LONG-TERM FITNESS EFFECTS OF ABIOTIC STRESS TOLERANCE TRANSGENES IN *ARABIDOPSIS THALIANA* POPULATIONS UNDER COMPETITIVE CONDITIONS

By

Patrick James Bigelow

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Breeding, Genetics and Biotechnology - Horticulture - Doctor of Philosophy

2013

ABSTRACT

LONG-TERM FITNESS EFFECTS OF ABIOTIC STRESS TOLERANCE TRANSGENES IN *ARABIDOPSIS THALIANA* POPULATIONS UNDER COMPETITIVE CONDITIONS

By

Patrick James Bigelow

The demands on agricultural lands from a growing world population will rise at the same time that climate change is predicted to increase the abiotic stresses (e.g. drought, heat-waves, frosts and salinity) that decrease crop yield today. Efforts to increase crop abiotic stress tolerance are ongoing, including via transgenic approaches. However, unlike past transgenes, abiotic stress tolerance genes function through indirect alterations to regulatory, signaling and metabolic pathways, increasing the possibility of complex secondary effects. Concerns have been raised that such genes could alter crop persistence and ferality and, through gene flow, the invasiveness and ecological range of recipient interfertile wild or weedy relatives. These possible ecological risks are influenced by fitness effects conferred by the transgene. Despite the link between competitive fitness and long-term environmental risks, competitive fitness has been rarely empirically determined in ecological risk assessment. In this study three transgenes, which increase salinity tolerance in *Arabidopsis thaliana* in growth chamber studies, were examined for impacts on plant fitness: (I) the abiotic stress response transcription factor C-repeat binding factor 3/drought responsive element binding factor 1a (*CBF3/DREB1a*), (II) the plasma membrane Na+/H+ antiporter Salt Overly-Sensitive 1 (*SOS1*), and (III) the mannitol biosynthetic enzyme mannose-6-phosphate reductase (*M6PR*). Transgene fitness impacts were examined across six field seasons and in the presence and absence of competition with the wild-type parental genotype at planting densities ($2600/m^2$) chosen to replicate conditions observed in wild populations. Fourteen replicate competitive populations, initially 1:1 transgenic:wild-type

mixes, of each transgenic line (2-3 lines/transgene) were maintained separately for six generations to allow transgene frequencies to fluctuate according to genetic drift and field selective pressures. Transgene frequencies were monitored each generation via phenotypic screening of progeny seed for presence of the co-integrated kanamycin resistance trait; low frequency populations were verified by qPCR analysis to rule out artifacts due to possible gene silencing. The fitness effects observed in competition with WT differed from relative fitness in pure populations. In pure populations, CBF3 lines showed moderately negative fitness relative to wild-type, but decreased to near extinction in direct competition. SOS1 lines performed equivalently to wild-type in pure populations but decreased in frequency by 50% in competition. The fitness of both M6PR lines was enhanced relative to wild-type in field pure populations, but in competition one line exhibited a competitive advantage while the other was selectively neutral and exhibited random drift. Selection and drift modeling, incorporating short-term non-competitive and competitive transgene fitness measurements, determined that only models which utilized competitive fitness values yielded long-term transgene frequency patterns comparable to trends observed in the field. Significant relative fitness gains were observed from all three transgenes under salt stress in the growth chamber, but from only SOS1 and M6PR lines in the greenhouse. In competition with wild-type plants no advantage was observed, indicating that like the field, competition reduced observed transgene fitness. The implications of these findings, together with prior transcriptomic analysis of the three transgenes, were examined in the context of environmental risk assessment (ERA) practices. Together these results indicate the important role competition has on the success or failure of a transgene to establish and support the use of competitive field assessments in estimating the risk of transgene establishment.

ACKNOWLEDGEMENTS

I would like to thank all my committee members Drs. Wayne Loescher, Jim Hancock, Paul Thompson, and Carolyn Malmstrom, with special thanks to my advisor Dr. Rebecca Grumet for the opportunity to design and conduct the experiments of this dissertation and for her guidance and support through the writing process.

I would also like to thank all current and past members of the Grumet lab, with special thanks to Zhulong Chan for the project's growth chamber ground work, Kaori for her advice as I was beginning research, Jessica for the humor needed to get through countless hours of seed screening, and Sue for help with said seed screening. I would also like to thank the undergraduate students who assisted, whether they wanted to or not, in the seed cleaning process and especially Jean for her many years of work in the field and lab with this project.

Thanks go out to all 'joint lab members' who have provided so much input and advice over the years, especially Veronica and Ann for being sounding boards for resolving strange issues.

Most importantly I would like to thank my wonderful family for their eternal patience with my long career as a student. To my mom Deb, dad Leon and brother Chris, I have finally made it. I know you are proud of me and I am so proud of all of you.

To my wife Erin, you have been there supporting me throughout this whole adventure with love, humor and wit. Thank you!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Literature Review

Introduction

The Green Revolution was driven in part by selection for a handful of genes for dwarfism, increased yield and shorter growing seasons (NRC 2002). While the new varieties carrying these genes fed the rising human population, the environmental, social, and cultural repercussions of the revolution remain to this day. Today plant breeders possess a much larger toolbox of genes, and the potential to move genes seemingly at will without species barriers. Given the sweeping and often unexpected changes that resulted from the use of a handful of genes (NRC 2002), what changes could result from today's ever expanding toolkit? The first generation of genetically modified (GM) crops, mostly herbicide- and pest-resistant varieties, targeted a specific problem with a single transgene (Chua and Tingey 2006). Herbicide tolerance was usually conferred by an altered enzyme and insect pest resistance by the production of proteins that while toxic to insects are merely inert within the plant cells (Warwick *et al.* 2009). Although there are now commercialized varieties with up to eight stacked transgenes to resist a variety of insect pests as well as herbicides, the transgenes involved remain simplistic in their function. The next generation of GM crops to be commercialized will target larger and more complex issues and arguably one of the most important of these will be increasing crop tolerance to abiotic stresses, including drought, heat waves, frost, and soil salinity that cause an estimated 50% global yield reduction (Wang *et al.* 2003).

Salinity Stress

Salinity stress is one of the fastest increasing abiotic stresses on arable land across the globe. Salinity currently affects at least 20% of crop land, but more than 50% of arable land is predicted to be under salinity stress by 2050 (Wang *et al.* 2003, Chinnusamy *et al.* 2005). Irrigation has added 110 million ha of agricultural land and played a vital role in increasing food resources globally, but this increase is not without side effects. Increased irrigation usage in arid and semi-arid environments has resulted in increasing salt stress levels. Arid and semi-arid areas

comprise 40% of the world's arable land, but do not receive enough annual rainfall to provide the necessary leaching needed to remove excess salts from the soil (Smedema and Shiati 2002).

Crop irrigation can affect the salinity levels of soils by several means. The use of irrigation water pumped from deep fossil aquifers, which tend to be more saline than typical surface or ground waters, directly applies additional salt loads to the soil surface. Overuse of irrigation can waterlog soils, which brings soil borne salts closer to the surface where they can accumulate due to evaporation, an issue currently impacting ~25% of irrigated land in semi-arid regions worldwide (Smedema and Shiati 2002). Lastly by altering water tables and affecting groundwater movement, irrigation can unlock fossil salt deposits, left behind from past marine events (Smedema and Shiati 2002). As market pressure to produce more food, feed, fiber and fuel rises, the high salinity issues facing farmers today will continue to increase as irrigation is adopted in previously non-irrigated regions and more salt is deposited in already irrigated lands. This will mean that salinity-tolerant varieties will become more important to farmers trying to meet the demands of a growing population.

Genetic engineering for abiotic stress tolerance

Submissions to the USDA for field trials of abiotic stress related traits have increased significantly in the last decade, indicating a concerted effort on the part of plant biotechnologists and breeders to produce crops with enhanced abiotic stress tolerance (Figure 1.1). Abiotic stresses can result from a broad range of environmental factors such as heat, drought, cold and salinity and numerous candidate genes have been described to potentially increase tolerance to one or more types of abiotic stress (Zhang *et al*. 2004, Sreenivasulu *et al.* 2007, Bhatnagar-Mathur *et al.* 2008, Warwick *et al*. 2009). The mechanisms of resistance supplied by these candidate genes vary and include such functions as membrane protection, stress signaling, protein and RNA stabilization, transcriptional activation, and the detoxification of toxic molecules and free radicals (Wang *et al.* 2003).

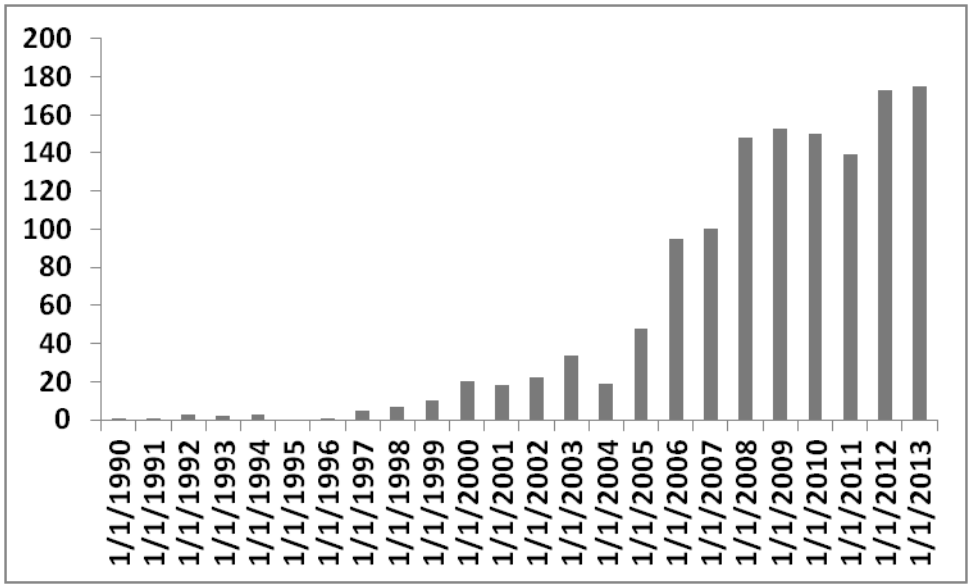


Figure 1.1. The yearly number of submissions to the USDA for field trial of abiotic stress related traits from 1990-2013. Submission data was collected from the Information Systems for Biotechnology (http://www.isb.vt.edu/search-release-data.aspx) on January 23, 2013.

Although the causes of abiotic stress differ, many have similar effects upon plant tissues. Drought, salinity and freezing all create osmotic stress resulting from the loss of water available for homeostasis and chemical reactions (Wang *et al.* 2003, Warwick *et al*. 2009). Oxidative stress results from reactive oxygen species or free radicals, which can be produced by plant cells under stress from high temperatures, drought or salinity. Therefore the introduction of one gene could potentially alter resistance against multiple stresses. In addition, multiple stresses, such as drought and heat, can combine to create greater levels of stress (Mittler 2006). These combinations could occur in many ways, any of which might influence the effectiveness of a given abiotic stress resistance gene.

At the time of writing, the first and only commercialized genetically engineered crop with enhanced abiotic stress tolerance is the corn line MON 87460 (APHIS 2011a,b), a parent to Monsanto's DroughtGard hybrid corn lines. These corn hybrids express a cold shock protein from *Bacillus subtilis* named CspB (Reeves 2010). This protein belongs to a class of RNA chaperones which prevent RNA misfolding, allowing cells to continue to produce vital proteins while under environmental stress (reviewed by Horn *et al*. 2007). Under water deficit stress, hybrids constitutively expressing CspB had increased growth rates, maintained higher chlorophyll content and photosynthetic rates and significantly out-yielded the untransformed conventional hybrids (Castiglioni *et al*. 2008). No cost of resistance was observed in high yielding environments. CspB was also shown to increase abiotic stress tolerance (cold, heat and drought) in *Arabidopsis thaliana* and rice (Castiglioni *et al*. 2008). With no reported cost of resistance and conferring increased tolerance to multiple abiotic stresses in both monocot and dicot species, cold shock proteins show potential to increase stress tolerance in many crops.

Another abiotic stress tolerant crop in the regulatory process is freezing tolerant hybrid Eucalyptus (*Eucalyptus grandis* x *E. urophylla*) (Nehra & Pearson 2011). Interest in Eucalyptus as a short rotation woody crop for fiber and biofuel production has been hindered by the tropical plant's low tolerance to cold temperatures. Previous research had shown that over-expression of Eucalyptus CBF1a and CBF1b homologs increased freezing tolerance; however, the transformed

plants showed significant negative phenotypic effects that rendered them unsuitable for commercialization (Navarro *et al*. 2011). The genetically engineered Eucalyptus lines submitted for deregulation express the abiotic stress response transcription factor C-repeat binding factor 2 (*CBF2*) gene from *Arabidopsis thaliana* under the cold inducible promoter rd29A. The transformation cassette also includes a barnase gene linked to an anther-specific promoter PrMC2 to confer pollen sterility. Transgenic lines showed significantly less winter die-back than the untransformed background: at multiple sites after 5 years the transgenic lines were 40-50 feet tall, while the background line had died back to less than 1 ft over the winter (Nehra & Pearson 2011). Under freeze-free conditions, the transgenic lines had small but significant reductions in growth compared to background. Flowers on the transgenic lines produced no viable pollen. In February 2013, the USDA released a notification of intent to perform an Environmental Impact Statement on the transgenic lines submitted for deregulation (APHIS 2013). Thus freeze-tolerant Eucalyptus could become the next abiotic stress tolerant crop to be released to the market. The addition of the pollen sterility trait could aid that process by reducing the ecological concerns related to feral crop plants and gene flow to compatible relatives that are frequently raised about crops with abiotic stress tolerance traits.

Ecological risk assessment of abiotic stress tolerance enhanced crops

Although many studies of the ecological risks of genetically modified crops are focused on the possibility of gene flow, gene flow in and of itself does not constitute an environmental harm (EFSA 2012). While exact definitions of ecological harm vary, most consider a reduction in biodiversity to be harmful (Sanvido *et al*. 2012). Thus, an ecological harm could result from gene flow if it resulted in a negative impact on biodiversity. A reduction in biodiversity could occur at the species level within an ecosystem or at the genetic level for an individual species (Hancock 2011). The effects of abiotic stress tolerance transgenes that could alter biodiversity include altered fitness, increased weediness or invasiveness, and range expansion (Tiedje *et al.* 1989, Hancock *et al.* 1996, Ellstrand 2003, Weaver & Morris 2005, Hails & Morley 2005). Unlike most first generation transgenes used for crop improvement, where the gene product

directly conferred the desired phenotype, e.g. herbicide resistance or Bt-mediated pest resistance, abiotic stress tolerance traits have the potential to affect a wide range of plant growth and developmental functions (Chua and Tingey 2006, Ellstrand 2003). Thus risk assessment of abiotic stress tolerant crops will require careful consideration of possible secondary and fitness effects due to transgene expression on recipient plants, for both the transformed crop and wild compatible relatives which could receive the transgene through pollen-mediated gene flow.

The transgene could confer significant competitive advantages allowing the transformed crop to become feral, growing along field margins and in ruderal areas, and possibly invasive in natural environments. Transgene movement into a compatible wild relative could give recipient plants a selective advantage under conditions of abiotic stress (Hancock *et al.* 1996, Ellstrand 2003). This advantage could result in the transgene establishing within wild germplasm. For a transgene that conferred a strong selective advantage, establishment could further alter the wild gene pool as other crop genes are also introgressed through linkage drag (Ellstrand 2003). The transgene, and possibly other crop genes, could allow the recipient plants to become weeds in agricultural systems and invasive in natural ecosystems. If the transgene instead conferred a selective disadvantage in natural ecosystems, high rates of gene flow from nearby transgenic crop fields could result in demographic swamping and in cases of transgenes with severely negative fitness effects, localized extinction of compatible relatives (Hancock 2011). However, even with a strongly negatively selected transgene, this effect would likely only occur to weedy or wild relatives that grow along field margins due to the inability of the transgene to persist outside of agricultural settings. Thus, local biodiversity could be impacted by the fitness effect of the transgene on recipient plants, whether those effects are positive or negative.

In breeding for abiotic stress tolerance, plant breeders will be potentially removing a factor that limits an individual plant's survival and fecundity, and a species range and distribution. Salt tolerance has been found to be a key attribute in the invasiveness of a number of plant species. Together with the increasing levels of salinity in rivers due to irrigation, and the use of salt as a de-icing agent, salt tolerance has enabled some species to invade habitats that

would otherwise be resistant to invasion (Thompson 1991, Bauer and Geber 2002, Glenn *et al*. 1998, Glenn and Nalger 2005). Proper risk assessment of these second generation crops expressing abiotic stress tolerance traits will be vital to their successful introduction while meeting ecological concerns over increased invasiveness.

Feral crops

The addition of transgenic traits to domesticated crops, has led to concerns that volunteers could become feral and become weedy or invasive in agricultural and natural ecosystems. Although feral or weedy versions of domesticated crops have been widely reported, the process by which domesticated crops become feral has not been extensively researched (Gressel 2005). This process of de-domestication has been classified into two categories dependent upon the source of the genetic changes needed to revert to a weedy phenotype. Endoferality occurs when a domesticated species is able to become feral on its own due to selection on existing or new genetic variation leading to the loss of domestication traits. While this has been reported and studied widely in animals, feral pigs for example, it has been less studied in plants (Gressel 2005) as most cases of crop ferality have been linked to the influx of wild genes and are thus the result of exoferality (Ellstrand 2003).

The best characterized example of endoferality occurred with wheat (*Triticum aestivum*) in the Tibetan highlands of China (Ghimire *et al.* 2006). This semi-wild wheat is found as an agricultural weed in barley and wheat fields and has been deemed a separate subspecies *Triticum aestivum* ssp. *tibetanum* (Shao *et al.* 1983). This subspecies is believed to have evolved its feral traits on its own without input from wild relative as the region is geographically isolated with no compatible relatives (Chen *et al.* 1991, Sun *et al.* 1998). Also the subspecies is hexaploid and there are no wild hexaploid progenitor species with from which it could have received genetic material (Ayal and Levy 2005).

The evolution of endoferality was examined in the cultivated radish (*Raphanus sativus*) (Campbell & Snow 2009). Experimental cultivated radish plots were established >1km from the nearest wild radish (*R . raphanistrum*) populations and were allowed to self seed for four

generations (Campbell & Snow 2009). Phenotypic assessments for traits associated with feral crops (early flowering, reduced root biomass, and high seed production) were conducted at each generation. Three populations went extinct, while two were observed to contain early flowering individuals by the third generation. However, these individuals were shown to be inadvertent wild-crop hybrids. To examine the endoferality potential of cultivated radish, greenhouse populations were grown under strong selective pressure for early flowering for two generations, with all but the first 10% of plants to flower culled. Two of three replicate populations had significantly earlier flowering and increased seed yield in common garden studies relative to control populations that had undergone random 90% culling. However, further gains in feral traits were not observed, leading the researchers to consider that the genetic variability of the cultivate radish was insufficient for rapid development of endoferality. The authors postulated that the domestication process appears to reduce genetic diversity and eliminate weediness traits (such as seed shattering) resulting in the rare occurrence of natural endoferality in plants and the difficulty in artificially inducing it. This indicates that hybridization and introgression with compatible wild relatives are the primary concerns for possible ecological impacts from the introduction of abiotic stress tolerance enhanced transgenic crops.

Crop x Wild hybridization

When outside genetic sources, i.e. wild relatives, supply the genes needed to de-domesticate, the process is termed exoferality. Hybridization between cultivated and wild relatives has been documented in a wide number of crop species. As early as 1965, crop-wild hybrids were found in wheat, sorghum and barley (Harlan 1965). From the 1970s through the 1990s, hybrids were found in almost every category of crop plants from grains to root crops (Chu and Oka 1970, Zohary 1971, Ladizinsky and Zohary 1971, Brunken *et al.* 1977a, Brunken *et al.* 1977b, Klinger *et al.* 1991). Hybridization between closely related crop and wild species has resulted in feral hybrid populations growing along the edges of fields or within the field from shed seed. Thus the risk of transgene flow, despite the species barriers between cultivated and wild plants potentially causing loss of fitness and partial sterility, exists where hybrid

populations can be maintained by high gene flow from crop sources (Wright 1969, Slatkin 1985, Hails and Morley 2005, Ellstrand 2003). Once hybrids form, introgression into the wild species can occur through repeated backcrossings. The conditions required for hybrids to form exist at any large farm where wild interfertile relatives persist as weeds within the field or along its margins.

One of the most well studied examples of wild-crop hybridization and its effects on plant phenotype and fitness is with sugar beet (*Beta vulgaris ssp. vulgaris*) and its wild relative sea beet (*B. vulgaris ssp. maritima*). Insufficient isolation distances in sugar beet seed production areas from natural sea beet populations resulted in an outbreak of weedy sugar beets in fields across Europe in the 1980s and 1990s (Hornsey & Arnold 1979, Longden 1989, Ford-Llyod 1995). Where sugar beet grows as a biennial, the hybrids bolted, flowered and set seed in one year (Boudry *et al*. 1994, Mucher *et al*. 2000). These weedy beets decreased farm yields and caused difficulties for processing facilities resulting in millions of dollars worth of economic losses. Control of these weedy beets proved difficult as seed banks rapidly built up in soils and remained for several years (Longden 1993). Later gene flow studies determined that the isolation distance for sugar beet seed production at the time (1km), fell far short the distances viable sea beet pollen was able to travel (up to nearly 10km) (Fenart *et al*. 2007). Genetic marker analysis not only confirmed the sea beet paternal origins of the weedy beets, but also found widespread multidirectional gene flow (wild↔weedy ↔cultivated) (Desplanque *et al*. 1999, Bartsch *et al*. 1999, Viard *et al*. 2002, Viard *et al*. 2004, Andersen *et al*. 2005, Fenart *et al*. 2008). This level of gene flow and the difficulties found in preventing it, raised ecological concerns related to the future release of transgenic sugar beets in Europe (Desplanque *et al*. 2002).

The evolutionary and ecological consequences of hybridization have been studied in sunflower (*Helianthus annuus*) and its compatible wild relatives. Sunflower is an annual native to the Great Plains and Southwest regions of North America which occurs in cultivated, weedy and wild types (Rieseberg & Seiler 1990). Hybridization between cultivated sunflower and wild *H. annuus* can occur at rates of 27-42% along field margins, with gene flow detectable out to

1km, and significant persistence of crop alleles in wild populations for at least five generations after initial hybridization (Arias & Rieseberg 1994, Whitton *et al*. 1997). Hybridization between related wild and weedy annual and perennial *Helianthus* species has been studied for over 60 years (Heiser 1947). In co-occurring populations of *H. annuus* and the wild annual prairie sunflower (*H. petiolaris*), hybrid swarms have been frequently observed despite differences in initial flowering times and high levels of pollen and seed sterility among hybrids. Backcrossed and fully introgressed individuals are also observed and genetically confirmed in hybrid zones, indicating bidirectional gene flow among both parental species and the hybrids (Heiser 1947, reviewed in Rieseberg *et al*. 2007). The impacts of such hybridization can include heterosis, increased genetic diversity, and reduced levels of negative mutations (Rieseberg *et al*. 2007).

These hybridization effects could increase the adaptability of the hybrids relative to the parent species, possibly resulting in speciation, while introgression of locally adapted germplasm has been proposed as a means of parental range expansion (Heiser 1947). Three species, *H. anomalus*, *H. deserticola*, and *H. paradoxus,* are the result of selection on transgressive hybrid (*H. annuus* x *H. petiolaris*) individuals, and are now ecologically isolated from the parent species inhabiting deserts, sand dunes and saline wetlands respectively (Rieseberg *et al*. 1990b, 1991a, 1991b, reviewed by Rieseberg *et al*. 2007). Expansion of *H. annuus* into Texas was facilitated by introgression of *H. debilis ssp. cucumerifolius* germplasm, resulting in the weedy subspecies *H. annuus ssp. Texanus* (Rieseberg *et al*. 1990a, 2007). These introgressed regions conferred indeterminate branching and resistance to damage from seed midges (*Neolasioptera helianthis*) which can destroy 90% of seed in a field of *H. annuus* in central Texas. Studies with sunflower have shown widespread hybridization between cultivated, weedy, and wild relatives resulting in evolutionary and ecological impacts including increased fitness, range expansion, and speciation.

Wild x GM crop hybridization

Examples of natural hybridization between genetically modified crops and compatible wild relatives are limited for two reasons. First, a relatively low number of crop species have

deregulated transgenic lines on the market (James 2012). Second, most transgenic crops are currently grown away from their origins of domestication and so are in regions low in compatible wild-relatives (e.g. the majority of GM maize and soybean acreage, Hancock 2012, James 2012). However, this trend is unlikely to continue as the acreage of genetically modified crops continues to increase and a more diverse range of transgenic crops become commercialized.

The first detection of transgene introgression into a wild relative was observed in 2005 in Canada between transgenic glyphosate-resistant canola, *Brassica napus*, and its weedy relative, *Brassica rapa* (Warwick *et al.* 2008). The first detected hybridization between GM canola and the wild species was observed in 2001 (Beckie *et al.* 2003). A follow-up study monitored populations of volunteer canola and wild *B. rapa* growing along commercial field margins from initial planting in 2000 to 2005, with glyphosate herbicide applied only once after 2000. Despite the lack of fresh seed input and low level of selective pressure, there was successful introgression of the herbicide resistance gene into the wild gene pool, as evidenced by occurrence of a plant isolated in 2005 that was morphologically and genetically, by AFLP analysis, *B. rapa*, but contained the gene for glyphosate resistance (Warwick *et al.* 2008).

While this detection occurred once, volunteer canola is widespread, with normal in-field seed loss estimated from 3,000 to 10,000 seeds per meter square (Harker *et al.* 2006). The planting of herbicide resistant canola in Canada began in 1995, and since then, double- and even triple-herbicide resistant canola volunteers have been found as farmers switched from one herbicide resistant line to another over time to control both volunteers, both in field and along margins, and developing resistance in other weeds (Simard *et al.* 2005, Knispel *et al.* 2008). The production of these multi-resistant volunteers and the successful introgression of the herbicide tolerance gene into a wild relative, indicates a high level of gene flow between compatible plants both within field and between the field and margins.

Transgene Fitness effects

While most risk assessments of first generation transgenic crops focused on the potential for transgene movement via pollen and seed, the rate of transgene establishment outside of cultivation is governed not by the stringency of containment procedures, but by the selective advantage or disadvantage resulting from transgene expression (Hancock *et al.* 1996, Ellstrand and Hoffman 1990, Haygood *et al.* 2004). Current GM crops include pathogen resistance and insect resistance, useful traits to any plant under attack by biotic agents (Warwick *et al*. 2009). Herbicide resistance, while unlikely to be of selective advantage in an environment without human presence, could be a great advantage in a weed trying to survive in an edge habitat in agricultural lands. The abiotic resistance genes currently under development for the second generation of GM crops pose potentially even greater advantages, as abiotic stress is often the greatest limiter of a plant species potential range (Davis and Shaw 2001, Ellstrand 2003, Thuiller *et al.* 2005, Warwick *et al*. 2009). Given the high impacts abiotic stress events like heat waves, droughts and freezes can have on plant reproduction, the selective pressures of such stresses could give a crop-wild hybrid possessing a transgene for abiotic stress tolerance a significant fitness advantage and thus hasten introgression of the transgene into the wild germplasm.

Measuring impacts on fitness

Fitness refers to the average contribution of an allele to succeeding generations. Changes in fitness have frequently been measured in one of two manners (Bourguet *et al.* 2004). First, a study can be performed comparing components of fitness (traits that directly influence fecundity) between wild-type lines and lines containing the gene of interest. This method was been widely used in the assessment of first generation GM crops to establish substantial equivalence for regulatory purposes (NRC 2002). Biomass, branching pattern, fruit set, seed production, timing of developmental stages and many other factors were reported in these papers and used to assess fitness.

The second approach calculates the contribution of an allele to future generations directly determining the fitness costs or benefits within a given population (Bourguet *et al.* 2004). Using this direct method requires a population of plants to be carried through several generations and

necessitates that the population possess a known number of homozygous positive and homozygous negative parent plants in the first generation (Gilliland *et al*. 1998, Roux *et al.* 2005). The fitness costs can then be calculated as percentage of total seed production of the population for each group. While this method could be problematic for studies involving plant species with long generation times, *Arabidopsis* is well suited to this method. With short generation times, high seed production and the ability to grow in high density stands, it combines all the traits needed for multigenerational risk assessment studies. These traits make *Arabidopsis thaliana* an excellent candidate to perform a primary assessment of the fitness costs of transgenes likely to be used in second generation abiotic stress resistant crops.

The duration of the experiment can be an important factor in the accuracy of a study's results. Most early GM trials designed to determine the selective advantage of a transgene were performed for only one or two generations (reviewed by Bergelson and Purrington 1996). Longer studies using a variety of mutants found fitness effects which would have gone undetected in shorter studies (Gilliland *et al.* 1998, Asmussen *et al.* 1998, Roux *et al.* 2005). In analyzing the functional role of three *Arabidopsis thaliana* actin genes, the fitness costs of their knockouts did not appear until the second and third generation (Gilliland *et al.* 1998, Asmussen *et al.* 1998). Another study used six herbicide resistant EMS mutant lines that had been studied previously, and tested them in competitive environments over seven generations (Roux *et al.* 2005). Fitness costs of resistance were found to change over time and across densities in some lines. One mutation found previously to cause negative fitness (Bergelson and Purrington 2002) was actually neutral over multiple generations (Roux *et al.* 2005). Another line exhibited density-dependent fitness costs. Its fitness cost increased from 38% to 64% as the prevalence of plants containing it decreased in the population. These studies show that when attempting to determine fitness costs it is best to use multiple generations, large populations, and a high number of replicates (Roux *et al.* 2005, Whitlock 2000). These considerations were used in the design of the field and greenhouse experiments of this dissertation.

Transgenes assessed

As discussed earlier, a variety of genes that function through differing modes of action are in development to improve crop abiotic stress tolerance. For the risk assessment studies described herein, three diverse transgenes shown to increase salinity tolerance in the growth chamber were selected: (i) the transcription factor [C-repeat binding factor 3/drought responsive element binding factor 1a (*CBF3/DREB1a*)], (ii) the plasma membrane Na+/H+ antiporter [Salt Overly-Sensitive 1 (*SOS1*)], and (iii) the metabolic enzyme [mannose-6-phosphate reductase (*M6PR*)]. Salinity stress related transgenes were chosen due to the large and increasing impact of salt stress on crop yield worldwide. While cold, heat and drought stress are generally transient stresses in farmer fields, occurring for generally a matter of days, in irrigated arid and semi-arid regions salt stress occurs continually and increases due to insufficient rainfall to leach excess salt from the soil (Smedema and Shiati 2002). The three transgenes were assessed for secondary effects of transgene expression that could impact plant fitness and transgene establishment in natural populations.

CBF3/DREB1a. Previous research has shown that upon exposure to cold or dehydration, a set of genes termed COR (cold-regulated) or DR (dehydration-responsive) genes is rapidly induced. Sequence analysis of the promoters of these COR/DR genes in *Arabidopsis thaliana* revealed the presence of a conserved motif, TACCGACAT, which was simultaneously discovered by two groups and named both the C-repeat element and the dehydration-responsive element (DRE) (Baker *et al*. 1994, Yamaguchi-Shinozaki *et al.* 1994). Yeast one-hybrid screening revealed this sequence to be the target of a family of abiotic stress-responsive transcription factors, named C-repeat binding factors (CBF) / drought responsive element binding factors (DREB) (Stockinger *et al*. 1997, Liu *et al*. 1998, Yang *et al*. 2005). Among the genes discovered in the family are CBF1/DREB1b, CBF2/DREB1c, and CFB3/DREB1a (Kasuga *et al*. 1999, Gilmour *et al*. 2000). All three transcription factors have been found to be functionally redundant, with the expression of each resulting in the induction of COR gene expression, phenotypic changes in growth and development, and increased plant tolerance to drought, cold, and salinity (Gilmour *et al.* 2004).

The CBF3/DREB1a transcription factor was found by northern and microarray analysis, to activate not only known genes in the cold regulated (COR) regulon controlled by the CBF family of transcription factors, but also a number of other genes closely linked to drought, salinity and osmotic stress tolerance (Jaglo-Ottosen *et al*. 1998, Kasuga *et al*. 1999, Gilmour *et al*. 2000, Seki *et al.* 2001, Fowler and Thomashow 2002, Maruyama *et al*. 2004, Chan *et al.* 2012). CBF3/DREB1a over-expression resulted in the constitutive expression of COR genes as well as other abiotic stress tolerance genes such as dehydrins and metabolic enzymes for the production of compatible solutes (Gilmour *et al*. 2000, Fowler and Thomashow 2002, Chan *et al.* 2012). Given that the CBF regulon includes other transcription factors, such as members of the ZAT and RAP gene families, over-expression of CBF3 results in a cascade of gene expression activations and repressions that influence over 1300 genes (Seki *et al*. 2001, Maruyama *et al*. 2004, Vogel *et al*. 2005, Chan *et al.* 2012).

Over-expression of CBF3/DREB1a increases abiotic stress resistance of transgenic *Arabidopsis* through this activation of stress tolerance genes and pathways. In abiotic stress experiments, increased freezing, drought and salinity tolerance were observed (Jaglo-Ottosen *et al.* 1998, Kasuga *et al.* 1999). Transgenic lines developed levels of soluble sugars and proline three and five times higher than the controls, compounds which have been demonstrated to increase tolerance to dehydration and salinity stress (Gilmour *et al.* 2000, Cook *et al*. 2004).

CBF3 over-expression was also associated with significant secondary effects resulting in a clear CBF3 over-expression phenotype for *Arabidopsis thaliana.* Varying degrees of dwarfism, delayed flowering, and reduced seed production, are seen in the majority of CBF3 over-expression lines (Kasuga *et al.* 1999, Gilmour *et al.* 2000, Chan *et al.* 2012). In addition, transgenic lines exhibited changes in plant architecture including decreased axillary shoot formation, shorter petiole lengths, and leaves that remain close to the ground (Gilmour *et al.* 2000, Chan *et al.* 2012). These phenotypic and transcriptomic effects could have impacts on plant fitness and the ability to establish under field conditions.

SOS1. Mutational screening of *A. thaliana* revealed a series of loci, *Salt Overly Sensitive 1* through *3* (*SOS1*-*SOS3*) that showed a highly salt-sensitive phenotype when mutated (Wu *et al.* 1996, Zhu *et al.* 1998). Crossing of the mutant lines revealed that the loci act together to regulate cellular sodium ion levels. Positional cloning, sequence, and protein analyses revealed SOS1 to encode a putative plasma membrane Na+/H+ antiporter (Shi *et al.* 2000, Shi *et al.* 2002). SOS3 was determined to encode a calcium binding protein, while SOS2 encodes a protein kinase (Halfter *et al.* 2000). The SOS pathway begins with high Na+ concentration inducing the release of a Ca^{2+} signal which binds to SOS3 causing it to complex with SOS2 (Halfter *et al.* 2000). The SOS2-SOS3 complex then activates SOS1 via phosphorylation causing the antiporter to begin the extrusion of Na+ ions from the cytosol to the extracellular space (Shi *et al*. 2000, Qiu *et al.* 2002, Guo *et al.* 2004). Recent work has also found that SOS1 can be activated by RCD1, an oxidative-stress regulator, bringing together *Arabidopsis thaliana*'s response to salinity stress and oxidative stress (Katiyar-Agarwal *et al.* 2006). SOS1 has also been found to be constitutively expressed in salt cress (*Thellungiella halophila*), a relative of *Arabidopsis* which grows in salt marshes, and may be one of the reasons why plants of the species are able to survive 500 mM NaCl concentrations (Taji *et al.* 2004).

Transformed *Arabidopsis* plants constitutively expressing the SOS1 gene showed higher levels of SOS1 transcript than wild-type plants with or without stress (Shi *et al.* 2003). SOS1 transcript levels in over-expression and wild-type lines increased in the presence of salt, indicating post-transcriptional regulation of SOS1 mRNA levels via increased mRNA stability in the presence of reactive oxygen species produced under salinity stress (Shi *et al.* 2003, Chung *et al*. 2008). Transcriptomic analysis of SOS1 over-expression lines detected roughly 600 genes with altered expression profiles in the absence of salinity stress, including down-regulation of a large number of oxidative and redox related genes (Chan *et al*. 2012). These results corroborated prior studies indicating interactions between the SOS response pathway and oxidative and redox signaling pathways (Katiyar-Agarwal *et al*. 2006, Verslues *et al*. 2007). Minimal overlap

between CBF3 and SOS1 transcriptomic changes indicate that the SOS signaling pathway is independent of CBF, ABA and MYB regulated pathways (Kamei *et al*. 2005, Chan *et al*. 2012).

SOS1 over-expression plants had reduced levels of sodium ions in both cellular tissues and xylem sap after salt exposure than wild-type plants under the same stress (Shi *et al.* 2003). The over-expression lines showed greater ability to germinate, grow and set seed under salinity stress than wild-type plants (Shi *et al.* 2003, Chan *et al*. 2012). Without the presence of salt stress, no phenotypic differences were observed between the transgenic and WT plants. In the presence of salinity stress, SOS1 over-expression lines were better able to maintain cellular functions than wild-type plants, with reduced impacts to root growth, total protein and chlorophyll levels, and photosynthetic efficiency (Shi *et al.* 2003). By maintaining cellular homeostasis under abiotic stress, SOS1 over-expression could alter plant fitness and establishment in the field.

M6PR. The mannose-6-phosphate reductase (M6PR) gene was cloned from celery, *Apium graveolens* L., a salt-tolerant plant whose tolerance has been linked to its production of mannitol as a primary product of photosynthesis (Everard *et al*. 1997). By catalyzing the first irreversible reaction in the mannitol biosynthetic pathway, M6PR was shown to be the key enzyme in the production of mannitol (Zhifang and Loescher 2003). Mannitol has been found to act as a compatible solute, counterbalancing the osmotic effects of sodium and chloride ions, as well as an osmoprotectant, reducing the damage caused by free radicals produced under salinity stress (Shen *et al.* 1997, Sickler *et al.* 2007, Zhifang and Loescher 2003). Additionally, transcriptomic analysis of M6PR over-expression lines in the absence of salt found over 1700 genes with altered expression levels relative to wild-type (Chan *et al*. 2011). Approximately 50% of these overlapped with those altered by CBF3 over-expression under the same conditions (Chan *et al*. 2012). Strong overlap was also detected between transcripts affected by M6PR and those induced in wild-type plants in response to salinity stress. Also observed was significant up-regulation of biotic stress responsive genes by M6PR over-expression (Chan *et al.* 2011). Mannitol is not commonly produced by plants but is produced by a number of pathogenic fungal

species and thus could be acting as an elicitor of biotic stress response. Together these indicate multiple levels of protection against the damaging effects of abiotic stress and suggest possible effects on the response to biotic stress as well.

Constitutive expression of M6PR in *A. thaliana* resulted in the accumulation of mannitol and increased salinity tolerance without visible phenotypic effects in the absence of salinity stress. Mannitol levels ranged from 0.5 to nearly 6 umol per gram fresh weight depending on the transgenic line, with the metabolite present in all tissues but highest in floral tissues and seeds (Zhifang and Loescher 2003). Dark room experiments showed no ability to catabolize mannitol by the transgenic *Arabidopsis thaliana* plants. Plants expressing M6PR were able to grow and set seed in salt concentrations up to 300mM NaCl, levels which prevent wild-type seed formation (Zhifang and Loescher 2003). Transgenic lines were also able to maintain their photosystem II efficiency at wild-type stress-free levels in the presense of salinity stress and showed reduced salt stress impacts on CO_2 assimilation (Sickler *et al.* 2007). In the absence of salt stress, the transgenic 35S-M6PR lines showed no observable differences in phenotype to the wild-type Columbia ecotype background (Sickler *et al*. 2007, Chan *et al*. 2011). These experiments demonstrated that M6PR confers primary and secondary effects which could significantly alter plant fitness and confer advantages that might increase the likelihood of transgene establishment in the natural environment.

Objectives of dissertation

The fitness effects conferred by a transgene play a significant role in the possible environment risks from a transgenic crop cultivar or recipient wild plants, and thus are closely evaluated during the environmental risk assessment conducted before any commercialization.

My first objective was to examine whether transgenes involved in different abiotic stress tolerance mechanisms conferred differing secondary fitness effects and whether those transgene fitness impacts differed among growing environments. I performed a series of experiments in the field and greenhouse to determine the secondary fitness effects of three transgenes; CBF3, SOS1 and M6PR, whose expression in *Arabidopsis thaliana* confers increased tolerance to salinity

stress. Each gene represents a different cellular mechanism for tolerance and a possible route for the production of salt tolerant genetically engineered crops. Given the potentially broad-reaching effects of altering abiotic stress tolerance via their effects on gene regulation, cellular ion balance, and carbon metabolism, we expected to find secondary effects of transgene expression including fitness impacts, either positive or negative. Transgene fitness was assessed under a variety of field growing environments ranging across spring, summer and fall (described in chapter 2). Additionally fitness effects were assessed in the greenhouse, in the presence and absence of salinity stress, and compared to previously performed growth chamber studies (described in chapter 3).

My second objective was to assess whether transgenic plant performance in competition with other genotypes, as would occur in the wild, differs from non-competitive pure line performance, as occurs in agriculture. Plant productivity and fitness in competition with wild-type plants was observed across six generations in the field (detailed in chapter 2) and in three repeated single-generation greenhouse competition experiments in the presence and absence of salinity stress (detailed in chapter 3).

