Bidirectional recurrent selection and endogenous production of geosmin in table beet

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Dissertation Abstract

The flavor profile of table beet is dominated by the earthy-flavored terpene derivative geosmin. Geosmin can be a desired characteristic in beet or a deterrent to the consumption of the vegetable. Geosmin is also produced by *Streptomyces* spp., various cyanobacteria, and fungi often found in soil and water environments. A long-held presupposition suggests that geosmin production in beets is due to an association with geosmin-producing microbes in the soil. This dissertation presents the results of breeding and experimental efforts to disentangle the source of geosmin production in table beet. Bidirectional half-sib recurrent selection for geosmin concentration over four breeding cycles resulted in the development of low (LGC) and high (HGC) geosmin concentration populations. From Year 1 to Year 3, the LGC mean shifted from 17.3 to 4.3 µg geosmin kg^{-1} tissue and the HGC mean shifted from 22.3 to 33.8 µg geosmin kg^{-1} tissue. Selected families in Year 4 continued this trend after evaluation, demonstrating that table beet is responsive to selection for geosmin concentration.

To separate the influence of potential geosmin-producing microbes from geosmin production *in planta*, four beet accessions were grown in an aseptic tissue culture environment. Lack of microbial contamination was demonstrated by sequencing 16S ribosomal RNA to identify potential contaminating microbes. Operational taxa units (OTUs) returned from this analysis were identified as either chloroplast (98%) or mitochondria (2%), demonstrating that beet plants free of microbes were capable of endogenous geosmin production. Geosmin was detected in 15 of the 16 tissue culture-grown beet samples used for the 16S rRNA aseptic validation. Geosmin concentration was higher in tissue culture grown beets than those grown in a greenhouse environment. A BLAST analysis of the recently published *Beta vulgaris* genome for

geosmin synthase from *Streptomyces coelicolor* resulted in two hypothetical proteins functionally annotated as either isoprenoid or terpene synthases.

The successful bidirectional selection efforts for low and high geosmin concentration and the identification of geosmin in aseptically grown beets is a strong indication that geosmin is endogenously produced by beets and is a heritable trait. As such, geosmin concentration is another selectable trait for beet breeders to consider.

Chapter 1: Review of Literature

History and taxonomy of table beet

Beet's center of origin is the Mediterranean, in a region extending to the Black sea and south into the Canary Islands. DNA evidence supports sea beet, a species with edible dark green leaves, as the ancestor to modern day beet cultivars (Dohm et al., 2014; Biancardi et al., 2005). Beet is in the order Caryophyllales, the family Amarantheaceae (formerly Chenopodiaceae), and the genus *Beta*. Genus *Beta* is comprised of four sections; Beta, Corollinae, Procumbentes, and Nanae that encompass 12 species in total. Within *B. vulgaris* there are six subspecies: *vulgaris* (sugar beet, table beet, fodder beet), *cicla* (swiss chard and leaf beet), *maritima* (wild sea beet), *adanensis*, *macrocarpa*, and *trojana* (Goldman and Navazio, 2003). Section Beta is the most economically important section, containing all the cultivated forms of the species (Ford-Lloyd, 2005). The cultivated and domesticated members of section Beta are used as both root and leaf crops for fresh market, processing, animal fodder, and as sources of sucrose and pigmentation in other processed foods.

The use and significance of beets have been documented throughout history. Early written records show beet consumption in the Mediterranean by the Ancient Greeks and Romans (De Bock, 1986). Other early writings of beet appear in 12th century Arabic text. During the Middle Ages, the mangel, a *B. vulgaris* sub-species, was used as a fodder beet for animal feed. The fodder beet became an important component of European agriculture by the nineteenth century (Pink, 1993). The first known European records of beetroot turn up in 16th century herbal literature. Beets may have been consumed in Europe long before this documentation, but there was a profusion of herbal writings during the 16th century documenting the diets of many European cultures. In the early 1600's engravings show that the leaf beet was the major beet crop in Germany, the Netherlands, England and France. The well-known swollen root of a dark red

beet was published in an engraving in 1608 (De Bock, 1986). It is unknown if the beet commonly known today was selected for by these ancient Mediterranean cultures or if selection occurred as the crop moved from the Mediterranean coasts into Europe. The advantages of the enlarged root include a larger root mass for consumption and longer storage duration (Goldman and Navazio, 2003). During the transition from the longer growing season in the Mediterranean to the shorter growing season and colder climate of northern Europe, the beet may have transitioned to a biennial crop to compensate for the environmental shift. Early hybridization between the swollen beet varieties and leaf varieties occurred across Europe. The beet may have been one of the first sources of plant based sugar used during the long European winter months (McGrath et al., 2007).

It is impossible to write about table beet without reference to the economically important sugar beet. The lack of crossing barriers between table beet, sugar beet, and some wild accessions not only allows for introgression of important traits, but also has provided genomic resources to table beet breeders. Many of the significant genes used to improve table beet germplasm have been integrated from sugar beet such as the biennial growth habit, cytoplasmic male sterility, self-fertility, and various forms of disease resistance that will be discussed in the breeding section of this literature review. The first person reported to extract sucrose from beet was German chemist Andreas Sigismund Marggraf in 1747. By 1801 the first sugar factory in Germany was established by one of Marggaf's students, Franz Achard, and selection for sucrose in a white fleshed fodder beet was underway (Harveson et al., 2009; De Bock, 1986). During the British blockade of Napoleonic France, only a limited supply of sucrose from the British controlled sugar cane market was available in France. Large-scale sugar beet production was

undertaken to alleviate the sugar demands in France. The white fodder beet 'White Silesian' is considered to be the main sugar beet germplasm source (McGrath et al., 2007).

Biology of the table beet

Table beet is known for its mild yet sweet propagule and a spiraled leaf rosette. Both the root and the leaves come in a variety of colors and shapes bred for a variety of uses such as a cylindrical shape for canning, fresh baby greens for salad mixes, and a variety of colorful roots for fresh market sale. Table beet freely crosses with swiss chard, fodder beet, sugar beet and some of the wild species. There is evidence showing gene flow between wild sea beet populations and cultivated beet seed production areas located nearby (Bartsch et al., 1999). Table beet is a diploid where $2n=2x=18$. It should be noted that tetraploids (both auto and allo), and allohexaploids exist within *Beta* (Ford-Lloyd, 2005). Triploid sugar beets have been developed with limited success in the market due to diploid hybrids preforming equally well. There are also high production costs associated with triploid production (Bosemark, 1993). Recently the sugar beet genome was published and over 17,000 genes were functionally annotated based on sequence homology (Dohm et al., 2014). Dohm et al., (2014) reported large regions of low nucleotide variability which indicates sugar beet derivatives from a limited number of haplotypes as well as selection pressure from breeders.

Early in beet development, cylindrical vascular cambium forms between the primary xylem and phloem cells leading to subsequent cambial ring formation. It is the manifestation of supernumerary cambia that occurs after the vascular cambium has encircled the primary xylem that gives rise to the swollen hypocotyl and storage root. Development continues with cell division, expansion and storage of carbohydrates. Like the sugar beet, the table beet's primary storage carbohydrate is sucrose. The concentration of sucrose is thought to be determined by the

ratio of large to small cells in the parenchymatous and vascular zones of each ring (Goldman and Navazio, 2003).

The zoning or color differences between the xylem and the phloem are due to varying levels of pigmentation. Betacyanin and betaxanthin cause the red and yellow pigmentations, respectively. Ratios of these pigments change between cultivar and environment (Rubatzky and Yamaguchi, 1997). A small number of genes control the pigmentation of beets which include *R* (red), *Y* (yellow) and *bl* (blotchy). Dominant and recessive combinations of these alleles result in an array of color in both the leaf and root tissue of beet (Goldman and Austin, 1999). The precursor for betalain production is tyrosine. Red pigmentation in beet is a combination of both red and yellow pigmentation (betacyanin and betaxanthin), but a higher ratio of betacyanin to betaxanthin results in red beet. A missing or dysfunctional *R* gene results in yellow pigmentation. Recessive alleles at the *R* and *Y* loci result in a white beet. In addition, the *bl* allele, when homozygous, causes in blotchy pigmentation of the beet.

The UW-Madison beet-breeding program has been very successful in creating high pigment red and yellow beet cultivars. However, breeding for high pigment yellow has not attained the same level of success as the red-pigmented population. Recent work by Wang et al. (2017) has proposed a model for this biochemical conundrum. In order to form betacyanin, two tyrosine molecules are needed. Wang et al. (2017) found when measuring tyrosine levels in red and yellow beet and chard cultivars, the betalain to tyrosine molar ratio was 36 times higher in the reds than in the yellows. This finding suggests that red beets are more efficient than yellow beets at utilizing tyrosine. Supporting this conclusion at the molecular level are the lack of particular enzymes, encoded by gene *CYP75AD1*, in the tyrosine-betalain pathway that oxidize tyrosine. Yellow beets are recessive at the *R* locus that encodes the gene *CYP75AD1*. Thus,

yellow beets are deprived of a key oxidation reaction in the biosynthetic pathway and a bottleneck is created for betaxanthin production.

Both annual and biennial growth habits exist in beet germplasm. The annual habit is controlled by dominance of the *B* allele, where the phenotype of the *bb* allele will extend the vegetative growth habit of the beet. The cultivated varieties of beets have a biennial life cycle requiring specific environmental cues to make the switch from vegetative growth to the reproductive life-stage. Vernalization, the exposure of the root to temperatures just above freezing (~40°F or ~4°C) for a short duration, followed by long day length is required to promote bolting and flowering in beet. Bolting is the emergence of the flower stock and the subsequent flowering from the vegetative state. The *B* locus was recently cloned from sugar beet and encodes a regulator gene bolting time control 1 (*BTC1*) (Pin et al., 2012). Paralogs to the Arabidopsis flowering locus T gene (*FT*) found in beet, *BvFT1* and *BvFT2* act antagonistically in the promotion of flowering in beet (Pin et al., 2010). The regulation of bolting time in beet is due to *BTC1* repressing *BvFT1* (a floral repressor) and activating *BvFT2* (a floral inducer). A modification to this proposed model includes a second gene, *BvBBX19*, acting epistatically to *BTC1* in the regulation of *BvFT1* and *BvFT2* (Dally et al., 2014). Standard vernalization time used in the University of Wisconsin-Madison beet breeding program is about 12 weeks. After the 12-week vernalization period, roots, also known as stecklings, can be planted in either a greenhouse if in a cold climate or back into the field for seed production. In more temperate environments, stecklings can be vernalized directly in the field if protected from freezing, usually by mounding soil above the roots.

Emergence of the flower stalk after planting occurs at approximately eight weeks. The flower stock of the beet can be up to 1.5 meters tall. Flowers are perfect with the five-part calyx

adhered at the base of the ovary and five stamens inserted at the base. The gynecium is composed of the pistil, tricarpellate ovary with the ovule and the style which is short with a three to four lobed stigma (McGrath et al., 2007). The inflorescences are green and sessile, cluster together in groups of two to many, and result in a multigerm seed when two or three flower axils bond together. The multigerm seed, sometimes called a seedball, has a cork like appearance and feel. Botanically, this structure is called a glomerule or utricle. The beet seed itself has a pigmented coat and maternally-derived perisperm, vestigial endosperm, and the embryo (McGrath et al., 2007). Monogerm varieties of table beet, conditioned by the *mm* genotype and introduced from sugar beet, exist although they are not widely available on the market. The multi-germ seed will often produce 2-3 seedlings when planted. An advantage of the monogerm seed is the increased uniformity of beets after seedling emergence, obviating the need for thinning.

Cytoplasmic male sterility

Beets are primarily wind pollinated, but have a low frequency of insect pollination (McGrath et al., 2007). Beets produce very fine pollen grains that can travel up to three miles. Self-fertilization is rare due to self-incompatibility, but the introduction of the self-fertility gene S^f by Owen in 1942 has limited the outcrossing behavior (McGrath et al., 2007). Additionally, the introduction of the cytoplasmic male sterility (CMS) from sugar beet was an important breeding achievement to produce hybrids. Owen (1942) first described CMS in which there is disruption of microsporogenesis and pollen development is aborted. A sterile beet will have sterile cytoplasm, denoted S, as compared to normal cytoplasm, denoted N. Markers found for S and N cytoplasm indicated that CMS is due to modifications at the transcription level (Kubo et al., 1999). Sterility is conditioned by the interaction between the cytoplasm and at least two

recessive nuclear alleles (Neelwarne and Halagur, 2013). A sterile beet is often denoted S*frfr*. The male sterile plants will produce pollen-less anthers that can be pigmented red brown or yellow depending on the root color. In order to maintain sterility a male fertile pollinator with a normal cytoplasm, but recessive at both the *Fr* loci is required (N*frfr*). Identifying a maintainer still requires much time and many resources as there are no publicly available markers available for table beet. Currently in table beet, fertile plants are crossed with a known sterile and their progeny tested to see if sterility is maintained.

Table beet breeding

One of the first records of mass selection in plant breeding came from the development of the sugar beet during the 18th century. As mentioned previously, over the course of breeding table beets in the United States, many agronomically important traits found in sugar beet have been introgressed back into table beet. The germplasm base populations used as the foundation for the United States table beet breeding programs is narrow. Cheng et al. (2010) found the mitochondrial haplotype diversity in garden beet to be low and the North American germplasm having only two haplotypes (derived from European germplasm). According to Goldman and Navazio (2003), three distinct groups form the genetic base populations in table beet. These include the 'Egyptian' group, the 'Detroit' group, and the 'Long' group. It appears these groups made up about 95% of the beets grown in 1940. The introduction of the inbred-hybrid method developed by D. F. Jones was crucial in the development F_1 hybrid beets started by W. H. Gableman at the University of Wisconsin-Madison in 1949. The success of this program was contingent on introgressing sterility from an outside source as well as identifying maintainers for the sterile lines. Also important for the development of beet inbred lines was the ability to introduce genes to overcome self-incompatibility. This was done by introducing the S^f allele into

the breeding program. Both CMS and S^f were identified and used in sugar beet first. Using the presence or absence of polymorphic RAPD bands to determine genetic distance between table beet and sugar beet accessions, multidimensional scaling plots revealed that inbred lines developed at the University of Wisconsin-Madison still retain an intermediate genetic distance between sugar beets and table beets (Wang and Goldman, 1999). Even after many generations of backcrossing and selection the UW-Madison inbred beets may still retain some linkage drag and recombination restrictions from the introgression of sugar beet germplasm (Wang and Goldman, 1999).

Recurrent selection in table beet

The popularity and depth of knowledge of the inbred-hybrid system have meant seed companies use this method to develop beet hybrids that are on the market today. However, there are still open-pollinated varieties that are developed and maintained. Recurrent selection is one technique used in beet breeding and other biennial crops such as carrot. First proposed in the 1940's, recurrent selection was offered as a method to improve the occurrence of favorable alleles in populations prior to inbreeding (Hull, 1945). Recurrent selection involves evaluation and selection of superior individuals and intermating among the selected individuals. This intermating increases the recombination potential of alleles in a population and increases the potential for favorable allelic combinations used in inbred development (Bernardo, 2010). The Illinois long term selection experiment (ILTSE), lasting over 100 years, observed remarkable gains and losses of protein and oil content in corn (Goldman, 2004). One of the advantages to recurrent selection is the maintenance of genetic variation with-in a population. There are many recurrent selection methods that depend on the biology of the crop, the traits that are of interest and expected gain assumed for a given trait. Response to recurrent selection depends on

heritability of the trait and the selection differential. Any number of variations to these components affects the end population characteristics and results can be confounded by the environment and experimental design, to name a few. The coefficient of additive variance (determined by family structure) is a gauge on determining the effectiveness of the selection method. It has been suggested that maximum increase in gain can be achieved by matching the number of individuals selected with the number of breeding cycles (Bernardo, 2010). Flavor is one trait that can be selected on using recurrent selection, as shown in a recent project to develop sweet corn populations for organic conditions (Shelton and Tracy, 2015). A well-known recurrent selection program was instigated in Iowa by G.F Sprague for the improvement of corn stalk strength (Edwards, 2011). The Iowa Stiff Stalk Synthetic has produced some of the most utilized inbred corn lines today.

A well-used method is half-sib recurrent selection, in which selection of roots with desired traits can be made before pollination. Root characteristics are selected upon at time of harvest and up to time of planting in either the greenhouse or field. This insures an increase of additive variation and increased gain from selection for a given trait. A recessive genotype at the B locus (biennial growth habit) makes recurrent half-sib selection possible by restricting the beet to a vegetative growth period during long photoperiods (Goldman and Navazio, 2003). The biennial growth habit was introgressed into table beet from Russian sugar beet material brought to the United States. The biennial growth habit allows for evaluation of fertility prior to pollination events. A half-sib recurrent selection scheme has been used by the University of Wisconsin beet breeding program to successfully increase the betalain pigments for both red and yellow beets (Gaertner and Goldman, 2005; Goldman et al., 1996; Wolyn and Gabelman, 1990).

Economics and production of table beet

Today table beet is considered a specialty crop in Europe, North America and Asian markets and consumed as both a fresh market and processed product (Goldman and Navazio, 2003; Kikkert et al., 2010). In the United States, over 90% of the table beets produced for processing (canning and frozen product) are grown in the states of Wisconsin and New York (Kikkert et al., 2010). The United States 2012 Census of Agriculture reported beet production increased from 8,412 harvested acres (3,404.2 ha) in 2007 to 12,933 acres (5233.8 ha) in 2012. Of the harvested acres in 2012, 7,288 acres (2949.3 ha) were harvested for processing and 5,644 acres (2284 ha) were harvested for fresh market. A growing interest in fresh market beets has included a demand for more novel color and variation than the typical red epidermis and red interior desired by the processing industry. Beet varieties that include yellow, white, and pink fleshed roots, as well as the striped interiors found in the cultivar ‘Chioggia’, are available for both the home gardener and farm production.

For processing beets, roots are graded on color, uniformity of size and shape, absence of defects and texture for a total score of 100. A score of 85 or above is considered grade A and a score between 70 and 85 is considered grade C. Anything below a score of 70 is considered substandard (USDA, 2017). Popular varieties grown for processing include ‘Ruby Queen’, ‘Red Ace’, and cylindrical shaped roots such as ‘Cylindrica’. Producing uniform beets is difficult for growers due to the multigerm beet seed, uneven seedling emergence, and a myriad of environmental factors such as moisture, growth space, soil crusting, and disease that drastically affect field stands (Kikkert et al., 2010).

Cultivated beets are subject to many pathogens (fungal, viruses, bacteria and nematodes) that affect both foliar and root development. Primary disease-causing pathogens in beet include

Cercospera betacola (Cercospera leaf spot), *Peronospora farinose* (downy mildew) *Aphanomyces cochliodes* (black root), *Phoma betae* (black root and mature root rot), *Pythium* spp. (black root), and *Rhizoctonia solani* (black root and mature root rot) (Harveson et al., 2009). Black root rot is often seen in seedling "damping-off" and could be caused by multiple species infecting the root either in the pre-or post-emergence growth stage. *Rhizoctonia* root rot is considered the most destructive to mature beets. Field roots become dry and brown at the crown and as the disease progresses, fissures of decay make the beet unmarketable (Goldman and Navazio, 2003). Sugar beet breeders have been successful in developing some partially resistant lines, but none exist yet in table beet. Viral diseases of beet include beet western yellows, curly top virus, and rhizomania (Harveson et al., 2009). However, these viral diseases are typically seen in sugar beet production areas and less so in table beet production. Insect pests include the leaf miner *Liriomyza huidobrensis* which punctures the beet leaf during feeding and egg-laying (Goldman and Navazio, 2003). Other insect pests depend on the production region, for instance, in the west beet leafhopper and aphids transmit viral diseases and must be controlled (Harveson et al., 2009).

The betalain pigments produced by beet can be produced by both field grown roots and produced in tissue culture. Due to the interest in the health-related properties of betalains and the linkage of betalain production to growth of vacuoles red beet is used as an experimental model (Neelwarne, 2013). Obtaining uniform clonal plant propagules and increasing the germination rate of wild beet species has led to a wide range in vitro techniques to accomplish these goals (Neelwarne, 2013; Felisberto-Rodrigues et al., 2010).

Geosmin

Bacteria and plants produce thousands of low weight molecular compounds through the course of their metabolic lifecycles. Of these compounds, few seem to have relevance to the immediate needs of developmental plant processes (Singh et al., 2009). However, these secondary metabolites have been found to be relevant in plant defenses and plant-to-plant communications (Jones and Elliot, 2017). Additionally, humans have exploited secondary metabolites from plants and bacteria for medicinal uses, culinary purposes and other marketable goods. The largest family of secondary metabolites produced by plants are terpenoids. These terpenoid compounds are the product of a catalytically diverse family of terpenoid synthase enzymes (Degenhardt et al., 2009). Terpenes occur in nearly every natural food and produce the largest group of natural products including hormones, antibiotics, flavor and odor constituents (Glasby, 1982; Wagner and Elmadfa, 2003). Geosmin, an aromatic double ringed sesquiterpene that gives off an earthy smell, is of interest due to its presence in drinking water supplies (Jüttner and Watson, 2007). It is produced by microorganisms, including gram-positive bacteria species, cyanobacteria, fungi, and liverworts (Jiang et al., 2006). Often the presence of geosmin in water confers a musty flavor that is undesirable by many consumers. Therefore, a large body of literature is devoted to the mitigation and treatment of geosmin in water supplies. Microbial presence in the water is the source of geosmin production, but geosmin may also be produced by certain species of plants including table beet.

Geosmin synthesis

To date, all literature regarding geosmin synthesis is primarily based on bacterial and fungal studies. The structure of geosmin (1,10-dimethyl-*trans*-9-decalol) has been known for some time (Figure 1.1) (Gerber, 1967). However, it was only recently that the biosynthetic

pathway of geosmin was resolved. Geosmin synthase is encoded by the gene SC9B1.20. This gene was discovered in *Streptomyces coelicolor*, an actinomycete from which geosmin can be extracted (Jiang et al., 2007). In *S. coelicolor SC9B1.20* is a 2,181 base pair ORF that encodes a protein 726 amino acids in length (Cane and Watt, 2003). This amino acid sequence shows high similarity to another 336 amino acid sequence that encodes for a sesquiterpene synthase called pentalenene synthase (Jiang et al., 2007).

