Spinach (*Spinacia oleracea* L.) Breeding: From Classical to Genomics-Centric Approach

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Abstract

The nutritious leafy vegetable, spinach (*Spinacia oleracea* L.) having diploid chromosome numbers, $2n = 2\times = 12$, is a versatile wind-pollinated crop which is rich in health-promoting minerals and vitamins. Majority of the spinach plants are dioecious in nature and it is gaining popularity throughout the world owing to nutrient content of this economically important cool season leafy crop. This crop is effected by several devastating biotic and abiotic stresses which need to be managed using the modern biotechnological tools. In this context, the breeding for overcoming these problems have gained momentum in the post-genomics era. Hence, numerous quantitative trait loci (QTLs), genes, and molecular markers linked with different phenotypic traits like leaf shape, flowering traits, nutritional traits, etc., have been identified in the past decades. But, still there is an urgent need to breed spinach for decreasing the anti-nutritional factors like oxalates, consumption of which can cause health issues. In the post-genomics era, plethora of genomic and sequence resources of spinach have been made available, which have the potential to accelerate spinach breeding program. Development of downy mildew-resistant cultivars of Spinach via introgression of NBS-LRR (nucleotide-binding site leucine-rich repeat) genes from wild allies have been made successful. In the past decade, the genomics have provided insight into sex evolution in spinach and various candidate miRNAs (micro RNAs) related to sex forms in spinach have been identified. In this chapter, we have provided detailed

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overview of progress made in spinach genetic improvement in the post-genomics era.

Keywords

Spinach · Breeding · Genetics · Spinach genomics · Spinach sex expression

6.1 Introduction

Cultivated Spinach (*Spinacia oleracea* L., 2n = 2x = 12) is a highly nutritious leafy vegetable crop that is consumed throughout the world (Morelock and Correll 2008a, b). Succulent leaves with its stems are edible either as cooked or in raw form. Recently, the development of baby leaf spinach with the fortification of nutrient content increased the popularity and consumption of this leafy vegetable. The word 'spinach' is derived from the Spanish word '*espinaca*' (Ryder 1979) which is an annual, diploid, dioecious, wind-pollinated, and highly heterozygous crop and belongs to the family *Amaranthaceae* under the Caryophyllales order. Many other important agricultural crops such as beet, quinoa, and amaranth are also member of this family (Hassler 2018). Typically, spinach has distinct vegetative and reproductive phases that form a rosette of leaves at the end of winter followed by induction of bolting with the arrival of warm-long summer days (Krarup and Moreira 1998). With the changes to various photoperiods and climatic conditions, few spinach cultivars show resistance to bolting under warm conditions which favors its cultivation even in summer conditions (Van der Vossen 2004). Although spinach is a dioecious crop, monoecious plants are also found in nature (Khattak et al. 2006). Further, it has tetramorphic sex expression which is classified based on sex and plant morphology (Rosa 1925). The phenomenon of sex reversion is also reported in several plants that results in the formation of gynomonoecious and andromonoecious plants derived from female and male plants, respectively (Komai and Masuda 2004; Morelock and Correll 2008a, b). Over the last few decades, spinach production is gradually increased reaching 30.1 million tons in 2019 with productivity of 32.36 t/ha. China accounts for major production (nearly 91%) of spinach followed by USA, Turkey, Japan, Kenya, Indonesia, France, Iran, Pakistan, and Italy (FAOSTAT 2019). Worldwide increased understanding of health and nutrition as well as growing habit towards intake of low-calorie diets have boost-up the demand for spinach over the past few decades as it is rich in several nutritional elements.

From a nutritional point of view, spinach is regarded as a superfood as it is rich in several minerals especially, iron, vitamins, folate, proteins, and flavonoids (Roberts and Moreau 2016) including a substantial amount of carotenoids vitamin A, lutein, and zeaxanthin (Bunea et al. 2008) and other antioxidant compounds such as vitamin C and vitamin E (Chun et al. 2005; Pandjaitan et al. 2005). Often the nutritive value of spinach is higher than many common leafy vegetables. Regular consumption of spinach facilitates several health benefits as it exhibits anti-inflammatory, anti-tumoral, and anti-obesity properties as well as able to overcome the problem of

anemia and age-related muscular disorder. Among all the vegetable crops, it exhibits one of the highest ORAC (oxygen radical absorbance capacity) values. However, spinach leaf possesses some anti-nutritional factor that causes some negative effect on the human body. It is one of the rich sources of oxalic acid which is reported to reduce the bioavailability of certain minerals such as Ca, Mg, and Zn (Kelsay and Prather 1983; Heaney et al. 1988; Noonan and Savage 1999; Bohn et al. 2004) and in severe cases may lead to the formation of kidney stones in human body (Noonan and Savage 1999; Ermer et al. 2016). Apart from oxalic acid, spinach leaf contains nitrate (NO_3) which converts into nitrite (NO_2) in the human digestive system by the activity of commensal bacteria (Tiso and Schechter 2015), and later, this NO_3 binds to hemoglobin to form methemoglobin that blocks the binding of oxygen to hemoglobin, potentially causing methemoglobinemia (Santamaria 2006). Moreover, the cultivation of spinach has potential for agricultural crop diversification in remote areas as well as having the ability to combat the nutrition-related disorder in the human body in many parts of the world.

Inspite of its high nutritional and economic value, the genetic resources of spinach have been less exploited, and very few initiatives have been taken towards its improvement. Very recently, the spinach genome has been sequenced fully and some advanced genomic resources including transcriptome sequences and genome variant data have become publicly accessible. Further information on spinach breeding history, related wild species, and the domestication process was very limited and not well reviewed except in a recent article published by Ribera et al. (2020). Keeping in view, this chapter deals with all the above-mentioned facts and summarizes the key information related to the genetic and genomic resources of spinach and the breeding goals achieved in the past and in these recent post-genomics eras.

