DIPLOMA THESIS

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2023

HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES INSTITUTE OF GENETICS AND BIOTECHNOLOGY BUDAPEST

Molecular Marker Based Comparison of Drought Tolerant and Sensitive Turkish Sunflower (*Helianthus annus* L.) Cultivars

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2023, Budapest

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1. INTRODUCTION

Helianthus annus L., known as sunflower, is belong to *Asteraceae* family. *Asteraceae* family, called as *Compositae*, is one of the biggest angiosperm plant families in between dicotyledons. In the world, 10% of all flowering plants have been constituted by the *Asteraceae* family which has 1620 genera and 23600 species (Funk et al. 2005; Funk et al. 2009). This family, which has 12 subfamilies, grows mostly in subtropical and temperate climates, especially in meadows, valleys, grassy plains, rolling plateaus, and mountain slopes (Funk et al. 2005; Bayer 2007).

The cultivation of sunflowers can be traced back to 4625 B.C., as evidenced by archaeological findings that suggest the American Indians were the first to engage in this practice (Crites,1993). After the discovery of America, the sunflower was brought to the botanical garden of Madrid by Spanish explorers (Putt, 1941). The history of sunflower as an oil crop dates back to 1716, when a patent for extracting oil from sunflower seeds was registered in England. However, the use of sunflowers for oil production was already widespread in Russia by 1697, thanks to Tsar Peter the Great's introduction of the crop due to its beauty. Sunflower cultivation as a field crop and for oil production truly began with the discovery of a method to extract oil from sunflower seeds by D.S. Bokarev from the Belgorod area in 1829 (Pustovoit, 1990). Scientific studies on sunflower breeding commenced in 1912 with the establishment of the Kruglik Plant Breeding and Experimental Station (Škorić, 1988).

Several sunflower breeding centres were established by the previous Soviet Union in 1932, with the first centre located in Krasnodar (VNIIMK), followed by centres in Rostov-on-Don, Kharkiv, and Odesa. These centres played a pivotal role in the development of high-yield and high-oil sunflower genotypes such as Peredovik, VNIIMK 8931, and Smena, which made significant contributions to the global expansion of sunflower as an oil crop and prompted advancements in sunflower production worldwide. North America's first sunflower breeding program started in Saskatchewan, Canada, in 1937. Meanwhile, in 1950, the United States established its sunflower breeding program at the Texas Experimental Station (Škorić, 2012).

Sunflower breeding has undergone significant advancements throughout history, with three key phases that include mass selection, individual selection for variety development, and hybrid development methods. The mass selection technique was a significant catalyst in the widespread cultivation of sunflowers, leading to the creation of numerous local varieties that were extensively grown in gardens by the late 1800s (Jocić et al., 2015). At the end of the 19th century and the beginning of the 20th century, mass selection techniques were instrumental in developing sunflower cultivars that demonstrated resistance to two major threats to sunflower production: the sunflower moth (*Homoeosoma nebulella* Denis and Schiffermüller) and broomrape (*Orobanche cumana* Wallr.). These pests posed significant risks to sunflower crops, and the creation of resistant cultivars marked a major achievement in sunflower breeding (Marinković et al., 2003). The mass selection method is widely used in sunflower breeding due to its simplicity and cost-effectiveness. However, its success depends on various factors such as the gene effects on the selected trait, trait heritability, genotype-environment interaction, and sample size. It is particularly useful for traits that are controlled by additive genes and have high heritability.

Although mass selection did not lead to an increase in sunflower yield, it did help to develop sunflower varieties that were resistant to diseases and insects and had higher oil content and earlier maturation (Morozov 1947, Vranceanu 1974). Sunflower breeding has benefited greatly from the individual selection method, which has been the most widely used and successful approach for variety development. V.S. Pustovoit introduced individual selection with seed reserve preservation to sunflower breeding in the early 1920s, and this method is often referred to as Pustovoit's method of reserves. This method has proven effective in improving various traits such as yield, oil content, and resistance to pests and diseases. The success of individual selection depends on factors such as trait heritability, genetic variability, and selection intensity. It is a more effective method for traits controlled by additive genes and with high heritability. With this method, individual plants with desired traits are selected from a population and used to create new varieties, preserving a reserve of seeds for future selection and breeding (Pustovoit, 1967). This breeding method is based on carefully selecting the best individual plants from a population to create a new variety. The selected plants are harvested one by one, and their seeds are divided into two portions: one for sowing and one as reserve. For this method, the original population is made up of super-elite plants from the best varieties, inter-varietal hybrids, and the most promising offspring from previous selection cycles (Jocić et al., 2015). The primary objective of using the method of hybridization in sunflower breeding is to achieve higher yields by exploiting the phenomenon of heterosis or F1 vigor. Heterosis is mainly the result of intra-allelic interactions such as domination and super domination, and to a lesser extent, inter-allelic interactions like epistasis from a genetic standpoint. By exploiting this phenomenon, breeders can develop sunflower hybrids that are more productive and resistant to various environmental stresses (Jocić et al., 2015). Sunflower breeding has been focused on utilizing heterosis to achieve higher yields. The first experiments in this field were carried out in the 1940s, resulting in a remarkable 60% increase in yield compared to traditional varieties (Morozov 1947, Unrau and White 1944).

Abiotic stresses have a significant impact on the cultivation and distribution of crops, and determine whether certain areas can be used for breeding. It is estimated that only 24.2% of the world's geographic area is suitable for cultivation. Out of this area, only 10.6% is currently being used for cultivation, while the remaining land is not suitable for growth due to one or more abiotic stresses. Among these stresses, drought is the most critical factor, affecting 26% of the arable land (Singh, 2006). Mineral toxicities and deficiencies rank second in terms of their impact, followed by freeze stress. Drought, on the other hand, is the most severe abiotic stress factor, affecting more than a third of all soils across the globe. Plants that are able to survive the effects of drought stress often exhibit reduced fertility, lower yield, and diminished crop quality (Monti, 1987).

The first investigation into the use of molecular markers to study sunflower's resistance to abiotic stress dates back to 1996 (Belhassen et al., 1996; Cellier et al., 1996). Arce and colleagues (2012) utilized various molecular biology techniques to investigate uncommon transcription factors and miRNAs in sunflowers that play a significant role in responding to abiotic stresses. These strategies included genomic DNA library screening, northern blots, western blots, qRT-PCR, and confocal microscopy, among others. Their findings revealed that transcription factors have the capability to recognize and bind specific DNA sequences in the regulatory regions

of target genes. A study was conducted by researchers to investigate the correlation between a set of molecular markers (AFLP and SSR) and leaf expansion parameters under water-deficit conditions. The study was performed on a cross of two public sunflower lines with contrasting response, their F2 and F2:3 progenies, and an independent F8 recombinant inbred line (RIL) population. The outcome of this study suggested that the findings could be useful in the improvement of molecular markers for assisted selection in breeding programs aiming to create new cultivars with enhanced adaptation to water stress conditions (Alberdi et al., 2012).

Sunflowers are subjected to various biotic stresses, among which fungi are the most severe stress factor. Following fungi, parasitic angiosperm Broomrape is the second most significant concern for sunflowers. Additionally, viruses and bacteria are also recognized as significant stress factors for sunflowers (Škorić, 2012).

The breeding of sunflowers involves the use of a diverse range of cultivated and wild germplasm, and molecular markers have become increasingly important in this process. By identifying markers linked to specific traits, breeders can select plants that have those desired traits. However, the efficacy of these markers must be validated in various genetic backgrounds and environmental conditions to ensure their widespread applicability (Rauf et al., 2020; Yadav et al., 2018). Sunflower genomic sequence information is now readily available, providing opportunities to develop new SNP-based markers that can be linked to economically important traits. These markers can be used to identify allelic variations associated with desired characteristics, such as seed oil content and disease resistance genes. By incorporating genes of interest into these new markers, breeders can more effectively select for desired traits in sunflower varieties (Rauf et al., 2020; Lyu et al., 2020). Sunflower-breeding programs have long aimed for the development of early maturing and high-yielding hybrids, which have traditionally been time-consuming and costly to produce. The selection of parents that have the potential to produce high-yielding hybrids, as well as their purity testing, have been considered as limiting factors in heterosis breeding. However, the application of molecular techniques has led to significant improvements in this process. Genotyping technologies can now be utilized to protect elite germplasm and enhance breeding programs (Duca et al., 2013). The molecular marker has greatly enhanced the sunflower breeding program. These markers are characterized by high polymorphism, co-dominance, strong correlation with the trait of interest, measurability at all growth stages, and phenotypic neutrality (Dimitrijević A et al., 2017). There have been numerous studies conducted in sunflowers that demonstrate the importance of marker-assisted selection (MAS), genetic diversity prediction, identification of inbred lines for heterosis breeding, and determination of heterotic patterns (Iqbal et al., 2010). Mapping of Quantitative Trait Loci (QTL) associated with economically important traits has led to the discovery of molecular markers that could be applied for marker-assisted selection (MAS) (Iqbal et al., 2010; Ahmed et al., 2021).

Genetic variation and species identification have been studied using molecular markers, including the start codon targeted (SCoT) marker, which targets regions around the ATG start codon that are conserved across plant genes (Collard and Mackill, 2009). The SCoT marker has already been utilized in analyzing genetic variation in various plant species, including both cultivated and wild populations of ramie (*Boehmeria nivea* L. Gaudich.) (Satya et al. 2015), Chinese *Elymus sibiricus* accession (Zhang et al. 2015), durum wheat (Etminan

et al. 2016), two different populations of bottle gourd (Bhawna et al. 2017), *Bletilla striata* (Guo et al. 2018), rose (Agarwal et al. 2019) and the Tunisian *Phoenix dactylifera* population (Soumaya et al. 2020).

2. OBJECTIVES

Until now there is not any published information about characterization of sunflower genotypes by SCoT marker. The first objective of this research is to test to SCoT polymorphism of sunflower genotypes. The second objective is to compare drought tolerant and sensitive Turkish cultivars using the SCoT marker system to find a linked DNA region that could be responsible for the tolerance.

3. LITERATURE REWIEV

3.1. SUNFLOWER (*Helianthus annus* L.)

The sunflower's Latin scientific name, *Helianthus annuus* L., originates from the Greek words "helios," meaning sun, and "anthus," meaning flower. The sunflower is a member of the *Asteraceae* family, also known as the daisy family, and is one of 67 species of plants in the *Helianthus* genus (Berglund 2007; Hu et al. 2010). The *Asteraceae* family is the largest family among dicots, with around 25,000 species that include plants of economic importance for human nutrition, bioenergy production, and floriculture. Among the daisy family, *Helianthus annuus*, the cultivated sunflower, holds a significant position as an oil plant. The genus has been in existence for a considerable period, estimated to be from 4.75 to 22.7 million years ago, based on the analysis of chloroplast DNA (Schilling 1997). The divergence of species within this genus occurred relatively recently, between 1.7 and 8.2 million years ago (Schilling 1997).

