "QTL"). With this approach, molecular markers unaffected by environment could be used for selection in breeding programs through marker-assisted-selection (MAS) (Flowers, 2004).

Cultivated sunflower (*Helianthus annuus* L.), the fourth most important annual crop in the world grown for its edible oil (Fernández-Martínez et al., 2009), has been classified as moderately salt sensitive based on water stress index (Katerji et al., 2003). Salinity delays sunflower germination and emergence due to the absorption of Na^+ and Cl^- through the hypocotyl (Katerji et al., 1994). Salt also affects leaf expansion and biomass accumulation in seedlings (Delgado and Sánchez-Raya, 1999; Rawson and Munns, 1984). Achene yield lost has been observed due to reduction in the number of seeds per head, while oil percent was unaffected (Francois, 1996).

Silverleaf sunflower (*Helianthus argophyllus* Torrey & Gray), the closest relative of common sunflower (Schilling and Heiser, 1981), has been widely used as donor of disease resistance alleles (Dussle et al., 2004; Heiser Jr, 1951; Miller and Gulya, 1991; Radwan et al., 2004; Seiler et al., 2007; Slabaugh et al., 2003; Wieckhorst et al.), fertility restoration to the PET1 cytoplasm (Chepurnaya et al., 2003), and cytoplasmic male sterility (Horn et al., 2002). It has been also suggested that it can be a source of favorable alleles for salt and drought tolerance (Rauf, 2008; Richards, 1992) and insect resistance (Rogers and Thompson, 1980; Rogers et al., 1987; Sujatha and Lakshminarayana, 2007). Our data have shown a high level of salt tolerance in different *H. argophyllus* accessions that originated from Florida, North Carolina and Texas. It has been observed that most of the accessions were able to tolerate a salt concentration of 460 mM of NaCl, equivalent to seawater salt concentration (Rey, unpublished data). These observations, together with the fact that it has been the most used wild species in sunflower breeding, makes *H. argophyllus* good candidate for the improvement of salt tolerance in cultivated sunflower.

The objectives of this study were to i) identify QTLs responsible for salt tolerance using Bayesian analysis in an interspecific cross between *H. annuus* and *H. argophyllus* ii) validate the QTL results through QTL comparison between two generations of the same cross, iii) compare our results with previous QTL studies for salt tolerance in sunflower, iv) elucidate the complexity of salt tolerance in sunflower through the identification of important epistatic QTL pairs contributing to salt tolerance, and vi) provide important information about the genomic regions involved in salt tolerance and discuss its implications for its use in sunflower breeding.

MATERIAL AND METHODS

Plant material

An interspecific cross was made between a nuclear male sterile *H. annuus* line (NMS377) and a *H. argophyllus* accession (ARG1820). NMS377 is an elite sunflower inbred line moderately susceptible to salt stress. ARG1820 is an accession that originated from the Gulf Coast of Texas and is highly tolerant to salt conditions (Rey, unpublished data). Two F_1 plants, one of them male sterile, were crossed and the resulting population was intermated during three cycles. In each cycle seeds from the male sterile plants were harvested and bulked for planting the next season. After these three recombination cycles, the intermated F_2 population of 185 individuals was genotyped and along with the two parents was evaluated for salt tolerance. Our ultimate objective after QTL mapping was the incorporation of the salt QTL into elite material through marker assisted backcrossing. With this objective in mind we decided to validate the QTL mapping results obtained in the F_2 in a group of BC2S1 families. This type of cross would give us a more precise estimate of the behavior of the QTL in the recurrent parent background and would provide the initial step toward the development of near isogenic lines (NILs). For this objective a single fertile F_1 plant from the first cross was crossed again to a single sterile

NMS377 plant. After this cross, fertile BC_1 plants were crossed again to sterile NMS377 plants and the resulting BC_2 plants were selfed to obtain BC2S1 families. Only 58 families were obtained due to problems with sterility and self incompatibility. These resulting BC2S1 families were planted in the greenhouse for assessment of salt tolerance.

Evaluation of salt tolerance

Seeds of parents, F_2s, and BC2S1 families were surface sterilized with 10% regular bleach (sodium hypochlorite, NaCLO) solution for 1 min and rinsed with double-deionized water. Seeds were then germinated in Petrie dishes in dark at 25°C for 3 d and healthy seedlings were transplanted into plastic 164 cm^3 cone-tainers (Ray Leach Containers, Tangent, OR) filled with washed sand. The method developed by Lee et al (2008) was used for salt tolerance screening in both populations. Plastic racks of cone-tainers (49 cone-tainers/racks) were placed in 39-L Steriliter boxes (Townsend, MA) containing 11 L of full strength Peters Excel CalMag solution (Fig. 1). The first salt treatment of 50 mM of NaCl (electric conductivity (EC) of ~53 ds/m) was applied when plants developed their first pair of true leaves and a 50 mM of NaCl was added every 2 d until reaching the target concentration of 300 mM of NaCl (~33 ds/m) to allow plants to gradually adapt to salinity. This salt concentration was chosen because it allowed the maximum differentiation between the parents of the populations (Rey, unpublished data). The EC and pH were monitored periodically, and if differences due to evaporation were found, tap water was added to maintain the experimental conditions. The solution was replaced every 4 d. The Steriliter boxes containing the cone-tainers were rotated on greenhouse benches every day in order to reduce the experimental error due to heterogeneity in light and temperature within the greenhouse. Greenhouse conditions were maintained at an average temperature day/night of 30/21°C and a 14 hr light during the experiment.

For the F_2 population a total of 210 F_2 plants along with the parents were evaluated for salt tolerance. For the 58 BC2S1 families, salt tolerance assessment was conducted using a randomized complete block design with two replications. For each replication seven plants per family and parents were grown together in nine boxes per replication. Plants in the F_2 population were evaluated for their salt tolerance using a salt tolerance rating (STR) of 1 to 4; where 1= indicates a healthy plant with no obvious symptoms of salt damage, and 4= indicates a dead plant (Fig. 2). Salt tolerance evaluation in the BC2S1 families was done using a weighted salt tolerance rating (WSTR). To estimate WSTR, we evaluate each plant from each family and assigned them a value in the scale previously used to evaluate the F_2 plants. This time we assigned a percentage to each value in the scale as follow; 1= 0%, 2= 1-33%, 3= 34-66%, and 4= 67-100% of damage. The total number of plants per family was recorded (TNPF), as well as number of plants per family in each value (i) of the scale (NPi), and then, the WSTR was calculated using the following formula:

$$WSTR = \frac{(NP2 \times 0.33) + (NP3 \times 0.66) + (NP4 \times 1)}{TNPF} \times 100$$

Leaf chlorophyll content (SPAD value) for each plant was estimated using a chlorophyll meter (Konica Minolta SPAD-502, Minolta corporation, Ltd., Osaka Japan). This SPAD value is proportional to the chlorophyll content in the leaves (Castelli et al., 1996). Evaluation was performed when most of the plants in the susceptible parent (NMS377) reached a STR of 3.

Marker genotyping and map construction

DNA was extracted from leaf samples collected from 94 4-wk-old BC_2 and 185 F_2 plants growing in the greenhouse. DNA was isolated from frozen tissue using a modified CTAB method (Murray and Thompson, 1980). DNA concentrations were estimated using the Quant-iT dsDNA BR kit (Invitrogen) measuring the Pico green fluorescence on a BioTek Synergy HT

Microplate Spectophotometer (BioTek Instruments, Winooski, VT) and concentrations for each sample were adjusted to 50 ng μL^{-1} for SNP genotyping.

Multiplexed genotyping was carried out using Illumina's BeadXpress™ assay (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. A custom 384 EST-SNP marker array was used. The assay involved the generation of hundreds of templates with specific target and address sequences using allele-specific extension followed by ligation and amplification with universal primers. Fluorescent products are hybridized to precoded beads on an array matrix from which the signal intensities are subsequently determined using Illumina's BeadArray Reader (Illumina Inc., San Diego, CA). Signal intensities were quantified and matched to specific alleles using GenomeStudio software (Illumina Inc., San Diego, CA). The software assigns three clusters, corresponding to the segregating genotypes, on a graph based on the fluorescence obtained. The homozygous and heterozygous clusters were checked visually and manual re-clustering was made as needed, determined by the expected allele transmission of markers in the F_2 or BC_2 population.

The SNP linkage maps for both populations were generated using a combination of colormapping (Kiss et al., 1998) and the program MapDisto v. 1.7 (Lorieux, 2007). SNP markers were grouped using colormapping and the order of the markers was determined using the commands Order and Ripple of MapDisto using a LOD=3. The Kosambi function (Kosambi, 1944) was used to convert the recombination fractions to centimorgans (cM). The deviation from the expected Mendelian ratio for each locus was determined by segregation X^2 tests.

Bayesian QTL mapping

Bayesian model selection (Yi et al., 2005) implemented in the package R/qtlbim (www.qtlbim.org) released by Yandell et al, (2007) was used to simultaneously detect main and

epistatic effects as well as QTL by QTL interactions. Each chromosome was divided into 1-cM grids and considered as possible QTL positions. Using the traditional QTL interval mapping (Jansen, 1993) as implemented in R/qtl (Broman et al., 2003), the number of QTL were estimated. Based on these estimations the prior number of QTL main-effect was set to be used in the Bayesian analysis. We simultaneously modeled main and QTL by QTL interactions. We fitted the models using R/qtlbim (Yandell et al., 2007), which implement a Markov chain Monte Carlo (MCMC) algorithm (Yi et al., 2005; Yi et al., 2007). The MCMC algorithm generated posterior samples from the joint posterior distribution of all parameters in the model, proceeding to draw each parameter from its conditional posterior distribution using the latest values of all other unknowns and the observed data. For all analysis the MCMC algorithm ran for 20,000 iterations after discarding the first 1,000 iterations as burn-in. To reduce serial correlation in the stored samples, the chain was thinned by one in 40 iterations, yielding 5000 samples for posterior analysis. Convergence diagnostics and mixing behavior of the chain was assessed using the CODA package (Plummer et al., 2006) incorporated in R/qtlbim. This showed that the simulation chains converged and mixed well. In posterior analysis, Bayes factors (BF) of main effects and epistasis per locus or pair of loci are individually calculated and compared with a BF threshold of 3, or 2ln(BF)=2.1, to claim the presence of a QTL (Kass and Raftery, 1995). The phenotypic variance explained by the genetics effects were estimated by its heritability.

RESULTS

Phenotypic trait distribution

The traits distributions for the F_2 plants and the BC2S1 families are shown in Figure 3. For STR in the F_2 population (upper left graph) the percent of the plants in each category are shown above each bar. The F_2 distribution showed that the majority of the plants are in the most

tolerant categories 1 and 2. Unlike the F_2 population, the BC2S1 families are skewed toward susceptibility (bottom left graph). For SPAD values we can observe a nearly normal distribution for F_2 plants and again a skewed distribution in the BC2S1 families toward high SPAD values. Transgressive segregation for STR or WSTR and SPAD values was only toward susceptibility side of the distribution (Fig. 1). The tolerant parent (ARG1820) has the lowest STR and WSTR and the highest SPAD value for both populations.

Genetic linkage maps

We constructed two linkage maps for the intermated-F_2 and the BC2 populations from the cross between the elite *H. annuus* line NMS377 and the *H. argophyllus* accession ARG1820. For the intermated-F_2 population the map was constructed with 306 EST-SNPs markers across 20 linkage groups spanning 1600 cM (Table 1).The BC2 map was constructed using 328 EST-SNPs markers across 17 linkage groups spanning 1829 cM.

QTL analysis in the F_2 population

Five significant ($2\log BF \geq 2.1$) main-effect QTL were found in the F_2 population for STR and SPAD value (Fig. 4). Two QTL on chromosome (chr) 1 and 6 corresponding to STR and three in chr 3, 6, and 8 corresponding to SPAD value (Fig. 4). For STR both QTL on chr 1 and 6, positions 65 cM and 61 cM respectively, the alleles for improved salt tolerance are inherited from the *H. argophyllus* parent. The QTL on chr 1, showed both a main effect, and a significant epistatic effect ($2\log BF=2.32$), but the proportion of the phenotypic variance explained by its epistatic-effect was much smaller than the phenotypic variance explained by the main-effect QTL in the same position (Table 2). For SPAD value, the three QTL were found on chr 3 (15 cM), 6 (61 cM) and 8 (66 cM), with their positives alleles contributed by the *H. argophyllus* parent. The QTL on chr 3 and 6 include significant epistatic effects in addition to their main

effects. The phenotypic variance explained by the QTLs for this trait varies between 5.6 and 6.7% for their main effects and 1.3 and 2.1% for their epistatic effects.

Significant QTL by QTL interactions were found for STR and SPAD value in the F_2 population (Fig. 5). For STR, the chromosomes pairs involved in the interactions were chr 3, 4 10, 13, 15 and 17 and each epistatic QTL pair explained between 36.1 and 45.6% of the phenotypic variance (Table 3). From all the loci involved in the interactions only the one on chr 4 is coincident with the epistatic-effect QTL found in the one-dimensional scan. Two loci, one on chr 3 and the other on chr 10A were involved in two pair interactions, and the remaining chr 4, 13 and 15 only on one interaction each (Table 3). For SPAD value we found three significant QTL by QTL interaction pairs (Fig. 7). The percent of the phenotypic variance explained by each interacting pair ranged from 14.4 to 15%.

QTL analysis in the BC2S1 families

In the BC2S1 families a total of six main-effect QTL were found for WSTR and SPAD value (Fig. 6). Additionally, two more epistatic-effect QTL were found for SPAD value. For WSTR we found QTL on chr 5 and 11 that inherited their salt tolerant alleles from *H. argophyllus* and on chr 8 a QTL with a favorable allele from *H. annus*. All QTLs for WSTR significant main and epistatic effects and explained from 6.1 to 8% of the phenotypic variance for main effects and from 9 to 14% for epistatic effects (Table 2). For SPAD value there were main-effect QTLs on chr 1, 10 and 11 and epistatic-effect QTL on chr 3 and 4. The favorable alleles for QTL on chr 1, 4, and 11 were contributed by the *H. argophyllus* parent and those on chr 3 and 10 from *H. annuus*.