6.2 Botanical Overview of the Genus *Spinacia*

Spinach is a biennial crop that produces leaf in first season followed by seed in the next season. During vegetative phase, it forms rosettes of fleshy leaves that can be crinkled or smooth. Bolting occurs when the plant shifts to the reproductive phase from vegetative phase by producing a peduncle of about one-meter tall terminal staminate flowers (Krarup and Moreira 1998) and/or pistillate blooms at bract axils with the onset of warm and long summer days (Uotila 1997). Number of flowers is varied from 6 to 12 per cluster. Male plants bolt earlier than female plants, and they die soon after flower production (Rosa 1925). Flowers may be staminate, pistillate, or hermaphrodite, and remain receptive for a week or longer. Pistillate flowers are borne in clusters in the leaf axis and have a single ovary with four or five styles borne on a two- to four-toothed calyx. Staminate flowers are clustered on a spike. The shape of the pistillate flowers distinguishes cultivated spinach from wild *Spinacia* species. Species in the wild have clusters of fused blooms that develop into spiny aggregated fruits with many seeds. Spinach is a dioecious species which is pollinated by the wind, yet, monoecious plants do exist (Khattak et al. 2006). As numerous

groups of spinach are established based on sex and morphology of the flower, spinach sex expression is versatile (Rosa 1925). They are (a) extreme male plants—these are smaller in size and earlier to flower than others; (b) vegetative male plants—these are larger in size; (c) female plants—these are larger in size and remain vegetative for a longer period; and (d) monoecious plants—these plants may be exclusively staminate, pistillate, or purely pistillate early and staminate later, or almost equally pistillate and staminate throughout the flowering season. The extreme male plants do not have any commercial use. In improved varieties, extreme male plants are almost eliminated. Often carbohydrates and pigment content of the plants are being utilized to differentiate male plants from female. Sugars, chlorophyll, and carotenoid contents are higher in female than in male plants (Sivtsev and Sizov 1972). Some dioecious plants may show sex reversion that results in the formation of gynomonoecious and andromonoecious plants derived from female and male plants, respectively (Komai and Masuda 2004; Morelock and Correll 2008a, b). Now-a-days, many cultivars exist with different leaf characteristics, ranging from round to hastate leaves as well as flat to crinkly (savoy) texture (Morelock and Correll 2008a, b). Seeds are of two types: round seeded (summer spinach) or prickly seeded (winter spinach). Adaptation to various photoperiods and climatic conditions is also evident. For example, certain cultivars are resistant to bolting under long and warm days that make them possible to cultivate under summer (Van der Vossen 2004).

6.3 Gene Pool and Genetic Resources of the Crop

Cultivated spinach consists of primary and tertiary gene pools where genus *Spinacia* belongs to the primary and genus *Blitum* belongs to the tertiary gene pool. Presently, genus *Blitum* contains 11 species and genus *Spinacia* consists of two species such as *S. tetrandra* and *S. turkestanica* (Table 6.1) (Vincent et al. 2013; Ribera et al. 2020). Earlier, it was assumed that *S. tetrandra* might be the wild ancestor of *S. oleracea* but a recent report suggests that phylogenetically *S. turkestanica* is more closer to

Table 6.1 Gene pool of genus *Spinacia*

Primary gene pool	Tertiary gene pool
Spinacia turkestanica Iljin	*Blitum asiaticum* (Fisch. & C.A. Mey.) S. Fuentes et al.
Spinacia tetrandra Steven ex M. Bieb	*Blitum atriplicinum* F. Mu¨ll.
	Blitum bonus-henricus (L.) Rchb
	Blitum litwinowii (Paulsen) S. Fuentes et al.
	Blitum nuttallianum Roem. & Schult.
	Blitum virgatum L.
	Blitum spathulatum (A. Gray) S. Fuentes et al.
	Blitum californicum
	Blitum capitatum L.
	Blitum hastatum Rydb.
	Blitum korshinskyi Litv.

S. oleracea rather than *S. tetrandra*. Further, reduced fertility both in direct and reciprocal crossing between *S. oleracea* and *S. tetrandra* arises the question whether it really belongs to the primary gene pool or secondary gene pool (Fujito et al. 2015).

Worldwide nearly 2208 accessions of the genus *Spinacia* are maintained of which 2052 accessions belong to *S. oleracea*, 93 belongs to *S. turkestanica*, and 63 belongs to *S. tetrandra* (Fig. 6.1) indicating poor representation of the *Spinacia* accessions which could be further improved by introducing accessions from unexplored regions, like *S. turkestanica* from South and South-West Asia and *S. tetrandra* from the Middle East and South-West Asia. Information on accessions from European countries could be collected from European Search Catalogue for Plant Genetic Resources (EURISCO) (https://www.ecpgr.cgiar.org/) and information of accessions about USA germplasms are maintained under National Plant Germplasm System (NPGS)—Germplasm Resources Information Network (GRIN) (https://www.ars-grin.gov/) system which are major source of *Spinacia* accessions. Further information on worldwide spinach collections can be found under Genesys system (https://www.genesys-pgr.org/).

Germplasms/accessions conserved under various gene banks were frequently utilized by different breeders around the world for carrying out pre-breeding activities that not only give an idea about the degree of genetic diversity and structure of the population but also characterize different quality traits (low oxalate, high carotene, high iron, high ascorbic acid, high micronutrients) as well as its tolerance against several biotic (e.g., fungal diseases such as downy mildew, white rust, leaf spot, fusarium wilt; viral diseases such as *Cucumber Mosaic Virus-CMV-1*, *Beet Mosaic Virus*, and abiotic stresses (e.g., osmotic stress, salinity stress, high temperature, heavy metal stress, nitrogen stress). Details of few of these characterize accessions are presented in Table 6.2. For genetic diversity studies, earlier SSR markers were widely used (Li et al. 2018; Göl et al. 2017) but now-a-days SNP markers are developed through Genotyping-by-Sequencing (GBS)-based approach (Shi et al. 2017; Gyawali et al. 2021) which is used to fulfill the aim as the cost of sequencing becomes very cheap. Recently, utilizing the already-available genomic resources of *Sp75* genome of Spinach and whole-genome sequencing (30×) of 21 accessions, novel SSR markers were developed which were further used for diversity analysis of multiple spinach accessions (Bhattarai et al. 2021).