Based on morphological, geographical, molecular, and archaeological evidence, it has been suggested that sunflowers were utilized by Indigenous peoples in North America for various purposes, such as food, medicine, and dye. According to this evidence, the cultivation of sunflowers occurred approximately 4,000 to 5,000 years ago in a region that spans from Mesoamerica through the United States and into southern Canada (Warburton et al. 2017; Sala et al. 2012). The sunflower was brought to Europe by Spanish explorers to the botanical garden in Madrid, after the discovery of America in 1510. The first recorded mention of the sunflower was made by Belgian Rembert Dodoens, a renowned botanist, in 1568. Although it was introduced as an ornamental plant, the first evidence of its industrial use was found in England in 1716, in a patent application for seed oil extraction. However, sunflower was first used for oil production in Russia in 1697 (Jocic et al. 2015).

Sunflower plants can grow up to 3 meters in height, with an inflorescence that can measure up to 30 cm in diameter. The inflorescence is a compressed raceme consisting of multiple sessile florets that all share the same receptacle, also known as the capitulum. The outside of the inflorescence is adorned with bright yellow ray florets, while the inside is filled with yellowish disc florets. Sunflowers exhibit a characteristic behaviour of turning their heads towards the sun during maturation, which is known as heliotropism. This movement ceases once the flowers begin to bloom (Harshavardan and Amendeep, 2021). The sunflower head is composed of

multiple circles of florets. The outermost circle consists of five petals on each flower, which are typically golden yellow and sterile. The disc florets, which cover the large disc in the centre of the head, are composed of individual flowers that contain both pistils and stamens. The fibres in the disc are more common in the outer ring than the inner ring (Hu et al. 2010). The sunflower's shape is similar to a plate with both concave and convex parts that slope towards the ground. The diameter of the sunflower head, or cup, typically falls within the range of 18 to 25 cm, but it can vary greatly between 5 and 50 cm across all genotypes. Furthermore, sunflowers have impressive yields, and head diameter is a parameter that can be greatly influenced by environmental factors, much like plant height (Kaya et al. 2012). The sunflower head is not a single flower, but instead comprises 1,000 to 2,000 individual flowers that are attached to the receptacle base. Sunflowers are self-fertile, relying on their own pollen for fertilization, but they also benefit from pollinators such as honey bees and other insects (Debaeke et al. 2017).

Sunflower is a significant industrial plant owing to its relatively short growth period (Sarazin et al. 2017), and it is also a model plant for comprehending sun tracking and flower development in plant science (Badouin et al. 2017).

Sunflower, a crop that originated in temperate North America, is the second most important oilseed crop worldwide, following soybean (Harshavardan and Amendeep, 2021). Native Americans were the first to domesticate sunflowers, using them for food, medicine, and as body paint in their rituals. Archaeological records indicate that Indians were cultivating sunflowers as early as 2300 BC, predating the domestication of maize, beans, and squash (Kaya 2012). Sunflower is among the four primary plants utilized for producing edible vegetable oil, along with canola, cotton, and soybean (Hu et al. 2010).

The growth and development of sunflowers are influenced by climatic conditions. Despite being native to North America, sunflowers can thrive in various regions globally due to their adaptability. Optimal growth of sunflowers occurs at warm temperatures of around 20-25°C during the day and 15-18°C at night. Inadequate sunlight may result in stunted growth and underdeveloped florets. To achieve optimal growth, sunflowers require sufficient water, particularly during germination and flowering stages. Sunflowers thrive best in nutrient-rich, well-drained soil, with a pH level between 6.0 and 7.5. Poor soil quality may lead to reduced growth and smaller buds (Karkanis et al. 2011; Laza et al. 2014). The root system of sunflowers is distinctive, as it extends both deeply and widely, allowing for efficient water and nutrient uptake. Sunflower roots grow faster than the leaves, and under favourable conditions, they can reach depths of more than 3 meters to access groundwater. The structure of the root system plays a significant role in the absorption of water and nutrients (Alberio et al. 2015). The blooming period for cultivated sunflowers usually lasts for 60-70 days, and they reach physiological maturity in 80-100 days. However, the total growth period can vary between 125-130 days depending on the genetic makeup of the sunflower and environmental factors (Schneiter et al. 1981).

The United States Department of Agriculture (USDA) predicts that the worldwide production of sunflower oil will reach 20.1 million tons in 2021, representing a 2.8% increase from the previous year. Ukraine,

Russia, and the European Union are expected to be the leading producers of sunflower oil in 2021, accounting for over 70% of global production. Other significant producers include Argentina, Turkey, and India (USDA 2021). The United States Department of Agriculture (USDA) predicts that global sunflower seed production will reach 52.2 million tons in 2022, a slight increase from the 51.8 million tons produced in 2021 by Ukraine, Russia, and Argentina, with expected productions of 16.5 million tons, 12 million tons, and 3.9 million tons, respectively. Turkey, France, China, Romania, Bulgaria, and Hungary are other significant sunflower seed producing countries in 2022 (USDA 2022).

The sunflower plant possesses a significant amount of high-quality unsaturated fatty acids. With its high yield potential, it is able to adapt to various environmental conditions despite being a highly cross-pollinated crop (Hilli 2021).

The seeds of the sunflower consist of both kernels and husks, and their oil content is 44% greater than that of canola and soybeans. Sunflower seeds contain 18% protein, 15% cellulose, 9% water, 14% minerals, and carbohydrates (Andrianasolo et al. 2016). The percentage of oil in sunflowers can vary depending on factors such as the variety of sunflower, the conditions in which it was grown, and the timing of the harvest. Generally, the weight of oil in sunflower seeds is between 35% and 50%. Scientists have reported the oil content of sunflowers ranging from 36.9% to 50.2% (Gómez et al. 2002).

Sunflower oil quality is determined by its fatty acid composition and the levels of tocopherols, sterols, carotenoids, and other compounds. Linoleic acid makes up 55-65% of typical sunflower oil, with oleic acid comprising 20-30%. The remaining 5-10% consists of palmitic and stearic acids. Sunflower oil is a rich source of polyunsaturated fatty acid, linoleic acid, and is known to contain calcium, phosphorus, niacin, and vitamin E, which is beneficial in reducing LDL cholesterol, boosting the immune system and providing protection against cardiovascular disease (Friedt et al. 1994; Joksimovic et al. 2006). Sunflower oil is known to have potential health benefits such as reducing low-density lipids, improving immunity, and preventing cardiovascular diseases (Staughton 2019).

The yield and quality of sunflower oil are determined by both genetic and environmental factors. Sunflowers require 500-600mm of rainfall during the growing season, with the 20 days before and after flowering being the most water-demanding periods. Water stress during these periods can negatively affect plant yield. Sunflower's water use efficiency has been determined to be 20% during the germination and plate formation stage, 60% during flowering and head formation, and the remaining 20% during the oil filling stage (Sabah 2010). Sunflowers have the potential to yield highly in soils that are deep, water-retentive, mineral-rich, and exposed to full sunlight. The growth and development of sunflowers require six macro elements, namely nitrogen, phosphorus, potassium, calcium, magnesium, and sulphur, along with seven trace elements such as iron, manganese, zinc, copper, boron, chlorine, and molybdenum. The uptake of these elements is influenced by several factors, including soil water content, availability of the elements in the soil, and root condition. Typically, the pH of the soil for optimal growth is between 6.5 to 7.5 (Kaya et al., 2012). Sunflower seeds are capable of

germinating at an average temperature of 5°C, however, they require temperatures of at least 14 to 21°C for optimal germination, growth, and development. Furthermore, they need a cumulative temperature of 2600-2850°C throughout the entire growing season (Anonymus, 2014).

The diverse range of economically important plants within this family has prompted extensive research in various fields of pure and applied plant science (Simpson, 2009).

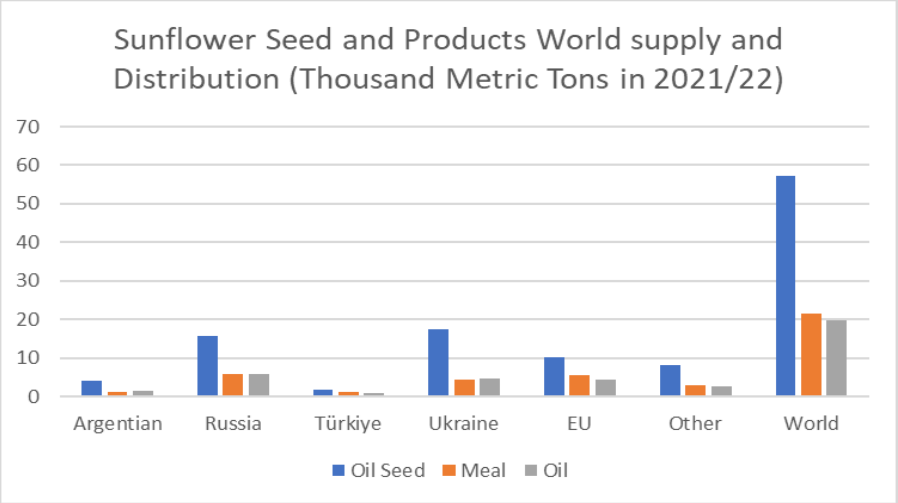

Figure 1. Sunflower Seed and Products World supply and Distribution (Thousand Metric Tons in 2021/22) (USDA, 2023).

3.2. BREEDING OBJECTIVES OF SUNFLOWER

There are multiple breeding objectives for sunflowers, including enhancing drought resistance, disease and pest resistance, and developing self-fertile and specific branching shape lines (Harshavardan and Amendeep, 2021). Sunflower genotypes are known for their extensive and deep root system, which can extract water from as deep as 270 centimetres (Hilli, 2021).

Crop improvement and breeding programs are essential components of agricultural development, and sunflower breeding has been a key focus for over 50 years. The initial efforts to develop sunflower varieties with higher oil content began in Russia in the 1960s and have continued ever since (Vear, 2016). In subsequent years, researchers focused on developing cytoplasmic male sterility (CMS) through a cross between *Helianthus petiolaris* and cultivated sunflower (Leclercq, 1969). Despite being an insect-pollinated species, sunflowers can experience unwanted genetic and phenotypic variation due to random crosses, leading to heterogeneity (Cveji et al., 2020). Furthermore, traditional breeding methods have some limitations such as the requirement of significant space and resources for plant selection, leading to breeding programs taking up to a decade to develop a new sunflower line (Davey and Jan, 2010). To overcome the limitations associated with heterogeneity in lines, true breeding lines can be established. This can be achieved through repetitive backcrossing to the parental line with the desirable trait and progeny selection, or by developing haploids and doubling their chromosomes to form doubled haploid (DH) lines (Dwivedi et al., 2015). Haploid plants have a single set of chromosomes and are incapable of undergoing meiosis, and therefore are infertile (Murovec et al., 2012).

