EVALUATING AN INTERSPECIFIC *Helianthus annuus* x *Helianthus tuberosus* POPULATION FOR USE IN A PERENNIAL SUNFLOWER BREEDING PROGRAM

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the *H. annuus* chromosomes (annual plants exhibit a higher dosage of *H. annuus* chromosomes). The model on the right associates perennialty with a single factor sufficient for tuber production segregating from one of the *H. tuberosus* sub-genomes. In this example, the single factor resides on one of the A_1 chromosomes. C) Backcrossing the F_1 hybrid with *H. annuus* yields progeny that are all annual. This result is consistent with both the dosage model or the single segregating factor model described in part (B).

Chapter 1

Perennial Grains and the Sunflower (*Helianthus annuus* L.)

Potential of Perennial grains

Over the past century many environmental problems have been associated with conventional agriculture practices (shortened crop rotation, frequent tillage, and increased use of pesticides). Problems include increased soil erosion and the leaching of nutrients into ground water and away from plants. Generally, these can be characterized as ecosystem disservices associated with annual agriculture (Costanza et al., 1997). One proposed way to mitigate these environmental problems is by increasing the year round groundcover through the use of perennial crops. Increased use of perennials could potentially provide significant ecosystem services. However, most ecosystem services are generally indirect, unmanaged, underappreciated and undervalued causing adoption of practices that enhance ecosystem services to be difficult (DeHaan et al., 2005). Ecosystem services include climate regulation, water regulation, aesthetic services, cultural services, soil fertility, land value that is both landscape and time specific (Costanza et al., 1997). Since the benefits of perennial habit are hard to quantify, the direct benefit to growers of being able to sell grain would add a tangible benefit to the indirect ecosystem services that are provided by perennial plants. Cropping practices could be changed resulting in reduced costs for famers. Such practices include:

1) Decreasing fall tillage during the multi-year lifetime of the crop stand, reducing input costs and soil erosion.
2) Cover cropping with perennials could reduce the need for herbicides.

3) The annual weed population will decrease, as the perennial crop will have an advantage over annual weeds in spring vigor, again decreasing herbicide use.

4) Perennial crops provide living ground cover for longer periods during the growing year allowing plants more time to produce photosynthate, take up soil moisture, and decrease loss of nitrogen from the soil. Decreasing the nitrogen loss would reduce surface water pollution.

A theoretical framework for explaining potential yield of perennial grains was developed by Cox et al. (2002) and elaborated upon by DeHaan et al. (2005). In essence, it proposes that while the natural condition of perennial plants may be to put more resources into perennial organs, there is no reason why a perennial plant cannot also be selected for seed yield as perennial plants have a longer time to assimilate resources than annual plants. In other words, they could divert more resources to seed production and total biomass due to the longer growing season. Although, the state of a high yielding perennial plant may be unlikely to occur in nature, it may be quite possible to maintain a potentially unstable state in a human-mediated system. Changes in management may alter plant development, which may change how perennial and annual plants produce seed relative to one another. Correlations that exist between perennial habit and seed production in a cultivated setting where biophysical constraints are different than in nature also require investigation.

Initial attempts to produce a perennial grain have been met with mixed results as initial yield of interspecific hybrids in managed systems have been reported 10-70% of the yield of annual check cultivars (Scheinost et al., 2001; Sacks et al., 2003; Sacks et al., 2006). These yields have been reported under ideal field conditions, however when the initial interspecific crosses in rice have been compared in high stress environments they

have performed well (Sacks et al. 2003). For example, interspecific rice populations performed as well as check cultivars during the dry season in the Philippines (Sacks et al., 2003). However, the gap between marginal and optimal environments must be addressed. Several perennial grain varieties (rice and wheat) have been released but they have had poor grain yields after the establishment year, with crops only being grown for forage after the first year. The theoretical economics of a perennial grain crop indicate that total yield could be reduced 30% and still be as economical as an annual crop under certain conditions (Bell et al., 2008); these conditions have yet to be accomplished in practice. Recent preliminary studies have caused perennial grains to gain widespread interest (Glover et al., 2010); this has led to the initiation of breeding programs in many institutions in many different crops (Table 1). One of the major thrusts of the University of Minnesota perennial breeding initiative is working to create a perennial sunflower.

Origin of domestic sunflower

Sunflower (*Helianthus annuus* L.) was domesticated from the wild *Helianthus annuus* approximately 5000 years ago (Arias and Rieseberg, 1995; Bohrer, 1970; Harter et al., 2004). Heiser (1955) was the first researcher to concluded that wild *H. annuus* was the progenitor of domestic sunflower based on examination of morphological characteristics (Heiser, 1955). This was later confirmed through a series of molecular genetic studies identifying wild annual sunflower as the progenitor of domesticated sunflower (Rieseberg and Seiler 1990; Arias and Rieseberg, 1995; Burke et al., 2002; Harter et al., 2004; Willis and Burke, 2006; Willis and Burke, 2007; Kolkman et al., 2007; Chapman et al., 2008; Blackman et al., 2011). The additional support for wild *H. annuus* being the progenitor of sunflower generally came from the genetic composition

and diversity of *H. annuus*. Specifically, linkage disequilibrium decays at a greater rate in wild sunflower (~200 bp;Lui and Burke, 2006) than in landraces (~1.1 kilobases; Lui and Burke, 2006) or modern cultivars (~5.5 kilobases; Kolkman et al., 2007). Domestic sunflower retains 40-50% of the total nucleotide diversity of the wild populations (Lui and Burke, 2006; Kolkman et al., 2007). A loss of diversity from that found in wild sunflower is expected in cultivated types. Considerable breeding of sunflower for specific uses has occurred over the history of the crop. Despite efforts to isolate cultivated sunflower from wild species, substantial gene flow occurs from domestic sunflower to wild relatives (Mercer et al., 2006; Snow et al., 2003), with the converse only occurring at the hands of breeders. Whereas gene flow can alter phenotypes, the significance of the impact on the genome appears somewhat more limited as is evidenced by all domestic sunflowers having the same chloroplast genome (Harter et al., 2004; Wills and Burke, 2006), and that all domestic lineages form a genetically distinct group when compared to wild relatives (Mandel et al., 2011).

Through comparisons of landraces and the wild progenitor (sampled across its entire geographic range) it is possible to identify the geographic origin of the domestic form. When this was done in *H. annuus*, all marker alleles found in *H. annuus* landraces represented a subset of marker alleles present in wild sunflower populations that had been sampled in the East Central United States (Harter et al., 2004; Blackman et al., 2011). If all members of a cultigen share the same set of alleles, that is strong evidence that the cultigen came from a single geographic region if those alleles are only present in that geographic region (Zohary, 1999). This provides support for the idea that extant genetic diversity in domesticated sunflower originated in a single geographic region. An

alternative interpretation is that many landraces were lost; however, as they cannot be sampled, a simpler explanation is that all existing domesticates were derived from a single geographic region. While gene flow between wild *H. annuus* and sunflower has been identified (Mercer et al., 2006; Snow et al. 2003), the domestic sunflowers make small contributions to the wild genome because of natural selection against introgression in wild populations and artificial selection against broad introgression of wild traits in domesticated populations. Additional support for a single domestication in East Central North America is provided by the genes HaFT1 and HaGA2ox; the Eastern North American haplotype present for these genes in Mexican landraces is not present in wild Mexican sunflower (Blackman et al., 2011).

Sunflower domestication has been a subject of the literature since the middle of the twentieth century. Different lines of evidence have provided different interpretations of how sunflower domestication occurred (Table 2). The genetic evidence strongly suggests a single domestication that occurred in east central United States. Archeological, linguistic, and ethnohistorical data point towards a complex history of use with the plant being well known for many millennia without necessarily having been a domestic cultivated plant (Lentz et al., 2001; Lentz et al., 2008). The history of widespread and complex use reopened a debate that had been considered closed. However, due to the history of trade, the native range of *H. annuus* and the fact that genetic data point to a single domestication, the most parsimonious explanation is a single domestication event followed by rapid cultural diffusion.

Historic sunflower cultivation

Helianthus annuus has a long history of production with many Native American

nations cultivating sunflower across much of the North American continent. By the time of European contact with the New World, and the beginning of the historical record, sunflower was in broad cultivation throughout much of North America, ranging from the Southwest desert to Eastern Canada (Heiser, 1951; Heiser, 1955). Within sunflower there were many regional and use-specific landraces and extensive folklore (Heiser, 1951; Nabhan and Richhardt, 1983) around crop origins and production. For example, the desert dwelling Hopi tribe selected for sunflower for types that produced specific dyes (Heiser, 1951; Willis et al., 2010).

While maize (*Zea mays* L.) is often thought of as the major staple in the Americas, sunflower also played a significant role and has been associated with many different agricultural Native American groups (Sturtevant, 1885; Jenks, 1916; Jones et al., 1933; Thone, 1936; Kaplan, 1963; Wasley, 1962; Fritz, 1990). Sunflower was an important crop to all the tribes of the American Southwest, particularly the Navajo, Apache, Pueblo and Hopi, and figures into the folklore and legends of these tribes (Wallis, 1936; Yarnell, 1965; Minnis, 1989), while in the American Southeast sunflower was integrated into polycropping agriculture (Scarry and Scarry, 2005). The use of sunflower in complex cropping systems has continued in modern times to increase the number of beneficial insects in organic farm fields (Jones and Gillett, 2005). The crop is useful largely because it can survive in adverse environments such as cold soil and air temperature, which decrease growth rate (Hanna, 1924), drought, and nutrient stress (Putnam et al., 1990). This robustness may have contributed to the initial adoption of this crop in many environments. For example, in the early twentieth century in the north central United States sunflower was used as a forage crop (Hanna, 1924; Putnam et al., 1990).

From the Americas, sunflower was introduced throughout Europe as early as the 16th Century and Africa and is now grown on every continent except Antarctica. First grown in Europe as an ornamental plant in the sixteenth century (Heiser, 1955), nineteenth century Russia recognized the potential of sunflower as an oilseed and began directed selection for high oil types that lasted well into the twentieth century (Dvoryadkin, 1976). In the early twentieth century sunflower was introduced to Africa where it was subsequently grown across the continent with production centering in East Africa (Shantz, 1940).

Current Cultivation

Current commercial sunflower is grown for the oil the seed produces. Oilseed varieties are in high demand as they include no trans-fat, high oleic oil types that are used to produce a healthy vegetable oil. This oil could also be used for production of biodiesel, but demand for the high-value vegetable oil product has limited this market. Confectionary sunflower also has a strong although smaller market. Sunflower in the production United States averaged 1.3 million metric tons since 2006 (Berglund 2007; USDA 2012). The majority of sunflower produced is the oilseed type, ranging from 75% to 90% oilseed between 1994 and 2006, with the remainder being confectionary (consumed as seeds) type (Berglund ed., 2007). Since 1998 acreage planted annually to sunflower has decrease in all but 2 years compared to the previous year (Berglund ed., 2007; USDA, 2012). Sunflower profitability has been perceived as risky due to potential for losses from pathogens such as *Sclerotinia sclerotiorum*, insects, and bird damage. At the same time, alternative crops were perceived to have greater profitability and ease of cultivation because of commercial transgenic events such as Bt and herbicide resistance.

While acres have decreased in the last decade, modern sunflower cultivars have continued to increase in productivity with average yield per hectare in U.S. from 1996 to 2005 rising from 1.28-1.75 metric tons (Berglund ed., 2007), a 36% increase, with yields remaining stable since (USA, 2012). Despite challenges to production, sunflower has the second largest worldwide acreage for a hybrid crop and is the fifth highest yielding oil seed crop in the world (Singh ed., 2007).

History of *Helianthus tuberosus*

Heliantus tuberosus is a perennial sunflower species native to North America (Kays and Nottingham, 2008). *Helianthus tuberosus* is an autoallohexaploid sharing one genome with *H. annuus* (Kostoff, 1934; Kostoff, 1939; Scribria, 1938). *H. tuberosus* is thought to be an ancient hybrid between {*H. decapetalus* L.*, H. hirsutus* Raf *or H. strumosus* L.} and {*H. gianteus* L., *H. grosseserratus* Marten, or *H. annuus*} (Heiser, 1978). *Helianthus tuberosus* was first described in Western literature in the early seventeenth century. *Helianthus tuberosus* has had an important history as a specialty crop, with spikes in cultivation at different times during history; for instance, World War II (Kays and Nottingham, 2008). The crop has been grown for its edible tuber, which has excellent nutritional properties, having often been used in gourmet cooking with some of the first recorded recipes emerging in England in 1617 (Kays and Nottingham, 2008). When *H. tuberosus* is commercially cultivated, it is grown as either a winter or summer annual and planted from tuber pieces.

Wild and semi-domestic *H. tuberosus* populations have great diversity. There are many proposed uses of *H. tuberosus*, as the plant produces a small amount of rubber (Seiler et al., 1991), the storage protein of tubers (inulin) has been investigated for use in

treating obesity and diabetes (Kays and Nottingham, 2008), and the nutrient profile suggests a potential as both a biofuel and forage crop (Seiler and Campbell, 2004; Seiler and Campbell, 2006; Rodrigues et al., 2007). However some of the desirable compounds are present at very low concentrations making production economically unfeasible (Seiler et al., 1991). Despite this widespread use, there has been little information developed on the genetics of wild *H. tuberosus*.

Interspecific hybridization between *Helianthus tuberosus* x *Helianthus annuus*

Interspecific hybridization has been employed as a strategy to transfer useful traits into crop plants for many years. Hybridization occasionally occurs in nature creating new species, which may be of use to humans. Three things are needed for interspecific hybridization to be useful: syngamy, non-zero fitness for the hybrid, and advantageous alleles in the wild background (Burke and Arnold, 2001; Rieseberg, 1997; Arias and Rieseberg, 1994). Interspecific hybridization provides a way for many genes to be changed simultaneously allowing for rapid divergence and subsequent adaptation to specific environments. Each hybridization event has the potential to create novel phenotypes not seen in the parental species. For example, interspecific perennial rice hybrids have differed from the parents in carbon exchange, assimilate partitioning, and photosynthetic capabilities (Zhao et al., 2008a; Zhao et al., 2008b). Occasionally, transgressive segregation occurs in interspecific hybrids such that hybrids that are more fit than their parents (Coyne, 1996). Transgressive segregation has been observed in *Helianthus*, with the hybrid species *Helianthus paradoxus* (with greater salt tolerance) having an advantage over its parent species (Schwarzbach et al., 2001). Gene complementation has also occurred in interspecific *Helianthus* hybrids creating

advantages for the hybrid in certain environments (Welch and Rieseberg, 2002a; Rieseberg et al., 2003). The best environment for a hybrid species may not be the favored environment of either parental species. However, if a hybrid genotype displays particular adaptations to an environment, it is likely that the characteristics providing variation are present in the parental species (Welch and Rieseberg, 2002b).

Helianthus is a large genus that contains 49 species, many of which are perennial (Kane et al., 2013). This provides many potential targets for hybridization to produce a perennial oil-seed sunflower. Chromosomal structural differences between *H. annuus* and other *Helianthus* have shown certain species to have limited ability to produce hybrids and pair during meiosis (Ceccarelli et al., 2007; Natali et al., 2008; Jan and Chandler, 1989). Hybrid polyploid organisms often display large variation in chromosome structure and pairing (Chester et al., 2012; Zhang et al., 2013). *Helianthus tuberosus* diverged from *H. annuus* relatively recently (approximently1.7-8.2 million years ago; Schilling, 1997). This close evolutionary relationship makes *H. tuberosus* an excellent potential donor for perennial habit. In addition, *H. tuberosus* has been used for nearly a century as a donor of useful genes in commercial sunflower (*H. annuus*) development (Hulke and Wyse, 2008; Kays and Nottingham, 2008).

The hybrid between *H. annuus* (2*n*=2*x*=34) and *H. tuberosus* (2*n*=6*x*=102) has been reported to have a stable intermediate number of chromosomes (2n=4x=68); however, it contains meiotic abnormalities that reduce fertility (Sujatha and Prakaran, 2006). Chromosomes of different *H. tuberosus* plants pair differently with *H. annuus* plants during meiosis due to different translocations and inversions among them, resulting in variable fertility in hybrid plants (Kostoff, 1939; Atlagic et al., 1995; Atlagic

et al., 1993; Natali et al., 1998). Pollen viability in interspecific *H. annuus* x *H. tuberosus* hybrids ranged from 1-90% depending on the cross and genetic background (Chandler et al., 1986; Atlagic et al., 1995). Reciprocal interspecific *Helianthus* crosses often result in different phenotypes (Reiseberg et al., 1995). Chromosome pairing at meiosis in *H. tuberosus* x *H. annuus* crosses was irregular, with the most efficient pairing plants exhibiting 85.9% bivalent pairing (Atalgic et al., 1995). Structural differences that have been found in different interspecific *Helianthus* hybrids have been shown to have chromosomal rearrangements that increase fertility over multiple generations (Reiseberg et al., 1995). Therefore, selection for meiotic stability and improvement of other phenotypes should be possible.

Differential fertility in interspecific hybrids

Differential fertility that causes decreases in fitness in interspecific hybrids has been postulated to be caused by incompatibilities that accumulate between at least two loci (Dobzhansky, 1933). In the initial model two interacting loci are needed for fertility. After a short time the loci accumulate mutations such that the two loci can no longer interact causing a loss of vigor or complete loss compatibility. These interactions, termed Dobzhanky-Muller interactions, have been observed in many species (most notably Drosophila) (Orr, 1996). Dobzhanky-Muller interactions may cause certain parental combinations to be unsuccessful, this can likely be overcome by selecting parents that produce the most vigorous hybrids and have high proportions of viable seed and pollen.

Rationale for this dissertation research

The idea for creating perennial grain crops originated in the early 20th century in many localities (former Soviet Union, United States, Germany, Canada, Sweden,

Australia, China, Hungary) in many crops (wheat, rye, oat, sunflower, sorghum, corn, and rice). Despite this interest, to date there has been little sustained effort to understand how perennial plants allocate resources in response to selection in managed environments, the potential for initial gains from selection, and the relationship between production environments and perennial habit. The combination of perennial habit from *H. tuberosus* with the marketability and agronomic characteristics of domesticated sunflower could result in a high-value, perennial crop that may benefit many different agroecosystems. The objective of this dissertation research was to use current genetics and plant breeding techniques to introgress genes for perennial habit from *Helianthus tuberosus* L. ($2n=6x=102$) into domesticated sunflower (*Helianthus annuus* L., $2n=2x=34$). To this end, this dissertation attempts to: 1) Increase the number of generations examined per year, 2) Identify important trait relationships in interspecific *Helianthus* hybrids, 3) Begin to understand the genetic architecture of perennial habit, and 4) Understand the best ways to select improved interspsecific hybrids.