6.4 Origin and Genomic Basis of Spinach Domestication

The primary center of origin of spinach is considered to be in the region of central Asia, most probably in Persia (presently in Iran) (Pandey and Kalloo 1993) and later, it was spread to other regions as evidence on the presence of the crop was not found in the ancient Greek or Roman civilizations (Boswell 1949). The oldest literature indicates that spinach was first time consumed in Mesopotamia in the fourth century AD. While, from written evidence it was found that cultivation of spinach begins in China in seventh century AD and arrived in China through India and Nepal (Laufer 1919). In the northern part of Africa, it was introduced by Arabs and later by the

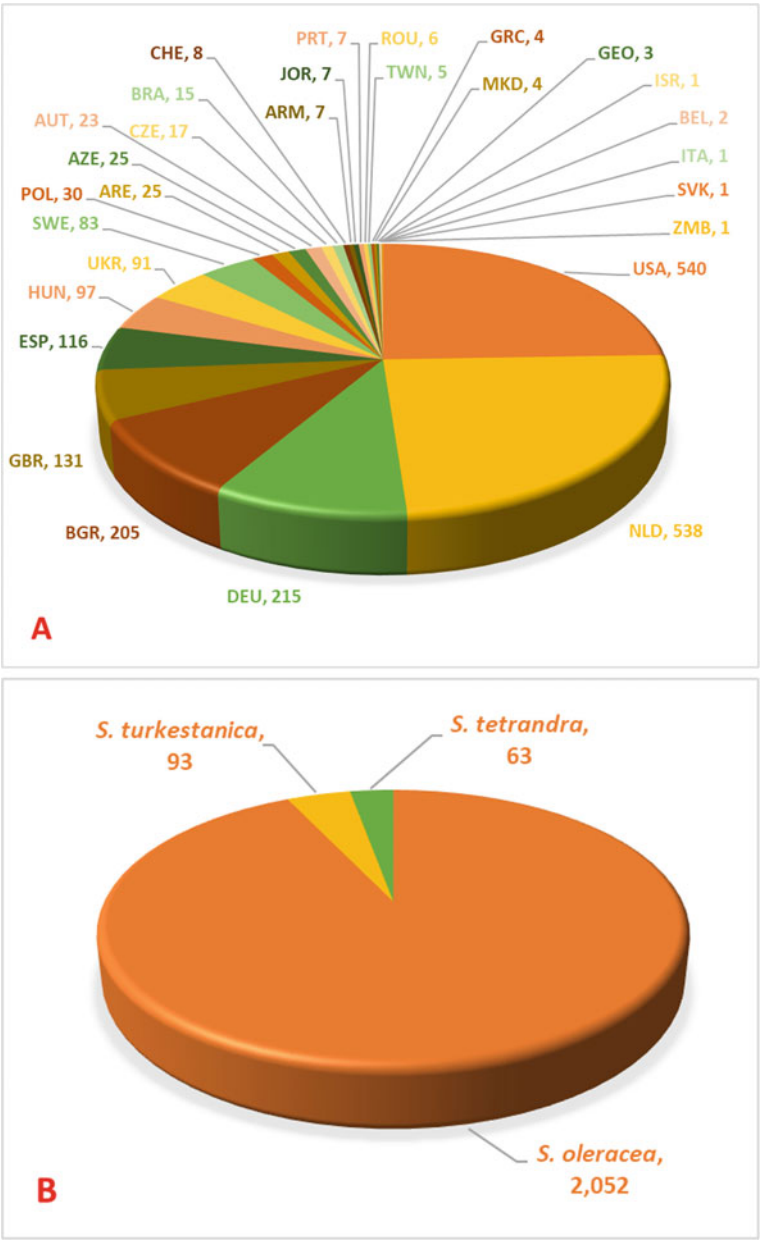

Fig. 6.1 (**a**) Number of spinach germplasms maintained in different countries. (**b**) Number of spinach accessions available under different species. Names of the country are denoted by their WIEWS code and data on number of accessions are downloaded on 4-5-21 from Genesys system (https://www.genesys-pgr.org/a/overview/v20RWL9OWaG)

Table 6.2 Details of different germplasms/accessions characterize for different resistant and quality traits

Resistant traits	Germplasm/variety	Reference
Leaf miner	PI274065 and PI1743854	Mou (2008a, b, c)
Leaf miner	PI220121, PI274059, PI358248, PI445783, PI449353, and PI531454	Shi and Mou (2016)
Downy mildew (*Peronospora farinosa* f. sp. *spinaciae*)	PI 140464 and PI 140467; *S. turkestanica* and *S. tetrandra*	Smith (1950); Smith and Zahara (1956); Correll et al. (1994); Qian et al. (2016)
Downy mildew	Races 1, 2: Nores Races 1, 2, 3: St. Helens and Polka	Brandenberger et al. (1992)
Downy mildew	Race 1: Symphonie Races 1, 2, 3, 4: Bolero, Palco, Previa, S. Carlos and Tamura	Nali (1998)
Downy mildew (*Pfs3* and *Pfs4*)	Race 3: Mazurka, Polka, and Rhythm Race 4: Bolero and Bossanova	Morelock (1999)
Downy mildew (against 10 races)	El Dorado, El Palmar, Emilia, Lazio and Lombardia	Irish (2004)
Downy mildew	Dolphin (races 1–7, 9) Lion (races 1–9) Resistofly (races 1 and 2) Califlay (races 1,3,5,8, and 9) Polka (races 1, 2, 3, and 5) Whitney (races 1,2,3,4) Rushmore (races 1,3,4,5,8,9) Campania (races 1, 2, 3, 4, 7)	Irish et al. (2007)
Downy mildew (*Pfs4*)	*S. oleracea* SPI 82/87 originating from Iraq, and *S. turkestanica* CGN09546 from Uzbekistan	Morelock and Correll (2008a, b)
White rust (*Albugo occidentalis*)	'Hybrid 178' and the USDA breeding line WRG 70-5, Fallgreen	Bowers (1972); Morelock (1999)
White rust	Green Stand, 'Green Valley 11' × PH 1452, 4C × 119, Coho, PI 165012, PI 17186, PI 173129, PI 174387, PI 217425, and PI 321020	Black and Dainello (1986)
White rust (moderate resistance)	Coho, Lessley, Padre, and Sassy	Morelock (1999)
White rust	NSL 6098, PI 175311, PI 220686, PI 224959, PI 226671, PI 227045, and PI 648958	Shi et al. (2022)
Leaf spot (*Stemphylium botryosum*) (moderate resistance)	PI 169685 and PI 173809	Mou et al. (2008)
Leaf spot	Shelby, Perentie and Goldeneye	Wadlington et al. (2018)
Cucumber Mosaic Virus-1 (*CMV-1*)	Dixie Market and Fall Green	Goode et al. (1988)
	Roga, Lavires and Mona Lisa	Chod (1985)
		Schmidt and Schubert (1980)

