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Smart Plant Breeding for Vegetable Crops in Post-genomics Era

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ISBN 978-981-19-5366-8 ISBN 978-981-19-5367-5 (eBook)
https://doi.org/10.1007/978-981-19-5367-5

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Dedicated
To
Indian Farmers and Our Beloved Parents

Preface

After the beginning of post-genomics era about two decades ago, rapid revolutionary evolution has occurred in the sequencing technologies. There is no doubt that the future plant breeding in the post-genomics era will efficiently utilize accumulated information from genomics research. The genomic selection (GS) based on whole-genome SNP markers, genomic resources, RNA-seq-based transcript analysis, QTL-seq, computational biology, and bioinformatics are becoming the crucial tools of plant breeding methodologies. Then recently, the accelerated acceptance of genome editing based on CRISPR/Cas9 systems has remarkably revolutionized the plant science to speed up the crop breeding. As most of the commercial traits are under the genetic control of polygenic loci, the quantitative genetics became an integral part of plant breeding. Traditional plant breeding has served for decades in the generation of new high yielding and disease resistance cultivars. With the advent of state-of-the-art techniques, plant breeding has evolved over time. Recently, plant breeders and molecular biologists are confronting the challenge to feed ten billion people by the next three decades in the era of climate change. Among the 17 United Nations (UNs) sustainable development goals (SDGs), mitigating hunger, poverty, and climate catastrophe is one of major challenges to accomplish by 2030. The population explosion and climate change-mediated escalated frequency of biotic and abiotic stresses on crop plants are potent impediments to accomplish SDSs. To address these challenges, much progress has been made with the smart breeding and genomics tools. After the era of molecular breeding using DNA markers, marker-assisted selection (MAS), traditional sequencing methods, the advent of next-generation sequencing (NGS) platforms, genomics, genome editing, and epigenomics have accelerated the crop improvement programmes to the next level to meet the SDGs.

Vegetable crops are important components of healthy diet and hold a promising position in building up a strong immune system. They are preferably called as protective foods as they are abundant source of vitamins, minerals, phytochemicals, and other nutraceutical compounds. In the wake of the current pandemic of Covid-19 which has engulfed the entire world, vegetable- and fruit-based diet has crucial role in building a healthy immune system. Thus, they are vital for a healthy lifestyle, but their production is significantly hampered by different biotic and abiotic stresses in the era of climate change. To meet the increasing demand of these nutritious crops is

a great challenge ahead of plant breeders, geneticists, and molecular biologists. Breeding designer vegetable crops to fight the challenges ahead in sustainable manner by keeping the harmony with nature is an important approach to fulfil the UNs SDGs. In this context, great efforts have been made across the world with the use of cutting-edge NGS, genomics, bioinformatics, genome editing, and genome engineering tools. Numerous monographs and edited books have been published in the last decade regarding crop-wise deliberations on genetic improvement via novel techniques. However, none of previously accomplished books provide the complete latest information of vegetable crop genetic improvement via next-generation breeding tools in the post-genomics era in a single publication. After CRISPR-cas9 system also new improved version of genome editing have been postulated like prime editing, CRISPR-cas13a and cas12, etc. Epigenetics has also solved the mysteries behind alteration of crop traits using DNA methylation and histone modification pathways. Therefore, we have made an effort in this volume of this book to provide comprehensive enumeration of latest developments and future prospects of vegetable crop improvement in post-genomics era in a single publication.

We have designed this volume by targeting the students, researchers, and scientists in academia and research industries to provide them comprehensive information of all the up-to-date developments on genetics and breeding of vegetable crops in the post-genomics era. Even the readers, academics, social activists, and others fond of reading will get a fair idea of journey travelled so far and future roadmap for fighting against challenges to meet sustainable development goals.

This volume will comprise all the major vegetable crops like potato, tomato, eggplant, Brassica vegetables, cucurbits, legume vegetables, leafy vegetables, root, and tuber crops. All the editors of this volume confess that it was really a quite difficult task to complete this venture. We are earnestly thankful to all our contributors from different parts of world who have given their valuable time to write the chapters of their expertise. Without their contribution, it would have been difficult to accomplish this volume, and we are also thankful to all the scientists, researchers, farmers, students, and teachers who have made significant contribution to the crop genetic improvement programmes and feeding the world.

Jhansi, Uttar Pradesh, India — Saurabh Singh
Almora, Uttarakhand, India — Devender Sharma
Imphal, Manipur, India — Susheel Kumar Sharma
Shimla, Himachal Pradesh, India — Rajender Singh

Book Summary

In the post-genomics era, rapid evolution has occurred in the advancement of sequencing approaches and genome engineering. The revolution in genetic and genomics research, epigenomics, genomic selection, computational biology and bioinformatics, genome editing, speed breeding, doubled haploidy, and other next-generation breeding methodologies has accelerated the plant breeding. This volume enumerate the latest applications of these post-genomics tools like genomics and genome editing, bioinformatics, genomic resources, epigenetics, and smart breeding to tackle the challenges in vegetable crop improvement. This volume is a fruitful and leading-edge resource for the researchers, students, scientists, teachers, and private players interested in smart plant breeding tools for crop genetic improvement. This is a leading-edge volume highlighting the modern results in vegetable breeding in the post-genomics era and forecasts crucial areas of future needs.

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About the Editors

Saurabh Singh is currently working as Teaching cum Research Associate at RLBCAU, Jhansi, India. He completed his Ph.D. from ICAR-IARI, New Delhi, India. His main research interests are genetic improvement of vegetable crops using molecular breeding, genome editing, and doubled haploidy. He has published research papers in peer-reviewed journals like PLOS ONE, Plant Reproduction, Euphytica, 3 Biotech, Frontiers in Plant Science, Genetic Resources, and Crop Evolution, PGR, Cambridge. He has published 6 book chapters, 20 popular articles, and 1 edited book. He is the recipient of Dr. B. R. Barwale Young researcher award by IAHS. He also holds the responsibility of independent peer reviewer for Scientia Horticulturae, Genes, Agronomy, Horticulturae, Biology, Plants, Life, Molecules and Frontiers in Genetics, etc.

Devender Sharma is currently working as a Maize Breeder [Scientist] at ICAR-VPKAS, Almora, Uttarakhand, India. He is the ICAR-Senior Research Fellowship recipient and completed his Ph.D. from GBPUAT Pantnagar, Uttarakhand, India. His main interest areas are the genetic improvement of cereal crops. He is currently working on the biofortification of maize for nutritional quality using genomics and genome-edited tools. He has published over 21 peer-reviewed research papers, 10 book chapters, and 12 popular articles. He is the consignee of the Young Scientist Award from UCOST, Dehradun. He is the recipient of Jagar Nath Raina Memorial All India Best Research Award-2020, in the recognition of his Doctoral research work. He is also the peer reviewer for reputed journals.

Susheel Kumar Sharma is currently working as Scientist at ICAR Research Complex for NEH Region, Manipur Centre, Imphal, Manipur. He is a recipient of University Gold Medal, Dr. J. S. Negi Gold Medal, ASPEE Gold Medal, and Prakash Singha Gold Medal. He completed Ph.D. from ICAR-IARI, New Delhi. He is recipient of IARI-Best Student Merit Medal. He has 12 years of research experience in viral genomics and host-pathogen interactions studies.

Dr. Sharma has handled five externally funded projects as Principal Investigator funded by NASF, DBT, and DST. He has published 45 research papers, 2 edited

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Rajender Singh is currently working as Research Associate at ICAR-CPRI, Shimla, Himachal Pradesh, India. He completed his Ph.D. from Thapar University, Patiala, and ICAR-DMR, Solan, Himachal Pradesh, India. He has also qualified ICAR-NET in Agricultural Biotechnology. He is the recipient of Junior Scientist of the Year Award 2010 NESA, New Delhi. He has more than 11 years of experience in research. He has published research papers and peer-reviewed journals like Bioresource Technology, 3 Biotech, and Indian Journal of Microbiology. He has published 11 book chapters and one edited book. Previously, he was associated with research and development in edible fungi. Currently, his main research interest is technology management and licensing in potato research at ICAR-Central Potato Research Institute, Shimla.

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1 The Role of Epigenetic Transcriptional Regulation in *Brassica* Vegetables: A Potential Resource for Epigenetic Breeding

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Abstract

The Brassica genus includes many economically important vegetables such as Chinese cabbage, cabbage, broccoli, cauliflower, pak choi, turnip, etc. The environment has a great impact on growth and development of *Brassica* vegetables. Epigenetic regulators are involved in various biological processes to regulate the transcription of genes related to agronomic traits in *Brassica* vegetables and are associated with their adaptation to the changing environments and stresses. Epigenetics refers to the modifications of chromatin states including DNA methylation and histone modification. Any of these modifications in chromatin states can epigenetically control transcriptional outputs, and the epigenetic regulators are involved in activation or silencing of many genes. In vernalization, accumulation of the repressive histone mark histone H3 lysine 27 tri-methylation by prolonged cold treatment causes the epigenetic silencing of a floral repressor, *FLOWERING LOCUS C*, to accelerate the flowering in *Brassica* vegetables. A contribution of epigenetic regulation in agronomically important traits such as hybrid vigor/heterosis or biotic and abiotic stress resistance is discussed. The dominance relationship between heterozygous alleles of pollen determinants of

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_1

self-incompatibility is controlled by transcriptional silencing by the de novo DNA methylation in the recessive allele. Transposition or transcription of transposable elements (TEs) is regulated by DNA methylation, and sometimes TEs in the adjacent genic region affect gene expression through DNA methylation or histone modifications. As heritable epigenetic mutations may contribute to generation of a new phenotype, this chapter discusses epigenetic breeding in *Brassica* vegetables.

Keywords

Brassica · Epigenetics · DNA methylation · Histone modification · Heterosis · Stress response · Self-incompatibility · Vernalization

1.1 Introduction

The genus Brassica is in the Brassicaceae family, and Brassicaceae consists of more than 330 genera and 3800 species (Bailey et al. 2006; Huang et al. 2016). Plants in the genus Brassica are known as cruciferous or cole crops and include many plants of economic importance; vegetables (Chinese cabbage, cabbage, napa cabbage, cauliflower, broccoli, kale, pak choi, mizuna, komatsuna, Brussels sprouts, kohlrabi, rutabaga, turnip), oilseeds (canola), the condiment (mustard), fodder, and many wild species growing as weeds. *Brassica* plants are studied due to their genomic characteristics and agricultural importance. Among the *Brassica* species, the evolution and genomic relationships of six *Brassica* species have been described by the "Triangle of U" (UN 1935). The "Triangle of U" described that three ancestral diploid *Brassica* species, *Brassica rapa* L. (AA genome), *Brassica nigra* L. (BB genome), and *Brassica oleracea* L. (CC genome), have undergone natural hybridization and developed three allotetraploid species, *Brassica juncea* (L.) Czern and Coss (AABB genome), *Brassica napus* L. (AACC genome), and *Brassica carinata* A. Braun (BBCC genome). Later, molecular studies proved the "Triangle of U" theory (Chalhoub et al. 2014; Kim et al. 2018; Xue et al. 2021; Yang et al. 2016). *Brassica* underwent a whole-genome triplication (WGT), a crucial event for the species diversification and intra-species morphotypes (Cheng et al. 2014, 2016). *Brassica* and *Arabidopsis thaliana* are thought to diverge from a common ancestor 14.5–20.4 million years ago and belong to same Brassicaceae family (Blanc et al. 2003; Bowers et al. 2003; Koenig and Weigel 2015). Two species of the genus *Brassica*, *B. rapa* and *B. oleracea,* consist of many economically important vegetables (Lv et al. 2020; Prakash et al. 2012).

Characteristics of any living organisms are determined by their gene expression. DNA sequences are the blueprint of all information as they are the determinants of gene expression and protein function. Alterations in the DNA sequences of genes can alter gene expression/function. Alteration in gene expression without changes in the DNA sequences is known as epigenetics, and different kinds of modifications to the DNA can make an epigenetically modified gene turn on or off. The epigenetic

modification is sometimes reversible, and it is sometimes heritable. Chromatin, DNA, and protein consisting in chromosome can be controlled by epigenetic regulators including DNA methylation, chromatin remodeling, and histone post-translational modification. These epigenetic regulators can participate in many biological processes at any stage of plant growth and development to regulate the gene expression. DNA methylation and histone modification can regulate the chromatin structure and significantly change gene expression in response to stresses (Kim et al. 2015; Meyer 2015). Genome-wide epigenetic states such as DNA methylation or histone modification have been studied in *A. thaliana* (Bernatavichute et al. 2008; Oh et al. 2008; Roudier et al. 2011; Turck et al. 2007; Zhang et al. 2007, 2009) and now have been investigated in *Brassica* vegetables (Akter et al. 2019; Chen et al. 2015; Mehraj et al. 2021a; Parkin et al. 2014; Takahashi et al. 2018a).

In this chapter, we introduce epigenetic research in *Brassica* vegetables and how epigenetics is involved in agronomically important traits such as heterosis/hybrid vigor, vernalization, stress response, and self-incompatibility. Through these, the future role of epigenetics in breeding is discussed.

1.2 DNA Methylation

DNA methylation is a covalent addition of the methyl group ($-CH_3$) to the fifth carbon position of cytosine (5mC) (Fig. 1.1). In plants, DNA methylation occurs in all sequence contexts of cytosines such as symmetric CG and CHG and asymmetric CHH (where H is A, C, or T) sites (Fig. 1.1). DNA methylation is observed in

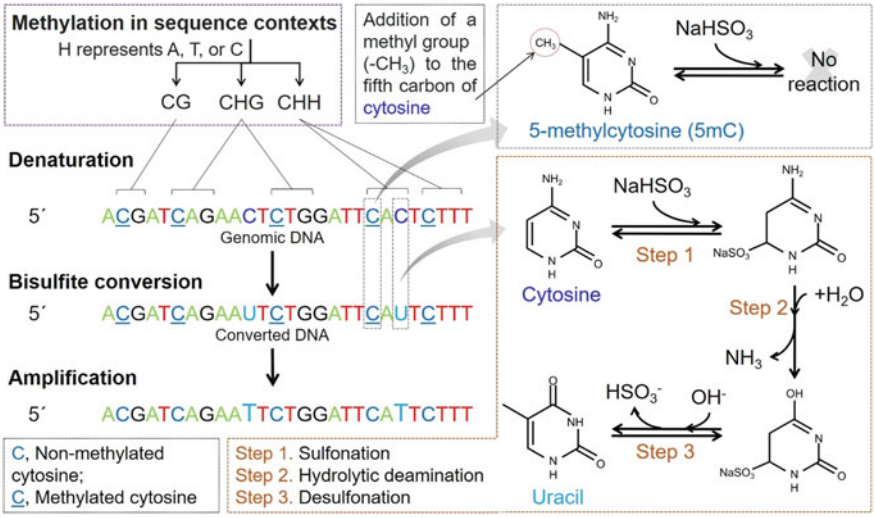

Fig. 1.1 DNA methylation, methylation in sequence contexts, and cytosine conversion by sodium bisulfite ($NaHSO_3$) treatment in whole-genome bisulfite sequencing (WGBS)

heterochromatic regions such as centromeric and pericentromeric regions, where many repetitive sequences and transposable elements (TEs) are included (Fujimoto et al. 2012a). DNA methylation is also found in repetitive sequences and TEs in euchromatin regions, as well as in genic regions. A phenomenon defined as gene-body methylation (gbM), where only CG methylation is found in exon regions, is also observed (Fujimoto et al. 2012a).

DNA methylation can be divided into two types, maintenance DNA methylation and de novo DNA methylation. The enzymes involved in maintenance DNA methylation have been identified by studies using *A. thaliana* hypomethylated mutants, and the enzymes involved in maintenance DNA methylation of the three contexts are known to be METHYLTRANSFERASE 1 (MET1) for CG, CHROMOMETHYLASE 3 (CMT3) for CHG, and CMT2 for CHH in *A. thaliana* (Kawakatsu and Ecker 2019; Osabe et al. 2012). De novo DNA methylation is mediated by the RNA interference (RNAi) pathway, which is termed RNA-directed DNA methylation (RdDM). RdDM consists of two phases, production of 24 nucleotide small-interfering RNAs (24 nt-siRNAs) and DNA methylation. Plant-specific RNA polymerase IV (Pol IV) produces single-stranded RNAs, and converted to double-stranded RNAs by RNA polymerase 2 (RDR2). Dicer-like 3 (DCL3) processed double-stranded RNAs into 24 nt-siRNAs that are incorporated into ARGONAUTE 4 (AGO4). This complex interacts with scaffold RNA transcribed by plant-specific Pol V to recruit DNA methylase, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which catalyzes DNA methylation in all sequence contexts (Kawakatsu and Ecker 2019; Matzke and Mosher 2014). DNA demethylation is catalyzed by DNA glycosylase/lyase, DME (DEMETER), ROS1 (Repressor of silencing 1), and DMLs (Demeter-like) in *A. thaliana* (Kawakatsu and Ecker 2019; Zhang et al. 2018).

Genes involved in DNA methylation have also been identified in *B. rapa* (Fujimoto et al. 2006a, 2008a; Grover et al. 2018). Three mutants, *nuclear rna polymerase d1* (*bra*A.*nrpd1*), *bra*A.*rdr2*, and *nuclear rna polymerase e1* (*bra*A.*nrpe1*) have been characterized in *B. rapa*; NRPD1 and NRPE1 are components of largest subunit of Pol IV and Pol V, respectively. Production of 24 nt-siRNAs was reduced in *bra*A.*nrpd1* and *bra*A.*rdr2*, while *bra*A.*nrpe1* showed a similar level of 24 nt-siRNAs production to wild type, indicating that Pol IV and RDR2 play a role in biosynthesis of 24 nt-siRNAs (Grover et al. 2018). This phenomenon is the same as in *A. thaliana* and maize (Li et al. 2015; Nobuta et al. 2008). DECREASE IN DNA METHYLATION 1 (DDM1) encodes a chromatin-remodeling factor, SWI2/SNF2, which is required for maintenance of DNA methylation (Vongs et al. 1993). A reduction of DNA methylation in all sequence contexts in *A. thaliana* occurs by the loss of DDM1 function. In *B. rapa, ddm1* knock-down transgenic plants produced by RNAi showed reduced DNA methylation levels indicating the involvement of DDM1 in the maintenance of DNA methylation (Fujimoto et al. 2008a).

1.3 Histone Modification

The basic unit of eukaryotic chromatin is a nucleosome, and this consists of 147 bp DNA wrapped around a core of histone proteins, the histone octamer (two each of the core histone H2A, H2B, H3, and H4) (Fig. 1.2) (Itabashi et al. 2018; Kim 2021; Talbert and Henikoff 2021). Modifications of the N-terminal tails of the core histones such as methylation, acetylation, phosphorylation, and ubiquitination regulate the chromatin structure (Bannister and Kouzarides 2011; Black et al. 2012; Li et al. 2007; Zhao et al. 2019). Lysine residues of core histones can be mono, di, or trimethylated (Fig. 1.2), and each histone methylation has different function in gene expression (Fuchs et al. 2006; He et al. 2011). In plants, histone deacetylation and methylation such as H3K9me2 (dimethylation of lysine 9 of histone H3) and H3K27me3 repress the gene expression and known as repressive histone marks, whereas histone acetylation and methylation such as H3K4me3 and H3K36me3 activate the gene expression and known as active histone marks (Fig. 1.2) (Kim et al. 2015; Quadrana and Colot 2016).

The acetylation of lysine residue of histones H3 and H4 (i.e., K9, K14, K27ac) is reversible; adding an acetyl group to a lysine residue of histones is catalyzed by histone acetyltransferase (HAT); and removal of the acetyl group is catalyzed by histone deacetylase (HDAC). Four subfamilies of HAT exit including general control nonrepressible 5 (GCN5)-related N-terminal acetyltransferases (GNAT), MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60), p300/CREB-binding protein (CBP), and transcription initiation factors $TAF_{II}250$ families. Fifteen HATs in these four subfamilies were identified in *B. rapa* (Eom and Hyun 2018). There are three subfamilies of HDAC, the reduced potassium dependence 3/histone deacetylase 1 (RPD3/HDA1), silent information regulator 2 (SIR2), and histone deacetylase 2 (HD2) families, and 20 HDAC genes categorized into these three subfamilies were identified in *B. rapa* (Eom and Hyun 2021).

Histone lysine methylation is catalyzed by histone lysine methyltransferase (HKMTase) that contains SET (SU(VAR)3–9, enhancer of zeste E(z), trithorax (TRX)) domains. H3K9me2 that is associated with heterochromatic regions is catalyzed by HKMTases, KRYPTONITE(KYP)/SU(VAR)3–9 HOMOLOG 4 (SUVH4), SUVH5, and SUVH6 in *A. thaliana* (Du et al. 2015). Addition of H3K27me3 that plays a role in developmental stage or tissue-specific transcriptional

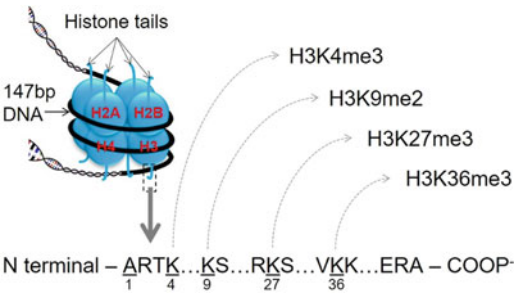

Fig. 1.2 A histone octamer showing major modifications on the N-terminal histone H3 (H3) tail

regulation is catalyzed by the POLYCOMB REPRESSIVE COMPLEX 2 (PRC2), which is composed of a subset of the polycomb group (PcG) (Zheng and Chen 2011). Histone demethylases (HDMases) have two major domains, JmjC and lysine-specific demethylase domains (LSD) (Shi and Tsukada 2013). In *B. rapa*, 60 HKMTases and 57 HDMases were identified, and HKMTases and HDMases are classified into seven and nine subgroups, respectively, based on domain organization and phylogenetic analysis (Liu et al. 2019).

1.4 Epigenome Analysis

The epigenome is defined as the map of epigenetic marks such as DNA methylation or histone modification decorating the genome (Lloyd and Lister 2022). The application of high-throughput sequencing technologies provides the genome-wide profiles of the epigenetic information.

1.4.1 Whole Genome Bisulfite Sequencing (WGBS)

WGBS, alternatively known as Bisulfite-Seq or BS-Seq, is a method to examine cytosine methylation across the genome at single-base resolution. In WGBS, sodium bisulfite is used to treat the genomic DNA before sequencing; sodium bisulfite converts unmethylated cytosine to uracil, but methylated cytosine is unchanged (Fig. 1.1). After treating with sodium bisulfite, whole-genome sequences are determined and the DNA methylation level at each cytosine can be calculated from the percentage of converted and unconverted cytosine ratio. WGBS has been successfully applied for genome-wide DNA methylation profiling at single-base resolution in *A. thaliana* (Cokus et al. 2008; Lister et al. 2008) and has been widely used in various plant species including *Brassica* vegetables (Niederhuth and Schmitz 2014).

WGBS can identify not only DNA-methylated regions or genes but also differentially methylated regions (DMRs) or genes (DMGs). The relationship of DNA methylation states and transcriptional levels can be examined by the integrative analysis of WGBS with the transcriptome data such as RNA-sequencing (RNA-seq) data. WGBS has been applied in *Brassica* vegetables, i.e., WGBS using 14-day first and second leaves in an inbred line of Chinese cabbage (*B. rapa*). In this study, the average level of methylation was 36.5% in CG, 13.4% in CHG, and 5.3% in CHH sites of the whole genome (Takahashi et al. 2018a). DNA methylation in 200 bp up or downstream regions of genic regions was negatively associated with the gene expression levels (Takahashi et al. 2018a). CHG and CHH methylation in genic region were negatively associated with expression levels, while CG methylation level in exon regions had no negative relationship with expression levels (Takahashi et al. 2018a). In the four-leaf stage of Chinese cabbage, the average level of methylation of the whole genome was 39.3% in CG, 15.4% in CHG, and 5.2% in CHH sites (Liu et al. 2018). There is a negative relationship between CG methylation in the 2 kb upstream regions and gene expression levels, but this was not observed in

CHG and CHH methylation in the 2 kb upstream regions (Liu et al. 2018). A higher level of CG methylation in internal exons of highly expressed genes suggests that CG methylation in internal exons might be associated with transcriptional activation (Liu et al. 2018). In exon regions, highly expressed genes had a lower level of CHG and CHH methylation, while low-expressed genes showed a higher level of CHG and CHH methylation (Liu et al. 2018). Results from these two groups suggest that CG methylation in the promoter region is associated with a decrease in gene expression and that CHG and CHH methylation in genic regions is negatively associated with gene expression in *B. rapa*. CG methylation in exon regions is associated with higher expression levels like in other species (Liu et al. 2018; Takahashi et al. 2018a). In *B. oleracea*, the average level of methylation of the whole genome was 54.9% in CG, 9.4% in CHG, and 2.4% in CHH sites (Parkin et al. 2014). There is a non-linear relationship between CG methylation levels in genic regions and expression levels, though a higher level of expression is associated with the lower CG methylation levels in *B. oleracea*. Genes having moderate level of gene expression tended to have gbM in *B. oleracea* (Parkin et al. 2014).

Genus Brassica have three subgenomes (as Brassica gemone has undergone WGT), and these are one least fractioned (LF) and two more fractionated (MF1 and MF2) subgenomes. There are significant correlations between paralogous gene (subgenomes) and the DNA methylation levels of gbM in *B. rapa*, indicating that gbM was conserved between paralogous genes (Takahashi et al. 2018a). There is a difference in DNA methylation level of genic regions among the three subgenomes in *B. rapa*, and the average DNA methylation level of genic regions in the MF1 subgenome is higher than in the LF and MF2 subgenomes (Chen et al. 2015). Transcription levels among the three subgenomes (LF > MF2 > MF1) are inversely related to DNA methylation levels in *B. rapa* (Chen et al. 2015). Differences of DNA methylation level among subgenomes were also observed in *B. oleracea* with the level of DNA methylation in LF subgenome being lowest. Of 588 triplets, 43% of genes showed higher expression in the LF subgenome, indicating that the inverse relationship between expression level (among paralogous genes) and DNA methylation was not observed in *B. oleracea* (Parkin et al. 2014).

1.4.2 Chromatin Immunoprecipitation Sequencing (ChIP-seq)

ChIP-seq is a method of chromatin immunoprecipitation (ChIP) followed by sequencing for genome-wide profiling of histone modifications where highly specific antibodies for histone modifications are used (Fig. 1.3) (Furey 2012; Park 2009). In the *Brassica* vegetables, genome-wide histone profiling of active histone marks (H3K4me3 and H3K36me3) and repressive histone marks (H3K9me2 and H3K27me3) were examined using 14-day leaves in inbred lines of Chinese cabbage (*B. rapa*) by ChIP-seq (Akter et al. 2019; Mehraj et al. 2021a; Takahashi et al. 2018a). About 45%, 30%, 3%, and 25% of genes had H3K4me3, H3K36me3, H3K9me2, and H3K27me3 marks, respectively, in *B. rapa* (Akter et al. 2019; Mehraj et al. 2021a; Takahashi et al. 2018a). H3K4me3, H3K36me3, and

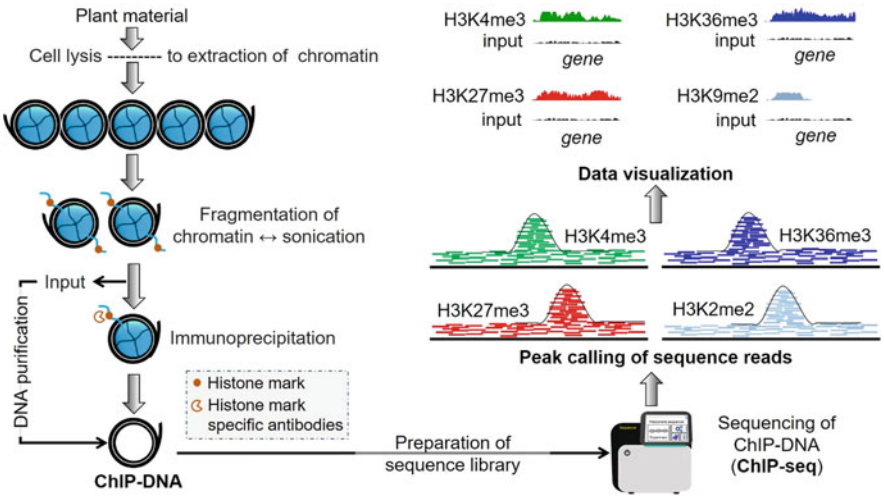

Fig. 1.3 An illustration of the chromatin immunoprecipitation (ChIP) and histone marks profiling procedure

H3K27me3 marks were found in euchromatic regions, while H3K9me2 was found in heterochromatic regions (Akter et al. 2019; Mehraj et al. 2021a; Takahashi et al. 2018a). Expression levels of H3K4me3 and H3K36me3-marked genes were associated with gene activation, while H3K9me2 and H3K27me3-marked genes were associated with gene repression.

At the whole-genome level in *B. rapa*, association between four histone modifications (H3K4me3, H3K36me3, H3K9me2, H3K27me3) and DNA methylation levels were compared; there is a positive association between H3K4me3 and H3K36me3 marks and a moderate positive association among the three histone modifications (H3K4me3, H3K36me3, and H3K27me3) at the whole-genome levels. There is a negative association between H3K9me2 and three histone modifications (H3K4me3, H3K36me3, and H3K27me3). There was a negative association between DNA methylation and three histone modifications (H3K4me3, H3K36me3, and H3K27me3), and there was a positive association between DNA methylation and H3K9me2. The average DNA methylation level in the region having H3K9me2 marks was higher than in the total genome, while the average DNA methylation level in the region overlapping the three histone modifications (H3K4me3, H3K36me3, and H3K27me3) was lower than in the total genome (Akter et al. 2019; Mehraj et al. 2021a; Takahashi et al. 2018a). In genic regions, H3K36me3 and H3K27me3 were antagonistically co-existing, while bivalent-active H3K4me3 and repressive H3K27me3 histone modifications were identified in some genes in *B. rapa* (Mehraj et al. 2021a). Co-existence of the H3K4me3 and H3K27me3 marks in some genes was confirmed by sequential ChIP or re-ChIP (Mehraj et al. 2021a). Overrepresentation of the biotic (*Fusarium oxysporum* f. sp. *conglutinans* inoculation) and abiotic stress (4 weeks vernalization) responsive

genes has been observed in bivalent-active and repressive histone modifications in *B. rapa*, suggesting that these bivalent-active and repressive histone modifications might be associated with higher transcriptional sensitivity to biotic or abiotic stress (Mehraj et al. 2021a).

Among paralogous paired genes of three subgenomes, comparison of the three histone modifications (H3K4me3, H3K36me3, and H3K27me3) that were observed in genic regions showed no significant difference between paralogs for their average expression levels with and without H3K4me3 marks (Mehraj et al. 2021a). In contrast, the average expression levels of paralogs with H3K27me3 and H3K36me3 marks tended to be lower and higher than those without H3K27me3 and H3K36me3 marks, respectively (Akter et al. 2019; Mehraj et al. 2021a), suggesting that both H3K27me3 and H3K36me3 have a role in gene expression variance between paralogous paired genes.

1.4.3 Epigenetic States in Regions Encoding Long Noncoding RNAs

RNA-seq has identified RNAs without protein-coding potentials, and RNAs devoid of protein-coding potential is termed noncoding RNAs (ncRNAs). ncRNAs are classified into two families; long ncRNAs (lncRNAs) having >200 nucleotides (nt) in length, and small RNAs (sRNAs) having ~18–30 nt in length (Fig. 1.4). Based on genomic location, three types of lncRNAs are classified, long intergenic noncoding RNAs (lincRNAs), natural antisense RNAs (NATs), and intronic noncoding RNAs (incRNAs) (Fig. 1.4). Developmental stage- and tissue-specific lncRNA expression or alteration of lncRNA expression under abiotic or biotic stress suggests that lncRNAs play a role in plant development and stress response. Thousands of lncRNAs have been identified in plants, but it has been elucidated only limited numbers of functional lncRNAs, i.e., vernalization (Heo and Sung 2011; Kim and Sung 2017; Swiezewski et al. 2009), plant immunity (Seo et al. 2017), photoperiod-sensitive male sterility (Ding et al. 2012), red-light-mediated seedling photomorphogenesis (Wang et al. 2014), and seed dormancy (Fedak et al. 2016). In *B. rapa*, lncRNAs showed lower expression levels, shorter transcript size, and lower number of exons than mRNAs and low sequence conservation between species (Mehraj et al. 2021b; Shea et al. 2019).

Although the identification of lncRNAs and transcriptional variation of lncRNAs in different tissues or under stress have been investigated, the epigenetic states of genomic regions encoding lncRNAs have not been well studied. In *B. rapa* using 14-day leaves, the average DNA methylation levels in regions encoding lincRNAs and incRNAs are similar to total genome, and average DNA methylation levels in regions encoding NATs are lower than in the total genome. DNA methylation levels increased when the region encoding three types of lncRNAs overlapped with the interspersed repeat regions (IRRs). About 60% lincRNAs, 85% NATs, and 50% incRNA-coding genomic region had H3K4me3 marks that are higher than the percentage of H3K4me3-marked genes (~45%). H3K4me3 marks were enriched in the transcribed regions of three types of lncRNAs. There is a positive association

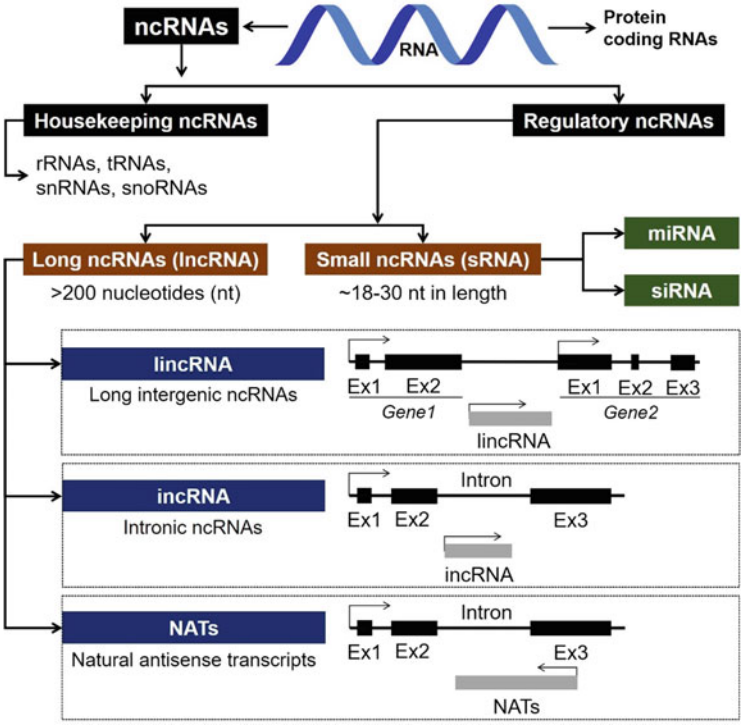

Fig. 1.4 Classification of noncoding RNAs (ncRNAs)

between expression levels of lincRNAs and having H3K4me3 marks, but this was not observed in NATs and incRNAs (Akter et al. 2022). About 50% lincRNAs, 70% NATs, and 40% incRNA-coding genomic region had H3K36me3 marks that are higher than H3K36me3-marked genes (~30%). H3K36me3 marks were also enriched in the transcribed regions of three types of lncRNAs. A positive association exists between the expression level of lincRNAs and having H3K36me3. In contrast, the transcription level of NATs and incRNAs was not associated with having H3K36me3 marks (Akter et al. 2022). About 10–16% of the three types of lncRNAs coding regions had H3K27me3 that is lower than the percentage of H3K27me3-marked genes (~25%). H3K27me3 marks were enriched in the transcribed regions of lncRNAs. Negative association between lncRNA expression levels and H3K27me3 marks was not observed, and incRNAs-encoding genomic regions having H3K27me3 showed higher expression levels than without H3K27me3 (Mehraj et al. 2021b).

The expression levels of lncRNAs in *A. thaliana* was positively correlated with the active H3K4me3 and H3K36me3 marks, while it was less correlated with repressive H3K9me2 and H3K27me3 marks (Hung et al. 2020). The same trend was observed in *B. rapa*, though there was no positive association between expression level and having H3K4me3 or H3K36me3 marks in NAT and incRNA-coding

region. Since the expression of lncRNAs varies among tissues or under stress, the analysis of lncRNAs by a single stage cannot identify all the regions encoding lncRNAs. In order to investigate the relationship between lncRNA expression and epigenetic states, it is necessary to identify lncRNA-coding regions under various conditions.

1.4.4 Epigenetic States in Transposable Elements

TEs constitute a large proportion of plant genomes, especially in species with large genome sizes. Transposition of TEs causes mutations, which is sometimes harmful to the host plant, but they are also a source of diversity of the genome. Among Brassicacea species, there is a difference of differentiation of TEs, and more TEs are observed in *B. oleracea* than in *B. rapa* (Fujimoto et al. 2008b; Parkin et al. 2014). Transposition of TEs into genes leads to disruption of gene function, while transposition into promoter regions can sometimes cause changes in gene expression levels (Akter et al. 2021; Fujimoto et al. 2006b). Transposons are also involved in changes in local genome structure between syntenic regions of different species (Fujimoto et al. 2006c, 2008c, 2011; Parkin et al. 2014). Therefore, a conflict between transposition of TEs and their repression is thought to occur in the host plant. Transposition of TEs is known to be epigenetically regulated, and repressed TEs are activated in hypomethylated mutants (Miura et al. 2001; Tsukahara et al. 2009). Epigenome analysis in *B. rapa* showed that TEs have higher levels of cytosine methylation in all contexts than in total genome (Liu et al. 2018; Takahashi et al. 2018a, b). Low levels of H3K4me3, H3K36me3, or H3K27me3 marks were observed in TEs, while H3K9me2 marks were overrepresented in TEs (Akter et al. 2019; Mehraj et al. 2021a; Takahashi et al. 2018a). Transcriptional activation of TEs and a preference of hypomethylation in TEs were observed in *ddm1* knock-down transgenic plants, suggesting that DDM1 plays a role in DNA methylation in TEs in *B. rapa* (Fujimoto et al. 2008a; Sasaki et al. 2011).

1.5 Epigenetic Regulation in Vernalization of *Brassica* Vegetables

The genus *Brassica* has flowering time regulation that is crucial for crop production and adaptation especially for leafy vegetables such as *B. rapa* and *B. oleracea.* Early bolting and/or complete inhibition of flowering that affect crop yield and seed production depend on *FLOWERING LOCUS C* (*FLC*) expression. Vernalization or promotion of flowering by prolonged cold is the strongest phenomenon for controlling flowering time. In *A. thaliana*, prolonged cold represses the *FLC* gene that represses the floral activators such as *FLOWERING LOCUS T* (*FT*) or *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Mouradov et al. 2002; Srikanth and Schmid 2011). VERNALISATION INTENSIVE3 (VIN3) and VERNALISATION2 (VRN2) are components of plant homeodomain-PRC2

(PHD-PRC2) complex, which represses *FLC* expression through the accumulation of H3K27me3 during the prolonged cold condition. After return to warm temperature, the maintenance of stable repression of *FLC* is mediated through the spread of H3K27me3 accumulation in the whole *FLC* locus (Berry and Dean 2015; Whittaker and Dean 2017).

The molecular mechanism of vernalization has been studied in the genus Brassica, which shows similar pattern to *A. thaliana*. (Akter et al. 2018, 2021; Itabashi et al. 2018; Shea et al. 2017). Due to WGT, *Brassica* has multiple copies of orthologs, which sometimes leads to subfunctionalization. *B. rapa* has two *FRI* paralogs (*BrFRIa*, *BrFRIb*) and four *FLC* paralogs (*BrFLC1*, *BrFLC2*, *BrFLC3*, *BrFLC5*) where *BrFLC5* is a pseudogene in some accessions (Wang et al. 2011). *B. oleracea* also has two *FRI* and four *FLC* paralogs and *BoFLC5* is a pseudogene (Irwin et al. 2012; Itabashi et al. 2019; Schranz et al. 2002). Three FLCs (FLC1, 2, and 3) act as floral repressors in *B. rapa* and *B. oleracea* (Itabashi et al. 2019; Kim et al. 2007; Takada et al. 2019). Quantitative trait loci (QTLs) affecting flowering time in different populations of *B. rapa* and *B. oleracea* have been identified (Shea et al. 2017), and some QTLs cover the regions including *BrFLC1, BrFLC2, BoFLC2,* or *BrVIN3.1,* indicating that these genes play a role in flowering time (Akter et al. 2021; Okazaki et al. 2007; Shea et al. 2017; Su et al. 2018; Zhao et al. 2010). Insertion of a TE in the first intron of *BrFLC2* or *BrFLC3* may cause the disruption of vernalization requitement leading to extremely late flowering in Tsukena No. 2 (Kitamoto et al. 2014).

In *B. rapa*, prior to cold condition all four *BrFLC*s had the active histone marks H3K4me3 and H3K36me3 (Mehraj et al. 2021a). During cold treatment, the accumulation of H3K27me3 around the transcription start site in all four *BrFLC*s was observed, and *FLC* expression was repressed (Akter et al. 2019, 2020). H3K27me3 accumulation spreads across the four *BrFLC* paralogs after returning plants into warm condition that maintain stable silencing (Akter et al. 2019; Kawanabe et al. 2016). In *A. thaliana*, *FLC* transcription can indirectly regulate histone acetyltransferases 1 (HAC1) to promote flowering. As for *B. rapa*, knock-down of *BrHAC1* expression by virus-induced gene silencing (VIGS) causes slow growth and delayed bolting in Chinese flowering cabbage. The expression level of *FLC* was increased, but there was no change of H3 histone acetylation level in *FLC*, suggesting that *BrHAC1* regulates epigenetic modification of an upstream factor of regulating *BrFLC* expression (Si et al. 2021). In addition, the loss of function of histone methyltransferase, *SET DOMAIN GROUP 8* (*SDG8),* which has an TRX domain, in *B. rapa* results in early bolting and downregulation of *BrFLC*s, suggesting that BrSDG8 is involved in the epigenetic regulation of *BrFLC*s; this may be activation of *BrFLC*s by adding H3K36me2/H3K36me3 (Fu et al. 2020). Furthermore, loss of function of the histone methyltransferase, *CURLY LEAF* (*CLF*) that has an E(z) domain and is a component of PRC2 showed early bolting in *B. rapa* (Huang et al. 2020; Payá-Milans et al. 2019; Tan et al. 2021). In the *brclf* mutant, expression levels of *BrFT* and *BrFLC*s were increased in Chinese cabbage, suggesting that increased BrFT activity might mask the effects of the upregulation of *BrFLC* expression (Huang et al. 2020).

Three cold-induced lncRNAs, COOLAIR, COLDAIR, and COLDWRAP, are involved in *FLC* silencing and maintaining stable repression in *A. thaliana*, and COLDAIR and COLDWRAP play a role in the recruitment of the PRC2 to the nucleation region of *FLC* (Heo and Sung 2011; Kim and Sung 2017; Swiezewski et al. 2009). In *B. rapa*, only COOLAIR-like transcripts (BrFLC2as) have been identified in the *BrFLC2* locus, while no COOLAIR-like transcript was detected from the other three *BrFLC* loci (Li et al. 2016; Shea et al. 2019). COLDAIR and COLDWRAP-like transcripts were not found from the promoter or first intron in the four *BrFLC* paralogs (Li et al. 2016; Shea et al. 2019). H3K27me3 accumulation was confirmed in all four *BrFLC* paralogs (Akter et al. 2019), how PRC2 is recruited to the *BrFLC* loci without lncRNAs during vernalization need to be shown in *B. rapa*.

1.6 Epigenetic Regulation Under Stress

Plants are sessile organisms, so they cannot escape biotic and abiotic stresses. To adapt to unfavorable environments, plants change the gene expression level. Epigenetic transcriptional regulation through DNA methylation or histone modification is essential in responses to stress (Ashapkin et al. 2020; Chinnusamy and Zhu 2009). Changes of expression levels in some *BrHAT*s, *BrHDAC*s, *BrHKMTase*s, and *BrHDMase*s under different types of stress conditions were identified (Eom and Hyun 2018, 2021; Liu et al. 2019), suggesting that change of expression levels of epigenetic modifiers under stress conditions might be involved in alteration of epigenetic states resulting in changes in stress-responsive genes expression.

In pak choi, both DNA methylation and demethylation in cold-acclimated plants or following vernalization were identified by Methylated DNA immunoprecipitation sequencing (MeDIP-seq) (Duan et al. 2017; Liu et al. 2017). Cold-acclimated plants exhibited increasing heat stress tolerance, and four genes (*BrMDH1*, *BrKAT2*, *BrSHM4*, and *Br4CL2*) showed demethylation and higher expression levels in cold-acclimated plants (Liu et al. 2017). Treatments with an inhibitor of DNA methylation, Azacytidine, cause demethylation in these four genes with activation of their expression. Overexpression of *BrMDH1* in *A. thaliana* enhanced heat stress tolerance. These results suggest that change of DNA methylation levels by cold acclimation in some genes may play a role in heat stress tolerance (Liu et al. 2017). Following vernalization, two subunits of casein kinase II, *BrCK2* and *BrCK4*, showed decreased DNA methylation levels and transcriptional upregulation. Under the vernalization conditions, the period of the clock gene, *BrCCA1*, expression was shortened, but this was not observed in double *BrCK2* and *BrCK4*-silenced plants, suggesting that demethylation and increased expression of *BrCK2* and *BrCK4* following vernalization might be involved in a shortened period of *BrCCA1* expression (Duan et al. 2017). The DNA methylation pattern under 4 h and 12 h heat stress was examined by WGBS, and DMRs between with and without heat stress were identified. CG-related DMRs were identified, and there were a few CHH-related DMRs. DMRs were found in genic regions and intergenic regions, but

there was less altered DNA methylation in TEs. The different gene ontology (GO) categories were overrepresented in genes associated with DMRs between at 4 h and 12 h heat stress; switching different sets of heat stress-induced genes was observed (Liu et al. 2018). Differences in DNA methylation levels in genic regions did not always cause changes in gene expression. Further research is needed to understand the effects of stress on gene expression caused by changes in DNA methylation.

Histone H4 protein, BrHIS4.A04, interacts with BrVIN3.1 and overexpression of this gene in *A. thaliana* (BrHIS4.A04^{OE}) causes early flowering with reduction of *FLC* expression and activation of *FT* expression. Drought induced *BrHIS4.A04* expression that was suppressed by heat, and BrHIS4.A04^{OE} showed hypersensitivity to drought stress treatments. The flowering time under drought conditions of plants carrying BrHIS4.A04^{OE} was similar to those grown in normal conditions, while wild-type Columbia-0 (Col) under drought conditions was earlier in flowering time than when grown under normal conditions, suggesting that overexpression of *BrHIS4.A04* masks the effect of drought treatment on flowering time. High levels of histone acetylation levels were observed in BrHIS4.A04^{OE} plants, suggesting that a change in expression of *BrHIS4.A04* by drought regulates the histone H4 acetylation levels in flowering time or drought-related genes (Xin et al. 2021). Genes showing a difference of H3K27me3 levels between with and without vernalization were identified in *A. thaliana* and *B. rapa*. Both species showed little change to H3K27me3 levels through genome-wide subseqently a return of the plants to 22 °C after vernalization; and two genes, *CYTOKININ-INDEPENDENT 1* and *PROTEIN KINASE FAMILY PROTEIN*, showed a lower levels of H3K27me3 (Akter et al. 2019).

1.7 Epigenetics in Hybrid Vigor/Heterosis

Heterosis is a complex biological phenomenon where heterozygous F_1 progeny shows superiority compared with the homozygous parental lines for the growth, yield, and tolerance of biotic or abiotic stress (Fujimoto et al. 2018; Itabashi et al. 2018; Lippman and Zamir 2007). Genetic approaches such as QTL analysis has been performed to explain molecular mechanisms of heterosis in plants (Fujimoto et al. 2018; Groszmann et al. 2013; Lv et al. 2020; Yu et al. 2021; Wu et al. 2021). Studies in *A. thaliana* and the *B. rapa* showed that parental genetic distance is not a strong predictor for the heterosis in F_1 hybrids (Kawamura et al. 2016; Vasseur et al. 2019; Yang et al. 2017). Epigenome analysis has also been performed to identify its association with heterosis as the epigenome of parental lines may have high levels of diversity even when there is genetic similarity between parental lines. In *A. thaliana*, there are several reports suggesting that epigenetics can contribute to explaining the molecular mechanism of heterosis (Dapp et al. 2015; Kawanabe et al. 2016; Lauss et al. 2018; Zhang et al. 2016).

Yield heterosis is an important trait in F_1 hybrid cultivars in *Brassica* vegetables (Fujimoto et al. 2018). In cabbage, head weight showed heterosis in F_1 hybrids.

Transcriptome analysis using the outer layer of cabbage head leaves in four F_1 hybrids and their parental lines showed that the expression pattern in F_1 hybrids was more similar to maternal lines than paternal lines. The number of overlapped genes between differentially expressed genes between F_1 hybrids and paternal lines was lower than between F_1 hybrids and the maternal lines, and a higher level of maternal expression dominance was observed in F_1 hybrids, suggesting that the maternal parent is a higher contributor to gene expression divergence of F_1 hybrids (Li et al. 2021). Early developmental and yield heterosis are observed in a commercial F_1 hybrid cultivar of Chinese cabbage (Li et al. 2021; Saeki et al. 2016). Heterosis levels was reduced in later seedling stages by the inhibition of photosynthetic process in the cotyledon, suggesting that the photosynthetic capacity at early developmental stages may be important for yield advantage in Chinese cabbage (Saeki et al. 2016). A similar possibility has been shown in *A. thaliana* (Fujimoto et al. 2012b). Heterosis not only in yield but also in soluble sugar and protein contents was observed in pak choi (Liu et al. 2020). Transcriptome analyses have been performed using F_1 hybrids and their parental lines using different stages or tissues in *B. rapa*, and the GO category, 'Photosynthesis,' was overrepresented in differentially expressed genes between F_1 hybrids and their parental lines (Kong et al. 2020; Li et al. 2021; Liu et al. 2020; Saeki et al. 2016). The transcriptome analysis of microRNAs (miRNAs) revealed that the majority of the miRNA clusters in the F_1 hybrid had lower expression levels compared with mid-parent values (MPVs) of parental lines both at the seedling and early-heading stages (Li et al. 2021). Target genes of differentially expressed microRNAs in F_1 hybrids are involved in leaf morphogenesis, leaf development, leaf shaping, photosynthesis, and chlorophyll synthesis, suggesting that miRNAs play a role in biomass heterosis (Li et al. 2021).

WGBS was performed in heterotic F_1 hybrids and their parental lines of pak choi, and DNA methylation levels in F_1 hybrids were higher than parental lines (Liu et al. 2020). This group identified *BrLhcb1* as a candidate for a contributor to the heterosis phenotype, but differences of expression levels of this gene among F_1 hybrids and their parental lines were independent from DNA methylation levels in its promoter region (Liu et al. 2020). Heterosis is also observed in broccoli (*B. oleracea* L. var. *italic*), especially in curd weight, and transcriptome and methylome using 70-day-old curds of hybrids and their parental lines were performed (Li et al. 2018). Methylation-dependent restriction-site-associated DNA (MethylRAD) methylome analysis revealed a slightly higher DNA methylation in F_1 hybrids than that in their parental lines and additive DNA methylation states in F_1 hybrids. The sites having different levels of DNA methylation in F_1 hybrids were predominantly in the intergenic regions, and alteration of DNA methylation in the genic region did not affect the gene expression level in the F_1 hybrids (Li et al. 2018). Differences of DNA methylation levels between F_1 hybrids and parental lines were observed, but extensive study is needed to explain the role of epigenetics in heterosis in *Brassica* vegetables.

1.8 Dominance Relationship of *S* Haplotypes in Pollen Is Caused by De Novo DNA Methylation in the *SP11* Promoter Region of a Recessive Allele

Self-incompatibility is a mechanism that prevents a self-fertilization, and self-incompatibility system existed in many diploid species of the *Brassica* genus. A single *S* locus with multiple alleles controls self-incompatibility (Fujimoto and Nishio 2007). *S* allele-specific interaction between female determinant, S receptor kinase (SRK), and male determinant, SP11/SCR (S-locus protein 11/S-locus cysteine rich) (SP11 hereafter) triggers inhibition of self-pollen germination on the stigma or pollen tube elongation, and these genes are closely linked in the *S* locus (Fujimoto et al. 2006c; Kachroo et al. 2001; Takayama et al. 2001). There are more than 30 *S* haplotypes, a set of alleles of *SRK* and *SP11*, in *B. oleracea* and *B. rapa*, and *S* haplotypes are classified into two groups, class-I and class-II, based on nucleotide sequence differences in *SRK* and *SP11* genes (Fujimoto and Nishio 2007). Self-incompatibility in the genus Brassica is sporophytically controlled, and alleles of *S* heterozygotes have dominance relationships (Thompson and Taylor 1966).

In the class-I/class-II *S* heterozygote plants of pollen, class-I *S* haplotypes are dominant over class-II *S* haplotypes (Nasrallah et al. 1991), and this is due to suppression of class-II *SP11* in class-I/class-II *S* heterozygotes (Shiba et al. 2002). Interestingly, this suppression of class-II *SP11* in class-I/class-II *S* heterozygotes was observed even when genomic fragments of whole class-I *SP11* of one allele was deleted in the *S* locus (Fujimoto et al. 2006b). Later, it was revealed that the suppression of class-II *SP11* results in the de novo DNA methylation of the promoter region of class-II *SP11* (Shiba et al. 2006), and this de novo DNA methylation is caused by 24 nt-small RNAs, *SP11* methylation inducer (*Smi*) that has sequence homology to the promoter region of class-II *SP11* and is expressed from the *SP11*-methylation-inducing region (SMI) located in the class-I *S* locus; class-I derived *Smi* silences the expression of class-II *SP11* by *trans*-acting de novo DNA methylation in the class-I/class-II *S* heterozygotes (Tarutani et al. 2010).

In pollen, there is a linear dominance order among the class-II *S* haplotypes (*BrS-44* > *BrS-60* > *BrS-40* > *BrS-29*), and this dominance relationship is also explained by the transcriptional silencing of recessive class-II *SP11* allele by de novo DNA methylation caused by 24 nt-small RNAs, *Smi2* (Kakizaki et al. 2003; Yasuda et al. 2016). Unlike the class-I/class-II *S* heterozygotes, the nucleotide sequences of the promoter region of *SP11* among four class-II *S* haplotypes were similar (Kakizaki et al. 2006), but there were nucleotide sequence polymorphisms in the *Smi2*-binding target among them (Yasuda et al. 2016). *Smi2* expressed from the most dominant *S* haplotype (*BrS-44*) has high sequence homologous to the three recessive *SP11* promoter region (*BrS-60, BrS-40, BrS-29*) and can bind to the *SP11* promoter region of these three recessive *S* haplotypes. On the other hand, *Smi2* expressed from *BrS-60*, the second dominant *S* haplotype has low homology with the promoter region of *BrSP11-44* and cannot bind to the promoter region of *BrSP11-44*. By contrast, *Smi2* expressed from *BrS-60* has high homology with the promoter region of *BrSP11-S40* and *BrSP11-S29* and can bind to the promoter region of *BrSP11-40* and *BrSP11-29*.

Smi2 expressed from most recessive *S* haplotype (*BrS-29*) has low homology with the three dominant *SP11* promoter regions and cannot bind to the *SP11* promoter region of dominant *S* haplotypes. This linear dominance hierarchy of the four class-II *SP11* alleles is dependent on the sequence diversity within *Smi2* among class-II *S* haplotypes (Yasuda et al. 2016).

B. rapa (AA genome) and *B. oleracea* (CC genome) are self-incompatible, while allotetraploid *B. napus* having A subgenome from *B. rapa* and the C subgenome from *B. oleracea* is self-compatible. However, artificially synthesized allotetraploids from crossing of *B. rapa* and *B. oleracea* are self-incompatible (Kimura et al. 2002; Okamoto et al. 2007), suggesting that the natural *B. napus* has lost self-incompatibility. 'Westar' in *B. napus* has class-I *BnS-1* haplotype in the A subgenome and class-II *BnS-6* in the C subgenome. Recognition specificity of self-incompatibility in the female side is functional, while both *BnSP11-1* and *BnSP11-6* are not expressed in 'Westar.' A 3.6 kb insertion in the *BnSP11-1* promoter region is considered to eliminate expression. Dominance relationship between class-I and class-II *S* haplotypes on a different chromosome also occurred in artificially synthesized *B. napus*, i.e., class-I *S* haplotype in the A subgenome is dominant over class-II *S* haplotype in the C subgenome (Kimura et al. 2002; Okamoto et al. 2007). There was no mutation causing the lack of *BnSP11-6* expression, suggesting that *BnSP11-6* expression could be repressed by a dominance relationship (Okamoto et al. 2007). These results suggest that mutation in the promoter region of *BnSP11-1* is the direct cause of self-compatibility of 'Westar' (Okamoto et al. 2007). The *B. napus* self-compatible line (326) has class-II *BnS-7* in the A subgenome and class-II *BnS-6* in the C subgenome and has low *BnSP11-7* expression. Knockout of *Smi2* of *BnS-6* by CSIRPR-Cas9 showed higher *BnSP11-7* expression and lower DNA methylation level in the *BnSP11-7* promoter region in original line (326) than in knockout mutant line, indicating that *BnS-6* in the C subgenome is dominant over *BnS-7* in the A subgenome in pollen and recessive *BnSP11-7* expression is repressed by *Smi2* that was expressed from *BnS-6*. This showed that *Smi2* derived from C subgenome silences the expression of recessive *SP11* from A subgenome by *trans*-acting de novo DNA methylation (Dou et al. 2021).

1.9 A Perspective of Epigenetic Breeding in *Brassica* Vegetables

An effective crop-breeding program needs an ideal selection of breeding material. Studies on epigenetic changes to plant adaptability will help improve crop traits. Genetic diversity cannot reflect the complete phenotypic diversity of any crop plants including *Brassica* vegetables (Tirnaz and Batley 2019). Transcriptional regulation of phenotypic traits by epigenetic modification such as DNA methylation and histone modification could expand phenotypic variation. Epi-mutants that produce new epigenetic alleles (epialleles) are a source of the phenotypic variation; this was clearly shown using epigenetic inbred lines that were genetically identical but

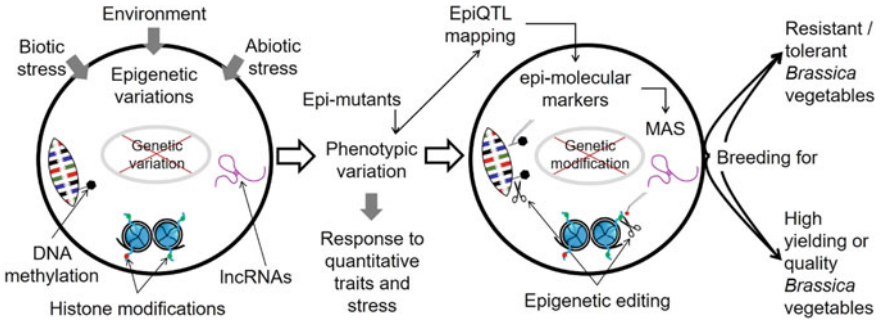

Fig. 1.5 Epigenetic source of phenotypic variation and prospects of the epigenetic breeding in *Brassica* vegetables. Red crosses represent without changes of DNA sequences

differed in DNA methylation states that had heritable phenotypic variation (Cortijo et al. 2014; Johannes et al. 2009; Roux et al. 2011). EpiQTL mapping using epialleles and epigenome-wide association studies might aid in the understanding of epigenetic variation in quantitative traits (Gahlaut et al. 2020). Moreover, the epiHybrids having the best parent heterosis phenotype were also developed using these epiRILs, suggesting a linkage of DMRs in the parental lines with epiHybrids performance (Dapp et al. 2015; Lauss et al. 2018). The development of epi-molecular markers will accelerate the identification of causative genes in EpiQTLs and marker-assisted selection of traits by epialleles (Fig. 1.5). Epigenetic editing is a potential tool for activation or repression of gene expression and might generate phenotypic variations (Fig. 1.5). Genetic and epigenetic knowledge with epigenetic resources will assist in improving agriculturally important traits of *Brassica* vegetables.

Acknowledgements This chapter includes results supported by Grant-in-Aid for Scientific Research (B) (19H02947 and 21H02163) and Grant-in-Aid for Challenging Exploratory Research (20K21313) from the Japan Society for the Promotion of Science (JSPS). We thank Dr. Elizabeth S. Dennis for her helpful comments and manuscript editing.

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Melon (*Cucumis melo* L.): Genomics and Breeding

2

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Abstract

Melon (*Cucumis melo* L.), belonging to the Cucurbitaceae family, is an economically important vegetable crop cultivated worldwide and highly valued for its fruit quality. Unfortunately, this crop is affected by several biotic and abiotic stresses that reduce yield and quality considerably. Melon breeding for fruit quality and disease resistance gained great achievements through Next-Generation Sequencing (NGS) technology. During the last decade, a rapid and huge development of genetic and genomics resources was achieved including draft genome assemblies, and high-density genetic maps, making it possible to accelerate translational research for melon breeding. The increasing availability of high-throughput sequencing technology has the potential to develop innovative genome-based strategies for the identification of loci involved in fruit quality and disease resistance. Advancements in genomics provide new opportunities to accelerate

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_2

classical breeding programs. We report here the major findings from these investigations and future perspectives in melon-breeding programs. Genomic tools used including genome editing, improvement of the melon genome assembly, identification, and molecular mapping of important genes or quantitative trait loci for disease resistance and fruit-quality traits are summarized, and the use of such knowledge in melon breeding is reported.

Keywords

Genomic tools · Genome assembly · Melon · Molecular mapping · Marker-assisted selection · Reference genome · Doubled haploids

2.1 Introduction

Melon (*Cucumis melo* L., 2n = 2x = 24) is a cross-pollinating crop with a small diploid genome size of 450 Mb (Garcia-Mas et al. 2012). It is an important horticultural crop worldwide, belonging to the Cucurbitaceae family, and exhibits high levels of diversity in morphological, physiological, and biochemical properties (Pitrat 2016). Based on ovary pubescence, the melon was classified into two subspecies, *melo* and *agrestis* (Whitaker and Davis 1962), and then further divided into 16 horticultural groups according to morphological variations of fruit (Pitrat 2016), with the *flexuosus*, *cantalupensis*, *inodorus*, and *reticulatus* being the most economically important ones in Mediterranean countries (Chikh-Rouhou et al. 2021a; Pitrat 2016). All of these groups are intercrossable. *C. melo* subsp. *melo* is cultivated worldwide, whereas *C. melo* subsp. *agrestis* is concentrated in East Asia (Liu et al. 2004).

Until a few years ago, the study of melon genome was limited to molecular markers analysis including AFLP, RAPD, and SSR associated with some morphological and pathogen-resistance traits (Chikh-Rouhou et al. 2021a, b, c; Lakshmana Reddy et al. 2016; Garcia-Mas et al. 2000). However, genetic and genomic information for this crop has increased significantly and a broad range of genomic tools are available nowadays (Grumet et al. 2021; Ezura and Fukino 2009). These tools are generating a lot of information about genes involved in various biological processes, such as plant resistance, fruit quality, and ripening (Zhang et al. 2022, Cao et al. 2021, Tamang et al. 2021; Branham et al. 2018; Argyris et al. 2017).

Several melon accessions have been sequenced and characterized using the Illumina short-read Next-Generation Sequence (NGS) platform (Zhao et al. 2019; Pavan et al. 2017). Third-generation sequencing technologies, such PacBio and Oxford Nanopore, which can generate long reads have been also used (Yano et al. 2020). The importance of next-generation technology is increasing in melon research, allowing several applications related to understanding genetic variation and facilitating marker identification and characterization (Zhang et al. 2022; Pereira et al. 2018; Pavan et al. 2017). Indeed, given the increasing genomic data availability for breeders, genomics is playing an important role in all aspects of melon breeding,

such as Quantitative Trait Loci (QTL) mapping and Genome-Wide Association Studies (GWAS), where genomic sequencing can allow gene-level resolution of agronomic variation (Wang et al. 2021; Liu et al. 2020; Zhao et al. 2019; Pereira et al. 2018; Phan and Sim 2017; Gur et al. 2017; Zou et al. 2016; Grumet et al. 2020, 2021).

In melon improvement, conventional breeding plays an essential role to generate superior genotypes through genetic recombination. It involves growing and examining large melon populations derived from cycles of phenotypic selection and crossing which is a labor-intensive process and time consuming, as it needs several phases of crossing, selection, and testing. Thus, the emergence of doubled-haploid (DH) technology has reduced dramatically the time required to generate pure homozygous lines which can be directly released as a new variety or used as parents in breeding programs (Sari and Solmaz 2020; Fayos et al. 2015). In addition, advances in genomics have provided new opportunities to accelerate classical breeding programs. Indeed, the availability of melon genome sequence has made it possible to identify genes and genetic variants that contribute to agronomic traits (Yano et al. 2020). Likewise, the high-throughput sequencing technology is enabling the development of innovative genome-based strategies for the identification of loci involved in disease resistance and fruit quality (Branham et al. 2018, 2021; Lian et al. 2021; Liu et al. 2020). For the rapid development of new cultivars to face climate change and food scarcity, Marker-Assisted Selection (MAS), molecular Marker-Assisted Breeding (MAB), and gene-editing are needed (Marsh et al. 2021; Veillet et al. 2019). Hence, novel molecular techniques, integrated with predictions based on the genome, might provide new strategies to breed plants more efficiently as reported in several vegetable crops (Bohra et al. 2019).

For crop improvement, Varshney et al. (2020) recommended the deployment of 5 Gs (Genome assembly, Germplasm characterization, Gene(s)/marker(s) associated with breeding trait, Genomic Breeding and Gene editing). In the case of melon breeding, whole-genome assemblies have become available, melon accessions/germplasm characterization is ongoing in several countries. Similarly, gene/marker identification was accelerated due to the genomic and genetic resources availability and genotyping platforms. However, a precise phenotyping is important for the germplasm used for trait mapping (Thudi et al. 2021). Comprehensive analyses of genotyping and phenotyping data can provide genes/markers, haplotypes, genomic-estimated breeding values that can be used in genomic breeding and gene-editing approaches (Bohra et al. 2019).

The revolution in genetic and genomics research, genomic selection, computational biology and bioinformatics, genome editing, and other next-generation breeding methodologies will accelerate melon breeding. The present chapter will enumerate the latest applications of the doubled-haploid technology, genomics and genome editing, bioinformatics, and genomic resources to tackle the challenges in melon crop and its improvement in the post-genomics era.

2.2 Doubled-Haploid Technology

Advanced biotechnological tools are being used to develop new varieties that meet the consumers' and the producers' needs and preferences. DH technology is a biotechnological method that can be successfully applied to melon. Commercial melons are produced using open-pollinated (OP) and hybrid cultivars. Hybrid melon cultivars are mainly developed by classical hybridization technique, obtaining a stabilized line with the trait of interest is extremely labor and time-consuming (Sari and Solmaz 2020). The development of inbred pure lines is the basic step in hybrid melon breeding; however, many rounds of selfing are required. The DH technology has several advantages such as reducing the period for generating inbred pure lines and increasing breeding efficiency (Zhu et al. 2020). This technology allows the development of completely homozygous lines from heterozygous plants, which can be used either as parents in breeding programs or directly can be released as homozygous varieties (Sari and Solmaz 2020; Germanà 2011; Solmaz et al. 2011; Sari et al. 1999b).

Haploid plants contain a gametophytic chromosome number whereas DH plants are haploid plants that are subjected to spontaneous or stimulated chromosome duplication (Germanà 2011). The haploid plants provide considerable benefits to conventional breeding studies which can be achieved by three different techniques: androgenesis, gynogenesis, and parthenogenesis.

Although androgenesis is known as in vitro culture of anthers or isolated microspores, used in several plant species, no successful results have been obtained in melon. Likewise, gynogenesis, which is the in vitro culture of ovules or ovaries, has been exploited in different plants. However, it has not been routinely applied in melons and a quite few studies have been reported (Sari and Solmaz 2020; Koli and Murthy 2013; Malik et al. 2011; Ficcadenti et al. 1999). Irradiated pollen technique (parthenogenesis) is the most effective technique widely used in melon and provides successful results (Hooghvorst et al. 2020; Godbole and Murthy 2012; Solmaz et al. 2011; Sari et al. 1992, 2010a, b; Lim and Earle 2009; Lotfi et al. 2003; Abak et al. 1996).

Melon is one of the species that responds best to haploidization studies. The most effective method for haploid embryo induction in melon is the pollination of female flowers with irradiated pollen one day before anthesis. Although different methods such as ovule/ovary and anther culture have been used before, the most successful method today is the development of haploid embryos by the parthenogenesis method. The most commonly used irradiation source for this purpose is the Co^{60} of gamma rays (Sari et al. 1992). However, it has been reported that Cesium (Cs137) and X-ray sources can also be used for irradiation (Dal et al. 2016).

Due to the wide diversity in melon, efficient DH protocols are variable for each botanical group and genotype (Hooghvorst et al. 2021). One of the factors affecting success in haploid plant production in melon is undoubtedly genotype selectivity. Another important issue is that melon plants to be induced with irradiated pollen should be grown in optimum environmental conditions and without stress. In the

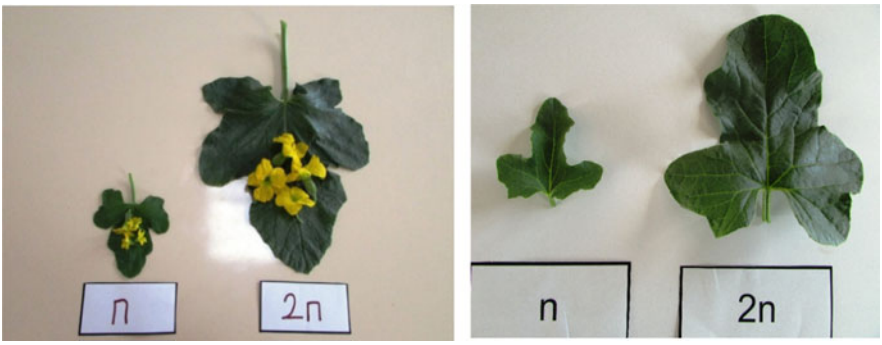

Fig. 2.1 Leaves and flowers of haploid and doubled-haploid melons

irradiated pollen technique, male and female flowers were used the day before anthesis (Sari et al. 2010a, b).

After the first successful study by Sauton and Dumas de Vaulx (1987) on the Charentais group melon, the most appropriate irradiation dose was determined as 300 gray in summer (Galia type) and winter (Kirkagac and Yuva type) melon varieties (Sari et al. 1999a). Various methods are used to determine the level of ploidy in the obtained melons, which can be divided into either direct (chromosome counts) or indirect methods (flow cytometry, stomata size, chloroplast count, morphological observations). The most classic among these is chromosome counts in plant parts where cell growth is the fastest. Abak et al. (1996) reported that morphological observations, pollen absence/presence check, a number of chloroplasts in stomatal guard cells also were used for ploidy level determination. Haploid plants have the feature of being miniature of the same plant with smaller leaves (Fig. 2.1).

DH technology has been used in Galia-type melon breeding. Haploid plants were obtained by irradiated pollen technique and then were duplicated by colchicine treatment. New F1 DH melon cultivars belonging to *C. melo* var. *cantalupensis* were developed, resistant to race 0 and 1 of *Fusarium oxysporum* f. sp. *melonis* (*Fom*) and with high yield and quality (Sari et al. 2010a, b). After agronomic performance tests for several years, the DH melon Sari F1, Yetisir F1, Solmaz F1, Emin F1, and Yucel F1 were registered in the new varieties catalog of the Republic Turkey Ministry of Agriculture and Forestry (Sari et al. 2010a, b). Besides, melon French cv Isabelle was crossed to Italian landrace, and the resulting F1 was subjected to parthenogenesis, haploid embryo rescue, and chromosome doubling. Two DH homozygous lines, Nad-1 and Nad-2, with strong resistance to FOM race 1.2 were obtained by Ficcadenti et al. (2002).

The parthenogenetic capacity of seven genotypes of *C. melo* var. *inodorus* 'Piel de Sapo' type was evaluated to obtain DH lines which might be used in further F1 breeding studies. These lines were assessed for agronomic traits and diseases (Fusarium wilt, powdery mildew, and MNSV). DH lines with high resistance to the pathogens were produced from melon donor genotypes (six genotypes were

inbred lines and one genotype was an open-pollinated cultivar) (Hooghvorst et al. 2020).

For genomic studies, the doubled-haploid homozygous line DHL92 was used in the sequencing and assembly of the draft reference genome (Garcia-Mas et al. 2012). DHL92 line derived from the cross between the Korean accession PI 161375 (Songwhan Charmi, spp. *agrestis*) (SC) and the 'Piel de Sapo' T111 line (ssp. *inodorus*) (PS).

2.3 Breeding in the Genomics Era

2.3.1 Genomic Resources

Advanced tools in genome sequencing, assembly, and bioinformatic fields were recently and massively used (Bevan et al. 2017; Chakradhar et al. 2017; Brenton et al. 2016; Martínez-Gómez et al. 2012; Pérez-de-Castro et al. 2012; Varshney et al. 2005). A burst of publications describing the draft genomes of important crops and an important number of sources of large and complex plant genomes databases have been published to date. These sources are available for public uses in online platforms such as the Potato Genome Sequencing Consortium (PGSC) (spuddb.uga.edu), the Tomato Genetic Resource Center (tgrc.ucdavis.edu), WheatGamp which is a comprehensive platform for wheat gene mapping and genomic studies (www.wheatgmap.org), and the CuGenDB platform for several Cucurbitaceae species including melon crop (https://www.cucurbitgenomics.org/) containing assembled genomes and annotations, genetic maps, transcriptomes, Expressed Sequence Tags (ESTs), and Genotyping By Sequencing (GBS) data along with analysis and visualization tools. Two other melon crop databases were developed: the MELOGEAN (Gonzalez-Ibeas et al. 2007) and the Melonomics (www.melonomics.net) platforms for melon functional genomics and the genome assembly and annotation version of the reference genome, respectively.

Crop and wild relatives' genomic analysis helps researchers to assess and to characterize species genetic diversity and genomic evolution under natural selection and domestication (Grumet et al. 2021; Coyne et al. 2020; Preece and Peñuelas 2019). Additionally, this analysis is a crucial step facing agriculture worldwide challenges, essentially population increase and climate changes by crop improvement and breeding programs releasing more resilient crops (Shivapriya et al. 2021; Chikh-Rouhou et al. 2021a, b, c; González et al. 2020; Maleki et al. 2018). Associated with the improved and automated phenotyping tools and functional genomic studies, genomics is providing new foundations for crop-breeding and improvement systems.

Melon is an attractive model for studying valuable biological characteristics, such as fruit ripening (Pech et al. 2008), sex determination (Boualem et al. 2008), and phloem physiology (Zhang et al. 2010). Compared to other Cucurbitaceae members, the melon genome is quite larger than the genome of the watermelon and cucumber but is relatively small in comparison to other crop species (Table 2.1).

Table 2.1 The genome size of different crops compared to melon (*Cucumis melo* L.)

Crop/species	Genome size (megabases) and Chromosome number	Reference
Melon (*Cucumis melo*)	450 Mb (x = 12)	Garcia-Mas et al. (2012)
Watermelon (*Citrullus lanatus*)	425 Mb (x = 11)	Guo et al. (2019, 2013)
Cucumber (*Cucumis sativus*)	367 Mb (x = 7)	Yano et al. (2020)
Tomato (*Solanum lycopersicum*)	900 Mb (x = 12)	The Tomato Genome Consortium (2012)
Pepper (*Capsicum annum*)	3480 Mb (x = 12)	Kim et al. (2014)
Wheat (*Triticum aestivum*)	17,000 Mb (x = 7)	Brenchley et al. (2012)

Recent research has increased the availability of genetic and genomic resources for melon, such as (1) the sequencing of ESTs (The Cucurbit Genomics (CucCAP) n.d.; Gonzalez-Ibeas et al. 2007); (2) the development of an oligonucleotide-based microarray (Mascarell-Creus et al. 2009); (2) the construction of BAC libraries (González et al. 2010a, b; Leeuwen et al. 2003; Luo et al. 2001); (4) the production of mutant collections for TILLING analyses (Dahmani-Mardas et al. 2010; Tadmor et al. 2007; Nieto et al. 2007); (5) the development of a collection of Near-Isogenic Lines (NILs) (Eduardo et al. 2005); (6) the construction of several genetic maps (The Cucurbit Genomics (CucCAP) n.d.; Harel-Beja et al. 2010, Deleu et al. 2009); and (7) the development of a genetically anchored BAC-based physical map (González et al. 2010a, b). High-density genetic maps have been also realized (Zhang et al. 2022; Pereira et al. 2018). The high-density genetic maps of melon were constructed by GBS (Oren et al. 2020; Branham et al. 2018), resequencing (Hu et al. 2018), or RNA-Seq. (Galpaz et al. 2018), which greatly improved the QTL mapping resolution for fruit-related traits and disease resistance (Sáez et al. 2022).

2.3.2 The Melon Genome

Since the development of next-generation sequencing technologies (NGS: 454, Illumina, SOLID), several draft genomes of important crops have been published. These genomes have been sequenced mostly using NGS technologies, sometimes complemented with Sanger sequencing. A high-quality reference genome assembly of melon was released for the first time by Garcia-Mas et al. (2012). However, EST'S, microarrays (Mascarell-Creus et al. 2009), genetic and physical mapping (Diaz et al. 2011), Bac sequencing (González et al. 2010a, b), and reverse genetic tools (González et al. 2011; Dahmani-Mardas et al. 2010) were early investigated on the melon crop.

Draft genomes of nearly a dozen cucurbit crops are now available (https://www.cucurbitgenomics.org/) and are constantly being revised using new technologies and experimental data. Garcia-Mas et al. (2012) sequenced the melon genome using the DHL92 line. The assembled genome organized in 12 chromosomes (pseudomolecules) comprised 27,427 annotated protein-coding genes, with 17% of

the genome being transposable elements (TEs) (Garcia-Mas et al. 2012). The melon reference genome v3.5.1 was obtained using 454 pyrosequencing technology. Based on a shotgun sequencing approach, several DNA fragments were produced and assembled in contigs which were grouped into larger portions (scaffolds). Shotgun sequencing involves breaking up DNA sequences into small pieces and then reassembling the sequence by looking for regions of overlap. The final sequence assembly covered 83.3% of the genome (a total of 375 Mb) in 29,865 contigs and 1594 scaffolds, with a scaffold N50 of 4.68 Mb (Garcia-Mas et al. 2012). Further, Ruggieri et al. (2018) improved the genome assembly using optical mapping to produce v3.6.1, and a new comprehensive annotation was also built composed of 29,980 protein-coding loci. However, v3.6.1 still contained 19% of gaps and more than 40 Mb unassigned sequences, probably missing complex repeat regions. Castanera et al. (2020) using PacBio single-molecule real-time (SMRT) sequencing produced an improved reference genome version v4.0. In that study, they used the hierarchical genome-assembly process 4 (HGAP4) pipeline techniques with concatenated steps to improve the assembly of the longest reads. DHL92 melon assembly v4.0 had an increase of the melon genome pseudomolecule size by 40 Mb with 90% of the v3.5.1 gaps being filled and transposable element (TE) coverage improved from 19.7% (v3.5.1) to 45.2% (v.4.0) due to the progress in TE annotation tools (Castanera et al. 2020). Specifically, 40% more full-length LTR retrotransposons, which represented the largest fraction of TE, were identified in the v4.0 assembly, mainly located in centromeric and pericentromeric regions, and a burst of these repetitive elements was found to occur less than two million years ago showing they are very young (Castanera et al. 2020). Young LTR retrotransposons have an impact on gene expression. Some of these elements are polymorphic among melon varieties and sit in the upstream regions of genes. Ito et al. (2016) evidenced that the expression of plant LTR retrotransposons (subfamilies gypsy and copia) exhibits stress-inducible transcription, i.e., up-regulated by abiotic and biotic stress (Ito et al. 2016; Grandbastien 1998). Figure 2.2 summarizes the melon reference genome assemblies and annotation analysis and the improved versions highlighting the principal outputs from each study.