However, their fertility can be restored through chemical or spontaneous chromosome doubling, resulting in 100% homozygosity in a single generation (Brit, 2016; Murovec et al., 2012). Doubled haploid (DH) lines eliminate the need for repeated backcrossing to a desirable parent line over multiple generations, thus greatly accelerating the generation of true-breeding lines (Ishii, 2016; Karimi-Ashtiyani et al., 2015). Doubled haploids (DHs) have several potential uses in plant breeding, such as accelerating the pyramiding of various mutants, facilitating forward mutagenesis screening, reducing ploidy levels (e.g., tetraploid to diploid), generating homozygotes for gametophyte-lethal mutations, and decreasing inbreeding depression associated with self-pollination. These applications have been supported by various studies (Murovec et al., 2012; Karimi-Ashtiyani et al., 2015). Chromosome substitution lines can be rapidly generated using doubled haploids as a starting point (Ishii, 2016).

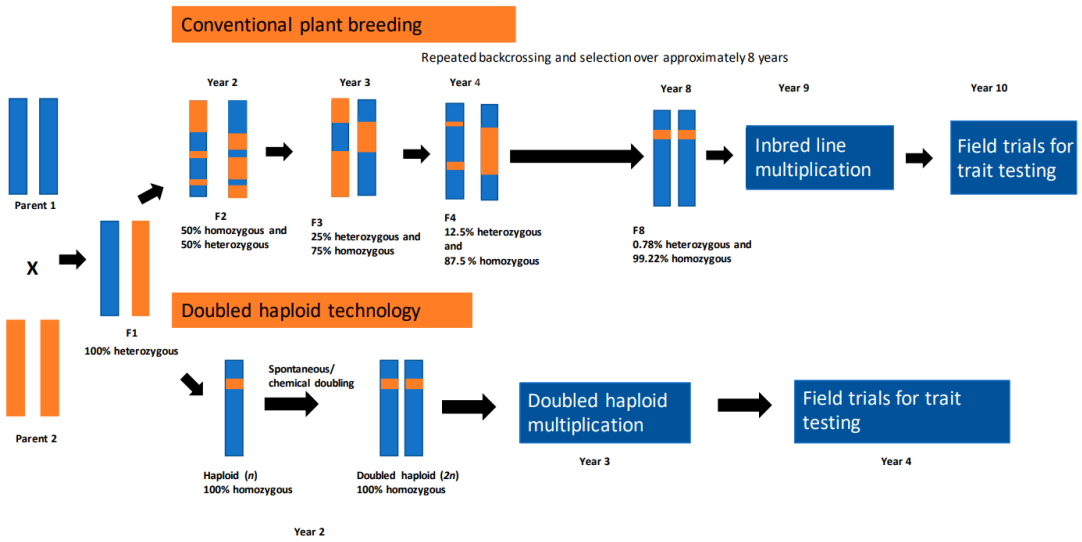

Figure 2. Comparison of conventional breeding and doubled haploid ('accelerated breeding') technology breeding methods (Eliby et al., 2022).

Sunflower breeding utilizes various haploid induction methods, including parthenogenesis. Experiments have been conducted to test the resistance of resulting haploid plants to broomrape, fungus, imidazoline, and downy mildew (Drumeva et al., 2014; Drumeva, 2017; Todorova, 1997). Another haploid induction method used in sunflower breeding is anther culture. This method has been applied for fertility restoration and has been studied for its effectiveness in improving resistance to broomrape, fungi, imidazoline, and downy mildew (Bohorov, 1985; Saji, 1998; Jonard, 1990).

In 1987, Ishino made a discovery while studying genes that are associated with the conversion of alkaline phosphatase's isozyme in *E. coli*, which led to the development of CRISPR (Ishino et al., 1987; Ishino et al., 2018). For the past four decades, the transfer of sunflower plants via *Agrobacterium* has been on the rise (Bidney et al., 1992; Laparra et al., 1995; Rao et al., 1999; Weber et al., 2003; Ikeda et al., 2005; Mohamed et al., 2006). Studying molecular biology to develop transgenic sunflowers with traits such as pest resistance, herbicide resistance, and increased oil yield is essential. It is also crucial to investigate the ecological impact of these modifications (Mayrose et al., 2011; Malnoy et al., 2016; Wang et al., 2017). Moreover, a research study

conducted a survey that resulted in the development of CAS-3 and CAS-5 mutants with high levels of stearic acid and palmitic acid contents, respectively (Osorio et al., 1995). CAS-14 mutants resulted in an increase of stearic acid content up to 37% (Fernández-Moya et al., 2002).

Helianthus annuus L. is a member of the *Asteraceae* family, which comprises 65 distinct species in the *Helianthus* genus. (Andrew et al., 2013). Sunflower *H. annuus* is commonly preferred as an annual plant by people. Its name is derived from the shape and image of the flower, which is often used to represent the sun. The plant has a hairy stem, broad and coarsely toothed leaves, and large inflorescence of circular flowers. It is generally considered an annual plant (Khaleghizadeh, 2011). The sunflower heads consist of numerous individual flowers that develop into seeds on a receptacle base (Seghatoleslami et al., 2012). The *Helianthus* genus comprises only 14 species that are annual. However, plant breeders have developed interspecific hybrids and transferred beneficial traits such as increased oil content, cytoplasmic male sterility for hybrid production, as well as resistance to pests and diseases in commercial sunflower (Fernández-Luqueño et al., 2014). The most commonly grown annual species of sunflower include *Helianthus annuus*, *Helianthus argophyllus*, *Helianthus maximiliani*, *Helianthus petiolaris* ssp. *petiolaris*, and *Helianthus annuus* ssp. lenticularis. Perennial sunflower species that are widely cultivated include Ashy sunflower (*Helianthus mollis*), Giant sunflower (*Helianthus giganteus*), Maximilian sunflower (*Helianthus maximiliani*), Paleleaf woodland sunflower (*Helianthus strumosus*), Purpledisc sunflower (*Helianthus atrorubens*), Sawtooth sunflower (*Helianthus grosseserratus*), Smooth sunflower (*Helianthus laevigatus*), Thin leaf sunflower, ten-petaled sunflower (*Helianthus decapetalus*), and Woodland sunflower (*Helianthus divaricatus*). Commercial sunflowers are self-compatible, meaning they do not require pollination by insects, although some studies have shown that bee pollinators can provide a small increase in yield (de Carvalho and de Toledo, 2008).

Sunflowers are not only used for human consumption but for various other purposes as well. As the world's fourth largest oil-seed plant, sunflowers have many uses. The seeds are commonly used as food, while the dried stalks are used as fuel. Sunflowers are also used as ornamental plants and have been used in ancient ceremonies (Harter et al., 2004; Muller et al., 2011). Additionally, sunflowers have a range of uses beyond human consumption. They have been found to have medicinal properties that help treat pulmonary afflictions. The plant's parts are also utilized in the textile industry for making dyes, as well as in body painting and other decorative practices. Sunflower oil, which is derived from its seeds, is used in salad dressings, cooking, and the production of margarine and shortening (Kunduracı et al., 2010). Roasted sunflower seeds can be used to create a coffee substitute. In some countries, the remaining seed cake after oil extraction is used as livestock feed. In the Soviet Union, sunflower stems were used to produce ethyl alcohol, plywood lining, and yeast, while the dried stems were utilized as fuel. The stems are also rich in phosphorous and potassium, making them a valuable source of fertilizer when composted and returned to the soil. Additionally, sunflower meal is a promising source of protein for human consumption due to its high nutritional value and absence of anti-nutritional factors (Fozia et al., 2008). Sunflower is gaining recognition as a valuable feedstock for biodiesel due to its positive agronomic characteristics, which are similar to other popular oil crops such as canola and soybean. It can be easily and

profitably grown in a range of conditions and scales, making it a promising source of oilseed for biodiesel production. Furthermore, sunflower is a versatile crop that can be used for both food and bioenergy production (Kibazohi et al., 2012). Integrated food and energy systems have the potential to create synergies between food and energy production under certain circumstances. Such systems could produce food crops while simultaneously addressing energy requirements (Bogdanski et al., 2010). Growing crops in short rotation or monocultures, such as sunflowers, is a common practice in traditional agriculture worldwide (Bennett et al., 2012).

Crop wild relatives (CWRs) are classified based on the degree of crossbreeding with related species. The first germplasm consisted of cultivated and wild types of *Helianthus annuus* and *Helianthus winterii*, while the secondary germplasm included species such as *H. anomalus, H. paradoxus*, *H. petiolaris*, and *H. deserticola*. The secondary species showed some level of genetic variation from the primary germplasm. Tertiary germplasm displays a high level of genetic and cytological divergence. The species belonging to the tertiary germplasm group are *H. hirsutus*, *H. tuberosus*, and *H. divericatus*. These interspecific hybridizations necessitated specialized techniques such as embryo rescue for the retrieval of interspecific hybrids (Warburton et al. 2017). Species variation can be characterized based on molecular, cytological, and morphological principles. The extent to which wild species can be utilized depends on various factors, such as ploidy level, growth habit, and reproductive barriers. Reproductive barriers can occur due to the evolution of any form of reproductive isolation, such as pre-zygotic and post-zygotic barriers. Pre-zygotic barriers are more common in plants than post-zygotic barriers, which is in contrast to animals where post-zygotic barriers are more prevalent, and hybrids have selective disadvantages (Maheshwari and Barbash 2011). Pre-zygotic barriers prevent pollen from germinating on the stigma, or the pollen tube from growing into the stylar tissues, leading to fertilization failure. These barriers are influenced by the differences and adaptation of species to their specific habitats. Post-zygotic barriers involve embryo death after fertilization, as well as hybrid viability or infertility due to negative interactions between loci. Hybrid sterility may occur due to the inability to produce viable gametes. The rate of gene transfer from wild species varies depending on the ploidy level, with the fastest transfer occurring in diploid species, followed by tetraploid and hexaploid species (Alix et al. 2017). The delay in gene transfer is attributed to the laborious process of eliminating extra chromosomes through backcrosses (Jan et al. 2014).The rapid restoration of chromosomes to 2n=34 can also be achieved by utilizing polyploid species as the male parent. However, the identification of wild species was not well-understood, and several sister species were not adequately characterized (Vanzela et al. 2002). The origin of many *Helianthus* species is attributed to various homoploid hybrid speciation events. The classification and rearrangement of chromosomes resulting from multiple homoploid events have led to the emergence of numerous species from a common ancestral species (Lai et al. 2005). Three distinct diploid hybrid species, namely *H. anomalus*, *H. paradoxus*, and *H. deserticola*, evolved due to the hybridization of *Helianthus annuus* and *Helianthus petiolaris*, followed by genome doubling resulting from unreduced gametes (Gross et al. 2003). These species have undergone significant differentiation and adaptation to specific environmental conditions as a result of the transgressive segregation of chromosomes. For example,

H. anomalus, H. paradoxus, and *H. deserticola* have adapted to dunes, salt marshes, and high deserts, respectively (Rosenthal et al. 2002).