QTL by QTL interactions for WSTR and SPAD value in the BC2S1 families are shown in Figure 7. For WSTR the chromosomes involved in the interactions included chr 3, 5, 6, 8 and

10, with chr 6 involved in three interactions and chr 3 in two interactions out of 4 interacting pairs. The percent of the phenotypic variance explained by these epistatic interactions varied between 41.2 and 66.3%. For SPAD value chr 1, 3, 4, 6, 11 and 14 were involve in the epistatic interactions. Chromosome 3 is involved in three interactions and 1 is involved in 2 out of 5 interacting pairs.

DISCUSSION

We used two different generations of the same cross between *H. annuus* and *H. argophyllus* to identify and validate QTL for salt tolerance. In our study we constructed two linkage maps that differed in the number of linkage groups, number of total markers, and linkage groups length (Table 1). For the construction of the F_2 map we use an intermated-F_2 population what it should increase mapping accuracy for tightly linked loci as it was suggested in maize (Lee et al., 2002) and *Arabidopsis thaliana* (Liu et al., 1996). It also has been shown that the construction of linkage maps from intermated populations presents some limitations when it is done using mapping methods developed for F_2 populations (Falke et al., 2006). When maps are constructed with the same mapping method but different types of populations (F_2 vs intermated-F_2) are compared, the length of the linkage groups in the intermated F2 tend to be shorter than in the F_2 (Falke et al., 2006). Our intermated-F_2 map was constructed using F_2 mapping methods. This could be one of the factors responsible for the differences observed between maps length for our F_2 and BC2S1 populations and the different intervals between markers.

Taking the differences in the relative position of markers into consideration, we can make inferences regarding the co-localization of QTL found in the different populations. The main-effect QTL on chr 1 for STR in the F_2 population co-localized with the main-effect QTL (it is pleiotropic) for SPAD value found in the BC2S1 families. There is also a suggestive peak

(2logBF=1.1) for WSTR in the BC2S1 families for the same marker interval. In a similar way the main-effect QTL on chr 6 for STR in the F2 population, has a suggestive peak (2logBF=0.8) in the same interval for SPAD value in the BC2S1 families. These peaks most likely correspond to the same QTL found in the F_2 population, but could not be declared as significant due to the lower power of detection caused by the relatively small population size in the BC2S1 families (Charmet, 2000; Vales et al., 2005). The main-effect QTL found in the F_2 population for SPAD value on chr 3 co-localized with the epistatic-effect QTL for SPAD value in the BC2S1 families. This could provide evidence for the validation of that QTL in the BC2S1 families. We were able to detect it from its interactions with other loci, but likely did not have adequate detection power to declare it as main-effect QTL. It is relevant to point out that epistatic-effect QTL in the BC2S1 families are more important that main-effect QTL (Table 2 and 3). It is possible that there is a significant background interaction from the recurrent parent in the BC_2 population. The percent of genetic background from the recurrent parent could alter the penetrance and/or the phenotypic variance generated by the specific QTL (Gibson and Dworkin, 2004). We can consider those QTLs in chr 1, 3, and 6 as partially validated, suggesting that those chromosomes regions could be playing key roles in the genetics of salt tolerance.

Only one other study has identified QTL conferring salt tolerance in sunflower. Lexer at al. (2003b) elucidated how the homoploid hybrid *H. paradoxus* established in saline habitats when its parental species *H. annuus* and *H. petiolaris* were not tolerant to salt stress. They studied an interspecific BC2 population from a cross between the parental species *H. annuus* and *H. petiolaris* and identified a total of 14 QTL for mineral ion uptake and survivorship under saline conditions. Their results, supporting previous findings from the same group (Lexer et al.,

2003a), showed that salt tolerance in sunflower was achieved through the exclusion of toxic mineral ions such as Na, B, Mg, and Mn and the preferential uptake of Ca and K.

Since the QTL mapping of Lexer et al. (2003 and 2003b) was done using a linkage map constructed from SSRs and our study with a map constructed using SNP markers, we utilized a consensus sunflower map build by Bowers et al (2011 unpublished data) which included both types of markers to compare the QTL locations between studies (Fig. 8). Lexer et al. (2003b) reported a QTL for survivorship on chr 1 that maps 16 cM from our QTL for STR in the F_2 and SPAD value in the BC2S1. Further studies by Lexer et al. (2004) found associations of candidate genes with some of the QTL previously found for salt tolerance. They found an association between a candidate gene for Ca uptake/transport with their QTL for survivorship on chr 1. Their candidate gene maps only 2 cM from our QTL for STR in the F_2 and SPAD value in the BC2S1 in the consensus map for chr 1. Their results, coupled with our findings, suggest that the candidate gene for Ca uptake/transport could be a candidate gene for salt tolerance in sunflower. They reported a QTL for Ca uptake located on chr 6, that overlaps with the location of our QTL for STR and SPAD values in the F_2 population. The work of Lai et al.(2005b) supports the importance of this region of chr 6, for salt tolerance in sunflower. They establish an association between an EST marker that putatively encodes an ER-type Ca pump protein with the QTL for leaf Ca concentration found by Lexer et al. (2003b). That EST marker maps in the middle of the marker interval delimitating our QTL for STR and spad value in the F_2 population in the consensus map. Finally, they also found a QTL for K uptake in the bottom part of chr 11 that co-localizes with our QTL for WSTR in the BC2S1 families.

A complex trait by definition is governed by many genes with small effects, the environment, and gene by environment interactions. Usually, the summation of additive effects

of individual loci does not account for the entire phenotypic variation observed. The inclusion of gene by gene interactions, known as epistasis in QTL models will improve the results of the analysis. Contributions of new epistatic QTL will help to explain more of the observed phenotypic variation than just the main-effects QTL alone. The exploration of epistasis using Bayesian analysis in our study yielded some interesting results. The use of this method allowed us to distinguish between main and epistatic-effect QTL. This is important since additive and epistatic effects are partially confounded and analyzing only main-effect QTL can detect a QTL that do not actually have a main effect, but instead interact epistatically with another QTL (Purcell and Sham, 2004). An example of this phenomenon is the epistatic-effect QTL on chr 3 and 4 for SPAD value in the BC2S1 families (Fig. 6) and the importance of the mentioned interacting pair explaining a large proportion of the phenotypic variance (Fig. 7, Table 3).

We can observe from Table 2, that chr 3 and 6 are involved in at least one interacting pair of loci in both populations across STR/WSTR and SPAD value. These results show the importance of these two chromosomes regions on salt tolerance. All interacting pairs of loci explain a greater proportion of the phenotypic variance than any main-effect QTL by itself. This indicates the importance of epistasis underlying salt tolerance in this sunflower population. We also found three types of epistatic interactions, i) when both QTLs (interacting positions) are also main-effects QTLs, ii) when only one locus is a main-effect QTL, and iii) when neither of the interacting loci are main-effect QTLs. Finding all these type of interactions showed that salt tolerance is a highly complex trait and that salt tolerance improvement within a breeding program will be a difficult.

Ignoring the importance of epistasis in MAS for salt tolerance could greatly affect genetic gains (Liu et al., 2003). Reaching the target genotype during the development of inbred

lines is also slower when digenic epistasis is present (Wang et al., 2004). As a positive aspect, epistasis could be an important source of new genetic variation within breeding programs (Rasmusson and Phillips, 1997). From our previous comparative mapping studies between *H. argophyllus* and *H. annuus* (Rey et al 2011, unpublished data) we can infer the result of the introgression of the salt tolerance QTLs into an elite sunflower background. For the QTL on chr 1 and 11, we would not expect any difficulty during their introgression, since those regions are co-linear between the genomes of both species. For the QTL on chr 6 we should expect more problems in its introgression due to a translocation on chr 15 between the two species.

H. argophyllus and *H. annuus* diverged from *H. petiolaris* around 0.75 to 1 MYA (Rieseberg et al., 1991). From an evolutionary point of view the QTL for salt tolerance coming from the *H. argophyllus* parent and co-localizing with the salt tolerant QTL from *H. petiolaris* (Lexer et al., 2003b) in Lexer et al., 2003a; Fig. 8), provides a clue about the evolution of salt tolerance within the genus. The shared QTL indicate that *H. annuus* lost those salt tolerant alleles at some point after diverging from *H. argophyllus*. Since Lexer et al. (2003b) used a wild *H. annuus* in their cross with *H. petiolaris* and we used cultivated *H. annuus* in the cross with *H. argophyllus*, we can infer that the lost of the salt tolerance alleles are not due to domestication. Our results coupled with those of Lexer et al. (2003b) suggest that the ancestral forms in the genus Helianthus were more salt tolerant.

In summary, we found important QTL for salt tolerance that were validated to some extent through their presence in both mapping populations or through their co-localization in the consensus map with QTL for salt tolerance in previous studies. We assessed and confirm through the use of Bayesian analysis the importance of epistasis in the genetics of salt tolerance and its

possible implicancies in the improvement of salt tolerance in sunflower. Finally, we also presented evidence suggesting ancestral salt tolerance in the genus Helianthus.

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TABLES AND FIGURES

Figure 3.1. Experimental layout used for the screening of salt tolerance in the F_2 population and BC2S1 families.

Figure 3.2. Salt tolerance rating (STR) scale (see bottom part of plants) used in the assessment of the F_2 population and for weigthed salt tolerance rating (WSTR) in BC2S1 families

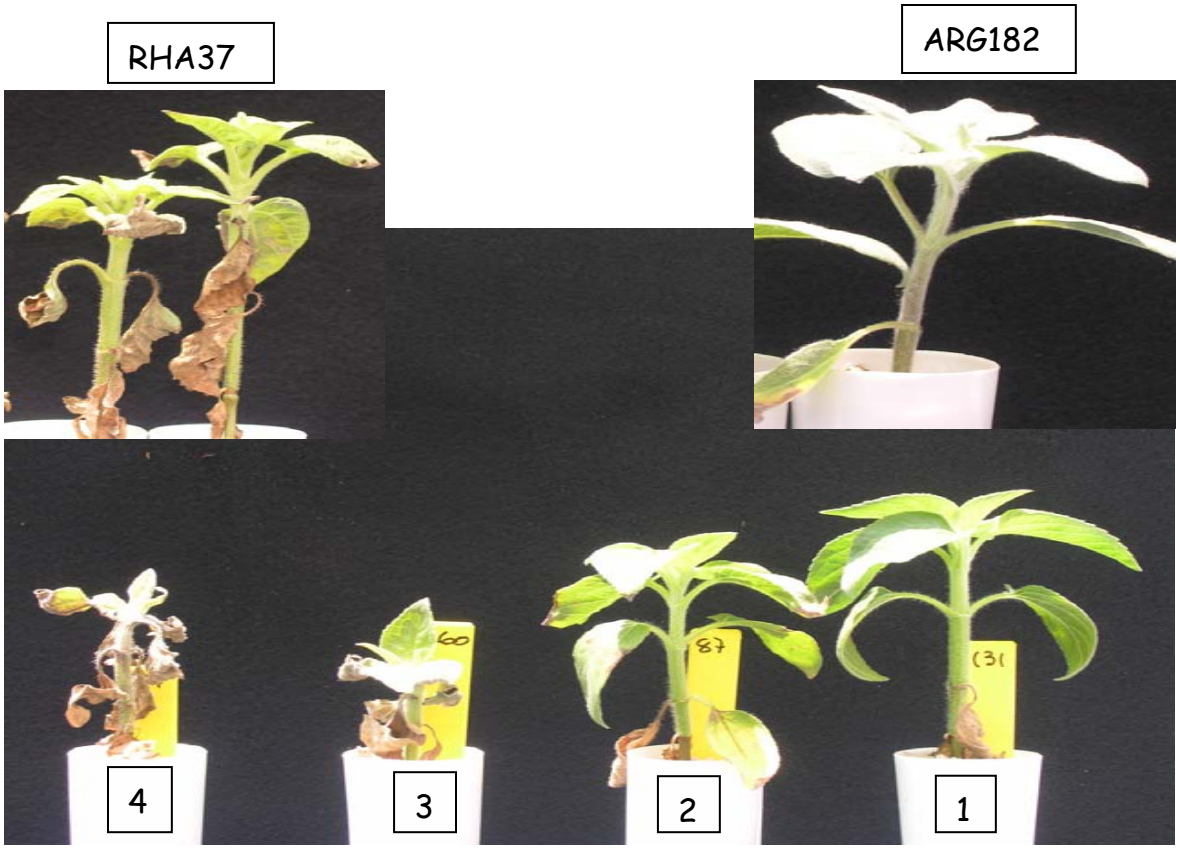

Figure 3.3. Phenotypic distribution of salt tolerance rate (STR) and SPAD values for F_2 plants (upper graphs) and weighted salt tolerance (WSTR) rate and SPAD values for BC2S1 families (bottom graphs).