(continued)

Table 6.2 (continued)

Resistant traits	Germplasm/variety	Reference
	Elsom's Special 23, Dominant Hunder up, Achille (Winter giant type), Parry (F1 hybrid) and Selandia Qsena	
CMV (all strains)	Jolina	Schmidt and Schubert (1980)
Beet Mosaic Virus	Roga and Lavires	Chod (1985)
Fusarium wilt (*Fusarium oxysporum* f. sp. *Spinaciae*)	EH 7, EH 10, Hybrid 50, EH 424, EH 425, 'Ozarka', 'Green Valley' and WRG 70-5	O'Brien and Winters (1977)
Quality traits	**Germplasm/variety**	**Reference**
Late bolting	Nobel, Viking	Sneep (1982)
Late bolting and erect leaves	PI103063, PI169678, PI169684, PI171863, PI171865, PI174386, PI175929, and PI648963	Shi et al. (2016a, b, c)
Winter-hardy	Ge'ant d'Hiver'	Sneep (1982)
Highest levels of Na, K, Mg and Ca	PI209644 from Iraq, PI531456 from Hungary, PI204732 from Turkey, and PI205231 from Turkey, respectively	Qin et al. (2017a, b)
Low oxalate concentration	PI445782 ('Shami') and PI 445784 ('Baladi')	Shi et al. (2016a, b, c)
High ascorbic acid content	PI 648953, PI 648959, and PI 648945	Rueda et al. (2021)
Higher carotene content	Bitola, Ohrid and Prilep	Cirkova-Georgieva et al. (1970)
High iron content	Bloomsdale long standing	Tronickova et al. (1965)

Moors eleventh century AD. Finally, it moved to other regions of Europe and USA in the fifteenth century AD and in the eighteenth century AD, respectively (Scheewe and Reimann-Philipp 1986). Spinach basically spreads into two different directions one to Southern and Eastern Asia and another to Africa, the Mediterranean region, and Northern Europe, from where it was introduced to USA. These two regions applied two different modes of selection pressure that results in the regeneration of two types of cultivars: (1) Asian type and (2) Western type (Simoons 1990; Van der Vossen 2004). Asian cultivars have narrow, hastate, smooth leaves with long petioles, whereas western cultivars have round expanding leaves with savoy leaf texture (Van der Vossen 2004).

As very less difference exist between wild and cultivated *S. oleracea* species, domestication syndrome traits cannot be differentiated properly. It was found that leaf shape and other morphological traits were not probably the domestication syndrome of spinach. While, sex forms like monoecism might be the potential domestication trait of spinach (Ribera et al. 2020). The appearance of smooth type of fruit was the first time evidence from Europe during fifteenth century AD (Bock

1539), which indicates that this trait was not the part of the domestication syndrome of spinach. Further, this smooth type of phenotype was also found in different landraces from the Middle East (Sabaghnia et al. 2014; Mohebodini et al. 2017). However, seed dormancy was one of the part of domestication syndrome traits as seed germination percentage was typically high in the cultivated spinach as compared to wild types (Van Treuren et al. 2019). This domestication trait was very common and was selected in parallel to a number of crop families (Rendón-Anaya and Herrera-Estrella 2018; Wang et al. 2018a). With an aim to investigate the changes within genome level during domestication of spinach Cai et al. (2021) compared the genome of *S. oleracea* with its closest wild relatives *S. turkestanica* and were able to identify total of 996 domestication sweeps within *S. oleracea* genome that spans over 17.6 Mb, harboring total 748 genes. This sweep coincides with many QTLs associated with many important vegetative and reproductive traits of spinach. This study also depicted that selection for savoy types of leaf might be highly desirable during human selection process of spinach. Further, high XP-CLR scores at the 52 Mb genomic region of chromosome three proved the occurrence of earlier differentiation of *S. turkestanica* into two groups in central asia which was attributable to the occurrence of a selective sweep at this region (Gyawali et al. 2021).

6.5 Genomic Resources of *Spinach*

The first draft genome sequence of spinach was assembled to 498 Mb using the cv. Viroflay representing half of the estimated genome size (989 Mb) of spinach determined through the C-value enigma (Dohm et al. 2014). However, recently sequenced draft genome of spinach line *Sp75* using the whole-genome shotgun approach combined with BioNano Genomics optical maps, available in SpinachBase (http://www.spinachbase.org), revealed the estimated genome size of 996 Mb with a scaffold (N50) size of 2.2 Mb that is closer to the genome size estimated based on the C-value enigma (Xu et al. 2017). The draft genome of *Sp75* (designated as *Spov1*) consisted of more than 70% of transposable elements (TEs) of which Copia and Gypsy retro-elements are predominant, as like many other plant genomes. The draft genome of spinach is one of the most highly repetitive plant genomes to date that contains 74.4% of repetitive sequences and approximately 25,500 protein-coding genes of which 139 NBS-LRR genes govern disease resistance in spinach plants. Further, the genome size of spinach is quite larger than its related species sugar beet, and analysis of Genome synteny between spinach and sugar beet suggests the occurrence of inter- and intra-chromosomal rearrangements during the genomic evolution of Caryophyllales order (Xu et al. 2017). The second draft genome assembly was constructed using cv. Viroflay (designated as *Spov2*) using Illumina short reads of Illumina and SMRT sequencing technique of Pacific Biosciences (PacBio), it comprised 968.8 Mb and nearly 26,862 genes (Hulse-Kemp et al. 2021). Further, PacBio libraries and Illumina PE data were assembled to 913.5 Mb (70× genome coverage) to generate a new assembly designated as *Spov3* (available at