Table 1. Organizations with perennial breeding programs and crops being examined for conversion to perennial types.

Organizations with perennial breeding programs	**Crops being examined**
• University of Minnesota	• Wheat, intermediate wheatgrass, sunflower, maize, flax, hazelnut
• Michigan State University	• Wheat, intermediate wheatgrass
• Cornell University	• Wheat, maize
• North Carolina State University	• Maize
• University of Georgia	• Maize
• University of Illinois	• Rice
• Kansas State University	• Wheat
• University of Manitoba	• Wheat, intermediate wheatgrass, sunflower,
• The Land Institute	• Sorghum, sunflower, wheat, intermediate wheatgrass, Illinois bundleflower, rice
• Charles Sturt University	• Wheat
• Washington State University	• Wheat, intermediate wheatgrass
• University of Chicago	• Maize
• Sustainable Ecosystems Agricultural Production Systems Research Unit; Commonwealth Scientific and Industrial Research Organization (Australia)	• Wheat
• Swedish University of Agricultural Sciences	• Rye, oat, wheat
• Yunnan Academy of Agricultural Science	• Rice
• USDA	• Wheat, sunflower, maize
• CIMMYT	• Wheat, Maize
• Universidad de Buenos Aires	• Wheat, Maize
• Texas A&M University	• Wheat Maize

Table 2.Different types of evidence that have informed the history surrounding sunflower domestication.

Evidence	Archeological Interpretation	Genetic Interpretation
Plant morphology	Wild *H. annuus* is the wild progenitor of domestic sunflower	Wild *H. annuus* is the wild progenitor of domestic sunflower
Geographic range	The wide distribution of wild sunflower indicates that multiple locals had the opportunity to domesticate the plant	The wide distribution of wild sunflower indicates that multiple locals had the opportunity to domesticate the plant
Linguistic	Sunflower is rarely a cognate among indigenous people of North America; this provides evidence for multiple domestications as local communities have individualized knowledge	NA
Carbonized seed	Domestic sunflower achenes have been found at archeological sites across North America at similar times in history	NA
Allelic / nucleotide diversity in domestic and wild *H. annuus*	NA	Allelic and nucleotide diversity in landraces is subset of diversity found in wild *H. annuus;* wild *H. annuus* is the wild progenitor of domestic sunflower
Geographic localization of diversity	NA	All allelic and nucleotide diversity found in extant domestic sunflower can be traced to wild populations sampled in the East Central United States; this indicates a single domestication
Individual genes	NA	Flowering time genes HaFT1 and HaGA2ox in Mexican landraces possess the Eastern North American haplotype not present in wild Mexican sunflower (Blackman et al. 2011).
Long distance trade	Long distance trade as seen by obsidian, shell beads and turquoise indicates that prehistoric contact occurred across much of the North American continent	NA

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Chapter 2

Breaking Tuber Dormancy in *Helianthus tuberosus* L. and Interspecific Hybrids of *Helianthus annuus* L. x *Helianthus tuberosus*

Summary

Tubers of *Helianthus tuberosus* L. are dormant following production in the late fall until the following spring. In the wild, tuber dormancy is broken after exposure to winter cold, resulting in sprouting and shoot development in the spring when conditions are favorable. The dormancy period typically limits *H. tuberosus* populations to one growth cycle per year. An efficient method for breaking tuber dormancy is needed to have an additional growth cycle per year in a breeding program, which could take place in winter nursery or greenhouse allowing for increased breeding efficiency. The objective of this research was to compare chemical and cold temperature treatments for artificially breaking tuber dormancy in twelve genotypes of *H. tuberosus* and interspecific hybrids of *Helianthus annuus* L. x *H. tuberosus*. Five cold exposures (2, 4, 6, 8, 10 weeks at 2°C), three plant hormones (ethylene, cytokinin, and gibberellic acid), and one untreated control were examined. Gibberellic acid was the best chemical treatment, initiating plant growth within 6.5-11.5 days in the majority of genotypes tested. The best cold treatment was exposure to 2°C for eight weeks, where plant growth began 63.6-67.5 days following treatment initiation. Although longer cold treatments shortened the time to emergence while in the greenhouse, the penalty of the long cold treatment *per se* was too long to be useful. The gibberellic acid treatment strategy described here may not need further optimization, as it is short enough to allow for two growth cycles of *H. tuberosus* per year.

Introduction

Plant propagule dormancy ensures that plant growth occurs during optimal environmental conditions. Plants utilize environmental cues such as shortening photoperiod, colder temperatures, and mild drought to regulate growth habit. Dormancy is generally broken by consistent changes in the environment that lead to favorable growing conditions (*i.e.* a response to erratic environmental changes could lead to plant death). Dormancy is defined as the suspension of active growth in meristematic tissue (Kalberer et al., 2006). There are three general types of dormancy (Lang, 1987): endodormancy, paradormancy, and ecodormancy. Endodormancy is related to physiological factors within plant organs, paradormancy is related to physiological factors outside the affected plant organs but within the plant, and ecodormancy is related to environmental factors. *Helianthus tuberosus* L. is thought to be regulated by endodormancy (Kays and Nottingham, 2008). The biochemical mechanisms that affect the tuber dormancy period have been well studied in *H. tuberosus* and these studies have implicated an increased level of polyamines in actively growing tuber cells relative to dormant tuber cells (Bagni and Serafini-Fracassini, 1985; Kays and Nottingham, 2008; Tassoni et al., 2010).

Breaking dormancy artificially is important for propagation of plants outside of field or wild conditions. The use of cold treatments to break dormancy among different ecotypes of *H. tuberosus* was studied during the first half of the 20th century (Boswell, 1932; Steinbauer, 1932; Steinbauer, 1939; Traub et al., 1929). *H. tuberosus* tubers quantify exposure to cold, and once the cold period has reached a certain length, the tubers initiate growth (Kays and Nottingham, 2008). The time required to break

dormancy for *H. tuberosus* is dependent on ecotype, and ranges between 30-200 days (Steinbauer, 1939; Traud et al., 1929). Cold acclimation temperatures between -2ºC and 5ºC have been recommended, as tubers rot above 5ºC and freeze below -2ºC (Whiteman, 1957).

Various chemical treatments have also been studied with regard to breaking tuber dormancy in both *Helianthus* and *Solanum* species. In potato (*Solanum tuberosum* L.) tubers, ethanol caused apical bud dormancy to be broken more quickly than in untreated controls (Claassens et al., 2005). In *H. tuberosus* an ethanol treatment caused apical bud dormancy to be masked (during treatment tuber cell growth occurred), but after the ethanol treatment stopped, cell growth stopped and dormancy was restored (Petal et al., 1993). 2,4-dichlorophenylacetic acid (2,4-D) has been shown to break dormancy in cultured *H. tuberosus* tuber cells (Bennici et al., 1982). In potato tubers, ethylene and gibberellic acid have been implicated in both breaking dormancy and initiating growth after dormancy, but the role of these hormones have not been clearly defined (Coleman, 1998). Cytokinins have been implicated in the maintenance of dormancy in potato tubers (Coleman, 1998). As these hormones have been implicated in potato tuber dormancy, they may have similar effects on the tubers of *H. tuberosus*.

H. tuberosus has an important history as a specialty food crop. Initial breeding efforts date back to the 17th century although the first systematic breeding program did not begin until the early 20th century (Kays and Nottingham, 2008). The major goal of these breeding programs has been to increase tuber yield and inulin content. Inulin, the primary storage carbohydrate of *H. tuberosus*, is proposed as a source of carbohydrate that will help fight the obesity epidemic as inulin is of low caloric value and helpful to

digestion (Kays and Nottingham, 2008). In addition, *H. tuberosus* has been utilized as a resource for disease and insect resistance in *Helianthus annuus* L. breeding programs (Charlet and Brewer, 1995; Hulke and Wyse, 2008; Miller and Gulya, 1987). Cultivated *H. annuus* is the world's second largest hybrid crop in acreage and is the 5th highest yielding oil seed crop (Jan and Seiler**,** 2007).

Recently, there has been interest in increasing landscape ecosystem services through the use of perennial crops (DeHaan et al., 2005; Hu et al., 2003; Hulke and Wyse, 2008; Sacks et al., 2003; Wang et al., 2009). One of the promising wild species being investigated for creating a perennial sunflower crop is *H. tuberosus*. Interspecific hybrids between *H. annuus* and *H. tuberosus* also show promise as breeding material for a perennial crop. Hybrids have been shown to have potential for both biomass and tuber production (Kays and Nottingham, 2008). In addition, hybrids have substantial variation in seed, agronomic, and tuber traits (Kays and Nottingham, 2008). Despite the large amount of research on tuber dormancy in *H. tuberosus*, the dormancy requirement has not been characterized for interspecific hybrids. It has been difficult to break dormancy in the interspecific *Helianthus* tubers, delaying the breeding process with these materials by making it difficult to grow more than one generation per year. The goal of this research was to determine a reliable method to break tuber dormancy in genotypes of wild *H. tuberosus* and in interspecific hybrids of *H. annuus* x *H. tuberosus* in order to have two full growth cycles per year.

Materials and Methods

Plant Material

Twelve genotypes corresponding to three different genomic backgrounds of *H. tuberosus* (wild *H. tuberosus*, *H. tuberosus x H. annuus,* and *H. annuus x* [*H. tuberosus x H. annuus*]*)* were examined (Table 1). Genotypes were selected from populations collected and developed in 2003 by Hulke and Wyse (2008). Briefly, the initial interspecific populations were created by crossing eighteen wild *H. tuberosus* parents collected from UMore Park in Rosemount, MN to three inbred cultivated *H. annuus* lines (CMS HA89 (Miller, 1997), HA89 (released by the USDA-ARS in 1971) and HA434 (Miller et al., 2004)). HA89 and HA434 were used as male parents and CMS HA89 was used as a female parent. The different genomic backgrounds exhibited variation in timing of floral initiation: early season flowering (before July 15), intermediate season (before August 15), or late season (after August 15) (Table 1). Annual parental inbreds flowered either early season or intermediate season. Tubers of all genotypes were morphologically similar and of similar size. Large tubers were selected as have been shown to have better germination (Kays and Nottingham, 2008). One tuber was planted per pot with all tubers of any given genotype being harvested from the same clone that had fully senesced and had produced viable seed.

Cold treatment

The first experiment was designed to identify the optimum duration of cold treatment needed to break tuber dormancy. The experimental design was a randomized complete block with a split plot arrangement of treatments with four replicates. The cold period duration treatments were arranged as the whole plots while genotypes were arranged as the sub plots. Genotypes exhibited flowering times covering the entire season

in Minnesota within *H. tuberosus* (Table 1). Tubers were collected from the field in mid-October 2009, planted in cone-tainers (Stuewe & Sons Inc., Tangent, OR) in Sunshine professional growing mix® (Sun Gro, Seba Beach, AB), and then placed in a cold acclimation room at 2ºC with no lights in early November 2009. Depth of planting was 1.25 cm below the soil surface. After being placed in the cold room, tubers were removed after 2, 4, 6, 8, and 10 weeks, and placed in the greenhouse with 14 hour daylength at approximately 24ºC. Control tubers received no cold acclimation and were placed directly into the greenhouse. All tubers were scored for number of days to sprouting after initiation of treatment. A positive score was a sprout visible above the soil surface. We did not dig up un-sprouted tubers at the end of the experiment, nor did we test them with a hormone to see if they were still viable. The control tubers were maintained until all of the plants from the cold treatments had flowered.

Hormone treatment

The second experiment examined breaking tuber dormancy by use of a chemical plant hormone treatment. The experimental design was a randomized complete block with three replicates in a split plot arrangement of treatment. Whole plots were hormone and sub plots were genotype. Hormone treatments were chosen as representative of treatments used to break seed dormancy in several species (Biddington and Thomas, 1976; Koyuncu, 2005; Miller, 1987; Rehman and Park, 2000) and tuber dormancy in *Solanum tuberosum* (Coleman, 1998). Three hormones were evaluated: ethylene, cytokinin and gibberellic acid. Ethylene was applied by soaking tubers overnight in a 1% aqueous ethephon solution (Proxy®, Bayer Environmental Science, Research Triangle PK, NC). Ethephon has been used to promote pre-harvest ripening in fruit and has been

shown to decompose to ethylene in plant tissue (Epstien et al., 1977). Cytokinin was applied by soaking the tubers for 90-120 seconds in a 2000 ppm aqueous cytokinin solution (6-benzylamino purine, Sigma-Aldrich, St. Louis, MO). Gibberellic acid was applied by soaking tubers for 90-120 seconds in a 2% aqueous gibberellic acid solution (4% ProGibb®, Valent, Memphis, TN). After treatment, the tubers were planted in 5 cm pots in Sunshine professional growing mix® (Sun Gro, Seba Beach, AB), 1.25 cm under the soil surface and placed in a greenhouse with 14 hour daylength at 24ºC. All tubers were scored for days to sprouting after initiation of the hormone treatment and scored in the same way as the cold experiment.

Statistical analysis

Statistical analysis was conducted using R Statistical software package (R Development Core Team, 2008). Treatment means were separated using a Fisher's protected LSD. A significance level of $P \leq 0.05$ was used to determine treatment differences. Two models were tested for each treatment program: one with a linear covariate for flowering date and one without this covariate. When replicate tubers did not germinate they were treated as missing data, however, the gibberllic acid, ethylene, 8 week and 10 week cold treatments had no missing data.

Results

Cold treatment

The effect of cold treatment was measured as the total time from treatment initiation to tuber sprouting in the greenhouse. The ideal cold treatment would be the minimum duration treatment that breaks dormancy of all genotypes. Not all genotypes sprouted under all cold treatments (Figure 1), most (67%) required a pretreatment to

stimulate growth. Not all genotypes sprouted in response to the shorter cold treatments (two through six weeks). These data support previous research showing that tuber dormancy varies among *H. tuberosus* genotypes (Steinbauer, 1932; Steinbauer, 1939). The two week treatment produced the earliest overall emergence (Figure 2). However, only nine of the twelve genotypes emerged following the two week treatment. The eight week treatment was the shortest cold treatment under which all genotypes sprouted. This emergence, however, was not until 63.6-67.5 days after treatment initiation. However, if the time of treatment is not considered, then the genotypes sprouted in 7.6-11.5 days in response to the eight week cold treatment. In addition, complete emergence (100%) from all genotypes and tubers was not observed until the eight week treatment (Figure 1; Figure 3). Plant growth proceeded normally through anthesis and reproduction after all cold exposures.

Hormone Treatment

All hormone treatments resulted in more rapid sprouting than the untreated control (Figure 2). Gibberellic acid treatment resulted in significantly faster tuber sprouting than ethylene treatment, which caused significantly faster tuber sprouting than cytokinin treatment (Figure 2). All genotypes sprouted under the ethylene and gibberellic acid treatment, which initiated growth within 9.8-20.5 and 6.5-11.5 days, respectively. The cytokinin treatment caused tubers to sprout in 14-25.2 days; however, not all genotypes sprouted (JA 318F failed to sprout) (Figure 4).

There was a genotype by hormone interaction indicating that genotypes responded differently to plant hormones. The interaction is mostly due to rank wise differences between the ethylene and the cytokinin treatment, except in the case of JA318F where the

interaction occurred among all three treatments. JA318F was the only genotype that did not sprout earliest under the gibberellic acid treatment (Figure 4). The percentage of *H. tuberosus* genome in the genotypes did not seem to have an effect on response to chemical hormone, as some of the earliest and latest plants to emerge were interspecific hybrids (Figure 4). Plant growth was somewhat atypical after each of the hormone treatments as multiple shoots were generated from a single tuber. However, if plants were trimmed to a single shoot per tuber, plant growth appeared to proceed normally through anthesis and reproduction.

Flowering time and dormancy

When genotypes were grouped by flowering time during the growing season (early, intermediate, or late season), there was no difference in how they developed after cold or hormone treatment. There was no interaction between hormone treatment or cold treatment and flowering time. The flowering time covariate was not significant with $P = 0.17$ and $P = 0.15$ for cold and hormone treatments, respectively.

Discussion

The goal of this project was to determine the fastest method for breaking dormancy in *H. tuberosus* tubers. Ideally, an optimized method would allow researchers to evaluate or increase materials under greenhouse or winter nursery conditions and have new mature tubers ready to induce and plant for the next growing season (*i.e.* allowing for two growth cycles per year rather than one) (Figure 5). Bearing this goal in mind, we chose to evaluate the treatments based on the total amount of time from treatment initiation to plant emergence in the greenhouse (Figure 2). By this measure, all three chemical treatments outperformed even the best cold treatment. Although longer cold

treatments shortened the time to emergence in the greenhouse, the penalty of the long cold treatment *per se* was too great to complete two growth cycles per year (Figure 5). Moreover, the ethylene and gibberellic acid chemical treatments induced sprouting among all genotypes and tubers tested, whereas only the longest cold durations (eight and ten week treatment) were able to break dormancy in all genotypes and tubers tested (Figure 3).

Among the chemical treatments, the most effective plant hormone for breaking dormancy was gibberellic acid. Nearly every genotype tested exhibited the most rapid sprouting under the gibberellic acid treatment, with the exception of JA 318F, which sprouted most rapidly in response to ethylene. It remains an interesting question whether altering the dosage of any of these hormonal treatments may shorten the time to break dormancy below that of our current gibberellic acid treatment (as a 2% aqueous solution). However, that question may be irrelevant as the gibberellic acid treatment broke tuber dormancy in less than three weeks and allowed us to achieve two full growth cycles on all genotypes in 2010-2011. The gibberellic acid treatment accomplishes the goal of getting two full growth cycles per year from *H. tuberosus* and interspecific *Helianthus* hybrids.

Table 1. Genotype name, annual parent, species, and flowering time for the plant material used in this study.