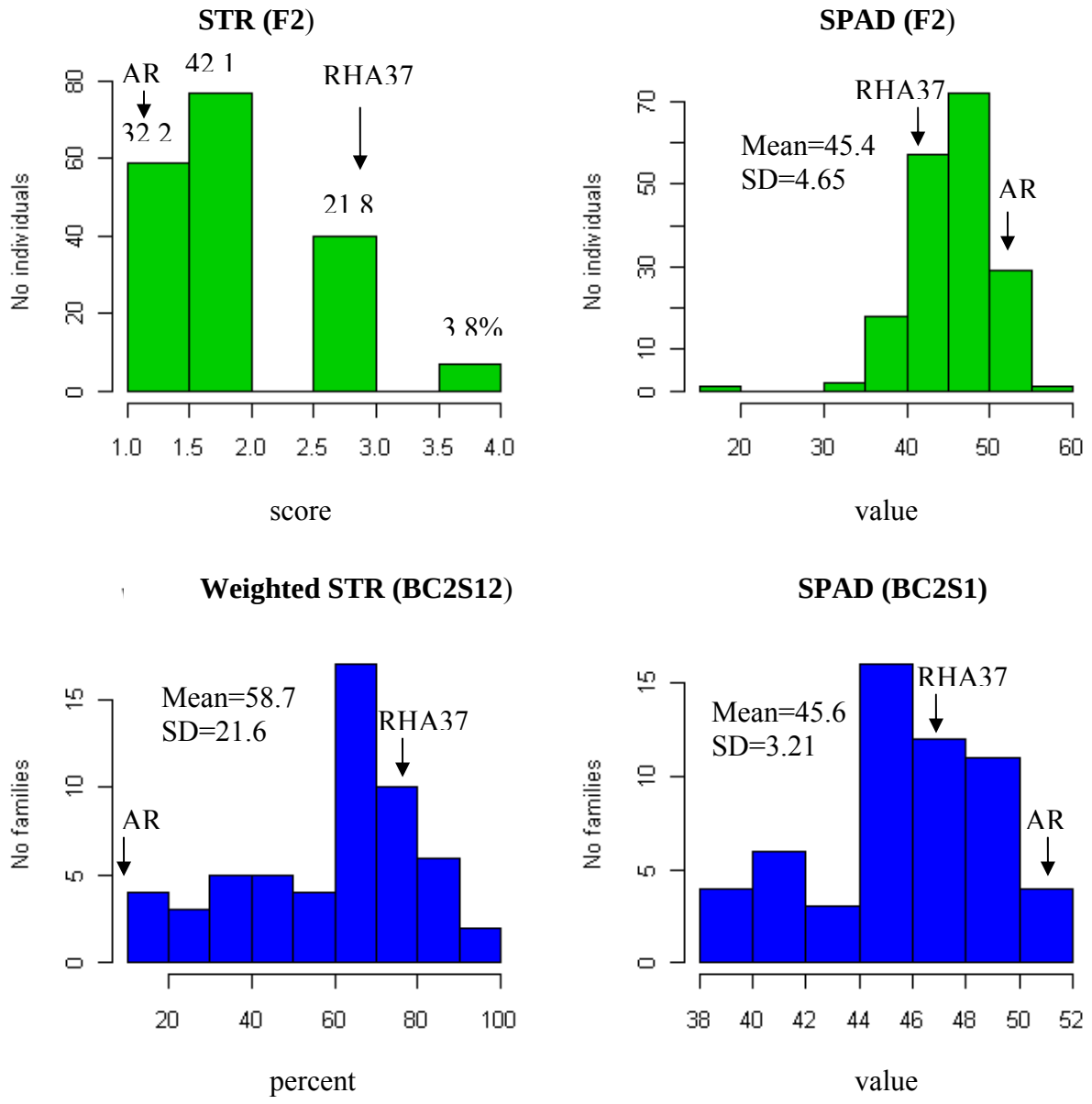

Figure 3.4. One-dimensional profiles of Bayes factors rescaled as 2logBF for main (solid black lines) and epistatic effects (dotted black lines) for intermated F_2 population. Salt tolerance rating (STR, upper) and SPAD (bottom). The horizontal lines represent the significance threshold of 2logBF=2.1.

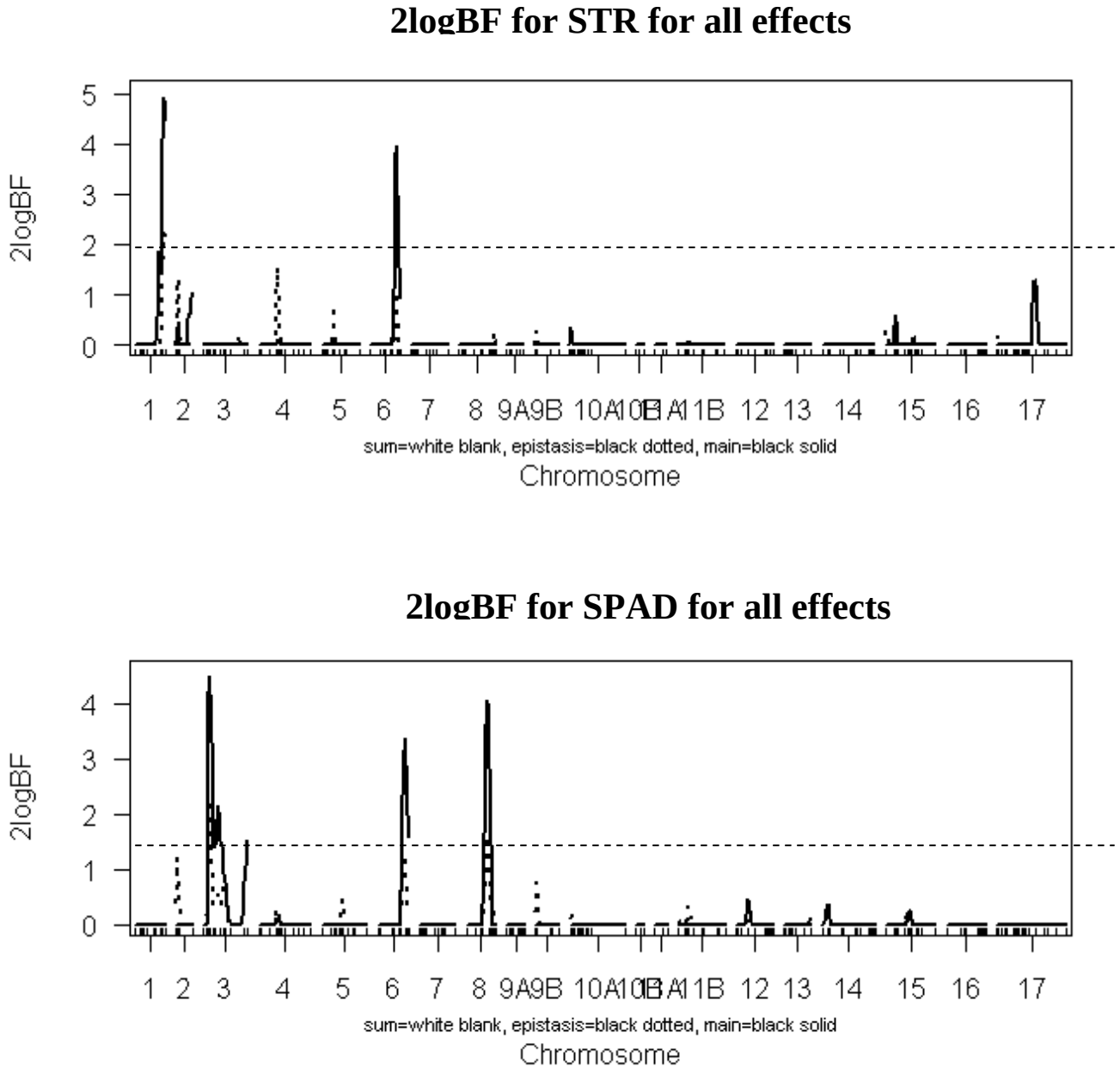

Figure 3.5. Two-dimensional profiles of Bayes factors recalled as 2logBF for intermated-F2 population for selected chromosomes. Salt tolerance rating (STR, upper figure) and SPAD (bottom figure), the upper diagonal of each figure shows the Bayes factor for the epistatic model, the lower diagonal shows the Bayes factor for the full model with epistasis compared with no quantitative trait loci. Color bar on the right side of the figure indicates the 2logBF value.

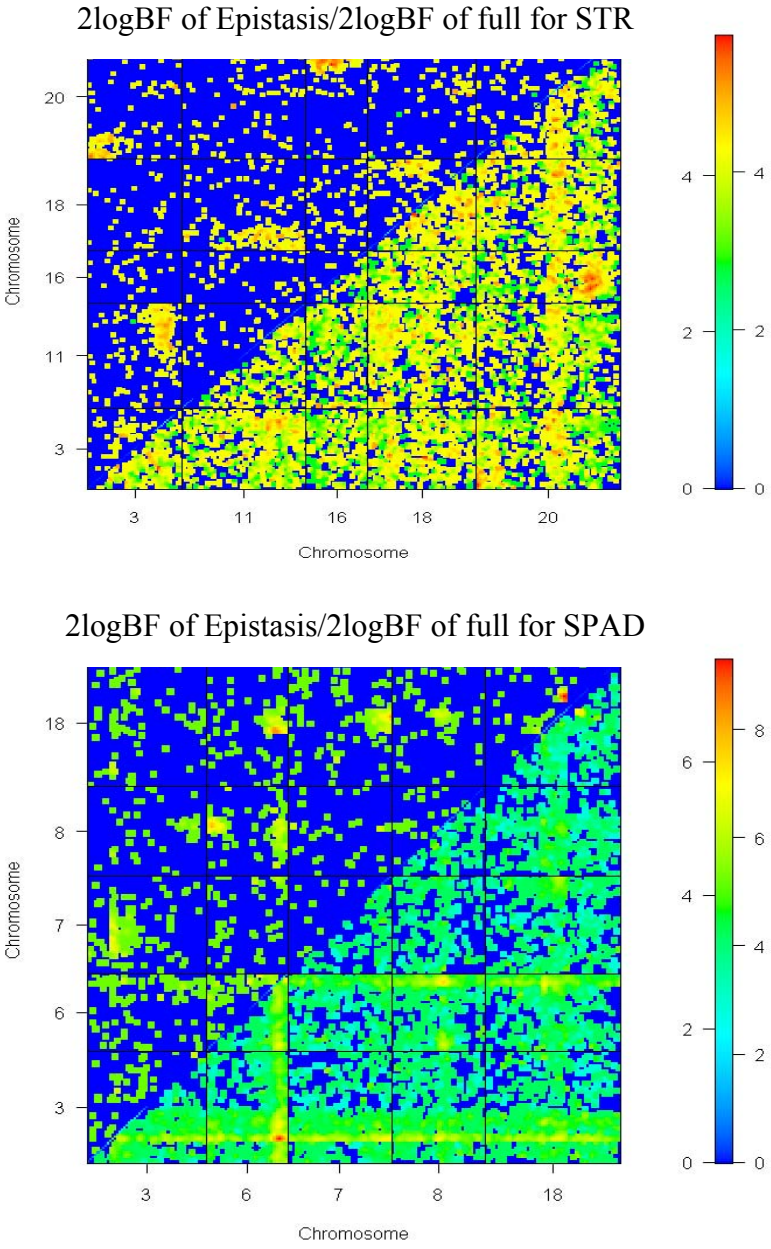

Figure 3.6. One-dimensional profiles of Bayes factors rescaled as 2logBF for main (solid black lines) and epistatic effects (dotted black lines) for BC2S1 families. Weighted salt tolerance rating (WSTR, upper) and SPAD (bottom). The horizontal lines represent the significance threshold of 2logBF=2.1.

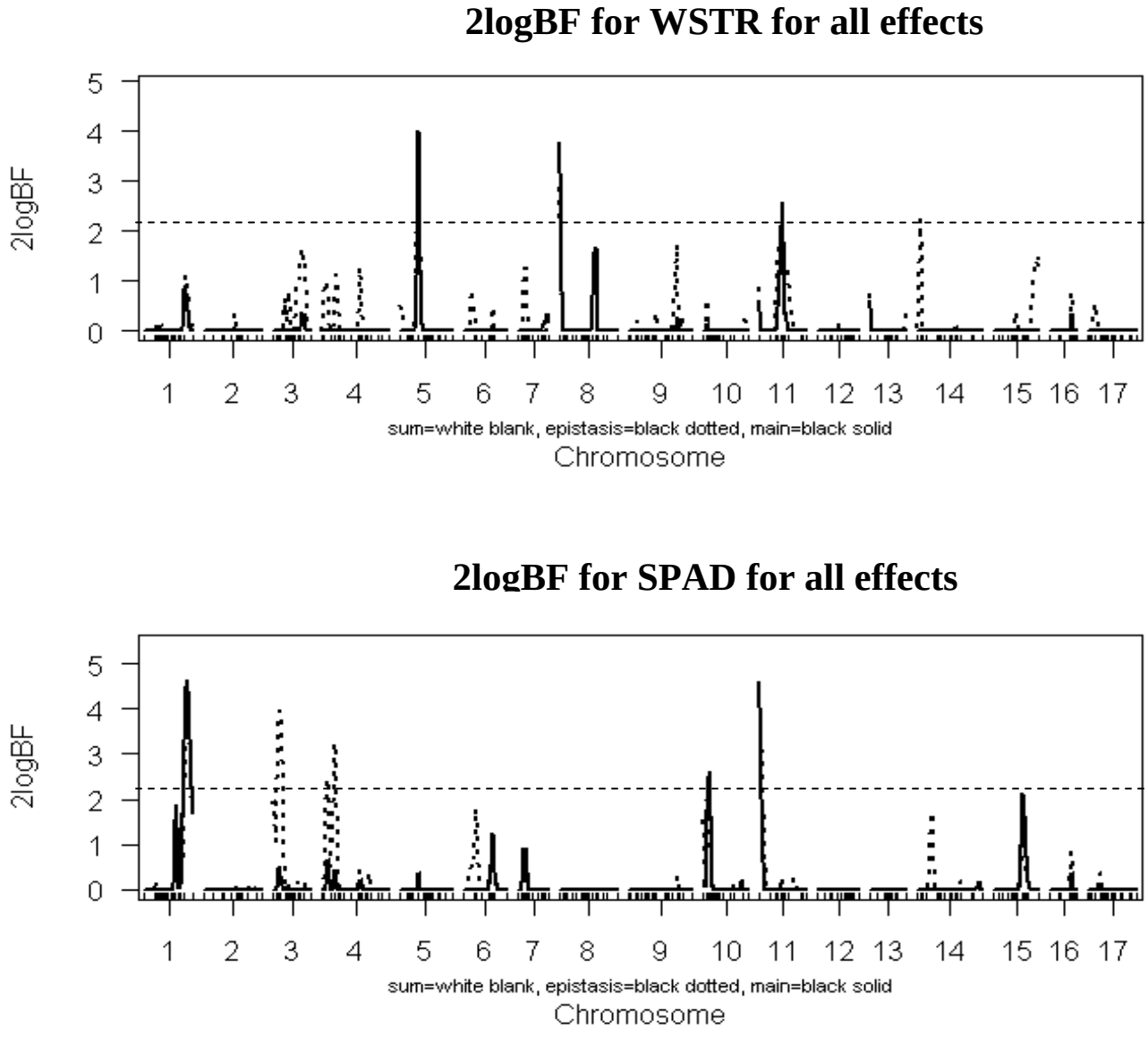

Figure 3.7. Two-dimensional profiles of Bayes factors recalled as 2logBF for BC2S1 families for selected chromosomes. Weighted salt tolerance rating (WSTR, upper figure) and SPAD (bottom figure), the upper diagonal of each figure shows the Bayes factor for the epistatic model, the lower diagonal shows the Bayes factor for the full model with epistasis compared with no quantitative trait loci. Color bar on the right side of the figure indicates the 2logBF value