https://phytozome-next.jgi.doe.gov/info/Soleracea_Spov3). The *Spov3* genome assembly consisted of 745 Mb which is 81.56% of the whole genome and contains 34,877 annotated genes, of which 1004 genes encode for disease resistance. Very recently, high-quality *de novo* reference genome of spinach (designated as *SOL_r1.1*) was published using PacBio long-read and Illumina short reads comprising 287 scaffolds with an estimated total genome size of 935.7 Mb (N50 = 11.3 Mb), which is 73.59% of the whole assembly anchored to six pseudo-chromosomes (Hirakawa et al. 2021). The complete annotated chloroplast genome of spinach is also available (Schmitz-Linneweber et al. 2001). A chromosome-scale reference genome assembly was also generated using an inbred line 'Monoe-Viroflay' comprising of 894.3 Mb (N50 contig = 23.8 Mb), which was around 98.32% of the whole assembly significantly higher than the previously released genome assemblies (Cai et al. 2021; Bhattarai and Shi 2021). In this assembly, nearly 28,964 protein-coding genes were predicted of which 115 genes belong to NBS-LRR groups. Further, NCBI database (https://www.ncbi.nlm.nih.gov/) stores 363 genomic sequences, 284 mRNA sequences, and 16 rRNA sequences. The database sequence information was used to create a set of 35 primer pairs of which 13 were used in a diversity study of cultivated spinach (Groben and Wricke 1998; Khattak et al. 2007). The genome sequencing of 305 wild (7 *S. turkestanica*, and 3 *S. tetrandra*) and cultivated spinach accessions (295 *S. oleracea*) are publicly available where 5,511,663 SNPs, and 55,330 structural variants (SVs) are detected within this spinach populations. Inspite of the genome sequencing approach of this large population, comparative genomics between cultivated spinach and its closest wild relatives such as *S. turkestanica* and *S. tetrandra* are hampered due to a lack of more detailed information on genome assemblies of these two wild species. However, some initiatives were taken in the recent past in collaboration with private breeding companies for the creation of reference genome assemblies of these two species (Ribera et al. 2020).

6.6 Genetics and Epigenetic Regulation on Flower-Sex Expression

From previous discussions, it is well known that spinach is a pre-dominantly dioecious plant that produces male and female plants in equal ratio. Apart from dioecy, monoecious plants also exist in spinach plants (Janick and Stevenson 1955). For this dioecious nature, it has been found that the mechanism of sex control is quite similar to the sex control mechanism of animals which is governed by a pair of sex chromosomes such as X and Y as per the report of Janick (1954) for the determination of sex in spinach. Selfing of male flower is always segregated into male and female plants while selfing of female monoecious plants does not produce any male plants. Hence, it can be suggested that maleness is determined by heterogametic sex (XY) and femaleness by homogametic sex (XX) (Janick 1954). The homomorphic and heteromorphic sex chromosomes are homologs to each other and evolved from the same ancestry. However, it is not clear that how these X and Y-linked genes were

diverged and dioecious plants are evolved. Based on the evolutionary theory of divergence of sexes and sex chromosomes, hermaphroditism is believed to be the ancestor of diocey, and the sex chromosomes are evolved from autosomes. Further, previous reports also suggest that two mutation models are actually responsible for the evolution of male and female plants. The recessive mutation causes male sterility and produces female plants and one dominant mutation causes female sterility, producing male plants (Charlesworth and Charlesworth 1978). Further, this X and Y locus is tightly linked which might be due to the suppression of recombination between two locus (Pannell and Gerchen 2018). In the course of evolution, these regions were continuously expanded with the accumulation of retrotransposons and other repetitive sequences (Charlesworth 2019). This non-recombining region is referred to as male-specific region (MSR).

In spinach, the mechanism of sex determination is also not very clear. Comparing linkage map between spinach and sugarbeet, it was found that chromosome 4 of spinach exhibited high synteny with chromosomes 4 and 9 of sugar beet for these sex chromosomes. Later, this sex-determining genes were mapped on chromosome IV of spinach using high-density linkage map constructed through Bulked Segregant Analysis (BSA) followed by specific-locus amplified fragment sequencing (SLAF-seq) technology (Super BSA) by Qian et al. (2017). After the publication of the spinach draft genome by Xu et al. (2017), 120 SLAF markers were mapped on chromosome IV between 21 and 110 mb, serving as a candidate region for sex-determining genes (She et al. 2021). Later, She et al. (2021) were able to conclude that this region (~21 kb on chromosome 4) could be MSR and also identified a tightly linked KASP marker, namely SponR with MSR locus. Further, RNA-seq analysis was done using male and female progeny generated from sib-mating of dioecious plants to identify the genes governing MSR locus by Okazaki et al. (2019) and identified 354 SNPs from 219 transcripts, of which 12 are found to be closely linked with MSR locus after validation using large-scale spinach population. In spinach, monoecism is governed by an independent incompletely dominant gene (X^m) which is allelic to X/Y locus (Janick and Stevenson 1955). Further, to narrow down the location of the monoecious governing gene, 19 AFLP markers were mapped of which four were converted into SCAR markers that are linked to both monoecious (M) and Y genes (Yamamoto et al. 2014).

In epigenome level, methylation at CpG and CpNpG domain is very common which ultimately alters the gene expression in higher plants. In papaya, significant association between DNA methylation and hetero-chromatinization with identification of sex at early stages was already reported by Zhang et al. (2008). Thus, it can be assumed that there might be any effect of DNA methylation patterns on sex determination of dioecious spinach. With this aim, Gao et al. (2014) tried to find out the effect of DNA methylation pattern on leaves of both male and female plants using demethylating agent 5-azac and reported that sexual dimorphism in spinach was not influenced by the DNA methylation pattern on vegetative parts such as leaves but on reproductive organs mainly in flowers.