Genotype	Annual Parent	Species	Flower initiation
JA 5	None	*H. tuberosus*	Late
JA 8	None	*H. tuberosus*	Late
JA 9	None	*H. tuberosus*	Late
JA 213	HA 89	*H. tuberosus x H. annuus*	Early
CMS 201 D	CMS 89	*H. tuberosus x H. annuus*	Early
JA 206 D	HA 89	*H. tuberosus x H. annuus*	Early
JA 312 F	HA 434	*H. tuberosus x H. annuus*	Early
CMS 201 C	CMS 89	*H. tuberosus x H. annuus*	Intermediate
JA 212 I	HA 89	*H. tuberosus x H. annuus*	Intermediate
JA 318 F	HA 434	*H. tuberosus x H. annuus*	Intermediate
JA213 x HA 89	HA 89	(H. *tuberosus x H. annuus)x H. annuus*	Early
CMS2xx x HA 89	CMS 89	(H. *tuberosus x H. annuus)x H. annuus*	Early

Figure 1. Influence of 2°C cold treatments on total days (treatment + days in greenhouse) to sprouting in nine interspecific sunflower hybrids and three *H. tuberosus* genotypes. Letters (a-d) above the bars indicate significantly different groups as indicated by least significant difference (LSD). An * indicates that the genotype did not germinate under this treatment.

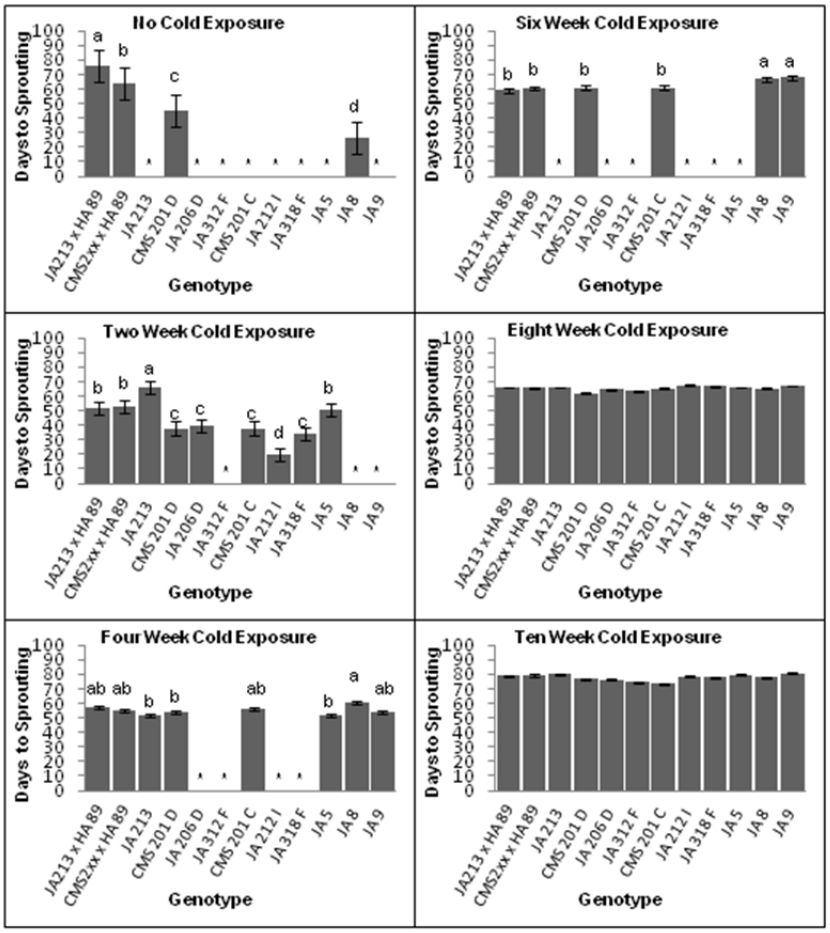


Figure 2. Influence of cold and chemical hormone treatments on time to sprouting averaged across nine interspecific sunflower hybrids and three *H. tuberosus* genotypes. Error bars indicate the standard error for each treatment. The separate cold treatment and chemical hormone treatment experiments were both compared to an untreated control where an * indicates that the treatment caused tubers to sprout in a significantly longer time than the untreated control and a ** indicates that the treatment caused tubers to sprout in a significantly shorter time than the untreated control.

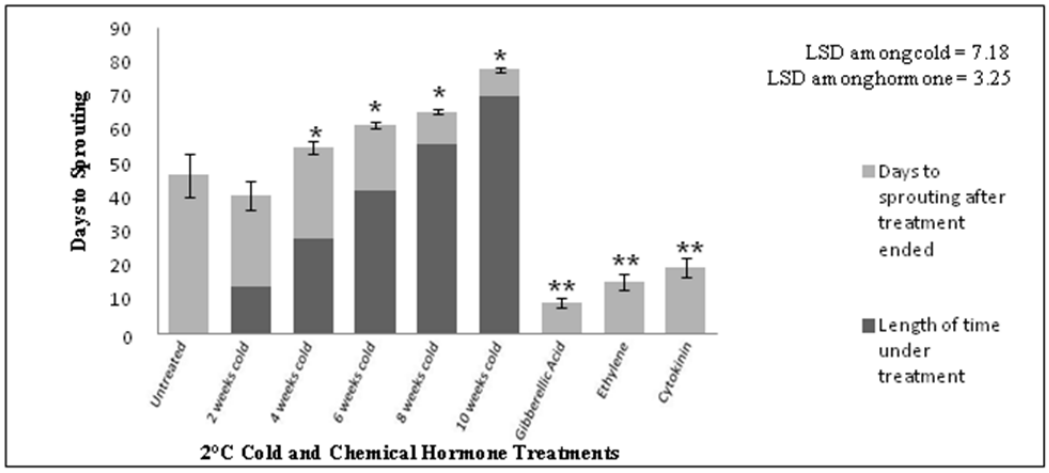


Figure 3. Percentage of treated tubers that sprouted under the influence of cold and chemical hormone treatments across nine interspecific sunflower hybrids and three *H. tuberosus* genotypes. The separate cold treatment and chemical hormone treatment experiments were both compared to an untreated control where an * indicates that the treatment caused significantly more tubers to sprout than the untreated control.

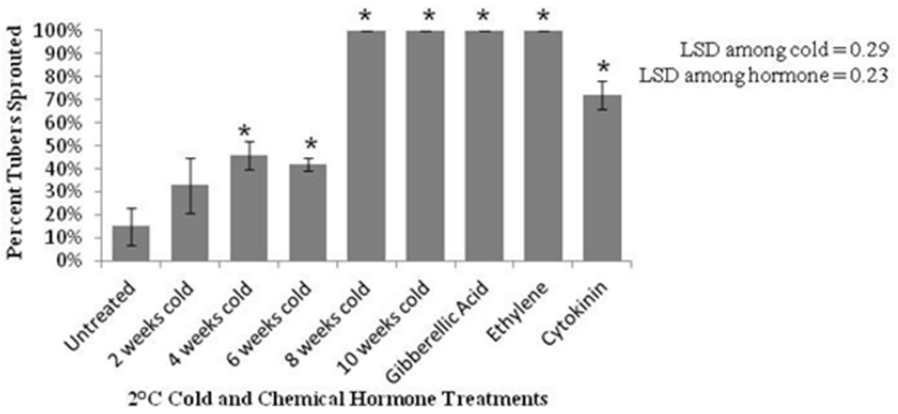


Figure 4. Influence of hormone treatments on total days (treatment + days in greenhouse) to sprouting in genotypes in nine interspecific sunflower hybrids and three *H. tuberosus* genotypes. Error bars indicate the standard error for each individual hormone. Letters (a-c) indicate significantly different groups as indicated by least significant difference (LSD). An * indicates that the genotype did not germinate under this treatment.

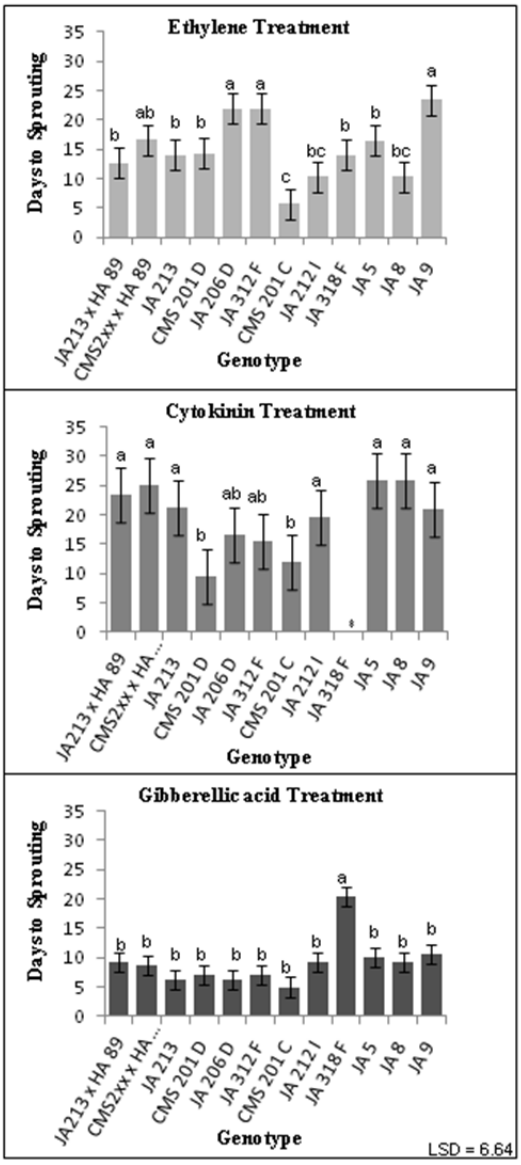
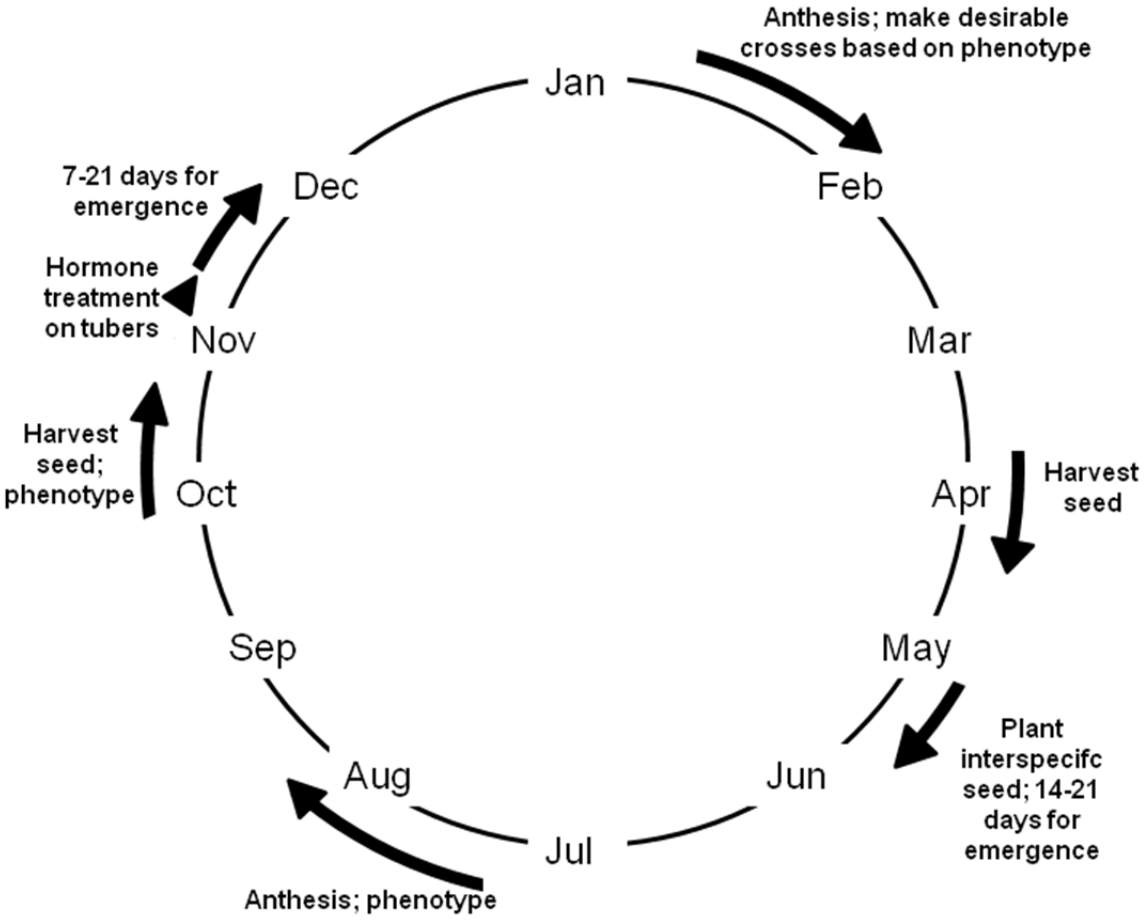


Figure 5. Potential breeding scheme to get two generations per year of *H. tuberosus* or *H. tuberosus* x *H. annuus* interspecific hybrids. Length of arrows indicates the approximate amount of time an activity will take.



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Chapter 3

Evaluating an interspecific *Helianthus annuus* x *Helianthus tuberosus* population for use in a perennial sunflower breeding program

Summary

Perennial crops show promise as a sustainable agricultural production tool that provides ecosystem services (maintaining healthy soil, controlling erosion, improving water quality, and enhancing wildlife habitat). Perennial crops could also provide economically viable cropping option to farmers. Sunflower (*Helianthus annuus* L.) is an ideal crop for perennialization because of existing genetic resources and a wide variety of end-uses. The objective of this research was to evaluate interspecific hybrids between perennial *Helianthus tuberosus* L. (2n=6x=102) and annual *Helianthus annuus* L. (2n=2x=34) for perenniality and agronomic traits; assessing their utility in developing a perennial seed crop. Field trials indicated that seed yield traits were positively correlated with head traits. Tuber traits, which are required for perenniality, and seed yield traits were not correlated, indicating that simultaneous selection may be able to target high yielding lines that also tuberize. The F_1 individuals were intermated for one generation and the intermated F_1 (IM_1F_1) showed increases in head size (up to 20%) compared to the best F_1 individual. The lack of correlation between tuber and seed traits coupled with phenotypic improvement after one generation of intermating suggest that the best improvement strategy for perennial sunflower is a recurrent selection program focusing on yield.

Introduction

Over the past century, agricultural research has contributed to dramatically increased crop yields and productivity, yet this increase in productivity has often come at the expense of long term environmental sustainability through greater use of fossil fuel-based fertilizers, the depletion of fresh water, and the reduction of arable land (Baulcombe et al., 2009; Tilman et al., 2002). Addressing environmental damage and enhancing ecosystem services such as climate regulation, water management, and soil fertility will be essential for the adequate production of food in the future (Baulcombe et al., 2009; Costanza et al., 1997; Tilman et al., 2002). Currently there are cultural practices such as zero tillage and cover cropping, which provide many ecosystem services without a yield reduction. Recently, the addition of perennial plants, particularly perennial crops, has been suggested as another tool for incorporating ecosystem services into the landscape while maintaining productivity (DeHaan et al., 2005; Baulcombe et al., 2009; Glover et al., 2010; Chia et al., 2012). The potential of perennial crops to reduce the environmental impact of agricultural systems through reduction in fall tillage, soil erosion, and nutrient runoff has long been ignored, but recently has regained popular interest (Glover et al., 2010). In addition, due to reduced input costs, perennial grains can be as profitable as annual counterparts over a three year life of the perennial crop if the market price is equal and the perennial yields at least 60% as much as the annual crop (Bell et al., 2008).

Recent research has shown that the genetics of perenniality (development of perennial organs) may not be as complex as previously thought, with several studies identifying only a few quantitative trait loci (QTL) necessary for perennial organ

development (Wang et al., 2009; Sacks et al., 2007; Hu et al., 2003). Hu et al. (2003) identified two QTL that controlled production of rhizomes in rice (*Oryza longisteminata x Oryza sativa* hybrids) with the segregation matching a dominant two gene model with complementary gene action. Stolon presence:absence segregated in a 3:1 fashion in F_2 families of the interspecific cross *O. sativa* x *O. rufipogon* (Sacks et al., 2007). Further, Wang et al. (2009) identified a single gene, perpetual flowering 1 (*PEP1*), which regulates perennial flowering in *Arabis alpina*. These findings suggest it may be possible to introduce perennial habit into annual crops without introducing large portions of wild relatives of the species.

Domesticated sunflower (*H. annuus* L., $2n=2x=34$) is an annual crop that produces a diverse range of products, including oilseed types (used to produce birdseed or high-quality vegetable oil) and confection seeds for direct human consumption. Sunflower is a compelling target for perennialization, as *Helianthus* includes 49 species, many of which are perennial (Kane et al., 2013). Breeders have used interspecific hybridization to introgress useful wild traits into *H. annuus* for disease resistance (Miller and Gulya, 1987), insect resistance (Charlet and Brewer, 1995), adaptation to distinct environments, abiotic stress (Rieseberg, 1997), and cytoplasmic male sterility (Kohler and Friedt, 1999). A similar approach could be used to introgress the perennial habit, as perennial *Helianthus* species can potentially be used as donor materials for transferring perennial habit into domesticated sunflower. Breeding for perennial seed crops poses a unique problem because perennials need to allocate photosynthetic resources to both the perennial organs used for carbon storage and the seed itself (DeHaan et al., 2005). It has been suggested that perennial plants can be selected for increased seed production while

maintaining asexual reproduction (Cox et al., 2002; DeHaan et al., 2005). Perennial plants have a longer growing season to assimilate nutrients and breeding can influence photosynthate utilization to optimize seed production and perennial habit. In addition, there is historical precedent as farmers who initially domesticated rice selected for perennial habit during low intensity production (Hill, 2010).

Helianthus tuberosus, a tuber-bearing perennial species, is a prime candidate for the introduction of perenniality into domestic sunflower. It has been used to introgress traits into *H. annuus* for nearly a century and has a separate history as a specialty crop (Hulke and Wyse, 2008). *Helianthus tuberosus* ($2n=6x=102$), is an autoallohexaploid with three sub-genomes. The three sub-genomes have been traditionally designated as A_1, A_2, and B_t (Kostoff, 1939). The B_t sub-genome is thought to be very similar to the *H. annuus* genome (Kostoff, 1934; Kostoff, 1939; Scribria, 1938), which may help stabilize meiotic chromosome pairing in interspecific hybrids between the two species. Through conventional hybridization it is possible to create large populations of interspecific *H. annuus x H. tuberosus* hybrids. The hybrids are perennial by way of tuber-sprouting and have good fitness. Commercial varieties have been released in Russia and Sweden for tuber production and forage purposes (Kays and Nottingham 2008). *H. annuus* x *H. tuberosus* hybrids generally have a stable intermediate number of chromosomes (2n=4x=68), although meiotic abnormalities can reduce fertility and decrease stability in initial generations (Sujatha and Prakaran, 2006; Chandler et al., 1986; Atlagic, 1993).