The DHL92 genome reference has been utilized for supporting transcriptome analyses as well as QTL studies of important agricultural traits, including fruit ripening, fruit morphology, and disease resistance (Branham et al. 2021; Tamang et al. 2021; Lian et al. 2021; Liu et al. 2020; Argyris et al. 2017). Additionally, assembled transcriptomes have been generated through the quantitative RNA sequencing (RNA-Seq) for mapping transcribed regions, in which complementary DNA fragments are subjected to high-throughput sequencing and mapped to the genome. Assembled transcriptomes of Cucurbits species were made available on the CuGenDB database (http://cucurbitgenomics.org/rnaseq/home) allowing exploration of a Cucurbit Expression Atlas (Andolfo et al. 2021). The transcriptomes can be used as a reference for gene expression analysis in different organs and tissues and under different environmental conditions (Andolfo et al. 2016).

C. melo genome showed a high level of synteny with cucumber (*C. sativus*), suggesting an ancestral fusion of five melon chromosome pairs in cucumber and

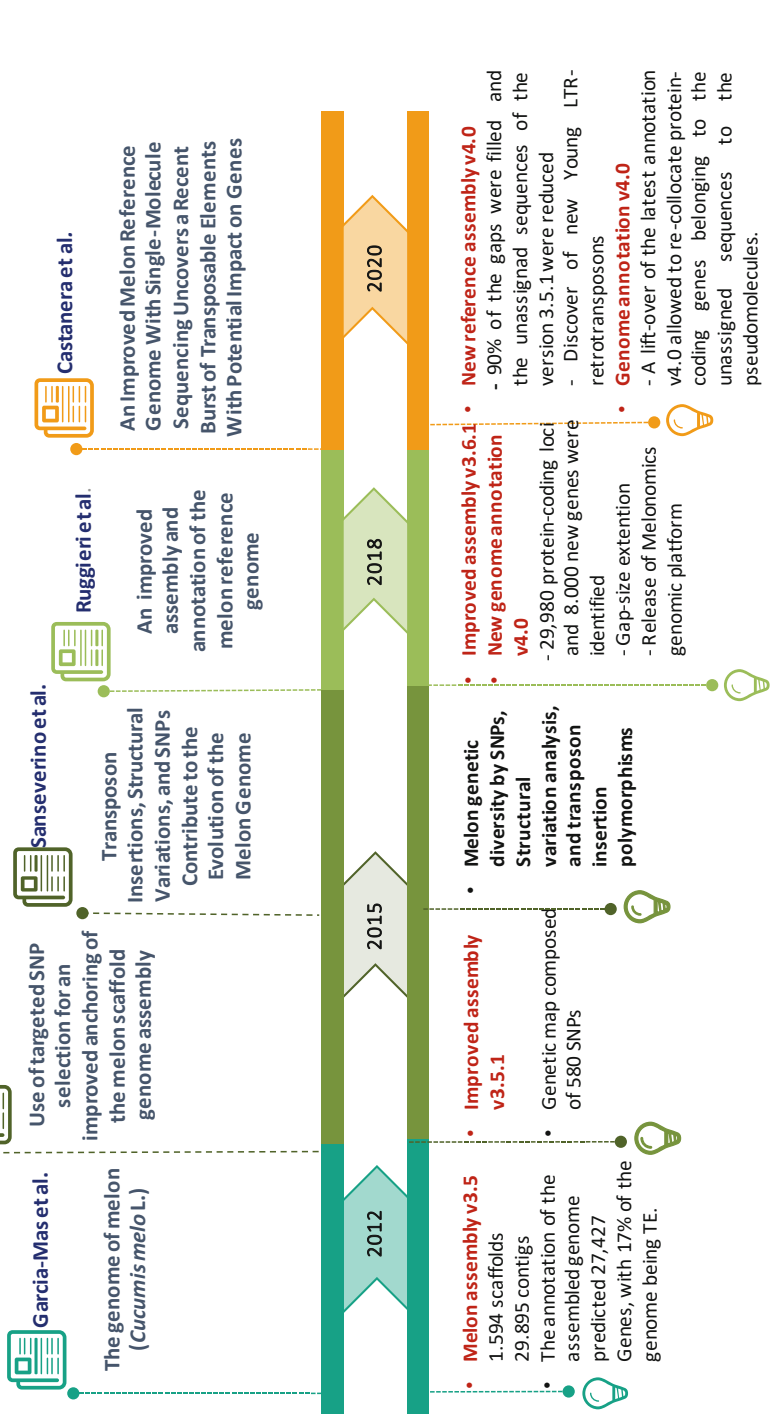

Fig. 2.2 Reference genome assemblies and annotation of the double-haploid melon line DHL92, its improvement in time scale highlighting the outputs and improved versions

Table 2.2 Technologies used for the sequencing of the melon DHL92 reference genome and other melon genomes

Sequencing technology	Genotype used	Assembled genome	Reference
Shotgun strategy based on 454 pyrosequencing + Sanger reads	DHL92	375 Mb	Garcia-Mas et al. (2012)
Optical mapping approach	DHL92	375.36 Mb	Ruggieri et al. (2018)
PacBio and Illumina sequencing	DHL92	357.64 Mb	Castanera et al. (2020)
PacBio combined with the Hi-C interaction analysis	Payzawat	386 Mb	Zhang et al. (2019)
Oxford nanopore	Harukei-3	378 Mb	Yano et al. (2020)

several inter- and intra-chromosome rearrangements (Garcia-Mas et al. 2012). Same authors showed that the melon genome increased size, compared to its relative cucumber, may be attributed to TE amplification and that the melon genome did not have any lineage-specific whole-genome duplication as in *C. sativus* (Huang et al. 2009).

Other melon genomes have been also assembled and/or re-evaluated using newer DNA-sequencing advanced technologies (Table 2.2), such the Chinese *inodorus* melon genome, which was sequenced using a PacBio long molecule sequencing (Zhang et al. 2019) and the genome of the Japanese semi climacteric *reticulatus* cultivar using the Oxford nanopore technology (Yano et al. 2020). Information regarding the Japanese 'Harukei-3' genome assembly, annotation, and transcriptome dataset is available in the Melonet-DB database (https://melonet-db.dna.affrc.go.jp/).

Determination of the complete melon genome also includes sequencing of the chloroplast (cpDNA) and mitochondrial (mtDNA) genomes (Cui et al. 2021). Few studies were reported regarding sequence analysis of the cpDNA and mtDNA genomes (Cui et al. 2021; Rodríguez-Moreno et al. 2011). The mitochondrial genome of melon is eight times larger than other cucurbits (Rodríguez-Moreno et al. 2011). The nucleotide sequences of chloroplast and mitochondrial genomes of PIT92 melon were determined by Rodríguez-Moreno et al. (2011), showing that the chloroplast genome of 156,017 bp included 132 genes, with 98 single-copy genes and 17 duplicated genes in the inverted repeat regions (IRR). Moreover, 2.74 Mb of mitochondrial sequence, using Roche-454 sequencing technology, were assembled into five scaffolds and four additional unscaffolded contigs (Rodríguez-Moreno et al. 2011). These same authors showed that melon mitochondrial genome contained a high number of repetitive sequences and a high content of DNA of nuclear origin. Indeed, DNA transfer from organellar genomes to nuclear genome, and vice versa, seems a common phenomenon (Cui et al. 2021; Kleine et al. 2009; Martin 2003).

2.3.3 Genomic Tools for Melon Breeding

In recent years, efforts were dedicated to building genomic tools to be applied for breeding programs in order to obtain better melon varieties. The use of genomic resources to better understand disease resistance, fruit morphology, and quality has been facilitated by the availability of a reference genome and the rapid advances in Next-Generation Sequencing (NGS) technologies, such as Whole-Genome Sequencing (WGS), Whole-Genome Resequencing (WGR), RNA-seq, and GBS (Pereira et al. 2018). NGS has been used to extend insights in genomic research for developing molecular markers, identification of genetic variation, and gene discovery using sequencing approaches (Natarajan et al. 2016). Among these technologies, WGS allows scientists and breeders with improved analysis based on bioinformatics; therefore, discovering and identification of genes regulatory sequences, molecular markers, and quantitative trait locus controlling fruit quality, biotic and abiotic threats as well as other agronomic traits were performed (Pérez-de-Castro et al. 2012). Genome-wide SNP markers developed by sequencing enable high-density genetic maps, greatly improving the QTL mapping resolution (Gur et al. 2017) as well as the selection of core collections to capture the maximum genetic diversity with minimal redundancy (Wang et al. 2021). Likewise, WGR is widely used to discover the genetic diversity and molecular markers in a variety of plant populations and to gain a better understanding of the relationship between genotypic and phenotypic changes (Xu and Bai 2015). In addition to the identification of genetic polymorphisms such as SNP and insertion/deletion polymorphism (InDel), WGR permits the detection of Copy Number of Variation (CNV), presence/absence variation (PAV), and QTLs associated with disease resistance genes for the re-sequenced variants based on available R-genes from the reference genome (Natarajan et al. 2016). The large-scale data generated by NGS, combined with powerful computational tools enabled a major technological leap from low-resolution to high-resolution QTL mapping (Galpaz et al. 2018).

Transcriptome sequencing using RNA-seq technology allows exploring gene expression changes in melon plants during fungal and viral infections (Sáez et al. 2022; Cao et al. 2021) and fruit-related traits (Zhang et al. 2022; Galpaz et al. 2018). This tool offers a global view of expression changed during the defense response and elucidates complex resistance mechanisms in plants through comparing gene expression upon infection in susceptible and resistant genotypes (Sáez et al. 2022) and also to elucidate genetic factors that determine melon fruit-quality traits (Galpaz et al. 2018).

In addition, all these tools and resources also facilitate the melon genetic diversity studies, which are important for the management, improvement, and enhancement of germplasm.

2.3.4 Genomic Selection

Creating a new melon cultivar might take 10–12 years, due to several stages of crossing, selection, and testing required in the traditional production of new melon varieties. Innovative molecular tools, marker-assisted selection (MAS) including marker-assisted backcross selection, 'breeding by design,' or new strategies, like genomic selection, molecular marker-assisted breeding (MAB), and gene-editing are needed for the rapid development of new cultivars (Salgotra and Stewart 2020).

2.3.4.1 MAS and FMs for Precision Breeding

MAS is a powerful genomic tool that assists phenotypic selection for the development of disease-resistant cultivars and allows breeders incorporate and pyramid resistance genes into breeding material (Zhu et al. 2020). An example is 'Carmen,' a new Yellow Canary-breeding melon line obtained by introgression of powdery mildew, CYSDV, and *A. gossypii* resistances of TGR-1551 into the genetic background of Bola de oro cultivar using molecular markers linked to *P. xanthii* races 1, 2, and 5 resistances and *Vat* gene (Palomares-Rius et al. 2018). MAS has also been extensively applied to search for the molecular markers that are linked to a specific trait during the development of disease-resistant cultivars (Teixeira et al. 2008), and it has been successfully applied to melon breeding, to improve, disease resistance and fruit quality, but these methods are not effective for detecting complex quantitative traits with small-effect QTL (Xu et al. 2012). Important genes and QTLs for disease resistance, fruit quality, and other traits in melon are listed in the Cucurbit Genetics Cooperative (https://cucurbit.info/home/gene-lists/).

Identifying genes and functional markers (FMs) that are highly associated with plant phenotypic variation is a challenge (Salgotra and Stewart 2020). Strategies to identify FMs for breeding goals include functional genomics approaches such as transcriptomics, targeting induced local lesions in genomes (TILLING), homologous recombinant (HR), association mapping, and allele mining (Salgotra and Stewart 2020). In comparison to other markers used in plant breeding, FMs had the advantage of the close genomic association with a phenotype, which may facilitate the direct selection of genes associated with phenotypic traits, and therefore, increase the selection to develop new varieties (Salgotra and Stewart 2020).

Advances in sequencing techniques enable the identification of SNPs and indels linked with various traits; FM development is, thus, enabled (Salgotra and Stewart 2020). Indels may cause phenotypic variation from extensive genomics, which are accompanied by chances of elimination from natural selection (Andersen and Lübberstedt 2003). Hence, SNP-derived FMs have advantages over indel-derived markers because of the widely distributed nature of FMs throughout the genome (Liu et al. 2012). Besides, the use of SNPs sets in high-throughput genotyping platforms is a powerful approach (due to their low cost, high genomic abundance, locus specificity, co-dominant inheritance, and low genotyping error rates (Cao et al. 2021)) for many genetic applications for breeding programs, such as germplasm characterization, quality control (QC) analysis, linkage mapping, linkage-based, and linkage disequilibrium-based QTL mapping, allele mining, marker-assisted

backcrossing (MABC), genomic selection (GS), and MAS (Salgotra and Stewart 2020; Cao et al. 2021).

2.3.4.2 *R*-Genes

The large amount of generated data in melon sequencing projects can be useful to promote the *in silico* identification of important classes of genes (Andolfo et al. 2021). In recent years, the identification of genome-wide resistance (*R*) gene candidates has become a popular research aim in several species due to the development of prediction tools based on the identification of distinctive structural domains (Andolfo et al. 2013; Garcia-Mas et al. 2012). To date, more than 150 (*R*-genes) have been cloned and characterized in plants (www.prgdb.org). In melon, only a handful *R*-genes have been cloned, including *Vat*, which confers resistance to melon aphid (Dogimont et al. 2014), *Fom*-2, conferring resistance to *Fom* races 0 and 1 (Joobeur et al. 2004), and the head-to-head oriented pair of *R*-genes, *Fom*-1 and *Prv*, which confer resistance to *Fom* races 0 and 2, and the potyvirus *Papaya ring spot virus*, respectively (Brotman et al. 2013). In addition, recessive resistance genes were also identified; *nsv*, controlling resistance to the *melon necrotic spot virus*, which encodes a translation elongation factor (Nieto et al. 2007), downy mildew resistance genes encoding photorespiratory amino transferases (Taler et al. 2004), and *cmv1*, which encodes a vacuolar-sorting protein (Giner et al. 2017).

In the reference genome, 411 putative disease *R*-genes organized in clusters were identified, among them, 81 may exert their disease resistance function as cytoplasmic proteins through canonical resistance domains, such as the NBS, the LRR, and the TIR domains (Garcia-Mas et al. 2012). Besides, 15 homologs to the barley *Mlo* (Büschges et al. 1997) and 25 homologs to the tomato *Pto* (Loh and Martin 1995) genes were also identified.

To gain access to information and to facilitate the analysis of melon *R*-gene repertoires, the exploration of resources could be an important starting point (Andolfo et al. 2021). Several methodologies such as BLAST search, domain matching, sequence alignment, and phylogenetic analysis methods can be employed for R-proteins identification (Andolfo et al. 2013; Garcia-Mas et al. 2012). To search for plant resistance genes in the plant genome, DRAGO (Disease Resistance Analysis and Gene Orthology), a robust prediction tool, is available on the PRGdb platform (Andolfo et al. 2021; Osuna-Cruz et al. 2018).

To accelerate *R*-gene discovery and localization, high-resolution genetic maps can be combined with sequence data. Andolfo et al. (2021) reported that knowing the location of a given *R*-gene locus is a great advantage for mining its nucleotide sequences using both recombination analysis and protein-function prediction.

2.3.4.3 Trait Mapping and Discovery of Candidate Genes

Linkage analysis has been extensively conducted to identify QTL using segregating populations derived from biparental crosses (Heffner et al. 2009). F2, backcross (BC), DH, and recombinant inbred line (RIL) populations are used as biparental mapping populations. However, Pérez-de-Castro et al. (2012) reported that low mapping resolution is provided when using biparental populations due to the

occurrence of only a few recombination events. In contrast, Pereira et al. (2018) reported that the GBS approach applied in a biparental RIL population is highly effective for QTL mapping studies highlighting that type and size of the population and map density are the main limiting factors for detecting QTLs.

The association mapping approach is a powerful method that uses historical recombination events for QTL detection in natural populations or germplasm collections via GWAS (Phan and Sim 2017; Zhu et al. 2008; Gupta et al. 2005). In comparison to linkage analysis, mapping approach is less time consuming, has a higher mapping resolution and a greater number of alleles to mine (Yu and Buckler 2006). NGS technologies facilitated the development of genome-wide molecular markers, especially SNPs, for high-throughput genotyping, providing the opportunity for association mapping (Zhu et al. 2008).

Bulked segregant analysis (BSA) is an important technique used to map QTLs and identify DNA markers. BSA provides a convenient and rapid method to identify resistance genes by generating two DNA bulks with a contrasting traits (Nie et al. 2015; Abe et al. 2012; Michelmore et al. 1991). Recently, whole-genome resequencing has been coupled with BSA to map the genes of interest that are associated with a given phenotype (Zou et al. 2016). The combined application of BSA with NGS (BSA-Seq) has accelerated the identification of tightly linked markers for gene identification and QTL mapping (Zou et al. 2016).

Linkage maps are an effective tool to study the genetic architecture of both monogenic and complex traits (Diaz et al. 2011). Recently, high-density maps (using hundreds to thousands of markers) have been constructed for QTL mapping of main traits (Pereira et al. 2018; Chang et al. 2017) demonstrating that a higher SNP density substantially increases the QTL mapping potential which affects the QTLs detection and resolution. These QTLs location in narrow genomic intervals could facilitate genes cloning and use in breeding programs by MAS (Pereira et al. 2018).

Disease Resistance

Melon is susceptible to several pathogens. In breeding programs, identification of disease resistances and associated molecular markers is a priority. We report here the last molecular mapping of host resistances against the most important fungal and viral pathogens in melon.

For QTL mapping of the genes involved in the resistance of powdery mildew, caused by the airborne fungus *Podosphaera xanthii* (*Px*), Branham et al. (2021) used a densely genotyped RIL population and identified two major QTLs associated with resistance to *Px* race 1 in chromosomes 5 and 12 (*qPx1-5* and *qPx1-12*) and two minor QTLs (*qPx1-4* and *qPx1-10*) in chromosomes 4 and 10. For marker development across the major QTLs and functional annotation of SNPs for candidate gene analysis, the authors used the WGR of the parents. Competitive allele-specific PCR (KASP) markers were tightly linked to the QTL peaks of *qPx1-5* and *qPx1-12* in the population which will enable efficient marker-assisted introgression of Px resistance into improved germplasm. Candidate genes were identified in both major QTL

intervals that encode putative R-genes with missense mutations between the parents. These candidate genes provide targets for future breeding efforts.

Cao et al. (2021) identified, for resistance to powdery mildew (PM), a novel QTL on chromosome 12 named *qCmPMR-12*. They used an F_2 segregating population to map major PM resistance genes using BSA-Seq analysis. Most likely candidate genes were predicted from RNA-Seq analysis which indicated that the *MELO3C002434* gene encoding an ankyrin repeat-containing protein was the most likely candidate gene that was associated with resistance. Moreover, they successfully converted 15 polymorphic SNPs around the target area to KASP markers. KASP is a high-throughput SNP-genotyping platform, which become a global benchmark technology and has been widely used for genetic mapping and trait-specific marker development, due to its low cost and genotyping error rates, and its high reliability and reproducibility (Cao et al. 2021). So, the novel QTL and candidate gene identified provide insights into the genetic mechanism of PM resistance, and the tightly linked KASP markers developed to this disease resistance can be used for MAS in melon-breeding programs.

Natarajan et al. (2016) investigated the genetic variation of 4 melon accessions to PM. The whole-genome resequencing using the Illumina HiSeq 2000 platform was done, to characterize the genotypic variation in terms of SNPs, InDels, and structure variations (SVs). QTLs associated with PM resistance genes were detected. In addition, 112 SNPs and 45 InDels, were identified, associated with defense genes that will serve as candidate polymorphisms in the search for sources of resistance against PM and could accelerate marker-assisted breeding in melon.

Fusarium wilt in melons is caused by *Fusarium oxysporum* f. sp. *melonis* (FOM) and is considered one of the most devastating soil-borne diseases (Oumouloud et al. 2013; González et al. 2020). Two major genes *Fom*-1 and *Fom*-2 have been genetically characterized (Risser et al. 1976) and tightly linked markers to these genes are available (Oumouloud et al. 2012, 2015). *Fom-1* confers resistance to races 0 and 2, whereas *Fom-2* confers resistance to races 0 and 1 of *Fom*. Branham et al. (2018) reported that four QTLs (a major QTL co-located with the previously validated resistance gene *Fom*-2, and three minor QTLs) and an epistatic interaction were associated with resistance to FOM race 1 in a RIL population of 172 lines (MR-1 × susceptible AY).

The *Cucurbit yellow stunting disorder virus* (CYSDV) is a Crinivirus of the family Closteroviridae (Martelli et al. 2000) that severely infects melon. Pérez-de-Castro et al. (2020) reported two major QTLs to CYSDV resistance in melon line TGR-1551, both located near each other in chromosome 5. A RIL population was used, for mapping the gene/s responsible for this resistance. The RIL population was evaluated for resistance to CYSDV and genotyped in a GBS analysis. SNP markers were identified, which will be useful in MAS of CYSDV resistance introgression in elite melon cultivars. Further, Tamang et al. (2021) reported the identification of two QTLs to CYSDV resistance on chromosomes 3 and 5 for potential use in MAS. Besides, 24,673 SNP markers were identified in GBS-SNP calls in $F_{2:3}$ TM × PI313970 population. The identified QTL region that conferred resistance to CYSDV in melon line PI 313970 by Tamang et al. (2021), confirmed the QTL

regions on chromosome 5 of TGR-1551 that were previously identified by Pérez-de-Castro et al. (2020). The tightly linked markers with the CYSDV resistance QTL in TGR-1551 and PI313970 can be used to expedite the development of CYSDV-resistant elite breeding lines and cultivars.

Tomato leaf curl New Delhi virus (ToLCNDV) is a severe disease on melon. Sáez et al. (2022) performed an RNA-seq assay to identify associated genes that are differentially expressed, during ToLCNDV infection, between resistant and susceptible melon genotypes and transcript levels were also compared. Differentially expressed genes (DEGs) were classified using gene ontology (GO) terms, and genes of the categories transcription, DNA replication, and helicase activity were down-regulated in the resistant genotype but up-regulated in the susceptible, suggesting that reduced activity of these functions reduces ToLCNDV replication and intercellular spread and thereby contributes to resistance. The expression levels of selected candidate genes were validated by qRT-PCR in resistant and susceptible genotypes and SNPs with an effect on structural functionality of DEGs linked to the main QTLs for ToLCNDV resistance have been identified.

Fruit Quality

Fruit quality is the main target for melon-breeding improvement. Morphology (external and internal color, shape, netting, sutures), aroma, nutritional content, sweetness, acidity, ripening, and post-harvest storage are complex traits that contribute to the final fruit quality in melon (Monforte et al. 2004). The availability of genomic resources in melon is contributing to the understanding of the processes that control fruit quality (Ramamurthy and Waters 2015; Monforte et al. 2004). In recent years, many loci involved in the genetic control of these traits have been described (Perpiña et al. 2016; Diaz et al. 2011; Fernandez-Silva et al. 2010). Once the genes underlying these traits are identified, the use of natural variation found in germplasm collections or induced variation through genome editing is a promising way for fruit-quality improvement.

Tomason et al. (2013) used 87 melon accessions from different geographic regions for association mapping study and identified 22 major QTLs for fruit shape, fruit length, fruit diameter, soluble solid content, and rind pressure.

Ramamurthy and Waters (2015) used an F_2 mapping population constructed from a cantaloupe orange-fleshed melon and a green-fleshed snake melon and identified a total of 31 QTLs associated with fruit quality and fruit morphological traits. They showed that most of the phenotypic variation for yield is explained by a small segment of LG8.

Argyris et al. (2017) reported that a valuable resource for QTL mapping is the NILs, which contains a single homozygous introgression of a donor line in the genetic background of a recipient line. NILs are a powerful tool that has advantages over other types of mapping populations in making possible the detection and estimation of QTL of small effect (Keurentjes et al. 2007). Dissection of QTL identified in NILs through the development of subNILs has been utilized to effectively map and clone QTL involved in melon fruit morphology (Fernandez-Silva et al. 2010), and fruit ripening (Rios et al. 2017). Fine-mapping of QTL involved in

sugar accumulation in melon has been reported (Argyris et al. 2017). Despite this, there is a correspondence of positions of QTL in different mapping populations, with clustering of QTL for SSC and soluble sugars identified on chromosomes 2, 3, and 5 (Diaz et al. 2011).

Sugar accumulation in melon flesh has been reported to have strong GxE interactions and low heritability (Perpiña et al. 2016) which complicates breeding of this trait. Argyris et al. (2017) identified a stable QTL, *SUCQSC5.1*, which reduced SSC and sucrose content. Through fine mapping with the subNILs, the authors accurately estimate its phenotypic effect and provide its function. Indeed, expression analysis of the candidate genes in mature fruit showed differences between the 'high' sugar and 'low' sugar phenotypes for *MELO3C014519*, encoding a putative BEL1-like homeodomain protein. The molecular markers linked to the QTL developed can be used in breeding programs with wild accessions to select against those alleles reducing SSC.

Galpaz et al. (2018), to elucidate genetic factors that determine melon quality, they used RNA-Seq-based QTL and eQTL mapping and identified *Thiol acyltransferase* (*CmThAT1*) gene, within the QTL interval of its product, the *S*-methyl-thioacetate which is a key component of melon fruit aroma, as well as a candidate major gene *CmPPR1* determining fruit white-flesh color in melon.

Zhao et al. (2019) reported a comprehensive map of the genomic variation in melon derived from the resequencing of 1175 diverse accessions. Resequencing of genomes is very useful for the genome-wide discovery of polymorphisms amenable for high-throughput genotyping platforms (Galpaz et al. 2018). Zhao et al. (2019) sheds light on the domestication history of melon suggesting that three independent domestication events occurred, two in India and one in Africa. In addition, using GWAS, 208 loci associated with fruit quality, and morphological characters were identified.

Liu et al. (2020), using GWAS, identified eight fruit size and seven flesh thickness signals overlapping with selective sweeps. *CmCLV3* was detected in most melon accessions, which has pleiotropic effects on carpel number and fruit shape. They also detected 233 and 159 potential selective signals in ssp. *agrestis* and spp. *melo*, respectively. Two alcohol acyltransferase genes (*CmAATs*) unique to the melon genome may have undergone stronger selection in ssp. *agrestis* for the characteristic aroma as compared with other cucurbits.

Amanullah et al. (2021) used an F_2 population and SNP-derived CAPs markers to map QTLs for seed traits (width, length, thickness, shape, and 100-seed weight), and identified three QTLs for seed width, seed length, and seed thickness on chromosomes 3 and 9. Besides, a major-effect QTL, *SW3.1*, was also detected on chromosome 3. Fine mapping or cloning of QTLs for fruit-related traits is still rarely reported in melon (Zhang et al. 2022)

The high-resolution genetic maps and QTLs analyses for fruit size described in Lian et al. (2021) provided a better understanding of the genetic basis of domestication and differentiation. Indeed, two loci for fruit size were identified on chromosomes 5 and 11. An auxin response factor and a YABBY transcription factor

were inferred to be the candidate genes for both loci. These findings could provide a valuable tool for map-based cloning and molecular marker-assisted breeding.

Zhang et al. (2022) illustrated the strength of a joint analysis combining resequencing-based genetic map for QTL mapping and a combination of KASP genotyping and RNA-seq analysis to facilitate QTL fine mapping. They reported a high-density genetic map of melon and nine major QTLs. Based on RNA-seq, *EVM0009818*, involved in cytokinin-activated signaling, was differentially expressed in the young fruits. Selective sweep analysis identified 152 sweep signals for seed size, including two seed-related QTLs and nine homologs that have been verified to regulate seed size in *Arabidopsis*.

2.3.5 Genome Editing

Sequencing techniques are able to provide important details on the position of functional elements of DNA, highlighting differences even of a few bases between genotypes of the same species; at the same time, great progress has been achieved in developing genomic engineering tools (Andolfo et al. 2016).

Genome-editing tools have the potential to modify genomic sequences with accuracy (Veillet et al. 2019). Some of these tools are Homologous Recombination (HR), Targeted Induced Local Lesions In the Genome (TILLING), Zinc Finger Nucleases (ZFN), Transcriptional Activator-Like Effector Nucleases (TALENs), or Clustered Regularly Interspaced Short Palindromic Repeats associated with nuclease Cas9 (CRISPR/Cas9).

Efficient gene editing in melon presents the possibility to study new gene functions for basic research, and new opportunities for melon productivity by improving biotic stress resistance, melon production, and post-harvest utilization (Bin et al. 2022; Hooghvorst et al. 2019; Dahmani-Mardas et al. 2010).

2.3.5.1 Tilling

The TILLING method is useful in identifying novel alleles in genes controlling agronomic traits of interest in melon (Dahmani-Mardas et al. 2010). Indeed, a TILLING platform generated from a monoecious climacteric cantalupensis genotype and andromonoecious non-climacteric inodorus genotype has become available and has proven to be useful for improving the melon shelf life and represented a useful resource for functional studies and melon breeding (González et al. 2011).

For ethylene biosynthesis, the conversion of aminocyclopropane-1-carboxylic acid (ACC) to ethylene by the ACC oxidase (ACO) is required (Ayub et al. 1996). In melon, *CmACO1* silencing inhibits fruit ripening and extends fruit shelf life, demonstrating that ACO is involved in ripening, growth, and development (Ayub et al. 1996). Dahmani-Mardas et al. (2010) have developed a reference ethyl methanesulfonate-mutagenized (EMS) mutant population and characterized *CmACO1* TILLING mutants that inhibit fruit ripening and extend fruit storage life.

2.3.5.2 CRISPR/Cas9

The new CRISPR/Cas9 genome-editing technique was developed in 2013 and has transformed genetic engineering, due to its efficiency, versatility, precision, and reduced costs (Andolfo et al. 2016; Hooghvorst et al. 2019). Precise changes are produced, at preselected genomic sites with no genetic footprints and no off-targets (Hooghvorst et al. 2019; Chandrasekaran et al. 2016). Genes function studies by knocking out genes that negatively affect fruit quality is also allowed using this technique (Tian et al. 2016).

CRISPR knockout mutants in melon have been reported for the first time in 2019 by Hooghvorst et al. using CRISPR/Cas9-mediated genome editing. In plants, the major uses of CRISPR/Cas9 have been gene knockouts to elucidate the function of a target gene–by-gene mutation and transcriptional regulation (Hooghvorst et al. 2019).

Hooghvorst et al. (2019) using CRISPR/Cas9 generated multi-allelic mutations in both genomic target sites of the phytoene desaturase gene (*CmPDS*), a key enzyme for the carotenoids production in melon. Chimeric albino phenotypes have been successfully regenerated.

Giordano et al. (2022), using CRISPR/Cas9, showed the generation of melon knockout mutants CTR1 and ROS1 for fruit ripening and reported for the first time the inheritance of the introduced mutations to the following generations. Two functionally validated genes (*CmROS1* and *CmCTR1-like*) are involved in the regulation of fruit ripening and showed the role of the DNA demethylase ROS1 in fruit ripening. The authors characterized the ETHQV6.3 QTL genomic interval, a QTL involved in climacteric ripening regulation, which allowed the identification of a negative regulator of ripening *CTR1*-like (*MELO3C024518*), and a demethylase *ROS1* (*MELO3C024516*) and evidenced the role of both genes in melon climacteric ripening. Indeed, in the CRISPR mutants, the authors reported the formation of abscission layer, aroma, and ethylene production. The *CmROS1* knockout mutant revealed that during fruit ripening, the balance of global DNA methylation/demethylation is altered, which is governed by DNA demethylases (Giordano et al. 2022).

Bin et al. (2022) provided new insight regarding CmNAC-NOR function in melon fruit ripening. Two CRISPR/Cas9-mediated mutants nor-3 and nor-1 in the climacteric Védrantais background were obtained. nor-3, containing a 3-bp deletion altering the NAC domain A, resulted in the delay of ripening without affecting fruit quality. In contrast, nor-1 containing a 1-bp deletion resulting in a fully disrupted NAC domain, completely blocked climacteric ripening (ethylene was not produced, abscission layer was not formed, and external color was not changed) suggesting it as a potential target to modulate shelf life in climacteric melon.

In summary, gene-editing technology has great potential. To date, no edited plants have been obtained for disease resistance in melon but such technology can strongly contribute to making the melon more resistant to biotic/abiotic stress and improving consequently yields. The use of CRISPR and genome-editing technologies will open new opportunities, potentially circumventing restrictions on Genetically Modified crops (Veillet et al. 2019).

2.4 Conclusion

The increasing of high-throughput sequencing technology has made possible huge progress through molecular, and genetic research on disease resistance, fruit development, and ripening of melon. The high-quality reference genome of melon has played a primary role in this advancement and has been used for the resequencing of diverse germplasm to explore genome-wide sequence variations, especially SNPs. Several QTLs with high mapping resolution have been discovered for disease resistance, and fruit traits in melon; which have enabled the development of useful resources, such as molecular markers for these QTLs, to improve selection efficiency in melon-breeding programs.

Genomics allow the identification of polymorphic loci responsible for variation in phenotypic traits. The release of a genome assembly and large-scale sequencing and resequencing data improved knowledge of the evolution, selection footprint, genetic architecture, and gene mapping and cloning of fruit-related traits. Besides, advancements in transcriptomics, plant defense mechanisms, and genomics will provide new opportunities to accelerate melon breeding programs. Indeed, integrating genetic and genomic data will help breeders to obtain a more durable resistance to diseases and a better fruit quality.

All the resultant data should be made available according to FAIR (findable, accessible, interoperable, and reusable) principles, and linked phenotype data should also be incorporated. Indeed, huge information from high-throughput phenotyping and genomics technologies are provided which helps researchers to guide their breeding programs to biotic and abiotic stresses. In this process, bioinformatics is fundamental to exploit and integrate these data, through association studies to detect genomic targets underlying key traits useful for melon breeders.

Acknowledgments The authors H.C.R and W.A are grateful to Wageningen University & Research (WUR)-Plant Breeding (Netherlands) and CRRGC-Béja (Tunisia) and also to the Orange Knowledge Programme (OKP)-Nuffic for offering the 3 months Tailor-Made Training Course 'Application of Genomic Plant Breeding for Challenging Climate Change in Tunisia'.

A.G.C is grateful to PID2020-116055RB-C22 I+D+I project funded by MCIN/AEI/10.13039/501100011003 and the A11-20R project funded by the Aragon Government.

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Ash Gourd Genomics: Achievements, Challenges and Future Perspectives

3

Hament Thakur

Abstract

Ash gourd is an important crop plant species belonging to family cucurbitaceae. This traditional vegetable is versatile in nature, which can be used as vegetable, medicine and for making post-harvest products like sweets and candies. Advanced molecular breeding strategies have been applied to improve yield, quality and disease resistance and to meet consumer and farmer requirements. The next-generation sequencing techniques have fast tracked the sequencing of all the vegetable crops. In 2019, Chinese researchers were able to sequence the ash gourd genome, which will aid researchers in determining the precise positions of corresponding genes and employing these connected markers in marker-assisted breeding procedures. Also, the sequencing methods have contributed for the development of molecular markers, gene identification and QTL mapping, transcriptomics and genome editing. This chapter highlights genome-based gene identification methodologies for numerous features and problems for future functional genomic studies, and this could provide as a theoretical foundation for current ash gourd breeding efforts.

Keywords

Ash gourd · Genome · Mapping · Transcriptomics · Sequencing

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_3

3.1 Introduction

Cucurbits are the popular vegetable crops belonging to Cucurbitaceae family. The major cucurbitaceous crops like cucumber, muskmelon, water melon and pumpkin are grown worldwide, while bottle gourd, bitter gourd, wax gourd and luffa are prevalent in many Asian and African countries. *Benincasa hispida*, is also known as ash gourd, wax gourd, white gourd and winter melon. The winter melon is the only member of the genus *Benincasa* (Chopra and Nayar 1956; Chen et al. 2021). Ash gourd is considered as the native of the Indo-China region (Rubatazky and Yamaguchi 1999) and is extensively cultivated in old world tropical countries viz. India, China and Japan whereas it is less common in New World. Ash gourd has huge popularity in Asia because of its medicinal and nutritional properties (Al-Snafi 2013). It is well known for long storage and value-added products as it is baked, fried, boiled, pickled and candied (Robinson and Decker-Walters 1999).

During last decade, there was a rapid development in genomics research including draft genomes and high-density genetic maps in cucurbit crops. Despite high economic importance, the genomic information in ash gourd is limited, thus, retarding the translational research in its breeding. Advanced molecular breeding tactics have been used in ash gourd not only to enhance yield but also to meet out consumer demands and to bring convenience to farmers. In ash gourd, the traits like plant architecture, increased yield, resistance to biotic and abiotic stresses, quality traits, pest resistance are some of the main targets of modern breeding. The biotechnological tools like next-generation sequencing have significantly accelerated the process of genome sequencing. The published genome sequence of cucurbits including *Cucumis sativus* (Huang et al. 2009), *Cucumis melo* (Garcia-Mas et al. 2012), *Citrullus lanatus* (Guo et al. 2013), *Momordica charantia* (Urasaki et al. 2017), *Cucurbita pepo* (Montero-Pau et al. 2018), *Lagenaria siceraria* (Wu et al. 2017), *Cucurbita maxima* (Sun et al. 2017), *Cucurbita moschata* (Sun et al. (2017), *Cucurbita argyrosperma* (Barrera-Redondo et al. 2019) and *Benincasa hispida* (Xie et al. 2019) have helped researchers in determining the gene position on chromosomes. These identified linked markers can be used for marker-assisted based molecular breeding.

To examine genetic variation and evolutionary links among related crops, new generation sequencing has been used to produce molecular markers (Hu et al. 2021; Levi et al. 2010). With the advancement in sequencing technology, the application of SNPs has surpassed the other molecular markers. Earlier, the sequencing was costly and time consuming when it was performed after the PCR amplification for genomic region of interest (Edwards and Batley 2010). Nowadays, a large number of sequences are generated through NGS methods, which are less costly with higher efficiency of SNP discovery. These markers have been used to create molecular genetic and physical maps, as well as to identify the genes or quantitative trait loci that govern economically significant traits (Varshney et al. 2009). In addition, resequencing approaches based on reference genomes are employed to investigate genome-wide diversity. Bulk segregant analysis (BSA) has been proposed as the primary method for whole genome sequencing. A number of approaches based on

BSA like QTL-seq (Takagi et al. 2013), MutMap (Abe et al. 2012), bulked segregant RNA-Seq (BSRseq) (Liu et al. 2012), specific locus-amplified fragment sequencing (SLAF-seq) (Xu et al. 2014) and genome-wide association studies (GWAS) (Saidou et al. 2014) have been used in many vegetable crops for identification of the linked gene and marker development.

The sequence data obtained from transcriptomic analysis as well as their expression profiles linked to various physiological situations will aid in the identification of genes that control various traits. This will unravel the regulatory mechanisms behind different traits and help to elucidate the complete pathway. For the past five decades, there have been significant advancements in molecular biological approaches. The discovery of sequence-specific nucleases (SSNs) and (CRISPR)/CRISPR-associated protein (Cas) system has taken gene editing to the next level, resulting in vegetables with modified functions and desired traits (Abdallah et al. 2015; de Caceres et al. 2020).

In this chapter, the author will summarize studies related to genome sequencing and genome-based techniques for the identification of genes and molecular breeding, which can serve as a theoretical reference for ash gourd and Cucurbitaceae crop improvement programmes.

3.2 The Ash Gourd Genome

The genome of the ash gourd was discovered to be larger than that of cucumber, melon and watermelon (Xie et al. 2019). Authors completed the sequencing of genome in 2019 by using ash gourd line B227 with large (80 cm length) dark green fruits, using Illumina and single-molecule real-time (SMRT) sequencing technologies. Based on k-mer analysis the estimated 1.03 Gb genome comprises 55.4 Gb high-quality cleaned sequences with ~50-fold coverage of the genome. De novo assembly of Illumina and PacBio sequences resulted in a 913 Mb long-draft genome. The number of scaffolds was 2197 with a scaffold N50 of 3.4 Mb, N90 scaffold of 0.9 Mb and longest scaffold of 14.5 Mb (Xie et al. 2019). Using the high-density genetic map, 859 Mb (94.1%), comprising 397 scaffolds, could be attached to the 12 linkage groups. The number of predicted coding genes was 27,467 in number with average gene length of 3962 bp. According to a comparative examination of the genomes of all the cucurbit species, the genome of wax gourd is the most ancestral karyotype studied so far.

3.3 Beyond Ash Gourd to Family Cucurbitaceae

The cucumber genome published in 2009 was the first ever published genome among the cucurbit species. The reference genome of cucumber '9930' was sequenced with a genomic coverage of 72.2-fold with a genome size of 367 Mb (Huang et al. 2009), subsequently genome of semi-wild cultivar 'GY14' and wild cultivar 'PI 183967' were sequenced (Qi et al. 2013). The genome size of melon

(450 Mb) is larger than its close relative cucumber (367 Mb), and this increased size may be due to the transposon amplification which may have led to duplications in the melon lineage. Moreover, the variations in phenotypic and quality traits and other stress-related genes in melon may also have occurred due to transposon amplification (Garcia-Mas et al. 2012). Guo et al. (2013) sequenced the genome of watermelon by using an inbred line '97103' using Illumina sequencing which resulted in a genome size of 353.5 Mb. The resequencing of watermelon genome in 2019 using inbred line '97103' have led to the genome size of 365.1 Mb, and authors indicated the role of selection during domestication and improvement of watermelon fruit size and fixation of a non-bitter allele in *C. lanatus* (Guo et al. 2019). A more confident gene set of bitter gourd genome (Cui et al. 2020) of line Dali-11(300 Mb) compared to line OHB3-1 (Urasaki et al. 2017) provided valuable insights into domestication of bitter gourd in Southern Asia. The evidence for allotetraploidization event in *Cucurbita* was provided by studying the high-quality genome sequences of *C. maxima* and *C. moschata* (Sun et al. 2017). The first high-quality genome of *Cucurbita argyrosperma* wild relatives (Barrera-Redondo et al. 2021) provided insight into domestication genes having their role in hormonal regulation, defence mechanisms, seed germination and development. The occurrence of shared alleles among *C. argyrosperma* and *C. moschata* suggested the introgression during domestication between both the taxa. The genome sequence of bottle gourd (313.4 Mb) provided understanding of the history of genomic evolution of the family and identified chromosome-level syntenic relationships between bottle gourd and other cucurbits (Wu et al. 2017). Snake gourd (*Trichosanthes dioca*) and chayote (*Sechium edule*) diverged from sponge gourd and have one of the largest genome sizes of 919.8 Mb (Ma et al. 2020) and 606.42 Mb (Fu et al. 2021), respectively, among all the cucurbit species. The Illumina HiSeq 4000 platform was used to sequence the whole chloroplast genome of wax gourd (156,758 bp). Based on the entire chloroplast genome and 72 genes, phylogenetic analysis revealed sibling ties between *B. hispida* and *Citrullus*, *Lagenaria* and *Cucumis* (Song et al. 2022). In 2019, The Cucurbit Genomics Database (CuGenDB; http://cucurbitgenomics.org) was created to utilize the large-scale datasets and to provide an essential gateway for the cucurbit research. The database provides information about all the available cucurbit genomes and genomic information with respect to expressed sequence tag (EST) sequences, genetic maps, transcriptome profile synteny blocks etc. (Zheng et al. 2019).

3.4 Molecular Markers in Ash Gourd Breeding

Molecular markers are the vital tools which have been used to study genetic diversity and variability, phylogenetic relationships, purity of F_1 hybrids and mapping the location of qualitative and quantitative traits. Randomly amplified polymorphic DNAs (RAPD) owe the qualities like simplicity, speed and comparatively less cost (Rafalski and Tingey 1993) due to which they are generally used for genetic diversity studies in ash gourd (Parkash et al. 2000; Singh 2002; Verma et al. 2007;

Pandey et al. 2008; Sikdar et al. 2010). The factors like number of molecular markers and per cent polymorphism are an important factor for DNA marker-based studies. Jiang et al. (2013) identified 6242 SSRs in ash gourd, and out of the 200 synthesized primer pairs, 28.8% markers showed polymorphism. SSR markers have played an important role in comparative genomics as they are conserved among closely related species and genus (Ghebretinsae et al. 2007; Yang et al. 2012; Guo et al. 2013). In a cross-species transformation study, ash gourd was found closely related to watermelon as the transferable SSR markers were more from watermelon than melon, pumpkin and cucumber (Hu et al. 2021). Pandey et al. (2021) checked the validity of 70 SSR markers developed from cucumber genome in 16 different species and reported 49.21% polymorphism in ash gourd, and these cross-transferable markers can be used for marker-based studies like molecular mapping and marker-assisted breeding. In another phylogenetic study using EST-SSR and EST-PCR primers, *B. hispida* was reported closest to *P. fistulosus* (Levi et al. 2010).

Pericarp colour, controlled by single gene, is an important trait as it represents the quality of a fruit. A total of 140 F_2 plants were used for SLAF sequencing, detecting 142,653 SLAF sequences with 22,151 (15.42%) polymorphic sequences (Jiang et al. 2015). In another study using BSA-seq data and distribution and density of the SNP physical sites, 14 KASP markers were identified linked with peel colour (Ma et al. 2021). Zhu et al. (2016) reported a primer ISSR-855 linked with the fruit colour trait at a genetic distance of 9.04 cM. Some winter melon germplasm has a pleasant aroma, which adds to its value-added potential. The 'pandan' like aroma found in rice and soybean due to 2-acetyl-1-pyrroline (2AP) was also reported in some of the landraces in ash gourd. A PCR-based marker AroGourd was identified to detect the 804 bp deletion in the aromatic ash gourd (Ruangnam et al. 2017). First female flower node is an important trait in all the cucurbitaceous crops as it leads to early harvest of the fruit, and markers linked with this trait were identified (Cheng et al. 2010).

3.5 Gene and QTL Mapping

Development of novel varieties by way of traditional breeding methods is labour intensive and time consuming. This becomes more difficult when the genes-controlling desirable traits are to be introduced from a donor source. Also, the negative correlation between desired traits obstructs the breeder's ability to select and breed effectively (Nicholson 1960). Introducing contemporary biotechnological technologies utilizing molecular markers in breeding programmes can alleviate these types of challenges. In cucurbits, numerous molecular markers linked with qualitative and quantitative traits have been identified and mapped on various chromosomes (Miao et al. 2011; Qi et al. 2013; Yang et al. 2013, 2019; Nimmakayala et al. 2014; Wei et al. 2014; Cui et al. 2015).

In ash gourd, nine QTLs related to fruit morphological traits namely fruit weight, length, diameter and flesh thickness were identified on chromosome 3, 4, 5, 6, 9, 10 and 11. Among these nine, four QTLs were showing large effect and were

responsible for 10.0% phenotypic variance (Liu et al. 2018). Fruit shape in wax gourd is controlled by a single-candidate gene *Bch02G016830* designated as BFS. The sequencing of a F_2 population by using BSASeq leads to the identification of the candidate gene located in the 17.18 Mb region on chromosome 2, and it was reduced to 19.6 Kb region by using a kompetitive allele-specific polymerase chain reaction (KASP) marker (Cheng et al. 2021). The QTLs controlling first female flower trait, viz. *fn1*, *fn2* and *fn3* were located on linkage group 1 (fn1) and linkage group 6 (fn2 and fn3). The length of map was 1651.9 cM with average distance of 11.47 between two markers. These QTLs viz., *fn1*, *fn2* and *fn3* accounted for 62.54%, 0.2% and 37.39% of the phenotypic variance, respectively (Cheng et al. 2010).

SLAf-seq technique is an important technique especially in those species where the sequenced genome is not available. Use of SLAF-seq leads to the identification of a gene-controlling pericarp colour on chromosome 5, map covered 2172.86 cM, with an average distance of 0.49 cM between neighbouring markers (Jiang et al. 2015). Similarly, bulk segregant analysis sequencing (BSA-seq) was also employed to uncover the candidate gene *Bch05G003950* (*BhAPRR2*), which encodes the *Arabidopsis* pseudo-response regulator2 (APRR2) protein that controls peel colour modulation. The coding sequence of *BhAPRR2* in green-skinned wax gourd had two bases (GA) that were missing in white-skinned wax gourd (Ma et al. 2021). The primary gene that gives winter melon its "pandan-like" scent is *BhAMADH*. This aroma is imparted by an aminoaldehyde dehydrogenase (AMADH), which is encoded by *BhAMADH*. When the aromatic and non-aromatic accessions of *BhAMADH* were compared, an 804-bp deletion-covering exon 11–13 was discovered in the aromatic accession. The genes and QTLs identified so far in ash gourd are presented in Table 3.1.

3.6 Transcriptomics in Ash Gourd

Transcriptome sequencing of different resistant species is proving to be an effective method for discovering linked resistant genes and identifying biological processes implicated in multiple stresses (Jiang et al. 2011; Pan et al. 2012; Garg et al. 2016). RNA sequencing has been widely employed in cucurbitaceous crops to study significant traits such as fruit growth, parthenocarpy, sex expression and responses of plants to biotic and abiotic challenges. (Grassi et al. 2013; Behera et al. 2016; Pawełkowicz et al. 2016; Guo et al. 2018; Wang et al. 2019). Tissue-specific expression of certain miRNAs was detected in a comparative analysis of five tissues, with considerably higher expression in the wax gourd fruit. Fruit had the highest level of MiR164-x expression, suggesting that miR164 may play a role in wax gourd fruit growth by making the miR164-NAC module (Yan et al. 2021). The transcriptome responses of heat-tolerant and heat-sensitive genotypes in chieh-qua genotypes (*Benincasa hispida* Cogn. var. Chieh-qua How) revealed significant change in differentially expressed genes (DEGs). DEGs related to heat shock proteins (HSPs), ubiquitin-protein ligase, transcriptional factors and pentatricopeptide repeat-containing proteins were significantly changed after heat

Table 3.1 Genes and QTLs Identified So Far in Ash Gourd

Trait	Approach	Population	Identified gene/ QTL	Compound/protein involved	Details	Reference
'pandan-like' aroma	Sanger sequencing	237, F_2 progenies	*BhAMADH*	2-acetyl-1-pyrroline (2AP)	804-bp deletion encompass exons in aromatic accession	Ruangnam et al. (2017)
Fruit shape	BSA-seq mapping	6461 F_2 individuals	*Bch02G016830* (BFS)	IQD protein	BFS expression was substantially higher in circular fruits than long cylindrical fruits.	Cheng et al. (2021)
Peel colour	BSA-seq mapping	6244 F_2 individuals	*Bch05G003950* (*BhAPRR2*)	*Arabidopsis* pseudo-response regulator2 (APRR2)	Significantly higher chlorophyll content and *BhAPRR2* expression	Ma et al. (2021)
First pistillate flower node	QTL mapping	115, F_2 individuals	*fn1*, *fn2*, *fn3*	–	Accounted for 62.54%, 0.2% and 37.39% of the phenotypic variance	Cheng et al. (2010)
Fruit related traits	QTL mapping	140 F_2 individuals	FW: *fw3.1*, *fw 6.1* FL: *fl4.4*, *fl10.1* FD*: fd3.1, fd11.1* FT*: ft3.1, ft5.1, ft9.1*	–	Four of the nine identified QTLs had a significant impact	Liu et al. (2018)
Pericarp colour	SLAF-seq	140 F_2 individuals	Single locus on chromosome 5	–	The map covered 2172.86 cM, with an average distance of 0.49 cM between neighbouring markers	Jiang et al. (2015)

stress. This will aid in the discovery of useful genes in heat stress and the study of the mechanisms involved in high-temperature tolerance. At the ovary development stage, gene expression analyses of the gene-determining fruit shape (BFS) in wax gourd revealed that BFS expression was significantly higher in circular fruits than the fruits with cylindrical shape. The BFS gene was thought to alter Ca2+ CaM signalling, cell division and the cytoskeleton, resulting in changes in fruit shape. The protein produced by the BFS gene was supposed to be a member of the IQD protein family, which has been reported to affect cell shape and number in arabidopsis and rice (Duan et al. 2017; Sugiyama et al. 2017), as well as the slenderness of tomato fruit (Wu et al. 2011). A gene called *BhAPRR2* regulates the colour of the fruit's peel in wax gourd. Green-skinned fruit had much higher chlorophyll content and *BhAPRR2* expression than white-skinned wax gourd fruit (Ma et al. 2021).

3.7 Role of Genomics in Detection of Viral Diseases

Diseases cause significant production and quality losses in a wide range of cultivated crops. Similarly, cucurbits are affected with a variety of diseases, but viral diseases are the most serious, since they are causing havoc on these crops. At least 59 distinct plant viruses affect cucurbitaceous crops (Lecoq and Desbiez 2012), majority of which are vector-borne (mostly aphids and whiteflies). The detection and identification of the virus is an important aspect for developing an effective management strategy. The use of molecular tools like RT-PCR has enhanced the rapidity and accuracy of viral diagnosis. Multiple virus detection methods have been developed, and (NGS) is now a major focus of this field since it allows for impartial and hypothesis-free assessment of plant samples in ash gourd, the viruses like *Cucumber mosaic virus* (CMV), *Cucurbit aphid-borne yellows virus* (CABYV), *Papaya ringspot virus* (PRSV), *Zucchini yellow mosaic virus* (ZYMV), Watermelon mosaic virus (WMV), *Cucumber mosaic virus* (CMV), *Cucurbit aphid-borne yellows virus* (CABYV), *Papaya ringspot virus* (PRSV), Watermelon mosaic virus (WMV), *Watermelon silver mottle orthotospovirus* (WSMoV) and *Cucumber green mottle mosaic virus* (CGMMV) contain RNA as a genetic material. The detection of these viruses is done following RT-PCR using a specific set of primers. The cloned and sequenced representative PCR products are submitted to GenBank, followed by the identification by comparing its sequence to that of other isolates. On the other hand, begomoviruses infecting ash gourd which contain DNA as their genetic material namely, *Squash leaf curl China virus* (SLCCNV), *Squash leaf curl Philippines virus* (SLCuPV), *Tomato leaf curl New Delhi virus* (ToLCNDV) and *Tomato leaf curl virus* (ToLCV) are identified by PCR using virus specific primers. The full-length genome is isolated by rolling circle amplification, followed by restriction enzyme digestion, vector ligation and vector transformation. The sequences are entered into a database of nucleotide sequences, and the sequence identities of different isolates are compared. The viruses infecting ash gourd along with their method detection are presented in detail in Table 3.2.

Table 3.2 Viral Diseases Infecting Ash Gourd at Various Locations

Virus	Genus	Family/ Species	Detection	Transmission	Location	Reference
CGMMV	Tobamovirus	*Virgaviridae*	–	Mechanical sap-inoculation	India	Kumar et al. (2017)
CMV	Cucumovirus	*Bromoviridae*	ELISA	Aphid	Western Samoa	Pearson and Liayanage (1997)
CABYV	Polerovirus	*Luteoviridae*	RT-PCR, Sequencing	Aphid, *Myzus persicae*, *M. euphorbiae*, and *Aphis gossypii*	China	Xiang et al. (2008)
CABYV	Polerovirus	*Luteoviridae*	RT-PCR, Sequencing	Aphid, *Myzus persicae*, *M. euphorbiae*, and *Aphis gossypii*	Taiwan	Knierim et al. (2010)
MYSV	Orthotospovirus	*Tospoviridae*	RT-PCR, Sequencing	*Thrips palmi*	Japan	Okuda et al. (2002)
PRSV	Potyvirus	*Potyviridae*	Dot Immuno Binding Assay, DIBA, RT-PCR, Sequencing	Aphid vectors, mechanical sap inoculation	India	Nagendran et al. (2017)
SLCCNV	Begomovirus	*Geminiviridae*	PCR with geminivirus-specific primers, sequencing	White fly (*Bemisia tabaci*)	Thailand	Sawangjit (2009)
SLCCNV	Begomovirus	*Geminiviridae*	PCR with geminivirus-specific primers, sequencing	White fly (*Bemisia tabaci*)	India	Mohammed-Riyaz et al. (2013)
SLCuPV	Begomovirus	*Geminiviridae*	PCR with geminivirus-specific primers, sequencing	White fly (*Bemisia tabaci*)	Taiwan	Liao et al. (2007)
ToLCNDV	Begomovirus	*Geminiviridae*	PCR with geminivirus-specific primers, sequencing	White fly (*Bemisia tabaci*)	India	Roy et al. (2013)
ToLCV	Begomovirus	*Geminiviridae*	PCR with geminivirus-specific primers, sequencing	White fly (*Bemisia tabaci*), Graft transmission	Thailand	Samretwanich et al. (2000)
WMV	Potyvirus	*Potyviridae*	–	Aphid vectors, *Myzus persicae* and *Aphis gossypii*, by mechanical sap-inoculation	India	Bhargava and Bhargava (1977)

(continued)

Table 3.2 (continued)

Virus	Genus	Family/ Species	Detection	Transmission	Location	Reference
WSMoV	Orthotospovirus	*Tospoviridae*	ELISA and Western blotting with polyclonal antibodies	Thrips vector, *Thrips palmi*, mechanical sap-inoculation and grafting	Taiwan Japan	Chen et al. (1995) Okuda et al. (2002)
ZYMV	Potyvirus	*Potyviridae*	–	Aphid and mechanical sap-inoculation	Japan	Fukumoto et al. (1993)

3.8 Summary and Future Perspectives

The genome sequencing of ash gourd has significantly accelerated molecular breeding practices. To find key qualitative and quantitative traits, tools such as next-generation sequencing, QTL mapping, gene mapping, genotyping and other newly identified technologies have been created and used. A lot of molecular markers directly connected to specific features have been identified and produced for marker aided breeding. The breeding methods for ash gourd, on the other hand, still need to be improved. For example, gene-editing technology has opened up new possibilities for development in a variety of vegetable crops, although no studies on this technique have been published in this crop yet. Approaches like GWAS, QTL-seq, SLAF-seq, BSA-seq, MutMap+ and MutMap-Gap have yet to be successfully implemented. These approaches have been proven to be reliable in other cucurbitaceous crops; thus, there is a great scope of utilizing them in ash gourd to discover target genes and associated molecular markers. For the identification of SNPs, considerable resequencing is required. The high cost of sequencing is the major hurdle. However, in recent years, the price has reduced dramatically and is expected to continue to do so in coming time. The techniques like microarray and RNA-seq can be utilized to monitor the gene expression of different physiological processes which can provide information regarding various functions of the gene. Before attempting gene transfer, however, thorough characterization is required. Pleiotropic side effects must also be taken into account (Salgotra et al. 2014). By using this knowledge to ash gourd breeding, crop variants with superior quality, resilience to biotic and abiotic stresses and increased yield could be developed.

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Understanding the Genetics and Genomics of Vegetable Grafting to Ensure Yield Stability

4

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Abstract

Climate change seems to have an impact on agricultural and horticultural production systems through biotic stresses such as new disease races and insect pests, as well as abiotic stresses such as drought, flood, salinity, and heavy metal stress. Vegetable grafting is one of the most important procedures for improving vegetable production under a variety of environmental situations, as well as increasing yield and product nutritional quality. Many crops, including watermelon, tomato, eggplant, pepper, and cucumber, are currently grafted on a commercial basis. This method is viewed as a quick alternative to the somewhat laborious process of breeding fruits and vegetables to raise their environmental stress tolerance. Despite the fact that this is used in the majority of vegetables, the genetics and genomic foundation of gene transfer, interaction, and epigenetics mechanisms are

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_4

unknown. Recent advances in molecular breeding and biotechnology techniques including as marker-assisted selection, genomic selection, and next-generation sequencing will help researchers to better understand the biological basis of root stock–scion interaction. In grafted scions, significant alterations in DNA methylation are seen, suggesting that these epigenetic pathways may be involved in grafting effects. Multiple resistant genes have a key role in the fight against different stresses when resistant rootstocks are used, whether intraspecific or interspecific. Genetic information is horizontally conveyed between the two grafted partners, either as DNA bits or plastids, according to transgenic lines with antibiotic markers in tobacco. Furthermore, the proteomics and transcriptomics research will reveal biochemical alterations in the products produced by the grafted plants. Mapping epigenetic markers and QTLs/genes in grafted crops can reveal fresh information about how to improve the crop. Vegetable grafting, thus, has a huge potential to improve the efficiency of modern and intelligent vegetable cultivation by increasing adaptability and resilience to various stress situations while also increasing yield.

Keywords

Abiotic stress · Biotic stress · Climate change · Genetics · Genomics · Marker-assisted selection · Molecular breeding · Proteomics · QTLs · Transcriptomics · Vegetable grafting · Yield stability

4.1 Introduction

Horticultural diversification is seen as critical to meeting the increasing demand for food and nourishment for an ever-increasing global population, and vegetables play a vital role in this direction (Jena et al. 2018). Vegetable production is hindered by biotic (pest and disease) and abiotic (environmental and soil stresses) factors (Pandey et al. 2017). These limits have been overcome by the development of new varieties or hybrids and the standardization of crop management procedures. Grafting has gain its popularity as a farming technique for fast improving modern vegetable cultivars flexibility or resistance to various conditions by grafting them onto stress-resistant rootstocks (Colla et al. 2013; Kumar et al. 2018). An important weapon in the fight against stresses appears to be the use of resistant rootstocks that are intraspecific (within the same species) selections with resistance genes or with resistance mechanisms that are not based on the host and are inter-specific (different species) and inter-generic (different genera) (Louws et al. 2010). Cucurbitaceous (cucumber, melon, and watermelon) and Solanaceous crops (eggplant, tomato, and pepper) employ grafting extensively (Kyriacou et al. 2016; Colla et al. 2008). It is possible to use natural genetic variation for specific root properties to alter the phenotype of the shoot by grafting (Kyriacou et al. 2017). Rootstock selection is an important step in grafting because it can influence the morphology and physiology of the scion, as well as the ability to manage environmental stresses, such as soil

and foliar pathogens, arthropods and viral diseases, weeds and nematodes, and abiotic stresses such as thermal stress, drought, and salinity, as well as adverse soil pH (alkalinity and acidity) (Kumar et al. 2017). Grafting has been practiced for millennia, yet there are still many unanswered questions about the process. To better understand grafting and screening from genetics and genomics perspective, as well as the epigenetics and problems that come along with grafting, we look at a variety of grafting methods and screening techniques in this chapter. In addition, it expresses the potential of vegetable grafting for further study.

4.2 Grafting, Its Purpose, Historical Background to Current Status

This is crucial to consider grafting as a way to combat biotic and abiotic stresses, as well as organic vegetable cultivation. When two plants of different genetic backgrounds are joined together, a new plant is formed. One gives a stem, or scion, and one supplies a root system (rootstock). Since the 1970s, vegetable grafting has been used for commercial purposes around the world. For fruit and vegetable crops, the major goal of grafting is to boost yield and quality in the face of high soil-borne disease and nematode density and unfavorable circumstances. Increased yield and production efficiency, as well as improved economic viability, can be achieved through the use of grafting to reduce pesticide use in sustainable vegetable production through organic agriculture (Lee et al. 2010). For breeding, grafting can be used to create new genetic combinations (e.g., pomato). Grafted plants are more able to withstand both biotic and abiotic stresses, which can result in higher yields. Watermelon yields increased by almost 106% in Australia after grafting a specific rootstock (Yetisir and Sari 2003). To combat soil-borne diseases, such as Fusarium wilt, Verticillium wilt, Ralstonia wilt, Pyrenochaeta and Phomopsis rots, and root-knot nematodes, grafting has become increasingly common in the growth of fruit crops in many countries (Collonier et al. 2001). By employing different grafting tools, plant vigor is improved, harvesting time is extended, yield and fruit quality are improved as well as shelf life is extended, and nutrient uptake is increased. Low- and high-temperature tolerance, salinity and heavy metal stress tolerance, drought, and waterlogging tolerance are all made possible through the use of this substance. Cucumber (*Cucumis sativus* L.) grafting began in the late 1920s, but little success was reached until the 1960s (Sakata et al. 2008). In the 1950s, eggplant (*Solanum melongena* L.) was grafted into scarlet eggplant (*Solanum integrifolium* Poir.), and tomato (*Lycopersicon esculentum* Mill.) was introduced commercially (Lee and Oda 2003). In Japan and Korea, the use of grafting in S

Solanaceous (eggplant, tomato) and Cucurbitaceous (cucumber, melon) crops had increased by 59% and 81%, respectively (Lee 1994). On the commercial scale, grafted vegetables have gained appeal across the globe and are most commonly grown in greenhouses in nations such as the United States and China. In Asia and Europe, the market for grafted vegetable plants has already begun to grow outside Asia and now includes North America (Kubota et al. 2008). Watermelon grafting is a

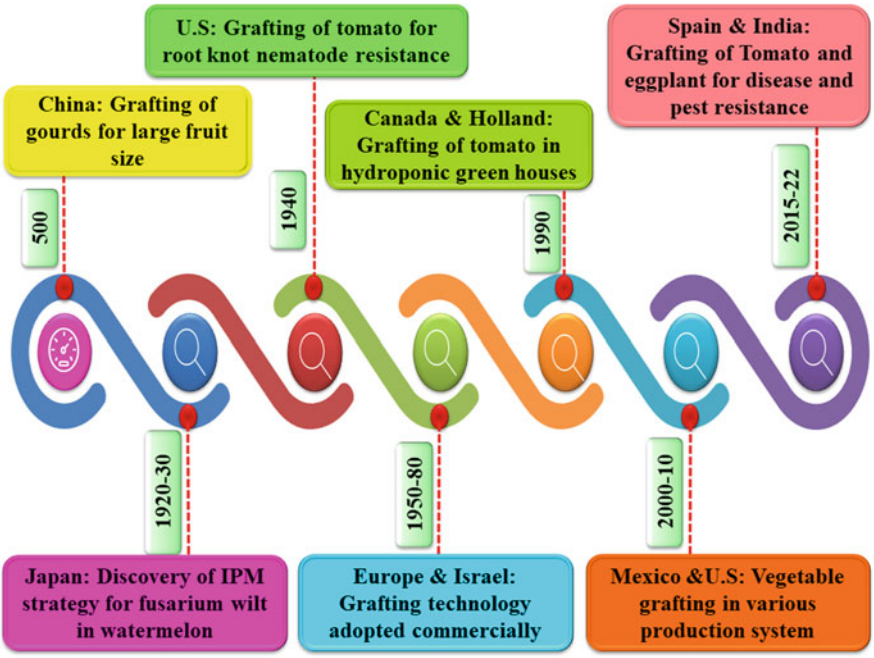

Fig. 4.1 A timeline showing the milestones of vegetable grafting in Worldwide

common practice around the world (Bekhradi et al. 2011). There were 40 million grafted seedlings utilized in greenhouse hydroponic tomato farming in North America, according to a study (Kubota et al. 2008). Watermelon, cucumber, eggplant, and 58% of the tomato plants grown in Japan were all grafted in the country (NARO 2011). Over the course of the last two decades, the number of plants produced by 16 Italian nurseries has climbed from ten million grafted plants to more than 60 million plants (Leonardi 2016). A timeline of vegetable grafting has been shown in Fig. 4.1. The use of desired rootstocks in vegetable grafting helps increase the plant's resistance to abiotic stressors. Among other things, it increases vigor and precocity; improves production and quality and reduces soil-borne pathogen infection (Gaion et al. 2018). To feed the world's ever-increasing population, grafting cucurbits, tomatoes, eggplants, and peppers onto hardy, disease-resistant rootstocks has become widely popular (Röös et al. 2017).

4.3 Genetic Basis of Vegetable Grafting

Numerous phenotypic polymorphisms in peppers have been described as a result of graft-induced changes in several plant traits (Tsaballa et al. 2011). In light of these findings, it is evident that genetic information is being traded between the grafted

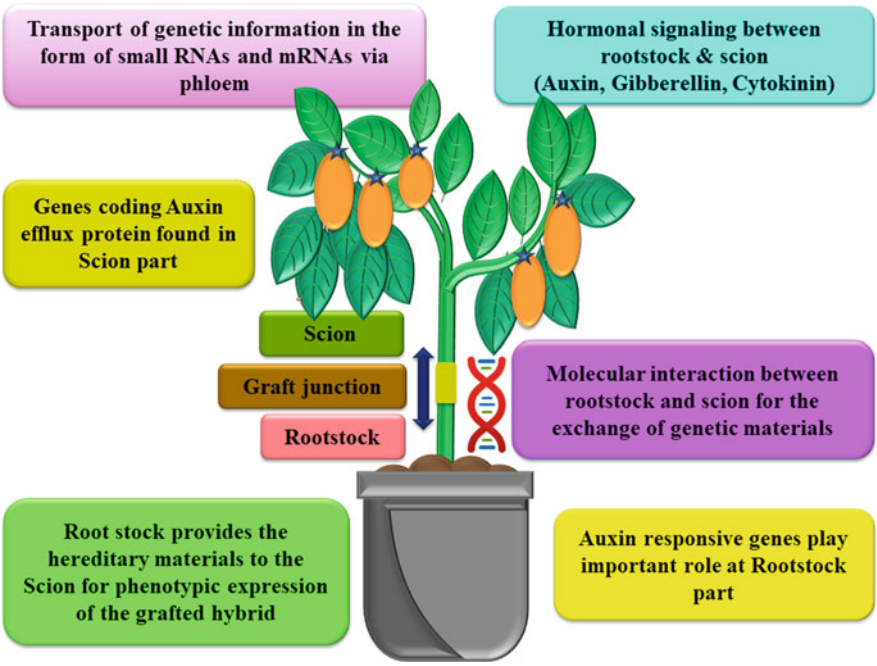

Fig. 4.2 A graphical representation of the molecular mechanism of vegetable grafting and the impact on phenotypic variation

partners (Fig. 4.2). One of the most important issues in recent literature has been the movement of genetic information in the form of short RNAs. Plants are known to transport messenger RNA (mRNA) molecules via the phloem for decades, and grafting has been employed in numerous related research to confirm just that (Spiegelman et al. 2013; Turnbull and Lopez-Cobollo 2013). Using micro-grafting experiments, researchers found the miR399, a phosphorus deficiency-induced miRNA, for the first time in the phloem sap of rapeseed and pumpkin (Pant et al. 2008). A broader range of miRNAs, including those found in the scion but not in the rootstock, have been proposed for rootstock-to-scion transfer (Bhogale et al. 2014). A new understanding of the genetic information traveling through grafted plants as siRNAs has recently been made possible thanks to basic studies in Arabidopsis. De novo methylation of transposable elements (TEs) and repetitive DNA has been reported using 24-nt heterochromatic siRNAs, which leads to transcriptional gene silencing (TGS). A Dicer-like protein, DCL3, generates 24-nt siRNAs that can be transferred from the shoot to the root of grafted plants, according to Molnar et al. (2010), who employed wild-type (WT) Arabidopsis plants and mutants that were grafted onto each other. 24-nt siRNAs have been related to DNA methylation of three TEs in the roots, despite the fact that 22-nt and 23-nt siRNAs are also mobile (Molnar et al. 2010). The phloem is responsible for transporting siRNA. Only the mobile 24-nt siRNAs directed DNA methylation at recipient meristematic tissues

and transgene promoter TGS in tests with Arabidopsis transgenic lines later on (Melnyk et al. 2011). Another finding was the ability of the non-exclusive 24-nt class of mobile siRNAs to go from shoot to root and directly modify hundreds of genomic locations related once more with TEs using mobile 24-nt siRNAs (Lewsey et al. 2016). For example, sRNAs that originate in the scion of a plant and travel toward the rootstock are more efficient at moving through the phloem and plasmodesmata than those that originate in the rootstock and travel toward the scion (Molnar et al. 2010; Melnyk et al. 2011). SiRNAs made in rootstock phloem partner cells have been shown to reach the WT scion and reduce viroid infection, even after lateral leaves and buds are removed (Kasai et al. 2013). In tomato 'LeFAD7' transgenic rootstocks, non-transgenic scions were used for grafting purposes. This study found that the LeFAD7 gene was under-expressed in grafted plants, indicating that the GM rootstock had transferred the gene to the scion. Before grafting, the scions in this project had their leaves removed as well (Nakamura et al. 2015). sRNA mobility in grafted plants could have significant practical ramifications, as can be seen from the evidence. Rootstock-to-scion transmission of resistance to viruses has been demonstrated in tomato. It has been shown that by grafting a tomato variety that is resistant to the TSWV virus onto another tomato variety that has a stronger RNA interference (RNAi) response to the viral infection, it is possible to create scions resistant to the virus. Roots of resistant grafted plants showed an increase in the expression of key RNAi genes, such as Argonaute (AGO) and RNA-Dependent RNA polymerase (RDR) genes. Researchers discovered that RNA silencing was significantly stronger in self-grafted plants, indicating that the mechanism might be activated by the act of itself (Spanò et al. 2015).

4.4 Crop Improvement via Vegetable Grafting

Plant breeding has largely focused on improving harvest and disease resistance, mechanical injury resilience, total postharvest performance, and quality attributes. It may take longer to create a high-yielding variety, and it may also necessitate sacrificing a desirable attribute in the name of productivity. Ethylene-dependent biosynthetic pathways have been connected to volatile fragrance components, as well as shelf-life performance (Pech et al. 2008). As a result, shelf-life breeding may have unintended side effects on sensory qualities that are otherwise beneficial (Causse et al. 2002). During the process of selecting for desirable features, undesirable impacts might impede breeding attempts. In some cases, independent selection of scion and rootstock traits may be possible by grafting, if the graft combination is compatible enough. It is also possible to boost yields through the use of marketable rootstocks and safe agriculture (Colla et al. 2011). Solanaceae and Cucurbitaceae grafting has been facilitated by using wild genetic resources to develop root physiological traits that are more tolerant to stress than scion characteristics under marginal conditions of salinity, nutrient stress, water stress, organic pollutants, and alkalinity (Schwarz et al. 2010; Borgognone et al. 2013). A new variety of vegetables can be

developed more easily by grafting appropriate rootstock and scions. An autonomous rootstock and scion breeding program can do trait stacking. To better understand root-to-shoot signaling, scientists have used reverse genetics, which involves grafting genetically separate rootstock and scion. In contrast, the epigenetic and molecular components of vegetable grafting are still mostly unsearched. Epigenetics is the study of changes in gene expression that are not produced by changes in the primary DNA sequence, but rather by changes in how the DNA is packed (Bender 2002). Many plant characteristics and interactions with the environment have been connected to epigenetics. Crop breeders may now make better crop varieties that are more resilient to climate change by increasing and utilizing genomic diversity through genome-wide mapping of markers for epigenetic markings and epigenetic target identification. If heritable epigenetic modifications are caused by plant grafting and have a considerable impact on gene expression variability, it is evident that this should be investigated.

4.5 Epigenetics Basis of Vegetable Grafting

A plant's genes can be activated or silenced by any one of three epigenetic mechanisms: DNA methylation/demethylation, histone changes, and non-coding RNA-mediated activity (Kapazoglou et al. 2018). There are three types of plant DNA methylation: the addition of the CH_3 methyl group (CH_3) to the nucleotides of DNA cytosines, which results in a 5-methylcytosine (He et al. 2011). To cleave or repress/inhibit the translation of homologous gene transcripts, non-coding RNA and specifically small non-coding RNAs (sRNAs) of 21–24 nucleotides (nt) (DCL) proteins target them. Four types of small RNAs exist in plants: micro-RNAs (miRNAs), which are post-transcriptional regulators; siRNAs, which are also engaged in transcriptional gene silencing; and snoRNAs, which are both. RNA-directed DNA methylation processes use siRNAs with a length of 24 nt to silence transposons (Chen 2009). DNA methylation and non-coding RNAs have been linked to epigenetic changes in grafting. When plants are grafted, they have been found to have different DNA methylation levels. Despite the fact that variations in DNA methylation have previously been connected to wound stress (Cao et al. 2016), it appears that the association between plant grafting and DNA methylation goes beyond wound stress. Using grafted tomato and eggplant plants, as well as pepper plants utilised exclusively as a rootstock for the tomato plants, Wu et al. (2013) reported a study indicating alterations in DNA methylation in these grafted Solanaceae plants.

Using MSAP analysis, no alterations in the global methylation of the grafted plants were seen. Only alterations in local methylation were found in the scions and pepper rootstocks, in a locus-specific manner. The self-pollinated grafted progeny carried a high proportion of the DNA methylation alterations from the scion. Bisulfite sequencing (BS) of specific loci indicated that while self-grafting can result in minor DNA methylation modifications, interspecies grafting in Solanaceae is accompanied by considerable heritable methylation changes. Genes all connected

to DNA methylation, such as Methyl Transferase (MET) 1, displayed dramatically altered expression profiles in tomato-to-eggplant grafted plants compared to their seed non-grafted controls, but these profiles were reversed in the progenies (Wu et al. 2013). In this study, epigenetics and DNA methylation were closely linked to grafting, particularly in scions. Other plant groups that often employ interspecies propagation, such as the Cucurbitaceae, may similarly rely on DNA methylation for grafting effects. On pumpkins, we have found that MSAP markers may be used to detect a large increase in DNA methylation in cucumber scions, but not watermelon scions, when grafted onto pumpkins (Avramidou et al. 2015). That epigenetic changes in grafting scions are specific to the interaction between the rootstock and the scion could be suggested by this finding. *Cucurbita pepo* scion fruit quality was studied via intraspecies/inter-cultivar grafting, and we conducted methylation and miRNA studies to monitor the fruit phenotypic changes after grafting in order to understand the effects of grafting. Results show Cucurbita grafting modifies DNA methylation patterns and the expression of particular miRNAs, as demonstrated by MSAP and qRT-PCR (Xanthopoulou et al. 2019). Using both homo- and reciprocal grafting techniques, Li et al. (2014) grew cucumber and pumpkin on one another. Heterogeneous and homogeneous grafts were compared for miRNA levels by analyzing RNA from their leaves and root tips. Most of the miRNAs were observed to be altered in the hetero-grafted compared to the homo-grafted (Li et al. 2014). However, stress cannot be ignored when it comes to the expression of miRNAs in grafted plants. During salt stress, cucumbers grafted onto pumpkin rootstocks showed different miRNA expression patterns, suggesting that salt stress adaptation may play a role in the control of miRNA expression (Li et al. 2016). The role of diverse rootstock and scion combinations in stress adaptations and the role of miRNAs in these adaptations are still unanswered. However, greater investigation is needed into the origin, transport, and participation of these sRNAs in epigenetic modifications. To our knowledge, there has been no research tying vegetable grafting to histone alterations to this date.

4.6 Methods of Vegetable Grafting

Crop type, grower expertise, and the availability of grafting facilities all influence the grafting method used. There are a number of ways to graft vegetables, and examples of each are provided here. A schematic representation of vegetable grafting technique has been shown in Fig. 4.3, and the list of different grafting methods and the rootstock used are listed in Table 4.1. The scion meristematic tissues are in direct touch with the rootstock meristematic tissues. Callus tissue is formed when cambium cells from the rootstock and the scion fuse together to form a callus tissue when they are in close proximity (Acosta Muñoz 2005). Incompatible grafts as well as compatible grafts experience this first phase of cohesion, which is analogous to wound healing and does not need communication between the rootstock and scion. When a graft is appropriate, the callus shows a differentiation of some phloem vessels and sieve tube parts that are not generated from the cambium and form the first bridging

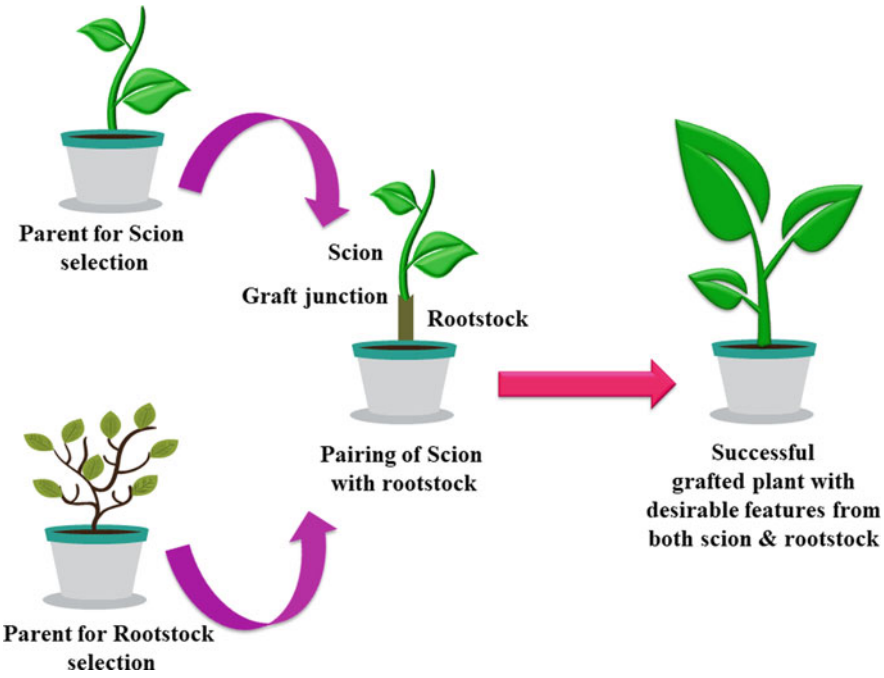

Fig. 4.3 A schematic representation of vegetable grafting to combine the desirable traits from both scion and rootstock

and uninterrupted union between the rootstock and the scion. During the last stages of the grafting process, new vascular tissue is generated by the cambium layer that has grown in the bridge of the callus. For the first time, it is possible to establish an intercellular contact between rootstock and seedling (Pina and Errea 2005).