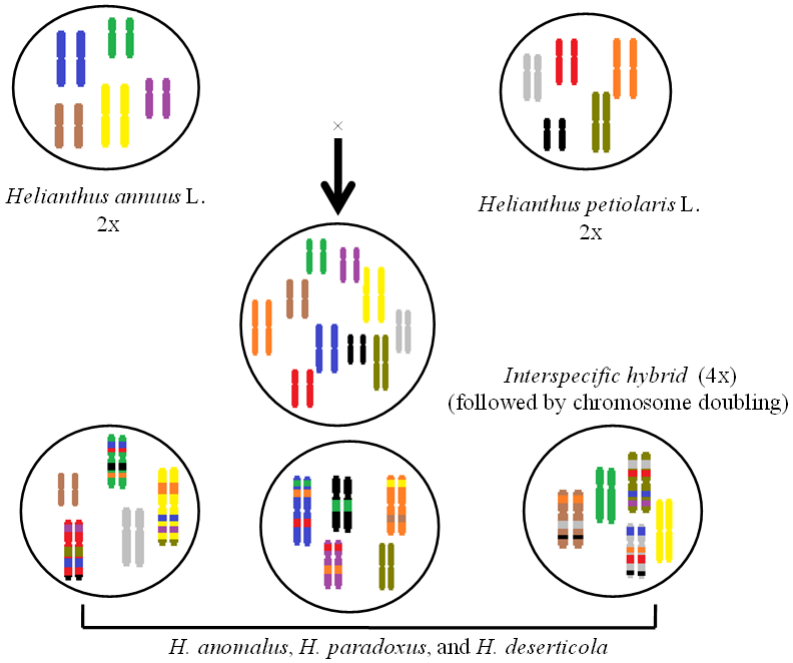

Figure 3. Origin of three homoploid species i.e. *H. anomalus*, *H. paradoxus*, and *H. deserticola* due to chromosome sorting and rearrangement (Gross et al. 2003).

3.3. MOLECULAR MARKERS

A molecular marker is a DNA sequence with a known position on the chromosome (Kumar 1999). A gene that shows easily recognizable phenotypic traits can be used to identify individuals, or as a probe to label a chromosome or locus (King and Stansfield 1990; Schulmann 2007). Molecular markers are indicative of polymorphism, which may arise due to chance nucleotide variations or mutations at specific genomic loci, (Hartl and Clark 1997) markers enable the identification of genetic variations between individual organisms or species (Collard et al. 2005). Molecular markers have diverse applications, ranging from genetic mapping, paternity testing, and detecting mutant genes linked to hereditary diseases, to identifying cultivars, marker-assisted crop breeding, investigating population history and epidemiology, ensuring food safety, and conducting population studies (Hartl and Jones 2005).

Biochemical and DNA markers are used to evaluate genetic diversity. DNA markers are nucleotide sequences that reveal polymorphism between the genomes of different individuals. Polymorphism can arise due to insertions, deletions, point mutations, duplications, and translocations. However, it does not affect the activity of specific genes (Mondini et al. 2009; Nadeem et al. 2018). DNA markers have proven to be valuable tools in determining polymorphism, genetic relationships, and diversity (Chalmers et al. 2001).

Using molecular markers for selecting resistance genes can aid in the creation of better germplasm. In various crops, PCR-based genetic markers have been widely used to map and analyse agronomic traits (Klein-Lankhorst et al. 1991; Quiros et al. 1991).

Molecular markers are categorized into different groups based on various criteria, such as gene activity (dominant or co-dominant markers), detection methods (hybridization-based or polymerase chain reaction-based techniques), and modes of transmission (maternal organelle inheritance, paternal organelle inheritance, bi-parental nuclear inheritance, or maternal-nuclear inheritance) (Semagn et al. 2006; Mondini et al. 2009; Nadeem et al. 2018).

3.4. MOLECULAR MARKERS STUDIES IN *HELIANTHUS ANNUS*

Sunflower is a model system for genomic studies in the *Asteraceae* family owing to its significance (Leclercq 1969; Paniego et al. 1999). The study of sunflower genetics is crucial due to the broad spectrum of traits present in their germplasm, including yield, plant height, seed number, earliness, and susceptibility to biotic and abiotic stresses (Thormann et al. 1994; Paniego et al. 1999). The genus *Helianthus* comprises of 49 to 67 species of perennial and annual herbaceous plants indigenous to North America, as per multiple estimations (Anashchenko, 1974; Heiser, 1978; Schilling and Heiser, 1981). Further investigation into the speciation of this taxon is necessary due to the significant variation in species numbers. Sunflowers consist of diploids, tetraploids, and hexaploids with a total of 17 major chromosomes (Heiser, 1978). Traditionally, the relationship between sunflower species has been determined through morphological and hybridological analyses (Chandler et al., 1986; Schillin and Heiser, 1969).

The diploid crop common sunflower has a chromosomal number of 2n=34 and a haploid genome size of 3000 Mb (Darvishzadeh et al. 2010).

The *Helianthus* genus comprises 51 wild species that possess valuable allelic variation and agronomic traits such as yield and resistance to biotic and abiotic stresses (Milton et al. 2013). Rieseberg and Seiler discovered in their analysis using RFLP that during domestication, cultivated sunflower genotypes were derived from a single source, and these lines had limited allozyme variability, and were all distinguished by a single cpDNA (Kaya et al.2012). The gene pool populations of cultivated sunflowers differ by 40-50% from those of wild sunflowers (Sala et al. 2012).

Sunflowers are fingerprinted using various molecular markers, such as AFLP, RAPD, and SNPs (Heesacker 2008) and SSRs are frequently utilized molecular markers in fingerprinting sunflowers, genome mapping, phylogenetic and population studies, estimation of genetic polymorphism, and marker-assisted selection because of their simplicity, co-dominant inheritance, reproducibility, high polymorphism, and low cost (Nadeem et al. 2018; Zimmer 2015). These properties of SSR markers promote different species such as barley (Varshney et al. 2007), maize (Nyaligwa 2016), wheat (Ahmed et al. 2017), rice (Singh et al. 2016), rapeseed (Kapoor et al. 2009), and others. The development of the first SSR genetic map has expanded the possibilities

for using SSR markers in sunflower experiments, including fingerprinting, genome mapping, and population studies.

The initial composite genetic map for sunflowers, which included 278 single-locus SSR markers and 379 additional markers (public and proprietary), covered 1423 cM. This map has become a reference for sunflower genetic studies and has been augmented with more SSR markers using three new mapping populations (Yu et al. 2003). Chapman and Heesacker (2008) developed over 2000 SSR markers from genomic sequences (gSSR) and ESTs (EST-SSR), which are now applicable for mapping and genotyping. Sunflower maps have been constructed using gSSRs, EST-SSRs, INDELs, and TRAP markers.

The initial genetic linkage map of cultivated sunflower was established using a combination of RFLP and randomly amplified polymorphic DNA markers (Berry et al. 1995, 1996, 1997; Genzbittel et al. 1995, 1999; Jan et al. 1998). Later on, amplified fragment length polymorphisms (AFLPs) were utilized to develop several genetic linkage maps (Peerbolte and Peleman 1996; Gedil et al. 2001b).

The development of several hundred microsatellite markers for sunflowers has significantly advanced the analysis of molecular genetic variation in this crop (Yu et al. 2002; Paniego et al. 2002).

Rieseberg and Seiler (1990) conducted a comprehensive study on various wild and domesticated sunflower lines and discovered that domesticated sunflowers showed decreased allozyme variability and had a single cpDNA restriction fragment length polymorphism (RFLP) haplotype. This finding suggests that the domesticated sunflowers have a single origin of domestication. However, the study's results are not conclusive, as the domesticated cpDNA haplotype was present at a relatively high frequency (27%) in the wild and was geographically widespread.

The genome size of sunflowers is 3.6 gigabase, containing approximately 3.6 billion nucleotides of A, T, C, and G, distributed among 17 chromosomes. However, the major obstacle with sunflowers is that over 75% of the genome is composed of lengthy repeated DNA segments known as long terminal repeat retrotransposons. These DNA stretches share a similar appearance, making it difficult to differentiate and categorize them properly (Renaut, 2017). In order to gain a deeper insight into the evolutionary trajectory of the sunflower genome, Badouin et al. (2017) conducted a comparative analysis of the sunflower genome with several closely related species such as lettuce and artichoke, as well as more distantly related species like coffee and grape. They confirmed a previous finding of a significant expansion in the sunflower genome size, which resulted in tripling its length.

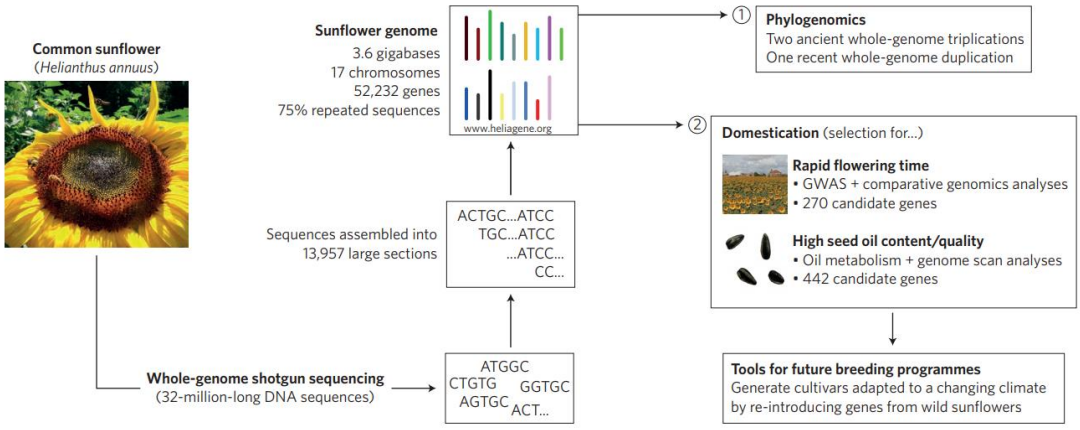

Figure 4. Summary of the experimental design used to construct the sunflower genome, from whole-genome shotgun sequencing to sequence assembly and the high-quality genome (Renaut, 2017).

3.5. START CODON TARGETED (SCoT) MARKER

SCoT (Start Codon Targeted) is a molecular marker that is dominant in nature and is used to study genetic polymorphism, diversity, and phylogenetic relationships. It targets the short-conserved region around the ATG start codon on both DNA strands. This marker employs a single 18-mer primer that anneals at a temperature of 50°C, as the region flanking the ATG start codon is highly conserved across plant species. Although a few SCoT markers can be codominant through insertion or deletion, they are rare. The amplification products of SCoT markers are distributed within gene regions, including genes, pseudogenes, and transposable elements that could also be primer binding sites. The spacing between the binding sites of the primer can be observed through agarose gel electrophoresis, making it a simple and cost-effective method (Davis et al. 1995; Collard and Mackill, 2009). The SCoT marker technology can detect the presence of both dominant and codominant markers (Nair et al, 2016). Zhang et al. (2015) noted that SCoT marker technology is simple to use and less expensive, making it widely applicable in laboratories with basic equipment. The technique's first validation was carried out in rice (Oryza sativa) (Collard and Mackill, 2009).