6.7 Genomics-aided Breeding for Quality Traits and Stress Resistance

6.7.1 Breeding for Improvement of Quality Traits

Spinach consumption can be encouraged through the optimization of nutrient concentrations along with consumer-preferred texture, color, and taste. Among the two types of leaf, savoy-leaved cultivars also known as Bloomsdale-type spinach and for distant markets these types are mostly preferred over smooth-leaved types as crinkles keep them less compact during packing and transporting that results in an extended shelf life (Sneep 1982; Rubatzky and Yamaguchi 1997). It is well established that growing methods and preservation processes have an impact on nutritional compositions of the leaf (Lester et al. 2010; Koh et al. 2012) as well as existence of wide range of nutritional compositions among cultivars (or types) facilitates a possibility of quality improvement through breeding program (Howard et al. 2002; Morelock and Correll 2008a, b; Wang et al. 2018). The objective of any quality breeding program is to maximize health-related compounds and to minimize anti-nutritional compounds. To achieve this goal, identification of QTLs and their associated linked marker are highly important. Recently, SNP markers were identified through Genome-wide Association Studies (GWAS) and were found to be associated with reduced oxalate concentration (Shi et al. 2016c) and with 14 important mineral elements in leaf (Qin et al. 2017a, b). A major QTL and three candidate genes were also found to be associated with leaf color (Cai et al. 2018). Further, SNP markers were also identified for plant size, bolting, and other leaf morphological traits such as petiole color, leaf texture, leaf margin shape, and leaf erectness in spinach (Chitwood et al. 2016; Ma et al. 2016). Chitwood et al. (2016) identified Three, eight, and four SNPs were reported to be associated with bolting, plant height, and erectness, respectively, and Ma et al. (2016) identified five, seven, and 14 SNPs found to be associated with leaf traits such as surface texture, edge shape, and petiole color, respectively. Further, 99 SNPs were shown to be strongly linked with important growth parameters through genome-wide association studies using a single-nucleotide polymorphism (SNP) panel generated by ddRADseq (Awika et al. 2019b).

6.7.2 Breeding for Biotic Stress Resistance

6.7.2.1 Downy Mildew

Downy mildew (DM) (or blue mold), caused by *Peronospora farinosa* f. sp. *spinaciae* Byford [= *P. effusa* (Grev.) Ces.] is the most widespread, destructive, and commercially important disease of spinach that results in serious economic and agronomic impacts on spinach production at the global scale (Correll et al. 1994, 2011). The typical symptoms are slightly yellow, irregular chlorotic lesions on leaves, leaf curling, and distortion. *S. turkestanica* and *S. tetrandra* are found to be potential source of resistance for downy mildew disease (Table 6.2). However,

evolution of new races at a rapid rate hinders spinach production. Race 1 of DM pathogen was first reported in 1824 (Greville 1824), and its resistance was identified in two accessions (PI 140467 and PI 140467) from Iran (Smith 1950). Resistance against race 3 (1976) was incorporated into hybrids of 'Mazurka,' 'Polka,' and 'Rhythm,' and these hybrids were released in 1978 (Morelock 1999).

The *RPF1* gene, which is one of the primary genes demonstrating resistance against a particular race of *P. effusa* in spinach, was identified using a SLAF-Seq technique paired with BSA. Out of six different *RPF* loci which govern resistance against the *P. effusa* races, *RPF1*, *RPF2*, and *RPF3* have been genetically characterized. The *RPF1* locus, which is regulated by a single dominant allele, is located on chromosome 3 and has been found to be associated with the co-dominant marker, DM1. In addition, 14 candidate R genes were found within a 0.89 Mb region, with three of the most likely candidate genes identified through amino acid sequence analysis and conserved domain analysis between resistant and susceptible inbred lines, which aids in determining the functionality of the resistant gene as well as developing suitable markers for spinach breeding (Bhattarai et al. 2021). The development of downy mildew-resistant cultivars through introgression of NBS-LRR genes from wild relatives as well as the use of loss of functional alleles controlling susceptibility to the disease plays a crucial role in spinach breeding (Ribera et al. 2020). Such type of hybrid spinach cv. Whale contains downy mildew resistance locus *RPF3* that showed resistance against *P. effusa* races 1–3, 5, 8–9, 11–12, 14, 16, and susceptible to *P. effusa* races 4, 6–7, 10, 13, 15, while the DM-resistant cv. Lazio contains the *RPF2* and *RPF4* loci (Bhattarai et al. 2021). Furthermore, Genome-wide Association Studies (GWAS) use segregating populations from DM-resistant cv. Whale able to identify six significant SNPs related to *P. effusa* race 16. The discovery of race-specific resistance markers and the identification of candidate genomic region linked with *P. effusa* race 16 would improve the efficiency as well as precision of breeding for downy mildew resistance cultivars (She et al. 2018).

6.7.2.2 White Rust (WR)

WR in spinach is caused by *Albugo occidentalis*, an oomycete obligate pathogen that damages the vegetative and flowering structures of the infected plants, causing severe yield losses in spinach. The symptom is characterized by yellow lesions on the upper leaf surface and white pustules on the abaxial side (Henning et al. 2016). Resistance to WR in spinach is reported to be polygenic (Correll et al. 2011; Awika et al. 2019a). The commercial 'Hybrid 178' and the USDA breeding line 'WRG 70-5' are the key players in developing resistant cultivars (Table 6.2). The University of Arkansas also developed open-pollinated cultivar 'Fallgreen' with a high resistance level in long back 1987 (Morelock 1999). Inspite of the development of resistant cultivars in recent years, these old resources were mostly preferred by the spinach breeding industry to create novel white rust-resistant hybrids till now (Morelock and Correll 2008a, b).

WR disease severity ratings (WR-DSRs) were used in a diversity panel of 267 spinach accessions to define resistant and susceptibility associated groups within

the distribution scores and then tested the SNP variants to interrogate the minor alleles (MA) prevalence in the most susceptible (MS) vs. most resistant (MR) individuals using genome-wide association studies. In the comparison of the 25% MS and 25% MR accessions, 448 minor alleles (MA) linked with WR severity were discovered, but the MA was largely similar between the two halves of the interquartile range. The minor alleles of MS groups were found on all six chromosomes of which 71% were found to be highly correlated with WR resistance in a newly generated association panel. These findings suggest that the disproportionate overrepresentation of minor alleles may play a significant role in determining susceptibility and that this information could be utilized to select resistant plants. Furthermore, by focusing on the distribution tails, allelic mapping plays a crucial role in the identification of plant markers associated with quantitative traits on the most desirable segments of the phenotypic distribution (Awika et al. 2019a). Subsequently, 9 SNPs located on chromosomes 2, 3, 4, and 6 were associated with white rust resistance in GWAS panel (Shi et al. 2022).