Three breeding strategies have been proposed to create perennial grain crops: direct domestication of perennial relatives of crop plants, transgenic modification of annual plants, and genetic introgression of perennial habit from wild relatives into

domesticated crops through wide hybridization (Glover et al., 2010). There is doubt regarding the feasibility of direct domestication of perennial sunflower relatives as QTL mapping studies within annual sunflower suggest that a larger number of loci contribute to domestication-related phenotypes in sunflower than in other species (Burke et al., 2002; Wills and Burke, 2007; Doebley & Stec, 1991), Furthermore, the most important domestication trait in sunflower is suppression of axillary flowers (single headed state) (Chapman et al., 2008), a trait present at low frequencies in wild sunflower populations. Transgenic modification is not possible at present, as no known "perenniality" genes have been identified for sunflower, and only one has been identified so far in other species (Wang et al., 2009). Moreover, sunflower is recalcitrant to regeneration and transformation (Lewi et al., 2006: Piqueras et al., 2010), and gene flow issues with weedy conspecifics have halted regulatory acceptance of transgenic sunflower (Snow et al., 2003). Introgression of perennial habit from wild relatives through wide hybridization may be the most feasible approach. The main advantage of this approach is that a copy of the domesticated genome is present in a hybrid, enabling the selection of existing domesticated or elite loci that may not be present at high frequency in the wild germplasm. This approach can be implemented in at least two different ways: (1) selection on a population backcrossed to the domesticated parent or; (2) recurrent selection on populations derived from intermating the hybrid materials.

This study evaluates an interspecific population of *H. tuberosus* x *H. annuus* as a base for developing a perennial oil-seed sunflower. We validate the interspecific origin of hybrid populations, examine parental diversity, and then evaluate the potential for improving the perennial populations based on the interactions between perennial,

agronomic, fertility and yield traits.

Materials and Methods

Populations

Five populations were investigated. The first population was 18 *H. tuberosus* individuals collected from UMore Park in Rosemount, MN. The second was a set of 187 interspecific F_1 hybrids between *H. annuus* and *H. tuberosus*. The interspecific hybrids were developed during the years 2003-2006 (Hulke and Wyse, 2008) by crossing the 18 *H. tuberosus* (perennial) parents with three inbred *H. annuus* (annual) lines (CMS HA 89 [PET1], HA 89 (released by the USDA-ARS in 1971) and HA 434 (Miller et al., 2004)). HA 89 and HA 434 were used as male parents and CMS HA 89 was used as a female parent (Table 1). The third population was a derivative of the second, as the F_1 hybrids were intermated to form an intermated F_1 population (designated as the IM_1F_1 population). This population was developed in 2007 by Hulke and Wyse (2008) (Table 1). The fourth population was a backcross of the interspecific F_1 to the inbred lines HA 434 and HA 89 (designated as the BC_1F_1 population). This population was developed in 2006 by Hulke and Wyse (2008) (Table 1). The fifth population was 31 *H. tuberosus* plants from the seed stocks of the United States Department of Agriculture Germplasm Resources Information Network (GRIN) that were collected from a diverse set of geographical locations (Table 1).

2.2 *Flow cytometry*

Individuals in the following populations were examined for genome size using flow cytometry: 187 interspecific F_1s, 170 IM_1F_1s, 120 BC_1F_1s, the 18 *H. tuberosus* parental lines and two of the *H. annuus* parental lines (HA 89 and HA 434).

Nuclear DNA content was assessed using a BD FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer. Two technical replicates of the same clone were performed (on different days) for each plant on 42 F_1 individuals and the inbred annual lines. A single measurement was performed on the other individuals. Fully expanded leaf tissue sections of 0.55 cm^2 were finely chopped in 500 ml of extraction buffer (Partec, CyStain PI Absolut P), followed by filtration through a 50 micron nylon mesh. Filtered nuclei were stained with 2 ml of propidium iodide staining solution (Partec, CyStain PI Absolut P), stored at 4 ºC, and examined within 12 hours of preparation. A commercial standard of trout erythrocytes (Partec, DNA Control UV, 25 ml) as well as the internal standard from diploid HA 89 were used to calculate DNA content. A minimum of 1000 nuclei were examined for each sample. DNA content was calculated by taking the ratio of the peak intensity of each sample to that of the known standard and then multiplying the ratio by the picogram (pg) genome size of the standard. The BC_1F_1 populations were characterized with freeze dried tissue, which has decreased fluorescence relative to fresh tissue (Doezel et al., 2007). Freeze dried samples were calibrated by identifying differences between freeze dried and fresh tissue of the same clone in a subsample of 40 F_1 individuals. Ploidy boundaries were assessed by constructing 95% confidence intervals around the mean genome content of each population and comparing the genome content to the known values in the literature. Confidence intervals were constructed by 1000 bootstrap replications from the empirical distribution of each population. Briefly, a random sample was drawn with replacement from genome sizes in each population, creating a new distribution from which the 2.5% and the 97.5% individuals were used to create a 95% confidence interval for each ploidy level (Efron and Tibshirani, 1993).

Phenotyping of the interspecific populations

F_1 phenotyping

The 187 F_1 hybrids (*H. tuberosus* x *H. annuus*) and 18 *H. tuberosus* parents were field grown in St. Paul (2009 and 2010) and Rosemount, Minnesota (2010). Plants were grown in a randomized complete block design with three replications per environment. The two environments were separated by approximately 35 km and differed in climate and soil: St. Paul (located at 45°00' N 93°05' W), has a soil type of fine-silty over sandy or sandy-skeletal, mixed, mesic Typic Hapludolls, and Rosemount (located at 44°44' N 93°01' W), has a soil type of a well-drained Waukegan silt loam (fine silty over sandy, mixed mesic Typic Hapludolls). F_1 hybrids were transplanted as young plants newly emerged from tubers in May in both St. Paul 2009 and Rosemount 2010 from a living collection maintained in St. Paul. F_1 hybrid tubers were harvested from the St. Paul 2009 planting and replanted in a different field in St. Paul in November 2009. All plants were grown one meter apart within rows and 1.8 meters apart between rows. Large tubers were used for planting, as sprouting and survival increase with larger tubers (Kays and Nottingham, 2008). Plants were scored for 13 traits. The traits and phenotyping procedures are outlined in Table 2.

Statistical analysis was conducted using the R Statistical software package (R Development Core Team, 2012). Analysis of variance was conducted using families and individuals as fixed effects and environment as a random effect. Replications were nested within environment. A significance level of α=0.05 was used to determine significant differences. Contrasts were performed with PROC GLM of SAS to test differences between *H. tuberosus* parents and F_1 progeny (SAS Institute, 2008). Means, ranges, and

heritability were calculated for each trait evaluated and phenotypic correlations estimated for each pair of traits. Narrow sense heritability was calculated using the 18 *H. tuberosus* half-sib families within each environment and then pooled across environments using parent offspring regression (Fehr, 1991). Parent-offspring regression coefficients were calculated by 1000 bootstrap replications from the residuals of an initial regression where wild parental values were used to predict the value of the interspecific progeny (Efron and Tibshirani, 1993).

IM_1F_1 phenotyping

F_1 individuals were intermated to generate an IM_1F_1 population. In 2009, 71 IM_1F_1 plants were grown in St. Paul. The 55 surviving IM_1F_1 plants were phenotyped for average head diameter, largest head diameter, pollen viability, seeds per head, seed yield and individual seed weight in 2010 in St. Paul. During the winter of 2010-2011, 151 IM_1F_1 plants were grown in the greenhouse in St. Paul and screened for tuber production, along with *H. tuberosus* plants as controls. Plants in the greenhouse were grown in 30 cm pots with 50-50 mix of Sunshine professional growing mix® (Sun Gro) and soil. Plants were grown in a greenhouse with 14 h day-length at 24^0C, they were given no supplemental light. In 2011-2012, 104 tuber-bearing IM_1F_1 plants out of the 151 IM_1F_1 plants were transplanted in late May and grown in the field at Rosemount. As the IM_1F_1 population exhibited segregation for perennial habit and tuber traits, Chi-square tests were used to test examine segregation ratios for tuber presence in the IM_1F_1 population. We tested a single gene model where the expected ratio is 3:1 and a two gene model having complimentary gene action where the expected ratio is 9:7, both cases assumed disomic inheritance and equal initial allele frequencies.

Parental diversity analysis

Thirty-one additional *H. tuberosus* accessions from the GRIN collection (Table 1) were used to assess the diversity of the *H. tuberosus* germplasm, and estimate the representation of this diversity in our 18 lines used for breeding. DNA was prepared from the 31 accessions, along with 14 of the *H. tuberosus* and two of the *H. annuus* breeding parents, to assess diversity using molecular markers. Genomic DNA was isolated from fresh or freeze dried leaf tissue on all accessions using either a Qiagen Plant DNeasy Mini kit according to the manufacturer's protocol or a modified CTAB procedure optimized for sunflower (Webb and Knapp, 1990).

Sixteen expressed sequence tag-simple sequence repeat (EST-SSR) markers previously identified by Heesacker et al. (2008) were polymorphic in our population and used to genotype. Following DNA extraction, samples were sent to Biogenetic Services, Inc. (Brookings, SD). Samples were multiplexed by combining two loci, each with a different color label into a single plate. Multiplexed samples were loaded into the ABI3100 genetic analyzer and were run according to the manufacturer's standard recommendation. Direct labeled primers tagged with ABI dyes 6-FAM or Hex were scored on an Applied Biosystems Inc. (ABI) 3730xl capillary instrument. The resulting electropherograms were scored using the GeneScan software package (ABI). A numerical base-pair size was assigned to each electropherogram peak. The program TANDEM (Matschiner and Salzburger, 2009) was used to bin raw allele sizes. The program PowerMarker (Lui and Muse, 2005) was used to analyze genotype data by calculating expected heterozygosity, observed heterozygosity and the average number of alleles per locus. The SSR markers utilized appeared to follow a stepwise mutation model

therefore we used R_{ST} to differentiate the populations. R_{ST} is a measure of population differentiation that accounts for SSR markers undergoing a stepwise mutation model (Slatkin, 1995). The stepwise mutation model postulates that an SSR marker will change (gain or lose) by only one repeat unit per generation (Di Rienzo et al., 1994). R_{ST} was calculated between *H. annuus* parents, *H. tuberosus* parents, and GRIN accessions utilizing the program GENEPOP (Rousset, 2008).The genetic distance between population pairs was calculated using the Nei73 coefficient (Nei, 1973). An unrooted neighbor-joining tree was constructed using MEGA 5 (Tamura et al., 2011). Genetic assignment of genotypes was performed with Structure version 2.3.4 (Prichard et al., 2000). STRUCTURE was run with a Markov Chain Monte Carlo (MCMC) burn-in of 20,000 steps, followed by an MCMC chain of 10,000 steps for clustering inference. The number of subpopulations was determined by performing ten runs for each *K* (number of subpopulations), with *K*=1 to *K*=8 examined. An admixture model (mixed ancestry from multiple populations was allowed) was used along with uncorrelated allele frequencies between subpopulations. We used StructureHarvester to identify the optimum *K* using the Evanno method (Earl and vonHoldt, 2012; Evanno et al., 2005). CLUMPP was used to integrate results across runs per *K* (Jakobsson and Rosenberg, 2007).

Results

Validation of interspecific origin of hybrid populations

We utilized flow cytometry to compare the genome sizes of hexaploid *H. tuberosus* and diploid *H. annuus* to the hybrid offspring. A portion of the putative hybrid individuals may have resulted from inadvertent self-pollinations or mating between *H.*

tuberosus individuals. Therefore, this analysis served to identify the individuals that were true interspecific hybrids (i.e. tetraploid plants).

The estimated 1C genome size of *H. annuus* is ~ 3.3 pg of DNA (Bennett and Leitch, 2010). The two diploid annual parents used in the present study exhibited similar values, as HA 89 and HA 434 were measured at 3.45 pg and 3.60 pg, respectively (Table 3). The average 1C genome size among the 18 *H. tuberosus* accessions was 14.52 pg, higher than the previously reported value of 12.55 pg (Bennett and Leitch, 2010).

Based on flow cytometry measurements, the expected genome size for an average tetraploid hybrid was approximately 9 pg. The vast majority of the putative F_1 hybrid plants (166 out of 187) exhibited flow cytometry readings near this value, indicating that these are likely true interspecific hybrids (Figure 1). However, 21 putative F_1 hybrids (12.2%) had DNA content equal or greater than the reported 1C genome size for *H. tuberosus* (12.55 pg). We inferred that these plants were hexaploid, resulting from either self-pollinated or intermated *H. tuberosus* plants. These plants were excluded from further analysis.

In the intermated F_1 (IM_1F_1) population, nearly all plants (167 out of 170) contained approximately 9 pg DNA per cell, indicating that these plants maintained a tetraploid chromosome number. Three individuals (1.7%) in the IM_1F_1 population had a genome size outside the expected range (two appeared pentaploid, the other octoploid) and were also excluded from the phenotypic analysis. All BC_1F_1 plants were triploid and displayed annual habit. As no perennial plants were recovered, the BC_1F_1 population was not investigated further.

Trait evaluation in interspecific H. annuus x H. tuberosus F_1 populations

Generally, F1 individuals were intermediate to the domesticated annual and the wild parents for each trait examined, with most traits having wide distributions but being more similar to the wild parent (Figure 2-3). Some traits in the F_1 hybrids were more similar to the domesticated *H. annuus* (e.g., seed traits), and others were more similar to wild *H. tuberosus* (Figure 2-3; Table 4). For most traits, a few F_1 individuals closely resembled the domesticated phenotype. Across environments yield traits were positively correlated and tuber traits were correlated with each other (Table 5). Seed weight and number were not correlated with tuber traits (Table 5). Correlations from individual environments were different from the correlation pattern across all environments (Table 5). For individual traits, heritability in the F_1 varied from 0.05-0.76 indicating differing selective potential for different traits in this breeding program (Table 6). For most traits, heritability was similar across environments, but varied for seed weight and tuber weight (Table 6). We observed individuals that were consistently superior for seed and agronomic traits compared to the wild *H. tuberosus* parent in all environments, particularly we observed increases in head size and seed yield.

F_1 plants typically flowered earlier than *H. tuberosus* (Figure 2; Table 7). Three architecture traits were measured: branching type, spreading ability, and head number. The F_1 hybrids were not statistically different from *H. tuberosus* for spread ability but were for branching type. While branching type was significantly different (less branching) between F_1 and *H. tuberosus,* it still was not unbranched like the domestic *H. annuus* (Figure 2). F_1 plant spreading and head number varied greatly between environments. Hybrids displayed fewer total flowers, but not the single head type

preferred in *H. annuus* cultivars. Three tuber traits were examined: tuber number, total tuber weight and individual tuber weight. The F_1 hybrids had greater tuber number, individual and total tuber weight than *H. tuberosus* (Table 4).

The F_1 families were different from their wild *H. tuberosus* parents for most yield traits (Table 4; Table 7; Figure 2). Head size exhibited a wide phenotypic range both within plants and among families. The largest heads among the F_1 individuals were 5-6 times bigger in diameter than *H. tuberosus* (Figure 2-3). The majority of F_1 plants had few seeds (0-25), yet some individual F_1 plants yielded ten times this amount. There was differential shattering or predation among environments that likely increased seed yield variability.

Trait evaluation in the IM_1F_1 population

F_1 individuals were intermated to generate an IM_1F_1 population. We continued to observe improvements for yield traits in this generation, despite the absence of artificial selection pressure in choosing parents for the IM_1F_1. However, differences in pollen fertility may indicate that inadvertent selection for viability of pollen occurred. In 2010, 55 IM_1F_1 plants were evaluated for head and seed traits. Three plants were ranked in the top 10% for largest head diameter, seed weight, and seed per ten head for the IM_1F_1 population. The largest head was 20% larger than any head observed in the F_1. Positive correlations between head size, seed weight, and seed per head were maintained. The best IM_1F_1 individuals exhibited numerically higher values than the best F_1 individuals for all seed traits, indicating the potential power of a recurrent selection program.

While all F_1 individuals exhibited perenniality through tuber sprouting, the IM_1F_1 population segregated for the perennial habit. In the winter of 2009-2010, 71 plants were

screened for winter survival with 77% (55) surviving, which did not differ from a 3:1 ratio (P=0.89) but did differ from other tested ratios (Table 8). Initially, it was unknown whether this was a result of segregation for tuber production, tuber survival (winter hardiness), or both. To confirm that the 3:1 segregation result was largely the result of tuber production segregation, 151 additional IM_1F_1 plants were grown in the greenhouse during the winter of 2010-2011 and screened for tuber production. All control *H. tuberosus* plants and 67% (104) of the IM_1F_1 produced tubers. Again, the IM_1F_1 segregation did not significantly differ from a 3:1 (P=0.39) ratio (Table 8). In 2011-2012, we grew these 104 tuber-bearing plants in the field at Rosemount and found that 51% died, largely attributable to winter kill. Detailed evaluation of tuber winter hardiness has not been conducted to identify the factors involved.

Parental diversity

Sixteen SSR markers were used to examine the genetic diversity in the parents of the Minnesota perennial sunflower breeding program and a subset of the accessions in the GRIN collection. Although diversity measures showed a moderate level of diversity (Table 9), they are difficult to interpret due to complexities related to polyploidy (Brown and Young, 2000; Luo et al., 2006; Akhunov et al., 2010; Stift et al., 2008). However, useful estimates of the relatedness of the breeding material to other germplasm can be made. The parents of the Minnesota breeding program did not cluster with the accessions from the GRIN collection (Figure 4). R_{ST} indicated that the Minnesota population is moderately different than the GRIN accessions when grouped as a population (Table 10). Structure analysis identified two subpopulations ($K = 2$) as the best fit, essentially dividing the Minnesota and GRIN accessions (Figure 4a) (Prichard et al., 2000; Evanno

et al., 2005). This was similar to the interpretation obtained from examining a phylogenetic tree based on genetic distance (Figure 4b). This indicates that the 18 *H. tuberosus* individuals used to develop the F_1 and IM_1F_1 populations represent a relatively narrow sampling of the genetic pool of the species.