4.6.1 Cleft Grafting

Cucurbit cleft grafting was once common in several places, but these days, it is more common in solanaceous crops like tomatoes and brinjal. Rootstock seeds are sown 5–7 days before the scion's seeds. They are cut at right angles 2–3 inches deep, with 2–3 leaves left on the stem of the seedlings that are selected for rootstock. One-quarter inch in diameter is the ideal diameter for the scion. To fit inside the vertical incision on the rootstock, the scion has two angled cuts on either side of the bottom end. Over the rootstock's top, a grafting wax or clip is applied to seal the wounds and stabilize the graft Cleft grafting is a basic technique that works well with rootstocks that have hypocotyls that are rather wide (Gaion et al. 2018).

Table 4.1 Grafting methods and rootstocks used in different vegetable crops

Sl. No.	Vegetables	Root stock used	Grafting method	References
1.	Eggplant	*Solanum torvum*	Tongue and Cleft	Arao et al. (2008)
		Solanum sisymbriifolium	Cleft method	Maurya et al. (2019)
		Solanum khasianum	Tongue and Cleft	Jacob and Malpathak (2004)
		Solanum integrifolium	Tongue and Cleft	Mozafarian et al. (2020)
2.	Tomato	*Lycopersicon pimpinellifolium*	Cleft	Asins et al. (2010)
		Lycopersicon hirsutum	Cleft	Khah et al. (2006)
		Lycopersicon esculentum	Cleft	Miskovic et al. (2016)
		Solanum nigrum	Tongue and Cleft	Wang et al. (2021)
3.	Cucumber	*Cucurbita ficifolia*	Tongue Approach	Zhang et al. (2010)
		C. maxima × *C. moschata*	Tongue Approach	El-Eslamboly and Deabes (2014)
		Cucumis sativus	Tongue Approach	Noor et al. (2019)
		Sicyos angulatus	Tongue Approach	Sugiyama et al. (2006)
		Cucurbita moschata	Hole insertion and Tongue	Baron et al. (2018)
		Cucurbita maxima	Tongue	Al-Debei et al. (2012)
4.	Water melon	*Benincasa hispida*	Hole insertion and Cleft	Singh (2021)
		Cucurbita moschata	Hole insertion and Cleft	Sun et al. (2009)
		Cucumis melo	Cleft	Ajuru and Okoli (2013)
		C. moschata × *C. maxima*	Hole insertion	
		Lagenaria siceraria	Splice Grafting	Bekhradi et al. (2011)
5.	Bitter gourd	*Sicyos angulatus*	Tongue Approach	Oda (2006)
		Cucurbita moschata	Hole insertion and Tongue	Ashok Kumar and Sanket (2017)
6.	Bottle gourd	*Cucurbita moschata, Luffa sp.*	Hole insertion and Tongue	Yetisri and Sari (2004)
7.	Melon	*Cucumis melo*	Tongue and Cleft	Fita et al. (2007)

4.6.2 Tongue Approach/Approach Grafting

Farmers and small nurseries utilize this method the most but novice farmers and those without a greenhouse with a robust microclimate management system tend to prefer it. If you are just starting out, you can get started with this strategy thanks to the excellent seedling survival rate. Rootstocks with hollow hypocotyls should not be utilized on members of the cucurbitaceous vegetable family. To achieve a

diameter of less than 1 inch, it is best if the rootstock and scion are grown at least 3 days apart. In order to prevent any further growth, the rootstock's shoot apex is cut off. Hypocotyls are cut at 45° angles on both the scion and rootstock, resulting in a 45° hypocotyl. When the scion is interlocked with the rootstock via interlocking, a second, vertical incision is made to form notches or tabs. In order to allow the hypocotyl of the scion to fully heal, it is cut off from its roots and partially trimmed below the graft for 3–4 days. Finally, grafting tape is wrapped around the graft to keep it in place as it grows (Thakur 2020).

4.6.3 Hole Insertion/Top Insertion Grafting

Cucurbits scion and rootstock with hollow hypocotyls are commonly grown this way. The scion should be sown 3–8 days after the rootstock, in order to acquire the same diameter as the rootstock. The chasm widened or narrowed according on the rootstock type (Lee et al. 2010). Using a bamboo or plastic gimlet, cut a hole at a slant angle is cut to the longitudinal direction of the rootstock and the genuine leaf and growing tip are removed. By slant cutting the hypocotyl of the scion, it has a thin end that makes it easier to implant into the plant. It is incredibly cost effective for small farmers to produce up to 1500 grafts each day from one individual. High success rates can be achieved with 95% relative humidity and a temperature range of 21–36 °C from healing to transplantation. Hole insertion hypocotyl grafting is preferred by many farmers across the country because to the lower seedling size of watermelons compared to their rootstock (often Squash or Bottle gourd) (Lee et al. 2010). In comparison to tongue grafting, this procedure is quite prevalent in China since it produces a stronger union and vascular connection.

4.6.4 One Cotyledon/Slant/Splice Grafting

Expert growers and commercial nurseries are well versed in splice grafting. Vegetables of all kinds can be treated with this method, which can be done by hand, machine, or robot. Rootstock should be sown 7–10 days prior to scion sowing in order to guarantee equal hypocotyl diameter and hold the scion in place on the rootstock properly. Intact or excised, i.e., root-removed rootstock seedlings can be employed, depending on the preference of the growers and farmers. It is possible to do grafting by making slant incisions on both rootstock and scion while retaining only one cotyledon leaf on the cucurbit rootstock, which is also known as one cotyledon grafting (OC-SG). Grafting is typically done at the lower epicotyl of solanaceous plants and secured with simple clips. For 3 days, grafted plants should be kept at 25 °C and 100% humidity to ensure a successful graft union. Gluing tubes together A tube connect the rootstock and scion, similar to a slant, but instead of using clips, this approach uses an elastic tube (Kubota et al. 2008). A 45° angle under the cotyledons and a 5- to 10-mm-deep cut in the scion are also necessary. At the cut end of the rootstock hypocotyl, one tube about midway is placed down the stem. It is

aimed for a flawless fit between scion and rootstock while inserting it into the grafting tube. Depending on the materials, the tube can be repurposed numerous times. Healing takes around 7 days. Tomatoes and brinjal are the most prevalent vegetables to contain it.

4.6.5 Pin Grafting

Splicing or slant grafting is the same procedure. Instead of grafting clips, specific pins are employed to keep the graft in place. Approximately 15 mm long and 0.5 mm wide, the ceramic pin has a hexagonal cross section. This pin is made of natural ceramic and can be left on a plant without any issues. Because ceramic pins are expensive, bamboo pins with rectangular cross-sectional shapes might easily replace them at a considerably reduced cost. Watermelons and other solanaceous plants may benefit from its application (Lee et al. 2010).

4.7 Diverse Applications of Vegetable Grafting

The leading objective of vegetable crop grafting is to develop resistant crops against serious diseases and insect pests as well as improve the fruit quality by using the desirable resistant root stock. Several advantages offered by vegetable grafting have been represented in Fig. 4.4, and stress resistance mechanism of grafted plants is schematically exemplified in Fig. 4.6.

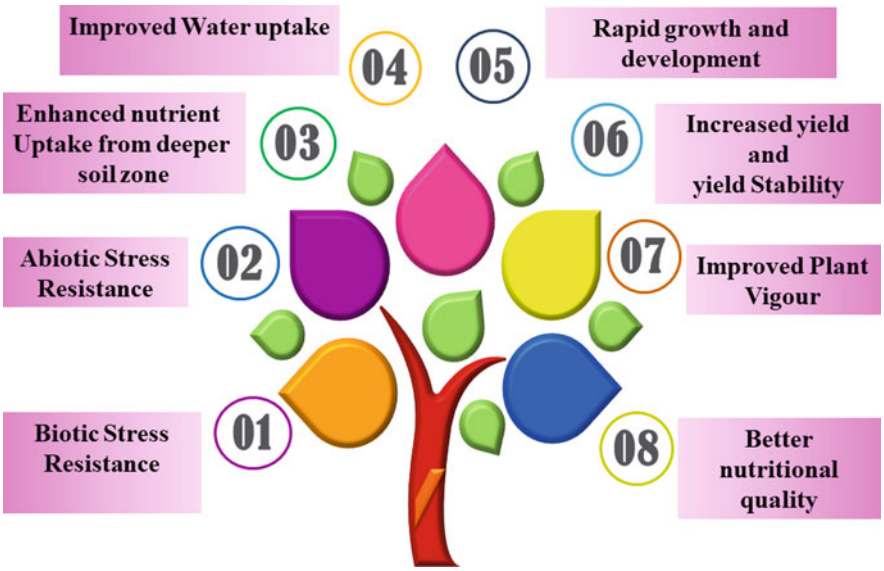

Fig. 4.4 Multiple advantages offered by vegetable grafting

4.7.1 Grafting Improves Biotic Stresses

Seedlings that are healthy and well established are essential to a successful vegetable farm's profitability. By limiting plant death and the transmission of disease to new areas, these robust seedlings increase vegetable yields while lowering production costs (Ventura et al. 2019). However, due to a dearth of cultivable land, vegetables like cucurbits and solanaceous crops are commonly cultivated in disease polluted soil and environmental circumstances because of their high demand and market price (Schwarz et al. 2010). Diseases can stunt the growth of vegetable seedlings, resulting in a decrease in output and a decrease in the quality of the fruit. Planting disease-resistant cultivars is the most efficient method for preventing vegetable illnesses (Ventura et al. 2019). Sources of resistance have yet to be discovered in many plants, yet resistance may be reduced or lost as new pathogens, strains, or races are introduced. The grafting of vegetables onto rootstocks that can restrict or avoid the detrimental impact of external biotic stress on the plant is one technique to decrease production losses caused by soil-borne illnesses (Colla et al. 2013). The use of disease-resistant rootstocks in grafting methods has been shown to protect vegetable crops against a wide range of soil-borne illnesses in varied locales and situations (Rivard and Louws 2011). A list of crop plants is mentioned in Table 4.2 which is used for grafting to eradicate the fungal, bacterial, viral, and nematode pathogens. The use of grafting to combat Verticillium wilt, Fusarium wilt, corky root rot, and bacteria wilt diseases has proved successful in a number of nations. Grafting technology has evolved into a unique component for the improvement of pest management and crop productivity techniques in the production of several solanaceous (tomato and brinjal) and cucurbitaceous vegetables. Here, we want to highlight one case study about vegetable grafting in eggplant (*Solanum melongena*) to fight against fruit and shoot borer (*Leucinodes orbonalis*) at Regional Research and Technology Transfer Station, Odisha University of Agriculture and Technology, Keonjhar district, Odisha. The parent variety is highly susceptible to the fruit and shoot borer. Thus, looking to the severity, we planned to graft the scion on the rootstock of *Solanum torvum* (Turkey berry, wild eggplant relatives). The *Solanum torvum* plant is a perennial evergreen shrub or small tree with plant height of 4–5 m, and it produces small berry fruits. The observations are recorded that the grafted plant is completely resistant to fruit and shoot borer and the fruit size. As the life span of *S. torvum* is more, the plant is able to uptake more nutrient and water from the deeper soil layer for a longer period and the yield also increased as compared to the non-grafted plant (Figs. 4.5 and 4.6). The average fruit weight of grafted eggplant is 200–210 g, whereas in non-grafted, this is observed 90–95 g. Worldwide, grafted seedlings are becoming more popular due to the availability of disease-resistant rootstocks and the development of grafting technology. A better understanding of grafting-induced safeguards from inherent resistance to mediate systemic resistance in rootstocks was also provided by the research studies (Guan et al. 2012).

Table 4.2 A list of crops shows biotic stress (disease) tolerance by using grafting techniques

Sl. No.	Crop plant	Disease name	Pathogens	Reference
Fungal Diseases				
1.	Tomato, pepper, watermelon, melon, cucumber	Fusarium wilt	*Fusarium oxysporum*	Álvarez-Hernández et al. (2015)
2.	Tomato, pepper, watermelon	Fusarium crown and root rot	*F. oxysporum, F. solani*	Vitale et al. (2014)
3.	Tomato, eggplant, watermelon, melon, cucumber	Verticillium wilt	*Verticillium dahliae*	Miles et al. (2015)
4.	Watermelon	Monosporascus sudden wilt	*Monosporascus cannonballus*	Park et al. (2013)
5.	Tomato, pepper, watermelon, Cucumber	Phytophthora blight	*Phytophthora capsici*	Jang et al. (2012)
6.	Tomato, eggplant, pepper	Corky root	*Pyrenochaeta lycopersici*	Al-Chaabi et al. (2009)
7.	Cucumber	Target leaf spot	*Corynespora cassiicola*	Hasama et al. (1993)
8.	Cucumber, Melon	Black root rot	*Phomopsis sclerotioides*	Shishido (2014)
9.	Melon	Gummy stem blight	*Didymella bryoniae*	Keinath (2013)
10.	Tomato	Southern blight	*Sclerotium rolfsii*	Rivard and Louws (2011)
11.	Tomato, eggplant, soybean	Brown root rot	*Colletotrichum coccodes*	Garibaldi et al. (2008)
12.	Tomato	Rhizoctonia damping off	*Rhizoctonia solani*	Gilardi et al. (2010)
13.	Cucumber, watermelon	Powdery mildew	*Podosphaera xanthii*	Kousik et al. (2018)
14.	Cucumber	Downy mildew	*Pseudoperonospora cubensis*	Wehner and Shetty (1997)
Bacterial Diseases				
15.	Tomato, pepper, eggplant	Bacterial wilt	*Ralstonia solanacearum*	Rivard et al. (2012)
Viral Diseases				
16.	Tomato	Tomato yellow leaf curl	Tomato yellow leaf curl virus	Mohamed et al. (2014)
17.	Tomato	Tomato spotted wilt	Tomato spotted wilt virus	Spanò et al. (2015)
18.	Tomato	Pepino mosaic	Pepino mosaic virus	Schwarz et al. (2010)
Nematode Diseases				
19.	Cucumber, melon, watermelon, tomato, eggplant, pepper,	Root-knot	*Meloidogyne spp.*	Owusu et al. (2016)

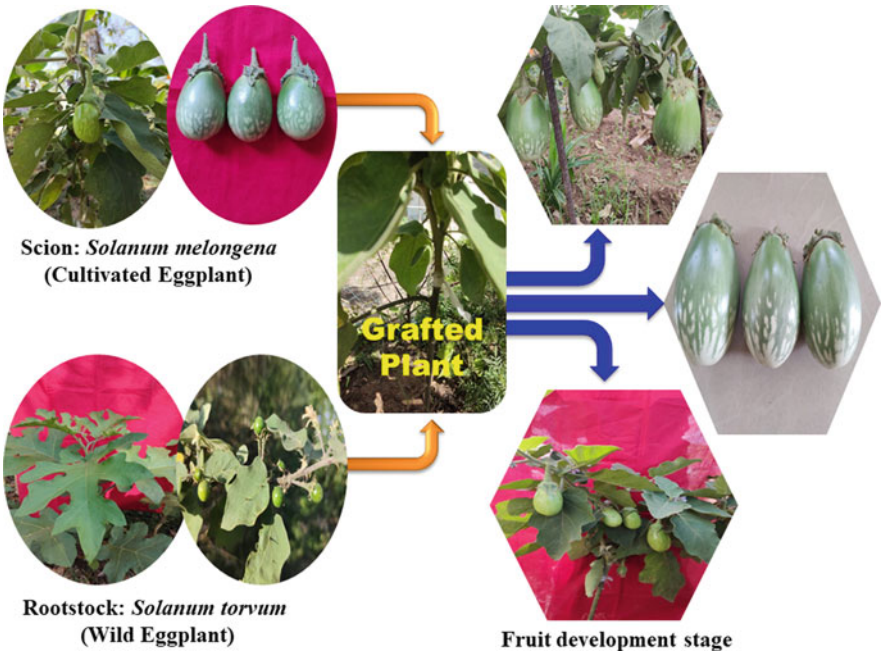

Fig. 4.5 A successful grafting between cultivated eggplant (*Solanum melongena*) and wild eggplant relative (*Solanum torvum*) to fight against fruit and shoot bores and improved yield

4.7.1.1 Vegetable Grafting to Induce Resistance Against Fungal Pathogens

In vegetable crops, soil-borne Fusarium and Verticillium wilt infections have been successfully avoided through the use of vegetable grafting (Louws et al. 2010). *F. oxysporum* formae speciales have not been found in many of the cucurbit rootstocks used for this purpose. This is why fusarium wilt disease in cucurbits was successfully managed by grafting (Louws et al. 2010). When Fusarium wilt first appeared in Japan in the 1920s, watermelon (*Citrullus lanatus*) was grafted onto bottle gourd (*Lagenaria siceraria*). The grafting method has since expanded to countries across the world. Watermelon grafts are used in nearly all of Japan and Korea's vegetable farms (Lee et al. 2010). Squash (*Cucurbita moschata*), bottle gourd, and interspecific hybrid squash (*C. maxima* × *C. moschat*) have all been grafted onto it, and it has shown a great affinity for connected rootstocks (Gaion et al. 2018). Squash and interspecific hybrid squash have a superior root system and are more resistant to Fusarium wilt than the others (Keinath and Hassell 2014). Interspecific hybrid squash 'Shintoza' or 'Super Shintoza' provided watermelon plants with resistance to Fusarium wilt when grown in the presence of polluted soils. The rootstocks increased fruit size and yield compared to non-grafted plants. *V. dahliae* wilts Solanaceae and Cucurbitaceae plants by damaging the vascular system (Paplomatas et al. 2000). Vegetables infected with the *V. dahliae* bacterium can be

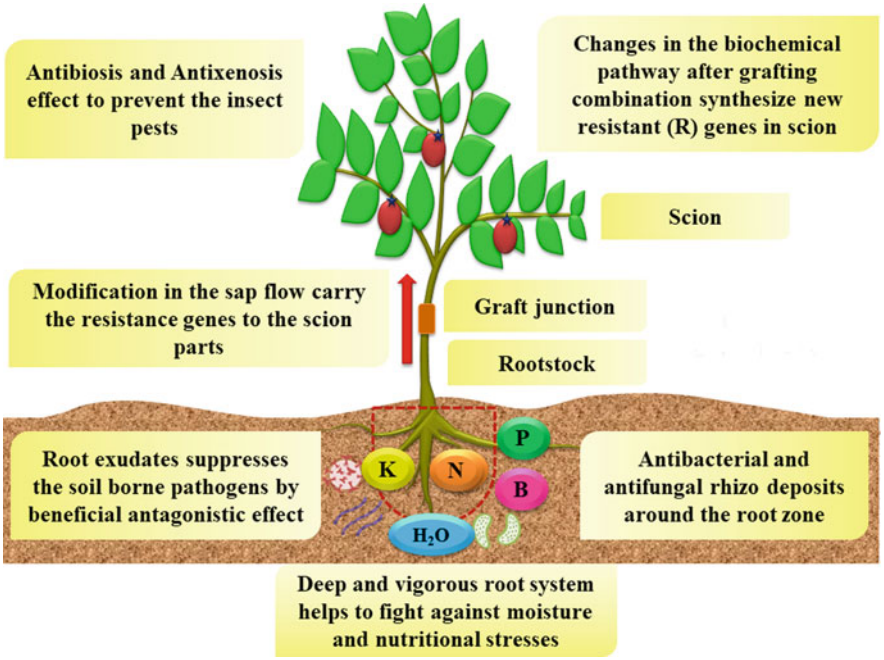

Fig. 4.6 A schematic representation of stress resistance mechanism of grafted plants

grafted onto agricultural rootstocks and a scion infected with *V. dahliae* to create disease resistance in melons, watermelons, cucumbers, and tomatoes (Solanum lycopersicum). Resistance to Verticillium wilt was demonstrated by the 'Super Shintoza' rootstock, which decreased *Verticillium microsclerotia* incidence (Gaion et al. 2018). Watermelon and melon plants can be affected by Monosporascus cannonballus, a soil-borne disease that causes watermelon and melon plants to suddenly wilt (Edelstein et al. 1999). Melon resistance to *M. cannonballus* was improved by grafting sensitive types onto *C. maxima* and interspecific hybrid squash rootstocks (Cohen et al. 2005). However, it was discovered that the increased tolerance and increased production of grafted plants were not constant. Variations in the rootstock and scion combinations, as well as the surrounding environment, could account for the inconsistent results. In cucurbit production, the *Phytophthora capsici* pathogen is considered one of the most devastating. Grafting *P. capsici* onto bottle gourd, *C. moschata*, and wax gourd (*Benincasa hispida*) rootstocks increased yields and vegetative development in areas afflicted with *P. capsici* (Nemati and Banihashemi 2015). Scions of watermelons grafted onto Lagenaria siceraria rootstocks also demonstrated resistance to *P. capsici* (Kousik and Thies 2010). When grafted onto 'Beaufort' (*S. lycopersicum*, *S. habrochaites*) rootstocks, grafted tomatoes, and eggplants (*S. melongena*) had lower incidences of corky root disease, better yields, and larger fruits (Hasna et al. 2009). Rootstocks derived from

watermelon, bottle gourd, pumpkin, and squash have been successfully grafted onto melon, and interspecific hybrids of C. melo, cucumber, and wax gourd have been developed, providing resistance to soil-borne diseases caused by *Monosporascus cannonballus*, *F. oxysporum*, and *Stagonosporopsis spp* (King et al. 2010; Lee et al. 2010; Zhou et al. 2014). Root and stem rot, caused by Fom, *M. cannonballus*, *Macrophomina phaseolina*, and *Stagonosporopsis spp*., are the primary opponents to rootstock generation (King et al. 2010). It has been difficult to establish melon cultivars that are completely resistant to all forms of Fom (Dhall 2015). Due to the discovery of rootstocks that are tolerant of all races, farmers in Fom-infested areas can utilize them to boost growth and development (Oumouloud et al. 2010). They can be used as rootstocks for melon because they are practically resistant to the Fusarium wilt disease. A variety of Fom races have been tolerated by interspecific hybrid rootstocks (SYTZ and NZ1), and their use has increased yields compared to non-grafted melon cv. Liyu's (Zhou et al. 2014). Squash, interspecific hybrid squash, and pumpkin are commonly grafted onto cucumbers to protect them against Fusarium Wilt (*C. ficifolia*) (Dhall 2015). A frequent method is to graft tomato onto tomato genotypes, as well as tomato interspecific hybrids, to protect them from soil-borne fungus, such as *Verticillium spp*. (Polizzi et al. 2015). Traditionally, natural species like *S. integrifolium* or hybrid tomato rootstocks have been used to graft eggplants. Fusarium wilt-resistant rootstocks from hybrid tomatoes are more commonly used by farmers who use them for grafting tomatoes (King et al. 2010). Traditional rootstocks have been shown to lose their resistance to grafting or suffer detrimental effects as a result (Kawaguchi et al. 2008), which necessitated the development of new rootstocks, such as interspecific hybrids and those that are more closely related to wild species (King et al. 2010). *S. sisymbriifolium* or *S. sisymbriifolium* for eggplant grafting, which has shown strong resistance to Fusarium or Verticillium wilt, has proved its promise. Vegetable cultivars that were grafted onto *S. sisymbriifolium* roots and grown in either infested or uninfested soil saw increased yields as well as increased resistance to Verticillium wilt (Bletsos et al. 2003). Eggplant types grafted onto the 'Beaufort' F_1 demonstrated enhanced yield and fruit output as a result of the increased vigor of the grafted plants A resistance to Verticillium wilt has been found in the grafted Epic eggplant cv. Beaufort F_1 rootstock (Johnson et al. 2014; Miles et al. 2015). On cucumbers, tomatoes, and eggplant, diseases like the black root rot of cucumbers, the target leaf disease of cucumbers, and the Southern Blight disease of tomatoes have all been successfully prevented by the use of grafting (Louws et al. 2010). While using specific rootstocks, grafting has been shown to boost crop resistance to foliar diseases such as powdery mildew and downey mildew on cucurbits.

4.7.1.2 Vegetable Grafting to Induce Resistance Against Bacteria Pathogens

Tomatoes are susceptible to bacterial wilt, which is caused by the bacterium Ralstonia solanacearum. Tobacco plant resistance to this wilt disease is a measurable trait that is closely tied to the size of the fruits itself (Louws et al. 2010). Tomato

varieties that are resistant to wilt are scarcely commercially stable (King et al. 2010). As produced on rootstocks that are resistant to the bacteria that cause tomato wilt, susceptible tomato cultivars (e.g., BHN 602 tomato line) were successfully grown to prevent the disease in tomato plants (Rivard et al. 2012). When grafted plants are more resistant to bacterial infections, it may be because the lower stems are less likely to become infected (Nakaho et al. 2000). To fight soil-borne bacterial wilt disease, the eggplant was grafted onto a wild scarlet eggplant rootstock (*S. integrifolium*) (King et al. 2010). Bacterial wilt resistance in wild relatives, such as *S. torvum* or *S. sisymbriifolium*, has been found to be higher (Gousset et al. 2005).

4.7.1.3 Vegetable Grafting to Induce Résistance Against Nematode

Root galling, which reduces nutrient and water intake, is a common sign of root-knot nematode (RKN) infection in vulnerable plants. *M. incognita* resistance was found in *Cucumis metuliferus*, *Cucumis ficifolius*, and bur cucumber (*Sicyos angulatus*) (Gu and Zhang 2006). Reduced root gall number and nematode infection were achieved by grafting *C. metuliferus* as rootstock on to RKN-susceptible melon cultivars (Sigüenza et al. 2005). In addition, a variety of melon cultivars have successfully been grafted onto *C. metuliferus* (Nisini et al. 2002). Using the bur cucumber as a rootstock, researchers found that it was more resistant to RKN (Zhang et al. 2006). The development of *M. incognita*-resistant rootstocks for wild watermelon has also shown promising results (*Citrullus lanatus*). RKN-resistant cucurbit rootstocks, on the other hand, are not widely available (Thies et al. 2010). The Mi gene was inserted into tomato rootstocks and cultivars, allowing for successful RKN control in tomatoes (Louws et al. 2010). It was possible to graft RKN-resistant rootstocks onto tomato cultivars because of the reduced RKN infestation in the field soils. However, it is possible that the temperature sensitivity of the Mi gene's resistance to RKN is not always constant (Cortada et al. 2009). The rootstocks of Capsicum annuum (*Capsicum annuum*) with the N gene have proven beneficial in controlling RKNs *(M. incognita*, *M. arenaria*, and *M. javanica*) in pepper (Oka et al. 2004). Root-knot nematodes are resistant to grafted melon seedlings (Zhou et al. 2014). Resistance to nematodes (*Meloidogyne incognita* and *M. javanica*) is induced by wax gourds and squash, which have been employed as rootstocks (Galatti et al. 2013). Resistance to RKN in wild species like *S. torvum* and *S. sisymbriifolium* suggests that they could be used as rootstocks for eggplant. Eggplant can be grown on interspecific rootstocks that have good compatibility, strong plant vigor, enhanced yield, and mild root-knot nematode tolerance without affecting the quality of the fruit. Root-knot nematode in sweet pepper on *Capsicum annuum* rootstocks is the primary cause of disease, and successful grafting may be a potential strategy for managing this (Oka et al. 2004). The grafted cv. Celica, which is resistant to RKNs (*M. incognita* and *M. javanica*), performed much better than non-grafted plants cultivated in infested soils (Oka et al. 2004).

4.7.1.4 Vegetable Grafting to Induce Resistance Against Virus

Due to a paucity of thorough studies in this field, research into vegetable grafting-based resistant to viral illnesses (Spanò et al. 2020) has yielded mixed findings. Wang et al. (2002) found that grafted seedless watermelon plants with an anti-virus function performed better than seeded watermelon plants. Rootstocks that are resistant to melon necrotic spot virus in cucurbits were successfully used in Israel instead of soil fumigation with methyl bromide to control a soil-borne virus in cucurbits (Cohen et al. 2007). Tomato yellow leaf curl virus, tomato-spotted wilt virus, and the pepino mosaic virus can all be managed via grafting (Louws et al. 2010). There have been numerous reports that grafted plants are far more susceptible to viruses, probably due to graft incongruity that harms the scion's health (Davis et al. 2008).

4.7.2 Grafting Improves Abiotic Stresses

In addition to salt, heat, soil alkalinity, heavy metals, and excess trace elements (Colla et al. 2013; Zhang et al. 2020; Huang et al. 2016a, b; Martínez-Andújar et al. 2017), vegetable crops are also subjected to numerous abiotic stresses, which have a substantial impact on crop growth and productivity. Grafting vegetables onto rootstocks can help lessen the effects of environmental stress on the shoots and help farmers to prevent or reduce production losses in times of bad weather (Schwarz et al. 2010). It is possible for both the scion and the rootstock to have a detrimental effect on the resistance of grafted plants to poor environmental conditions (Colla et al. 2010). When grown in difficult conditions, grafted plants were able to grow more quickly than un-grafted or self-grafted plants. They also had a greater photosynthetic rate and a lower concentration of heavy metals and a considerable number of trace necessary elements in their shoots (Siamak and Paolo 2019). Table 4.3 lists the abiotic stresses that have been reported to be alleviated by vegetable grafting.

4.7.3 Grafting Improving Yield Stability

Many fruit vegetables benefit significantly from grafting, even if they are infected with soil-borne illnesses. An increase in fresh fruit weight of 25–55% in oriental melons over own-rooted plants has been found. In addition to disease resistance, these yield increases were directly linked to high plant health throughout the growing season. Fusarium-infected plants produced virtually no commercial products. With tomato, the same results were achieved. Rootstocks 'Kagemusia' and 'Helper' increased tomato marketable output by up to 54% and 51%, respectively (Chung and Lee 2007). In comparison to the own-rooted 'Seokwang' tomato, plants grafted to most rootstocks had much less aberrant fruits. Watermelon, cucumber (Lee and Oda 2003), melon, pepper, and eggplant have all seen similar increases in output.

Table 4.3 A list of crops shows abiotic stress tolerance by using grafting techniques

Sl. No.	Crop plant	Root stock	Special feature	Reference
1.	Tomato	Maxifort, He-Man (*S. lycopersicum* × *S. habrochaites*)	Salt tolerant	Borgognone et al. (2013)
		S. lycopersicum cv. Zarina	Higher fruit number, fruit having phenol and flavonoid content	Sánchez-Rodríguez et al. (2012)
		Tomato introgression line LA3957	High total plant dry matter and leaf area	Poudyala et al. (2015)
2.	Chili	*C. annuum* × *C. chinensis*	Superior yield and fruit quality	Lee et al. (2010)
		Chili cv. Alante, Creonte and Terrano	High water use efficiency	
3.	Egg plant	S. torvum Sw. × S. sanitwongsei	Salt tolerant	Colla et al. (2010)
		S. melongena × S. aehtiopicum gr. gilo	Improve fruit quality	Sabatino et al. (2019)
4.	Water melon	Jingxinzhen No.4 (*Cucurbita moschata* Duch.)	Low-temperature tolerant	Huang et al. (2016a, b)
		Ferro, RS841 (*Cucurbita maxima* × *C. moschata*)	Both high- and low-temperature tolerant	Yetisir and Erhan (2013)
		Interspecific hybrid (Cucurbita maxima × C. moschata)	Higher lycopene vitamin-c, high water use efficiency	Rouphael et al. (2008)
5.	Cucumber	Luffa cv. Xiangfei No. 236	Higher water use efficiency	Liu et al. (2016)
		Cucumber cv. Power, ferro, strong tosa	Tolerant to heavy metal toxicity (Ni, Cd, Zn)	Savvas et al. (2010)
		Cucumber cv. Kalaam F_1 with ridge gourd, bitter gourd, pumpkin, bottle gourd	Higher growth, Yield and Quality of Cucumber	Noor et al. (2019)
6.	Melon	Self-grafted melon Proteo, or grafted onto three interspecific (RS841, Shintoza, and Strong Tosa) and two intraspecific hybrids (Dinero and Magnus)	Arsenic tolerant	Allevato et al. (2019)

4.7.4 Improving the Fruit Quality

Several studies (Proietti et al. 2008; Flores et al. 2010) disagree over whether grafting improves fruit quality or has the opposite impact. A variety of factors, including the type of rootstock/scion combination utilized and harvest timing, could explain the discrepancies in results reported. When compared to watermelons from

intact plants, the fruit size of grafted watermelons with strong root systems is sometimes greatly increased. This is why many growers employ grafting. Fruit shape and skin color; rind thickness; and soluble solid concentration are all influenced by the rootstock used in the cultivar. When it comes to exporting cucumbers, color and bloom development are critical. Despite the fact that these traits are typically viewed as cultivar specific, the rootstock can have a significant impact. As a result, rootstocks can negatively affect various aspects of cucumber fruit quality, including lower soluble solids and thicker peel as well as the flavor of rootstocks, as well as undesirable internal breakdown in mature fruit. Most new advice for producing grapes are geared at mitigating rootstock's impact on fruit quality. Table 4.3 lists studies that show grafting improves fruit quality in vegetable crops.

4.8 Problems Associated with Vegetable Grafting

A wide range of issues arise while dealing with grafted grafts. The procedure is time-consuming and requires the expertise of qualified professionals. For graft healing, a regulated environment, and efficient grafting equipment and robots are all necessary. Time management for sowing rootstock and scion seeds is also necessary (Fig. 4.7). Scion fruit quality and output can be dramatically impacted if transplants develop too quickly in the field (Huang et al. 2015). Rootstock–scion incompatibility may be found in the early stages or during field transplantation. Depending on the soil and environmental circumstances, it is necessary to select rootstock and scion combinations with care. Seeds for both the rootstock and the scion must be purchased, and the cost of hybrid and special seed might be prohibitive. Suckers and offshoots of the rootstock that form during the healing process or in the field (following transplantation) must be removed. The spread of pathogens, especially seedborne ones (e.g., *Clavibacter michiganensis subsp. michiganensis* in tomato, bacterial fruit blotch caused by *Acidovorax citrulli* in watermelon and melon, charcoal rot caused by *Macrophomina phaseolina* in melon and bottle gourd, and tomato mosaic virus and pepino mosaic virus infections in tomato) can be increased by grafting, particularly in the nursery. This is because a grafted plant is made from two seeds and is grafted with cutting devices. In order to prevent the spread of pathogens in the nursery, it is necessary to use seeds that have been certified free of pathogens, disinfect cutting instruments, use clean clothing and sterilized hands by grafting workers, disinfect grafting areas and plant growing environments, and constantly monitor the phytosanitary status of seedlings. Vegetable grafting may provide several career opportunities, but researchers have discovered a number of hazards that may endanger the health of those who work in nurseries. During the months of April–June, September, and October, employees in greenhouses and growth chambers experience heat stress and pain while grafting plants (Lee et al. 2010; Marucci et al. 2012). However, workers' health and safety can be improved by the use of cooling pads, blowers, and covering sheets, but better facilities (such as

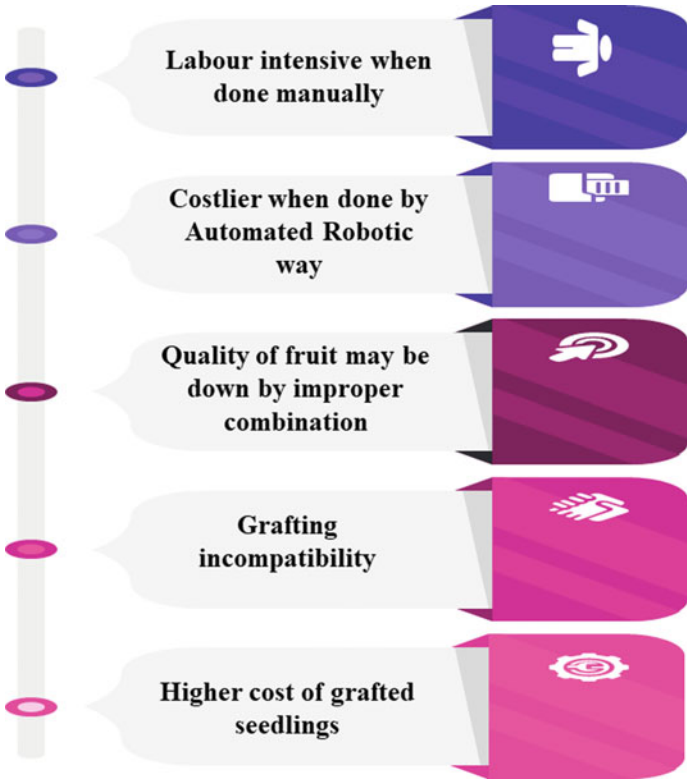

Fig. 4.7 Problems associated with vegetable grafting

air-conditioned settings) are still needed. However, careful management approaches can greatly lessen the severity of these issues.

4.9 Conclusion and Future Prospects

Some soil-borne illnesses can have a dramatic impact on the production of vegetables, particularly tomatoes, in rural areas. There are many diseases that can damage tropical tomato plants, but one of the most common and most devastating is bacterial wilt. In the case of cucurbits, such as eggplant, this has also been the case. Soil-borne illnesses and nematodes can affect yields, but grafting sensitive cultivars onto wild rootstock has been shown to reduce such risks. It is necessary to conduct location-specific research in order to analyze and identify the most compatible rootstocks. As a vegetative growth technique, grafting can be used to overcome the incompatibility between two different species. Another major application of this approach is to offset yield losses due to abiotic stresses. Identification of appropriate disease-resistant rootstocks with tolerance to biotic and abiotic stressors is vital for

the sustained success of grafting. As the molecular mechanism of gene flow between rootstock and scion is still not clearly understood, it needs further advanced study by using molecular markers and genome sequence study. The proteomics and metabolomics study can further clarify the fruit nutritional quality status in the grafted hybrid. The level of beneficial and anti-nutritional factors should need to analyze as most of the rootstocks are used from wild sources. In coming future, the technique will explore with more precision and innovation. The key to widespread adoption is the availability of reasonably priced, healthy grafted seedlings. It takes a lot of time and effort to grow and manage grafted plants in the nursery. Unemployed individuals may be able to find work as a result of this initiative. Preparation of the bed soil and planting for the creation of grafted nursery plants are just a few of the many stages involved. Farmers should use low-cost grafting techniques to ensure the long-term viability of grafted seedlings. Improvements in grafting technique and the healing environment are required before commercial use. Farmers need to be made more aware of grafted vegetable processes and advantages. Fruit and vegetable grafting can help boost agricultural output. A growing number of organic farmers are using vegetable grafting as a way to expand their harvests. To feed the predicted 10–11 billion people on the planet by 2050, agricultural production must rise by 60%. Sustainable use of natural resources can attain this goal. An growing number of biotic (soil-borne disease and nematode problems) and abiotic (salinity, drought, heat, waterlogging) challenges are having an effect on vegetable output. Grafting techniques and nursery management practices should be improved in future study to ensure organic farm growers receive high-quality grafted transplants. Grafting can be used in both breeding and research to advance sustainable agricultural production, as can many other methods.

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Biotechnological Implications in Tomato for Drought Stress Tolerance

5

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Abstract

Tomato growing is one of the utmost profitable farming in India. Many abiotic stresses like drought, extreme temperature, and high salinity affect greatly tomato production. It causes about upto 70% yield loss. Drought can have an impact on plant water metabolism and causes major changes. Drought stress in tomatoes affects physiological activities like photosynthesis, relative water content, and osmotic adjustment. In addition, drought stress damages membrane lipid composition that increases the reactive oxygen species and modulation of protein synthesis. However, improving drought tolerance through traditional genetic improvement techniques has less efficient, is more time consuming, and requires large resources. Recently, the *omics* approaches were found to be the most efficient tool to understanding the mechanism of drought tolerance more precisely. Modern sequencing technologies have greatly accelerated genomics and transcriptomics studies in tomatoes. Particularly, transcriptomic analysis can be more useful in understanding the genes and pathways involved in stress tolerance. Also, it allows identifying more microsatellites, which are the traits that can aid in large-scale genotypes. New genomics-based breeding tools such as genotyping by sequencing, genome-wide association studies, genomic selection, and SNPs are powerful tools for genotyping the genetic resources for various trait improvement, including drought stress tolerance. Drought-tolerant QTLs can also be identified by these techniques for efficient QTL introgression to the elite lines. In addition, genome-editing techniques such as Crisper/Cas9 and RNAi become a major tool for improving drought stress tolerance in tomato at various levels. Further, overexpression of drought-responsive genes and TFs played major role in developing resistance tomato cultivar. This review describes mechanistic

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_5

insights for drought stress based on genomic and biotechnological techniques, as well as effective morpho-physiological, biochemical, and biotechnological solutions for overcoming drought stress detrimental consequences. Altogether, these techniques would help in tomato genetic improvement for drought stress tolerance by increasing productivity under arid and semi-arid conditions.

Keywords

Tomato · Genomics · RNAseq · Drought tolerance · Physiology and *omics* tools

5.1 Introduction

Tomato belongs to the Solanaceae family (Gebhardt 2016) and originated from the Andean region now encompassed by part of Chile, Bolivia, Ecuador, Colombia, and Peru (Bai and Lindhout 2007). Tomato is one of the most important crops worldwide and has a high demand in the fresh fruit market and processed food industries (Chaudhary et al. 2019). Tomato production and area in India are projected to be at 350 thousand hectares and 5.3 million tons. The cultivation area accessible for tomato production in India for the year 2021 was expected to be 852 thousand hectares. Unfortunately, abiotic stresses such as drought, extreme temperature, and high salinity affect almost every stage of the tomato lifecycle (Cui et al. 2020; Shao et al. 2015). Depending upon the plant stage and duration of the stress, abiotic stress causes about 70% yield loss (Krishna et al. 2019). As a result of the lower P, N, leaf area, shoot fresh, and dry weight, drought stress may induce a more severe drop in tomato output than heat stress. However, further study is needed to determine the effects of single and combined stress on tomato flowering, fruit set, and yield (Zhou et al. 2017). Drought can have an impact on plant water metabolism and produce major changes in plant morphology, physiology, and biochemistry of tomato (Torres-Ruiz et al. 2015). Therefore, this chapter mainly focused on how drought stress affects tomato and efficient tools for genetic improvements of tomato for drought stress tolerance.

5.2 Drought Stress Responses of Tomato

Drought can have a negative impact on both the vegetative and reproductive phases of tomato cultivars, inhibiting seed formation and reducing stem and fruit growth (Bartels and Sunkar 2005; Nuruddin et al. 2003). General observations on morphological (anatomical) changes of drought resistance mechanisms are as follows: (1) Leaf rolling, folding, and reduced evaporation of leaf surface area, as well as reduced absorption radiation, (2) a deeper and more productive root system for rooting (Massimi 2021; Rao et al. 2016).

Drought stress inhibits plant development by lowering the photosynthetic rate (Kebbas et al. 2015; Liang et al. 2020; D. Zhang et al. 2018). The primary causes of

reduced photosynthesis might be due to stomatal (due to decreasing CO_2), nonstomatal (decreased photosynthetic activity in mesophyll tissue), or both attributes (Liang et al. 2020). Drought greatly affected tomato photosynthesis, as evidenced by a considerable decrease in the net photosynthetic rate. Both stomatal and nonstomatal limitations are to blame for the decrease in photosynthesis (Liang et al. 2020). Plants can minimize the generation of extra electrons under stress by using energy dissipation processes during photosynthetic electron transfer. In terms of photosynthesis in leaves, the gas exchange index shows its apparent properties, whereas chlorophyll fluorescence reflects its intrinsic properties (Jiang et al. 2006; Long and Bernacchi 2003). Drought tolerance in plants is mostly demonstrated by effects such as osmotic material buildup, changes in membrane lipid composition, scavenging of reactive oxygen species (ROS), and modulation of protein synthesis and hormonal balance (Cuming et al. 2007; Martìnez et al. 2004), also photosystem II (PSII) is a heat-sensitive component (Čajánek et al. 1998) and chlorophyll fluorescence is an effective and non-destructive approach for measuring the photochemical efficiency of PSII and, as a result, detecting stress damage in PSII (Baker and Rosenqvist 2004). The maximum potential quantum efficiency of PSII (Fv/Fm) estimates PSII's maximum quantum efficiency, which is mostly impacted by stress (Sharma et al. 2012; Zhou et al. 2015).

Drought stress disrupts the equilibrium of ROS generation and scavenging in plants (Ajithkumar and Panneerselvam 2014). ROS buildup damages membranes and induces membrane lipid peroxidation (Xu et al. 2006). Several antioxidant enzymes and osmotic substances (such as soluble sugars, proteins, free prolyls, and so on) in plants form antioxidant enzyme systems (Sairam et al. 2011), which scavenge ROS and protect macromolecules in plant cells (Ennajeh et al. 2009; Liang et al. 2020). Abiotic stresses cause biochemical changes, such as enzyme inactivation, changes in the water and ion uptake, and altered hormone concentrations plant system aid in tissue water changes. It is vital to recognize the indirect relationship between drought stress and other forms of stress, and how high temperatures promote drought stresses and salinity stress. Pillay and Beyl (1990) discovered a reduction in cytokinin content in a drought-tolerant tomato cultivar. According to Maggio et al. (2010), GA3 treatment in tomato-reduced stomatal resistance and enhanced plant water usage (Fig. 5.1).

5.3 Molecular Mechanisms of Drought Tolerance in Tomato

Drought stress is a major crop yield limiting factor all around the world. Changes in gene expression promote plant physiological responses to drought stress (He et al. 2020). Plants adapt to water scarcity by going through a sequence of physiological, cellular, and molecular processes that lead to stress tolerance. In Arabidopsis, rice, and other plants, molecular and genomic analyses have found several drought-inducible genes with varied roles, including a variety of transcription factors that regulate stress-inducible gene expression (Shinozaki and Yamaguchi-Shinozaki 2007). Likewise in tomato SlGRAS4, a drought stress-responsive GRAS gene

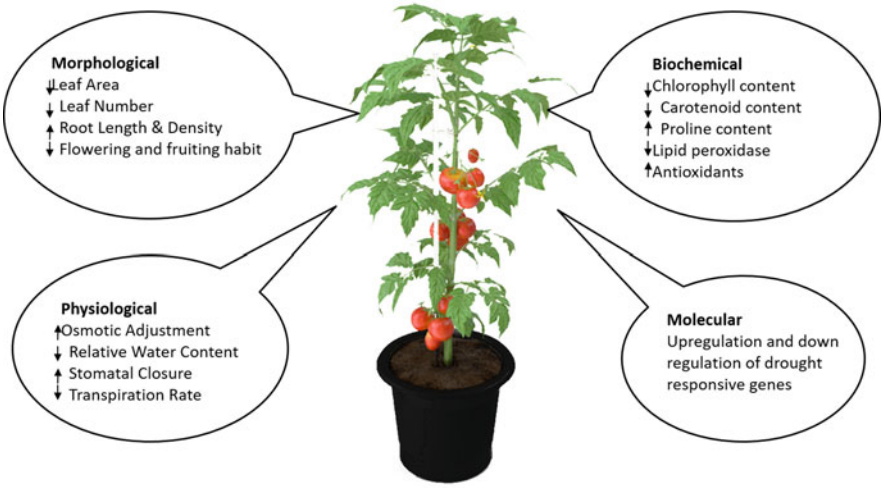

Fig. 5.1 Whole plant drought responses of tomato

from tomato (*Solanum lycopersicum*) was functionally characterized. Repressing SlGRAS4 (SlGRAS4-RNAi) increased sensitivity to drought stress, whereas overexpressing SlGRAS4 (SlGRAS4-OE) in tomato-enhanced tolerance of this stress (Liu et al. 2021). Also, HsfA1a heat-shock transcription factor, is involved in the induction of autophagy in tomato tolerance to drought stress (Wang et al. 2016). Downregulating the expression of the ZF-HD TF, SL-ZH13, reduces tomato drought tolerance (Zhao et al. 2019). Under drought stress, a functionally recognized tomato GATA gene family member, several GATA TFs have been linked to photosynthesis, germination, circadian rhythm, and other processes (Zhao et al. 2021). In addition, we review recent progress in tomato on drought response regulation by drought-responsive genes and transcription factor (TF) families, including the ABA-responsive element/ABRE-binding factors (ABRE/ABF), WRKY, and ABA-independent AP2/ERF and NAC families (Karkute et al. 2018). Some of the major drought-responsive genes TFs studied in tomato are shown in Table 5.1.

5.4 Genome-Editing Approaches in Tomato for Drought Tolerance

Researchers have been able to apply genome-editing methods for the functional evaluation of many genes useful for crop improvement owing to developments in genome sequencing and high-throughput approaches. This chapter focuses on genome-editing approaches in tomato for increasing drought stress tolerance. RNA interference, insertional mutagenesis, and clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) have all been used to successfully modify candidate

Table 5.1 Some of the major drought-responsive genes and TFs reported in tomato

Gene/TF	Stress response	Reference
ATAF1	Drought stress	Awais et al. (2018)
AnnSp2	Drought and salt	Ijaz et al. (2017)
AtNHX1 and TVP1	Drought and salt	Khoudi et al. (2009)
SlWRKYs	Biotic and/or abiotic stress	Bai et al. (2018); Hichri et al. (2017); Karkute et al. (2018)
SlAREB1	Drought stress	Bian et al. (2019)
SlHA1, 2 and 4	Related to stomatal opening	
LeERF1	Drought stress	Lu et al. (2010)
P5CS	Drought stress	
LEA	Drought stress	
ltpg2	Drought stress	
tdi-65	Drought stress	
WRKY	Drought stress	Karkute et al. (2018)
ABRE	Drought stress	
HSE	Drought stress	
MBS	Drought stress	
SlSR1L	Drought stress	Li et al. (2014)
SlSR2L	Drought stress	
SlGRAS4	Drought stress	Liu et al. (2021)
SpUSP	Drought stress	Loukehaich et al. (2012)
ASR1	Drought stress	Ricardi et al. (2014)

genes giving tolerance to abiotic stimuli such as heat, cold, drought, and salinity stress (Salava et al. 2021). Among the methods, CRISPR/Cas9 has been extensively utilized to alter many crop plants due to its precision, efficiency, and cost effectiveness. Several researches in Arabidopsis, tobacco, rice, cucumber, maize, tomato, wheat, cassava, and potato have reported genome editing utilizing CRISPR/Cas9 to generate superior crop varieties in terms of biotic and abiotic stress responses, as well as nutritional and other characteristics (Jaganathan et al. 2018). ABA is essential for ABA homeostasis and is conjugated to glucose by uridine diphosphate glycosyltransferases (UGTs) (Priest et al. 2006). Sun et al. 2017 used RNAi to mute SlUGT75C1 in tomato and found that SlNCED1 (a key enzyme in ABA biosynthesis) levels were unaffected, whereas SlCYP707A2 (a key enzyme in ABA breakdown) levels were up regulated in the silenced line. The knockdown mutants also exhibited tolerance to drought stress. bZIP is involved in a variety of physiological and signaling activities, as well as the stress response. ABRE-binding factors (ABFs) and ABA-responsive element-binding proteins (AREBs) are bZIP proteins that control ABA-dependent transcription and abiotic stress responses (Jakoby et al. 2002; Umezawa et al. 2010). Some of the major genome editing for drought tolerance in tomato is shown in Table 5.2 (Fig. 5.2).

Table 5.2 Some of the important genome-editing techniques in tomato for drought stress tolerance

Target gene	Functions	Method	Reference
SlLBD40	Increased tolerance to drought	CRISPR/Cas9	Liu et al. (2020)
SlMAPK3	SlMAPK3 is induced by drought stress	CRISPR/Cas9	Wang et al. (2017)
SlNPR1	The mutants exhibited reduced drought tolerance	CRISPR/Cas9	Li et al. (2019)
SlAO	Increased photosynthetic activity under drought stress	RNAi	Zhang et al. (2011)
ShATL78-Like	Susceptible to cold and drought	RNAi	Song et al. (2016)
SlUGT75C1	Increased tolerance to drought stress	RNAi	Sun et al. (2017)
SlPP2C1	Resistant to drought stress	RNAi	Zhang et al. (2018)
SlMBP8	Enhanced tolerance to drought and salt stress	RNAi	Yin et al. (2017)
SlbZIP1	Reduced tolerance to salinity and drought stresses	RNAi	Zhu et al. (2018)
SlAOX1a	Susceptible to drought stress	RNAi	
SpPKE1	Susceptible to drought stress	RNAi	Li et al. (2019)
SlSnRK2.1, SlSnRK2.2	Enhanced tolerance to osmotic stress	RNAi	Yang et al. (2015)
SlNAC11	Susceptible to salinity and drought stress	RNAi	Wang et al. (2017)
SlPti4	Susceptible to drought and weak resistant to B. cinerea infection	RNAi	Sun et al. (2018)
SlJUB1	Susceptible to drought stress	VIGS	Thirumalaikumar et al. (2018)
SlSR1, SlSR3L	Enhanced resistance to B. cinerea and susceptible to drought stress	VIGS	Liu et al. (2014)
tos1, and tss2	The mutants are hypersensitive to osmotic stress	EMS mutagenesis	Borsani et al. (2002)

5.5 Applications of Biotechnological Tool for Tomato Improvement

Tomato production, productivity, and quality are adversely affected by abiotic stresses, which account for about 70% of yield loss (Krishna et al. 2019). Among the abiotic stresses, drought is the major one. The complex nature of drought tolerance limits its management through conventional breeding methods for many reasons (Gosal et al. 2009). Advancement biotechnological tools would provide the potential for increasing the efficiency of breeding by reducing the genotype × environment interaction and facilitating efficient introgression of superior alleles from wild species. Since advances in genetics and genomics have improved the understanding of plant sequencing and functional aspects of drought stress tolerance.

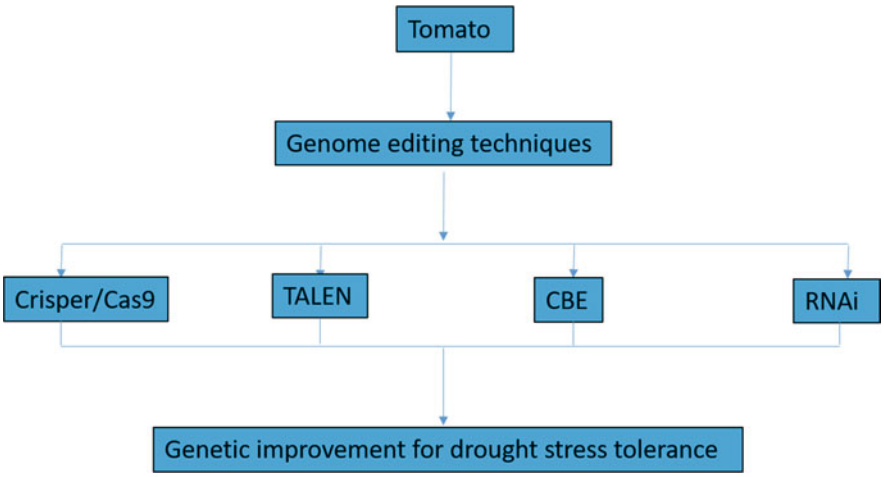

Fig. 5.2 Schematic representation of genome-editing techniques in tomato for drought stress tolerance

Advances in sequencing analysis platforms had enabled the decoding of the genome of tomato. With the help of next-generation sequencing (NGS) approaches, including Illumina Hiseq, Miseq, Truseq, Pac Bio, Hic, and oxford nanopore, several genomes and transcriptomes are sequenced and made available in the public domain.

The availability of genome-sequencing serves for data facilitates identifying genes, alleles, and QTLs underlying key traits through functional genomics and/or GWAS-based approaches. Candidate genes and gene families can be characterized using in sillico tools such as HMM profiling, domain architecture analysis expansion profiling in publicly available RNA sequence data homology modeling, etc. These exercises allow pinpointing the candidate genes which can be further functionally characterized to elucidate their role in particular traits. Presently genomic selection has also been seen as a promising tool in tomato trait improvement, for example, Macadamia sp. and nut yield has improved using GWAS in GS approaches without compromising breeding value (O'Connor et al. 2018). In *C. arabica*, genome-wide SNP analysis offered an efficient parental selection to obtain improved varieties (Sousa et al. 2017). Several traits such as flowering, fruiting traits, and disease resistance are governed by multiple genes or QTLs (Quantitative Trait Loci) exhibiting the quantitative distribution of phenotypes that can be identified using genomics tools (Khan and Korban 2012; Savolainen et al. 2007). More importantly, techniques like RNA seq could allow us to mine for microsatellite motifs results in identifying SSR markers useful in genotyping purposes. These markers are also valuable for studying genetic diversity and its relationship, population structure, linkage disequilibrium, etc. These studies accentuate the importance of NGS to dissect the genetic determinates of complex traits by sequencing the genomes and transcriptomes of tomato. Application of genomics in tomato is to discover novel and high-throughput genetic and molecular technologies as an indirect selection tool,

for facilitating efficient introgression of alleles from wild species into cultivated plants for promoting gene-pyramiding, and enhancing the development of stress-tolerant and higher-yielding in tomato. The CBF/NHX1/DREB1 genes have been used successfully to engineer drought tolerance in tomato, these genes are transcription factors implicated in tomato response to drought and heat stress (Solankey et al. 2014). Finally, the recent advancement in sequencing platform results in low-cost and high-throughput approaches that can effectually be used in tomato improvement.

5.6 Tomato Genomics Approaches for Drought Stress Tolerance

Omics approaches were found useful in detecting the genes actually involved in salinity and drought tolerance. Notably, next-generation sequencing (NGS) has paved a way for a new generation of different *omics*, such as genomics, transcriptomics, and proteomics, and has also been well documented in vegetables like tomato (Chaudhary et al. 2019; Yang et al. 2021). In tomato, Pineda et al. (2012) reported that salt stress resulted in the altered synthesis and accumulation of a number of prominent polypeptides in roots. Transcriptomic analysis can be useful for identifying stress-related pathways and genes regulated by stress encoding unknown proteins (and putatively new functions) and for inferring the main mechanisms responsible for different stress tolerances between cultivated and wild species in tomato (Bohnert et al. 2006; Cuartero et al. 2006). These advancements facilitate Quantitative trait loci (QTL) mapping, genome-wide association studies (GWAS), and genomic selection (GS) in tomato. However, limited efforts have been made in other *omics* branches like proteomics, metabolomics, etc. for a better understanding of the molecular mechanism in tomato (Chaudhary et al. 2019). However, these tools help understand the plant responses and the genetic regulatory networks involved in abiotic stress tolerance and efficient utilization of *omics* resources for tomato crop improvement (Chaudhary et al. 2019).

Several crop genomes, like tomato, have been sequenced using both Sanger and NGS approaches (Kumar et al. 2016; Varshney et al. 2009). Bolger et al. (2014) used high-quality genome sequencing to identify potential genes related to stress tolerance in introgressed lines of *S. pennellii* x *S. lycopersicum* (Bolger et al. 2014). The sequencing of two tomato landraces, COR and LUC chosen for their drought tolerance and fruit quality attributes, revealed hundreds of thousands of SNPs and hundreds of structural alterations (Tranchida-Lombardo et al. 2018).

The sequence variations are likely to explain the genotype's remarkable drought resistance and adaptation to low water regimes in tomato. Further examination of potential genes revealed around 122 genes with high-efficient SNPs. Because both genotypes are drought resistant, the genes with the most frequent variation were chosen as the most promising possibilities. Pan-genome sequencing has grown in importance in recent years because it adds depth and completeness to the reference genome. Recently, genome sequences of 725 accessions were used for tomato pan-genome sequencing, which indicated that 4873 genes were not detected in the

reference genome. In tomato, the researchers performed presence/absence variation studies to better understand significant gene loss and intensive negative selection of genes and promoters during tomato domestication and improvement (Gao et al. 2019). Apart from transcriptomics, proteomics also plays an important role in understanding the genetic variation between genotypes. Recently Zhongcheng et al. (2019) performed a proteome study to explore the molecular variations between two tomato genotypes that differ in salt tolerance to salinity. They discovered 23 salt stress response proteins that were categorized into six functional categories and nearly all of these proteins increased their abundance in the salt-tolerant phenotype. Also, Chen et al. (2016) investigated the impact of exogenously administered glycine–betaine on tomato growth inhibition caused by abiotic stress by altering the expression abundance of six proteins in the salt-tolerant phenotype and two proteins in the salt-sensitive phenotype compared to salt-stressed seedlings.

Genome-wide association studies (GWAS) have an advantage over linkage mapping as investigate the genetic diversity and recombination events found in germplasm collections and offered a greater mapping resolution (Fukushima et al. 2009). Also, Pasam et al. (2012) employed GWAS to find SNPs for agronomic variables in a global tomato germplasm collection. In tomato, GWAS used 182 SSR markers to determine the chromosomal regions related to the 28 different volatile compounds that define tomato flavor (Zhang and Yin 2016). In addition, there have been GWAS studies for fruit metabolic characteristics and other phenotypes. QTL mapping is a marker-based method that necessitates a large amount of genotype data. In tomato, discovering marker-trait connections, linkage mapping, and association mapping were used to the discovery of QTL (Cockram and Mackay 2018). A linkage map with 1345 markers separated at an average spacing of 1.68 cM, comprising 524 distinct map sites, was used for QTL mapping. The genetic map, which measures 2,156 cM and covers more than 84% of the 900 Mb tomato genome (Pascual et al. 2015). Several QTL mapping experiments in tomato have been conducted, specifically to find loci influencing stress tolerance (Pascual et al. 2015). Also, the genomic selection (GS) technique addresses the problem of QTL mapping-based breeding, which makes it difficult to identify the tiny effects of QTLs. Importantly, the modest effect of QTLs may yield bigger effects on economically significant abiotic characteristics when combined (Crossa et al. 2017). Genotyping by sequencing (GBS) is a quick and low-cost approach for genotyping breeding populations, allowing plant breeders to apply genomic diversity studies, genetic linkage analysis, molecular marker identification, and genomic selection (GS) in large-scale plant-breeding programs (Narum et al. 2013; Poland and Rife 2012). An NGS technique was used to uncover 8784 SNPs in a tomato GBS investigation. SNPs (88%) are often detected in tomato germplasm (Sim et al. 2012). GBS has been used to a variety of agricultural species due to its low-cost and advanced technology, according to (Kim et al. 2016; Poland and Rife 2012). In tomato, GEBV (genomic-estimated breeding value) is mostly utilized to increase production and flavor, fruit weight and SSC (soluble solid content) were determined and provide the highest predictability in tomato (Yamamoto et al. 2016) (Table 5.3).

Table 5.3 Some of the important transcriptome studies on tomato for drought stress tolerance

S. No.	Total no of DEGs	Enriched functional annotation	Tissue type used	Reference
1	3850	Plant hormone signal, transduction, phototransduction, and calcium signaling pathway	Leaf	Yu et al. (2021)
2	12213	Hormone signal transduction	Leaf	Yang et al. (2021)
3	14065	DNA binding, protein binding, structural constituent of ribosome, DNA-binding transcription factor and phosphorylation	Leaf and Root	Diouf et al. (2020)
4	1850	Reactive oxygen species, phytohormone signal, transduction photosynthetic, electron transport, carbohydrate transport	Leaf	Zhou et al. (2019)
5	4455	Plant hormone signal transduction	Leaf and Root	Zhang et al. (2019)
6	213	In response to biotic stimulus, lipid transport, response to wounding, response to extremal stimulus, response to stimulus	Root, stem, leaf, flower, fruit, seedling and vegetative parts	Dai et al. (2017)
7	400	Transcription factors and signaling proteins growth and development processes	Leaf	Gong et al. (2010)
8	152 microRNA 3875 Genes	Genes-encoding transcription factor and protein kinase.	Leaf	Liu et al. (2017)
9	2787	ABA-signaling pathway, genes related to stress tolerance, pathogen resistance, and stress-responsive TFs	leaf	Wang et al. (2013)

5.7 Transgenic Tomato for Drought Tolerance

Transgenic tomatoes developed for conferring abiotic stress resistance are the need of the hour for meeting the continuous demand for tomato fruits, due to the severe shift in temperature and uncertain rainfall patterns (Krishna et al. 2019). Drought resistance was demonstrated in transgenic tomato plants with a unique 66-kD BSP (boiling stable proteins) gene from *Populus tremula*. It has been reported that H1-S, a drought-induced tomato linker histone, plays a structural role in protecting DNA from damage during drought stress as well as a functional role in gene regulation

Table 5.4 Some of the important engineered genes for drought stress tolerance in tomato

Gene/origin	Function	References
mt1D/E. coli	Mannitol synthesis	Khare et al. (2010)
Osmotin/tobacco	Osmotin accumulation (provides osmotolerance)	Goel et al. (2010)
ScTPS/yeast	Involved in trehalose biosynthesis	Cortina and Culiáñez-Macià (2005)
TPSP (TPS/TPP fusion gene)/E. coli	Involved in trehalose biosynthesis	Lyu et al. (2013)
MdVHA-B/apple	Maintenance of ion homeostasis	Hu et al. (2012)
SlTIP2;2/tomato	Involved in plant water balance	Sade et al. (2009)
katE/E. coli	Oxidative stress (catalase)	Mohamed et al. (2003)
Osmyb4/rice	Transcription of stress-related gene	Vannini et al. (2007)
ATHB-7/A. thaliana	Transcriptional regulation; a TF induced during drought stress via a mechanism that requires production of ABA	Mishra et al. (2012)
AtDREB1A/CBF3/ A.thaliana	TF, transcriptional regulation; influence on enzymatic antioxidant system	Rai et al. (2013)
ZAT12/B. carinata	TF, encodes a C2H2 zinc finger protein, transcriptional regulation	
tas14/tomato	Accumulation of protein with chaperone-like and detergent properties	Muñoz-Mayor et al. (2012)
ShDHN/S. habrochaites	Accumulation of protein with chaperone-like and detergent properties	Liu et al. (2014)

during stress (Scippa et al. 2000). Also, in transgenic tomato plants, overexpression of the osmotin gene improves salt and drought tolerance (Goel et al. 2010). Further, the gene osmotin has played a major role in alleviating negative impacts of environmental changes such as drought and salinity. In addition, the transgenic tomato which carries the ATHB-7 gene (DTL-20) exhibits greater drought tolerance as compared with wild-type tomato plants (Mishra et al. 2012). Tomato plants overexpressing the SlGATA17 gene were more drought tolerant than wild-type plants, and overexpression of the SlGATA17 gene increased phenylpropanoid biosynthesis pathway activity, which influence tomato plant drought resistance (Zhao et al. 2021) (Table 5.4).

5.8 Conclusion

No doubt, drought stress, have a negative impact on crop production, reducing growth, development, and productivity of tomato. On the other hand, plants have tremendous stress-resistance mechanisms in the form of adaptation. One of the most difficult problems is to understand the drought tolerance mechanisms at whole plant level as biochemical, physiological, and morphological. For this purpose, molecular

biology approaches allow precise gene alterations to be introduced without eradicating natural genetic features. Their efficiency in terms of time and applicability to a wide range of species are also obvious advantages. Particularly, genomics-based breeding techniques such as genomic selection, genome-wide association studies, and genotyping by sequencing in tomato would offer efficient genotyping at large scale. Using NGS drought-responsive genes can be identified and characterized at whole genome level. Further, these genes functions can be modified by genome-editing techniques for improved drought stress tolerance in tomato. In addition, modifying the genes involved in these processes resulted in tomato with higher tolerance to abiotic stresses. The strategy of choice appears to be genetic engineering of genes encoding transcription factors involved in the control of stress-responsive genes, transcription factors, and metabolic pathways for improving the tomato drought stress tolerance.

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

6

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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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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_6

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

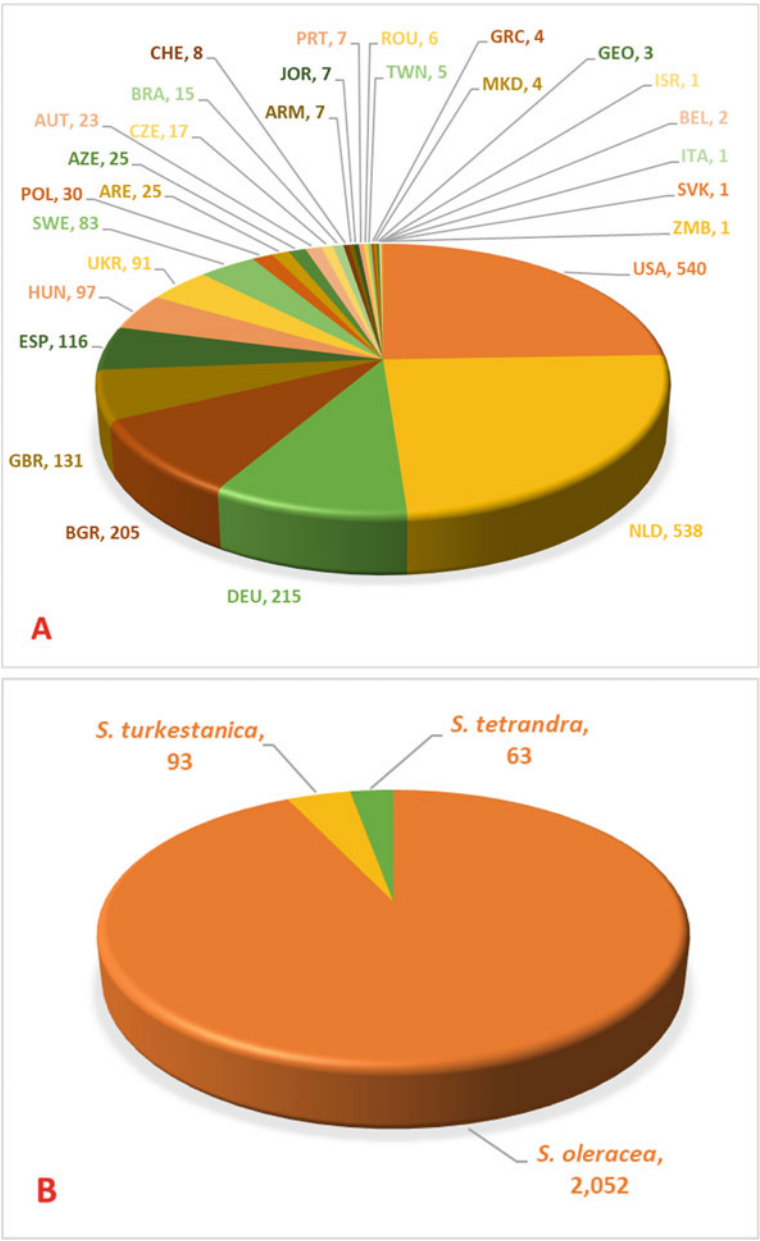

Fig. 6.1 (**a**) Number of spinach germplasms maintained in different countries. (**b**) Number of spinach accessions available under different species. Names of the country are denoted by their WIEWS code and data on number of accessions are downloaded on 4-5-21 from Genesys system (https://www.genesys-pgr.org/a/overview/v20RWL9OWaG)

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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Impact of Biotic and Abiotic Stresses on Onion Production: Potential Mitigation Approaches in Modern Era

7

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Abstract

Onion is one of the most commonly consumed vegetable crops in the world, following tomato. Biotic and abiotic stresses are known to affect onion production, causing significant yield losses, similar to other vegetables and main crops. It is estimated that about 40% of overall losses in agricultural production is related to insects, pests, and weeds. Likewise, environmental fluctuation and climate change are threatening the production rate of onion. Strangely, in the era of omics approach to understand plants' responses to environmental changes, there is limited literature regarding onion response to biotic and abiotic stresses. This chapter enlightens the impacts of biotic and abiotic stresses with the potential exploitation of modern plant improvement techniques.

Keywords

Biochemical · Breeding tools · Climate changes · Onion · Stresses · Morpho-physiology

7.1 Introduction

The bulb onion (*Allium cepa* L.) is the second most important vegetable crop after tomato. Domestication of bulb onion came into being from a wild species from Vavilov's Central Asiatic Center encompassing Pakistan, Afghanistan, and Central Asian regions of former USSR (Havey 1997). The supposed onion ancestor had probably migrated to the Near East (Grubben and Denton 2004; Bagali et al. 2012).

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_7

Onion is famous and is used in world's every region, its bulb is used raw, sliced for seasoning salads, and cooked with other vegetables and meat. Onion bulbs are essential ingredients in many African sauces and relishes. Versatile use of onions and their storage durability made them acceptable in every culture and tradition (Griffiths et al. 2002). The leaves, whole immature plants called 'salad onion' or leafy sprouts from germinating bulbs are used in the same way. It is also known that sliced raw onions have antibiotic properties, which can reduce contamination by bacteria, protozoa, or helminths in salads (Grubben and Denton 2004). Consumption of Onion has numerous benefits including cardiovascular stability because of their anti-diabetic, anti-hypertensive, hyper-cholesterolemic, and antithrombotic effects. Onion also contain biological activities including anti-microbial, anti-cancerous, anti-asthmatic, antimutagenic and prebiotic activities (Corzo-Martínez et al. 2007). It is also known that their anti-depressant and therapeutic effects have been observed and they have potential to be used in the medicine industry.

Onion is day length sensitive; different onion types depend on the latitude at which they grow. It is estimated that around the world, over 104 m tons of onions are grown annually (FAOSTAT 2020). Onion is widely distributed in world and within the last 10 years its production has increased significantly. Annual onion trade constitutes about 20 million USD (FAO 2017). Leading producer of onion in the world is India (26 m tons) followed by China, USA, Egypt, Iran, Turkey, and Pakistan, respectively (FAOSTAT 2020). The worldwide onion exports are estimated at around seven million Metric tons. The Netherlands is the world's largest onion exporter with a total of around 220,000 Metric tons followed by India (FAO 2013). Annual production of onion is 88 million tons approximately. Best suitable areas for onion growth are temperate and subtropical regions (Brewster 2008). Annual gross production value of onion is $42,743 million (FAO 2017).

Continuously increasing world population and climate change are undeniable threat to onion growth and production (Brewster 2008). Onion production faces biotic and abiotic stresses and suffers heavy yield losses, and to overcome these problems, modern plant improvement techniques/approaches are being implied to enhance onion yield, quality, adaptability, and tolerance to diseases and insect pests. In this chapter, we highlight the major biotic and abiotic factors that influence onion production, and different approaches to develop resilient onion cultivars are discussed.

7.2 Climate Change and Overview of Major Biotic and Abiotic Stresses

Climate change is a change in the statistical distribution of weather patterns when that change lasts for an extended period (i.e., decades to millions of years). It affects agro ecosystems through changes over the long term in key variables affecting plant growth (e.g., rising temperatures) (Lorenzoni and Pidgeon 2006). Global warming is constantly increasing CO_2 levels in atmosphere, resulting in decreased available land and quality (Peters et al. 2011). Agricultural yields and productivity are estimated to

fall by 3–16% till 2080, especially in under-developed countries that are already suffering from higher average crop temperatures. Contrarily developed countries having lower temperatures are expected to face lower decline in yields varying between 8% expansion of productivity and 6% decline till 2080 (Mahato 2014). Various affects are caused by climate change including changes in CO_2 level, variability in sea levels, fluctuation in precipitations, and continuous higher temperatures. Climate change influences by rising temperatures and changes in soil moisture which increase pest and disease occurrence in plants (Junaid et al. 2021).

According to Lawande (2010), temperature above 40 °C seriously damages bulb onion and an increase of 3.5 °C significantly reduces onion yield. Similarly, in other vegetable crops 10–20% yield losses have been reported due to increasing atmospheric temperature. Furthermore, the duration of onion gets shortened due to high temperature leading to reduced yields (Daymond et al. 1997; Wheeler et al. 1996). Plants including onion respond via morpho-physiological mechanisms against such climatic anomalies (Malhotra 2017).

7.3 Biotic Stress Factors Affecting Onion Growth

Agriculture sector is coping with gradual economic losses due to diseases and pests, the damages caused by pests are leading factor in the reduction of world's crop yields (Mahato 2014). Overall damages in the world by insect pests are hard to estimate (Culliney 2014). It is estimated that 38% of overall losses in agriculture are caused by insect pests (Cerda et al. 2017). Pests mainly arthropods destroy an estimated 18–20% of annual production globally, which is valued at more than US $470 billion (Sharma et al. 2017). Along with these losses, measure taken to avoid their attacks also needs high expenses, causing burden on economy and it also pollutes environment (Mahato 2014). According to Oliveira et al. (2014) about 7.7% losses to crop production caused in Brazil are due to pests. Crop losses due to pests and diseases for major food and cash crops (rice, wheat, barley, maize, potatoes, soybeans, cotton, and coffee) were estimated between 20 and 40% at country and regional levels in different continents (Oerke 2006). In onion and allied crops number of insect pests threaten its production, it is estimated that 25% of total world production is lost to pest attacks in onion (Rabinowitch 2018). In onion, the major destructive insect pests are briefly described in the next section.

7.3.1 Onion Thrips (*Thrips tabaci*)

Thrips tabaci Lindeman (Thysanoptera: Thripidae) was first reported by a Russian entomologist Karl Lindeman in 1889 with their acute attack on tobacco plants causing severe damage. *T. tabaci* belongs to order Thysanoptera and family Thripidae (Mound and Walker 1982). Its biology, management, and ecological behavior have been described by T. Lewis in his book "Thrips as Crop Pests."

T. tabaci is an increasingly difficult pest to manage overall the world. *T. tabaci* is native to Eastern Mediterranean region (Mound 1997). It has a wide range of host plants, i.e., 141 plant species in 41 families, way more when compared to other thrips species (Ghabn 1948). Moreover, its attack on 355 flowering species was also reported (Morison 1957). Despite its wide range of hosts, onions are its favorite hosts, and they are attacked in different parts of the world by T. tabaci. It attacks onions at very diverse regions from sea level to 2000 m above sea level (Lewis 1997). *T. tabaci* is found in North America, Europe, South America, Asia, Africa, and Australia (Mound 1997). *T. tabaci* has great economic significance on various plant species in temperate and subtropical regions, especially in dry weather (Morison 1957).

7.3.2 Leek Moth (*Acrolepiopsis assectella*)

Leek moth was first reported in Ottawa, Canada in 1993 (Landry 2007). It is native to Europe and has devastating effects on onion crop. It has widespread through eastern Ontario to southern Quebec, New York State, New Brunswick, and Prince Edward Island (Mason et al. 2011). Leek moth has vast worldwide distribution extending from Asia, Europe, and Africa to Pacific Islands (Lecomte 1976). All over the European countries, the presence of moth had been observed, i.e., from Italy to Sweden and Spain to Norway (Frediani 1954; Tullgren 1918; Gaedike 1997; Velitchkevitch 1922).

7.3.3 Onion Maggot (*Delia antiqua*)

Delia antiqua commonly known as onion maggot or onion fly is a devastating pest of onion, which is mostly found in continents of Europe, Asia, and North America (Ishikawa et al. 2000). It was first reported in Wisconsin region of USA during the twentieth century. It is distributed globally. Although it is present in higher distribution, and it is also predicted that with change in climate due to global warming its infestation rate will increase near Caspian and black sea especially in southern England, southern Turkey, and southern Kazakhstan in between 2030 and 2080 (Ning et al. 2017). It also destroys the other species of the Allium genus such as leeks, shallots, chives, and garlic and they are also its host plants (Straub and Emmett 1992).

7.3.4 Weeds

Weeds are the unwanted, undesired, unsuitable, and harmful plants. They are mostly C_4 fast-growing plants. Owing to their tremendous fast-growing ability they challenge the economic growth of vegetable crops, cereals, sugar crops, fiber crops as well as floricultural crops for place, space, air, CO_2, water, light, and moisture

(Leghari et al. 2015). They are more detrimental to agricultural productivity when compared to pests and pathogens under different situations. Along with abiotic factors, weeds have great potential to cause crop losses (Oerke 2006). Weeds compete with economic crop production for water, fertilizers, and other resources affecting agro-biodiversity, yield, and quality; increasing the cost of production (Zimdahl 2018). It is reported that about 31.5% losses to agricultural production occur due to weeds. In natural ecosystem, there are different weed species with different competitive abilities. It is difficult to approximate crop losses due to one weed type so it is measured as allied sufferings by all weeds. Globally, about 34% agricultural losses are caused by weeds in contrast to 18–16% losses caused by both pathogens and pests (Gharde et al. 2018). According to Milberg and Hallgren (2004), 31% reduction in cereal production occurred due to competition of main crop with weeds. Weed losses in rice are extreme than pests as 60–70% of yield losses occurred. About 27–77% yield losses caused in oilseed crops are due to weeds infestation (Leghari et al. 2015).