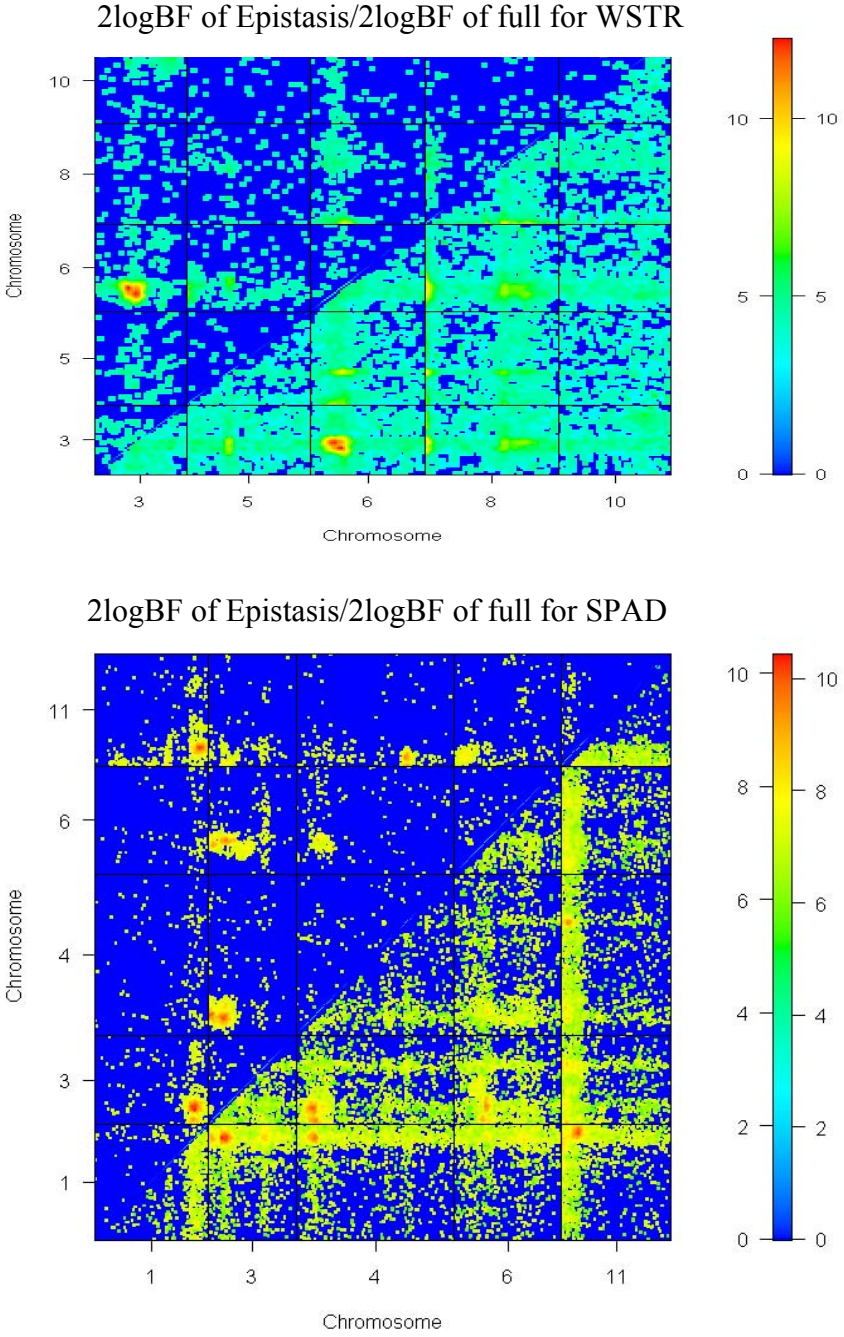

Figure 3.8. Comparison of the salt tolerance QTL found in this study and those found by Lexer et al. (2003)

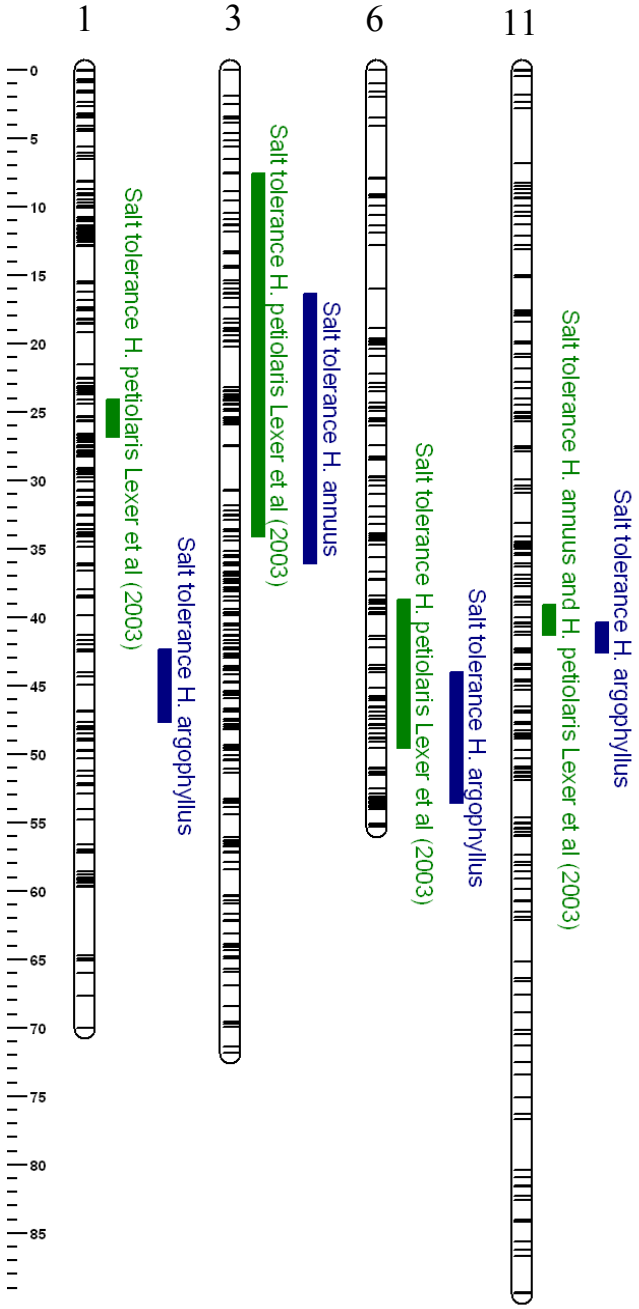

Table 3.1. Statistic of linkage maps for the intermated-F_2 and BC2 populations

Linkage	Length			Loci		
group	F_2	BC2S1	Difference	F_2	BC2S1	Difference
Chromosome	cM	cM	cM	no.	no.	no.
1	68.2	108	39.8	23	27	4
2	35.8	129	93.2	14	17	3
3	102	85	17	18	18	0
4	120	150	30	17	22	5
5	125	115	10	21	22	1
6	69.4	99.7	30.3	12	12	0
7	82.6	79.4	3.2	18	17	1
8	84.7	128.7	44	22	21	1
9A	40.2	143.5	48.2	11	28	17
9B	55.1	NA	NA	11	NA	NA
10A	34.6	100	47.8	16	22	6
10B	17.6	NA	NA	5	NA	NA
11A	28.7	104	31.7	4	12	8
11B	107	NA	NA	13	NA	NA
12	84.8	92	7.2	12	17	5
13	62.5	81	18.5	13	13	0
14	120	146	26	18	21	3
15	115	100	15	17	17	0
16	89.7	60	29.7	18	20	2
17	158	108	50	23	22	1
Total	1601	1829	228	306	328	22

Table 3.2. Summary of statistics for main and epistatic QTLs obtained with Bayesian analysis for salt tolerance rating (STR) and weighted salt tolerance rating (WSTR) and SPAD value in the intermated-F2 and BC2S1 families.

			2logBF			Effects			Heritability[c]	
Trait[a]	Generation	LG	pos.	Main	Epistasis	Additive[b]	Dominance	Epistasis	Main	Epistasis
		chr	cM						%	%
str	F2	1	65.9	5.04	2.32	-0.03	-0.22	0.006	7.87	0.93
str	F2	6	61	3.69	0.81	-0.001	0.2	0.006	6.5	0.71
spad	F2	3	15.1	4.84	2.41	+0.012	1.94	-0.56	5.8	1.3
spad	F2	6	61	4.18	2.36	-0.052	-1.92	0.04	5.6	2.1
spad	F2	8	66.3	4.46	1.61	+0.011	-1.86	0.011	6.7	0.82
wstr	BC2S1	5	41	2.12	2.32	-6.36	-	16.6	6.1	13.6
wstr	BC2S1	8	0	4.04	3.52	+10.76	-	13.4	9.8	10.2
wstr	BC2S1	11	51.1	2.56	2.32	-9.51	-	-14.2	7.7	8.9
spad	BC2S1	1	96	4.74	498	-1.48	-	6.05	10.3	38.3
spad	BC2S1	3	18.2	1.13	4.25	+0.164	-	2.67	2.5	69
spad	BC2S1	4	16.6	0.7	2.25	-0.35	-	-5.46	3.64	41.2
spad	BC2S1	10	11.7	2.52	3.31	+0.959	-	-6.63	10.3	36.8
spad	BC2S1	11	0	4.4	3.86	-1.81	-	3.42	11.3	14.6

[a]Trait abbreviation: salt tolerance rating (*str*), chlorophyll content (*spad*) and weighted salt tolerance rating (*wstr*).
[b]Negative additive value indicates that the *H. argophyllus* parent is contributing with the favorable allele.
[c]Heritability is the proportion of the phenotypic variance explained by main-effect and epistatic QTL

Table 3.3. Summary of statistics for epistatic interactions obtained with Bayesian analysis for salt tolerance rating (STR) and weighted salt tolerance rating (WSTR) and SPAD value in the intermated-F2 population and BC2S1 families.

Trait[a]	Generation	Pair[b]	pos1	pos2	2logBF	aa[c]	ad[d]	da[e]	dd[f]	Heritability [g]
		chr	cM	cM						%
str	F2	10A:15	84	4	7.63	0	0	0.37	0	45.6
str	F2	3:04	80.4	44.4	8.15	0	0	0	-0.57	43.3
str	F2	13:17	26.1	124.4	8.08	0	-0.4	0	0	36.1
str	F2	3:10A	80.4	87.2	7.6	0	-0.28	0	0	37.5
spad	F2	3:07	15.1	16.5	6	0	0	-2.49	0	14.6
spad	F2	6:08	3.6	66.4	5.9	0	3.03	0	0	14.4
spad	F2	6:15	54.4	55.4	5.6	-1.14	0	0	0	15
wstr	BC2S1	3:06	27.8	26.8	12.3	59.1	-	-	-	64.3
wstr	BC2S1	5:06	41.1	35.3	9.02	31.5	-	-	-	66.3
wstr	BC2S1	6:08	28.8	0	9.09	22.5	-	-	-	41.2
wstr	BC2S1	3:10	71.5	76	8.4	38.9	-	-	-	63.2
spad	BC2S1	1:03	96	16	10.7	7.83	-	-	-	57
spad	BC2S1	3:04	0	30.3	9.6	-7.06	-	-	-	61
spad	BC2S1	1:11	26.7	0	6.5	4.45	-	-	-	59
spad	BC2S1	3:06	13.9	29.9	7.5	-5.44	-	-	-	60.8

[a]Trait abbreviation: salt tolerance rating (*str*), chlorophyll content (*spad*) and weighted salt tolerance rating (*wstr*).
[b]Pair of loci with significant interaction
[c]aa is the effect of additive by additive interaction between QTLs
[d]ad is the effect of additive by dominant interaction between QTLs
[e]da is the effect of dominant additive interaction between QTLs
[f]dd is the effect of dominant by dominant interaction between QTLs
[g]Heritability is the proportion of the phenotypic variance explained by QTL-QTL interaction

CHAPTER 4

QTL ANALYSIS OF MORPHOLOGICAL TRAITS UNDER SALINE AND NON-SALINE CONDITIONS DURING VEGETATIVE GROWTH IN SUNFLOWER[1]

[1] Rey, J.I., J.M. Burke, E.R. Boerma and S.J. Knapp to be submitted to *Eupphytica*

ABSTRACT

The effect of salinity on nine morphological traits in an interspecific cross between *Helianthus annuus* and its closet relative *Helianthus argophyllus* was investigated by QTL (quantitative trait locus) analysis. A total of 22 and 26 QTL related to productivity and salt tolerance under saline and non-saline conditions respectively were found. The comparison of the QTL found in the BC2Test Cross families growing under saline and non-saline conditions allowed us to identify only two QTL in common under saline and non-saline conditions. The large genotype by salinity interaction could complicate the improvement of salt tolerance and productivity under saline conditions. A relatively small proportion of the phenotypic variance was explained for QTL for all traits under saline condition limiting the use of marker assisted selection (MAS). *H. argophyllus* was confirmed to be a good source for the improvement of salt tolerance and productivity under saline conditions. Due to the limitation of MAS for salt tolerance, other approaches for the exploitation of the salt tolerance found in *H. argophyllus*, such as genomic selection should be investigated.