6.7.2.3 Leaf Spot

Ascomycete fungi are the major causal agents for the occurrence of leaf spot diseases (Koike et al. 2007). *Colletotrichum dematium* (anthracnose), *Stemphylium botryosum*, and *Cladosporium variabile* are mainly responsible for causing leaf spots in spinach (De Visser 2015; Liu et al. 2018). However, *Cercospora beticola*, *Colletotrichum truncatum*, *Colletotrichum coccodes*, and *Myrothecium verrucaria* are the minor agents for the emergence of leaf spot diseases in spinach (Liu et al. 2018). In spinach, resistance to *Stemphylium* leaf spot is complex in nature. Association analysis revealed that eight SNP markers were strongly associated with *Stemphylium* leaf spot resistance, with a LOD score of 2.5 or above. Thus, these SNP markers could be a potential tool in marker-assisted backcross breeding (MABB) program for the development of *Stemphylium* leaf spot resistance line (Shi et al. 2016a).

6.7.2.4 Anthracnose

Anthracnose (*Colletotrichum dematium*) is one of the most emerging diseases in spinach that affects major spinach-growing regions in the world (Correll et al. 1994; Awika et al. 2020). To address this issue, genomics-governed disease trait characterization has been less exploited in spinach. Recently, a diverse group of 276 spinach accessions was screened for anthracnose disease severity by Awika et al. (2020) and reported that resistance to spinach anthracnose is governed by polygenes. Further, the group was also able to identify 49 significant marker-trait associations using various marker-identification strategies [single-SNP (sSNP), pairwise haplotype (htP), and multi-marker haplotype (htM)] that prove the power of haplotype-based association analysis over any single-SNP (sSNP) analysis.

6.7.2.5 Wilt

Wilt in spinach is caused by fungus *Fusarium oxysporum* f. sp. *spinaciae* and *Verticillium spp*. that threatens spinach cultivation worlwide (Correll et al. 1994;

Koike et al. 2007). Although *Verticillium* wilt is caused by *V. dahliae* in many vegetable and field crops but earlier it is not recognized in the case of spinach (du Toit et al. 2005). However, later Villarroel-Zeballosa and his associates (2012) were able to isolate *V. dahliae* types from the seeds of spinach accessions. O'Brien and Winters (1977) screened 205 PI accessions for *Fusarium* wilt of which six showed a moderate level of resistance and the resistance was found to be governed by a single dominant gene. However, Goode et al. (1988) reported polygenic resistance against *Fusarium* wilt pathogen using cv. 'Fall Green.' Recently, molecular markers associated with *V. dahliae* types were also identified by Shi et al. (2016b).

6.7.2.6 Leaf Miner

Leaf miner (*Liriomyza langei*) is one of the major insect-pest of spinach that possesses serious threats in spinach-growing areas around the world especially in USA. Although many species of *Liriomyza* are reported in many crops but *L. langei* act as a principal causal agent in spinach that was identified through polymerase chain reaction (PCR) amplification of its mitochondrial DNA (Scheffer et al. 2001). Due to variable responses among the spinach genotypes, resistance against leaf miner is found to be complex in nature (Mou 2008b). Till date, no information on the inheritance of leaf miner resistance is available in public domain and only two resistant germplasms of spinach have been released so far (Mou 2007a, b). Through conventional breeding techniques, it is very difficult to transfer these complex traits within a short time. Therefore, recently, 5 strongly associated SNP markers were identified for this trait through GWAS using an association panel of 300 US spinach accessions (Mou 2008b; Shi and Mou 2016). The group also reported that resistance against leafminer in spinach was governed by polygenes with minor effects.

6.7.3 Breeding for Abiotic Stress Resistance

In addition to biotic stresses, spinach is highly susceptible to various abiotic stresses such as water and salinity stress (Bagheri et al. 2015; Zuccarini and Savé 2016; Ors and Suarez 2016, 2017; Ferreira et al. 2018), heavy metal stress (Fagioni et al. 2009; Bagheri et al. 2015), and temperature stress (Mogren et al. 2012; Chitwood et al. 2016; Ors and Suarez 2016). The increased H^+ concentration in thylakoid membranes is one of the primary effects of oxidative stress (La Haye Yergeau and Samson 2021). Additionally, salinity stress is positively correlated with water stress which results in reduced relative yield (Ors and Suarez 2017). Upregulation of antioxidants and osmoprotectants is also positively correlated with the level of tolerance to drought in spinach (Jabeen et al. 2019). Moreover, the genetics governing tolerance to different abiotic stresses in spinach is complex and has not been explored fully to date. However, genes governing tolerance to osmotic stress (Weretilnyk and Hanson 1988; Burnet et al. 1995; Hibino et al. 2002) and two QTLs associated with the growth under poor nitogen conditions have been already identified (Chan-Navarrete et al. 2016). In response to early heat stress, the calcium

signaling molecule and certain transcription factors play significant role to overcome the negative impact of the stress. Recently, through comparative transcriptome analysis 896 differentially expressed genes were identified in spinach (Yan et al. 2016).

6.8 Transcriptomes-Based Approach for Functional Characterization of Genes

Transcriptomics is a branch of genetics to study the expression of the genes specially and temporally in various tissues (Wang et al. 2009). Transcriptomes are analyzed for interpreting molecular constituents of the functional elements of the genome in the cells and tissues. In transcriptomics, measurement of messenger RNA (mRNA) expression was studied for the array of genes localized genome wide using various hybridization-based and next-generation sequencing (NGS)-based approaches (Brady et al. 2006; Gomase and Tagore 2008). Quantification of transcriptome allows experimental scientists to study a complete set of transcripts in cells in the context of their abundance of a particular transcript in a specific developmental stage and physiological condition (Wang et al. 2009). The primary aims of transcriptomics are to study various transcripts including mRNAs, small RNAs, and non-coding RNAs (nc RNAs) to analyze multiple transcriptional status of genes such as the determination of 5′ end and 3′ end sites of the genome, splicing patterns, and posttranscriptional modifications (Wurtzel et al. 2010). Therefore, transcriptomic study imparts a significant impact on various branches of biological sciences as it offers the ability to analyze differential expression of genes both quantitatively and qualitatively (Tan et al. 2009). Various technologies such as hybridization-based approaches, sequence-based approaches, and RNA-sequencing-based approaches are being used for genome-wide transcriptomic studies (Tan et al. 2009; Wang et al. 2009). In hybridization-based approaches, custom-made or commercial high-density oligos were tagged with fluorescently-labeled cDNA on the microarrays for gene expression profiling (Nowrousian 2007). Probes designed using sequence neighboring the exon junction in the microarrays were used for quantification and detection of distinct spliced isoforms and thereby offers high-throughput resolution for quantifying large genomes inexpensively (Clark et al. 2002; Yamada et al. 2003; David et al. 2006). However, hybridization-based approaches depend on the prior knowledge about the genome sequences understudy and thereby restricted their use for its dynamic range of species (Okoniewski and Miller 2006; Royce et al. 2007). In contrast, direct determination of cDNA sequences is possible using sequence-based approaches across the cross species. Even though Sanger sequencing comes with high accuracies, it has low throughput as well as high cost in the quantification process, thereby limiting its use in large-scale transcriptome study (Gerhard et al. 2004). Therefore, various tag-based methods such as serial analysis of gene expression (SAGE) (Hu and Polyak 2006), cap analysis of gene expression (CAGE) (Shiraki et al. 2003), and massively parallel signature sequencing (MPSS) (Brenner et al. 2000) were used for parallel sequencing and gene expression study (Kodzius