Geosmin is formed from the cyclization of the substrate farnesyl diphosphate (FDP or FPP) by geosmin synthase, also known as germacradienol/germacrene D synthase. FDP is an intermediate in the mevalonate pathway and used as a building block in sterol and terpene biosynthesis processes (Miller and Allemann, 2012). Sesquiterpene synthases use C_{15} isoprenoids as the basis for production of their final compounds (Harris et al., 2015). This is in comparison to monoterpene synthases that use C_{10} skeleton precursors or diterpene synthases that use C_{20} skeleton precursors (Nieuwenhuizen et al., 2013). The cyclization of FDP by geosmin synthase results in a mixture of germacradienol and germacrene D at a ratio of 85:15 (Jiang et al., 2006). Geosmin is an unusual product of this particular cyclization because geosmin is a C_{12} bicyclic structure, not a C_{15} structure that is normally produced by a sesquiterpene synthase.

Geosmin synthase is an enzyme of interest due to its two bifunctional αα domains (Chen, 2016). Geosmin synthase uses both the N and C terminals to convert FDP to geosmin. By using site directed mutagenesis, researchers have shown that both the N and C terminals have an independent functioning active sites (Jiang et al., 2006). The N terminal domain catalyzes the reaction of FDP to cyclic sesquiterpenes germacradienol and germacrene D. This step requires a Mg^{2+} cofactor. Once thought to be catalytically silent, the C-terminal has been shown to further catalyze the reaction of the germacradienol/germacrene D to geosmin (Cane and Watt, 2003;

Giglio et al., 2008). Germacradienol must fully dissociate from the N-terminal active site into solution before it re-binds to the C-terminal. It is during this second step of the process that fragmentation occurs resulting in geosmin and C_3 acetone, which is an unusual reaction for a terpenoid cyclase (Christianson, 2017). A perplexing question left in understanding the full geosmin synthesis pathway is the role of the Mg^{2+} cofactor during the catalysis performed by the C-terminal. There is a conserved binding pocket for the metal ion, but a metal ion is not needed for a fragmentation reaction (Christianson, 2017).

Role of geosmin and where it is found

Geosmin has been described in many ways depending on the context in which it is detected. Some of the descriptors include earthy, muddy, moldy, camphoric and musty. Frequently geosmin is an isolated metabolite from the microorganisms living in, on or around the product of consumption and is considered an unfavorable quality in water facilities as well as in the production of wine and beer (La Guerche et al., 2005; McGarrity et al., 2003; Darriet et al., 2000). Geosmin has also been detected in beans, fish, cheese and nuts (Buttery et al., 1976; Frisvad et al., 1997; Paterson et al., 2007; Freidig and Goldman, 2014). Geosmin itself has no ill effects on human health, but there is often public concern by consumers about their water quality if they detect the musty flavor of geosmin in their water (Giglio et al., 2008).

The purpose of geosmin production is unknown. Geosmin given off by *Streptomyces* bacteria can act as both a deterrent (in flies) and attractant (camels in the desert). Drosophila flies are alerted to harmful microbes on potential bacterial food sources by detecting geosmin using specialized olfactory receptors (Stensmyr et al., 2012). It is thought that camels can detect geosmin in potential water sources, leading them to water in the desert. An advantage for bacteria would be the ability to spread their spores beyond the original water source (Simmons,

2003). Dehydrogeosmin has been documented as a floral volatile given off by cactus flowers and has been hypothesized to be a pollinator attractant (Schlumpberger et al., 2004).

Geosmin and beets

Geosmin provides a defining sensory characteristic of the table beet. One hypothesis made by Buttery and Garibaldi (1976) was that the earthy flavor of beet was due to the soil. Geosmin was first identified as the earthy compound in beets by Acree and Lee in 1976 using gas chromatography and mass spectrometry. Subsequent work by Parliment et al. (1977) again identified geosmin as a volatile from cooked beets as one of 17 other compounds. In their work geosmin was detected at a low threshold. Archer and Stokes (1978) examined geosmin concentration content in beets varying in maturity levels, cultivar type, storage history, and the effect of peeling and coring beets. Their work found that geosmin concentration is directly related to the surface area of the beet and only a small amount of variation observed between cultivars was due to increased surface area. Archer and Stokes (1978) observed that the peels of the beets had a higher geosmin concentration than the core. Many of the findings from this study provided the basis for future hypotheses and the opportunity to expand upon their work. Additionally, Archer and Stokes (1978) found that geosmin concentration decreased in beet roots after they entered storage.

The improvement of methodology by Lu et al. (2003a and b) to measure volatile compounds using headspace solid-phase micro-extraction (HSPME) in combination with GC-MS allowed for the development in geosmin analysis in beet. Up until this study, other techniques were used to trap geosmin before analysis, but these methods required large samples or specialized expensive equipment (Lu et al., 2003b). Tyler et al. (1978) used gas chromatography to quantify geosmin concentration in beet, but the method required a large

amount of beet juice. Lu et al. (2003b) found that HSPME was an efficient way to recover geosmin from a small amount of beet tissue. Another advantage to using HSPME is the lack of organic solvent needed. Using this method, they went on to quantify geosmin in four different beet cultivars where the range was from cultivar 'Detroit Dark Red' which had the lowest geosmin concentration of 9.69 ± 0.22 (μg/kg) to 'Chioggia' which had the highest geosmin concentration of 26.7 ± 1.01 (μg/kg).

A second study done by Lu et al. (2003a) began to examine the biosynthetic origin of geosmin in beet. Due to the higher concentration of geosmin in the skin of the beet one hypothesis was that beets could absorb external geosmin through the epidermal layer. Lu et al. (2003a) grew beets grown on Murashige and Skoog basal salt (MSBS) media with added geosmin and found no difference in geosmin concentration to beets grown on regular MSBS media. Lu et al. (2003a) also found that beets grown in tissue culture produced geosmin. Cultivar 'Detroit Dark Red' had a geosmin concentration of 65.2 ± 25.9 (μg/kg) and the cultivar 'Chiogga' had a concentration of 135 ± 30.6 (μg/kg). Additionally, they noted that geosmin concentration remained constant for five months of growth and increased between five and six months of growth.

In a more comprehensive study to understand the variation of geosmin in beet cultivars and related species, Freidig and Goldman (2014) reported geosmin concentrations for eight beet cultivars and related three table beet relatives (Swiss chard, sugar beet and mangel) as well as one carrot cultivar. These cultivars were grown in three environments that included a field environment and two different greenhouse environments using autoclaved and non-autoclaved soil in 2011 and 2012. There were no significant differences in cultivar rank for geosmin

concentration across environments and years in the beet, chard, and mangel accessions, while the carrot accession did not produce geosmin.

Geosmin and microorganisms

To date, no explanation for the role of geosmin production in bacteria has been discovered. Geosmin is considered a volatile organic compound (VOC). Both plants and bacteria give off VOCs and these VOCs can be utilized as signaling molecules for communication. VOCs in plants are used to attract pollinators and send plant-plant and within-plant SOS signals from (Ueda et al., 2012). A new exploratory behavior by *Streptomyces venezuelae* is triggered by the interaction with *Saccharomyces cereisiae* and other fungi and has been associated with the production of an alkaline VOC trimethylamine (TMA). TMA when released by one bacterium triggers the exploration ability of other *Streptomyces* (Jones and Elliot, 2017). Schöller et al. (2002) noted a strong correlation between geosmin production and spore number produced by *Streptomyces albidoflavus*. In another study by Blevins et al. (1995) it was documented that a stressful environment could be the cause of geosmin production in *Streptomyces halstedii* and cyanobacterium *Anabaena* sp. Stressful environmental conditions included changes in temperature, light and nutrient composition (nitrate and ammonium-nitrogen). Temperature and oxygen effects also altered the amount of geosmin produced by *Streptomyces tendae* and *Penicillium expansum.* Geosmin concentration increased at a higher temperature and oxygen enrichment in *S. tendae* that produced higher biomass. In contrast, geosmin concentration did not associate with increased biomass of *P. expansum* that occurred at a higher temperature (Dionigi and Ingram, 1994).

In addition to *S. coelicolor,* geosmin synthase has been found in model cyanobacteria *Nostoc punctiforme* and *Anabaena circinalis* (Giglio et al., 2008; Giglio et al., 2011). There is

some discussion as to whether geosmin production is correlated to isoprenoid synthesis and this was further investigated with *Oscillatoria brevis* (Naes et al., 1985; Giglio et al., 2011). An indicator of isoprenoid synthesis is chlorophyll *a* (chl *a*) where an increase in chl *a* correlated with an increase in geosmin production (Giglio et al., 2011). Some evidence points to preferential channeling of carbons into the isoprenoid pool for geosmin synthesis where an inverse relation between chl *a* and geosmin synthesis was reported (Giglio et al., 2011).

In a study done by Singh et al. in 2009, a sesquiterpene synthase with similarity to geosmin synthase was isolated from *Streptomyces peucetius* called *spterp13*. However, in this species of *Streptomyces* geosmin was not detected while its close homologues in *S. coelicolor* A3(2) and *S. avermitilis* are strong producers of geosmin. Further investigations revealed that geosmin could be produced with the deletion of approximately 18kb of the doxorubicin gene cluster. Doxorubicin uses nine acetyl-CoAs in the production of each molecule. By deleting the doxorubicin gene, the intracellular acetyl-CoA pool increased. Acetyl-CoA is essential to many metabolic reactions, residing at the beginning of many biosynthetic pathways that result in secondary compounds, including geosmin. Singh et al. (2009) observed that with the increased production of acetyl-CoA an available acetyl-CoA pool formed followed by an increase in geosmin production, cellular growth and longer survival of the cell culture. The geosmin concentration was also increased by inserting more copies of *spterp13* into *S. peucetius*. The geosmin production was 2.4±0.4 fold higher than the original mutated *S. peucetius* that increased the acetyl-CoA pool.

Methodology for geosmin detection

Measuring secondary metabolites in plants is often a costly and time-consuming endeavor. Common analytic tools have included gas chromatography (GC), mass spectrometry

(MS) and high-performance liquid chromatography (HPLC). In order to study a secondary volatile compound such as geosmin, the use of the gas chromatograph mass spectrometer (GC-MS) has been vital, specifically the use of headspace solid phase micro extraction (HSPME). Solid phase micro extraction (SPME) was developed in the early 1990s to address the need for on-site and more efficient sample preparation for volatiles and semi-volatiles (Lord and Pawliszyn, 2000; Souza-Silva et al., 2015). Other GC-MS techniques such as purge and trap methods and gas chromatography- olfactometry (GC-O) have been used to identify malodors in sugar produced from sugar beet, specifically identifying geosmin as a major defect in beet sugar (Pihlsgård et al., 2001; Marsili et al., 1994).

In SPME, a thin polymer-coated silica fiber can be injected directly into the GC after it is used to absorb the sample either in liquid form or the headspace over the chemical of interest (Ai, 1997). In this way, all steps of sample preparation (sampling, extraction, concentration and sample introduction) are performed at one time (Risticevic et al., 2009). Since SPME's introduction, continuous improvements have been made to this method including sampling the analytes above the headspace of a sample. SPME was first developed by Zhang and Pawliszyn (1993) to overcome long extraction times due to the kinetic limitation occurring between the extraction fiber coating and the liquid sample. For sampling geosmin in beet a HSPME GC-MS protocol was developed by Lu et al. (2003a and b) and adapted by Freidig and Goldman (2014).

Health benefits of beet

A recent trend of consuming beet juice for enhanced athletic performance has resulted in a body of literature testing this potential. Beet juice is often sold as a natural supplement. The efficacy of beet juice has been tested on athletes for a range of aerobic activities including cycling, running, rowing and kayaking (Jones, 2014; Murphy et al., 2012; Ormsbee et al., 2013;

Wylie et al., 2013). The reason for this interest has been the high concentration of nitrate in beet juice. Along with beet, other vegetables containing high NO_3^- include spinach, celery, lettuce and carrot juice (Ormsbee et al., 2013). When NO_3^- is consumed, facultative anaerobic bacteria in the body reduce NO_3^- to NO_2^-. NO_2^- is then converted into NO and other reactive nitrogen compounds used by the body (Wylie et al., 2013). Among some of the documented benefits of consumption of foods high in nitrate is the lowering of resting blood pressure and improved oxygen consumption (VO_2) (Ormsbee et al., 2013; Wylie et al., 2013). This is an ongoing area of study, where other properties of beet consumption such as antioxidant and betaine properties on athletic performance are limited in their documentation.

Table beet, roots and leaves, are also high in oxalic acid ($C_2O_4^{2-}$). Other foods high in oxalic acid include spinach, tea (*Camellia sinensis*), and chocolate. Oxalic acid consumption is associated with increased risk of kidney stone formation and decreased bioavailability of other nutrients (Brogren and Savage, 2003). Freidig and Goldman (2011) found significant variation in 24 Chenopodiaceae (now Amaranthaceae) members for total and soluble oxalic acid levels. They concluded, however, that breeding for lower oxalic acid levels in beet would be a difficult task and may have limited results due to the need to substantially decrease oxalic acid levels to be categorized a low oxalate food.

Beet pigmentation, as mentioned previously, is due to tyrosine-derived betalains. These pigments are used for natural food dyes and beets have been bred for increased betalains used by the natural food dye industry. Concerns about the use of synthetic colorants in food has prompted an increase in the use of natural food dyes. Betalains have been shown to have antioxidant properties (Belhadj Slimen et al., 2017). There is some evidence as well to the chemo

preventative properties of betalains, but these studies have only been performed using red beet extract on laboratory murine species (mouse and rat) (Kapadia and Rao, 2013).

Flavor of beet

Geosmin is a defining characteristic of the table beet, but other taste components include sweetness from differing sugar levels, bitterness from saponins and the sensory mouth-feel from oxalate acid (Acree and Lee, 1976; Mazza and Chubey, 1985; Freidig and Goldman, 2011; Mikolajczyk-Bator and Kikut-Ligaj, 2016). Geosmin is also a divisive taste compound where consumers can have strong favorable or unfavorable reactions to the consumption of the compound. Culinary preparation of beet affects the different sensory features present. Cooking beets decreases the amount of geosmin up to 60% (Tyler et al., 1979). Bach et al. (2014) evaluated the culinary preparation of beet using both a trained sensory panel and a semi-trained consumer panel for raw, pan-fried, and boiled beets. Among their many findings, Bach et al. (2014) found cooking beet diminished the ability of the consumers to differentiate between beet cultivars, but the earthy flavor of beet was a desirable trait for boiled beet. In addition, they found raw beet associated with high scores for crispness, beet flavor and juiciness.

An important flavor component of table beet is the sugar level. In fruits and vegetables sugar is measured using Brix. Named after a German scientist, the Brix measurements is used to quantify the percentage of soluble solids (sucrose) in plant juice. Brix are measured using a refractometer. For table beet the percent soluble solids falls between 6 and 14 (Harrill, 1998). According to Harrill (1998) the percent of sucrose considered to be of poor quality falls around 6%, average quality at 8%, good quality at 10%, and excellent quality at 12%. In studies done by Feller and Fink (2004) and Mazza and Chubey (1985), cultivated varieties of beets were reported to be within the excellent range suggested by Harrill (1998).

Recently, studies have been published regarding the presence of triterpene compounds called saponins in beet. Saponins are glucosides that have a bitter taste and are known for their foaming or soap-like characteristic. Using reverse-phase liquid chromatography and negative-ion electrospray ionization quadrupole mass spectrometry 44 triterpene saponins were detected from the cultivar Nochowski (Mikołajczyk-Bator et al., 2015). In a sensory analysis of red beet cultivars statistically significant differences were found between bitter taste perceived at differing intensity levels attributed to the cultivar and root parts (Mikolajczyk-Bator and Kikut-Ligaj, 2016).

Breeding for flavor

A recent review by Folta and Klee (2016) highlighted the growing consumer desire for increased flavor and aromas in their fruits and vegetables. The underlying basis for this review is that while plant breeders have been extremely successful improving the genetics of crops, the traits that have been improved have focused only in certain areas. Both the processing industry and plant breeders have lowered the cost of crop production and moved towards a more efficient system by increasing fruit and vegetable size and uniformity, raising yields and improving disease resistance and shipping quality. While these improvements have certainly made an impact on the price and availability of fruits and vegetables, the genetic cost of mass production has been on nutrition and flavor (Folta and Klee, 2016). Breeding for flavor is a difficult task not only from a genetic standpoint where flavors are often complicated metabolic pathways, but also because of the great variation in human perception of taste and sensory qualities. Olfaction, the sense of smell, is made possible by molecular sensors called olfactory receptor (OR) proteins (Hasin-Brumshtein et al., 2009). These genes have undergone a wide range of changes from deleterious single nucleotide polymorphisms, copy number variation, and deletions resulting in

large phenotypic variation within the human population. Almost every person has a unique combination of ORs. There is also some indication that inhalation of geosmin produces differing electroencephalogram (EEG) activity within the brains of both males and females. These differences were measured using a variety of EEG indicators with some types of these indicators differing between genders (Kim et al., 2017).

Trained taste panels are one way to effectively describe odors and can be used as an analytical instrument. A typical sensory panel will contain 10-15 trained assessors that describe products objectively. This is not to be confused with a consumer panel that assesses the degree of liking of a certain product (Naes et al., 2010). Rashash et al. (1997) evaluated a trained panel on a variety of odors found in drinking water and found it to be an effective way to identify odors and concentrations, such as geosmin. The triangle test is often used to determine differences between samples and has been used for vegetables. In cabbage grown under different irrigation environments, a triangle test successfully identified differences between cabbages grown in these environments (Radovich et al., 2004).

The task of breeding for flavor is complicated by the hundreds to thousands of chemicals produced by plants that contribute to aroma and taste. Plant volatile organic compounds can differ by plant organ and developmental life stage. Over 7,000 plant volatiles have been identified and categorized in food and beverages (Goff and Klee, 2006). These in turn interact with other plant compounds such as sugars and fatty acids producing overall taste perception. The tremendous task of breeding for flavor involves understanding some genetic basis for the flavor of interest, trained consumer taste panels, and access to expensive analytical tools to measure the compounds (Folta and Klee, 2016).

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Figures

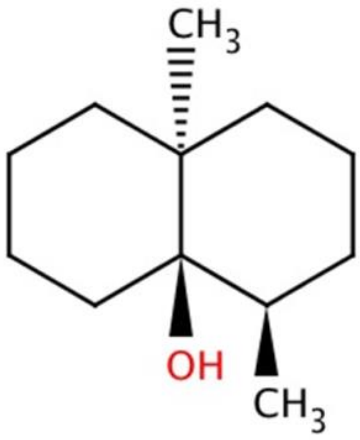

Figure 1.1 Geosmin (1,10-dimethyl-*trans*-9-decalol) is a sesquiterpene derivative produced by cyanobacteria, actinonmycetes, fungi and is a defining flavor of table beet.

Chapter 2: Bidirectional Recurrent Half-Sib Family Selection for Geosmin Concentration in Table Beet

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Abstract

Geosmin (trans-1,10-demethyl-trans-9 decalol) is a terpene derivative produced by *Streptomyces* spp., various cyanobacteria, and fungi in the soil. Geosmin is also responsible for the earthy flavor of the table beet. Geosmin in beet, spinach, and Swiss chard can be a deterrent to the consumption of these foods, while other consumers desire their earthy flavors. While it is known that microbes are capable of producing geosmin, it is not clear if geosmin associated with beets is microbial in origin. The objective of this investigation was to determine if table beet populations were responsive to selection for geosmin concentration. Four cycles of bidirectional half-sib recurrent selection for geosmin concentration were conducted in table beet, resulting in low (LGC) and high (HGC) geosmin concentration populations. The LGC mean shifted from 17.3 µg geosmin kg^{-1} tissue in Year 1 to 4.3 µg geosmin kg^{-1} tissue in Year 3. The HGC mean shifted from 22.3 µg geosmin kg^{-1} tissue in Year 1 to 33.8 µg geosmin kg^{-1} tissue in Year 3. This trend continued after evaluation of selected families in Year 4. Positive and negative response to bidirectional recurrent selection for geosmin concentration is one indication that geosmin may be endogenously produced by beets.

Abbreviations

AS, beet roots remaining after attrition in breeding population; C1–C4, Cycles 1–4 of a half-sib recurrent selection program; FDP, farnesyl diphosphate; GC-MS, gas chromatography-mass spectrometry; h^2, narrow-sense heritability; HGC, high geosmin concentration; HSPME, headspace solid phase microextraction; ISO, International Organization for Standardization; LGC, low geosmin concentration; OS, beet roots in original selected breeding population; RR, relative recovery.

Introduction

The unique scent of soil after a rainstorm and the undesirable smell of musty water is due to a volatile terpene derivative called geosmin (Lu et al., 2003a). Geosmin (trans-1,10-dimethyl-trans-9 decalol) is produced by *Streptomyces* spp. in the soil and, in turn, these bacteria produce the characteristic earthy smell of soil (Jiang et al., 2006). Geosmin is a secondary metabolite and is formed during the Mg^{2+} dependent cyclization of farnesyl diphosphate (FDP). An intermediate in the HMG-CoA reductase pathway, FDP is used as a building block in sterol and terpene biosynthesis (Miller and Allemann, 2012). The chemistry of this reaction was discovered in *Streptomyces coelicolor* in which a bifunctional sesquiterpene synthase called geosmin synthase was found to possess two functionally different catalytic N and C domains (Jiang et al., 2006). The N terminal half of geosmin synthase converts FDP into an 85:15 mix of germacradianol and germacrene D. This is followed by the conversion of germacradianol to geosmin by the C terminal half of geosmin synthase (Jiang et al., 2007). Overall sequence homology in the general class of sesquiterpene synthases found in plants, insects, fungi, and bacteria is lacking, but these synthases share a general chemical outcome resulting in more than 300 sesquiterpene derivatives (Miller and Allemann, 2012). To date, geosmin has been isolated from gram-positive bacteria species, cyanobacteria, fungi, and liverworts (Jiang et al., 2006). The functionality of geosmin in *Streptomyces* spp. is unknown.