Discussion

Prospects and Limitations for perennial grain breeding

The combination of traits comprising the perennial *Helianthus* seed crop ideotype includes seed and head traits contributing to high seed yield (high pollen fertility, high seed weight, large head size), plant architecture traits enabling uniform seed maturity (no branching and one central flower, or all heads having synchronous flowering), and tuber traits contributing to a manageable perennial habit (low tuber number, high individual tuber weight). Therefore, the perennial ideotype is the domesticated phenotype with the addition of perenniality. This ideotype for the initial perennial sunflower lines would be targeted toward marginal landscapes with high potential for degradation to maximize the environmental benefit. In addition, the lines could be used as a trap crop near production fields to help mitigate bird predation.

The straightforward way to produce a perennial sunflower would be to backcross perennial habit into annual sunflower. Based on our investigation, we found that when F_1 plants (tetraploid) were crossed with *H. annuus* plants (diploid), weak annual triploid plants were generated. This was also found in other species where backcrossing approaches have generally led to a loss of perenniality, so breeding programs have focused on domestication of interspecific hybrids or wild relatives (Cox et al., 2010).

Therefore, we refocused our efforts on selection and intermating of the best F_1 individuals.

From a physiological perspective, there is reason for optimism regarding our refocused efforts on selecting for domestication phenotypes in the intermated individuals. With the potential to allocate resources to both sexual and asexual reproduction, the expectation is that both types of reproduction will compete for resources (Darwin, 1876; Van Noordwijk and De Jong, 1986). Empirical studies have identified negative (Westley, 1993) and positive phenotypic correlations (Cheplick, 1995) between sexual and asexual reproduction, with occasional genetic correlations as well as large environmental effects on resource allocation (Westley, 1993; Piquot et al., 1998). However, we observed few negative correlations between tuber and seed traits with total seed weight being significantly positively correlated with tuber traits; this may be due to the resource rich environments in which the plans were grown (Cheplick, 1995). The wide phenotypic distributions (with some favorable types and transgressive segregants) in the F_1 hybrids described here indicate that selection for both perenniality and yield in this population may be possible. Furthermore, wider ranges were observed in the IM_1F_1 compared to the F_1 for some traits, indicating that recurrent selection on intermated materials may successfully take advantage of the wide genetic variation for further improvement. Key improvement targets for the breeding program are summarized in Table 11.

From a genetic perspective, the situation is more nuanced and depends on currently unknown factors. It is attractive to consider the past utilization of the *Helianthus* wild germplasm as an indicator of future success for introgressing perenniality. Additionally, Baack et al. (2008) made the promising observation that

selection for domestication traits proceeds rapidly in progeny from domestic x wild annual *Helianthus* matings. We have observed this first hand, as improvements in seed and head traits were achieved while maintaining perennial habit after one generation of intermating. However, our breeding design is unique in that it uses interploidy hybridization and maintains the populations as polyploids. The ability to drive the phenotypic traits toward the ideotype using our intermating scheme will depend on the rate at which the *H. tuberosus* genetic material can be purged from the genome (and replaced by the *H. annuus* genetic material), while maintaining perenniality. There are two factors that will determine this limitation: 1) The amount of *H. tuberosus* genetic material that is required for perenniality and 2) The meiotic pairing behavior of homeologous chromosomes.

Best case scenarios require that very little *H. tuberosus* genetic material is necessary to confer perenniality in the intermated progeny. This would reduce the linkage drag associated with introgressing the perenniality loci. Furthermore, an ideal scenario would presume that the A_1, A_2, and B_t sub-genomes of *H. tuberosus* are all capable of pairing with the *H. annuus* chromosomes. In this case, it would be possible to continuously increase the proportion of the *H. annuus* genome with each successive generation of intermating possibly increasing the speed of producing the ideotype. However, if the B_t genome exclusively pairs with the *H. annuus* chromosomes, then it would be impossible to purge the A_1 and A_2 chromosomes, regardless of the number of generations of intermating. In this case, all of the intermated tetraploid plants would contain at least 50% *H. tuberosus* genome, severely limiting the progress that could be made towards achieving the ideotype.

If conventional breeding is severely limited by these genetic limitations, it may be possible that genetic transformation could provide an avenue to create a plant more similar to the ideotype. If the gene(s) underlying the seemingly simple segregation in the IM_1F_1 for tuber development (discussed in the next section) can be identified, they may be cloned and transformed for this purpose. It is unclear if such an approach would be acceptable to consumers, and the approach is likely to be subject to regulatory assessment due to the invasive potential of transgenic sunflowers. The intriguing question also arises whether coupling genetic transformation to ecosystem services will make the technology more palatable.

Segregation of perenniality in Helianthus IM_1F_1 and other species

Variation in perennial traits (including the lack of perenniality) has been reported in populations derived from perennial rice, sorghum, wheat grass, and teosinte crossed with their annual crop relatives (Hu et al., 2003, Murphey et al., 2009; Lammer et al., 2004; Westerbergh and Doebley, 2004; Sacks et al., 2006a; Sacks et al., 2006b; Sacks et al., 2003a; Sacks et al., 2003b). In the present study, all *Helianthus* F_1 plants were perennial and the IM_1F_1 population segregated approximately 3:1 for tuber production with tuber survival (over-wintering ability) differing among seasons. This finding implies that it may be relatively simple to identify the genetic factors that are most essential for perennial organ development.

Segregation for perenniality in the IM_1F_1 has several potential explanations (Figure 5). The first and simplest explanation is that a single dominant gene in the *H. tuberosus* genome is necessary for tuberization (Figure 5b), but it is probably not that simple because the BC_1F_1 generations did not produce tubers. Second, segregation for

perenniality may result from sub-genome dosage effects. It has been observed that interspecific hybrids with genomic composition less than 50% of the perennial parent rarely maintain pereniality (Cox et al., 2002; Cox et al., 2010). This suggests that there may be a stoichiometric regulatory balance, and that certain proportions are necessary for phenotypic stability (Birchler and Veitia, 2007; Birchler et al., 2001). This scenario is supported by the lack of perenniality in the BC_1F_1 individuals, which had a reduced relative proportion of the *H. tuberosus* sub-genomes (Figure 5c). This may present a problem as we continue to attempt to enrich for rare recombination events to purge as much as possible of the *H. tuberosus* genome, but currently we see improvement in phenotypes. Thirdly, the interspecific hybrid may be viewed as a neopolyploid, which may cause multivalent pairing, homoeologous recombination, aneuploidy, and/or large de novo structural variants (Chester et al., 2012; Tate et al., 2009). If this scenario is true genome stability may be increased by intermating for several generations. While these phenomena may result in inconsistent transmission for many traits, including perenniality, imposing selection may lead to more stable plants allowing us to identify those individuals that have the perennial ideotype.

Conclusion

Based on the examination of the *H. tuberosus* parents, the F_1, IM_1F_1 and BC_1F_1, hybridization followed by selection for domestication traits appears feasible to improve *Helianthus* for use as a perennial oil-seed crop. The development of perennial oil-seed is a long term endeavor; however, there are checkpoints along the way such as use as a trap crop that provide value during the development. As these checkpoints are reached new agronomic and disease challenges (likely due to the lack of crop rotation) will need to be

addressed in order for perennial crops to be adopted. In addition, much can be learned about the biology of perennial habit and about interspecific hybridization. The intermating (IM_1F_1) approach exhibited the greatest potential, as domestication traits were improved in the IM_1F_1 while maintaining perenniality in a high proportion of the population. The improved phenotypic traits in the IM_1F_1 may be indicative of the loss of wild chromosomes or portions of chromosomes in favor of domestic chromosomes. Therefore, recurrent intermating and selection of advanced intermated lines appears to be a promising approach for further improvement. Perenniality may segregate in a relatively simple way even if the underlying genetics are complex. Our data indicate that we have started our program with limited diversity, but despite this we have seen gains in initial generations (Table 8; Table 11). If we do not see continued gains, the addition of more parents to the University of Minnesota perennial sunflower breeding program would likely be beneficial. There is potential to eventually develop a perennial *H. annuus*-like plant that produces tubers and yield grain consistently over the life of a stand, leading to a crop that produces ecosystem services while having a commercially viable yield.

Future Directions for the University of Minnesota Perennial sunflower Breeding Program

In order to continue developing the perennial sunflower breeding program at the University of Minnesota there are four major areas of study that should be pursed in the future. These broadly fit into the categories of 1) Continued population development through recurrent selection; 2) Development of molecular genetic resources; 3) Better understanding of the physiological relationship between photosynthate partitioning in herbaceous perennial seed producing plants; and 4) Understanding the economic potential of perennial sunflower and policy initiatives that may accelerate adoption.

Continued population development through recurrent selection

During this research, the highest yielding individuals in the IM_1F_1 population were planted in an isolated crossing block to create an IM_2F_1. An IM_3F_1 was created from half-sib families of the IM_2F_1. An additional IM_1F_1 was created from the highest yielding F_1 plants and families. These materials will be used to continue the breeding program. There are four areas that these populations will be used to examine: The first is to phenotype for seed composition and oil quality to identify differences from market quality the oil. This will inform potential use and provide additional targets for selection for continued improvement of the populations.

Next, there should be continued selection and intermating based on selection of plants based on yield and architecture characteristics. Currently it is unclear if the domestic architecture is needed to improve the population or if synchrony of flowering would also provide similar improvement. Understanding what the "best" architecture is will inform the selection and allow progress to be made more quickly. In addition, a synthetic variety should be created from the best individuals. Briefly, IM_2F_1 half-sib families will be plant with the best individuals from among the families selected using these individuals as the basis for random mating seed to use for further testing. The population was your tester and pollen source, and so the progeny rows are single plant x population crosses that you can use to judge performance with relation to the unselected population itself. Seed of the best 20% of the rows will be used for random mating in the greenhouse. This random mated seed will be Syn1 and can be grown as an increase to produce Syn2.

The third area that needs to be examined should be conducted by our collaborators in the USDA-ARS (Dr. George Linz). This involves testing the improved seeds on large plots for their ability to mitigate bird predation near sunflower production fields.

Lastly, it has been identified that tuber production does not mean plants are winter hardy. Therefore the variation in freezing tolerance in Interspecific *Helianthus* tubers needs to be examined to see if this needs to be a characteristic that should be selected for in the breeding program. Briefly, twelve genotypes corresponding to different genomic backgrounds of H. tuberosus (*H. tuberosus* and *H. tuberosus* × *H. annuus*) will be examined. One tuber will be planted per pot with all tubers of any given genotype being harvested from the same clone that had fully senesced and had produced viable seed. Analysis will include LD50 curves with *H. tuberosus* serving as a control. Germination after freezing can be tested with GA treatment.

Development of molecular genetic resources

Currently, a study is underway in collaboration with the University of British Columbia in Vancouver to assess the molecular genetic basis of the tuber production in the segregating IM_1F_1 population. Briefly, ~80 IM_1F_1 plants are being submitted for genotype by sequencing (GBS), these data will provide a large number of single nucleotide polymorphism (SNP) markers that can be used to characterize the perennial sunflower population. There is the potential that a single genomic region that confers the ability to produce tubers in sunflower populations will be identified.

These SNP markers can then repurposed for several other projects that would provide basic information about the perennial sunflower breeding program more general

information about *H. tuberosus* and interspecific *Helianthus* hybrids. To this end the markers could be used to ssess diversity in *H. tubersosus*, and assess how diversity is maintained in the IM populations. They could be used to build sequence based karyotypes of *H. tuberosus* which could then be compared based on structural differences with *H. annuus* and the interspecific hybrids. If a single gene for tuber production is identified then the following could be explored: 1) clone the gene, 2) develop a working transformation protocol for sunflower, and 3) transform the tuber production gene into annual sunflower. Finally, a linkage map of *H. tuberosus* and of the interspecfic hybrids could be created as the F_1 population can be used to simplify the genetics of *H. tuberosus*.

Better understanding of the physiological relationship between photosynthate partitioning in herbaceous perennial seed producing plants

The first step in this future direction will be explore the literature and develop a model that compare annual seed production vs. perennial seed production given an annual seed harvest. This model will focus on the potential photsynthate partitioning a forward simulation with selection over many generations. Utilizing previously published data as potential physiological boundaries, a forward simulation can be created examining how different potential genetic interactions between above and below ground biomass production will affect yield and yield improvment from selection in a perennial grain over time (20-40 generations of selection).

The veracity of this simulation can then be examined by conducting a photosynthate partitioning experiment in *H. tuberosus*, *H. annuus* and interspecific *Helianthus* hybrids. This experiment would examine harvest index, above ground

biomass at sequential time points, below ground biomass at sequential time points, carbon use, nitrogen use, light absorption, and water use. This empirical data can then be compared to the simulation, and it can also be used to modify selection targets in the perennial sunflower breeding program.

Understanding the economic potential of perennial sunflower and policy initiatives that may accelerate adoption

This initiative will help evaluate if under the current system whether or not perennial grain crops can be feasible adopted. This could be done by creating economic goals for the plant material and creating a decision tree and risk analysis given the different parameters of different perennial crops. This model could then be used to analyze many different perennial crops and help assess which crops should receive the most resources. Ideally such a model would be able to answer questions such as:

- What are changes can be made to the system to achieve perennial crop adoption?
- What are the relative costs compared to other crops in Minnesota/Upper Midwest?

This dissertation has made a preliminary examination of a perennial oilseed crop. There is significant potential to ask both scientifically and societally relevant questions with the next generation of plant material.

Table 1. Parentage of interspecific F_1 hybrids, IM_1F_1 plants, BC_1F_1 plants, and GRIN accessions.

Interspecific F_1 parantage			IM_1F_1 parantage		BC_1F_1 parantage			GRIN Accessions for diversity assessment
Paternal Parent	Maternal Parent	Hybrid	Maternal Parent	Intermated F1 Hybrid	Maternal Parent	Paternal Parent	BC1F1	LENNINGRAD
JA1	CMS HA89	CMS 201 A	JA213B	JA213B6_6.3	JA306	HA434	JA306 x HA434	PI650098
JA1	CMS HA89	CMS 201 B	JA314C	JA314C12_4.2	JA312	HA434	JA312 x HA434	PI650150
JA1	CMS HA89	CMS 201 C	JA201A	JA201A_12.2	JA316D	HA434	JA316D x HA434	TUB1904
JA1	CMS HA89	CMS 201 D	JA207F	JA207F3_8.3	JA316D	HA434	JA316D x HA434	AMES2067
JA3	CMS HA89	CMS 203 B	JA201A	JA201A2_12.3	JA316D	HA434	JA316D x HA434	AMES2229
JA5	CMS HA89	CMS 205 A	JA307C	JA307C9_18.3	JA305C	HA434	JA305C x HA434	TUB1783
JA5	CMS HA89	CMS 205 B	JA207B	JA207B4_4.3	JA305C	HA434	JA305C x HA434	TUB2024
JA5	CMS HA89	CMS 205 C	JA306D	JA306D9_12.2	JA210E	HA89	JA210E x HA89	TUB2061
JA5	CMS HA89	CMS 205 D	JA207C	JA207C4_5.3	JA210E	HA89	JA210E x HA89	TUB1774
JA6	CMS HA89	CMS 206 A	JA305C	JA305C9_7.3	JA210E	HA89	JA210E x HA89	TUB1775
JA6	CMS HA89	CMS 206 B	JA207C	JA207C4_5.4	JA316C	HA434	JA316C x HA434	TUB2051
JA8	CMS HA89	CMS 208 A	JA305A	JA305A9_5.2	JA213A	HA89	JA213A x HA89	TUB1775
JA8	CMS HA89	CMS 208 B	JA207C	JA207C9_18.2	JA305D	HA434	JA305D x HA434	TUB1786
JA8	CMS HA89	CMS 208 C	JA216G	JA216G7_13.2	CMS208C	HA89	CMS208 C x HA89	TUB1776
JA8	CMS HA89	CMS 208 D	JA209B	JA209B4_15.2	CMS208C	HA89	CMS208 C x HA89	TUB49
JA8	CMS HA89	CMS 208 E	JA216C	JA216C7_13.3	CMS208C	HA89	CMS208 C x HA89	TUB1799
JA9	CMS HA89	CMS 209 A	JA211B	JA211B5_7.2	CMS208C	HA89	CMS208 C x HA89	TUB2047
JA9	CMS HA89	CMS 209 B	JA216G	JA216G7_7.4	CMS201B	HA89	CMS201 B x HA89	AMES2747
JA9	CMS HA89	CMS 209 C	JA211F	JA211F5_11.3	CMS201B	HA89	CMS201 B x HA89	TUB33
JA12	CMS HA89	CMS 212 B	JA213E	JA213E6_4.2	JA313B	HA434	JA313B x HA434	TUB7318
JA12	CMS HA89	CMS 212 C	JA211F	JA211F5_11.4	JA313B	HA434	JA313B x HA434	AMES7318
JA16	CMS HA89	CMS 216 C	JA213B	JA213B6_6.6	CMS208C	HA89	CMS208 C x HA89	TUB1635
JA18	CMS HA89	CMS 218 B	JA211F	JA211F5_11.6	CMS208C	HA89	CMS208 C x HA89	AMES22746
JA18	CMS HA89	CMS 218 C	JA213B	JA213B6_6.4	CMS208C	HA89	CMS208 C x HA89	TUB2062
HA89	JA1	JA 201 A	JA212C	JA212C5_16.5	CMS208C	HA89	CMS208 C x HA89	AMES8387
HA89	JA1	JA 201 B	JA213B	JA213B6_6.3	CMS208C	HA89	CMS208 C x HA89	TUB1769
HA89	JA1	JA 201 C	JA212C	JA212C5_16.5	JA213	HA89	JA213 x HA89	TUB2066
HA89	JA1	JA 201 D	JA213B	JA213B6_6.2	JA213	HA89	JA213 x HA89	TUB2062
HA89	JA1	JA 201 E	JA314A	JA314A2_11.2	JA213	HA89	JA213 x HA89	TUB1277
HA89	JA1	JA 201 F	JA315E	JA315E12_12.2	JA213	HA89	JA213 x HA89	TUB2052
HA89	JA1	JA 201 H	JA306G	JA306G9_15	CMS218C	HA89	CMS218 C x HA89	
HA89	JA2	JA 202 A	CMS205B	CMS205B1_7	CMS218C	HA89	CMS218 C x HA89	
HA89	JA2	JA 202 B	JA306D	JA306D11_7.2	JA210	HA89	JA210 x HA89	
HA89	JA3	JA 203 B	CMSxxxB	CMSxxxB2_3.2	JA309B	HA434	JA309B x HA434	
HA89	JA5	JA 205 D	JA306D	JA306D11_7.1	JA309B	HA434	JA309B x HA434	
HA89	JA6	JA 206 A	CMS201B	CMS201B1_2	JA309B	HA434	JA309B x HA434	
HA89	JA6	JA 206 B	JA303A	JA303A9_2	CMS208C	HA89	CMS208 C x HA89	
HA89	JA6	JA 206 C	CMS205B	CMS205B1_7.2	CMS208C	HA89	CMS208 C x HA89	
HA89	JA6	JA 206 D	JA216B	JA216B7_8	CMS208C	HA89	CMS208 C x HA89	
HA89	JA6	JA 206 E	JA201C	JA201C2_14	CMS208C	HA89	CMS208 C x HA89	
HA89	JA6	JA 206 G	JA215D	JA215D7_5	CMS208C	HA89	CMS208 C x HA89	
HA89	JA6	JA 206 H	JA201D	JA201D2_15.2	CMS208C	HA89	CMS208 C x HA89	