INTRODUCTION

Salinity is a major abiotic stress threatening food production in many areas around the world, especially in arid and semi-arid climates. Around 6% of the world's land is affected either by salinity or sodicity, which is a secondary effect of salinity in clay soils (www.fao.org). Almost 20% of the irrigated and 2.1% of the dryland agricultural land is affected by salt (Yamaguchi and Blumwald, 2005). This estimation does not take into account the land that is salt affected and cannot be cultivated due to high salinity levels. Salinisation can take one of two forms; primary salinity due to natural causes or secondary salinity (human-induced salinity) due to practices such as irrigation or deforestation (Irrigation, 2002). With a projected increase of world population by 1.5 billion over the next 20 years (Yamaguchi and Blumwald, 2005), increasing productivity of crops in non-saline as well as saline soils is imperative for feeding the growing world.

Efforts to improve crop productivity under saline conditions through conventional breeding have been conducted in several crops with limited success (Flowers, 2004). One of the main problems that conventional plant breeders face is the limited genetic variability for salt tolerance existing in the elite gene pools of most crop species (Ashraf and Akram, 2009). In order to overcome this problem, breeders have utilized salt tolerant wild relatives of crops to introgress salt tolerance genes into elite germplasm. The use of inter-specific crosses in plant breeding could bring undesirable genes in combination with the desirable ones, this phenomenon known as linkage drag limits the success of this approach (Chinnusamy et al., 2005). Marker-assisted-selection (MAS) has been proposed as a solution to reduce linkage drag during the introgression of wild alleles into elite germplasm (Frisch and Melchinger, 2001; Frisch et al., 1999). Furthermore, methods such as advanced backcross QTL mapping (AB-QTL) (Tanksley

and Nelson, 1996) and introgression libraries (Zamir, 2001) have been developed for an efficient use of wild and exotic germplasm in breeding programs.

Another problem that plant breeders have to face when they breed crops for salt tolerance is the spatial and temporal heterogeneity of salinity in the field (Cetin and Kirda, 2003). There has been disagreement for a long time among breeders about the best environment to make evaluations and selections of the breeding material to improve salt tolerance. Richards (1983; 1992; 1995), argues that due to the heterogeneity of salinity in soils breeders should concentrate their efforts on selecting for yield in non-saline environments and disregard salt tolerance. He suggests breeders should take the high yield from the areas with low salinity and accept the yield losses in the saline patches in the field. In this way we will obtain more yield than planting salt tolerant cultivars with low potential yield. Isla et al., (2003) agreed that on moderately saline soils the best strategy was to breed for high yield potential, but argued that under higher salinity breeding for both yield and salinity, tolerance was important. If the target environment is of moderated salinity, the breeder should select for high yield in non-saline environment. On the contrary, if the environment is of high salinity the breeder should select in the target environment for high yield and salt tolerance. These authors have not mentioned the interactions between genotype and salt treatment that exist and have been documented in some studies (Igartua, 1995; Monforte et al., 1997; Singh et al., 2009; Zhou et al.). Monforte et al.(1997) approached this problem using QTL mapping for yield components in tomato under saline and non-saline conditions and assessing genotype x environmental interaction. This strategy combines QTL information from both environments to select for "broad adaptation" through MAS.

Cultivated sunflower (*Helianthus annuus* L.), the fourth most important annual crop in the world and grown for its edible oil (Fernández-Martínez et al., 2009), has been classified as

moderately salt sensitive based on water stress index (Katerji et al., 2003). Salinity affects leaf expansion and biomass accumulation in seedlings (Delgado and Sánchez-Raya, 1999; Rawson and Munns, 1984) and results in yield loss due to reduction in the number of seeds per head, while oil percent is unaffected (Francois, 1996). There is only a single release of sunflower lines bred specifically for salt tolerance from an interspecific cross between *H. annuus* and *H. paradoxus* (Miller, 2003). Silverleaf sunflower (*Helianthus argophyllus* Torrey & Gray), the closest relative of common sunflower (Schilling and Heiser, 1981), has been widely used in sunflower breeding and it is suggested as a source of favorable alleles for salt and drought tolerance (Rauf, 2008; Richards, 1992). Our data have shown a high level of salt tolerance in different *H. argophyllus* accessions surviving to a seawater level of salt concentration (Rey, unpublished data). These observations, together with the fact that it has been the most used wild species in sunflower breeding, makes *H. argophyllus* a good candidate for the improvement of salt tolerance in cultivated sunflower.

The objectives of the present study were to: i) identify QTL for morphological traits in an interspecific population between *H. annuus* and *H. argophyllus* growing under saline and non-saline conditions, ii) make comparisons of the QTL found for the morphological traits under different saline conditions, iii) assess the importance of *H. argophyllus* as donor of favorable alleles for salt tolerance to sunflower breeding, and iv) using the information generated here, make inferences about the best strategy for the improvement of salt tolerance in sunflower.

MATERIAL AND METHODS

Plant material

An interspecific cross was made between a nuclear male sterile *H. annuus* line (NMS377) and a *H. argophyllus* accession (ARG1820). NMS377 is an elite sunflower inbred line

moderately susceptible to salt stress. ARG1820 is an accession that originated from the Gulf Coast of Texas and is highly tolerant to salt conditions (Rey, unpublished data). A single fertile F_1 plant was identified and crossed with a sterile NMS377 plant. The fertile BC1F1 plants were crossed again with sterile NMS377 plants. Finally, the BC2F1 plants were crossed to a tester, CMS412 High Oleic (HO) line, which is salt sensitive (Rey, personal observation) to produce the BC2 TestCross (TC) families that were used in this study.

Evaluation of salt tolerance

Seeds of parents, F_1, and BC2TC families were surface sterilized with 10% bleach (sodium hypochlorite, NaCLO) solution for 1 min and rinsed with double-deionized water. Seeds were then germinated in Petrie dishes in the dark at 25°C for 3 d and healthy seedlings were transplanted into plastic 164-cm^3 cone-tainers (Ray Leach Containers, Tangent, OR) filled with washed sand. The method developed by Lee et al (2008) was used for salt tolerance screening in both populations (Fig. 1). Plastic racks of cone-tainers (49 cone-tainers/rack) were placed in 39-L Steriliter boxes (Townsend, MA) containing 11 L of full strength Peters Excel CalMag solution. The boxes were arranged in a split-block treatment design where genotype was the main plot and salt treatment was the sub-plot. The experiment was repeated three times, where each time represents one replication. The first salt treatment of 50 mM of NaCl (electric conductivity (EC) of ~5.3 ds/m) was applied when plants developed their first pair of true leaves and a 50 mM of NaCl was added every 2 d until reaching the target concentration of 150 mM of NaCl (~18 ds/m). The EC and pH were monitored periodically, and if differences due to evaporation were found, tap water was added to maintain the experimental conditions. The solution was replaced every 4 d. The Steriliter boxes containing the cone-tainers were rotated on greenhouse benches every day in order to reduce the experimental error due to heterogeneity in

light and temperature within the greenhouse. Greenhouse conditions were maintained at an average temperature day/night of 30/21°C and a 14 hr daylenght by use of supplemental lighting during the experiment.

When plants reach the R1 phenological stage (Schneiter, 1981), leaf chlorophyll content (SPAD value) for each plant was estimated using a chlorophyll meter (Konica Minolta SPAD-502, Minolta corporation, Ltd., Osaka Japan). This SPAD value is proportional to the chlorophyll content in the leaves (Yamamoto et al., 2002). Plants were then harvested and fresh shoot (fws) and root (fwr) weights were recorded. All plants in each BC2TC family were put in paper bags and dried in an oven at 60 °C for 3 d, then dry shoot (dws) and root (dwr) weights were recorded. The ratio shoot:root for fresh (fws/fwr) and dry (dws/dwr) weights, as well as the ratio dry:fresh weights for shoots (dws/fws) and roots (dwr/fwr) were calculated.

Statistical analysis

Data analysis was conducted using SAS PROC GLM (SAS 9.1; Cary, NC) to determinate the significance of main effects and interactions for all traits measured in this study. A mixed model with genotype and salt treatment as a random, replication and border as fixed effects. The border term for each family growing on the border of the racks was added in the model to be able to capture the differences in growth due to this effect. Number of plants in each row (family) was used as covariate in the model. The broad sense heritability (h^2) was estimated on family basis as $\sigma^2_G/(\sigma^2_{G+} \sigma^2_{e/3})$ x 100 for both saline conditions. In the formula σ^2_G is the genotypic variance and σ^2_e is the error variance estimated by the REML method of SAS PROC MIXED. Least squares means for each family in both salt treatments were estimated by SAS PROC MIXED for use in the QTL analysis, where family effect was fixed and replication and border were random effect. Pearson's correlation between traits was estimated by SAS PROC CORR.

Genetic linkage map and QTL analysis

An EST-SNP genetic linkage map constructed in a previous study for the NMS377 x *H. argophyllus* ARG1820 BC2 population, was use to map the QTL involved in the expression of the morphological traits under saline and non-saline conditions (Rey et al., 2011, unpublished data). The map contained 328 EST-SNP markers spanning 1829 cM across 17 linkage groups.

Three analyses were performed to identify QTL; single-marker analysis (SMA), multiple-loci analysis (MLA), and Bayesian interval mapping (BIM). SMA and MLA were conducted using PROC GLM (SAS 9.1; Cary, NC). Association between markers and traits were considered to be significant at the P=0.05 level. Coefficients of determination (R^2) were obtained for each marker associated with a trait. The marker with the highest R^2 value in each chromosomal region was then chosen and a multiple linear model was fitted using stepwise regression for the MLA.

Bayesian interval mapping (Yi et al., 2005) implemented in the package R/qtlbim (www.qtlbim.org) released by Yandell et al, (2007) was used to map main-effect QTL for validating the results from MLA. Each chromosome was divided into a 1-cM segment and considered as possible QTL positions. Based on these estimations the prior number of QTL identified by MLA main-effect QTL was set to be used in the Bayesian analysis. We fitted the models using R/qtlbim (Yandell et al., 2007), which implements a Markov chain Monte Carlo (MCMC) algorithm (Yi et al., 2005; Yi et al., 2007). The MCMC algorithm generated posterior samples from the joint posterior distribution of all parameters in the model, proceeding to draw each parameter from its conditional posterior distribution using the latest values of all other unknowns and the observed data. For all analysis the MCMC algorithm ran for 20,000 iterations after discarding the first 1,000 iterations as burn-in. To reduce serial correlation in the stored

samples, the chain was thinned by one in 40 iterations, yielding 5000 samples for posterior analysis. Convergence diagnostics and mixing behavior of the chain was assessed using the CODA package (Plummer et al., 2006) incorporated in R/qtlbim. This showed that the simulation chains converged and mixed well. In posterior analysis, Bayes factors (BF) of main effects and epistasis per locus or pair of loci are individually calculated and compared with a BF threshold of 3, or 2ln(BF)=2.1, to claim the presence of a QTL (Kass and Raftery, 1995). The phenotypic variance explained by the genetic effects was estimated by its heritability. A QTL was identified as putative if it was detected using the three analysis.

RESULTS

Phenotypic distribution

Analysis of variance for genotypes (the BC2TC families, their parents NMS377, ARG1820, and the F1 RHA377 x CMS412HO) growing in saline and non-saline conditions showed significant differences between salt treatments and significant (P=0.05) genotype-treatment interactions for all the nine traits studied (data not shown). For the analysis of the BC2TC families growing under saline conditions, there were significant (P=0.05) differences for all traits except for dws/dwr and dwr/fwr (Table 1). For the BC2TC families growing under non-saline conditions we observed significant (P=0.05) differences for all traits except for dwr/fwr. Comparison of the F_1 mean with the maximum values for BC2TC families indicated that for all traits there were superior BC2TC families in both saline and non-saline conditions. Coefficient of variation (CV) ranged from 11 to 30% for the traits in saline conditions and from 7 to 48% in non-saline conditions (Table 1).

Heritabilities and phenotypic correlations

Correlation coefficients among the morphological traits under, saline and non-saline conditions are presented in Table 2. There were a few pairs of traits such as dws/dwr-dws/fws, and SPAD with fws/fwr and dws/fws that were not significant ($P>0.05$), but most comparisons were significant. For saline conditions, correlation among most trait pairs were highly significant ($P\leq0.001$), except for fwr-fws, fws/fwr with fws and fwr, dwr/fwr with dws, fws and fwr, and SPAD with dwr fws, and dws/dwr (Table 2). For dws (an important trait related to productivity) there is a positive correlation between dwr, fws, and fwr under both saline ($r=0.60$, $r=0.95$ and $r=0.78$) and non-saline conditions ($r=0.85$, $r=0.98$ and $r=0.93$). Whereas there is a positive correlation between dws and dws/fws ($r=0.53$) under non-saline condition and that correlation become negative ($r=-0.15$) under saline condition. There is also positive correlation between dws and dwr/fwr ($r=0.53$) under non-saline conditions while there is not significant correlation ($P>0.05$) for the same pair of traits under saline conditions ($r=0.02$). Another important trait related to productivity is fws and we observed some changes in relationship to other traits as product of salinity. The fws is negatively associated with fws/fwr ($r=-0.30$) and spad ($r=-0.20$) under non-saline conditions, while it is not significantly correlated ($P>0.05$) under saline conditions. Furthermore, fws is positively correlated with dwr/fwr ($r=0.5$) under non-saline conditions and not correlated under saline conditions. We can also observe a positive correlation ($r=0.54$) and a negative correlation ($r=-0.63$) between fwr and dws/fws under non-saline and saline conditions respectively. These results indicated that saline conditions change the association among traits. Then, families with higher fws and dws under saline conditions have reduced dws/fws ratio than under non-saline conditions.