How geosmin is often described is in the context in which it is detected. Humans have a very low threshold of detection (10–100 parts per trillion, Cook et al., 2001). Geosmin is considered a contaminant to water quality, beer, wine, and other foodstuffs (Darriet et al., 2000; Paterson et al., 2007). Geosmin is also considered a defining flavor component of beet (Beta

vulgaris subsp. vulgaris), imparting earthy flavors. No other reports show a root crop producing a detectable amount of geosmin (Buttery et al., 1968).

Geosmin can be a deterrent to the consumption of crops such as beet, spinach (Spinacia oleracea L.), and Swiss chard (Beta vulgaris subsp. vulgaris), all members of the family Amaranthaceae (Acree and Lee, 1976). The interest in food and water contamination and geosmin synthase functionality in microorganisms has led to the development of analytical protocols using gas chromatography-mass spectrometry (GC-MS; Acree and Lee, 1976; Jiang et al., 2006; Lu et al., 2003a). These have been further refined using headspace solid phase micro extraction (HSPME) as a component of the GC-MS protocol (Lu et al., 2003a, 2003b). A defined HSPME protocol to measure geosmin in table beet was adapted from Lu et al. (2003b) by Freidig and Goldman (2014) to analyze geosmin concentration in various beet cultivars across multiple environments.

Thus far, geosmin has been associated with plants in the Amaranthaceae family and interestingly, in the Cactaceae family. Dehydrogeosmin, when in a particular configuration, is identical to geosmin and has been documented as a floral volatile in six genera in the Cactaceae (Schlumpberger et al., 2004). These families are closely related in the order Caryophyllales. As in bacteria, the functional significance of geosmin is unknown in planta. It has been suggested by Schlumpberger et al. (2004) that dehydrogeosmin many play a role in pollination in certain species in Cactaceae. While it is known that microbes are capable of producing geosmin, it is not clear if geosmin associated with plants is entirely microbial in origin. The characteristic earthiness of certain beet cultivars and not others suggests defined geosmin levels for particular genotypes (Freidig and Goldman, 2014); however, no studies have been conducted to examine whether geosmin concentration in plants has a genetic component.

There are three possibilities for the presence of geosmin in beets. Geosmin production could be a result of associations between the plant and microorganisms in the soil. Geosmin is concentrated in the outer skin of beet at a concentration six times that of the inner core, suggesting that geosmin may be absorbed through the epidermal layer from an outside source (Lu et al., 2003b). This scenario was tested by Lu et al. (2003b) in which geosmin was added to Murashige and Skoog basal salt (MSBS) media on which beet seedlings were grown for 4, 5, and 6 mo. Lu et al. (2003b) found no significant differences in geosmin concentration between beets grown on the adjusted geosmin media and those grown on regular media. In addition, the aseptically-grown cultivars exhibited higher levels of geosmin production than those same cultivars grown in a field environment (Lu et al., 2003a, 2003b).

A second explanation for geosmin production in beet is via endophytic microorganisms within the beet plant. Jacobs et al. (1985) and Lu et al. (2003b) observed actinomycete filaments in beet both in the inner cellular region and outer tissues of a beet root. It was unclear if the actinomycetes observed were true endophytes or were of a species that could produce geosmin (Lu et al., 2003b). In such a scenario, certain beet genotypes might favor the growth of specific geosmin-producing microorganisms, leading to higher or lower geosmin concentrations.

Finally, it is possible that beets are capable of endogenous production of geosmin. In a study by Lu et al. (2003b), beets were grown in aseptic conditions on MSBS media and tested for geosmin concentration. ‘Round Red’ beet seedlings produced detectable amounts of geosmin that increased from a mean of approximately 14 µg kg–1 of plant tissue at 4 mo of growth to 28 µg kg–1 of plant tissue at 6 mo of growth. In addition, geosmin concentration differed among cultivars grown under aseptic conditions. ‘Chioggia’ beet had a concentration of 135 ± 30.6 µg kg–1 of plant tissue, whereas ‘Detroit Dark Red’ had a concentration of 65.2 ± 25.9 µg kg–1

plant tissue. It was noted that the growth habits of these cultivars in tissue culture differed, which may have contributed to this large difference. One of the limitations of these findings is that the aseptic cultures were not assayed for the presence of microbes.

Cultivar variability for geosmin has been discovered. Lu et al. (2003a) analyzed field-grown beets for geosmin concentration via a HSPME method using GC-MS. The four cultivars ('Detroit Dark Red', 'Crosby Green Top', 'Lutz Green Leaf', and Chioggia) differed significantly in geosmin concentration, ranging from 9.69 ± 0.22 µg kg–1 plant tissue to 26.7 ± 0.27 µg kg–1 plant tissue. Freidig and Goldman (2014) compared eight beet cultivars, three cultivars of close table beet relatives (mangel, sugar beet, and Swiss chard, all *Beta vulgaris* subsp. *vulgaris*), and one carrot (*Daucus carota* L.) cultivar for geosmin concentration. Cultivars were grown across 2 yr in three environments which consisted of a greenhouse environment using autoclaved soil and nonautoclaved soil, and a field environment. They found no significant differences in cultivar rank for geosmin concentration between years in the field environments and greenhouse environments for the beet, mangel, and Swiss chard cultivars. Their work suggested that beet cultivars contain characteristic signatures of geosmin concentration, lending support to the idea of endogenous production of geosmin by the plant; or perhaps the possibility of associations between host plant genotype and microbial populations capable of geosmin synthesis.

Recurrent[1] and bidirectional selection have been used effectively in beet for improving quantitative traits such as betalain pigment concentration and total dissolved solids (Gaertner and Goldman, 2005; Wolyn and Gabelman, 1990). Half-sib selection is an effective selection method

[1] Traditionally bidirectional selection has a single starting population. For this study, two founding populations were used, but the means of both populations were relatively close beginning the selection process.

in beet because the biennial growth habit allows for selection before pollination, thus doubling the expected rate of gain per cycle through controlled pollination (Wolyn and Gabelman, 1990). Bidirectional selection has been used as an innovative way to gauge selection's effectiveness and historically has a precedent in the Illinois Long Term Selection Experiment (Dudley and Lambert, 2010; Goldman, 2004). Additionally, the products of bidirectional selection for geosmin concentration in table beet have the benefit of appealing to different consumer bases (Bach et al., 2014). The outcome of such a selection program, if successful, would result in both a population of mild, low earthy tasting beets and a population of intense high earthy flavor. The taste of geosmin is a fundamental characteristic of beet, and thus a potential breeding target. If the concentration of geosmin could be manipulated by selection, it would provide an exciting and unique component to the culinary quality of beet. Therefore, our objective was to determine if table beet populations are responsive to selection for geosmin concentration.

Materials and Methods

Selection Experiment

Population Formation

In 2012, two populations, defined by overall geosmin concentration, were formed based on the work of Freidig and Goldman (2014). The LGC base population was comprised of individual plants from the open-pollinated beet cultivars 'Blankoma' (Johnny's Selected Seeds, Windslow, ME) and 'Touchsone Gold' (Alf Christianson Seed Co., Mount Vernon, WA). The HGC base population was comprised of individual plants from the open-pollinated beet 'Bull's Blood' (equal mix of Johnnys Selected Seeds, Winslow, ME, and Atlee Burpee and Co., Warminster, PA) and Chioggia (Johnny's Selected Seeds). Plants were grown for approximately 12 wk at the Arlington Horticulture Farm in Arlington, WI. After harvest, beet roots were

vernalized at 5°C for approximately 12 wk. Post vernalization, 5 roots from each cultivar were individually planted in the greenhouse under 16 h of daylength. At flowering, each population was moved into an isolated greenhouse to open-pollinate. Seed bulked from each individual plant was considered a half-sib family. Each population was intermated for two seasons of recombination in 2011 and 2012 before selection was initiated in the fall of 2013. The populations in 2013 were considered Cycle 0 of the recurrent selection scheme, where LGC0 represents the low population and HGC0 represents the high population at Cycle 0.

Cycles 1 through 4

Beet half-sib families were grown in a randomized complete block design with two replications. Half-sib family was considered an experimental unit. Beet seeds were planted at Arlington Horticulture Research Farm (Arlington, WI) 3 cm apart using a Planet Junior seeder with a cone attachment in rows 3.7 m long, with 46 cm between rows the first week of June. Six to 10 roots were harvested during the last 2 wk of August at random from each plot, and tops were trimmed to 2.53-cm stubs. After sampling, beets were vernalized at 5°C for 12 to 14 wk. Post vernalization, selected roots were planted in the greenhouse (Walnut Street Greenhouse, University of Wisconsin-Madison, Madison, WI) in pots 13.8 cm in diameter × 15.2 cm tall, containing approximately two-thirds field soil and one-third Metro-mix 366-P by volume (Sun Gro Horticulture, Agawam, MA). Beets are wind pollinated and, to prevent cross contamination, beets were moved into two isolation chambers at the onset of bolting (flower stalk emergence). Inflorescences were shaken periodically to disperse pollen. Mature seed from these isolations were hand harvested by half-sib family.

Selection Criteria

Half-sib families were comprised of seed from an individual female plant. The HSPME/GC-MS analysis was conducted on each of the families (for details, see "Sample Preparation" section below). Selection was based on the geosmin concentration of individual roots within and among half-sib families. Following completion of the HSPME/GC-MS analysis, 80 roots comprising of the highest and lowest gesomin concentrations were put into separate isolations to open-pollinate during Cycle 1 (C1).

Sample Preparation

In C1 to C3, four beet roots from each field plot were sampled approximately 1 wk after harvest. Each root was individually labeled after sampling. The first year of selection, one-half of the beet root was sampled. Due to variation in epidermal and core geosmin levels, sampling in Years 2 and 3 was conducted using a core borer 1 cm in diameter. Root cores were frozen at –20°C until processed. Six 30-g cores (six 1-cm epidermal layer plus remainder secondary vascular cambia) were ground at a 1:1 ratio of fresh weight grams of tissue/grams of Milli-Q water (Millipore, Bedford, MA) for 2 min. For selection years 2013, 2014, 2015, n = 290, 324, and 325, respectively, for HGC populations; and n = 421, 404, and 405, respectively, for LGC populations. Analysis for C4 was conducted on the five highest and lowest geosmin concentration families, thus one core from each of six roots was combined in the homogenate. Homogenate was analyzed using HSPME and GC-MS (Shimadzu model QP2010SE with AOC-5000 Shimadzu autoinjector, Shimadzu, Kyoto, Japan).

Samples for HSPME were thawed in a water bath, and 5 g of homogenate was added to a glass vial with 1 g of NaCl and capped immediately with a screw cap containing PTFE(polytetrafluoroethylene)/silicone septum. Internal standard, menthone, was added at a

concentration of 2.82 µg geosmin kg–1 root tissue (Sigma-Aldrich, St. Louis, MO). Geosmin standards in 5 mL water blanks were 5, 10, and 21.6 µg geosmin kg–1 root tissue. Relative recovery (RR) rates were determined using ground beet tissue spiked with 5, 10, 15, and 21.6 µg geosmin kg–1 root tissue using the calculation from Lu et al. (2003a):

$$RR = \frac{\mu g\ G_{total} - \mu g\ G_{originally\ in\ beet}}{\mu g\ G_{spiked}} \times 100\%$$

Relative recovery was 37.46% in 2013, 34.52% in 2014, 35.80% in 2015, and 39.4% in 2016. While RR was low, it remained consistent over each year of selection.

Standards and Reagents

Standards of menthone [(1R,4S)-p-menthan-3-one] and geosmin (2β,6α-dimethylbicyclo[4.4.0]decan-1β-ol) were purchased from Sigma-Aldrich. Milli-Q water was used for sample preparation and standards. Weed control included preemergent herbicides cycloate (Ro-Neet, Stauffer Chemical Co., Louisville, KY) and chloridazon (Pyramin, Nufarm Australia, Laverton North, VIC, Australia) applied to the field at recommended rates before planting (cycloate), and before emergence (chloridazon).

HSPME and GC-MS Analysis

Analysis of samples was conducted as described in Freidig & Goldman (2014). The GS-MS total program time per sample was 10.43 min. Beet samples were incubated for 3 min at 60°C and agitated at 500 rpm. Following incubation, extraction was done using PDMS/DVB (polydimethylsiloxane/divinylbenzene) fibers conditioned according to manufacturer specifications (Sigma-Aldrich) for 10 min under the same conditions. The column was a 30 m SHRXI-SMS (Shimadzu, Tokyo, Japan) with 0.25 mm i.d. and 0.25 mm df. The temperature

protocol was 40°C for 1 min, increased to 35°C min–1 and to 300°C, and held for 2 min. During sample process by GC-MS, the fiber was thermally desorbed at 250°C for 1 min. Injection mode was split (split ratio of 10), and a split/splitless liner was used.

Statistical Analysis

Data were analyzed using R version 3.2.3 (R Development Core Team, 2015). Data were adjusted for RR. Data were log transformed for Welch's two-sample t test and ANOVA. Variances were compared using the Bonferroni correction.

Triangle Taste Test

Root tissue from the progeny of C3 selections were used for triangle tests on 8 and 9 Sept. 2016 at the Babcock Hall Sensory Analysis Lab, Madison, WI. The triangle taste test is a standard protocol for determining differences in culinary characteristics of food products (ISO, 2004). Four triangle tests were conducted for three sets of comparisons; two HGC vs. LGC (one on 8 September and a second on 9 September) and 1 each of LGC and HGC versus check cultivar Red Ace. Each triangle test consisted of 36 participants. Roots for the test were chosen from the five highest and five lowest families and Red Ace, with an attempt to find roots of matching color. Roots used for both HGC vs. LGC triangle tests were from the same family, as were the roots used in the LGC vs. Red Ace. The HGC roots used in the HGC vs. Red Ace were from two different families. Roots were washed, peeled, and cut into equal matchstick-size pieces. The International Organization for Standardization (ISO) triangle test protocol was followed and red filters were used in each of the sampling booths to mask color differences (ISO, 2004). The remaining beet was frozen at –20°C. Beet samples were analyzed for geosmin concentration (see "Sample Preparation") with the modification that the beet epidermis was removed. In addition, samples for soluble solids analysis were prepared by thawing frozen tissue

and measuring beet exudate samples in triplicate on a refractometer (Wolyn and Gabelman, 1990).

Results

Geosmin Concentration Differences between and within Families and among Cycles of Selection

Analysis of variance revealed significant differences among the families in each population (LGC and HGC) in each selection cycle (Table 2.1). Thus, the half-sib families comprising the LGC and HGC populations remained significantly variable for geosmin concentration throughout the selection experiment. The overall population mean for the LGC decreased after each cycle of selection and the overall population mean for the HGC increased after each year of selection (Fig. 2.1, Table 2.2). The LGC mean shifted from 17.3 µg geosmin kg–1 tissue in Year 1 to 4.3 µg geosmin kg–1 tissue in Year 3. The HGC mean shifted from 22.3 µg geosmin kg–1 tissue in Year 1 to 33.8 µg geosmin kg–1 tissue in Year 3. Welch's two-sample t test on log transformed data showed significant differences between overall population mean of LGC and HGC in each year (Table 2.2). The LGC population showed a larger decrease in geosmin concentration between the first and second cycles and a smaller decrease in geosmin concentration between the second and third cycles. In contrast, the HGC population showed a larger shift in geosmin concentration between the second and third cycles (Fig. 2.1). In 2016, the progeny of the five highest and five lowest families were evaluated for geosmin concentration. One high family was removed due to high attrition[2] rate of roots in the field, resulting in a low

[2] Survival rate for roots both in the field and greenhouse environments varied for each population. The largest decline in survival was in the HGC population, particularly when roots were taken from storage into the greenhouse environment for recombination. The parental lines used in the founding of the HGC population may not have been adapted to the recurrent selection methods used in this study, thus applying added selection pressure beyond selection for geosmin concentration.

number of samples. Concentrations for the high families were between 39.0 and 41.4 µg geosmin kg–1 tissue, and between 2.3 and 3.0 µg geosmin kg–1 tissue (Fig. 2.1). In addition, check cultivars Bull's Blood and Red Ace, and two breeding lines (red and yellow) with 'Touchstone Gold' in the pedigree, were analyzed. Touchstone Gold is an open-pollinated cultivar with low geosmin levels (Freidig and Goldman, 2014). The geosmin levels were 20.6, 3.3, 15.8, and 4.0 µg geosmin kg–1 tissue, respectively (Fig. 2.1).

Within half-sib family variation for geosmin concentration was also evaluated by measuring geosmin concentration of individual roots in all families (Fig. 2.2 and 2.3). Some HS families showed little variation for geosmin concentration among individual roots, while others were more variable. The variances of each family were compared within each population within each year using the conservative Bonferroni correction to control for false discovery. Significant differences were found between seven different pairs of families in LGC 2013 and 16 family pairs in HGC 2013. In 2014, no significant differences between family variances were detected in either LGC or HGC. In 2015, 11 family pairs in LGC and one family pair in HGC 2015 were counted as significantly different. Of these pairs of families, the same few families were repeatedly represented, encompassing the extreme ends of within-family variation. For example, in the LGC 2013 population, only seven families were represented out of a possible 14. The high within-family variation encouraged an initial selection method on an individual root basis for the three cycles of selection. This method has also been employed for increasing pigment in table beets work by UW-Madison (Goldman et al., 1996). Concern for a narrowing genetic pool was mitigated after observation that selected individuals in both populations represented over 50% of the families.

Field Observations

Field observations of the HGC and LGC populations showed a visual difference in leaf color, susceptibility for bolting, and varying degrees of vigor. During the four selection cycles, it was observed that the HGC population had darker leaves and petioles overall than families in the LGC. In the 2014 field season, families in the HGC population showed a high susceptibility for early flowering during vegetative growth. However, families in the HGC population also varied for bolting susceptibility. A portion of the families had no individuals bolting during vegetative growth, while a majority of individuals bolted in other families. No individual plants that were bolting during vegetative growth were chosen for geosmin analysis in the 2014 selection year, as this is an undesirable trait for beet root production. Little to no flowering during vegetative growth was observed in the 2013 and 2015 field seasons.

During the greenhouse seed production phase of each cycle, the attrition rate of the HGC, as measured by the early death of individual roots, was much higher than that of the LGC. During the selection process, attrition affected many of the families, resulting in 12 to 16% of selected roots surviving each cycle of breeding in both populations. Because of varying attrition rates from cycle to cycle, the contributing percentage of selected roots for the low geosmin population was 13% in 2013 (C1), 13% in 2014 (C2), and 16% in 2015 (C3). The contributing percentage of selected roots for the high geosmin population was 16% in 2013 (C1), 13% in 2014 (C2), and 15% in 2015 (C3). In the seed production phase of 2015, 100 individuals were principally selected and planted to compensate for the high attrition rate observed over the first two breeding cycles and to increase seed production for future projects.

Realized Heritability for Geosmin Concentration

Realized heritability was calculated using the ratio of the cumulative selection response to the cumulative selection differential from C1 through C3 (Table 2.3). This was done for the LGC and the HGC populations. Due to the high attrition rate in each cycle of selection, realized heritability was calculated for both the original selected (OS) population and the population after the atrophied individuals were removed (AS). The OS realized heritability for the HGC was 0.23 and the AS realized heritability was 0.24. The realized heritability for the LGC in both the OS and the AS was 0.7.

Taste Test

Two separate tastes test for roots from the HGC vs. LGC comparison were conducted; however, geosmin levels were not assessed until after the taste tests were completed. Geosmin levels from the first taste test did not appropriately discriminate HGC and LGC populations and are not presented here[3]. The second test, where geosmin levels did discriminate between HGC and LGC populations, resulted in 13 of 36 participants correctly identifying the differing sample. No statistically significant difference was identified. The geosmin levels for the roots were 10.12 µg geosmin kg–1 tissue for the HGC and 1.04 µg geosmin kg–1 tissue for the LGC. Soluble solids were 11.4 for HGC and 11.93 for the LGC (Table 2.4).

The LGC vs. check test was conducted over 2 d, for a total of 36 responses. Roots were taken from the same families for both tests. Five of 25 responses were correct on Day 1 and

[3] Roots used in the taste tests were measured for geosmin content following the taste test. The first HGC vs LGC taste test conducted resulted in roots with similar geosmin levels. The purpose of this HGC vs LGC taste test was to distinguish between beet populations bred for different levels of geosmin concentration. The similarity of geosmin concentration in this particular taste test did not appropriately represent the taste test initially sought, thus these data were not used. The variation within families remains high and the epidermal lay of the HGC beet was removed (which contains higher levels of geosmin), so it was not unexpected to see geosmin concentration of similar levels.

seven of 11 responses were correct on Day 2. Therefore, respondents were unable to differentiate between LGC and check cultivar for this test. The geosmin levels for the roots in these tests were 1.62 and 1.3 µg geosmin kg–1 tissue for the LGC and 6.69 and 4.59 µg geosmin kg–1 tissue for the check, respectively. Soluble solids were 12.6 and 11.23 for the LGC family and 15.1 and 14.17 for the check, respectively (Table 2.4).

The HGC vs. check test was also conducted over 2 d. It was difficult to find roots of similar color in the top five high families to that of Red Ace, thus more roots of smaller size were used for this test. A total of three roots from two families were used from the HGC population. Sixteen of 24 responses were correct on Day 1 and six of 12 responses were correct on Day 2 (Table 2.4). Overall, 22 of 36 participants correctly picked the odd sample. Therefore, at the $\alpha = 0.001$ level, it can be concluded that a perceptible difference exists for this test. The geosmin levels for the roots were 16.75, 17.5, and 8.66 µg geosmin kg–1 tissue for the HGC, and 1.9 and 5.19 µg geosmin kg–1 tissue for the check, respectively. Soluble solids were 9.63, 11.67, and 15.1 for the HGC, and 14.43 and 13.2 for the check, respectively.