HA89	JA7	JA 207 B	JA213B	JA213B6_6	CMS208C	HA89	CMS208 C x HA89
HA89	JA7	JA 207 C	JA201E	JA201E2_6.2	CMS208C	HA89	CMS208 C x HA89
HA89	JA7	JA 207 D	JA213A	JA213A6_5.3	CMS208C	HA89	CMS208 C x HA89
HA89	JA8	JA 208 A	JA201E	JA201E2_6.2	CMS208C	HA89	CMS208 C x HA89
HA89	JA8	JA 208 B	JA213A	JA213A6_5.2	CMS208C	HA89	CMS208 C x HA89
HA89	JA8	JA 208 D	JA201E	JA201E2_16.4	CMS208C	HA89	CMS208 C x HA89
HA89	JA209	JA 209 B	JA213A	JA213A6_5.1	JA313B	HA434	JA313B x HA434
HA89	JA209	JA 209 C	JA202B	JA202B3_3.2	JA313B	HA434	JA313B x HA434
HA89	JA209	JA 209 D	JA211F	JA211F5_11.5	JA313B	HA434	JA313B x HA434
HA89	JA209	JA 209 E	JA204A	JA204A3_8.2	JA313B	HA434	JA313B x HA434
HA89	JA10	JA 210 B	JA211F	JA211F5_11.2	JA313B	HA434	JA313B x HA434
HA89	JA11	JA 211 B	JA204B	JA204B7_8	JA313B	HA434	JA313B x HA434
HA89	JA11	JA 211 E	JA211F	JA211F5_11.1	JA313B	HA434	JA313B x HA434
HA89	JA11	JA 211 G	JA205C	JA205C3_11.2	JA213	HA89	JA213 x HA89
HA89	JA12	JA 212 A	JA208C	JA208C4_15	JA213	HA89	JA213 x HA89
HA89	JA12	JA 212 B	JA207C	JA207C4_5.2	JA213	HA89	JA213 x HA89
HA89	JA12	JA 212 C	JA316B	JA316B12_15	JA213	HA89	JA213 x HA89
HA89	JA12	JA 212 D	JA216xx	JA216xx7_13	CMS208C	HA89	CMS208 C x HA89
HA89	JA12	JA 212 F	CMS208C	CMS208C1_14	CMS208C	HA89	CMS208 C x HA89
HA89	JA12	JA 212 G	JA201A	JA201A2_12	CMS208C	HA89	CMS208 C x HA89
HA89	JA12	JA 212 H	JA211xx	JA211xx5_7.2	CMS208C	HA89	CMS208 C x HA89
HA89	JA12	JA 212 I	JA201A	JA201A2_12.2	CMS208C	HA89	CMS208 C x HA89
HA89	JA13	JA 213 A	JA314C	JA314C12_4	CMS208C	HA89	CMS208 C x HA89
HA89	JA13	JA 213 B	JA208E	JA208E4_12	CMS208C	Ha89	CMS208 C x Ha89
HA89	JA13	JA 213 C	JA314C	JA314C12_4	CMS208C	HA89	CMS208 C x HA89
HA89	JA13	JA 213 D	JA209C	JA209C4_16	CMS208C	HA89	CMS208 C x HA89
HA89	JA13	JA 213 E	JA312F	JA312F11_16.2	CMS208C	HA89	CMS208 C x HA89
HA89	JA13	JA 213 F	JA210D	JA210D5_4.2	CMS208C	Ha89	CMS208 C x Ha89
HA89	JA14	JA 214 A	JA312F	JA312F11_16	CMS208C	HA89	CMS208 C x HA89
HA89	JA14	JA 214 B	JA211A	JA211A5_12	CMS208C	HA89	CMS208 C x HA89
HA89	JA14	JA 214 C	JA310B	JA310B12_6	CMS208C	HA89	CMS208 C x HA89
HA89	JA14	JA 214 E	JA211A	JA211A5_13.2	CMS208C	HA89	CMS208 C x HA89
HA89	JA14	JA 214 G	JA211A	JA211A5_13.2	CMS208C	HA89	CMS208 C x HA89
HA89	JA15	JA 215 A	JA307xx	JA307xx9_18.2	JA313B	HA434	JA313B x HA434
HA89	JA15	JA 215 B	JA211B	JA211B5_7	JA313B	HA434	JA313B x HA434
HA89	JA15	JA 215 C	JA307xx	JA307xx9_16	JA313B	HA434	JA313B x HA434
HA89	JA15	JA 215 D	JA212B	JA212B5_15	JA313B	HA434	JA313B x HA434
HA89	JA15	JA 215 E	JA309A	JA309A9_5	JA313B	HA434	JA313B x HA434
HA89	JA15	JA 215 F	JA212C	JA212C5_16	JA313B	HA434	JA313B x HA434
HA89	JA16	JA 216 A	JA218D	JA218D8_7	JA313B	HA434	JA313B x HA434
HA89	JA16	JA 216 B	JA212H	JA212H6_3	JA313B	HA434	JA313B x HA434
HA89	JA16	JA 216 C	JA216F	JA216F7_12	JA313B	HA434	JA313B x HA434
HA89	JA16	JA 216 D	JA213E	JA213E6_9	JA213	HA89	JA213 x HA89
HA89	JA16	JA 216 E	JA214H	JA214H6_3	JA213	HA89	JA213 x HA89
HA89	JA16	JA 216 F	JA213E	JA213E6_9.2	JA213	HA89	JA213 x HA89

HA89	JA16	JA 216 G	JA214E	JA214E6_15	JA213	HA89	JA213 x HA89
HA89	JA17	JA 217 A	JA307xx	JA307xx9_18	JA313B	HA434	JA313B x HA434
HA89	JA17	JA 217 B	JA204D	204D2_15	JA313B	HA434	JA313B x HA434
HA89	JA17	JA 217 C	JA204A	204A2_10	JA313B	HA434	JA313B x HA434
HA89	JA17	JA 217 D	JA204A	204A3_8	JA313B	HA434	JA313B x HA434
HA89	JA17	JA 217 E	JA205C	205C3_1	JA313B	HA434	JA313B x HA434
HA89	JA17	JA 217 F	JA206D	206D3_16	JA313B	HA434	JA313B x HA434
HA89	JA17	JA 217 G	JA207B	207B4_4.1	JA313B	HA434	JA313B x HA434
HA89	JA17	JA 217 H	JA207B	207B4_4.2	JA313B	HA434	JA313B x HA434
HA89	JA18	JA 218 A	JA208D	208D4_4	JA313B	HA434	JA313B x HA434
HA89	JA18	JA 218 B	JA209A	209A4_14	JA313B	HA434	JA313B x HA434
HA89	JA18	JA 218 C	JA210B	210B5_5	JA313B	HA434	JA313B x HA434
HA89	JA18	JA 218 D	JA216B	216B7_10.2	JA313B	HA434	JA313B x HA434
HA89	JA18	JA 218 E	JA305C	305C9_7	JA313B	HA434	JA313B x HA434
HA89	JA18	JA 218 G	JA305C	305C9_7.2	JA313B	HA434	JA313B x HA434
HA89	JA18	JA 218 H	JA309A	309A10_16	JA313B	HA434	JA313B x HA434
HA434	JA1	JA 301 A	JA318D	318D13_13	JA313B	HA434	JA313B x HA434
HA434	JA1	JA 301 B	JA308A	308A10_7	JA313B	HA434	JA313B x HA434
HA434	JA1	JA 301 C	JA317G	317G13_9.2	JA313B	HA434	JA313B x HA434
HA434	JA1	JA 301 D	JA305C	305C9_7.3	JA313B	HA434	JA313B x HA434
HA434	JA1	JA 301 E	JA310D	310D13_13	JA313B	HA434	JA313B x HA434
HA434	JA1	JA 301 F	JA317G	317G13_9	JA313B	HA434	JA313B x HA434
HA434	JA2	JA 302 A	JA314A	314A2_21	JA313B	HA434	JA313B x HA434
HA434	JA3	JA 303 A	JA312E	312E11_15	JA313B	HA434	JA313B x HA434
HA434	JA3	JA 303 B	JA312F	312F11_16	JA313B	HA434	JA313B x HA434
HA434	JA3	JA 303 C	CMS201C	CMS201C1_3.1	JA313B	HA434	JA313B x HA434
HA434	JA5	JA 305 A	CMS201C	CMS201C1_3.2	JA213	HA89	JA213 x HA89
HA434	JA5	JA 305 B	CMS201D	CMS201D1_4	JA213	HA89	JA213 x HA89
HA434	JA5	JA 305 C	JA208D	208D4_11	JA213	HA89	JA213 x HA89
HA434	JA5	JA 305 D	JA214E	214E6_15.2	JA213	HA89	JA213 x HA89
HA434	JA6	JA 306 B	JA207D	207D7_10	JA210	HA89	JA210 x HA89
HA434	JA6	JA 306 C	CMS212C	CMS212C2_4	JA210	HA89	JA210 x HA89
HA434	JA6	JA 306 D	JA207C	IM.383	CMS201B	HA89	CMS201 V x HA89
HA434	JA6	JA 306 E	JA213D	IM.395			
HA434	JA6	JA 306 F	JA216A	IM.396			
HA434	JA6	JA 306 G	JA317G	IM.398			
HA434	JA7	JA 307 A	JA316B	IM.431			
HA434	JA7	JA 307 B	JA201B	JA201B2_11			
HA434	JA7	JA 307 D	JA202B	JA202B3_3			
HA434	JA7	JA 307 E	JA202B	JA202B3_3.2			
HA434	JA7	JA 307 F	JA207C	JA207C4_5.1			
HA434	JA7	JA 307 G	JA208B	JA208B4_9			
HA434	JA7	JA 307 I	JA208E	JA208E4_12			
HA434	JA8	JA 308 A	JA210D	JA210D4_2.2			
HA434	JA8	JA 308	JA212C	JA212CB5_15			

		B		
HA434	JA8	JA 308 D	JA212E	JA212E5_18
HA434	JA8	JA 308 E	JA122G	JA122G6_2
HA434	JA8	JA 308 G	JA216A	JA216A7_7.1
HA434	JA8	JA 308 H	JA216A	JA216A7_7.2
HA434	JA8	JA 308 I	JA217C	JA217C4_5.2
HA434	JA9	JA 309 A	JA309C	JA309C10_18
HA434	JA9	JA 309 C	JA309C	JA309C10_18
HA434	JA9	JA 309 D	JA313A	JA313A11_18
HA434	JA9	JA 309 E	JA207A	JA207A4_3
HA434	JA9	JA 309 F	JA211A	IM.330
HA434	JA9	JA 309 G	JA303A	IM.331
HA434	JA10	JA 310 A	JA214E	Im.333
HA434	JA11	JA 311 A	JA214E	IM.334
HA434	JA11	JA 311 B	JA306H	IM.348
HA434	JA12	JA 312 A	JA203D	IM.351
HA434	JA12	JA 312 B	CMS208C	IM.354
HA434	JA12	JA 312 C	CMS208C	IM.355
HA434	JA12	JA 312 D	CMS205A	IM.359
HA434	JA12	JA 312 E	JA201B	Im.361
HA434	JA12	JA 312 F	JA201B	IM.362
HA434	JA12	JA 312 G	JA215D	IM.371
HA434	JA13	JA 313 A	JA313A	IM.373
HA434	JA13	JA 313 B	JA209C	IM.380
HA434	JA14	JA 314 A	JA205C	IM.390
HA434	JA14	JA 314 B	CMS210B	IM.401
HA434	JA14	JA 314 C	JA210D	IM.404
HA434	JA14	JA 314 D	JA212E	IM.406
HA434	JA14	JA 314 E	JA211F	IM.407
HA434	JA14	JA 314 F	JA201F	IM.408
HA434	JA15	JA 315 A	JA201F	IM.409
HA434	JA15	JA 315 B	JA206D	IM.411
HA434	JA15	JA 315 C	JA205D	IM.415
HA434	JA15	JA 315 D	JA205D	IM.424
HA434	JA15	JA 315 F	JA301E	IM.443
HA434	JA16	JA 316 A	JA206E	IM.446
HA434	JA16	JA 316 B	JA206E	IM.447
HA434	JA16	JA 316 C	JA2156B	IM.448
HA434	JA16	JA 316 D	JA307C	IM.450
HA434	JA16	JA 316 F		
HA434	JA16	JA 316 G		
HA434	JA17	JA 317 A		
HA434	JA17	JA 317 B		
HA434	JA17	JA 317 C		
HA434	JA17	JA 317 D		
HA434	JA17	JA 317		

		E
HA434	JA17	JA 317 F
HA434	JA17	JA 317 G
HA434	JA18	JA 318 A
HA434	JA18	JA 318 B
HA434	JA18	JA 318 C
HA434	JA18	JA 318 D
HA434	JA18	JA 318 E
HA434	JA18	JA 318 F
HA434	JA18	JA 318 G
HA434	JA18	JA 318 H

Table 2. Description of phenotyping methodology for each trait examined.

Days to flowering	Counted from date of emergence in the spring to the appearance of the first flower
Total tuber number Total tuber weight (grams) Average tuber weight (grams)	Tubers were harvested in November 2009 by digging, washing, counting and weighing all tubers in a 0.5 m radius around each individual plant, as we observed that most tubers were centered in this area. To improve efficiency in data collection, in 2010, a subsample was taken at Rosemount and St. Paul that consisted of six 15 cm soil cores taken in a 0.5 m radius around each individual plant. The subsamples were calibrated to whole plot measurements by harvesting a row of plants (41 in total) in 2010 using the 2009 method. When the measurements of all tubers per plant in 2009 were compared to the partial sampling measured in 2010, the R2= 0.71 for tuber number and R2= 0.63 for tuber weight (based on individual plant), with the relationship between years fitting a linear model better than a quadratic model.
Pollen viability	Scored by staining pollen with Alexander stain and scoring 300 pollen grains from each replication (Alexander, 1980)
Flower number	ounted for each plant at physiological maturity
Branching type	Scored on a scale of 0-4 according to Hockett and Knowles (1970), with 0 being no branching.
spreading ability	Above ground plant spreading ability was scored on a 1-5 scale with 1 indicating the domestic phenotype of spreading to 15 cm, 2 indicated that plants spread 15-30 cm, 3 indicated intermediate spreading of 31-60 cm, 4 indicated vigorous spreading of 61-90 cm, 5 had spread greater than 90 cm.
Maximum head diameter (cm)	Measured in cm after plant physiological maturity
Average head diameter (cm)	Measured in cm after plant physiological maturity. Ten randomly selected heads, including the central head, were measured to calculate average head diameter.
Number of seeds per head	Calculated by dividing the total number of seeds by the ten heads harvested.
Seed weight (grams)	Calculated by threshing ten random heads from each plant, including the central head, and weighing the resulting seeds.Heads were randomly chosen on plants. All plants were not harvested at the same time but all heads were harvested from the same individual plant on the same day. Plants were harvested as they reached physiological maturity, so early maturing individuals were harvested earlier.
Average seed weight (grams)	Calculated by weighing the seed from the ten heads and dividing by the total number of seed.

Table 3. 1C DNA content ranges (pg) for *H. annuus* and *H. tuberosus* accessions in the perennial sunflower breeding program and their F_1, IM_1F_1, and BC_1F_1 derivatives that were tested and literature values.

Accession	No. Individuals	Range (pg)	Mean (pg)	Mean (Gb)	Reported Value (pg)†
HA89	108	3.14-3.82	3.45	3.37	1.78-3.98
HA434	98	3.15-4.11	3.6	3.52	1.78-3.98
Helianthus tuberosus	18	12.95-15.58*	14.52	14.20	12.55ǂ
F_1	187	6.92-16.92	9.98	9.76	NA
IM_1F_1	170	7.53-19.03	9.5	9.29	NA
BC_1F_1	120	4.89-6.28	5.45	5.33	NA

†Reported values are all based flow cytometry for *H. annuus* and on Feuglen Densitometry for *H. tuberosus*, and were reported in Bennett and Leitch, 2010. Conversion to base pairs was done using the equation from Doezel et al., 2007.

ǂFeugelin microdensitometry was used in the initial measurement, which may underestimate genome size in the presence of secondary metabolites (Doezel et al., 2007).

*Variation in genome size was greater among the hexaploid *H. tuberosus* individuals than among diploid *H. annuus*, similar to high ploidy accessions of switchgrass (Costich et al., 2010).

Table 4. Analysis of Variance for the phenotypic traits of the interspecific F_1 hybrids and *H. tuberosus* parents.