In Northern and Central America, food losses due to weeds were observed in various crops, 148 ton of corn (50% of corn in America) was wasted due to weed growth in fields causing direct 26.7 billion USD loss to economy (Soltani et al. 2016). Weeds drastically reduce food resources of plants, hence, dropping crop yields because plants cannot uptake nutrients and fulfill their energy needs (Shad 1987). They compete with plants extricate resources from soil (moisture and minerals) and environment (light and air). Especially in critical growth periods, weeds negatively affect their growth and their competition cause reduction in the yield (Leghari et al. 2015). Weeds are difficult to control as they multiply fast and thousands of seeds are produced from a single plant (Anderson 1983).

7.4 Abiotic Stress Factors Affecting Onion Growth

Drought can be defined as prolonged absence or deficiency of precipitation or periods of dry weathers causing hydrological imbalances (Trenberth et al. 2014). Water shortages are increasing day by day; losses caused by these shortages are way higher than other factors due to its extent and mildness. Drought aligns at first in natural catastrophes in terms of total number of affected people worldwide (Hewitt 1997). Drought mainly disturbs plant water association and diminishes water-use efficiency. Almost all the plant's mechanisms get affected by water scarcity, e.g., phenology, growth rates, photosynthesis, respiration rates, reduced leaves, and stem size (Farooq et al. 2009). All geographic regions of the world are virtually affected by drought (Wilhite 2000). Literature suggests that due to shallow onion root system, it is prone to water scarcity. In onion, drought at early stages of growing season causes more than 26% yield losses in the final harvest (Malhotra 2017).

Salts are naturally present in all type of soils and many of them act as essential and helpful nutrients for the agricultural productivity. But, if their concentration in the soil increases from a specific value, they hinder the production and quality of crops. This depends upon the quantity and type of salt present in that soil along with the

Table 7.1 Summarized table reviewed from past studies elucidating various factors and their damages to overall plant production

Factors	Damages	Reference
Insect pests and pathogens	7.7% (Brazil) 38% (Global) 20–40%(Global) 50% (In American Corn)	Oliveira et al. (2014) Cerda et al. (2017) Oerke (2006) Soltani et al. (2016)
Weeds	34% (Global)	Oerke (2006)
Soil erosion	80% (Global Agricultural land)	Pimentel et al. (1995)
Salinity	20% (Global Agricultural land)	Pitman and Läuchli (2002)
Post-harvest losses	10–30% (Global)	Hodges et al. (2011)
Anthropogenic	20% (Greenhouse gas emissions)	Aydinalp and Cresser (2008)

environmental factors. Thus, when there are excessive salts in the soils that can impair its efficiency are called saline soils (Qadir et al. 2000). Several crops in the world are sensitive towards salt stresses and show low productivity. Global agricultural losses due to salinity are estimated to be 12 billion $/year (Pitman and Läuchli 2002). Salinization of irrigated lands is a major issue; about 17% of world's cropland receive irrigation and produce up to 30% of total agricultural production (Hillel 2000). It is reported that in onion, bulb yield reduces with per unit increase in soil salinity, and thus, onion is categorized as a salt-sensitive crop (Shannon and Grieve 1998). Moreover, early flowering also takes place under high salinity in onion (Pasternak et al. 1979). Salinity decreases bulb traits, plant height, and number of leaves in onion plant (Shannon and Grieve 1998). It was also reported that onion crop significantly shows change in its morphological and physiological traits including gaseous exchange, and chlorophyll traits in response to salt stress (Chaudhry et al. 2020). Shallow root system of onion makes it prone to drought and salt stress, which results in smaller bulb size and affects important economic traits (Hanci and Cebeci 2015). Onion production suffers yield losses due to water scarcity and salinity.

Soil erosion is the process of detachment and transport of soil particles by erosive agents (Ellison 1944). About 99.7% of human food came from land and 0.3% from aquatic system or ocean. Loss of land due to erosion is a serious threat for sustainable agriculture. It is approximated that every year 75 billion metric tons of soil particles are detached and moved from land via wind and precipitations (Myers 1993). As a result, arable land is degrading day by day and production is declining. Inappropriate practices used by farmers is also a big reason of soil erosion, efficient use of land and proper management practices play a key role (Lal 1990) (Table 7.1).

7.4.1 Physio-biochemical Changes in Onion

Onion is a shallow-rooted vegetable; its roots generally lie at 0.18 m depth, and therefore water in deeper soil pockets is barely available for onion. Decrease in water availability causes drought stress conditions. Additionally, salt deposits in the upper

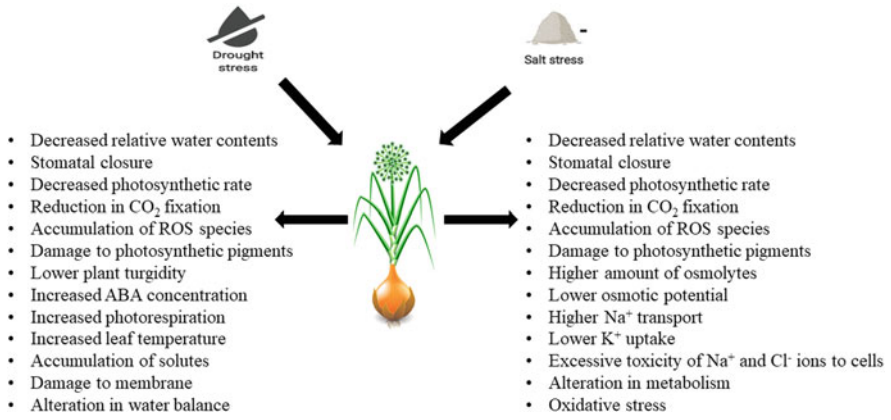

Fig. 7.1 Drought and salt stress effect on physiological changes in onion

soil surface also causes serious salinity problems at the bulbification stage (Chaudhry et al. 2020). Reduction in soil moisture and higher sodium toxicity in vicinity of plant roots disturbs the physiological functioning of the plant (Gokçe et al. 2021). Decreased absorption of water increases osmotic stress in the plant. It affects turgor pressure, solute potential, and water potential in plant cell. Salt stress further aggravates ionic stress by disturbing ionic homeostasis. It favors the higher uptake of Na^+ and Cl^- and resulting in suppression of essential nutrients uptake. For instance, higher Na^+ uptake decreases the availability of K^+ ion that effects the stomatal control resulting in excessive water loss, while Cl^- ions damage the photosynthetic pigments that ultimately cause chlorotic toxicity. Impact of drought and salt stress on physiological changes in onion is illustrated in Fig. 7.1.

7.4.2 Abiotic Stresses and Photosynthesis

Plants' photosynthesis is a complex process characterized by drought and salinity stress in both the short and long terms. Photosynthesis is complicated because it incorporates multiple aspects such as photosystems, photosynthetic pigments, electron transport systems, and CO_2 absorption. As a result, abiotic stress results in loss of photosynthetic activity. It is mostly harmed because of reduced stomatal efficiency and CO_2 absorption. Even after only a few hours of dehydration and salt stress, it causes rapid growth decrease (Asim et al. 2021). Reduced stomatal activity benefits plant by minimizing the loss of water and limiting harmful ion transport to the roots. It was demonstrated that inhibition during the early stages of salt stress reduces the inflow of harmful ions into the transpiration stream. The internal water contents of the leaf correlate with closure of stomata, revealing strong link between a leaf's water content and stomatal action during drought stress. Stomatal conductance causes photosynthesis to slow down immediately. Several studies have revealed that

the fluctuation is a gaseous exchange trait of the vegetables to single and combined stresses (Demirel et al. 2020; Altaf et al. 2022). However, literature regarding onion physiological behavior is still limited (Chaudhry et al. 2020; Semida et al. 2020).

7.4.3 Production of Oxidative Stress

Reactive oxygen species (ROS) are oxygen derivatives that are capable of reacting. Singlet oxygen radicals are independent oxygen radical elements superoxide anions ($O2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) (Gökçe et al. 2022). The ROS production occurs in a variety of cell components, the most common of which are the chloroplast and mitochondria. Numerous metabolic processes in mitochondria, chloroplasts, and peroxisomes also contributed to their formation (Mittler 2002). The photosystems PSI and PSII are the primary sites of ROS generation in chloroplasts located in the thylakoid membrane. Environmental adversities can cause change in physiological responses that have a significant impact on ROS generation. Furthermore, the rate of its creation increases due to excessive photon energy that is greater than that necessary for CO_2 fixation by plants (Asada 2006). In drought stress, restricted water availability and limited CO_2 accessibility accelerates electron transfer to molecular oxygen, commencing Mehler's reaction and resulting in the formation of superoxide anion in PSI. The singlet oxygen ($^1O_2$) is produced in the PSII by stimulation of the triplet state of chlorophyll at the reaction center of P680 (Asada 2006). The univalent reduction of superoxide anion, as well as protonation, results in the formation of H_2O_2. The H_2O_2 is essential at a lower rate for several physiological functioning of the plants, whereas its higher production causes harmful effects and known as ROS (Breeze and Mullineaux 2022). Reactive oxygen species are involved in the signaling pathway that controls ionic channel function and gene expression. Out of a plant's total O_2 consumption, at least 1% is transformed to ROS (Mano et al. 1987). With salinity stress, stomatal closure occurs, resulting in a decrease in CO_2 availability for the plant. As carbon fixation decreases, chloroplast is exposed to excitation energy, which encourages the slowing down of photosynthetic electron transport, leading to an increase in ROS formation. In all the major vegetables, several studies have revealed the functioning of plants with the exposure to stress resulting in ROS accumulation. Contrarily, recent studies are focusing on the response of onion to drought and salt stresses (Ghodke et al. 2018; Ghodke et al. 2020; Chaudhry et al. 2021a, b; Gökçe et al. 2022).

7.4.4 Role of Antioxidant Enzymes for ROS Mitigation

Plants are exposed to multifarious abiotic stresses that result in oxidative burst, however, higher production of antioxidant enzymes results in the suppression of oxidative stress (Hussain et al. 2018). The major antioxidant enzymes include Superoxide dismutase (SOD), and catalase (CAT), and ascorbate peroxidase

(APX). Superoxide dismutase catalyzes the breakdown of superoxide radical (O_2) to H_2O_2 and O_2 in the cytosol, acting as a first in the antioxidant system. SODs are divided into three groups based on their prosthetic metals: copper-zinc, iron, and manganese-containing SODs. SOD localization in plants varies depending on plant species and stress conditions. CuZn-SOD and FeSOD are therefore found in chloroplasts (Bowler et al. 1994), MnSOD is found in mitochondria and many kinds of peroxisomes. As a result, at least one copy of MnSOD will be present in each plant genome, and this enzyme assists in the defense of mitochondria against ROS damage in plants (Moller 2001). During stress, ascorbate peroxidase is the key participant in the detoxification of hydrogen peroxide in plant chloroplasts, mitochondria, cytosol, and peroxisomes. To decrease H_2O_2 to H_2O, ascorbic acid is used as an electron donor. By converting H_2O_2 to H_2O, catalase also helps to prevent oxidative damage (Demiral and Türkan 2009). Plants may endure drought and salt challenges by sustaining activities of antioxidant enzymes to deal with oxidative stress generated by environmental stressors in diverse plant species. In onion, studies have revealed the positive correlation of antioxidant enzyme accumulation to cope with stress conditions (Chaudhry et al. 2021a, b). The higher expression of genes involved in the synthesis of antioxidant enzymes also results in conferring tolerance to onion (Ghodke et al. 2020).

7.5 Molecular Approaches for Stress Tolerance in Onion

Plantsmolecular processes are altered because of abiotic stress that results in differential regulation of genes. The study of the actions of genes that are activated by stress is a useful method for deciphering the molecular mechanism of plant stress resistance (Chaudhry et al. 2022). Abiotic stress tolerance is a polygenic trait that influences the gene expression. The genes are categorized as one that are directly involved in plant cell defense, i.e., LEA proteins, chaperones, osmoprotectants, free radical scavengers, and detoxification enzyme (Divya et al. 2021). Transcriptional regulation and signaling pathways are included in the second category, i.e., transcriptional factors, calcium-dependent protein kinase (CDPK), mitogen-activated protein kinase (MAPK), SOS kinase, and phospholipases. Ion transporters and aquaporins, which are involved in ion, water, and transport in plants, make up the third category (Razi and Muneer 2021). Onion genome size is large that is the major hurdle for quantification of molecular responses of onion to cope with future climatic changes. Recently, Ghodke et al. (2020) showed the contrasting regulation of genes in onion in response to drought stresses. Further attention of this important crop is required to reveal molecular responses to other major abiotic stresses as reported in tomato and potato (Mushtaq et al. 2022; Şanlı and Öztürk Gökçe 2021).

7.5.1 Stress Signaling and Positive Role of Sensors

Abiotic stressors have a deleterious impact on plant function. Plants manage a system of signals pathway to begin nuclear gene expression in response to adverse conditions. Plants express distinct gene expression in response to stress. Plant cells were previously thought to be capable of sensing a variety of stress signals. Just a few reports of potential sensors have been reported, despite various efforts. Activated nuclear genes stimulated the expression of several genes. The receptors detect the first stress signal at the membrane level. The most important receptors are G-protein-coupled, histidine kinase, and ionic channels. As a result, secondary signal molecules are initiated, i.e., inositol phosphate, Ca^{2+} channels, ROS formation, and synthesis of abscisic acid. The abiotic stress signaling pathway is widely understood, but understanding all of the bridge is immensely difficult. The basic explanation is that abiotic stress signaling is a complicated feature controlled by a number of genes. However, research in the last two decades have clarified the significance of single or multiple gene expression, allowing for a genome-wide expression method to fully understand complex features. Even though abiotic stress activates a variety of signaling pathways, the mitogen-activated protein kinase (MAPK) signaling pathway is one of the most important one. It connects the behaviors of intrinsic cells to environmental stimulation. MAPK is a signaling module found in all eukaryotes. It is essential for the transmission of numerous types of signals. There were roughly 10 MAPKKs, 20 MAPKs, and 80 MAPKKs in the Arabidopsis genome (Saijo and Loo 2020). The stress signal activates stress-responsive genes in the nucleus, allowing for stress adaption. There are two types of genes that respond to stress. Early responsive genes are a type of gene that activates within moments after being exposed to a stress. Another set of genes is delayed genes, which activates over time and in response to the degree of the stress. A variety of abiotic stress-related genes, regulatory sequences, and transcription factors have been discovered and researched in several vegetables to confer stress tolerance (Ba et al. 2021; Zaynab et al. 2021).

7.5.2 Role of Transcription Factors

Transcription factors are proteins that are recognized to be primary regulators of gene expression. Abiotic stress-responsive transcription factors include WRKY, bZIP, NAC, AP2, MYB, bHLH, and DREB (Chaudhry et al. 2021a, b). By binding to cis-acting regions in the promoter region, such signaling pathway genes can influence the expression of target genes. In this method, the candidate genes' gene expression is upregulated. Transcription factors are ABA-independent and ABA-dependent signal pathway. Each of these pathways is important in regulating gene expression in response to abiotic stress (Xiong et al. 1999). The expression of levels of NAC, MYB, and WRKY transcription factors has been reported in tolerant onion cultivarunder drought stress (Ghodke et al. 2020). Important crops have shown higher transcriptional regulations that help in coping with abiotic stress. In

response to salt, the transcription factors ZFP179 and NAC5 were upregulated. It controls the creation and accumulation of proline, LEA protein, and sugar, resulting in stress tolerance.

7.5.3 Role of Transporters

Plant cells are subjected to ionic stress as well as water deficiency circumstances as a result of salinity stress. Because of increased sodium toxicity and a comparably larger amount of solution in soil, plants are unable to retain moisture required for optimal development. Furthermore, it disrupts the plant's ionic balance, culminating in ionic stress. Plant cells are exposed to a harsh atmospheric due to changes in K^+/Na^+ ratio and increased Na^+ and Cl_- ions. It is because of the increased input of Na^+ ions through the same routes which develop potassium. Another idea is that the similarity of hydrated ionic radii makes it difficult for plant transporter proteins to distinguish between K^+ and Na^+. It is thought to be the primary cause of Na+ toxicity in plants. Moreover, Na^+ and K^+ compete for K^+ binding sites. Plant cytosol is unable to thrive at high concentrations of salt, most likely because cytosolic enzymes are poor in dealing with the input of Na^+(Bernstein 2019).

Glycophytes developed a salinity resistance approach that included cytosol with a reduced Na^+ content and a higher K^+ level. It keeps a larger concentration of K^+ ions in the cytosol through sodium cellular compartments as well as extruded, which is a dynamic system. Na^+/H^+ antiporters aid in compartmentalization of Na^+ vacuole and extrusion from the cell. So, it assists in osmotic balance, which is necessary for salinity tolerance. Even though the method of Na^+ entrance into the plasma membrane is unknown, it can be carried via K+ carriers. Furthermore, when compared to Na^+, various selective pumps prefer K^+ uptake. To ingest K^+ extracellular media, plant cells use high- and low-affinity K^+ transporters. Low-affinity K^+ transporters, AKT1, are inward channels critical for the induction of a K^+ influx when the plasma membrane is hyperpolarized. External Na^+ and K^+ levels are extremely selective for it. However, due to an excess of Na^+ in the soil because of salinity stress, it may also promote increased Na^+ absorption. HKT1 is a high-affinity K^+transporter that initiates at a very low level of exogenous K^+. It was first classified as an H^+/K^+ and Na^+/K^+ substitutability. When Na^+ levels in the soil are within normal limits, K^+ transport is stimulated. In many plants, the high-affinity potassium transporter (HKT) protein was the first potassium-specific carrier involved in K^+/Na^+ absorption (Schachtman and Schroeder 1994). Its major function is to keep Na^+ out of plant leaves and to help maintain K^+ homeostasis. $HKT_{2:1}$ has been shown to confer salt resistance in tomato (Ali et al. 2021). The tonoplast contains the Na^+/H^+ transporter, which is involved in the outward migration of Na^+ from the cytosol to the apoplast or vacuole. It is, however, an energy-intensive operation for cells, and proton pumps provide force for transferring Na^+ in the opposite direction of the electrochemical gradient (Blumwald 2000). Its positive effect in cotton has been found to impart tolerance by rejecting greater Na^+ concentrations and favoring a larger H^+ input. In

the plasma membrane, the salt excessively sensitive pathway (SOS) also functions as an exchanger. Its activation under salt stress also prevents increased Na^+ concentrations from causing plants to become salinity tolerant. The role of transporters in onion has not been studied to combat with salinity stress. Contrarily, it has been thoroughly studied in other vegetables (Wang et al. 2019, 2020).

7.6 Modern Plant Breeding Approaches in Onion

Development of climate resilient onion cultivars in modern era is crucial, and in this section, we discuss some potential approaches, which can play important role in onion stress-resilient cultivars development. Marker-assisted breeding has played a huge role in vegetable and crop development in recent history, since the development of first genetic map of onion (King et al. 1998; Van Heusden et al. 2000), a number of molecular studies are done and markers associated with valuable traits are identified, which further enabled plant breeders to incorporate desired traits into vegetables through different approaches. Moreover, identification of codominant markers including SSRs and SNPs and their affordable sequencing cost has made their identification easier than before (Fischer and Bachmann 2000; Jakše et al. 2003; Havey and Ghavami 2018), affordable sequencing of markers also leads to the development of genetic maps (Duangjit et al. 2013; Damon et al. 2014). The genetic improvement of onion will benefit not only against climate changes but genetic markers associated with desired traits in onion will also help in onion improvement, moreover, the constraints of long growing time of onion and problems with its harvesting and storage can also be minimized by using these approaches (Havey 2018). Markers linked to desirable traits in onion may also help in the identification of superior plants prior to their full growth in green house condition, so at early stages of plant growth they can be selected and then used in crossing procedure, which can further be used in the selection of desired plant types.

In vitro plant propagation approaches are useful tools in vegetable breeding, in the recent past, successful callus culture and micropropagation of onion and its relatives were reported (Luthar and Bohanec 1999). However, these approaches primarily depend on their cost effectiveness and suitability. First successful genetic transformation in onion plant was reported by Dommisse et al. 1990. Moreover, onion was also transformed with herbicide resistance gene (Eady et al. 2008) but due to commercial and consumer constraints these products were not commercialized, furthermore, these products had also high regulatory costs. Recently identified gene-editing technologies such as CRISPR/cas system may help in the addition of desired traits in inbred onion lines. Furthermore, new NGS technologies to understand population genetics and devise genomic scans are also way move forward for resilient cultivars development (Matz 2018). In onion, GBS method has also been implied to develop reference-free genetic maps, moreover, application of pool sequencing for population genetic analysis has also great scope in onion cultivars development (Schlötterer et al. 2014). Recent advancements in the next-generation sequencing technologies and many genomic resources in onion

have been developed, this might help in the improvement of economic traits (Finkers et al. 2015). Studies have reported that from onion gynogenic haploids were extracted from flowering buds on simple growth media (Martınez et al. 2000). One major constraint in onion haploid production is that this technique is inconsistent among onion germplasm (Geoffriau et al. 1997), furthermore, haploids may need chemical treatment to avoid unnecessary diploidy (Alan et al. 2007). Double haploids have been used in several vegetable cultivars development including eggplant, melon, pepper, rapeseed, rice, tobacco, triticale, and wheat. Moreover, haploids and double haploids have also implication in the genetic studies, marker-assisted studies, gene mapping, QTLs, and identification of targets for transformation (Murovec and Bohanec 2011). QTL mapping in addition to tracing a gene for resistance to assist in understanding the foundation of genetic resistance of each QTL and their associated expression regarding plant defense, they emerged as potent tool for plant breeders and entomologist to grasp genetic basis for developing insect resistance in germplasm (Yencho et al. 1996). The Mi gene has been mapped to confer aphid resistance in potato (Rossi et al. 1998). Moreover, resistance to corn earworm was also unraveled by QTL mapping (Byrne et al. 1996). Likely by exploiting QTL mapping, sorghum midge resistance was achieved (Tao et al. 2003). Leaf epicuticular waxes amount is beneficial for insect resistance in numerous crops (Kunst and Samuels 2003). It is efficient against onion thrips control. Two main pathways are involved in the production of epicuticular waxes: conversion of fatty acid into alcohols and esters via acyl reduction pathway and the second includes synthesis of ketones and alchols via decarbonylation pathway (Millar et al. 1999). Naturally, the amount of epicuticular waxes varies in onion (Damon et al. 2014), with the intermediate amount that it depicted resistance to onion thrips (Diaz-Montano et al. 2012), therefore, QTLs controlling the amount of these waxes were mapped in onion to develop thrips-resistant onion cultivars (Damon and Havey 2014). Endogenous defense strategies are the plant ability to cope with pest attack by secreting defense molecules including the production of primary and secondary metabolites that play direct and indirect roles in plant defense (Kessler and Baldwin 2002). Insects bite plant and antimetabolite starts to alter their digestive system with changes in lectins, chitinase, and biotin-binding protein (Vandenborre et al. 2011; Wang et al. 2005). Lectin causes alterations in epithelial mid-gut cells resulting in inhibition of absorption of nutrients in insects (McCafferty et al. 2008). Furthermore, lectins are toxic for insects which were observed to negatively influence the performance of numerous insects of different orders (Coleoptera, Lepidoptera, Hemiptera, and Diptera). Lectin's role against legume pod borer successfully effected its development and delayed with higher mortality rate (Machuka et al. 1999). Transgenic plants have been developed to overexpress protein to work as antimetabolite to combat with pests (Wang et al. 2005). Plant protein proteinase works against wounding response which is a pivotal defensive compound (Green and Ryan 1972). Protein proteinase inhibitor gene expression was upregulated by genetic engineering exhibited higher level of insect resistance observed in transgenic tobacco (Ren and Lu 2006). Molecular pathways involved in biotic and abiotic resistances need to be explored for onion to protect them from devastating

production losses. From literature it is evident that these modern approaches are in practice for various important agricultural crops and fruitful results have been achieved by employing these techniques, however, in the case of onion these gaps need to be filled for its protection from stresses.

7.7 Potential of Smart Agriculture in Onion Production

Sustainable growth of vegetables including onion depends on the adequate availability of nutrients and water in the soil. In the modern era, climate change poses a major threat in inadequate availability of water and suitable temperature. Now, techniques including the use of temperature and moisture sensors are implemented to cope climate change and sustainably develop plants (Nandurkar et al. 2014). Similarly, for appropriate vegetables growth including onion, systems and software are being developed to control and measure water quantities by using microcontroller-based gateways. This system can be connected to cellular internet and can communicate among farmers using photovoltaic panels so that they can schedule the irrigation timings via online system (Gutiérrez et al. 2013). Furthermore, there is potential of the use of Wireless Sensor Networks in greenhouses for onion growth, for their timely control of temperature humidity and other parameters (Devi and Kumari 2013). Irrigation systems which are controlled by remote sensing networks are helpful in those areas which are hit by drought or face water scarcity, this may help in real-time field sensing, site-specific irrigation, and variable rate irrigation, and this system may ensure the adequate and minimal use of water by the plants (Kim et al. 2008). Global positioning system (GPS) is another useful system that helps in the collection of field data with the help of sensors and collects it in one main database from where necessary actions can be taken if needed. This system is suitable for precision agriculture, and it is also a low-cost wireless system (Kim et al. 2008). Moreover, soil sensors can be used for communication protocols which may help in better lifetime of soil monitoring system. This system also known as universal asynchronous receiver transmitter (UART) is helpful in several ways but its major drawback is its high-cost management and when sensors are placed under the soil, it might cause disturbance to radio frequency signals (Wang et al. 2010). In onion production, all mentioned modern techniques can be implied for its growth in such areas where there is water scarcity and other abiotic or abiotic stresses. These techniques are continuously evolving and have potential to be used to cope against climate change.

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Advances in Summer Squash (*Cucurbita pepo* L.) Molecular Breeding Strategies

8

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Abstract

Summer squash (*Cucurbita pepo* L.) is a self-pollinated crop belonging to Cucurbitaceae. It is an annual crop that is grownup in tropical and subtropical areas. It is one of the most vital and economical vegetables in cultivation. Globally, squash is used as food and medicine for the presence of vitamins and antioxidants. *C. pepo* plants vary in shape, color and size, with several varieties and landraces. There are many types of *Cucurbita* species with diverse genomes and chromosome numbers. The number of summer squash chromosomes (2n) ranges from 40 to 48. Squash harbors a great diversity dependent on ploidy, regional and morphological characteristics. The introduction of new alleles

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_8

through the crossing of different genetic resources for the *C. pepo,* for example, crossing common genotypes with locally developed genotypes with those developed locally increases genetic diversity and the preselection of characteristics of interest. A fair natural variability in the degree of phenotypes must be ensured. The main targets of squash breeders and geneticists are to enhance various desirable morphological characteristics, including tolerance to biotic and abiotic stress and crop characteristics. Achieving these targets can be comforted by using modern genomics methods to enhance the traditional breeding program. This chapter provides an outline of the shortcomings of the summer squash origin and historical background, botanical description, economic and health importance, photochemistry, cultivation requirements, biodiversity and conservation of germplasm, cytogenetics, aims and stages of squash breeding programs and traditional approaches of breeding. Additionally, it discusses new plant breeding methods involving marker-assisted breeding and genetic engineering.

Keywords

Biodiversity · Biotechnology · *Cucurbita pepo* · Genetic improvement · Genetic map · Modern breeding · Summer squash (*Cucurbita pepo* L.) · Traditional breeding

8.1 Introduction

Summer squash (*Cucurbita pepo* L.) is an essential component of a balanced and safe diet. It contains large quantities of vitamins, antioxidants and other ingredients which avoid disease and promote healthier living standards. Summer squash is the edible green fruit of the Cucurbitaceae family of gourds, a very diverse genus of *C. pepo*. It becomes ready for harvest when it is shiny. Typically, the recommended size varies from 100 to 200 grams. This size is often reached between 2–5 days after anthesis depending on growing conditions. They keep growing and start losing their shininess unless the fruits are harvested on time. Usually, oversized fruit is unsaleable. Summer squash is a short-season crop, simple to grow and ideal for tropical and subtropical areas, with some species extending to the temperate zone (Ittah and Kwon-Ndung 2019; Ndoro et al. 2007; Subrahmanyam 2004). According to the last taxonomical treatment, cucurbits belong to Cucurbitaceae and contain about 118 genera and 825 species (Jeffrey 1990). Cucurbits are found in both the new and old worlds and are amongst the essential plant families that produce edible foods and beneficial fibers to humans. Cucurbits are composed of five subfamilies, i.e., *Fevilleae*, *Melothrieae*, *Cucurbitaceae*, *Sicyoideae* and *Cyclanthereae*. *Cucurbita*, *Cucumis*, *Citrullus*, *Lagenaria* and *Luffa* were the most important cultivated genera in the subfamily Cucurbitaceae and *Sechium* of the Sicyoideae subfamily (Whitaker and Davis 1962).

The summer squash is *C. pepo* eatable undeveloped fruit that contains various species belonging to the Cucurbitaceae family. Squash is one of the easy and simple

crops to plant, has the yield of a short season and can grow in tropical and subtropical regions (Paris 1996). The *C. pepo* is considered one of the Middle East's most well-known vegetable crops and its common name is Kosa, well-known among Egyptians, who coined this (Abdein 2016). Summer squash belonging to the species *C. pepo* contains numerous varieties. These varieties show variations in their vegetative features, flowering times, yield and components traits. The fruits are also highly edible in length, diameter, form and color. Thus, squash breeders will find a great variation among all summer squash germplasm collections (Abdein 2016; Moon et al. 2019).

8.2 Origin and Distribution

Bailey (1929) coined a cucurbit term for cultivated species within the Cucurbitaceae family. In the Arabic language, the word Qaraayat is used to identify a Cucurbitaceae cultivated species. Many species of Cucurbitaceae have been domesticated in prehistoric times (Kochhar 1981). Cucurbits have been cultivated for centuries. Several recent cultivated types are unknown in the wild and their origin has been difficult to trace (Herklots 1972).

The cucumber is of Asian descent, first found in Nepal's Himalayan foothills. Cucumber remains dated from the third millennium BC in Eastern Iran. Early travelers took cucumber to Mediterranean countries 3000–4000 years ago, where the fruits were revered by the ancient Romans and Egyptians. Since the ninth century, the cucumber was known in France and was cultivated in the early days of the fourteenth century in Britain. Columbus introduced it to the New World, planting it in Haiti in 1494 and it was presumably transported to the USA afterward (Decker-Walters et al. 1999; Thompson and Kelly 1957; Wehner and Robinson 1991; Whitaker and Davis 1962).

Sinnott and Durham (1922) made the first attempt at a thorough genetic study of the cucurbits. Many botanists claim that two *Cucurbita* species, vegetable marrow *C. pepo* and squash *C. moschata* Duch are of American origin and pumpkin *C. maxima* are of Asian origin (Thompson and Kelly 1957). Archaeological evidence positions wild populations of *C. pepo* about 10,000 and 30,000 years ago in Mexico and the eastern USA, respectively (Decker 1988).

Summer squash is belonging to the *Cucurbita* genus and the Cucurbitaceae family (https://www.itis.gov). *Cucurbita pepo* L. subsp. ovifera (L.) D. S. Decker var. ovifera (L.) Harz; *Cucurbita pepo* L. subsp. pepo var. pepo; *Cucurbita pepo* L. var. montia duch; *Cucurbita pepo* L. var. patisson duch; *Cucurbita pepo* var. giraumontia Filov; *Cucurbita pepo* var. melopepo (L.) Harz; *Cucurbita pepo* var. styriaca Greb are all synonyms for *Cucurbita pepo*. Summer squash, vegetable marrow, field pumpkin, jack-o-lantern pumpkin, zucchini, cocozelle, citrouille, (*Cucurbita pepo* L.) is a flowering plant within the class Magnoliopsida, order Cucurbitales and family Cucurbitaceae (gourds, squashes, citrouilles, gourdes). Summer squash (*Cucurbita pepo* L.), vegetable marrow, field pumpkin, jack-o-lantern pumpkin, zucchini, cocozelle, citrouille is of the *Cucurbita*.

Kingdom: Plantae—Plantes, Planta, Vegetal, plants
Subkingdom: Viridiplantae—green plants
Superdivision: Embryophyta
Division: Tracheophyta—vascular plants, tracheophytes
Spermatophytina—spermatophytes, seed plants, phanérogames
Class: Magnoliopsida—Dicotyledons
Superorder: Rosanae
Order: Cucurbitales
Family: Cucurbitaceae—gourds, squashes, citrouilles, gourdes
Genus: *Cucurbita*L.—gourd
Species: Cucurbita pepo L.–vegetable marrow, field pumpkin, jack-o-lantern pumpkin, zucchini, cocozelle, citrouille
Subspecies Cucurbita pepo ssp. pepo L.
Summer squash

8.3 Botanical and Distribution

Cucurbits are annual herbaceous or perennials that take care of the roots and usually the wet vines. The plants grow either level along the ground or use rings to ascend. They rarely grow as trees, shrubs or hedges. The curls may render fanned or guided, produced at the base of the petiole. Four twisted strands were usually curling with a concrete surface. Take-off has changed from essential to a compound of palmate. Those get out that, grow a while later, are lobed even more comprehensively and extra-natural nectars are found now and again. The sprouts are unisexual, and the plants are male or female, to be hermaphroditic occasionally.

Numerous perpetual cultivars have tuberous roots or pachypodia and in an annual period, their herbaceous shoots kick the bucket and re-develop (Hemicryptophyte life frame). Pachypodia can reach the broadness of one meter (Olson 2003). There are also several shrub species within the family (*Acanthosicyos horridus* Welw. ex Hook. f., *Corallocarpus glomeruliflorus* (Deflers) Cogn. *Momordica Spinoza* (Gilg) Chiov and lianas up to 10 cm (Carlquist 1992) with woody and perennial stems. Neel et al. (2017) point out that *C. pepo* is a creep or climbing annual plant with five diagonal stems up to 15 m long. Branches outside the shallow root chain, grow from well-developed roots. The stems are rough and hairy at the nodes, branching between 6 to 24 cm long, and usually rooted. The plant has tendrils at 90°when inserting the rolled and branched leaf 1–6. Tendrils can be formed poorly on dense plants. The leaves are flat, alternating, wide oval to deltoid, generally cardioid, apical pointed, palm ally lobulated with 5–7 lobes, slightly dented, rough, palm-veined, 20–30 cm length and 10–35 cm wide leaves with 5–25 cm small leaves which are oval elliptical heart to suborbicular cordate, with or without white spots on the surface and have 3 to 5 rounded or obtuse lobes, apical lobes, middle lobes. Petioles that cordately support sub-orbicular-cordate, superficially with or without white spots and have 3–5 modified or unnatural, apiculate lobules, the focal lobules invited

Fig. 8.1 Differences of some new, fresh squash leaves. (Source: Photo by M. A. Abdein)

than level ones. Squashes are monoecious and have single, nectar-giving actinomorphic blooms. This species of flowers usually have three stamens. The female flowers have thick peduncles, 3 to 5 cm long. It is also elliptical to the curved, multilocular ovary, sepals which are rarely fallacious and have a larger corolla than the male, with flowers having three lobate stigmas (Fig. 8.1). Cucurbit fruits are distinct in shape, color and measure (Yadav et al. 2017). On 1 or 2 closes it may be oval, round and hollow, scalloped, globular, fusiform, straight or possibly decreasing to a twisted or straight neck. Also, fruits have different colors (white, yellow, light to dark green, nearly black, creamy and/or orange) with smooth or rough skin. The pulp also varies in color (white, yellow or orange) and thickness (Figs. 8.2 and 8.3). Different *C. pepo* characteristics are shown in Table 8.1.

8.4 Economic Importance, Uses and Health Benefits

As a vegetable crop squash has valuable economic importance. World pumpkin, squash and gourd production quantities are 27.45 million tons over a total area of approximately 20.78 million hectares. Pumpkins, squash, and gourds are produced worldwide commercially in many countries and are cultivated in most places as a vegetable (FAO 2018) (Fig. 8.4a). It is especially important in Asia, Europe, America and Africa as a commercial crop (FAO 2018) (Fig. 8.4b). China, India, Russia, Ukraine, USA, Egypt, Mexico, Malawi, Italy and Spain are the top ten pumpkins, squash and gourds producers worldwide in 2018 (FAO 2018) (Fig. 8.4c). The pumpkin, squash and gourd production are shown in (Table 8.2).

Fig. 8.2 Male and female flowers of *Cucurbita pepo*. (Source: Abdein 2016)

The Cucurbitaceae family includes pharmaceutically important plants. Cucurbits are a group of sound nourishments. Cucumbers are used as nourishment, significantly consuming fewer calories. It has 96% water, a little sugar and multiple calories figuratively speaking. By extension, it provides an incredible wellspring of nutrients A, K and C as well as full potassium. National cancer coordinated considered cucumbers as one of the pharmaceutical properties to have disease defense advantages. Cucurbits are the most important basic plant families which offer appropriate things and important filaments to individuals. Cucumbers are either rough or salted to exhaustion. Pickling may be a standard approach for sparing the cucumber for long times. It allows them to be available long after the creating regular season. Cucumbers are always soaking in brine, vinegar or some other flavor. The cucumber jam was obtained, just as it was instilled with special flavors (Perez Gutierrez 2016). This family has huge medicinal and pharmaceutical properties that are hostile to HIV, anxiolytic, pyretic, diarrhea-hostile, carminative, cancer-preventive, diabetic, antibacterial, diuretic, anthelmintic, tuberculosis-hostile and laxative. It is used as an abortive, diuretic and cardiotonic head to boot. They do tend to have strong properties soothing, antitussive, cytotoxic and expectorant (Saboo et al. 2013). Cucurbitaceae includes vegetables such as squashes, melons, cucumbers and luffas. Cucurbits shape a vitally important and huge group of vegetables produced in the subtropical and tropical nations. This family has many pharmaceutical and nutritional plants (Gill and Bali 2012). In standard and alloxan-diabetic rats (Kolawole et al. 2011), natural products of cucumber flowers function

Fig. 8.3 Various fresh fruits color and shape of *Cucurbita pepo*. (**a**) Various fresh fruits and (**b**) Different shapes of ripening fruits. (Source: Abdein 2016)

Table 8.1 Different summer squash *Cucurbita pepo* characteristics

Traits	Degree of traits
Cotyledon leaves shape	Oval, Wide circular rectangular, Circular rectangular narrow
Nature of plant growth	Short, Medium Stretch, Dispenser
Discharge	Exists, Does not exist
Degree of Discharge	Poor, Medium, Strong
Direction of petiole	Erect, Semi erect, Horizontal
Stem length cm	Short (>50), Medium (50–100), Long (<100)
The green color pigment of the vein	Light green, Medium, Dark green
The presence of the veins	Absent, Archaeological, Developing well
Number of plant leaves	Little (15–25), Average (25–35), Many (>35)
Leaves pin nation	Absent, Weak, Medium, Strong, Very strong
The green color pigment of the upper layer of leaves	Light green, Medium, Dark green
Leaf area (cm^2)	Small (>500), Medium (500–1000), Large (>1000)
Greenness of the petioles	Light green, Medium, Dark green
Length of petioles (cm)	Short (<30), Medium (30–50), Long (>50)
The thickness of petioles (mm)	Thin (<30), Medium (30–40), Thick (>50)
Cross-section of petiole	Polygon, circular
The presence of thorns (spines) on the petiole	Little, Average, Many
Length of masculine syphilis (cm)	Short (<25), Medium (25–30), Long (>30)
Color of the male flower	Yellow, Orange
Color of the female flower	Yellow, Orange
Fruit length cm (age combined)	Short (<10), Medium (10–15), Long (>15)
Fruit diameter cm (age combined)	Thin (>3), Medium (3–5), Wide (>5)
Fruit shape index (fruit length/fruit diameter)	Small (<0.5), Medium (0.5–1), Large (>1)
The presence of a neck with fruit	Absent, Exist
Young fruit color	White, Yellow, Creamy, Dark green, Light green; Green-striped
The shape of the cross-section of the young fruit	Lobular, Ribbed, Circular
The shape of the fruit	Cylindrical, Rectangular, Synthetic, Disc, Thumb, Spherical, Cardiac, Twisted Neck
Color of mature fruit	White,Yellow, Creamy, Dark green, Light green, Green, striped
Color density in mature fruit	Light, Medium—Bold
The length of the fruit is mature (cm)	Very short (<15), Short (15–20), Medium (20–25), Long (25–30), Very long (>30)
Fruit diameter cm (ripe fruit)	Small (<10), Medium (10–15), Wide (>15)
Fruit mature shape index (the length of mature fruit/diameter of mature fruit)	Small (<0.5), Medium (0.5–1), Large (>1)
Seed shape	Rectangular Rectangle, Rectangular, Rectangular Rectangle
Seed color	White, Yellow

Prepared by M. A. Abdein

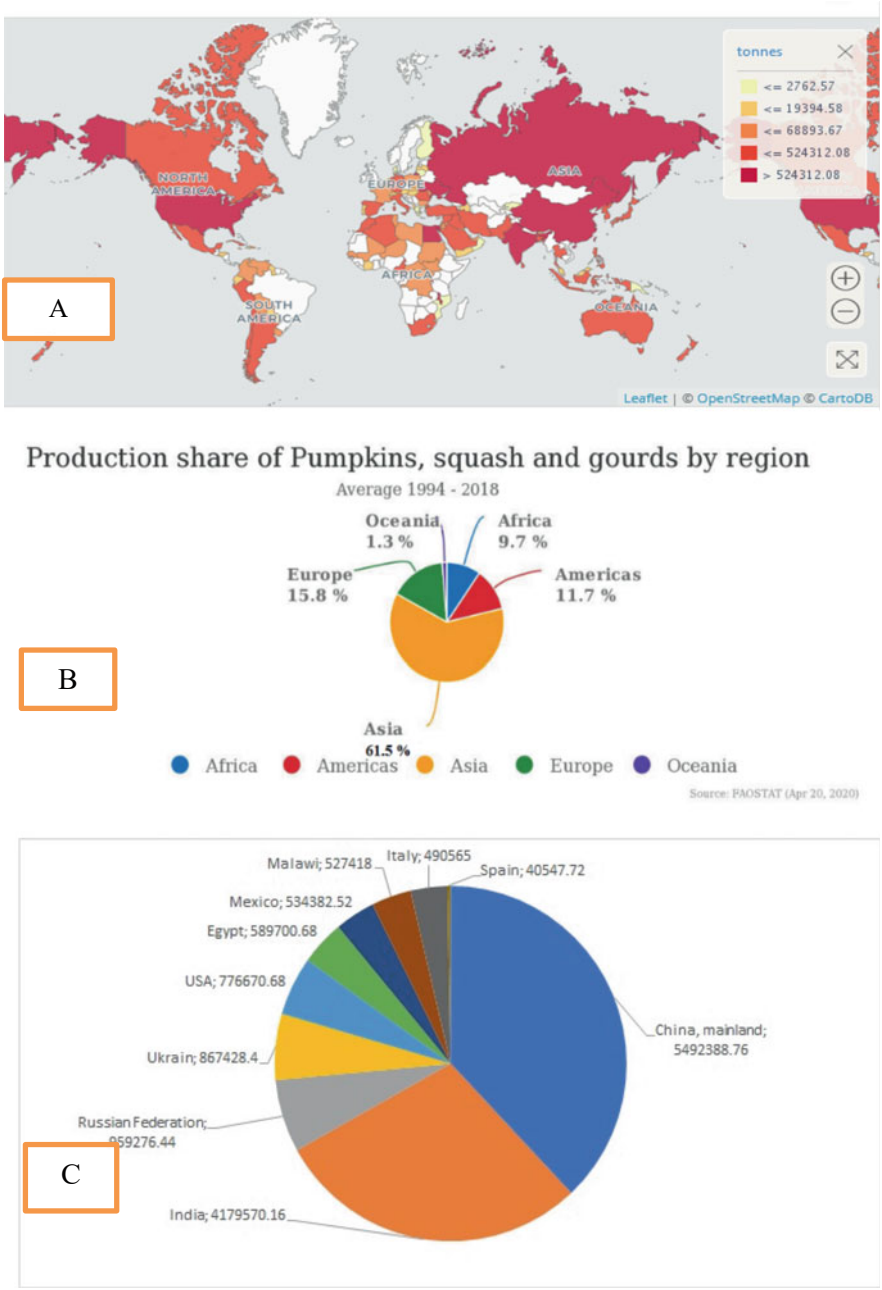

Fig. 8.4 Pumpkins, squash and gourds worldwide production. (**a**) Top ten producers of pumpkins, squash and gourds worldwide, (**b**) Map of World pumpkins, squash and gourds production and (**c**) Important region of pumpkins, squash and gourds. (Source: FAO 2018)

Table 8.2 World pumpkins, squash and area harvested, production and yield in 2017

Location	Area harvested (ha)	Production (tons)	Yield (hg/ha)
World	2,078,450	27,449,481	132,067
Americas	210,115	3,427,339	163,118
Asia	1,332,071	16,623,732	124,796
Europe	177,266	4,213,743	237,708
Africa	344,755	2,938,604	85,238
Australia and New Zealand	11,785	190,417	224,403

Source: FAO, FAOSTAT agricultural database. http://apps.fao.org 2017

as an antidiabetic specialist and can be utilized as an antiviral treatment (Puri et al. 2009). In addition, it has antimicrobial, cell reinforcement (Leelaprakash et al. 2011) and anti-tumor activities on human nasopharyngeal carcinoma cells in vitro and in vivo (Fang et al. 2011). Cucurbit plants have been successfully used to treat different diseases as traditional, homegrown cures. They also demonstrated exercises that are anti-inflammatory, anti-tumor, hepatoprotective, cardiac and immunoregulatory. In common, these family individuals are well off the protein source, with various natural works being performed as antibacterial, antiviral, antifungal, antitumor, antidiabetic and anti-aids. Various bioactive compounds are found in cucurbits such as triterpenes, cucurbitacin, sterols and alkaloids (Ajuru and Nmom 2017).

Cucurbita pepo is used in many nations as an anti-inflammatory, antiviral, analgesic, antiulcer, antidiabetic and antioxidant, for example, to treat a few diseases. *C. pepo* is known for its natural product and seeds used for consumption. It has white seeds embedded within the shell. These seeds are chewable and have a slightly sweet taste. The benefit of handling squash seed comes from its stimulating effect on dislocation of the bladder and sphincter. Its seeds have been shown to infringe mononuclear blood cells in vitro by the immunosuppressive movement (Winkler et al. 2005). Furthermore, as antibacterial agents (Obi et al. 2009). Gill and Bali (2012) reported that seeds of *C. pepo* have antiulcer and antioxidant activities due to the tetracyclic and triterpenoids (cucurbitacin) material. Uses of different parts of cucurbits were illustrated in Table 8.3 and Fig. 8.5.

8.5 Photochemistry

Squash is one of the most critical health sources as it includes many diverse bioactive elements, for example, polysaccharides, corrosive para-aminobenzoic, settled oils, sterols, proteins and peptides (Caili et al. 2006; Murkovic et al. 2002; Salehi et al. 2019). *C. pepo* seeds have squalene concentrations of 583.2–747 mg/100 g. Squalene can be a triterpene produced by humans, plants and animals. In their biosynthesis inside the human body, it could be a predecessor of steroid hormones, cholesterol and vitamin D. Squalene also has significant effects on various cancer treatment

Table 8.3 Squash parts and common uses

Parts	Traditional uses	References
Fruit	Cooling and astringent to the bowels, cure fatigue and thirst	Acosta-Patino et al. (2001)
	Increases appetite, cures leprosy and purifies the blood	Saboo et al. (2013)
Seed	Cure sore chests, hemoptysis, bronchitis and fever	Winkler et al. (2005)
	Biochemical immune mechanisms are caused by interferons, hyperplasia of the prostate and antioxidant capacity	Abdel-Rahman (2006)
	Oil from squash seeds has long been used to alleviate challenges related to an expanded prostate organ and touchy bladder-related micturition issues	Williams et al. (2006) Obi et al. (2009)
	Treatment decreases the side effects of micturition but does not decrease the increased prostate organ volume. It has antioxidant movement (inhibitory movement against lipid peroxidation and free radical rummaging), the androgenic activity of the insects, immunological movement, antiviral movement, cardiovascular movement, anti-inflammatory activity, and hepatoprotective motion	Gill and Bali (2012)
	Often, it triggers inhibition of prostate hyperplasia caused by testosterone	Müller and Bracher (2015)
Leave	Reduced fever, nausea medication and a boost to blood hemoglobin content	Ratnam (2017)

Prepared by A. A. Ibrahim

Fig. 8.5 Fresh *Cucurbita pepo* seeds from mature fruit. (Source: Photo by M. A. Abdein)

types (Martha and Gutierrez 2016). The chemical components of the different parts of the squash are shown in (Table 8.4).

Table 8.4 Plant part and chemical composition in squash

Plant part	Chemical composition	References
Fruit	Low fat content (2.3%), carbohydrate content (66%), protein content (3%), high carotenoid content 171.9 μg/g	Elinge et al. (2012)
Seed	Natural source of phytosterols, proteins, polyunsaturated fatty acids, antioxidant vitamins, tocopherols, carotenoids and other elements	Phillips et al. (2005)
	Fatty corrosive components such as palmitic (10.68%), palmitoleic (0.58%), stearic (8.67%) oleic (38.42%) linoleic (39.84%), linolenic (0.68%), gadoleic (1.14%), add up to immersed greasy acids (19.35%) and add up to unsaturated greasy acids (80.65%). Approximately 50% corrosive oil (generally linoleic and oleic), with the most active compounds being Δ7 sterols (avenasterol, spinasterol) and Δ5 sterols (sitosterol, stigmasterol). Triterpenoids, sesquiterpenoids, squalene, and tocopherols (α tocopherol) are also added. Elements include phosphorus, potassium, magnesium, calcium, iron, zinc and trace elements)	Glew et al. (2006); Sabudak (2007)
	Fiber, protein, β-carotene, carbohydrates, minerals and greasy acids were reported in the coat, tissue, seeds and fatty seeds, and triglyceride greasy corrosive blend, tetrahydrothiophene, linoleic corrosive, cholesterol and 13(18)-Oleanen-3-ol. Vitamins (thiamine, riboflavin, niacin, pyridoxine and pantothenic acid), phenolic glycosides and lignan	Stevenson et al. (2007); Ukiya et al. (2002)
Leave	Secondary bioactive compounds such as alkaloids, flavonoids, carbohydrates, phytosterols, tannins, saponins, steroids, gums, mucilage, fixed oils and fats, proteins and amino acids	Kalaiselvi and Selvi (2016)

Prepared by D. M. Hikal

8.6 Nutritional and Genetic Studies

Squash fruit and its peels contain moderate amounts of mineral salts as nutritional compounds used to feed humans. Because of its high content of fibers and vitamins, it can be eaten or cocked as an immature fruit or consumed as a good source of fats and proteins from mature seed.

In a new study, Hikal and Abdein (2018) used the dried rinds of some squash genotypes as antioxidants when applied to the treated cake (Fig. 8.6). It can be eaten as an immature fruit due to its high amount of fibers and vitamin content or used from fully grown seeds as a good resource of fats and proteins. They used four Egyptian and exotic squash varieties Escandarani (Egyptian), Siyah Kabuk (Turkey), Erbil Garden (Iraq) and Zucchino Alberallo Di Sarzana (Italy). The sugar content from the cake and organoleptic properties have been reported under the effect of various peel additives. According to 4 × 4 half mating crosses, different

Fig. 8.6 Samples of cake treated with squash peels for several genotypes. (**a**) Control cake, (**b**) Cake treated with the squash parent 3 peels (10/250 g and 5/250 g) from left to right, (**c**) Cake treated with squash parent 4 peels (10/250 g and 5/250 g) from left to right, (**d**) cake treated with squash parent 2 peels (10/250 g and 5/250 g) from left to right and (**e**) cake treated with squash parent 1 peel (10/250 g and 5/250 g) from left to right. (Source: Hikal and Abdein 2018)

cultivars were also coated to obtain 6 F_1 crosses. The results showed that different pumpkin skins improved their acceptability and sugar content. On the other hand, the amounts of hybrid activity showed the great importance of all the traits studied compared to the intermediate parents. Estimates of the strength of the hybrid versus the best kinship were very important.

8.7 Cultivation and Conventional Breeding

8.7.1 Recent Cultivation Procedures of the Summer Squash

Depending on the relations between the genotype (the distinctive features of the crop), the environment (climate and soil conditions), management, crop growth and yield can be improved. In warm weather, cucurbits had to be developed to increase the best production. It can also grow in different types of soil, in well-fertilized soil, giving maximum yield. Suitable soil pH ranged from 6 to 6.5. It needs well irrigation for seed germination, with a continuous water supply (Napier 2009). For the preeminent reasonable seed germination, the soil cannot be colder than 16 °C and must be between 18.3 °C and 24 °C. Soil temperatures from 18.3 °C to 29.4 °C, are idealized for the most exceptional growth of the plants. Squash is one of the most sensitive vegetables to rainfall and rainfall deficiency and over-irrigation (OECD 2016).

8.7.2 Current Agricultural Challenges

Squash is manually harvested. It is regularly planted in the soil in glasshouse conditions utilizing a drip irrigation technique in the spring, summer, and fall. The physical properties are fundamental in optimizing the productivity of squash seeds for different variables, sifting skills, and moving pneumatics. The bulk thickness of squash seed and part of the various dampness ranges changed individually from 350–475 to 406–460 kg/m^3. Simultaneously, the porosity increased from 22.2 to 24.0% as the dampness grew significantly from 6.46 to 52.9%. The burst quality of the dampness material was excessively subordinate (Rouphael and Colla 2009).

Generally, seeds had to be fragile at high humidity content and consequently, cracking required less restriction. Higher temperatures and sun-powered radiation from spring to summer and less concentrated fertilizer arrangements should be used to keep the average electrical conductivity (EC) increasing to the required degree to avoid a delivery drop. In expansion, our consideration has appeared that in the summer to fall season both water system frameworks can be adopted using supplement arrangement, composition 1.8 deci-Siemens per meter (dS/m), but under certain water systems, higher natural product quality (dry matter and carbohydrates) and a decrease in component concentration in the arrangement in the course of the growth process leading to the rearrangement of the closed circle supplement solution administration (Bahlgerdi 2014).

To induce the most noteworthy natural product to abdicate in the summer squash established in field conditions, the impacts of irrigation water quantity, plant water utilization and interim water system are fundamentally critical. The interim water system and the coefficient of skillet essentially influenced the natural product to give up summer squash. Summer squash is delicate too and may be harmed from seed sowing to growing over the topsoil water. Since the depth of the establishment of summer squash is relatively shallow, soil water needs to be kept up to more than 65%

of the soil water capacity that can be reached to bypass and avoid water shortage. (Mario et al. 1997).

With the sub-water subsystem, the EC of the growth medium rises significantly quicker than with the drip irrigation frameworks. Fewer concentrated fertilizer arrangements should be used to keep the EC of the growth medium at the specified point to expect a decrease in *Cucurbita* surrender. Seed quality for squash edit generation is influenced by several indoor and natural components during seed progression (Araujo et al. 1982). The surrender and quality of squash seeds can be a move to sufficient dispersal of populace plants and the amount of dust that affects the quality of pollination and fertilization (Lima et al. 2003).

8.7.3 Tolerance for Environmental Stress

Summer squash is moderately sensitive to salinity stress (Blaylock 1994). The rootstocks consumption has increased offspring vigor via soil pathogen resistance and the resilience of *C. pepo* to moo soil temperatures or salted (Ruiz et al. 1997). The usage of rootstock is an effective approach to reduce sodium toxicity by increasing salt tolerance. Sevengor (2010) developed salt-tolerant genotypic variations among local squash and pumpkin genotypes in Turkey (Balkaya and Kandemir 2015). In many regions of Turkey, winter squash and pumpkin can grow on non-irrigated soil. Winter squash and pumpkin growth can also be considered an acceptable solution to the salinity or drought problem (Kurtar et al. 2016; Sevengor et al. 2011). Winter squash and pumpkins which can be cultivated without irrigation are a strong choice in arid and semiarid ecosystems for the soils with salinity problems. Many researchers are working on the abiotic stress tolerance of *C. pepo* (Table 8.5).

8.7.4 Genetic Improvement Objectives

Varieties of squash had a broad range of differences. Vegetative qualities and early yield are the most important traits in squash. Abdein (2016) analyzed seven yield and quality characteristics for *C. pepo*, as shown in Table 8.6. For all called characteristics, the heterosis versus mid-parents tended to be very significant values. The heterosis over mid-parents tended to be highly noteworthy values. For most of

Table 8.5 Abiotic stress tolerances of *Cucurbita pepo*

Tolerance type	References
Pollen water stress	Gay et al. (1987)
Drought and salt stress associated with mycorrhiza	Harris-Valle and Esqueda (2011)
Salt stress	Balkaya et al. (2016)
Low-temperature	Carvajal et al. (2018)

Prepared by A. A. Ibrahim

Table 8.6 The mean performances for yield and its components traits of seven squash varieties

Parents	Fruit length (cm)	Fruit diameter (cm)	Fruit shape index	Total soluble solid % (%)	Fruit weight (g)	Number of fruits per plant	Fruit yield per plant (g)
Parent 1	12.93	33.13	4.24	3.33	117.6	223.4	2.73
Parent 2	4.73 L	7.77 H	0.61 L	5.63 H	130.1	115.9	2.11 L
Parent 3	10.93	2.63 L	4.15	3.53	93.9 L	224.8	2.33
Parent 4	13.07 H	2.83	4.61 H	4.37	98.9	30.2 H	2.87
Parent 5	11.67	3.07	3.81	3.37	107.7	26.9	2.81
Parent 6	8.63	6.53	1.32	2.43 L	134.9 H	15.1 L	2.23
Parent 7	12.47	2.93	4.26	3.57	112.7	28.2	3.15 H

Where: H = The highest value L = The lowest value. Source: Abdein (2016)

the traits tested, the estimation of heterosis over the superior parent appeared of profound importance. None of the crosses showed the most extreme heterogeneity for all the characteristics. Still, for the different traits, the critical and alluring degree of heterosis over mid-parents and superior parents was found in a few crosses.

8.8 Germplasm Diversity and Conservation

8.8.1 Plant Germplasm Conservation

Germplasm conservation is a highly important and attainable technique for studying and genomic conservation of the most valuable crops. Germplasm contains entire genes of this species and can preserve this information for a long time which can be used in the future. To conserve genetic information for new species, endangered species and unidentified species, germplasm has been put in the genebank. Gene libraries or genebanks can assist in vitro in evaluating germplasm genetic variation (transgene) and in selecting the best germplasm (Reitsma et al. 2014).

For the first generations, genetic diversity may be influenced by the loss of genetic information, so germplasm is used as a success factor to retain genetic information. Genetic diversity may be lost due to climate change variability, hybrid use and enhanced landraces. Genebanks have a crucial role in the perseverance of landraces and alleles and can be used for the genetic conservation of valuable and economic crops afterward. The conservation of germplasm includes cultivated plants, commercial genotypes or landraces and breeding lines (Byrne et al. 2018; Cruz-Cruz et al. 2013).

8.8.2 In-situ Conservation

Squash genetic resources are the natural genetic materials for squash breeding and a source of continued growth in production, biotic resistance, abiotic tolerance, yield and its components and quality traits improvement. Genetic erosion has been recorded in the crib regions of yield domestication, where the loss of traditional cultivars parallels the specialization and intensification with which new high-yielding varieties are introduced and disseminated (FAO 1996; Hammer and Teklu 2008). In situ preservation represents one form of preservation of crop genetic resources, which means conserving genetic resources in natural environments (Brush 1995; Nabhan 1985; Maxted et al. 2013). Maintaining the genetic diversity of crops in farmers' fields, in in-situ, is important to preserve crop breeding gains and provide the possibility of further development in the future. The genetic conservation of species using natural individuals in biosphere reserves such as national parks as conserve props in their natural habitat (Spataro and Negri 2013). Research models on the potential effect of climate change on pumpkin allocation indicate that all pumpkin distributions are expected to decline dramatically over the next 60 years. (Lira et al. 2009).

8.8.3 Ex-situ Preservation

Preservation of genetic information for critical species (endangered species, rare species, and national crops outside its natural habitats. This conservation can be preserved species by using different methods such as in vitro seeds and stored in the genebank for a long time till use (Engelmann 2004). Large collections of *cucurbits are preserved* in Genebanks worldwide (Bolivia, Brazil, Colombia, Costa Rica, Czech Republic, Italy, Mexico, Portugal, Russia, Spain, Turkey and USA) (Clark et al. 1991; Diez et al. 2002; Ferriol and Pico 2008; Karlova 2008; Lebeda et al. 2007; Nuez et al. 2000). Restricted funding for Genebank activities may limit the restoration efforts required to make collections accessible to breeders for distribution. A huge amount of ex-situ *Cucurbita* germplasm was collected and maintained across the globe in various agricultural centers. These centers and the international consortium for squash genetic resources in Europe, the USA, Africa, Asia and Australia. A significant proportion of *Cucurbita* is kept in the U.S. national plant germplasm network (https://npgsweb.ars-grin.gov/gringlobal/search.aspx).

8.8.4 Cryopreservation

The cells or tissues used in this process are stored in a frozen state. That occurs by using very low temperatures, using solid CO_2 (−79 °C). Usage also of nitrogen vapor (−150 °C) and liquid nitrogen vapor (−196 °C) (Kartha and Engelmann 1994). This process involves multiple stages such as freezing, thawing and re-culturing. This freezing temperature can preserve the cells in inactive form so

Table 8.7 Listing of valuable genebanks for squash genetic resources

Genebank	Country	Website
Institute of Crop Science, Chinese Academy of Agricultural Sciences	China	http://www.cgris.net/photobase/default.html
Czech Republic Gene Bank	Czech Republic	https://grinczech.vurv.cz/gringlobal/search.aspx
Leibniz Institut für Pflanzengenetik und Kulturpflanzen forschung (IPK)	Germany	https://www.ipk-gatersleben.de/en/gbisipk-gaterslebendegbis-i/
Leibniz-Institut für Pflanzengenetik und Kulturpflanzen forschung (IPK)	Europe	https://eurisco.ipk-gatersleben.de/apex/f?p=103:4:::NO:RP:P4_NATIONAL_INVENTORY:3
Nordic Genetic Resource Centre, NordGen	Sweden	https://sesto.nordgen.org/sesto/index.php?thm=sesto
USA Plant Germplasm Introduction and Testing Research Station, Pullman	USA	https://npgsweb.ars-grin.gov/gringlobal/search.aspx

that they can be saved for a long time (Jang et al. 2017; Kartha 1981). The cryopreservation method can be used as embryos, meristems, ovules and seeds on any tissue from the crop. Specific compounds such as dimethyl sulfoxide, glycerol, ethylene, propylene, sucrose, mannose and glucose can be used to protect the cells from damage (Kaviani 2011). Table 8.7 shows a list of the most significant gene banks in the world for preserving the genetic resources of squash.

8.8.5 Cytogenetics

Basic chromosome number in genus in the genus *Cucurbita* (2n = 2x = 40) (Domblides et al. 2018; Gong et al. 2013). Whitaker and Flory (1955) and Koxyxob (1925) identified several 2n chromosomes 2n = 40 and 2n = 44–48, respectively. Therefore, there has been a misunderstanding of the exact number of 2n chromosomes in such varieties until now. Functional genome regions, variation in structure as an inversion and translocation between genome regions can be revealed by similarities and differentiation between the genomes of different *Cucurbita* (Chaney et al. 2016; Morrell et al. 2012). The chromosome size is very small (Fig. 8.7). The numbers of chromosomes of different C. pepo cultivars vary according to heredity. The morphology of chromosomes is undifferentiated where, in addition to the chromosome, a long arm or short arm of individual chromosomes may not be identified as small spots from each other, so it is difficult to estimate karyotype (Weeden 1984; Weeden and Robinson 1990).

Flow cytometry provided strong knowledge about the *C. pepo* haploid genome was about 500 million base pairs long (Arumuganathan and Earle 1991). The nucleus of the vegetative cell *C. pepo* (2n) contains about 1.04–1.08 pg of DNA. Because of the morphological characteristics of this crop, several molecular markers have been utilized to study the genetic map (Havey et al. 1998). To monitor the

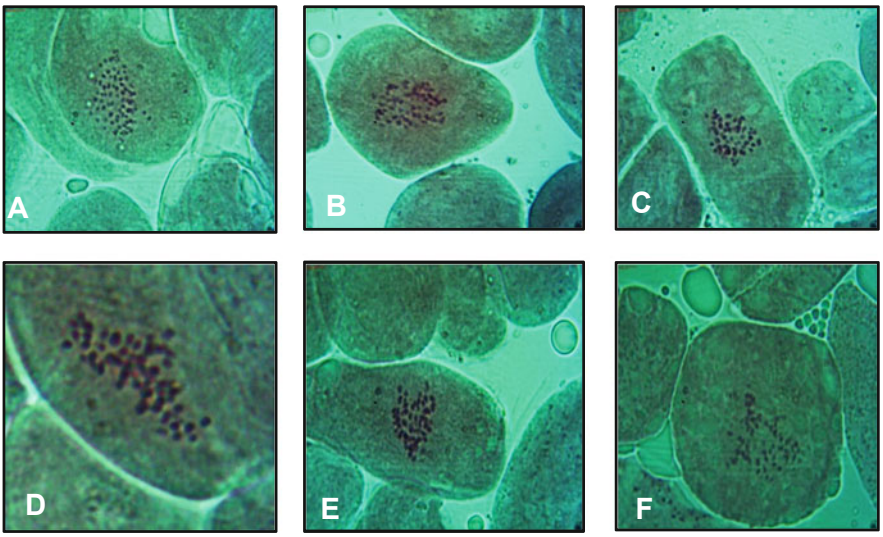

Fig. 8.7 Chromosome number of various *Cucurbita pepo* cultivars. (**a–c)** Chromosome number of *C. pepo* (Eskandarani) and (**d–f**) Chromosome number of *C. pepo* (Coppi). (Source: Photo by A. A. Ibrahim)

genetic variability, self-pollination for four consecutive generations of *C. pepo* inbreeding depression was performed. The self-pollination ratio indicated a range from 0.16 to 0.54, and this result varies by character, year and condition (Hayes et al. 2005). Whitaker and Robinson (1986) suggested that the response of different types of cucurbits to inbreeding could reflect these different types of pollination. Variations in *C. pepo* gene sequences resulted in variations in phenotypes that assisted in successful landrace selection. Gene and allele-specific markers are related rather than random markers to the appropriate genes.

8.9 Traditional Breeding of Summer Squash

Inter-species hybridization is utilized to enhance yields by imparting certain attributes from their wild relatives. (Bowley and Taylor 1987). In the *Cucurbita* genus, there have been a few challenges in granting interspecific crossbreeds between five developed species (*C. maxima, C. pepo*, *C. moschata*, *C. ficifolia* and C. *argyrosperma*) and some wild species. The success of hybridization is dependent on species within this genus. Curtis (1940) recorded that the summer squash F_1 has been observed to produce female flowers 10 days earlier than either parent. He added that more female flowers were produced by F_1 than either parent.

Hutchins and Croston (1941) also found that winter squash *C. maxima*, the first female flower in F_1 opened much earlier than their parents.

Fig. 8.8 Four squash *Cucurbita pepo* varieties. Eskandarani (Parent 1), Zucchino Mezza Lung Bianco (Parent 2), White Bush Scallop (Parent 3) and Zucchino Nano Verde di Milano (Parent 4). (Source: Abdein 2005)

Table 8.8 The parent genotypes and their origins

No.	Parent name	Origin
1	Eskandarani	Egypt
2	Zucca Patisson custard white	France
3	All Green Bush	United Kingdom (UK)
4	Courgette Orelia	Germany
5	Sakiz	Turkey
6	Copi	Egypt
7	Gapla	Syria

Source: Abdein (2016)

Abdein (2005) has studied the nature of heterosis and evaluated the genetic behavior of certain quantitative squash traits, demonstrating the genetic materials utilized to study genetic diversity along with the different genotypes of squash. Abd El-Hadi et al. (2014) and Abdein (2016) considered the species to have a place in the genetic materials of seven squash lineages. The following parent lines are Eskandarani (P1), Zucca Patisson custard white (P2), All Green Bush (P3), Courgette Orelia (P4), Sakiz (P5), Copi (P6) and Gapla (P7) (Fig. 8.8). Seeds of these lines were taken from diverse origins, as shown in Table 8.8. Heterosis for some squash economic traits was estimated (Abdein 2016; Abd El-Hadi et al. 2005; Al-Araby 2010; Al-Ballat 2008). For both yield and its components traits, they observed that heterosis was detected only over the mid-parents.

8.9.1 Inbreeding Depression and Selection

Three generations of inbreeding with selection were used to improve the summer squash variety Eskandarani. Self-pollination can be utilized in reproductive species of the genus *Cucurbita*, although the plants interbreed, there is almost no loss of durability due to inbreeding. (Allard 1971; Robinson 2008; Whitaker and Robinson 1986). Selection is a methodology that allows again in quantitative traits inheritance, resulting in a new population than the original, as much as on the average performance of the best individuals (Fehr 1987).

Abd El-Al and Khalaf-Allah (1973) perused inbreeding depression (ID) in the summer squash variety Eskandarani. They noted that early yield inbreeding depression and the number of fruits per plant appeared in the F_2 generations. They perused the deviation of F_2 generations against F_1 hybrids and reported the inbreeding depression manifestation for stem length in F_2 generations.

Borghi et al. (1973) studied inbreeding depression of the anthesis day of the earliest female flower. Results confirmed that inbreeding affects *Cucurbita* species.

In another study, Schuster et al. (1974) recorded that the total fruit yield decreased by 96% relative to open-pollinated plants after 11 generations of self-pollinated in the variety Giessener. In this sense, Hassanein et al. (1975) noted that local variations in summer squash differed in their response to inbreeding concerning stem length and the number of plant leaves. They clarified that no detectable deviations between summer squash inbred lines and their ancestral parents regarding yield per plant.

El-Gazar (1981) recorded significant inbreeding depression with values of 39.27, 84.03 and 83.45% for fruits number per plot, fruit weight for each story, and fruit weight per plant, respectively. Metwally (1985) recorded highly significant inbreeding depression for total yield in squash. The results showed that ID values for each plot's number of fruits and fruit weight per plot were 17.7 and 19.7%, respectively. He registered various ID values as follows −7.0, −1.41, −2.31 and −3.61% of the nodes for the first female flower, date of birth of the first male flower, and the first date of forming a female flower and the number of male flowers per plant, respectively. In their investigation of *C. pepo*, El-Diasty and Kash (1989) stated that the ID values were 11.91, 37.71 and 9.91% for the leaves number, the male flowers number and the stem length, respectively.

Within 10 years in *C. Pepo*, El-Gendy (1999) determined ID values for plant length, number of plant leaves, first female flowering nodes' number, number of male flowers per plant and time of the first female flower as follows 0.162, −17.11, 1.98, −7.24 and 6.73%, respectively for F_2 generations. She estimated ID in squash and recorded that the estimates were as follows −21.49, 20.97, −1.71, −2.49 and −3.98% for the plant fruits number' weight, the fruit length, the fruit thickness and the fruit shape index.

Abd El-Hadi et al. (2004) and Gabr (2003) suggested that most studied traits of ID (F_1, F_2), ID (F_{1r}, F_{2r}) and ID ($F_{1,1r}$, $F_{2,\ 2r}$) had inbreeding depression (Fig. 8.9). For those traits which had large estimates of heterosis, the estimated values were greater. The performances of all F_2 generations ($F_{2,\ 2r}$) were better than the parental varieties not only for vegetative traits but also for yield and its components traits. Mohan et al.

Fig. 8.9 Inbreeding depression in squash F_2 generations (P_3 ♀ × P_4 ♂). (Source: Gabr 2003)

(2012) and Xanthopoulou et al. (2019) found significance in all crosses for most of the vegetative traits in ash gourd. Concerning earliness traits, all crosses showed significant inbreeding depression for all traits except the number of days to first flowering in crosses 2 and 3 and the days are taken for first fruit production in cross two.

8.9.2 Heritability

El-Gazar (1981) found that the narrow-sense heritability was 53.91, 42.50, 31.59 and − 32.92% for stem height, the number of plant leaves, the number of male flowers and the number of first female flowering nodes, respectively. Also, the broad-sense heritability was 48.06, 67.37, 94.75 and 10.44% for the same traits, respectively. In squash, El-Gazar and Zaghloul (1983) found that heritability values were moderate and ranged from 50 to 63% for the node position of the first female flower. Metwally (1985) recorded high heritability values in a broad sense for the number of the plant's male flowers and the number of days to the first female flowering.

Abd El-Maksoud (1986) investigated 20 F_1 hybrids including five varieties of summer squash. In both wide and narrow sense values, he calculated the heritability for virgin traits like the number of nodes for the first female flower, the number of each plot's fruits in the first seven selections and the fruits' weight per plot in the first seven picks were 59.94, 97.4 and 45.85%. El-Shawaf et al. (1986) used a 4 × 4

partial diallel cross to test heritability for yield and its constituents. They concluded that heritability estimations in the wide-ranging sense (h^2_b%) were above average for all traits except yield and its components traits in the limited sense (h^2_n%).

Metwally et al. (1988) used partial diallel crosses (5 × 5) to estimate heritability in the seed yield and its associated characteristics in the summer squash. They found that d that heritability assessments in a narrow sense were mild for seed index and mean length of ripe fruit of44.3% and 35.0%, respectively. El-Diasty and Kash (1989) found a heritability of a narrow-sense ranging from 15.59 to 31.58% for the plant fruits' number and the fruit weight.

Dahiya et al. (1990) revealed that plant height showed high heritability values. The maximum rate in the broad sense was 97.64% for the fruit diameter, while it was 87.93% in the narrow sense.

Abd El-Hadi et al. (2004, 2005) found that heritability in the wide-ranging sense (h^2_b%) was more significant than the narrow sense heritability (h^2_n%). For the first picking date (1stPD), the approximate heritability values in a limited context (h^2_n%) ranged from 0.00 to 60.38%.

Al-Jebory (2008) used a co-mating project of six female strains × two male lines to produce 12 first-generation hybrids. To determine heritability, all genotypes of vegetative traits were evaluated r. Fayeun et al. (2012) recorded a high predicted genetic gain in vine height, leaf's fresh weight, vine weight, the leaves number and viable yield. Broad-sense heritability for vegetative and qualitative characteristics was examined in crossbreeds from fruit-type groups in *C. pepo* and *C. moschata* (Darrudi et al. 2018; Davoodi et al. 2016).

8.9.3 Genotypic and Phenotypic Correlation

Total marketable fruit weight per plant was significantly correlated with the total fruit weight. The recurrence dissemination polygons for an open-pollinated summer squash called Eskandarani recommended the major gene(s) with a calculable natural impact that controls the blossom sex expression (Brewbaker 1964; Mohamed 1996). Latent qualities appeared to have an impact on the propensity to gentility, number of fruits per plant and earliness (El-Tahawy 2007).

Abd El-Maksoud et al. (2003) and Abdein (2005) found that most sets of traits showed a negative genetic and phenotypic correlation. However, the following pairs of features exhibited positive correlation coefficients such as sex percentage, times to the first female flower head, prematurely yield in terms of weight and number of fruits in 7 harvests per plant. They also added that a breeding program system to improve one or more of these characteristics must enhance the others. Abdel Sayyed et al. (2003), in sweet melon, assessed correlations between the fruit's quality characteristics. They found flesh texture to be negatively associated with flesh flavor and aroma, while positive genotype associations between fleshy flavors were observed. Al-Jebory (2006) studied genotypic, phenotypic and environmental correlations for several characters of summer squash. Sanín et al. (2015) used

open-pollinated introductions to study the genetic correlation, relationship and path analysis for the squash yield and fruit components of diallel crosses.

8.10 Molecular Breeding

8.10.1 SDS-PAGE Electrophoresis

Choudhary and Ram (2000) described 65 strains of melon germplasm using a seed protein extract. A total of 15 seed protein bands that were supplied into four different zones A, B, C and D could be resolved. Banding patterns in C and D zones were not polymorphic.

Using a single seed, Singh and Ram (2001) suggested that SDS-PAGE has examined seed storage protein of the cucumber genotypes under reduced conditions. Seed proteins consisting of 19 germplasm genotypes were expressed in a total of 17 domains divided into 3 regions (A, B and C), A region containing six bands, region B had seven bands and region C had four bands. The 19 lines can be divided into eight groups, based on protein profiles. Therefore, some lines of germplasm may be differentiated on protein profile bases.

Abd El-Salam et al. (2010) tested five snake cucumber genotypes. The four genotypes showed band patterns differing. The variety Ismailia 2 provided the highest number of six bands, and the minimum number of three bands was present in the Fayoom 5 and Kalyoubia varieties. On squash, El-Shamy (2009) showed protein pattern variation and content of stable and cucumber mosaic virus (CMV) symptoms infected squash leaves using SDS-PAGE.

Al-Tamimi and Attyaf (2014) applied SDS-PAGE techniques to fingerprint seven squash genotypes. SDS electrophoresis pattern of total seed protein demonstrated low polymorphism due to four monomorphic bands from six bands produced. SDS electrophoresis technique produced 33.3%, polymorphic bands.

Abdein (2016) discriminated against biochemical fingerprints using the protein electrophoresis technique for squash leaves. Protein electrophoresis successfully produced reproducible polymorphic bandsThe profiles produced showed high polymorphism among studied parents.

8.10.2 Molecular Marker-assisted Breeding

Molecular markers of DNA such as restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs) and inter simple sequence repeats (ISSRs) have already been evaluated for diversity within *C. pepo* (Dalda-Şekerci et al. 2020; Esteras et al. 2011; Lebeda et al. 2007). Much of the opinions focused on determining the genetic and developmental relations between the wild–tamed species, between the two subspecies and between the cultivar bunch, counting as many European landrace agents (Ferriol et al. 2003; Paris et al. 2003).

Despite the growing economic importance of squash varieties for seed production, no information is available on population structure within and between accessions with modified seed coats and the range of inbreeding found in those accessions is not available. Earlier genomic studies were based primarily on intra-species diversity within the genus *Cucurbita* or on genetic diversity between subspecies *C. Pepo*, focusing on certain groups of cultivars (Ferriol et al. 2003; Formisano et al. 2012; Gong et al. 2008, 2012; Paris et al. 2003).

Using random amplified polymorphic DNA (RAPD) markers to analyze genetic variants in cucurbits has been widespread and more effective (Cuevas-Marrero and Wessel 2008; Ntulia et al. 2015). Previously, genetic variation within *C. pepo* was evaluated using allozyme and various DNA marker systems and inter-simple sequence repeats (ISSRs) (Dalda-Şekerci et al. 2020; Esteras et al. 2011; Lebeda et al. 2007). It is widely known that, at the level of plant genomes, using DNA-based markers is very effective in assessing genetic diversity and identifying plant accessions. Inter simple sequence repeats (ISSR) markers are easy-to-use, PCR. Polymorphic random amplified polymorphic DNA (RAPD) is used to verify unique genetic marks and differences in a wide variety of plants such as the genus *Cucurbita* (Heikal and Hadia 2008). Mohamed et al. (2018) explored squash landraces that compete with RAPD markers to further assess genetic relations and similarity among squash landraces, established and assessed these genetic constitutions under Egyptian conditions in an exposed field experiment and finally stated that these molecular markers can be applied to classify squash landraces in molecular breeding. RAPD markers are thus valuable for assessing genetic variation within and between squash species.

El-Adl et al. (2012) used 21 RAPD primers, which produced high polymorphism levels among the seven cultivars of summer squash. Heikal and Hadia (2008) examined two PCR molecular marker strategies, arbitrary opening of polymorphic DNA (RAPD) and connecting basic grouping rehashes (ISSR) to clarify the polymorphisms and the connections between 14 landraces with three distinctive *Cucurbita* species (*C. pepo*, *C. moschata* and *C. maxima*).