The SCoT marker system is based on the short-conserved region surrounding the ATG translation start codon in plant genes, and it is a simple marker system. This marker system has several advantages over other marker systems, including RAPDs, ISSRs, and SSRs, as it focuses on genic regions that typically have low recombination levels between marker alleles and the gene or trait. Consequently, SCoT markers are often considered more reliable and advantageous, as noted by Hajibarat et al. (2015). SCoT markers offer several advantages, including high polymorphism, specificity, repeatability, simplicity of use, and rich genetic information (Xiong et al. 2009). SCoT markers have been effectively utilized in diverse areas such as genetic diversity analysis, cultivar identification, quantitative trait loci (QTL) analysis, differential gene expression, stress-related gene screening, and evaluation of genetic fidelity in tissue culture plants, in various plant species, including grape (Guo et al., 2012), mango (Luo et al., 2011, 2014), chickpea (Amirmoradi et al., 2012), sugarcane (Que et al., 2014), wheat (Hamidi et al., 2014), *Albizia spp*. (Rahmani et al., 2015) and bottle gourd (Bhawna et al., 2017).

The use of SCoT markers extends to the evaluation of genetic diversity in Mentha. Additionally, SCoT markers are important for gene tagging and have the ability to locate quantitative trait loci (QTL) in various crops. With the ability to investigate genetic variation at the gene level, SCoT markers can also identify new alleles (Khan and Dhawan, 2016).

In a study on cultivated flax genotypes (*Linum usitatissimum* L.), SCoT markers were employed to evaluate genetic diversity and relationships. A total of 120 DNA fragments were detected with an average of 12 bands per primer. Out of the total bands, 69 were found to be polymorphic, while 51 were monomorphic. The percentage of polymorphic bands ranged from 27% to 92% with an average of 64.8% in the analyzed group of nine flax genotypes (*Linum usitatissimum* L.) (Mohamed Z.S. Ahmed et al., 2018). In study of Gorji et al., (2011), The authors noted that the use of SCoT analysis was more efficient for creating fingerprints of both a population of tetraploid potatoes and 24 potato varieties. Xiong and colleagues (2011) demonstrated that the SCoT technique is a valuable marker for studying genetic variation and relationships within domesticated peanut (*Arachis hypogaea* L.), through the analysis of DNA polymorphisms. Agarwal et al. (2019) employed the SCoT marker to assess genetic diversity in 29 rose germplasm accessions. According to the findings of the study conducted by Agarwal et al. (2019), the SCoT marker technique was found to be a preferable option over RAPD, ISSR, AFLP, SRAP, and TRAP techniques for studying genetic relationships among different rose germplasms. This is due to the SCoT marker's higher polymorphism, greater reliability, ease of access, time-saving, and lower cost.

Genetic diversity in tetraploid durum wheat genotypes was studied using the SCoT marker. The analysis showed that the SCoT marker has a higher level of polymorphism compared to the Inter-Simple-Sequence-Repeat (ISSR) marker, making it an important tool for detecting genetic variation in the collection. Furthermore, the SCoT marker produced the most accurate analysis of the cluster pattern (Etminan et al. 2016).

The SCoT marker has also been utilized to assess the genetic diversity of the wild population of *Maytenus emarginata*. The findings of this study indicated that the SCoT analysis is an efficient tool for the rapid characterization of *M. emarginata*. The results showed a high percentage of genetic diversity within the population, as well as high levels of gene flow and genetic differentiation. Furthermore, the study revealed that all genotypes were primarily associated based on geography and habitat (Shekhawat et al., 2018).

The SCoT marker has also been utilized for evolutionary research and assessing the genetic diversity of *Bletilla striata*. The findings demonstrate that SCoT markers serve as highly precise and effective tools for examining the genetic diversity of populations. Furthermore, this study provides crucial foundational data on the population genetics of *B. striata* in China, making it valuable for conservation, germplasm evaluation, building a core collection, and disseminating regional varieties and substitutions (Guo et al. 2018).

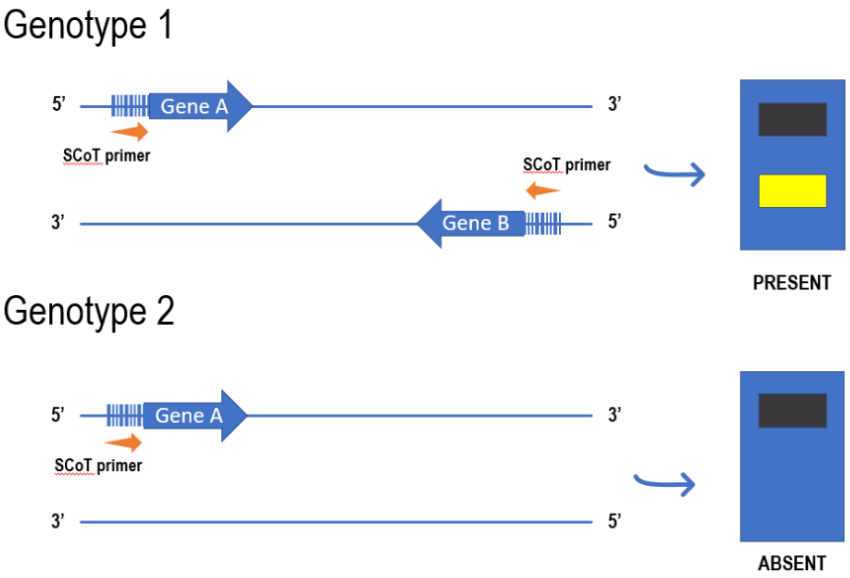

Figure 5. The principle of the SCoT primer in PCR amplification (Collard and Mackill, 2009)

3.6. CHLOROPLAST MARKERS

The investigation of complete chloroplast genomes has significantly contributed to the comprehension of plant biology, plant diversity, domestication history, and evolutionary relationships (Rieseberg and Burke, 2008; Daniell et al., 2016). Over the years, more than 7,000 land plant plastid genomes have been publicly released since the initial publication of the first *Nicotiana tabacum* (Shinozaki et al., 1986) and *Marchantia polymorpha* (Ohyama et al., 1986) plastid genome sequences. Most of these genomes were identified within the last ten years using Next-Generation Sequencing (NGS) technology. The abundance of complete chloroplast genome sequences has proved valuable for contemporary taxonomy and phylogenetics (Jansen and Ruhlman, 2012).

Only a limited number of molecular studies have been conducted on chloroplast DNA (cpDNA) to investigate the interrelationships within the Helianthus genus (Rieseberg, 1991; Strasburg and Rieseberg, 2008; Wills and Burke, 2006; Timme et al., 2007; Bock et al., 2014). Azarine et al. (2014) conducted a study where they compared seven perennial *Helianthus* species with the reference sequence (GenBank: MK341449.1) and identified 414 polymorphic sites, of which 274 (66.2%) were SNPs, 73 (17.6%) were INDELs, and 67 (16.2%) were SSRs. The LSC regions had the highest number of polymorphisms, while the IR regions had the lowest number of polymorphic sites in the chloroplast genomes. Furthermore, the intergenic regions showed a higher average frequency of polymorphisms compared to the genic regions. This study provides valuable insights into the genetic diversity of Helianthus species using chloroplast DNA.

3.7. GENETIC BACKGROUND OF DROUGHT TOLERANCE

The definition of drought can vary depending on the location, timing, and amount of precipitation. In the context of plant breeding, drought is characterized as either a deficiency of water that results in a decrease in

crop yield, or a prolonged period of little to no rainfall or irrigation that causes insufficient soil moisture, which can lead to stunted crop growth and reduced yield (Fukai and Cooper, 1995; Blum, 2011). Drought is considered the most destructive abiotic stress that reduces crop fertility, and it is the most difficult one for breeders to address. In the past, attempts to increase drought tolerance through breeding have been limited by the quantitative genetic nature of drought tolerance and our incomplete understanding of the physiological mechanisms underlying crop response to water-limited conditions (Passioura, 2002; Blum, 1988). Drought is considered the most complex among all abiotic stresses, and is greatly influenced by factors such as rainfall patterns, soil types, availability of irrigation resources during a given season and across seasons, as well as the stage of crop growth (White and Singh, 1991). Drought stress is a common and severe ecological condition that can significantly reduce crop productivity. Developing crop resistance to drought stress is a cost-effective approach to improve agricultural productivity and reduce the consumption of freshwater resources. Hence, plant biologists and crop breeders have focused on understanding the mechanisms of drought tolerance and breeding drought-resistant crops. However, drought tolerance is challenging to study at the molecular level due to our limited knowledge of the traits associated with drought tolerance. Additionally, conducting drought stress treatments in a consistent and quantitative manner is difficult. These complexities have hindered research on plant drought tolerance, and the biological basis for drought tolerance remains unclear. As a result, only a few drought tolerance determinants have been identified (Ludlow and Muchow, 1990; Bohnert et al., 1995; Araus et al.,2002; Bruce et al., 2002). Despite our limited understanding of the mechanisms underlying drought tolerance, various physiological and molecular biological investigations have documented several plant responses to drought stress (Bohnert et al., 1995; Blum, 1996; Ingram and Bartel, 1996; Bray, 1997; Schroeder et al.,2001; Luan, 2002). Drought stress can cause stomata closure and trigger an increase in the production of the stress hormone abscisic acid (ABA), leading to the activation of genes that respond to drought and ABA. Over the past decade, molecular and biochemical research has revealed several of these genes that respond to stress and ABA, along with some transcription factors that are responsible for their activation, both in model plants and crop plants (Ingram and Bartel, 1996; Hasegawa et al.,2000; Thomashow, 2001; Finkelstein et al., 2002; Oztur et al., 2002; Shinozaki et al., 2003; Yu and Setter, 2003; Buchanan et al., 2005; Poroyko et al., 2005). Plant responses to ABA and stress have been studied extensively using genetic approaches to dissect complex cellular processes. These studies aim to identify the mechanisms involved in stress signalling and stress tolerance. Several studies have explored the ABA response in seed germination, gene expression, and guard cell movement, leading to the identification of some of the components involved in ABA signalling (Finkelstein et al., 2002). Researchers have used molecular mapping methods and found that drought tolerance in crop plants is governed by several quantitative trait loci (QTLs), with each locus contributing only a small percentage to the variations in drought tolerance (Sanchez et al., 2002; Diab et al.,2004; Lanceras et al., 2004; Yue et al., 2005). Transcription factors are important regulatory proteins that can precisely control the activation and suppression of target genes. In response to drought stress, plants can reduce water loss by partially closing stomata, efficiently absorb water from the soil through their roots, and adjust their metabolic processes to match available carbon resources. As drought intensifies, osmolytes like proline and soluble sugars accumulate to maintain cell