The last objective was to examine the implications of the data and methodology, including previous transcriptomic analysis of the three transgenes, for ecological risk assessments of abiotic stress tolerance enhancing transgenes (described in chapter 4). This project measured transgene fitness effects not predicted from prior growth chamber assessments, determined that competition with wild-type significantly affected the observed fitness of transgenic plants and developed methodology for the analysis of secondary fitness effects of future abiotic stress resistance transgenes using the model species *Arabidopsis thaliana.*

LITERATURE CITED

LITERATURE CITED

Andersen, N. S., Siegismund, H. R., Meyer, V., & Jørgensen, R. B. (2005). Low level of gene flow from cultivated beets (*Beta vulgaris L. ssp. vulgaris*) into Danish populations of sea beet (*Beta vulgaris L. ssp. maritima*).Molecular Ecology, 14(5), 1391-1405.

APHIS (Animal and Plant Health Inspection Service of the United States Department of Agriculture), (2011a). Monsanto Company Petition (07-CR-191U) for determination of non-regulated status of event MON 87460. Draft Environmental Assessment http://www.aphis.usda.gov/brs/aphisdocs/09_05501p_dea.pdf (accessed March 2013).

APHIS (Animal and Plant Health Inspection Service of the United States Department of Agriculture), (2011b). Determination of Nonregulated Status for MON 87460 Corn *(Zea mays* L). http://www.aphis.usda.gov/brs/aphisdocs/09_05501p_det.pdf (accessed March 2013).

APHIS (Animal and Plant Health Inspection Service of the United States Department of Agriculture), (2013). Notice of intent to prepare an environmental impact statement for determination of nonregulated status of freeze tolerant Eucalyptus lines and notice of virtual public meetings. Federal Register**,** 78 (39) 13309- 13312. http://www.aphis.usda.gov/brs/fedregister/11_01901p_fr_noi.pdf (accessed April 2013).

Arias, D. M., & Rieseberg, L. H. (1994). Gene flow between cultivated and wild sunflowers. Theoretical and Applied Genetics, 89(6), 655-660.

Asmussen, M. A., Gilliland, L. U., & Meagher, R. B. (1998). Detection of deleterious genotypes in multigenerational studies. II. Theoretical and experimental dynamics with selfing and selection. Genetics, 149(2), 727-737.

Ayal, S., Levy, A. A., & Gressel, J. (2005). Wheat Domestication and Dedomestication—What Are the Odds?. In *Crop ferality and volunteerism*. CRC Press.

Baker, S. S., Wilhelm, K. S., & Thomashow, M. F. (1994). The 5′-region of *Arabidopsis thaliana* cor15a has cis-acting elements that confer cold-, drought-and ABA-regulated gene expression. Plant molecular biology, 24(5), 701-713.

Bartsch, D., Lehnen, M., Clegg, J., Pohl-Orf, M., Schuphan, I., & Ellstrand, N. C. (1999). Impact of gene flow from cultivated beet on genetic diversity of wild sea beet populations. Molecular Ecology, 8(10), 1733-1741.

Beckie, H. J., Warwick, S. I., Nair, H., & Séguin-Swartz, G. (2003). Gene flow in commercial fields of herbicide-resistant canola (*Brassica napus*). Ecological applications, 13(5), 1276-1294.

Bergelson, J., Purrington, C. B., Palm, C. J., & LOpez-Gutierrez, J. C. (1996). Costs of resistance: a test using transgenic *Arabidopsis thaliana*. Proceedings of the Royal Society of London. Series B: Biological Sciences, 263(1377), 1659-1663.

Bergelson, J., & Purrington, C. B. (2002). Factors affecting the spread of resistant Arabidopsis thaliana populations. In *Genetically engineered organisms: assessing environmental and human health effects*. CRC Press.

Brauer, J., & Geber, M. A. (2002). Population differentiation in the range expansion of a native maritime plant, *Solidago sempervirens L*. International Journal of Plant Sciences, 163(1), 141-150.

Bhatnagar-Mathur, P., Vadez, V., & Sharma, K. K. (2008). Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Reports, 27(3), 411-424.

Boudry, P., Wieber, R., Saumitou-Laprade, P., Pillen, K., Van Dijk, H., & Jung, C. (1994). Identification of RFLP markers closely linked to the bolting gene *B* and their significance for the study of the annual habit in beets (*Beta vulgaris L*.). Theoretical and Applied Genetics, 88(6-7), 852-858.

Bourguet, D., Guillemaud, T., Chevillon, C., & Raymond, M. (2004). Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. Evolution, 58(1), 128-135.

Brunken, J., de Wet, J. M., & Harlan, J. R. (1977a). The morphology and domestication of pearl millet. Economic Botany, 31(2), 163-174.

Brunken, J. N. (1977b). A systematic study of *Pennisetum* sect. *Pennisetum* (Gramineae). American Journal of Botany, 161-176.

Campbell, L. G., & Snow, A. A. (2009). Can feral weeds evolve from cultivated radish (*Raphanus sativus, Brassicaceae*)?. American Journal of Botany, 96(2), 498-506.

Chan, Z., Grumet, R., & Loescher, W. (2011). Global gene expression analysis of transgenic, mannitol-producing, and salt-tolerant *Arabidopsis thaliana* indicates widespread changes in abiotic and biotic stress-related genes. Journal of Experimental Botany, 62(14), 4787-4803.

Chan, Z., Bigelow, P. J., Loescher, W., & Grumet, R. (2012). Comparison of salt stress resistance genes in transgenic *Arabidopsis thaliana* indicates that extent of transcriptomic change may not predict secondary phenotypic or fitness effects. Plant Biotechnology Journal, 10(3), 284-300.

Castiglioni, P., Warner, D., Bensen, R. J., Anstrom, D. C., Harrison, J., Stoecker, M., ... & Heard, J. E. (2008). Bacterial RNA chaperones confer abiotic stress tolerance in plants

and improved grain yield in maize under water-limited conditions. Plant Physiology, 147(2), 446-455.

Chen, P.D., D.J. Liu, G.Z. Pei, L.L. Qi & L. Huang, 1991. The chromosome constitution of three endemic hexaploid wheats in western China. Proc 7th International Wheat Genetics Symposium, pp. 75–80.

Chinnusamy, V., Jagendorf, A., & Zhu, J. K. (2005). Understanding and improving salt tolerance in plants. Crop Science, 45(2), 437-448.

Chu, Y. E., & Oka, H. I. (1970). Introgression across isolating barriers in wild and cultivated *Oryza* species. Evolution, 24(2), 344-355.

Chua, N. H., & Tingey, S. V. (2006). Plant biotechnology: Looking forward to the next ten years. Current Opinion in Biotechnology, 17(2), 103-104.

Chung, J. S., Zhu, J. K., Bressan, R. A., Hasegawa, P. M., & Shi, H. (2008). Reactive oxygen species mediate Na+-induced *SOS1* mRNA stability in *Arabidopsis*. The Plant Journal, 53(3), 554-565.

Cook, D., Fowler, S., Fiehn, O., & Thomashow, M. F. (2004). A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America, 101(42), 15243-15248.

Davis, M. B., & Shaw, R. G. (2001). Range shifts and adaptive responses to Quaternary climate change. Science, 292(5517), 673-679.

Desplanque, B., Boudry, P., Broomberg, K., Saumitou-Laprade, P., Cuguen, J., & Van Dijk, H. (1999). Genetic diversity and gene flow between wild, cultivated and weedy forms of *Beta vulgaris* L.(Chenopodiaceae), assessed by RFLP and microsatellite markers. Theoretical and Applied Genetics, 98(8), 1194-1201.

Desplanque, B., Hautekèete, N., & Van Dijk, H. (2002). Transgenic weed beets: possible, probable, avoidable?. Journal of Applied Ecology, 39(4), 561-571.

EFSA (2012) Scientific Opinion on a request from the European Commission related to the prolongation of prohibition of the placing on the market of genetically modified oilseed rape events Ms8, Rf3 and Ms8 x Rf3 for import, processing and feed uses in Austria. EFSA J 2878:1-33, http://www.efsa.europa.eu/en/efsajournal/doc/2878.pdf (accessed April 2013).

Ellstrand, N. C., & Hoffman, C. A. (1990). Hybridization as an avenue of escape for engineered genes. BioScience, 40(6), 438-442.

Ellstrand, N. C. (2003). *Dangerous Liaisons?*. Johns Hopkins University Press.

Everard, J. D., Cantini, C., Grumet, R., Plummer, J., & Loescher, W. H. (1997). Molecular cloning of mannose-6-phosphate reductase and its developmental expression in celery. Plant physiology, 113(4), 1427-1435.

Fenart, S., Austerlitz, F., Cuguen, J., & ARNAUD, J. F. (2007). Long distance pollen-mediated gene flow at a landscape level: the weed beet as a case study. Molecular Ecology, 16(18), 3801-3813.

Fénart, S., Arnaud, J. F., De Cauwer, I., & Cuguen, J. (2008). Nuclear and cytoplasmic genetic diversity in weed beet and sugar beet accessions compared to wild relatives: new insights into the genetic relationships within the *Beta vulgaris* complex species. Theoretical and Applied Genetics, 116(8), 1063-1077.

Ford-Lloyd, B. V. (1995). Sugarbeet and other cultivated beets. In *Evolution of crop plants.* Longman Sinagpore, 2nd edition.

Fowler, S., & Thomashow, M. F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. The Plant Cell, 14(8), 1675-1690.

Gilliland, L. U., McKinney, E. C., Asmussen, M. A., & Meagher, R. B. (1998). Detection of deleterious genotypes in multigenerational studies. I. Disruptions in individual Arabidopsis actin genes. Genetics, 149(2), 717-725.

Gilmour, S. J., Sebolt, A. M., Salazar, M. P., Everard, J. D., & Thomashow, M. F. (2000). Overexpression of the *Arabidopsis CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology, 124(4), 1854-1865.

Gilmour, S. J., Fowler, S. G., & Thomashow, M. F. (2004). *Arabidopsis* transcriptional activators *CBF1*, *CBF2*, and *CBF3* have matching functional activities. Plant molecular biology, 54(5), 767-781.

Glenn, E., Tanner, R., Mendez, S., Kehret, T., Moore, D., Garcia, J., & Valdes, C. (1998). Growth rates, salt tolerance and water use characteristics of native and invasive riparian plants from the delta of the Colorado River, Mexico. Journal of Arid Environments, 40(3), 281-294.

Glenn, E. P., & Nagler, P. L. (2005). Comparative ecophysiology of *Tamarix ramosissima* and native trees in western US riparian zones. Journal of Arid Environments, 61(3), 419-446.

Ghimire, S. K., Akashi, Y., Masuda, A., Washio, T., Nishida, H., Zhou, Y. H., ... & Kato, K. (2006). Genetic diversity and phylogenetic relationships among East Asian common wheat (*Triticum aestivum L.*) populations, revealed by the analysis of five isozymes. Breeding Science, 56(4), 379-387.

Gressel, J. (2005). Introduction-the challenges of ferality. *Crop ferality and volunteerism*, 1-7.

Guo, Y., Qiu, Q. S., Quintero, F. J., Pardo, J. M., Ohta, M., Zhang, C., ... & Zhu, J. K. (2004). Transgenic evaluation of activated mutant alleles of *SOS2* reveals a critical requirement for its kinase activity and C-terminal regulatory domain for salt tolerance in *Arabidopsis thaliana*. The Plant Cell, 16(2), 435-449.

Hails, R. S., & Morley, K. (2005). Genes invading new populations: a risk assessment perspective. Trends in Ecology & Evolution, 20(5), 245-252.

Halfter, U., Ishitani, M., & Zhu, J. K. (2000). The *Arabidopsis SOS2* protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proceedings of the National Academy of Sciences, 97(7), 3735-3740.

Hancock, J. F., R. Grumet, and S. C. Hokanson. (1996). The opportunity for escape of engineered genes from transgenic crops. HortScience 31:1080–1085.

Hancock, J. (2011). Factors influencing the genetic diversity of plant species and the potential impact of transgene movement. In *Environmental safety of genetically engineered crops*. Michigan State University Press.

Hancock, J. F. (2012). *Plant Evolution and the Origin of Crop Species*. Cabi.

Harker, K. N., Clayton, G. W., Blackshaw, R. E., O'Donovan, J. T., Johnson, E. N., Gan, Y., ... & Van Acker, R. C. (2006). Persistence of glyphosate-resistant canola in western Canadian cropping systems. Agronomy Journal, 98(1), 107-119.

Harlan, J. R. (1965). The possible role of weed races in the evolution of cultivated plants. Euphytica, 14(2), 173-176.

Haygood, R., Ives, A. R., & Andow, D. A. (2004). Population genetics of transgene containment. Ecology Letters, 7(3), 213-220.

Heiser Jr, C. B. (1947). Hybridization between the sunflower species *Helianthus annuus* and *H. petiolaris*. Evolution, 1(4), 249-262.

Horn, G., Hofweber, R., Kremer, W., & Kalbitzer, H. R. (2007). Structure and function of bacterial cold shock proteins. Cellular and molecular life sciences,64(12), 1457-1470.

Hornsey, K. G., & Arnold, M. H. (1979). The origins of weed beet. Annals of Applied Biology, 92(2), 279-285.

Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O., & Thomashow, M. F. (1998). *Arabidopsis CBF1* overexpression induces COR genes and enhances freezing tolerance. Science, 280(5360), 104-106.

James, C. (2012). Global Status of Commercialized Biotech/GM Crops: 2012. *ISAAA Brief* No.44. ISAAA: Ithaca, NY.

Kamei, A., Seki, M., Umezawa, T., Ishida, J., Satou, M., Akiyama, K., ... & Shinozaki, K. (2005). Analysis of gene expression profiles in *Arabidopsis* salt overly sensitive mutants *sos2− 1* and *sos3− 1*. Plant, Cell & Environment, 28(10), 1267-1275.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., & Shinozaki, K. (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature biotechnology, 17(3), 287-291.

Katiyar-Agarwal, S., Zhu, J., Kim, K., Agarwal, M., Fu, X., Huang, A., & Zhu, J. K. (2006). The plasma membrane Na+/H+ antiporter *SOS1* interacts with *RCD1* and functions in oxidative stress tolerance in *Arabidopsis*. Proceedings of the National Academy of Sciences, 103(49), 18816-18821.

Klinger, T., Elam, D. R., & Ellstrand, N. C. (1991). Radish as a model system for the study of engineered gene escape rates via crop-weed mating. Conservation Biology, 5(4), 531-535.

Knispel, A. L., McLachlan, S. M., Van Acker, R. C., & Friesen, L. F. (2008). Gene flow and multiple herbicide resistance in escaped canola populations. Weed Science, 56(1), 72-80.

Ladizinsky, G., & Zohary, D. (1971). Notes on species delimination, species relationships and polyploidy in *Avena L*. Euphytica, 20(3), 380-395.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., & Shinozaki, K. (1998). Two transcription factors, *DREB1* and *DREB2*, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. The Plant Cell, 10(8), 1391-1406.

Longden, P. C. (1989). Effects of increasing weed-beet density on sugar-beet yield and quality. Annals of Applied Biology, 114(3), 527-532.

Longden, P. C. (1993). Weed beet: a review. Aspects of Applied Biology. 35:185-194.

Maruyama, K., Sakuma, Y., Kasuga, M., Ito, Y., Seki, M., Goda, H., ... & Yamaguchi-Shinozaki, K. (2004). Identification of cold-inducible downstream genes of the *Arabidopsis DREB1A/CBF3* transcriptional factor using two microarray systems. The Plant Journal, 38(6), 982-993.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. Trends in Plant Science, 11(1), 15-19.

Mücher, T., P. Hesse, M. Pohl-Orf, N.C. Ellstrand, and D. Bartsch. 2000. Characterization of weed beets in Germany and Italy. Journal of Sugar Beet Research, 37: 19–38.

National Research Council (NRC) Staff & Board on Agriculture and Natural Resources Staff. (2002). *Environmental effects of transgenic plants: the scope and adequacy of regulation*. National Academies Press.

Navarro, M., Martinez, Y., Laur, J., El Kayal, W., Marque, and C., Teulieres, C. (2011). Two *EguCBF1* genes overexpressed in *Eucalyptus* display a different impact on stress tolerance and plant development. Plant Biotechnology Journal, 9(1), 50-63.

Nehra, N.S. & Pearson, L. (2011). Petition for Determination of Non-regulated Status for Freeze Tolerant Hybrid *Eucalyptus* Lines. ArborGen Inc. Summerville, SC.

Qiu, Q. S., Guo, Y., Dietrich, M. A., Schumaker, K. S., & Zhu, J. K. (2002). Regulation of *SOS1*, a plasma membrane Na+/H+ exchanger in *Arabidopsis thaliana*, by *SOS2* and *SOS3*. Proceedings of the National Academy of Sciences, 99(12), 8436-8441.

Reeves, W. R. (2010). Petition for the Determination of Non-Regulated Status for MON 87460. The Monsanto Company, St. Louis MO.

Rieseberg, L. H., & Seiler, G. J. (1990). Molecular evidence and the origin and development of the domesticated sunflower (*Helianthus annum*, Asteraceae). Economic Botany, 44(3), 79-91.

Rieseberg LH, Beckstrom-Sternberg S, Doan K (1990a) *Helianthus annuus* ssp. *texanus* has chloroplast DNA and nuclear ribosomal RNA genes of *Helianthus debilis* ssp. *cucumerifolius*. Proceedings of the National Academy of Science USA, 87:593–597

Rieseberg LH, Carter R, Zona S (1990b) Molecular tests of the hypothesized hybrid origin of two diploid *Helianthus* species (Asteraceae). Evolution 44:1498–1511

Rieseberg LH, Beckstrom-Sternberg S, Liston A, Arias D (1991a) Phylogenetic and systematic inferences from chloroplast DNA and isozyme variation in *Helianthus* sect. *Helianthus*. Systematic Botany 16:50–76.

Rieseberg LH, Choi H, Ham D (1991b) Differential cytoplasmic versus nuclear gene flow in *Helianthus*. Journal of Heredity, 82:489–493.

Rieseberg, L. H., Kim, S. C., Randell, R. A., Whitney, K. D., Gross, B. L., Lexer, C., & Clay, K. (2007). Hybridization and the colonization of novel habitats by annual sunflowers. Genetica, 129(2), 149-165.

Roux, F., Camilleri, C., Bérard, A., & Reboud, X. (2005). Multigenerational versus single generation studies to estimate herbicide resistance fitness cost in Arabidopsis thaliana. Evolution, 59(10), 2264-2269.

Sanvido, O., Romeis, J., Gathmann, A., Gielkens, M., Raybould, A., & Bigler, F. (2012). Evaluating environmental risks of genetically modified crops: ecological harm criteria for regulatory decision-making. Environmental Science & Policy, 15(1), 82-91.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., ... & Shinozaki, K. (2001). Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. The Plant Cell, 13(1), 61-72.

Shao, Q., LI, C. and Basang, C. (1983) Semi-wild wheat from Xizang (Tibet). Proc. 6th Int. Wheat Genet. Symp. Kyoto, 111-114.

Shen, B., Jensen, R. G., & Bohnert, H. J. (1997). Mannitol protects against oxidation by hydroxyl radicals. Plant Physiology, 115(2), 527-532.

Shi, H., Ishitani, M., Kim, C., & Zhu, J. K. (2000). The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na+/H+ antiporter. Proceedings of the National Academy of Sciences, 97(12), 6896-6901.

Shi, H., Quintero, F. J., Pardo, J. M., & Zhu, J. K. (2002). The putative plasma membrane Na+/H+ antiporter *SOS1* controls long-distance Na+ transport in plants. The Plant Cell, 14(2), 465-477.

Shi, H., Lee, B., Wu, S.J. and Zhu, J.K. (2003) Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nature Biotechnology. 21, 81–85.

Sickler, C. M., Edwards, G. E., Kiirats, O., Gao, Z., & Loescher, W. (2007). Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. Functional Plant Biology, 34(4), 382-391.

Simard, M. J., Légère, A., Séguin-Swartz, G., Nair, H., & Warwick, S. (2005). Fitness of double vs. single herbicide-resistant canola. Weed Science, 53(4), 489-498.

Slatkin, M. (1985). Gene flow in natural populations. Annual Review of Ecology and Systematics, 16, 393-430.

Smedema, L. K., & Shiati, K. (2002). Irrigation and salinity: a perspective review of the salinity hazards of irrigation development in the arid zone. Irrigation and Drainage Systems, 16(2), 161-174.

Sreenivasulu, N., Sopory, S. K., & Kavi Kishor, P. B. (2007). Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene, 388(1), 1.

Stockinger, E. J., Gilmour, S. J., & Thomashow, M. F. (1997). *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proceedings of the National Academy of Sciences, 94(3), 1035-1040.

Sun, Q., Ni, Z., Liu, Z., Gao, J., & Huang, T. (1998). Genetic relationships and diversity among Tibetan wheat, common wheat and European spelt wheat revealed by RAPD markers. Euphytica, 99(3), 205-211.

Taji, T., Seki, M., Satou, M., Sakurai, T., Kobayashi, M., Ishiyama, K., ... & Shinozaki, K. (2004). Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt cress using *Arabidopsis* microarray. Plant Physiology, 135(3), 1697-1709.

Tiedje, J. M., Colwell, R. K., Grossman, Y. L., Hodson, R. E., Lenski, R. E., Mack, R. N., & Regal, P. J. (1989). The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecology, 70(2), 298-315.

Thompson, J. D. (1991). The biology of an invasive plant. BioScience, 41(6), 393-401.

Thuiller, W., Lavorel, S., Araújo, M. B., Sykes, M. T., & Prentice, I. C. (2005). Climate change threats to plant diversity in Europe. Proceedings of the National Academy of Sciences of the United States of America, 102(23), 8245-8250.

Verslues, P. E., Batelli, G., Grillo, S., Agius, F., Kim, Y. S., Zhu, J., ... & Zhu, J. K. (2007). Interaction of SOS2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and H2O2 signaling in *Arabidopsis thaliana*. Molecular and Cellular Biology, 27(22), 7771-7780.

Viard, F., Bernard, J., & Desplanque, B. (2002). Crop-weed interactions in the Beta vulgaris complex at a local scale: allelic diversity and gene flow within sugar beet fields. Theoretical and Applied Genetics, 104(4), 688-697.

Viard, F., Arnaud, J. F., Delescluse, M., & Cuguen, J. (2004). Tracing back seed and pollen flow within the crop–wild Beta vulgaris complex: genetic distinctiveness vs. hot spots of hybridization over a regional scale. Molecular Ecology, 13(6), 1357-1364.

Vogel, J. T., Zarka, D. G., Van Buskirk, H. A., Fowler, S. G., & Thomashow, M. F. (2005). Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. The Plant Journal, 41(2), 195-211.

Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 218(1), 1-14.

Warwick, S. I., Legere, A., Simard, M. J., & James, T. (2008). Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population. Molecular Ecology, 17(5), 1387-1395.

Warwick, S. I., Beckie, H. J., & Hall, L. M. (2009). Gene flow, invasiveness, and ecological impact of genetically modified crops. Annals of the New York Academy of Sciences, 1168(1), 72-99.

Weaver, S. A., & Morris, M. C. (2005). Risks associated with genetic modification:–An annotated bibliography of peer reviewed natural science publications. Journal of Agricultural and Environmental Ethics, 18(2), 157-189.

Whitlock, M. C. (2000). Fixation of new alleles and the extinction of small populations: drift load, beneficial alleles, and sexual selection. Evolution, 54(6), 1855-1861.

Whitton, J., Wolf, D. E., Arias, D. M., Snow, A. A., & Rieseberg, L. H. (1997). The persistence of cultivar alleles in wild populations of sunflowers five generations after hybridization. Theoretical and Applied Genetics, 95(1-2), 33-40.

Wright, S. (1969). *Evolution and the genetics of populations: Vol. 2. The theory of gene frequencies*. University of Chicago Press.

Wu, S. J., Ding, L., & Zhu, J. K. (1996). SOS1, a genetic locus essential for salt tolerance and potassium acquisition. The Plant Cell, 8(4), 617-627.

Yamaguchi-Shinozaki, K., & Shinozaki, K. (1994). A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. The Plant Cell, 6(2), 251-264.

Yang, T., Zhang, L., Zhang, T., Zhang, H., Xu, S., & An, L. (2005). Transcriptional regulation network of cold-responsive genes in higher plants. Plant Science, 169(6), 987-995.

Zhang, J. Z., Creelman, R. A., & Zhu, J. K. (2004). From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant Physiology, 135(2), 615-621.

Zhifang, G., & Loescher, W. H. (2003). Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer. Plant, Cell & Environment, 26(2), 275-283.

Zhu, J. K., Liu, J., & Xiong, L. (1998). Genetic analysis of salt tolerance in *Arabidopsis*: evidence for a critical role of potassium nutrition. The Plant Cell, 10(7), 1181-1191.

Zohary, D. (1971). Orgin of south-west Asiatic cereals: wheats, barley, oats and rye. In *Plant life in south-west Asia*. Botanical Society of Edinburgh.

Chapter 2

Multigenerational study of the establishment of abiotic stress tolerance transgenes in *Arabidopsis thaliana* populations under competitive field conditions.

Introduction

Reducing crop losses due to abiotic stresses is a major target of agricultural biotechnology. However, concerns have been raised about the potential impact of such traits on native ecosystems (Davis and Shaw 2001, Ellstrand 2003, Thuiller *et al.* 2005). A prerequisite for ecological impacts is establishment of self-sustaining populations containing the transgene, which in turn depends on transgene movement rates, population sizes, and fitness effects. Although gene flow has been the subject of numerous modeling and empirical studies, transgene establishment has received less attention.

Establishment could occur by two processes. Transgenic plants could become feral and form a self-sustaining population outside of agricultural plantings, perhaps first along field margins and later expanding into natural areas. Alternatively, the transgene could enter a compatible wild relative through hybridization and introgression and then be maintained due to a selective advantage conferred by the transgene. Establishment alone, however, does not constitute harm; the transgenic populations would need to behave differently from the untransformed crop or wild relative (EFSA 2012). If the transgenic crop behaves the same as wild-type or its relative, with no increase in invasiveness or toxicity, the risk of a feral/hybrid population is not unique to the presence of the transgene, but is instead, a risk posed by cultivation of the crop in general.

In the case of transgenes increasing abiotic stress tolerance, environmental concerns are generally linked to two possible effects (Tiedje *et al.* 1989, Hancock *et al.* 1996, Dale *et al*. 2002, Ellstrand 2003, Hancock 2003, Hails and Morley 2005, Snow *et al*. 2005, Weaver and Morris 2005, Weebadde & Maredia 2011). First, expression of the transgene will confer a competitive advantage under stressful conditions allowing plants to outcompete non-transgenic

plants within that environment. Second, the transgenic plants, feral or hybrid, could invade regions where that plant was previously constrained due to environmental stresses. In either case, the fitness effects of the transgene would have an important influence on the success of transgene establishment outside of cultivation and would be a key value in any environmental risk assessment seeking to prevent that outcome.

In this study, I sought to determine the long-term fitness effects of abiotic stress tolerance genes and their potential for transgene establishment within competitive populations. Extensive research over the past two decades on plant responses to their environments has identified a wide variety of genes with possible applications for enhancing abiotic stress tolerance (Bhatnagar-Mathur *et al.* 2008). Examples include chaperone proteins, membrane stabilization proteins, metabolic and detoxification enzymes, stress signaling pathway genes, and transcriptional activators. For this study, three genes representing diverse mechanisms shown to confer salinity tolerance were selected for analysis: (i) an abiotic stress associated transcription factor [C-repeat binding factor 3/drought responsive element binding factor 1a (*CBF3/DREB1a*)]; (ii) a plasma membrane Na+/H+ antiporter [Salt Overly-Sensitive 1 (*SOS1*)]; and (iii) a mannitol biosynthesis enzyme [mannose-6-phosphate reductase (*M6PR*)].

The CBF (C repeat binding factor)/DREB (drought responsive element binding factor) genes are a family of transcription factors, that induce a regulon of cold responsive (COR) or drought responsive (DR) genes upon exposure to abiotic stresses such as cold or dehydration (Baker *et al*. 1994, Yamaguchi-Shinozaki *et al*. 1994, Stockinger *et al*. 1997, Liu *et al*. 1998, Kasuga *et al*. 1999, Gilmour *et al*. 2000, Yang *et al*. 2005). Expression of CBF3/DREB1a results in the induction of COR gene expression, phenotypic changes in growth and development, and increased plant tolerance to drought, cold, and salinity (Gilmour *et al.* 2004). Over-expression of CBF3/DREB1a results in a cascade of gene activations and repressions that influence over 1300 genes (Jaglo-Ottosen *et al*. 1998, Kasuga *et al*. 1999, Gilmour *et al*. 2000, Seki *et al.* 2001, Fowler and Thomashow 2002, Maruyama *et al*. 2004, Vogel *et al*. 2005, Chan *et al.* 2012). This cascade of gene expression changes results in increased freezing, drought and salinity tolerance

(Jaglo-Ottosen *et al.* 1998, Kasuga *et al.* 1999). These changes, however, also result in significant phenotypic changes in transgenic *Arabidopsis,* including varying degrees of dwarfism, delayed flowering, reduced seed production, and altered plant architecture (Kasuga *et al.* 1999, Gilmour *et al.* 2000, Chan *et al.* 2012). These phenotypic and transcriptomic effects could have impacts on plant fitness and the ability to establish under field conditions.

The SOS1 gene encodes a plasma membrane Na+/H+ antiporter (Shi *et al.* 2000, Shi *et al.* 2002). High Na+ concentrations induce the release of a Ca^{2+} signal which binds to the calcium binding protein SOS3, allowing it to complex with the protein kinase SOS2 (Halfter *et al.* 2000). The SOS2-3 complex then phosphorylates SOS1, which extrudes cytosolic Na+ ions into the apoplast (Shi *et al*. 2000, Qiu *et al.* 2002, Guo *et al.* 2004). SOS1 can also be activated by RCD1, an oxidative-stress regulator, bringing together salinity and oxidative stress response pathways (Katiyar-Agarwal *et al.* 2006). SOS1 transcript levels are post-transcriptionally regulated resulting in increased mRNA stability in the presence of reactive oxygen species produced under salinity stress (Shi *et al.* 2003, Chung *et al*. 2008). Transcriptomic analysis of SOS1 overexpression lines detected roughly 600 genes with altered expression profiles in the absence of salinity stress, with the down regulation of oxidative and redox related genes the most characteristic impact (Chan *et al*. 2012). SOS1 over-expression plants had reduced levels of sodium ions in cellular tissues and sap after salt exposure, as well as greater ability to germinate, grow and set seed under salinity stress than wild-type plants (Shi *et al.* 2003, Chan *et al*. 2012). In the absence of salt stress, no phenotypic differences were observed between the transgenic and WT plants. By maintaining cellular homeostasis under abiotic stress and with no apparent cost of resistance, SOS1 overexpression could alter plant fitness and establishment in the field.

Mannose-6-phosphate reductase (M6PR) catalyzes the first committed step in mannitol biosynthesis. The *M6PR* gene was cloned from celery, *Apium graveolens* L., a salt tolerant plant whose tolerance has been linked to its production of mannitol as a primary product of photosynthesis (Everard *et al*. 1997, Zhifang and Loescher 2003). Mannitol can act as a compatible solute, counterbalancing the osmotic effects of sodium and chloride ions, as well as

an osmoprotectant, reducing the damage caused by free radicals produced under salinity stress (Shen *et al.* 1997, Sickler *et al.* 2007, Zhifang and Loescher 2003). Additionally, transcriptomic analysis of M6PR overexpression lines in the absence of salt found over 1700 genes with altered expression levels, with strong overlap with genes affected by CBF3 overexpression and an additional up regulation of biotic stress responsive genes (Chan *et al*. 2011, Chan *et al*. 2012). Constitutive expression of M6PR in *A. thaliana* resulted in the accumulation of mannitol and increased ability to germinate, grow and set seed under salinity stress without visible phenotypic effects in the absence of salinity stress (Zhifang and Loescher 2003, Chan *et al*. 2011). Transgenic lines were also able to maintain their photosystem II efficiency at wild-type stress-free levels in the presence of salinity stress and showed reduced salt stress impacts on CO_2 assimilation (Sickler *et al.* 2007). These experiments demonstrated that M6PR confers primary and secondary effects which could significantly alter plant fitness and confer advantages that might increase the likelihood of transgene establishment in the natural environment.

In this study we compared the fitness effects of these three different types of abiotic stress resistance genes and their influence on, and ability to predict, transgene establishment using the model species *Arabidopsis thaliana*. We measured fitness relative to wild-type in non-competitive populations and tracked the transgene frequency within competitive, transgenic verses wild-type, populations over six generations in the field. Both non-competitive and competitive fitness was modeled to determine the predictive ability of those fitness values. When in competition with wild-type plants, CBF3 plants reached near-extinction within four generations, SOS1 plants showed reduced fitness relative to wild type, and M6PR lines showed either selective neutrality or a competitive advantage over wild-type plants. Modeling of expected gene frequency based on early generation testing in competition allowed for greater accuracy in predicting observed gene frequency after six generations than did relative fitness assessments from pure line populations. These results illustrate the role of competition in influencing gene frequency and indicate that competitive field trials may facilitate evaluation of ecological risk in cases where there are concerns regarding transgene establishment.

Materials and Methods

Arabidopsis lines for field experiment

To determine the long-term fitness effect of transgene expression under field conditions, pure line and mixed competitive populations were grown for six generations. *Arabidopsis thaliana* over-expression lines A28, A30 and A40 for the CBF3/DREB1a transcription factor in the Wassilewskija ecotype (WS) (Gilmour *et al.* 2000) were provided by Dr. Michael Thomashow of Michigan State University. Lines 1-1 and 7-6, overexpressing SOS1 in the Columbia glabrous ecotype (Col(gl)) (Shi *et al.* 2003), were provided by Dr. Huazhong Shi from Texas Technological University. The M6PR overexpression lines M2-1, M5-1, and M7-6 in the Columbia ecotype (Col) (Zhifang and Loescher 2003) were provided by Dr. Wayne Loescher of Michigan State University. All lines represented unique transformation events. Each of the abiotic stress resistance transgenes was expressed constitutively using the Cauliflower Mosaic Virus (CaMV) 35S promoter. Each line also contains the neomycin phosphotransferase II (NPTII) gene for kanamycin resistance as a selectable marker expressed under the nopaline synthase (NOS) promoter. The wild-type backgrounds are all rapid-cycling *Arabidopsis thaliana* ecotypes and each was used in the experiment for both competitive and non-competitive phenotypic comparisons between background and transgenic populations.

The initial parent plants used for seed production were first verified to contain and express their transgene (and only their intended transgene) by PCR, Southern and northern blot analysis (Figure 2.1, Chan *et al.* 2012). All seeds used for the initial multigenerational fitness experiments were from greenhouse grown seed stocks produced during the winter of 2008.

Experimental design for field experiment

The experimental design for the multigenerational field study was adapted from a greenhouse experiment by Roux *et al*. 2005 who examined the long-term fitness effects of *Arabidopsis* mutations under competitive conditions. For our study, gene frequency of the CBF3, M6PR and SOS1 transgenes was monitored over six generations from a starting allelic frequency

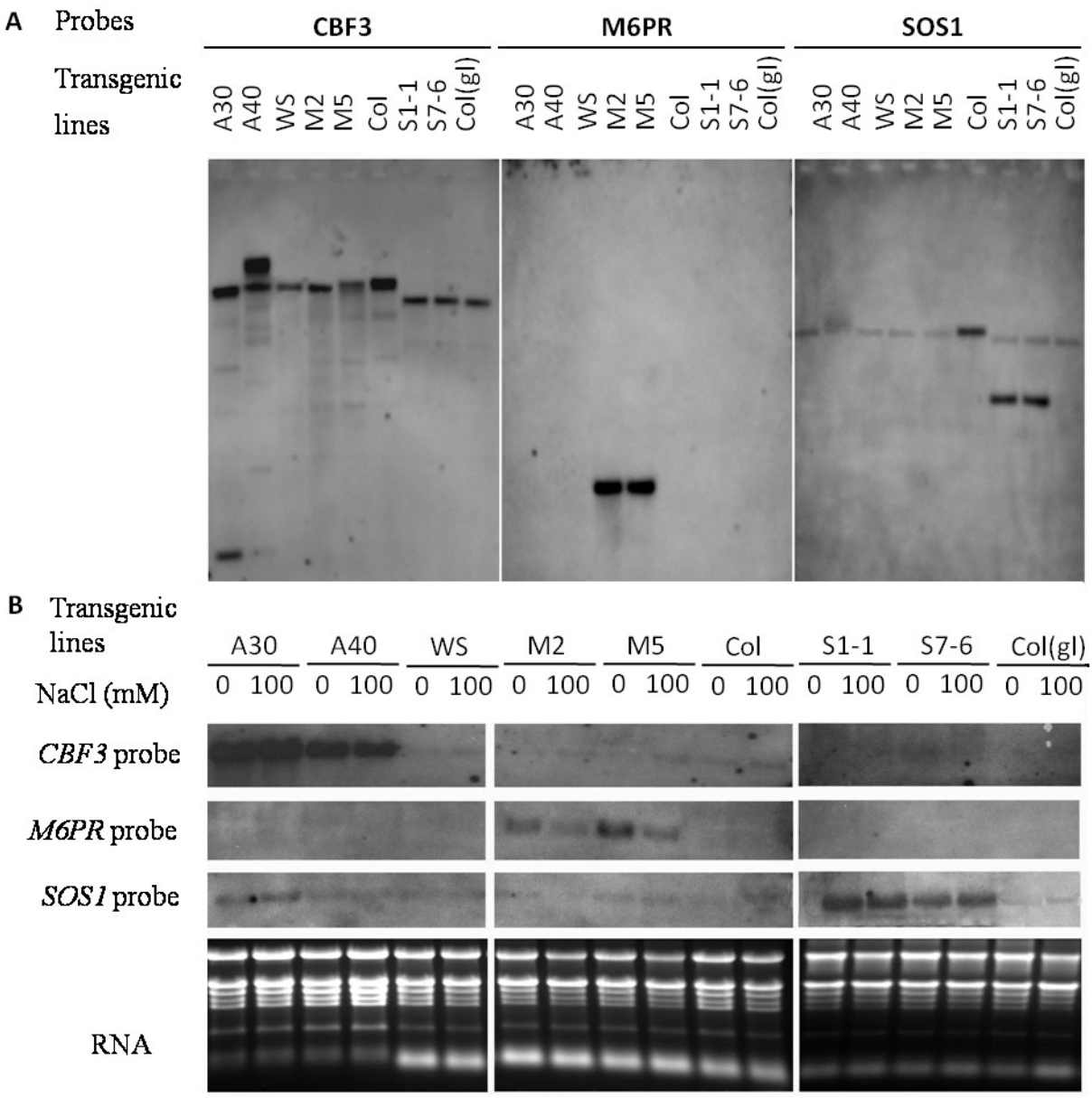


Figure 2.1. Verification of transgenic Arabidopsis thaliana lines via Southern (A) and northern analysis (B). SOS1 and CBF3 lines show the respective transgene and the endogenous gene, while M6PR lines show only the transgene since the gene is not endogenous to *Arabidopsis thaliana.* Verification was performed by Zhulong Chan.

of 50% transgenic and 50% wild-type within populations of ~180 individuals. The planting density, 2600 seeds/m^2, was equivalent to that used in Roux *et al*. 2005 and comparable to the natural density of an *Arabidopsis thaliana* population studied in 2008 (appendix). In each subsequent generation, each population was started with enough viable seeds for 180 seedlings. The number of seeds planted for each population was based upon viability tests of seeds derived from the prior generation. Fourteen replicate competitive populations were established for each transgenic line and background wild-type mix. In addition to the mixed competitive populations, five pure populations of each genotype were included for comparative purposes. A total of 167 populations were grown for each of the six generations: 112 mixed populations and 55 pure populations (Table 2.1).

Seeds were counted using custom-made counting plates, 20 gauge galvanized steel plates with multiple 5/64 inch (1.98 mm) holes drilled into them. Calibration counts were performed to determine the number of seeds that fit into one hole of that size for each genotype at each generation, and this calculation was then used to determine the correct number of holes of seed needed for proper planting density of the replicates. This calibration step compensated for changes in seed size due to genotypic and/or environmental effects. The counted seeds were then mixed with sterile dry white laboratory sand which aided planting by improving seed scatter and seeding visibility on the dark soil.

All seeds were first planted in the greenhouse utilizing 26.0x26.0 cm trays, (L-HFT NCR, Landmark Plastics Corporation; Akron, OH) with perforated bottoms filled with Baccto potting soil (Michigan Peat Company), pre-moistened and lightly compacted to create a firm seed bed. Soil choice was based on preliminary growth studies performed in the greenhouse (appendix). Slow-release (3-4 month) 14-14-14 Osmocote Classic® fertilizer (The Scotts Company LLC, Marysville, Ohio) was mixed into the Baccto potting soil at the rate of 2 g/L soil prior to planting. Seeds were stratified at 4°C for 48-60 hours and then directly seeded onto the soil surface. Once planted, all watering was performed via subsoil irrigation. The trays were placed, two per flat, into 27.4x53.9 cm greenhouse flats (L-1020NCRN, Landmark Plastics Corporation;

Table 2.1. Transgenic *Arabidopsis thaliana* lines engineered with abiotic stress tolerance genes and the number of replicate populations tested over six generations in the field.

Wild-type[1]	Transgene	Transgenic line	Number of Reps	Initial Mix Ratio
WS			5	Unmixed
Col			5	Unmixed
Col(gl)			5	Unmixed
	CBF3	A28	5	Unmixed
	CBF3	A30	5	Unmixed
	CBF3	A40	5	Unmixed
	SOS1	S1-1	5	Unmixed
	SOS1	S7-6	5	Unmixed
	M6PR	M2-1	5	Unmixed
	M6PR	M5-1	5	Unmixed
WS	CBF3	A28	14	1:1
WS	CBF3	A30	14	1:1
WS	CBF3	A40	14	1:1
Col(gl)	SOS1	S1-1	14	1:1
Col(gl)	SOS1	S7-6	14	1:1
Col	M6PR	M2-1	14	1:1
Col	M6PR	M5-1	14	1:1

[1]Wild-types are Wassilewskija (WS) ecotype, Columbia glabrous (Col(gl)) ecotype and Columbia (Col) ecotype

Akron, OH) filled with water to approximately 2.5 cm. This depth was maintained daily to ensure sufficient soil moisture for germination. After germination, watering was reduced to 20-30 minutes of subsoil irrigation on an as needed basis, with any excess water dumped to prevent water-logging of the soil.