et al. 2006). However, only a small portion of the transcript can be analyzed using tag-based methods (Brenner et al. 2000). In contrast, RNA-sequencing has received great momentum in differential gene expression studies as it offers high throughput across the species inexpensively (Strickler et al. 2012). In this approach, the entire population of total or fractionated RNA in a particular tissue at a specific developmental stage of the plant is first converted to the library of cDNA using adaptors sequences attached to both the ends followed by sequencing in high-throughput manner to obtain the short sequence reads (Holt and Jones 2008; Vera et al. 2008). This technology offers many advantages such as no restriction of detection for transcripts with respect to the existing reference genome, precise positioning of the transcription boundaries, low background signal, and no upper limit of quantification (Imadi et al. 2015). This method allows the entire transcriptome to be surveyed quantitatively in a high-throughput manner (Cloonan et al. 2008; Mortazavi et al. 2008; Nagalakshmi et al. 2008).

In the case of spinach, there is a huge gap of knowledge about the genetic architecture of spinach germplasms, and thus, it paves the way to explore the wild relatives for spinach improvement program (Xu et al. 2015). Developing suitable genomic resources to study the genetic diversity will provide valuable information for better germplasm utilization and for facilitating the breeding of new spinach varieties. Transcriptome characterization of cultivated and wild spinach (three from cultivated *S. oleracea*, three from wild *S. tetrandra*, and three from wild *S. turkestanica*) using the high-throughput Illumina sequencing technology was conducted to identify the phylogenetic relationship and genetic diversity of cultivated and wild spinach for its use in marker-assisted breeding (Xu et al. 2015). Genes associated with the signaling pathways of vernalization, gibberellin, photoperiod/circadian clock, autonomous, and aging pathways were identified while studying genetic networks controlling spinach bolting using RNA-seq analysis on early bolting accession and late-bolting accession at both reproductive and vegetative stages (Abolghasemi et al. 2021). Molecular mechanisms influenced by nitrogen stress were also analyzed using RNA-Seq to identify the differential expression of gene influencing photosynthesis, amino acid profiles, biomass accumulation, and partitioning of nitrogen across tissues (Joshi et al. 2020). In another study, a total of 2308 leaf-spcific and 1686 root-specific differentially expressed genes were investigated using RNA-Seq analysis of the leaf and root tissues of two spinach genotypes with contrasting oxalate phenotypes (Joshi et al. 2021). Recently, a detailed study was conducted using transcriptome sequencing of 120 cultivated and wild spinach where multiple SNP variants were identified to study inter- and intra-chromosome rearrangements during the Caryophyllales genome evolution (Xu et al. 2017). Heat adaptation mechanism in a spinach inbred was also investigated using physiological and proteomic approaches where 911 heat stress-responsive proteins with phosphorylation level changes of 45 phosphoproteins were identified suggesting the involvement of heat-induced calcium-mediated signaling, reactive oxygen species, cross-membrane transport pathways, and endomembrane trafficking in the heat homeostasis (Zhao et al. 2018). The information together provides insights to study various responsive metabolic atlas in spinach to elucidate

the detailed investigations of sophisticated molecular mechanisms for translating them into molecular breeding initiatives.

6.9 Future Prospects

Spinach is a low-calorie vegetable crop which reqires very low input for cultivation. In addition, it has high concentration of beneficial nutrients and other health-promoting compounds but rich in undesirable oxalic acid that produces calcium oxalate crystals (kidney stones) with the reaction to calcium. Due to increasing interest towards healthier diets, demand for spinach as a salad crop is growing throughout the world. However, its production is decreasing due to major diseases, particularly downy mildew, fusarium wilt, white rust, and leaf spot as well as due to other abiotic stresses, especially heat and drought. Plant breeders are consistently trying to overcome these negative impacts on the crop and maintain stable production. The investigation of genetic diversity and phenotypic variation within cultivated and wild types is the foundation of the spinach improvement program. The advancement of next-generation sequencing technologies in the recent decade to sequence large panel of germplasms and commercial cultivars has also accelerated genetic investigations and created significant molecular biological knowledge as well as genomic resources. Recently published whole-genome sequence data of spinach along with other publicly available reference genome sequences and ongoing sequencing data are able to mine key SNPs associated with many disease-resistant loci. Precise phenotyping through automated high-throughput image-based platforms will also facilitate GWAS and linkage mapping studies including QTL-seq to identify linked markers through mapping of desirable QTLs. Furthermore, studies of pan-genome on crop communities have also gained popularity in recent years to find out core genes (present in all members of the panel) and variable genes (not present in all the members of the panel). Proteomics and metabolomics approaches may be further used to elucidate the gap between genetic and phenotypic relationships in order to improve crop nutritional quality and overcome various biotic and abiotic stresses. Constant effort on comparative transcriptomic study between contrasting genotypes (resistant *vs* susceptible) also provides useful pieces of information and increases genomic resources for spinach. In addition, the availability of genome-enabled tools such as marker-assisted selection (MAS), genomic selection, haplotype-based selection facilitates the section process for the novel cultivar development which improved the breeding efficiency of commercially valuable traits, benefiting the whole spinach breeding community.

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