Using various molecular markers, for example, RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), SRAP (sequence-related amplified polymorphism), ISSR (inter simple sequence repeat) and AFLP (amplified fragment length polymorphism), molecular markers were embraced to consider an inherited variety in and among a few species of *Cucurbita* (Baranek et al. 2000; Dalda-Şekerci et al. 2020; Gwanama et al. 2000; Katzir et al. 1998, 2000; Montes-Hernandez and Eguiarte 2002; Ramon et al. 1991).

Michael et al. (2019) used 39 simple repeats of sequences (SSRs) to explain *C. pepo* genetic relationship among resistance accessions. Some researchers have utilized various molecular markers to study genetic variation among *C. pepo* (Table 8.9).

Table 8.9 Different DNA markers used in the genetic diversity of *Cucurbita pepo*

Molecular markers	No of primers	References
Simple sequence repeats (*SSRs*)	27	Formisano et al. (2012)
	18	Murovec (2015)
	10	Ntulia et al. (2015)
Random amplified polymorphic DNA (RAPD)	9	Ntulia et al. (2015)
	7	Tsivelikas et al. (2009)
	6	Heikal and Hadia (2008)
	5	Khalil and Hassan (2013)
	20	Santos et al. (2012)
	5	Mohamed et al. (2018)
Inter Simple Sequence Repeat (ISSR)	7	Heikal and Hadia (2008)
	5	Khalil and Hassan (2013)
	5	Abdein (2018)
	15	Santos et al. (2012)
	6	Xanthopoulou et al. (2015)
Start Codon Targeted (SCoT)	6	Abdein (2018)
	7	Xanthopoulou et al. (2015)
Single-nucleotide polymorphism (SNP)	10	Xanthopoulou et al. (2019)

8.10.3 Genomic Resources

Genomic resources include numerous tools such as molecular markers, genotyping-by-sequencing (GBD), and genome-wide association studies (GWAS) that prioritize the establishment of single nucleotide polymorphism (SNP) that monitors economic traits. *C. pepo* was analyzed using the simple sequence repeats (SSRs) molecular marker and confirmed that *C. pepo* had the greatest genetic diversity in the Cucurbitaceae family. In *C. pepo*, genomic tools were improved using SNP to detect quantitative trait loci (QTLs). The *C. pepo* subsp. *pepo* genome morphotype was about 93% of the genome size and divided into 20 pseudo chromosomes. Xanthopoulou et al. (2019) recorded full *C. pepo* subsp. *pepo* and subsp. *ovifera* genome distinguished by various SNPs (Fig. 8.10).

Dense Dark genetic maps are important implements for effective molecular breeding. Several gene binding maps were generated in *Cucurbita*. The *Cucurbita* binding maps have been established in recent decades using both interspecific *C. pepo* × *C. moschata* and intraspecific crosses *C. pepo* ssp. *pepo* × ssp. *pepo* and *C. pepo* ssp. *pepo* × ssp. *ovifera* the related populations. Such maps were first constructed using randomly generated DNA amplification polymorphic (RAPD) dominant markers and amplification polymorphism (AFLP) (Brown and Myers 2002; Lee et al. 1995; Zraidi et al. 2007), and SSRs were completed (Gong et al. 2008, 2012). Zucchini and scallop genotypes were utilized to produce the *C. pepo* transcriptome and were earlier used as parents of the F_2 mapping group which was used to create the first SNP-based gene map and map of quantitative trait loci (QTLs), flower and fruit characteristics (Esteras et al. 2012).

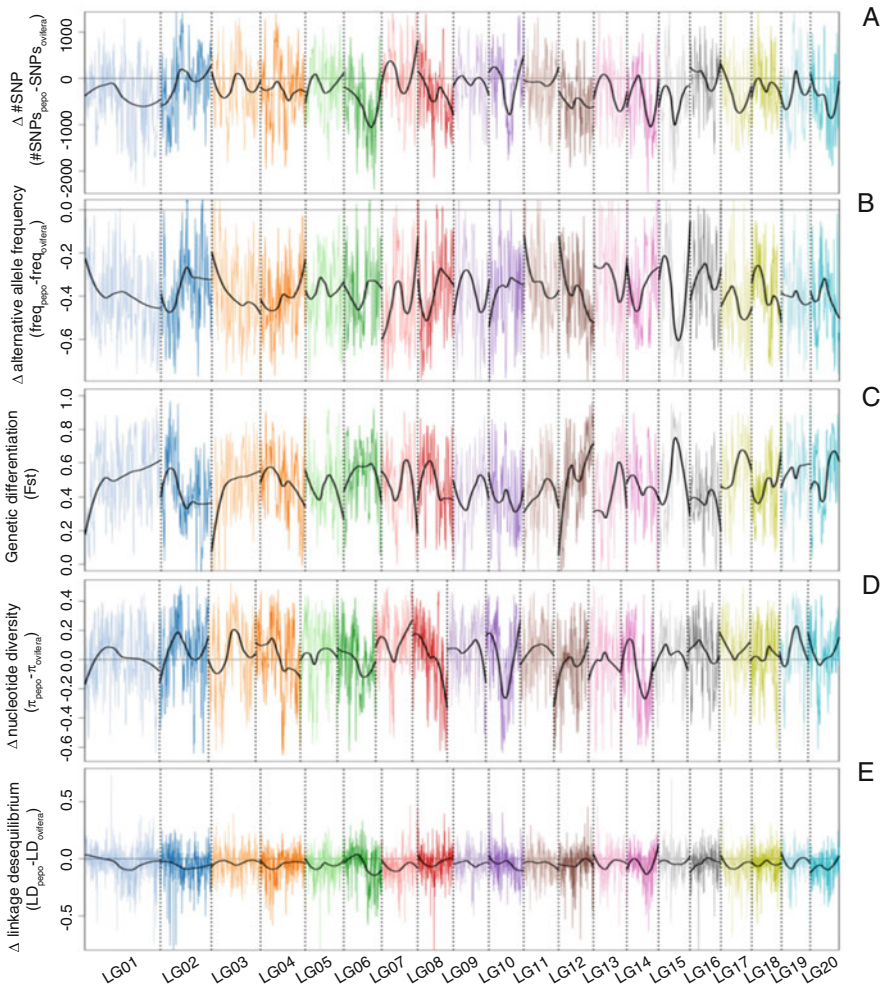

Fig. 8.10 Broad genome variations between *Cucurbita pepo* subsp. *pepo* and subsp. *ovifera*. (**a**) Variations in SNPs, (**b**) Frequency of the alternative allele (allele doses not present in the reference genome), (**c**) genetic differentiation, (**d**) nucleotide diversity and (**e**) linkage disequilibrium between *Cucurbita pepo* subsp. *pepo* and subsp. *ovifera* along the chromosomes. Vertical dashed lines indicate the end/beginning of a chromosome. The solid horizontal line marks the absence of differences. (Source: Xanthopoulou et al. 2019)

Esteras et al. (2012) formed a saturated gene map based on 315 high-quality markers (304 SNPs and 11 SSRs) from a *C. pepo* subsp. *pepo* var. Zucchini MU-CU-16 × *C. pepo* subsp. *ovifera* var. Scallop UPV-196 cross (Fig. 8.11). Also, Montero-Pau et al. (2017) constructed a saturated genetic map based on genotyping-by-sequencing from a Zucchini × Scallop (ssp. *pepo* × ssp. *ovifera*) inter-sub-specific cross. Several thousand SNP markers were detected and

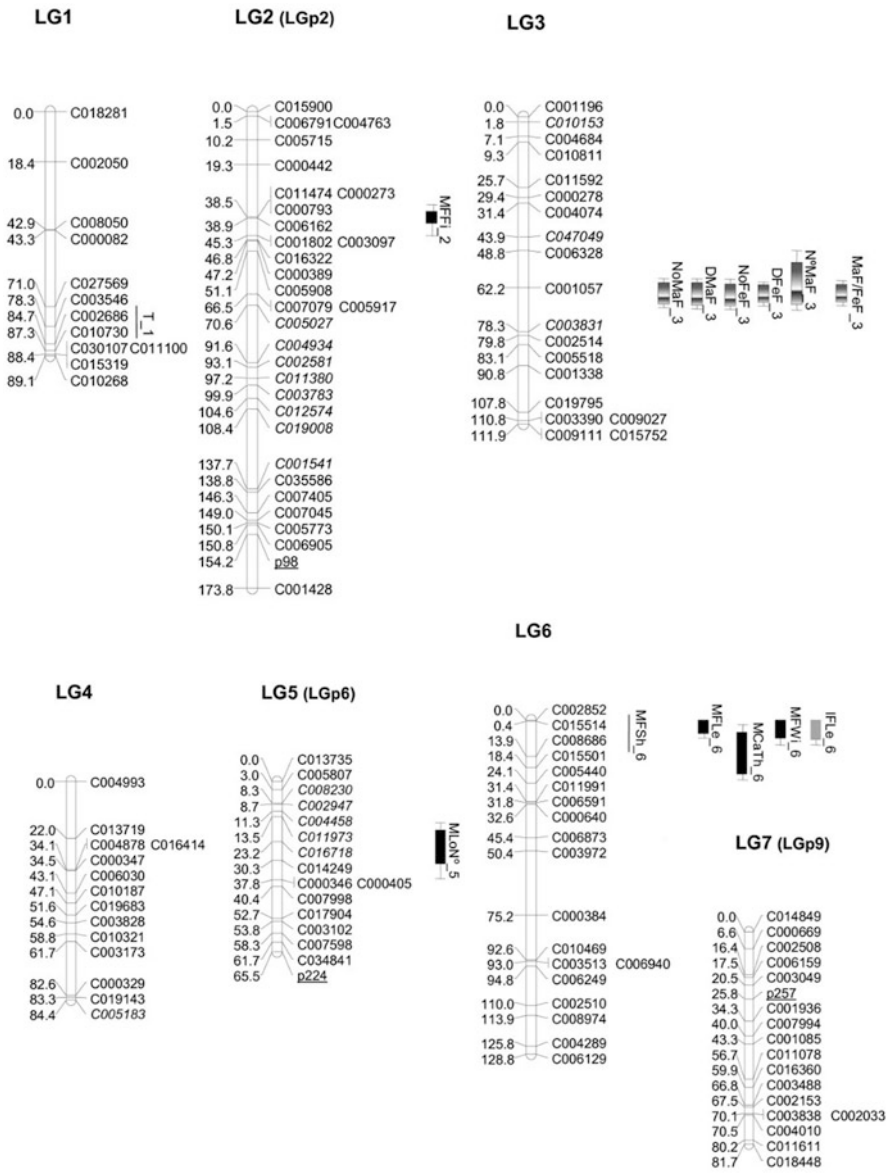

Fig. 8.11 Genetic map of Zucchini × Scallop F_2 population. Linkage map and locations of quantitative trait loci (QTL) whose impacts have been validated in the backcross populations linked with vine improvement, flowering and fruit quality based on 146 F_2 plants resulting from a Zucchini × Scallop cross. QTL indicated in light grey, grey or black correspond to flowering and immature mature fruit traits, respectively. (Source: Esteras et al. 2012)

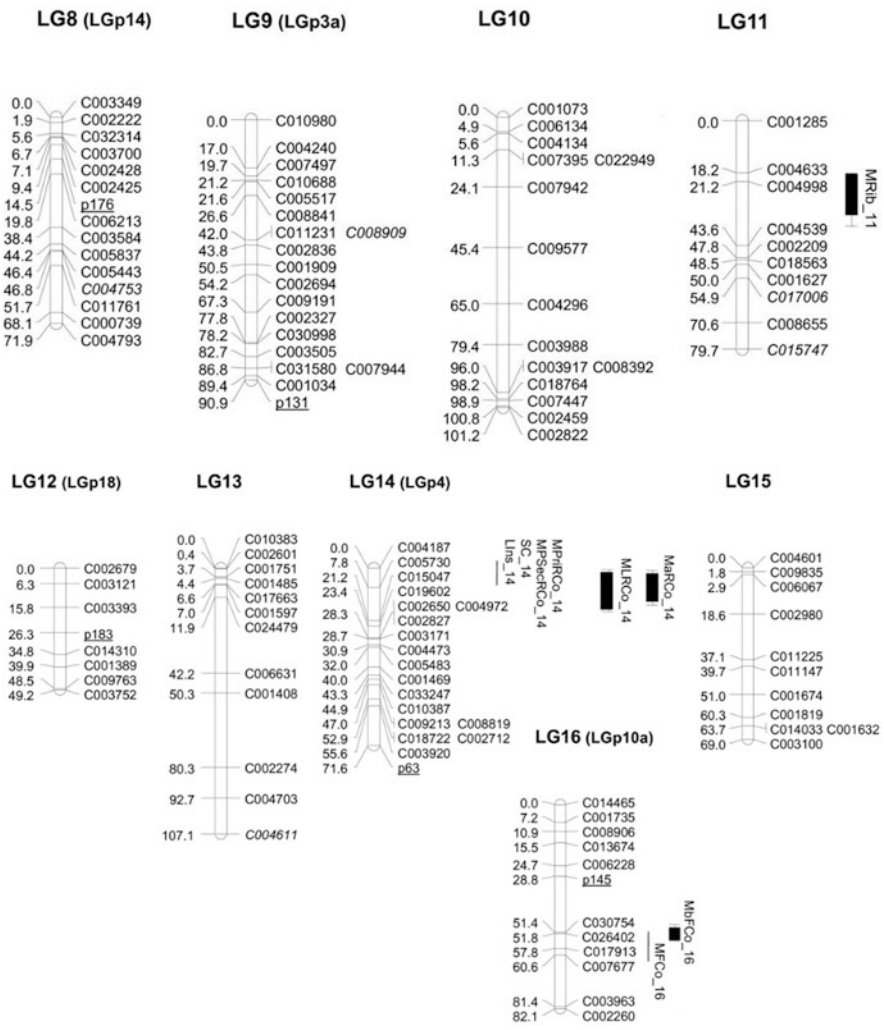

Fig. 8.11 (continued)

genotyped, supported by the creation of a high-density linkage map based on 7718 SNPs (averaging 386 markers per binding group) covering 2817.6 cm of the entire genome, which is a significant improvement compared to earlier maps. Especially different mapping populations were used to map Quantitative and qualitative characteristics (Esteras et al. 2012), Montero-Pau et al. 2017; Zhong et al. 2017), dwarf trait (Xiang and Duan 2018), resistance to tomato leaf curl New Delhi virus (Sáez et al. 2020), fruit and agronomic traits (Del Valle Echevarria et al. 2020) and seed traits (Wang et al. 2020). In *Cucurbita* spp. much QTL mapping was performed utilizing several types of DNA markers. Some QTL mapping for the various squash

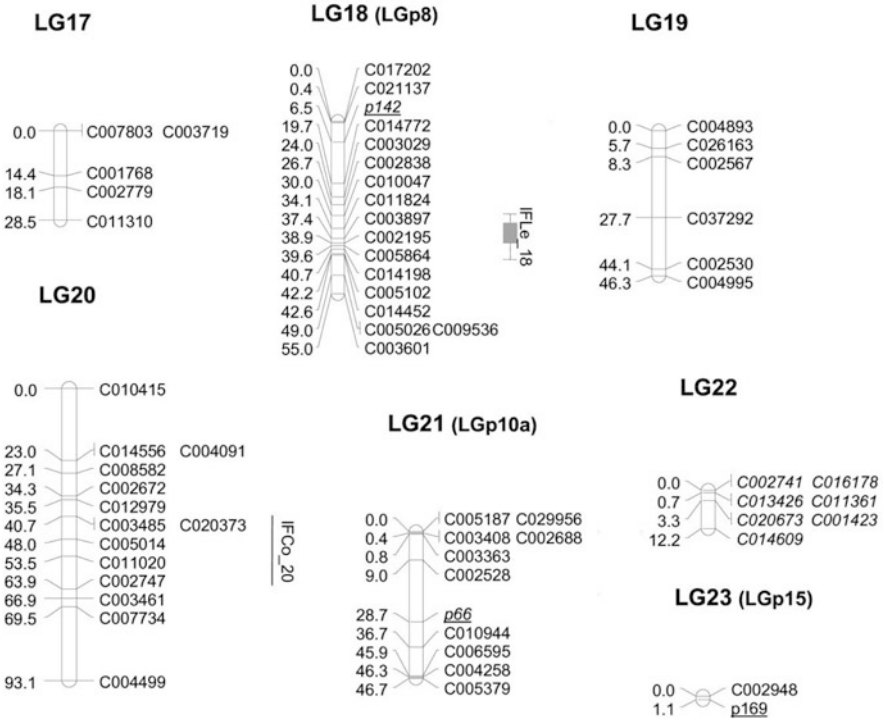

Fig. 8.11 (continued)

characteristics is shown in Table 8.10. Using QTL mapping s speeds up squash breeding as it makes it easier for the squash breeder to a) identify specific genes that control the quality, b) understand the impact of genes/QTLs that control traits, c) determine the gene/QTL location and d) screen of relationships between the diverse genes/QTL of attention. Each of these goals should improve the pyramid of various target genes into one genotype and reflect the variability of the examined germplasm.

8.11 Tissue Culture Applications

Advancing recovery engineering for in vitro culture is part of management development. In a few species of the cucurbit family, recovery by physical embryogenesis is limited (Debeaujon and Branchard 1993). Embryogenesis is the one that was a reliably established form of recovery for *C. pepo* (Chee 1991, 1992; Gonsalves et al. 1995; Jelaska 1972, 1974). Indirect organogenesis is a pathway to recovery, reported in summer squash *C. pepo* (Pal et al. 2007).

Induction of gynogenesis by culturing unfertilized ovary/ovule is an efficient way to speed up breeding programs by getting homozygous materials in a short period,

Table 8.10 Listing of various kinds of DNA markers identified for QTL in *Cucurbita* spp.

Trait	Mapping population	QTLs	Marker	Reference
Quantitative and qualitative traits	F_2 individual	59 QTL	Single nucleotide polymorphisms (SNPs)	Esteras et al. (2012)
Fruit-related traits	F_2 individual	29 QTLs	Single nucleotide polymorphisms (SNPs)	Zhong et al. (2017)
Fruit-related traits	Recombinant inbred lines (RILs)	48 QTLs	Genotyping-by-sequencing	Montero-Pau et al. (2017)
Fruit-Associated	Recombinant inbred lines (RILs)	QTLs	Simple-sequence repeats (SSRs), Single nucleotide polymorphisms (SNPs) and diversity array technology (DArT)	Kaźmińska et al. (2020)
Dwarf trait	F_2 individual	3 QTLs	Expressed sequence tags (EST-SSRs) and Simple-sequence repeats (SSRs)	Xiang and Duan (2018)
Resistance to tomato leaf curl New Delhi virus	F_2 individual	Major QTL	Single nucleotide polymorphisms (SNPs)	Sáez et al. (2020)
Fruit and agronomic traits	Backcross (BC1)	QTLs	Single nucleotide polymorphisms (SNPs)	Del Valle Echevarria et al. (2020)
Seed traits	F_2 individual	10 QTLs	Specific length amplified fragment (SLAF)	Wang et al. (2020)

Prepared by Prof. Dr. K.F.M. Salem

and significant advances have been made in recent years with the culture of cucurbitaceous crops (Min et al. 2016). Shalaby (2007) investigated the impact of genotype, female flowers' position on the stem of a plant, temperature and sucrose concentration on induction gynogenesis of squash in vitro and stated that genotype is the main factor affecting in vitro reproduction in squash.

The highest ratio of ovules reacting, and the number of plantlets per plate was found in the second female. Incubated ovules developed a better embryogenic response at 4 or 32 °C for 4 days. The significant result was obtained from the ovules in the MS culture medium of Murashige and Skoog (1962) containing 30 g/L of sucrose. Zou et al. (2020) observed that induction levels for both embryo-like structures (ELSs) and plantlets differed for different lines present in the same culture conditions, and various lines had various optimal initiation media. Genotype and initiation media may have important impacts on ELS initiation, but they performed a more significant role in their interaction.

Kathiravan et al. (2006) investigated the cultivation and propagation of *C. Pepo* in vitro through direct organogenesis from cotyledons. They noted that, in addition to the impact of ploidy in cucurbit populations, a close association exists between the

propagation of chimeric plants and seed measurements correlating with the medium composition.

Previously, plant regeneration of the *Cucurbita* was reported through direct and indirect organogenesis. The development of somatic embryos in six squash *C. pepo* cultivars was also recorded by Carol et al. (1995) using cotyledon segments. Similarly, cotyledons may be utilized for the regeneration of squashes *C. pepo* and *C. maxima* through direct organogenesis (Ananthakrishnan et al. 2003).

Kintzios et al. (2002) stated that the use of 2,4-dichlorophenoxyacetic acid (2,4-D) for 24–48 h in the treatment of *C. pepo* leaf improved plant regeneration and growth by 143% and proposed that this approach be used in the regeneration of *C. pepo* from embryos.

A few molecular markers play critical roles in the study of clonal dedication and among them, start codon targeted (SCoT) and randomly amplified polymorphic DNA (RAPD) markers were commonly applied (Rathore and Rai 2014). *C. pepo* embryogenic callus is done on media complemented with one of a few manufactured auxins (Jelaska 1974). Choosing adjusted sublimes from long-term squash societies which developed in an auxin-free medium was conceivable (Krsnik-Rasol et al. 1982). Figure 8.12 occurs in distinctive stages of *C. pepo* Egyptian cultivar Eskandarani tissue culture (Badawi et al. 2008).

8.12 Genetic Engineering and Gene Editing

Genetic engineering is an effective method used to recombine DNA technology in the improvement of new genotypes resistant to many diseases. Production of new genotypes had particular genes as a result of the combination of millions of crosses-translation of undesirable genes with beneficial genes through conventional breeding. Often, the loss of one desired gene through the rearrangement of parent genes during the crossing (Danida 2002).

Conventional breeding took place through exchanges between very closely related plant species and results from unwanted genes during gene transfer, and this process takes a long time to achieve the best result. The genetic engineering method is capable of transferring one or two genes, at least among related or distant organisms. Genetic engineering assists in definite time crop improvement by removing or switching undesirable genes (Lopez-Bucio et al. 2000). There are about 70 genes reported from *C. pepo*, 30 for *C. moschata* and 19 for *C. maxima* (Paris and Padley 2014).

The genetic transformation was used in *C. pepo* for virus-resistant (Clough and Hamm 1995; Collinge et al. 2010; Fuchs et al. 1998; Gaba et al. 2004; Tricoli et al. 2002). Genetically engineered zucchini and crookneck squash with resistance to cucumber mosaic virus, watermelon mosaic virus, and yellow zucchini mosaic virus has been widely commercially distributed in the United States (Fuchs and Gonsalves 2007). Resistances are originated from genes that code for the virus envelope proteins that are incorporated into the squash genome. Genes like these can be introduced into elite breeding lines and recombined with other attributes.

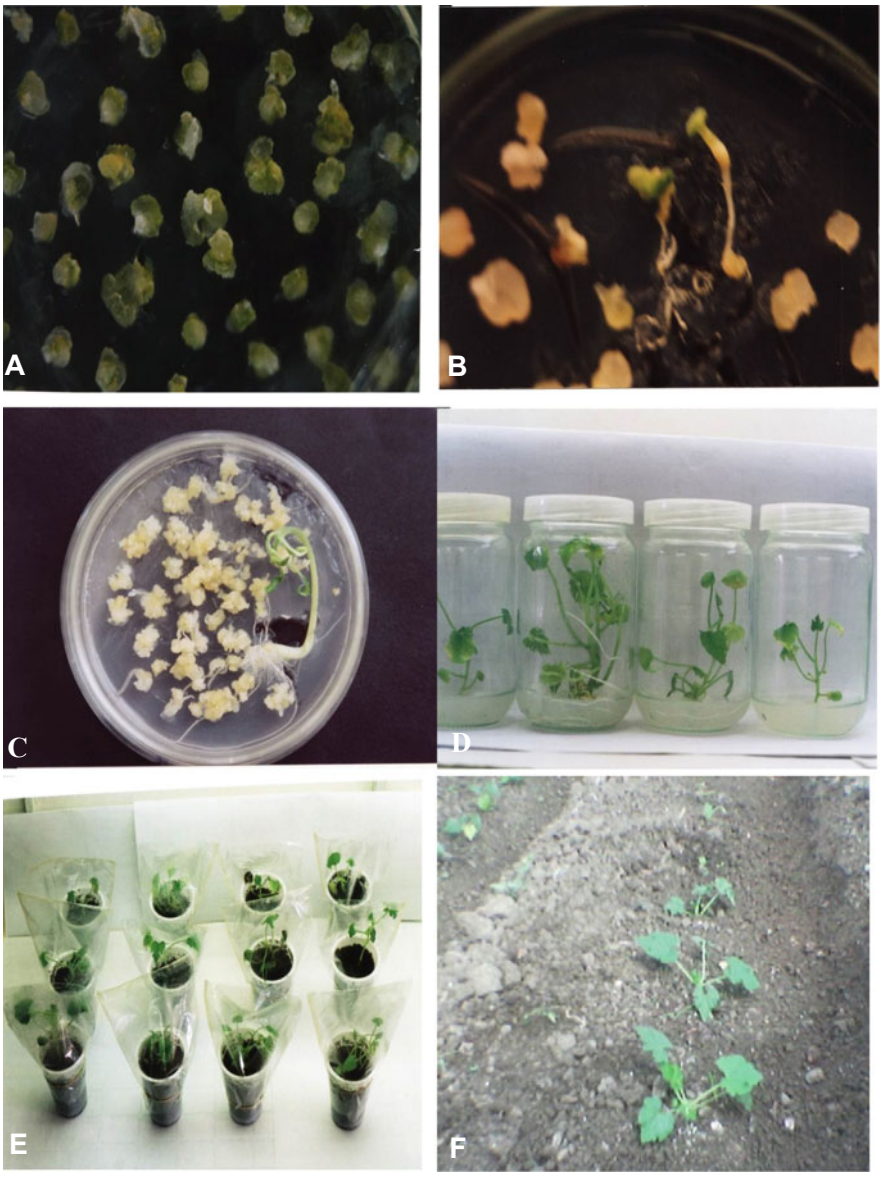

Fig. 8.12 Different phases of ovules culture in squash by tissue culture. (**a**–**c**) propagated plant from an ovule, (**d**) propagated plant with storage root, (**e**) propagated plant with a plastic bag and (**f**) plant transferred to soil. (Source: Badawi et al. 2008)

Very high rates of resistance to viruses have been found, in both artificial inoculation trials and the field (Fuchs et al. 1998). The appearance of recent viruses causing heavy losses on cucurbit are emerging every few years, genetic modification

techniques can provide an economically viable way to counter them if they are relatively cheap.

Recently, a new type of genome-editing technology has been established, namely the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) method. The CRISPR/Cas system theory was developed from a prokaryotic immune system adaptive to the type II organism (Jinek et al. 2012). They were first recognized as an unusual sequence feature in the *Escherichia coli* genome in 1987, comprising of a series of 29 nucleotide repeats separated by distinct 32 nucleotide spacer sequences (Ishino et al. 1987; Wiedenheft et al. 2012). Later, repeated sequences with similar interval repeat patterns were established in the genomes of bacteria and archaea, but the functions of these repeats were not clear until 2005 when three separate studies observed that the spacer sequences were identical to a portion of the virus and plasmid sequencing (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005) (Fig. 8.13).

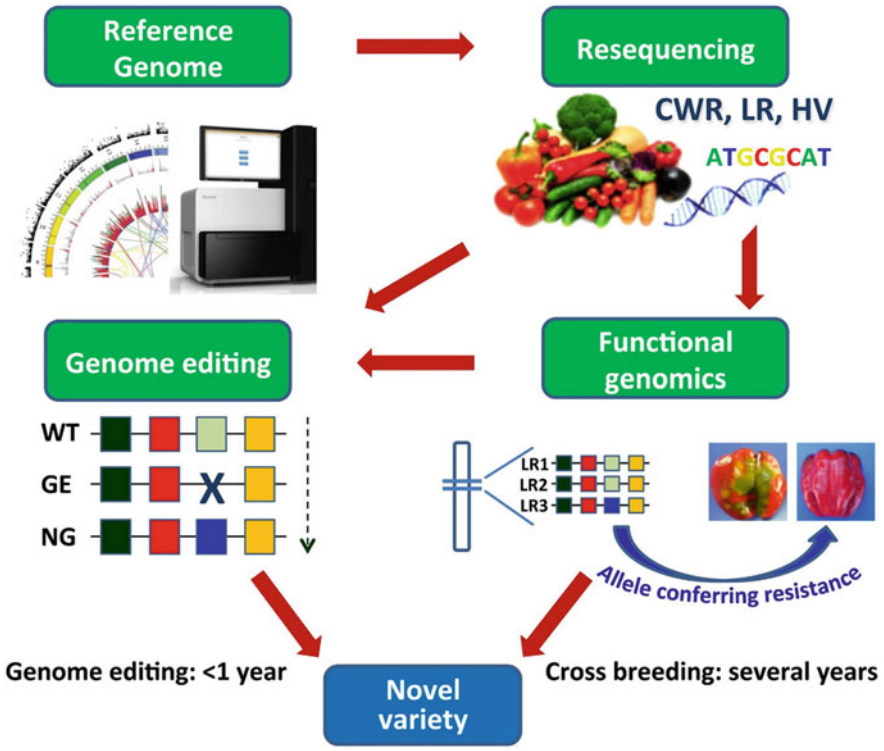

Fig. 8.13 Enhancement and manipulation of genomic resources by cross-breeding and by genome editing. Resequencing of crop wild relatives (CWR), landraces (LR) and heirloom varieties (HV) in vegetables and subsequent detection of the allelic variant associated with relevant attributes permit the introduction of precise genome modifications accelerating the breeding of novel varieties. Wild type (WT), genome editing (GE) process, new genome (NG) edited genotype, landraces (LR 1, 2, 3). Alleles are shown by colored boxes. (Source: Cardi et al. 2017)

8.13 Mutation Breeding in Summer Squash

8.13.1 Conventional Mutagenesis (Seeds)

Artificial mutation induction has proved to be a valuable method to produce variation in many species. In addition, mutation induction techniques may be used to alter a genetic pattern of crop plants, with a strong probability of success. There are few studies on the use of mutagenesis in summer squash or the family Cucurbitaceae to induce mutation. A few numbers of investigations discussed the effect of EMS on germination rate. Most results indicated that the low concentrations did not impact the percentage of germination, although high concentrations lowered it. Among the various mutagenesis gamma rays and ethyl methane sulfate (EMS) were successfully used in several plant species to induce variability in both quantitative and qualitative characteristics (Ahirwar et al. 2014).

A few numbers of studies addressed the impact of gamma irradiation on germination time and percentages. Most results showed that the low doses increased germination percentage and decreased germination period, while the high doses decreased the first trait and increased the second. Several types of chlorophyll mutants were isolated in the M_2 generation after treating seeds of several crops with mutagenesis (Melki and Marouani 2009).

In *C. pepo* Whitwood and Weigle (1978) reported that two compact mutants induced in early prolific Straight Neck with a 0.035 M solution of ethyl methane sulphate (EMS), which segregated for habit in the M_2, were phenotypically similar to a naturally occurring extreme dwarf of *C. pepo* data obtained from the F_2 of the mutants induced and the extreme dwarf and from their crosses and backcrosses to the F_1 of the normal height crossed with the extreme dwarf, revealed that the mutations induced were operated by a single suppressed gene that controlled the extreme dwarf. Kawaide and Matsuura (1996) exposed seeds and pollen of the monoecious inbred line of cucumber T_1 to gamma-rays and M_2 lines obtained after self-pollination of seed irradiation and non-irradiation-derived M_1 plants. Eleven mutants from seed irradiation and two from pollen irradiation were identified among the M_2. Segregation in the M_2 indicated that one recessive gene regulated the mutations.

Low doses of gamma rays will stimulate the effect, whereas high doses have an inhibitory effect on the cucumber (Zagorcheva et al. 1985). From another point of view, some significant reports showed that radiation had influenced the number of leaves per plant. The low doses yielded the best results concerning the number of leaves produced, while the high doses reduced this trait. In winter squash *C. maxima* and pumpkin *C. moschata* genotypes, Kurtar et al. (2017) determined the semi-lethal gamma-ray doses for initiation of a genetic variant. The seeds were therefore irradiated at five separate gamma-ray doses (50, 100, 200, 300 and 400 Gy) by a cobalt-60 (^{60}Co) source and the efficacy of irradiation doses was checked for seed and seedling observations. García et al. (2019) examined the phenotypes of single and double-modified homozygous and heterozygous plants showing that the two ethylene receptors help in regulating the ethylene reaction.

8.13.2 Enhanced Traits and Improved Cultivars

In any economic crop, the yield is the main goal. This critical object was improved by various means. The radiation toll has played a major role in this issue. Treatments with low doses of gamma rays were used to stimulate early and total yield, while the higher doses reduced yield and its component. Li et al. (1994) treated *C. moschata* seeds with gamma rays of ^{60}Co. In plants of M_1 generation, there was a marked variation in fruit shape and weight, even within a single plant.

Soltysiak and Kubicki (1988) stated that in cucumber, the EMS-induced line of the cultivar Borszczagowski, characterized by a hypocotyl two-thirds shorter than the normal and reduced length of internode, was crossed with lines of different habit, the color of the fruit spine and type of sex.

The segregation ratio indicated that the mutation expression was dependent upon a recessive gene. In the other study, Jiawang et al. (1997) irradiated inbred cucumber line dry seeds with a gamma rays dose of 90,000 Roentgens, and mutants that had excellent comprehensive traits were developed. The inbred line $M_{8,}$ which had many beneficial traits, was produced after three generations of inbreeding and selection. A new F_1 line, tolerant to low temperature and low-intensity light and suitable for greenhouse plastic cultivation, was derived by crossing M_8 with another inbred line. The hybrid was earlier maturity, higher yield and was more disease resistant than its parents.

There was almost no branching, and the morphology of the flower differed significantly from the previous species mutation. Abd El-Rahman (2000) also treated the *Citrullus colocynthis* seeds with gamma rays of 0, 5, 10, 15 and 20 KR. He found that the variations between mutant lines in the M_3 generation were highly considerable for stem length, the number of leaves and branches for each plant and plant dry weight. For at least one of the four character measures, all mutant lines varied significantly from the original parent. Asbah (2007) studied the efficacy of mutagenesis in especially ethyl methane sulphate (EMS), and gamma-rays in the mutation process in two summer squash cultivars, namely Eskandarani as well as causing genetic variation for mutant selection with desirable characteristics. Test the mutagenesis effect of both 10, 20, 30 and 40 Kr gamma irradiation and EMS at 0.025, 0.05, 0.1 and 0.2% concentrations on two summer squash cultivars, Eskandarani and Gabla as an attempt to induce valuable productivity mutations. García et al. (2018) registered 20 randomly distributed chromosomes in squash. The mutations were most influencing intergenic zones, but 7.9 and 6.0% of the mutations were found in L_1 and $L_{2.}$

8.14 Hybridization

8.14.1 Conventional Hybridization

Hybridization is the best way to improve cross-pollinated crops such as summer squash. Several researchers have used squash inbred lines have been used to produce F_1 hybrids and study the nature of the genetics and characteristics of these hybrids. The breeders of summer squash have effectively exploited heterogeneity in yield characters to develop desirable hybrids through the crossing of inbred lines. Increased F_1 hybrid performance has encouraged the seed men to spend many dollars on developing novel F_1 hybrids. The breeders must examine and evaluate a huge number of cultivars and inbred lines in cross groups and evaluate them in different seasons and sites to select those hybrids that have shown high yield, good characteristics and high stability (Mulualem and Abate 2016).

Cucurbit plants are joined to various species of rootstock and assortments using a set of strategies for uniting. The usually unified cucurbit crops include watermelon, melon and cucumber. Cucurbit breeders have always been curious about crosses between species of *Cucurbita*. The collection of the most amazing interspecific cross combinations is crucial to the breeding of vegetable rootstocks. The inter-specific rootstocks are nowadays used widely to join the watermelon, melon and cucumber (Lee et al. 2010). Other strategies, bridge cross counting (Zhang et al. 2012), bud fertilization (Hayase 1961), rehashed fertilization (Yongan et al. 2002) and using development controllers (Nascimento et al. 2007) were used in efforts to advance the victory of interspecific crossing (Lebeda et al. 2007).

The variable evidence shows that selection during the sale is not particularly effective, raising the frequency of desirable genes of small effect, especially genes involved in controlling quantitative inheritance traits (Allard 1971). *C. pepo* exhibits great differences in fruit size, shape and color. Paris proposed a classification of the species comprising eight groups of cultivars based on the fruit shape, a polygenic characteristic. Numerous botanists have described the successful, refined embryos from the production of natural products at *C. pepo* (Kwack and Fujieda 1987; Loy 2012; Sisko et al. 2003). Planted *Cucurbita* species do not have the same inbreeding and heterosis behavior as other cross-pollinated crops such as Zea *mays* and *Allium cepa* (Jansi et al. 2018; Whitaker and Davis 1962).

8.14.2 Heterosis and Hybrid Vigor

Different breeding strategies depend on the selection of suitable hybrids, in addition to the degree of heterosis, which required a unique specific linking power. The breeders often challenge the selection of parents and hybrids (Bocianowski et al. 2015).Breeding for high-yielding crop varieties,

The heterogeneity of the superior parent in the number of fruits per plant and earliness was found within the genetic examination of the summer squash cultivar Eskandarani (Abdein 2005; Metwally et al. 1988). Abdein (2005) studied the

variations in fruits, shapes and the size of certain crosses and their reciprocals from the photographic images (Fig. 8.14).

There are several studies for estimating heterosis on summer squash. Heterosis is considered to be a common occurrence in all plants. Firpo et al. (1998) crossed between 10 selected summer squash inbred lines. They found that for the number and diameter of extended leaves and plant length, most crosses exhibited significant values of heterogeneity compared to normal parents. Obiadalla (2006) recorded heterosis in squash for days until the emergence of the first female flower, and the sex ratio was evaluated as the number of female flowers/number of total flowers. In the summer squash, Al-Hamdany and Al-Lelah (2010) measured four parents and their hybrids to estimate heterogeneity using a 4 × 4 cross over the entire night.

Marie et al. (2012) studied heterosis concerning the better parent. For the hybrid, IL 3 × IL 8, negative heterobeltiosis of −13.71% was obtained for the number of nodes to the first flower trait. El-Khatib (2013) observed that the evaluated quantity of heterogeneity in the mid-parents demonstrated positive and negative high significant values, except that some hybrids were negligible for vegetative attributes at both sites and their pooled data. In a 7 × 7 half-diallel cross mating pattern, Albrifcany (2015) studied heterosis in early traits. More recently, summer squash F_1 hybrids have been used to enhance production and earliness characteristics (Habiba et al. 2015; Hussien 2015; Hussien and Hamed 2015; Karipçin and İnal 2017).

8.14.3 Genetic Parameters and Nature of Gene Action

Summer squash belonging to the species *C. pepo* contains different varieties. These varieties are different in vegetative characters, flowering times, yield and its components traits. Also, the fruits that make up the edible plant component vary significantly in length, thickness, shape and color. Thus, among all collections of various varieties, breeders would find great variations.

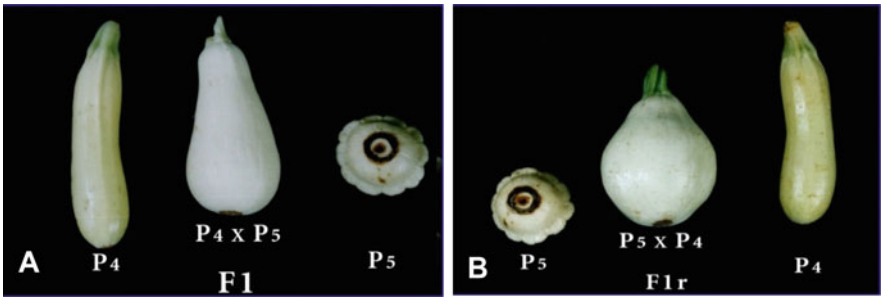

Fig. 8.14 *Cucurbita pepo* fruits from hybridization, (**a**) F_1 hybrid $P_4^{\varphi} \times P_5^{\sigma}$ and (**b**) F_{1r} reciprocal hybrid $P_5^{\varphi} \times P_4^{\sigma}$. (Source: Abdein 2005)

A lot of investigation on summer squash has been performed to identify the types of gene action associated with this phenomenon. Hence, studying the degrees of difference and nature of gene behavior among several selected varieties that are being utilized would be very useful. In this area, three major matching systems are used as follows, full diallel, half-diallel and line × tester matching designs (Bocianowski et al. 2015; El-Shoura and Abed 2018; Patel et al. 2010).

8.14.3.1 Complete Diallel Mating Design

The complete diallel cross has the advantage of generating F_1 and their reciprocal F_1. The reciprocal hybrids benefit from modifying the parental at the cross by utilizing one parent as a female in one cross and utilizing it as a male parent in its reciprocal cross. So, it would be very important to evaluate and study the differences in F_1 hybrid performance and F_{1r} reciprocal performance for all traits. The obtained differences were positive for their reciprocals from certain crosses and negative for each other since the complete diallel crosses mating design presents estimates of general combining ability (GCA), specific combination ability (SCA) and reciprocal results. Those components will develop into conditions with genetic variation. GCA estimates additive (δ^2 A), while SCA is a non-additive approximation or dominance (δ^2 D) (Abdein 2016). Al-Ballat (2008) reported that the additive component was important for stem length and the number of leaves per plant and highly significant for both traits was the dominance of genetic variations.

Al-Hamdany and Al-Lelah (2010) assessed four parents and their hybrids to assess the genetic variability for vegetative characteristics by utilizing four summer squash varieties with complete diallel crosses. Douglas et al. (2011) confirmed the effects of an additive and non-additive gene on parthenocarpy expression and papaya ringspot virus (PRSV-W) resistance were very important. Sanin et al. (2014) examined the predominance of additive gene action on the dominant type for the early traits.

El-Shoura and Abed (2018) studied heterosis and the combined ability for squash hybrid development. GCA was important for all characteristics except early yield per plant and shape index. SCA was significant for all characteristics except for the number of female flower nodes and the initial yield of the plant. The determined mean GCA/SCA squares showed that the effects of the additive gene, except for the first yield per plant and vitamin C, were the main influence on the inheritance of all traits studied. GCA estimates were also larger for most of the traits studied than their respective SCA estimates.

8.14.3.2 Half Diallel Mating Design

The half diallel analysis is a type of matting system that assists and enables the breeder to obtain estimates of the GCA and SCA. These estimates are indicators of additive and non-additive genetic differences, respectively. The half-diallel mating design may be used for GCA and SCA estimation. Thus, diallel crosses are sometimes used in genetic studies to assess the inheritance nature (additive and non-additive) of the traits examined (Abdein et al. 2017; Al-Araby 2010; Hussien 2015; Obiadalla 2006). Abou El-Nasr et al. (2010) observed that, for all characters

studied, the proportion between the mean squares of GCA and SCA showed that the additive component of genetic variation played a major role in heredity. Abd El-Hadi et al. (2013) showed in the summer squash that the parental variety Zucchino Nova Verde di Milano was the best compound for the number of leaves per plant.

Albrifcany (2015) used seven squash varieties in a half-diallel cross-mating design and observed yield traits. Hussien and Hamed (2015) found that GCA magnitude was always greater than SCA and the GCA/SCA ratios were higher than unity. They accept the significance of additive and non-additive genetic variation in the inheritance in summer squash hybrids of important traits (El-Shoura and Abed 2018).

8.14.3.3 Factorial (Line × Tester) Mating Design

Sprague and Tatum (1942) utilized the terms GCA and SCA to the average performance of the parental line and hybrid combination. Ahmed et al. (2003) used the summer squash line × tester procedure to evaluate the combined power magnitudes by measuring the components of factorial mating designs. Analysis of line × testers provides information about GCA and SCA effects of combining ability, and this would help estimate different types of genetic variation actions. The x-line tester analysis method is also used to assess beneficial parents and hybrids (Bocianowski et al. 2015).

Ghai et al. (1998) measured a factorial mating design of 10 lines × 4 testers and their 40 top crosses during the summer squash. El-Sharkawy (2000) researched the combining capability of seven inbred lines obtained from the Eskandarani genotype. She utilized three testers and eight parental inbred lines to regenerate 24 F_1 hybrids. The consequences of σ^2_{sca} of hybrids, which indicate the non-additive genetic variation, were observed to be greater than those of the other two components in most vegetative traits, i.e. σ^2_{gca} of lines and σ^2_{gca} of testers.

Ahmed et al. (2003) assessed combining ability in 7 lines × 2 tester crosses in a factor layout of summer squash cultivars. He utilized eight inbred summer squash lines from Al-Jebory (2006 and 2008) to approximate genetic parameters of earliness traits. He also utilized eight inbred lines of summer squash and crossed them with six lines as female parents and two lines as male parents and their 12 F_1 hybrids.

Tamilselvi et al. (2015) used line × tester mating design to analyze combining abilities for earliness in squash. On squash, Davoodi et al. (2016) examined standard heterosis in vegetative and qualitative traits in *C. pepo* and *C. moschata* fruit-type hybrids. Recently, Al-Araby et al. (2019) studied the heterosis and combining ability in cucumber (*Cucumis sativus* L.) using line × tester analysis.

8.14.3.4 Homogeneity Test

Abdein (2016) studied the homogeneity test for the mean square from factorial breeding design and complete diallel crosses mating design. The results of the homogeneity test revealed that the mean squares of GCA from the complete diallel crosses mating design were more effective and bigger in amounts than their elements of mean squares from the factorial mating design for males and females. These

results showed that additional genetic variation appears to be significant when evaluated by utilizing male or female components. Instead, the predominant genetic variance appears to be crucial when assessed from a factor mating design, since the mean square of the male by the female was greater than the mean square of SCA. Generally, in light of this analysis, calculating the additional genetic variance will be more reliable than the nature of factorial mating, and the opposite for dominance genetic variance that will be calculated to use the diallel crosses more precisely. Consequently, all patterns of mating can be used to predict genetic variances with a variable degree of efficiency.

8.15 Conclusions and Prospects

Many efforts should be spent on genetic conservation of different landraces and developing cultivars resistant to the pathogen, especially *Cucurbita* is one of the most essential crops in a developing country. A gene bank in each country should cooperate with the ministry of agriculture in developing and evaluating the new genetic engineering tools in increasing yield productivity. Karyotype, in addition to genetic map to produce quantitative trait loci (QTL) should be utilized for the advancement of qualitative and quantitative characteristics. The report concludes that people as of now utilize about half of the plant arrive for nourishment generation and, as worldwide populace levels rise, agricultural arrive is reaching to be in exceptionally brief supply. Typically, one or more of the impacts of climate change will be a decline in agrarian efficiency over the tropics, meaning that we are going to have to cut down woodlands and change over unused arrive into farmland. This deforestation will lead to indeed more carbon emanations, coming full circle in a horrendous cycle of expanding warming.

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Enhancing *Spinacia oleracea* L. Breeding in the Post Genomics Era

9

Eman Tawfik

Abstract

Spinach is a popular cool-season green vegetable that is eaten all around the world. Spinach is a highly heterozygous dioecious, wind-pollinated plant. Spinach plant is a six-chromosome diploid crop ($2n = 2x = 12$). The spinach plant is characterized by rich nutrients which are high in health-enhancing chemicals and nutrients. Spinach is a rich-nutrient vegetable that is high in minerals and vitamins. To improve the varied qualities of spinach cultivars, several breeding procedures were used. At the level of genome sequencing and annotation, proteins, RNA, and metabolic pathways, many databases were used for the bioinformatics of spinach. Spinach breeders and geneticists are primarily interested in improving spinach for a variety of desirable features, such as abiotic and biotic stress tolerance and high yield. Modern genomics tools and traditional breeding approaches can help achieve these goals. One of the challenges in spinach plant regeneration is establishing full, healthy plants, which is related to plant transplanting to the greenhouse. As a result, tissue culture techniques using variable plant growth regulators can be used to solve the problem.

Keywords

Biotechnology · Genetic improvement · Genetic map · Genomics · *Spinacia oleracea* · Spinach · Breeding · Tissue culture

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_9

9.1 Introduction

A high-fiber, fruit-and-vegetable-rich diet is associated with a lower disease effect, including diabetes, cardiovascular disease, and cancer. One of these vegetable crops is Spinacia oleracea, also known as spinach (Morelock and Correll 2008; Fuhrman 2014; Metha and Belemkar 2014).

Spinacia oleracea L. (commercially spinach) is a green, leafy, valuable vegetable that grows as an annual plant (Fig. 9.1). It can be planted in both the spring and the autumn (Jaiswal 2020). The stem is 30–60 cm tall, smooth, spherical, and succulent. The lower leaves are long-petioled and variably lobed. Male and female flowers are the two types of flowers. Male flowers are found on long terminal glomerate spikes, calyx has 4 sepals, antheridium has 4 stamens, twin anthers, big. Where, the female flowers are axillary, sessile, and dense. When the seeds are formed, they out from protruding horns on each side of the calyx. In general, there are four white tapering styles. Capsules are one-celled, one-valved, armed, and capped with the little surviving calyx (Kirtikar and Basu 2005).

9.1.1 Taxonomy

Long thought to belong to the Chenopodiaceae family, common spinach was merged under the family Amaranthaceae, order Caryophyllales in 2003. Spinach is a member of the Chenopodioideae subfamily of the Amaranthaceae (Rubatzky and Yamaguchi 1997; Dawling 2013).

PLANT PROFILE *Spinacia oleracea* Linn Scientific Classification (Subhash et al. 2010) as follow:

Fig. 9.1 The Geographical distribution map of different spinach accessions. (Source: Ribera et al. 2021) (permission: Rightslink® by Copyright Clearance Center)

"Kingdom: Plantae
Subkingdom: Tracheobionta
Division: Magnoliophyta
Class: Magnoliopsida
Order: Caryophyllales
Family: Chenopodiaceae
Genus: *Spinacia* L.
Species: *Spinacia oleracea* L."

9.1.2 Origin and Distribution

Spinacia oleracea is an annual herb that originated from Central and Southwest Asia with therapeutic properties. It was imported to Europe in the fifteenth century and is cultivated for its succulent leaves. In the winter, it is the most popular dish among Indians. It goes under a variety of names in different traditional medicinal systems. It is known as 'Paalankikaa' in Ayurvedic medicine, 'Paalak' in Unani medicine, and 'Vasaiyila-keerai' in Siddha medicine (Kirtikar and Basu 2005; Khare 2007; Guha and Das 2008; Subhash et al. 2010). According to some sources, spinach is native to Southwest Asia and is grown all over the world. (Khare 2007; Subhash et al. 2010). The geographical distribution of different *Spinacia spp* all over different locations were illustrated in Fig. 9.1.

It's still a mystery how spinach got from its origins to other parts of the world (Hallavant and Ruas 2014). Spinach extended to the West may have occurred as a result of Muslim territory expansion (Sneep 1983). According to current evidence, the Moors brought spinach to Europe via the Iberian Peninsula (Al-'Awwam 2000). Even less distinct is the spread of spinach cultivation into Central and Eastern Asia. According to the oldest documented documents, spinach was brought to China from Nepal in 2000 (Laufer 1919).

9.1.3 Spinach Nutritional Value

Because spinach is high in vitamins, carotene, starch, oxalates, and minerals, which limit calcium absorption, it is not suggested for persons with urolithiasis. β-Carotene is an antioxidant that helps to protect against cancers of the lungs, mouth, prostate, and stomach. Spinach is high in fiber and digestable, with low carbohydrate, protein, and fat contents and calories. Spinach is high in vitamin K, which helps to strengthen bones. Vitamins play a key role in human consumption. The chemical composition of spinach is presented in Table 9.1 (Butnariua and Butub 2014; Park et al. 2013; USDA 2017).

Table 9.1 The nutritional value of fresh spinach

No.	Content	Percentage (%)/100 g	Reference
1	Moisture	9	Subhash et al. (2010); Jaiswal (2020)
2	Lipid	0.4 and 0.6	
3	Protein	2.9	
4	Carbohydrate	2–10	
5	Fiber	2.2	
6	Calcium	10	Butnariua and Butub (2014)
7	Iron	21	
8	Magnesium	22	
9	Manganese	43	
10	Phosphorus	7	
11	Potassium	12	
12	Sodium	5	
13	Zinc	6	

Table 9.2 Chemical constituents of some secondary metabolites in spinach

No.	Secondary metabolites	Constituents	Reference
1	Flavonoids	querecetin; 5,3′,4′-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4′-glucuronide; myricetin; 5,4′-dihydroxy-3.3′-dimethoxy-6:7-methylene dioxyflavone-4′-glu-curonide; kampeferol; apigenin; luteolin; patuletin; spinacetin; jaceidin; 3,5,7,3′,4’pentahydroxi-6-methoxiflavone	Ferreres et al. (1997); Annonymus (2004); Sultana and Anwar (2008)
2	Phenolics	ferulic acid, para-coumaric acid, ortho-coumaric acid	Andjelkovic et al. (2008)
3	Carotenoids	β-carotene, lutein, violaxanthin and 9′-(Z)-neoxanhin	Guha and Das (2008)
4	Vitamins	vitamin A, C, E, K, oxalic acid, folic acid	Guha and Das (2008)
5	Minerals	iron, magnesium, zink, manganese, calcium, phosphorus, and copper	Guha and Das (2008); Subhash et al. (2010)

9.1.4 Primary and Secondary Metabolites

Medicinal plants contain a high amount of secondary metabolites (potential drug sources) and therapeutically significant essential oils. The main advantages of using medicinal plants to treat various disorders are their safety, as well as their price, effectiveness, and accessibility (Prakash and Gupta 2005; Subhash et al. 2010). Some essential secondary metabolites and their constituents were listed in Table 9.2. These metabolites are cooling, sweet, laxative, carminative, and beneficial in blood and brain illnesses. Seeds have a laxative and cooling flavor. Also treat

things like respiratory problems and liver inflammation. Urinary calculi are treated using the green plant (Chopra et al. 1956; Kirtikar and Basu 2005).

9.2 Pharmacological Activities

9.2.1 Protection Against Gamma Radiation

The methanolic extract of *Spinacia oleracea* produces lipid peroxidation (LPO) products and glutathione. They work against radiation-induced oxidative stress. At all intervals, LPO readings were considerably lower in pre-treated irradiated mice compared to untreated-irradiated ones but returned to normal by day 7 (Subhash et al. 2010). Radiation caused a considerable increase in LPO readings, which could be reduced by supplementing SE before the investigated irradiation intervals (Verma et al. 2003).

9.2.2 Antioxidant Activity

Grossman examined the chemical proportion of natural antioxidants in *Spinacia oleracea* in 2001. The antioxidant activity in the spinach leaves was extracted with a water:acetone (1:9) solution and analyzed on HPLC. When examined using three different assays, all the fractions obtained demonstrated antioxidant activity (Grossman et al. 2001; Subhash et al. 2010).

9.2.3 Hepatoprotective Activity

Gupta and Singh (2006) found that the alcoholic extract of spinach leaves alleviated the hepatosuppression caused by carbon tetrachloride (CCl4). The serum-marker enzymes like AST, GGT, LDH, SDH, ALT, ALP, and GDH, were measured in both serum and the liver, to assess the spinach effect.

9.2.4 Anticancer Activity

The inhibition of DNA polymerases was tested by spinach ethanol extract and 3 fractions were obtained by hydrophobic column chromatography. The dose of spinach glycoglycerolipid fraction decreased pol activity with an IC50 value of 43.0 g/ml, but the fat-soluble fraction very mildly inhibited pol activity, while the water-soluble fraction had no impact. Although the ethanol extract from spinach includes pol inhibitory glycoglycerolipid, it had no effect on pol (Maeda et al. 2008).

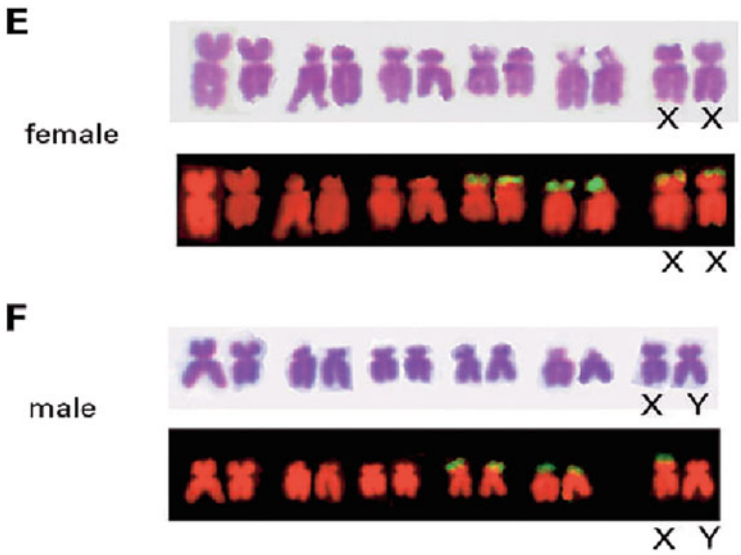

Fig. 9.2 Female and male spinach chromosomes in Giemsa and FISH pictures. The karyograms of female and male spinach (2n) chromosomal complements are E and F, respectively. Bar = 5 μm. (Source: Lan et al. 2006)

9.3 Cytogenetics

Spinach is a dioecious herb with 2n = 12 chromosomes classified as 2 large metacentric, 2 long sub-telocentric, 2 short sub-telocentric, 2 acrocentric, and 4 sub-metacentric in both male and female plants (Fig. 9.2) (Lan et al. 2006; Morelock and Correll 2008).

9.4 Traditional Breeding

9.4.1 Spinach Breeding History

Due to a lack of documentation, determining the shape of spinach in the past is challenging. Sneep gathered evidence about the breeding and domestication history of spinach in the 1950s. Sneep's study is perhaps the greatest and most comprehensive source of information on spinach breeding prior to the 1950s.

There are numerous kinds of spinach that were established based on sex and morphology, spinach sex expression is versatile. In male and female plants, sex reversion can occur, resulting in gynomonoecy and andromonoecy, respectively (Morelock and Correll 2008).

Only 1959 of the almost 2097 *Spinacia* accessions in genetic resources collections around the world are categorized as *S. oleracea*, suggesting the scarcity

of wild spinach is found in gene banks. Only 14 *S. turkestanica* accessions and 12 *S. tetrandra* accessions were collected in 2012 in the gene bank. To date, genetic resources collections contain 89 *S. turkestanica* accessions and 49 *S. tetrandra* accessions, with an additional 10 *S. tetrandra* accessions expected to be accessible by the end of 2020 (Van Treuren et al. 2012, 2019). Accessions from hitherto undiscovered places, such as *S. tetrandra* from the Middle East and South-West Asia and *S. turkestanica* from South-West Asia, could help to enrich spinach collections. The organized collection has become increasingly problematic for many countries, not least due to tougher access and benefit-sharing rules.

9.4.2 Crop Breeding

The spinach genome is available in the SpinachBase database (http://www.spinachbase.org). The genome of spinach is assessed to be 1000 megabytes in size, with 74.4% repetitive sequences and about 25,500 protein-coding genes. Former studies discovered a total of 93 genomic sites linked to wild species introgressions, all of which could be linked to spinach breeding (Xu et al. 2017).

9.4.3 Methodologies and Limitations of Traditional Breeding

Mendel's law became the foundation of plant breeding science in 1900. Plant breeding is the process of combining traits that have been identified and chosen in a single plant. These features may result in larger and heavier heads, higher seed yields, improved color, heat and drought tolerance, pest and disease resistance, and increased agronomic quality. To generate a novel plant, several procedures must be taken, including describing and diagnosing the problem with the present germplasm, as well as creating breeding goals and objectives. The manipulation of how chromosomes combine is what traditional plant breeding is all about. To manage plant chromosome combinations, there are two primary approaches. To begin with, the most ancient technique in plant breeding is the selection of germplasm that satisfies the breeding objectives (Gupta et al. 2008; Tashi et al. 2010). Second, cross-pollination of desired traits found in various plants to produce plants with the required features. Cross-pollination is used to combine the obtained features into a single plant line (Michelmore 1995). Traditional breeding techniques have some drawbacks, such as the difficulty of maintaining offspring purity, the time commitment, the inability to produce many offspring, and the development of negative characteristics which can be eradicated through time-consuming backcrossing.

9.4.4 Examples for Breeding

Breeding spinach is primarily focused on improving its quality and resistance to different diseases. Biotechnology and genetic engineering approaches, including

gene transfers, are employed to obtain such features. Using these new approaches, researchers were able to save several genotypes, create a clean environment, and overcome the limits of in vivo procedures by lessening breeding programmers. Hapolidization, shoot organogenesis, somatic hybridization, and other techniques are now being applied. Pure lines can be produced by haploidization. Shoot organogenesis increases crucial lettuce properties. Somaclonal variation is another source of genetic diversity. Regeneration by somatic embryogenesis is also used to grow lettuce, and it offers a lot of benefits (Sariçam et al. 2017).

Ribera et al. (2021) crossed spinach with the wild species *S. turkestanica* and *S. tetrandra* assumed to be the crop's progenitor; and concluded that *S. turkestanica* is the most close ancestor of cultivated spinach.

9.5 Breeding and Climate

Climatic changes are projected to have a significant negative effect on crop domestication around the world. Because of the unexpected heat and drought seasons brought on by rising global temperatures, spinach agriculture faces significant hurdles. Besides heat and drought stress, increased soil water evaporation is projected to result in an increase in mineral salts in topsoil layers, causing salt stress. Because considerable output losses are projected, new spinach cultivars which are more resistant to these abiotic stressors are urgently required (Ribera et al. 2020).

9.6 Molecular Breeding

9.6.1 Molecular Marker-Assisted Breeding

Identifying linked markers or connected genomic areas affecting phenotypic expression has long been a goal of genome-wide association studies (GWAS) and genetic linkage mapping. Bhattarai et al. (2020) stated that "the generation of progeny segregating for a trait of interest is required for biparental QTL mapping, whereas GWAS allows mapping the trait in varied germplasm or a mixed population. The GWAS method has been used to map several traits in plants and animals (Guo et al. 2017; Minamikawa et al. 2018). Multiple biparental populations were studied using the GWAS method to find genetic areas regulating resistance to the downy mildew pathogen. The goal of this work was to fine map race 13 resistance loci from several segregating populations to discover SNP markers linked to resistance. The potential genes in imparting resistance to the downy mildew pathogen were identified and refined using associated genomic areas."

9.6.2 Functional Genomics

The germplasm of spinach (wild accessions and cultivars) has a wide range of physiological varieties like carotenoid, folate, oxalate, and nitrate content, indicating that there is a lot of room for development. Pests, diseases, weeds, drought, salt, and heat have all impeded the production of spinach, as they have many other crops. Introducing nucleotide-binding site leucine-rich repeat (NBSLRR) resistance genes from wild relatives is the most common strategy for developing downy mildew resistant cultivars, whereas using loss-of-function alleles of susceptibility genes may provide a long-term strategy for developing resistant cultivars (Ribera et al. 2020).

Comparative genomics, genetic mapping, and gene cloning research still require a chromosome-level reference-grade genome for spinach. With 98.3% of the six spinach chromosomes attached and structured, the assembly is extremely precise, complete, and continuous. To better understand Chenopodiaceae's evolution, the primordial karyotype is rebuilt. Then used genome resequencing to create a genomic variation map for 305 farmed and wild spinach accessions (Hirakawa et al. 2021; Hulse-Kemp et al. 2021; Hassan et al. 2021).

The Monoe-Viroflay inbred line had only a homozygous peak in the k-mer distribution, whereas the heterozygous peak seen in the previously sequenced sibling inbred line Sp75 was absent, confirming the Monoe-Viroflay inbred line's high homozygosity (Cai et al. 2021). Where Xu et al. (2017) used a complete genetic map to anchor 439 scaffolds comprising 463.4 Mb (47%) of the assembled genome to the six linkage groups (Fig. 9.3).

9.6.3 Quantitative Trait Locus (QTL)

To evaluate the potential of breeding cultivars with improved Nitrogen use efficiency (NUE), it is essential to estimate the genetic diversity in the germplasm of spinach for characteristics that control NUE (Baligar et al. 2001). In a hydroponics system, Chan-Navarrete et al. (2014) investigated features linked to growth and photosynthesis, as well as their relationship with NUE. Leaf area and specific leaf area (SLA) were found to be important predictors of variation in NUE in spinach.

A dedicated segregating population's quantitative trait locus (QTL) investigation can provide insight into the genetics of a complex trait like NUE. Although the genome sequence is not yet accessible, the chloroplast and mitochondrial genomes of spinach have been sequenced (327 and 150 kb, respectively) (Arumuganathan and Earle 1991; Correll et al. 2011). A genetic linkage map with enough molecular markers dispersed over the genome is required for QTL analysis in a segregating population. Only one genetic linkage map for spinach had been published before, with a small number of SSR and AFLP markers (Khattak et al. 2006). It was used to investigate the genetic variation associated with sex expression. Onodera et al. (2011) used the same molecular marker data to create a map of dioecism and monoecism genes in spinach.

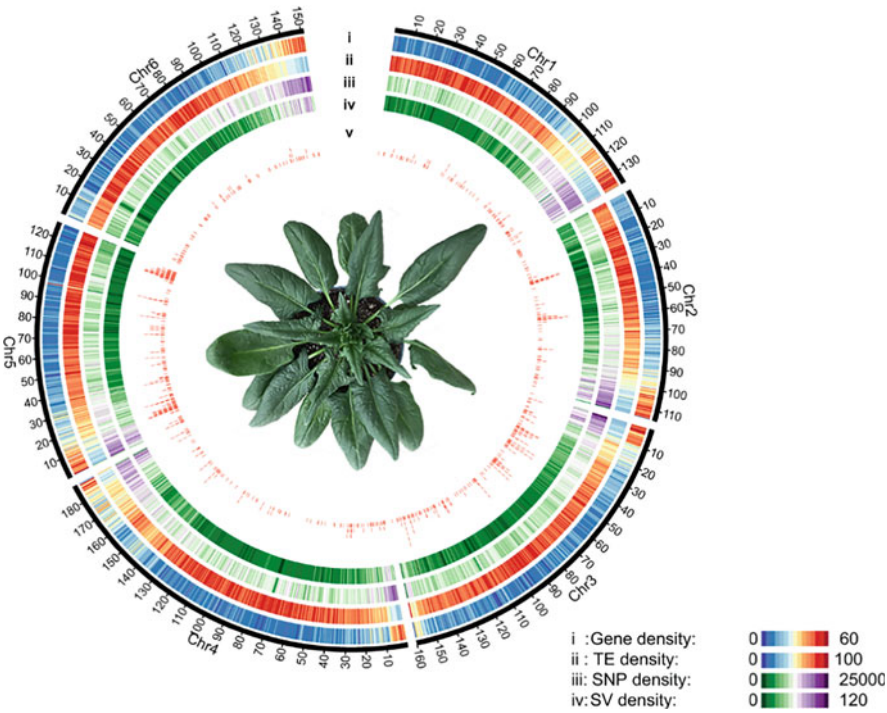

Fig. 9.3 Genome of the spinach Monoe-Viroflay Showing gene density, transposable element density, SNP density, and Distribution of domestication sweeps across the genome. (Source: Cai et al. 2021)

Van Ooijen (2006) used JoinMap 4.1 software to generate the genetic linkage map, which included 283 relevant markers and 320 genotypes. This is the same as the number of chromosomes in spinach.

9.7 Bioinformatics and Gene Bank

A reference spinach genome was just published, and more spinach genetic resources are being generated rapidly. To help with research and breeding in spinach by SpinachBase which gives the centralized public access to genetic data and analytical tools. The spinach reference genome sequence and functional annotations for protein-coding genes predicted from the genome are currently available in the database. Besides, the database includes gene expression profiles generated by RNA-Seq experiments and highly co-expressed genes and genetic variants determined from sequences of 120 farmed and wild Spinacia accessions transcriptomes. SpinachBase is a pathway database (SpinachCyc) that contains biochemical pathways predicted from spinach protein-coding genes. SpinachBase includes

BLAST sequence similarity searches, genome browser, functional classification studies, and functional enrichment (Collins et al. 2019).

The genome sequence and annotation: Xu et al. (2017) stated that "A total of 25 495 protein-coding genes were predicted from the draft genome of spinach 'Sp75'. The spinach genome sequences, coding sequences (CDS) and protein sequences of predicted spinach genes, a genome feature file in gff3 format (generic feature format), and functional descriptions of predicted genes can be downloaded from the 'download' page of SpinachBase. The sequences of chromosomes, scaffolds, CDS, and proteins were imported into the Chado database using the Tripal 'Data Loader' module."

Protein Database: Camacho et al. (2009) and Mitchell et al. (2014) mentioned that "the protein sequences of spinach genes were compared against the GenBank nr, UniProt (TrEMBL/SwissProt) and *Arabidopsis* protein databases using the BLAST program. Gene ontology (GO) terms were assigned to spinach protein-coding genes using the Blast2GO program (Conesa and Götz 2008) based on the comparison results against the nr and the InterPro domain databases. A set of concise and informative functional descriptions were assigned to spinach genes using the AHRD program (https://github.com/groupschoof/AHRD) based on the BLAST results against the UniProt (TrEMBL/Swiss-Prot) and Arabidopsis protein databases. With the assistance of the Tripal 'Analysis' extension modules, the homologs derived from top BLAST hits, GO terms, and InterPro domains assigned to the protein-coding genes were deposited into the SpinachBase and displayed on the gene feature page."

Genetic Variants: Van der Auwera et al. (2013) mentioned "transcriptome sequences of the 120 *Spinacia* accessions were also used to call genetic variants, mainly single nucleotide polymorphisms and small indels, using GATK following the online best practices protocol with recommended parameters for RNA-Seq data (https://software.broadinstitute.org/gatk/best-practices/)." The cleaned RNA-Seq reads were aligned to the spinach reference genome via 2-pass technique with STAR (Dobin et al. 2013). Using Picard (http://broadinstitute.github.io/picard), the alignment files in SAM format (Li et al. 2009) were further processed to add read groups, highlight duplicates, and construct an index. Among the 120 *Spinacia* accessions, 751,189 variations were discovered. SpinachBase was updated to include these variations, then presented in the genome browser.

The properties and structure of spinach antioxidant proteins were studied using bioinformatics and molecular modeling. PROCHECK and WHAT IF, two protein structure checking tools, validated the models. These structures will serve as a solid foundation for functional analysis of crystal structures generated experimentally.

9.8 Tissue Culture

Plant regeneration from the leaf is applied for breeding propagating for desired features. In spinach breeding operations, digging plants from selection plots and bringing them to greenhouses to make crosses in isolation is common. In the

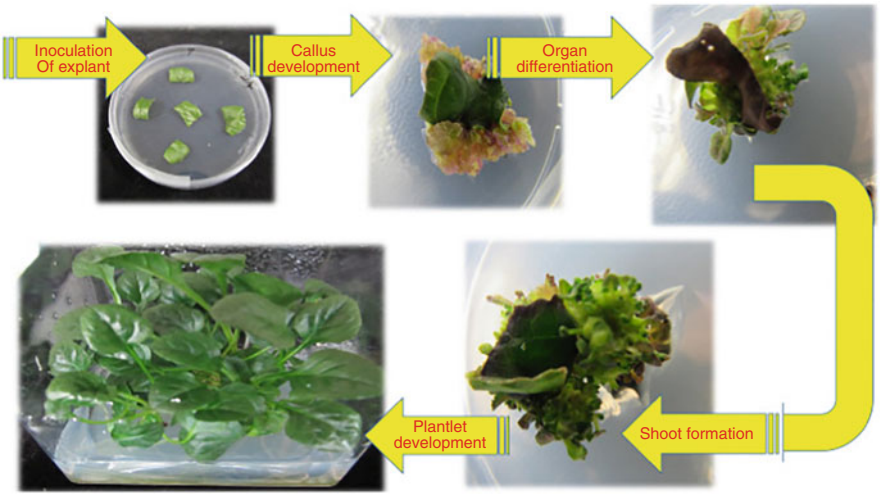

Fig. 9.4 Schematic workflow of spinach tissue culture. (Source: Chun et al. 2020)

greenhouse, limited transplant survival could be a serious issue. The time-consuming method may be removed with the availability of a micropropagation system, and a single leaf from a chosen plant can be used to regenerate multiple plants, giving a huge number of seeds. The original plant's genome is conserved in the regeneration; therefore, its existence is no longer required (Goode et al. 1988).

Cultivars and explant types control plant regeneration in spinach (Leguillon et al. 2003). Root, hypocotyl, and leaf explants were employed for embryogenesis, with root explants proving to be preferable for somatic embryogenesis (Komai et al. 1996).

Because of plant bolting, in vitro-grown spinach plants are difficult to transplant into the soil (Ishizaki et al. 2002). During the shoot induction stage, a low ACR is required for plant regeneration via organogenesis, while auxin is needed to induce root development (Geekiyanage et al. 2006; Chin et al. 2009). Auxin therapy for root induction is time demanding and can result in plant bolting. Plant regeneration by somatic embryogenesis, on the other hand, was successful in a high ACR medium, and auxin was not required during the rooting stage (Komai et al. 1996; Zdravkovic-Korać and Neskovic 1999; Ishizaki et al. 2001). As a result, plant creation via somatic embryogenesis could be an effective way of establishing complete plants without the use of seeds. The general steps included in the micropropagation of the spinach plant were represented in Fig. 9.4.

9.9 Conclusions

The leafy vegetable spinach is a member of the Chenopodiaceae family. *Spinacia oleracea* has been shown to have antioxidant, antiproliferative, anti-inflammatory, antihistaminic, antihistaminic, CNS depressant, gamma radiation protection, and hepatoprotective properties. This plant has been found to contain a variety of secondary metabolites including flavonoids, carotenoids, and phenolic chemicals. As a result, more phytochemical, pharmacological, and clinical research on Spinacia oleracea is needed to establish a viable natural medicine that can deliver therapeutically effective lead compounds or extracts. The SpinachBase provides a central portal for data on spinach genomic. It is one of many databases dedicated to spinach bioinformatics. Root and root-derived callus, as well as the callus induction period, were used to build an efficient system for spinach plant regeneration. This technique can be used to improve crop breeding and spinach genetic transformation. Many breeding strategies were applied to enhance the different characteristics of spinach.

Appendix 9.1: Genetic Variability in Spinach

Spinach major varieties

Variety	Type	Important traits
Savoy Spinach		thick, crinkled leaves that are difficult to clean leaves
	Hammerhead Spinach	Savoy spinach comes in both round and curled varieties. They have dark green leaves with high fiber content and are delectable
	Bloomsdale Spinach	A favorite among gardeners with a green thumb. Their leaves are strongly curled with a medium-dark greenish tint
	Palco Spinach	They have dark greenish leaves that are deeply curled, circular, and cupped
	Regiment Spinach	The uncooked leaves of this spinach cultivar are big, delicate, curly, and deep green
Semi-Savoy Spinach		Leaves that aren't as crinkled. This spinach variety has partially straight leaves that are very easy to clean. The majority of the time, they are grown in backyard gardens
	Carmel Spinach	Spinach varieties are fast-growing, upright, uniform, and semi-savoy. The leaves of this spinach variety are beautiful and dark green
	Emperor Spinach	This spinach plant boasts dark green leaves and a tall stem, making bunching a breeze
	Kookaburra Spinach	They are typically found in residential gardens and have slightly curled dark greenish leaves
	Acadia Spinach	The leaves are oblong, lustrous, and dark green
	Tasman Spinach	leaves of medium green color and grow quickly and can be harvested in 4 weeks
	Reflect Spinach	They have medium green leaves that are relatively straight and round

(continued)

Variety	Type	Important traits
	Kolibri Spinach	The medium-dark green leaves of this spinach are silky and gently curved
	Teton Spinach	This spinach variety is semi-Savoy and upright. When cooked as a salad, it features oval and deep green leaves that are wonderful and flavorful
	Indian Summer Spinach	Appropriate for all three seasons (fall, summer, and spring season). They have dark greenish leaves that are flat and somewhat curved
	Catalina Spinach	It has dark greenish leaves that are thick, somewhat curved, and spear-shaped
	Tyee Spinach	It has large, round, dark green leaves with a wonderful aroma
	Crocodile Spinach	Crocodile Spinach
	Avon Spinach	Its flavor is ideal for salads. It features huge, slightly curled, dark green leaves that are good for freezing
Smooth Leafed Spinach		Leaves that are smooth and flat. They're quite easy to clean and are commonly utilized in processed foods
	Gazelle Spinach	They have dark greenish, mid-oval, and spherical leaves. They have long stems that make bunching easier
	Corvair Spinach	They have dark greenish leaves that are round and erect
	Flamingo Spinach	These spinach varieties have smooth, homogeneous, and straight-leafed leaves. Their leaves are dark greenish and lengthy, with a pointed arrow tip
	Red Kitten Spinach	They have medium green leaves with dark red veins that are straight, homogeneous, and smooth
	Wood Pecker Spinach	They are medium-dark green in color and grow at a medium rate
	Seaside Spinach	They have dark green leaves that are tiny and spade-shaped
	Renegade Spinach	Renegade spinach is the ideal choice for salads because of its dark green leaves and soft stems

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Breeding Strategies of Beetroot and a Future Vision in the Post-genomic Era'

10

Eman Tawfik

Abstract

The beetroot is considered a cross-pollinated plant that follows the family Chenopodiaceae. It is an important and cost-effectively grown vegetable. Beetroot is used for salads, food, juice, and pickles rather than sugar production around the world. The root can also be used to extract edible sugar. Beetroot is highly nutritive and is one of the primary sources of many essential metabolites in the diet. Beets are high in dietary fibres, minerals (iron, potassium, copper, magnesium, sodium, calcium, zinc, and phosphorus), vitamins (B-complex, ascorbic acid, retinol, and), antioxidants, betalains, and phenolics. Beetroot is investigated as a potential medicinal treatment for a variety of clinical conditions linked to oxidative stress and inflammation. In vitro and in vivo, its constituents, particularly the betalain pigments, show potent antioxidant, anti-inflammatory, and chemopreventive activity. Beetroot genetic resources, including wild species, have been saved by gene banks. There are a variety of species with different chromosome counts. Beta is diverse in terms of geographic regions and morphological features. It's critical to have enough natural phenotypic variation. Beetroot is being improved for variable desirable characters, including biotic and abiotic stress tolerance, quality, and yield, by breeders and geneticists. We can get closer to our goals by using recent genetic tools to enhance traditional breeding programmes. This chapter covers the biodiversity and conservation of beetroot germplasm, as well as the goals and stages of beetroot conventional and modern breeding programmes. It also discusses modern plant breeding techniques, such as marker-assisted breeding.

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_10

Keywords

Secondary metabolites · Genetic improvement · Genetic map · Genomics · *Beta vulgaris* · Beetroot · Breeding · Tissue culture

10.1 Introduction

The main root and smaller roots that can reach a depth of 60 cm and branch out laterally make up the plant's root system. It also has a tuberous, purplish-red, globular-shaped part that develops almost on the soil's surface and has a sweet taste (Ravichandran et al. 2013). The beetroot is a biennial plant that requires a time of extreme cold to complete the reproductive stage of its life cycle. During the vegetative phase, elongated leaves appear near the stem and tuberous part, whereas floral tassel emission occurs during the reproductive stage when glomeruli seeds are produced. Cultivation conditions in high temperature and humidity encourage the spread of pests and diseases, altering the plant's internal colour and taste, making it less sweet, and reducing plant productivity by about half. The cycle lasts from 60 to 100 days, according to the cultivation mode and cultivar, whether in the summer or winter (Sediyama et al. 2011; Baião et al. 2017).

10.1.1 Taxonomy, Origin and Distribution

The Chenopodiaceae family includes three species of the beetroot as shown in Table 10.1 (Baião et al. 2017; Lange et al. 1999).

Beetroot is a vegetable that originated in North Africa and Europe, where it is grown in mid-cold temperatures (10–20 °C) (Fig. 10.1) (Sediyama et al. 2011; Tullio et al. 2013).