Once the study populations reached the rosette stage (five to six true leaves) they were transported from the greenhouse to the field in an enclosed vehicle. A tray-in-flat potting method was developed for the field, in which a single planted 26x26 cm tray was placed in the center of a 27.4x53.9 cm flat, thus allowing for subsoil irrigation as the flat filled with water via trickle hose (Fig. 2.2a, appendix). The larger flat also allowed for secure anchoring to the ground, using 15.2 cm landscaping stakes, to prevent movement or tipping of the planted trays. To protect the exposed plants during extreme weather conditions and prevent movement of plants or seeds from the trial site, the trays were covered with clear plastic lids secured to the anchor stakes by bungee cords. The lids were deployed no more than six hours ahead of damaging weather and were removed once the weather passed. Four drainage holes were drilled into the large flat approximately 2.5 cm from the bottom to allow excess rainwater to drain off. This system successfully protected the plants from driving rains, sleet, hail, early frosts and snows, and sustained high winds. Plants were grown in the field until approximately 75% of siliques had begun to lose chlorophyll and yellow, a change immediately prior to maturation (the beginning of stage 18 as determined by Roeder and Yanofsky 2006). The trays were then returned to the greenhouse for final maturation and dry down prior to harvest. This system was designed to reduce seed loss prior to harvest which aided in both the accuracy of seed yield measurements and seed containment.

Developmental data collected on all populations included germination rate, days to germination, days to two true leaves, days to rosette formation (5-6 true leaves), days to initial bolting and flowering, days to ~75% bolting and flowering, days to 75% of siliques maturing, and percentage of plants that survived to population maturity. At harvest, all aboveground plant

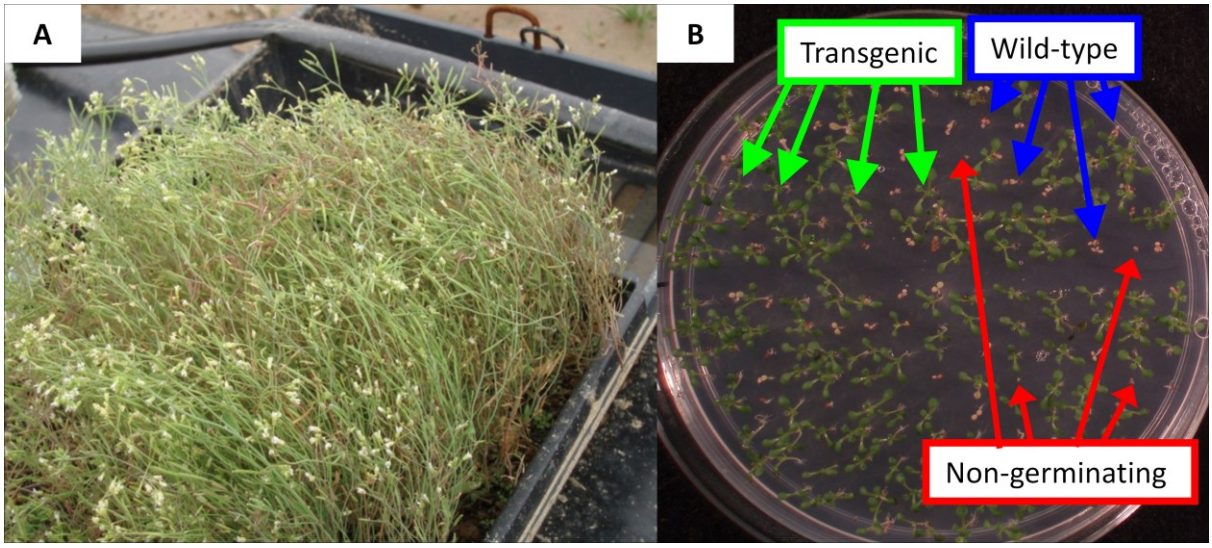


Figure 2.2. The tray-in-flat field planting method showing the trickle hose for irrigation and the high density of planting for interplant competition (A) and the screening of progeny seed on ½ MS 1% agar 100mg/L kanamycin containing media (B). Germination rates and transgene frequencies can be calculated from the number of: bleaching of wild-type seedlings (blue arrows), healthy transgenic seedlings (green arrows) and non-germinating seeds (red arrows). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

material was removed and temporarily stored in 10lb-sized brown paper bags for transport back to campus. In lab, the dried plant materials were cleaned of seed and total plant dry weight and seed mass were recorded. Prior to any further analysis, a subset of the seed produced by each replicate population was set aside for planting the next generation. This protocol allowed each replicate population to be separately maintained and carried forward through six generations.

Climatic data was recorded at the Horticultural Teaching and Research Center weather station, which was located less than 400m from the field site. The weather station recorded air temperature, precipitation, solar radiation, potential evapotranspiration, and wind speed across all six field growing seasons.

Genotyping field grown progeny via kanamycin screening

Kanamycin screening of progeny seed was performed on each replicate population in every generation to determine relative gene frequency of the NPTII selectable marker gene. A ~75 uL aliquot of seed was taken from each mixed competitive population to test for kanamycin resistance. Each seed aliquot was sterilized with 95% EtOH for 10 minutes followed by three cycles of 15% commercial bleach plus 0.000025% SDS for 10 minutes. The seed samples were vortexed during each 10 minute period to ensure uniform sterilization. Sterilized seed were then washed 4 times in sterile dH_2O. These seeds were then screened by being plated, via 20uL pipette, onto ½ MS media + 1% agar containing 100 mg/L kanamycin, with the exception of lines A40 and S1-1 which were plated onto media containing 75mg/L kanamycin due to a lower level of resistance than the other transgenic lines. The kanamycin screenings were performed in triplicate with the objective of scoring 100 seedlings in each replicate. For each mixed population screening, seed from the respective WT were included on the same plate as negative controls to verify kanamycin effectiveness. Wild-type seedlings showed complete bleaching while transgenic seedlings grew normally. Resistant and susceptible individuals were easily distinguished from each other and from any seeds that failed to germinate (Fig. 2.2b). In cases of low germination rates, the plating was repeated with increased numbers of seeds to achieve a total of three replicates of 100 scored seedlings.

The competitive fitness of each transgenic line in each generation was calculated from the mean of the transgene frequencies of the 14 replicate populations as determined by kanamycin screening. The relative fitness of transgenic lines was derived from comparison of the mean seed yields of the pure transgenic populations to the mean yields of the corresponding WT background (each with 5 replicate pure populations).

qPCR verification of transgene frequency calculated from kanamycin screening

Due to the possibility of transgene silencing, the transgene frequencies of the competitive populations determined via kanamycin screening were verified by qPCR analysis. Progeny seed were sterilized as described above and plated via pipette on to ½ MS media + 1% agar. A set of standards was created using confirmed transgenic or WT seed. Seedlings were grown to the two true leaves stage in the growth room under fluorescent lights for 16 hours light and 8 hours dark, ~8-12 days depending on genotype. Seedlings were then removed by hand in batches of 100, weighed and flash frozen in liquid nitrogen, yielding ~150-200 mg fresh weight. For the standards, seedlings were removed and mixed by hand to result in 100 seedling batches with 0% transgenic, 10%, 20%, 50% and 100% transgenic seedlings. These standardized batches were then frozen and processed following the same protocol as the seedlings from competitive populations.

The frozen material was processed using a modified version of the protocol described by Vorwerk in 2001, the protein purification step was performed twice, using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). DNA quality was confirmed via gel electrophoresis and DNA quantity was calculated using the Qubit® fluorometric quantification system (Life Technologies Corporation, Invitrogen™, Grand Island, NY, USA). The qPCR was performed using primers for the NPTII gene on a Stratagene Mx4000 (now Agilent Technologies Inc., Santa Clara, California) (forward 5'-CGGCTGCATACGCTTGATC-3' and reverse 5'-GATGCGATGTTTCGCTTGGT-3') and SYBR® Green master mix (Life Technologies Corporation, Applied Biosystems®, Grand Island, NY, USA). Transgene frequency within the mixed populations was calculated by

comparison of each replicates Ct values to the Ct values of the known transgene frequency standards.

Determination of selective pressure under competitive conditions

The variance in mean allele, or transgene, frequencies predicted to be associated with random genetic drift at the *t* generation was calculated based on theoretical drift distributions using the following formula; $V_{qt} = q_0 * p_0 * (1-(1-1/(2N_e))^t)$ (Falconer & Mackay 1996). The initial frequencies of transgenic and wild-type plants are q_0 and p_0 respectively and N_e is the effective population size. Confidence intervals can then be derived as the expected mean ± [1.96*(*Vqt*/14)^0.5] with 14 being the number of replicate populations and the expected mean equal to the starting frequency, 0.5, due to the assumption of no selective pressure.

Modeling transgene frequency

The PopGene.S^2 modeling program (Hamilton 2011) was used to model transgene frequency within 100 theoretical populations across six generations. Each population began at a starting transgene frequency of 0.5. The number of plants within each population was 100, the mean number of plants surviving to maturity per population across the six field seasons. Due, however, to the highly selfing nature of *Arabidopsis thaliana*, 98-99% (Snape & Lawrence 1971), the effective population size was calculated based on the following formula, Ne = N/[1+(β/(2- β))] (Caballero 1994) where β is the proportion of selfing (0.98) and N is the observed population size (100), for an effective population size of 51.

The program incorporated both genetic drift, through a Monte Carlo simulation to introduce random chance into the probability of producing progeny, and natural selection based on user-entered fitness values. For this study, measured fitness values for two transgenic lines per transgene under three growing conditions were used: non-competitive relative fitness values from yield data from growth chamber pure populations (Chan *et al.* 2012), non-competitive relative fitness values from field generation 1 pure populations, and competitive fitness measured from selectable marker screening of progeny seed from field generation 1 competitive populations.

Statistical Analysis

All statistical calculations and comparisons were performed with the SAS version 9.2 and R version 2.14.0 statistical programs (SAS Institute Inc., Cary, NC and R Foundation for Statistical Computing, Vienna, Austria). Differences were considered statistically significant at $P<0.05$ and Duncan's Multiple Range Test was used for mean separation.

Results

These experiments examined the fitness effects of three abiotic stress tolerance transgenes over six generations in the field in the presence and absence of competition from their respective wild-type background. All seed used to initiate the experiments were from plants that were verified by Southern and northern analysis for presence and expression of each transgene (Figure 2.1, Chan *et al*. 2012). Continued presence and expression of the transgene in pure-line populations, as determined by kanamycin resistance, was verified after each of the six generations in the field. The absence of the transgenes in wild type lines also was verified after each of the six generations.

The planting density (2600 seeds/m^2) was selected to ensure a high level of competition between plants. This density had been used previously by Roux *et al*. 2005 to assess the fitness impacts of herbicide tolerance mutations on *Arabidopsis thaliana* and was also determined to be within the range of natural population densities we observed growing in ruderal habitat south of East Lansing, Michigan (769-3254 plants/m^2). Although *Arabidopsis thaliana* is generally classified as a winter annual (Pigliucci *et al.* 2002), i.e. germinating and forming a rosette in the fall and flowering and setting seed the following spring, the observed Michigan populations contained individuals with both spring and fall annual growth habits. This 'bet-hedging strategy' of plant development has been previously observed in *Arabidopsis* by Montesinos-Navarro *et al*. (2012), who found such life-history polymorphisms within 17 natural populations in Spain. Our field experiments used both spring and fall plantings.

Effects of seasonal differences on plant development and productivity

Environmental influences on plant development and productivity were observed across the six growing seasons (Tables 2.2 and 2.3). Plants grown in the late fall (field generation 2) were the slowest to reach every developmental stage, vegetative or reproductive, regardless of genotype (Table 2.2). This season was also marked by the lowest average maximum and minimum air temperatures of the six field seasons (Figure 2.3a).

The different seasons also showed a 4-fold range in yield capacity measured by average seed yield across all genotypes and 2.4-fold differences in biomass capacity, as measured by total aboveground dry weight, and seed partitioning, the proportion of total aboveground dry weight that is seed (Table 2.2, Figure 2.4). For example, field generation 3 spring-grown plants out yielded those of all other seasons (all $P<0.001$), while another spring-growing season (generation 5) tied with a fall season (generation 2) for the worst yield regardless of genotype (Table 2.2). Seasonal influences on productivity were not correlated with temperature or solar radiation levels (all R^2 values <0.1); thus, other unmeasured environmental factors or combinations of factors likely contributed to the seasonal effects on productivity. Additionally, not all seasonal influences had uniform effects on the performance of populations containing the three different transgenes, indicating significant genotype by environment (GxE) interactions ($P<0.01$, Table 2.3). Seed yield of the SOS1 and M6PR transgenic lines largely paralleled yield capacity of the environment (Figure 2.4). There did not appear to be a yield cost to the expression of these transgenes in high-yielding environments under the conditions encountered in these experiments. The CBF3 transgenic lines underperformed except under the least favorable conditions (Figure 2.4).

Effects of transgene expression on plant development

Overexpression of the transcription factor CBF3 delayed plant reproductive development across all six field seasons from initial bolting through to maturation (stages 4-8, $P<0.001$, Figure 2.5a). No delays in vegetative development from germination to rosette formation (stages 1-3) occurred but CBF3 plants did show the characteristic dwarf phenotype observed in previous

Table 2.2. Seasonal effect on development and productivity averaged across all lines and populations.

			Days to reach Lifecycle Stage								Measures of Productivity		
Field Generation	**Time in field**	**Season**	**Germination**	**2 True Leaves**	**5-6 True Leaves**	**First Bolting**	**Majority Bolting (75%)**	**First Flowering**	**Majority Flowering (75%%)**	**Mature (75% dry siliques)**	**Dry Weight**	**Seed Yield**	**Partitioning to Seed**
1	June - July 08	Summer	4.31c[1]	12.13b	16.25a	20.18a	NA	23.58a	NA	44.71a	12.33b	1.33d	0.11c
2	Sept - Nov 08	Fall	7.82d	17.51d	28.02c	37.31b	55.64c	46.02c	67.52d	118.8e	10.6bc	0.91e	0.09c
3	May - June 09	Spring	4.02c	12.9c	19.06b	20.74a	27ab	24.66a	32.14ab	53.18b	17.36a	3.56a	0.21a
4	Sept - Oct 09	Fall	3.06a	11.58a	18.88b	20.96a	25.22a	26.22a	30.98a	55.0b	9.95c	2.34c	0.21a
5	May - June 10	Spring	4.0c	12.38c	18.64b	22.95a	28.85b	32.74c	37.47c	57.83c	7.45d	0.83e	0.1c
6	Sept - Oct 10	Fall	3.48b	12.91c	18.5b	19.74a	27.48ab	27.53a	34.66b	68.25d	18.72a	2.72b	0.15b

[1]Each value is the mean of all non-competitive populations grown during a season (n=50). Means with the same letter are not significantly different from each other at $P<0.05$ (Duncan's).

Table 2.3. Analysis of variance showing the effects of transgene, transformation event, environment and their interactions on seed yield from non-competitive pure populations grown for six seasons in the field.

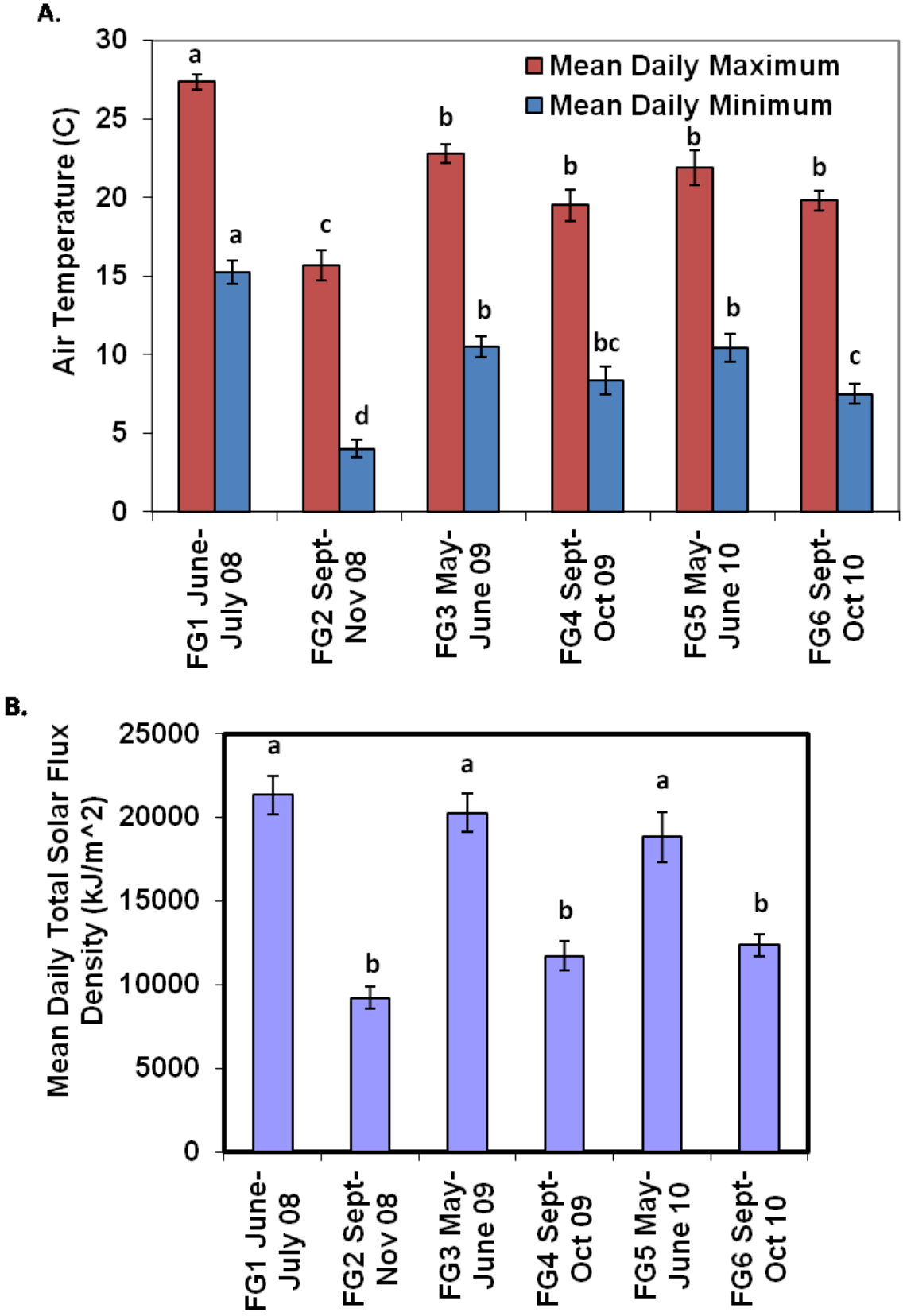
Source	Degrees of freedom	Sum of Squares	Mean Square	F Value	Pr>F
Model	58	487.24	8.4	10.43	<0.0001
Environment[1]	5	285.36	57.07	70.83	<0.0001
Genotype[2]	5	71.95	14.39	17.86	<0.0001
Transgenic line	4	29.47	7.37	9.14	<0.0001
Genotype*Environment	25	72.44	2.9	3.6	<0.0001
Transgenic line*Environment	19	28.02	1.47	1.83	0.02
Error	230	185.33	0.81		
Corrected Total	288	672.58			

[1]Environment includes seasonal effects.

[2]Includes transgenes and wild-type ecotypes

Figure 2.3. Seasonal differences in maximum and minimum air temperature (A) and daily total solar flux density (B) across the six field generations. Each value is the mean ± SE across all days populations were in the field for that growing season. Bars with the same letter were not significantly different from each other at $P<0.05$ (Duncan's).

Figure 2.3 (cont'd)



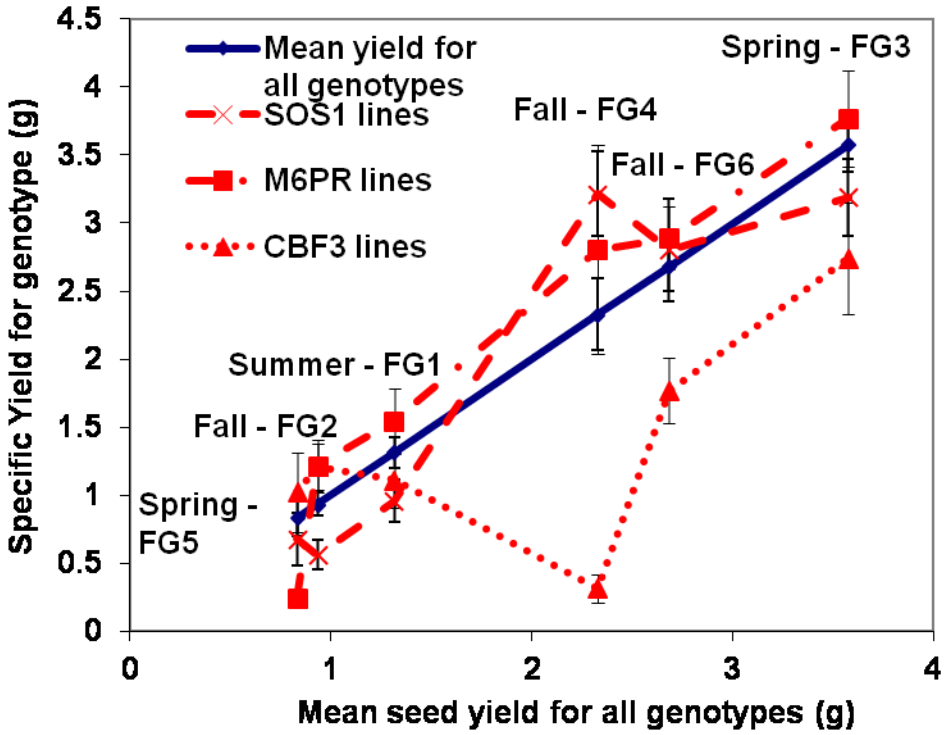
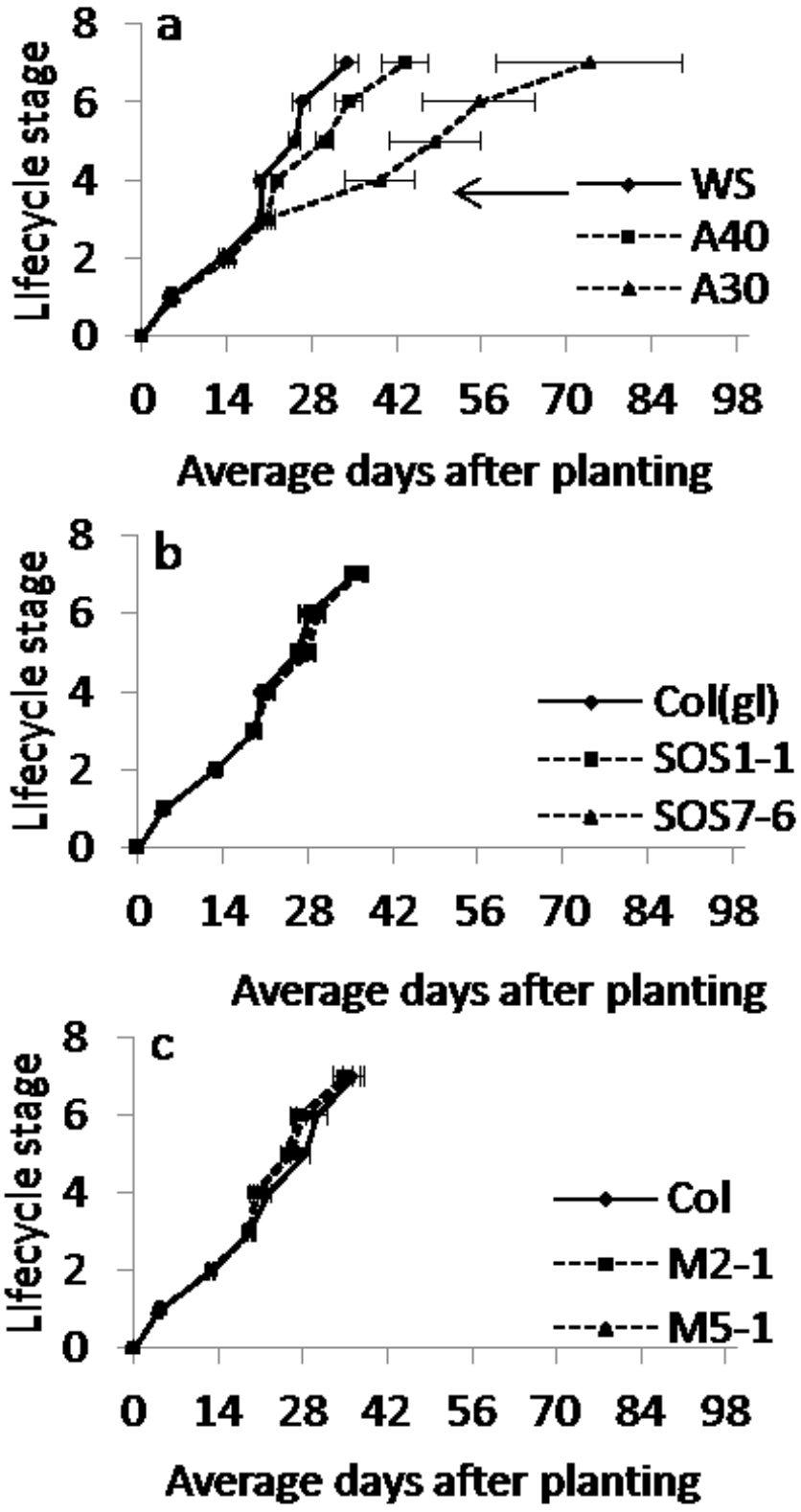


Figure 2.4. Mean genotype yield in each season in relation to the environmental yield capacity of that season. The environmental yield capacity was the mean yield for all genotypes grown in pure line plots (n=50) (dark line). The CBF3, SOS1 and M6PR values are the mean of 15, 10 and 10 populations, respectively (three, two and two transgenic lines per transgene, with five populations per line).

Figure 2.5. Mean days to reach various lifecycle stages for pure populations of wild-type (solid) and transgenic plants (dashed) CBF3 overexpression lines (A40, A30) and their background ecotype WS (A), M6PR lines (M2-1, M5-1) and their wild-type Col (B), and SOS1 lines S1-1 and S7-6 with their wild-type Col(gl) (C). Values are the mean of five field generations with five replicate populations per genotype. The stages are days to: first germination (1), 75% of the population with two true leaves (2), 75% of the population with five to six true leaves forming a rosette (3), first bolting (4), first flowering (5), 75% reaching bolting (6) , 75% flowering and 75% mature (75% of siliques drying down). The transition from vegetative to reproductive growth is indicated (black arrow), highlighting the effect of CBF3 overexpression.

Figure 2.5 (cont'd)



studies (Chan et al. 2012). The other two transgenes studied, the Na+/H+ antiporter SOS1 and M6PR, for mannitol production, had no effect on the rate of vegetative or reproductive development across the six field growing seasons ($P>0.8$, Figure 2.5b,c).

Effects of transgene expression on non-competitive plant productivity

To ensure that all productivity parameters for pure populations were accurate, seed samples from all pure populations, transgenic and wild-type, were screened on kanamycin-containing media. This process would detect via phenotype, contamination by seed or outcrossing between wild-type and transgenic populations (and vice versa) or silencing of the transgene in the transgenic lines.

The three transgenic CBF3 lines exhibited reduced aboveground biomass and seed yield, resulting in reduced fitness relative to the wild-type WS background (Table 2.4). The semi-dwarf CBF3 line A40 was less severely impacted in productivity compared to fully dwarfed lines A28 and A30, indicating transgene position effects. Similar to the lack of observable effect on development, overexpression of SOS1, did not significantly impact any measure of plant productivity when averaged across lines (all $P>0.8$, Table 2.4). While seed yield was not significantly increased for line S1-1, the derived value of relative fitness was increased compared to wild-type and the other SOS1 line, S7-6 (Table 2.4). Expression of M6PR did not significantly change dry weight or seed yield ($P>0.8$), however it did significantly increase partitioning to seed and fitness relative to wild-type in both lines ($P<0.03$, Table 2.4).

Productivity, fitness and transgene frequency in competition with wild-type plants

Each of the transgenic lines was also grown in competitive populations with their wild-type background starting with an initial transgene frequency of 50%. Fourteen replicate populations of each line were maintained separately over six generations. Transgene frequencies, tracked by selectable marker screening of progeny seed, were determined for each population at every generation. Observed changes in transgene frequency from the initial 50% could indicate either selection for or against the presence of the transgene, or the effects of genetic drift. A mean transgene frequency either above or below 95% confidence intervals based on theoretical

Table 2.4. Transgene effect on productivity as observed in non-competitive populations averaged across all field seasons.

Line	Genotype	Dry weight (g)	Seed yield (g)	Partitioning to seed	Relative fitness[2]
WS	WT	16.32a	3.26a[1]	0.20a	
A28	CBF3	7.29d	0.73c	0.10f	0.22*
A30	CBF3	10.18c	1.17c	0.11ef	0.36*
A40	CBF3	13.78ab	2.22b	0.17b	0.68c*
Col(gl)	WT	13.91ab	1.81b	0.13cde	
S1-1	SOS1	12.54b	2.10b	0.15bcd	1.16*
S7-6	SOS1	13.51b	1.70b	0.13cde	0.94
Col	WT	13.71ab	1.82b	0.12def	
M2-1	M6PR	13.18b	2.06b	0.15bc	1.13*
M5-1	M6PR	11.17bc	2.10b	0.16b	1.15*

[1] Each value is the mean of 5 replicate populations per genotype per season. Mean values with the same letter are not significantly different from each other at $P<0.05$ (Duncan's).

[2] The relative fitness value for each transgenic line is its seed yield relative to the seed yield of the corresponding wild-type background. Mean transgene relative fitness values significantly higher or lower than wild-type at $P<0.05$ are indicated (*).

drift distributions would be significantly different from predicted means influenced by drift alone, indicating selective pressure on the transgene within those replicate populations (Falconer & Mackay 1996).

In competition with WT, the proportion of plants overexpressing CBF3 decreased quickly and went extinct in most replicate populations within four generations in all three transgenic lines (Figure 2.6abc). To rule out the possibility that apparent reduction in transgene frequency as measured by kanamycin screening was an artifact due to gene silencing, progeny seed from the sixth field generation was tested by qPCR analysis. All samples from CBF3 lines under competition showed genomic levels of the nptII selectable marker gene below the detection limit of 1%, confirming the near extinction of this transgene in all three lines by the sixth generation (Table 2.5). Thus the dwarf phenotype, delayed transition to reproductive growth, and reduced relative seed production seen in the pure population studies of the CBF3 lines is manifested in a very low competitive fitness and strong selective disadvantage.

Changes in transgene frequency were also observed in the competitive populations with the SOS1 transgene and its Col(gl) background. The mean values for both lines fell below the 95% confidence interval indicating negative selection against the transgenic plants over the six generations (Figure 2.7ab).

Unlike CBF3 and SOS1, M6PR showed positive or neutral selection depending on the transgenic line involved. M6PR line M2-1 maintained a high mean frequency, above the 95% confidence interval, for six generations, indicating a positive selection for this line across a range of field environmental conditions (Figure 2.8a). In contrast, the transgene frequencies of the individual replicate populations of M5-1 diverged after the first generation and continued to separate in subsequent generations (Figure 2.8b). When averaged across all 14 replicate populations the mean fell within the 95% confidence interval indicating that genetic drift, and not selection, was affecting transgene frequency as might be expected for small population sizes in the absence of selection.

Figure 2.6. Transgene frequency within mixed populations of wild-type WS and three CBF3 overexpression lines, A40 (A), A30 (B) and A28 (C), as determined by selectable marker screening. All populations began at 50% starting frequency (FG0) and were maintained separately in subsequent generations. Changes in transgene frequency from one generation to the next in each of the fourteen replicate mixed populations are shown by the black lines, with the mean of all fourteen replicates indicated by the gray line. Dashed lines indicate 95% confidence intervals predicted for mean transgene frequency undergoing solely genetic drift.

Figure 2.6 (cont'd)

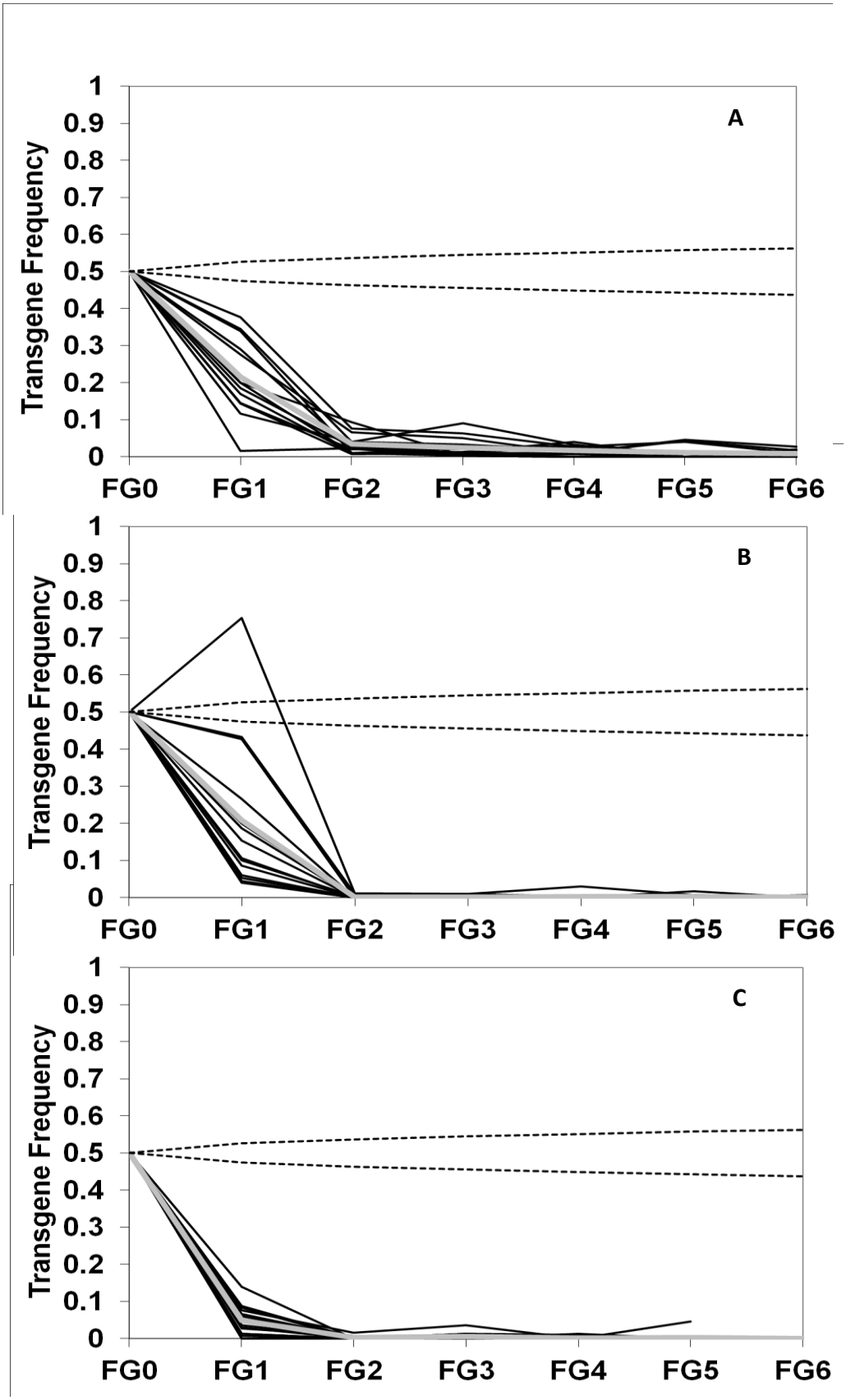


Table 2.5. Comparison of transgene frequencies estimated by phenotypic selectable marker screening (nptII) and qPCR analysis.

Transgene	**Sampled populations**[1]	**nptII screening**[2]	**qPCR analysis**[3]
M6PR	Positive control	100.0	>90.0
None (WT)	Negative control	0.0	<DL[4]
CBF3	A30 #7	0.007	<DL
CBF3	A28 #5	0.0	<DL
CBF3	A40 #2	0.027	<DL
SOS1	S1-1 #6	0.003	<DL
SOS1	S1-1 #11	0.0	<DL
SOS1	S1-1 #14	0.003	<DL
SOS1	S7-6 #1	0.007	<DL
SOS1	S7-6 #5	0.05	<DL
SOS1	S7-6 #6	0.133	<0.1
M6PR	M2-1 #14	0.117	<0.1
M6PR	M5-1 #3	0.08	<0.1
M6PR	M5-1 #8	0.127	<0.1
M6PR	M5-1 #14	0.107	<0.1

[1]All sixth generation competitive populations estimated to have < 15% transgenic individuals by phenotypic selectable marker (nptII) screening were tested by qPCR.

[2]Each value is the mean frequency estimated from three replicate screenings of 100 seedlings each.

[3]Each value is the mean frequency from three replicate samples with three technical replicates each. Frequency estimates were calculated based on a standard curve containing 0%, 10%, 20%, 50% and 100% transgenic seedlings.

[4]Frequency below detection limits (DL) of 0.1.

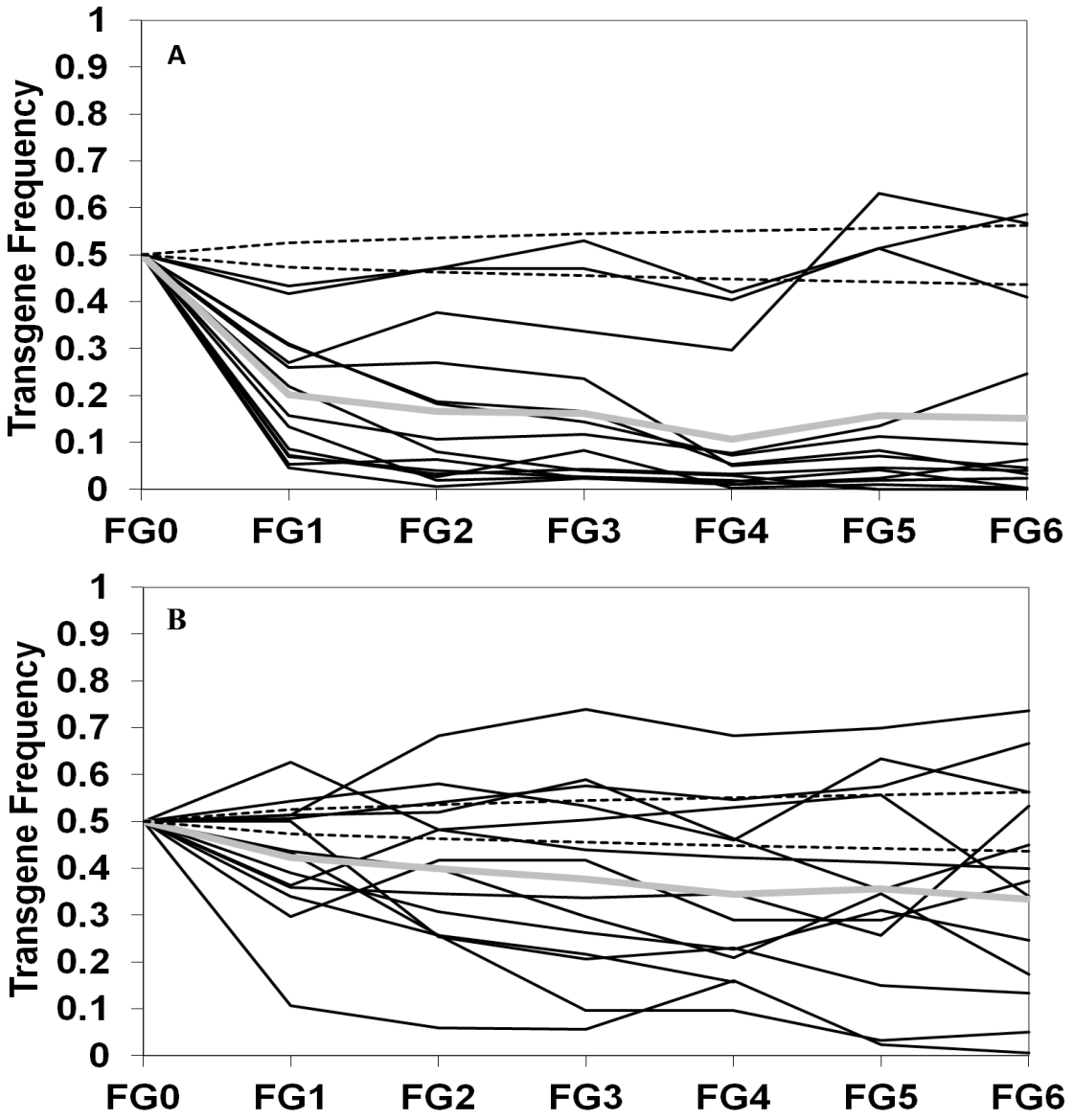


Figure 2.7. Transgene frequency within mixed populations of wild-type Col(gl) and two SOS1 overexpression lines, S1-1(A), S7-6 (B), as determined by selectable marker screening. All populations began at 50% starting frequency (FG0) and were maintained separately in subsequent generations. Changes in transgene frequency from one generation to the next in each of the fourteen replicate mixed populations are shown by the black lines, with the mean of all fourteen replicates indicated by the gray line. Dashed lines indicate 95% confidence intervals predicted for mean transgene frequency undergoing solely genetic drift.