turgor pressure. Additionally, various antioxidants, such as superoxide dismutase, reduce the toxic effects of reactive oxygen species (Ereful et al., 2020). Delivery network policies for quality play a crucial role in the response of plants to drought. Various transcriptions govern different crops, and transcription analysis has shown different responses to drought stress in two wheat genotypes (Faghani et al., 2015). According to the findings, wheat responded to drought stress by upregulating auxin and abscisic acid receptors, which are associated with reactive oxygen species and cell wall biosynthesis, while downregulating ethylene receptors. In contrast, maize showed a moderate response to drought, with DNA and cell cycle related genes being affected. The study further revealed that metabolic pathways and the biosynthesis of alternative digestion were also influenced by drought, and the expression of several genes related to ABA signal transduction was induced (Fracasso et al., 2016). Drought resistance or tolerance is a complex trait that is controlled by multiple genes with low to medium heritability (Ekanayake et al., 1985). In the past twenty years, plant scientists have made progress in understanding the molecular and genetic basis of plant responses to drought, which has been a complex trait to study and elucidate (Maazou et al., 2016). Plants have developed various strategies to adapt and survive under conditions of water deficit, such as closing stomata, inhibiting cell growth, and increasing respiration (Shinozaki and Yamaguchi-Shinozaki, 2007). During periods of drought stress, plants accumulate high levels of soluble sugars and sugar alcohols (such as sucrose, trehalose, and raffinose) in order to help maintain the stability and integrity of their cell membranes (Ahanger et al., 2018). Recent advancements in molecular biology have led to high-throughput genome sequencing, genome annotation, and gene expression analyses becoming faster, more cost-effective, and accessible. Many of these methods can now be routinely utilized in breeding programs, leading to significant progress in plant genetics and genomics (Collard et al., 2008). Galactinol synthase (GolS), which is a crucial enzyme for the production of galactinol and raffinose family oligosaccharides (RFOs), is accumulated under drought, heat, and salt stress conditions (Takahashi et al., 2020). *Arabidopsis* has a unique seven-gene family that encodes for AtGolS genes, and the expression of these genes is regulated both spatially and developmentally (Salvi et al., 2016). Microarray analysis in *Arabidopsis* and rice has identified numerous stress-inducible genes that play a vital role in plant stress response and ultimately improve stress tolerance. In rice, 73 stress-inducible genes have been identified, and 51 of those genes have a similar function to those identified in *Arabidopsis* (Rabbani et al., 2003). The morpho-physiological traits that influence crop drought tolerance are quantitatively inherited, and the identification of QTLs (quantitative trait loci) is essential for their improvement through marker-assisted selection (MAS) (Blum, 1998). Rice possesses over 20,000 SSR markers and more than one million SNPs and Indels, comprising functional as well as non-functional markers (Mcnally, et al., 2009, McCouch et al., 2010) and the availability of a large number of molecular markers in rice, including SSRs, SNPs, and Indels, presents numerous opportunities for their application in varietal analysis, gene/QTL mapping for various agronomic traits under drought stress, and their utilization in marker-assisted breeding. Additionally, these markers can aid in the positional cloning of QTLs to identify candidate genes for complex traits (Salvi and Tuberosa, 2005). Yang et al. (2004) A study was conducted on tissue-specific gene expression in drought-stressed rice roots, which identified 66 transcripts. Among these, four transcripts were found to be located within the QTLs for root growth under water shortage. In recent years, much research has been focused

on isolating and testing the expression of genes related to drought tolerance in sunflowers. Through subtractive hybridization of cDNA synthesized from RNA isolated from both drought-stressed and unstressed sunflower plants, new stress-responsive genes have been identified. These include SunTIP, HaDhn1, HaDhn2, Sdi (sunflower drought induced), Gdi15, Hahb-4, and HAS1 or HAS1.1. The transcript levels of these genes were found to be highly expressed under drought stress, suggesting their role in the sunflower response to drought. (Ouvrard et al., 1996, Cellier et al., 1998; Sarda et al., 1999, Gopalakrishna et al., 2001, Liu and Baird, 2003, Dezar et al., 2005; Herrera Rodriguez et al., 2007). A study was conducted to investigate the genetic variability between cultivated and wild sunflowers for the dehydrin gene (Dhn1) related to drought stress. Results showed diversification of biochemical properties of the expressed protein between annual and perennial *Helianthus* species. However, cultivated sunflowers showed lower genetic variability for dehydrin genes compared to wild sunflowers. The study also proposed that Dhn1 can be used to study the phylogeny of *Helianthus* (Giordani et al., 2003; Natali et al., 2003).

4. MATERIALS AND METHODS

4.1. PLANT MATERIALS

This research conducted with five common sunflower (*Helianthus annus* L.) genotypes that from three of them was drought tolerant, two of them was drought sensitive genotypes. Five of plant materials were provided from Trakya Agricultural Research Institute in Türkiye. Drought resistance genotypes name were “TUNCA, 8129R, P64LL62”, drought sensitive genotypes names were “9718A, 97251A”.

P64LL62, a variety of sunflower, was developed in Turkey in 2009 with a grain yield of 348.8 kg/da, which was 10.1% higher than the average yield of 316.9 kg/da. It also exhibited an oil content of 50.0%, which was 7.4% higher than the standard varieties' average oil content of 46.5%. It reaches physiological maturity within 93-103 days. Pioneer company registered this variety in 2016. On the other hand, TUNCA was registered by Limagrain in 2008 and is known for its high yield potential, high oil content, and exceptional drought resistance (Çan E. 2019). In 2012, 8129R was registered at the Trakya Agricultural Research Institute in Türkiye. Later, in 2015, 9718A was also registered at the same institute. Finally, in 2019, 97251A was registered at the Trakya Agricultural Research Institute in Türkiye as well.

Leaves samples were obtained from seeds. For each genotype, seeds were sowed, leaves were reached 3 or 4 cm length and then leaves collected for DNA extraction.

4.2. DNA EXTRACTION

The DNeasy Plant Mini Kit (Qiagen 2016) was used to isolate DNA from fresh leaves in the study. To begin the process, approximately 100 mg of leaf samples were ground with liquid Nitrogen in a sterile mortar and pestle until a fine powder was obtained. The powder was transferred to a tube and 400 µl of AP1 and 4µl of RNase A were added. The mixture was then vortex homogenized and incubated at 65ºC for 10 minutes, with the tube being upturned 2-3 times during incubation. Following this, 130 µl of buffer P3 was added to the tube, mixed thoroughly, and then incubated on ice for 5 minutes.

After centrifuging the lysate at 1400 rpm for 5 minutes to separate it from the tissue debris, it was transferred to a QIAshredder spin column and then placed in a 2 ml collection tube. Next, the tube containing the lysate and QIAshredder spin column was centrifuged at 1400 rpm for 2 minutes. The resulting flow-through was then carefully transferred to a new tube, and 675 µl of buffer AW1 was added. The mixture was mixed thoroughly by pipetting, and then 650 µl of this mixture was transferred to a DNeasy Mini Spin Column, which was then placed in a 2 ml collection tube. Afterward, the mixture was centrifuged at 8000 rpm for 1 minute and the resulting flow-through was discarded. This step was repeated with the remaining flow-through.

First, the spin column was placed into a new 2 ml collection tube, and then 500 µl of buffer AW2 was added to it. After that, the mixture was centrifuged at 8000 rpm for 1 minute, and the resulting flow-through was discarded. Next, another 500 µl of buffer AW2 was added, and the tube was centrifuged at 1400 rpm for 2

minutesTo extract the DNA from the spin column, 100 µl of buffer AE was added and left at room temperature (15-25ºC) for 5 minutes. The spin column was then centrifuged at 8000 rpm for 1 minute, and another 100 µl of buffer AE was added. The resulting DNA solution was considered ready to use and was stored at -20ºC.

4.3. PCR AMPLIFICATION AND GEL ELECTROPHORESIS

A total of 25 primers were evaluated, out of which 13 SCoT primers were chosen for further data analysis due to sufficient polymorphism. PCR amplification was conducted with a reaction volume of 14 µl consisting of 5x DreamTaq red buffer at a final concentration of 1x, 0.4 µl dNTPs at a final concentration of 200 µM, 0.5 µM of the SCoT, 1 U Polymerase Phire, 0.6 µl DMSO at a final concentration of 3%, and 8.6 µl Milli-Q water. The PCR amplification was carried out using a 2720 thermocycler with about 20-40 genomic DNAs.

Table 1. The sequences of SCoT primers (Collard and Mackill, 2009)

No.	Primer	Sequences (5' - 3')
1	SCoT1	CAACAATGGCTACCACCA
2	SCoT2	CAACAATGGCTACCACCC
3	SCoT7	CAACAATGGCTACCACGG
4	SCoT8	CAACAATGGCTACCACGT
5	SCoT17	ACCATGGCTACCACCGAG
6	SCoT18	ACCATGGCTACCACCGCC
7	SCoT19	ACCATGGCTACCACCGGC
8	SCoT20	ACCATGGCTACCACCGCG
9	SCoT23	CACCATGGCTACCACCAG
10	SCoT24	CACCATGGCTACCACCAT
11	SCoT27	ACCATGGCTACCACCGTG
12	SCoT29	CCATGGCTACCACCGGCC
13	SCoT30	CCATGGCTACCACCGGCG

To amplify the SCoT primers, the PCR reaction was initiated with an initial denaturation step at 94ºC for 5 minutes. Subsequently, 35 cycles were performed with denaturation at 94ºC for 1 minute, annealing at 50ºC for 1 minute, and extension at 72ºC for 2 minutes. The final extension was carried out at 72ºC for 5 minutes.

The SCoT PCR products were subjected to electrophoresis on a 1% TBE agarose gel and stained with ethidium bromide (EtBr) for 20-60 minutes at 90-100 voltage. The DNA bands were visualized under UV illumination using a gel documentation system (Bio-Rad). To determine the approximate fragment size, the GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific) was used for comparison.

4.4. DATA ANALYSIS

A binary data matrix was created based on the presence (1) or absence (0) of clear, unambiguous, and reproducible bands generated by the selected SCoT primers. Smudged and poor bands were excluded from the analysis to ensure the reliability of the data. The resulting banding profiles were used to construct a dendrogram of primers and perform principal component analysis (PCA) using Past 4.04 software (Hammer et al. 2001).

5. RESULTS AND DISCUSSION

5.1. CHARACTERISTICS OF POLYMORPHIC SCoT MARKERS IN Helianthus annus L.

Altogether 25 primers were tested and 13 SCoT primers successfully amplified 3 droughts tolerant and 2 drought sensitive genotypes of *Helianthus annus* L. The total number of bands from 13 SCoT primers was 161, with 125 bands showing polymorphism and the average percentage of polymorphic bands was 76.79%. The size range of bands was from 250-8000 bp. The total number of bands range from 4 to 27 an average of 12.38 bands, while the number of polymorphic bands varied from 2 to 21 an average of 9.61 bands per primer (Table 2).

Table 2. Characteristics of SCoT amplification bands of *Helianthus annus* L.

Primer Name	Size Range (bp)	Total Number of Bands	Number of Polymorphic Bands	Percentage of Polymorphic Bands (%)
SCoT1	250-4000	8	6	75
SCoT2	250-8000	27	21	77.7
SCoT7	250-8000	8	5	62.5
SCoT8	250-8000	9	5	55.5
SCoT17	500-6000	8	7	87.5
SCoT18	250-5000	9	9	100
SCoT19	500-8000	18	16	88.8
SCoT20	500-4000	14	10	75.42
SCoT23	500-3000	4	2	50
SCoT24	250-5000	16	11	68.75
SCoT27	250-8000	15	13	86.6
SCoT29	250-5000	17	12	70.5
SCoT30	250-3000	8	8	100
Total		161	125	
Average		12.38	9.61	76.79

SCoT2 gave the highest total number of bands (27), while SCoT23 gave the lowest total number of bands (4). The highest number of polymorphic bands observed SCoT18 and SCoT30 with 100%. The lowest number of polymorphic bands observed SCoT23 with 50%.