Table 10.1 Classification of beetroot species and groups

No.	Sub-Species	Characters	Groups
1	*Beta vulgaris* ssp. *adanesis*	"has a distinct group of semi-annual plants with a significant drop in auto-fertilization and distinctive morphological characteristics"	
2	*Beta vulgaris* ssp. *maritima*	"formed by a diverse range of morphological types found across a large geographic area"	
3	*Beta vulgaris* ssp. *vulgaris*	"All domesticated cultivars are grouped together in this category"	Leaf Beet
			Sugar Beet
			Fodder Beet
			Garden Beet

Fig. 10.1 The Geographical distribution map of different beetroot accessions. (Source: http://www1.biologie.uni-hamburg.de/b-online/schaugarten/varconditiva/Beetroot.html)

10.1.2 Beetroot Nutritional Value and metabolites

Carotenoids, flavonoids, ascorbic acid, and phenolic acids are the essential phytochemical compounds found in beetroot (Table 10.2). (Lee et al. 2005; Georgiev et al. 2010; Wootton-Beard and Ryan 2011). Lee et al. (2005), Ninfali and Angelino (2013) and Vulić et al. (2014) say that "beetroot is one of the few vegetables that contain betalains, a group of highly bioactive pigments. Betacyanin pigments, which are red-violet in colour, and betaxanthin pigments, which are yellow-orange in colour, are both members of the betalain family." In vivo and in vitro animal models, betalains have been shown to have high anti-inflammatory and antioxidant capabilities (Tesoriere et al. 2004; Pavlov et al. 2005; Zielińska-Przyjemska et al. 2009; Vidal et al. 2014; Vulić et al. 2014).

There was a list of some important secondary metabolites and their constituents. These metabolites are cooling, sweet, laxative, and carminative, and they can help with blood and brain problems. The seeds have a cooling and laxative flavour. They've been used to treat respiratory issues, liver inflammation, and jaundice, among other things. Urinary calculi are treated using the green plant (Chopra et al. 1956; Kirtikar and Basu 2005).

Red beets are the tenth most antioxidant-rich vegetable on the planet. These antioxidants are free radical scavengers and protect proteins, DNA, and lipoproteins from oxidative damage. Chronic diseases like cardiovascular disease, cancer, neurodiseases, cataractogenesis, and stroke are caused by oxidative damage to macromolecules, which can be inhibited by antioxidant compounds found in red beets. Secondary metabolites are abundant in red beets as well (ascorbic acid,

Table 10.2 Nutrient composition of raw beetroot (Source: Mirmiran et al. 2020)

No.	Content	Measure unit	Amount/100 g
1	Water	g	87.58
2	Energy	kcal	43
3	Protein	g	1.61
4	Total fats	g	0.17
5	Carbohydrate	g	9.56
6	Fiber	g	2.8
7	Sugars	g	6.76
8	Calcium	mg	16
9	Iron	mg	0.8
10	Magnesium	mg	23
11	Phosphorus	mg	40
12	Sodium	mg	78
13	Zinc	mg	35
14	Vitamin C	mg	4.9
15	Thiamin	mg	0.031
16	Riboflavin	mg	0.04
17	Niacin	mg	0.334
18	Folate	µg	109
10	Total phenolics	mg/g	225
20	Total flavonoids	mg/g	260

Table 10.3 Chemical constituents of some secondary metabolites in beetroot

No.	Secondary metabolites	Constituents
1	Flavonoids	"Myricetin; Neringenin; Kaempferol; Apigenin"
2	Phenolics	"Gallic acid; Ferulic acid; Catechol; p-Comuaric acid; o-Coumaric acid; Cinnamic acid"
3	Vitamins	"Vitamin C (Ascorbic acid); Vitamin B3 (Niacin); Vitamin B6 (Pyridoxine); Vitamin B9 (Folic acid)"

flavonoids, phenolic acids) (Halliwell and Gutteridge 1999; Chang et al. 2008; Uttara et al. 2009; Georgiev et al. 2010; Vulić et al. 2012, 2014).

Beetroot contains many active phytochemicals such as flavonoids, betalains, Saponins, polyphenols, and inorganic Nitrate (NO_3), as well as several metals (Table 10.3). Various food cultures consume it in the form of supplemental powder, juice, bread, boiled, gel, oven-dried, pureed, pickled, or jam-processed form. Beetroot is actually one of the ten plants with the highest antioxidant activity (El-Beltagi et al. 2018).

10.2 Pharmacological Activities

The discovery that dietary nitrate sources may have an essential effect on cardiovascular health management has reignited interest in beets. Beetroot, on the other hand, contains several other bioactive compounds that may be beneficial to one's health, especially in cases of chronic inflammation. As a result, beetroot's potential as a complementary treatment for a variety of clinical conditions will be discussed. The following are the review's specific objectives: "(1) to highlight recent evidence of beetroot's physiological and biological actions; and (2) to assess its use as a nutritional intervention in health and disease, with a focus on experimental studies relating to oxidative stress, inflammation, endothelial function, and cognition" (Lundberg et al. 2008; Ninfali and Angelino 2013; Clifford et al. 2015).

Beetroot supplementation could help to boost antioxidant defences and protect cellular components from oxidative damage. Reactive oxygen and nitrogen species (RONS) are oxidation-capable molecules produced in cellular metabolism on a continuous basis (Kannan and Jain 2000; Netzel et al. 2005). RONS are involved in a wide range of cellular and biochemical processes at these low concentrations, including cell proliferation, gene expression, muscular contraction, and apoptosis. Excessive exposure of a cell to exogenously generated RONS (xenobiotics, UV radiation) or endogenously synthesised RONS (abnormal cell metabolism, inflammation) can overwhelm the cell's antioxidant defences, resulting in a redox homeostasis imbalance, which leads to oxidative stress (Baker et al. 2001; Schinella et al. 2002; Madamanchi et al. 2005; Powers and Jackson 2008; Reuter et al. 2010).

10.3 Cytogenetics

All species of beetroot, including red petioles, white petioles, and green petioles leaf beet, are dioecious plants with $2n = 18$ chromosomes. Figure 10.2 shows the karyotype and metaphase chromosomes of 3 types of green leaves leaf beet roots. The three types of green leaves leaf beet had the same chromosome number: $2n = 18$.

10.4 Traditional Breeding

10.4.1 Beetroot Breeding History

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*, cultivar group Sugar Beet) is one of the most important crops, accounting for 68% of global production in 2013 according to "Food Agricultural Organization of the United Nations 2016". Sugar beet production accounts for 20% of all sugar production worldwide. The genus also includes the cultivar groups Garden Beet, Fodder Beet, and Leaf Beet (Lange et al. 1999). In Biancardi et al. (2012) "the origin and domestication of sugar beet were thoroughly examined." Sugar beet's breeding pool was small because it was one of the newest

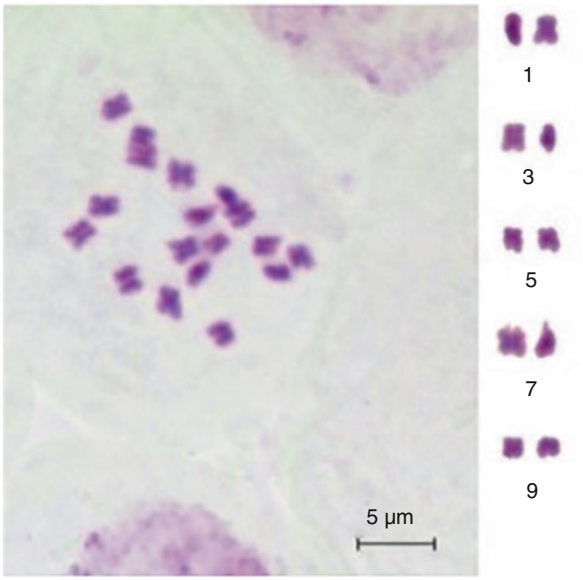

Fig. 10.2 Red leaf beet root tip metaphase chromosomes and karyotype. (The numbers 1 through 9 represent the number of chromosomes). (Source: Sun et al. 2018)

crops, limiting breeding progress (Bosemark 1979). *B. vulgaris* L. ssp. *maritima* L. (Arcang.) has been widely used as a source of resistance gene to specifically complement the breeding pool (Biancardi et al. 2012). Sugar cane competes with sugar beet on the global market. It's no longer enough to make incremental breeding progress to stay competitive. Rather, as the French AKER project aims for, a performance leap is required (Frese et al. 2001; AKER 2017).

10.4.2 Crop Breeding

The sequencing of the sugar beet genome was completed, which provided genomic resources to prob. molecular breeding (Dohm et al. 2014). However, identifying agronomic characters related to adaptive phenotypic capacity via assessment of in situ CWR populations that occur under abiotic stresses should be a key next step in the search for "new genetic variation" that can help sugar beet breeding.

The traditional approach to marker-assisted introgression only allows for the incorporation of a marker (molecular, morphological or biochemical) linked to the desired character (e.g., quality and productivity), ignoring the entire genomic panorama required to understand a plant's adaptive capacity and crop transferability (Monteiro et al. 2018).

10.4.3 Methodologies and Limitations of Traditional Breeding

The first season of early flowering is one of the most challenging issues for beetroot growers. Abu-Ellail et al. (2021) stated that "beet plants are ready to bloom when exposed to low temperatures, then begin to flower when the temperature rises and day length increases, thereby reducing yield. Early management of weeds is critical in reducing competition with the small tender seedlings. Mechanical and hand weeding is usually carried out. Thereafter, plant leaf cover helps to prevent weed growth. The pre-emerging herbicide Chloridazon, marketed as Pyramin, is approved for use on beetroot to control specific broad-leafed grasses each year. A total of 56 different types of beet pests have been identified although, under normal conditions, beetroot has relatively few pests and disease problems. Nematodes and cutworms are the most important pests. Other beetroots pests include beet weevil, beet-leaf fly and beet butterfly, aphids, red spider mites and various leaf-eating insects but no chemicals are reportedly used for their control. However, beetroot can be infected with numerous diseases, the most common being rust, wilt and root rot, as well as mosaic beet virus (Schrader and Mayberry 2003), leaf spot disease, downy mildew and powdery mildew (Harveson et al. 2009). These diseases can become a concern in hot and humid conditions. The use of high-quality disease-free seeds and crop rotation and sanitation help to reduce disease incidence. One measure is to stop growing the crop during months when illness issues occur. According to Abu-Ellail et al. (2019), the salinity of soil and irrigation water is a constraint to growing beets, where the roots are affected and eventually lead to a decrease in the root yield unit area."

10.4.4 Examples for Breeding (Domestication and Selection)

Beetroot has been cultivated and domesticated as a root and leaf vegetable crop for thousands of years. Roots are a cultivated crop that originated as a leaf vegetable developed by the Romans from wild Beta species found in the region of Mediterranean (Goldman and Navazio 2003). The familiar leaf (chard), table, fodder, and sugar beet are all part of the *Beta vulgaris* species complex, which consists of divergent lineages (crop types) (Galewski and McGrath 2020). Domesticated members of the *Beta* complex include leaf crops (leaf beet and Swiss chard), root crops (mangel beet and beetroot), animal feed (fodder beet), and a sugar source (sugar beet) (sugar beet). Beetroot is one of two vegetable species belonging to the *B. vulgaris* genus. Beet breeders created modern beetroot when this root crop spread to northern Europe.

In some cases, F1 hybrids have provided advantages over open-pollinated crops, such as higher root yield (Pink 1993). Both open-pollinated and F1 hybrids are effective methods for creating new varieties that are highly adaptable (Fig. 10.3).

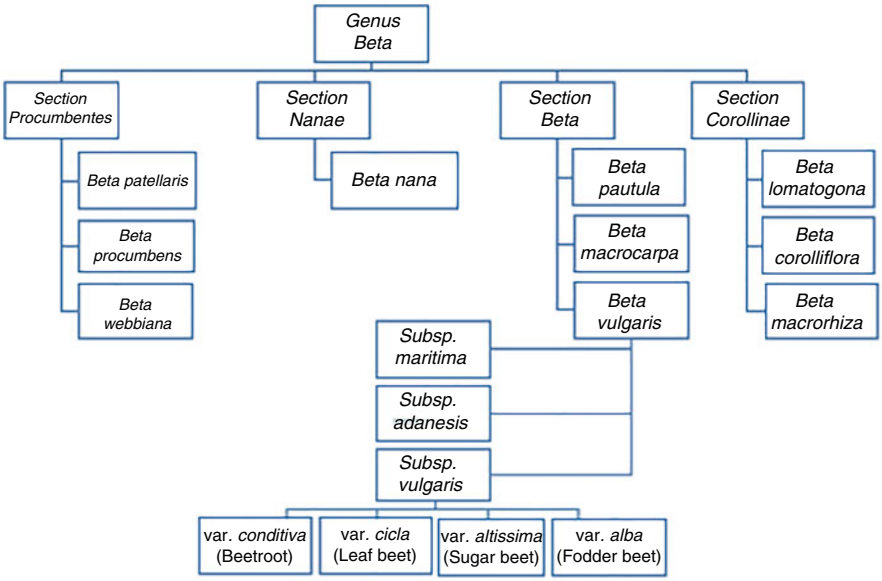

Fig. 10.3 A proposed genomic-assisted breeding system combines genetic and genomic tools. (Source: Abu-Ellail et al. 2021)

10.5 Breeding and Climate

Climatic changes are projected to have a significant negative influence on crop domestication around the world. Because of the unexpected heat and drought seasons brought on by rising global temperatures, spinach agriculture faces significant hurdles. Besides heat and drought stress, increased soil water evaporation is projected to result in an increase in mineral salts in topsoil layers, causing salt stress. Because considerable output losses are projected, new beetroot cultivars which are more resistant to these abiotic stressors are urgently required (Ribera et al. 2020).

10.6 Molecular Breeding

10.6.1 Molecular Marker-Assisted Breeding and Quantitative Trait Locus

Several studies on marker-assisted selection (MAS) have been conducted as a result of using molecular markers in the cultivated beet. Over the last decade, marker-assisted selection has been widely used in a variety of crops. Abu-Ellail et al. (2021) stated that "the related marker is used to perform an indirect selection for desired traits of the target gene (Hospital 2009; McGrath 2010). DNA markers are an

effective tool for genetic studies and significantly contribute to the study of genes and genome mapping (Dohm et al. 2014; Jiang et al. 2015; Wang et al. 2018) and quantitative trait loci (QTLs) (Grimmer et al. 2007, 2008; Wang et al. 2018) and their transmission upon gene transformation. Molecular markers have been used including random amplified polymorphic DNA (RAPD) (Amiri et al. 2009; Tomaszewska-Sowa and Olszewska 2019), restriction fragment length polymorphism (RFLP) (Barzen et al. 1995), amplified fragment length polymorphism (AFLP) (Grimmer et al. 2008; Stevanato et al. 2010), expressed sequence tagged (EST) (Bellin et al. 2002), simple sequence repeats (SSRs) (Abbasi et al. 2015), single-nucleotide polymorphism (SNP) (Grimmer et al. 2008) and specific-locus amplified fragment (SLAF) (Jiang et al. 2015).

Several linkage maps were constructed in *Beta vulgaris* ssp. *vulgaris*, but the availability of markers tends to restrict the utility of genetic maps in public programs. McGrath et al. (2007) constructed a new genetic map from a sugar beet × table beet cross consensus functional map based on many markers. Using linkage group special markers validated in other populations, the primary markers used were AFLPs, which were anchored to the Butterfass chromosome-nomenclature groups. Thus, a common structure was formed which anchors 331 markers, including 23 newly mapped SSRs markers, having a combined total of 526.3 cM among the nine beet linkage groups. Grimmer et al. (2007) developed a new *B. vulgaris* ssp. *vulgaris* linkage map based on several markers."

10.7 Bioinformatics and Gene Bank

Because of beets' unique taxonomic position, its genome sequencing will affect society and science, helping to improve economies by allowing for developing genomic bioinformatics, advanced chemical processing and new beet applications not found in other crops. Furthermore, comparative genomics will enable the development and testing of environmentally friendly yet effective herbicides for chenopod weeds, thereby enhancing agricultural sustainability. Complete genomes have been published by scientists all over the world, and more genome research is recently underway (Rodríguez del Río et al. 2019). Experiments such as these have aided in the understanding of plant evolution and are now being used to enhance new cultivars. To handle, interpret, and use all of this genetic data, scientists require databases and related resources. Bioinformatics is a field of study that combines mathematical algorithms, computers, and statistics with biology principles to analyse different genomes. Abu-Ellail et al. (2021) stated that "Bioinformatics deals with the following data (a) DNA, RNA and protein sequences (nucleotide sequences in DNA or RNA and amino acid sequences in protein) (Bansal 2010); (b) a higher molecular structure (these data are obtained by integrating thermodynamic data and computer simulation with data laboratory technology) (Li et al. 2009); (c) expression data on when and where genes are expressed as well as general gene expression in other cells or under different environmental conditions (Manichaikul et al. 2010; Paradis and Schliep 2018) and (d) bibliographic data such as research projects and genome

sequencing programs collected in public databases. Several libraries of genomic DNA were installed for beet but only a small number of SSR markers were established (Cureton et al. 2002; Richards et al. 2004). An alternative approach solution to Wnd SSRs is the use in public repositories of the growing number of available EST sequences. Reports for the Beta vulgaris ssp. vulgaris genome reference sequence (RefSeq) (https://www.ncbi.nlm.nih.gov/refseq/about) are annotated by the NCBI Eukaryotic Genome Annotation Pipeline, an automated pipeline that annotates genes, transcripts and proteins on draft and completed genomes assemblies. The report provides information on the products of annotation, input data used in the pipeline and the results of the intermediate alignment. The annotation products are available on the sequence databases (www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=3555)."

10.8 Tissue Culture

Plant regeneration from the leaf is applied for breeding propagating for desired features. In spinach breeding operations, digging plants from selection plots and bringing them to greenhouses to make crosses in isolation is common. In the greenhouse, limited transplant survival could be a serious issue. The time-consuming method may be removed with the availability of a micropropagation system, and a single leaf from a chosen plant can be used to regenerate multiple plants, giving a huge number of seeds. The original plant's genome is conserved in the regeneration; therefore, its existence is no longer required (Goode et al. 1988).

Explant sterilisation and seedling formation in the MS nutrient medium, followed by propagation and root evolution, and finally adaptation are the four stages of micropropagation (Fig. 10.4). Mineral element constituents and concentrations in MS growth medium are critical to the technique's success (Adlak et al. 2019). The medium containing 1 mg/L benzyladenine (BA) formed callus from *Beta vulgaris* seedling explants, according to Gürel et al. (2001).

Plant regeneration in spinach is influenced by cultivars and explant types.

Because of plant bolting, in vitro-grown spinach plants are difficult to transplant into the soil. During the shoot induction stage, a low ACR is required for plant regeneration via organogenesis, while auxin is needed to induce root development. Auxin therapy for root induction is time demanding and can result in plant bolting. Plant regeneration by somatic embryogenesis, on the other hand, was successful in a high ACR medium, and auxin was not required during the rooting stage. As a result, plant creation via somatic embryogenesis might be an efficient way of establishing complete plants without the use of seeds. The general steps included in the micropropagation of the beetroot plant were represented in Fig. 10.4.

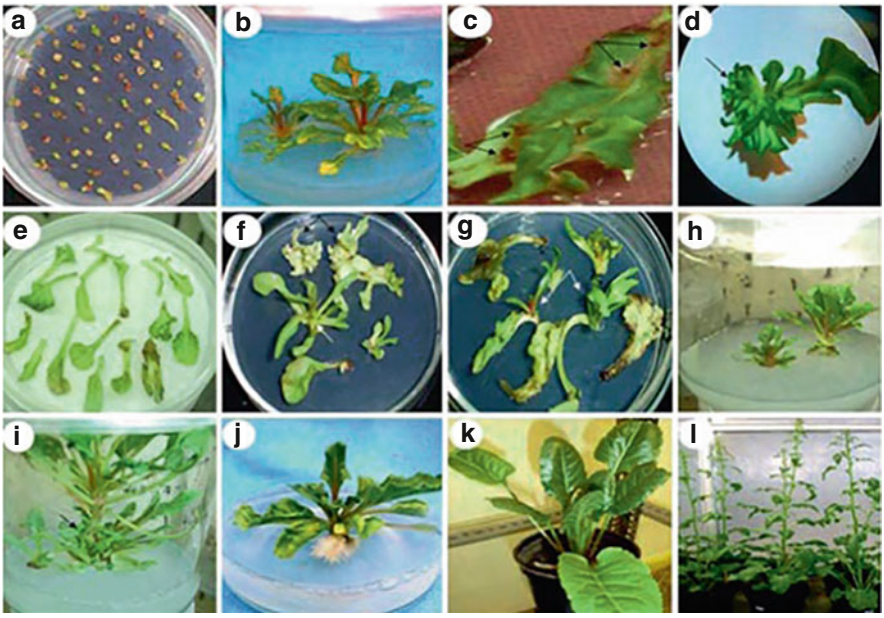

Fig. 10.4 Techniques to modify and regenerate sugar beet. (Source: Abu-Ellail et al. 2021)

10.9 Conclusions

Because of their constituents, which include fibres, bioactive compounds, minerals, and vitamins, vegetables are important parts of a balanced diet. *Beta vulgaris* is economically valuable, and it is used for seed, feed, food, and industrial purposes all over the world. Traditional breeding techniques are expensive and consume time. The modern techniques include introducing new alleles by crossing plants from various plant genetic resources to increase genetic variation. While advances in molecular methods and DNA sequences have attracted beet breeders and geneticists to use new approaches to beetroot biotechnology, traditional breeding methods are still important for the production of new beetroot cultivars with specific characters. Many favourable varieties developed that are resistant to change in climate as well as abiotic and biotic stress. New beetroot breeding pathways are needed to develop new varieties with higher root yields and resistance to abiotic and biotic stress.

There are numerous reasons for more applications in the beetroot development that will lead to various improvements in qualitative and quantitative characteristics. DNA marker production must be closely linked to abiotic and biotic stress, crop yield, physiology, and component characters. The QTLs for both quantitative and qualitative attributes should be identified to improve these characteristics. In addition, germplasm and biotechnology should be enhanced to accelerate and simplify the development of newly qualified genotypes.

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Advances in Lettuce (*Lactuca* spp.) Molecular Breeding Strategies

11

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Abstract

Lettuce (*Lactuca* spp.) is a characteristic annual crop of the Asteraceae family (Compositae). This is one of the most important economical vegetables and is used in salads and sandwiches. Leaves of *Lactuca* are also used to make nicotine-free cigarettes. The seeds and shoots include the consumption of oil and dry latex.

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_11

The Genebanks maintained a huge collection of lettuce germplasm from wild species with the same chromosome number 2n = 18. They have a wide variety of *Lactuca* based on geographical areas and agronomic traits. Promoting new alleles by hybridizing commercial cultivars with the help of national modified genotypes increases genetic diversity and facilitates state filing of interesting traits. The main goal of breeders is to improve lettuce for various desired properties, including stresses and high yield. These goals can be achieved with modern genomic tools using classical breeding methods. The current chapter provides an outline of origin and distribution, economic importance, germplasm diversity and conservation, conventional breeding, molecular breeding, gene editing and genetic engineering, mutation breeding beside hybridization.

Keywords

Genetic diversity · Genetic engineering · Gene editing · Lettuce · Conventional and modern breeding

11.1 Introduction

Lettuce (*Lactuca sativa* L.) is a common leafy vegetable with fresh leaves. Salads and sandwiches are common uses for the leaves. From the Mediterranean to Europe, it grows in a variety of climate and soil conditions (Lebeda et al. 2007). The Middle East is the focal point of the origin of lettuce (Ryder 1986). There are 100 *Lactuca* species, but only four types (*L. sativa* L., *L. virosa* L., *L. serriola* L. and *L. saligna* L.) can be hybridized using traditional methods (Lebeda and Astley 1999; Lebeda et al. 2007). These are self-fertile with chromosome number of 2n = 18 (Doležalová et al. 2002; Grulich 2004). The lettuce crop is an annual and self-pollinating crop that belongs to the *Lactuca* genus, Cichoreae tribe, Asteraceae (Compositae) family, and order Asterales. Nevertheless, up to 5% cross-pollination occurs in lettuce genotypes (George 1999). It absorbs water and minerals through a deep-rooted, horizontal root that is densest at the soil surface. The stems are frequently shorter, and the leaves are grouped in a thick rosette. Different types and shapes of lettuce come in a range of colors, shapes, surfaces, margins, and surface leaves.

The surface of the leaf might be downy, savoy, or curling. Leaves range in color from yellow to dark green. The anthocyanins could be combined to coat the entire leaf or just a piece of it. The late vegetative stage and the beginning of the reproductive stage are signaled by the elongation of the stem. Generally, an inflorescence forms on a stem, a dense red cuticle consisting of numerous heads, each of which consists of various flowers. The number of small flowers is commonly 12–20 but can be 7–35. A flower produces a seed that is ribbed and covered with pappus hair. White, grey, yellow, brown, and black seeds are among the many colors available. Freshly harvested seeds go into dormancy quickly, and nearly all strains have varying degrees of thermal dormancy. Lettuce is an economically valuable vegetable as it is used as a mix in salads and sandwiches, from its leaves for making

nicotine-free cigarettes, from its seeds as cooking oil, and from the dried latex contained in its stems as a sedative. Histology (Dupont et al. 2000). Lettuce is grown from seed under irrigation and rain situations. Conventional breeding contributes to the integration of new alleles by combining genotypes from various genetic sources, such as modern genotypes with national improved genotypes to increase genetic variation and select interesting characteristics like high yields and early maturity, quality improvement and resistance to biotics and tolerance to abiotic stresses related pressures. Even though innovative biotechnological methods utilizing DNA sequencing and advanced DNA techniques are attracting lettuce farmers and geneticists, traditional farming methods are still needed and represent the first entry point for the improvement of promising lettuce genotypes with desirable traits. This chapter provides an outline of the center of origin, the aims and steps of a breeding program, conventional breeding approaches, genetic diversity and new breeding approaches for the development of promising lettuce genotypes with desirable characters.

11.2 Origin, Classification and Distribution

Lettuce belongs to the *Lactuca* (lettuce) genus and the Asteraceae (sunflower or dahlia) family (https://www.itis.gov). Charles Linnaeus previously described the species in the second volume of Species Plantarum in 1753. *Lactuca scariola* var. sativa, *L. scariola* var. integrata, and *L. scariola* var. integrifolia are all synonyms for *L. sativa*. Lettuce (*L. sativa* L.) is a flowering plant within the class Magnoliopsida, order Asteralesand and family Asteraceae. Many edible plants, including sunflowers (*Helianthus annuus* L.) are also in the Asteraceae (https://www.itis.gov). Lettuce (Lactuca sativa L.), lettuce or garden lettuce is of the Lactuca and species.

Kingdom: *Plantae*—Plants
Subkingdom: Viridiplantae—green plants
Infrakingdom: Streptophyta—land plants
Superdivision: Embryophyta
Division: Tracheophyta—vascular plants, tracheophytes
Subdivision: Spermatophytina—spermatophytes, seed plants, phanérogames
Class: Magnoliopsida
Superorder: Asteranae
Order: Asterales
Family: Asteraceae—sunflowers, tournesols
Genus: Lactuca L.—lettuce
Species: *Lactuca sativa* L.—garden lettuce, lettuce"
Common names: Lettuce, garden lettuce

Lettuce is primarily a European also Southwest Asian plant (Pitrat 2012). The origins of lettuce can be traced back to Southwest Asia, although the Mediterranean

Fig. 11.1 Inscriptions on the tomb wall of the Choi church in Abydos (1800 BC, photo from the Archaeological Museum E-Ost, Leiden). (Source: Harlan 1986)

is the epicenter of its cultivation. It has been in use since the year 2000 BC (Hancock 2004). Whitaker (1969) proved that lettuce's origins originated in the eastern Mediterranean, likely in Egypt. Zeven and De Wet (1982) reported that part of the European region of Siberia is an important center of origin for *L. sativa*. Rulkens (1987) understood that the lettuce plant came from Kurdistan-Mesopotamia, not Egypt. According to Boukema et al. (1990), the domestication of lettuce in Southwest Asia took place between Egypt and Iran. This is because (a) many related wild species are found between the Tigris and the Euphrates, while only one related wild species is observed in the Nile Valley: *L. serriola*; (b) The culture of wheat cultivation in Kurdistan-Mesopotamia was identified long before the emergence of the first Egyptian wheat culture, indicating the ancient roots of agriculture (Rulkens 1987). This crop was obtained from the wild lettuce *L. serriola* (De Vries 1997). In old Egyptian farming, lettuce was grown for a variety of reasons, involving extracting oil from seeds, consuming the leaves, or for religious reasons (Pitrat 2012). The history of lettuce is extensive. Salad frescoes in Egyptian tombs and temples, as depicted in Fig. 11.1, are mentioned by Lindqvist (1960a). Lindqvist (1960b) affirmed the presence of some early types of *L. sativa* in Egypt, classifying them as semi-wild rather than cultivated. Lactuca L. has about 100 species, with 17 species in Europe, 10 in North America, 33 in tropical eastern Africa, and 40 in Asia (Lindqvist 1960a). The origin and distribution of lettuce in Egypt are seen in Fig. 11.2. According to Kew Gardens, Table 11.1 displays various types of lettuce.

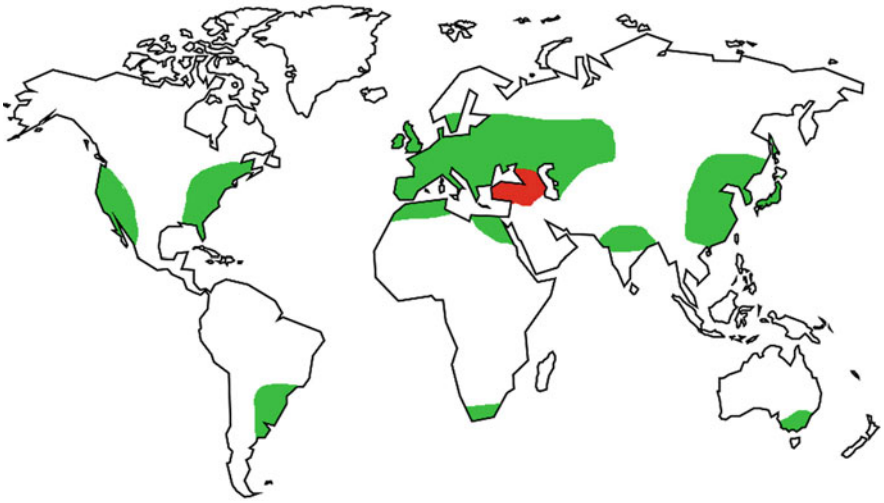

Fig. 11.2 Origin and history of different cultivar groups. The origin of lettuce and its distribution in the world. (Area of Origin: ■ Cultivation Area: ■). Text by Wolfgang Schuchert; Adapted from HTML by R. Saedler (http://www1.biologie.uni-hamburg.de/b-online/schaugarten/LactucasativaL/Lettuce.html). (Source: Harlan 1986)

11.3 Economic Importance

Lettuce (*L. sativa* L.), one of the oldest native horticultural crops (8000–4000 BC), is a popular food crop belonging to the staple crop group (Hancock 2004). It is widely used in salads that are consumed more since they are perceived as beneficial food (DuPont et al. 2000). A wide array of antioxidant compounds like vitamins and fibers are linked with its health benefits (Llorach et al. 2008; Nicolle et al. 2004; Noumedem et al. 2017; Serafini et al. 2002). Anthocyanins and chlorophyll are two phytochemicals that have a role in health qualities (Li et al. 2010). Anthocyanins are abundant in red anthocyanins (Llorach et al. 2008). Metals like calcium and iron are also present (Romani et al. 2002).

Lettuce has a medicinal benefit, and according to traditional Chinese medicine, it is used to treat neuropathy, insomnia, rheumatic pain, dry cough, and anxiety (Araruna and Carlos 2010; Harsha and Kumar 2012, 2013; Katz and Weaver 2003). Guaianolide conjugated with 15-oxalyl and 8-sulfate and deoxy lactucin, which is neuroprotective (Sadeghnia et al. 2012), antioxidants, and anti-inflammatory properties, are the primary components of *L. sativa* extract (Chu et al. 2002; Mulabagal et al. 2010). It's high in vitamins A and C as well as in minerals including calcium, iron, magnesium, potassium, and sodium. If lettuce is cooked for too long, then it would lose vitamin C. It's mostly grown for the young leaves and heads, which are sliced and served in salads. Lettuce, as is customary, is a common foliage plant. The stalk group eats the stems, and the seeds can be used to

Table 11.1 Species of *Lactuca* according to Kew Botanical Garden

Origin	Species	Origin	Species
Crete, Turkey	*Lactuca acanthifolia* Willd Boiss	Asia Minor	*Lactuca aculeata* Boiss
Americas	*Lactuca graminifolia* Michx	Europe	*Lactuca alpina* (L.) Benth. and Hook. f.
Eurasia	*Lactuca altaica* Fisch and C.A. Mey	Africa	*Lactuca attenuata* Stebbins
Asia	*Lactuca dolichophylla* Kitam	Iran	*Lactuca azerbaijanica* Rech. f.
Zaire, Zambia	*Lactuca mwinilungensis* Pope	Africa	*Lactuca nana* (Baker) Chiov.
Asia, Egypt	*Lactuca orientalis* Boiss	Canary Islands	*Lactuca palmensis* C. Bolle
Europe	*Lactuca perennis* L.	North America	*Lactuca biennis* (Moench) Fernald
Africa	*Lactuca calophylla* Jeffrey	Tajikistan	*Lactuca spinidens*
Congo	*Lactuca corymbosa* L.	Eurasia	*Lactuca quercina* L.
Asia	*Lactuca raddeana* Maxim	Europe	*Lactuca aurea* (Vis. and Pančić) Stebbins
North America	*Lactuca canadensis* L.	Crete	*Lactuca alpestris* (Gand.) Rech. fil.
Azores	*Lactuca watsoniana* Trelease	Eurasia	*Lactuca saligna* L.
Western Asia	*Lactuca scarioloides* Boiss	South Africa	*Lactuca dregeana* DC
Angola, Cameroon, Zaire	*Lactuca schulzeana* Büttner	Pakistan	*Lactuca erostrata* Bano and Qaiser
North America—Florida	*Lactuca floridana* L. Gaertn	Cyprus	*Lactuca cyprica* (Rech.f.) N. Kilian and Greuter
Asia	*Lactuca georgica* Grossh	Africa	*Lactuca setosa* Stebbins ex C. Jeffrey
Asia	*Lactuca glaucifolia* Boiss	Africa	*Lactuca imbricata* Hiern
Turkestan	*Lactuca crambifolia* (Bunge) Boiss	Iran	*Lactuca rosularis* Boiss
Africa, Asia, Europe	*Lactuca serriola* L.	North America	*Lactuca hirsuta* Muhl. ex Nutt.
Zaire, Zambia	*Lactuca homblei* De Wild	Asia	*Lactuca indica* L.
Africa, Arabian Peninsula	*Lactuca inermis* Forssk	Eurasia	*Lactuca sibirica* (L.) Maxim.
Albania, Greece, Turkey	*Lactuca intricata* Boiss	Spain	*Lactuca singularis* Wilmott
Jamaica	*Lactuca jamaicensis* Griseb	Angola	*Lactuca stebbinsii* N. Kilian
North America		Africa	

(continued)

Table 11.1 (continued)

Origin	Species	Origin	Species
	Lactuca ludoviciana (Nutt.) Riddell		*Lactuca lasiorhiza* (O. Hoffm.) C. Jeffrey
Armenia	*Lactuca takhtadzhianii* Sosn	Northern Hemisphere	*Lactuca tatarica* (L.) C. A. Meyer
Southern Europe, Morocco	*Lactuca tenerrima* Pourr	Cyprus	*Lactuca tetrantha* B. L. Burtt and P. H. Davis
Asia	*Lactuca triangulata* Maxim	Eurasia	*Lactuca tuberosa* Jacq.
North America	*Lactuca ludoviciana* (Nutt) Riddell	Asia	*Lactuca undulata* Ledeb.
Europe, northern Africa	*Lactuca virosa* L.	Africa	*Lactuca schweinfurthii* Oliv. and Hiern
Africa, Asia, Europe	Lactuca viminea (L.) Presl and Presl		

Source: Bano and Qaiser (2011); Lebeda et al. (2019)

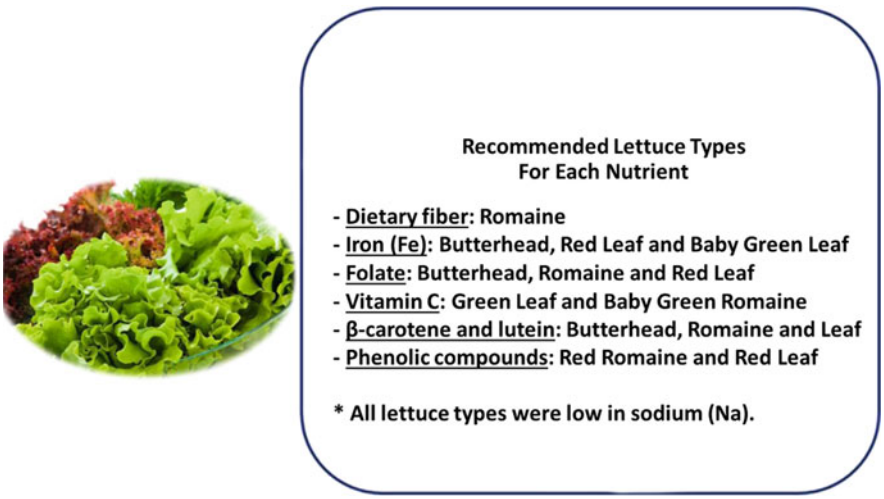

Fig. 11.3 Lettuce nutritional value. (Source: Noumedem et al. 2017)

extract the oil. Because lettuce is grown both outdoors and in greenhouses, it is available all year (De Vries 1997).

The existence of bioactive composites in lettuce has been shown to have anti-inflammatory, hypocholesterolemic and antidiabetic characteristics in vivo and in vitro investigations. Folic acid is prevalent in lettuce leaves. In addition, lettuce is analgesic, diuretic, and expectorant. Lettuce has a high water content (95%) and is low in calories. This is shown in Fig. 11.3 and the nutritional benefits of lettuce expected to its impact on nutritive fiber and the existence of many valuable nutritive

Table 11.2 Nutritional content of lettuce per 100 g

Component	Value	
Energy	55 kj (13 kcal)	
Carbohydrates	2.23 g	
Sugars	0.94 g	
Dietary fiber	1.1 g	
Fat	0.22 g	
Protein	1.35 g	
Water	95.63 g	
Nutrients	**% DV**†	**Quantity**
Vitamins		
Beta-Carotene	40%	166 µg
Lutein zeaxanthin	18%	1223 µg
Thiamine (B1)	5%	0.057 mg
Riboflavin (B2)	5%	0.062 mg
Vitamin B6	6%	0.082 mg
Folate (B9)	18%	73 µg
Vitamin C	4%	3.7 mg
Vitamin E	1%	0.18 mg
Vitamin K	97%	102.3 µg
Minerals	**% DV**†	**Quantity**
Calcium	4%	35 mg
Iron	10%	1.24 mg
Magnesium	4%	13 mg
Manganese	9%	0.179 mg
Phosphorus	5%	33 mg
Potassium	5%	238 mg
Sodium	0%	5 mg
Zinc	2%	0.2 mg

Units: lg = micrograms, mg = milligrams, IU = International units, **DV** = Daily value
Source: Noumedem et al. (2017)

metals, numerous vitamins, and bioactive composites (carotenoids and phenolic compounds) are presented in Table 11.2.

Lettuce is grown as a vegetable in many regions of the world and is produced economically in many more. In Asia, America, and Europe, it is highly valuable as a crop (FAO 2019). The majority of these are dry plants from the Central African mountains, endemic Liana-like species (Stebbins 1937). The crop is grown in a cold climate and can be grown with considerable caution during the warmer season. It grows best in sandy, loamy soils with plenty of organic manure and lime. Excessive soil salt can also harm lettuce, especially during the germination stage. As a vegetable crop, lettuce has commercial value (FAO 2019) (Table 11.3).

Table 11.3 Top producers of lettuce worldwide in 2019

Countries that produce the most lettuce			
Total top 25 countries		28,108,484	
Rank	Countries	Tonnes	% Of Top 25
1	China	16,314,499	58.04%
2	United States	3,688,520	13.12%
3	India	1,262,702	4.49%
4	Spain	1,009,810	3.59%
5	Italy	758,980	2.70%
6	Japan	582,416	2.07%
7	Iran	547,590	1.95%
8	Belgium	527,250	1.88%
9	Mexico	515,647	1.83%
10	Turkey	499,766	1.78%
11	France	469,340	1.67%
12	Netherlands	290,650	1.03%
13	Germany	240,450	0.86%
14	Colombia	239,820	0.85%
15	Niger	216,594	0.77%
16	Australia	133,525	0.48%
17	United Kingdom	111,026	0.39%
18	Tunisia	104,534	0.37%
19	South Korea	91,647	0.33%
20	Guatemala	89,504	0.32%
21	Chile	88,034	0.31%
22	Canada	84,902	0.30%
21	Egypt	81,994	0.29%
24	Mali	79,772	0.28%
25	Peru	79,512	0.28%

Source: FAO (2019)

11.4 Genetic Diversity and Conservation

11.4.1 Genetic Diversity

The importance of genetic supplies in enhancing long-term explanations for fundamental dietary limits and applied on occasion, however, these genetic resources are underutilized due to large-scale issues and a lack of appropriate quantification and classification. Maintaining, evaluating, and characterizing commercial valuable attributes are important needs of a breeding program for any crop. Genetic diversity in horticultural crops is critical for choosing the greatest genotypes to improve production. Both qualitative and quantitative parental characteristics can be selected for outcrossing by heterozygosity or for selecting the desired segregation in subsequent generations. Knowing how the different properties are related forms a

Table 11.4 Analysis of variance for different characters in lettuce

Traits	Source of variation		
	Genotypes (26)[a]	Replication (2)[a]	Error (52)[a]
Days to marketable maturity	152.86[b]	8.160	9.814
Number of non-wrapper leaves	13.02[b]	0.984	0.529
Gross head weight (g)	13.716[b]	3.603	1.786
Equatorial diameter (cm)	2.445[b]	0.118	0.069
Polar diameter (cm)	3.254[b]	0.323	0.248
β-carotene content (μg/100 g)	5.357[b]	0.101	0.058
Iron content (mg/100 g)	0.227[b]	0.025	0.020
1000-seeds weight (g)	0.0016[b]	0.0002	0.0005
Seed germination (%)	47.302[b]	0.405	5.215
Seed vigor index-I	0.000284[b]	0.000005	0.000006
Seed vigor index-II	72.955[b]	1.337	0.612
Severity of bacterial soft rot (%)	67.465[b]	2.942	2.840
Yield per plot (kg)	4.543[b]	0.547	0.396

[a] Value in parentheses is the degree of freedom. Source: Kumar et al. (2016)
[b] Significant at a 5% level of significance

basis for selecting products and components for product improvement. Genetic variability is a fundamental requirement of any horticultural breeding program. Evaluation of variation revealed important differences among genotypes for all characteristics examined in Table 11.4. These differences showed that there is diversity and room for development in the yield and quality characteristics of lettuce (Kumar et al. 2016). DNA markers are useful in determining genetic variation in *L. sativa*. The utilization of several DNA marker techniques for diverse genetic aims for L. *sativa* species is summarized in Table 11.5. Genetic variety, taxonomy, and genetic diversity are among the goals. Figure 11.4 shows the morphology of some valuable lettuce species (Mao et al. 2014).

11.5 Conventional Breeding

11.5.1 Improvement Strategies

Even though current breeding procedures that take advantage of advances in DNA technology have piqued the interest of lettuce breeders, traditional breeding methods remain the most significant and largest stage in producing new lettuce varieties with desirable features. The main goal of lettuce breeding is to create a genotype of lettuce that developed and thrives in a variety of environments. Crop adaptations, abiotic and biotic stress, quality enhancement, and output expansion must all be considered when global concerns develop (Hartman et al. 2014; McCabe et al. 2001). These worldwide lettuce difficulties can be solved by using breeding approaches that allow

Table 11.5 Survey of molecular marker methods used for lettuce (*L. sativa* L.) characterization

Molecular marker	Purpose of work	Reference
Restriction Fragment Length Polymorphism (RFLP)	To construct of lettuce genetic map	Landry et al. (1987)
	To study relations between cultivated lettuce and five wilds-related lettuce; study the origin of cultivated lettuce ((*L. sativa* L.)	Kesseli et al. (1991)
	To study relationships among Cichorium species and related genera of the tribe Lactuceae	Vermeulen et al. (1994)
	Construction of a genetic map of lettuce (*L. sativa*)	Kesseli et al. (1994)
	Molecular genetic markers for assessing the genetic variation and relationships in *Lactuca* germplasm	El-Esawi (2015)
Random Amplified Polymorphic DNA(RAPD)	To analyze the genetic map of lettuce	Kesseli et al. (1994)
	To determine DNA polymorphism in lettuce	Yamamoto et al. (1994)
	Genetic variation with RAPD markers in lettuce (*L. sativa* L.)	Tardin et al. (2003)
	Molecular genetic markers for assessing the genetic variation and relationships in *Lactuca* germplasm	El-Esawi (2015)
Amplified Fragment Length Polymorphism (AFLP)	To study genetic relationships in *Lactuca* spp.	Hill et al. (1996)
	Study of an integrated AFLP map between lettuce species *L. sativa* × *L. saligna* F_2 populations	Jeuken et al. (2001)
	To study the relationship between species in *Lactuca* spp.	Koopman et al. (2001)
	To estimate molecular characterization of a lettuce germplasm collection	Van Hintum (2009)
	Assess genetic diversity within gene bank accessions of lettuce (*L. sativa* L.) using AFLP markers	Jansen et al. (2006)
	To compare molecular markers for the estimation of genetic diversity of lettuce (*Lactuca* spp.)	Van Treuren and Van Hintum (2009)
	Molecular genetic markers for assessing the genetic variation and relationships in *Lactuca* Germplasm	El-Esawi (2015)
Microsatellite	Identify, genetic localization, and allelic diversity SAMPL in lettuce and wild relatives lettuce (*Lactuca* spp.)	Witsenboer et al. (1997)
	To identify variation in wild and cultivated lettuce	Van de Wiel et al. (1998)

(continued)

Table 11.5 (continued)

Molecular marker	Purpose of work	Reference
	To distinguish lettuce cultivars and screen diversity of genetic resources	Van de Wiel et al. (1999)
	To compare targeted molecular markers in ex-situ conserved *Lactuca* variety	Van Treuren and Van Hintum (2009)
	Development of genomic SSR markers of lettuce and mapping genes	Rauscher and Simko (2013)
	Molecular genetic markers for assessing the genetic variation and relationships in *Lactuca* germplasm	El-Esawi (2015)
	Analysis of genetic diversity in purple lettuce by SSR markers	Rui et al. (2020)
Expressed Sequence Tags—Single Sequence Repeats (EST-SSR)	Development of EST-SSR markers for the study of population structure in lettuce	Simko (2008)
	EST-SSR development from 5 lettuce species and their use in genetic variation among *L. serriola*	Riar et al. (2011)
Single Nucleotide Polymorphism (SNP)	SNP-based codominant markers for a recessive gene of resistance in lettuce	Moreno-Vázquez et al. (2003)
	Development of molecular markers for marker-assisted selection of lettuce	Simko et al. (2010)
	Molecular genetic markers for assessing the genetic variation and relationships in *Lactuca* germplasm	El-Esawi (2015)
	New sources of resistance to the lettuce aphid	Walley et al. (2017)
Sequence-related Amplified Polymorphism (SRAP) and Sequence-specific Amplified Polymorphism (SSAP)	To compare anonymous and targeted molecular markers for the estimation of genetic diversity in *ex-situ* conserved *Lactuca* using different molecular markers microsatellite and SARP	Van Treuren and Van Hintum (2009)
	SRAP markers are used to test the distinctiveness, uniformity, and stability of lettuce	Liu et al. (2011)

Source: Hassan et al. (2021)

for the selection of lines with needed genes/QTLs that manage important features (Hartman et al. 2014).

11.5.2 Conventional Breeding Methods and Limitations

In 1900, Mendel's law laid the foundation for the science of plant breeding, which is achieved by combining certain selected traits in a single plant. These features can

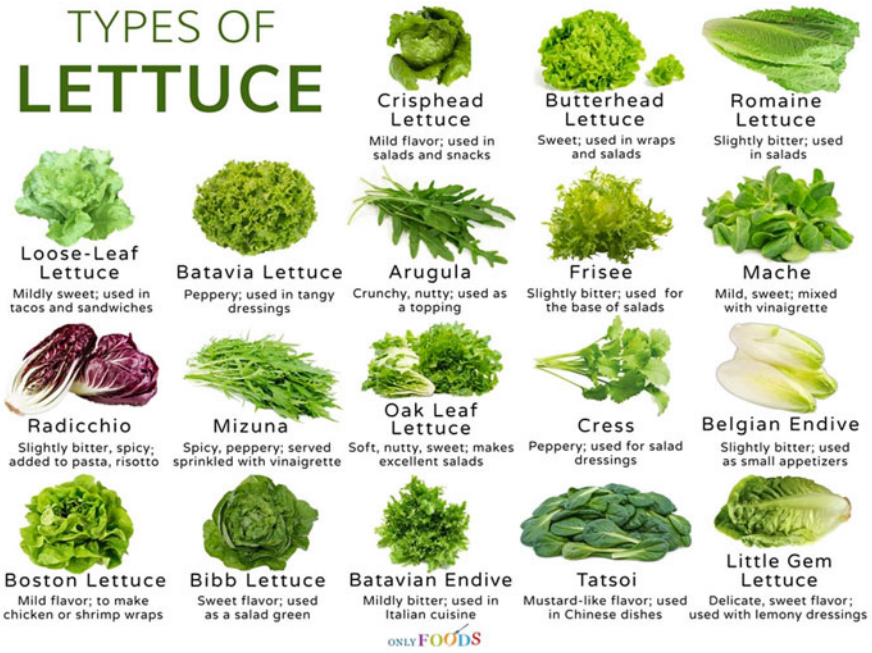

Fig. 11.4 Morphology of some common Lactuca species and their relatives. Botanical names are listed in Table 11.5. (Source: https://www.reddit.com/r/coolguides/comments/j61rd7/types_of_lettuce)

improve head sizing and weight, seed yield, pigment development, biotic and abiotic tolerance and agronomic characteristics. Classical breeding can be identified as the processing of a mixture of chromosomes. In general, there are two main methods of manipulating the chromosomal structures of plants. The first is the selection of germplasm corresponding to reproductive purposes (Gupta et al. 2008; Ragheb 2015; Tashi et al. 2010). The second method is to cross the required attributes discovered in various plants to acquire plants containing the preferred attributes. Crossing aims to combine desired traits in a single plant line by crossing. The most common breeding strategies to improve lettuce are mass selection and selection (Gupta et al. 2008; Ragheb 2015; Tashi et al. 2010), backcrossing (Michelmore 1995; Ryder 1991) and pedigree (Ryder 1986). These approaches aim to provide attractive characteristics, superior yields, great quality, and tolerance to different stresses (Michelmore 1995; Ryder 1986, 1991).

11.5.3 Biotechnology Role

Lettuce (*L. sativa* L.) breeding is primarily focused on improving quality and resistance to early wilt, diseases, and pests. Genetic engineering methods, including

biotechnology and gene transfer, are used to preserve these traits. With the use of these modern methods, a healthy environment has been created by protecting different genotypes, and the limitations of in vivo methods have been overcome by reducing breeding programs. Currently, numerous approaches are applied, including haploid consolidation, organelle formation and somatic hybridization. Haploid transformation can give pure lines. At a similar time, the formation of organogenesis in the stem increases the important properties of lettuce. Somatic hybridization through protoplast fusion gives access to foreign and sexually incompatible germplasm. Somaclonal variation is a different source of a genetic variant. In lettuce production, somatic embryogenesis regeneration is also used, and it has numerous advantages (Sariçam et al. 2017a).

11.6 Molecular Breeding

11.6.1 Marker-Assisted Selection

Quantitative trait loci (QTL) in lettuce have been linked to biotic stress (Christopoulou et al. 2015; Simko et al. 2013) and abiotic stress (Hartman et al. 2014; Jenni et al. 2013) and potential for improvement has been discovered. Since the development of DNA markers in the 1980s, molecular replication has been used (Rafalski and Tingey 1993). Many DNA markers, such as AFLP, RAPD, RFLP, SSRs, SCAR, and SNPs, were created to design a genetic map for crop development. Regions of quantitative trait loci (QTL) containing genes for a specific quantitative trait (plant height) within the genome can be detected utilizing DNA tags and genetic mapping (Collard et al. 2005). When QTLs and their associated DNA markers for important agricultural traits have been confirmed, the DNA markers can be applied as the molecular procedure for marker-assisted selection (MAS) in a breeding program (Collard and Mackill 2008). Positions of quantitative parameters linked with lettuce seedling root growth (Roberts et al. 2020). The lettuce genome resource recently released an annotated lettuce genome sequence alignment (Reyes-Chin-Wo et al. 2017; https://lgr.genomecenter.ucdavis.edu). Biochemical markers were also utilized to find out the relationships between wild lettuce and lettuce germplasm (Cole et al. 1991; Collard and Mackill 2008; Dziechciarková et al. 2004). In inbreeding research, molecular markers can be used in a variety of ways. Landry et al. (1987) used 41 RFLP loci and three phenotypic markers to create a lettuce linkage map. Kesseli et al. (1994) found a genetic association map for RFLP and RAPD in *L. sativa*. Jeuken et al. (2001) developed an interspecies AFLP map for lettuce that included F_2 populations of *L. sativa* x *L. saligna*. Truco et al. (2007) created a high-intensity map of 2744 DNA markers by combining seven lettuce linkage maps.

11.6.2 Functional Genomics

In lettuce, little research was done on efficient genomics. The expression of genes responsible for stress tolerance has been developed using functional genomics. The genome of Lactuca sativa was recently published (Reyes-Chin-Wo et al. 2017), however references to the genomes of *L. saligna, L. serriola,* and *L. virosa* should be available to the public shortly, and large-scale resequencing studies to conserve *Lactuca* germplasm sources are currently underway. A large amount of DNA sequencing information should open the entrance to functional genomics methods that will finally lead to the selection of plants into improved lettuce genotypes for more sustainable yield and better nutritious quality for customers. But, such methods can only be effective if comprehensive, high-quality information on the phenotype is available (Still 2007).

11.6.3 Bioinformatics

Lettuce bioinformatics is available on the internet and is easily accessible. Several databases are available for this plant, involving the "Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg)", where transcriptome and proteome data can be browsed including the structure of the protein and gene function. On the other hand, the Uniport database (https://www.uniprot.org/) provides information on the complete genomes of unassembled Shotgun. Meanwhile, NCBI (https://www.ncbi.nlm.nih.gov/) can be applied for all bioinformatics functions. It also collects information on microsatellites.

11.7 Genetic Engineering and Gene Editing

11.7.1 Improved Methods and Features

Genetic engineering is done to directly control lettuce genes. It is supposed to maintain conventional breeding approaches by improving the efficiency of lettuce yield and preventing damage due to biotic and abiotic stress by developing highly tolerant strains under various conditions. Genetic engineering presents the occasion to enhance lettuce productivity and yield by applying for advances in genome sequencing and molecular breeding. It starts with extremely effective and vigorous transformation approaches that target sequence-specific nucleases. Transformation systems like Agrobacterium tumefaciens have been effectively utilized for lettuce. The results obtained in genome editing with tools such as Cas9 technology promise higher productivity and yields, so the genetic engineering of lettuce must surpass conventional transformation approaches. Numerous findings on lettuce have recently been issued (Chen et al. 2018; Woo et al. 2015).

11.7.2 Genetically Modified Lettuce Varieties

Gene transformation can be applied to expand the genetic base of germplasm accessible for conventional methods. It is applied to reduce the period it takes to introduce traits of one gene interested in an economically important plant. Lettuce (*L. sativa* L.) is one of the greatest essential leafy horticulture crops in the world. It is highly reactive to a variety of plant growth hormones in tissue culture and many genotypes representing different genotypes achieve stem regeneration. Genetic manipulation of lettuce requires a reliable, efficient and genotype-independent transformation method (Curtis et al. 1994; Dan et al. 2014). Plant diseases and pests are among the obstacles to the farming and production of lettuce crops. Plant diseases greatly affect the quality and yield of the lettuce crop. For example, *L. sativa* Sclerotinia was introduced into the lettuce world, severely damaging the central part of the stems and leaves. For this reason, numerous specialists have dedicated themselves to the analysis of genetically modified lettuce. Different sorts of genes have been transmitted to lettuce and are stably expressed and inherited from the offspring (Dan et al. 2014). Many genetic transformation processes have to withstand many biotic or abiotic stresses. Additionally, genetic transformation in lettuce served to improve vegetative traits. The whole is summarized in Table 11.6.

The goals of modern lettuce cultivation begin with the development of genotypes that can resist insects and disease, enhance quality and increase productivity. For instance, lettuce transformed through the *ipt* gene, resulting in a significant delay in leaf growth and senescence in mature tufts (McCabe et al. 2001). Cotyledons grown 48 h after seed germination were transferred to an MS medium augmented with 0.1 mg/L BA and 0.1 mg/L IBA for callus induction and the callus was transferred to an MS medium comprising 0.1 mg/L of BA for indirect regeneration (Mohebodini et al. 2011). Two genotypes were used in this experiment, Central (Yazd) and Southwest (Ahvaz), which have great potential for biotic and abiotic stress. The symptoms of swelling and stretching occurred 3 days after cultivation. Table 11.7 shows the influence of the explant stage and numerous concentrations of growth

Table 11.6 Effects of genotype, explant age and growth regulators on callus formation (%) of lettuce (*L. sativa* L.) after 3 weeks of sowing

	Genotype			
Medium	Ahvaz		Yazd	
0.1 mg/L BA and 0.1 mg/L IBA	3 days	7 days	3 days	7 days
M1	10i	73bcd	60de	86ab
M2	22ghi	32gh	89ab	37 g
M3	40 fg	37 g	56ef	34gh
M4	19 hi	27ghi	95a	23ghi
M5	23ghi	36gh	73bcd	28gh
M6	38 g	56ef	60de	74bcd
M7	66cde	62de	81ab	83abc

BA benziladenine; *IBA* indole-3-butyric acid
Source: Mohebodini et al. (2011)

Table 11.7 Transgenesis in lettuce for multiple purposes

Gene	Expression profile	Expression system	Vector	Reference
GUS-intron gene	Genotype-independent transformation	*A. tumefaciens* LBA4404	pMOG23	Curtis et al. (1994)
Mutated *P5CS* gene	Freezing Resistance	*A. tumefaciens* LBA4404	pBIF414	Pileggi et al. (2001)
rbcL and accD	Achieve plastid transformation	Gene bombardment	pRL200	Kanamoto et al. (2006)
pCR2·*1TOPO*	Expression of an oxalate decarboxylase gene	*Agrobacterium* strain EHA 105	pUC19-35SAMVNOS	Dias et al. (2006)
GmPIP and *LsPIP* genes	Drought tolerance	*E. coli* (DH5 α)	pGEM	Porcel et al. (2006)
pta gene	Provide an alternative method for the molecular analysis of gene functions in lettuce	*A. tumefaciens* LBA4404	pBIPTA	Ahmed et al. (2007)
CTB-Pins	Expression of cholera toxin B-proinsulin fusion protein	Gene bombardment	pLD-5′UTR-	Ruhlman et al. (2007)
Genes codes for the surface antigen *HBsAg*	Resistant to Hepatitis B. I-113 V	*A. tumefaciens*	PG35SHBsAg	Jackson and Ekkehard (2008)
Neomycin phosphotransferase II gene and *ESAT6*	Lettuce plants containing anti-tuberculosis genes (bacterial antigen).	*A. tumefaciens* (strain GV3101)	pCB063 and pCB064	Matvieieva et al. (2009)
fbp/*sbp*	Spectinomycin-resistant shoots	Gene bombardment	pRL200	Ichikawa et al. (2010)
GCHI	Enhance shooting rate	*A. tumefaciens* strain pGV3101	pBI121	Fallah-Ziarani et al. (2013)
*Pro*A-Pins fusion gene	Express *IgG*-binding protein A and human pro-insulin as a fusion protein	*A. tumefaciens* LBA4404	pCAMINS	Mohebodini et al. (2014)
Brassinosteroid insensitive 2 (*BIN2*)	Plant development	Polyethylene glycol (PEG)	CRISPR/Cas9	Woo et al. (2015)
Flowering locus T (*LsFT*)	Analysed the Flowering locus T (*LsFT*) gene during bolting regulation in lettuce	*A. tumefaciens*	pFGC1008	Chen et al. (2018)

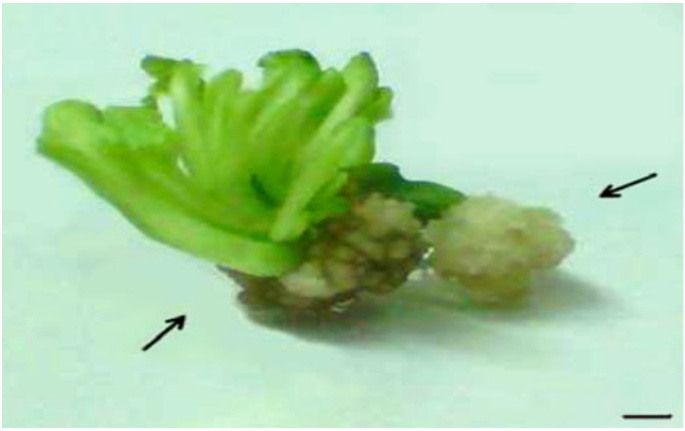

Fig. 11.5 The best place for direct regeneration of plants in lettuce explants is near the cotyledons. (Source: Mohebodini et al. 2011)

regulators on the mean callus ratio. Callus initiation started one week after the initiation of culture and was seen in the whole media. The impacts of genetic resource, explant stage and different growth regulator concentrations on immediate plant regeneration are presented in Table 11.7. Direct shoot regeneration was observed within 14 days of cultivation. The shoot buds appeared at the very cut ending of the area close to the petiole of the cotyledon explants. Several buds formed within 21 days of starting cultivation Fig. 11.5.

The major goal of the breeding programs is to breed a promising high-yielding variety that is resistant to biotic and tolerant to abiotic stress. To reach this, breeders tried to modify the plant's genome by enhancing random mutations or by modifying certain genes in the genome by applying various methods such as EMS mutagenesis, T-DNA insertion, zinc finger nucleases (ZFN) and the TAL effector (TALENs). Nevertheless, various complexities were encountered in utilizing the above approaches, as well as high costs and long lead times due to the requirement for protein engineering. A recent genome-editing procedure known as "Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein 9 (Cas9)" was developed to defeat the complexities of earlier approaches. The CRISPR/Cas9 endonuclease procedure has become common for accurate genome editing of organisms for precision and comfort of use. CRISPR/Cas systems can lyse in living cells with different genetic material. Among these, the nucleoprotein procedure is of particular importance for the enzymatic action in a quicker time and the control of its action (Park et al. 2019).

11.8 Spread of Mutation Breeding

The mutation was defined by Koornneef (2002) as the procedure by which genes are permanent, natural or induced. It can happen at the chromosomal or genetic level. A trait can be missing or acquired due to mutation, so breeders applied the mutation procedure in plant selection to improve new promising lines with certain characteristics. Mutations can be caused by chemical or physical factors. The greatest common mutagens are physical mutagens like X-rays and ultraviolet light. Till et al. (2007) give examples of chemical mutants such as "nitric acid, sodium azide, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), and diethyl sulfate (DES)". They have also been applied to cause mutations in various plants. Recently, Bertier et al. (2018) reported that CRISPR/Cas9 is a transformative implement for aimed genetic modifications. Great mutation efficacies of primary transformers have been described in various plants. Nevertheless, various of the mutations evaluated were autosomal and consequently not genetic.

11.8.1 Traditional Mutagenesis

Genetic differences are important for lettuce breeding programs. The inherited habitat of lettuce results in relatively little genetic variation in yield compared to cross-pollinated plants. A variation is a useful implement for creating new attributes in lettuce plants and can be categorized as congenital and pathogenic mutations. Natural mutations also occur in the crop plant and its wild ancestors, albeit slowly, and the resultant valuable traits can be chosen for human demands. Mutagens are applied to raise mutation rates or to create mutations that are unavailable from natural sources (Mou 2011). Michelmore et al. (2002) reported that EMS, fast neutrons and gamma rays were applied to treat seeds of 'Diana' and 'Saffier' lettuce cultivars to find mutagenic tolerance to some herbicides like carfentrazone, rimsulfuron, imazamox, glucosinate and glyphosate.

The mutation frequency of chemical mutations in physical space is 103 times higher than that of natural changes. Studies on lettuce breeding focus on leaves, narrow head, existence or lack of anthocyanins, disease resistance and soil mass, great quality under stressed situations, early maturing, performance and acclimatization to various regions and ecological situations (De Vries 1997; Mou 2011). The current work was performed to create a list of lettuce mutagenicity analyses (cv. Cervantes). For this objective, "surgical rate of Co60 (gamma rays) of 0, 50, 100, 200, 300, 400, 500 and 600 were treated on lettuce seeds as somatic mutations. Thirty seeds were utilized per dose. Thirty days later the treatment, the germination and growth of the carnation buds were assessed. The effective modulator (EMD50) was calculated by linear regression analysis. Based on the findings, a dose of 372.66 Gy was determined as the EMD50" (Sariçam et al. 2017b).

11.8.2 Induced Mutagenesis In Vitro

Mou (2011) reported that *L. serriola* and *L. sativa* can interbreed and that the chromosomes of the two species are morphologically very similar. Some consider these two species to be two subspecies of identical species. The mutation-induced variations in *L. serriola* likely resulted in promising attributes that were attractive to humans, especially the non-spiny shapes on stems, leaves and large seeds. These signs are "the number of spines on the stems, leaves and large seeds". They were then chosen to reproduce and further modify themselves to gather human requirements. These primary forms may have suited the practice of seed oil for household use in goods and services. The majority of these grow and improve quickly and contain non-reflective materials to avoid seed cracking, large seeds and superior seed oil content (35%).

11.8.3 Mutation Molecular Analysis

There are two main approaches to molecular mutation analysis in molecular mutation analysis (Candela et al. 2015), (1) mass DNA sequencing and detection of mutations with connections to polymorphous lineages, and (2) identification of mutations by bulk DNA sequencing and backcrossing isogenic lines. Huo et al. (2016) reported a case molecular study of mutant lettuce lines as follows, two independent lines of mutant lettuce seed lines, TG01 and TG10, were generated by mutations in ethyl methanesulfonate (EMS). Physiological and genetic analyzes showed that mutants were allelic and recessive. Apply a separate pooled analysis to the entire genome sequence to identify causative genes. For every mutant, DNA samples collected from heat-stable (mutant) spacer seeds were sequenced and analyzed for homozygous single nucleotide polymorphisms (SNPs). Classical genetic mapping proved that the causative mutations were positioned near the ZEP/ABA1 gene, but a separate genome-wide sequencing approach more efficiently identified the specialized gene liable for the phenotype.

11.8.4 Improved Traits and Varieties

Mutations played a critical role in the domestication of the crop. Various attributes originating from natural and stimulated mutations, like early flowering, dwarfism, chlorophyll deficiency, and male infertility are beneficial for physiological and genetic analyses. The mutations have also been utilized to improve recent lettuce products, which contain herbicide-tolerant and miniature cultivars. Mutation evaluation has been instrumental in lettuce genetic analyses, containing the detection and cloning of disease-resistant genes. Mutations mixed via genomic technology can offer great implements for discovering new alleles. Mutations have profound effects on the development of self-pollinating plants like lettuce. Essentially, it is a procedure of detecting genetic differences that can later be applied in physiological or

genetic analysis and cultivar improvement. Editing qualitative traits driven by key genes like disease resistance and quality is more effective than quantitative attributes such as productivity and adaptability (Mou 2011). Various mutagens have been used to enhance the properties of lettuce like EMS, gamma rays, and fast neutrons. It has been applied to treat the seeds of some lettuce genotypes that increase tolerance to herbicides: imazamox, glufosinate, rimsulfuron, carfentrazone and glyphosate (Michelmore et al. 2002). EMS produced and described mutant biosynthetic flavonoids in lettuce with promising health advantages. These flavonoid profiles described by a recording accumulation of kaempferol and naringenin chalcone can turn lettuce into food with health advantages. Nevertheless, clinical trials in animals and humans are required to prove the health advantages of the superior flavonoid lettuce genotypes discussed here. Advanced mutagenization and choice approaches that produce superior amounts of useful phytochemicals might be an essential approach to enhancing the value of conventional crops (Gurdon et al. 2019).

11.9 Hybridization

11.9.1 Classical Hybridization

Attempts to breed F1 hybrids have been unsuccessful because *L. sativa* pollen is heavy, sticky, and does not transfer simply. Hybridization was therefore based on three principles: (1) pedigree, (2) backcrossing, and (3) single plant or mass selection. For all plants to flower, different parents stumble on hot sunny days, flowers bloom in the morning (Acquaah 2012). The weather will be delayed on cloudy and cool days. Lettuce contains 10 to 20 florets that form the flower head (Mou 2008). The hybridization took place (Hooftman et al. 2005; Ryder 1997). F2 seeds can be produced by self-tapping from a single F_1 plant. F_2 seeds are planted and 200 seedlings are randomly chosen, transplanted and genotyped. The plants are self-pollinated and F_3 seeds are collected for every F_2 plant (Uwimana 2011).

11.9.2 Somatic Hybridization

Electrical processes and polyethylene glycol (PEG) could be applied to allow the lettuce protoplast to fuse simply (Siddiqui 2014; Taniguchi et al. 1990). The protoplasts could be achieved from lettuce grown in glasshouses and growth chambers and form shoots, roots or cotyledons of seedlings grown in vitro (Berry et al. 1982). The somatic crossing was carried out using polyethylene glycol (PEG) and electrofusion to allow culture work in four types of media. While protoplast damage has been observed with other treatments, chemistry and electrofusion of micro and macro colonies have been shown to produce significant results for combined protoplasts when treated with numerous treatments for every replication. Electrofusion has also been shown to cause protoplast fusion at a frequency of 40.51% compared to PEG (Siddiqui 2014).

11.9.3 Hybrid Varieties

Lettuce contains a wide variety of landraces and ancient varieties held in global genebanks, and seven major genotype groups grown in different regions (Lebeda et al. 2007). They are often defined as morphotypes. Traditional and modern breeding methods provide new varieties for the certain requirements of growers and customers. The principal gene pool of *L. sativa* is represented by its various genotypes, primitive landraces, and non-transitional wild species: cosmopolitans (*L. azerbaijanica*, *L. aculeata*, *L. serriola*, *L. scarioloides*, *L. azerbaijanica*, *L. georgica*, *L. Taica*) from Asia and *L. dregeana* from South Africa (Zohary 1991).

11.10 Conclusion and Prospects

Lettuce is a common and inexpensive vegetable. It has several regions of origins. It is stated in the ancient Egyptian civilization. Lettuce contains various nutrients such as vitamin A, potassium, calcium and folic acid. Because of the commercial value of lettuce, many findings have concentrated on the domestication of several varieties. Quantitative trait loci (QTLs) associated with biotic and abiotic stress were detected in the lettuce plant, resulting in better tolerance of lettuce to various stress factors. There are still various explanations for future investigation into the improvement of lettuce. These explanations concentrated only on nutritional adequacy and vitamin supplement. It is necessary to work on improving the qualitative and quantitative qualities of lettuce. These properties may contain improvement and rising vitamin content and regulate mineral absorption through an osmotic balance in extracellular and intracellular elements. There may be other cross-sectional studies linking different vegetables with consumption. You can also increase your intake of this nutritious vegetable, as lettuce is high in fiber, sugar, protein, vitamins and minerals.

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Integrated Use of Molecular and Omics Approaches for Breeding High Yield and Stress Resistance Chili Peppers

12

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Abstract

In a rapidly changing environment along with the growing population, crop production and improvement are the most sought-after sectors in the world. Pepper (*Capsicum spp.*) is a highly consumed plant species in the form of spice or whole fruit and is widely popular for its pungent flavour and vibrant colours worldwide. Like other crop plants, pepper is also severely affected by environmental and genetic constraints and reflects a massive loss in yield, quality and production rate. Therefore, it is imperative to immediately change our focus towards the development of stress-resilient and high-yielding *Capsicum* varieties. This chapter discusses the integrated use of conventional, molecular and multi-omics tools towards meeting the end goal of *Capsicum* trait improvement. Conventional breeding programmes have utilized natural sources of genes and QTLs attributed to biotic and abiotic stress tolerance and other important horticultural traits in pepper improvement which involve time-consuming multiple and tedious crossing cycles followed by selection. The development of reliable molecular markers like SSRs, SNPs, InDels etc. have enabled genetic and biparental QTL mapping, and genome-wide association studies which all have not only helped in the rapid identification of significant QTLs governing important traits but also marker-assisted selection of desired phenotypes. Several candidate genes for *Capsicum* plant and fruit morphology, metabolite content,

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_12

disease resistance and stress tolerance have been identified and are available for introgression via marker-assisted breeding and genetic engineering into elite genetic backgrounds. Further, genome and transcriptome sequencing projects have changed the tides for advancement in *Capsicum* research and provided new insights into *Capsicum* genome structure, size, features and function. Other omics approaches like epigenomics, proteomics and metabolomics are rapidly evolving to reveal transcriptional, post-transcriptional and post-translational modifications in *Capsicum* genes and proteins. In brief, established breeding strategies combined with new technological hues can be successfully exploited for breeding high-quality, better performing, stress-resistant and sturdy *Capsicum* varieties to meet the needs of the growing human population.

Keywords

Capsicum · Stress resistant · Genomics · Transcriptomics · Metabolomics · Proteomics · Epigenomics

12.1 Introduction

The genus *Capsicum* belonging to Solanaceae is a main vegetable and spice crop originating from tropical regions of central and south America and is now grown all over the world (Basu et al. 2003; Bosland and Votava 1999; Davenport 1970). Commonly known as chili peppers, this genus consists of more than 35 species out of which only five, i.e., *Capsicum annuum, C. chinense, C. baccatum, C. pubescens,* and *C. frutescens* are cultivated (Kim et al. 2014; Ramchiary et al. 2014). Chili peppers propagate through seeds, and are perennials although nowadays also grown as annual crops. Flowers of Capsicums are hermaphrodite and protogynous, usually self-pollinated, with some percentage of cross-pollination being reported. The presence of wide diversity of chili peppers for traits such as fruiting habits, fruit size and shape, metabolites content and other agronomic traits are reported (Ramchiary et al. 2014, Sarpras et al. 2016). Chili peppers are important component of the human diet due to their high content of various metabolites and health-beneficial compounds (Shetty 2013; Sarpras et al. 2016; Ahmad et al. 2021).

12.2 Economic Importance of *Capsicum*

Pepper or chili is consumed by humans all around the world. Some parts of the eastern countries prefer the spicy trait and wholly consume chili for its pungency. Global production of chilli has considerably elevated in the past two decades and both dry, as well as fresh chili, are produced and marketed in million tons. Countries like China and Mexico are leading producers of chilli (www.fao.org/faostat). Fresh

pepper is mostly produced by African, European and American countries (upto 12%) and Asian countries are the highest producers of dry chili contributing up to 70.3% (Di Dato et al. 2015). They are consumed as spice or condiments as powder or salsa in several diverse dishes in many countries. Fruits are processed as sauces, pickles, paste, beer and depending on the type of fruits and their nutritional contents, they are commercially marketed as bell peppers, jalapeno, pasilla, Hungarian wax, Jwala, cayenne and several others, depending on the country. Pepper extracts are used in cosmetics, pharmaceuticals, used as a spice, and also grown as ornamental plants in pots or gardens.

12.2.1 Unique Properties of Chili and Their Economic and Pharmaceutical Uses

Chili plants are defined by the presence of specific alkaloids, capsaicinoids, in their fruits which impart pungency. Pungency is highly variable among fruits depending on the genotype and the influence of the environment. Capsaicinoids are formed from the integration of two pathways, a phenylpropanoid and a fatty acid pathway, catalyzed by a condensation reaction by a final enzyme *CAPSAICIN SYNTHASE (CS)*, an acyl transferase (Stewart et al. 2005). Capsaicin and dihydrocapsaicin are the two primary capsaicinoids that contribute upto 90% of pungency to the fruits of chili. Some studies have reported the pharmacological properties of Capsaicinoids as anti-inflammatory, anti-oxidant, anti-cancerous and anti-obesity (Luo et al. 2011). Apart from that, the fruits of *Capsicum* are highly rich in compounds that impart colour such as flavonoids, capsanthin, capsorubin, violoxanthin, lutein and beta-carotene (Gómez-García and Ochoa-Alejo 2013). Chili peppers are also reported to have antimicrobial and antifungal properties. They are also used in cosmetics, as pepper spray and for pest management (Muhyi and Bosland 1995). The powder obtained from chili fruits is also used as food colouring agents, as paprika and in food processing in industries (Kumar et al. 2006).

12.3 Diversity of Capsicum Species

12.3.1 Morphological and Genetic Diversity Among Different *Capsicum* Species

Genetic diversity is important for crop improvement. Several studies around the world reported wide diversity present within and between *Capsicum* species (Finger et al. 2010; Datta and Das 2013; Ramchiary et al. 2014; Sarpras et al. 2016; Dutta et al. 2017; Andrade et al. 2020). Of the five species reported to be domesticated, however, mostly *C. annuum, C. baccatum, C. frutescens* and *C. chinense* are cultivated (Kim et al. 2014). These species differ in their morphology, especially the fruit size, shape and metabolite content including the level of pungency which vary among species and within genotypes (Fig. 12.1). *C. chinense* harbours the

Fig. 12.1 *Capsicum* germplasm showing variations in fruit morphology, colour, shape etc.

highest levels of pungency among the different species of chili. Finger et al. (2010) reported the presence of diversity in 49 *C. chinense* accessions of Amazonian regions, while Sarpras et al. (2016) reported the morphological and metabolite diversity in 136 *Capsicum* germplasm belonging to *C. annuum*, *C. chinense* and *C. frutescens*. Sudré et al. (2010) in their study reported the presence of variation in 56 *Capsicum* germplasm from Brazil. Their study aided in differentiation and categorization of different *Capsicum* varieties into their respective species i.e., *C. annuum, C. frutescens, C. baccatum* and *C. chinense* (Sudré et al. 2010). Along with morphological diversity studies, molecular markers have been widely used to characterize and understand the genetic diversity present in different *Capsicum* species (Dias et al. 2013; Jaiswal et al. 2020; Chhapekar et al. 2020). Also, using Unigene Pepper GeneChip, the polymorphism of diverse *C. annuum* fruits could be studied which showed the presence of population structure and genetic relatedness/distances (Hill et al. 2013). Some cultivated species of chili such as *C. baccatum*

have been reported to be highly variable in their traits due to the influence of its geographical distribution (Albrecht et al. 2012). Around 116 *C. baccatum* germplasms were assayed for understanding the presence of genetic variation using AFLP markers and fruit morphological traits in Brazil (Cardoso et al. 2018).

The diversity at the genome level was studied in 373 accessions belonging to 11 species of chili peppers collected from 51 countries by sequencing approx. 1.8% genome (Colonna et al. 2019). This study identified 746 K polymorphic sites which helped into subdivision of those accessions into different clades and species. Further they have identified the variations present between the large and small fruit accessions of *C. annuum* (Colonna et al. 2019). Du et al. (2019) reported re-sequencing of 35 different *C. annuum* lines and identified the genetic variations present among those lines including 92 SNPs that determine the fruit shape. A diallel genetic analysis has also been adopted to evaluate the morphological variation and fruit biochemical traits in chili peppers (Aiswarya et al. 2020). Several QTLs that were associated with resistance to fungal pathogen *Phytophthora capsici,* and also the formation of corolla in floral development were identified in commonly cultivated species of *C. annuum* (Lu et al. 2012). SSR markers spanning the whole genome from 51 accessions of nine *Capsicum* species have also validated the 381 allele populations that represented the morphological species variations among *Capsicum* varieties in Spain (González-Pérez et al. 2014). In a large-scale genotyping of 3821 accessions of different *Capsicum* species, *C. annuum* showed the highest frequency of genetic diversity compared to other germplasms (Lee et al. 2016a, b). Some high-resolution melting analysis of chili accessions was performed in Ethiopian germplasms to categorize one *C. baccatum*, 9 *C. frutescens* and 132 *C. annuum* among the 142 germplasm studied (Solomon et al. 2019). Twenty SCAR markers were also developed to determine the morphological variation associated with male sterility in peppers (Jo et al. 2019).