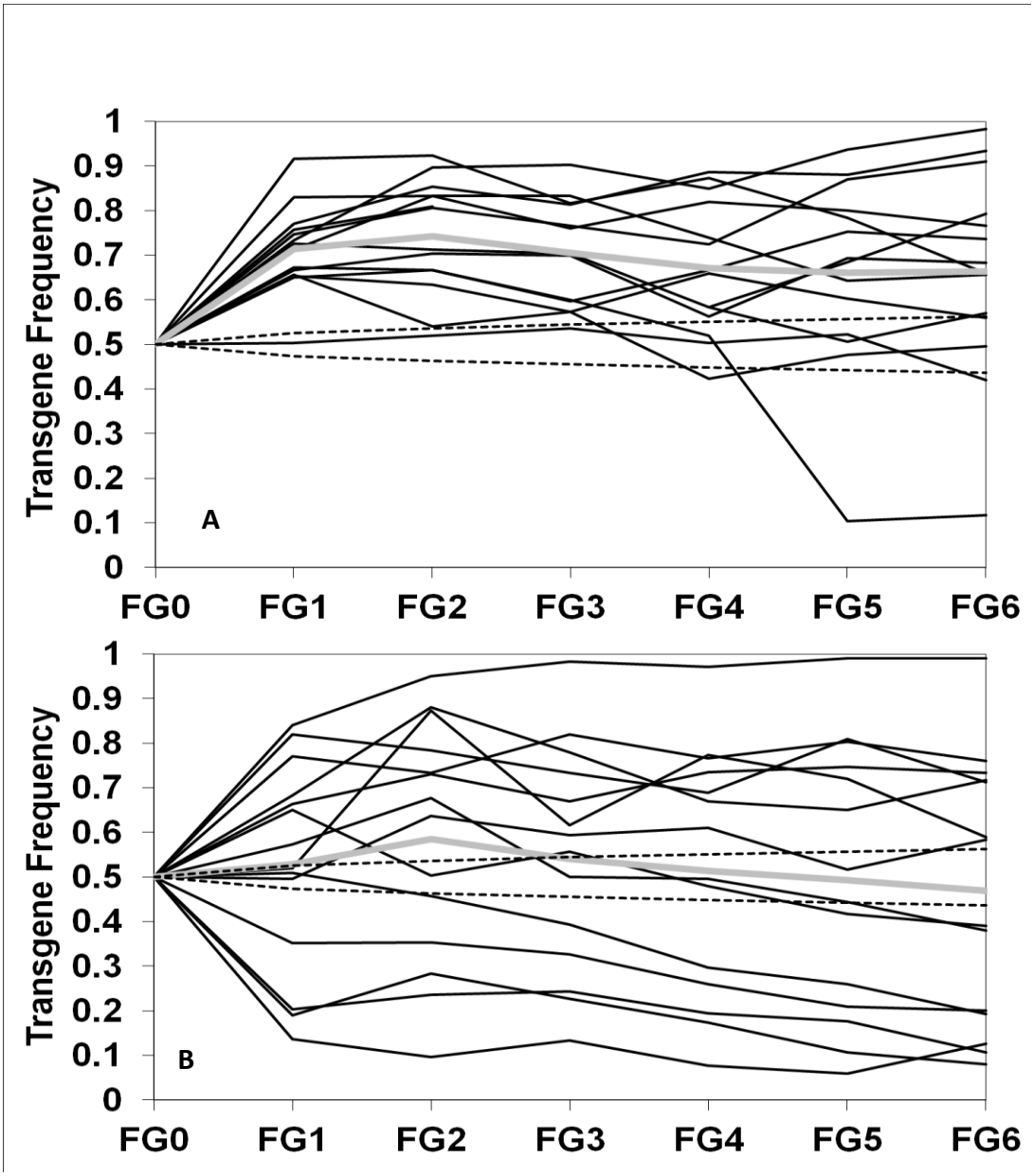


Figure 2.8. Transgene frequency within mixed populations of wild-type Col and M6PR overexpression lines, M2-1(A) andM5-1(B), as determined by selectable marker screening. All populations began at 50% starting frequency (FG0) and were maintained separately in subsequent generations. Changes in transgene frequency from one generation to the next in each of the fourteen replicate mixed populations are shown by the black lines, with the mean of all fourteen replicates indicated by the gray line. Dashed lines indicate 95% confidence intervals predicted for mean transgene frequency undergoing solely genetic drift.

All SOS1and M6PR populations with kanamycin resistant phenotypic frequencies below 0.1% also were verified to have low transgene frequencies by qPCR analysis (Table 2.5). Thus observed low values by kanamycin screening in these populations reflect loss of transgene from the populations rather than gene silencing.

Relative fitness in non-competitive verses competitive conditions

There were significant differences between the fitness values of the transgenic lines determined by performance relative to their wild-type counterparts in pure stands verses their performance in direct competition (Figure 2.9). All three CBF3 lines showed decreased fitness in pure stands relative to wild-type WS in the field, as was observed previously in growth chamber studies (Chan *et al*. 2012). However, their competitive fitness in the field was even more reduced (Figure 2.9). Both SOS1 lines also had this marked difference, with non-competitive fitness comparable to WT or better in pure populations but with significantly lower competitive fitness. The relative fitness of M6PR lines was comparable whether in the presence of competition or not.

Modeling transgene frequency

Single generation fitness values, determined under field and growth chamber conditions, were used to model transgene frequency across six theoretical generations and these predictions were then compared to the observed competitive populations. The PopGene.S^2 modeling program (Hamilton 2011) was used to model the effects of selection and genetic drift on transgene frequencies within competitive populations. Estimated fitness values from first generation field competitive populations, and field and growth chamber pure populations (Table 2.6) were input into the models to predict transgene frequencies after six generations. In cases of negative or positive selection (e.g. CBF3 line A40 or M6PR line M2-1 respectively), field-derived competitive fitness values predicted transgene frequencies more comparable to the observed frequencies under competition than models utilizing fitness values measured in pure populations under either growth chamber or field conditions (Table 2.6, Figure 2.10a-f). When the transgene was neutral (i.e. relative fitness~1.0, M6PR line M5-1) and

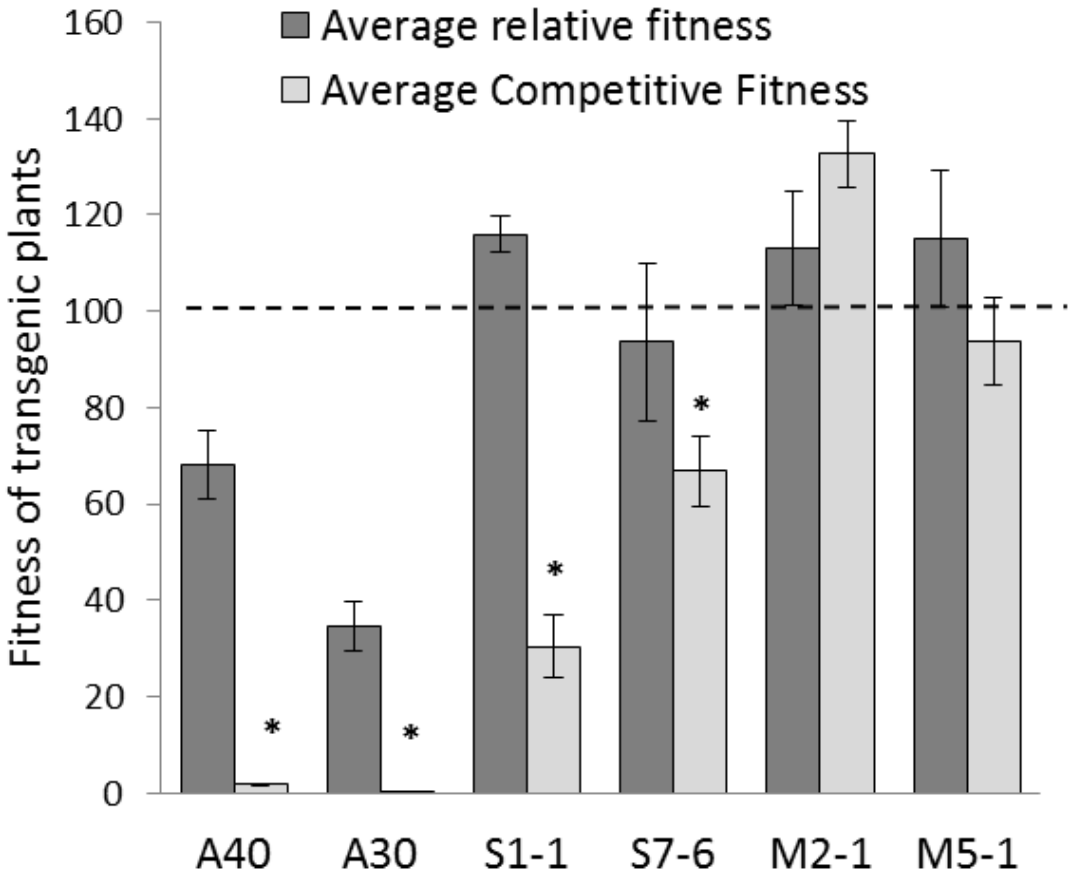


Figure 2.9. Comparison of non-competitive fitness estimates to competitive fitness estimates for the three abiotic stress tolerance enhancing transgenes. A fitness value of 100% (dashed line) indicates fitness equal to wild-type. Each non-competitive fitness value is the mean ± SE transgene fitness, calculated from seed yields relative to WT, of five replicate pure populations averaged across all six field seasons. Each competitive fitness value is the mean ± SE transgene fitness, calculated by selectable marker screening of fourteen replicate, but separately maintained, populations which began as 1:1 wild-type:transgenic mixes. Lines where non-competitive and competitive fitness values significantly differ at $P<0.05$ are marked (*).

Table 2.6. Measured single generation fitness values, from non-competitive growth chamber (GC), non-competitive field (NCF), and competitive field (CF) populations, used in transgene frequency modeling with PopGene.S[2] modeling program as well as predicted and observed sixth generation competitive fitness values.

		Measured single generation fitness values			**Predicted sixth generation competitive fitness values[4]**			**Measured sixth generation competitive fitness values**
Gene	**Line**	**GC[1]**	**NCF[2]**	**CF[3]**	**GC**	**NCF**	**CF**	**CF**
CBF3	A30	0.38	0.33	0.21	0.02	0.01	0.001	0.001
CBF3	A40	0.82	0.76	0.44	0.66	0.59	0.04	0.02
SOS1	S1-1	0.80	0.78	0.41	0.62	0.58	0.02	0.30
SOS1	S7-6	0.89	0.87	0.85	0.81	0.76	0.76	0.67
M6PR	M2-1	1.14	2.00	1.43	1.16	1.53	1.41	1.33
M6PR	M5-1	1.06	1.09	1.06	1.08	1.10	1.10	0.94

[1]Growth chamber fitness values were calculated as transgenic seed yield relative to wild-type seed yield under control conditions (Chan *et al*. 2012).

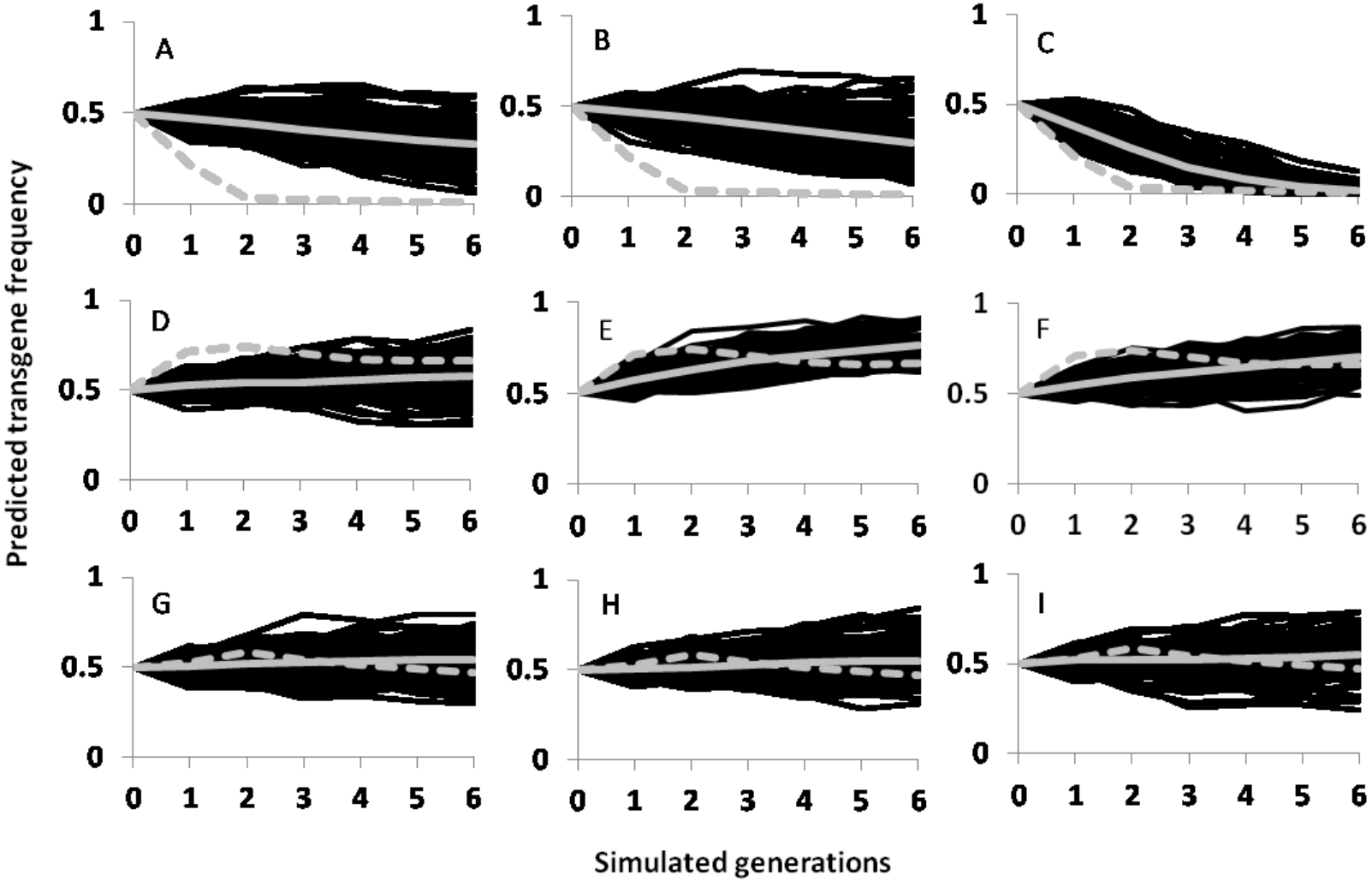
[2] NCF fitness values were calculated via mean seed yield of non-competitive transgenic populations relative to mean wild-type seed yield in the first field season.

[3]CF fitness values were calculated from selectable marker (nptII) screening of progeny seed from competitive populations grown in the first season.

[4]Predicted sixth generation fitness values are the mean fitness, after six simulated generations, of 100 modeled competitive populations using the respective measured single generation fitness values in the model.

Figure 2.10. Examples of predicted transgene frequencies for 100 modeled mixed populations (black lines) of wild-type and transgenic lines. Negatively selected CBF3 line A40 (top row, a-c), positively selected M6PR line M2-1 (middle row, d-f) and neutral M6PR line M5-1 (bottom row, g-i). Also indicated are the mean predicted transgene frequency (solid gray line) and the mean field observed frequency (dashed gray line). The model incorporated both genetic drift and selection based on growth chamber derived fitness values (left column, a,d,g), non-competitive fitness estimates from field generation 1 (middle column, b,e,h) and competitive fitness estimates from field generation 1 (right column, c,f,i).

Figure 2.10 (cont'd)



undergoing genetic drift the models were comparable to observed trends regardless of growth conditions used to assess the fitness values (Table 2.6, Figure 2.10g-i).

Discussion

The objectives of this study were to assess fitness effects that might influence transgene establishment in native populations for three transgenes previously shown to confer salinity tolerance under growth chamber conditions. The experiment was designed to perform long-term multigenerational assessments of transgene effects under field conditions. Sets of 14 mixed competitive populations of each transgenic line and its wild-type parent, and pure populations of each line were grown in the field for six generations, with growing seasons spanning from early spring to late fall. The seed planted for each generation was a subset of the progeny seed produced in the previous generation, allowing the impacts of one season/transgene combination to influence the transgene frequency of the population growing in the next season. This method follows the natural processes of selection and drift that would be occurring during establishment of any new genotype in the natural environment and allows direct comparison of predicted outcomes obtained from models based on short-term assessments with empirical observation of long-term transgene establishment patterns.

The ability of a transgene to become established in wild populations will require that the genotype survive both intraspecific competition and the full range of environmental conditions present at that site. With the range of growing conditions experienced over the six growing seasons, environmental effects as well as genotype x environment interactions were expected and observed. The different growing seasons provided environments with a greater than 4-fold range in yield capacity as measured by the mean yield across all genotypes. This range of seasonal yield capacity was likely due to a number of environmental factors including variations in temperature, day length and intensity of sunlight. None of the abiotic stresses for which these genes have been shown to improve tolerance to were specifically applied in this study (e.g. salinity stress for SOS1, CBF3 and M6PR or freezing and drought for CBF3). While it would be

expected that relative fitness results would be influenced by presence of the target stress, as has been observed in first generation traits like herbicide, insect and disease resistance (reviewed by Warwick *et al*. 2009) and in abiotic stress tolerance traits (reviewed by Mittler & Blumwald 2010), the measured transgene effects, both positive and negative, on plant development and productivity observed in these environments indicate secondary fitness effects and GxE interactions not predicted by the direct effect of the transgene's expression. Given the function of the introduced transgene products (transcription factor, sodium antiporter, compatible solute and possible pathogen signal molecule), and thus their capacity to modify a range of cellular functions; their extensive influence on the transcriptome (Chan *et al*. 2012); and the known interplay among plant responses to multiple abiotic and biotic stresses (Warwick *et al*. 2009), it was anticipated that the transgenes would cause secondary effects not evident in controlled environments. It was also expected that effects would vary among transgenes. Although the strongest effects were observed for CBF3, as has been the case in growth chamber studies, fitness effects in the field also were observed for SOS1 and M6PR plants which did not show differential performance relative to wild type when grown in the absence of salt stress in the growth chamber.

Numerous studies examining fitness effects of the first generation of transgenes (e.g., herbicide tolerance, Bt-mediated insect resistance, and virus resistance) have been performed over the past two decades (reviewed by Hails & Morley 2005, Warwick *et al*. 2009), and more recently with abiotic stress related transgenes (reviewed by Mittler & Blumwald 2010). These studies were typically performed as pure-line comparisons to wild-type plants or conventional cultivars. Our results indicate that fitness estimates in the absence of competition do not accurately predict performance in the presence of competition. Differences were observed in both direction and severity of transgene effect. Field grown SOS1 plants showed either equivalent or increased relative fitness, but when under competition with wild-type, both transgenic lines showed a selective disadvantage. The negative effects of CBF3, when measured as relative seed yield, were more pronounced in the presence of competition with wild type

plants than when grown as pure lines. In competition, the CBF3 plants, which exhibited observable-dwarf phenotypes, were easily overgrown by their wild-type competitor and were largely unable to reproduce. While showing no obvious changes in plant development, both M6PR lines showed elevated fitness relative to wild-type in pure populations through increased partitioning to seed; however, under competition only line M2-1 exhibited a competitive advantage while line M5-1 was selectively neutral.

The other studies that have compared effects of transgences in the presence and absence of competition also found differences in fitness estimates in competition. Insect resistant (Bt) rice exceeded yield of wild-type under insect pressure in pure populations but lost that advantage in competition (Yang *et al*. 2012). Transgenic insect resistant canola produced more seed in pure populations, but did not show increased seed yield at all sites when grown in 1:1 competitive populations with wild-type canola (Ramachandran *et al*. 2000). Other studies compared effects of transgenic plants when subjected to inter-specific competition. Herbicide-tolerant oilseed rape, grown in the greenhouse without herbicide application, performed as well as to non-transgenic lines in non-competitive conditions, but when grown in competition with barley, some transgenic lines exceeded non-transgenic lines in fitness (Fredshavn & Poulsen 1996). Virus-resistant sugarbeet yielded more biomass than wild-type in an infected field, but when in competition with *Chenopodium album,* both transgenic and wild-type sugarbeets had equal reductions in biomass (Bartsch *et al*. 1996).

The competition studies mentioned above were replicated in locations and years, but did not provide longitudinal data following populations over generations. Classic long term studies of potential invasiveness were performed by Crawley *et al*. (1993, 2001) comparing survival of transgenic herbicide tolerant oilseed rape, maize and sugarbeet and insect-resistant sugarbeet to their non-transgenic counterparts in paired plots under natural conditions over a ten year period. In these studies, in which competition arose from natural vegetation in the surrounding area, none of the transgenic lines exhibited increased survival. However, the authors cautioned that

the traits tested were not expected to increase plant fitness in natural habitats, and that traits such as abiotic stress resistance, would require further analysis.

Multigenerational assessments of fitness in competition with wild type plants were performed with non-transgenic herbicide resistant *Arabidopsis thaliana* EMS mutants in the greenhouse by monitoring resistance allele frequency over seven generations in the absence of herbicide application (Roux *et al*. 2005). This study showed discrepancies between fitness estimates from short and long-term competitive experiments. A prior single generation competitive fitness assessment showed a significant cost of resistance (Bergelson & Purrington 2002), while the multigenerational study observed the allele to be selectively neutral (Roux *et al*. 2005). These results indicate that the data used to model long-term transgene establishment risk could be improved by the use of multigenerational studies.

The side-by-side design in this study incorporated both transgenic pure line performance over six field seasons and monitoring of transgene frequency in competitive populations against wild-type plants for six generations. The three transgenes tested in this study followed different trajectories, likely reflecting the different secondary effects of the transgenes. CBF3 plants were quickly driven to near-extinction while the SOS1 transgene showed a slow decline in frequency. The mean transgene frequency of line M5-1 indicated that genetic drift as the primary influence on transgene frequency, indicative of no cost or benefit from expression under the environmental conditions experienced in the field, while line M2-1 maintained a high transgene frequency across six field seasons.

A number of models have been developed to examine gene flow from transgenic crop varieties into non-transgenic varieties and interfertile wild relatives (Maxwell *et al*. 1990, Lavigne *et al*. 1996, Squire *et al*. 1997, Colbach *et al*. 2001a,b, Baker & Preston 2003, Thompson *et al*. 2003, Haygood *et al*. 2004, Weekes *et al.* 2005, Ghosh *et al*. 2010, 2012). For example, the GeneSys modeling program, which incorporated both empirical and theoretical life-history parameter values, was used to predict gene flow patterns between commercially cultivated and volunteer oilseed rape populations over time and landscapes (Colbach *et al*.

2001a,b). Single generation hybridization rates between transgenic and conventional oilseed rape were predicted from experimentally determined gene flow rates (Baker & Preston 2003, Weekes *et al.* 2005), and the influence of domestication QTLs on theoretical transgene introgression into wild soybean (*Glycine soja*) was modeled using empirical QTL fitness impacts on wild soybean (Kitamoto *et al.* 2012). While the above models and others have been used to examine a variety of parameters influencing transgene establishment (e.g. pollen flow, hybridization, and introgression), a review of the literature did not find studies that paired modeling based on short-term fitness assessments with empirical observation of long-term transgene establishment patterns.

This study compared the ability of selection and drift models to predict transgene frequencies and levels of long-term establishment based on first generation fitness values vs the actual values observed in the field over six competitive generations. The fitness values employed in the models were based on the non-competitive and competitive assessments in the field and from previous non-competitive growth chamber assessments (Chan *et al*. 2012). For the line in which the transgene exhibited selective neutrality, M6PR M5-1, all measures of fitness were comparable and predicted observed transgene frequencies equally well. The first generation non-competitive fitness assessments for the positively selected M6PR line M2-1 either under or over-estimated the transgenic plant's competitive fitness (growth chamber and field assessments, respectively). For negatively selected transgenes CBF3 and SOS1, models using non-competitive relative fitness values, either growth chamber or field measured, predicted transgene frequencies higher than those observed. In contrast, while models based on non-competitive fitness measurements fared poorly, models using first generation competitive fitness measurements yielded predicted frequencies comparable to those observed in the field after six generations. Given that transgene establishment in a natural environment would take place in a competitive environment, the ability of transgenic plants to establish could be over- or under- estimated if only non-competitive assessments have been performed. The discrepancy between non-

competitive and competitive assessments was more pronounced with greater deviation from neutrality.

In conclusion, this study performed a multigenerational field assessment of the secondary fitness impacts of three abiotic stress tolerance enhancing transgenes: the transcription factor CBF3, the metabolic enzyme M6PR and the ion transport protein SOS1. Secondary effects of transgene expression were observed on plant development and productivity as well as GxE interactions that were not evident in growth chamber experiments. Significant differences between non-competitive fitness and competitive fitness estimates were observed for all transgenes. For transgenic lines which were selectively advantaged (M6PR line M2-1) or disadvantaged (all CBF3 and SOS1 lines), models based on non-competitive fitness estimates did not predict changes in transgene frequencies observed after the sixth generation, while models using competitive fitness estimates matched observed patterns. Together these results indicate the important role competition has on the success or failure of a transgene to establish and support the use of competitive field assessments in estimating the risk of transgene establishment.

LITERATURE CITED

LITERATURE CITED

Baker, S. S., Wilhelm, K. S., & Thomashow, M. F. (1994). The 5′-region of *Arabidopsis thaliana* cor15a has cis-acting elements that confer cold-, drought-and ABA-regulated gene expression. Plant Molecular Biology, 24(5), 701-713.

Baker, J., & Preston, C. (2003). Predicting the spread of herbicide resistance in Australian canola fields. Transgenic Research, 12(6), 731-737.

Bartsch, D., Schmidt, B., Pohl-Orf, M., Haag, C., & Schuphan, I. (1996). Competitiveness of transgenic sugar beet resistant to beet necrotic yellow vein virus and potential impact on wild beet populations. Molecular Ecology, 5(2), 199-205.

Bergelson, J., and C. B. Purrington. (2002) Factors affecting the spread of resistant *Arabidopsis thaliana* populations. In *Genetically engineered organism: assessing environmental and human health effects*. CRC Press.

Bhatnagar-Mathur, P., Vadez, V., & Sharma, K. K. (2008). Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Reports, 27(3), 411-424.

Caballero, A. (1994). Developments in the prediction of effective population size.Heredity, 73(6), 657-679.

Chan, Z., Grumet, R., & Loescher, W. (2011). Global gene expression analysis of transgenic, mannitol-producing, and salt-tolerant *Arabidopsis thaliana* indicates widespread changes in abiotic and biotic stress-related genes. Journal of Experimental Botany, 62(14), 4787-4803.

Chan, Z., Bigelow, P. J., Loescher, W., & Grumet, R. (2012). Comparison of salt stress resistance genes in transgenic *Arabidopsis thaliana* indicates that extent of transcriptomic change may not predict secondary phenotypic or fitness effects. Plant Biotechnology Journal, 10(3), 284-300.

Chung, J. S., Zhu, J. K., Bressan, R. A., Hasegawa, P. M., & Shi, H. (2008). Reactive oxygen species mediate Na+-induced *SOS1* mRNA stability in *Arabidopsis*. The Plant Journal, 53(3), 554-565.

Colbach, N., Clermont-Dauphin, C., & Meynard, J. M. (2001). GeneSys: a model of the influence of cropping system on gene escape from herbicide tolerant rapeseed crops to rape volunteers: I. Temporal evolution of a population of rapeseed volunteers in a field. Agriculture, Ecosystems & Environment, 83(3), 235-253.

Colbach, N., Clermont-Dauphin, C., & Meynard, J. M. (2001). GeneSys: a model of the influence of cropping system on gene escape from herbicide tolerant rapeseed crops to

rape volunteers: II. Genetic exchanges among volunteer and cropped populations in a small region. Agriculture, Ecosystems & Environment, 83(3), 255-270.

Coll, A., Nadal, A., Palaudelmas, M., Messenguer, J., Mele, E., Puigomenech, P. and Pla, M. (2008) Lack of repeatable differential expression patterns between MON810 and comparable commercial varieties of maize. Plant Molecular Biology 68, 105–117.

Crawley, M. J., Hails, R. S., Rees, M., Kohn, D., & Buxton, J. (1993). Ecology of transgenic oilseed rape in natural habitats. Nature, 363(6430), 620-623.

Crawley, M. J., Brown, S. L., Hails, R. S., Kohn, D. D., & Rees, M. (2001). Biotechnology: Transgenic crops in natural habitats. Nature, 409(6821), 682-683.

Dale, P. J., Clarke, B., & Fontes, E. M. (2002). Potential for the environmental impact of transgenic crops. Nature Biotechnology, 20(6), 567-574.

Davis, M. B., & Shaw, R. G. (2001). Range shifts and adaptive responses to Quaternary climate change. Science, 292(5517), 673-679.

EFSA (2012) Scientific Opinion on a request from the European Commission related to the prolongation of prohibition of the placing on the market of genetically modified oilseed rape events Ms8, Rf3 and Ms8 x Rf3 for import, processing and feed uses in Austria. EFSA J 2878:1-33, http://www.efsa.europa.eu/en/efsajournal/doc/2878.pdf (accessed April 2013).

Ellstrand, N. C. (2003). *Dangerous Liaisons?*. Johns Hopkins University Press.

Everard, J. D., Cantini, C., Grumet, R., Plummer, J., & Loescher, W. H. (1997). Molecular cloning of mannose-6-phosphate reductase and its developmental expression in celery. Plant Physiology, 113(4), 1427-1435.

Falconer, D. S., & Mackay, T. F. C. 1996. *Introduction to quantitative genetics*,*4th ed*. Longman Press.

Fowler, S., & Thomashow, M. F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. The Plant Cell, 14(8), 1675-1690.

Fredshavn, J. R., & Poulsen, G. S. (1996). Growth behavior and competitive ability of transgenic crops. Field Crops Research, 45(1), 11-18.

Gilmour, S. J., Sebolt, A. M., Salazar, M. P., Everard, J. D., & Thomashow, M. F. (2000). Overexpression of the *Arabidopsis CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology, 124(4), 1854-1865.

Gilmour, S. J., Fowler, S. G., & Thomashow, M. F. (2004). *Arabidopsis* transcriptional activators *CBF1*, *CBF2*, and *CBF3* have matching functional activities. Plant Molecular Biology, 54(5), 767-781.

Ghosh, A., & Haccou, P. (2010). Quantifying stochastic introgression processes with hazard rates. Theoretical population biology, 77(3), 171-180.

Ghosh, A., Serra, M. C., & Haccou, P. (2012). Quantifying time-inhomogeneous stochastic introgression processes with hazard rates. Theoretical Population Biology, 81(4), 253-263.

Guo, Y., Qiu, Q. S., Quintero, F. J., Pardo, J. M., Ohta, M., Zhang, C., ... & Zhu, J. K. (2004). Transgenic evaluation of activated mutant alleles of *SOS2* reveals a critical requirement for its kinase activity and C-terminal regulatory domain for salt tolerance in *Arabidopsis thaliana*. The Plant Cell, 16(2), 435-449.

Hails, R. S., & Morley, K. (2005). Genes invading new populations: a risk assessment perspective. Trends in Ecology & Evolution, 20(5), 245-252.

Halfter, U., Ishitani, M., & Zhu, J. K. (2000). The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proceedings of the National Academy of Sciences, 97(7), 3735-3740.

Hancock, J. F., R. Grumet, and S. C. Hokanson. (1996). The opportunity for escape of engineered genes from transgenic crops. HortScience 31:1080–1085.

Hancock, J. F. (2003). A framework for assessing the risk of transgenic crops. BioScience, 53(5), 512-519.

Hamilton, M. (2011). *Population genetics*. Wiley-Blackwell.

Haygood, R., Ives, A. R., & Andow, D. A. (2004). Population genetics of transgene containment. Ecology Letters, 7(3), 213-220.

Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O., & Thomashow, M. F. (1998). *Arabidopsis CBF1* overexpression induces COR genes and enhances freezing tolerance. Science, 280(5360), 104-106.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., & Shinozaki, K. (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology, 17(3), 287-291.

Katiyar-Agarwal, S., Zhu, J., Kim, K., Agarwal, M., Fu, X., Huang, A., & Zhu, J. K. (2006). The plasma membrane Na+/H+ antiporter *SOS1* interacts with *RCD1* and functions in oxidative stress tolerance in *Arabidopsis*. Proceedings of the National Academy of Sciences, 103(49), 18816-18821.

Kitamoto, N., Kaga, A., Kuroda, Y., & Ohsawa, R. (2012). A model to predict the frequency of integration of fitness-related QTLs from cultivated to wild soybean. Transgenic Research, 21(1), 131-138.

Lavigne, C., Godelle, B., Reboud, X., & Gouyon, P. H. (1996). A method to determine the mean pollen dispersal of individual plants growing within a large pollen source. Theoretical and Applied Genetics, 93(8), 1319-1326.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., & Shinozaki, K. (1998). Two transcription factors, *DREB1* and *DREB2*, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. The Plant Cell, 10(8), 1391-1406.

Maruyama, K., Sakuma, Y., Kasuga, M., Ito, Y., Seki, M., Goda, H., ... & Yamaguchi-Shinozaki, K. (2004). Identification of cold-inducible downstream genes of the *Arabidopsis DREB1A/CBF3* transcriptional factor using two microarray systems. The Plant Journal, 38(6), 982-993.

Maxwell BD, Roush ML *et al*. (1990) Predicting the evolution and dynamics of herbicide resistance in weed populations. Weed Technology 4: 2–13.

Mittler, R., & Blumwald, E. (2010). Genetic engineering for modern agriculture: challenges and perspectives. Annual Review of Plant Biology, 61, 443-462.

Montesinos-Navarro, A., Picó, F. X., & Tonsor, S. J. (2012). Clinal variation in seed traits influencing life cycle timing in *Arabidopsis thaliana*. Evolution,66(11), 3417-3431.

Pigliucci, M. (2002). Ecology and evolutionary biology of Arabidopsis. In *The Arabidopsis Book.* American Society of Plant Biologists.

Qiu, Q. S., Guo, Y., Dietrich, M. A., Schumaker, K. S., & Zhu, J. K. (2002). Regulation of SOS1, a plasma membrane Na+/H+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proceedings of the National Academy of Sciences, 99(12), 8436-8441.

Ramachandran S, Buntin GD, All JN, Raymer PL, Stewart CN (2000) Intraspecific competition of an insect-resistant transgenic canola in seed mixtures. Agronomy Journal 92:368–374

Ricroch, A.E., Berge, J.B. and Kuntz, M. (2011) Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. Plant Physiology 155, 1752–1761.

Roeder, A. H., & Yanofsky, M. F. (2006). Fruit development in Arabidopsis. In *The Arabidopsis Book.* American Society of Plant Biologists.

Roux, F., Camilleri, C., Bérard, A., & Reboud, X. (2005). Multigenerational versus single generation studies to estimate herbicide resistance fitness cost in Arabidopsis thaliana. Evolution, 59(10), 2264-2269.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., ... & Shinozaki, K. (2001). Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. The Plant Cell, 13(1), 61-72.

Shen, B., Jensen, R. G., & Bohnert, H. J. (1997). Mannitol protects against oxidation by hydroxyl radicals. Plant Physiology, 115(2), 527-532.

Shi, H., Ishitani, M., Kim, C., & Zhu, J. K. (2000). The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na+/H+ antiporter. Proceedings of the national academy of sciences, 97(12), 6896-6901.

Shi, H., Quintero, F. J., Pardo, J. M., & Zhu, J. K. (2002). The putative plasma membrane Na+/H+ antiporter *SOS1* controls long-distance Na+ transport in plants. The Plant Cell, 14(2), 465-477.

Shi, H., Lee, B., Wu, S.J. and Zhu, J.K. (2003) Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nature Biotechnology. 21, 81–85.

Sickler, C. M., Edwards, G. E., Kiirats, O., Gao, Z., & Loescher, W. (2007). Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. Functional Plant Biology, 34(4), 382-391.

Snape, J. W., & Lawrence, M. J. (1971). The breeding system of *Arabidopsis thaliana*. Heredity, 27, 299-302.

Snow, A. A., Andow, D. A., Gepts, P., Hallerman, E. M., Power, A., Tiedje, J. M., & Wolfenbarger, L. L. (2005). Genetically engineered organisms and the environment: current status and recommendations. Ecological Applications, 15(2), 377-404.

Stockinger, E. J., Gilmour, S. J., & Thomashow, M. F. (1997). *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proceedings of the National Academy of Sciences, 94(3), 1035-1040.

Squire, G. R., Burn, D., & Crawford, J. W. (1997). A model for the impact of herbicide tolerance on the performance of oilseed rape as a volunteer weed. Annals of Applied Biology, 131(2), 315-338.

Thuiller, W., Lavorel, S., Araújo, M. B., Sykes, M. T., & Prentice, I. C. (2005). Climate change threats to plant diversity in Europe. Proceedings of the National Academy of Sciences of the United States of America, 102(23), 8245-8250.

Thompson, C. J., Thompson, B. J. P., Ades, P. K., Cousens, R., Garnier-Gere, P., Landman, K., ... & Burgman, M. A. (2003). Model-based analysis of the likelihood of gene introgression from genetically modified crops into wild relatives. Ecological Modeling, 162(3), 199-209.

Tiedje, J. M., Colwell, R. K., Grossman, Y. L., Hodson, R. E., Lenski, R. E., Mack, R. N., & Regal, P. J. (1989). The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecology, 298-315.

Vogel, J. T., Zarka, D. G., Van Buskirk, H. A., Fowler, S. G., & Thomashow, M. F. (2005). Roles of the *CBF2* and *ZAT12* transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. The Plant Journal, 41(2), 195-211.

Vorwerk, S. Wizard Genomic DNA Purification Kit and the Isolation of Plant Genomic DNA. [Internet] 2001. Available from: http://www.promega.com/resources/articles/pubhub/enotes/wizard-genomic-dna-purification-kit-and-the-isolation-of-plant-genomic-dna/ (accessed July 2012).

Warwick, S. I., Beckie, H. J., & Hall, L. M. (2009). Gene flow, invasiveness, and ecological impact of genetically modified crops. Annals of the New York Academy of Sciences, 1168(1), 72-99.

Weaver, S. A., & Morris, M. C. (2005). Risks associated with genetic modification:–An annotated bibliography of peer reviewed natural science publications. Journal of Agricultural and Environmental Ethics, 18(2), 157-189.

Weebadde, C. & Karim M.M. (2011). Environmental biosafety issues associated with genetically engineered crops. In *Environmental safety of genetically engineered crops*. Michigan State University Press.

Weekes, R., Deppe, C., Allnutt, T., Boffey, C., Morgan, D., Morgan, S., ... & Henry, C. (2005). Crop-to-crop gene flow using farm scale sites of oilseed rape (Brassica napus) in the UK. Transgenic Research, 14(5), 749-759.

Yamaguchi-Shinozaki, K., & Shinozaki, K. (1994). A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. The Plant Cell, 6(2), 251-264.

Yang, T., Zhang, L., Zhang, T., Zhang, H., Xu, S., & An, L. (2005). Transcriptional regulation network of cold-responsive genes in higher plants. Plant Science, 169(6), 987-995.

Yang, X., Wang, F., Su, J., & Lu, B. R. (2012). Limited fitness advantages of crop-weed hybrid progeny containing insect-resistant transgenes (Bt/CpTI) in transgenic rice field. PloS one, 7(7), e41220.

Zhifang, G., & Loescher, W. H. (2003). Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer. Plant, Cell & Environment, 26(2), 275-283.

Chapter 3

The fitness effects of three abiotic stress tolerance transgenes in *Arabidopsis thaliana* in the presence of salinity stress and competition

Introduction

With an increasing world population (FAO 2011) and a changing world climate, increasing crop yields is an important target for plant breeders. While breeding for increased intrinsic yield is important, another method for increasing overall yield is to reduce crop losses due to abiotic stresses. Submissions to the USDA for field trials of transgenic crops with altered abiotic stress response have steadily increased in past decade (chapter 1, Figure 1.1). These field trial requests demonstrate that increasing abiotic stress tolerance is an important objective of breeding programs both private and public. Any time a plant's response to its environment is altered, however, the possibility of unintended secondary effects of transgene expression arises. These secondary effects could affect the growth and development of the transformed plant itself or how it behaves in its environment. (Davis and Shaw 2001, Ellstrand 2003, Thuiller *et al.* 2005).

Methodologies for examining secondary effects of transgene expression have been used extensively to assess some types of genetically engineered crops, especially insect resistance through the use of various *Cry* genes from *Bacillus thuringiensis* (reviewed by Andow and Hilbeck 2004, Thies & Devare 2007, Ricroch *et al*. 2011). These experiments testing for secondary effects examined, for example, changes in plant development, productivity, fitness, transcriptome, proteome and metabolome. The secondary effects of the next generation of transgenic crops engineered to express more complex traits may, however, be more difficult to detect and quantify. With abiotic stress related transgenes, the secondary effects of transformation are related to a plant's behavior within its environment. A commonly raised ecological concern about transgenic plants with increased abiotic stress tolerance is the possibility of increasing the ecological range (e.g. invasiveness) of the crop or interfertile wild

relatives that receive the transgene by gene flow from crop pollen. (Hoffman 1990). Expansion of ecological range could result from a geographic range expansion in which the transgene allows the recipient plant to survive previously limiting abiotic conditions, an opening of new niche environments within the current geographic range, or increasing competitiveness against other species in the current range.