Discussion

Bidirectional Selection Successfully Altered Geosmin Concentration in Table Beet

Until recently, the operating assumption for geosmin production in table beet has been thought to be an association with geosmin-producing organisms found the soil in which the beet is grown. Geosmin, a distinguishing trait in beet and other members of the Amaranthaceae and Cactaceae families, is produced by many microorganisms, but its functional significance is yet unknown. Some of the documented geosmin-producing microbes also produce toxins (Suurnäkki et al., 2015). Drosophila have a specialized olfactory receptor that detects geosmin, alerting flies to harmful microbes on potential food sources (Stensmyr et al., 2012). Humans are also

remarkably sensitive to geosmin, and a large body of literature is devoted to water quality and the mitigation of geosmin production in water quality control (Jüttner & Watson, 2007). The consumption of foodstuffs that contain geosmin is more nuanced, whereas the earthy quality produced by geosmin can be both desired and disliked in wine, beer, and cheese. These differences in preference holds true for beet. These results are the first to demonstrate that geosmin concentration in table beet responds to selection. Furthermore, our results demonstrate that selection for both increased and decreased geosmin levels is effective. Thus, through bidirectional selection, two breeding groups defined by characteristically low and high geosmin concentrations have been developed in our breeding program.

Progress made by this selection scheme is one indication that geosmin may be endogenously produced by beets. There is a wide variation of geosmin production in microbes and within their own species. It is also possible that this work has selected for environments that are both inhibitory and advantageous to geosmin-producing microbes that associate with beets. Alternatively, if the geosmin production is an endogenous trait of beet, selection for geosmin could be from increasing or decreasing substrate, geosmin synthase, or some yet other undefined component in the geosmin production pathway.

While mean geosmin concentration in these populations changed significantly across cycles of selection, the distribution of geosmin concentrations in each population was large. The variation of geosmin concentration within family was the reason why selection was practiced on an individual root basis rather than on a family mean basis. By doing so, we were able to more efficiently increase and decrease the geosmin concentration means of the populations during three cycles of recurrent selection. Selection on individuals prompted consideration of the number of families represented by the tails of the geosmin concentration distribution to avoid a

bottleneck. Selected individuals represented at least 50% of the families in both populations. While selection on an individual plant basis was effective in this experiment, selection on a family mean basis would be a more efficient method for future breeding purposes. Similar methods have been used for successful recurrent selection for betalain pigment concentration in table beet. Initially, the variable nature of the trait led the breeders to select on an individual plant basis until distinct families emerged, when selection was practiced on a family mean basis (Goldman et al., 1996). Relative recovery for geosmin concentration was low, but consistent. It is possible that the protocol used for geosmin extraction underestimates geosmin bound up in protein rather than cytosol (Jüttner and Watson, 2007), thereby underestimating total geosmin present in table beet. Further analytical work will be necessary to determine absolute amounts of geosmin in this species and for comparisons across plant and microbial species.

Prior to this selection experiment, Freidig and Goldman (2014) surveyed known open-pollinated table beet cultivars for geosmin concentration. Their conclusions that geosmin is a variable trait in beet cultivars led to the formation of the two populations in this study. The parental populations of five individuals from each cultivar is a small genetic pool, but because these individuals were from open-pollinated cultivars, it is possible they may have represented a substantial amount of genetic variation. This is also a possible explanation for the color segregation found between the two populations where the starting population for the HGC was the dark red leafed and rooted Bull's Blood and the striped white and red Chioggia. The LGC population had the yellow Touchstone gold and the white rooted Blankoma. The control of the primary pigmentation of beets is partially known. Red-pigmented roots are the result of dominance at the R and Y loci, and white roots are the result of recessive alleles at the Y locus. Additionally, a blotchy allele conferring irregular pigmentation has been linked to the R and Y

loci (Goldman and Austin, 2000). In mangel, the gene *p* has been characterized as a color suppressor, and is not known to be present in table beet (Goldman and Austin, 2000).

We found that there was high attrition within the selected roots during pollination and subsequent seed production. The amount of attrition in these populations was higher than that observed in other recurrent selection studies in our laboratory (data not shown). The HGC population seemed especially sensitive, where the attrition rate was upward of 50%. However, realized narrow-sense heritability (h2) between the originally selected individuals and the remaining individuals at planting was negligible. The LGC had a much higher h2 than the HGC. The geosmin biosynthetic pathway has yet to be described in table beet, but our observations suggest the high attrition rate and low realized h2 of the HGC may mean it is more resource intensive for plants to produce high levels of geosmin.

Five of the highest and lowest geosmin families (from C3 data) were compared with cultivars Bull's Blood and Red Ace, and two breeding lines from our program with Touchstone Gold in the pedigree in 2016 (Fig. 2.1). These families did not deviate from the previous selection cycle trend. The HGC families were much higher than Bull's Blood, one of the parents for the HGC population. Similarly, the LGC families were lower than both Touchstone Gold (yellow and red pigmented) lines and Touchstone Gold was a parent for the LGC population. A popular variety, Red Ace, was quite low in geosmin concentration, like the LGC population.

Taste Test

In the HGC vs. check taste test on Day 1, the geosmin levels were much higher than the Red Ace check. The soluble solids were lower in the HGC roots than the check. The tasters found a significant difference between these roots. The roots used for the second day were closer in their geosmin concentrations and soluble solids and the tasters found a moderate difference

between the roots. No difference was found between the Red Ace and LGC root, which is supported by the cultivar comparison (Fig. 2.1). The HGC vs. LGC test had very similar soluble solids and a large difference between geosmin concentrations as compared to the LGC vs. check test, yet tasters could not tell the difference between HGC and LGC roots. Soluble solids levels were consistent with levels found in the literature (Feller and Fink, 2004). It was observed that the geosmin levels in beets from the HGC population were much lower than the family means. This may be due to the method of sampling where the tasted beets had the epidermis removed for both the taste test and for the GC-MS analysis. This set of data make it difficult to make any conclusions regarding the high, low, and check comparisons. It could be there is some other factor that tasters are perceiving, such as not masking color sufficiently or another culinary quality yet to be determined. A recent body of work regarding saponins, a bitter taste, could be an influencing characteristic (Mikoajczyk-Bator and Kikut-Ligaj, 2016). Another important variable might be the epidermis and should remain on for taste tests (Archer and Stokes, 1978; Bach et al., 2014). Additional taste tests with these populations, perhaps with a trained taste panel, might be able to distinguish the true differences between these populations.

In conclusion, a significant shift in geosmin concentration was achieved through half-sib recurrent selection resulting in distinct populations defined by a high geosmin concentration and a low geosmin concentration. These results might be an indication that geosmin is genetically controlled by beet and not a microbial association. Further efforts to select for desirable agronomic traits within these populations are underway.

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Tables

Table 2.1. Analysis of variance for geosmin concentration averaged over families within the high geosmin concentration (HGC) and low geosmin concentration (LGC) populations in each of three cycles of selection.

Cycle	Population	d.f.	Sum of Squares	Means squared	F-value	p>F	Significance
1	LGC	59	40229	681.8	8.549	<2e-16	***
	HGC	39	34109	874.6	6.018	<2e-16	***
2	LGC	53	988.1	18.644	2.585	1.39e-07	***
	HGC	45	8282	184.05	2.04	0.000274	***
3	LGC	52	955.6	18.377	2.176	2.00e-05	***
	HGC	41	21194	516.9	2.417	1.31e-05	***

*** Significance at the $p < 0.001$ level

Table 2.2. Mean geosmin concentration of high geosmin concentration (HGC) and low geosmin concentration (LGC) populations from Selection Cycles 1 to 4 and comparison of population means in Selection Cycles 1 to 3.

	Mean geosmin concentration, μg kg^{-1} tissue		
Cycle	**HGC**	**LGC**	**Significance**[†]
1	22.3±0.92	17.26±0.62	***
2	22.7±0.56	5.3±0.15	***
3	33.85±0.88	4.27±0.15	***
4[‡]	42.75±3.1	2.55±0.14	

*** Significance at the $p < 0.001$ level.

[†]Welch two-sample t-test between HGC and LGC geosmin populations cycles 1-3.

[‡]Cycle 4 mean is the average of the highest and lowest five families in the HGC and LGC.

Table 2.3. Geosmin concentration means for populations per se and realized heritabilities of selected individuals and individuals remaining following pollination in three cycles of selection.

	Geosmin concentration, µg kg^{-1} tissue					
Cycle	**HGC population†**			**LGC population†**		
	Pop Mean	**Selected Mean**	**Attrition Mean**	**Pop Mean**	**Selected Mean**	**Attrition Mean**
1	22.297	41.546	44.094	17.256	4.726	4.748
2	22.7	35.72	32.469	5.301	2.068	2.029
3	33.847	52.322	51.086	4.271	1.57	1.459
h^2‡		**0.23**	**0.24**		**0.7**	**0.7**

†LGC, low geosmin concentration population; HGC, high geosmin concentration population.

‡h^2, narrow-sense heritability.

Table 2.4. Geosmin concentration (µg geosmin kg^{-1} tissue), soluble solids and results of participant triangle test to identify differences among table beet samples of HGC, LGC populations and check variety.

Test		Tasted Beets	Geosmin	Soluble Solids	Correct of total	Significance
HGC vs LGC	Day	*HGC*	10.12	11.4	**13/36**	**NS†**
		LGC	1.04	11.93		
LGC vs Check	1	*LGC*	1.62	12.6	5/25	NS
		Red Ace	6.69	15.1		
	2	*LGC*	1.3	11.23	7/11	0.1
		Red Ace	4.59	14.17		
Overall					**12/36**	**NS**
HGC vs Check	1	*HGC*	16.75, 17.5	9.63, 11.67	10/18, 6/6	0.1, **
		Red Ace	1.9	14.43		
	2	*HGC*	8.66	15.1	6/12	0.2
		Red Ace	5.19	13.2		
Overall					**22/36**	*******

**Significance at the $p < 0.01$ level.

***Significance at the $p < 0.001$ level.

†NS, not significant.

Figures

Figure 2.1. Response to four cycles of bidirectional selection for geosmin concentration in two table beet populations, HGC and LGC. Regression for HGC is $y = 5.81x - 11{,}673$, $R^2 = 0.94$; and for LGC is $y = -3.38x + 6816.2$, $R^2 = 0.76$. †Cycle 4 includes the lowest (No. 1–5) and highest (No. 2–3, 5–6) families for geosmin concentration from that cycle. ‡Check cultivars, including two breeding lines (red and yellow pigmented) with 'Touchstone Gold' in the pedigree.

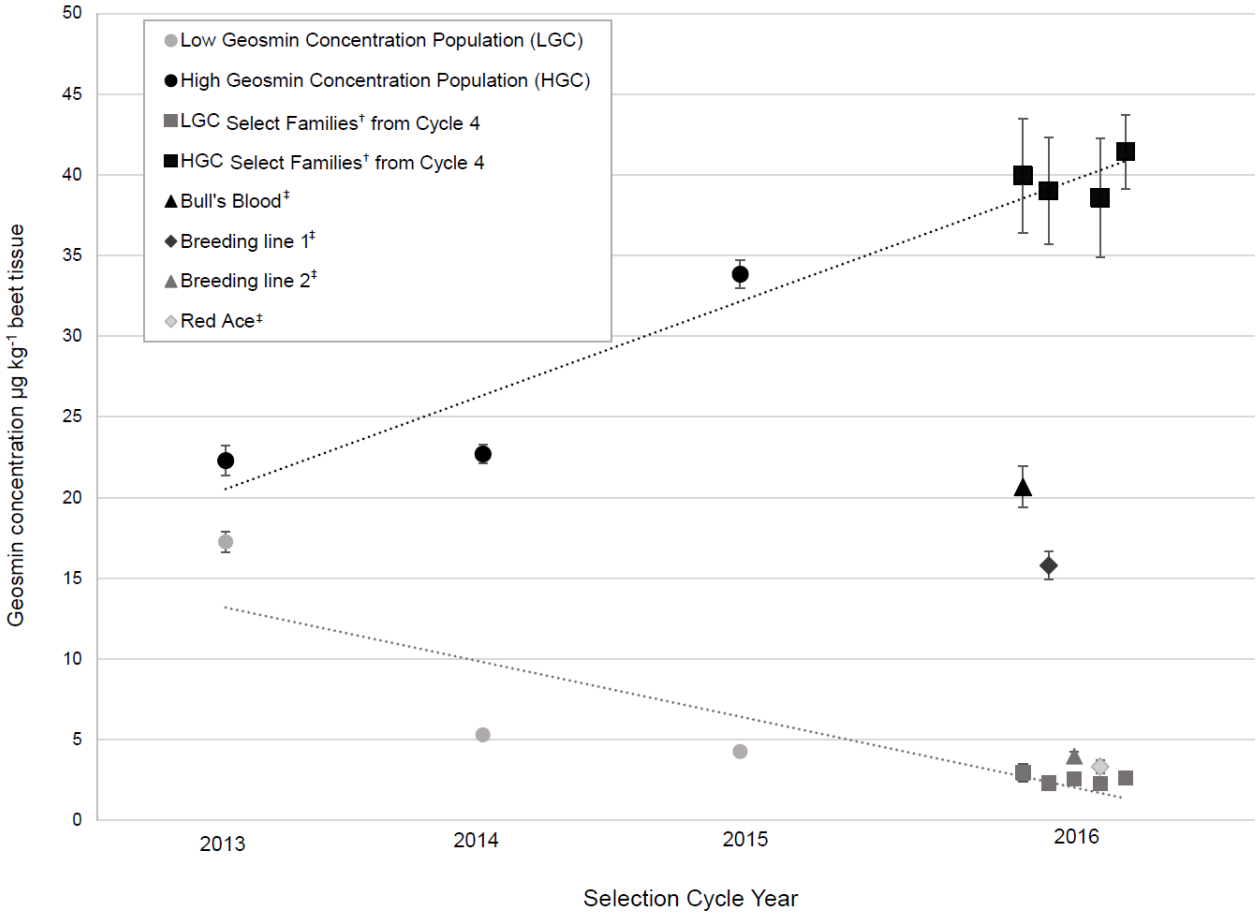


Figure 2.2. Box and whisker plots of geosmin concentration in three selection cycles of high geosmin concentration (HGC) population organized by family mean averaged over replications.

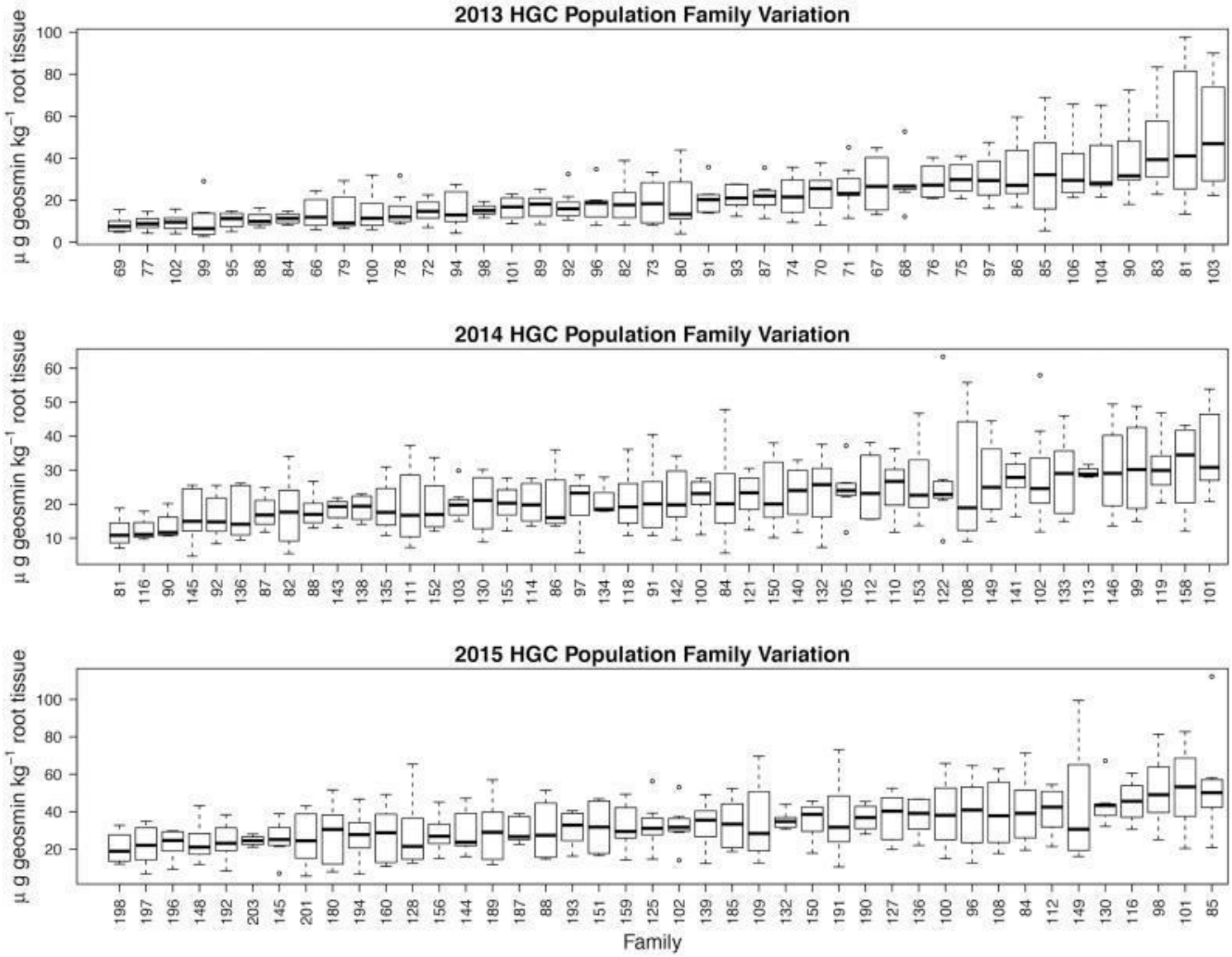
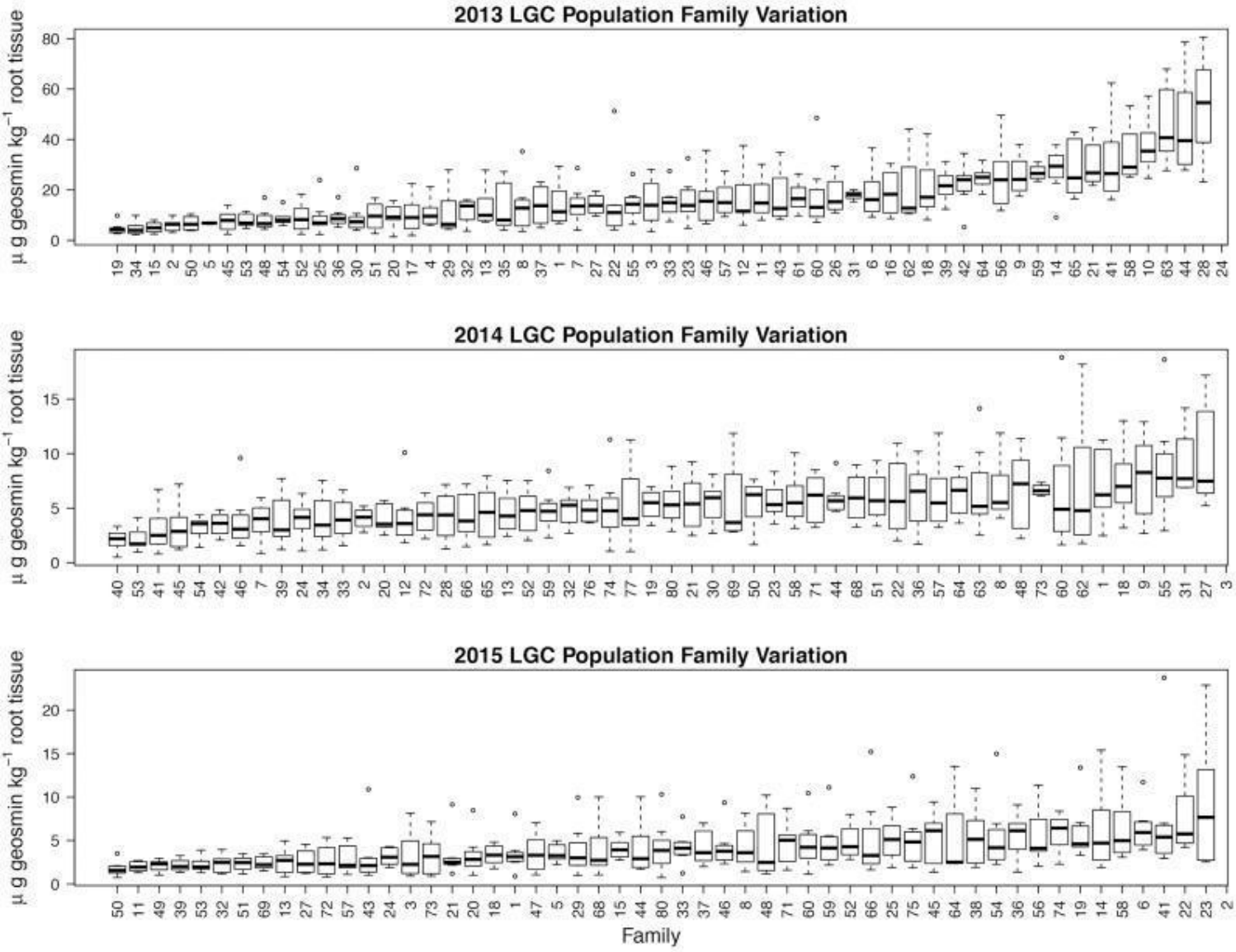


Figure 2.3. Box and whisker plots of geosmin concentration in three selection cycles of low geosmin concentration (LGC) population organized by family mean averaged over replications.