Broad-sense heritabilities ranged from 10% for dws/dwr under non-saline conditions to 62% for dws under saline conditions (Table 1). Inheritance was moderated and similar for fws

under both saline conditions (57% saline and 55% non-saline conditions).Inheritance was also moderate for fwr (38%), spad (43%), fws/fwr (40%) and dws/fws (42%) under saline conditions. The lowest heritabilities were 23% for dwr under saline conditions and 25% and 10% for dwr and dws/dwr under non-saline conditions, respectively.

QTL analysis for morphological traits

QTL mapping showed the presence of 22 QTL on 11 different chromosomes for seven traits under saline conditions (Fig. 2, Table 3). We found QTL on all chromosomes except on 1, 5 7 11, 12, and 14. We found three QTL per trait except for dwr and spad that we found 2 and 4 QTL, respectively. We found a QTL for fwr on chr 9 (122 cM) that co-localized (pleiotropic) with a QTL for dws/fws. Another QTL for fwr on chr 10 (86 cM) also co-localize with the QTL for dws and spad. The QTL for fws on chr 16 (57 cM) co-localize with the QTL for fws/fwr and dws/fws. Finally, the QTL for fws/fwr on chr 10 (20 cM) co-localize with the one for dw/fws. The percent of the phenotype variance explained by the QTL were relatively low, the values varied between 4 and 11% in SMA, 3.8 and 14% in MLA and 3 and 10% in BIM (Table 3). The highest R^2 values corresponded to *dws/fws.10.1* (13%), *dws/fws.16.1* (9.3%), and for *spad.3.1* (10%). While the smallest R^2 values of the phenotypic variance explained by the QTL corresponded to *fwr.9.1* (4%), *dws.10.1* (4%), and *dwr.4.1* (3%). Both parents contributed favorable alleles to various traits. The *H. argophyllus* parent contributed the positive alleles at 13 QTL, while the *H. annuus* parent contributed positive alleles at nine QTL (Table 3, Fig. 2).

The analysis of marker and phenotypic data for eight traits under non-saline conditions found 26 QTL (Fig. 2, Table 4) These QTL were distributed across all chromosomes except on 1, 2, 5, 6, 12, 13, and 15. We found four QTL for fwr, dws, dwr and spad, three QTL for fws and fws/fwr, and two QTL for dws/dwr and dws/fws. The percent of the phenotype variance

explained by the QTL (R^2) varied from 5 to 22% for SMA 5 to 22% for SMA and from 5 to 20% for BIM . We found the highest R^2 values for *fws.14.1* (18%), *fwr.16.1* (17%), and *fws/fwr.16.1* (19.7%). While the smallest R^2 values were found for *dws.9.1* (5.5%), *dwr.16.1* (4.4%), and *dwr.11.1* (4.8%). As under saline conditions both parents contributed favorable alleles for the various traits. Both, the *H. argophyllus* and the *H. annuus* parents contributed positive alleles at 13 QTL. (Table 4). We detected more QTL for the morphological traits under non-saline than saline conditions. In general the values for the proportion of the phenotypic variance explained by the QTL were larger under non-saline conditions. The *H. argophyllus* parent contributed a greater number of positive alleles at QTL under saline conditions.

DISCUSSION

Saline conditions in our study reduced fresh and dry weight of shoot and roots, as shown in Table 1 and Figure 1 by comparing mean fresh and dry weights of shoot and roots of BC2TC families, F1, and parents under saline and non-saline conditions. Salinity reduces plant growth through the osmotic effects of the salt in the external solution around roots. A consequence of this decrease in the osmotic potential is reduction of cell expansion in root tips, young leaves, and closure of stomata (Munns and Tester, 2008a). Reduction in growth decreases the volume of the biomass where the excess of toxic ions, such as Na^+ and Cl^- can be accumulated (dilution effect of the ions). With the continuous accumulation of salt and limited growth, toxics ions can reach noxious levels within plant cells producing irreversible damage (Munns, 1993). Neumann, (1997) suggests that for high-input crops that grow under intermittent irrigation with moderate salinity breeders should focus their efforts on increasing the capacity for cell expansion (growth) under moderate osmotic stress.

Ashraf and Tufail (1995) found that cultivated sunflower seedlings producing the highest biomass under saline conditions also produced the highest seed yield under saline conditions. They concluded that salt tolerance in sunflower does not change with the stage of plant development and that selection for salt tolerance could be achieved during the initial growth stage through the selection of the seedlings with the highest biomass. We found genetic variation for fresh and dry weight of shoots and roots among the BC2TC families (Table 1). Based on this hypothesis we would expect that selection for high seedling biomass under saline conditions will lead to higher seed yield. This hypothesis should be validated growing diverged selected BC2TC families in yield trials and correlating seed yield with seedling biomass from this study.

Salinity increased SPAD values associated with chlorophyll content in the BC2TC families, parents, and F1. This increase in SPAD values could be associated with reduction in growth. As previously indicated reduction in osmotic potential reduces cell elongation and cell division. Appearance of leaves will be delayed and leaf size will be reduced. The largest reduction will be in cell area rather than in cell volume, as a consequence leaves will be smaller but thicker. This change in leaf anatomy results in higher chloroplast density per unit area increasing SPAD values in the BC2TC families, parents, and F1 (Munns and Tester, 2008a). The increase in SPAD value was higher for the tolerant parent ARG1820 than for the susceptible RHA377 (Table 1). Salinity reduces chlorophyll content in sunflower through the increase of chlorophyll degradation (Santos, 2004). Chlorophyll content estimated through SPAD values, was shown to be associated with salt tolerance in soybean (Hamwieh and Xu, 2008), wheat (Munns and James, 2003), and sunflower (Rey et al., 2011, unpublished data). Cuin et al., (2010) found that SPAD value was a reliable method to screen for salt tolerance in wheat. These studies

suggest that the use of SPAD value would be a useful method for screening sunflower for salt tolerance in breeding populations.

Our data also shows that the fws/fwr ratio decreased and the dws/fws ratio increased as a consequence of salinity (Table 1). Shoot:root ratio (fws/fwr) has been reported to be affected by salinity in other crops and it has been suggested to be a good indicator of salt tolerance (Albacete et al., 2008; Flowers and Hajibagheri, 2001). This morphological parameter is an important adaptative response and is due to a rapid inhibition of shoot growth and maintenance of root growth (Gama et al., 2007). A smaller reduction in the fws/fwr ratio would be a desirable characteristic to improve salt tolerance. The dws/fws ratio is directly related with the content of water in the plant and our data shows that it was increased as an effect of the salt treatment (Table 1). A smaller dws/fws ratio will imply greater content of water within the plant also refered as succulence. It has been shown that dilution of salts through succulence is an important adaptative trait that can be used as an indicator of salt tolerance in plants (Foolad, 2007; Haq et al., 2008). We found that the tolerant parent ARG1820 has a smaller dws/fws ratio than the susceptible RHA377 parent, indicating more succulence (Table 1). The fws/fwr and dws/fws ratios are important indicators of salt tolerance, but not good indicators of productivity under saline conditions. These ratios should be used in association with another trait indicator of productivity such as fresh shoot weight to be effective in improving productivity under saline conditions.

The broad-sense heritability estimates indicated that most traits were low to moderately heritable (Table 1). In general, heritabilities of the traits in saline conditions were a little higher than under non-saline conditions. There were also traits with big differences in heritability estimates, such as for dws/fws with 42% under saline and 28% under non-saline conditions. With

these heritability estimates, it would be more efficient to select for the traits under saline than non-saline conditions. Furthermore, the equation for the efficiency of MAS over phenotypic selection developed by Lande and Thompson (1990) indicates that the smaller the h^2 of the trait, the greater efficiency of MAS when compared with phenotypic selection. By only considering the heritability values, the use of MAS should increase efficiency of selection for these traits. Phenotypic correlations were high and positive among fresh and dry weight of shoot and roots under, saline and non-saline conditions (Table 2). The dws/fws ratio, or succulence, under saline conditions is significantly and negatively associated with fws (r=0.2) and fwr (r=0.63). As previously indicated succulence would be a good parameter to select for salt tolerance but is not a good indicator of productivity under saline conditions. Since this trait is negatively associated with other productivity traits such as fws and dws, it would be beneficial to select for these two traits at the same time. Selection for a desirable low dws/fws ratio will be associated with an increase in fws and dws. On the contrary, fws/fwr ratio and SPAD value, are good indicators of salt tolerance, but are not associated with fws (P>0.05). These results suggest that selection for a high fws/fwr ratio and/or high SPAD value should have no effect on fws but reduce dws. SPAD value and dws/fws ratio are both good traits for selection together with fws to increase productivity and salt tolerance simultaneously. Both traits have similar heritabilities and dws/fws is negatively correlated with fws and dws, while SPAD value is not associated with fws (no negative effect on fws), and is simple to measure. Given the trait heritabilities and the association of SPAD value and fws these traits would be the suggested traits to increase both salt tolerance and productivity.

The QTL analysis identified 48 QTL for the morphological traits measured under the two saline conditions in this study (Table 3 and 4; Fig. 2). These QTL were located across all

chromosomes except for 1 and 12. There were two important genome regions on chr 9 and 16 in common for both saline and non-saline conditions (Fig. 2). There were only two QTL, for *fws/fwr* ratio and *fwr* on chr 16 (57 cM) in common for both saline conditions. There was a big change in the proportion of the phenotypic variance explained for this QTL from 20% under non-saline conditions to 9% in saline conditions for *fws/fwr* ratio and from 17% to 4.5% for *fwr*. Overall, the proportion of the phenotypic variance explained was bigger for QTL under non-saline conditions. The positive allele at the QTL for *fwr* changed with the salt treatment. The RHA377 parent contributed with the favorable allele under non-saline condition and the ARG1820 parent under saline conditions. This phenomenon of "altered QTL" has been observed previously in other QTL studies for salt tolerance in tomato (Monforte et al., 1997). It is possible that the *fws/fwr*.16.1 QTL is orthologous present in the wild and cultivated sunflower. This locus is also pleiotropic for the *dws/fws* ratio under saline conditions and for *dwr* under non-saline conditions. Another QTL identified for both saline conditions but for different traits is on chr 9 (115 cM). Under saline conditions a QTL for *fwr* was found at this locus and under non-saline conditions for *dws*. There are two other QTL under saline conditions for *fws* and *dws/fws* ratio which were only 7 cM from this QTL (chr 9, 122 cM). Even if these two regions on chr 9 and 16 are important for both saline and non-saline conditions, the QTL varied greatly in effect and proportion of the phenotypic variance explained. This means that the genotype x salt treatment interaction can affect the intra-locus relationships. The *fws.16.1* QTL changed the direction of its effect due to salt treatment. The large genotype by salt treatment interaction presented here it would make very difficult to breed for crop productivity outside the saline target environment.

Under saline conditions we found 22 QTL across all chromosomes expect on chr 1, 5 7 11, 12, 14 and 17 (Table 3, Fig. 2). The QTL *fwr.9.1* co-localized (pleiotropic) with *dws/fws.9.1*

and is located 7 cM from *fws.9.1*., the QTL *fwr.10.1* co-localized with *dws.10.1* and *spad.10.1*. the QTL *fwr.16.1* co-localized with *fws/few.16.1* and with *dws/fws.16.1*., and the QTL *fws/fwr.10.1* co-localized with *dws/fws.10.1*. The traits fws/fwr and dws/fws ratios, share two QTL out of a total of three for each trait, which would explain the high positive correlation of 0.9 between these traits (Table 2). These results indicated that these four regions on chr 9, 10, and 16 may contain clusters of QTL affecting morphological traits pleiotropically under saline conditions that could be elucidated through fine mapping. The *H. argophyllus* parent contributed the positive alleles at 13 of the 22 QTL found under saline conditions. These data indicate that the wild parent is contributing favorable alleles for the improvement of the elite hybrid CMS412 HO x RHA377 under saline conditions. This is also seen from the phenotypic distributions in Table 1. None of the QTL present here were found in our previous study with the same cross under higher salt concentration (300 mM NaCl) (Rey et al., 2011, unpublished data). This suggests that there may be interaction not only between control and salt conditions, but between different levels of salt concentration.

One of our goals was the development of a strategy for salt tolerance improvement using the information generated in this study. Some QTL explaining between 9 and 13% of the phenotypic variance, such as *spad.3.1*, *spad.10.1*, *dws/fws.16.1* and *dws/fws.10.1*, could be useful in the application of MAS for salt tolerance. The relatively low proportion of the phenotypic variance explained by these QTL (results from BIM in Table 3) could limits the applicability of MAS since many small effect QTL are inconsistent (Bernardo, 2008). Previous to their utilization the QTL should be validated in different genetic backgrounds and/or populations to confirm their utility for MAS (Xu and Crouch, 2008). Once we validate the QTL and have them introgressed into an elite breeding line, we could incorporate them into sunflower breeding. An

efficient strategy would be the use of MAS for the QTL conferring salt tolerance during early generations and a posterior phenotypic selection for yield under optimal conditions. MAS is not as effective when selection is required for three, four, or more QTL as the population size required to obtain a target genotype increase rapidly with additional QTL. F2 enrichment followed by inbreeding as suggested by Bonnett et al., (2005) would be a good alternative to increase the frequency of the favorable alleles for salt tolerance in the base population and increase the probabilities to obtain target genotypes. With this approach we can develop a set of target genotypes segregating for productivity.