While for most crop plants the domestication process has reduced fitness in natural ecosystems, some crop species can become feral and many have compatible wild relatives that could potentially benefit from transgenes that increase resistance to drought, freezing, cold, heat, or salinity stress (Ellstrand 2003). Assessing the risk of these effects requires determining whether the expression of the transgene alters how the plant responds to its environment, to competition from other plants, and to interactions between those factors. Pure population comparisons between transgenic and wild-type plants have found a number of genes which, when expressed via a constitutive or inducible promoter, increase tolerance to drought, cold, heat, or salt stress (reviewed Wang *et al*. 2003, Bhatnagar-Mathur *et al*. 2008, Warwick *et al*. 2009, Mantri *et al*. 2012). But, based on an extensive search of the literature, none of these numerous studies of abiotic stress tolerance enhanced transgenic plants were, to our knowledge, performed in competition with wild-type plants as would be necessary for transgene establishment in natural populations.

This study examined the competitive fitness effects of over-expression of three genes shown to confer salinity tolerance. C-repeat binding factor 3/drought responsive element binding factor 1a (*CBF3/DREB1a*) is an abiotic stress associated transcription factor that activates a regulon of stress responsive genes (Kasuga *et al*. 1999, Gilmour *et al*. 2000). Salt Overly-Sensitive 1 (*SOS1*) is a plasma membrane bound Na+/H+ antiporter that facilitates removal of toxic Na+ ions from the cytoplasm (Shi *et al*. 2000 &2002). The biosynthetic enzyme mannose-6-phosphate reductase (*M6PR*) allows the production of the sugar alcohol mannitol, which acts as a compatible solute and osmoprotectant (Zhifang and Loescher 2003, Sickler *et al*. 2007). The ability to increase salinity tolerance makes these genes candidates for crop abiotic stress

tolerance improvement, but this could also raise ecological concerns during the risk assessment process.

Each of these three transgenes has been demonstrated to confer increased salinity stress tolerance under growth chamber conditions (Chan et al. 2012), and these transgenic *Arabidopsis thaliana* lines were also assessed previously for secondary effects in the field for six growing seasons (spring and autumn for 3 years) in Michigan (Chapter 2). These field studies measured transgene effects on plant development and productivity with a focus on transgene influences on fitness relative to wild-type, as well as fitness under direct competition with wild-type, across a diverse range of environmental conditions. The multigenerational field assessment determined that relative fitness, as determined by pure line performance relative to wild-type, was a poor predictor of a transgenic plant's ability to compete and establish within a population in the field. However, these experiments did not assess the competitive performance of the transgenic lines in the presence of salinity, the stress they had been engineered to better tolerate.

For this study, these three abiotic stress tolerance enhancing transgenes were examined for secondary fitness effects while under salinity stress and in the presence and absence of competition from wild-type plants in the greenhouse. Salinity impacts were more severe than observed in the growth chamber and significant differences were observed in the transgene effects in pure populations between the two growing conditions, with growth chamber results for all three transgenes predicting greater relative fitness under salinity stress than was observed in the greenhouse. Additionally, gains in fitness relative to wild-type observed in salinity-stressed pure populations in either the growth chamber or greenhouse did not translate into a competitive advantage against wild-type plants when grown together under salinity stress. Together these results indicate that non-competitive assessments may poorly predict the fitness impacts of abiotic stress enhancing transgenes under competitive conditions and emphasize the importance of the effects of interplant competition.

Materials and Methods

Arabidopsis lines

Mixed and pure line populations of transgenic *Arabidopsis thaliana* were grown in replicated greenhouse experiments to assess transgene fitness impacts under saline and control treatments. SOS1 overexpression lines 1-1 and 7-6, with Columbia glabrous (Col(gl)) parents, were supplied by Dr. Huazhong Shi from Texas Technological University (Shi *et al.* 2003). M6PR overexpression lines M2-1 and M5-1, with a Columbia (Col) background, were provided by Dr. Wayne Loescher of Michigan State University (Zhifang and Loescher 2003). Three lines, A28, A30 and A40, which overexpress the CBF3/DREB1a transcription factor in the Wassilewskija ecotype (WS) were provided by Dr. Michael Thomashow of Michigan State University (Gilmour *et al.* 2000). Each line resulted from separate transformation events and all constructs used the Cauliflower Mosaic Virus (CaMV) 35S promoter for constitutive expression of the abiotic stress resistance transgene. The selectable marker gene neomycin phosphotransferase II (NPTII) for kanamycin resistance under the control of the nopaline synthase (NOS) promoter was also present in all transgenic lines. The three wild-type backgrounds are rapid-cycling *Arabidopsis thaliana* ecotypes and were utilized in each greenhouse experiment for both competitive and non-competitive phenotypic comparisons between each untransformed background and the transgenic lines.

The initial parent plants used for seed production were first verified to contain and express their transgene (and only their intended transgene) by PCR, Southern and northern blot analysis (see chapter 2, Figure 2.1). All seeds used for the three replicated greenhouse experiments were from greenhouse grown seed stocks produced during the winter of 2008.

Experimental design and salt treatment

The greenhouse salinity stress experiments used a randomized complete block design. Twenty-eight replicate competitive populations were planted for each transgenic line with a 50/50 mix of the transgenic line and its background ecotype. Ten non-competitive replicate populations were planted for each transgenic and WT line for comparison. Planting density (at 2600 seeds/m^2) and planting method were the same as described in chapter two. Once the plants reached the rosette stage (5-6 true leaves) half of the populations were randomly selected for the

salinity stress treatment while the remaining half continued to be watered normally as described below. A total of 167 populations were maintained for each treatment: 112 mixed populations (8 transgenic lines x 14 replicate populations/line) and 50 pure populations (10 genotypes (transgenic and wild-type) x 5 replicates/genotype) (Table 3.1).

Growth conditions used in the greenhouse were based on our established growth chamber conditions with respect to seed stratification, direct seeding to soil, and subsurface irrigation (Chan *et al*. 2011) and an assessment of optimal conditions for *Arabidopsis thaliana* in the greenhouse using a factorial design for two soil media and a range of pot-sizes (appendix). All greenhouse plantings were performed using 26x26x6 cm trays with perforated bottoms (L-HFT NCR, Landmark Plastics Corporation; Akron, OH), 4.0 L Baccto potting soil (Michigan Peat Company) mixed with 2 g/L slow-release (3-4 month) 14-14-14 Osmocote Classic® fertilizer (The Scotts Company LLC, Marysville, Ohio), pre-moistened and lightly compacted via rolling a 1 kg cylinder across the soil surface to create a firm seed bed. Seeds were counted as described in chapter two, stratified at 4°C for 24-48 hours and then directly seeded onto the damp soil surface. Subsoil irrigation was used for all watering by placing the 26x26x6 cm trays into larger trays (L-1020-NCR, Landmark Plastics Corporation; Akron, OH), maintained with approximately 2.5 cm of water to ensure sufficient soil moisture for germination. After germination, but prior to the beginning of salt treatment, watering was performed on an as needed basis, approximately every 1-2 days until soil was saturated. To minimize greenhouse positional effects, pot locations were re-randomized three times each week. Salt treatment was initiated once the majority of plants reached the rosette stage (5-6 true leaves). All saline solutions were diluted to the desired concentration from 3.2 M NaCl stock solution. Salinity stress treatment populations were incrementally exposed to salinity stress beginning with a 25mM NaCl solution applied via subsoil and increased by 25mM every two days until 75mM NaCl was reached. The decision to use 75mM as the concentration of the salinity stress treatment was based on previous growth chamber work and preliminary greenhouse studies using 50, 75, and 100mM NaCl treatments (Chan *et al*. 2012 and appendix). Once the final concentration of

Table 3.1. Transgenic lines of *Arabidopsis thaliana* and the number of replicate populations tested in three greenhouse experiments under control and salinity stressed conditions (75mM NaCl)

Wild-type[1]	Transgene	Transgenic line	Number of Reps +salt	Number of Reps -salt	Initial Mix Ratio
WS	CBF3	A28	14	14	1:1
WS	CBF3	A30	14	14	1:1
WS	CBF3	A40	14	14	1:1
Col(gl)	SOS1	S1-1	14	14	1:1
Col(gl)	SOS1	S7-6	14	14	1:1
Col	M6PR	M2-1	14	14	1:1
Col	M6PR	M5-1	14	14	1:1
WS			5	5	Unmixed
Col			5	5	Unmixed
Col(gl)			5	5	Unmixed
	CBF3	A28	5	5	Unmixed
	CBF3	A30	5	5	Unmixed
	CBF3	A40	5	5	Unmixed
	SOS1	S1-1	5	5	Unmixed
	SOS1	S7-6	5	5	Unmixed
	M6PR	M2-1	5	5	Unmixed
	M6PR	M5-1	5	5	Unmixed

[1]Wild-types are Wassilewskija (WS) ecotype, Columbia glabrous (Col(gl)) ecotype and Columbia (Col) ecotype

75mM NaCl was reached, further treatments were applied as needed to ensure that the soil remained well watered. Each 75mM salt treatment was performed in two stages. First, the 75mM NaCl solution was applied via sub-soil irrigation and after 20 minutes any excess unabsorbed solution was dumped out of the bottom trays. Then 1 L of 75mM NaCl was applied gently from above to leach any excess NaCl from the soil. All leachate was then removed. This reduced a buildup of excess salt in the topsoil that would have occurred due to evaporation. All control populations were watered via sub-soil irrigation each time a salt treatment was performed on the other populations to ensure equal saturation between treatments so that differences in performance between treatments would be due to the presence of the salt and not soil water content.

Lifecycle parameters including: the number of days until germination, initial and majority bolting, initial and majority flowering and maturation was collected on all populations. All lifecycle stages were scored in the same manner as the field experiment (Chapter 2). After harvest, total population aboveground dry weight and seed mass were measured as described in chapter 2. Progeny seed viability was determined during the kanamycin screening described below. Pure population relative fitness was calculated as seed yield of each transgenic line compared to its wild-type background under the same treatment. The full greenhouse salinity experiment was repeated three times.

For comparison purposes, two transgenic lines from each of the three transgenes (CBF3 lines A40 and A30, SOS1 lines S1-1 and S7-6, M6PR lines M2-1 and M5-1) and the three wild-type backgrounds were grown in the growth chamber under control and salinity stressed (100 mM NaCl) conditions as described by Chan *et al*. 2011. These experiments were performed by Zhulong Chan.

Genotyping progeny from competitive populations via kanamycin screening

Genotyping of the progeny seed from competitive populations was based on phenotypic assessment for the presence of the neomycin phosphotransferase II (NPTII) selectable marker gene for kanamycin resistance present in all transgenic lines following the protocol described in

chapter two. In cases of low germination the screening procedure was repeated with increased numbers of seeds plated with the objective to score 300 seedlings. Competitive fitness was calculated as the transgene frequency within the progeny seed from each population relative to the planted transgene frequency (50%). Mean competitive fitness was the mean fitness across the 14 replicate competitive populations for each transgenic line for each treatment.

Determination of selective pressure under competitive conditions

To determine if changes in transgene frequency in competitive populations were due to selective pressure or genetic drift, the following formulas were used to calculate 95% confidence intervals. Mean transgene frequencies outside these intervals would indicate selection on the transgene, either negative or positive. Based on theoretical drift distributions, the following formula $V_{qt} = q_0*p_0*(1-(1-1/(2N_e))^t)$ (Falconer & Mackay 1996) calculates the variance in mean transgene frequencies predicted to be associated with random genetic drift after t generations. The frequencies of transgenic and wild-type plants in the initial population are q_0 and p_0 respectively while the effective population size is N_e. From the starting frequency of 0.5, and assuming no selection effects, with 14 replicate populations, the confidence intervals are then derived as $0.5 \pm (1.96*(Vqt/14)^0.5)$.

Statistical Analysis

All statistical analyses were performed with the SAS (version 9.3) and R (version 2.14.0) statistical programs (SAS Institute Inc., Cary, NC and R Foundation for Statistical Computing, Vienna, Austria). To reduce heteroscedasticity in the data and meet the normality assumptions required for regression analysis, Box Cox transformations were performed and productivity values were transformed according to the resulting λ values. For dry weight, seed yield and partitioning to seed values were transformed ($x^{0.25}$), while viability of progeny seed values were transformed ($x^{0.5}$). Mean separation was performed with Duncan's Multiple Range Test on these transformed values and differences were considered statistically significant at $P<0.05$. Mean values were back-transformed for clarity in all tables and figures.

Results

Effects of salinity stress on plant development and productivity

The levels of salinity stress applied in the greenhouse experiments (75mM NaCl) was chosen to be comparable to effects observed in our prior growth chamber experiments (100mM; Chan *et al*. 2011, 2012). The growth chamber experiments were performed at 23/18°C and 70% relative humidity with a 12 h light/12 h dark cycle providing 350 μmol m^{-2} s^{-1} (Chan *et al*. 2011, 2012). Similar conditions were used by other laboratories working with these transgenes (e.g. Gilmour *et al*. 2000, Shi *et al*. 2002, Sickler *et al*. 2007). In the greenhouse supplemental lighting was used to provide a minimum 12h daylength, but variations in sunlight resulted in differing levels of mean maximum daily solar flux density ranging from 522 to 837 W/m^2.

The performance of the transgenic lines in the presence or absence of salinity stress was tested in three repeated greenhouse experiments. These experiments included both pure line populations and competitive populations with a mixture of transgenic and wild-type plants. The planting density of both non-competitive pure populations and mixed competitive populations was 2600 seeds/m^2. This density was same as that used previously in field studies of these transgenic line (Chapter 2) and within the range of natural *Arabidopsis thaliana* population densities in Michigan (appendix). Due to variable environmental conditions among the three greenhouse experiments (such as light intensity and temperature), the environmental capacity for viable seed yield per pure population (the mean for all genotypes transgenic and wild-type) ranged from 0.6 to 2.26 g under control conditions and from <0.01 g to 0.46 g under salinity stress (Figure 3.1). Despite these differing conditions, fitness values of the transgenic lines relative to wild-type (as measured by viable seed yield comparisons) were equivalent between experiments (all $P>0.05$). For all subsequent analyses, unless specifically mentioned, all data were pooled between the repeated experiments.

The timing of early reproductive stages, bolting through flowering, were not significantly affected by salinity stress in the greenhouse (all $P>0.05$); however, salinity-stressed plants exhibited a more rapid maturation of siliques, observed as a decrease in the number of days until

75% of siliques had begun to dry down (Table 3.2). In the growth chamber, salinity stress delayed reproductive development for both days to bolting as well as flowering (Chan *et al*. 2012). Regardless of genotype, wild-type or transgenic, salinity stress decreased above ground dry weight and seed yield in the greenhouse (Figure 3.2ab, Table 3.2, all $P<0.05$). Mean dry weight for all genotypes was reduced by 52%, while overall mean seed yield decreased 73%. Mean biomass partitioning to seed was also decreased by salinity stress by 66% (Figure 3.2c, Table 3.2, all $P<0.001$). Progeny seed viability was also markedly reduced by salinity stress, regardless of genotype (Figure 3.2d, Table 3.2, $P<0.01$).

Salinity stress in the growth chamber (100mM NaCl) resulted in decreases in dry weight (by 64%) and seed yield (by 72%), comparable to those observed under 75mM NaCl stress in the greenhouse (Figure 3.3ab, all $P<0.05$). Mean partitioning of biomass to seed decreased 45% (Figure 3.3c). Progeny seed viability in the growth chamber was reduced under 100mM NaCl salinity stress for all genotypes, except CBF3 line A40 and SOS1 line S7-6 (Figure 3.3d, $P<0.01$ and $P>0.05$ respectively). Averaged across all genotypes, the reduction in progeny seed viability due to salinity stress in the growth chamber was considerably less severe (an 11% reduction) than that observed in the greenhouse (a 47% reduction).

Effects of transgene expression on plant development and productivity in the absence of salinity stress

The three CBF3 overexpression lines exhibited delayed reproductive development at all stages from initial bolting to maturation of the siliques under greenhouse and growth chamber conditions in the absence of salinity stress (Figure 3.4a, Table 3.3, Chan *et al*. 2012, all $P<0.05$). The delayed development, dwarf phenotype and change in leaf architecture described in previous CBF studies was strongest in the fully dwarfed A28 and A30 lines, while the semi-dwarf A40

Figure 3.1. Viable seed yield of pure populations of transgenic and wild type plants in relation to yield capacity of the environment for six growing conditions, three seasons ±salt (closed and open symbols respectively). The environmental yield capacity was calculated as the mean viable seed yield of all genotypes wild-type and transgenic (n=50, gray solid line). The yield capacity for specific genotypes was the mean viable seed yield for each transgenic line (n=5, black dashed lines) and wild-type background (n=5, black solid line). The three CBF3 lines and their WS wild-type background (A), the two SOS1 lines and their wild-type Col(gl), and the two M6PR lines and their wild-type Col (C).

Figure 3.1 (cont'd)

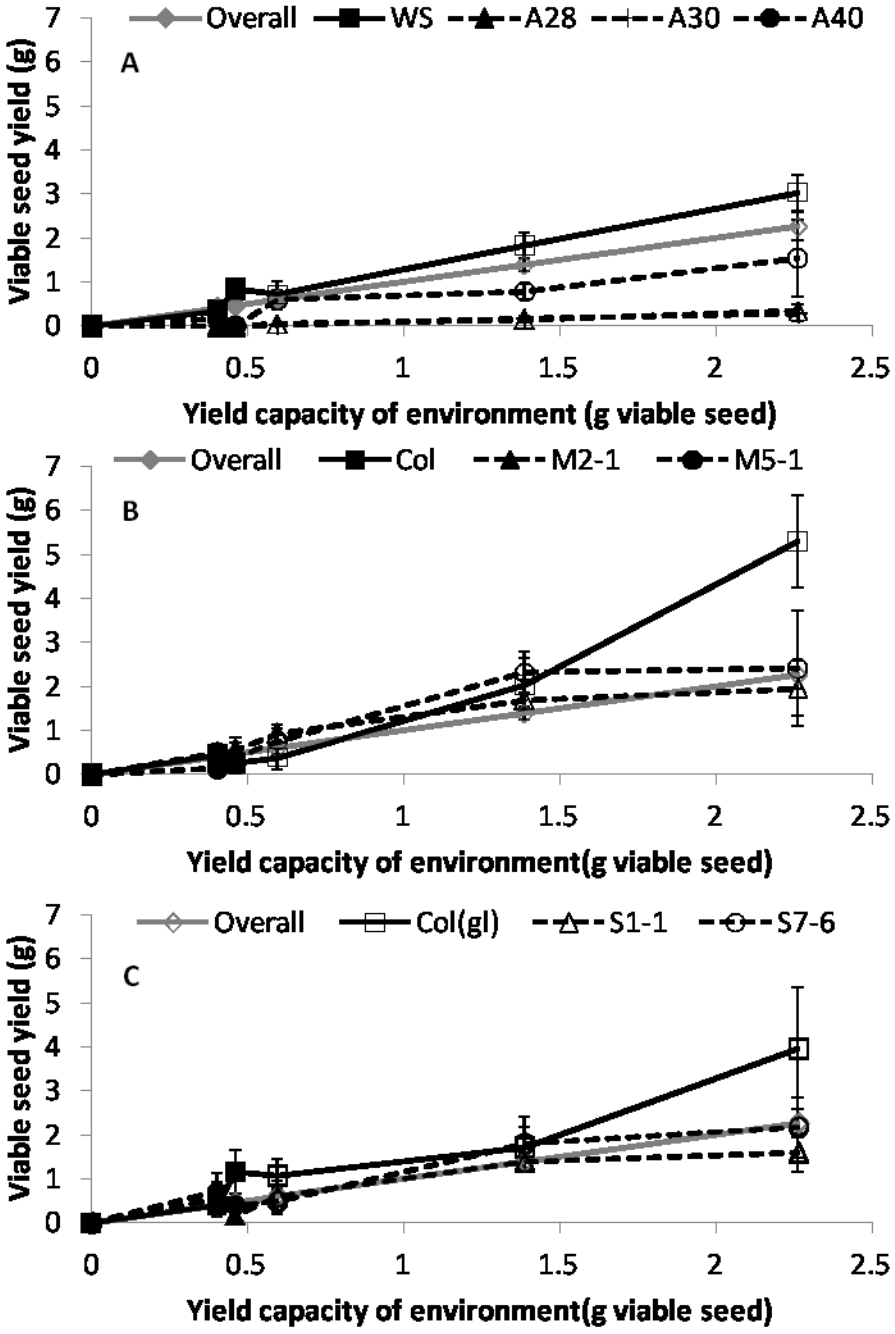


Table 3.2. Salinity treatment effect on development and productivity averaged across all lines, populations and experiments.

Treatment	Days after planting to reach lifecycle stage[1]					Measures of productivity[1]			
	First bolting	**Majority bolting (75%)**	**First flowering**	**Majority flowering (75%)**	**Mature (75% dry siliques)**	**Dry weight (g)**	**Seed yield (g)**	**Partitioning to seed**	**Seed viability**
Control	38.6a	49.1a	45.3a	59.7a	94.3b	11.73a	1.58a	0.14a	0.81a
Salt	40.1a	48.8a	45.1a	61.2a	85.5a	5.61b	0.43b	0.05b	0.40b

[1] Each value is the mean of five replicate populations per genotype per treatment per experiment. Data are pooled from three replicate experiments; equivalent trends were observed in each experiment. Mean values with the same letter were not significantly different from each other at $P<0.05$.

Figure 3.2. Mean productivity measures for greenhouse-grown pure line populations under control and salinity stress (75mM NaCl); aboveground dry weight (A), viable seed yield based on germination rates of progeny seed (B), partitioning to seed (C) and progeny seed germination rate (D). Salt treatment reduced dry weight, seed yield, partitioning to seed and progeny seed viability across all genotypes ($P<0.01$). Each value is the mean from five replicate populations per genotype per treatment per experiment. Data are pooled from three replicate experiments; equivalent trends were observed in each experiment. Mean values with the same letter were not significantly different from each other at $P<0.05$. (Duncan's).

Figure 3.2 (cont'd)

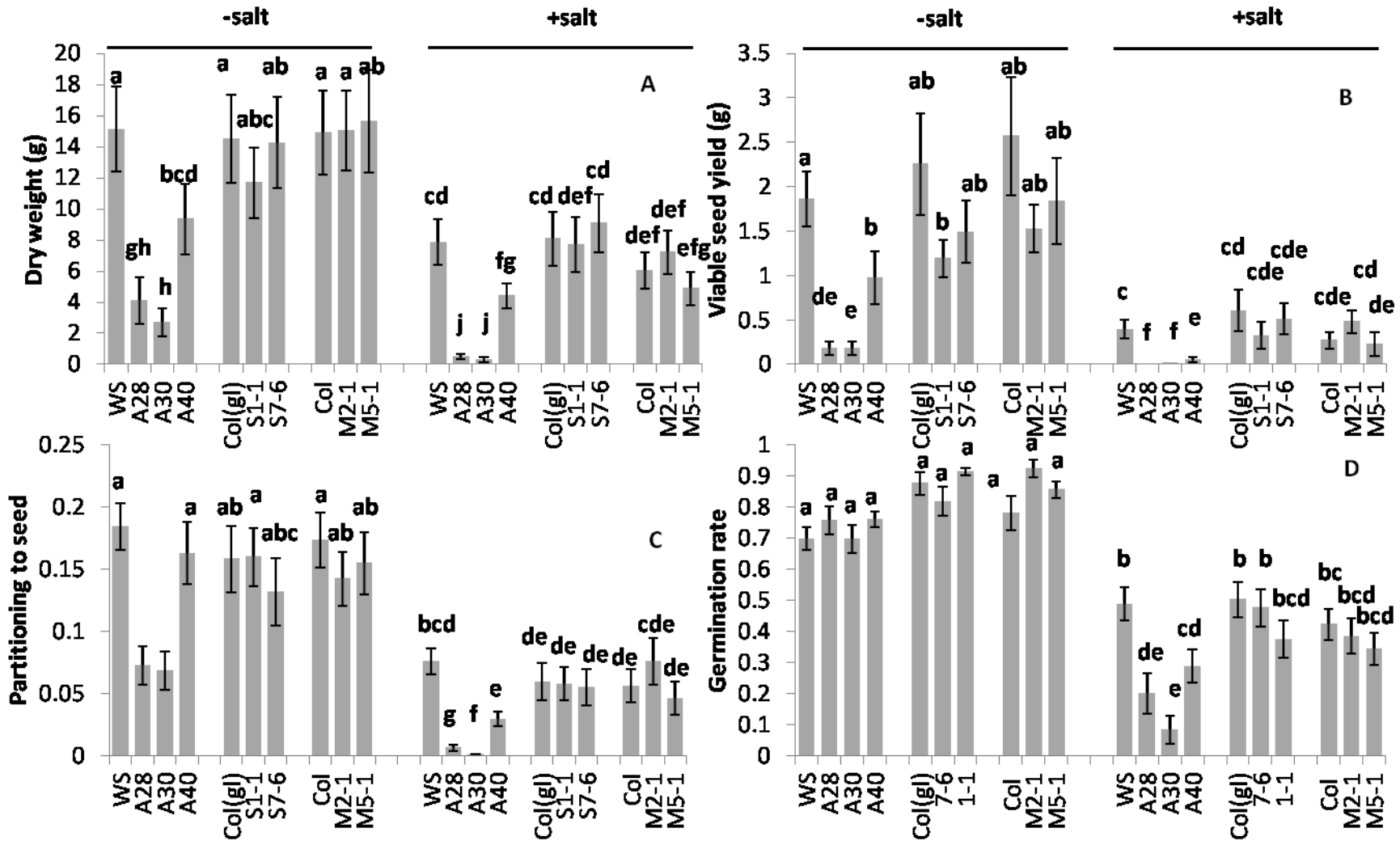


Figure 3.3. Mean productivity measures for growth chamber grown pure line populations under control and salinity stress (100mM NaCl); aboveground dry weight (A), viable seed yield based on germination rates of progeny seed (B), partitioning to seed (C) and progeny seed germination rate (D). Each value is the mean relative fitness from three replicate populations per genotype per treatment . Mean values with the same letter were not significantly different from each other at $P<0.05$. (Duncan's). The dry weight data for Col and M6PR lines were previously included in Chan *et al*. 2011 and were gathered by Zhulong Chan.

Figure 3.3 (cont'd)

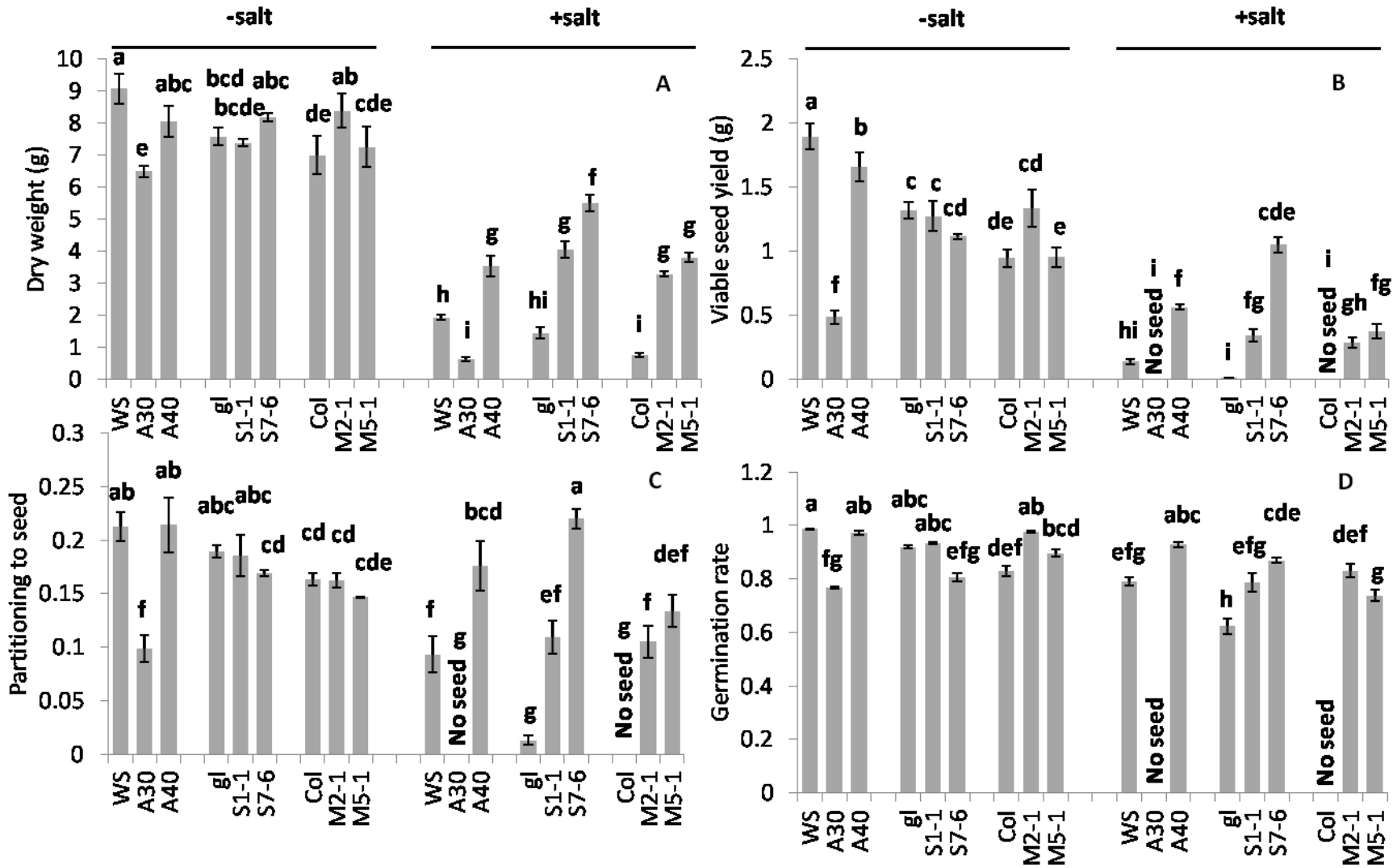
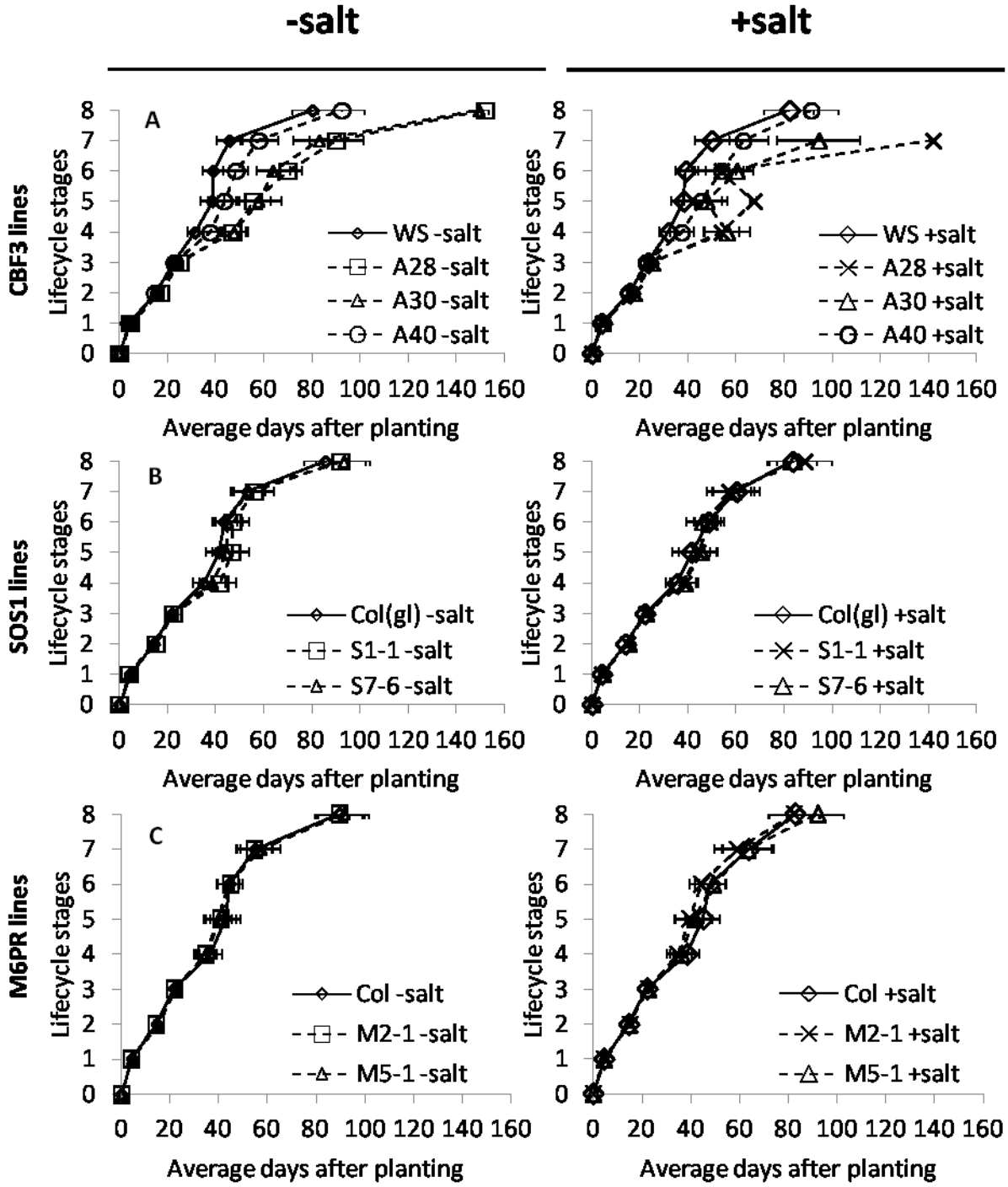


Figure 3.4. Mean days to reach various lifecycle stages under control (black line) and salt treated (75mM NaCl, grey lines) conditions for pure populations of wild-type (solid) and transgenic plants (dashed). The development of CBF3 overexpression lines (A40, A30, A28) and their background ecotype WS (A), M6PR lines (M2-1, M5-1, M7-6) and their wild-type Col (B), and SOS1 lines (S1-1, S7-6) with their wild-type Col(gl) (C). Values are the mean of three experiments with five replicate populations per genotype. The stages are days to: first germination (1), 75% of the population with two true leaves (2), five to six true leaves forming a rosette (3), first bolting (4), first flowering (5), 75% reaching bolting , flowering and maturation(6, 7 & 8 respectively).

Figure 3.4 (cont'd)



line was more intermediate to the WS wild-type (Figure 3.4a). The mean seed yield from pure CBF3 populations in each of the six greenhouse environments (three repeated experiments ±salt) only marginally increased as environmental conditions improved, falling well below both the environmental yield capacity and their wild-type background (Figure 3.1, $P<0.05$). In almost all cases mean dry weight, viable seed yield and partitioning to seed of the CBF3 overexpression lines were reduced compared to WS wild-type in both the greenhouse and growth chamber (Table 3.3, Figures 3.2abc & 3.3abc, all $P<0.05$). Progeny seed viability was equal to wild-type under control conditions in the greenhouse, while line A30 was reduced relative to wild-type in the growth chamber (Figures 3.2d & 3.3d, Table 3.3, $P>0.05$ and $P<0.05$ respectively).

The sodium antiporter SOS1overexpression lines had wild-type development except for a delay in initial bolting and days to maturity (Figure 3.4b, Table 3.3, $P<0.05$). In the growth chamber SOS1 lines were unaffected in development compared to their respective wild-type Col(gl) (Chan *et al*. 2012). The seed yield of SOS1 lines was not significantly different from the environmental yield capacity measured across the six greenhouse growth conditions (Figure 3.1, $P>0.05$). Averaged over all environments, lines overexpressing the salt antiporter SOS1maintained wild-type levels of dry weight, seed partitioning and progeny seed viability in the greenhouse (Figures 3.2acd, Table 3.3, $P>0.05$). Viable seed yield trended to be lower than Col(gl) wild-type (Figures 3.2b, Table 3.3, $P<0.1$). In the growth chamber, in the absence of salinity stress, SOS1lines were comparable to wild-type across all measures of productivity (Figure 3.3abcd, all $P>0.05$). Under the highest yielding environment in the absence of salinity stress, the wild-type line Col(gl) exceeded the yield of the SOS1 lines (Figure 3.1b, all $P<0.05$).

The mannitol biosynthetic enzyme M6PR had no effect on development under control conditions in either the greenhouse (Figures 3.4c, Table 3.3, all $P>0.05$) or growth chamber (Chan *et al*. 2012). M6PR seed yield was not significantly different from the environmental yield capacity measured across the six greenhouse growth conditions (Figure 3.1, $P>0.05$). Likewise M6PR expression lines were overall not significantly different from their Columbia wild-type in the absence of salinity stress in dry weight, viable seed yield, or partitioning to seed in either the

Table 3.3. Transgene effect on development and productivity averaged across lines and populations.

Treat-ment	Trans-gene	Days to reach lifecycle stage[1]					Measures of productivity[1]			
		First bolting	Majority bolting (75%)	First flower-ing	Majority flower-ing (75%)	Majority mature (75% dry siliques)	Dry weight (g)	Seed yield (g)	Partitioning to seed	Seed viability
Control	WS-WT	31.7a	39.0a	38.7a	46.1a	80.4a	15.1a	2.6a	0.184a	0.70a
	CBF3	44.3d	60.8d	52.5c	75.5f	120.2f	5.4c	0.54c	0.101bc	0.74a
	Col(gl)-WT	35.5ab	43.7ab	41.6ab	53.5bc	85.9abcd	14.5a	2.3ab	0.158ab	0.88a
	SOS1	40.2c	46.1b	45.8b	54.8bcd	92.9e	13.0a	1.46b	0.146ab	0.87a
	Col-WT	36.7bc	44.5b	42.7ab	54.8bcd	89.4cde	14.9a	2.76ab	0.174a	0.78a
	M6PR	34.6abc	44.5b	41.6ab	55.4bcd	89.4cde	15.3a	1.89ab	0.155a	0.88a
Salt	WS-WT	31.8a	39.0a	38.3a	50.1ab	82.2ab	7.9b	0.61c	0.076cd	0.49b
	CBF3	49.3e	56.1c	53.4c	75.4f	91.2de	1.7d	0.05d	0.014e	0.26c
	Col(gl)-WT	35.0ab	48.4b	41.5ab	60.2de	83.5ab	8.1b	0.75c	0.059cd	0.5b
	SOS1	38.4bc	47.4b	44.8b	58.1cde	86.1bcd	8.4b	0.64c	0.056d	0.43b
	Col-WT	38.3bc	47.8b	44.8b	63.5e	82.7ab	6.0bc	0.38c	0.056d	0.42b
	M6PR	35.2bc	46.1b	41.8ab	60.9de	85.1abc	6.4bc	0.62c	0.064cd	0.39b

[1] Each value is the mean of five replicate populations per genotype per treatment per experiment. Data are pooled from three replicate experiments, equivalent trends were observed in each experiment. Mean values with the same letter were not significantly different from each other at $P<0.05$ (Duncan's).

greenhouse or growth chamber (Figures 3.2abcd, 3.3abc, Table 3.3, all P>0.05). As was observed for SOS1, in the highest yielding environment, seed yield of wild-type Col exceeded the M6PR lines (Figure 3.1c, all P<0.05). In the growth chamber, progeny seed viability was increased relative to wild-type (Figure 3.3d, P<0.05).

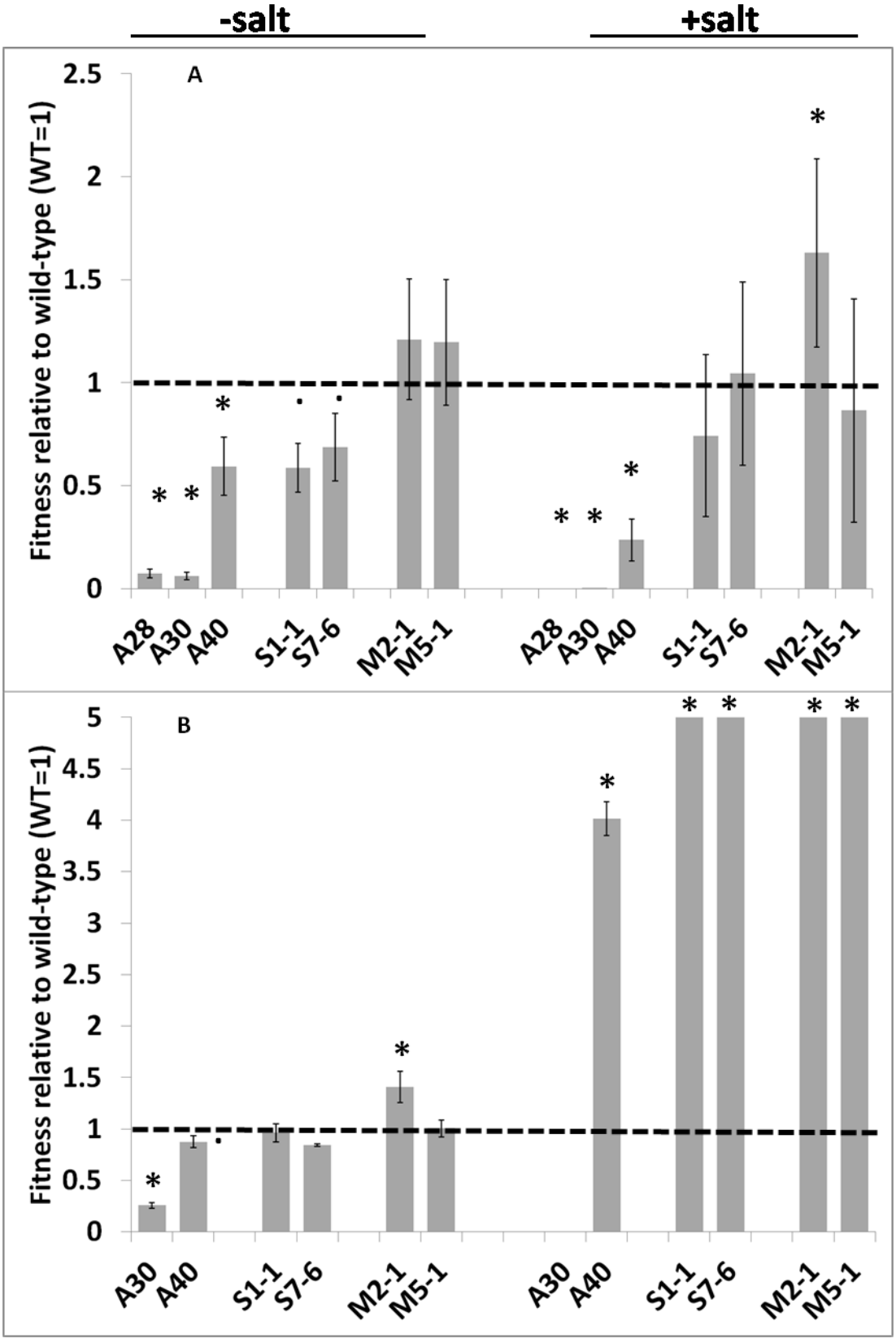
Viable progeny seed yield is the measure for plant fitness in a self-pollinated, seed-propagated species like *Arabidopsis thaliana*. The relative fitness of the transgenic lines was determined by comparing the viable seed yield between pure transgenic and pure wild-type populations. In the absence of salinity stress in the greenhouse and growth chamber, CBF3 lines overall had reduced fitness relative to their WS wild-type (Figures 3.5ab, all P<0.05). SOS1 lines trended to be lower in relative fitness in the greenhouse (P<0.1) while maintaining wild-type fitness in the growth chamber (P>0.05). In the greenhouse, M6PR lines had a relative fitness comparable to wild-type under the same conditions (all P>0.05); however, in the growth chamber line M2-1 exceeded wild-type fitness (P<0.05).