Chapter 3: Endogenous Production of Geosmin in Table Beet

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Abstract

The earthy flavor of table beet is due to an aromatic terpene derivative called geosmin. It has been hypothesized that geosmin presence in beet is due to geosmin producing bacteria such as *Streptomyces* spp. that exist in the soil. However, recent findings suggest that beet may produce geosmin endogenously without microbial influence. The purpose of this study was to determine whether such endogenous production of geosmin occurred in beet by making use of an aseptic tissue culture environment to remove potential microbial influences on geosmin production. Four table beet accessions ('Bull's Blood', 'Touchstone Gold', 'W364B' and 'Pacemaker III') were grown in three separate tissue culture experiments and in the greenhouse and measured for geosmin concentration via Gas Chromatography – Mass Spectrometry (GCMS). Sequencing of 16S ribosomal RNA was used to identify potential microbial contaminants in tissue culture. Operational taxa units (OTUs) classification resulted in RNA sequences with homology to bacterial RNA of either chloroplast (98%) or mitochondria (2%). Other OTUs identified were considered with-in range of sequencing error. In 15 of the 16 tissue culture-grown samples used for the 16S rRNA aseptic validation and in all the greenhouse-grown plant samples, geosmin was detected. Geosmin concentrations from bulked beet tissue of each accession were higher in the tissue culture environment than the greenhouse environment. The lack of microbial detection in the tissue culture environment and the subsequent identification of geosmin from beets grown in the aseptic environment is a strong indication that geosmin is produced endogenously by beets. This finding raises a number of interesting questions about the functional significance of this molecule for *Beta vulgaris*.

Additional index words. *Beta vulgaris, Streptomyces* spp., 16S rRNA, geosmin

Introduction

The aromatic earthy compound geosmin is a characteristic flavor and scent to which humans are remarkably sensitive. The detection rate for geosmin and another musty-earthy compound, 2-methylisoborneol (MIB), is as low as 6-10 ng L^{-1} (Rashash et al., 1997). Many microbial species produce geosmin including the majority of *Streptomyces*, cyanobacteria and some fungal species (Jiang et al., 2006). The abundance of *Streptomyces* species in the soil is the primary cause of the quintessential earthy aroma of soil (Jones and Elliot, 2017). Geosmin producing microbial species, including those in the genus *Streptomyces*, have been found as the source of geosmin contamination in wine, beer and other foodstuffs, conferring an undesirable musty taste (Paterson et al., 2007). Also drawing consumer complaints are instances of musty and earthy tasting water caused by geosmin-producing microbes in water supplies. A large body of literature is devoted to the mitigation of geosmin and geosmin producing microbes in drinking water supplies (Jüttner and Watson, 2007). While geosmin is often a negative quality in food, it can sometimes be a desirable characteristic for consumers seeking earthy flavors. The earthy taste of beet (*Beta vulgaris* L.), caused by geosmin, elicits strong favorable and unfavorable reactions from consumers.

The interest in monitoring food and water quality due to volatiles like geosmin led to the development of analytical protocols using gas chromatography- mass spectrometry (GCMS) (Acree and Lee, 1976; Jiang et al., 2006; Lu et al., 2003b). Further refinements to the GCMS protocol for analysis of geosmin in beet have used headspace solid phase micro extraction (HSPME) (Lu et al., 2003a; Lu et al., 2003b). A HSPME protocol to measure geosmin in table beet was adapted from Lu et al. (2003a and b) by Freidig and Goldman (2014) and Maher and Goldman (2017) to analyze geosmin concentration in various beet cultivars across multiple environments.

To date, the presence of geosmin in root vegetables other than beet has not been recorded. However, geosmin does occur in other subspecies of *Beta vulgaris* such as swiss chard and sugar beet (Freidig and Goldman, 2014; Maga, 1987). Additionally, dehydrogeosmin, when in a particular configuration, is identical to geosmin and has been recorded as a floral volatile of some species of Cactaceae (Schlumpberger et al., 2004). It is interesting to note that these species all belong to the order Caryophyllales.

The biosynthetic pathway for geosmin, first elucidated in *Streptomyces coelicolor*, is an Mg^{2+} dependent cyclization reaction (Jiang et al., 2006). Geosmin synthase, a unique bifunctional sesquiterpene synthase, first converts farnesyl diphosphate (FDP) into an 85:15 mix of germacradianol and germacrene D via the N terminal unit. The C terminal then converts germacradianol into geosmin (Jiang et al., 2007). The purpose of geosmin production in *Streptomyces* spp. is unknown, but many *Streptomyces* species produce geosmin suggesting that the trait is conserved and has some importance (Seipke et al., 2012). Schöller et al. (2002) screened 26 different *Streptomyces* spp. and characterized 120 different volatile organic compounds (VOCs). They observed that geosmin and sporulation were strongly correlated. VOCs could potentially be used as communication signals between bacteria. A recent study by Jones and Elliot (2017) found that *Streptomyces* exploration, a novel form of growth and development by the bacteria when in a multi-species culture, was stimulated by the VOC trimethylamine.

The functional significance of geosmin in beet is unknown. It was initially hypothesized that the presence of geosmin may be due to the interaction between the beetroot and soil microbes over the course of the growing season (Buttery and Garibaldi, 1976). Geosmin levels are much higher in the beetroot epidermal layer than the inner hypocotyl layers (Lu et al.,

2003a). Lu et al. (2003a) tested the possibility of geosmin accumulating in the beet epidermal layer by adding pure geosmin to Murashige and Skoog basal salt (MSBS) media and testing the differences between beet seedlings grown on non-adjusted MSBS media for 4, 5 and 6 months. They found no significant difference in geosmin concentration between beet plants grown with and without exogenously applied geosmin.

An alternative explanation for geosmin production in beet could be the result of endophytic bacteria within the beetroot. Both pathogenic and symbiotic *Streptomyces* spp. are known to invade and colonize plant hosts (Seipke et al., 2012). A plant colonized by endophytic *Streptomyces* spp. may not show disease symptoms that are common with pathogenic *Streptomyces* spp. (Coombs and Franco, 2003). Known plant endophytic *Streptomyces* species can provide growth promotion via auxin production and increased nutrient assimilation. The prolific production of antimicrobial compounds by the endophytic *Streptomyces* species can also provide protection from phytopathogens to the host plant (Seipke et al., 2012). Actinomycete filaments were observed by Jacobs et al. (1985) and Lu et al. (2003a) in the inner cellular region and outer tissue of beets, but it was unclear if those actinomycetes were true endophytes or could produce geosmin.

Lastly, geosmin in beets could be the result of endogenous production. Recent work by Freidig and Goldman (2014) showed that geosmin concentration in beet is cultivar specific. They grew eight beet cultivars, three cultivars of close table beet relatives (mangel, sugar beet and Swiss chard) and one carrot cultivar over two years in three environments. The environments consisted of a greenhouse environment using autoclaved soil and non-autoclaved soil and a field environment. No significant differences in cultivar rank for geosmin concentration between years in the field environments and the greenhouse environments were found for beet and related

cultivars. Although they identified repeatable geosmin signatures in beet cultivars, they were not able to determine whether this cultivar specificity was due to endogenous production *in planta* or associations between the host plant genotype and the geosmin producing soil microbes. Maher and Goldman (2017) recurrently selected two table beet populations for high and low geosmin concentrations. They found significant responses to selection, suggesting either endogenous production of geosmin or selection for improved microbial associations with this host plant. Theirs was the first study to show that geosmin concentration could be manipulated by selection in beet.

Plant material is often grown in sterile culture to obtain disease free material, but it is also a method to eliminate microbial influence. For example, Dai et al. (2016) examined the effects of endophytic bacteria *Bacillus* sp. on an invasive plant species *Wedelia trilobata* and its native congener *W. chinensis* by eliminating all other bacterial influencing factors in an aseptic culture system. Through this system, they were able to conclude that the plant growth-promoting effects of the endophytic bacteria enhanced the growth of the invasive species, but not the native species. To address the question of whether geosmin levels in beet are due to microbial associations or endogenous production, Lu et al. (2003a) grew 'Round Red' beets aseptically on MSBS media and reported geosmin concentration of 14 µg kg^{-1} of plant tissue at 4 months of growth. Lu et al. (2003a) also reported geosmin concentrations of 135 ± 30.6 µg kg^{-1} and 65.2 ± 25.9 µg kg^{-1} of plant tissue beet for cultivars 'Chioggia' and 'Detroit Dark Red', respectively. A limitation of this study was the lack of a microbial assays beyond visual inspection to confirm sterility of the beet cultures. Bacterial populations that associate with plants may be present in the root zone (rhizosphere), the above ground biomass (phyllosphere), and inside the plant (endophytes). The challenge for studying microbial communities in any of these locations is that

as few as 1% of bacteria can be identified using traditional culturing techniques. Advances in sequencing technology allow for a greater understanding of the diversity of microbial communities in marine systems, soil and agricultural environments (Jackson et al., 2015). Developments in metagenomic sequencing tools such as 16S ribosomal RNA gene (rRNA) sequencing provide a powerful method to characterize these microbial communities. 16S rRNA gene sequencing has been used to characterize the endophytic bacteria of sugar beets at various developmental periods grown on Tianshan Mountain in China (Shi et al., 2014). The diversity of the bacterial communities varied with developmental period, generating the greatest number of operational taxonomic units (OTUs) during rosette and root growth of the beet. Alphaproteobacteria represented the largest taxonomic group identified in that study.

Results from the Maher and Goldman (2017) selection study showed that geosmin concentration is a trait that can be selected in beet, yet the exact targets of selection remain unknown. Further understanding of the nature of geosmin production in beet is essential for future breeding efforts. The objective for this study was to characterize geosmin concentrations in root and shoot tissues from *Beta vulgaris* plants grown in sterile tissue culture, and to verify whether these cultures contained geosmin-producing microbes using 16s rRNA sequencing.

Methods

Plant sterilization and propagation

Four beet accessions, ‘Pacemaker III’ (hybrid, Territorial Seed Company, Cottage Grove, OR), Touchstone Gold’ (open pollinated, Johnny’s Selected Seeds, Winslow, ME), ‘Bull’s Blood’ (open pollinated, Johnny’s Selected Seeds, Winslow, ME), and W364B (inbred, University of Wisconsin-Madison, Madison, WI) were propagated in 25mm glass tubes with 36 tubes per accession. All seeds were multigerm where multiple embryos exist within the corky

seed ball due to 2-5 inflorescences clustered together. The seed sterilization protocol was adapted from Yildiz et al., (2007). Seeds were soaked in 100% concentrated Clorox® bleach (8.5% sodium hypochlorite) for 5 hours while shaken at room temperature on a New Brunswick G10 gyrotory shaker® at low speed. Seeds were rinsed 3-4 times and soaked in Milli-Q water (Millipore, Bedford, MA, USA) for 24 hours. Prior to entry into tissue culture, seeds were treated with 70% ethanol and rinsed 3-4 times in Milli-Q water. Seeds were germinated on Murashige and Skoog basal (MSBS) media (media mix with vitamins and sucrose from Phytotechnology Laboratories) prepared as directed by manufacturers. Beets were grown for 12 weeks (June 15-September 7, 2016) in a walk in controlled culture chamber at 22ºC under cool fluorescent light (average 15.94 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 24 hours of light. Vials were visually checked for sterility (cloudy media, bacterial or fungal growth) and removed if contaminated. Leaf tissue was sampled for DNA extraction and sequencing and the remaining plant material frozen at -20°C until processed for geosmin analysis. Beets ('Touchstone Gold', 'Pacemaker III', 'Bull's Blood', '364B') were grown for one month in Pro-mix HP potting soil in the greenhouse (Walnut Street Greenhouse, University of Wisconsin-Madison, Madison, WI) at 16-hour day length. Greenhouse-grown beet seedlings were identical in maturity to tissue culture-grown beets when harvested. The level of maturity was the beet growth stage where the beet hypocotyl had elongated, but minor swelling of the root had yet to occur. Beet tissue samples included the leaf tissue and hypocotyl. Plant materials were frozen at -20°C until processed for geosmin concentration analysis. These same beet accessions ('Touchstone Gold', 'Pacemaker III', 'Bull's Blood', '364B') were also grown in 2014 and 2015 in tissue culture and greenhouse environments and visually checked for contamination. The plant material produced in all three years was frozen at -20°C until analyzed for geosmin concentration. Samples were prepared for

bulk analysis as described below. Sequencing of 16S ribosomal RNA was performed only once with plant materials from the final experiment.

DNA extraction, construction and sequencing of V3-V4 16s metagenomic libraries

6-10 mg lyophilized beet leaf tissue was used for DNA extraction. The CTAB method, described in Saghai-Maroof et al. (1984) with minimal modification, was used to extract the DNA. DNA was eluted and a final DNA cleaning step was applied to remove any remaining inhibitory compounds with a 1.5:1 by volume ratio of Axygen Clean-Seq beads (Corning Life Sciences, Corning, NY, USA). Quant-IT PicoGreen fluorescent dye (Thermo Fisher, Waltham, MA, USA) was used for DNA quantification. Purified genomic DNA concentration was verified using the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, California, USA) and the samples were prepared by the University of Wisconsin-Madison Biotechnology Center for the 16S Metagenomic Sequencing Library Preparation Protocol, Part # 15044223 Rev. B (Illumina Inc., San Diego, California, USA). Modifications to the protocol included amplification of the 16S rRNA gene V3/V4 variable region with fusion primers (forward primer 341f: 5'-ACACTCTTTCCCTACACG ACGCTCTTCCGATCTCCTACGGGNGGCWGCAG-3', reverse primer 805r: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGT ATCTAATCC-3') and region-specific primers, previously described in Klindworth et al. (2013) were modified by an addition of Illumina adapter overhang nucleotide sequences to gene-specific sequences. A 0.7x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences, Union City, CA) was used to clean reactions after first amplification. Illumina dual indexes and sequencing adapters were added by primers (forward primer: 5'-AATGATACGGCGACCA CCGAGATCTACAC[55555555]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', reverse primer: 5'-CAAGCAGAAGACGGCATACGAGAT[77777777]GTGACTGGAGTT

CAGACGT GTGCTCTTCCGATCT -3', where bracketed sequences are equivalent to the Illumina Dual Index adapters D501-D508 and D701-D712) in a later PCR. Another 0.7x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences) was used to clean the reactions after PCR. Finished libraries were assessed for quality with an Agilent DNA1000 chip and quantity with a Qubit® dsDNA HS Assay Kit. Before sequencing, libraries were pooled after standardization to 2nM. Illumina MiSeq Sequencer and a MiSeq 500 bp (v2) sequencing cartridge were used for paired end, 250 bp sequencing. Images were analyzed using the standard Illumina Pipeline, version 1.8.2. OTU assignments and diversity plots were created using QIIME analysis pipeline (Caporaso et al, 2010).

Sample preparation for geosmin concentration analysis

Samples were prepped for two different analyses; single plant geosmin concentration and bulk plant geosmin concentration. All plants were ground in liquid nitrogen. Single plants, tissue culture grown and greenhouse grown, were ground and blended with 5ml Milli-Q water and frozen at -20°C until analyzed. This deviation from a 1:1 ratio was to ensure adequate sample for HSPME protocol (for details see *HSPME and GC-MS Analysis* below). A 1:1 ratio of tissue to water was used for the bulk plant geosmin concentration analysis. Four g of tissue cultured plants and greenhouse grown plants were bulked by accession and environmental treatment and blended with 4ml Milli-Q water and frozen at -20°C until analyzed. Four g of beet root was also ground with liquid nitrogen and blended with 4ml Milli-Q water. Approximately 7-9 young beets were used for each bulked sample.

Beet homogenate samples were thawed in a water bath and 5g of homogenate was added to a glass vial with 1g of NaCl and capped immediately with a screw cap containing PTFE/silicone septum. Menthone [(1R,4S)-p-menthan-3-one], used as the internal standard, was

added at a concentration of 2.82 µg geosmin (kg root tissue)$^{-1}$. For the bulk tisue analysis geosmin (2β,6α-dimethylbicyclo[4.4.0]decan-1β-ol)) standards of 5, 10, and 21.6 µg geosmin (kg root tissue)$^{-1}$ added to Milli-Q water blanks. Menthone and geosmin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Relative recovery rates, determined by the calculation from Lu et al. (2003a), used beet leaf tissue spiked with 5, 10, 15, and 21.6 µg geosmin (kg root tissue)$^{-1}$.

$$Relative\ Recovery = \frac{\mu g\ geosmin\ total - \mu g\ geosmin\ originally\ in\ beet}{\mu g\ geosmin\ spiked}\ x\ 100\%$$

HSPME and GC-MS Analysis

Analysis of samples was conducted as described in Freidig and Goldman (2014). Total program time per sample was 10.43 minutes. Samples were incubated for 3 min at 60 °C and agitated at 500 rpm. Following incubation, extraction under the same conditions for 10 minutes, used PDMS/DVB fibers conditioned according to manufacturer specifications (Supelco: Sigma-Aldrich, St. Louis, MO, USA). Column used was a 30 m SHRXI-SMS with 0.25 mm i.d. and 0.25 mm df (Shimadzu). Injection mode was split (split ratio of 10), and a split/splitless liner was used. The temperature protocol was as follows: 40 °C for 1 min, increased to 35 °C min−1 and to 300 °C, and held for 2 min. During the sample process, the fiber was thermally desorbed at 250 °C for 1 min. Menthone and geosmin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Results

Beet growth in tissue culture and greenhouse environments

Through preliminary studies, a basic, yet intensive bleach wash and treatment with ethanol of the multigerm seed ball was determined to be enough to create sterile beet seeds that preserved the seed coat and retained a high germination rate. The treatment resulted in few vials

with visible contamination. These were confirmed with regular visual checks. In comparison to methods used in other studies (Lu et al., 2003a; Yildiz et al., 2007), we found that the removal of the corky seed ball exterior to expose the true seed was a difficult process that increased the potential of further ambient contamination and resulted in a greater likelihood of damaged embryos. The majority of germination occurred over an 11-day window beginning five days after introduction into culture. Overall, 0.018% of the vials with germinated seed were removed due to contamination with the visual assessment. Minimal swelling of the hypocotyl into what would be considered a beet was observed over the 12 weeks in culture, but the entire root-hypocotyl axis was evaluated in our assays (Figure 3.1). Limited space in the growing environment is a probable cause for this restricted root development in comparison to unconstrained beets grown in normal soil conditions. Greenhouse grown beets were harvested after four weeks of growth. These beets were at similar maturity (no hypocotyl swelling) to the tissue culture grown beets. We observed fewer leaves on the greenhouse grown beets, but the leaves were much larger than their tissue cultured counterparts.

16S Amplicon Sequencing Analysis

Illumina sequencing of the V3-V4 region of the bacterial 16S rRNA gene for a selected subset of 16 beets grown in a tissue culture environment resulted in a total of 553,372 amplicons across all samples with an average of 34,586 amplicons per sample after quality filtering. The median length of amplicons was 441 bp for each sample. The majority of the operational taxa units (OTUs) were classified as chloroplast, classified by the QIIME pipeline as Streptophyta, ranging between 96.8% and 99.0% over the 16 samples (Figure 3.2, Table 3.2). The second largest group were mitochondria ranging between 1.0% and 3.0% over the 16 samples classified

as *Rickettsiales*. Total percentage was 98% chloroplast and 2% mitochondria for all 16 beet samples.

Following initial sequence processing, reads/OTUs were filtered to remove chloroplast and mitochondria reads. The remaining OTUs resulted in an average of 0.037% of the total OTUs. Subsequently, while these OTUs were classified, they did not contribute to the rounded percentages found in Table 3.2 and these classifications do not appear in Figure 3.2. The number of OTUs are reported for each of the negligible bacterial classifications in Table 3.2.

Geosmin concentrations

A total of 16 individual samples were used for the 16S rRNA analysis to verify the sterility of the beets grown in tissue culture. Four individual beet plants from a total of four accessions: open-pollinated 'Bull's Blood' and 'Touchstone Gold', hybrid 'Pacemaker III', and an inbred from the University of Wisconsin-Madison, 'W364', were selected at random. All 16 plant samples returned negligible amounts of classifiable bacteria from the 16S V3-V4 rRNA. The lack of amplification indicated no endophytic bacterial presence. After the verification of sterility, the HSPME protocol outlined in the methods resulted in geosmin detection in 15 of the 16 beet samples. One sample, from the hybrid 'Pacemaker III', did not register geosmin presence (Table 3.1).

Geosmin concentration levels were compared for each of the accessions that were grown in both the tissue culture and greenhouse environments using bulked beet leaf and hypocotyl tissue at pre-root development stage (Figure 3.3). Geosmin levels were measured from beetroot tissue ground with liquid nitrogen, the same preparation used for the leaf and hypocotyl tissue. Beetroot tissue was from the low geosmin population described in Maher and Goldman (2017). This was to insure the method of preparation for beet leaves, altered from Maher and Goldman

(2017) which measured geosmin concentration in roots, was similar to the method used for roots. Relative recovery was 28.1%, within range of previously reported recovery rates taken from beet root samples (Maher and Goldman, 2017). The geosmin concentrations for the greenhouse grown beets for each of the four accessions was lower than their tissue culture grown counterparts (Figure 3.3). Interestingly, the cultivar 'Bull's Blood' grown in tissue culture had a substantially higher geosmin concentration with a mean of 61.8 μg kg^{-1} beet tissue as compared to the greenhouse grown 'Bull's Blood' which had a mean of 8.35 μg kg^{-1} beet tissue. The greenhouse grown 'Bull's Blood' was also slightly lower than the tissue culture grown hybrid 'Pacemaker' with a mean of 10.89 μg kg^{-1} beet tissue and inbred 'W364B' with a mean of 9.98 μg kg^{-1} beet tissue. Overall 'Bull's Blood' grown in tissue culture had a much higher mean than any of the other beets grown both in tissue culture and the greenhouse. Geosmin concentrations from plants grown in 2016 were within the range of geosmin concentrations measured in tissue cultured and greenhouse grown plants for these same accessions in 2014 and 2015 (data not shown).