In this study we did not take into consideration the epistatic effect that could be present among QTL and could significantly contribute to the variation of the traits. In our previous study (Rey et al., 2011, unpublished data) we showed the high complexity of the salt tolerance trait due to epistatic interactions in the same cross. Other approaches to improve salt tolerance and productivity under saline conditions such as genomic selection (Heffner et al., 2009; Meuwissen et al., 2001) should be explored. Methods taking into account interaction have been also develop for genomic selection (Gianola and van Kaam, 2008). These methods are specifically design to deal with complex traits affected by many genes with small effects Jannink et al., (2010).

In summary, we have shown that the wild species *H. argophyllus* is a good candidate for improving salt tolerance and productivity in cultivated sunflower under saline conditions. Improvement for salt tolerance and productivity through MAS would be complicated due to the high genetic complexity of the morphological traits under saline conditions. More powerful tools to improve complex abiotic stresses, such as genomic selection should be further studied.

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TABLES AND FIGURES

Figure 4.1. Scheme of the cone-tainers method used to screen the BC2TC families, parents, and F1 for salt tolerances. Sunflower plants in cone-tainers and racks immersed in Sterilite containers Larger plants are plant growing under non-saline conditions (0 mM NaCl) while smaller plants are growing under saline conditions (150 mM NaCl).

Table 4.1. Least square means and minimum, and maximum values for nine morphological traits for the 94 BC2TC families, parents, and the F_1 and family-mean broad sense heritabilities (h^2) and coefficient of variation (CV) growing under saline and non-saline (control) conditions for the 94 BC2TC families.

Treatment	Trait (units)		Parents		BC2TC Families					
		F1	ARG1820	RHA377	Mean	Min	Max	Significance	CV	h^2
									— % —	
Salt	*fws* (g)	42.0	13.9	33.1	43.5	13.8	60.7	***	14.8	57
	fwr (g)	10.9	6.5	8.1	11	6.6	23.7	***	30.6	38
	dws (g)	5.7	1.9	4.7	6.09	1.9	8.7	***	10.9	62
	dwr (g)	1.2	0.6	0.1	1.36	0.4	3.6	*	45	23
	spad	42.5	49.2	44.8	43.85	38.8	50.7	**	6.0	43
	fws/fwr	4.1	2.9	4.8	4.26	2.2	5.7	***	18.7	40
	dws/dwr	4.6	4.1	5.2	5.03	2.4	8.6	ns	24	NA
	dws/fws	0.5	0.4	0.6	0.6	0.3	0.8	***	20	42
	dwr/fwr	0.1	0.1	0.1	0.12	0.06	0.1	ns	33.8	NA
Control	*fws* (g)	174.9	56.6	153.6	188.3	56.7	249.2	***	20	55
	fwr (g)	41.6	16.9	29.3	40.4	16.9	62.4	***	27.2	49
	dws (g)	18.4	5.3	15.9	19.9	5.2	30.8	***	24	50
	dwr (g)	7.5	3.5	5.7	7.62	0.7	17.5	*	48	25
	spad	36.9	37.2	35.5	37.7	27.8	42.7	*	7.2	29
	fws/fwr	4.4	3.9	6	4.99	3.5	6.5	**	15	39
	dws/dwr	3	3.4	4.5	3.79	1.9	6.8	**	37	10
	dws/fws	0.1	0.09	0.09	0.11	0.08	0.1	**	11.2	28
	dwr/fwr	0.16	0.2	0.14	0.16	0.09	0.2	ns	34.6	NA

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Table 4.2. Pearson's correlation coefficients (*r*) between nine traits based on the mean of the BC2TC families growing under saline conditions (upper diagonal) and non-saline conditions (lower diagonal).

Trait	*dws*	*dwr*	*fws*	*fwr*	*dws/dwr*	*fws/fwr*	*dws/fws*	*dwr/fwr*	*spad*
dws		0.68***	0.95***	0.78***	-0.17**	-0.16**	-0.15**	0.02^{ns}	-0.12*
dwr	0.85***		0.66***	0.82***	-0.61***	-0.46***	-0.46***	0.41***	-0.09^{ns}
fws	0.98***	0.85***		0.78***	-0.18***	-0.09^{ns}	-0.20***	-0.03^{ns}	-0.09^{ns}
fwr	0.93***	0.93***	0.94***		-0.45***	-0.60^{***}	-0.63***	-0.09^{ns}	-0.19**
dws/dwr	-0.28***	-0.43***	-0.30***	-0.37***		0.62***	0.66***	-0.54***	0.01^{ns}
fws/fwr	-0.34***	-0.52***	-0.30***	-0.55***	0.41***		0.90***	0.11*	0.20***
dws/fws	0.65***	0.49***	0.53***	0.54***	-0.07^{ns}	-0.31***		0.18**	0.13*
dwr/fwr	0.53***	0.79***	0.52***	0.56***	-0.57***	-0.41***	0.30***		0.12*
spad	-0.19***	-0.25***	-0.22***	-0.23***	0.11*	0.05^{ns}	0.02^{ns}	-0.16**	

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Table 4.3. Molecular markers that were associated with morphological traits (QTL) by single marker analysis (SMA), multiple-loci analysis (MLA), and Bayesian interval mapping (BIM) for the BC2TC families growing under saline conditions.

					SMA	MLA	BIM	
QTL	Marker	Favorable allele	Chromosome	Position	R^2	R^2	2logBF	Heritability
			no	cM	%	%		%
fws.6.1	SFW08930	ARG	6	60	7**	7**	4	5
fws.9.1	SFW03325	ARG	9	115	6**	7**	3	6
fws.13.1	SFW06114	ANN	13	0	4*	6**	2	5
fwr.10.1	SFW04382	ARG	10	86	9**	9**	5	7
fwr.9.1	SFW03481	ANN	9	122	5*	4*	2	4
fwr.16.1	SFW09058	ARG	16	57	5*	3*	2	4
dws.15.1	SFW02014	ARG	15	9.6	8**	8**	3	7
dws.10.1	SFW04382	ARG	10	86	4*	4*	2	4
dws.8.1	SWF04997	ANN	8	82	4*	4*	2	4
dws.9.1	SFW04672	ARG	9	5.3	4*	4*	2	4
dwr.8.1	SFW06050	ARG	8	16	6**	6*	2	6
dwr.4.1	SFW02626	ARG	4	96	5*	5*	2	3
spad.3.1	SFW01787	ANN	3	10	9**	14***	5	10
spad.10.1	SFW04382	ARG	10	86	7**	13***	5	8
spad.2.1	SFW05340	ANN	2	129	10**	4*	4	7
spad.17.1	SFW00982	ARG	17	73	8**	4*	5	8
fws/fwr.16.1	SFW09058	ARG	16	57	9**	9**	4	8
fws/fwr.10.1	SFW01483	ANN	10	20	6**	7**	4	8
fws/fwr.3.1	SFW03936	ANN	3	0	4*	5*	2	4
dws/fws.16.1	SFW09058	ARG	16	57	11***	9**	5	9
dws/fws.10.1	SFW01483	ANN	10	20	8**	8**	5	13
dws/fws.9.1	SFW03481	ANN	9	122	6**	4*	2	5

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

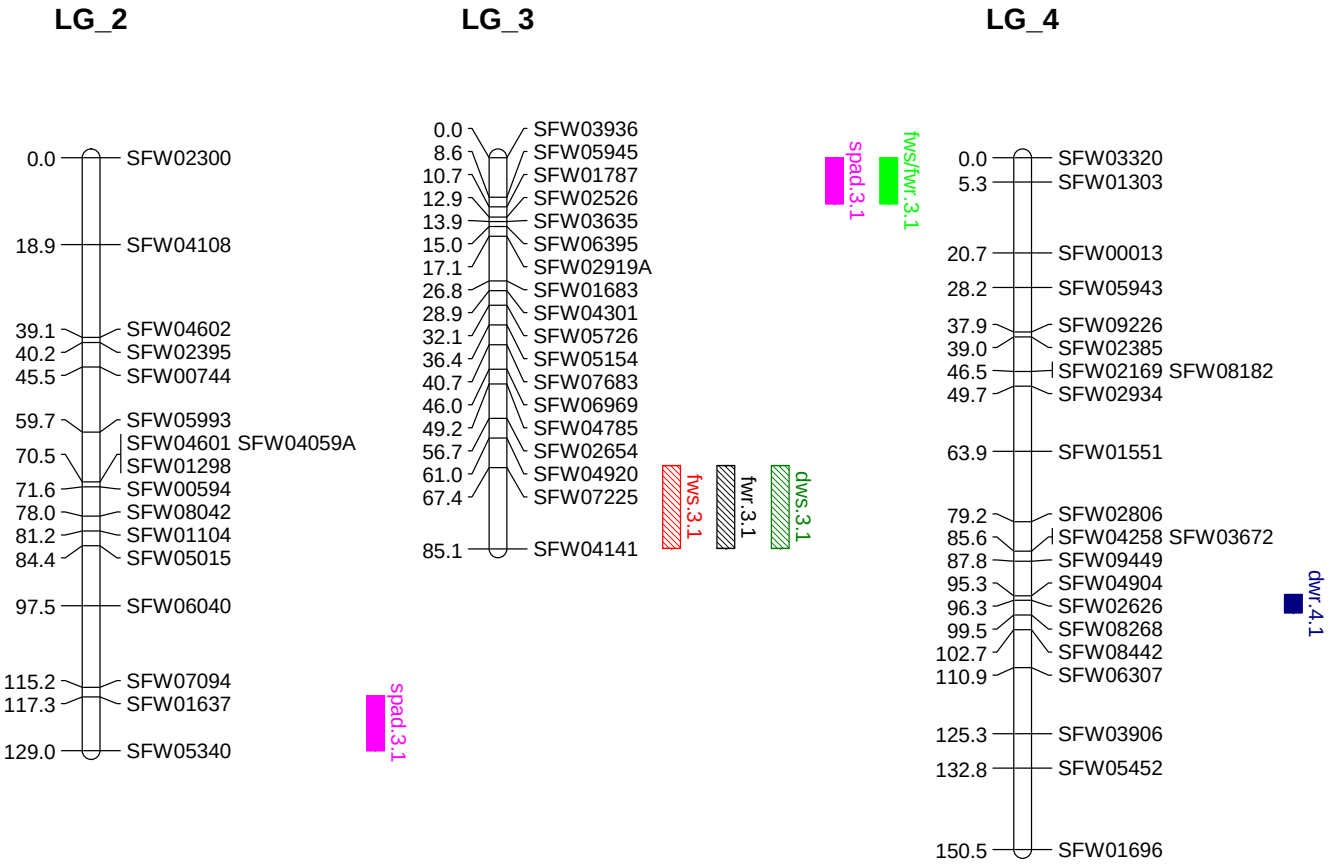
Table 4.4. Molecular markers that were associated with morphological traits (QTL) by single marker analysis (SMA), multiple-loci analysis (MLA), and Bayesian interval mapping (BIM) for the BC2TC families growing under non-saline conditions.

					SMA	MLA	BIM	
QTL	Marker	Favorable allele	Linkage Group	Position	R^2	R^2	2logBF	Heritability
			chr	cM	%			%
fws.14.1	SFW00598	ARG	14	54	19***	21***	6	18
fws.3.1	SFW04141	ANN	3	85	15***	6.6**	3	7
fws.11.1	SFW08036	ARG	11	50	5*	4.2*	5	10
fwr.16.1	SFW09058	ANN	16	57	19***	19***	10	17
fwr.14.1	SFW01544	ARG	14	60	13***	13***	6	13
fwr.3.1	SFW04141	ANN	3	85	8**	3.5*	3	7
fwr.7.1	SFW01024	ARG	7	52	11***	2.8*	3	6
dws.3.1	SFW04141	ANN	3	85	8**	9**	5	8
dws.14.1	SFW01915	ARG	14	0	8**	9**	5	7
dws.11.1	SFW01688	ARG	11	55	8**	8.5***	5	9
dws.9.1	SFW03481	ARG	9	122	5*	4.7**	2	5
dwr.17.1	SFW06481	ARG	17	3	11***	11***	4	7
dwr.16.1	SFW09058	ANN	16	57	8**	6.6**	3	4
dwr.14.1	SFW08544	ARG	14	74	7**	6**	4	8
dwr.11.1	SFW01688	ARG	11	55	4*	4**	2	4
spad.14.1	SFW02864	ANN	14	46	15***	14***	6	12
spad.11.1	SFW06339	ANN	11	0	6**	7**	4	6
spad.4.1	SFW02626	ANN	4	96	8**	5**	4	8
spad.8.1	SFW05227	ANN	8	36	7**	5**	3	6
fws/fwr.16.1	SFW09058	ARG	16	57	22***	22***	9	19
fws/fwr.17.1	SFW03974	ANN	17	0	8**	6**	4	7
fws/fwr.8.1	SFW03221	ARG	8	94	5**	6**	4	10
dws/dwr.14.1	SFW03746	ANN	14	60	6*	5**	7	6

dws/dwr.16.1	SFW05325	ARG	16	21	5**	5**	12	5
dws/fws.14.1	SFW02864	ANN	14	46	9**	9**	4	8
dws/fws.10.1	SFW06042	ANN	10	42	5*	5*	2	5

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Figure 4.2. Location of morphological QTL on the NMS377 x ARG1820 BC2 genetic linkage map. Filled rectangles represent QTL found in the BC2TC families growing under saline conditions and diagonal stripe rectangles in non-saline conditions.