Effects of transgene expression on plant development and productivity in the presence of salinity stress

In the presence of salinity stress, whether imposed in the greenhouse (at 75mM NaCl) or growth chamber (at 100mM NaCl), CBF3 lines were delayed in all reproductive stages (Figures 3.4a, Table 3.3, Chan *et al*. 2012, P<0.05). Under salinity stress in the greenhouse, the three CBF3 overexpression lines also had reduced mean dry weight, viable seed yield and partitioning to seed relative to WS wild-type (Figure 3.2abc, Table 3.3, all P<0.05). CBF3 lines were the only transgenic lines to have greater reductions in progeny seed viability under salinity stress in the greenhouse than their wild-type counterpart (Figure 3.2d, Table 3.3, P<0.05). Performance under salinity stress in the growth chamber showed some differences from the greenhouse, where the semi-dwarf line A40 had increased dry weight, viable seed yield and

Figure 3.5. Mean relative fitness for pure populations of CBF3 (lines A40, A30 and A28), SOS1 (lines 1-1 and 7-6) , and M6PR (lines M2-1 and M5-1) transgenic plants under control and salt treated (75mM NaCl) conditions in the greenhouse (A) and control and salt treated condions in the growth chamber (100 mM NaCl) (B). Relative fitness was calculated as the proportion of viable transgenic seed yield to viable wild-type seed yield under the same treatment. The relative fitness of SOS1 and M6PR lines under salinity stress in the growth chamber exceeded 20 and were cut off for figure clarity. Each value is the mean relative fitness from replicate populations, with n=15 in the greenhouse and n=3 in the growth chamber. Mean fitness values which differ from wild-type at $P<0.1$ or $P<0.05$ are marked by (**.**) or (*****) respectively.

Figure 3.5 (cont'd)



partitioning to seed relative to wild-type and had no significant reduction in progeny seed viability (Figure 3.3abcd).

In the greenhouse in the presence of salinity stress, pure populations of lines overexpressing the Na+/H+ antiporter SOS1 had wild-type development as well as wild-type levels of dry weight, viable seed yield, partitioning to seed and progeny seed viability (Figure 3.4b, Figure 3.2abcd, Table 3.3, all $P>0.05$). In the growth chamber under salinity stress, line S7-6 had increased bolting and flowering relative to wild-type Col(gl) while line S1-1 had wild-type development (Chan *et al*. 2012). In contrast to greenhouse results, in the growth chamber SOS1confered an advantage in the presence of salinity stress with markedly increased dry weight, viable seed yield, partitioning to seed and progeny seed viability relative to wild-type Col(gl) (Figure 3.3abcd, all $P<0.05$).

Similar to control conditions, under salinity stress in the greenhouse M6PR lines were not significantly different from Col wild-type in development, dry weight, viable seed yield, partitioning to seed or progeny seed viability (Figures 3.4c and 3.2abcd, Table 3.3, all $P>0.05$). Under salinity stress in the growth chamber, the M6PR lines had increased bolting and flowering relative to Col wild-type (Chan *et al*. 2012) and produced over 4-fold more biomass than wild-type and yielded viable seed while Col was unable to do so (Figure 3.3abd, all $P<0.05$).

The relative fitness, as measured by mean viable seed yield of pure transgenic populations compared to viable yield from pure wild-type populations, of all the transgenic lines was increased in the presence of salinity stress. While the CBF3 lines under salinity stress in the greenhouse continued to have reduced fitness relative to the WS wild-type (Figure 3.5a, all $P<0.05$), in the growth chamber CBF3 line A40 had increased fitness relative to WS (Figure 3.5b, $P<0.05$). SOS1lines under salinity stress in the greenhouse had fitness levels equal to wild-type (Figure 3.5a, all $P>0.05$), but had significantly increased relative fitness in the growth chamber under salt stress (Figure 3.5b, all $P<0.05$). In the greenhouse with salt, M6PR line M2-1 had increased relative fitness while line M5-1 had wild-type fitness (Figure 3.5a, $P<0.05$ &

P>0.05 respectively). In the growth chamber with salt both M6PR lines had increased relative fitness (Figure 3.5b, P<0.05).

Effects of salinity and transgene expression on competitive ability

Competitive advantages conferred by the transgene in the presence of salt stress were predicted from pure population studies in the growth chamber (Chan *et al*. 2012). To quantify competitive advantage each transgenic line was grown in competition in the greenhouse with their wild-type counterpart in populations (14 replicates/line/treatment in each of 3 repeated experiments) that were established as 1:1 mixes of each transgenic line and their wild-type background. Transgene frequencies were determined by selectable marker screening of progeny seed from all salinity and control populations. To assess whether changes in observed transgene frequency from the initial 50% were likely the result of selection or genetic drift, 95% confidence intervals were calculated based on theoretical drift distributions (Falconer & Mackay 1996). Mean transgene frequencies above or below those thresholds indicated a competitive advantage or disadvantage due to the transgene.

In competition under no salt stress control conditions, CBF3 overexpression lines showed a significant competitive disadvantage against their WS wild-type background (Figure 3.6, P<0.05). In contrast, neither SOS1or M6PR lines experienced a competitive advantage or disadvantage under control conditions in competition (Figure 3.6, P>0.05). Under salinity stress no transgenic line showed a competitive advantage against wild-type plants. In the presence of salinity stress, CBF3 lines continued to be disadvantaged (Figure 3.6, P<0.05). SOS1 transgenic plants under salinity stress experienced a disadvantage in competition with Col(gl), while M6PR transgenic plants were competitively the equal of Col (Figure 3.6, P<0.05 and P>0.05 respectively).

To determine whether any transgene conferred a competitive advantage under specific growth environments in the greenhouse, transgenic seed yield was compared to 50% of the mean seed yield of all mixed populations in that environment (i.e. if all plants from the starting 1:1 mix

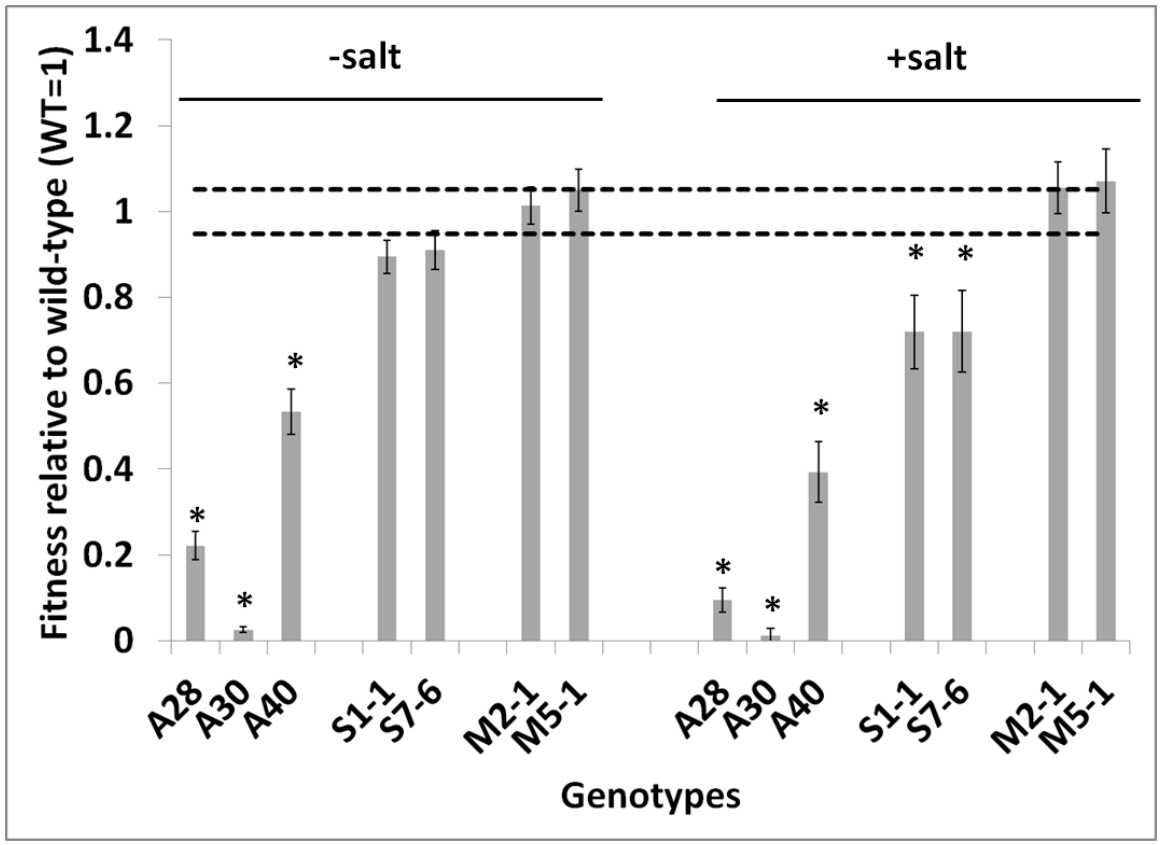
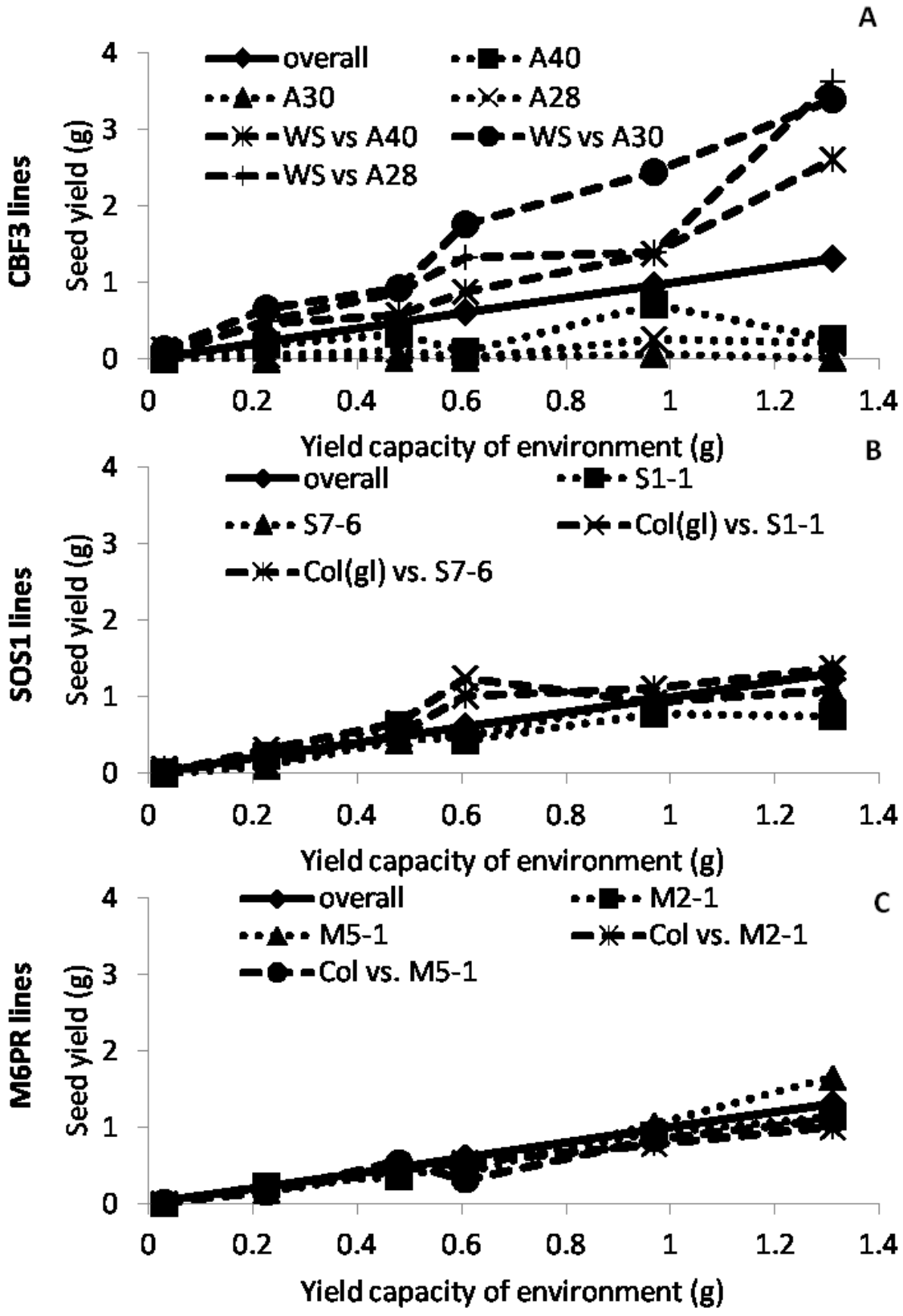


Figure 3.6. Mean competitive fitness of transgenic lines, CBF3 lines (A40, A30 and A28), SOS1 (lines 1-1 and 7-6) and M6PR (lines M2-1, M5-1), in mixed populations under control and salt treated (75mM NaCl) conditions. All populations were planted as 1:1 wild-type:transgenic mixes, with each transgenic line planted with its respective wild-type background: WS with CBF3 lines, Col(gl) with SOS1 lines and Col with M6PR lines. Competitive fitness was calculated from selectable marker screening of progeny seed. Each value is the mean fitness from fourteen replicate populations per treatment in three repeated greenhouse experiments. Mean competitive fitness values outside the 95% confidence intervals, calculated based on theoretical drift distributions, indicate negative selective pressures (marked by *).

Figure 3.7. Seed yield of competitive mixed populations of transgenic plants and their wild type backgrounds in relation to yield capacity of the environment for six growing conditions, 3 seasons ±salt (closed and open symbols respectively). The three CBF3 lines in competition with their WS wild-type background (A), the two SOS1 lines in competition with wild-type Col(gl), and the two M6PR in competition with wild-type Col (C). All values are the mean of 14 replicate populations, each planted with an initial transgene frequency of 50%. Overall yield capacity was calculated as mean seed yield of all genotypes for each of the three experiments multiplied by 50% (the starting transgene frequency). Yield capacity of each transgenic line was calculated as the mean seed yield multiplied by the mean transgene frequency measured by selectable marker phenotyping of progeny seed.

Figure 3.7 (cont'd)



yielded equivalently; half the seed would be transgenic) (Figure 3.7). CBF3 lines were shown to be at greater disadvantages vs. their wild-type as the environment improved, while SOS1 and M6PR lines generally averaged around that capacity measurement. Thus the fitness increases under salinity stress seen in some SOS1 and M6PR lines in pure populations in the greenhouse and growth chamber were not observed while in competition with wild-type plants in the greenhouse.

Discussion

This study compared the fitness effects of three abiotic stress tolerance transgenes in salt stress experiments in the greenhouse in the presence and absence of competition with wild-type parents. Prior field experiments with these transgenic lines indicated the importance of assessing transgene fitness effects in the presence of competition rather than pure line performance (Chapter 2). However, the field competitive experiments were not performed with salinity stress.

As expected from previously published growth chamber studies (Chan *et al*. 2011, 2012), salinity stress impacted reproductive development rates, and reduced vegetative and reproductive productivity. Another impact of salinity stress was a marked reduction (>50%) in progeny seed viability, to the extent that it was not possible to obtain sufficient viable seed to perform multi-generational fitness assessments as had been done in the field experiments (Chapter 2). As is most common in the literature, our previous studies in the growth chamber did not measure seed viability; however, the low viability observed in progeny seed from salt stressed plants in the greenhouse indicates the importance of this criterion in making accurate fitness assessments.

Transgene impacts on plant development in the greenhouse in the presence and absence of salt stress were consistent with previous non-competitive growth chamber experiments (Chan *et al*. 2011, 2012); however, differences were observed in the effect of the transgenes on productivity. While CBF3 line A40 showed enhanced dry weight and seed yield under salinity stress in the growth chamber, it did not in the greenhouse. The significant gains in productivity, both vegetative and reproductive, from SOS1 and M6PR overexpression shown under salinity

stress in the growth chamber, were reduced in the greenhouse. SOS1 lines were comparable to wild-type, and the M6PR plants showed elevated relative fitness in only one line. Interestingly this was the same line observed to have a competitive advantage over wild-type across six generations in the field (Chapter 2).

Observed differences between growth chamber- and greenhouse-grown pure populations may be due to the combinations of stresses that can occur in the less tightly controlled environment of the greenhouse. Light levels in the greenhouse were 4-fold higher than those in the growth chamber and peak light levels also corresponded to increased air temperatures in the greenhouse. Research has shown that the effects of combinations of abiotic stresses can be more severe than those suffered under the stresses individually (reviewed by Mittler 2006). A recent transcriptomic analysis of *Arabidopsis thaliana* ecotypes under paired combinations of abiotic stresses (such as high light and salt stress or high temperature and salt) revealed that over 60% of expression patterns under stress combinations were not predicted by their individual stress expression profiles (Rasmussen *et al*. 2013). These findings may indicate that the combined conditions of salinity, high light and temperature contributed to the lower relative fitness of the greenhouse-grown transgenic plants than was observed in the tightly controlled and relatively mild environment of the growth chamber.

In competition with their wild-type background in the greenhouse, the performance of the transgenic lines differed from both growth chamber and greenhouse pure line assessments. No transgene conferred a competitive advantage over wild-type plants in the presence or absence of salinity stress. CBF3 and SOS1 lines were less competitive in the presence of salinity stress than they were under control conditions, indicating that competition remains an important factor on transgene fitness even under significant salinity stress. Competitive assessments of transgenic plants have been performed previously for traits such as herbicide, virus, and insect resistance. Transgenic insect resistant hybrids of cultivated, *Oryza sativa* L., and weedy red rice, *O. sativa f. spontanea*, showed an increased seed yield under natural insect pressures in pure populations; however, in competition with non-transgenic hybrids the transgenic hybrids were equal to wild-

type (Yang *et al*. 2011, 2012). Similarly, the increased productivity in disease-stressed pure populations of virus-resistant sugar beet was reduced when planted in competition with *Chenopodium album* (Bartsch *et al*. 1996). Herbicide tolerant oilseed rape, *Brassica napus*, has been shown under interspecific competition in field plots (Fredshavn *et al*. 1995), field margins (Warwick *et al*. 2008) and roadsides (Knispel *et al*. 2008), to have productivity equal to or less than wild-type. Thus, in general, first generation transgenic traits conferred significant fitness gains relative to wild-type under the target stress (reviewed by Warwick *et al*. 2009), but relative fitness values were reduced in the presence of competition whether in the presence or absence of the target stress.

The reduced relative fitness observed from the salt-stressed transgenic lines when in competition with wild-type plants may be linked to the natural responses of *Arabidopsis thaliana* to intraspecific competition, which has been found to be significantly different from abiotic or biotic stress responses (Geisler *et al*. 2012). Transcriptomic analysis of Columbia ecotype *Arabidopsis* plants at varying levels of competition (i.e. planting densities), found that in response to increasing competition, the plants down-regulated genes involved in abiotic stress response, pathogen defense and secondary metabolism, and up-regulated photosynthesis related genes. Thus, *Arabidopsis* plants appear to attempt to outgrow their competition at the expense of defense. These changes in transcriptional responses relative to the absence of competition were seen in densities ranging from 1400 seeds/m^2 to over 15,000 seeds/m^2; our plant density of 2600 seeds/m^2 was within this range. Given that densities higher than 2600 seeds/m^2 were observed in natural populations in MI, these transcriptional changes in response to competition likely occurred in our field (Chapter 2) and greenhouse studies as well in natural populations. It is possible that our transgenic lines, due to constitutive transgene expression, were transcriptionally locked into an abiotic stress response (Chan *et al*. 2012), and thus may have been unable to compete effectively with wild-type plants.

While the transgenic pure line performance vs. competitive performance trends observed in this study were similar to those observed in transgenic plants expressing first generation traits

(e.g herbicide or insect resistance), there are some important differences between these traits and traits increasing abiotic stress tolerance. Herbicide, virus and insect resistance in current transgenic crops have been conferred by genes encoding alternate forms of existing plant enzymes, viral coat proteins, or small RNAs, or Bt proteins respectively (Warwick *et al*. 2009). These gene products function directly to confer the trait of interest; the introduced genes encode enzymes that degrade or are unaffected by herbicide, or RNAs and proteins that only interact with their target pest. Expression of these traits has resulted in few secondary effects on the transformed plant as measured by the timing of life-cycle parameters, plant architecture, biomass, and seed yield (Thies and Devare 2007, Cheng *et al*. 2008, Zolla *et al*. 2008). However, microarray analysis of the genes in this study showed that expression of the three transgenes had complex transcriptional effects; *CBF3* and *M6PR* both affected genes involved in ABA signaling, cell wall biosynthesis, ion and ABC transport, redox, and osmoprotectant biosynthesis, while the effect of *SOS1*caused a general down-regulation of oxidative stress response genes (Chan *et al*. 2012). Thus transgenes enhancing abiotic stress tolerance can result in complex changes to regulatory, signaling, and metabolic pathways that could impact plant fitness.

Furthermore, although both disease and insect pressures can, like abiotic stresses, limit plant populations (reviewed by Warwick *et al*. 2009), the fitness gains from resistance to biotic stresses are density dependent. Each resistant plant, by not serving as a host, reduces the pressure on its neighbors, as has been observed for insect resistance (Yang *et al*. 2012). This effect does not occur in abiotic stress conditions, as all plants in an affected area experience the stress regardless of how many of their neighbors may be more tolerant to it. This lack of density dependence in abiotic stress tolerance has important implications on transgene establishment and further supports the need for competitive assessment of genes enhancing this trait.

Overall, these studies indicate the importance of assessment of abiotic stress tolerance enhancing transgenes under a range of environmental conditions and corroborate the effects of competition observed over six generations in the field in the absence of salinity stress (Chapter 2). The secondary fitness effects of transgene expression under salinity stress were influenced by

the presence of other abiotic stresses, and by the presence of competition. Although significant fitness gains in the presence of salt stress were observed in pure population relative to wild-type in the growth chamber, these gains were reduced in greenhouse pure populations. These differences in fitness relative to wild-type may be the result of possibly differing transcriptomic and physiological responses under combined abiotic stresses (as occurs in a greenhouse) than when a stress is experienced individually (as occurs in a growth chamber). In direct competition with wild-type plants, these small gains were lost, resulting in no transgenic line showing a competitive advantage under salt stress. These results indicate that risk assessments and modeling based on non-competitive assessments, especially those under tightly regulated environmental conditions, may poorly estimate the risks of abiotic stress tolerance enhancing transgene spread and establishment. Future studies on the secondary fitness impacts of abiotic stress tolerance enhancing transgenes should strive to more closely mimic natural conditions by including competition and a range of environmental conditions to gain more accurate and useful results for risk assessments.

LITERATURE CITED

LITERATURE CITED

Andow, D. A., & Hilbeck, A. (2004). Science-based risk assessment for nontarget effects of transgenic crops. Bioscience, 54(7), 637-649.

Bartsch, D., Schmidt, B., Pohl-Orf, M., Haag, C., & Schuphan, I. (1996). Competitiveness of transgenic sugar beet resistant to beet necrotic yellow vein virus and potential impact on wild beet populations. Molecular Ecology, 5(2), 199-205.

Bhatnagar-Mathur, P., Vadez, V., & Sharma, K. K. (2008). Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Reports, 27(3), 411-424.

Chan, Z., Grumet, R., & Loescher, W. (2011). Global gene expression analysis of transgenic, mannitol-producing, and salt-tolerant *Arabidopsis thaliana* indicates widespread changes in abiotic and biotic stress-related genes. Journal of Experimental Botany, 62(14), 4787-4803.

Chan, Z., Bigelow, P. J., Loescher, W., & Grumet, R. (2012). Comparison of salt stress resistance genes in transgenic *Arabidopsis thaliana* indicates that extent of transcriptomic change may not predict secondary phenotypic or fitness effects. Plant Biotechnology Journal, 10(3), 284-300.

Cheng, K. C., Beaulieu, J., Iquira, E., Belzile, F. J., Fortin, M. G., & Strömvik, M. V. (2008). Effect of transgenes on global gene expression in soybean is within the natural range of variation of conventional cultivars. Journal of Agricultural and Food Chemistry, 56(9), 3057-3067.

Davis, M. B., & Shaw, R. G. (2001). Range shifts and adaptive responses to Quaternary climate change. Science, 292(5517), 673-679.

Ellstrand, N. C. (2003). *Dangerous Liaisons?*. Johns Hopkins University Press.

FAO (Food and Agriculture Organization of the United Nations), (2011). The state of the world's land and water resources for food and agriculture: managing systems at risk. http://www.fao.org/docrep/017/i1688e/i1688e.pdf (accessed March 2013).

Falconer, D. S., & Mackay, T. F. C. 1996. *Introduction to quantitative genetics*,*4th ed*. Longman Press.

Fredshavn, J. R., & Poulsen, G. S. (1996). Growth behavior and competitive ability of transgenic crops. Field Crops Research, 45(1), 11-18.

Geisler, M., Gibson, D. J., Lindsey, K. J., Millar, K., & Wood, A. J. (2012). Upregulation of photosynthesis genes, and downregulation of stress defense genes, is the response of *Arabidopsis thaliana* shoots to intraspecific competition. Botanical Studies, 53(1), 85-96.

Gilmour, S. J., Sebolt, A. M., Salazar, M. P., Everard, J. D., & Thomashow, M. F. (2000). Overexpression of the *Arabidopsis CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology, 124(4), 1854-1865.

Hoffman, C. A. (1990). Ecological risks of genetic engineering of crop plants. BioScience, 40(6), 434-437.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., & Shinozaki, K. (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology, 17(3), 287-291.

Knispel, A. L., McLachlan, S. M., Van Acker, R. C., & Friesen, L. F. (2008). Gene flow and multiple herbicide resistance in escaped canola populations. Weed Science, 56(1), 72-80.

Mantri, N., Patade, V., Penna, S., Ford, R., & Pang, E. (2012). Abiotic stress responses in plants: present and future. In *Abiotic Stress Responses in Plants* (pp. 1-19). Springer New York.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. Trends in Plant Science, 11(1), 15-19.

Rasmussen, S., Barah, P., Suarez-Rodriguez, M. C., Bressendorff, S., Friis, P., Costantino, P., ... & Mundy, J. (2013). Transcriptome responses to combinations of stresses in *Arabidopsis thaliana.* Plant physiology. Plant Physiology, 161(4), 1783-1794.

Ricroch, A.E., Berge, J.B. and Kuntz, M. (2011) Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. Plant Physiology, 155, 1752–1761.

Shi, H., Ishitani, M., Kim, C., & Zhu, J. K. (2000). The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na+/H+ antiporter. Proceedings of the National Academy of Sciences, 97(12), 6896-6901.

Shi, H., Quintero, F. J., Pardo, J. M., & Zhu, J. K. (2002). The putative plasma membrane Na+/H+ antiporter *SOS1* controls long-distance Na+ transport in plants. The Plant Cell, 14(2), 465-477.

Shi, H., Lee, B., Wu, S.J. and Zhu, J.K. (2003) Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nature Biotechnology. 21, 81–85.

Sickler, C. M., Edwards, G. E., Kiirats, O., Gao, Z., & Loescher, W. (2007). Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. Functional Plant Biology, 34(4), 382-391.

Thies, J. E., & Devare, M. H. (2007). An ecological assessment of transgenic crops. Journal of Development Studies, 43(1), 97-129.

Thuiller, W., Lavorel, S., Araújo, M. B., Sykes, M. T., & Prentice, I. C. (2005). Climate change threats to plant diversity in Europe. Proceedings of the National Academy of Sciences of the United States of America, 102(23), 8245-8250.

Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 218(1), 1-14.

Warwick, S. I., Legere, A., Simard, M. J., & James, T. (2008). Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population. Molecular Ecology, 17(5), 1387-1395.

Warwick, S. I., Beckie, H. J., & Hall, L. M. (2009). Gene flow, invasiveness, and ecological impact of genetically modified crops. Annals of the New York Academy of Sciences, 1168(1), 72-99.

Yang, X., Xia, H., Wang, W., Wang, F., Su, J., Snow, A. A., & Lu, B. R. (2011). Transgenes for insect resistance reduce herbivory and enhance fecundity in advanced generations of crop–weed hybrids of rice. Evolutionary Applications, 4(5), 672-684.

Yang, X., Wang, F., Su, J., & Lu, B. R. (2012). Limited fitness advantages of crop-weed hybrid progeny containing insect-resistant transgenes (Bt/CpTI) in transgenic rice field. PloS One, 7(7), e41220.

Zhifang, G., & Loescher, W. H. (2003). Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer. Plant, Cell & Environment, 26(2), 275-283.

Zolla, L., Rinalducci, S., Antonioli, P., & Righetti, P. G. (2008). Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. Journal of Proteome Research, 7(5), 1850-1861.

Chapter 4

Crop improvement utilizing abiotic stress tolerance enhancing transgenes and the implications for ecological risk assessments.

Introduction

The next generation of transgenic crops will express a range of traits designed to reduce crop yield loss from the abiotic stresses that can devastate fields today, and to meet the future challenges of a changing global climate over the next century. The Food and Agriculture Organization of the United Nations has estimated a world population of 9.1 billion people by the year 2050 which will require an estimated increase of 70% in total crop yield over 2007 levels (Bruinsma 2009). Climate modeling predicts that during this same time period farmers will experience significant increases in the severity and frequency of extreme weather events (e.g. heatwaves, droughts, or heavy precipitation) (Goodness 2013).

The 2012 drought across much of the United States affected 80% of US agricultural land, making it the most severe American drought in a half century, with significant reductions in yield observed in corn, soybean, and sorghum as well as negative impacts on meat and dairy livestock (ERS 2013). In the 2012 drought, the world's first commercialized abiotic stress tolerance enhanced crop, Monsanto's DroughtGard corn, faced a natural trial by fire alongside millions of acres of conventional and genetically engineered corn expressing first generation traits like herbicide and insect resistance. The farms of the next century are predicted to face even greater challenges from abiotic stress, as climate change is expected to increase the frequency and severity of extreme weather events such as heat waves, droughts, floods, and frosts (Goodness 2013). Combining with these climate-imposed threats will be increases in agricultural soil salinity that will occur in arid and semi-arid regions due to farm irrigation applied to protect against drought, and from rising sea levels that will push saline waters farther into coastal regions (Smedema and Shiati 2002, Chinnusamy *et al.* 2005). Facing these threats

and meeting the additional challenge of feeding a growing global population, will require the planting of crops able to sustain yield under these extreme conditions.

Approaches to developing such crops incorporate both conventional and molecular breeding techniques, such as marker assisted selection, as well as genetic engineering approaches (Mittler and Blumwald 2010). While progress has been made in identifying quantitative trait loci and selectable markers for abiotic stress tolerance traits (Collins *et al*. 2008, Mittler and Blumwald 2010, Varshney *et al*. 2011), breeding for improved varieties has been hindered by the complex nature of abiotic stress tolerance, the yield drag that commonly results from crosses with more stress tolerant wild relatives, and the germplasm diversity constraints caused by reproductive incompatibility (Varshney *et al*. 2011). Since crops developed using marker assisted selection do not require ecological risk assessments under the biosafety regulatory systems of most countries, this chapter will focus on the implications on ecological risk assessments of abiotic stress tolerant crops developed with genetic engineering.

Analysis of implications for ecological risk assessments

The current status of abiotic stress tolerant crops

Although abiotic stress results from a range of climatic and soil conditions, there is significant overlap in impacts on plant tissues, resulting in osmotic and oxidative stress at the cellular level (Wang *et al.* 2003, Warwick *et al*. 2009, Mittler and Blumwald 2010). Low water availability in cells causes a loss of turgor pressure, negative conformational changes to proteins and RNA, and a reduction in chemical reactions including photosynthesis. Abiotic stress conditions, such as high temperatures, drought and high salinity, can affect cellular metabolism resulting in elevated production of damaging reactive oxygen species. These impacts at the cellular level exert significant selective pressure on plants and have led to complex, targeted and overlapping plant abiotic stress response mechanisms. This overlap in response indicates the potential for enhanced resistance to multiple stresses through regulatory, signaling or metabolic changes.

Significant interest, both private and public, in the development of enhanced abiotic stress tolerance is evident in the increasing number of submissions to the USDA for field trials of abiotic stress related traits over the previous decade (Chapter1). These field trials have examined a range of candidate genes for abiotic stress tolerance, with an even greater array being assessed in growth chambers and greenhouse. The functions of these genes range from membrane, protein and RNA stabilization to stress signaling and transcriptional regulation (Wang *et al*. 2003, Zhang *et al*. 2004a, Sreenivasulu *et al.* 2007, Bhatnagar-Mathur *et al.*2008, Munn & Tester 2008, Warwick *et al.* 2009, Hirayama & Shinozaki 2010, Mittler and Blumwald 2010). A number of these abiotic stress tolerance genes have been transformed into crop species and expressed either constitutively or under stress inducible promoters resulting in elevated abiotic stress tolerance relative to untransformed controls or conventional cultivars; a proof of concept critical for the movement of lab discoveries to the farmer's field (reviewed by Mittler & Blumwald 2010, Deikman *et al*. 2012, Mantri *et al*. 2012).

Experimentally assessed abiotic stress tolerance enhanced crops include monocots and dicots, transformed with a broad range of genes with differing resistance mechanisms. The use of stress responsive transcription factors in crop species to enhanced abiotic stress has been demonstrated in rice (Hu *et al*. 2006 &2008, Oh *et al.* 2009, Xiao *et al.* 2009), canola (Jalgo *et al.* 2001, Savitch *et al.* 2005), tomato (Hsieh *et al*. 2002, Zhang *et al.* 2004b), tobacco (Kasuga *et al*. 2004), wheat (Pellegrineschi *et al*. 2004), potato (Behnam *et al*. 2006 & 2007) and forage grass (James *et al*. 2008). Transgenes encoding ion transporters have been utilized to increase salinity tolerance in tobacco (Gao *et al*. 2006, Yue *et al*. 2012), tomato (Olías *et al*. 2009), sweetpotato (Gao *et al*. 2012), barley (Mian *et al*. 2011), and rice (Xiao *et al*. 2009, Batelli *et al*. 2007). Introgression of a sodium transporter from a wild relative into durum wheat increased yields 25% in fields with saline soil compared to the non-introgressed background line (Munn *et al*. 2012). Transgenes increasing production of compatible solutes, such as proline, inositol, sorbitol and glycine betaine, have increased abiotic stress tolerance in tobacco (Sheveleva *et al*. 1997 & 1998, Konstantinova *et al*. 2002), wheat (Abebe *et al*. 2003), and maize (Wei *et al*.

2011). While some of these stress tolerant transgenic crops have been tested in the growth chamber, greenhouse and field, only two crops have been submitted to the USDA for deregulation.

The first and currently only commercialized abiotic stress tolerant crop cultivar is Monsanto's MON 87460 (APHIS 2011ab). This line expresses the gene *CspB* which encodes a cold shock protein from *Bacillus subtilis* (Reeves 2010). This cold shock protein is naturally upregulated in response to environmental stress and acts as an RNA chaperone preventing the misfolding of RNA that can disrupt translation (reviewed by Horn *et al*. 2007). CspB has been demonstrated to increase cold, heat and drought tolerance when constitutively expressed in *Arabidopsis thaliana* and rice (Castiglioni *et al*. 2008). Transgenic maize lines expressing *CspB* yielded significantly higher than non-transformed lines by maintaining higher chlorophyll content and photosynthetic rates under drought stress in the field, while exhibiting no cost of resistance in high yielding environments (Castiglioni *et al*. 2008). In spring 2013, DroughtGard maize was made available to farmers for wide-scale planting across the Western Great Plains region of the United States.

At the time of writing, a petition for deregulation had been submitted to the USDA for only one other abiotic stress tolerant GE crop: freeze-tolerant hybrid Eucalyptus (*Eucalyptus grandis* x *E. urophylla*) (Nehra & Pearson 2011). Eucalyptus is grown in tropical regions around the world, largely for its high quality fiber used in paper production. Eucalyptus is also being planted as a source of biomass for celluloisic biofuel production. In temperate climates, its low freezing tolerance has prevented its planting in managed forest plantations. Constitutive over-expression of Eucalyptus CBF1a and CBF1b homologs has been shown to increase freezing tolerance (Navarro *et al*. 2011), but these lines also showed significant negative phenotypic effects. To overcome these negative effects, the abiotic stress response transcription factor C-repeat binding factor 2 (*CBF2*) gene was expressed under the stress inducible promoter *rd29A*. The transgenic Eucalyptus lines submitted for deregulation also express a barnase gene under the anther-specific promoter, *PrMC2*, to cause the production of sterile pollen (Nehra & Pearson

2011). After 5 years, transgenic lines grown at multiple sites were 40-50 ft tall, while the background line continued to experience severe winter die-back. The transgenic lines did show a small but significant cost of resistance, visible as a slower rate of growth under freeze-free conditions compared to background, as well as the intended male sterile phenotype. The USDA has begun to assemble an Environmental Impact Statement, indicating that freezing tolerant Eucalyptus may be the second deregulated abiotic stress tolerant crop in the US (APHIS 2013).

Challenges in the development and implementation of abiotic stress tolerant crops

A number of issues have delayed the successful development and implementation of transgenic crops with enhanced abiotic stress tolerance. Barriers to market for genetically engineered crops have been described as: demonstration of trait efficacy in the field, development of product concepts, intellectual property concerns, establishing support of stakeholders, recordkeeping requirements, regulatory approval, and consumer communication and acceptance (Rommens 2010). Importantly only two of these barriers can be directly addressed through scientific data, demonstrating trait efficacy and regulatory approval. Regulatory approval, however, does not hinge on scientific evidence alone, as it, like the other hurdles on the way to market, incorporates legal and socioeconomic concerns. Althought these barriers are factors affecting the commercialization of all transgenic crops, they have also slowed the commercialization of abiotic stress tolerant crops.

Abiotic stress tolerance traits face significant hurdles from the first of these barriers to market, demonstrating trait efficacy in the field. Abiotic stress field trials are expensive to conduct and suffer from inconsistent stress levels across field sites (Mittler & Blumwald 2010, Richards *et al*. 2010, Deikman *et al*. 2012, Weber *et al*. 2012). Although greenhouse stress trials can be used to pre-screen transformed lines, promising transformed lines may not show the same level of tolerance in the field that was observed in the greenhouse (Deikman *et al*. 2012, Saint Pierre *et al*. 2012,Tavakkoli *et al*. 2012, Witt *et al*. 2012). The difficulty in phenotyping for abiotic stress tolerance traits has hindered plant breeders ability to select resistant germplasm, although recent technological advances in field-based phenomics (e.g. spectral imaging for leaf

temperature, water, nitrogen and chlorophyll content) may aid breeders in finding new selectable phenotypes strongly linked to stress tolerance (White *et al*. 2012). The next scientific barrier to the commercialization of abiotic stress tolerant crops is the focus of this chapter, the ecological risk assessments that occur during the regulatory approval process.

Ecological risks of abiotic stress tolerant crops

The ecological implications of a genetically engineered crop considered during the risk assessment process are the result of interactions between four factors: the gene inserted, the trait conferred by that gene, the crop transformed, and the location where the crop will be grown (Grumet *et al*. 2011). Environmental risks could result if the transgene affects: non-target species, the ferality of the transformed crop, the genetic diversity or invasiveness of compatible wild or weedy relatives, or the ecological range of recipient plants (Tiedje *et al.* 1989, Hoffman 1990, Hancock *et al.* 1996, Conner *et al*. 2003, Ellstrand 2003, Weaver and Morris 2005, Hails and Morley 2005). Non-target impacts, a concern for insect resistant traits, are not an ecological risk for abiotic stress tolerance enhancing transgenes. The remaining environmental risks are all related to the fitness and ecological range of the recipient plants.