Discussion

The earthy flavor of beet, caused by the aromatic compound geosmin, would be a desirable breeding target due to its defining flavor in the beet taste profile (Bach et al., 2014). However, the degree to which this trait is under genetic control is currently unknown. Three potential explanations for geosmin production in beet have been proposed. First, beets could produce geosmin endogenously. Support for this explanation may be found in the studies by Lu et al (2003a), Freidig and Goldman (2014), and Maher and Goldman (2017). Each of these studies pointed to geosmin levels that were either seemingly independent of microbial associations or repeatable with particular cultivars and environments. In addition, Maher and

Goldman (2017) demonstrated that geosmin concentration responded to bi-directional selection, strongly suggesting a heritable component for this trait.

The second two explanations for geosmin presence in beet pertain to interaction between microbes and beets. Geosmin production could be due in part or in whole to an association between soil microbes and beets over the course of a growing season or, alternatively, the result of endophytic geosmin producing bacteria within the beet root. Disentangling the potential contribution or association by microbial species requires the verification of the sterility of the beets grown in tissue culture. Lu et al. (2003a) grew beet cultivars 'Round Red', 'Chioggia' and 'Detroit Dark Red' in aseptic conditions and successfully measured geosmin from all cultivars. However, a limitation to their study was an assessment for microbial presence beyond a visual assay.

Our negative results from the 16S rRNA sequencing of the bacterial V3-V4 region and the subsequent geosmin detection in beet tissue presents a strong claim for endogenous production of geosmin in beet. The majority of amplicons were classified as chloroplast (grouped in the order *Streptophyta*) or mitochondria (grouped in the family *Rickettsiales*). Streptophyta broadly contains archegoniate and postarchegoniate plants and some algae groups. We attribute the chloroplast and mitochondrial classification to the amplification of beet chloroplast and mitochondrial 16S rRNA. Evolutionarily, chloroplasts are derived from bacteria and thus the homology of bacterial 16S rRNA and plant chloroplast 16S rRNA result in the amplification of the non-bacterial sequences (Hanshew et al., 2013).

After the chloroplast and mitochondria OTUs were filtered from the data set, the remaining OTUs had an average of 0.037% percentage of the total. Sequencing error for Illumina has been reported to be less than 0.1% and this can vary depending on the methods used (Glenn,

2011). Dominant sources of Illumina error come from substitution-like miscalls (Schirmer et al., 2015). In addition, microbial growth in the vials was minute at 0.018% confirmed by visual inspection. The lack of classification for the amplified OTUs beyond chloroplast and mitochondria is an important verification of the aseptic environment in which the beets were grown. An added benefit of sequencing DNA from tissue cultured beet plants was that should microbial detection have occurred; the species identification would have been used to implement an appropriate application of antibiotics into the tissue culture media in an attempt to create an aseptic environment. For this study, it was not necessary as the tissue cultures proved to be a sterile environment.

Other interesting observations from this study include the high level of geosmin in tissue culture grown beets as compared to greenhouse grown beets measured at similar developmental growth stages. Secondary metabolites have been shown to be higher in tissue culture environments in both beet (betalain production) and other plants (berberine, nicotine, etc.) than in field environments (Neelwarne, 2013). In commercial settings, increasing secondary metabolite production will sometimes use elicitors that are biological, chemical or physical applications to the plant growth environment. One such abiotic factor used to increase betalain production in beet was the addition of calcium chloride (Savitha et al., 2006). The type of media can also affect the growth rate and betalain production in beet (Thimmaraju et al., 2003). Media, pH, light regiment, and limited space may have effected geosmin concentration differential found between the bulk greenhouse and tissue culture grown beets. The limiting space constraints of the tissue culture tube and perhaps some yet unknown other environmental factors caused slower than normal developmental growth in the beet and lack of hypocotyl swelling that would be expected after 3 months of growth. To prepare samples of similar tissue developmental

stage for geosmin concentration, beets grown in the greenhouse environment were harvested after one month. Geosmin concentrations found in this study were lower than the Lu et al. (2003a) study, but different growth times and cultivars may have contributed to this difference.

In conclusion, verification of the sterility of the tissue culture environment provides an important clue to understanding geosmin production in beet. We showed that beets grown in an aseptic environment produced geosmin and demonstrated a lack of microbial contamination in beets grown in this environment. This result challenges the long-held presupposition that geosmin production in beet is due to microbial interaction between soil microbes and the root vegetable. Our findings point toward the high likelihood that beets produce geosmin endogenously. Additionally, the determination that table beet produces geosmin offers a new flavor breeding target in beet. This breeding work has recently begun in the University of Wisconsin- Madison's table beet breeding program. Finally, this study lays the groundwork for future genetic studies of geosmin production in table beet. The lack of geosmin detection in other root vegetables raises the interesting question of the functional significance of geosmin production in beet. One possibility for the presence of this trait could be horizontal gene transfer from geosmin producing bacteria. An evaluation of both the family Amaranthaceae and order Caryophyllales, of which the beet belongs, for geosmin production would be an area for further research to determine if and where horizontal gene transfer might have occurred.

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Tables

Table 3.1. Geosmin presence (+) or absence (-) in beet tissue† grown in tissue culture in single 25mm test tubes. Four accessions replicated four times (R1-R4).

Cultivar	**Sample**			
	R1	R2	R3	R4
364B	+	+	+	+
Pacemaker III	+	+	-	+
Touchstone Gold	+	+	+	+
Bull's Blood	+	+	+	+

† Geosmin concentration was measured on beet leaf and hypocotyl tissue. (+) or (-) represents tissue taken from one 25mm test tube that contained 2 multigerm beet seeds.

Table 3.2. Percentage and classification of operational taxa units found in aseptically grown beet accessions

Bacterial Classification	Total %	Cultivar and Replication															
		Bull's Blood				*Pacemaker III*				*Touchstone Gold*				*W364*			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Cyanobacteria																	
Chloroplast	98.0	96.8	98.7	97.1	97.8	98.6	97.9	98.1	97.2	98.6	98.0	97.3	99.0	97.5	98.7	98.1	98.4
Streptophyta																	
Proteobacteria																	
Alphaproteobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Rhizobiales																	
Methylobacteriaceae														2 OTU			
Rickettsiales	2.0	3.1	1.3	2.9	2.2	1.4	2.1	1.9	2.7	1.4	2.0	2.7	1.0	2.4	1.2	1.8	1.6
Mitochondria																	
Betaproteobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Burkholderiales									3 OTU	1 OTU							
Oxalobacteraceae																	
Gammaproteobacteria																	
Pseudomonadales	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pseudomonadacea									2 OTU								
Pseudomonas																	
Xanthomonadales	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Xanthomonadaceae									2 OTU								
Luteimonas																	
Firmicutes																	
Bacilli	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillales															2 OTU		
Streptococcaceae																	
Streptococcus																	
Unassigned	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
		35 OTU	7 OTU	18 OTU	10 OTU	16 OTU	12 OTU	17 OTU	5 OTU	5 OTU	15 OTU	16 OTU	1 OTU	3 OTU	5 OTU	13 OTU	7 OTU

Figures

Figure 3.1. Table beet accession ‘Touchstone Gold’ grown in tissue culture and greenhouse. (A) Example of tissue culture beets in culture. (B) Germination of beet multigerm seed ball in tissue culture. (C) Greenhouse grown beet tissue rinsed for geosmin concentration analysis.

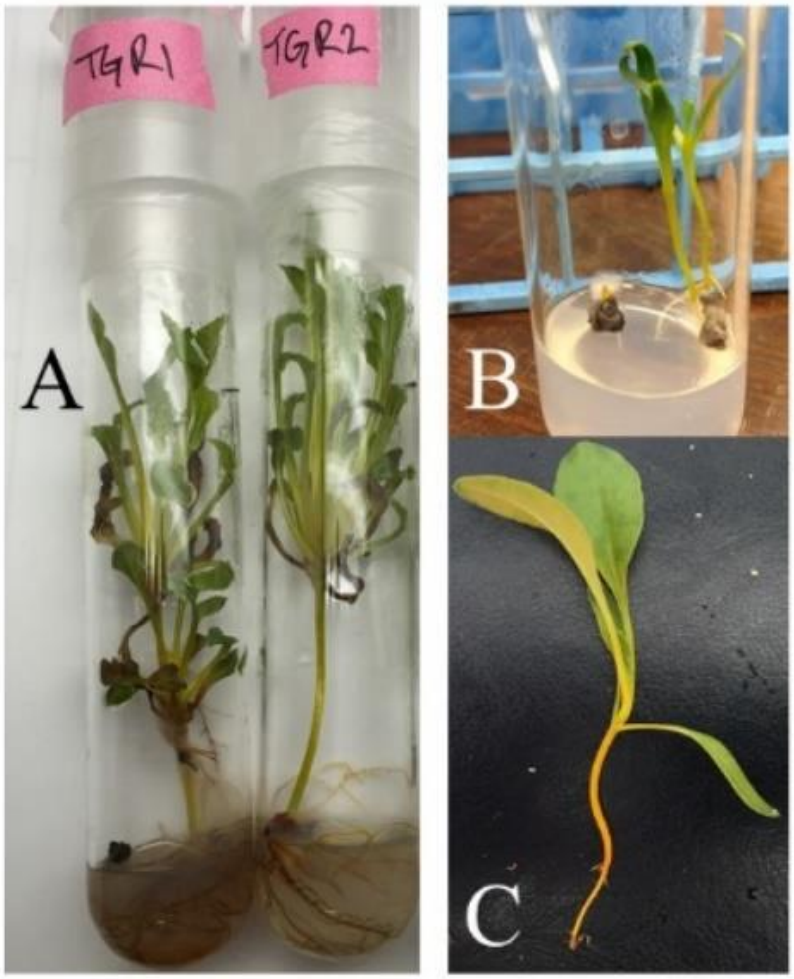
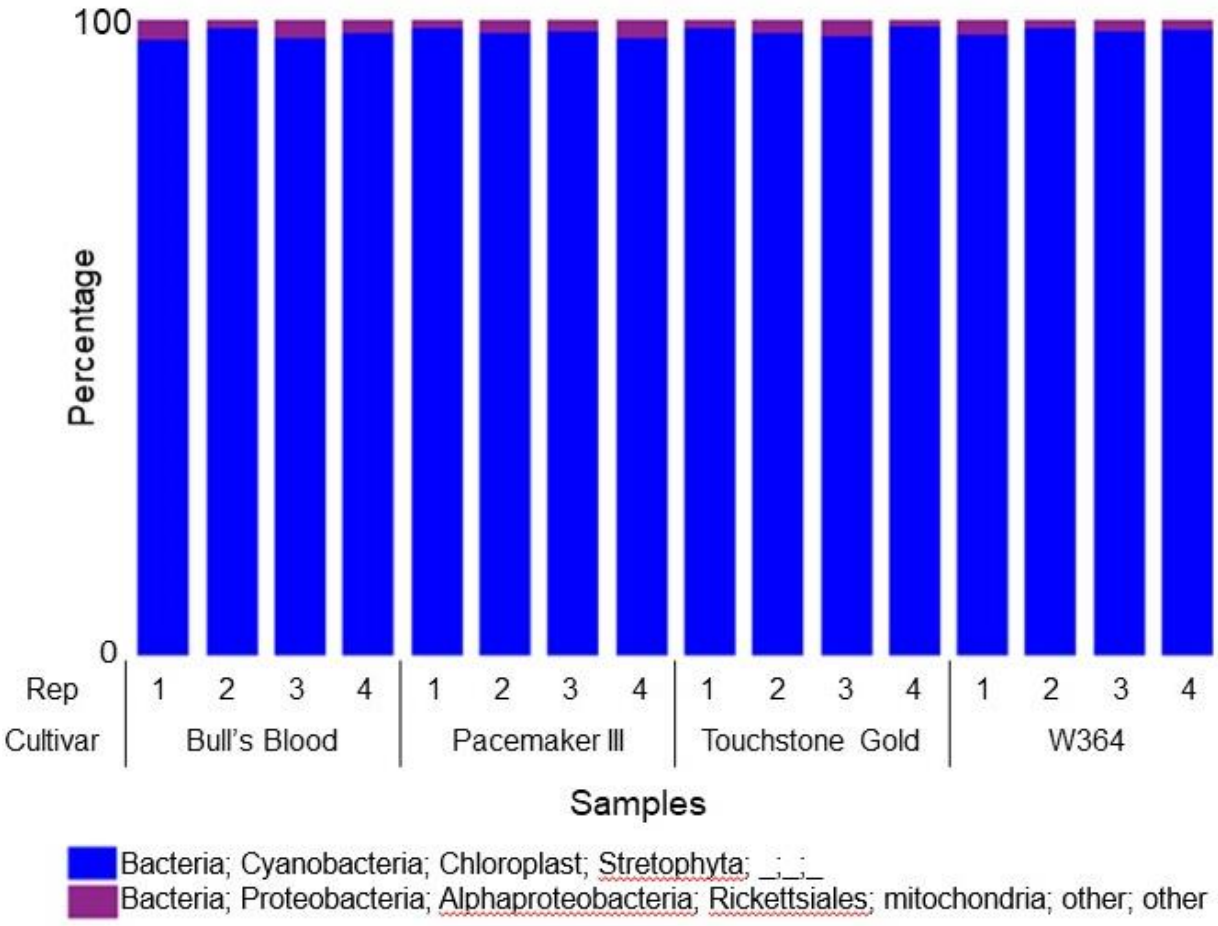
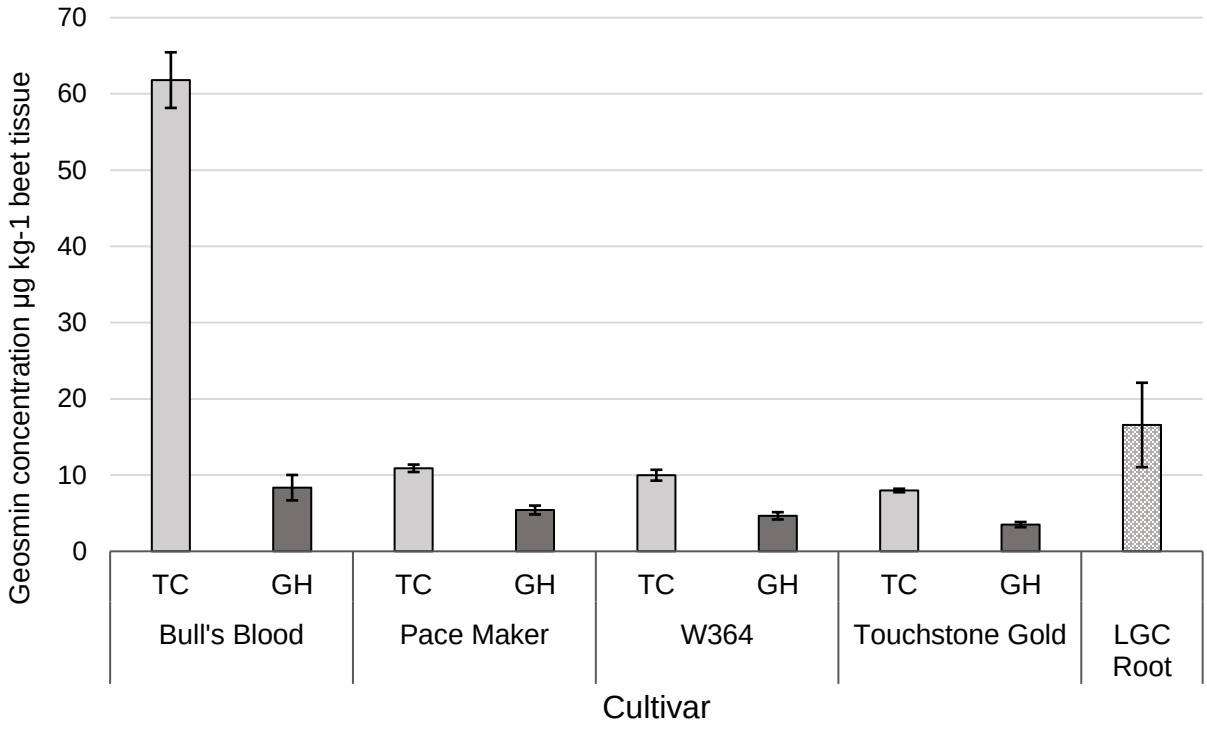


Figure 3.2. Percentage of classified bacteria in 16 aseptically grown beet accessions. Taxonomic classification by kingdom; phylum; order; class; family; genus; species†.



†Amplicons returned from the 16S rRNA analysis were run through the QIIME pipeline, an analysis program for bacterial classification. The classification of chloroplast/Streptophyta and Rickettsiales/mitochondria is an indication that beet plant organelles were amplified. The classification of these plant organelles as bacteria happens due to their close ancestry to bacteria.

Figure 3.3. Geosmin concentration µg kg^{-1} of bulked beet leaf tissue (n=3) of open pollinated (‘Bull’s Blood’ and ‘Touchstone Gold’), hybrid (‘Pacemaker’), and inbred (‘W364’) beets grown in either aseptic tissue culture (TC) or greenhouse conditions (GH). LGC Root is beetroot tissue from a low geosmin beet population and prepared for analysis under the same conditions as beet accession leaf tissue.



Chapter 4: Preliminary search for geosmin synthase in *Beta vulgaris*

Abstract

Recent evidence has indicated that endogenous production of sesquiterpene geosmin by beets. Geosmin synthesis has been studied almost exclusively in microbes, specifically *Streptomyces coelicolor* where the biosynthetic and enzymatic properties are well documented. Geosmin synthase from *S. coelicolor* was used in a PSI-BLAST search of the recently published sugar beet genome resulting in two hypothetical proteins located adjacent to each other on chromosome 8. Interestingly these two proteins, *BVRB8g184050* and *BVRB8g184060*,

were also identified in a previous study searching for prenyltransferases and terpene synthases adjacent to one another in published plant genomes. Other probable sesquiterpene synthases were identified in beet using germacrene D synthase from *Solidago canadensis* as the query. Further experimental evidence is necessary to identify geosmin synthase in beet, but these findings are another indication of endogenous production of geosmin in beet.

Introduction

Geosmin biosynthesis has been described in prokaryotes but has not yet been described in plants (Jiang et al., 2007), yet table beet (*Beta vulgaris* L. *vulgaris*) is responsive to bi-directional selection for geosmin concentration and appears to be capable of endogenous geosmin production (Maher and Goldman, 2017). To date, no biosynthetic pathway for geosmin in *Beta* vuglaris has been identified in table beet. The enzyme responsible for geosmin biosynthesis in prokaryotes is geosmin synthase (Cane and Watt, 2003; Jiang et al., 2006; Jiang et al., 2007). Table beet is a progenitor of sugar beet. The recently published reference sugar beet genome by the Max Planck Institute for Molecular Genetics and their collaborators now provides a database in which to search for geosmin synthase *in planta* (Dohm et al., 2014).

Beet is a diploid with 2n=2x=18 and has a genome size of about 714-758 megabases (Dohm et al., 2014). Dohm et al. (2014) predicted over 27,000 protein coding genes and were able to functionally annotate over 17,000 genes based on sequence homology. Repetitive DNA consisted of 42.3% of the genome and was comprised of a large number of retrotransposons. Large regions of low variability over all chromosomes were identified in four doubled haploid accessions. This suggests mating between a limited number of haplotypes and artificial selection by breeders for specific genes. The table beet is ancestral to the sugar beet but both crops are closely related and were domesticated for different end uses (Ford-Lloyd, 2005). The modern era of table beet breeding has introgressed genes from sugar beet, such as sterile cytoplasm, biennial growth habit, self-fertility, and monogerm seed (Wang and Goldman, 1999). The close genetic relationship between table beet and sugar beet should allow us to relate information regarding the presence or absence of geosmin synthase in sugar beet to that of table beet. Sugar beet contains geosmin at relatively high levels as compared to table beet cultivars and related subspecies and can sometimes remain at notable levels after sucrose production (Marsili et al., 1994; Pihlsgård et al., 2001; Freidig and Goldman, 2014).

The genus *Beta* is placed in the Amaranthaceae family within the Caryophylalles order. Sugar beet is the first Caryophyllales species to be sequenced of 11,510 species contained in the order (Dohm et al., 2014). Interestingly, other crops that have been reported to have an earthy flavor or produce geosmin also are contained in this order including spinach, amaranth (anecdotally), and cactus (Schlumpberger et al., 2004). While geosmin has been documented and measured in plants, an *in planta* mechanism for geosmin synthesis has not been identified. To date, all literature regarding geosmin synthase comes from bacterial and fungal studies, where the primary emphasis has been on water quality and the elimination of geosmin from drinking

water. Geosmin has been isolated from gram positive bacteria and cyanobacteria species, fungi, and liverworts (Jiang et al., 2006). It has also been detected and considered a contaminant in beer, wine and other foodstuffs (Darriet et al., 2000; Paterson et al., 2007). This may be due, however, to the presence of microorganisms on the foodstuffs. For example, geosmin producing fungi *Botrytis cinerea* and *Penicillium expansum* on wine grapes cause an undesirable earthy smell in both grape juice and wines made from grapes infected with these pathogens (La Guerche et al., 2004).

Geosmin is just one compound in the large and diverse family of terpenoids (Figure. 4.1). The largest group of natural plant products are terpenoid compounds produced by plants through terpene synthases. These compounds are used for flavoring, fragrances, spices, and medical purposes (Singh and Sharma, 2015). Terpene enzymes together make up an impressively large family with a diversity of catalytic functions (Degenhardt et al., 2009). Isopentenyl diphosphate (IPP) is a C_5 carbon chain and is the basis for production of C_{15} isoprenoids such as farnesyl diphosphate (FPP) which is used as the substrate in geosmin synthesis (Harris et al., 2015). FPP is an intermediate product found in the later steps of the mevalonate pathway (Miller and Allemann, 2012). Geosmin synthase is classified as a sesquiterpene synthase; a cyclase that uses precursor base carbon skeleton C_{15} as compared to monoterpene synthases using C_{10} precursor or diterpene synthases that use C_{20} precursors (Nieuwenhuizen et al., 2013). It performs an unusual cyclization reaction where a C_{12} bicyclic structure, geosmin, is produced instead of a C_{15} compound. This is unique in that the enzyme mediates this structural change rather than substrate modification before or after enzyme processing (Harris et al., 2015).