12.3.2 Characterization of Different *Capsicum* Species Based on Metabolic Diversity

Capsicum species are highly variable in their metabolites content (Wahyuni et al. 2014; Sarpras et al. 2016, 2019). Species of *Capsicum* are rich in many bioactive compounds. They possess many bioactive properties due to the presence of bioactive compounds. Considerable variation in the quality and quantity of bioactive compounds in various species of *Capsicum* is observed (Rodríguez-Burruezo et al. 2009; Vera-Guzmán et al. 2011). Rodríguez-Burruezo et al. (2009) have found that *C. baccatum* is rich with health beneficial antioxidant compounds. Notable signature metabolites include the carotenoids, capsaicinoids, flavonoids and vitamins. Apart from morphological differences such as fruit colour, size, shape etc. and genetic diversity analysis using molecular markers such as AFLP, SSRs, SNPs, etc. categorizations of different chili accessions were also performed based on various fruit metabolites content (Wahyuni et al. 2014; Sarpras et al. 2016, 2019). The metabolites such as carotenoids, capsaicinoids, glycosides, presence and

absence of vitamins and pro-vitamins also served as factors that differentiate chili plants into different cultivars. The presence of wide variations in different metabolites content in fruits in 32 accessions of chili peppers were studied and reported by Wahyuni et al. (2011, Wahyuni et al. 2014). Sarpras et al. (2016) reported the variations of global metabolites content including capsaicinoids (pungency) content in fruits of 136 *Capsicum* germplasm belong to *C. chinense*, *C. frutescens* and *C. annuum*. They reported the diversity of metabolites content within and between species. Dutta et al. (2017) studied 72 *C. frutescens* accessions from Northeast India and observed variations of total flavonoids and phenolics content. Andrade et al. (2020) in their study of 192 *Capsicum* accessions, reported the diversity present in metabolites contents such as total flavonoids and phenolics content which also influenced the antioxidant assays.

12.4 Development of Molecular Markers in *Capsicum*

As in other crop species, in *Capsicum* crop also, several molecular markers have been developed (for details see review by Ramchiary et al. 2014; Ibarra-Torres et al. 2015). Of the several markers developed, simple sequence repeats markers (SSRs) are highly preferred owing to their ease to genotype, presence of polymorphism, robustness and less cost involved. Cheng et al. (2016b) reported the identification of a total of 876,580 SSRs in the Zulna-1 genome of which 739,723 could be used as potential SSR markers. Each chromosome had about 56,901.77 SSR units and the highest density of SSRs was found on Chromosome 2. Dubey et al. (2019) developed 49 SSRs gene based SSRs markers in *C. annuum* which could help in marker assisted selection (MAS) of fruit development and ripening genes. In a first of its kind study, a total of 623 non coding RNA (ncRNA) based SSRs were identified including 119 microRNA based and 504 long non-coding RNA based, out of which 120 SSRs were utilized to conduct genetic diversity analysis of 96 *Capsicum* accessions (Jaiswal et al. 2020). In a study, a SNP-based genetic map from the F_2 population of 84 individuals derived from a cross between *C. annuum* 'NB1' (female parent) and *C. chinense* 'Jolokia' (male parent) was developed and 1.76 million SNPs (HRM) markers were identified between the parents. Among them, a total of 116 SNP markers were located on a 1167.9 cM linkage map of 12 linkage groups and were developed (Lee et al. 2013). Two *C. annuum* varieties A1 (CMVP1 resistant line) and 2602 (CMVP1 susceptible line) were used as maternal and paternal parents to create a single seed-descent (SSD) F_3 population of 174 individuals (Eun et al. 2016). Using GBS analysis, a total of 22,446 SNPs were identified. Only 906 of the 19,099 SNP markers from the SNP matrix were selected for a more precise linkage map. The minimum number of SNPs mapped per chromosome was 42 on chromosome 8 and the maximum was 98 on chromosome 3.

A total of 1910 potential SNPs were developed and mapped on *C. annuum* chromosomes, out of that 412 SNPs were tested for polymorphism in 27 *Capsicum* accessions (Kang et al. 2014). Two markers for *Phytophthora* root rot resistance-(M3-2 and M3-3), five for Anthracnose disease resistance (CcR9, CA09g12180,

CA12g17210, CA09g19170, and CA12g19240), three for powdery mildew resistance (Ltr4.1-40344, Ltr4.2-585119 and Ltr4.2-5630), one for bacterial spot disease (Bs2), one for CMV (Cmr1-2), one for Pepper mild mottle virus (PMMoV) (L4), two for Pepper mottle virus (PepMoV) (pvr1 and pvr2-123457) and finally five for capsaicinoids content (qdhc2.1-1335057, qdhc2.2-43829, qcap3.1-40134, qcap6.1-589160 and qcap6.1-299931) were developed (Kim et al. 2017a). Whole genome-resequencing of two *C. annuum* inbred lines BA3 and B702 led to the prediction of a total of 14,498 InDel sites between BA3 and B702. Among them, 251 InDels were validated and mapped onto a 1178.01 cM InDel-based linkage map (Li et al. 2015).

12.5 Development of Genetic Maps in *Capsicum*

Capsicum possesses huge phenotypic variation for morphological and horticultural traits that are inherited quantitatively. Quantitative trait loci (QTLs) maps have been developed extensively for *Capsicum* plant traits, growth parameters, shape of fruit, size, diameter, weight, colour, quality, pericarp thickness, metabolites content and resistance to biotic as well as abiotic stresses. Most of the mapping studies were conducted in intra species population derived from crossing between different accessions of *C. annuum*, and interspecies populations derived from crossing *C. annuum* with *C. frutescens* and *C. chinense*. Quite a few studies have reported on interspecific crosses between *C. annuum* and *C. baccatum* to utilize its potential for the improvement of fruit yield and quality. Few mapping populations have been developed in other *Capsicum* spp. like *C. baccatum* and *C. pubescens*. Recent genotyping techniques like genotyping-by-sequencing (GBS) have enabled the generation of ultra-high-density QTL maps in *Capsicum* spp. to aid in mapping and diversity analysis (Elshire et al. 2011). Further, candidate genes which govern these variations and the molecular markers linked to them have been identified, which can be employed for introgression of resistance and fruit-related genes in elite species. Previous reports have summarized the development of genetic maps and mapping of QTLs for economically important traits in *Capsicum* spp. for applications in pepper breeding and improvement (Ben-Chaim et al. 2001; Ben-Chaim et al. 2006; Paran 2013; Ramchiary et al. 2014). This chapter reviews more recently developed QTL maps and genetic tools for mapping plant architecture, vigor and fruit associated traits.

A pepper genetic linkage map of about 1486·6 cM was constructed using an intraspecific cross between YCM334 (*C. annuum*) and Tean (*C. annuum*) to investigate *Phytophthora capsici* resistance in pepper. A total of 249 markers were developed including 136 Amplified Fragment Length Polymorphisms (AFLPs), 112 Simple Sequence Repeats (SSRs) and one Cleaved Amplified Polymorphic Sequence (CAPS) (Truong et al. 2012). The F_8 population consisting of 126 RILs was utilized for composite interval mapping and several QTLs for *P. capsici* were detected on pepper chromosomes 5, 10 and 11. Table 12.1 provides a summary of genetic mapping populations along with markers identified in *Capsicum*.

Table 12.1 Some of the Genetic maps constructed in *Capsicum* genomes and the molecular markers developed for marker assisted selection in *Capsicum* improvement and breeding programmes

Mapping population	Markers	Major trait difference between the parental lines	Reference
F_3 Maor (susceptible) × Perennial (resistant	RFLP and AFLP	Cucumber mosaic virus resistance	Ben-Chaim et al. (2001)
NuMexRNaky (*C. annuum)* × BG 2814-6 (*C. frutescens)*	728 (SSR, AFLP and RFLP)	Capsaicinoids	Ben-Chaim et al. (2006)
YCM334 (C. annuum) × *Tean (C. annuum)*	249 markers (136 AFLPs, 112 SSRs and one CAPS)	*P. capsici* resistance	Truong et al. (2012)
C. annuum (AC1979) × *C. chinense (No. 4661)*	15 AFLP and 56 SSRs	Flavonoids	Wahyuni et al. (2014)
C. chinense Jacq. 'Bhut Jolokia' × *C. annuum* 'NB1'	246 markers (212 HRMs, 21 SSRs, 2 CAPS and 11 Capsaicinoid biosynthesis pathway genes)	Capsaicinoids	Lee et al. (2016a, b)
$F_{2:3}$ YW × DLL	326 markers: 13 SSRs, 312 SNPs, and 1 SCAR	Root-knot nematode resistance	Barbary et al. (2016)
C. annuum A1 (CMVP1 resistant line) × 2602 (CMVP1 susceptible line)	19,000 SNPs	Cucumber mosaic virus P1 strain	Eun et al. (2016)
C. frutescens (IL PBC688) × *C. annuum* (IL G29)	36,847 SLAF markers	Cucumber mosaic virus resistance	Guo et al. (2017a, b)
Perennial × Dempsey	1,000,000 SNPs	Plant height, width, main stem length, internode length, leaf length, width, flower size, fruit shape, weight, diameter etc.	Han et al. (2016)
Tabasco (*C. frutescens*) × blocky-type P4 (*C. annuum*)	5546 SNPs	Fruit types- bell/blocky, chile/hot and Lamuyo	Hulse-Kemp et al. (2016)
BA3 (*C. annuum*) × YNXML (*C. frutescens*)	5828 SNPs.	Fruit orientation-pendent and erect	Cheng et al. (2016a)
C. baccatum × *C. baccatum*	395 SNPs	Capsaicinoids	Lee et al. 2016a, b

(continued)

Table 12.1 (continued)

Mapping population	Markers	Major trait difference between the parental lines	Reference
PG, CHB-F_3 (*C. annuum* 'A1' × '2602') and JN-F_5 (*C. annuum* 'NB1' × *C. chinense* 'BhutJolokia')	20 SNP type assays	*Phytophthora* root rot, Anthracnose, CMV, Bacterial spot, PMMoV, PepMoV, powdery mildew and capsaicinoids	Kim et al. (2017a)
Perennial (*C. annuum)* × Dempsey (*C. annuum)* RIL and TF68 (*C. chinense)* × Habanero RIL	109,610 SNPs	Capsaicinoids	Han et al. (2018)
C. annuum IL BJ0747 (resistant) × IL XJ0630 (susceptible)	210,077 SLAF markers	Cucumber mosaic virus resistance	Li et al. (2018)
FL201 (*C. annuum*) × TC 07245 (*C. galapagoense*)	400 SSR	Fruit length- very small, small, medium long, long, very long and extra long	Arjun et al. (2018)
Habanero (*C. chinense*) × Jolokia (*C. chinense*) and SNU11-001 (non-pungent *C. chinense*) × Jolokia (pungent *C. chinense*)	1718 and 8297 SNPs	Capsaicinoids	Park et al. (2019)

The recent availability of databases for *Capsicum* reference genomes and cutting-edge genomic tools such as Next Generation Sequencing (NGS) have enabled development of ultra-high-density genetic maps for pepper (Kim et al. 2014; Qin et al. 2014). Han et al. (2016) constructed a 1372 cM highly saturated bin map for *C. annuum* recombinant inbred lines (RILs) obtained from a cross between Perennial and Dempsey. About 1,000,000 SNPs were found between the two parents distributed in about 2578 bins. Each chromosome had about 154-370 bins with an average density of 1 bin per 0.5 cM. A number of 86 QTLs for around 18 plant and fruit traits were identified including several QTLs that were associated with fruit weight and shape (Chunthawodtiporn et al. 2018).

Another high-density bin map was generated using RIL populations from an intraspecific (*C. annuum* Early Jalapeno × *C. annuum* CM334) and an interspecific cross (*C. frutescens* BG2814-6 × *C. annuum* NuMex RNaky, Hill et al. 2015). A pepper gene chip exhibiting 31,196 Expressed Sequence Tags (ESTs) (Ashrafi et al. 2012) was used to detect 3878 and 16,167 EST markers distributed among 783 and 2105 genetic bins in the two populations, respectively. Syntenic analysis allowed comparative mapping of common traits in pepper with other Solanaceae members. Translocation, recombination rate and marker distortion were also analyzed between the two maps.

Re-sequencing of 22 *Capsicum* lines led to the development of an array of 16,000 SNPs (Hulse-Kemp et al. 2016). The SNP array was utilized to construct a high-density-map from a cross between Tabasco (*C. frutescens*) and blocky-type P4 (*C. annuum*) and about 5546 markers were identified in 1362 bins arranged in 12 linkage groups. Further, using a set of common markers, this map was compared with the genetic map developed earlier from interspecific crossing between *C. frutescens* BG2814-6 × *C. annuum* NuMex RNaky and was found to be highly similar (Hill et al. 2015).

Another SNP Infinium array of 15,000 SNPs was generated using re-sequencing data of two pepper cultivars BA3 and B702 (Cheng et al. 2016a). A total of 8200 SNP loci were mapped to the Zunla reference genome (Qin et al. 2014) and used for phenotypic scoring. F2 mapping population developed from an interspecific cross between *C. annuum* (BA3) and *C. frutescens* (YNXML) was genotyped using 5828 SNPs. A fruit orientation controlling locus *Up12.1* was mapped to a 4.5 Mb genetic region on chromosome 12 and about 65 genes were identified in the locus. Genetic variation in 399 *C. annuum* lines was evaluated using this SNP array, however, low diversity obtained indicated the need for a broader genetic pool for breeding objectives.

A genome-wide association study (GWAS) of 94 *Capsicum* accessions was conducted to identify QTLs responsible for capsaicinoid content and fruit weight (Nimmakayala et al. 2016). GBS was used to identify 66,960 SNPs in the *Capsicum* accessions that were mapped to the CM334 reference genome (Kim et al. 2014). A total of 30, 56 and 14 SNPs were associated with capsaicin, dihydrocapsaicin and both, respectively, especially in the genomic regions with selective sweep signatures.

Recent efforts have been made for genetic mapping in other *Capsicum* species like *C. baccatum* which exhibits wide variations in fruit morphology and disease resistance (Kim et al. 2010; Lee et al. 2010; Mahasuk et al. 2016). An intraspecific cross in *C. baccatum* was used to develop a mapping population diversity analysis, Linkage Disequilibrium (LD) and QTL identification (Lee et al. 2016a, b). About 395 SNPs were mapped to the *C. annuum* reference genome CM334 and translocation events were detected that may serve as genetic barriers between the two species. GWAS of a panel of 283 *C. baccatum* cultivated and wild accessions revealed 13,000 SNPs (Nimmakayala et al. 2016). Significant associations for variation in peduncle length in the population were observed on at least 10 chromosomes.

An interspecific cross between *C. baccatum* and *C. annuum*, aided with multi-parent backcrossing and embryo rescue techniques, was used to construct BC_2S_1 population to study fruit-related traits like morphology, chemical composition, volatile and sensory properties and QTL mapping (Eggink et al. 2014). A QTL for fruit colour in the immature fruit originated from *C. annuum* and another for volatile nature and flavour of the fruit from *C. baccatum* were detected. Further, QTLs for improved flavour (sugar) and terpenoids were identified on chromosome 3, 1 and 10, respectively.

12.6 QTL Mapping for Economically Important Traits

Major horticultural traits used for pepper breeding programmes have included high yield, desired fruit quality, morphology, resistance to biotic and abiotic stresses, enhanced metabolite content etc. With the advancement of molecular breeding methods and genetic tools, the QTL regions and the candidate genes responsible for major agricultural traits have been identified and are available for utilization in *Capsicum* breeding as summarized in Table 12.2.

12.6.1 Plant Architecture

Plant traits such as plant height and width, early flowering, main stem length and thickness, lateral branching, internode length, number of leaves etc. determine the plant growth, vigor and development. QTLs for at six plant growth traits were detected in a population of RILs developed from a cross between Yolo Wonder and CM334 (Barchi et al. 2009). At least five QTLs for axis length, three for internode length, four for speed of axis growth, two for time of internode growth, five for flowering earliness and four for number of leaves were identified on various pepper chromosomes or linkage groups in the whole population. Tight co-localization of QTLs was found on chromosomes for P2, P4, P9 and LG47. In a population of RILs obtained from a cross between two *C. annuum* cultivars Perennial and Dempsey, 32 QTLs for plant height and width, stem length, lateral branches, internode length, leaf length and width and stem colour were detected on different pepper chromosomes (Han et al. 2016). A double haploid population obtained by anther culture of the F_1 individual of the cross between *C. annuum* accessions LS2341 and California Wonder was used for QTL mapping of five plant traits (Mimura et al. 2010). Twelve QTLs for plant growth characters like length of primary axis, axillary shooting, number of leaves, internode length and flowering date were mapped on pepper chromosomes or linkage groups. QTLs named *AS3.1*, *AS2.1* and *AS8.1* located on P3, P2 and LG8, respectively, explained the phenotypic variation for axillary shooting, *FD8.1* on LG8 for flowering date and *NL8.1* and *NL12.1 on LG8* and *P12*, respectively, explained most of the variation in number of leaves. Two QTLs for internode length *NL8.1* and *NL12.1* were located at the same positions as *AXL8.1* and on LG8 and P12, respectively. Also, QTL for axis length *AXL12.1* was co-localized with the QTLs for flowering date and number of leaves on P12 (Yarnes et al. 2013).

An interspecific cross between *C. frutescens* (var. 2814-6) and *C. annuum* (NuMexRNAKY) was used to develop RILs to study plant traits such as plant height, branching and leaf density and vigor (Yarnes et al. 2013). Overall, 17 QTLs including *2.1, 3.1, 3.2, 4.5, 4.6, 4.8, 4.9, 5.3, 5.4, 5.5, 10.1, 10.4, 11.3,* and *11.10* were found to be associated with plant morphology traits on chromosome 2,3,4,5,10 and 11, respectively. Negative correlations between QTLs for branching habit and leaf density and QTLs for plant height and vigor were observed, although phenotypic correlations pertain between them. Height of plant and fruits per plant

Table 12.2 QTLs mapped for important traits in Capsicum genome using different mapping populations

Trait	Population	QTLs	Chromosome	References
Plant height	Perennial (pungent *C. annuum*) × Dempsey (non-pungent *C. annuum*)	*PH-2*	2	Han et al. (2016)
	Maor (*C. annuum*) × Perennial (*C. annuum*)	*ph3.1, ph4.1* and *ph6.1*	3, 4 and 6	Ben-Chaim et al. (2001)
Immature fruit colour	PEN45 (*C. baccatum* var. *pendulum*) × SM, GNM (*C. annuum*) and Perennial (*C. annuum*) × Dempsey (*C. annuum*)	*LG10.1* and *IFC-10.2*	3, 6, 10	Eggink et al. (2014) and Han et al. (2016)
Mature fruit colour	*C. chinense* (PI 152225) × *C. annuum* (Kelvin and 4751) and PEN45 (*C. baccatum* var. *pendulum*) × SM, GNM (*C. annuum*)	*c1, c2* and *y*	3	Eggink et al. (2014) and Popovsky and Paran (2000)
Fruit position	Perennial (*C. annuum*) × Dempsey (*C. annuum*)	*FP12.2*	12	Han et al. (2016)
Fruit length	PEN45 (*C. baccatum* var. *pendulum*) × SM,GNM (*C. annuum*) and Perennial (*C. annuum*) × Dempsey (*C. annuum*)	*LG10.1, FL-3.1, FL-3.2* and *FL-3.3*	3	Eggink et al. (2014) and Han et al. (2016)
	Yolo Wonder (YW) × CM334	*Frl4.1*	4	Barchi et al. (2009)
	TF68 (*C. annuum*) × Habanero (*C. chinense)*		3	Lee et al. (2011a)
	California Wonder (*C. annuum*) × LCA235 (*C. annuum*)	*Qfl.iivr.3.2*	2	Dwivedi et al. (2015)
	Maor (*C. annuum*) × Perennial (*C. annuum*)	*fl2.1* and *fl3.1*	2 and 3	Ben-Chaim et al. (2001)
Fruit width	Perennial (*C. annuum*) × Dempsey (*C. annuum*))	*LG1_8, 10.1* and *9*		Han et al. (2016)

Fruit weight	Perennial (*C. annuum*) × Dempsey (*C. annuum*)	*FW-1*	1	Han et al. (2016)
	California Wonder (*C. annuum*) × LCA235 (*C. annuum*)	*Qtofw.iivr-1.1*	1	Dwivedi et al. (2015)
	NuMexRNaky (*C. annuum)* × BG 2814-6 (*C. frutescens)*	*fw2.1*and *fw3.1*	2 and 3	Ben-Chaim et al. (2006)
	Maor (*C. annuum*) × Perennial (*C. annuum*)	*fw2.1, fw3.1, fw3.2, fw4.1* and *fw8.1*	2,3,4 and 8	Ben-Chaim et al. (2001)
Fruit shape	Perennial (*C. annuum*) × Dempsey (*C. annuum*)	*FS-3.2*	3	Han et al. (2016)
	Maor (*C. annuum*) × Perennial (*C. annuum*)	*fs3.1, fs8.1*and *fs10.1*	3, 8 and 10	Ben-Chaim et al. (2001) and Borovsky and Paran (2011)
Fruit diameter	Perennial (*C. annuum*) × Dempsey (*C. annuum*)	*FD1, FD-3.2*	3	Han et al. (2016)
	Yolo Wonder (YW) × CM334	*Frd11.1*	11	Barchi et al. (2009)
	Maor (*C. annuum*) × Perennial (*C. annuum*)	*fd2.1, fd3.1, fd8.1* and *fd10.1*	2,3,8 and 10	Ben-Chaim et al. (2001)
Pericarp thickness	Maor (*C. annuum*) × Perennial (*C. annuum*)	*pt3.1, pt4.1, pt8.1* and *pt10.1*	3,4,8 and 10	Ben-Chaim et al. (2001)
Terpenoids	PEN45 (*C. baccatum* var. *pendulum*) × SM, GNM (*C. annuum*)	*LG10.1* and *LG1*		Eggink et al. (2014)
Malate	PEN45 (*C. baccatum* var. *pendulum*) × SM, GNM (*C. annuum*)	*LG1*		Eggink et al. (2014)
Chlorophyll	*C. annuum* and *C. chinense*	*pc8.1 and pc10.1*	8 and 10	Wahyuni et al. (2014)
Carotenoid	*C. annuum* and *C. chinense*	*pcr8.1*	8	Wahyuni et al. (2014)
Flavonoids	*C. annuum* AC1979 (no. 19) × *C. chinense* No. 4661	*mQTL*	9	Wahyuni et al. (2014)
Capsaicinoids	Perennial (*C. annuum)* × Dempsey (*C. annuum)* RIL	*PD-cap1, PD-cap3*and *PD-cap10*	1,3 and 10	Han et al. (2018)

(continued)

Table 12.2 (continued)

Trait	Population	QTLs	Chromosome	References
	C. annuum 'Maor' × *C. frutescens* BG2816	*cap*	7	Blum et al. (2003)
	TF68 (*C. chinense)* × Habanero RIL	*TH-cap1.4, TH-cap2.2 and TH-cap4*	1, 2 and 4	Han et al. (2018)
Dihydrocapsaicinoids	Perennial (*C. annuum)* × Dempsey (*C. annuum)* RIL	*PD-dicap1.1, PD-dicap2.1* and *PD-dicap10.2*	1,2 and 10	Han et al. (2018)
	TF68 (*C. chinense)* × Habanero RIL	*TH-dicap10*	10	Han et al. (2018)
Total capsaicinoids	Perennial (*C. annuum)* × Dempsey (*C. annuum)* RIL	*PD-total1.1, PD-total4.2* and *PD-total10.2*	1,4 and 10	Han et al. (2018)
	TF68 (*C. chinense)* × Habanero RIL	*TH-total2, TH-total3.2* and *TH-total10*	2,3 and 10	Han et al. (2018)
	NuMexRNaky (*C. annuum)* × BG 2814-6 (*C. frutescens)*	*total3.1,total4.1, total4.2 total7.1and total7.2*	3, 4 and 7	Ben-Chaim et al. (2006)
	Habanero (*C. chinense*) × Jolokia (*C. chinense*) F3	*HJ16-qtlseq, HJ16-tcp6.1, HJ16-tcp6.2, HJ16-tcp6.3*and *HJ16-tcp6.4*	6	Park et al. (2019)
	SNU11-001 (non-pungent *C. chinense*) × Jolokia (pungent *C. chinense*) F2	*SJ-tcp6.1* and *SJ-tcp6.1*	6	Park et al. (2019)
Capsaicin	NB1 (*C. annuum*) × Bhut Jolokia (*C. chinense*)	*qcap3.1*and qcap6.1	3 and 6	Lee et al. (2016a, 2016b)
	SNU11-001 (non-pungent *C. chinense*) × Jolokia (pungent *C. chinense*) F2	*SJ-cap6.1* and *SJ-cap6.1*	6	Park et al. (2019)
	SNU11-001 (non-pungent *C. chinense*) × Jolokia (pungent *C. chinense*) F2	*SJ-cap6.1* and *SJ-cap6.1*	6	Park et al. (2019)
	C. frutescens (2814-6) × *C. annuum* (NuMexRNAKY)	*4.13, 4.14* and *4.15*	4	Yarnes et al. (2013)
	NuMexRNaky (*C. annuum)* × BG 2814-6 (*C. frutescens)*	*cap3.1,cap4.1* and *cap7.1*	3, 4 and 7	Ben-Chaim et al. (2006)

Dihydrocapsaicin	Habanero (*C. chinense*) × Jolokia (*C. chinense*) F3	*HJ16-dhc3*	3	Park et al. (2019)
	SNU11-001 (non-pungent *C. chinense*) × Jolokia (pungent *C. chinense*) F2	*SJ-dhc6.1, SJ-dhc6.2* and *SJ-dhc6.3*	6	Park et al. (2019)
	NB1 (*C. annuum*) × Bhut Jolokia (*C. chinense*)	*qdhc2.1*and *qdhc2.2*	2	Lee et al. (2016a, 2016b)
	NuMexRNaky (*C. annuum)* × BG 2814-6 (*C. frutescens)*	*dhc4., 1dhc4.2, dhc7.1* and *dhc7.2*	4 and 7	Ben-Chaim et al. (2006)
	C. frutescens (2814-6) × *C. annuum* (NuMexRNAKY)	*3.1*	3	Yarnes et al. (2013)
	C. frutescens (2814-6) × *C. annuum* (NuMexRNAKY)	*4.2, 5.4* and 6.8	4, 5 and 6	Yarnes et al. (2013)
Nordihydrocapsaicin	NuMexRNaky (*C. annuum)* × BG 2814-6 (*C. frutescens)*	*ndhc7a.1*	7	Ben-Chaim et al. (2006)
P. capsici resistance	CM334 × Yolo B, *C. annuum* CM334 and *C. annuum NuMex Joe E. Parker* (CM334 × JEP) and *C. annuum NuMex RNaky* × *C. chinense* PI 159234	*Phyto.5.2*		Livingstone et al. (1999), Paran et al. (2004) and Quirin et al. (2005)
Powdery mildew resistance	Doubled-haploid (resistant 'H3' × susceptible 'Vania')	*Lt5.1, Lt6.1, Lt9.1, Lt10.1* and *Lt12.1*	5,6,9,10 and 12	Lefebvre et al. (2003)
	Resistant 'VK515R' × susceptible 'VK515S'	*PMR1*	4	Jo et al. (2017)
Phytophthora root rot	F_8 population YCM334 (resistant) × Tean (susceptible)	*Ph051-5·1, Ph051-5·2, Ph051-5·3, Ph051-5·4, Ph127-5·5, Ph127-5·6, Ph127-5·7, Ph051-10·1, Ph051-10·2, Ph051-10·3, Ph051-10·4* and *Ph127-11·1*	5 and 10	Thabuis et al. (2003)

(continued)

Table 12.2 (continued)

Trait	Population	QTLs	Chromosome	References
Cucumber Mosaic Virus resistance	F3 Maor (susceptible) × Perennial (resistant	*cmv 4.1, cmv6.1, cmv11.1* and *cmv13.1*	LG4, LG6, LG11 and LGUL[c]	Ben-Chaim et al. (2001)
	C. frutescens (IL PBC688) × *C. annuum* (IL G29)	*qCmr2.1*	2	Guo et al. (2017a, 2017b)
	C. annuum IL BJ0747 (resistant) × IL XJ0630 (susceptible)	*qcmv11.1, qcmv11.2 and qcmv12.1*	11 and 12	Li et al. (2018)
Root-knot nematode	YL (partially resistant) × DLL (highly susceptible)	*Minc-P1*, *Mare-P1, Mjav-P1* and *Mjav-P9*	P1 and P9	Barbary et al. (2016)
Anthracnose	PBC932 (*C. chinense*; resistant) × 77,013 (*C. annuum*; susceptible)	*AnRGO5, AnRGT5, AnRGD5, AnRRO5, AnRRT5* and *AnRRD5*	5	Sun et al. (2015)

were associated with *Qpht.iivr.5.1* and *Qnfp.iivr-2.1* on chromosome 5 and 2, respectively, in RIL population of an intraspecific cross between California Wonder (C. *annuum*) and LCA235 (*C. annuum)* (Dwivedi et al. 2015). In the near-isogenic lines (NILs) obtained from a cross between variable flowering time *Capsicum* accessions, a major QTL for flowering repression was identified on chromosome 2 (Borovsky et al. 2015).

12.6.2 Fruit-Related Traits

12.6.2.1 Fruit Morphology

Several fruit latitudinal and longitudinal morphology traits including width, height, area, perimeter, pericarp thickness etc. were studied in a population of interspecific cross between *C. frutescens* and *C. annuum* (Yarnes et al. 2013). Forty and twenty-three QTLs were associated with fruit latitudinal and longitudinal traits, respectively. For instance, lobedness degree of the fruit was associated with *2.6, 2.7, 2.9, 6.4, 9.4, 9.6* and *9.8* on chromosome 2,6 and 9, fruit height with *2.3, 2.4, 2.6, 2.9, 3.9, 3.10, 3.11, 4.4, 4.5, 4.6, 4.8, 11.10, 12.4* and *12.5* on chromosome 2,3,4,11 and 12 and fruit perimeter with *2.6, 2.8, 2.9, 3.6, 3.9, 4.3, 4.4, 4.5, 4.10, 5.6, 9.8, 9.9, 11.4, 11.6, 11.7* and *11.10* on chromosome 2,3,4,5,9 and 11. In another mapping population of Yolo Wonder × CM334, seven fruit-related traits like fruit weight, size, shape, pericarp thickness, pedicel length and number of locules were investigated for their association with QTL regions on *Capsicum* chromosomes (Barchi et al. 2009). Most of the QTLs were concentrated on P3, P4 and P12, for example, fruit weight and length (*Lfw4.1 and Frl4.1*), fruit shape, pericarp thickness and diameter (*Frs3.1, Pet3.1* and *Frd3.1*) and number of locules (*Nlo12.1* and *Nlo12.2*).

Eight significant QTLs for fruit phenotypes including firmness, shape and pericarp were identified in a RIL population developed from Early Jalapeno × CM334 on seven *Capsicum* chromosomes (Naegele et al. 2014). Five QTLs for fruit shape on chromosomes 1,2,4,5 and 10 were responsible for almost 50% of the variation in the population while the QTL for fruit firmness on chromosome 12 explained approximately 30% of the variation. Three QTLs for fruit weight (*Qtofw.iivr-1.1*, *Qtofw.iivr-2.1* and *Qtofw.iivr-3.1),* two for fruit length *(Qfl.iivr.3.2* and *Qfl.iivr.3.4),* one for fruit width (*Qfw.iivr-2.1)* and one for pericarp thickness (*Qpt.iivr-2.1)* were attributed for variation in 74 RILs obtained from a cross between *C. annuum* cultivars- California wonder and LCA235 (Dwivedi et al. 2015). The maximum phenotypic variance was explained by *Qfw.iivr-2.1 (*50%) and the minimum by *Qtofw.iivr-2.1* (8%). Five QTLs (*Qtofw.iivr-2.1, Qtfw.iivr-2.1, Qfw.iivr-2.1, Qnfp. iivr-2.1* and *Qpt.iivr-2.1*) were colocalized on chromosome 2 which may explain the pleiotropic effects of the QTLs. Significant QTLs for fruit weight (*fw2.1),* fruit length (*fl2.1),* fruit diameter (*fd2.1* and *fd3.1),* fruit shape (*fs3.1),* pericarp width (*perwd3.2* and *perwd11.1),* number of fruits (*fno2.1),* fruit maturity (*mat1.1* and *mat4.1),* yield (*yld8.1*) and seed weight (*swt2.1* and *swt8.1)* were detected in a population of interspecific cross between *C. annuum* (Maor) and *C. frutescens* (BG 2816) (Rao et al. 2003).

Significant QTLs for fruit length, width and shape using two F_2 mapping populations (5226 × PI159234 and 100/63 × IL 179) were determined (Ben Chaim et al. 2003a). One of the QTLs responsible for more than 40% variation in the fruit shape index, *fs10.1*, was found to be closely linked to an anthocyanin accumulation locus *A* on pepper chromosome 10. Fine genetic mapping of the QTL *fs10.1* using the population of NILs obtained from round-fruited *C. annuum* line 5226 and elongated-fruited *C. chinense* line PI 159234 led to the determination of another close marker CT11 which was 0.3 cM away from *fs10.1* (Ben Chaim et al. 2003b).

12.6.3 Pungency

A major QTL namely *cap* for capsaicin and dihydrocapsaicin contents explained 34-38% variation in the capsaicinoids content in the F_2 mapping population derived from *C. annuum* 'Maor' (non-pungent) and pungent *C. frutescens* BG2816 (Blum et al. 2003). This QTL was mapped on chromosome 7 in the interval between two molecular markers $UBC20_{2200}$ and CT84. F2 and F3 mapping populations were derived by crossing *C. annuum* cultivar-NuMexRNaky (large fruit and low pungent) with *C. frutescens* accession-BG 2814-6 (small fruit and highly pungent) (Ben-Chaim et al. 2006). QTLs for Capsaicin- *cap3.1, cap4.1* and *cap7.1*, dihydrocapsaicin- *dhc4., 1dhc4.2, dhc7.1* and *dhc7.2*, nordihydrocapsaci-*ndhc7a.1* and total capsaicinoids- *total3.1, total4.1, total4.2 total7.1* and *total7.2* were identified using QTL analysis on chromosomes 3,4 and 7, respectively. Twelve QTLs for capsaicinoids content were identified in C. *frutescens* (var. 2814-6) × *C. annuum* (NuMexRNAKY) population on seven *Capsicum* chromosomes, namely, *3.1* and *4.16* for dihydrocapsaicin, *4.2, 5.4, 6.8, 7.3, 10.2, 10.3* and *11.8* nordihydrocapsaicin and *4.13, 4.14* and *4.15* for capsaicin (Yarnes et al. 2013). *C. chinense* Jacq. Bhut Jolokia (highly pungent) and *C. annuum* NB1 (moderately pungent) were used as paternal and maternal parents to develop a F_2 mapping population to identify capsaicin (*qcap3.1*and qcap6.1) and dihydrocapsaicin (*qdhc2.1*and *qdhc2.2)* QTLs (Lee et al. 2016a, b).

A population of 120 RILs (*C. annuum* 'Perennial' × Dempsey; PD) was used for QTL identification for capsaicin (*PD-cap1, PD-cap3* and *PD-cap10*), dihydrocapsaicin (*PD-dicap1.1, PD-dicap2.1* and *PD-dicap10.2*) and total capsaicinoids (*PD-total1.1, PD-total4.2* and *PD-total10.2*) (Han et al. 2018). In the same study, another RIL population of 85 individuals (*C. annuum* 'TF68' × *C. chinense* 'Habanero'; TH) was utilized for detection of the following QTLs- *TH-cap1.4, TH-cap2.2 and TH-cap4* (capsaicin), *TH-dicap10* (dihydrocapsaicin) and *TH-total2, TH-total3.2* and *TH-total10* (total capsaicinoids). Habanero (Pungent placenta; *C. chinense*) was crossed with Bhut Jolokia (pungent placenta and pericarp; *C. chinense*) to develop a F_2 population (HJ) of 87 plants while SNU11-001 (Non-pungent; *C. chinense*) and Bhut Jolokia were used as parents for another F_2 population (SJ) of 124 plants (Park et al. 2019). Fifteen QTLs for individual and total capsaicinoids including- *HJ16-qtlseq, HJ16-tcp6.1,*

HJ16-tcp6.2, HJ16-tcp6.3, *HJ16-tcp6.4, HJ16-dhc3, SJ-tcp6.1, SJ-tcp6.1 SJ-cap6.1*, *SJ-cap6.1, SJ-dhc6.1, SJ-dhc6.2* and *SJ-dhc6.3* were detected on three *Capsicum* chromosomes- 3, 6 and 11, respectively.

12.6.4 Disease Resistance

A single QTL for pericarp that explained at least 26% of the variation in the population of the cross Early Jalapeno × CM334, was also found to be linked with a P*hytophthora capsici* (isolate 13,709 at 5 dpi) resistance QTL (Naegele et al. 2014). Five QTLs distributed across five pepper chromosomes (*Lt5.1, Lt6.1, Lt9.1, Lt10.1* and *Lt12.1*) conferred resistance to Powdery mildew manifested by *Leveillula taurica* in *C. annuum* as detected using a doubled-haploid population derived from a cross between 'H3' (resistant) and 'Vania' (susceptible) (Lefebvre et al. 2003). Another QTL *PMR1*, was mapped on pepper chromosome 4 in the genetic region between two molecular markers CZ2_11628 and HRM4.1.6 using the $F_{2:3}$ population obtained from VK5151R × VK5151S (Jo et al. 2017). Three SNP molecular markers (ZL1_1826, KS16052G01, and HRM2_A4) were located 0 cM away from the *PMR1* locus and co-segregated with the powdery mildew resistant phenotype.

Three *C. annuum* intraspecific crosses between H3 × Vania (HV), Perennial × Yolo Wonder (PY) and Yolo Wonder × CM334 (YC) were used to derive two doubled-haploid and one F3 population for evaluation for resistance to powdery mildew (Thabuis et al. 2003). Comparative genetic mapping in the pepper genomes revealed several conserved loci for resistance components against *Phytophthora capsici* in the three mapping populations. Fifteen other isolate-specific QTLs for *P. capsici* root rot has been identified using the F_8 population from an intraspecific cross between *C. annuum* lines- YCM334 (resistant) and Tean (susceptible) (Truong et al. 2012). Seven QTLs (*Ph051-5·1, Ph051-5·2, Ph051-5·3, Ph051-5·4, Ph127-5·5, Ph127-5·6* and *Ph127-5·7)* were mapped on pepper chromosome 5 between two AFLP markers a015_7 and a170_1 that explained more than 40% of the resistance against both the *P. capsici* isolates used in the study. Eight other QTLs were detected on chromosome 10, 11, Pb and Pc respectively, namely- *Ph051-10·1, Ph051-10·2, Ph051-10·3, Ph051-10·4, Ph127-11·1, Ph127-b, Ph051-c·1* and *Ph051-c·2*.

QTL analysis for root-knot nematode resistance using a $F_{2:3}$ population of the cross YW (partially resistant) × DLL (highly susceptible) revealed a *Meloidogyne incognita* resistance QTL named *Minc-P1* and a *M. arenaria* resistance QTL- *Mare-P1* on chromosome P1 and two *M. javanica* resistance QTLs named *Mjav-P1* on P1 and *Mjav-P9* on P9 (Barbary et al. 2016). Four Cucumber Mosaic Virus (CMV) resistance QTLs- *cmv 4.1, cmv6.1, cmv11.1* and *cmv13.1* were identified in *C. annuum* using a population derived from two cultivars Maor (susceptible) and Perennial (resistant) (Ben-Chaim et al. 2001). Two CMV resistance QTLs- *qCmr2.1* on chromosome 2 and *qCmr11.1* on chromosome 11 in C. *frutescens* were detected using F_2 populations derived from PBC688 (resistant) and G29 (susceptible) (Guo et al. 2017a, b). Three QTLs- *qcmv11.1* and *qcmv11.2* on chromosome 11 and

qcmv12.1 on chromosome 12 showed 10.2%, 19.2% and 7.3% variation in CMV resistance, respectively, in a population developed from pepper inbred lines- BJ0747 (CMV-resistant) and XJ0630 (CMVsusceptible) using Specific Length Amplified Fragment sequencing (SLAF-seq) (Li et al. 2018). A major cluster of QTLs and four minor QTLs (*AnRGO5, AnRGT5, AnRGD5, AnRRO5, AnRRT5* and *AnRRD5)* on pepper chromosome 5 confer resistance to anthracnose (*Colletotrichum acutatum*) in *Capsicum* mature green and ripe fruit (Sun et al. 2015).

12.7 Genome Sequencing and Identification of Genes/Genetic Loci Governing Important Traits

The genome size of *Capsicum* ranges from 3-3.8 Gb and approximately 80% of the genome contains repetitive elements. *Capsicum* varieties mainly from *C. annuum* were forerunner among *Capsicum* species for genomic and genetics research. The first draft genome sequence was reported for *C. annuum* cultivar (cv.) *Criollo de Morelos 334* (*CM334*) in early 2014 with an estimated genome size of 3.48 Gb (Kim et al. 2014). The *CM334* cv. was selected due to its high levels of resistance against several diseases including pepper mottle virus (PMV), *Phytophthora capsici*, root-knot nematodes. To provide a broader view of genomic and genetic diversity/variations, the same study also reported the resequencing of two *C. annuum cv.* '*Perennial*' and '*Dempsey*' and *de novo* sequencing of a wild accession *PI159236* belonging to *C. chinense*. The genome of cv. from the same species i.e., from *C. annuum* was observed to less diverge with a proportion of >0.4%, while comparatively high divergence (~1.8%) between two species i.e., *C. annuum* cv. *CM334* and *C. chinense,* was observed. Contemporary to the first study, a new reference genome of *C. annuum* accession *Zunla-1* and *C. annuum var. glabriusculum* aka *Chiltepin* (wild ancestor of *C. annuum*) with an estimated genome size of 3.26 and 3.07 Gb was reported by Qin et al. (2014). The *Zunla-1* was inbred cultivar and found to be adapted in different agro-climatic conditions and yield. This study also performed resequencing of 20 *C. annuum* accessions (including 1 semiwild accession from *C. chinense* and 2 wild accessions from *C. annuum*) and provided a comprehensive understanding of evolution, divergence and domestication of different *Capsicum* species. Further, this study emphasized that a selected genomic region among *Capsicum* related to its domestication encodes a set of genes (511) linked to regulation of transcription, stress/disease response, growth/development and protein-DNA interaction. In addition, this study also reported several proteins with NB-ARC domain provides resistance to several diseases in *Capsicum*, while few specific genes including *PepEST (Capana04g001148), CALTPI (Capana10g001225)* and *RGA15 (Capana01g004043)* promoted resistance in *Capsicum* against pathogens and environmental stresses (Qin et al. 2014), which would be useful for future crop genetic improvement to develop stress/disease resilient and high yield *Capsicum* varieties.

In 2016, an important haplotype map (HapMap) based on SNPs was generated using resequencing 22 phenotypically different *C. annuum* lines and 6 inbred lines of

C. annuum along with 78 interspecific hybrid lines generated from cross between *C. frutescens* (Tabasco; chile type) and *C. annuum* (P4; blocky type) were used to perform genotyping by array. Thip HapMap paved a way as standardized genetic analysis tools to analyze important traits associated with genomics regions/loci known as QTLs for *Capsicum* crop improvement and breeding programmes worldwide (Hulse-Kemp et al. 2016). Another study based on resequencing approach used two *Capsicum* cultivars (*YCM344* and *Taean*), which are parental recombinant inbred lines (RIL). The cv. *YCM344* is highly resistant against bacterial wilt (bw) while *Taean* is vulnerable to bw. Using both resistant and vulnerable *Capsicum* variety, this study identified genomic regions with novel SNPs and insertion/deletions (InDels) related to bw resistance (Kang et al. 2016). Such information could be deciphered for marker assisted breeding for selection of resistant varieties from a large pool of germplasm.

Again in 2017, an improvised reference genome of *CM334* and along with high-quality *de novo* reference genome assembly with an estimated genome size of 3.8 Gb for *Capsicum* baccatum (*PBC81*) and 3.2 Gb for *C. chinense* (*PI159236*), was published (Kim et al. 2017b). This study utilized a anthracnose (*Colletotrichum spp.*) resistance *C. baccatum* variety and identified 64 nucleotide-binding and leucine-rich-repeats proteins (NLRs) as candidate anthracnose-resistant (*Colletotrichum capsici*) loci located across 3.8 Mb region on chromosome (chr) 3. In earlier study, the same anthracnose-resistant loci located on chr 9 was reported in *C. baccatum* (resistant) and *C. annuum* (susceptible) parental lines and their interspecific progenies (Lee et al. 2011b). Also, this study, apparently established the role of transposable-elements (TEs) in evolution and expansion of disease resistance gene families including *NLRs* in *Capsicum* (Kim et al. 2017b). In succession of *Capsicum* genome sequencing, another study reported a highly contiguous genome assembly with a size of 3.21 Gb, using single Linked-Read sequencing approach for a heterozygous F_1 obtained from cross of *CM334* and non-pungent blocky cultivars of *C. annuum*. This study targeted F_1 heterozygous to evaluate the potentiality to obtain both haplotypes via assembling the genome in a phased manner and identified a pungency gene with a large 2.5 kb InDel (Hulse-Kemp et al. 2017).

Afterwards, another study performed resequencing of powdery mildew (PM) resistant and susceptible *Capsicum* lines *PRH1* and *Saengryeg* belonging to *C. baccatum* and *C. annuum*, respectively (Ahn et al. 2018). This study identified several polymorphic SNPs located on chr 4 were associated with disease resistance genes and observed that *PRH1* lines harboured the highest SNPs associated with *Nucleotide-binding site leucine-rich repeat (NBS-LRR)* genes (Ahn et al. 2018). The genomes of different *Capsicum* varieties (majority from *C. annuum*) have been reported, however, a single reference genome does not represent the diversity within species, which led to increasing adoption of pan-genome concept for *Capsicum*. Through the pan-genome, the genomic and genetic variations and diversity within and between species can be extensively explored, thereby the *Capsicum* pan-genome (CPG) was constructed in 2018 using *Zunla-1* genome (Ou et al. 2018). The CPG consist of 610,292 novel contigs with a total pan-genome size of 956.43 Gb (28.4% higher than to 3.26 Gb of *Zunla-1*) and was constructed using the data from a total of

383 *Capsicum* cultivars (4, 11 and 13 from *C. baccatum*, *C. chinense* and *C. frutescens*, respectively, while remaining 355 from *C. annuum*). Further, the study utilized gene presence–absence variations (PAVs) and genome-wide association study (GWAS) and identified few key genes associated with capsaicinoids and carotenoids biosynthesis pathway and deciphered that several traits including fruit colour, shape and pungency are governed by genes (Ou et al. 2018). The assembly features of different *Capsicum* genomes are outlined in Table 12.3.

Recently, in two separate studies phenotypically different *Capsicum* genotypes/lines from Italian landraces were analysed using genome resequencing (Acquadro et al. 2020; Esposito et al. 2022). The first study was conducted on four inbred *C. annuum* genotypes namely *Quadrato* (large sized blocky with sunken apex), *Cuneo* (heart shaped), *Corno* (elongated) and *Tumaticot* (small sized) with distinct fruit shape/size analyzed against *CM334* reference genome. The study observed palpable variations in resistance gene analogues (RGAs) and susceptibility genes (S-genes). Also, it noticed structural variations in RGAs and in *capsanthin/capsorubin* gene (*Ccs*) gene among the four distinct inbred lines, which indicated its potentiality in serving as resource for phenotypic variations and for pre-breeding (Acquadro et al. 2020). The later one utilized four phenotypically (fruit pungency, colour, weight, width and shape) and nutritionally different *C. annuum* genotypes collected from two different locations of south Italy. Two sweet genotypes namely *Corno di Toro* (CDT; horn-shaped) and *Papaccella* (PAP; roundish type) from Campania region, while remaining two pungent genotypes from Calabria regions namely *Sigaretta* (SIG; horn-shaped) and *Ciliegino* (CIL; cherry shaped). Using resequencing, the study identified substantial variations in terms of SNPs, InDels and observed that *Capsicum* genotypes with high fruit weight and no pungency showed higher polymorphism across chr 9 and chr 11 (Esposito et al. 2022). Overall, the sequencing of reference genome and genome re-sequencing from different *Capsicum* genotypes or inbred lines from different species provided an important genomic/genetic resource in shaping out the prospective functional important genes (especially resistance related) and sequence divergence in understanding and in development of future breeding program for stress resilient and high yield *Capsicum* varieties.

12.8 Application of Molecular and Omics Approaches for Breeding High Yielding and Stress Resistant Chili Peppers

Adverse climatic fluctuations result in major plant stresses that pose a major threat to sustainable agriculture by inhibiting plant growth (Raza et al. 2021). Plants themselves adopt various signalling cascades and mechanisms that make use of molecular, cellular and physiological changes in the plants, to cope with these stresses (Hasanuzzaman et al. 2013; Kang et al. 2020). Molecular techniques can be employed to increase the yield of the crop. Molecular breeding can be used for the selection of quantitatively inherited complex characters (Srivastava and Mangal

Table 12.3 Features of sequenced and assembled *Capsicum* genomes

Capsicum species	Common name/ accessions	Important feature	Haploid chromosomes	Estimated genome size (Mb)	Assembly size (Mb)	No. of Genes	Repeat (%)	Reference
C. annuum cv. Criollo de Morelos 334	CM334	Resistant against diseases including Pepper mottle virus (PMV), Phytophthora capsici, root-knot nematodes	12	3.48 Gb	3.06 Gb	34,951 in v1.0 and 35,884 in v2.0	76.4	Kim et al. (2014), Kim et al. (2017b)
C. chinense	PI159236	Wild type, resistant against Tomato Spotted Wilt Virus (TSWV), Pepper Mottle Virus (PMV), Tobacco Etch Potyvirus (TEV) and Potato Virus Y (PVY)	12	3.2 Gb	3.0 Gb	35,009	79.55	Kim et al. (2014), Kim et al. (2017b)
C. baccatum	PBC81	Anthracnose (Colletotrichum spp.) resistant	12	3.9 Gb	3.2 Gb	35,874	86.2	Kim et al. (2017a, 2017b)
C. annuum var. Zunla-1	Zunla-1	Adapted in different agro-climatic conditions, resistant against Fusarium wilt, Phytophthora spp. and anthracnose	12	3.35 Gb	3.26 Gb	34,476	80.9	Qin et al. (2014)
C. annuum var. Glabriusculum	Chiltepin	wild type	12	3.48 Gb	3.07 Gb	35,336	81.4	Qin et al. (2014)

(continued)

Table 12.3 (continued)

Capsicum species	Common name/ accessions	Important feature	Haploid chromosomes	Estimated genome size (Mb)	Assembly size (Mb)	No. of Genes	Repeat (%)	Reference
Capsicum pan-genome	Total 384 Capsicum accession used (334 C. annuum, 4 C. baccatum, 11 C. chinense and 13 C. frutescens)	Covers genomic and genetic diversity of Capsicum	12	956.4 Gb	–	89,181	–	Ou et al. (2018)

2019). DNA-based molecular markers are utilized to produce new cultivars (D'Amelia et al. 2018). Breeding programmes like marker-assisted selection, marker-assisted backcross breeding for trait introgression, genomic selection and gene editing require DNA markers of the desired properties so that the process of genetic breeding will have higher efficiency (Rao and Anilkumar 2020). *Capsicum* breeders also use techniques like mutation breeding, haploid breeding, polyploidy and embryo rescue (Srivastava and Mangal 2019). Probes for RFLP markers can be developed from the cDNA clones of the genes involved in capsaicinoid biosynthesis (Blum et al. 2003). One of the most practical, economic and environmental strategies is to develop pepper cultivars resistant to pests and diseases, thereby reducing yield loss (Dhall 2015). Method of development of the new cultivar depends upon the genetic variability in the population (Ramalho do Rêgo and Monteiro do Rêgo 2016). A representative diagram of different omics approaches employed for *Capsicum* crop improvement program integrated with conventional methods is given in Fig. 12.2.

12.8.1 Transcriptomic Analysis to Identify Gene(s) for Stress Resilient *Capsicum* Breeding

Transcriptomic studies along with genome assembly uplifted the understanding of functional behaviour/diversity of genomic regions at intra- and interspecific level. In early 2011, before the release of first draft genome of *Capsicum*, Hwang et al. (2011) investigated transcript diversity using microarray technique between *Chilbok* and *KC350*, a susceptible and resistance *C. annuum* genotypes, respectively, against *Ralstonia solanacearum*, which causes soil-borne vascular disease in plants. The study identified several key genes specifically differentially expressed to resistant and susceptible genotypes. For instance, expression of cell-wall organization and xyloglucan biosynthesis genes including *xyloglucan endotransglycosylase/hydrolase* (*XTH*) were upregulated in *KC350* genotype, while genes related to stress response and cell death including *β-galactosidase* were more expressed in susceptible *Chilbok* genotype. Later in 2015, after release of *Capsicum* draft genome (Kim et al. 2014; Qin et al. 2014), a ultra-high transcript based intra- and inter-specific genetic map was constructed (Hill et al. 2015) using two different RIL populations derived from *C. annuum* (cv. Early Jalapeño) × *C. annuum* (cv. *CM334*) cross (Sy et al. 2008) and *C. frutescens* (cv. BG2814-6) × *C. annuum* (cv. NuMex RNaky) cross, respectively (Ben-Chaim et al. 2006). Since the intraspecific RIL populations segregate broadly for resistance to various diseases including *P. capsici* from *CM334* (Sy et al. 2008; Rehrig et al. 2014), therefore, the genetic map will facilitate the identification of candidate genes involved in resistance.

In addition, the generated transcriptomic based genetic map also showed its utility for future trait associated selection useful for future *Capsicum* crop improvement and breeding programmes (Hill et al. 2015). Thereafter, in the last few years, a wide range of transcriptome-based studies have been reported in *Capsicum*, which have identified and profiled the expression of several candidate target genes in various

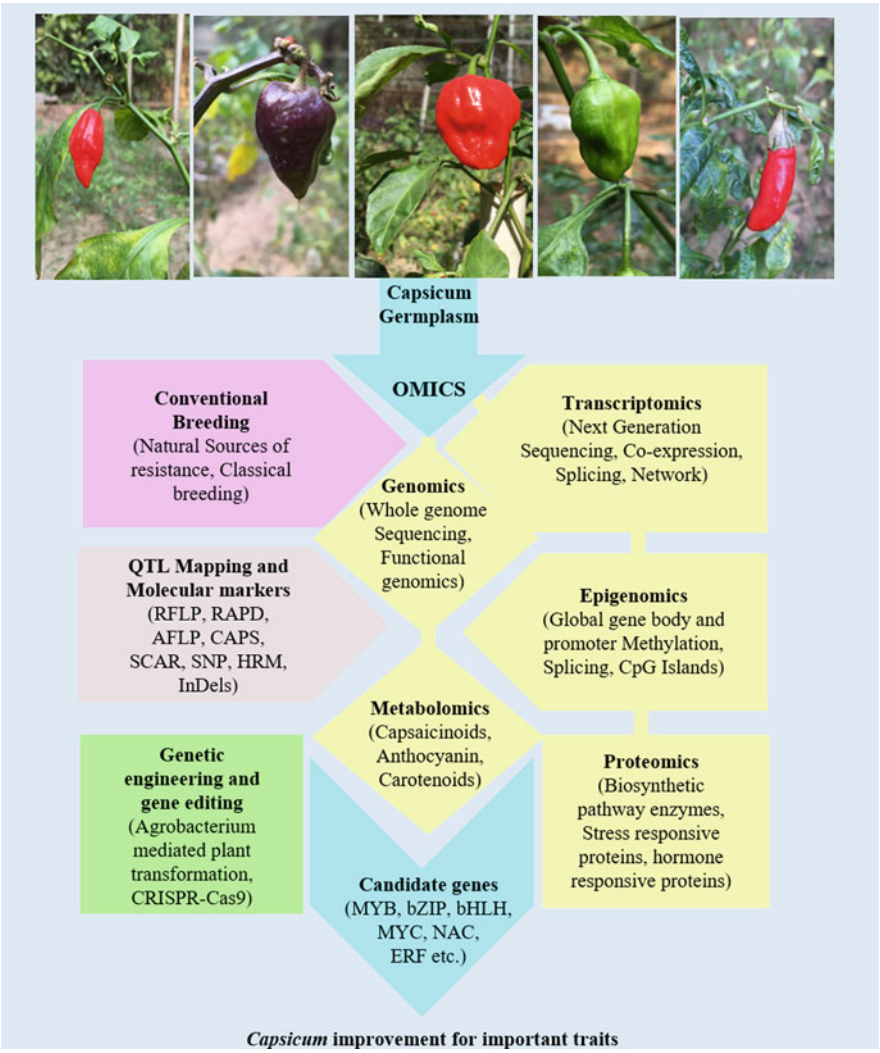

Fig. 12.2 Multi-omics approach for *Capsicum* breeding and improvement

stress responses. For instance, in 2016, Li et al. reported the role of *24-epibrassinolide* (*EBR*) from brassinosteroids (BRs) gene family in response to chilling stress (Li et al. 2016), while Widana Gamage et al. (2016) identified diverse set of genes related to pathogenesis, cell death, hormone signalling and defense response, which were upregulated in *Capsicum* chlorosis virus (*CaCV*) resistant *Capsicum* genotypes. Likewise, another study performed a global transcriptome profiling in massive datasets generated in response to several abiotic response (heat, cold, salt and osmotic stress) in *Capsicum*, which provided a basis for development of future stress-resistant *Capsicum* varieties (Kang et al. 2020). In

this order, another study identified a key member *CaChiVI2* of the chitin-binding protein family, whose high expression was found to be associated with stress resistance against heat and *P. capsici* in *Capsicum* (Ali et al. 2020). Further, another study performed transcriptome profiling during fruit development in *C. annuum*, *C. chinense* and *C. frutescens* of northeast India landrace and developed polymorphic SNP markers from transcripts with high utility and applicability in *Capsicum* breeding programmes for improved *Capsicum* varieties with various agronomic traits (fruit shape, size, pungency) (Chhapekar et al. 2020). Recently, a study deciphered role of genes in formation trichome toward providing defense response against various biotic and abiotic stresses using transcriptome profiling of a hairy (*GZZY-23*) and hairless (*PI246331*) *C. annuum* genotypes (Gao et al. 2021a, b). The study observed that many DEGs belonging to gene families including trichome development, hormone signalling, resistance related genes and transcription factors (from *MYB, bHLH, HD-Zip, zing finger* etc) were upregulated in hairy genotypes (Gao et al. 2021a). In addition, another study conducted a transcriptome wide survey in two bell pepper genotypes, two bell pepper (*C. annuum*) genotypes, *VI037601* and *Early Calwonder (ECW)*, which are resistant and susceptible to *Xanthomonas campestris pv. Vesicatoria (Xcv)*. The study found that genes related groups including *NBS-LRR, oxidoreductase, WRKY* and *NAC* transcription factors were upregulated in resistant genotypes when compared to susceptible one (Gao et al. 2021b). Similarly, Kang et al. (2022) identified that transcription factor families including bHLH and protein kinases were abundantly expressed in *P. capsici* resistant genotypes compared to susceptible genotypes of *C. annuum* (Kang et al. 2022). This can be epitomized that potential gene differential expressed during stress response in various transcriptome studies can serve as a highly beneficial resource useful for *Capsicum* crop improvement worldwide.

12.8.2 Sequencing of Non-coding RNAs in *Capsicum*

Non-coding RNAs (microRNAs: miRNAs, small interfering RNAs: siRNA, long non-coding RNAs: lncRNAs and circular RNAs: circRNAs) found to have regulatory roles in diverse biological functions including plant growth, development and response to various stresses (Jones-Rhoades et al. 2006; Kim et al. 2011; Khraiwesh et al. 2012; Chekanova 2015). Though, several ncRNAs studies have been reported so far in *Capsicum* and most of them just were related to identification and expression profiling of ncRNAs (mostly miRNAs, lncRNAs) in diverse tissues (Hwang et al. 2013; Liu et al. 2017a; Taller et al. 2018; Zuo et al. 2019; Chhapekar et al. 2021; Baruah et al. 2021), still functional basis of miRNAs/siRNA/lncRNAs in stress-response and/or in fruit development remained unclear. The first miRNA transcriptome was reported in early 2013, which profiled miRNAs from 10 different tissues including root, stem, leaf, flower and six fruit developmental stages (Hwang et al. 2013). Later, in 2017, another study profiled miRNAs in fruit tissues from three different fruit stages (mature green, breaker and red-ripe fruit stage) from two different hot pepper (*C. annuum*) varieties '*Luosijiao*' and '*06 J19-1-1-1-2*'. The

study identified 310 novel and 59 known miRNAs whose predicted targets were involved in starch and sucrose metabolism during fruit development (Liu et al. 2017a). In a similar approach, attempts were made to dissect miRNA/siRNA expression during fruit expansion in seed, placenta and fruit pericarp tissues of non-pungent *C. annuum* using a small RNA sequencing approach. The study found that miRNA mediated gene regulation is more active in expanding fruit, which is accompanied by increased accumulation of ARGONAUTE1 in expanding fruit pericarp tissues. The study also reported that heterochromatin-associated 24 nucleotide (nt) siRNAs were highly expressed in seeds, while 21-nt and 24-nt siRNAs were most expressed in seed and placenta tissues (Taller et al. 2018). Such study could be further explored for manipulation of sRNA pathways to improve the yield and quality of *Capsicum* fruit. Further, the plausible biological functions of ncRNAs during low temperature stress was also studied in chilled and unchilled *C. annuum* fruit (Zuo et al. 2018). The study identified a set diverse differentially expressed (DE) ncRNAs including of lncRNA (380), miRNAs (18), circRNAs (36) and 4128 DE mRNAs in response to chilling and observed that targets of these ncRNAs were involved different biological pathways related to stress-response, cell-wall metabolism, oxidative phosphorylation and were from different TFs groups including *MYB, bHLH, ERFs*. In addition, the study extended to explore expression diversity during bell pepper fruit ripening and observed that around 366 lncRNAs, 43 miRNAs, 125 circRNAs and 3266 mRNAs were DE in mature green and fruit tissues. The study observed that DE targets of DE ncRNAs were from different class of TFs (*ERF, WRKY, NAC, bZIPs, MYB* and *ARF*), cell-wall metabolism, fruit colouration, flavour, aroma and ripening related enzymes (Zuo et al. 2019). Then the feasibility of ncRNA based SSRs were also assessed in Northeast Indian accessions from three different *Capsium* species i.e. *C. annuum*, *C. chinense* and *C. frutescens* (Jaiswal et al. 2020). Around 623 ncRNA (504 lncRNAs and 119 miRNAs) based SSRs were developed and around 75% of them were polymorphic and could differentiate efficiently among the accessions of three *Capsicum* species, which suggested their potential utility in future *Capsicum* crop breeding for diverse traits and in mapping genes/QTLs (Jaiswal et al. 2020).

Moreover, the plausible role of ncRNA in heterosis were also deciphered in *Capsicum chinense* accessions *HNCc16, HNCc22* and their hybrid HNCy01, during seedling and flowering stages (Shu et al. 2021). Around 2525 lncRNAs, 47 miRNAs and 71 circRNAs were identified in hybrid *HNCy01* and found that 74 lncRNAs related to plant-pathogen interaction pathway were upregulated between HNCy01 vs. HNCc16, while miRNAs *miR156, miR169* and *miR369* were downregulated in hybrid *HNCy01* (Shu et al. 2021). Furthermore, a comprehensive study was performed using diverse set of tissues (stem, leaf, flower and fruit tissues) from two different Northeast Indian *Capsicum* landrace Kon jolokia (*C. frutescens*; small fruit and moderate pungent) and Bhut jolokia (*C. chinense*; larger fruit and highly pungent) (Chhapekar et al. 2021). The study showed that around 279 and 254 miRNAs were conserved, while 490 and 155 miRNAs were novel from Bhut jolokia and Kon jolokia accessions. Additionally, miRNA target prediction suggested that the predicted miRNAs plausibly regulated the fruit ripening,

carotenoid biosynthesis, cellular metabolism, and fruit aroma differently between *Capsicum* species (Chhapekar et al. 2021). Thereafter, the miRNA profiling in tissues including flower, fruit, early and mature green fruit was investigated in domesticated (*C. annuum* and *C. baccatum*) and wild (*C. chacoense* and *C. eximium*) accessions to understand their plausible functional roles in wild and domesticated Capscium species (Lopez-Ortiz et al. 2021). The study identified 22 and 27 known and novel miRNAs, respectively DE among tissues and *Capsicum* species. In addition, the study identified that miRNA-mRNA targets pairs including *miRNA156/157-SPL* gene, *miR159-GaMYB*, *miR-160-ARF, miR172-AP2-like transcription factor,* and *miR408-CLAVATA1* regulates flowering time and fruit development, while novel miRNAs may regulate fruit quality and plant pathogen defense in *Capsicum* (Lopez-Ortiz et al. 2021). Recently, in *C. annuum*, a bulk number of lncRNAs (12807) were identified and their expression profiling was investigated across tissues under abiotic stress conditions such as heat, cold, salt and osmotic (Baruah et al. 2021). The study observed that during abiotic stress, differentially expressed lncRNAs interacts with various miRNAs and stress-response related TFs including *WRKY, MYB*, *bZIP* and *ERF,* which indicated their active role in abiotic stress-response in *C. annuum* (Baruah et al. 2021).

12.8.3 Epigenomic or Whole Genome Bisulfite Sequencing

Cytosine methylation is one of the most important epigenetic marks which regulates plant growth/development and stress responses via influencing the integrity and expression profiling of genomes, TEs and genes, respectively (Law and Jacobsen 2010). In *Capsicum*, limited studies/reports on methylation are available. In 2004, the transcriptional activation as well as DNA methylation in embryo tissues of germinating and dry *Capsicum* seeds were investigated using methylation sensitive polymorphism (MSAP) analysis and suggested that active demethylation is involved during germination (Portis et al. 2004). Using the same MSAP technique, adjustment of methylation marks in two *Capsicum* genotypes (*D85* purple and *D34* green cotyledon) and in their reciprocal F_1 hybrid were observed (Xu et al. 2015). While, another study conducted in *C. annuum* observed that siRNA mediated *de novo* epigenetic stances regulate shape/size of fruits in grafted hybrids (Tsaballa et al. 2013). Further, genome-wide methylation instances were also deciphered in fruits of phenotypically different *Capsicum* accessions (2 *C. annuum* and one each from *C. chinense* and *C. frutescens*) (Rawoof et al. 2020). The study suggested that intragenic DMRs are most abundant in *Capsicum* genome, while *C. frutescens* (small fruit and moderate pungent) was overall hypomethylated compared to *C. annuum* (large fruit, low pungent) and *C. chinense* (large fruit and highly pungent). Further the study observed genome-wide overall significant negative correlation between CG, CHG methylation and gene expression in *Capsicum* and suggested that methylation might regulate fruit development/ripening in *Capsicum* (Rawoof et al. 2020). Furthermore, another study analyzed the methylation and expression regulation of fruit ripening related genes using McrBC PCR, bisulfite

sequencing (Xiao et al. 2020). The study attributed that hypo-methylation at promoter region of fruit ripening genes in breaker fruit stage is due to increased expression of *CaDMR2-like* and decreased expression of *CaMET1-like* (1 & 2), *CaCMT2-like*, and *CaCMT4-like* methyltransferase genes, which suggested the DNA methylation mediated regulation of fruit ripening in *Capsicum* (Xiao et al. 2020). However, it is not clear how methylation and demethylation patterns affect the fruit ripening process in fruit (Zhong et al. 2013; Lang et al. 2017). Also, the impact of salt (salinity) stress on DNA methylation patterns as well as on enzyme activity, nutrient uptake, malondialdehyde and hydrogen peroxide (H_2O_2) uptake was analyzed among the three *C. annuum* cultivars namely *Dolmalik*, *Carliston* and *Maras* from Turkey (Shams et al. 2020). The study observed that with increased salt stress, the level of DNA methylation ratio was increased by ~11% in *Carliston*, 10% in *Dolmalik* and 5.45% in *Maras* cultivars. The *Maras* cultivar was salt tolerant, while the remaining two were susceptible. The study indicated that de-methylation with increased enzyme activity could be one of the possible reasons for salt-tolerance in Maras cultivar (Shams et al. 2020). Recently, Araz et al. (2022), showed the impact of low temperatures on various attributes including plant growth, enzymes activities, H_2O_2 content, DNA methylation and chlorophyll content in four different *C. annuum* cultivars (cv. *AK, KD, YC* and *TK*). Based on genomic DNA methylation ratio during low-temperature stress, the study identified that cv. *AK* showed a comparatively decreased methylation ratio than the rest of 3 cv. *KD, YC* and *TK*, while it was more tolerant to low-temperature stress (Araz et al. 2022).

12.8.4 Proteomics

Proteomic study in *Capsicum* is a novel topic and a potential area to explore the mechanisms of regulation of biosynthetic pathways that are catalyzed by enzymes. As proteins regulate different developmental stages of growth and fruit ripening processes, identification of proteins at the proteome level or specific proteins is crucial to understand the growth mechanism of chili to impart tolerance to varying environmental stress, process of fruit development and ripening as well as the myriad synthesis of carotegenic compounds. A recent review on *Capsicum* proteomics was updated by Momo et al. 2022, where they have discussed the various proteomic investigations conducted in *Capsicum* over the last two decades (Momo et al. 2022). Several proteomic investigations have been carried out in different tissues such as seedlings (Pessoa-Filho et al. 2004; Manivannan et al. 2016; Zhang et al. 2019), roots (García-Fontana et al. 2020), flowers/anthers/buds (Wu et al. 2013; Guo et al. 2017a, b; Cheng et al. 2019), leaves (Lee et al. 2006; Elvira et al. 2008; Choi and Hwang 2011; Mahajan et al. 2014; Jaswanthi et al. 2019; Florencio-Ortiz et al. 2021), fruits (Lee et al. 2006; Siddique et al. 2006; Sánchez-Bel et al. 2012; Wang et al. 2013a; Aizat et al. 2013; Camejo et al. 2015; Chaki et al. 2015; Rodríguez-Ruiz et al. 2019) and also in callus suspension cultures (Sabater-Jara et al. 2010).

Many proteins were identified by researchers in the span of a couple of decades to identify tissue specific expressions. Proteins such as PR-1 like, chaperones Hsp 90-5, Hsp 83-like and signalling proteins MAPK homolog MMK2, CDPK SK5-like, Glycerol kinase, Chitin elicitor receptor kinase 1-like isoform X1, MAPK homolog NTF4 and CDPK4 were identified in early stages of floral development and young buds that shoot out from the meristematic stem (Patavardhan et al. 2020). As development progresses, the proteomic makeup also changes. In some of the cytoplasmic sterile pollens of *Capsicum*, proteins such as the Polyphenol oxidase F and ATP synthase subunit beta, ATP synthase CF1 alpha chain, glutelin and some triose phosphate isomerases were also observed (Wu et al. 2013). During fruit ripening and development, early green stages of fruit showed the expression of some proteins such as dihydroxy acid dehydratase, chaperonin 60 beta subunit, T-complex beta subunit, amino aldehyde dehydrogenase 2, ketol reductoisomerase and TCP domain transcription factor (Aizat et al. 2013). While transition from green to mature stage (breaker), XTH group of proteins (Aizat et al. 2013) as well as Annexins *ANN4*, cellulose synthase and pectin methyl esterase were observed which could possibly play role in the chloroplast to chromoplast transition during fruit development (Liu et al. 2019). The mature stage of fruit ripening displayed the presence of cell wall reconstruction enzymes, cellulose synthases, pectin methyl esterases, cysteine/spermidine synthases, kinases and phosphatases (Aizat et al. 2013). Moreover, carotenoid and terpenoid biosynthetic enzymes like *GGPS, CCS, HPT, DXS, DXR* along with glucose metabolism related isomerases were also detected (Siddique et al. 2006).

During biotic and abiotic stress, chili plants display the expression of PR proteins such as glucanases, chitinases, osmotin and germin like proteins that combat the invading pathogen and portray the first line of defense by inducing overall hypersensitive response (Elvira et al. 2008). ROS enzymes also play a significant role in detoxifying oxide radicals formed from oxygen and nitrogen derivatives. ROS detoxifying enzymes such as Catalase (*CAT*), Superoxide dismutase (*SOD*) and Peroxidase (*POX*) are usually upregulated during hyperoxide stress in plants (Jung et al. 2008). Other proteins like annexin (*ANN4*), Pyrabactin resistance (*PYR*), calreticulin (*CRT3*) and some phloem specific *SEOR1* as well as phosphoglycerate kinases were observed in chili plants infected with insects/herbivores (Wu et al. 2019). Some cold responsive proteins were also reported that specifically upregulate in response to cold stress. Proteins like annexin cap32, RNA processing maturase K and phosphoglucomutase were relatively acetylated and expressed as a response toward cold stress in transgenic plants that were resistant to cold (Liu et al. 2021b). Some reports in 'Bungwang' variety of chili, stated the abundance of signalling proteins that were upregulated in response to salinity. They include calcium binding protein CML17-like, some ubiquitin ligases, MADS-box transcription factor, vacuolar protein sorting associated 53 A isoform X2, Ras-related protein RABH1-b like and other receptor like protein kinases along with some F-box/kelch repeat proteins (Manivannan et al. 2016). Some signature proteins of the apoplast of chili during drought conditions were revealed that impart drought tolerance. They include miraculins, germin-like proteins, pin II type proteinase inhibitors and several redox

metabolizing enzymes like peroxidase, somatic receptor kinases, defensins and some heat shock cognate proteins (Jaswanthi et al. 2019). Similarly, in heat stressed chili plants, the expression of ROS scavengers was prevalent with the abundance of heat specific manganese stabilizing proteins, ATP synthase CF subunits ML domain proteins and activation of several WRKYs (Dang et al. 2014) and HSL1 along with other families of diverse heat shock cognate proteins (Kim and Hwang 2015; Feng et al. 2019; Huang et al. 2019).

Some reports for post-translational regulation of proteins were done in some chili cultivars. Abundance of phosphopeptides from *C. annuum* 'SJ11-3' depicted the role of signalling response mediated by phosphorylation at the pSer and pThr sites which are important motifs for switching the active state of cytosolic and nuclear enzymes that impart signals throughout the cell (Liu et al. 2020). As post-translational modifications dictate the regulation of proteins for their functional target, it is essential to understand the post regulation of protein synthesis to gain insights into understanding the protein regulation machinery within the cell. Not much work is done in chili in regard to post translational modifications and it is recommended that research should be focused in this aspect too.

12.9 Candidate Genes for *Capsicum* Breeding

Fruit orientation either erect or pendant is controlled by *up* locus on the chromosome 12 in *Capsicum* spp. (Lefebvre et al. 1995). Chlorophyll content in the green fruit is attributed to the GOLDEN2-like (*CaGLK2*) gene (Brand et al. 2014). An early-flowering mutant was isolated to determine the underlying gene responsible for transition to flowering in pepper (Borovsky et al. 2015). Genetic mapping using NILs developed from differential flowering time *Capsicum* accessions and sequencing of candidate genes could reveal an *APETALA2* transcription factor family gene *CaAP2* that explained about 52% of the variation in the phenotype. Although gene sequencing revealed no significant differences, differential expression of *CaAP2* could result in variation in the flowering time in the two pepper accessions. A SNP (A to G^{709} transition) in the coding region of β-*CAROTENE HYDROXYLASE2* gene completely co-segregated with the orange-fruit phenotype in *C. annuum* (Borovsky et al. 2013). Also, the expression analysis reveals possible involvement of β-*CAROTENE HYDROXYLASE2* in the red to orange transition in the carotenoid biosynthesis pathway. Fruit ripening in *C. annuum* was studied using transcriptomic and gene expression analysis and some of the WRKY genes (*CaWRKY28, CaWRKY, CaWRKY51, CaWRKY11, CaWRKY01* and *CaWRKY52)* were differentially expressed during the fruit maturation (Cheng et al. 2016c).

During fruit ripening, *chlorophyll retainer* (*cl*) mutant is associated with the inhibition of the chlorophyll degradation and retention of the green colour of the fruit. In a population of green fruited *C. annuum* (IL 4590) × red fruited *C. chinense* (PI 159234), the *C. annuum* stay-green (*CaSGR*) gene co-segregated with the *cl* mutant indicating complete linkage of the two loci (Borovsky and Paran 2008). Additionally, a SNP (W^{114} to R) was detected in the protein coding sequence of the

CaSGR gene that differentiated between the wild type and mutant allele and could serve as a selection marker in the pepper breeding.

The gene expression of *Acl, Fat* and *Kas* was positively correlated with the pungency levels in the placenta of *Capsicum* fruits (Aluru et al. 2003). The genetic map positions of *Acl, Kas* and *Fat* were determined on LG 1 and LG 6, respectively, using three F2 mapping populations- *C. annuum* cv. NuMex RNaky × *C. chinense* PI 159234 (AC), *C. frutescens* BG 2814-6 × *C. annuum* cv. NuMex RNaky (FA) and *C. frutescens* BG 2814-6 × *C. chinense* PI 159234 (FC). Chromosomal positions of other capsaicinoid biosynthetic genes like *Pal*, *b-ZIP2, bZIP, KasI* etc. were determined on chromosome 9, 1 and 12, respectively, using the AF population in another study (Blum et al. 2003). None of these genes however, was linked to pungency locus *C* on LG 2. Based on gene expression analysis, MYB TFs such as *CcMYB16*, *CcMYB100* and *CcMYB106* can potentially regulate capsaicinoid and anthocyanin biosynthesis in *C. chinense* (Islam et al. 2021).

Genetic controls of purple anthocyanin biosynthesis and accumulation in pepper fruits and flowers were investigated in two F_2 mapping populations- one from a cross between purple-fruited *C. annuum* (5226) and green fruited *C. chinense* (PI159234) and another from a cross between IL 579 and 100/63 (*C. annuum*) (Borovsky et al. 2004). Anthocyanin production in pepper leaves, flower and fruits was found to be controlled by a MYB transcription factor gene *A* locus which is also homologous to *Petunia Anthocyanin2* (*An2*). Genetic mapping of the *A* locus has placed it on chromosome 10 in the F_2 population resulting from the cross between *C. annuum* (5226) and *C. chinense* (PI 159234) (Ben Chaim et al. 2003a). The variation in the phenotype can be attributed to the upstream promoter region and resulting differential expression of the A locus in the two *Capsicum* accessions as no significant variations were observed in the coding sequence.

Resistance gene (R) clusters were co-localized with the resistance QTLs for *P. capsici* on pepper chromosome 11 in the genetic map generated using F_3 Yolo Wonder × CM334 (YC) population (Thabuis et al. 2003). Two *NBS-LRR-type* genes were speculated in the *PMR1* locus in powdery mildew resistant *C. annuum* 'VKR515R' (Jo et al. 2017). Using a F_2 population of a cross [PI201234 (resistant) × PI201234 (susceptible)] of two *C. annuum* lines, a dominant gene *CaPhyto* was identified as a candidate for resistance against *P. capsici* race 2 and was mapped to a 3.3 cM region on chromosome 5 with the help of 10 SSR and two CAPS markers (Wang et al. 2016). A highly reliable SSR marker named ZL6726, 1.5 cM away from *CaPhyto,* was developed for MAS for *P. capsici* race 2 resistant phenotypes. Another single dominant gene for *P. capsici* resistance- *PHR10* was mapped to an interval of 2.57 Mb between two SSR markers P52-11-21 and P52-11-41 on pepper chromosome 10 using two BC1 and one F_2 population obtained from CM334 (resistant) × NMCA10399 (susceptible) (Xu et al. 2016). Several Chitin-binding proteins like *CaChiI3*, *CaChiIII1*, *CaChiIII2*, *CaChiIII7*, *CaChiIV1* and *CaChiVI1* were expressed highly in response to *P. capsici* inoculation in *C. annuum* (Ali et al. 2018).