Abiotic stress tolerance enhancing transgenes could enable recipient plants to better tolerate one or multiple abiotic stresses, and thus could confer a fitness advantage over wild-type plants (Hancock *et al.* 1996, Ellstrand 2003). While increased survival and fitness, in terms of seed yield under agricultural production, is a desired transgene impact in the transformed crop variety, this attribute could also have implications outside of crop fields. Increased fitness under abiotic stress could alter the ferality of the crop variety and the invasiveness of recipient wild relatives that acquire the transgene through pollen-mediated gene flow. Either result could lead to the displacement of wild, non-recipient competitors reducing ecosystem biodiversity and possibly resulting in localized extinctions of non-recipient plants (Hancock, 2011). Additionally, a transgene conferring a strong selective advantage could cause a selective sweep of compatible wild germplasm. This sweep could reduce the diversity of the recipient plants' gene pool as the transgene and other linked crop genes or alleles became introgressed. A transgene conferring a

significant negative fitness effect (presumably only under non-agricultural conditions or else it would be unlikely to be planted) could reduce the fitness and genetic diversity of nearby ruderal recipient populations of compatible wild or weedy relatives resulting in a 'demographic swamping' of the wild germplasm.

Abiotic stress tolerance transgenes could, depending the promoter used, alter the plant's response to the environmental conditions experienced from germination to reproduction, and so could alter the ecological range the recipient plant is able to successfully inhabit (Ellstrand 2003). This change in ecological range could be an intended impact on the transformed crop, as is the case for freezing-tolerant Eucalyptus, or an unintentional effect on recipient plants if the transgene enters wild germplasm. The ability of introduced genetic material, through hybridization and introgression, to cause ecological range expansion has been documented and is attributed to the acquisition of locally adapted germplasm (Rieseberg *et al*. 2007). Thus abiotic stress tolerance transgenes by increasing plant survival and fitness under previously range-limiting conditions, could confer increased ecological range in recipient plants. Given the pressures of a changing global climate and rising market demand for food, feed, fiber and fuel due to a growing world population, the ability to increase crop yield and planting range under abiotic stressed conditions will be critical to future farmers. On the other hand, these abiotic stress tolerance transgenes will pose potentially greater environmental risks than the first generation of genetically engineered crops.

Implications of abiotic stress tolerant GE crops for current ecological risk assessment methodologies

Around the globe governmental policies on genetically engineered crops are implemented by regulators who must weigh the potential ecological risks (and in some systems, benefits) of deregulating a new transgenic crop for the commercial market. Ecological risk assessment alone does not encompass the full range of factors considered as socio-economic considerations are also included in the decision-making process for the deregulation of transgenic crops (Devos *et al*. 2008). The considered factors differ from country to country, particularly socio-economic

ones, resulting in a variety of regulatory frameworks for genetically engineered crops. Nonetheless, all these systems are all built around the same general scientific principles for the assessment and management of risk (Regal 1994, Hill & Sendashonga 2003, Jaffe 2004).

Risk is defined as the combination of hazard, harms which could result from a given action, and exposure, which influences the likelihood of the hazard occurring (Suter 1993, EPA 1998, Thies & Devare 2007). The first step in a risk assessment is problem formulation, during which regulators must define environmental harms and set assessment endpoints which define what is to be protected from harm (EPA 1998, Suter 2000, Nickson 2008, Wolt *et al*. 2010). Although ecological harm has not always been clearly defined, one of the most frequently addressed harms is the reduction of biodiversity, at both the species and ecosystem level (Sanvido *et al*. 2012). As described earlier, the environmental risks from abiotic stress tolerant crops could negatively impact biodiversity with a scope potentially larger than that of the first generation transgenes whose target stresses are generally specific to agricultural systems.

Regardless of the regulatory system, the hazard characterization stage of an ecological risk assessment of transgenic crops attempts to predict the secondary effects of the transgene on recipient plants, both the transformed crop itself and any future hybrids from intercrosses with wild or weedy relatives (EPA 1998, EFSA 2010). These predictions incorporate measurable phenotypic effects of the transgene as well as baseline data pertaining to the crop species. This baseline data includes the biology of the crop plant, the cropping system currently used with conventional varieties of that crop and gene flow and hybridization data between the crop and any compatible wild or weedy relatives. The risk characterization for a transgenic crop introduction incorporates both the characterized potential hazards and the characterized exposure level for each of those hazards. In addition, regulators consider risk management; whether the characterized risks could be reduced or mitigated by certain management practices (e.g. refugia in insect resistant crop plantings to slow the evolution of resistance in target insect species). These methods have been highly successful in guiding the deregulation and commercialization of the first generation of transgenic crops expressing herbicide, insect and virus resistance with little

negative environmental impact (Thies & Devare 2007). The traits which will be present in the next generation of transgenic crops, such as abiotic stress tolerance, may however, confer more complex secondary and fitness effects on the transformed plants than has been examined previously (Grumet *et al*. 2011). Questions about the ability of current risk assessment methodologies to assess the ecological risks from these potentially more complex traits, have led to suggestions for regulatory changes. These proposed changes include suggestions for altered field trial requirements to better assess fitness effects, experimental testing of lab created crop-wild hybrids, and utilizing 'omic' approaches to characterize transgene impacts at the transcript, protein and metabolite level (EFSA 2008). Regulatory changes may be needed address the new more complex traits; but, as regulatory systems adapt to these new crops they must also continue protecting their countries' ecosystems while not placing an undue burden on plant breeders and farmers. This can be done by ensuring that the regulatory system's data requirements are proportionate with the level of potential risk posed by a new submission (Jaffe 2004).

European Food Safety Association guidance report on the ERA of transgenic plants

In June of 2007, a colloquium was held by the European Food Safety Association (EFSA) to examine the future challenges and approaches for the ecological risk assessment of transgenic crops (EFSA 2008). The colloquium gathered one hundred stakeholder participants, including regulators, public and private researchers, industry experts and members of non-governmental organizations, from 19 EU countries to discuss topics related to risk assessment and the current EU regulatory system for genetically engineered crops which is based on 2001 legislation (EC 2001). These topics examined how current scientific research addressed aspects of risk assessment and included: measuring non-target effects, modeling the impacts of GE cultivation, assessing long-term and large-scale environmental effects, and the consideration of environmental benefits during risk assessments (EFSA 2008). The recommendations made during this colloquium could have implications for the ecological risks assessments of future abiotic stress tolerant crops in the EU.

Based in part on the recommendations from the 2007 colloquium, the European Food Safety Association has issued new guidance on the ecological risk assessment of genetically engineered plants (EFSA 2010). The risk assessment methodology changes addressed in this report could significantly impact the regulatory burden on the developers of abiotic stress tolerant crops. The guidance document outlines multiple broad areas to be considered in an ecological risk assessment: persistence and invasiveness including the effects of plant to plant gene flow, interactions with target organisms, interactions with non-target organisms, impacts of cultivation and harvest methods, and effects on biogeochemical processes. Each of these areas of risk will be examined as they relate to the ecological risk assessment of abiotic stress tolerance enhanced crops.

Persistence, invasiveness and plant-to-plant gene flow

The 2010 guidance report outlines problem formulation and hazard identification steps to be used by EU regulators in considering the environmental harm from transgene persistence and gene flow to compatible wild species. The report establishes a tiered assessment method for this area of risk which requires more information to be submitted for transgenic crops which potentially place the ecosystems of the European Union at greater risks (Figure 4.1). According to the guidance report, in addition to the standard molecular characterization of the transgenic plant, all applications should provide basic species-specific information including reproductive biology, known weediness traits or invasiveness, range limiting factors and the presence of known compatible relatives within the EU (EFSA 2010). This information is then to be used to determine whether the transformed plant could survive, reproduce, and possibly hybridize, under the environmental conditions present within the EU (described as stage 1).

For plants that are potentially able to do so, additional transformation event-specific information is then required (Figure 4.1). This information compares the transformed plant to its conventional counterpart in terms of: seed viability, germination, and persistence; plant phenotype under agronomic conditions; response to abiotic and biotic stress; and reproductive

Figure 4.1. The tiered hazard characterization system used by EFSA to address ecological concerns related to persistence, ferality, and invasiveness of transgenic plants, whether the genetically engineered crop itself or recipient wild plants due to gene flow. Reproduced from the EFSA 2010 report, Guidance on the environmental risk assessment of genetically modified plants.

Figure 4.1 (cont'd)

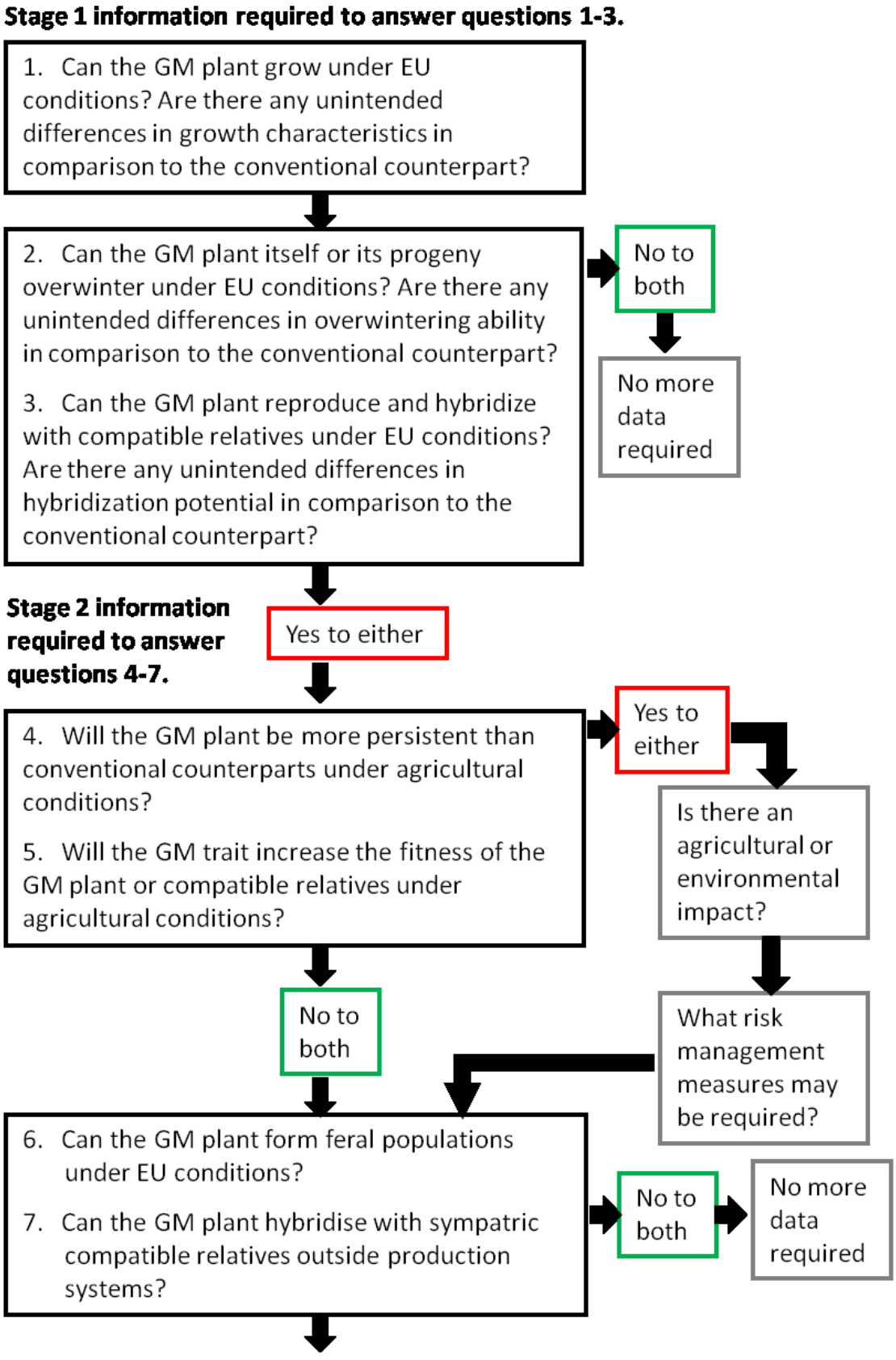
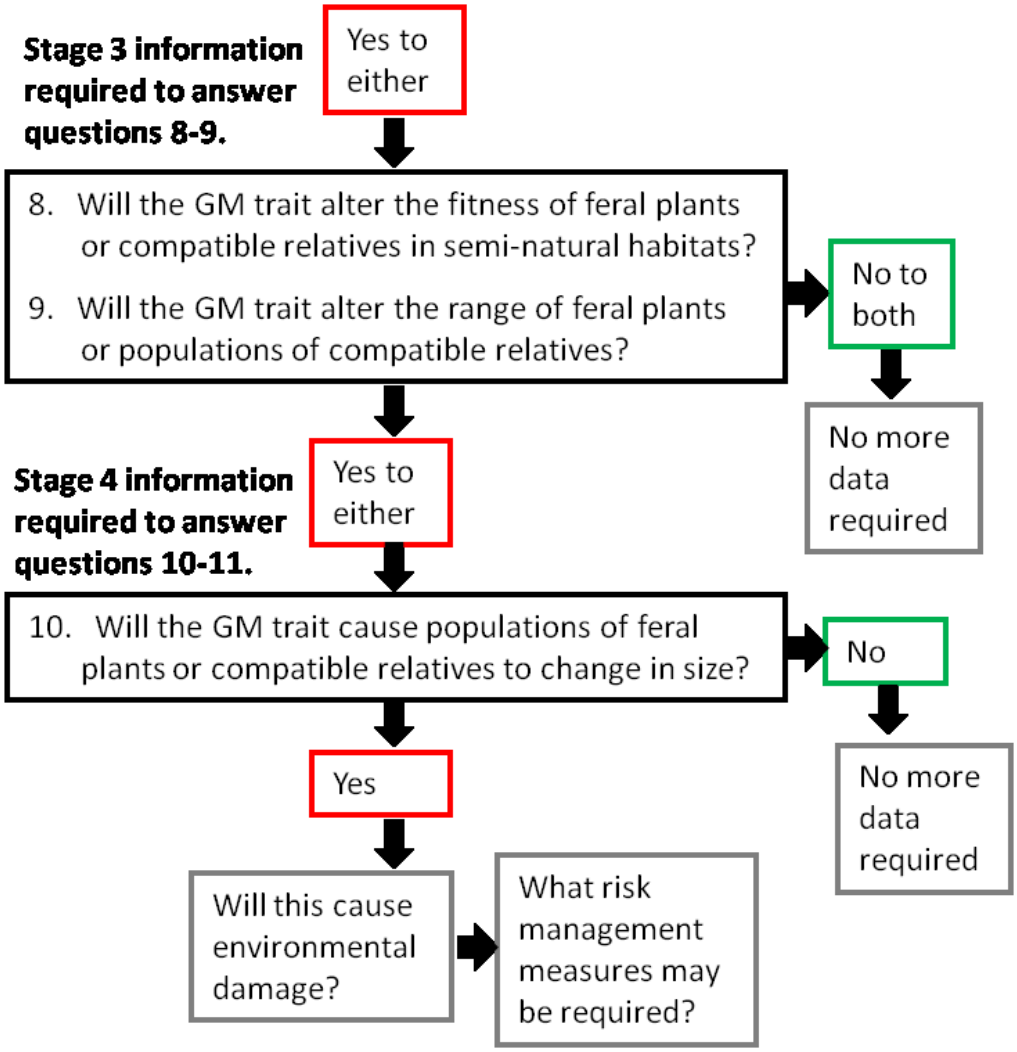


Figure 4.1 (cont'd)



biology. These data are used to answer questions related to the transgenic crop's fitness and persistence in agricultural settings and thus its ability to become feral and/or hybridize (stage 2).

If the transformed plant has altered fitness or is more able to persist than its counterpart, the protocol then examines trait-specific information to address the next two stages of questions. Stage 3 questions examine whether the transgene would confer altered fitness in semi-natural settings to either feral crops or recipient plants (hybrids or introgressed wild relatives) (Figure 4.1). This portion also acknowledges that fitness effects may differ outside of agricultural settings and in the presence of intra- and inter-specific competitors. Multi-year (2+) field experiments mimicking the disturbed sites common to ruderal areas are recommended. In addition, field and greenhouse treatments that include biotic and abiotic stresses should be performed to test for selective advantages in certain situations with data collection focused on plant survival and fecundity. Modeling is then incorporated to examine worst case scenarios.

When fitness effects are detected in semi-natural conditions or where the transgene could increase the range of recipient plants, additional modeling and experiments are suggested to determine whether the transgene could alter the population size of feral or recipient wild populations (stage 4, Figure 4.1). The report acknowledges effects on population dynamics from both enhanced and decreased fitness. Population sizes could increase as recipient plants become better competitors or gain access to a larger range due to increased fitness under adverse conditions, while populations could diminish due to outbreeding depression in semi-natural areas undergoing genetic swamping from nearby agricultural fields. The combination of growth chamber, field, modeling and background ecological data is then used to assess potential impacts on ecosystems from changing population sizes in recipient wild species.

Interactions with target and non-target species

As described in the guidance document, target species are generally pests or pathogens of the transformed crop, so these sections are specifically written for insect and pathogen resistant transgenic crops. Since generally abiotic stress tolerance transgenes do not impact pest or pathogen response, this section will not be reviewed in detail in this chapter. However, some

abiotic stress transgenes involved in regulatory or signaling pathways could activate pest or pathogen responsive genes. The production of the sugar alcohol mannitol in transgenic *Arabidopsis thaliana* lines expressing the biosynthetic enzyme M6PR, was shown to activate pathogen responsive genes, an unintended impact proposed to be due to the mannitol being perceived as a signal of fungal infection (Chan *et al*. 2011). Transcriptomic analysis could be utilized to determine whether biotic stress response genes have been affected by abiotic stress tolerance transgenes, and guide whether biotic stress hypothesis testing should be conducted. This aspect will be discussed below in greater detail.

Cultivation and management changes

Environmental concerns related to changes in crop cultivation and management could be significant for abiotic stress tolerant crops that have been engineered to allow successful cultivation of a crop in a region where it has not been grown previously. Using the freezing tolerant Eucalyptus example mentioned earlier, regulators would have to consider the environmental effects of not just the crop itself, but of managed short rotation forestry. Thus while the transgenic crop and its management system may be new to a country or region, cultivation, harvest and management practices are likely well established and documented elsewhere with known and predictable environmental impacts. The EFSA Guidance report specifies that regulators are to consider scientific and technical literature, field trials, data from cultivation in other countries and modeling studies in predicting the environmental impacts due to changes in cultivation methods due to the planting of the genetically engineered crop (EFSA 2010). These possible cultivation impacts are to then be considered in the context of existing known cultivation impacts on the environment from crop management systems across the EU.

The 2010 EFSA Guidance report on the ERA of genetically engineered plants provides specific guidelines for each step in an ecological risk assessment from problem formulation to risk management across multiple broad categories of sources of ecological harm with significant alterations from the prior system under the 2001 EC directive (EFSA 2010, EC 2001). In 2012 the EFSA GMO Panel issued its first recommendation on an abiotic stress tolerant crop,

Monsanto's DroughtGard maize (MON 87460) (EFSA 2012). While the panel did recommend this crop to the EU member states, the application was for food and feed only and so a full ecological risk assessment was not performed. Based on the examples discussed in the 2010 Guidance report, although the ecological risk assessment of abiotic stress tolerance enhanced crops would address multiple sources of environmental risk as detailed earlier, the ERA would likely focus on the possibility and possible harms from transgene persistence or gene flow to wild species (EFSA 2010).

The tiered hazard characterization system detailed in the guidance report section pertaining to persistence, invasiveness and gene flow from transgenic crops is a significant change from the past method (EC 2001), which has been termed the "bucket" method for the sheer quantity of detailed information required in a submission for the environmental release of a transgenic crop (Raybould 2010). This critical mass approach developed as regulators dealt with the first generation of transgenic crops which were generally one of four crops, maize, soybean, cotton and oilseed rape, transformed with a limited number of transgenes conferring either herbicide, insect, or virus resistance (Wilkinson & Tepfer 2009). But, with newer more complex traits, such an approach of gathering specific detailed data pertaining to every possible risk of release would significantly slow or halt commercialization efforts and increase the cost of completing the regulatory process. A tiered regulatory approach has been advocated for at least the past decade (Hancock 2003, Wilkinson *et al*. 2003) to focus data collection and analysis on crops that for which the combination of trait, gene, crop and location present elevated risks to the environment. For abiotic stress tolerance enhancing transgenes, one of the most critical aspects for the risk assessment will be the determination of the transgene's fitness impacts, whether detrimental, neutral or advantageous (Wilkinson & Tepfer 2009). The importance of this characterization is stressed in the guidance report as well as prior proposed tiered risk assessment systems (Hancock 2003, Wilkinson *et al*. 2003).

Assessing risk of abiotic stress tolerance enhancing transgenes by the measurement of secondary fitness effects

Given the importance of fitness to the environmental risks described earlier, such as the biodiversity, ferality, and invasiveness of recipient plants, the accurate measurement of transgene fitness impacts is, and will remain, critical for ecological risk assessments regardless of the method used, whether under current regulatory systems or revised systems like the one proposed by the EFSA Guidance report. First generation transgenes directly encoded the conferred traits (e.g. *Cry* genes encoded Bt toxins for insect resistance, altered enzymes for herbicide tolerance, viral coat proteins for virus resistance) (reviewed by Warwick *et al*. 2009). These traits were also of limited advantage outside of agricultural monocultures that generate the high insect, weed and disease pressures that necessitated the introduction of the transgenes. Unlike those transgenes, abiotic stress tolerance enhancing transgenes can operate indirectly by manipulating regulatory, signaling and metabolic pathways and target the environmental stresses that decrease plant survival and fitness in both agricultural and natural ecosystems (Grumet *et al*. 2011). Fitness assessments of first generation transgenic crops used a number of developmental and reproductive parameters to compare the performance of transgenic lines relative to untransformed lines and conventional cultivars (NRC 2002).

A second approach to measuring fitness involves measuring directly the contribution to the next generation from known genotypes growing in mixed competitive populations (Bourguet *et al.* 2004). This method requires knowing the starting genotypic frequency (i.e. the percent of each homozygous positive and negative) within the population and a method for determining the genotype of progeny seed. Used previously to assess the fitness effects of specific T-DNA insertions and EMS mutations (Gilliland *et al*. 1998, Roux *et al*. 2005), this fitness measurement method was also utilized to assess three transgenes which confer abiotic stress tolerance through differing methods (Chapter 2, 3).

The ability of these two fitness measurement approaches to predict the long-term risk of transgene establishment by three abiotic stress tolerance genes; *CBF3*, *SOS1* and *M6PR*, was compared using the model species *Arabidopsis thaliana* (Chan *et al*. 2012, Chapter 2, Chapter 3). These three transgenes were selected for their differing modes of action in conferring increased

salinity stress tolerance. CBF3 is an AP2 family transcription factor which activates an abiotic stress response regulon (Gilmour *et al*. 2000). SOS1 is a plasma membrane bound Na+/H+ antiporter which is activated in response to elevated levels of sodium ions (Shi *et al*.2000), and M6PR is a biosynthetic enzyme allowing the production of the compatible solute and osmoprotectant mannitol (Zhifang & Loescher 2003). Multiple independent lines for each transgene were assessed for fitness impacts in pure non-competitive and mixed populations, where transgenic competed against wild-type plants, across six field environments and in the three repeated experiments in the presence and absence of salinity stress. Pure line performance indicated significantly reduced fitness relative to wild-type in CBF3 overexpression lines, while SOS1 had relative fitness equal to WT. The M6PR lines had relative fitness levels which averaged better than wild-type across the six field seasons (Chapter 2). Either of these transgene effects on relative fitness, increased or decreased, would necessitate experiments to address stage 3 questions in environmental risk assessment in the EU.

Competition with wild-type plants was shown to alter the observed the fitness effects of the transgenes, however, with measured competitive fitness lower than predicted by pure line performance in all CBF3 and SOS1 transgenic lines assessed in the field (Chapter 2). In competition in the field one M6PR line was neutral while the other was observed to confer a competitive advantage across a range of field conditions. A similar competition effect was observed in the presence of salinity stress, where fitness gains observed in pure populations were not observed in competition with wild-type under the same conditions, such that none of the transgenes showed a competitive advantage against their wild-type competition in repeated greenhouse studies (Chapter 3). This difference in observed transgene fitness effects highlights a key factor in ERA field trial designs, and how significantly a difference in design can impact the data collected.

Given the importance of assessing transgene fitness impacts to the ecological risk assessment process, regulators will need to carefully consider and provide input on the design of field trials. The EFSA guidance report details suggestions for field trial location selection, study

design, choice of conventional comparator, and data analysis (EFSA 2010). It also acknowledges that the relative fitness effect of a transgene influenced by environment, the presence of inter- and intra-specific competition and the presence of abiotic or biotic stresses. To examine stage 3 questions pertaining to ferality, the report mandates the inclusion of disturbance field trials, in which perennial plant species have been removed prior to planting. Further examination of fitness effects on hybrid or introgressed wild relatives and transgene establishment is also based on modeling incorporating data from pure line assessments and the disturbance trials.

The study of CBF3, SOS1 and M6PR overexpression lines also compared observed changes in transgene frequency over six generations to the predicted frequencies from stochastic models incorporating a range of measured fitness values (Chapter 2). Non-competitive pure line performance fitness measurements were found to poorly predict transgene frequency, while models incorporating fitness values observed under competition, produced frequencies similar to observed trends. Given that transgenic plants that have been characterized to stages 3 and 4 are considered by the EFSA to be the most ecologically hazardous, these results have strong implications about the source of the data to be used in the suggested modeling. Based on the data collected about the fitness effects of the three abiotic stress tolerance transgenes (Chan *et al*. 2012, Chapters 2 & 3), field trials and modeling that substantially rely on pure line performance measures could miss critical information needed to predict the long-term performance of transgenic plants in competitive wild populations. Competitive fitness assessments could be crucial to predicting whether an abiotic stress tolerance transgene will increase the ferality of the transgenic crop or the invasiveness of recipient wild relatives, both unintended effects with significant environmental risks.

Assessing risk of abiotic stress tolerance enhancing transgenes using 'omic' approaches

Given the possibility of complex and unintended secondary effects of transgene expression, a variety of 'omic' analyses have been proposed for non-targeted molecular profiling and assessment of genetically engineered crops (Kuiper *et al*. 2003, Cellini *et al*. 2004, Hoekenga 2008, Ricroch *et al*. 2011). Concerns that these secondary effects could manifest at the

transcript, protein or metabolic levels has led some to suggest that ecological risk assessments incorporate transcriptomic, proteomic and metabolomic analyses as unbiased approaches to examine for unintended effects. These techniques have also been considered as means to confirm at the molecular level, the 'substantial equivalence' of transgenic lines undergoing the risk assessment process prior to deregulation (Millstone *et al*. 1999, Baudo *et al.* 2009, Beale *et al.* 2009).While not required in petitions for deregulation by current regulations, if utilized these 'omic' analyses would need to be carefully considered within the context of the varietal range and phenotypic impacts upon which selection could act.

First generation transgenic crops which utilized simple directly encoded traits were observed to have less impact at the molecular level than the differences between existing commercial varies. Transcriptomic analysis, via 9K cDNA microarray, of glutenin overexpressing wheat lines at multiple stages of development found the differences between the transformed and untransformed wheat lines was significantly less than the difference between the untransformed line and a commercial variety (0.06% and 5.59% respectively) (Baudo *et al*. 2006). Similar results were observed in transcriptional analysis of two transgenic glyphosate resistant soybean varieties and three conventional varieties that showed greater differences between the conventional varieties than between the transgenic varieties and the closest related conventional variety (Cheng *et al*. 2008). Greater transcriptomic effects were detected between radiation mutagenised rice lines and the non-mutagenised control lines, than between transformed rice lines and their respective controls (Batista *et al*. 2008). Differences in gene expression were greater between non-transgenic maize varieties than between insect resistant *cryIA(b)* expressing maize and its untransformed background (Coll *et al*. 2009, 2010). Expression differences due to environment (high and low nitrogen) were also found to exceed the effect of the transgene. Proteomic comparison of 12 independent transgenic *Arabidopsis thaliana* lines to their untransformed background in the presence and absence of cold stress found that the number of protein differences due to transgene insertion and expression was lower than the effect of the cold treatment on the wild-type background (Ren *et al*. 2009). Thus,

overall, the first generation transgenic crops caused lower molecular effects than the differences between conventionally bred varieties, lower than differences due to environment and less than that caused by mutagenesis. While these results do not directly apply to abiotic stress tolerant crops, they do indicate the importance of placing all 'omic' changes in the correct context.

The next generation of transgenic crops will express traits like abiotic stress tolerance using genes involved in regulatory, signaling and metabolic pathways, increasing the potential for unintended secondary effects which could alter a plant's molecular profile. Overexpression of the abiotic stress response transcription factor *CBF3* in *Arabidopsis thaliana* results in changes in expression level for 1350 genes in the absence of salinity stress and 1037 genes in the presence of salt stress (Chan *et al*. 2012). Overexpression of the sodium antiporter SOS1 under the same conditions altered, 619 and 845 transcript levels respectively, corresponding to previous evidence that the antiporter is stabilized and activated in response to salt stress (Qiu *et al*. 2003, Chung *et al*. 2008). The mannitol biosynthetic enzyme M6PR influenced the expression of 1719 genes in the absence of salt and 1001 in its presence (Chan *et al*. 2011). In comparison, salt stress on three wild-type ecotypes WS, Col and Col(gl) altered the expression levels of 1093, 2552 and 138 genes respectively, indicating that in terms of the number of transcripts affected all three transgenes were within the range caused by environmental effects (Chan *et al*. 2012). For CBF3 and M6PR substantial overlap was observed in the genes affected by the transgene and those affected by salt stress in wild-type plants, again stressing the importance of context, and not assuming risk purely based on the number of transcriptional changes.

For CBF3 and SOS1, the pathways of genes influenced by transgene expression matched known target genes and previously researched interactions (Chan *et al*. 2012). The biosynthetic enzyme M6PR, however, was shown to activate even more pathways than the transcription factor CBF3, including pathogen responsive genes. As mentioned earlier, this connection may be due to mannitol acting as a molecule signaling a fungal attack on the transformed plant (Chan *et al*. 2011). Whether the up-regulation of these pathogen responsive genes would confer a selective advantage under disease pressure has not yet been determined. In addition, the total

number of transcript expression differences was compared to changes in plant performance to examine whether transcriptomic analysis could predict the level of secondary phenotypic impact from transgene expression (Chan *et al*. 2011, 2012). But, the magnitude of transcriptomic changes relative to wild-type in the presence or absence of salinity stress was not indicative of the performance of a transgenic line relative to wild-type under the same conditions.

The use of transcriptomic analysis to test for unintended effects has also been utilized in the assessment of drought tolerant *Arabidopsis thaliana* overexpressing the abiotic stress responsive transcription factor *ABF3* (Abdeen *et al.* 2010). This analysis found that only under drought stress did the transcriptome of the transgenic lines differ from wild-type. Significant overlap was observed between the genes affected by drought stress in wild-type plants and those affected by drought in the ABF3 transgenic lines, with most showing an enhanced response in the transgenic plants compared to wild-type. The transgene-affected genes were shown to be in expected pathways responsive to abiotic stress, indicating no unintended pathway activations or repressions. The studies of ABF3 and M6PR transgenic plants demonstrate the capacity of transcriptomic analysis to detect unintended expression changes at the pathway and gene level. These results indicate that molecular profiling through 'omic' approaches may be able to guide further assessment inquiry but do not by themselves indicate levels of risk, and so must be considered in the context of the natural range of differences that exist between cultivars and within the same cultivar across differing environmental conditions.

Discussion

The agricultural challenges of the next century, both in terms of climate change and a growing world population, will require a diverse tool set. While significant advances in plant breeding have allowed selection to be performed at the molecular and genetic level and advanced imaging allows in field selection on a host of new trait linked phenotypes, these approaches are still limited by the complexity of stress responses and the availability and speed at which stress tolerance traits can be introgressed from sexually compatible germplasm (Mittler and Blumwald 2010, Varshney *et al*. 2011). The last decade of research has unveiled a broad suite of abiotic

stress tolerance genes which operate over many differing cellular and tissue level mechanisms to confer tolerance (Wang *et al*. 2003, Zhang *et al*. 2004a, Sreenivasulu *et al.* 2007, Bhatnagar-Mathur *et al.* 2008, Munn & Tester 2008, Warwick *et al.* 2009, Hirayama & Shinozaki 2010, Mittler and Blumwald 2010).

Concerns have been raised that the various national regulatory procedures, which capably addressed the ecological risks of the first generation of transgenic crops (Thies & Devare 2007), might not be able to handle to the more complex secondary effects of abiotic stress tolerance. This analysis examined the implications of abiotic stress tolerance crops on the ecological risk assessments critical to regulatory decision on transgenic crops. The EU system, considered one of the most stringent in the world, was examined for how the regulations would address the ecological risks from abiotic stress tolerant crops, namely the risks of ferality in the transformed crop, negative impacts on the genetic diversity of compatible wild relatives, enhanced invasiveness in wild or weedy relatives due to gene flow, or range expansion due to ecological release of recipient transgenic plants (Tiedje *et al.* 1989, Hoffman 1990, Hancock *et al.* 1996, Conner *et al*. 2003, Ellstrand 2003, Weaver and Morris 2005, Hails and Morley 2005).

The EU system addressed perceived weaknesses in its regulatory practices related to next generation traits and implemented, at least in part, a tiered ecological risk assessment system, long called for by scientific experts (Hancock 2003, Wilkinson 2003, Raybould 2010). The implementation of the 2010 system in the ecological risk assessment of abiotic stress tolerant crops is as of yet untested, as the recent EFSA GMO Panel approval of drought tolerant maize was a submission for import for feed and food only and so did not require a full ERA. In addition, the EU system allows for member states to object to EFSA approvals under the safeguard clause, resulting in protracted legal disputes over the legal status of genetically engineered crops in specific countries. For example, Austria has invoked the safeguard clause multiple times over the past five years to prevent the importation of genetically engineered oilseed rape lines, each time requiring substantial time and resources to examine their concerns, and each time their concerns have been found to be without scientific merit (EFSA 2013). Thus,

although the risk assessment system may have improved under the 2010 Guidance report on the environmental risk assessment of genetically modified plants, the EU system as a whole still leaves much to be desired from a biotechnological crop developer's perspective.

The use of 'omic' approaches to perform non-targeted review of transgene secondary effects has merit in guiding further lines of inquiry, but not in directly assessing ecological risk from phenotypic changes. For *CBF3*, *SOS1* and *M6PR* the total number of genes influenced by a transgene's expression in the presence or absence of salinity stress was not indicative of the performance of the transgenic plant under the same condition (Chan *et al*. 2012). This phenotypic buffering was also observed in an assessment of 162 recombinant inbred *Arabidopsis thaliana* lines bred from a cross between two ecotypes which differ genetically by at least 500,000 SNPs (Fu *et al*. 2009). These lines were assessed for over 40,000 molecular traits including transcript, protein and metabolite levels and examined for phenotypic changes across 139 morphological, developmental and stress response traits; however, only six loci for systemic change were found, indicating widespread buffering. The effects of this phenotypic buffering could decrease the phenotypic impact of almost any genetic change whether due to mutation or DNA insertion. On the other hand, by detecting the unintended up-regulation of biotic stress response genes in M6PR lines, transcriptomic analysis did demonstrate the potential for 'omic' approaches to guide inquiries into secondary transgene effects (Chan *et al*. 2011).

Field trials have been the most critical aspect of ecological risk assessments since the first transgenic crop was deregulated. The new European system has addressed changing environmental concerns due to new traits by altering the field trial requirements to better address transgene fitness effects on both the transformed crop and on wild interfertile recipients (EFSA 2010). Fitness effects are to be assessed across multiple sites and a minimum of two years, and transgenes which show fitness impacts are required to be examined under a range of field treatments including disturbance to mimic the effects of growing in disturbed habitats such as field margins or road sides. Although this treatment addresses interspecific competition, which affects the persistence and ferality of transgenic crops and has been shown to influence observed

transgene fitness effects (Fredshavn *et al*. 1995, Bartsch *et al*. 1996), it does not address the fitness effects under intra-specific competition from wild-type plants of the same species. Performance under intra-specific competition determines the long-term population dynamics that will occur after a transgene has introgressed into a wild species (Ellstrand 2003). In regulatory terms, for any environmental harm to occur the exposure rate has to increase (i.e. the transgene frequency within the wild population will need to increase). Under the EU system, transgene fitness effects on wild relatives, due to hybridization or introgression, are to be assessed in controlled environments (growth chamber or greenhouse) and predicted using models which incorporate the data collected in the field, greenhouse or growth chamber (EFSA 2010). Given the fitness differences observed in the study of three abiotic stress tolerance transgenes between field and controlled environments and between non-competitive and competitive assessments, and the effects those differences had on the accuracy of model predictions (Chapter 2 & 3), regulators will need to carefully consider and interpret the results of fitness studies in the context of realistic agricultural scenarios.

The abiotic stress tolerance traits that have the potential to aid farmers in feeding a growing world population while encountering increasing climatic uncertainty will pose greater challenges to the regulatory systems conducting ecological risk assessment than were faced from the limited and relatively low impact traits of first generation transgenic crops. With the use of ERAs that incorporate phenotypic and molecular approaches to assess for secondary transgene effects, the potential ecological risks in terms of ferality, genetic diversity, invasiveness and range expansion can be manageable. Tiered risk assessments should allow the focusing of resources on the gene/trait/crop/location combinations that are most likely to cause unintended environmental effects, without greatly hindering commercialization of transgenic crops which pose insignificant risks to the environment and potentially substantial benefits to farmers in need of crops capable of enduring a changing climate.

LITERATURE CITED

LITERATURE CITED

Abdeen, A., J. Schnell, and B. Miki, 2010: Transcriptome analysis reveals absence of unintended effects in drought-tolerant transgenic plants overexpressing the transcription factor *ABF3*. BMC Genomics 11, 69.

Abebe, T., Guenzi, A. C., Martin, B., & Cushman, J. C. (2003). Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiology, 131(4), 1748-1755.

APHIS (Animal and Plant Health Inspection Service of the United States Department of Agriculture), (2011a). Monsanto Company Petition (07-CR-191U) for determination of non-regulated status of event MON 87460. Draft Environmental Assessment http://www.aphis.usda.gov/brs/aphisdocs/09_05501p_dea.pdf (accessed March 2013).

APHIS (Animal and Plant Health Inspection Service of the United States Department of Agriculture), (2011b). Determination of Nonregulated Status for MON 87460 Corn *(Zea mays* L). http://www.aphis.usda.gov/brs/aphisdocs/09_05501p_det.pdf (accessed March 2013).

APHIS (Animal and Plant Health Inspection Service of the United States Department of Agriculture), (2013). Notice of intent to prepare an environmental impact statement for determination of nonregulated status of freeze tolerant Eucalyptus lines and notice of virtual public meetings. Federal Register**,** 78 (39) 13309- 13312. http://www.aphis.usda.gov/brs/fedregister/11_01901p_fr_noi.pdf (accessed April 2013).

Bartsch, D., Schmidt, B., Pohl-Orf, M., Haag, C., & Schuphan, I. (1996). Competitiveness of transgenic sugar beet resistant to beet necrotic yellow vein virus and potential impact on wild beet populations. Molecular Ecology, 5(2), 199-205.

Batelli G., Verslues P.E., Agius F., Qiu Q., Fujii H., Pan S., Schumaker K.S., Grillo S., Zhu J.K. (2007). SOS2 promotes salt tolerance in part by interacting with the vacuolar H^+-ATPase and upregulating its transport activity. Molecular and Cellular Biology 27: 7781–7790.

Batista, R., Saibo, N., Lourenço, T., & Oliveira, M. M. (2008). Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion. Proceedings of the National Academy of Sciences, 105(9), 3640-3645.

Baudo, M. M., Lyons, R., Powers, S., Pastori, G. M., Edwards, K. J., Holdsworth, M. J., & Shewry, P. R. (2006). Transgenesis has less impact on the transcriptome of wheat grain than conventional breeding. Plant Biotechnology Journal, 4(4), 369-380.

Baudo, M. M., Powers, S. J., Mitchell, R. A., & Shewry, P. R. (2009). Establishing substantial equivalence: transcriptomics. In *Transgenic Wheat, Barley and Oats* (pp. 247-272). Humana Press.

Beale, M. H., Ward, J. L., & Baker, J. M. (2009). Establishing substantial equivalence: metabolomics. In *Transgenic Wheat, Barley and Oats* (pp. 289-303). Humana Press.

Behnam, B., Kikuchi, A., Celebi-Toprak, F., Yamanaka, S., Kasuga, M., Yamaguchi-Shinozaki, K., & Watanabe, K. N. (2006). The Arabidopsis DREB1A gene driven by the stress-inducible rd29A promoter increases salt-stress tolerance in proportion to its copy number in tetrasomic tetraploid potato (Solanum tuberosum). Plant Biotechnology, 23(2), 169-177.

Behnam, B., Kikuchi, A., Celebi-Toprak, F., Kasuga, M., Yamaguchi-Shinozaki, K., & Watanabe, K. N. (2007). Arabidopsis rd29A:: DREB1A enhances freezing tolerance in transgenic potato. Plant Cell Reports, 26(8), 1275-1282.

Bhatnagar-Mathur, P., Vadez, V., & Sharma, K. K. (2008). Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Reports, 27(3), 411-424.

Bourguet, D., Guillemaud, T., Chevillon, C., & Raymond, M. (2004). Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. Evolution, 58(1), 128-135.

Bruinsma, J. (2009). The resource outlook to 2050: by how much do land, water and crop yields need to increase by 2050?. In *Proceedings of the Expert Meeting on How to Feed the World in 2050 24-26 June 2009, FAO Headquarters, Rome.* ftp://ftp.fao.org/docrep/fao/012/ak542e/ak542e00.pdf (accessed August 2012).

Chan, Z., Grumet, R., & Loescher, W. (2011). Global gene expression analysis of transgenic, mannitol-producing, and salt-tolerant *Arabidopsis thaliana* indicates widespread changes in abiotic and biotic stress-related genes. Journal of Experimental Botany, 62(14), 4787-4803.