Geosmin synthase is a multi-domain terpenoid cyclase comprised of two αα domains (Chen et al., 2016). Plant terpenoid cyclases are often comprised of multiple domains, but

frequently bacterial terpenoid cyclases only consist of one α domain (Harris et al., 2015). Over the years many studies have determined the bifunctionality of the domains in the synthesis of geosmin in *Streptomyces coelicolor* (Harris et al., 2015; Jiang et al., 2007; Jiang et al., 2006). The N and C domains of geosmin synthase have a 36% sequence identity. This high sequence identity suggests a gene duplication event, but over time divergence of the two domains has led to a novel protein (Chen, 2016).

The first catalytic event requires the N terminal domain to cyclize FPP to form an 85:15 mix of germacradienol and germacrene D. Germacradienol is then used as the substrate to form geosmin through a series of chemical reactions by the C terminal domain (Figure. 4.2) (Jiang et al., 2007). Harris et al. (2015) determined the crystal structure of the N-terminal domain and a homology model of the C-terminal domain. They showed that while the active sites were not oriented towards each other, the physical proximity of the active sites of these domains may give some catalytic advantage.

Among sesquiterpene synthases there is low overall amino acid sequence identity from plants, bacteria and fungi (Christianson, 2006). However, there are some common structural entities that define the group and are important features to identify in a sequence search including two αα domains, a specific protein fold and aspartate rich Mg^{2+} binding motifs. Geosmin synthase is categorized by a class I terpenoid cyclase fold (Christianson, 2006). Both N and C domains of geosmin synthase require Mg^{2+} for catalysis and their metal ion-binding motifs are characteristic of class I terpenoid cyclases (Harris et al., 2015). Terpene cyclases are traditionally categorized by two different mechanisms that initiate carbocation cyclization cascades. Class I enzymes catalysis begins by ionization of a diphosphate group initiated by the recruitment of 3 Mg^{2+} ions (Chen, 2016). These motifs are the aspartate-rich DDXXD and the

NSE sequence Mg^{2+} binding motif. In geosmin synthase the DDXXD sequences are D^{86}DHFLE^{91} and D^{455}DYYP^{459} and the NSE binding motifs are N^{229}DLFSYQRE^{237} and N^{598}DVFSQKE^{606} (Miller and Allemann, 2012; Harris et al., 2015).

Magnesium binding sesquiterpene cyclases have been characterized in various species of plants. One of the early x-ray crystal structures of a sesquiterpene synthase came from an epi-aristolochene synthase from *Nicotiana tabacum*, a double helical structure made up of α domains (Christianson, 2006). The metal binding sites of the (+)-δ-cadine synthase from *Gossypium arboretum* and (+)-germacrene D synthase from *Solidago canadensis* show some alterations from the conserved binding motifs, but still bind the required three metal cations characteristic of sesquiterpene synthases (Miller and Allemann, 2012). Substitutions at these active sites are thought to lead to the evolution of new cyclases. A small alteration in the trinuclear magnesium cluster or between the hydrogen bonds that recognize the diphosphate substrate can result in different cyclization products (Christianson 2006).

The diversity of sesquiterpene chemical reactions and protein structures creates a variety of volatile terpenoids. Geosmin synthase is just one of many proteins with a unique function in which it uses a chemical approach to eliminate acetone from an intermediate alcohol and forms geosmin through two functional catalytic domains (Miller and Allemann, 2012). The biochemistry of geosmin synthesis has been documented in *S.coelicolor*, but there is recent evidence to support endogenous production of geosmin in table beet (Lu et al., 2003a; Freidig and Goldman, 2014; Maher and Goldman, 2017). Geosmin has received little study in sugar beet, but the genome has recently been sequenced, providing an opportunity to search for geosmin synthases. This primary purpose of this study was to examine the sugar beet genome for protein

sequences matching that of geosmin synthase from *Streptomyces coelicolor* as well as any known geosmin synthases from eukaryotes.

Methods

The PSI-BLAST search of *Beta vulgaris* included sub-species *maritima*, *adanensis*, *macrocarpa*, and *vulgaris* (sugar beet and cicla group). Hereafter this grouping will be referred to as *Beta vulgaris*. Besides *Beta vulgaris*, various species of the Amaranthaceae family including *Spinacia oleracea*, *Chenopodium quinoa*, and *Amaranthus* L. were queried for geosmin synthase.

All protein sequences were obtained from the National Center for Biotechnology Information (NCBI) database. A general search for geosmin synthase was conducted in the identical protein group database. These genera were mapped to a phylogenetic tree using the Interactive Tree of Life (iTOL) (Letunic and Bork, 2016). Due to the large evolutionary distance between bacterial and plant proteins, geosmin synthase sequence from *Streptomyces coelicolor* A3(2) was used for a general search in the Eukaryotic group to find other potential geosmin synthase candidates to use for queries. The initial search of the Eukaryote group resulted in protein sequences from bryophytes which were then used as search queries in *Beta vulgaris* (Table 4.1). Additionally, these groups were also searched for germacrene D synthase using the protein sequence from model organism *Solidago canidensis*.

Searches were conducted in the non-redundant database using the position-specific iterated (PSI) BLAST algorithm with an e-value threshold of 0.005. This threshold is recommended by the NCBI PSI-BLAST tutorial (Bhagwat and Aravind, 2007). The PSI algorithm first uses the BLASTp program to generate alignments which are later used to calculate a position-specific

score matrix (PSSM). This matrix is used to detect further conserved regions during the subsequent database search. The strength of the iterative profile generation process used by PSI-BLAST is that the matrix uses conservation information from many related sequences enabling detection of more distant sequence similarity. Furthermore, PSI-BLAST has been shown to detect protein relationships by comparison of 3D structures, which can be conserved even after substantial erosion of the protein sequence (Bhagwat and Aravind, 2007).

Results

A search of the NCBI identical protein group database gives over 800 results for geosmin synthase with a majority in bacteria (Figure 4.3). The majority of this group are within the gram-positive phylum of actinobacteria. The largest genus in this group is the *Streptomyces* with almost 600 genera represented. Additional phyla represented are cyanobacteria and gram-negative proteobacteria.

There were 671 hits for geosmin synthase in the Eukaryotic group representing 205 organisms of which 661 hits representing 201 organisms were fungi. The remaining organisms were a moss species (*Sphagnum lescurii*) and 2 species of liverworts (*Marchantia polymorpha* and *Scapania nemorea*). These bryophyte proteins were used to search for similar proteins in *Beta vulgaris*, but no results less than the 0.005 e-value threshold were returned.

The PSI-BLAST of *Beta vulgaris* with geosmin synthase sequence from *Streptomyces coelicolor* A3(2) yielded three results that had a better e-value than the set threshold of 0.005. One of the three results was a sequence described to be a predicted linalool synthase-like protein and two results were hypothetical proteins (Table 4.2). These hypothetical proteins, *BVRB8g184050* and *BVRB8g184060* are located next to each other on chromosome 8 (Figure 4.4a). The predicted linalool synthase gene appears to be spanning the same region as the

hypothetical genes and there is little documentation for annotation in NCBI. No hits were found for geosmin synthase in the Amaranthaceae family (*Spinacia oleracea*, *Chenopodium quinoa*, and *Amaranthus*). The search of the *Beta vulgaris* genome for germacrene D synthase from *Solidago canidensis* resulted in a total of 56 hits with 39 hits having a better than e-value of 0.005. Many of the proteins have predicted terpenoid function including predicted sesquiterpene synthase proteins (Table 4.3).

Discussion

It is likely that beets produce the sesquiterpene volatile geosmin endogenously, however the determination of the genetic foundations are still unknown (Lu et al., 2003a; Freidig and Goldman, 2014; Maher and Goldman, 2017). Searching for geosmin synthase in the recently published sugar beet genome is a first step in determining the origin of geosmin production in beet.

One of the challenges presented when searching for a bacterial gene in plants is the low sequence similarity between microbial proteins (bacterial and fungal) and plant proteins (Cao et al., 2010; Jia et al., 2016). The largest body of literature regarding geosmin production in plants is in beet (Tyler et al., 1978; Lu et al., 2003a; b; Freidig and Goldman, 2014; Maher and Goldman, 2017). Unfortunately, reports of geosmin production in other plants is limited to Cactaceae, amaranth (anecdotally) and plants contaminated with geosmin producing bacteria such as grapes used for wine production (Schlumpberger et al., 2004; La Guerche et al., 2004). Therefore, representative geosmin synthase protein sequences useful for BLAST queries has been limited to microbial sequences. The PSI-BLAST evaluation of the sugar beet genome for geosmin synthase using a geosmin synthase sequence from a representative geosmin producing microbial species resulted in two potential candidates classified as hypothetical proteins located

next to each other on chromosome 8 (Table 4.2). The homology to bacterial geosmin synthase was low (e-values 0.002 and 0.003), however, this is not unexpected due to the large evolutionary distance between bacteria and plants.

As described in the introduction, terpenoid synthases in bacteria, fungi and plants retain key conserved domains. Highly conserved metal binding pockets for magnesium ions are a quintessential characteristic of terpenoid enzymes as well as aspartate rich regions. The two hypothetical proteins found, *BVRB8g184050* and *BVRB8g184060*, have both of these characteristics (Figure 4.4a). Taken alone, those observations are probably too broad to derive meaningful conclusions due to the ubiquitous nature of those features in terpenoid synthases.

Interestingly these genes were also identified in a study by Huang et al. (2017). Their study was interested in the detection of sestererpenoids, C_{25} terpenoids, in fungal and plant species by way of a customized algorithm identifying biosynthetic gene clusters using the program plantiSMASH. PlantiSMASH uses Hidden Markov Models (pHMMs) for enzyme families and clustering of predicted proteins belonging to the same family to predict gene clusters (Kautsar et al., 2017). The purpose of identifying these gene clusters was due to recent studies in plant species such as *Oryza sativa*, *Arabidopsis thaliana* and *Avena strigosa* indicating that plant biosynthetic genes are often clustered together on in localized regions on a chromosome (Boutanaev et al., 2015; Shao et al., 2017). Boutanaev et al. (2015), Wang et al. (2016) and Huang et al. (2017) used this idea of metabolic gene clustering to mine various plant genomes for terpenoid synthases resulting in a wide range of positive terpenoid identification.

Huang et al. (2017) looked specifically for proteins coding for prenyltransferases (PTs) and terpene synthases (TPS) located next to each other. PTs catalyze the formation of the carbon chains used as the backbones for terpenoid synthesis (Christianson, 2017). There is some thought

that the colocalization of PT and TPS proteins could lead to the more efficient production of terpenes (Brodelius et al., 2002). The *Beta vulgaris* genome was one of many plant genomes used for this analysis (Huang et al., 2017). *BVRB8g184050* and *BVRB8g184060* were identified in cluster 22 of the *Beta vulgaris* genome as potential candidates for terpene synthesis, but were discarded as candidates for the study due to the desire to retain PTs and TPSs of closer proximity.

Geosmin synthase is of interest to researchers due to the unique bifunctionality of the N and C domains which is a key feature in in bacterial producers of geosmin. In bacteria the two functional domains are fused together (Harris et al., 2015). It is unclear if two or more domains are present in either of the hypothetical beet genes. The Simple Modular Architecture Research Tool (SMART) generated what appears to be two domains for each of the hypothetical proteins (Figure 4.4b and 4.4c) (Letunic et al., 2015). However, the NCBI conserved domains database identified *BVRB8g184050* as an isoprenoid synthase and *BVRB8g184060* as a terpene synthase. It could be possible that geosmin synthesis in beet is due to two separate proteins performing the cyclization of FPP. It has been suggested that the disassociation of germacradienol from the N-terminal active site to the C-terminal active site does not have a direct channel (Jiang et al., 2007). At this time models of geosmin synthase from *Streptomyces* are unable to determine if germacradienol produced from the N-terminal site is used by the same geosmin synthase molecule (Harris et al., 2015). Therefore, it is possible that geosmin produced by beets could be the result of two separate proteins.

Probable sesquiterpene synthase sequences were discovered when searching for germacrene D synthase, another sesquiterpene synthase, from *Solidago canadensis* (Table 4.3). Minute changes in the residues of enzymes involved in terpenoid synthesis can result in a

plethora of new products (Christianson, 2017). While *BVRB8g184050* and *BVRB8g184060* were identified in the PSI-BLAST search, it is entirely possible that another protein or cluster of proteins are responsible for geosmin synthase. Transformation and expression of *BVRB8g184050* and *BVRB8g184060* into a model plant such as *Nicotiana benthamiana* and testing for geosmin production could be an efficient method to test their viability as geosmin gene candidates in leu of mapping. Mapping populations are being developed with segregating beet populations for geosmin concentration and this should provide another avenue for locating potential geosmin synthase loci.

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Tables

Table 4.1. Species used for PSI-BLAST query in *Beta vulgaris*

Species	***Protein***	***Accession***
Streptomyces coelicolor A3(2)	geosmin synthase	Q9X839.3
Sphagnum lescurii	terpene synthase 2	APB88775.1
Marchantia polymorpha	terpene synthase-like	APP91789.1, APP91788.1, APP91786.1, APP91790.1
Scapania nemorea	terpene synthase 4	APB88774.1
Solidago canidensis	germacrene D synthase	AAR31144.1

Table 4.2. *Beta vulgaris* sequence hits for geosmin synthase from *Streptomyces coelicolor A3(2)*

Sequence description	Max Score	Total Score	Query cover	E-value	Identity	Accession	Amino Acid Number
Predicted: S-linalool synthase-like	42	73.2	58%	0.002	22%	XP_010686682.1	840
hypothetical protein BVRB_8g184060	41.6	72.4	29%	0.002	21%	KMT04331.1	712
hypothetical protein BVRB_8g184050	41.2	72.0	58%	0.003	22%	KMT04330.1	590

Accession Number Q9X839.3 of *Streptomyces coelicolor* was used for the PSI-BLAST search.

Table 4.3. *Beta vulgaris* sequence hits for (+)-germacrene D synthase from *Solidago canadensis*

Sequence Description	Max Score†	Total Score‡	Query Cover§	E-value¶	Identity¥	Accession	Amino Acid Number
PREDICTED: probable sesquiterpene synthase [Beta vulgaris subsp. vulgaris]	397	397	98%	1.00E-132	40%	XP_010694277.1	551
PREDICTED: viridiflorene synthase isoform X1 [Beta vulgaris subsp. vulgaris]	389	389	98%	1.00E-129	38%	XP_010683546.1	552
PREDICTED: probable sesquiterpene synthase [Beta vulgaris subsp. vulgaris]	388	388	98%	4.00E-129	40%	XP_010675322.1	553
PREDICTED: probable sesquiterpene synthase [Beta vulgaris subsp. vulgaris]	380	380	97%	7.00E-126	38%	XP_019106190.1	556
PREDICTED: valencene synthase-like isoform X1 [Beta vulgaris subsp. vulgaris]	323	323	96%	4.00E-104	36%	XP_019105806.1	551
PREDICTED: (-)-germacrene D synthase [Beta vulgaris subsp. vulgaris]	270	270	57%	3.00E-86	44%	XP_010694308.1	325
hypothetical protein BVRB_4g075540 [Beta vulgaris subsp. vulgaris]	251	251	98%	3.00E-76	31%	KMT14789.1	557
PREDICTED: tricyclene synthase TPS4, chloroplastic-like [Beta vulgaris subsp. vulgaris]	251	251	94%	4.00E-76	32%	XP_010666655.1	583
PREDICTED: tricyclene synthase TPS4, chloroplastic-like isoform X1 [Beta vulgaris subsp. vulgaris]	251	251	98%	8.00E-76	31%	XP_010673967.1	594
PREDICTED: tricyclene synthase TPS4, chloroplastic-like isoform X2 [Beta vulgaris subsp. vulgaris]	240	240	87%	9.00E-73	32%	XP_019104433.1	495
hypothetical protein BVRB_003570 [Beta vulgaris subsp. vulgaris]	239	239	83%	2.00E-72	33%	KMS95949.1	496
PREDICTED: probable sesquiterpene synthase [Beta vulgaris subsp. vulgaris]	235	235	60%	2.00E-72	39%	XP_010674144.1	358
hypothetical protein BVRB_8g201930 [Beta vulgaris subsp. vulgaris]	224	224	98%	6.00E-66	29%	KMS96558.1	551
PREDICTED: (E,E)-alpha-farnesene synthase-like [Beta vulgaris subsp. vulgaris]	223	223	98%	9.00E-66	29%	XP_010665803.1	559
PREDICTED: (3S,6E)-nerolidol synthase 1 [Beta vulgaris subsp. vulgaris]	219	219	83%	2.00E-64	31%	XP_010686920.1	541
hypothetical protein BVRB_4g085480 isoform B [Beta vulgaris subsp. vulgaris]	222	393	97%	4.00E-64	37%	KMT13289.1	692
PREDICTED: (3S,6E)-nerolidol synthase 1 [Beta vulgaris subsp. vulgaris]	207	207	83%	9.00E-60	30%	XP_010665800.1	555
PREDICTED: probable sesquiterpene synthase [Beta vulgaris subsp. vulgaris]	188	188	43%	1.00E-55	46%	XP_019104895.1	262
hypothetical protein BVRB_8g186610 [Beta vulgaris subsp. vulgaris]	181	181	82%	2.00E-50	30%	KMT04064.1	515
PREDICTED: (3S,6E)-nerolidol synthase 1-like isoform X1 [Beta vulgaris subsp. vulgaris]	181	181	82%	9.00E-50	30%	XP_010686917.1	581
PREDICTED: probable terpene synthase 6 isoform X2 [Beta vulgaris subsp. vulgaris]	168	315	92%	5.00E-46	34%	XP_010683547.1	479
hypothetical protein BVRB_6g140070 [Beta vulgaris subsp. vulgaris]	160	160	42%	3.00E-45	40%	KMT08736.1	249
PREDICTED: probable sesquiterpene synthase isoform X2 [Beta vulgaris subsp. vulgaris]	164	259	82%	2.00E-44	40%	XP_010681160.1	480
PREDICTED: (3S,6E)-nerolidol synthase 1-like isoform X2 [Beta vulgaris subsp. vulgaris]	165	165	82%	4.00E-44	29%	XP_010686918.1	567
hypothetical protein BVRB_6g140080 [Beta vulgaris subsp. vulgaris]	115	115	45%	7.00E-29	30%	KMT08737.1	259
PREDICTED: ent-kaur-16-ene synthase, chloroplastic isoform X3 [Beta vulgaris subsp. vulgaris]	113	113	86%	2.00E-26	22%	XP_019107535.1	574
PREDICTED: ent-kaur-16-ene synthase, chloroplastic isoform X2 [Beta vulgaris subsp. vulgaris]	114	114	86%	2.00E-26	22%	XP_019107534.1	740
PREDICTED: ent-kaur-16-ene synthase, chloroplastic isoform X1 [Beta vulgaris subsp. vulgaris]	114	114	86%	2.00E-26	22%	XP_010690598.1	799
PREDICTED: ent-copalyl diphosphate synthase, chloroplastic isoform X1 [Beta vulgaris subsp. vulga	95.1	95.1	41%	3.00E-20	28%	XP_019106321.1	819
PREDICTED: ent-copalyl diphosphate synthase, chloroplastic isoform X2 [Beta vulgaris subsp. vulga	95.1	95.1	41%	3.00E-20	28%	XP_010683963.1	818
hypothetical protein BVRB_8g184050 [Beta vulgaris subsp. vulgaris]	94.4	94.4	80%	4.00E-20	24%	KMT04330.1	590
PREDICTED: S-linalool synthase-like [Beta vulgaris subsp. vulgaris]	94.4	94.4	80%	5.00E-20	24%	XP_010686682.1	840
hypothetical protein BVRB_8g184060 [Beta vulgaris subsp. vulgaris]	84.7	84.7	79%	6.00E-17	22%	KMT04331.1	712
PREDICTED: sesquiterpene synthase-like [Beta vulgaris subsp. vulgaris]	75.9	75.9	30%	2.00E-15	29%	XP_010679763.2	229
PREDICTED: (E,E)-geranyllinalool synthase-like [Beta vulgaris subsp. vulgaris]	79.7	79.7	86%	2.00E-15	22%	XP_019106684.1	844
hypothetical protein BVRB_6g129140 [Beta vulgaris subsp. vulgaris]	73.6	131	46%	5.00E-14	31%	KMT09442.1	309
PREDICTED: sesquiterpene synthase [Beta vulgaris subsp. vulgaris]	57.4	89	26%	7.00E-10	37%	XP_010694298.1	131
PREDICTED: ent-kaur-16-ene synthase, chloroplastic isoform X4 [Beta vulgaris subsp. vulgaris]	53.5	53.5	26%	3.00E-07	26%	XP_019107536.1	458
PREDICTED: S-linalool synthase-like [Beta vulgaris subsp. vulgaris]	48.5	48.5	28%	3.00E-06	23%	XP_010686683.1	200

†The alignment score for a set of aligned segments from the same subject sequence ordered by highest alignment
‡The sum of alignment scores of all segments from the same subject sequence.
§Percent of query length included in the aligned segments.
¶Number of alignments expected by chance with a particular score or better.
¥The extent to which two sequences have the same residues at the same positions in an alignment.

All definitions for metrics from the NCBI ("New Database…", 2007)

Figures

Figure 4.1. Geosmin is a degraded sesquiterpene derivative catalyzed by the enzyme geosmin synthase.