On this study 25 primers were tested and 13 SCoT primers gave 100% amplification with polymorphism, while the other twelve SCoT primers were either monomorphic or did not give 100% amplification products. This consequence was also parallel with Mulpuri et al. (2013), who performed SCoT primers for *Jatropha curcas* accessions 26 out of 36 SCoT primers tested gave 100% amplicon with polymorphism. According to this study observed that SCoT primers could be utilized for diverse materials with genetic variation and give an informative and highly reproducible amplification product.

Satya et al. (2015) used twenty-four start codons targeted (SCoT) markers to determine the genetic diversity and population structure of indigenous, introduced, and domesticated ramie (*Boehmeria nivea* L. Gaudich). The Indian ramie populations exhibited high SCoT polymorphism, high genetic differentiation, and moderate gene flow. This result put forwards that SCoT primers can be beneficial for DNA fingerprinting,

population analysis, genetic diversity studies, parentage specification, and effective management of ramie genetic resources. In addition, the present study indicates the efficacy of employing SCoT markers in a cross-pollinated heterozygous species like *Boehmeria*, and would be helpful for further studies in population genetics, conservation genetics, and cultivar improvement.

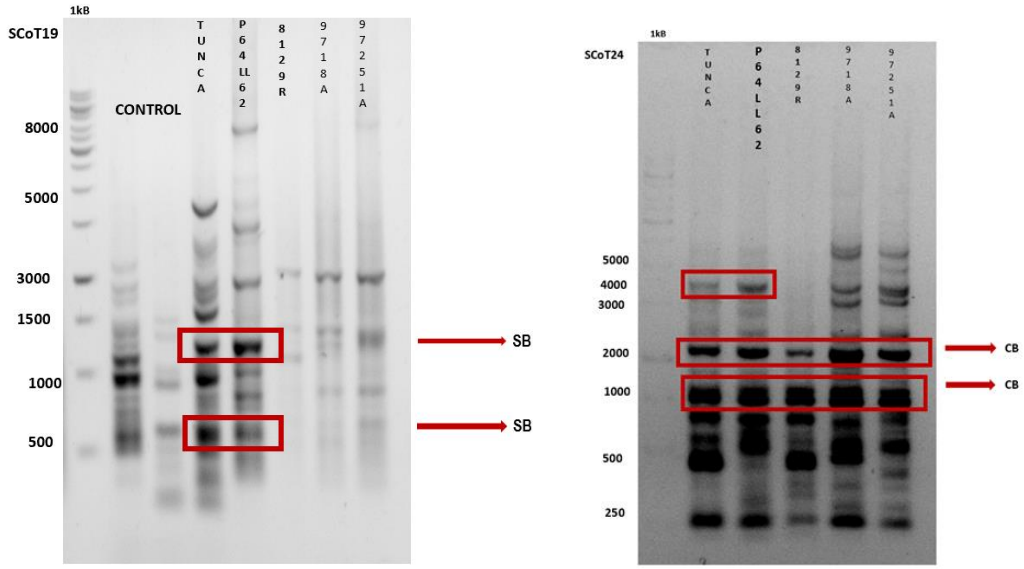

Figure 6. SCoT amplification profile with SCoT19 and SCoT24 primers.

TUNCA and P64LL62 drought tolerant cultivars showed specific band (SB) characteristics, while the other drought tolerant cultivar did not show any specific band characteristic with SCoT19 primer. On the other hand, both drought tolerant and sensitive cultivars showed common band (CB) characteristics with SCoT24 primer (Figure 6).

According to our results, this is a preliminary study to find DNA sequence linked to drought tolerant. Especially, drought tolerant cultivars TUNCA and P64LL62 showed similar characteristics in terms of genetical background of drought tolerant.

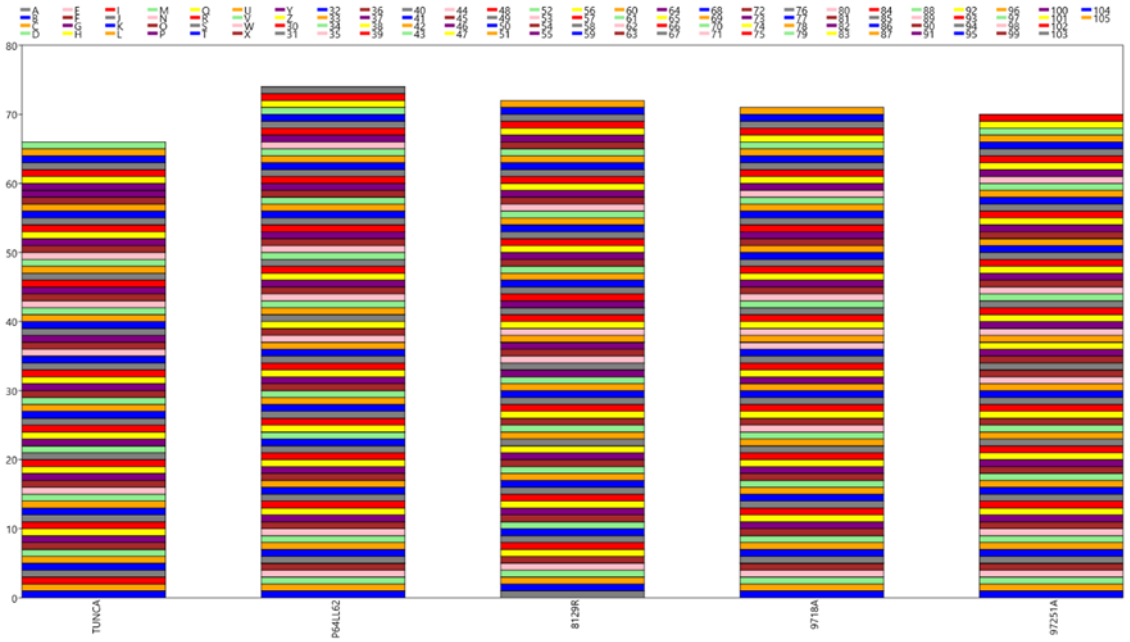

Figure 7. Stacked chart analysis of *H.anuus* cultivars using 13 SCoT primers (Past 4.04 software; Hammer et al. 2001).

Figure 7 demonstrates the PCR banding pattern in case of all amplified fragments generated by 13 SCoT primer. Each colour can be matched to a given PCR product. It could be seen that the smallest number of DNA fragments was produced in TUNCA, while the most in P64LL6.

5.2. CLUSTER ANALYSIS USING SCoT PRIMERS

A neighbour joining dendrogram (Past 4.04 software; Hammer et al. 2001) between 3 drought tolerant and 2 drought sensitive cultivars of *H.annus* using 13 SCoT primers similarity is shown Figure 8. The tolerant cultivars are marked by brown colour.

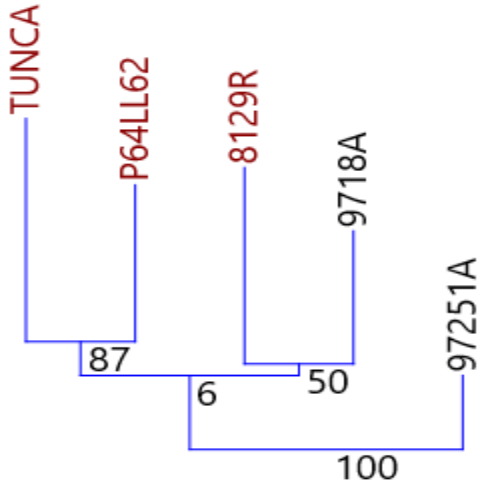

Figure 8. A neighbour-joining dendrogram with Euclidean distance between 3 drought tolerant and 2 drought sensitive cultivars of *H.annus* using 13 SCoT primers. The bootstrap values (%, n = 1,000) are indicated. Tolerant cultivars are signed by brown while sensitive cultivars are signed by black colour.

A main group was divided into two groups containing and then divided into another sub-main group with a bootstrap value of 100. The bootstrap value is essential in the study of phylogenies to assess the level of confidence in the estimated tree. According to Berry and Gascuel (1996), high bootstrap values (close to 100%) mean uniform support. The bootstrap value for a certain clade is close to 100%, nearly all of the characters informative for this group agree that it is a group. In addition, a bootstrap value of more than 50% can also be used to support the clade, although it is considered to be of low accuracy (Soltis and Soltis, 2003).

Table 3 has shown that the highest similarity value was belong to drought tolerant cultivars 8129R with 6,48 and the lowest value was belonged to drought sensitive 97251A with 4,123. In addition, drought sensitive genotypes 9718A and 97251A have shown closely related values. This highest similarity value could be due to the condition pedigree but no information is available on the origin of the varieties. We assume that the drought tolerance was introduced from the same gene source, and because of this, these genotypes have similar genetical background.

Table 3. Similarity and distance matrices of *H.annus* genotypes

	TUNCA	**P64LL62**	**8129R**	**9718A**	**97251A**
TUNCA	0	5,2915026	6,4807407	5,5677644	5,6568542
P64LL62	5,2915026	0	5,6568542	4,5825757	4,6904158
8129R	6,4807407	5,6568542	0	4,5825757	4,8989795
9718A	5,5677644	4,5825757	4,5825757	0	4,1231056
97251A	5,6568542	4,6904158	4,8989795	4,1231056	0

In their 2021 study, Zarei and Erfani-Moghadam used SCoT markers to examine genetic diversity, population structure, and phylogenetic relationships among three Pistacia species found in Iran. The researchers found that *P. vera* and *P. khinjuk* exhibited higher similarities. They also determined that environmental factors may contribute to phenotypic variations observed in different regions, and that plants growing in the same climate tend to have higher phenotypic similarities than those from distant regions.

Rayan and Osman (2019), examined phylogenetic relationships between Egyptian soybean cultivars using SCoT markers and protein patterns. This investigation indicates that the highest similarity value between two soybean varieties means that they are closely related, while the lowest similarity value means that they are genetically distant from each other. The result also shows that SCoT markers are potent markers for distinguishing and identifying different soybean cultivars.

5.3. PRINCIPAL COMPONENT ANALYSIS (PCA)

The polymorphic bands generated by 13 SCoT primers were analysed using principal component analysis (PCA) to determine the clustering pattern based on the characteristics of the bands obtained, thus making it easier to display the distribution of the data of each genotype in the form of a plot.