Chan, Z., Bigelow, P. J., Loescher, W., & Grumet, R. (2012). Comparison of salt stress resistance genes in transgenic *Arabidopsis thaliana* indicates that extent of transcriptomic change may not predict secondary phenotypic or fitness effects. Plant Biotechnology Journal, 10(3), 284-300.

Castiglioni, P., Warner, D., Bensen, R. J., Anstrom, D. C., Harrison, J., Stoecker, M., ... & Heard, J. E. (2008). Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiology, 147(2), 446-455.

Cellini, F., Chesson, A., Colquhoun, I., Constable, A., Davies, H. V., Engel, K. H., ... & Smith, M. (2004). Unintended effects and their detection in genetically modified crops. Food and Chemical Toxicology, 42(7), 1089-1125.

Cheng, K. C., Beaulieu, J., Iquira, E., Belzile, F. J., Fortin, M. G., & Strömvik, M. V. (2008). Effect of transgenes on global gene expression in soybean is within the natural range of

variation of conventional cultivars. Journal of Agricultural and Food Chemistry, 56(9), 3057-3067.

Chinnusamy, V., Jagendorf, A., & Zhu, J. K. (2005). Understanding and improving salt tolerance in plants. Crop Science, 45(2), 437-448.

Chung, J. S., Zhu, J. K., Bressan, R. A., Hasegawa, P. M., & Shi, H. (2008). Reactive oxygen species mediate Na+-induced SOS1 mRNA stability in Arabidopsis. The Plant Journal, 53(3), 554-565.

Coll, A., Nadal, A., Collado, R., Capellades, G., Messeguer, J., Melé, E., ... & Pla, M. (2009). Gene expression profiles of MON810 and comparable non-GM maize varieties cultured in the field are more similar than are those of conventional lines. Transgenic Research, 18(5), 801-808.

Coll, A., Nadal, A., Collado, R., Capellades, G., Kubista, M., Messeguer, J., & Pla, M. (2010). Natural variation explains most transcriptomic changes among maize plants of MON810 and comparable non-GM varieties subjected to two N-fertilization farming practices. Plant Molecular Biology, 73(3), 349-362.

Collins, N. C., Tardieu, F., & Tuberosa, R. (2008). Quantitative trait loci and crop performance under abiotic stress: where do we stand?. Plant Physiology,147(2), 469-486.

Conner, A.J., Glare, T.R. and Nap, J.P. (2003). The release of genetically modified crops into the environment. II. Overview of ecological risk assessment. Plant Journal 33, 19–46.

Deikman, J., Petracek, M., & Heard, J. E. (2012). Drought tolerance through biotechnology: improving translation from the laboratory to farmers' fields. Current Opinion in Biotechnology, 23(2), 243-250.

Devos, Y., Demont, M., & Sanvido, O. (2008). Coexistence in the EU—return of the moratorium on GM crops?. Nature Biotechnology, 26(11), 1223-1225.

EC (European Community). (2001). Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities L106, 1-39.

Ellstrand, N. C. (2003). *Dangerous Liaisons?*. Johns Hopkins University Press.

EFSA (European Food Safety Authority). (2008). EFSA scientific colloquium summary report: environmental risk assessment of genetically modified plants - challenges and approaches. http://www.efsa.europa.eu/en/search/doc/colloquiagmoera.pdf (acessed April 2013).

EFSA (European Food Safety Authority). (2010). Guidance on the environmental risk assessment of genetically modified plants. http://www.efsa.europa.eu/en/efsajournal/pub/1879.htm (accessed April 2013).

EFSA (European Food Safety Authority). (2012). Scientific Opinion on an application (EFSA-GMO-NL-2009-70) for the placing on the market of genetically modified drought tolerant maize MON 87460 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto. http://www.efsa.europa.eu/en/efsajournal/doc/2936.pdf (accessed April 2013).

EFSA (European Food Safety Authority). (2013). Scientific Opinion on a request from the European Commission related to the prolongation of prohibition of the placing on the market of genetically modified oilseed rape events Ms8, Rf3 and Ms8 □ Rf3 for import, processing and feed uses in Austria. http://www.efsa.europa.eu/en/efsajournal/doc/2878.pdf

EPA (Environmental Protection Agency). (1998). Guidelines for Ecological Risk Assessment. EPA 630/R-95-002F, Washington, D.C.

ERS (Economic Research Service of the United States Department of Agriculture), (2013). U.S. Drought 2012: Farm and Food Impacts. http://www.ers.usda.gov/topics/in-the-news/us-drought-2012-farm-and-food-impacts.aspx (accessed April 2013).

Fredshavn, J. R., & Poulsen, G. S. (1996). Growth behavior and competitive ability of transgenic crops. Field Crops Research, 45(1), 11-18.

Fu, J., Keurentjes, J. J., Bouwmeester, H., America, T., Verstappen, F. W., Ward, J. L., ... & Jansen, R. C. (2009). System-wide molecular evidence for phenotypic buffering in *Arabidopsis*. Nature genetics, 41(2), 166-167.

Gao, F., Gao, Q., Duan, X., Yue, G., Yang, A., & Zhang, J. (2006). Cloning of an H+-PPase gene from *Thellungiella halophila* and its heterologous expression to improve tobacco salt tolerance. Journal of Experimental Botany, 57(12), 3259-3270.

GAO, S., YUAN, L., ZHAI, H., LIU, C. L., HE, S. Z., & LIU, Q. C. (2012). Overexpression of *SOS* genes enhanced salt tolerance in Sweetpotato. Journal of Integrative Agriculture, 11(3), 378-386.

Gilliland, L. U., McKinney, E. C., Asmussen, M. A., & Meagher, R. B. (1998). Detection of deleterious genotypes in multigenerational studies. I. Disruptions in individual *Arabidopsis* actin genes. Genetics, 149(2), 717-725.

Gilmour, S. J., Sebolt, A. M., Salazar, M. P., Everard, J. D., & Thomashow, M. F. (2000). Overexpression of the *Arabidopsis CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology, 124(4), 1854-1865.

Goodess, C. M. (2013). How is the frequency, location and severity of extreme events likely to change up to 2060?. Environmental Science & Policy. 27(s1), s4–s14.

Grumet, R., Wolfenbarger, L., & Ferenczi, A. (2011). Future possible genetically engineered crops and traits and their potential environmental impacts. In *Environmental safety of genetically engineered crops*. Michigan State University Press.

Hails, R. S., & Morley, K. (2005). Genes invading new populations: a risk assessment perspective. Trends in Ecology & Evolution, 20(5), 245-252.

Hancock, J. F., R. Grumet, and S. C. Hokanson. (1996). The opportunity for escape of engineered genes from transgenic crops. HortScience 31:1080–1085.

Hancock, J. F. (2003). A framework for assessing the risk of transgenic crops. BioScience, 53(5), 512-519.

Hancock, J. (2011). Factors influencing the genetic diversity of plant species and the potential impact of transgene movement. In *Environmental safety of genetically engineered crops*. Michigan State University Press.

Hill, R. A., & Sendashonga, C. (2003). General principles for risk assessment of living modified organisms: lessons from chemical risk assessment. Environmental Biosafety Research, 2(2), 81.

Hirayama, T., & Shinozaki, K. (2010). Research on plant abiotic stress responses in the post-genome era: past, present and future. The Plant Journal,61(6), 1041-1052.

Hoffman, C. A. (1990). Ecological risks of genetic engineering of crop plants. BioScience, 40(6), 434-437.

Hoekenga, O. A. (2008). Using metabolomics to estimate unintended effects in transgenic crop plants: problems, promises, and opportunities. Journal of Biomolecular Techniques, 19(3), 159.

Horn, G., Hofweber, R., Kremer, W., & Kalbitzer, H. R. (2007). Structure and function of bacterial cold shock proteins. Cellular and Molecular Life Sciences, 64(12), 1457-1470.

Hsieh, T. H., Lee, J. T., Charng, Y. Y., & Chan, M. T. (2002). Tomato plants ectopically expressing Arabidopsis *CBF1* show enhanced resistance to water deficit stress. Plant Physiology, 130(2), 618-626.

Hu H, DaiM, Yao J, Xiao B, Li X, Zhang Q, Xiong L (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proceedings of the National Academy of Sciences USA, 35: 12987– 12992.

Hu H, You J, Fang Y, Zhu X, Qi Z, Xiong L (2008) Characterization of transcription factor gene *SNAC2* conferring cold and salt tolerance in rice. Plant Molecular Biology, 67: 169–181.

Jaffe, G. (2004). Regulating transgenic crops: a comparative analysis of different regulatory processes. Transgenic Research, 13(1), 5-19.

Jaglo K.R., Kleff S., Amundsen K.L., Zhang X., Haake V., Zhang J.Z., Deits T., Thomashow M.F. (2001). Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiology, 127:910–917.

James V.A., Neibaur I., Altpeter F. (2008). Stress inducible expression of the DREB1A transcription factor from xeric, *Hordeum spontaneum* L in turf and forage grass (*Paspalum notatum Flugge*) enhances abiotic stress tolerance. Transgenic Research, 17, 93–104.

Kasuga, M., Miura, S., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2004). A combination of the *Arabidopsis DREB1A* gene and stress-inducible *rd29A* promoter improved drought-and low-temperature stress tolerance in tobacco by gene transfer. Plant and Cell Physiology, 45(3), 346-350.

Konstantinova T, Parvanova D, Atanassov A, Djilianov D. (2002). Freezing tolerant tobacco, transformed to accumulate osmoprotectants. Plant Science, 163:157–164

Kuiper, H. A., Kok, E. J., & Engel, K. H. (2003). Exploitation of molecular profiling techniques for GM food safety assessment. Current Opinion in Biotechnology, 14(2), 238-243.

Mantri, N., Patade, V., Penna, S., Ford, R., & Pang, E. (2012). Abiotic stress responses in plants: present and future. In *Abiotic Stress Responses in Plants* (pp. 1-19). Springer New York.

Mian, A., Oomen, R. J., Isayenkov, S., Sentenac, H., Maathuis, F. J., & Véry, A. A. (2011). Over-expression of an Na+-and K+-permeable HKT transporter in barley improves salt tolerance. The Plant Journal, 68(3), 468-479.

Millstone, E., Brunner, E., & Mayer, S. (1999). Beyond 'substantial equivalence'. Nature, 401(6753), 525-526.

Mittler, R., & Blumwald, E. (2010). Genetic engineering for modern agriculture: challenges and perspectives. Annual Review of Plant Biology, 61, 443-462.

Munns, R. and Tester, M. 2008. Mechanism of salinity tolerance. Annual Review of Plant Biology. Vol.59, 651-681.

Munns, R., James, R. A., Xu, B., Athman, A., Conn, S. J., Jordans, C., ... & Gilliham, M. (2012). Wheat grain yield on saline soils is improved by an ancestral Na+ transporter gene. Nature Biotechnology, 30(4), 360-364.

Navarro, M., Martinez, Y., Laur, J., El Kayal, W., Marque, and C., Teulieres, C. (2011). Two EguCBF1 genes overexpressed in Eucalyptus display a different impact on stress tolerance and plant development. Plant Biotechnology Journal, 9(1), 50-63.

Nehra, N.S. & Pearson, L. (2011). Petition for Determination of Non-regulated Status for Freeze Tolerant Hybrid *Eucalyptus* Lines. ArborGen Inc. Summerville, SC.

Nickson, T. E. (2008). Planning environmental risk assessment for genetically modified crops: problem formulation for stress-tolerant crops. Plant Physiology, 147(2), 494-502.

National Research Council (NRC) Staff & Board on Agriculture and Natural Resources Staff. (2002). *Environmental effects of transgenic plants: the scope and adequacy of regulation*. National Academies Press.

Oh, S. J., Kim, Y. S., Kwon, C. W., Park, H. K., Jeong, J. S., & Kim, J. K. (2009). Overexpression of the transcription factor AP37 in rice improves grain yield under drought conditions. Plant Physiology, 150(3), 1368-1379.

Olías, R., Eljakaoui, Z., Pardo, J. M., & Belver, A. (2009). The Na+/H+ exchanger SOS1 controls extrusion and distribution of Na+ in tomato plants under salinity conditions. *Plant signaling & behavior*, *4*(10), 973-976.

Pellegrineschi, A., Reynolds, M., Pacheco, M., Brito, R. M., Almeraya, R., Yamaguchi-Shinozaki, K., & Hoisington, D. (2004). Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. Genome, 47(3), 493-500.

Qiu, Q. S., Barkla, B. J., Vera-Estrella, R., Zhu, J. K., & Schumaker, K. S. (2003). Na+/H+ exchange activity in the plasma membrane of Arabidopsis. Plant Physiology, 132(2), 1041-1052.

Raybould, A. (2010). The bucket and the searchlight: formulating and testing risk hypotheses about the weediness and invasiveness potential of transgenic crops. Environmental Biosafety Research, 9(03), 123-133.

Reeves, W. R. (2010). Petition for the Determination of Non-Regulated Status for MON 87460. The Monsanto Company, St. Louis MO.

Regal, P. J. (1994). Scientific principles for ecologically based risk assessment of transgenic organisms. Molecular Ecology, 3(1), 5-13.

Ren, Y., Lv, J., Wang, H., Li, L., Peng, Y., & Qu, L. J. (2009). A comparative proteomics approach to detect unintended effects in transgenic< i> Arabidopsis</i>. Journal of Genetics and Genomics, 36(10), 629-639.

Ricroch, A. E., Bergé, J. B., & Kuntz, M. (2011). Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. Plant Physiology, 155(4), 1752-1761.

Rieseberg, L. H., Kim, S. C., Randell, R. A., Whitney, K. D., Gross, B. L., Lexer, C., & Clay, K. (2007). Hybridization and the colonization of novel habitats by annual sunflowers. Genetica, 129(2), 149-165.

Richards, R. A., Rebetzke, G. J., Watt, M., Condon, A. T., Spielmeyer, W., & Dolferus, R. (2010). Breeding for improved water productivity in temperate cereals: phenotyping, quantitative trait loci, markers and the selection environment. Functional Plant Biology, 37(2), 85-97.

Rommens, C. M. (2010). Barriers and paths to market for genetically engineered crops. Plant Biotechnology Journal, 8(2), 101-111.

Roux, F., Camilleri, C., Bérard, A., & Reboud, X. (2005). Multigenerational versus single generation studies to estimate herbicide resistance fitness cost in Arabidopsis thaliana. Evolution, 59(10), 2264-2269.

Saint Pierre, C., Crossa, J. L., Bonnett, D., Yamaguchi-Shinozaki, K., & Reynolds, M. P. (2012). Phenotyping transgenic wheat for drought resistance. Journal of experimental botany, 63(5), 1799-1808.

Sanvido, O., Romeis, J., Gathmann, A., Gielkens, M., Raybould, A., & Bigler, F. (2012). Evaluating environmental risks of genetically modified crops: ecological harm criteria for regulatory decision-making. Environmental Science & Policy, 15(1), 82-91.

Savitch, L. V., Allard, G., Seki, M., Robert, L. S., Tinker, N. A., Huner, N. P., ... & Singh, J. (2005). The effect of overexpression of two *Brassica* CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. Plant and Cell Physiology, 46(9), 1525-1539.

Sheveleva E, Chmara W, Bohnert HJ, Jensen RG (1997) Increased salt and drought tolerance by D-ononitol production in transgenic Nicotiana tabacum L. Plant Physiol 115:1211–1219

Sheveleva EV, Marquez S, Chmara W, Zegeer A, Jensen RG, Bohnert HJ (1998) Sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco. Plant Physiol 117:831–839

Shi, H., Ishitani, M., Kim, C., & Zhu, J. K. (2000). The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. Proceedings of the National Academy of Sciences, 97(12), 6896-6901.

Smedema, L. K., & Shiati, K. (2002). Irrigation and salinity: a perspective review of the salinity hazards of irrigation development in the arid zone. Irrigation and drainage systems, 16(2), 161-174.

Sreenivasulu, N., Sopory, S. K., & Kavi Kishor, P. B. (2007). Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene, 388(1), 1.

Suter, G.W. II. (1993). *Ecological Risk Assessment*. Lewis Publishers.

Suter, G.W. II. (2000). Generic assessment endpoints are needed for ecological risk assessment. Risk analysis, 20(2), 173-178.

Tavakkoli, E., Fatehi, F., Rengasamy, P., & McDonald, G. K. (2012). A comparison of hydroponic and soil-based screening methods to identify salt tolerance in the field in barley. Journal of Experimental Botany, 63(10), 3853-3867.

Tiedje, J. M., Colwell, R. K., Grossman, Y. L., Hodson, R. E., Lenski, R. E., Mack, R. N., & Regal, P. J. (1989). The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecology, 70(2), 298-315.

Thies, J. E., & Devare, M. H. (2007). An ecological assessment of transgenic crops. Journal of Development Studies, 43(1), 97-129.

Varshney, R. K., Bansal, K. C., Aggarwal, P. K., Datta, S. K., & Craufurd, P. Q. (2011). Agricultural biotechnology for crop improvement in a variable climate: hope or hype?. Trends in Plant Science, 16(7), 363-371.

Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 218(1), 1-14.

Warwick, S. I., Beckie, H. J., & Hall, L. M. (2009). Gene flow, invasiveness, and ecological impact of genetically modified crops. Annals of the New York Academy of Sciences, 1168(1), 72-99.

Weber, V. S., Melchinger, A. E., Magorokosho, C., Makumbi, D., Bänziger, M., & Atlin, G. N. (2012). Efficiency of managed-stress screening of elite maize hybrids under drought and low nitrogen for yield under rainfed conditions in Southern Africa. Crop Science, 52(3), 1011-1020.

Weaver, S. A., & Morris, M. C. (2005). Risks associated with genetic modification:–An annotated bibliography of peer reviewed natural science publications. Journal of Agricultural and Environmental Ethics, 18(2), 157-189.

Wei, A., He, C., Li, B., Li, N., & Zhang, J. (2011). The pyramid of transgenes TsVP and BetA effectively enhances the drought tolerance of maize plants. Plant Biotechnology Journal, 9(2), 216-229.

White, J. W., Andrade-Sanchez, P., Gore, M. A., Bronson, K. F., Coffelt, T. A., Conley, M. M., ... & Wang, G. (2012). Field-based phenomics for plant genetics research. Field Crops Research, 133, 101-112.

Wilkinson, M. J., Sweet, J., & Poppy, G. M. (2003). Risk assessment of GM plants: avoiding gridlock?. Trends in Plant Science, 8(5), 208-212.

Wilkinson, M., & Tepfer, M. (2009). Fitness and beyond: Preparing for the arrival of GM crops with ecologically important novel characters. Environmental Biosafety Research, 8(1), 1.

Witt, S., Galicia, L., Lisec, J., Cairns, J., Tiessen, A., Araus, J. L., ... & Fernie, A. R. (2012). Metabolic and phenotypic responses of greenhouse-grown maize hybrids to experimentally controlled drought stress. Molecular Plant, 5(2), 401-417.

Wolt, J. D., Keese, P., Raybould, A., Fitzpatrick, J. W., Burachik, M., Gray, A., ... & Wu, F. (2010). Problem formulation in the environmental risk assessment for genetically modified plants. Transgenic Research, 19(3), 425-436.

Xiao, B. Z., Chen, X., Xiang, C. B., Tang, N., Zhang, Q. F., & Xiong, L. Z. (2009). Evaluation of seven function-known candidate genes for their effects on improving drought resistance of transgenic rice under field conditions. Molecular Plant, 2(1), 73-83.

Yue, Y., Zhang, M., Zhang, J., Duan, L., & Li, Z. (2012). *SOS1* gene overexpression increased salt tolerance in transgenic tobacco by maintaining a higher K+/Na+ ratio. Journal of Plant Physiology,169(3), 255-261.

Zhang, J. Z., Creelman, R. A., & Zhu, J. K. (2004a). From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant Physiology, 135(2), 615-621.

Zhang, X., Fowler, S. G., Cheng, H., Lou, Y., Rhee, S. Y., Stockinger, E. J., & Thomashow, M. F. (2004b). Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant Arabidopsis. The Plant Journal, 39(6), 905-919.

Zhifang, G., & Loescher, W. H. (2003). Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer. Plant, Cell & Environment, 26(2), 275-283.

Conclusions and future work

The changing global climate will pose significant environmental challenges to the farmers of the next century. Whether bred through conventional methods including molecular breeding techniques, or produced using transgenesis, crops better able to tolerant abiotic stress will be critical to feeding a growing human population under these difficult conditions. While there have been environmental concerns about all transgenic crops, the genes conferring abiotic stress tolerance function in more indirect manners (by altering regulatory, signaling or metabolic pathways) than the transgenes which have been previously commercialized (e.g. herbicide, insect and virus resistance). These indirect modes of action could result in more complex secondary effects than those observed with prior transgenic traits. Both the primary and possible secondary effects of abiotic stress tolerance transgenes raise a number of environmental concerns about the potential for the transgene to alter the fitness or ecological range of the crop or, through gene flow, recipient compatible wild species.

This dissertation examined the fitness effects of three abiotic stress tolerance transgenes which function by differing modes of action to confer increase salinity tolerance to *Arabidopsis thaliana* and assessed whether the observed transgene fitness impacts differed between a variety of environmental conditions and the presence or absence of competition from wild-type plants. The field studies of Chapter 2 determined that the fitness effects observed in lines from all three transgenes were significantly reduced in competition from what would be predicted from their performance relative to wild-type in pure populations. These differences led to significantly different outcomes in models that predicted the frequency of transgenic plants in simulated competitive populations. Models incorporating fitness values from non-competitive fitness values overestimated transgene frequency compared to the transgene frequency trends observed

across six generations in the field. Ecological risk assessments models designed to predict the likelihood of transgene establishment could have limited accuracy if they utilize transgene fitness measurements assessed under non-competitive conditions.

Chapter 3 examined whether the presence of salinity stress would increase the competitive ability of the transgenic lines. Although significantly increased fitness relative to wild-type was observed in pure populations subjected to salt stress in the growth chamber (by Zhulong Chan) and in the greenhouse, the presence of wild-type competitors again limited transgene fitness, resulting in no transgenes conferring a competitive advantage under competitive conditions. The lower transgene fitness observed in pure greenhouse populations compared to pure growth chamber populations may be due to the combinations of stresses (i.e. salt, high light and temperature) that co-occur in the less tightly controlled greenhouse environment. Recent transcriptomic analyses indicate that the reduction in fitness observed in the transgenic lines under competition in the field and greenhouse may be due transcriptional and physiological changes which occur in *Arabidopsis thaliana* plants in response to competition, these include an up-regulation of growth and photosynthesis related genes and the down regulation of stress response genes. Due to constitutive overexpression of abiotic stress response genes, our transgenic lines likely would have been unable perform the same changes as wild-type plants, possibly resulting in differing growth patterns in competition than in pure populations where all plants have the same genotype.

The implication of this research and abiotic stress tolerance traits in general for ecological risk assessments were examined in chapter 4 as part of the requirements for my USDA National Needs Fellowship to examine genetic engineering for abiotic stress tolerance in a broader societal context. Given the calls for regulatory changes to better assess next generation

biotechnological traits, the recently updated EFSA system for the environmental risk assessment of genetically modified plants was examined in the context of abiotic stress tolerant crops. The recommended adoption of tiered ecological risk assessment systems are intended to allow greater focus on gene/trait/crop/location combinations that are most risky to the receiving environment while allowing transgenic crops which pose negligible risks to be commercialized in a reasonable time span. For example, M6PR overexpression was shown to have the potential to increase plant fitness compared to wild-type. However, transgenic M6PR maize for planting in Europe would likely pose insignificant environmental risks due the inability of maize to persist and become feral and the lack of compatible wild relatives, while planting the same transgenic cultivar in Mexico, the crop's center of origin, should necessitate further risk assessment. Although assessing transgene fitness impacts on recipient plants is critical for an ecological risk assessment, these results must be considered within the context of all four environmental risk factors; gene, trait, crop and location.

The results of this dissertation research indicate significant competitive impacts on the observed fitness of transgenic plants with enhanced abiotic stress tolerance. Although this affect was observed in the field across six seasons and in the greenhouse in the presence and absence of salt stress, these results will need to be confirmed in a crop species. The methodology utilized in this dissertation, designed to mimic a worst case scenario of transgene introgression into a wild population, can be easily adapted to most crops. The most critical aspect is the ability to genotype progeny seed from competitive populations. Given that most transgenic plants co-express some form of selectable marker (e.g antibiotic or herbicide resistance) it should be fairly simple to adapt this method to a new crop/transgene combination. Corroboration of these findings in a crop species would strengthen the need to more closely consider the effects of

competition in ecological risk assessments. Given that, in this study, competition reduced the observed fitness effects of all three transgenes compared to pure line performance, abiotic stress tolerance may be a less hazardous trait under competitive non-agronomic conditions than has previously been assumed from non-competitive assessments.

This methodology has the additional potential for plant breeders utilizing molecular breeding strategies to enhance abiotic stress tolerance in crop species. Traditional breeding strategies for this trait have been limited by the uneven abiotic stress levels present in field plots. This stress heterogeneity and variability reduces the gains from selection and increases the chance that promising material may be culled due to where and when its plot was located. By planting with a mix of genotypes at known proportions, all plants will experience the full range of stress present in that field, regardless of underlying soil variations. The field could then be harvested normally and a subset of the bulked seed genotyped by either 'seed chip' technologies or screening of progeny seedlings. Changes in the genotypic ratio of progeny seed relative to the known planting ratios would indicate a yield advantage under the environmental conditions present in that field site.

Lastly, given that it was the only one of three transgenes which showed beneficial effects across six field seasons and in the greenhouse, M6PR warrants further inquiry. Pure populations overexpressing this transgene consistently showed enhanced partitioning to seed resulting in increased fitness relative to wild-type. M6PR lines were also the only lines which performed equal to or better than wild-type in direct competition. The prior transcriptomic analysis of M6PR lines indicated significant changes in gene expression, resulting in the pre-activation of stress response genes and an unexpected up-regulation of biotic stress response genes. Whether this up-regulation confers a disease resistant phenotype remains to be determined, but M6PR, by

demonstrating increased abiotic stress tolerance with no observed cost of resistance and enhancement of harvest index, demonstrated its potential for crop improvement and the need for further research.

APPENDIX

Supplemental material related to field study design

The measurement of plant density in natural Arabidopsis thaliana populations

To determine whether the planting density utilized in the field and greenhouse experiments (see Chapter 2, 3) was within the range of densities found in natural populations, ten natural *Arabidopsis thaliana* populations were located with the assistance of Dr. Brainard in late September 2009. These populations were distributed in disturbed field margins surrounding a field of broccoli. All populations were at most ~3 months old, as the entire site was sprayed with glyphosate prior to planting the vegetable field in mid-June. Since the purpose of the density measurements was to determine the highest natural population densities, the tem most dense patches of *Arabidopsis thaliana* were selected. A 26x26 cm quadrant, matching the pot size utilized in the field and greenhouse studies, was placed down and all *Arabidopsis thaliana* plants from cotyledon to senescence were counted. Population densities ranged from 770 to 3250 plants/m^2, with a mean density of 1845±219 SEM (Figure S.1). All counted populations contained plants at multiple stages of vegetative and reproductive development (Figure S.2). Some populations were visibly younger containing no mature plants (3/10 populations), however most contained mature plants which were already setting seed. Seedlings were visible beneath some senesced plants indicating that two generations of plants were present within the population. Given the time constraint from the vegetable field preparation in June, these populations contained at least some individuals with no vernalization requirement and low seed dormancy.

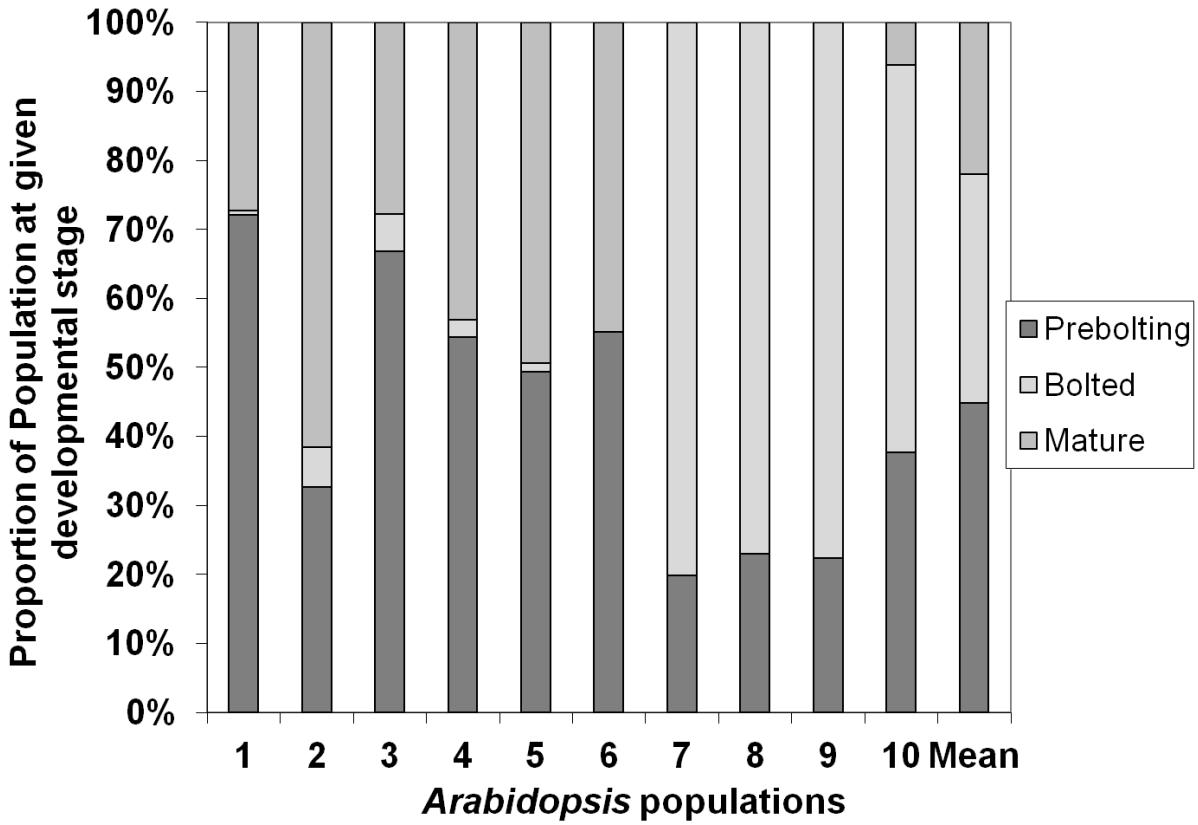


Figure S.1. The proportion of plants within ten natural *Arabidopsis thaliana* populations at three broad developmental and reproductive stages. These categories were: pre-bolting, which included all plants from the cotyledon to rosette stages of vegetative development; bolted, which included plants which had bolted but not yet flowered and flowering plants without developed siliques; and mature, which included flowering plants with well developed siliques and plants undergoing senescence.

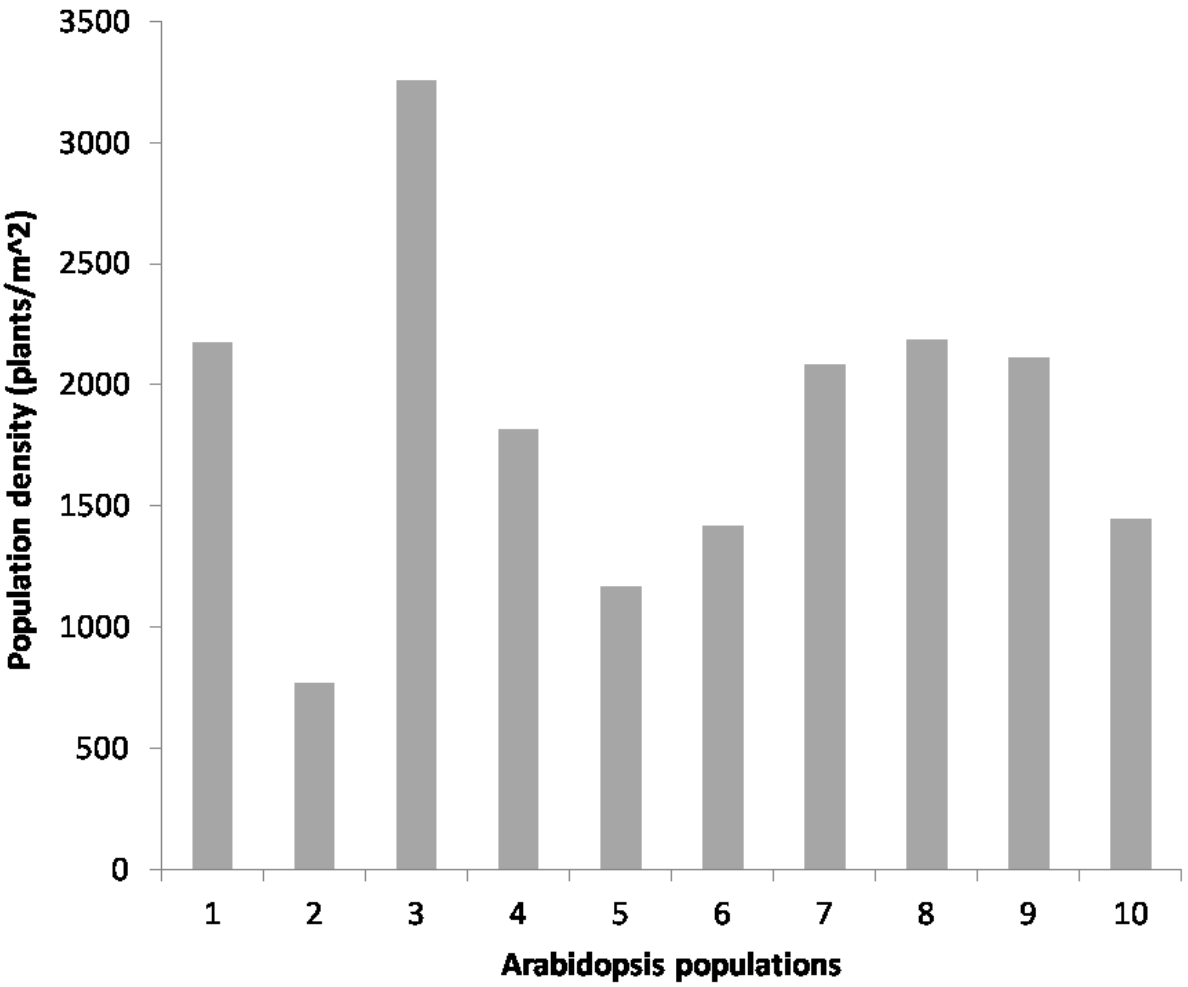


Figure S.2. The density in plants per square meter of ten natural *Arabidopsis thaliana* populations near East Lansing, MI. All ten populations were in disturbed ground along the margins of a broccoli field at the 'Sand Hill site' near the Michigan State University Tree Research Facility. Populations were distributed over several acres. Population density was determined by counting all *Arabidopsis thaliana* plants within a 26x26 cm quadrant.

Variability study to improve the growth of *Arabidopsis thaliana* in the growth chamber and greenhouse

To optimize lab protocols for the growth *Arabidopsis thaliana* in the growth chamber and greenhouse, an experiment was performed using two wild-type ecotypes Wassilewskija (WS) and Columbia (Col), two soil mixtures Baccto potting soil (Michigan Peat Company) and Redi-Earth Plug & Seedling mix (Sun Gro Horticulture Canada Ltd.), and three pot sizes ranging from a standard greenhouse flat (L-1020, Landmark Plastics) to small pots which fit 18 (L-1801, Landmark Plastics) or 36 pots per flat (L-3601, Landmark Plastics) . The seeds were sterilized using 1 mL 75% ethanol for 1 minute followed by 1 mL 15% bleach with 0.0025% SDS for 15 minutes. After washing five times with sterile dH_2O, all seed was stratified at 4°C for 4days prior to planting. Seed from each ecotype was planted into each soil/pot combination. Five seeds were direct seeded to damp soil in each small pot and then thinned to one after the development of four true leaves. The experiment was also performed in the growth chamber, excluding the full sized flats. For the growth chamber seeds were germinated on sterile ½ MS media and transplanted at the four true leaf stage into damp soil. All watering was performed via subsoil irrigation using 1/2 strength Hoagland's solution. Pots were allowed to saturate prior to removal of excess solution, with watering performed as needed. All pots were randomized after each watering to reduce location effects.

The days till germination, four-leaf stage, bolting, flowering, and senesce were recorded for plant. Rosette diameter was measured twice weekly and seed yield determined at harvest. WS wild-type seed production was found to exceed that of Col by 1.5-2 fold under the same condition (location, pot size and soil type). While WS performed equally well on either soil mix, Col yielded ~50% less when grown in Red-Earth than in Baccto. After controlling for the affects of soil type, pot-size was found to affect fecundity and plant growth with the larger pot-sizes,

full-tray and 18 pot flats out growing and out yielding the 36 pot flats. To prevent ecotype bias Baccto potting soil was utilized in all greenhouse and field experiments.

Preliminary field study to assess viability of experimental design and methods

To develop the necessary methodology to perform a large scale field study with *Arabidopsis thaliana*, a three-quarters scale field study was performed under APHIS permit in the summer and fall of 2007 at the Michigan State University Horticultural Teaching and Research Farm. The design for the greenhouse experiments was adapted for field conditions. Three CBF3/DREB1a lines (A28, A30, and A40) and three M6PR lines (M2-1, M5-1, M7-6) were used in the pilot experiment. Lines from a third transgene, the vacuolar sodium transporter *NHX1*, were also assessed however these lines were later determined to have been silenced by Zhulong Chang. Seed from each transgenic line was mixed at a 50/50 ratio with seed from its corresponding wild-type background, WS for CBF3/DREB1a and Col for M6PR, for a total of 100 seeds per population and a total of 14 replicates were planted for each mixed population. All seed was stratified at 4°C for 3 days prior planting. Seed mixes were scattered randomly on to 26x26 cm trays in pre-moistened standard Baccto potting soil mix on 7/28/07. Seeded trays were germinated in a hoop house and then transported to the field after reaching the four-leaf stage of development.

A tray-in-tray potting method was developed for the field to allow for subsoil irrigation via trickle hose (Figure S.3a). The larger lower tray also allowed for secure anchoring to the ground, using 6 inch landscaping stakes, to prevent movement or tipping of the trays. To protect the exposed plants from extreme weather conditions and prevent movement of plants or seeds from the trial site, the trays were covered with clear plastic lids secured to the anchor stakes by bungee cords. The lids were deployed no more than six hours ahead of damaging weather and

removed once the weather broke. Four drainage holes were drilled into the irrigation tray approximately 1 inch from the bottom to allow excess rainwater to drain off. This system successfully protected the plants from driving rains, sleet, early frost, and sustained high winds.

Plants were allowed to mature in the field; mature stems were harvested by hand prior to shattering of the siliques to prevent in-field seed dispersal. Each tray was harvested 3 to 4 times from 9/12/07 to the final harvest on 10/24/07. Harvested plant matter was placed into paper bags and allowed to dry for at least two weeks before the seed was removed. Each bag represented the mature seed for a single mixed population from a specific date. The seed from each bag of dried plant material was harvested individually and the seed stored in separate Eppendorf tubes. The separate seed collections were scored individually to develop a series of time points for each mixed population to determine the relative seed production of the wild-type and transgenic lines over time in competitive environments. These data give a record of the average production of seed over time and enable us to determine the most appropriate time(s) to harvest future studies for each line.

Soil overheating was considered to be a possible design problem, so a set of trays planted with wild-type plants, Columbia ecotype, were buried into the soil up to the rims of the trays while a control set was set up in the manner described earlier. Temperatures of the soil and of the base trays were recorded on sunny days. The largest difference between buried and unburied trays was found to be soil temperature difference of 0.5°C. This difference was not considered significant to necessitate the labor intensive burial process which would significantly slow field setup if used on the full experiment.

The summer/fall of 2007 field trial demonstrated that the tray-in-tray system could be utilized to grow populations of *Arabidopsis thaliana* under field conditions and with some

modification to the experimental methods was used in all other field trials (see Chapter 2). Due to the time intensive and potentially damaging process of in-field harvest of mature siliques, the methods were revised to have populations removed from the field and transferred to the greenhouse when 75% of the siliques begin turning yellow, the first sign of senescence, to prevent seed loss in the field. Harvesting of the mixes containing either of the late-maturing dwarf lines, A28 or A30, would include the removal of mature WS wild-type biomass, prior to allowing the dwarf material further time to mature. This method was selected to prevent sampling bias against the dwarf lines which would lead to an underestimation of their competitive fitness. The removal of mature populations and the in greenhouse harvest method also reduced in field seed loss, and thus volunteers.

Due to the preliminary nature of this study, only three transgenic lines were selected for kanamycin screening to determine the transgene frequency of progeny seed, CBF3 lines A28 and A40 and M6PR line M2-1. All CBF3 populations showed significantly reduced fitness in competition with WS wild-type (Figure S.4ab), while M6PR line M2-1 showed significant gains in fitness (Figure S.4c).

A. **B.**

Figure S.3. Photographs of the summer/fall 2007 preliminary field trial. The field layout with the tray-in-tray design allowing subsoil watering by trickle hose (A) and the protective lids temporarily deployed prior to inclement weather which could endanger the plot (B).

Figure S.4. Fitness of transgenic plants in competition with their wild-type background ecotype in a preliminary field study fall 2007. Fitness was calculated based on selectable marker screening of progeny seed from mixed populations of CBF3 lines A28 and wild-type WS and line A40 and WS (A and B respectively) and M6PR line M2-1 and wild-type Col (C). A fitness of 1 would indicate seed production equal to wild-type (red line). Both CBF3 lines showed significantly reduced fitness in competition ($P<0.05$), while M6PR line M2-1 showed increased fitness ($P<0.05$).

Figure S.4 (cont'd)