	Yield Traits																	
	Largest Head Diameter			Average Head Diameter			Seed Per Head			Seed Weight (g)			Individual Seed Weight (g)			Pollen Fertility		
	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value
Environment	2	60.94	0.00	2	55.21	0.00	2	49.44	0.31	2	0.84	0.07	2	0.09	0.42	2	0.06	0.71
Replication (Environment)	6	1.08	0.00	6	0.26	0.03	6	34.03	0.00	6	0.20	0.40	6	0.09	0.04	6	0.17	0.00
genotype	183	1.14	0.00	183	0.73	0.00	183	15.98	0.00	183	0.25	0.01	183	0.04	0.00	183	0.08	0.00
H.tuberosus	17	0.19	0.20	17	0.18	0.07	17	2.78	0.97	17	0.01	0.92	17	0.00	0.69	17	0.17	0.06
F_1	165	0.70	0.00	165	0.45	0.00	165	17.47	0.00	165	0.27	0.02	165	0.05	0.00	165	0.06	0.00
Tuberosus Half Sib Family	16	1.59	0.19	16	1.14	0.05	16	13.11	0.07	16	0.29	0.94	16	0.02	0.73	16	0.16	0.04
Annual Half Sib Family	2	4.65	0.00	2	2.33	0.00	2	106.41	0.00	2	1.81	0.00	2	0.03	0.32	2	0.23	0.01
Full-Sib Family	23	0.71	0.00	23	0.40	0.01	23	22.25	0.01	23	0.40	0.26	23	0.02	0.46	23	0.07	0.01
tuberosus vs. F_1	1	93.85	0.00	1	57.79	0.00	1	3.02	0.46	1	0.86	0.04	1	0.01	0.55	1	1.81	0.00
genotype x environment	364	0.25	0.87	364	0.16	0.68	365	8.99	0.70	365	0.19	1.00	365	0.03	0.00	366	0.04	0.00
Environment x H.tuberosus	34	0.13	0.80	34	0.10	0.66	34	6.44	0.25	34	0.02	1.00	34	0.00	1.00	34	0.09	0.00
Environment x F_1	328	0.25	0.00	328	0.16	0.00	329	9.14	0.00	329	0.20	0.29	329	0.03	0.99	330	0.03	0.04
Environment x Annual Half-Sib Family	4	0.64	0.05	4	0.20	0.30	4	2.78	0.88	4	0.81	0.04	4	0.03	0.17	4	0.02	0.50
Environment x Tuberosus Half-Sib Family	32	0.26	0.53	32	0.26	0.03	32	10.18	0.35	32	0.28	0.67	32	0.02	0.28	32	0.04	0.03
Environment x Full Sib Family	46	0.27	0.01	46	0.17	0.02	46	9.41	0.00	46	0.32	0.00	46	0.02	1.00	46	0.03	0.51
Environment x (tuberosus vs. F1)	2	1.81	0.00	2	0.65	0.00	2	24.66	0.01	2	0.00	1.00	2	0.00	0.95	2	0.19	0.00
Error	761	0.17		762	0.11		915	5.59		915	0.19		915	0.04		1006	0.03	

	Agronomic Traits															Tuber Traits					
	Days From Germination To Flowering			Flower Number			Branch Score			Spread Score			Tuber Number			Tuber Weight (g)			Individual Tuber Weight (g)		
	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value	DF	MS	P-value
Environment	2	35790.53	0.01	2	224508307	0.00	2	170.41	0.00	2	58.23	0.02	2	2627613.05	0.00	2	24807416.01	0.01	2	1243.72	0.00
Replication (Environment)	6	2820.02	0.00	6	50932.78	0.00	6	0.35	0.83	6	7.48	0.00	6	166817.67	0.00	6	2301589.73	0.00	6	37.59	0.00
genotype	183	590.78	0.00	183	7150.85	0.00	183	1.18	0.13	185	1.17	0.01	185	78687.35	0.00	185	1043912.30	0.00	185	13.49	0.00
H.tuberosus	17	221.45	0.33	17	2302.97	0.63	17	0.54	0.24	17	0.57	0.43	17	5481.02	0.45	17	46021.12	0.76	17	9.55	0.24
F_1	165	271.54	0.00	165	7646.59	0.00	165	1.13	0.21	165	1.22	0.01	165	73953.46	0.00	165	920377.00	0.00	165	13.55	0.01
Tuberosus Half Sib Family	16	390.32	0.17	16	10255.66	0.04	16	1.01	0.28	16	1.74	0.73	16	108402.81	0.48	16	1645698.11	0.12	16	8.96	0.98
Annual Half Sib Family	2	488.72	0.08	2	16705.86	0.07	2	1.06	0.36	2	4.28	0.03	2	70150.44	0.15	2	329240.73	0.51	2	84.27	0.01
Full-Sib Family	23	357.64	0.02	23	7519.20	0.12	23	0.88	0.79	23	1.15	0.28	23	127394.52	0.00	23	954790.07	0.00	23	15.04	0.46
tuberosus vs. F_1	1	61311.04	0.00	1	4916.18	0.25	1	16.57	0.00	1	0.15	0.64	1	2005206.40	0.00	1	36629126.89	0.00	1	70.49	0.01
genotype x environment	362	166.65	0.55	366	4734.60	0.75	366	1.02	0.97	366	0.88	0.77	366	21530.15	0.57	366	310150.80	0.07	366	9.77	1.00
Environment x H.tuberosus	34	187.37	0.01	34	2695.87	0.88	34	0.41	0.98	34	0.54	0.83	34	5312.90	1.00	34	63529.32	1.00	34	7.20	0.78
Environment x F_1	326	155.03	0.00	330	4905.90	0.00	330	1.02	0.00	330	0.87	0.01	330	23222.09	0.00	330	335144.70	0.00	330	10.07	0.08
Environment x Annual Half-Sib Family	4	140.43	0.50	4	1527.55	0.88	4	0.53	0.78	4	2.47	0.03	4	90590.56	0.00	4	484260.75	0.13	4	36.64	0.04
Environment x Tuberosus Half-Sib Family	32	175.65	0.40	32	5741.87	0.27	32	1.00	0.73	32	1.11	0.23	32	34399.16	0.02	32	480754.17	0.01	32	15.62	0.38
Environment x Full Sib Family	45	160.51	0.03	46	5027.58	0.06	46	1.20	0.00	46	0.94	0.07	46	21876.51	0.01	46	273036.66	0.12	46	14.76	0.00
Environment x (tuberosus vs. F1)	2	1542.01	0.00	2	9519.16	0.08	2	10.36	0.00	2	8.36	0.00	2	22753.74	0.19	2	168659.42	0.46	2	0.14	0.98
Error	930	115.55		1071	3716.71		1071	0.72		1070	0.70		1035	13770.50		1035	217228.50		1033	8.93	

Table 5. Pearson correlation coefficients for all traits examined in the F_1 population. Bold text indicates a significant correlation. DTF= Days to flowering from germination; PF=Pollen fertility; SA=Spreading ability; BT=Branching type; HN=Head number; ITWG=Individual tuber weight; TN= Tuber number; TWG=tuber weight; AHD= Average head diameter; LHD=Largest head diameter; SW=Seed Yield; SPH=Seed per Head; ISW=Individual seed weight; GS= genome size. A) Combined across environments B) St. Paul 2009; C) St. Paul 2010; D) Rosemount 2010.

A)

	PF	SA	BT	HN	ITW	TN	TW	AHD	LHD	SW	SPH	ISW	GS
DTF	0.01	**0.38**	**0.27**	**0.45**	**0.42**	**0.10**	**0.09**	**-0.28**	**-0.25**	0.08	-0.01	**-0.40**	-0.06
PF		0.03	0.02	0.07	-0.02	-0.01	-0.01	0.07	0.07	**0.11**	0.10	-0.01	-0.06
SA			**0.33**	**0.45**	**0.22**	**0.18**	**0.24**	0.05	-0.01	0.10	0.06	**-0.18**	0.04
BT				**0.54**	**0.57**	**0.30**	**0.29**	-0.47	**-0.47**	0.07	-0.08	**-0.55**	0.03
HN					**0.56**	**0.28**	**0.32**	**-0.26**	**-0.28**	0.10	-0.05	**-0.45**	-0.01
ITWG						**0.33**	**0.50**	**-0.52**	**-0.46**	**0.12**	**-0.09**	**-0.66**	-0.01
TN							**0.83**	**-0.24**	**-0.20**	**0.17**	0.02	**-0.35**	0.00
TWG								**-0.16**	**-0.14**	**0.19**	0.06	**-0.29**	0.00
AHD									**0.90**	**0.19**	**0.36**	**0.58**	-0.02
LHD										**0.19**	**0.34**	**0.51**	-0.03
SW											**0.88**	-0.07	-0.02
SPH												0.07	-0.03
ISW													0.02

B)

	PF	SA	BT	HN	ITWG	TN	TWG	AHD	LHD	SW	SPH	ISW	GS
DTF	-0.11	**-0.23**	-0.09	**-0.29**	-0.03	-0.09	**-0.16**	-0.09	0.02	0.14	0.10	-0.09	-0.12
PF		0.07	0.04	0.07	-0.04	0.04	0.10	0.06	0.01	0.07	0.08	0.00	0.08
SA			**0.22**	**0.62**	-0.11	**0.21**	**0.36**	**0.30**	0.12	0.08	0.14	0.08	0.09
BT				**0.33**	-0.02	-0.02	0.09	-0.05	**-0.15**	0.06	0.06	-0.07	0.07
HN					-0.08	0.12	**0.26**	0.12	-0.09	-0.07	-0.01	0.03	0.00
ITWG						0.07	**-0.23**	-0.07	-0.02	-0.08	-0.06	-0.04	-0.11
TN							**0.79**	0.14	0.13	-0.03	-0.01	-0.01	0.01
TWG								**0.19**	0.13	0.03	0.05	0.07	0.04
AHD									**0.83**	**0.34**	**0.34**	**0.18**	0.05
LHD										**0.35**	**0.33**	**0.18**	0.03
SW											**0.88**	0.14	-0.05
SPH												0.03	-0.10
ISW													0.04

C)

	PF	SA	BT	HN	ITWG	TN	TWG	AHD	LHD	SW	SPH	ISW	GS
DTF	-0.03	**-0.16**	0.02	-0.14	0.12	-0.13	-0.04	-0.03	-0.07	0.08	0.14	-0.01	-0.13
PF		-0.05	0.08	0.04	-0.10	-0.05	-0.13	0.05	0.06	0.10	0.12	-0.11	0.03
SA			**0.15**	**0.25**	-0.01	0.12	0.08	0.04	0.02	-0.09	-0.06	-0.04	0.15
BT				**0.28**	0.03	0.08	0.04	**-0.25**	**-0.29**	**-0.21**	**-0.16**	-0.03	-0.02
HN					0.05	**0.29**	**0.27**	0.03	-0.02	0.09	0.08	0.04	0.00
ITWG						-0.02	**0.45**	0.11	0.10	0.07	0.09	-0.01	-0.04
TN							**0.82**	0.11	0.12	0.09	0.09	0.05	0.07
TWG								**0.17**	**0.17**	**0.15**	**0.15**	0.06	0.03
AHD									**0.92**	**0.53**	**0.49**	0.02	-0.07
LHD										**0.53**	**0.48**	0.03	-0.05
SW											**0.93**	0.13	-0.03
SPH												-0.04	0.00
ISW													0.06

D)

	PF	SA	BT	HN	ITW	TN	TW	AHD	LHD	SW	SPH	ISW	GS
DTF	0.06	**-0.37**	**-0.19**	-0.04	-0.05	0.02	-0.01	-0.13	-0.07	0.02	0.03	0.01	-0.03
PF		-0.04	0.00	-0.01	-0.07	0.06	0.03	0.09	0.08	0.08	0.04	0.05	-0.11
SA			**0.28**	**0.29**	0.05	0.12	**0.18**	**0.31**	**0.17**	0.02	**0.16**	-0.07	-0.08
BT				**0.20**	-0.03	0.03	-0.01	-0.08	-0.09	-0.06	0.01	-0.07	0.00
HN					**0.20**	0.11	**0.25**	0.11	0.03	0.04	0.13	-0.03	-0.13
ITW						**-0.18**	**0.37**	0.10	0.08	-0.05	0.01	-0.06	0.02
TN							**0.78**	**0.18**	0.14	**0.17**	**0.16**	0.07	-0.11
TW								**0.25**	**0.20**	0.09	**0.15**	-0.01	-0.12
AHD									**0.64**	**0.19**	**0.45**	-0.08	-0.11
LHD										**0.17**	**0.31**	-0.01	-0.11
SW											**0.52**	**0.84**	-0.02
SPH												-0.02	-0.02
ISW													-0.01

Table 6. Narrow sense heritability (h^2) based on parent offspring regression for each individual environment as well as for all environments together in the F_1. R = Rosemount, SP = Saint Paul.

Trait	All locations combined	R2010	SP2010	SP2009
Days from germination to flowering	0.33	0.25	0.12	0.25
Head number	0.18	0.19	0.26	0.15
Spread score	0.12	0.24	0.18	0.16
Pollen fertility	0.05	0.11	0.21	0.06
Tuber number	0.66	0.43	0.87	0.47
Tuber weight (g)	0.61	0.56	0.69	0.51
Individual tuber weight (g)	0.48	0.43	0.44	0.55
Largest head diamater	0.48	0.36	0.49	0.21
Average head diamater	0.60	0.33	0.51	0.22
Seed per head	0.31	0.20	0.21	0.23
Seed weight (g)	0.16	0.70	0.22	0.22
Individual seed weight (g)	0.22	0.61	0.17	0.14
Branch Score	0.32	0.14	0.17	0.35

Table 7. Summary statistics of all traits examined in the F1. Average and range of values observed across environments for *H. tuberosus* parents, as well as domestic annual half-sib families, *tuberosus* half-sib families, and full-sib families.

Trait	*H. tuberosus*			Annual half sib families			*H. tuberosus* half sib families			Full sib families		
	Average	Range		Average	Range		Average	Range		Average	Range	
		Min	Max		Min	Max		Min	Max		Min	Max
Days from germination to flowering	90.47	81.71	99.88	67.94	66.91	69.44	67.95	60.59	80.09	68.53	58.63	84.00
Head number	115.42	82.13	146.67	105.26	95.64	110.78	105.57	81.83	122.60	106.08	57.00	150.11
Spread score	3.57	2.78	2.78	3.49	3.32	3.58	3.50	3.15	3.15	3.51	3.00	4.00
Branch Score	3.50	3.11	4.00	3.13	3.08	3.20	3.11	2.81	3.30	3.10	2.67	3.44
Tuber number	124.31	82.25	196.63	248.60	236.44	260.31	251.82	172.91	351.96	259.42	153.44	483.14
Tuber weight (g)	463.14	294.75	645.88	949.35	865.91	991.31	1005.71	731.76	1532.00	999.26	497.89	1810.67
Individual tuber weight (g)	2.81	2.00	6.80	3.33	2.87	3.82	3.50	2.67	4.44	3.43	1.98	5.96
Largest head diameter	2.38	2.07	2.63	3.30	3.18	3.36	3.23	2.65	3.60	3.22	2.48	4.16
Average head diameter	2.00	1.72	2.28	2.72	2.64	2.77	2.66	2.16	2.91	2.65	2.04	3.24
Seed per head	2.70	1.48	3.38	2.88	2.27	3.38	2.60	1.33	3.26	2.67	0.43	5.72
Seed weight (g)	148.82	107.22	213.50	255.91	174.38	353.49	211.96	113.07	392.36	224.51	41.43	911.67
Individual seed weight (g)	0.013	0.006	0.047	0.025	0.019	0.032	0.022	0.012	0.079	0.022	0.009	0.166
Pollen fertility	0.50	0.30	0.71	0.38	0.35	0.40	0.37	0.27	0.43	0.37	0.24	0.52

Table 8. Segregation ratios for tuber production in IM_1F_1 plants

	3:1 ratio for tuber production		9:7 ratio for tuber production	
	Winter 2009-2010	Winter 2010-2011	Winter 2009-2010	Winter 2010-2011
Plants examined	71	151	71	151
Observed number of tuber producers	55	104	55	104
Expected number of tuber producers	53.25	113.25	39.94	84.94
Chi-square value	0.019	0.75	5.63	4.24
p-value	0.89	0.39	0.02	0.04

Table 9. Summary statistics for 16 SSR markers in the University of Minnesota perennial sunflower breeding program and a subset of the GRIN *H. tuberosus* collection.

Marker	Major Allele Freq	Allele No.	Exp. Heterygosity	Obs. Heterozygosity
HT283	0.607	5	0.581	0.262
HT298	0.408	13	0.781	0.421
HT315	0.242	12	0.863	0.636
HT314	0.271	10	0.845	0.458
HT385	0.362	12	0.798	0.345
HT421	0.362	10	0.776	0.276
HT364	0.362	7	0.777	0.276
HT1055	0.250	7	0.817	0.706
HT555	0.470	6	0.694	0.758
HT591	0.294	12	0.843	0.706
HT285	0.641	6	0.529	0.103
HT440	0.311	12	0.835	0.757
HT466	0.353	9	0.765	0.824
HT1021	0.924	4	0.144	0.121
HT538	0.333	13	0.812	0.818
HT948	0.286	9	0.814	0.238
Average	0.405	9	0.730	0.481

Table 10. Pairwise R_{ST} of parental populations and GRIN collection based on 16 SSR markers derived from the *H. annuus* collection.

	GRIN	MN Parents
Annual	0.33	0.46
GRIN		0.15

Table 11. Moving the University of Minnesota Perennial Sunflower Breeding Program toward the Perennial Ideotype.

Trait	ideotype	Elite F_1	IM_1F_1	Potential
Flowering Time	• Early to intermediate • Flowering time during the year	• Resembled the domestic type in flowering time	• Flowering was similar to F_1	• Flowering time was identified as an important domestication trait with initial domestication favoring early flowering (Blackman et al., 2011) and is already starting to resemble the ideotype
Plan Architecture	• No branching • Single head • Minimal Spread	• Some branching • Multiple heads • Exhibited heterosis for biomass leading to more spread and vigor relative to the H. tuberosus plants	• Less branching than F_1 • Fewer flowers than F_1 • Variation similar to the F_1 but had more extreme types for plant spread	• Extreme individuals and moderate heritability for architecture traits indicate that selection for improved types is possible • Flower number may be under relatively simple genetic control (Hockett and Knowles, 1970; Putt, 1964) • Spreading of interspecific hybrids showed variation, including types that did not spread indicating progress toward the ideotype
Tuber Traits	• Few tubers • Intermediate tuber yield high • Individual tuber weight • This would be ideal because larger tubers would reliably germinate but if numbers were low there would likely be minimal spread	• High tuber numbers • Heterosis for tuber yield • Tubers were generally small	• Variation similar to the F_1	• Tuber number had high narrow sense heritability, indicating that genetic effects may be easy to select for and that it may be easy to select individuals with low tuber number • Not all F_1 may be persistent and genotypes can be selected for decreased weed potential as individuals with a phenotype similar to the perennial ideotype were identified
Yield Traits	• Large headed like *H. annuus* • Large number of seed per head • High total yield • High individual seed weight • High pollen fertility	• Low family means compared to the range for yield traits with many individual outliers, similar to interspecific perennial rice populations (Sacks et al., 2006a).	• Variation similar to the F_1 but had more extreme individuals	• There was no relationship between pollen fertility and perennial traits, mirroring studies in interspecific rice (Sacks et al., 2006a) • Pollen fertility was not a goo predictor of yield, likely as here is little cost to the plant, as increased pollen production can account for low viability • In the F_1 tuber and yield traits were not correlated indicating that there may not be an antagonistic relationship between pereniality and yield • Head size had a high narrow sense heritability indication selection for larger heads may progress rapidly • The low narrow sense heritability in seed weight indicates that many genes and/or genes with large non-additive effects are involved in its control (Fehr, 1991). • Progress has been made toward he perennial ideotype

Figure 1. The relationship between observed and predicted F1 genome size based on the average of the two parents for each cross. Individuals that had a genome size above 12 pg were considered to be hexaploid and were discarded from phenotypic analysis.