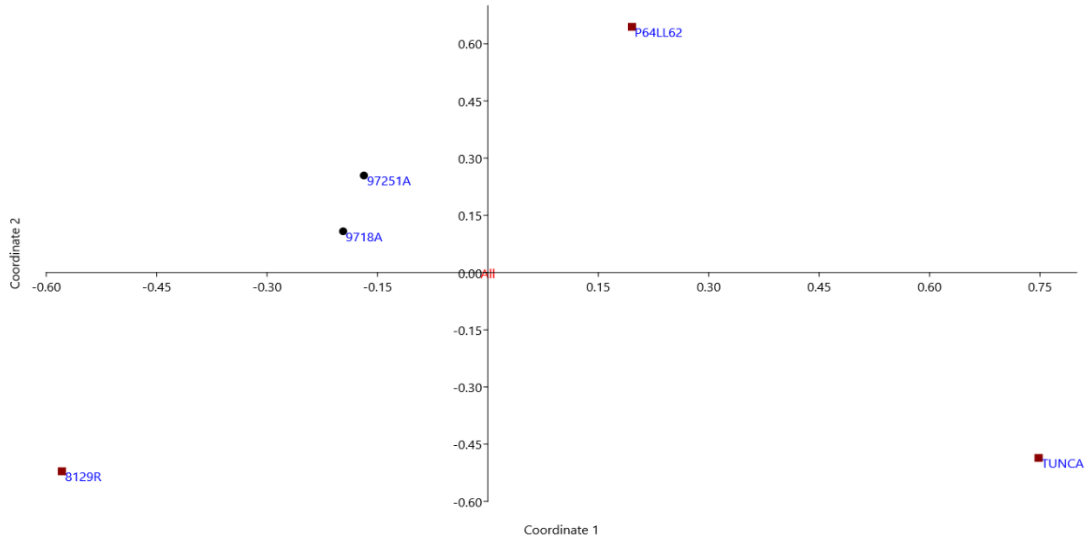

Figure 9. Principal component analysis (PCA) of *H. annus* genotypes using 13 SCoT primers. Tolerant cultivars are marked with a brown square, while sensitive ones are marked with a black circle.

Figure 9 shows the distribution of 3 drought tolerant and 2 drought sensitive genotypes of *H. annus*. The samples from drought tolerant named with TUNCA and 8129R contrast by coordinate 1. These two cultivars were found quite far from other genotypes and from each other. In coordinate 2, one of the droughts tolerant cultivars named as P64LL62 and the other drought sensitive cultivars named with 9718A and 97251A were located. In coordinate 2, one of the drought tolerant species P64LL62 and the drought sensitive species 9718A and 97251A are located. In addition, species of 9718A and 97251A were closely related to each other than the other accessions. This PCA result supports the results of similarity and distance matrices, which shows that 9718A and 97251A are closely related.

Principal Component Analysis (PCA) is a statistical technique used to analyse a dataset consisting of inter-correlated variables describing observations. The aim of PCA is to extract meaningful information from the dataset and represent it as a set of new orthogonal variables known as principal components. By doing so, PCA allows for visualization of the similarities between observations and variables by plotting them as points on maps (Jolliffe 2002; Saporta and Niang 2009).

Igwe et al. (2017) utilized PCA with SCoT markers to investigate the genetic diversity in *Vigna unguiculata* L. (Walp). They amplified five SCoT markers, resulting in 52 alleles and 80 polymorphic loci. The study concluded that this method was effective in generating genetic fingerprints and could potentially be used for applications such as seed purity determination, efficient utilization, and management of genetic resources in cowpea.

Karagöz et al. (2022) conducted a study to analyse the genetic diversity and population structure in oregano (*Origanum acutidens* L.) based on agro-morphological properties using SCoT markers. A total of 70 oregano genotypes were collected from different regions of Türkiye. PCA analysis was performed, and the first principal component explained 66.86% of the total variation and was strongly correlated with fresh herb yield,

dry herb yield, dry leaf yield, leaf stem rate, number of branches, canopy diameter, plant height, and chlorophyll content. The second principal component accounted for 12.69% of the total agro-morphological variation and was mainly influenced by essential oil ratios. Based on the results of the PCA, the authors were able to identify superior genotypes. They concluded that SCoT markers could be effectively used for genetic diversity and molecular analysis of oregano genotypes.

Table 4. Data set of principle coordinate analysis of 5 *H.annus* genotypes

Axis	Eigenvalue	Percent
1	22.544	41.748
2	44998	24.629
3	9.9895	18.499
4	8.1669	15.124
5	-6.0279E-16	-1.1163E-15

Principle coordinate analysis of *H.annus* generated with using 13 SCoT pirmers. The maximum eigenvalue was determined on the axis 2 with 44998, while the maximum data was belonged to axis 4 with 8.1669. In addition, maximum percentage was belonged to axis 1 with 41.748%, on the other hand minimum percentage was belonged to axis 4 with 15.124%.

The principal eigenvalue of an operator is a principal concept in modern analysis. The principle of eigenvalue is used to characterize the steadiness of the equilibrium of a reaction-diffusion equation enabling the description of perseverance criteria (Cantrell and Cosner 1989; Cantrell and Cosner 1998).

Karagöz et al. (2022) studied that genetic diversity and population structure in oregano (*Origanum acutidens* L.) on agro-morphological properties with SCoT markers. According to results of PCA analysis indicated that with eigenvalues accounted for 79.56% of the total variation.

Mandal et al. (2023) studied that genetic dissection, relationship and population structure of drumstick (*Moringa oleifera* Lam.) using agromorphological and with 36 SCoT markers. 20 primers showed polymorphic characterisation. Molecular variance using principal component analysis (PCA) showed 30.25% variations in the first three axes (axis 1-13.33%, axis 2- 9.33%, and axis 3- 7.59%, respectively).

6. CONCLUSION

According to our results, we can state that SCoT marker system can be useful in case of sunflower genotype diversity analysis. This is a preliminary study to find DNA sequence linked to drought tolerance. Especially, drought tolerant cultivars TUNCA and P64LL62 showed similar characteristics according to our SCoT diversity analysis. We assume that the drought tolerance was introduced from the same gene source, and because of this, these genotypes have similar genetical background. We could identify specific DNA fragments appearing only in tolerant cultivars. Further experiments are needed, these PCR amplicons must be sequenced and BLAST searches can give direct answer about the character of these bands. However, further research is needed to confirm and ensure that the samples of sunflower genotypes by using more molecular markers analysis.

SUMMARY

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Molecular Marker Based Comparison of Drought Tolerant and Sensitive Turkish Sunflower (*Helianthus annus* L.) Cultivars Using SCoT Markers

MSc in Agricultural Biotechnology

Helianthus annuus L. is the second most notable oilseed crop all around the world, after soybean, and comes from temperate North America. Sunflowers use for not only human consumption it uses also for many purposes. Morphological, geographical, molecular, and archaeological data indicate that sunflowers used as food, medicine, body painting in rituals, bioenergy and dye. Sunflower is a self-fertilize plant and it needs to pollen activity and honey bee for fertilization.

There are some objectives in terms of sunflower breeding. These are drought resistance, resistance to disease and pets, breeding for self-fertile lines and branching shape. The pioneering sunflower breeding study was belonged to develop varieties with increased oil content. This was followed by the development of cytoplasmic male sterility (CMS) and haploid induction methods. In order to produce new cultivars with classical breeding methods is time consuming. To reduce this period uses double haploid technology and molecular marker-based technology.

Molecular markers have been employed in a wide range of fields, including genetic mapping, paternity testing, identification of mutant genes related to hereditary diseases, cultivar identification, marker-assisted crop breeding, population history, epidemiology, food safety, and population studies. Among these, the start codon targeted (SCoT) marker has gained popularity for its ability to target a specific region around the ATG start codon, which is conserved across all plant species. SCoT markers are considered useful tools for studying genetic diversity in various plant species due to their simplicity, cost-effectiveness, high polymorphism, reproducibility, and time-saving attributes.

Drought is defined as geographic location, amount, and time of precipitation. Also, it is defined as a shortage of water availability sufficient to cause a loss in yield or a period of no rainfall or irrigation that results in insufficient soil moisture leading to reduced crop growth and yield. Sunflower is one of the plants that needs

a high amount of water during the development period. Studies of sunflower mainly focuses on drought response.

This study aims to compare drought tolerant and sensitive Turkish cultivars using the SCoT marker system to find a linked DNA region that could be responsible for the tolerance.

This research conducted with five common sunflower (*Helianthus annus* L.) genotypes that from three of them was drought tolerant, two of them was drought sensitive genotypes. The DNA of each sample was isolated from fresh leaves using the DNeasy Plant Mini Kit. Altogether 25 primers were tested and 13 SCoT primers gave enough polymorphism and PCR amplification was performed in a 2720 thermocycler. DNA bands were visualized by UV illumination using a gel document system (Bio-Rad). The approximate fragment size was compared with the GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific).

Banding profiles generated by SCoT primers were compiled into a data binary matrix based on the presence or absence of the selected band. The dendrogram and principal component analysis (PCA) were conducted using Past 4.04 software.

Altogether 25 primers were tested and 13 SCoT primers successfully amplified 3 droughts tolerant and 2 drought sensitive genotypes of *Helianthus annus* L. and 13 SCoT primers gave 100% amplification with polymorphism. The total number of bands from 13 SCoT primers was 161, with 125 bands showing polymorphism and the average percentage of polymorphic bands was 76.79%. The size range of bands was from 250-8000 bp. The total number of bands range from 4 to 27 an average of 12.38 bands, while the number of polymorphic bands varied from 2 to 21 an average of 9.61 bands per primer.

Based on the similarity and difference matrices highest value was belonged to drought tolerant cultivar with name 8129R. On the other hand, drought sensitive genotypes 9718A and 97251A have shown closely related values. The PCA result supports the results of similarity and distance matrices, which shows that 9718A and 97251A are closely related.

In conclusion, the SCoT marker can be used as an effective molecular marker in case of sunflower genetic diversity analysis. Especially, drought tolerant cultivars TUNCA and P64LL62 showed similar characteristics in terms of genetical background of drought tolerant.

ACKNOWLEDGEMENTS

The author is grateful to Mustafa Kemal ATATÜRK, the founder of the Turkish Republic and who gave equal citizenship rights to Turkish women.

The author would like to thank to all the people who helped and supported her in this work:

1. Prof. Júlia Halász, as supervisor, for her support, guidance, care, patience, knowledge, comments, and suggestions during the laboratory work and writing process.
2. Dr. Zsuzsanna Benyóné György for her help, support, guidance, care, patience, and knowledge during laboratory work.
3. Prof. Attila Hegedűs, Head of the Department of Plant Biotechnology, for his support and knowledge.
4. Republic of Türkiye Ministry of National Education.
5. Republic of Türkiye General Directorate of Higher Education and Foreign Education.
6. Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policies.
7. Trakya Agricultural Research Institute.
8. My beloved husband Dr. Hasan Alp ŞAHİN my precious parents Müşerref and Yusuf SOLDAMLI, and my beloved siblings Zeynepnur and Ahmet SOLDAMLI for their support the author to complete this thesis and study well.

This author has a financial scholarship from Republic of Türkiye.

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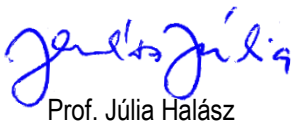

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