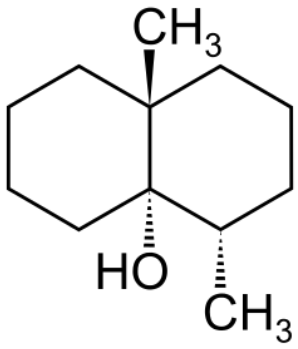


Figure 4.2. Geosmin synthase catalyzes cyclization of FPP to geosmin in prokaryotes. The N-terminal catalyzes FPP cyclization (green arrows) followed by the C-terminal catalyzing the reaction of germacradienol into the final product of geosmin (blue arrows).

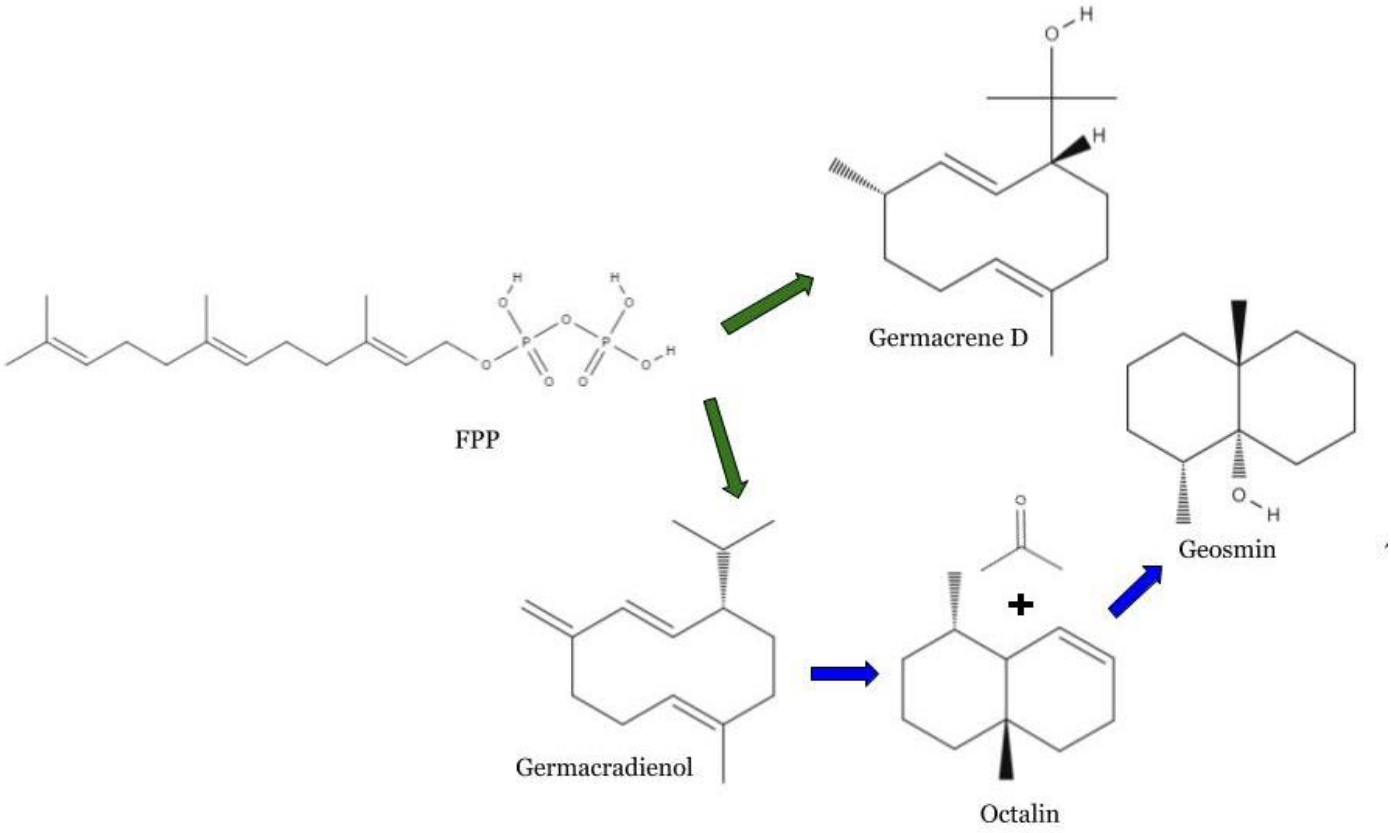


Figure 4.3. Number of species represented in each genera from NCBI protein database for search term ‘geosmin synthase’. Figure generated with Interactive Tree of Life (iTOL) version 3.

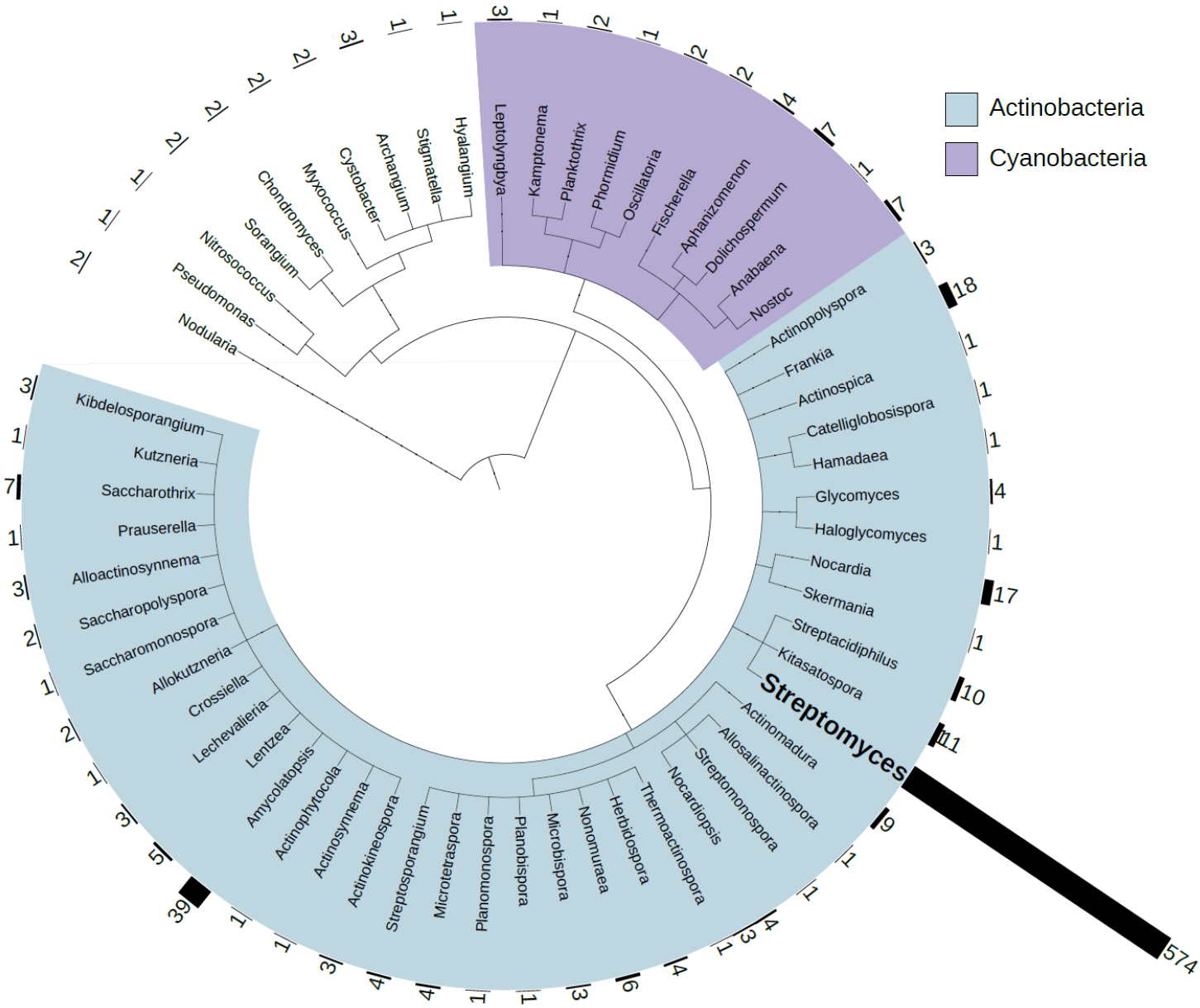
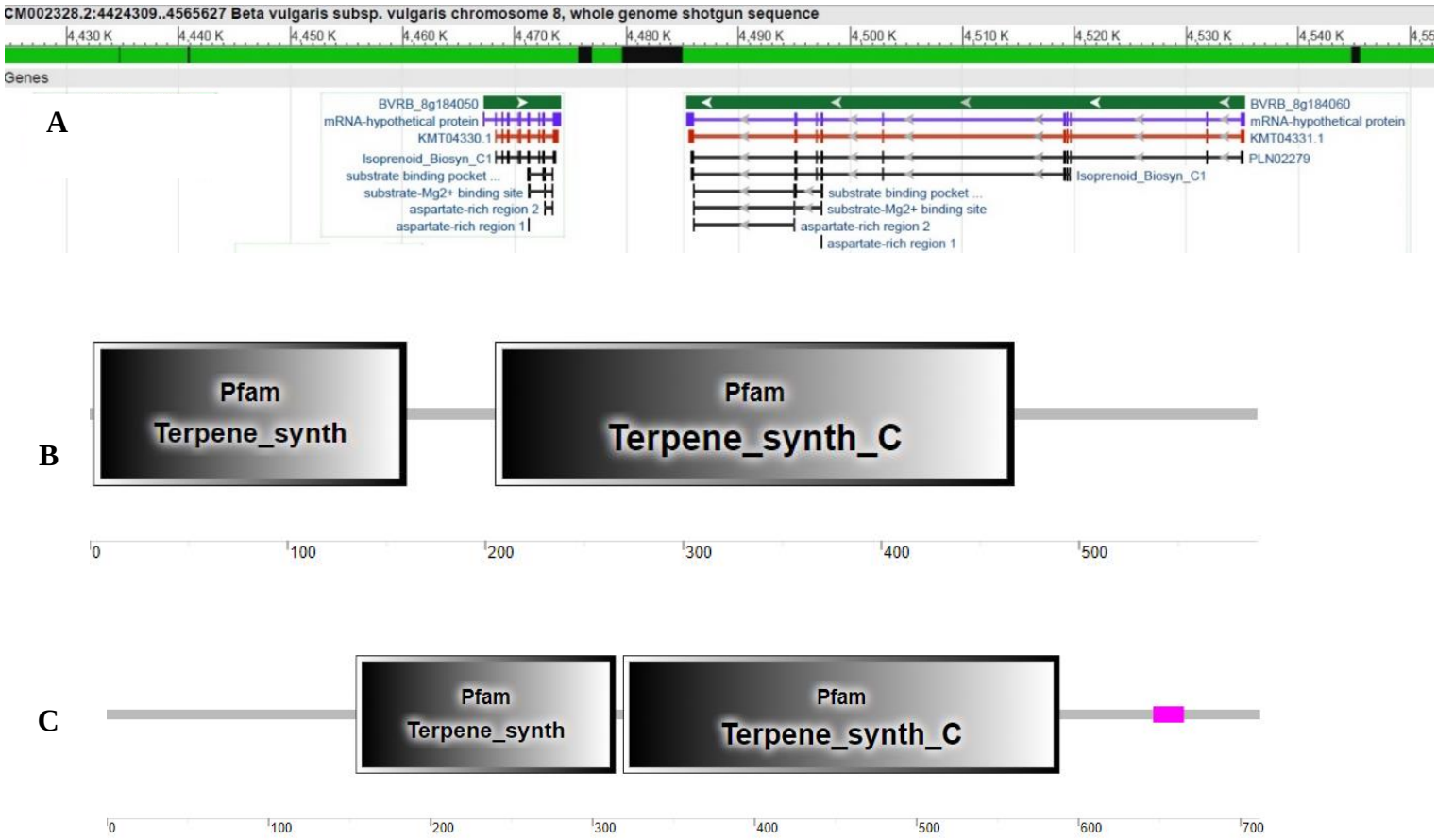


Figure 4.4. Hypothetical genes *BVRB8g184050* and *BVRB8g184060* (a) located next to each other on chromosome 8 with predicted functional annotation for Mg^{2+} binding sites and aspartate rich regions. (b) *BVRB8g184050* and (c) *BVRB8g184060* predicted domains using Simple Modular Architecture Research Tool (SMART).



Future Directions

At the outset of this work there was very limited knowledge regarding geosmin and its association with beet. A few studies indicated that geosmin might be endogenously produced, but there were limitations to their conclusions (Lu et al., 2003a; Freidig and Goldman, 2014). The successful bidirectional selection of geosmin concentration in beet combined with the verification of geosmin production by beets grown in an aseptic environment presented in this thesis shows strong evidence that geosmin is endogenously produced by beets.

While the bidirectional half-sib recurrent selection study was an effective proof of concept, the resulting populations lack other agronomic qualities desired in a finished variety. Selection within the high geosmin concentration (HGC) and low geosmin concentration (LGC) populations to regain agronomic traits such as uniform interior color, smaller crown diameter and overall palatability will be necessary should release of these populations be feasible. Selection for uniform color groups within the HGC and LGC populations began in December 2016. The LGC population was sub-divided into groups based on interior pigmentation of yellow, red and white. Similarly, the HGC population was sub-divided into interior pigmentation groups of red, coral and white. The red LGC population could be a particularly interesting population for the market. Currently the yellow cultivars available have relatively low geosmin concentrations compared to the red cultivars (Freidig and Goldman, 2014). A high geosmin red cultivar would be a unique addition to the market. Further agronomic selections were made in August 2017 where increased selection pressure for round root shape, small crowns and smooth exteriors was applied, especially on the yellow LGC and red HGC populations.

In regard to taste perception, there is a still much unknown on consumer preference to geosmin and how that might affect selection goals. The taste test conducted with the HGC and

LGC populations was inconclusive and further taste tests, perhaps with a trained panel might better differentiate between the populations. Other taste qualities may also play a role in consumer differentiation including soluble solids and saponin content as well as mouth feel characteristics such as oxalic acid.

The GC-MS methodology used for measuring geosmin concentration in beet used in this thesis is resource intensive in cost of consumables and time. Flavor phenotyping is an expensive endeavor and is often cost prohibitive to many breeding programs (Tieman et al., 2017; Folta and Klee, 2016). The method described in chapters 2 and 3 was used for consistency of measurements from year to year, but variations to the type of consumables and adjustment to parameters might be advised in the future. Geosmin contamination in drinking water has been of concern for many municipalities. The body of literature regarding the detection and mitigation of geosmin in drinking supplies may be of some use in discovering a more cost and time efficient method of measuring geosmin in beet.

This thesis identified potential geosmin synthase genes through a BLAST search using geosmin synthase from *Streptomyces coelicolor.* Much research could be done regarding the identification of geosmin synthase(s) responsible for geosmin production in beet. The LGC and HGC populations provide the genetic resources for future mapping populations. The hypothetical proteins identified in chapter 4 could also be transformed into a model plant, such as *Nicotiana benthamiana*, to see if geosmin production occurs.

Many intriguing questions are raised with the conclusion that geosmin is produced endogenously by beet. Is beet the only member of the Amaranthaceae family to produce geosmin? Anecdotally there have been reports of certain accessions of amaranth that produce an earthy aroma. If beet is the only member of its family to produce geosmin then evolutionary

questions as to how and when geosmin synthase was attained in beet would be fascinating to know. One possibility for geosmin in beets may have been horizontal gene transfer. It could also be possible that geosmin production in beet was a chance occurrence or as a byproduct of production of another essential terpenoid. Minute changes to terpene synthases can lead to a multitude of different terpenoid products and some terpenoid synthases are prolific in synthesizing multiple terpenoid compounds (Schifrin et al., 2016). Historically we have often referred to geosmin as microbial phenomenon rather than a beet phenomenon. Perhaps a reversal of this narrative could be influenced by determining the origin of geosmin synthase.

As to the function of geosmin in beet, there are many more questions than answers and a selective advantage for geosmin presence in beet is unclear. In bacteria the function of geosmin is also unknown. In *Streptomyces*, Jones and Elliot (2017) found that the volatile trimethylamine was used for communication between bacteria to promote exploration growth. Perhaps geosmin is another volatile signal used for communication either for other bacteria or another organism. For example, it has been hypothesized that camels can detect geosmin produced by bacteria in water sources in the desert, perhaps with the selective advantage of furthering bacteria distribution (Simmons, 2003). It has also been reported that cactus flowers produce dehydrogeosmin as a floral volatile and is hypothesized to be a pollinator attractant (Schlumpberger et al., 2004). To our knowledge, geosmin has not been documented as a floral volatile in beet. As beets are wind pollinated, pollinator attractants would be unnecessary and likely selected against.

As stated previously, breeding for volatiles is an expensive and time consuming endeavor, but could be an exciting new area for breeders. There has been a small but growing trend in utilizing chefs and consumers early in the breeding process to select for flavor in

vegetables (Beans, 2017). In tomato, commercial varieties have lost many volatile producing genes through selection and are much less flavorful than heirloom varieties that retain these genes (Tieman et al., 2017). Perhaps geosmin concentration was originally much higher in beet, but selection pressure has slowly reduced these levels. It has been suggested that breeding for increased volatiles could be achieved with minimal metabolic penalty due to the low concentration of volatiles needed to make a perceptible impact (Tieman et al., 2017). While this statement may be true, our observations of the LGC and HGC populations showed a high attrition rate in the HGC population. The genotype by environment interaction for geosmin concentration in beet is largely unknown and could influence the direction of new studies.

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Appendix 1.

Table A.1.1 Low geosmin concentration population (LGC) yellow selection August 2017 family phenotypic data. Families selected for yellow interior color, small top size, round root shape and smooth exterior.

	Leaf[†]				Petiole[‡]		
Family[§]	Color	Vein	Length(cm)	Width(cm)	Color	Length(cm)	Width(cm)
1	1,2	2	22.33	11.33	2	18.67	0.97
2	1,2	2	23	11.5	2	20.3	0.97
3	1,2	2	18.33	10.33	2	21	0.86
4	1,2	2	21.67	10.67	2	22.33	0.9
5	1,2	2	24.67	11.67	2	21	1.1
6	1,2	2	21.67	12	2	22.67	1.17
7	1,2	2	19.33	12.17	2	17.33	0.77
8	1,2	2	20	11	2	22.67	0.87
9	1,2	2	23	11.33	2	23.33	0.97
10	1,2	2	22.67	12.167	2	26	0.97
11	1,2	2	20	12	2	21.3	0.9
12	1,2	2	21	12.167	2	17.5	0.9
13	1,2	2	16.67	11.67	2	24	1.17
14	1,2	2	21.33	14.33	2	24.3	0.97
15	1,2	2	20.67	12.33	2	21.17	0.87
16	1,2	2	20.67	12	2	16.67	1
17	1,2	2	22	11	2	21	1.1
18	1,2	2	21	8	2	20	0.9
19	1,2	2	25	12.33	2	18	0.97
20	1,2	2	21.67	10.33	2	19.33	0.93
mean			21.33	11.52		20.93	0.96

[†] Color specified by U.S. Department of Agriculture Plant Variety Protection submission form.

Leaf: 1=Light green, 2=Green, 3=Purplish Green, 4=Crimson, 5=Other

Vein: 1= White, 2= Yellow, 3=Green, 4=Orange, 5=Red, 6=Other

[‡] Color specified by U.S. Department of Agriculture Plant Variety Protection submission form.

Petiole: 1= White, 2=Yellow, 3=Green, 4=Orange, 5=Red, 6= Crimson

[§]n=3 for each family

Table A.1.2 High geosmin concentration population (HGC) red selection August 2017 family phenotypic data. Families selected for a solid red interior color, small top size, round root shape and smooth exterior.

	Leaf[†]				Petiole[‡]		
Family[§]	Color	Vein	Length(cm)	Width(cm)	Color	Length(cm)	Width(cm)
1	2, 3, 4	5	26.6	15.67	4, 5	27.67	1.6
2	3	5	21.3	15.3	4,5	24.3	1.1
3	3	5	26	14.43	5,7	32	1.43
4	2,3	5	19.53	14	4,5	31.6	1.37
5	3,4	5	21.6	16.3	4,5	33	1.5
6	2,3	5	20.1	14.6	1,4,5	28	1.5
7	3	5	19.5	na	4,7	33	1.3
8	2,3	5	23.3	13.1	4,5	28.6	1.1
9	2,4	5	na	15.3	4,5	na	na
10	2,3	5	20.67	13.67	4,5	29.67	1.27
11	3,4	5	24	16.3	4,5	18	1.34
12	2,3	5	23	14.78	4,5	28.67	1.4
13	2,3	5	23.67	12.89	4,5	23.67	1.13
14	3	5	24	17	4,5	23.67	1.4
15	2,3,4	5	22.67	13.33	4,5	28.67	1.6
16	2,3	5	25.3	18.3	4,5	na	na
17	3	5	19.4	14.6	4,5	29.3	1.56
18	2,3	5	24	15	4,5	22.33	1.16
19	3,4	5	25	13.67	4,5	26.33	1.13
20	2,3	5	24	19	4,5	28.67	1.23
mean			22.82	15.18		27.62	1.34

[†] Color specified by U.S. Department of Agriculture Plant Variety Protection submission form.

Leaf: 1=Light green, 2=Green, 3=Purplish Green, 4=Crimson, 5=Other

Vein: 1= White, 2= Yellow, 3=Green, 4=Orange, 5=Red, 6=Other

[‡] Color specified by U.S. Department of Agriculture Plant Variety Protection submission form.

Petiole: 1= White, 2=Yellow, 3=Green, 4=Orange, 5=Red, 6= Crimson

[§]n=3 for each family where na is missing data.

Figure A.1.1 Low geosmin concentration (LGC) yellow families (top) and high geosmin concetration (HGC) red families (bottom) before selection for solid interior color, small top size, smooth exterior and round root shape in August 2017.