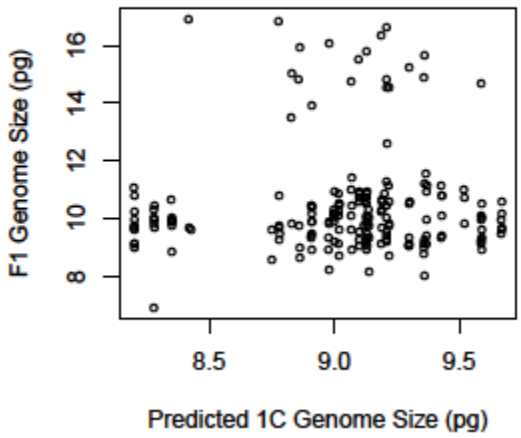
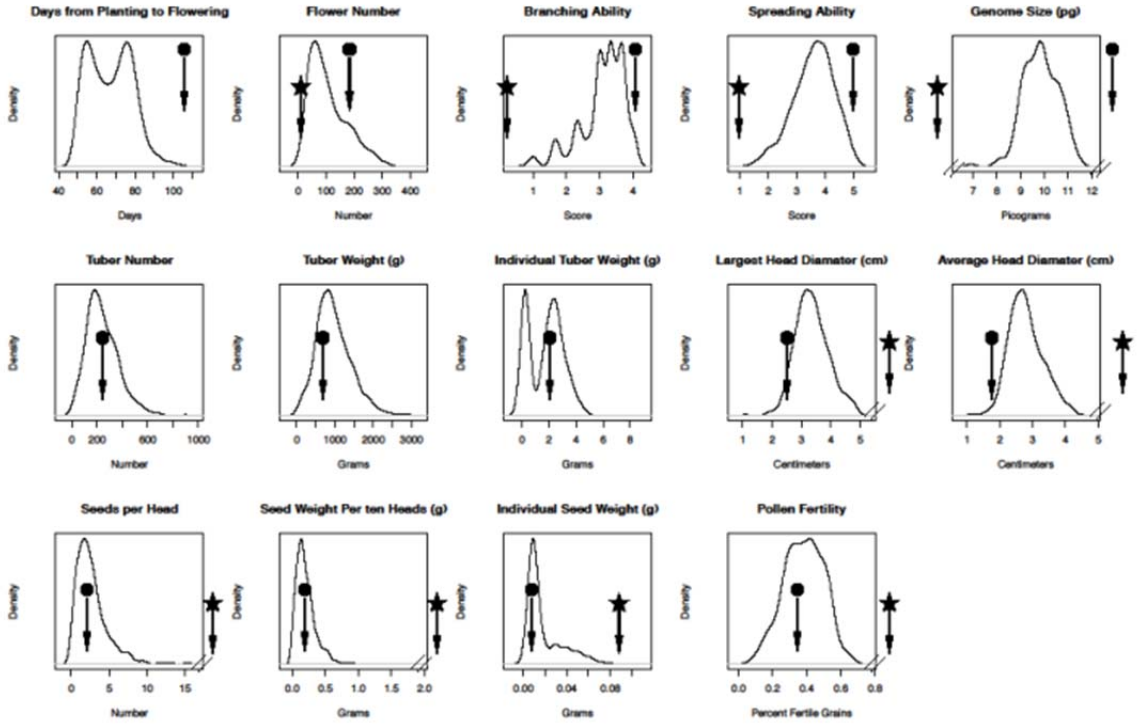


Figure 2. Trait distributions in the F1 hybrids. The symbol ★indicates where domestic annual *Helianthus annuus* is on the distribution [1]. The symbol ● indicates the trait value for wild perennial *Helianthus tuberosus* on the distribution with boxes under arrows indicating the value if it is outside the depicted distribution.



[1]Pollen fertility, genome size, largest head diameter, and average head diameter were measured in rows of *H. annuus* planted adjacent to this experiment, while seed per head and seed weight per ten head were known to be outside the depicted distribution based on previous published and unpublished experiments.

Figure 3. Phenotypic variation within and among the different populations. These images show the range of phenotypes in the F_1, from wild like to domestic like and the improvement seen with one cycle of intermating in the first intermated generation of F_1 individuals (IM_1F_1). (A) Variation in tuber size and shape among F_1, IM_1F_1, and *H. tuberosus* (HT). (B) Variation in seed size among F_1, IM_1F_1, HT, and *H. annuus* (HA). (C) Variation in head size among F_1, IMF_1, and HT. (D) Variation in leaf shape in the F_1, from lanceolate to ovate. (E) Variation in branching in the F_1: (1) no branching (2) minimal branching (3) Profuse branching. (F) The crossing scheme used to develop the populations.

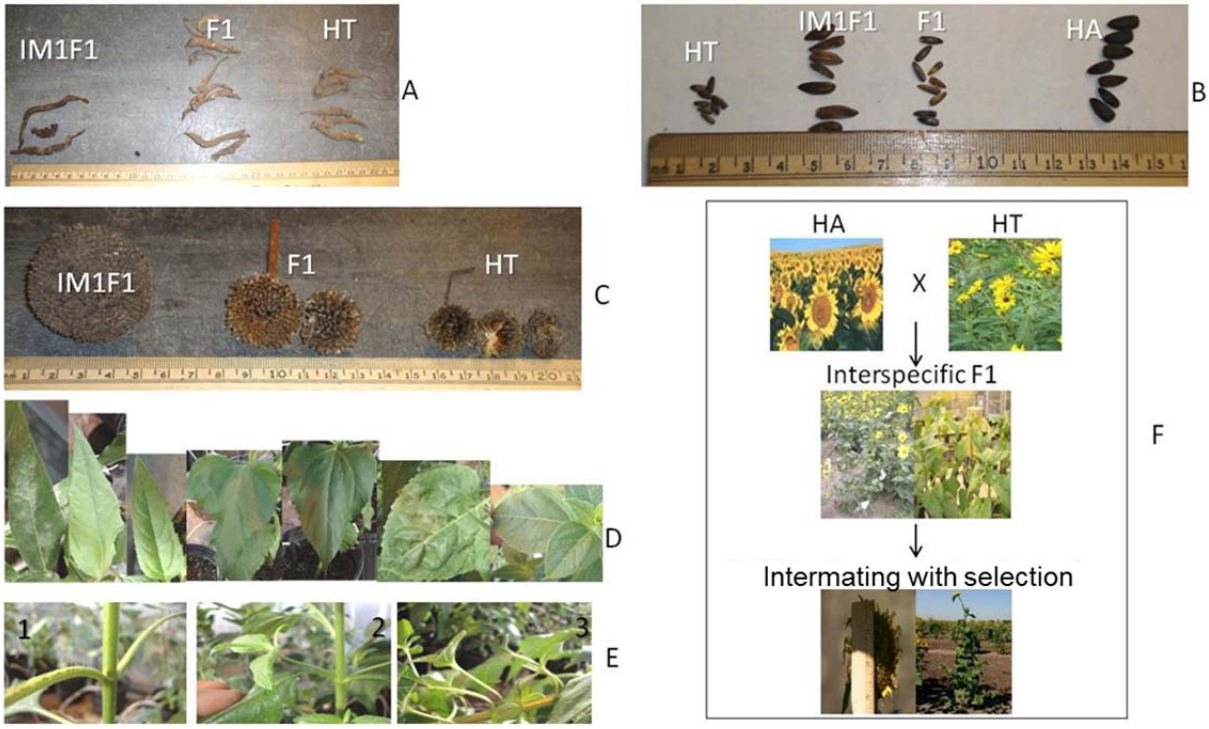


Figure 4. A) Structure clustering based on the same 16 SSR markers with colors indicating different sub-populations (Red indicating membership in the GRIN collection and Blue indicating membership in the Minnesota wild collected material), partial coloring indicated mixed ancestry for an individual accession, with percent membership indicated by the axis. B) Neighbor Joining Tree based on 16 SSR markers. Dark blue indicates *H. tuberosus* parents within the University of Minnesota perennial sunflower breeding program, light blue indicates *H. annuus* parents within the University of Minnesota perennial sunflower breeding program, and red indicates accessions from the GRIN database.

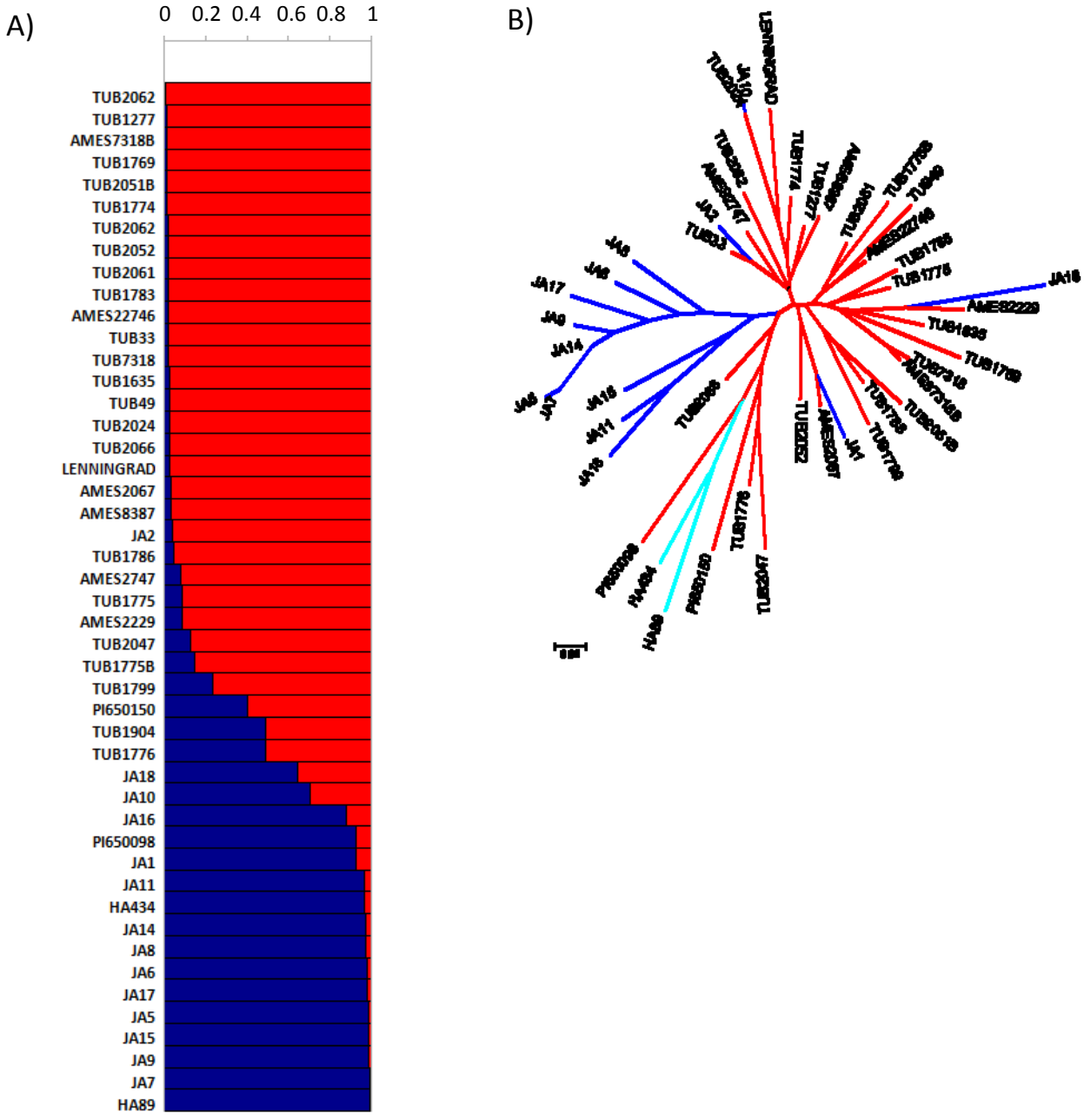
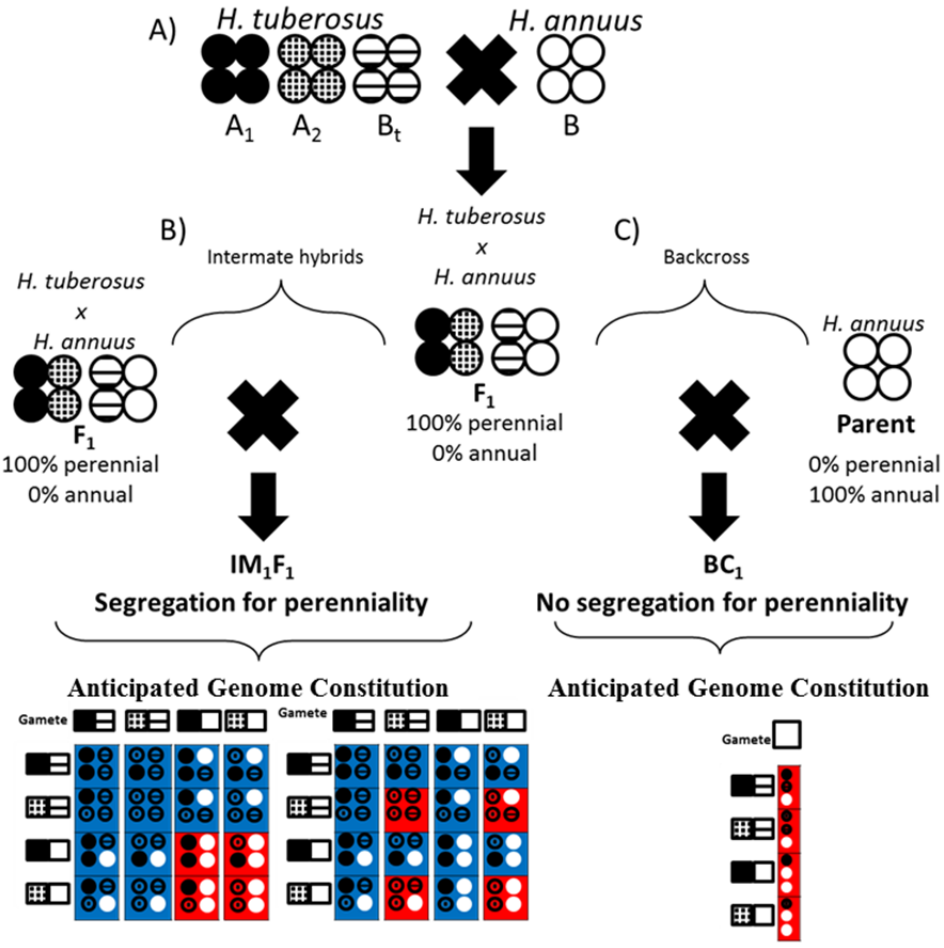


Figure 5. Hypothetical models of chromosome segregation that lead to perennial and annual progeny. A) *H. annuus* x *H. tuberosus* hybridization leads to 100% perenniality. White indicates the *H. annuus* chromosomes; chromosomes from the three sub-genomes of *H. tuberosus* are indicated by solid black (A_1), checkered (A_2) or lined (B_t) patterns. B) Subsequent intermating of F1 hybrids yields progeny that segregate for perenniality. Blue background indicates the genotype for perennial plants and red indicates the genotype for annual plants. Two models are shown to explain the ~3:1 segregation pattern. The model on the left associates perennialty with the dosage of the *H. tuberosus* chromosomes relative to the *H. annuus* chromosomes (annual plants exhibit a higher dosage of *H. annuus* chromosomes). The model on the right associates perennialty with a single factor sufficient for tuber production segregating from one of the *H. tuberosus* sub-genomes. In this example, the single factor resides on one of the A_1 chromosomes. C) Backcrossing the F1 hybrid with *H. annuus* yields progeny that are all annual. This result is consistent with both the dosage model or the single segregating factor model described in part (B).



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Appendix 1. Publication list.

Peer Reviewed Papers

Michael B. Kantar, Kevin Betts, James Luby, Peter Morrell, Brent S. Hulke, Robert M. Stupar, and Donald Wyse. *Evaluating an interspecific Helianthus annuus x Helianthus tuberosus population for use in a perennial sunflower breeding program*. Field Crops Research. Accepted

Kantar, M., Betts, K. ., Hulke, B.S., Stupar, R.M., and Wyse, D. 2012. *Breaking Tuber Dormancy in Helianthus tuberosus L. and Interspecific Hybrids of Helianthus annuus* L. x *Helianthus tuberosus.* HortScience: 47:1342-1346

Curtin SJ, **Kantar MB**, Yoon HW, Whaley AM, Schlueter JA, Stupar RM. 2012. *Co-expression of soybean Dicer like genes in response to stress and development*. Funct Integr Genomics 12: 671–682.

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Kantar, M., Sheaffer, C., Porter, P., Krueger, E., and Ochsner, T. E. 2011. *Growth stage influences forage yield and quality of winter rye*. Online. Forage and Grazinglands doi:10.1094/FG-2011-0126-01-RS.

Krueger, E., Ochsner, T., **Kantar, M.,** Sheaffer, C., and Porter, P. 2010. *Growth stage at harvest of a winter rye cover crop influences soil moisture and nitrogen*. Online. Crop Management doi:10.1094/CM-2010-1014-01-RS.

Non-peer reviewed papers

M.E. Mangan, Fernandez, A.L., Van Roekel, R.J., **Kantar, M.B**., Kluver III R.W., Yost, M.A. and Ries L. (2010, November). 21st Century Agriculture: Balancing Productivity and Conservation in a Changing Environment. CSA News 16-19