LG_6
0.0 SFW01802
26.8 SFW03480A
30.0 SFW02446
39.7 SFW06648
59.8 SFW08930
62.0 SFW00406
66.2 SFW02182
67.3 SFW08305 SFW01921
68.4 SFW06642
71.5 SFW07508
99.7 SFW01083
fws.6.1
LG_7
0.0 SFW00433
5.3 SFW03161
10.7 SFW08207 SFW05421
12.8 SFW06967
13.9 SFW00156
17.1 SFW01772
18.1 SFW04423 SFW00247
23.5 SFW08270
31.0 SFW09237
42.9 SFW08159
52.6 SFW01024
56.9 SFW02564
60.1 SFW03746
67.6 SFW01923
fwr.7.1
LG_8
0.0 SFW01113
6.4 SFW08543
11.8 SFW08421
16.0 SFW06050
29.2 SFW08527
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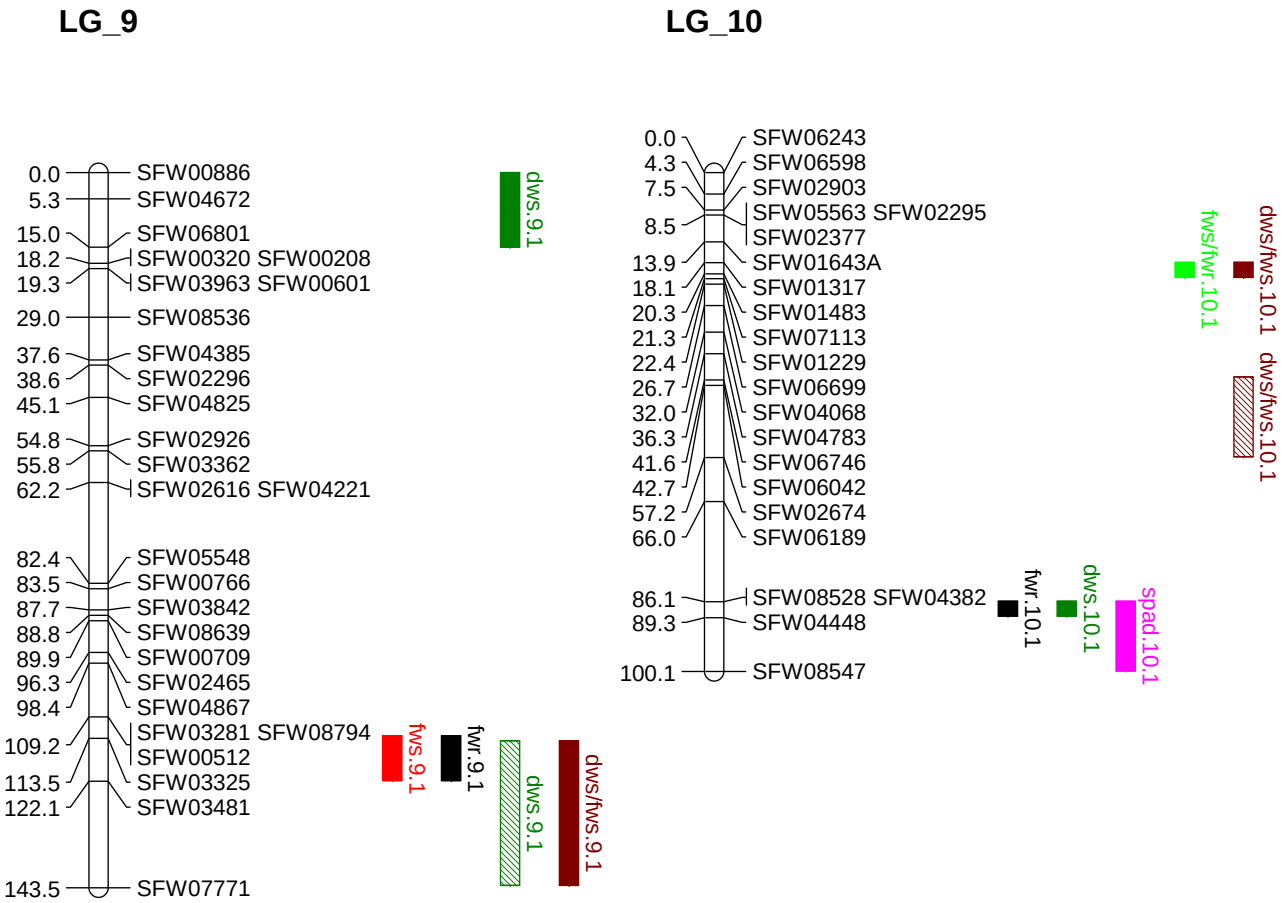
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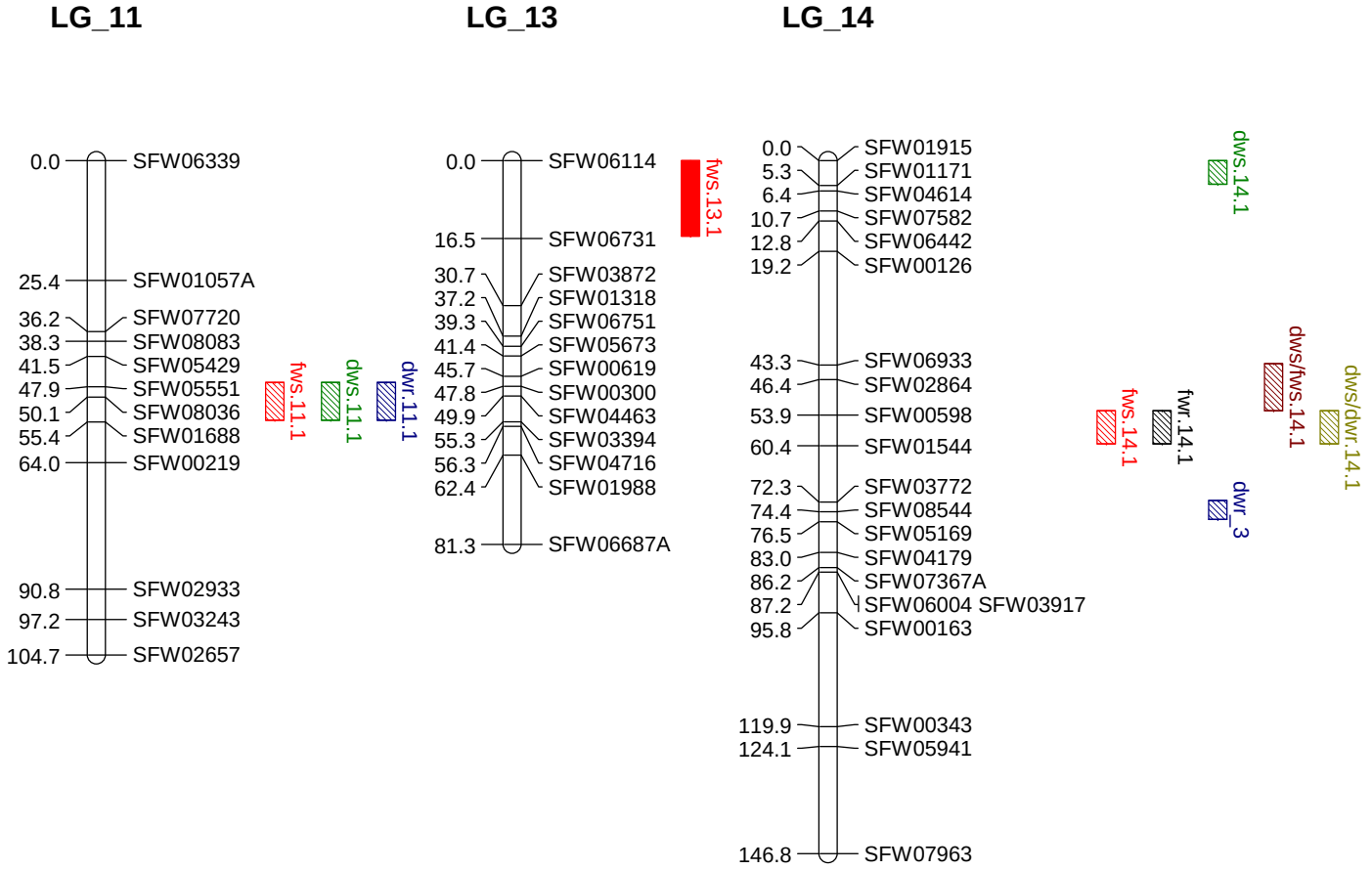
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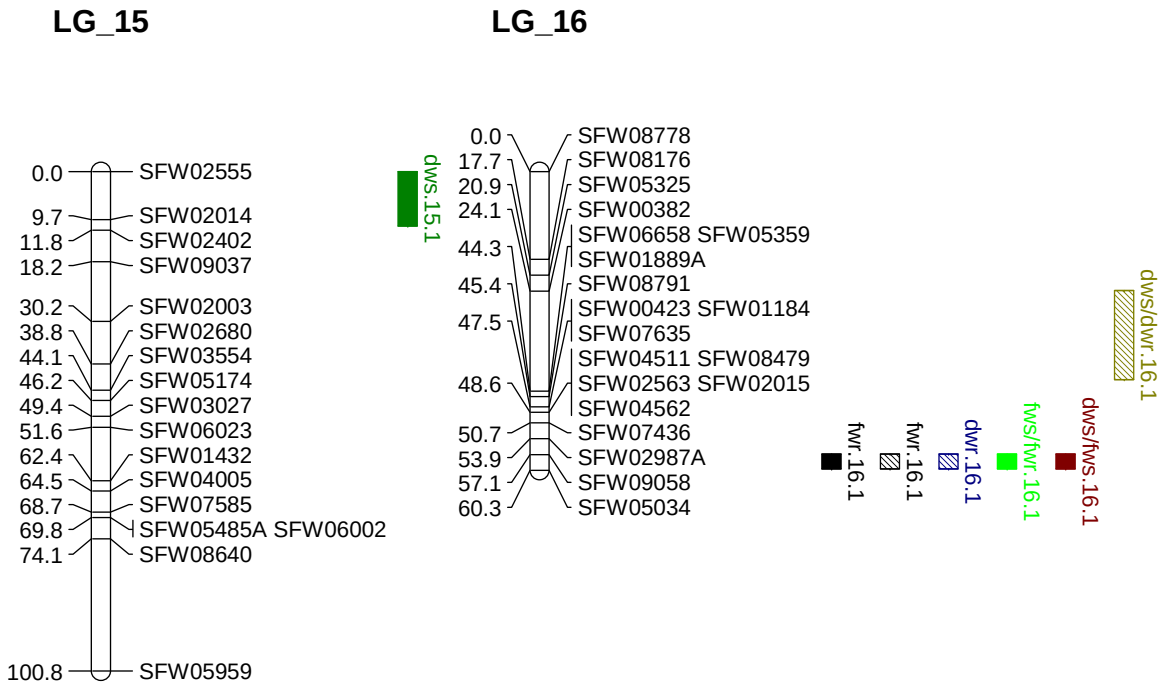
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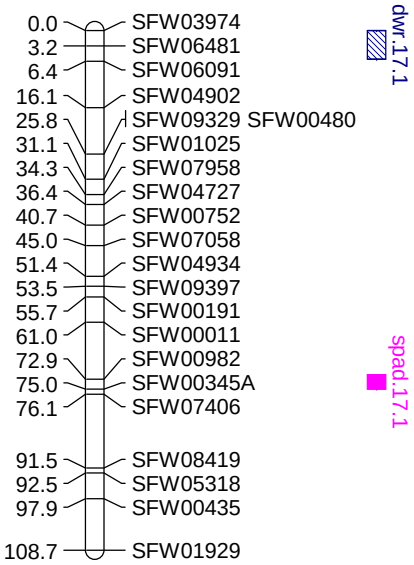
dws/fws.16.1
dws/dwr.16.1

LG_17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dwr.17.1
spad.17.1

CHAPTER 5

CONCLUSIONS

In this study we developed the first high density linkage map of *Helianthus argophyllus* using EST-SNP markers. Through comparative mapping with the *H. annuus* consensus map we were able to identify four chromosome rearrangements and provided insight into sunflower evolution along with the implications for the use of interspecific crosses between *H. annuus* and *H. argophyllus*. The presence of the four chromosome rearrangements could complicate the introgressions of genes located within these rearrangements. Most of the genome of *H. argophyllus* is colinear with the *H. annuus* genome which implies the introgressions will be facilitated in these colinear regions. The development of an introgression library will be very helpful for the utilization of the wild species in sunflower breeding and will be facilitated by the large extent of colinearity between the two genomes. Our high density comparative mapping also allowed us to observe a reduction in recombination in some linkage groups of *H. argophyllus* compare with *H. annuus*. This reduction in recombination will have implications in the fine mapping of QTL located on these regions. We will need a greater population size to find recombinants in these regions. This phenomenon also will have negative consequences for gene introgression, since it will favor "linkage drag". Our results support conclusions from previous studies confirming a rapid karyotypic evolution in the genus Helianthus. In addition, evolution within the genus has been mainly through the translocation type or rearrangements as opposed to other genera where the main difference has been through inversions.

The results from our salt tolerance study under a high salt concentration (300 mM of NaCl), using the interspecific cross *H.annuus* by *H. argophyllus*, indicated that salinity is a very

complex trait in sunflower. Few QTL with relatively small main effects were identified and epistasis played a key role in the genetics of the trait. Marker assisted selection (MAS) for salt tolerance will be difficult and we should consider the impact of the interactions among the QTL (epistasis) during selection. From the results of our experiment of the BC2TC families under saline and non-saline conditions we concluded that there is large genotype by saline level interaction. The main-effect QTL identified in this study explained a relatively small proportion of the phenotypic variance among the seedling traits, implying limitations for the use of MAS. As a general conclusion we found that salt tolerance in sunflower is a complex trait with a large environmental component, making its improvement difficult in a breeding program through either phenotypic selection or MAS. New selection methods such as genomic selection should be explored for their feasibility as tools in selection for salt tolerance in sunflower.