An Abscisic acid-responsive protein *C. annuum DRought Tolerance 1* (*CaDRT1*) was identified as a positive modulator of drought tolerance in *C. annuum* following

decreased transpiration and increased leaf temperature, stomatal closure and gene expression of drought responsive genes in the *CaDRT1* overexpression lines (Baek et al. 2016). A *CaF-Box* protein was upregulated twofold with respect to control plants upon treatment with ABA and displayed reduced tolerance to cold stress upon Virus Induced Gene Silencing (VIGS) in *C. annuum* (Chen et al. 2014). Chitin-binding proteins like *CaChiIII2, CaChiIII7* and *CaChiVI2* were upregulated in response to salinity (NaCl) treatment and *CaChiI3* and *CaChiIV1* in response to drought (mannitol) treatment in *C. annuum* and may play important roles in imparting tolerance to abiotic stresses (Ali et al. 2018). The gene expression levels of 22 *C. annuum* NAC (*CaNAC*) transcription factor gene family were analysed and it was observed that *CaNAC72* was upregulated in response to salt, that of *CaNAC13, CaNAC20, CaNAC29,* and *CaNAC53* in response to heat, *CaNAC*20, *CaNAC*23, *CaNAC*35, *CaNAC*53 upon *P. capsici* infection and *CaNAC*27, *CaNAC*37, *CaNAC*41, *CaNAC*61, and *CaNAC*72 upon ABA treatment (Diao et al. 2018). Several Dehydrin family genes were induced by abiotic stress treatments like- *CaDHN7, CaDHN1, CaDHN2, CaDHN3* and *CaDHN4* during cold stress, *CaDHN5, CaDHN7 CaDHN1, CaDHN2* and *CaDHN3* during salt stress, *CaDHN5* and *CaDHN7* upon osmotic stress in *C. annuum* (Jing et al. 2016). Among the negative regulators of drought tolerance in *Capsicum* is *C. annuum Mildew resistance locus O* 2 (Ca*MLO2*) which upon silencing leads to increased stomatal closure, lower transpiration and lipid peroxidation in dehydrated pepper leaves (Lim and Lee 2014). Overexpression of *C. annuum Lipooxigenases 1* (*CaLOX1)* led to increased tolerance to salinity, drought and osmotic stress via reduced H_2O_2 production and altered expression of ABA and stress responsive genes (Lim et al. 2015a). Other positive regulators of ABA and drought stress include- *C. annuum Ring finger protein gene 1* (*CaRING1)* whose overexpression provides higher sensitivity to ABA and increased stomatal closure in *C. annuum l*eaves (Lim et al. 2015b). Table 12.4 summarizes the candidate genes for breeding high yield and stress resistant *Capsicum* plants.

12.10 Breeding for Stress Resistance in *Capsicum*

In many countries, depending on the region and climate, vegetable crop production is limited by various abiotic and biotic stresses. These stresses alter the plant's transcriptomic, cellular, and physiological metabolism (Atkinson and Urwin 2012). Adopting classical breeding techniques for stress resistance like hybridization and backcross breeding were time consuming, expensive and hectic, because of which molecular approaches like genomics, mutation, MAS, recombinant DNA technology, targeted induced local lesions in genome (TILLING), and virus-induced gene silencing (VIGS) have been adapted by breeders (Hussain 2015). Exposure to various stresses produces pathogenesis-related proteins in the plants (Subramanyam et al. 2011). The studies show that virus and herbivore resistance of the *Capsicum* can be associated with the trichome formation in the plant (Kim et al. 2012). Certain genes like *CaChiVI2* gene provide both biotic (to *Phytophthora capsici*) and abiotic

Table 12.4 Candidate genes for important traits in *Capsicum* for introgression, genetic engineering and gene modulation in desired backgrounds for developing improved *Capsicum* varieties

Species/ population	Candidate gene	Trait	References
C. annuum	*CaAP2*	Flowering time	Borovsky et al. (2015)
C. chinense	*WD-40, CLAVATA1 and Auxin receptor, TTL3, EAR1, SEC8* and *PDR11*	Fruit shape and size	Nimmakayala et al. (2021)
C. annuum	*OVATE Family Protein (CaOFP20)*	Fruit elongation	Borovsky et al. (2021)
C. annuum	*CaWRKY28*	Fruit Ripening	Cheng et al. (2016a)
C. annuum	*LOC107847473* (ortholog of *MADS-RIN)*	Fruit Ripening	Dubey et al. (2019)
C. annuum	β-*CAROTENE HYDROXYLASE2*	Carotenoid composition	Borovsky et al. (2013)
C. annuum	*CabHLH032* and *CabHLH095*	Carotenoid	Liu et al. (2021a)
C. annuum	*CaERF66, CaERF97, CaERF101* and *CaERF107*	Carotenoid	Song et al. (2020)
C. annuum	Chlorophyll retainer (*cl*)/ stay-green (*SGR*)	Chlorophyll retention	Borovsky and Paran (2008)
C. annuum	*CaMYB12* and *Flavonol Synthase* (*Fs-2)*	Flavonoids content	Wahyuni et al. (2014)
C. annuum	*A* locus	Anthocyanin	Borovsky et al. (2004)
C. annuum	*MYB A, MYB1, MYB2, CaMYB, CaAn2* and *CaAN3*	Anthocyanin	Li et al. (2011), Zhang et al. (2020), Jung et al. (2019); Byun et al. (2022)
C. annuum × *C. chinense*	*pAMT*, *C4H, CSE* and *4CL*	Capsaicinoid	Han et al. (2018)
C. chinense × *C. annuum*	*FatA* (CA06g26640)	Capsaicinoid	Han et al. (2018), Park et al. (2019)
C. chinense × *C. annuum*	*KETOACYL-ACP REDUCTASE* (*KR*), *C4H*	Capsaicinoid	Park et al. (2019)
C. annuum × *C. frutescens*	*CCoAOMT*, *BCKDH-E1α* and *ACS2*	Capsaicinoid	Yarnes et al. (2013)
C. annuum × *C. frutescens*	*KasIIIα, KasI*	Capsaicinoid	Yarnes et al. (2013), Blum et al. (2003)
C. annuum × *C. frutescens*	*b-Zip2, BCAT, AC11, Pal*	Capsaicinoid	Blum et al. (2003)

(continued)

Table 12.4 (continued)

Species/ population	Candidate gene	Trait	References
C. chinense	*Fat, Acl, Kas*	Capsaicinoid	Aluru et al. (2003)
C. annuum	*CaMYB31*	Capsaicinoid	Zhu et al. (2019), Arce-Rodríguez and Ochoa-Alejo (2017), Han et al. (2019)
C. annuum	*CaMYB48*	Capsaicinoid	Sun et al. (2020)
C. annuum	*CaMYB108*	Capsaicinoid	Sun et al. (2019)
C. annuum	*CaPhyto* (Capana05g000764 and Capana05g000769)	*Phytophthora capsici* blight	Wang et al. (2016)
C. annuum	*PHR10*	*Phytophthora* root rot	Xu et al. (2016)
C. annuum	*CaChiI3*, *CaChiIII1*, *CaChiIII2*, *CaChiIII4*, *CaChiIII6*, *CaChiIII7*, *CaChiIV1*, *CaChiVI1* and *CaChiVI2*	*Phytophthora capsici* resistance	Ali et al. (2018)
C. annuum	*Resistance genes (R)*	*Phytophthora capsici* root rot	Thabuis et al. (2003)
C. annuum	*Nucleotide-binding site leucine-rich repeat (NBS-LRR)-type*	Powdery mildew resistance	Jo et al. (2017)
C. annuum	*NBs-LRR repeats* and *R genes*	Root-knot nematode resistance *(Meloidogyne incognita, M. arenaria,* and *M. javanica)*	Barbary et al. (2016)
C. frutescens	*CA02g19570* and *CA02g19600*	Cucumber Mosaic Virus resistance	Guo et al. (2017b)
C. annuum	Defensins- *CanThio* and *CanDef*	Inhibition of insect pest *Helicoverpa armigera* and antifungal activity against *F. oxysporum*	Mulla et al. (2021)
C. annuum	*CaChiIII2*, *CaChiIII7*, *CaChiVI1* and *CaChiVI2*	Salinity, cold and drought stress	Ali et al. (2018)
C. annuum	*CaDRT1*	Drought stress	Baek et al. (2016)
C. annuum	*CaF-Box*	Drought, cold and salinity	Chen et al. (2014)
C. annuum	*CaNAC72 and CaNAC27*	Salt and drought	Diao et al. (2018)
C. annuum	*CaDHN3*	Salt	Jing et al. (2016)
C. annuum	*CaMLO2*	ABA-mediated Drought tolerance	Lim and Lee (2014)
C. annuum	*CaLOX1*	Drought, salinity and osmotic stress	Lim et al. (2015a)
C. annuum	*CaRING1*	Dehydration tolerance	Lim et al. (2015b)
C. annuum	*CaPHT1;3, CaPHT1;7, CaPHT3;1*and *CaPHT3;4*	Phosphate stress	Ahmad et al. (2021)

stress (heat stress) (Ali et al. 2020). Similarly, peroxidase gene *CanPOD* was reported to present a dual role in plant defense response against *Phytophthora capsici* and abiotic stress (Wang et al. 2013b). SAR8.2 genes are also induced upon induction of both biotic and abiotic stresses (Lee and Hwang 2003). Ochoa-Alejo and Ramirez-Malagon (2001) did genetic engineering studies in chili plants that were resistant to virus and other abiotic stress. Overexpression genes such as *CaNAC1* have resulted in higher defense response to infection of biotrophic pathogens as well as drought stress than the wild type (Tweneboah and Oh 2017). An exposure to a pathogen X. ag 8ra and cold stress were also reported to induce increase in the *CaKR1* transcript levels (Seong et al. 2007).

12.10.1 Biotic Stress Resistance

Plant cells under biotic stress experience and display hypersensitive response, cellular senescence, defense activation and ion-flux change (Chhapekar et al. 2018). Biotic stress resistance can be provided by one or more genes and this genetic basis should be exploited by breeders for attributing the resistance to the plants (Hussain 2015). Researchers can also take advantage of NGS technologies to study the whole-genome sequencing of the pathogen and thereby confer resistance to it (Parisi et al. 2020). LeUCP gene, isolated from tomato, is studied to be responsible for attributing stress tolerance to these plants (Chowdhury et al. 2020). MAS can be used to identify the genotypes susceptible as well as resistant to anthracnose disease (Ridzuan et al. 2016). CaRGA2 isolated from a wild plant is proven to show resistance against *Phytophthora capsici* (Majid et al. 2017). Polygalacturonase-inhibiting proteins (PGIPs) were observed and isolated from chili peppers to play a crucial role in the inhibition of fungal endopolygalacturonase (Wang et al. 2013c).

12.10.2 Abiotic Stress Resistance

Drought stress affects the quality of crops (Kopta et al. 2020). The presence of low moisture and high temperature are major constraints that affect productivity in chili plants (Reddy et al. 2016). The abiotic stresses like heat, cold or salinity are sensed by receptors that induce a signalling cascade within the cell which later activates ion channels, kinase cascades, and sometimes produce reactive oxygen species and by some other means such as accumulation of hormones, i.e., salicylic acid (SA), ethylene (ET), jasmonic acid (JA), and ABA (Fujita et al. 2006). Pea DNA Helicase 45 (*PDH45*) is shown to impart resistance to multiple abiotic stresses in *Capsicum* (Shivakumara et al. 2017). Hence, overexpressing *PDH45* in *Capsicum* can provide resistance to the plant. Heat shock proteins (HSPs) that provide heat stress tolerance can be introduced into the plants to resist heat stress (Kunchge et al. 2012). Dehydrins (DHNs), which increase the abiotic stress resistance in plants, also play a major role in the growth and development of pepper and were also found to be induced upon exposure to stresses and also by signalling molecules (Jing et al.

2016). *CASAR82A* gene transcription was rapidly induced by abiotic stresses like high salinity, drought and low-temperature stresses (Lee and Hwang 2003).

12.10.2.1 Temperature Stress

High temperature affects the photosynthetic pigments in the leaf and thereby reduces the photosynthetic activity of chili pepper plants (Ghai et al. 2016). Under controlled growth conditions, the capsaicin content was increased during high temperature treatment than low temperature (Rahman et al. 2012). Low temperature is found to induce nitrosative and oxidative stress in *Capsicum* plants (Airaki et al. 2011). *LTSF1* and *LTSF2* are the F-Box genes that activate the antioxidant enzyme activities and are responsible for tolerance to low temperature in *C. chinense* (Venkatesh et al. 2020). *Serratia nematodiphila* PEJ1011 provides cold stress to *C. annuum* L., thereby adapting the plants to grow under adverse climatic conditions (Kang et al. 2015). The antioxidant system of chili pepper is reported to respond against the temperature changes occurring in the plant (Mateos et al. 2013).

12.10.2.2 Water Stress

Water stress reduces fruit quality in bell pepper (Delfine et al. 2002). Turner (1985) found that low water levels in the soil can lead to reduced uptake of phosphate from the soil by the plant. Silencing of *CaHsp25.9* reduces while overexpression of the gene enhances the tolerance to water stress in chili peppers (Feng et al. 2019). Transcription factors *CaNAC072* and *CaNAC104* are induced by drought stress and are downregulated on provision of water (Borràs et al. 2021).

12.10.2.3 Salinity Stress

Supplementation of silicon increased salinity stress resistance and also improved the physiology and photosynthesis of *Capsicum* plants under saline stress (Manivannan et al. 2016). Wheat Na+/H+ antiporter gene (TaNHX2) showed increased levels of proline, chlorophyll content, relative water content, enzymes like SOD, ascorbate peroxidase and reduced levels of hydrogen peroxide (H_2O_2) and malondialdehyde, which further imparts salinity tolerance to chili plants (Bulle et al. 2016). Application of ascorbic acid mitigated salinity stress and improved the growth and yield parameters of *C. annuum* L. plants (El-Beltagi et al. 2022). Exogenous application of the combination of osmolytes like proline and L-tryptophan increased the growth of pepper under control as well as saline conditions (Jamil et al. 2018). Plant growth-promoting bacterial species like Rhizobacteria alleviates the harmful effects of salinity stress in pepper plants (Hahm et al. 2017). Application of organic matter and gibberellic acid increased the resistance to abiotic stress caused by saline water in these plants (Altae 2017).

12.11 Genetic Engineering for Improvement of *Capsicum* Crop

There have been recurrent problems in engineering new traits in *Capsicum* through transgenesis majorly because of the recalcitrancy of *Capsicum* growth *in vitro*. Genetic engineering enables transfer of novel useful traits in *Capsicum* that could be beneficial for *Capsicum* to adapt to changing environments or to form new compounds. Genetic engineering program usually involves the overexpression of a single or multiple genes into a host plant through a constitutive promoter (CaMV 35S) or through knock-out of target genes which could be mediated by RNAi suppression, miRNA or latest CRISPR related technologies. In *Capsicum*, genetic constructs that carry 35S CaMV promoters with NOS terminator genes have been employed to study and characterize genes (Liu et al. 1990; Kim et al. 2001; Lee et al. 2004). These plasmids carry reporters such as GFP/YFP or GUS to study the expression or tissue specific localization of target genes. The transformation and regeneration of *Capsicum* is mostly done from young meristematic hypocotyls and cotyledons of seedlings which are most responsive for direct organogenesis of shoots and roots (Manoharan et al. 1998; Kim et al. 2001; Shin et al. 2002; Lee et al. 2004). Some transgene expression of foreign genes like the *OsMADS1* from rice have been conducted to induce dwarfism in *Capsicum* (Kim et al. 2001). Overexpressing a tobacco pathogenesis (PR) gene has been reported in *Capsicum* to display broad spectrum resistance against the pathogen, *Xanthomonas campestris* pv. *vesicatoria* (Shin et al. 2002). Additionally, by suppression of target genes using RNAi or other gene silencing mechanisms, transgenic *Capsicum* have been obtained for endo-1,4-beta-glucanase (Harpster et al. 2002), and also for a novel gene ketoacyl-ACP reductase (*CaKR1*) for the generation of non-pungent *Capsicum*. Some transgenic studies have been performed using the cotyledons in genotypes such as Yolo Wonder (Liu et al. 1990), Golden tower (Kim et al. 1997), Pusa Jwala (Manoharan et al. 1998; Shivegowda et al. 2002), some regional specific cultivars Zhongjiao and F1 Xiangyan (Mihálka et al. 2003) as well as some hybrids F1 Fiesta, Ferrari and Spirit (Heidmann et al. 2011). Hypocotyls have also been used as explants to regenerate transgenic plants in genotypes such as Nockkwang (Kim et al. 2001), Arka Gaurav and Arka Mohini (Kumar et al. 2012), Pusa Jwala (Mahto et al. 2018) and Liberty Bell (Liu et al. 1990). Other tissues like the young embryonic tissues, leaves, and shoot tips were also used for transformation (Harpster et al. 2002). The past and recent genetic engineering studies in chili crops are listed in Table 12.5.

As observed, genetic transfer has been successfully carried out despite the challenges in transformation and regeneration consistency of the growth of explants. Therefore, *Capsicum* presents a potential new model for exploring various molecular mechanisms for secondary metabolites biosynthetic pathways, metabolic synthesis, carotenoid accumulation and several other stress tolerance related pathways. Though *Capsicum* is not as feasible for genetic transformation as its relative tomato, and is not yet recognized as a model organism, it can be ascertained that the relevance of its agronomic and pharmacological importance worldwide depicts its exploration for study in fields of molecular and genetic research.

Table 12.5 Genetically engineered genes in *Capsicum* spp. for resistance and other desirable traits

Target gene	Biosynthetic pathway/Gene family	Background of engineering	*Capsicum* spp.	References
Sesquiterpene cyclase	Isoprenoid pathway	Synthesis of phytoalexins for protection against excessive UV	*C. annuum*	Back et al. (1998)
Hydroxycinnamoyl-coA:tyramine N-(hydrxyxinnamoyl)transferase	N-(hydroxycinnamoyl)-amines synthesis	UV-C protection of tissues by generation of a fortified cell wall	*C. annuum*	Back et al. (2001)
MK1 and *MK2*	MAP kinases	Cell signalling in response to wounding, UV-C and cold treatment	*C. annuum*	Shin et al. (2001)
Ferrodixin-like protein (pflp)	Synthesis of Oncidium	Resistance against Erwinia corotovora	*C. annuum*	Liau et al. (2003)
pvr1 locus	eIF4E	Resistance against potyvirus	*C. chinense*	Kang et al. (2007)
CaMNR1 [menthone (+) -(3S)-neomenthol reductase	short-chain dehydrogenase/ reductase (SDR) superfamily	Resistance to biotrophic pathogens (*Hyaloperenospora parasitica* and *Pseudomonas synringae* pv tomato DC(3000))	*C. annuum*	Choi et al. (2008)
Alpha mannosidase/beta-D-N-acetylhexoseaminidase	N-glycan processing enzymes	Fruit softening during ripening	*C. annuum*	Ghosh et al. (2011)
ChilV3	chitin binding protein family (CBP)	Resistance to *Phytophthora capsici*	*C. annuum*	Liu et al. (2017a)
CaChilV1	chitin binding protein family (CBP)	Increased resistance to *Phytophthora capsici*	*C. annuum*	Ali et al. (2019)
CaMYB108	R2-R3 MYB family	Jasmonic acid induced regulation of Capsaicinoid biosynthesis and stamen development	*C. annuum*	Sun et al. (2019)
PSY2	Carotenoid biosynthesis	Colour development in fruits of Capsicum	*C. annuum*	Jang et al. (2020)

12.12 *Capsicum* Genomic Database Resources

Till now, several genome assembly and transcriptomic studies have been conducted using various accessions of different *Capsicum* species. Most information about assembled genomes and genomic features are not available on single unified database resources but are available on different databases (such as Solgenomics, PepperHub etc.) hosted by individual institutions or authors of the specific genome assembly. Among the available databases, the National Center for Biotechnology Information (NCBI), facilitates a common platform for all published *Capsicum* genomes' assembly and their genomic features in a unified way. Using NCBI, one can search and obtain sequence information of nucleic acid (DNA, RNA) and proteins related to specific *Capsicum species* as well as accessions. The website also allows users to perform homology search for *Capsicum* sequences using various BLAST programmes to obtain homologous genes (https://www.ncbi.nlm.nih.gov/).

The database Solanaceae Genomics Network (https://solgenomics.net/) aka Solgenomics is a comprehensive server platform which provides genomic resources (complete and draft genomes) for various Solanaceae crop plants including tomato, potato, *capsicum*, tobacco, petunia etc. The online web server provides different tools from browsing genome to accessing, analyzing and comparing genomic sequences using BLAST, gene ID conversion, motif finding, sequencing alignment analyzer, genome and genomic sequence (mRNA, CDS, protein) download and annotation. Specific to *Capsicum*, the database provides information about the genetic maps named as Pepper *Capsicum*, Pepper-Acc99, Pepper-FAO7 and Pepper-COSII, Pepper-FAO3 from inter-specific F2 population of C. *annuum* A44750157 × C. *chinense* PI 152225, C. *annuum* cv. *NuMex RNaky* × C. *chinense* var. *PI159234*, C. *annuum* cv. *NuMex RNaky* (FA07) × C. *frutescens* var. *BG 2814-6* and C. *annuum* cv. *NuMex RNaky* × C. *frutescens* var. *BG 2814-6*, respectively (Mueller et al. 2005).

Similar to Solgenomics, another centralized web-resource specific to *Capsicum* was developed as Pepper Informatics Hub (http://www.hnivr.org/pepperhub/) and information of this database was migrated on PepperHub (http://pepperhub.hzau.edu.cn/index.php) and complete information is still under update. Overall, the information available on PepperHub database is partitioned in five different modules including Genome, Proteome, SRNome, Transcriptome and Variome of *Capsicum*. Under genome modules users can browse reference *Capsicum* genomes (CM334 and Zunla-1) and can perform BLAST analysis for genomic sequence or gene specific sequence. In proteome module section, user can perform different operations including prediction of TFs, identifying interacting partners of proteins and their interaction network. The SRNome module section provides annotated *Capsicum* miRNA and siRNA from published studies. In addition, this section also provides miRNA genomic loci and their target information. Further, with transcriptome module, the database also provides expression information (heatmap, barplot and tissue expression cartoon) off various genes expressed at different tissues (root, leaf, stem, flower and different fruit developmental stages) under different abiotic stress conditions identified using transcriptomic studies (Liu et al. 2017b). While under

variome section, user can search SNP or InDels for a gene or given chromosome location in tabular format and additionally user can also access *Capsicum* accession specific SNP or InDels from re-sequencing data reported in earlier studies (Liu et al. 2017b).

Ensembl Plants, a comprehensive plant-centric database of genome/genomic information retrieved from sequencing of genomes (Bolser et al. 2017). It hosts a large amount of genomic and genetic information for a larger number of plant species including Arabidopsis, rice, *capsicum*, maize, tomato, wheat, citrus plants etc. (https://plants.ensembl.org/). Recently, Ensembl plants incorporated the *CM334* genome (Kim et al. 2014) and its genomic regions information. Users can browse *Capsicum* genome and can retrieve sequence as well as genomic location information of exons, introns, transcripts, mRNA or genes.

The Kyoto Encyclopedia of Genomes and Genes (KEGG; https://www.kegg.jp/) is a database which provides diverse information including biological pathways, enzymes, metabolic pathways, gene functions for a genome of a species (Kanehisa et al. 2017). Recently, the database incorporated genome information of C. *annuum* var. Zunla-1 (Qin et al. 2014). A total of 32,546 (31,458 protein-coding) genes were mapped to 354 different pathways (140 KEGG pathway and 214 KEGG Module). Genes mapped to pathways were represented using NCBI's entrez ID. Using ensembl biomart and blast service users can convert the gene Ids from both *Capsicum annuum* genome assembly (*CM334* and *Zunla-1*) to perform pathway functional annotation and enrichment.

12.13 Conclusions and Future Perspectives

Conventional ways to develop stress/disease resilient and higher yield breeding lines are always tedious and exhaustive work. Capsicum breeding assisted with high-throughput omics approaches could meet the current demands and can improve the pace for development of improved breeding lines. In recent years, a huge chunk of genomic (sequencing, re-sequencing) and transcriptomics (microarray, RNA-seq) data generated for diverse genotypes or varieties of *Capsicum* has empowered the execution of novel approaches to understand the molecular basis of phenotypic variations and resistance against various stresses. Genomic regions of the *Capsicum* chromosomes such as chr 4 (harboured highest disease associated polymorphic SNPs and NBS-LRR genes), chr 9 and chr 11 (harboured substantial SNPs, InDels associated with fruit weight, pungency etc.) could be analyzed in-depth to develop more precise trait and candidate gene associated markers to facilitate easy and quick investigation and selection across larger populations. The genome sequencing, resequencing and transcriptomic profiling opened up pandora box of genes associated with various stress-resistance (*NB-ARC, RGAs, NLRs, NB-LRRs, XTH, EBRs, MYB, bHLH, HD-Zip, oxidoreductase, WRKY, NACs* etc.), SNPs, InDels, and QTLs associated with diverse phenotypic features (fruit shape, size, aroma, flavour, ripening, secondary metabolites). Such information could be used in future to develop an array-chip embedded with oligos or specific probe sequences of such

genes, which would speed-up our pace in identifying better germplasm pools for improved cultivar that would yield higher and would be less arduous, cost-effective. Such array-chips could also be customized for gene-set specific to class of disease, agro-climatic conditions (low or high temperature, low irrigation etc.) to facilitate selection of germplasm for specific demographic regions, which in turn might help us to furnish with suitable genotypes/cultivars to farmers in limited time. In the near future, with remarkable progress in different high-throughput sequencing techniques, the *Capsicum* pan-genome equipped with multi-omics facilitated genotyping and phenotyping would play pivotal roles in genomic assisted *Capsicum* crop improvement.

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Smart Plant Breeding for Potato in the Post-genomics Era

13

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Abstract

Ceaseless development of plant breeding and genetic endeavors have resulted in accidental plant selection, successive harvest training plus urge of food and food item. The headway made toward this objective explained plant genome composition and prompted deciphering the sequence of full DNA of plant genomes controlling the whole plant life. Each crop improvement program is based on broad usage of wild germplasm and opening the genetic diversity repository. Potato (*Solanum tuberosum* L.) is considered as the most important non-grain vegetation worldwide. It is a significant staple food and has the potential to provide a lot of macro/micronutrients and vitamins when contrasted to other potential food crops, especially in many developing countries. These characters enable engineered potato to gain the scientific attention for nutrition improvement. Very few genes with their known functions have been reported, while the Potato Genome Sequencing Consortium has recognized many genes having unknown functions. Therefore, it is important to assign systematically the functions of expected genes in order to improve the potato cultivars by using different functional genomic techniques. Such loss-of-function and gain-of-function experimental techniques are helpful for producing the mutants with phenotypic variation. So, potato cultivars improve as "future feed" by generating the desired cultivar after revealing the unknown function of mutants. The commercial

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_13

deployment of engineered products has become a challenge due to administrative/moral limitations and consumer inclination. In this specific situation, new smart breeding technologies have been discussed to create sans transgene items in a more meticulous, expeditious, and viable way along with their advantages and limitations. Hence, this effect could significantly contribute to the genetic improvement of potato with reference to nutritional/food security.

Keywords

Genomic era · Potato · Conventional plant breeding · Smart plant breeding

13.1 Introduction

Potato (*Solanum tuberosum* L.) is autotetraploid and heterozygous, ranked as the third most important staple food next to wheat and rice, and originates from the Andean Mountains of South America (Zaheer and Akhtar 2016). Potato tubers are storage organ and adaptable, used in vegetative propagation. Currently, ~5000 varieties are cultivated in 125 countries worldwide, showing the global production to be >374 million tons (www.cipotato.org). Interestingly, developing countries are more prone to produce potatoes as compared to economically developed countries. In 2009, China was the leading potato-harvesting country producing 91.81 million tons trailed by India (50.19 million tons), collectively accounting for almost one-third of the potato production worldwide (www.fao.org/faostat). In nutritional point of view, starch in potato supports the growth of beneficial gut microflora after fermentation, acts as prebiotics, decreases insulin and glucose responses, and helps in small fatty acid chain production. In addition to carbohydrates, potatoes are also rich in proteins, fats, vitamins B6/C, potassium, magnesium, and dietary fibers (Zhao et al. 2018). The year 2008 was declared as the "International Year of the Potato" by the UN regarding its contribution to global food security. It is the most favorite edible and used in the paper/wood, textile, and pharmaceutical industries (Clasen et al. 2016; Fritsch et al. 2017). Potato cultivars are prone to many devastating pests and pathogens. Moreover, their germplasms are very limited, and they have weak genetic basis (Xu et al. 2011). So, the need of the hour is to establish novel genetic techniques for the successful breeding of potatoes by understanding the genetic traits. Likewise, nutritionally ironic potatoes can alleviate the global hidden hunger, but many biotic/abiotic factors cause hindrance in such inferences. In recent years, many genomic studies, *viz.*, transcriptome profiling for novel candidate genes, expression of genes involved in biotic-abiotic stress resistance, development of tubers, and starch metabolism, have been conducted (Jeevalatha et al. 2017; Singh et al. 2015; Tiwari et al. 2015; Siddappa et al. 2014; Ferreira et al. 2010). However, smart plant breeding techniques for potato production are required in the post-genomic era. For this purpose, many high-throughput and functional technologies have been implied by producing the functional mutants, either loss or gain of functions, to elucidate the relevant functional gene/s as shown in Table 13.1. Loss-

Table 13.1 Advantages and disadvantages of different functional post-genomic era techniques in potato

Post-genomic era techniques	Advantages	Disadvantages
Techniques involved in loss of functions: VIGS, RNAi, SIGS, TILLING, ZFNs, TALENs	1. Rapid	1. Off-target effects
	2. Heritable in RNAi, VIGS, TILLING	2. May require transformation
	3. Applicable in polyploid crops	3. Silencing is variable
	4. Targeted (RNAi, VIGS, SIGS, ZFNs, TALENs)	4. Require prior knowledge of sequence
	5. No PAM requirement	5. Effectiveness of SIGS is dependent on the dsRNA uptake by the pathogens
Mutagenesis and CRISPR	1. Heritable/ nonheritable	1. Screening is difficult, time consuming in mutagenesis
	2. May lead to complete knockout	2. PAM motif is required in CRISPR
	3. Capable of incorporating mutations at multiple sites	3. May obtain unintended effects if genome sequence
	4. CRISPR is used for precision genome engineering	4. Information is not available in both the cases
	5. May upregulate the expression of genes	
Techniques involved in loss of functions: activation tagging	1. Heritable	1. Require large number of transformants
	2. Dominant phenotypes	2. May produce complex phenotype
	3. Useful for terminated gene function identification	
	4. Gain-of-function phenotypes are easily identified	

of-function [(virus-induced gene silencing (VIGS), RNA interference (RNAi), spray-induced gene silencing (SIGS), TILLING] and gain-of-function techniques (activation tagging) have faced limitations that have led to the introduction of clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein (Cas) that confers both gain of function and loss of function by enabling targeted insertion, replacement, or disruption of genes in plants. But this technique is poorly understood in clonally propagated polyploids like potato. Here, in this chapter, we provide an understanding of nine different smart functional genomic techniques

along with their pros and cons that lead to the introduction of improved and resistant varieties as shown in Table 13.1.

13.2 VIGS

For potato functional genomics, virus-induced gene silencing (VIGS) is a current and high-throughput technique that encompasses low cost. It can be explained by means of figuring out a lack of partticular phenotype of specific gene inside single generation only. Additionally, it can allow the large-scale selection for a particular evaluation in polyploidy plants. VIGS lets potato in the era of genotypically indistinguishable silenced plants, especially for those cultivars that are challenged to transform (Burch-Smith et al. 2004; Singh et al. 2018). So due to this targeted silencing, a solo potato plant is adequate to trail the phenotype, thus making VIGS an effective tool for deciphering the practical significance of genes (Becker and Lange 2010). In VIGS, a binary vector (modified viral genome devoid of pathogenicity-determined gene) is cloned with cDNA of targeted gene, multiple cloning sites (MCS), and CaMV35S promoter, followed by transfection in host plant via DNA bombardment, *Agrobacterium tumefaciens*-mediated transformation, or virus sap inoculation. After that, PTGS is activated due to degradation of dsRNA (plant Dicer-like enzyme) into homologous siRNA target gene that leads to loss of gene function (Voinnet 2001; Ramegowda et al. 2014).

Potato virus X (PVX) and TRV are the most appropriate VIGS-mediated silencing viral vectors in potatoes. Resistance/candidate (R) genes [RB in *S. bulbocastanum*; R1 and Rx in *S. tuberosum*] have been silenced and assessed functionally with VIGS system by obtaining the susceptible phenotypes (Brigneti et al. 2004; Faivre-Rampant et al. 2004). It is found that suberization-associated anionic peroxidase and lipoxygenase gene provide resistance to potato against *Phytophthora infestans* by using leaf detached assay and TRV vector (Du et al. 2013). In future, heat tolerance and tuber signaling in potatoes will be assessed by using VIGS technology (Tomar et al. 2021).

Nevertheless, VIGS has certain limitations; obtained phenotypes are not applicable to genetic engineering as they are nonheritable. Potatoes are tetraploid in nature, so it is impossible for the gene function to be completely knocked down. Moreover, production of functional protein and expression of phenotypes in silence plants could be achieved with less gene expression, and silencing level fluctuates between the growth conditions and construct-dependent experiments and potato varieties. However, many potato varieties are non-acquiescent for VIGS system for *in vivo* experiments (Burch-Smith et al. 2004; Gilchrist and Haughn 2010; Senthil-Kumar and Mysore 2011).

13.3 RNAi

Downregulation of genes by mRNA degradation is a natural RNA-mediated interference (RNAi) multistep process, also called PTGS, and depends on the entry of DNA construct and production of dsRNA complementary to target genes, which are later chopped into 21–25-nts-containing short fragment (siRNAs) overhangs by Dicer or DCL enzymes through 2 nts at 3′. siRNA contains sense (passenger) strand, which later due to cytoplasmic cellular events is broken, and other antisense (guide) strand that triggers RNA-induced silencing complex (RISC) that will now be part of siRNA-RISC complex, ultimately binding to targeted complementary mRNA. Another vital component of RISC complex, named Argonaute (AGO) endonuclease, hinders translation of target mRNA after chopping it (Majumdar et al. 2017). Another 21-nts-containing ssRNA fragment named microRNA (miRNA) is formed by DCL1 from particular hairpin precursor transcripts causing nonspecific PTGS of many mRNA, while siRNAs target single mRNA in a homology-dependent way, showing that both siRNAs and miRNAs are variable in biogenesis and effects (Lam et al. 2015; Moin et al. 2017). Artificial miRNA (amiRNA) and hpRNA are other RNA silencing means in plants for better understanding of gene functions and genetic engineering for crop improvements. The construct of hpRNA includes the sense and antisense sequences of the target gene mRNA in the form of inverted repeats (IR) lying in terminator and promoter regions of a plant.

A noncomplementary spacer region separates the inverted repeats to stabilize the construct in order to increase the silencing potential. Hairpin RNA structure is developed via complementary antisense and sense sequences of transcribed RNA and gets treated by DCL4 creating 21-nts-long siRNAs, later guiding to RISCs for target gene's inactivation (Guo et al. 2016). Development of amiRNA construct is done by the substitution of endogenous miRNA as well as miRNA* sequences part of miRNA precursor along cautiously developed amiRNA* and amiRNA sequences via PCR overlapping, while stem-loop structural preservation is required for the actual precursor of miRNA.

In amiRNA construct, miRNA is complementary to the targeted mRNA sequence, although the miRNA* strand's sequence is developed to preserve miRNA precursor's duplex (miRNA*:miRNA) structure (Guo et al. 2016). So, naturally found miRNA is used for gene silencing by amiRNAs. For appropriate AGO binding, designing of amiRNA requires cautious assortment of sequence (Guo et al. 2016).

Importance of RNAi is due to its heritable expression observed in T1 generation, dominant character turning potato transformant selection easy, sequence-specific nature which does not require a huge number of individuals for screening, and invention and validation of numerous gene functions accompanied by genetic engineering-based studies, which can never be denied. By using merely one construct, several genes can easily be silenced, so for a polyploidy crop like potato, gene discovery and its usefulness for various resistant cultivar-developing programs are increasing. Genetically mended cultivars have offered appropriate solutions to risk assessment issues and are commercially sanctioned (Arpaia et al. 2020) (Small 2007;

Eamens et al. 2008; McGinnis 2010). Alongside this technique, there has also been a formation of severity-varying phenotypes due to partial characteristics, functional loss, as well as analysis of vital genes whose suppression can cause extremely severe phenotypes or lethality (Small 2007; Eamens et al. 2008; McGinnis 2010).

Along with the advantages of RNAi, we have to also face a few of its disadvantages as in plant transformation, RNAi constructs act as transgenes, which require approval of GMO regulatory compliance policies for commercial use (Arpaia et al. 2020). However, due to variable gene copy numbers in genome, silencing is divergent leading to partial target silencing; sometimes, trials for screening of gene function turn unfavorable due to creation of modified phenotypes because of unwanted target's silencing (Wang et al. 2005; Small 2007; Gilchrist and Haughn 2010). Because of its double-stranded structure, exogenous RNA cannot silence a few genes; sometimes, due to sequence homology with some other genes, it can bind off target genes, thus making it less reliable and increasing regulatory concerns (Gebremichael et al. 2021).

The RNAi approaches in *S. tuberosum* have been extensively used for imparting resistance as well as recognizing genes accountable for defense mechanism against viruses, insects, and other pests which cause yield losses (Table 13.1). By considering its importance, highly resistant transgenic lines against three strains of potato virus Y (PVY) were developed by Missiou et al. in 2004 via hpRNA using 3′ end of viral coat protein-encoding gene. Bhaskar et al. in 2009 exhibited a double-agroinfiltration protocol for RNAi-dependent silencing construct that can be applied for the identification of such genes that contribute to the pathway for late blight resistance-facilitated gene RB. Eschen-Lippold et al. (2012) in another study have analyzed the utilization of processes like callose deposition and vesicle fusion for defense responses as secretions against *Phytophthora infestans* in potato.

With this, they developed transgenic lines exhibiting RNAi-based constructs, which were targeted against the potato's plasma membrane-localized syntaxin-related 1 gene (StSYR1), which led to *P. infestans* growth reduction in potatoes. This increase of late blight resistance was due to the downregulation of gene (syntaxin) expression in potatoes. Later cytological investigations exposed that infections produced by *P. infestans* were correlated with anomalous callose deposition and decreased papilla formation, representing secretory defense response due to syntaxins's contribution to potatoes. Furthermore, other fungi including oomycete plant pathogens synthesize various proteins like effectors into potato cells, modulating innate immunity of host as well as infection occurrence. Avr3a is a virulence gene against late blight-causing *P. infestans*. Silencing of Avr3a effector gene was utilized in popular cultivars of potato for development of resistant varieties by Sanju et al. (2015) via hpRNA construct. They used siRNA against merely Avr3a gene-conversed limited *P. infestans* resistance, so broader resistance creation requires targeting of cumulatively many effector genes.

In another study conducted in 2015 by Jahan et al., they indicated that they designed and introduced GFP marker-based hpRNA construct in potato. Work performed by them indicated that hp-PiGPB1 silences pathogenicity causing β-subunit of G-protein and retarded disease. Likewise, silencing of Avr3a effector

gene via amiRNA turned *P. infestans* into nonvirulent or imparted late blight resistance and caused its death (Thakur et al. 2015). Transgenic potato cultivars exhibiting nearly 100% resistance than severely infected untransformants were developed by Hameed et al. in 2017 using coding sequences of PVX, PVY, and potato virus S (PVS) protein coat via hairpin loop configuration to develop dsRNA expression cassette.

In another study, hairpin loop construct was developed by Tomar et al. (2018) to create resistance against the curling disease of apical leaf in potato, via PTGS of ToLCNDV-potato virus AC1 gene, which is required for replication. StSP6A, a gene in potato for tuberization signal SELF PRUNING 6A, was silenced to understand relatedness among flower bud development as well as tuberization; an observation noted by Plantenga et al. in 2019 indicated that flower bud development was inhibited by StSP6A signal occurrence and vice versa. Recently, ecdysone receptor (EcR)-encoding gene in Colorado potato beetle accountable for molting was significantly silenced using the RNAi construct harboring Agrobacterium transformation; when the larvae of beetle fed on these plants, tremendous reduction in EcR product supported dsRNA significance (Hussain et al. 2019).

Additionally, in transgenic plants, regulation of spatial and temporal gene silencing was observed, based on the expression of synthetic promoter (it encompasses synthetic motif and core promoter as well as ensures transgene expression specificity and strength and is considered ideal for engineering studies of potato) in dsRNAs by Liu and Stewart in 2016.

Li et al. (2013) utilized pCL synthetic promoter to get antisense expression of target gene (acid vacuolar invertase, StvacINV1) obtained from potato. In transgenic potato, sweetness caused by low temperature was inhibited due to the expression and particular activity regulation of target gene.

13.4 SIGS

Spray-induced gene silencing (SIGS) is an environment-friendly, reckless, and RNAi-based strategy to support the gene function in plants. As the name indicates, dsRNA/sRNA* related to pathogenic genes are sprayed on plant surfaces topically in order to confer the effective crop protection, leading to infection inhibition by silencing the target gene. This technique is especially designed for RNAi harboring pests and pathogens. After spraying, dsRNA either digested into sRNA by DCL of pathogen cell directly or spread systemically in plant and dice into sRNA by DCL (Wang and Jin 2017). In potatoes, SIGS is considered as a highly efficient method in controlling insecticidal action of CPB mesh gene (dsMESH) by spraying dsRNA that resulted in a high rate of beetle larval mortality in laboratory experiments (Petek et al. 2020). Recently, late blight of potatoes has been effectively reduced by topical spraying of targeted dsRNA to *Phytophthora infestans* genes causing sporulation and infection (Sundaresha et al. 2021).

SIGS method has many advantages due to its environment-effective nature. Moreover, it is administered and ethically legal, contrary to GMOs, and applicable

to those potato cultivars by identification and validation of responsible disease development genes, which are difficult for gene editing. For instance, dsRNA sprays are target sequence orientated and so are harmless to mutated strains of pathogens (Vetukuri et al. 2021). Nonetheless, dsRNA is less stable in the environment and difficult for uptake. Furthermore, length and duration of dsRNA sprays also affect the topical application (Dubrovina et al. 2019). Sundaresha et al. (2021) reported that irrespective of the difficult and expensive application of nanoparticle-based dsRNA sprays, it is helpful for the less progression of late blight in potatoes by enhancing and boosting the RNAi delivery and action correspondingly. Further research is required for efficient delivery of RNAi-based dsRNA for authentication of gene function to control the diseases in potatoes.

13.5 Mutagenesis

In an organism, various means as chemical, physical, and biological heritable alterations can be done, which are called mutagenesis; mostly, various breeding programs use two (insertional and induced) mutagenesis processes. According to Oladosu et al. in 2016, induced mutagenesis is activated by using various chemical compounds like ethyl nitrosourea, sodium azide, methyl methanesulfonate, and ethyl methane sulfonate, and X-rays, fast neutron, and gamma radiations. Single-nucleotide polymorphisms (SNPs) or one-nucleotide-based alteration is created more conveniently by chemicals. The effect of such radiations is variable; small deletion-based point mutations are created by gamma rays, while large deletions, chromosomal loss, and translocation are caused by fast neutron exposure. The random distribution of such induced mutations is turning gene function identification more easy in genome due to its high saturation in mutant population (Gilchrist and Haughn 2010).

According to Li et al. (2005), different characters like increased microtuber harvest bearing potato mutants were created by γ-irradiation, and improved textural and histological characters (Nayak et al. 2007), novel allele identification, and improved synthesis of starch were obtained using ethyl methanesulfonate (Muth et al. 2008).

In some other way, DNA randomly gets inserted as transposons; T-DNAs and retrotransposons in chromosomes are type of insertional mutagenesis, which can be used for endogenous gene's activation, new gene introduction, or specific gene function disruption and identification, as mutagen. Radhamony et al. (2005) and Tadele (2016) suggest that due to identified insertional element sequence, it is easy to restore disruptive gene via thermal asymmetric interlaced PCR (TAIL-PCR) technique. Various phenotypes as plant stature and morphology of leaf were acquired in *S. chacoense* (diploid wild tuber) Tnt1-carrying lines, during mutation screening caused by Tnt1 retrotransposon insertional mutagenesis created by Duangpan et al. in 2013. They also explained insertional mutation library development, which offers access to allele for gene function studies; gene contribution to

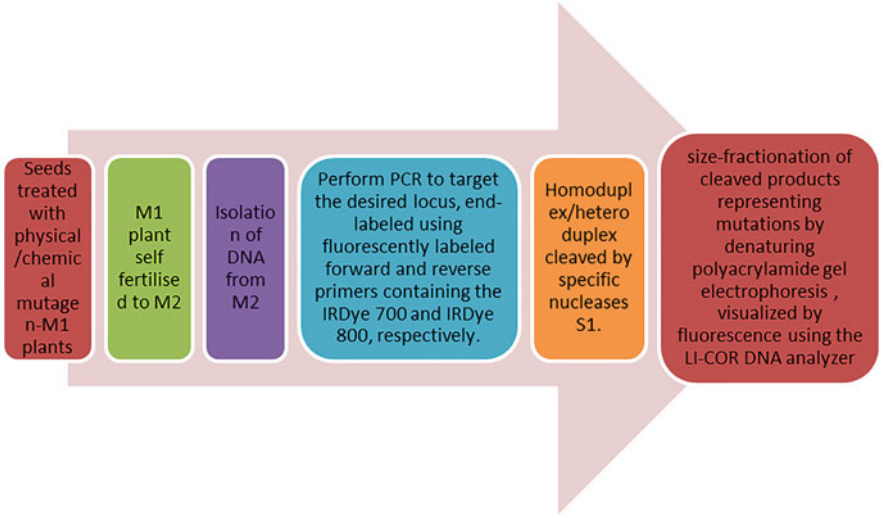
organ; biochemical, cellular, and tissue-based trait control; and tagging of every gene in potato genome (Duangpan et al. 2013; O'Malley et al. 2015).

Conclusively, these mutagenesis approaches are a valuable tool not only in being different from the tedious transformation technique but also in knocking out gene function and for low cost, innateness, easy PCR-based recognition of mutation, exploration of variation to improve traits, as well as correlation of biological function with gene sequence (Oladosu et al. 2016; Penna and Jain 2017; Kolakar et al. 2018). Along usefulness in some cases, as in a polyploid crop, the insertions may end up in one or some of the four homologous chromosomes, keeping other genes intact, which may lead to unexpected phenotypes. Though mutations are randomly dispersed in the whole genome, special care is necessary while handling different mutagens; also gene function analysis requires huge mutagenized population, making this process labor intensive and difficult (Kutscher and Shaham 2014).

13.6 TILLING

Targeting-induced local lesions in genomes (TILLING) is used to screen induced point mutants among chemically/physically mutagenized individuals using reverse genetics (Tadele 2016). It is the combination of traditional mutagenesis with high-throughput mutation discovery.

The detailed procedure is depicted in the following figure (McCallum et al. 2000; Elias et al. 2009; Fondong et al. 2016). Moreover, sequence-based TILLING and EcoTILLING are the extensions of TILLING to recognize INDEL (insertion/deletion) and SNPs. In potatoes, these techniques are employed for mutation identification (gain/loss of function, missense, and nonsense) and characterization of tetraploid germplasm to determine the functions of gene (Elias et al. 2009).



TILLING can be used for identifying those mutations unable to be detected from forward genetics and helps to use headstrong potato cultivars in contrary to transgenic varieties. However, its limitation includes the requirement of locus-specific amplification sequence and complex system to detect mutations because of polymerase slippage (Fondong et al. 2016; Tadele 2016).

13.7 Genome Editing

Genome editing is nifty and engaged for knockdown or overexpression of genes, assisted by protein-guided nucleases, viz., transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) systems. In genome editing, gene function is identified and studied after ensuring mutation in target sites by modifying and deleting the genes, thus inducing DSBs* which are repaired by homologous and nonhomologous recombination.

13.7.1 ZFNs

ZFN is a specific, efficient, and highly targeted technique, introduced with the discovery of IIS- and FokI-engineered endonucleases to separate the DNA-binding and cleavage domains. This is successfully useful in the gene modification of maize, Arabidopsis, and tobacco (Lloyd et al. 2005; Osakabe et al. 2010; Townsend et al. 2009; Shukla et al. 2009). ZFNs identify a unique ββα-configuration of 30-amino-acid Cys2-His2 ZF domain (Pavletich and Pabo 1991; Kim et al. 1996; Pabo et al. 2001) and manipulate the targeted gene by reducing nontarget cleavage as shown in Fig. 13.1.

13.7.2 TALENs

TALENs replace the ZFNs due to its rapid T-DNA integration and *Agrobacterium*-mediated delivery for targeted genes in potato cultivars that encode "acid invertase" and "starch branching enzymes" (Forsyth et al. 2016; Ma et al. 2017). TALENs are time effective and easy to design and generate in large numbers. The basis of TALENs is shown in Fig. 13.2. Recently, sterol side chain reductase 2 (SSR2) gene that controls the level of toxic metabolites in potatoes has been targeted by a very dynamic platinum TALEN expression vector construction system (Yasumoto et al. 2019). In order to reduce the anti-nutritional sterol glycoalkaloid production in potato tubers, four alleles of SSR2 have been knocked out by Sawai et al. (2014). Additionally, acetolactate synthase 1 (ALS1) gene knockout lines of tetraploid potatoes were developed by the transient expression of TALENs (Nicolia et al. 2015). TALENs also successfully enhance the processing traits and cold storage of "Ranger Russet" potato tubers by targeting vacuolar invertase (VInv) and having

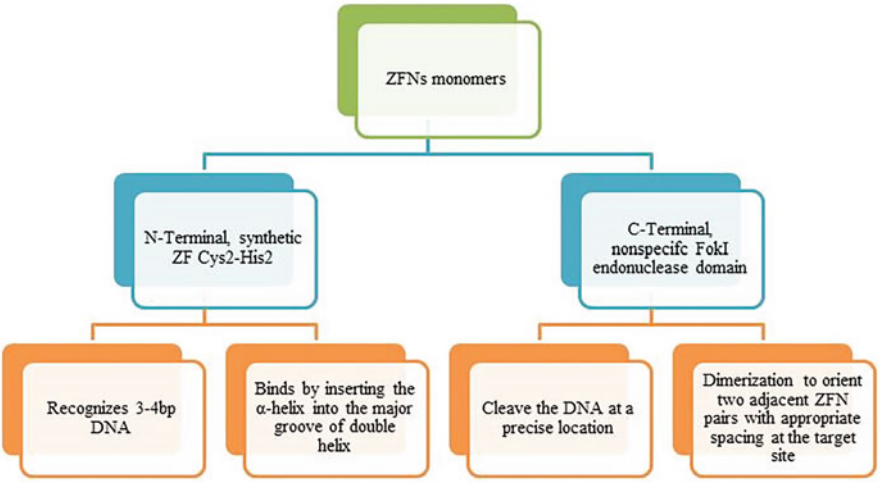


Fig. 13.1 Illustration of ZNFs. *DSBs: double-stranded breaks

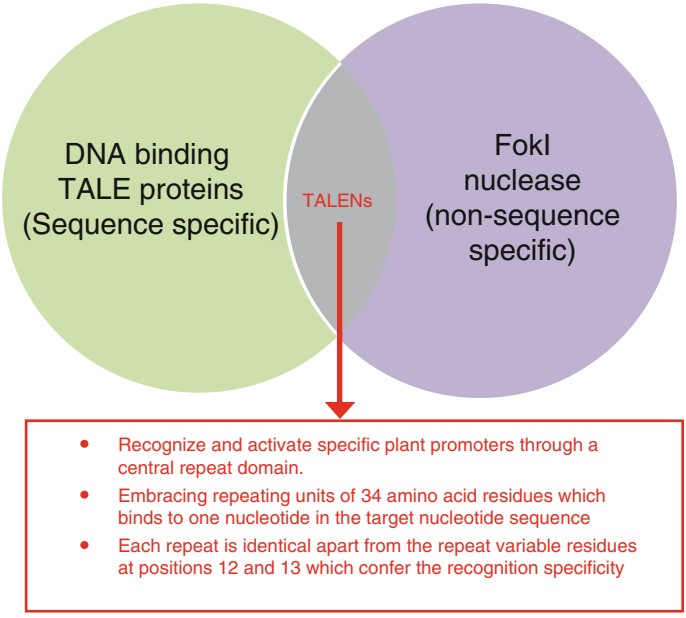


Fig. 13.2 Illustration of the basis of TALENs

reduced and undetectable level of acrylamide and reducing sugars, respectively (Clasen et al. 2016).

13.7.3 CRISPR

A specific, efficient, and easy substitution to TALENs and ZFN for target-specific genome editing induction, CRISPR/Cas, an RNA-dependent DNA cleaving system, represents a novel genome editing tool that has been newly developed. Its the characteristic of bacteria and archea to recognize the complementary sequences of invading phages, viruses and plasmids and cleave them (Wiedenheft et al. 2012; Malzahn et al. 2017). Based on the configuration of Cas genes and target, CRISPR/Cas is of three types, *viz*., type I, type II, and type III DNA editing performing types; except type I, the other two types can also target RNA. Type II complex has one Cas9 protein with two dissimilar RNA-based subunits; it is conspicuous and commonly utilized for gene editing of eukaryotes as here only one huge Cas9 protein is enough for identification and target DNA cleavage, while type I as well as type III contain one RNA subunit with several Cas proteins (Makarova et al. 2011; Unniyampurath et al. 2016).

CRISPR RNA (crRNA), Cas nuclease, as well as transactivating crRNA (tracrRNA) are three components constituting CRISPR/Cas of bacteria. A complex of tracrRNA and crRNA is developed via base pairing that stimulates and guides Cas9 toward targeted genes, which have 20 nucleotide sequences complementary to the crRNA. Cas9 has RuvC and HNH, two nuclease domains, and is an endonuclease introducing DSB into the targeted part of DNA. RuvC domain cleaves similar strand of the double-stranded DNA, while HNH cuts the complementary strand of the crRNA. Cas9 for efficient cleavage relies on protospacer adjacent motif (PAM) sequence downstream of targeted DNA having 5′-NGG-3′ sequence (Soda et al. 2018). In bacteria, CRISPR/Cas system can recognize among self and nonself sequences through interference by PAM recognition. This process led to the development of gene editing tool labeled as CRISPR/Cas system relying on crRNA target specificity and tracrRNA structural characteristics in a chimeric single-RNA guide (sgRNA), thereby reducing the system using Cas9 and sgRNA in place of three components (Doudna and Charpentier 2014). Target DNA sequence can easily be reprogrammed by altering 20 nts in sgRNA. Various mechanisms as homology-directed repair (HDR) as well as nonhomologous end joining (NHEJ) were commenced after DSB generation; mostly for gene knockout, NHEJ repairs DSB, creating gene INDELs and mismatches. HDR causes replacement of gene or knock-in of foreign DNA, when homology-bearing oligo template is near DSB sequence (Liu et al. 2017). By altering Cas proteins, CRISPR/Cas is further mended to make a more effective technique covering many genes. A more effective and simple gene editing alternative of CRISPR/Cas is CRISPRCpf1; here, Cpf1 is an RNA-guided nuclease making DSB specified via only required crRNA and introduces 5 bp cut, to find PAM, present at 5′ end protospacer (Alok et al. 2020; Zetsche et al. 2015). It is found to be remarkable for epigenetic modulation, multiplex gene targeting, base

editing, and transcription (Safari et al. 2019). Another variant is dCas9 (dead or catalytically inactive Cas9), which can strongly and specifically attach to the target via sgRNA guidance. Amendment of the action of target sequence is being done by the fusion of transcription activators and repressors to CRISPR/dCas9 through transcription increase or inhibition (Adli 2018). Another form, dimeric RNA-guided FokI nucleases (RFNs: effective gene editing needs dimerization of RFNs), depends on the fusion of FokI nuclease domain and inactive dCas9 (Khatodia et al. 2016). Similarly, there is another form which is independent of foreign DNA and directly transfers targeting sgRNA- and Cas9-based ribonucleoprotein (RNP) complex by biolistic and transfection method, useful in transgene and marker-free plants (Soda et al. 2018). Recently developed CRISPR-based system after Cas9 fusion with reverse transcriptase (RT; CRISPR/dCas9 H840A) was named prime editing based on new sequence insertion without DNA template and search-and-replace target editing form (Anzalone et al. 2019). It is important for functional genomics; relying on its ability to intrude deletions, 12 types of base substitution and insertion and being independent of DSB construction along need of donor template DNA, many traits of interest conferred via point mutation keeping intact gene loss of function led to accurate editing (Anzalone et al. 2019).

Butler et al. (2015) and Wang et al. (2015) described the usefulness of CRISPR/Cas for targeting StIAA2 gene encoding for Aux/IAA protein as well as ALS1 gene in various potato cultivars. In order to improve starch quality of tetraploid tuber, all four alleles of granule-bound starch synthase (GBSS) were knocked out using CRISPR/Cas9 by Andersson et al. (2017). In 2018, they again created amylopectin starch potatoes after inhibition of amylose formation via CRISPR/Cas9 RNP by knocking out amylose-producing GBSS enzyme. Moreover, GBSS gene was further exploited by Johansen et al. (2019) at protoplast level for editing efficiencies by substituting endogenous U6 promotor in potato with U6 promoter of *Arabidopsis* thus to drive the expression of CRISPR component. In another example, Ye et al. (2018) previously synthesized self-compatible diploid potatoes using CRISPR/Cas9 technique, when self-incompatibility causing Stylar ribonuclease S-RNase gene was knocked out.

After this, González et al. (2020) demonstrated the use of CRISPR/Cas9 for the polyphenol oxidase (PPO) mutation induction, which converts phenolic substrates to quinones responsible for dark-colored potato's precipitation by targeting StPPO2 gene, which results in less PPO activity in potato leading to browning reduction. In a very recent study by Kieu et al. (2021), loss of gene function-mediated CRISPR/Cas9 system was described; three genes StDMR6-1, StCHL1, and StDND1 were found accountable for late blight susceptibility, representing a pavement for novel resistant potato cultivar breeding. Likewise, prime editing via transversion and transition mutations was also utilized in potato's ALS1 gene, which encodes enzyme for branched-chain amino acid biosynthetic pathway (Veillet et al. 2020).

Lots of studies have shown evidences for the usefulness of CRISPR/Cas for potato's genome editing, due to its fast, efficient, and easy handling and being a potent source of multiple gene editing through one transformation, in addition to targeting methylated DNA and creating stable mutation for next generation (Cong

et al. 2013; Gaj et al. 2013; Xiong et al. 2015; Feng et al. 2014). Additionally, chances of off-target effects need genome scanning for mutation detection at sites where sequence matches to the gRNA target sequence that could be hard to get detected (Boettcher and McManus 2015; Unniyampurath et al. 2016; Malzahn et al. 2017). Significantly, gRNAs, due to potatoes' high heterozygosity, are required to be selected from the gene's conservative regions. So, editing creates biallelic, homozygous, heterozygous, and chimeric plants; homozygous state is more required but it is difficult to have in potato because of polyploidy (Fizikova et al. 2021).

13.8 Activation Tagging

For gene function analysis, activation tagging is the prevailing method used for screening of gene function's mutant loss in some plants by providing gain-of-function mutants. Hayashi et al. (1992) indicated the use of first T-DNA based on CaMV 35S gene's multimerized transcriptional enhancers (four tandem copies) inserted in T-DNA, which enhances either side flanking region expression after insertion into plant genome. Kakimoto (1996) identified cytokinin signaling pathway-based genes in the tissue culture of Arabidopsis. Weigel et al. (2000) described that huge, transformed plants conferring resistance against herbicide glyphosate or kanamycin were developed via such novel vectors. Various genes controlling special traits in important crops like tomato, barley, petunia, poplar, and Arabidopsis can be characterized (Weigel et al. 2000; Zubko et al. 2002; Mathews et al. 2003; Aylife et al. 2007; Busov et al. 2011). Regan et al. (2006) described that in Canadian Potato Genome Project, activation-lagged potato lines were developed and screened for tuber health and quality-based traits. In another study on chromosome 12, flanking genes and T-DNA insertion were used to observe cytosine methylation in mutant, revertant, and wild plants. Similarly AT615 (potato-activation tagged mutant) was characterized that differentially expressed 1632 genes in wild and mutant types (Aulakh et al. 2014, 2015). Currently used activation technology has turned out to be very useful for possible T1 generation dominant phenotype screening as well as redundant genes, which require only single allele to be activated to express phenotypically. Use of 35S enhancers rather than constitutive promoters could avoid ectopic expression, and gain-of-function screens can easily be recognized for further exploitation as compared to conventional screening.

For functional analysis of whole genes, a complete set of tagged mutants is difficult to get (Tomoko et al. 2010); expression of many genes simultaneously makes gene saturation difficult due to complex phenotypes, and its dependency on laborious transformation protocol hinders mutant line creation (Ichikawa et al. 2003).

13.9 Conclusion and Future Prospects

Potato genome is quite unknown in spite of its major role in global food security. Consequently, knowing the biological functions of candidate gene of potato genome has now become a big challenge. In order to address the issues related to hidden hunger and introduction of new biotic/abiotic stress-resistant varieties, many novel functional genomic techniques are coming into being to develop new breeding programs. Comparative phenotypic analysis of wild type and mutant is a key to identify the function of genes. Many techniques with their advantages and disadvantages have been discussed in this chapter. Among them, VIGS and RNAi are the cost-effective techniques used to identify the unknown function of genes by creating the mutants of targeted genes. However, sequence-based off-target effects are still puzzling for RNAi in spite of its ability to silence multi-targeted genes. This problem could be curtailed by using *in silico* tools for designing the construct. In SIGS, RNA is directly taken up by pathogens, but more optimized methods and delivery vectors are required for a range of pathogens. Complete gene knockdown is achieved by mutagenesis, but this technique is hard and time consuming due to the screening of large mutagenized population for succeeding the genome saturation. TILLING requires background information for locus-specific sequence amplification. Irrespective of these, ZFNs, TALENs, and CRISPR are genome editing techniques and are efficient, cost effective, and specific for knockout potato-targeted genes. Ever since potato has been characterized with genome heterozygosity and tetraploidy, avoidance of off-targets during gRNA selection has been very difficult. Many gain-of-function mutations could be achieved by activation tagging that could be recognized easily besides redundant gene analysis. Therefore, abovementioned smart plant breeding techniques after the sequencing era have the potential to breed resistant, healthy, and nutrition-rich potato cultivars.

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Current Overview of Breeding and Genomic Studies of White Button Mushroom (*Agaricus bisporus*)

14

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Abstract

Agaricus bisporus is a popular edible mushroom that is cultivated worldwide. *Agaricus bisporus* is the model fungus which acts as an important component of the human diet for over 200 years. Repetitive DNA elements are ubiquitous constituents of eukaryotic genomes and the availability of whole genome sequence leads to draw a picture of the genome-wide distribution of genes of interest. This also provides insights into potential mechanisms of genome arrangement and their expression pattern. The genomic data played an important role in assessing the evolution, adaptation of mushrooms and will enhance the scope of future genetic improvements of *A. bisporus*. Several microsatellites appeared widely and distributed over the whole genome sequence of *A. bisporus*. Molecular markers techniques help the researchers for accurate identification and differentiation of cultivars/strains of white button mushroom. These markers were developed by mining the genome sequence and an efficient technique for the identification of *A. bisporus* cultivars and have adequate potential to facilitate the marker-assisted breeding in the future.

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_14

Keywords

Mushroom · Genome · *Agaricus bisporus* · Microsatellites · Molecular markers

14.1 Introduction

Agaricus bisporus (Lange) Imbach (white button mushroom) is an extensively cultivated edible mushroom throughout world. *Agaricus bisporus* is a widely cultivated mushroom known for its significant economic value and abundant nutritional and medicinal attributes (Beelman et al. 2003). White button mushroom (*Agaricus bisporus*) is considered one of the most widely consumed and popular edible mushrooms, not only for delicious taste but also for its rich nutrition and medicinal value. This mushroom is one of the best source of vitamins, dietary fibre, protein, minerals, amino acids and bioactive compounds (Khan et al. 2014). Business related to edible mushrooms is estimated around US$42 billion per annum (Prescott et al. 2018). Besides consumption of fruit bodies of *Agaricus bisporus*, the spent substrate generated after cultivation of *A. bisporus* has been utilized for treatment of textile effluents decolourization (Singh et al. 2012) and bioremediation of 4 structurally different azo dyes (Ahlawat and Singh 2009). The white-rot fungi, viz., *Schizophyllum commune* and *Pezizomycotina* sp. Exhibit in the spent waste of this mushroom has also reported to decolourize the structurally different textile dyes (Singh and Chauhan 2017). The two strains of *Agaricus bisporus* (U3 and S11) were screened for their mycelia growth in petri plates for decolourization against 9 structurally different textile dyes (Singh et al. 2013; Singh 2014). The present chapter will highlights the advancement made in the field of, molecular breeding, improvement in germplasm and genomics pertaining to *A. bisporus*.

14.2 Genome Sequencing of *Agaricus bisporus*

The whole genome of white button mushroom (*Agaricus bisporus* var. *bisporus* ARP23) was sequenced and assembled with genome sequencing platform, viz., Illumina and PacBio sequencing technology. Morin et al. in 2012 sequenced and published the genome of *A. bisporus*. The two genomes H-97 and JB137-s8 have sizes of 30.4 and 32.8 Mb with 10,438 and 11,289 protein-coding genes estimated and reported. The fruiting ability of *A. bisporus* var. *burnettii* at 25 °C in have been reported by combination of QTL mapping, transcript analyses and candidate gene studies to unravel the genetic and molecular mechanisms. Numerous candidate genes have been identified and are analysed for potential targets and for functional analysis. The *A. bisporus* genome contains a full set of genes for polysaccharide-degrading enzymes similar to other fungi growing on plant wastes or wood, and two Mn peroxidases for lignin breakdown. Motifs pertaining to genome-sequenced soil-inhabiting or lignocellulosic fungi occurs at a higher rate in genomes of *A. bisporus*

which are 4.2 and 3.1 times more frequent in JB137-s8 and H97 (Morin et al. 2012). The combination of various factors, viz., physiology, genome composition and transcriptional regulation, does not allow *A. bisporus* to be considered as white-rot or brown-rot fungi. This edible fungi is well adopted to humic-rich environments and is the only genome-sequenced organism with this adaptation; it is therefore the 'type organism' or model species for this environment (Morin et al. 2012). In past several researchers have identified many genes and ESTs associated with mushroom growth and development both while attached to the mycelium and harvested mushroom fruit bodies (De Groot et al. 1997; Ospina-Giraldo et al. 2000; Eastwood et al. 2001). Analysis of the A. *bisporus* genome suggested that some of the regulatory switches are shared with other *Agaricus* species, while others are clade specific (Morin et al. 2012).

14.3 Expression of Genes and Their Linkage with White Button Mushroom

The overexpression of *c2h2* in *A. bisporus* mushroom results in faster production of mushrooms due to faster mycelia run in the cultivation substrates. The c2h2 gene is also involved in faster pin head formation and fruit body development. The c2h2 orthologue of *Agaricus bisporus* was overexpressed and forming basidiomycete using *Agrobacterium*-mediated transformation. Several important parameters, like morphology, cap formation rate and total number and biomass of mushrooms, were not affected by overexpression of c2h2. The crop of mushroom strain having c2h2 overexpression picked 1 day earlier as compared to control. The gene c2h2 impacts timing of mushroom formation at an early stage of development, making its encoding gene a target for breeding of commercial button mushroom strains (Pelkmans et al. 2016).

In another study, the expression of carbohydrate active enzyme (CAZyme)-encoding genes in compost casing layer and fruit body development during commercial cultivation of *A. bisporus* suggested a clear tissue-type related regulatory system (Patyshakuliyeva et al. 2013). Sufficient diversity of CAZy genes has been expressed in compost-grown mycelium which is related to the degradation of plant biomass components, while fruiting bodies mainly expressed CAZy genes which synthesized and modified the cell wall of this edible fungi. Differences were also visible at the metabolic level as the compost-grown mycelium-expressed genes of a wide variety of sugar catabolic pathways, while in the fruiting body, only glycolysis-related genes were expressed (Patyshakuliyeva et al. 2013). This showed the diversity of sugars released by the CAZymes is being converted simultaneously by *white button mushroom*, but in fruiting bodies only glucose and derivatives of glucose, such as trehalose or sorbitol and mannitol, are converted into fungal biomass. Other monosaccharides or other sugar alcohols could not be traced in the fruiting bodies which suggested that only these compounds are transported into the fruiting body from the mycelium of *A. bisporus*. This suggested that sugar transport to the fruiting

Table 14.1 Different types of genes encoding for necessary traits in *A. bisporus*

S. N.	Name of genes	Traits	Name of authors
1.	PPO and PAL genes *AbPPO1, AbPPO2, AbPPO3, AbPPO4, AbPPO5, AbPPO6, AbPAL1* and *AbPAL2*	Browning development	Xiaochen Qian et al. (2021)
3.	Cys2His2 (c2h2) zinc finger protein gene	c2h2 gene of *Schizophyllum commune* overexpressed for fruit bodies formation/earliness in *A. bisporus*	Pelkmans et al. (2016)
4.	Urea	Encoding urease in *A. bisporus*	Matthijs et al. (2006); Wagemaker et al. (2005)
5.	Riboflavin-aldehyde-forming enzyme (raf) gene	Transcriptional regulation of the raf gene during *A. bisporus* morphogenesis	Sreenivasaprasad et al. (2006)
6.	hom2 (homeodomain gene)	hom2 gene of *Schizophyllum commune* overexpressed in *A. bisporus*	Ohm et al. (2011)
7.	Gat1	Played a role in expansion of the fruiting body	Pelkmans et al. (2016); Ohm et al. (2011)
8.	Carbohydrate active enzyme (CAZyme)	Played a role in carbohydrate utilization by *A. bisporus*	Patyshakuliyeva et al. (2013)
9.	Heat shock protein (HSP70) gene	High temperature tolerance genes	Hao et al. (2021)
10.	Para-aminobenzoic acid (PABA) synthase	–	Lu et al. (2014)

body is not solely an osmotically driven process but involves either specific transporters or carrier proteins.

Comparative transcriptomics of mycelium grown on casing soil, defined medium and compost revealed genes encoding enzymes involved in pectin, cellulose, xylan and protein degradation are highly expressed in cultivation substrates. There is need to intregrate the output pertaining to mapping of quantative trait loci (QTL), transcript analysis and expression of candidate genes will make the genetic and molecular mechanism more understable. The specific growing temperature may be easily optimized and identified for cultivation of white button mushroom at industrial scale. Several candidate genes have been also identified and are potential targets for further functional analysis. Heat shock protein (HSP70) is high temperature tolerance genes of *A. bisporus* may release the high temperature tolerance varieties (Table 14.1).

14.4 Pangenome Genes of *Agaricus bisporus*

Pangenome is defined as the union of all genes observed across all strains/isolates of a species. A study was conducted for *A. bisporus* species and pangenome was constructed using the synteny-dependent PanOCT method implemented in Pangloss with the default parameters (Fouts et al. 2012; McCarthy and Fitzpatrick 2019a, b). PanOCT clusters homologous sequences into synthetic orthologous clusters (SOCs) based on BLAST score ratio (BSR) assessment of sequence similarity and on proportions of relative synteny (conserved gene neighbourhood, CGN) among the orthologues (Fouts et al. 2012; Rasko et al. 2005).

14.5 Gene Editing in *Agaricus bisporus*

Researcher from Penn state university engineered the common white button mushroom (*Agaricus bisporus*) to resist browning. They targeted the family of genes that encodes polyphenol oxidase (PPO) that causes browning. Yang et al. knocked out one of 6 PPO genes which ultimately led to reducing the 30% of enzyme's activity. Man-made gene editing techniques include zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) systems and recently developed hottest tool, CRISPR-Cas9. Yang et al. 2000 applied the CRISPR/Cas9 tool to edit the white button mushroom (*Agaricus bisporus*). This genome editing tool does not contain any foreign DNA from pests, viruses, fungi and bacteria. Mushroom that reduce browning are beneficial because they keep their colour for longer period when silenced which enhanced their shelf life. In September, 2015, Penn state university filed the provisional patent application for the protection of this innovative technology.

14.6 Molecular Markers Developed from Genome Sequences

DNA-based molecular markers developed in last two decades for *A. bisporus* are used to analyses and identification of important agronomic traits. Several simple sequence repeat (SSR) markers were also developed by mining the genome sequences of *A. bisporus* (Table 14.2). SSRs, also known as microsatellites or short tandem repeats (STRs), constituted from DNA sequences of length one to six base pairs (bp) (Jany et al. 2006; Dettori et al. 2015). These markers are multiallelic and co-dominant in nature and are considered more informative than other markers (Selkoe and Toonen 2006). These markers are frequently in use and have been used successfully in past for genetic studies and varietal identification of *A. bisporus* (Foulongne-Oriol et al. 2009, 2011; Rokni et al. 2015; Fu et al. 2016; Wang et al. 2016). In similar study, Wang et al. (2019) has identified 3134 SSRs markers and out of these 1644 are distributed in the intergenic regions and 1490 were reported from gene models. A total of 17 polymorphic primer pairs were produced and SSR fingerprints were constructed for all the commercial genotypes. The

Table 14.2 Different types of molecular markers used to study the *A. bisporus*

S. N.	Type of molecular marker/traits	Name of mushroom/strain	Name of authors
1.	SNPs	Linkage analysis in *A. bisporus*	Foulongne-Oriol (2012); Gao et al. (2015, 2016); Sonnenberg et al. (2016)
2.	RAPD	To identify button mushroom cultivars	Moore et al. (2001)
3.	Restriction fragment length polymorphism (RFLP)	To elucidate the life cycle of *A. bisporus*, to genotype commercial and wild lines and to generate the first linkage map	Summerbell et al. (1989); Loftus et al. (1988); Kerrigan et al. (1993)
4.	SSR	Demonstrated that microsatellite markers were more powerful to generate linkage maps for *A. bisporus*.	Foulongne-Oriol et al. (2009)
5.	Inter-simple sequence repeat marker (ISSR)	*A. bisporus* for strain differentiation	Barroso et al. (2000); Guan et al. (2008)
6.	Directed amplification of microsatellite-region DNA (DAMD)	*A. bisporus* for strain differentiation	Barroso et al. (2000); Guan et al. (2008)

variation in the number of repeats among genotypes and deletion or insertion of base pairs leads to showing polymorphism (Feng et al. 2016).

Mining of more and more SSR markers from the whole genome sequences may generate more accurate and informative SSR markers and these supposed to be more cost-effective than the markers developed by others methods (Zhao et al. 2012; Du et al. 2013; Chen et al. 2015). The aim of the above-mentioned study was to determine the SSR profile in the whole genome sequences of *A. bisporus* and develop a set of SSR markers for testing different genotypes of *A. bisporus*. Several researchers have already used the SSRs markers to study the genetic diversity, strain identification, genetic mapping and population structure of different fungi (Goodwin et al. 2007; Albertin et al. 2014; Masneuf-Pomarede et al. 2016). Similarly, ISSR markers provide an efficient alternate for identification of homokaryons and suggest these markers be considered as new tools for the survey of *Agaricus* species (Barroso et al. 2000; Guan et al. 2008). Others molecular markers, viz., directed amplification of microsatellite-region DNA (DAMD) and inter-simple sequence repeat marker (ISSR), are based on the amplification of a genomic region between two copies of a microsatellite sequence by using a single primer defined on the repeated motif. They were successfully developed in *A. bisporus* for differentiation of genotypes (Barroso et al. 2000; Guan et al. 2008).

Molecular markers provide an efficient technique for the identification of *A. bisporus* cultivars and this study will also facilitate the molecular identification and marker-assisted breeding of other mushrooms in the future.

After the publication of the whole genome sequence of *Agaricus bisporus* (Morin et al. 2012; Sonnenberg et al. 2016), SNP markers were designed and used because they appear to be very useful in generating linkage maps (Gao et al. 2015, 2016) and to study the precise location of meiotic crossovers (Sonnenberg et al. 2016).

14.7 Conclusion

Breeding programmes exploiting the variability in *Agaricus* germplasm with the aim to develop varieties, which may fulfil the broader objectives, such as resistance to disease, adaptation to climate changes or response to cultural conditions. Molecular markers are key tools to support and speed up the breeding programmes. There is need to develop more and more markers in *A. bisporus* for marker-assisted selection, linkage mapping, and strain fingerprinting and population diversity analysis. Presently, SSR markers are being utilized as an efficient and reliable technical support for the protection of mushroom varieties. Wild germplasm resources generally exhibit better genetic diversity and carry superior traits compared to commercial lines, which should be exploited and utilized to develop new varieties with improved agronomic and quality traits of button mushrooms. The SSR markers described in this study will lead to protect the breeders' rights of mushroom varieties, and they will also enhance the activities related to marker-assisted selection in future breeding practices. As co-dominant markers, SSRs are interesting for various genetic studies because they display a high level of heterozygosity and transferability; they are then key tools for genotyping individuals from natural populations or from a collection of cultivated strains. Sequencing the genome of *A. bisporus* has opened the way to understand the transcriptomics analysis & expression pattern of the specific strains towards cultivation substrates over the time. Post-genomic study needs to focus on solving the problem of wet bubble and dry bubble diseases in *Agaricus bisporus*. There is need to develop more and more strains of biotic and abiotic resistance in near future.

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Insight into Carrot Carotenoids in Post-genomic World for Higher Nutrition

15

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Abstract

Carrot is one of the major root crops having nutritional and economic value belonging to eudicot plant family Apiaceae. Carrot root is the reservoir of various kinds of pigments, like β-carotene, lycopene, anthocyanins and lutein. With respect to provitamin A content, carrot is considered as one of the major sources globally. In this context, carotenoid content and root colour are the major breeding goals of carrot breeders in the post-genomic era. Carotenoids in carrot roots impart huge diversity of different root colours, like orange, yellow and red. Hence, the extensive studies have been made towards understanding carotenoid biosynthetic genes and their accumulation across the genetic backgrounds. Much progress has been made in determining genomic regions and development of linked molecular markers for root colour traits in carrot. The active functioning of genes in the carotenoid pathway in carrot roots of all colours should be expected since pathway products serve as precursors for hormones important in plant development. Some of the genes like, DXS (1-Deoxy-d-xylulose-5-phosphate

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_15

synthase) under the MEP pathway, PSY (the phytoene synthase) and LCYB (lycopene b-cyclase (PSY, LCYB), have been reported to exhibit high up-regulation in the carrots with high carotenoid contents. Further, the variability in carotenoid content of white, orange and red colour carrots is attributed to genetic polymorphism in the Y and Y2 genes. In the post-genomic era the regulation of these genes is becoming clear with the advent of next-generation sequencing approaches. Based on the chromosome scale genome assembly, DCAR_032551, a candidate gene for carotenoid accumulation in carrot taproot has been identified which is not a member of carotenoid biosynthesis pathway. Rather it is the regulator of photosystem development. Recently, in carrot based on QTL analysis in genomics era, the *Or* gene for accumulation of α- and β-carotene contents and *CYC-B* (chromoplast-specific lycopene β-cyclase) gene for the β/α carotene ratio was determined. One of the remarkable fruits of carrot genome sequencing is the identification of carotenoid accumulation genes in carrot beyond the carotenoid biosynthetic pathway.

Keywords

Carotenoid biosynthetic pathway · Carrot root colour · Carrot genomics · Orange carrot · Carotenoid genetics

15.1 Introduction

Besides the main challenges of enhancing agricultural productivity and developing climate resilient cultivars, combating the menace of malnutrition is also one of the major challenges before the plant breeders in post-genomic era. Molecular plant breeding has been successful in the development of elite cultivars which have replaced the obsolete ones rapidly. Currently, the site-specific modification of genomes via innovative and rapidly evolving genome editing tools is gaining popularity in the plant community with tangible results (Holmes et al. 2016). The revolutionary genome editing technology is providing solutions to questions of enhancing food security, nutritional security, and resistance to biotic and abiotic stresses and plant architecture in the era of climate change. In the post-genomic world, the synergies of state-of-the-art technologies and genome editing platforms are attaining positive impact for crop research and development (Jiang et al. 2022). In the last decade, major crops, including carrot, have encountered unprecedented progress in the development of genomic resources and reference genome sequences owing to availability of next-generation sequencing (NGS) platforms (Iorizzo et al. 2016).

Carotenoids (C-40 isoprenoids) are one of the important member of secondary metabolites and substantially play a crucial role in overcoming degenerative diseases, like several types of cancer, and have anti-inflammatory and anti-hyperlipidemic properties as well (Iorizzo et al. 2016; Ghemeray et al. 2021; Kowarska-Starska 2022). These C-40 isoprenoids having antioxidant and anticancer value are widely present in nature, including plants, animals and bacteria and fungi as well

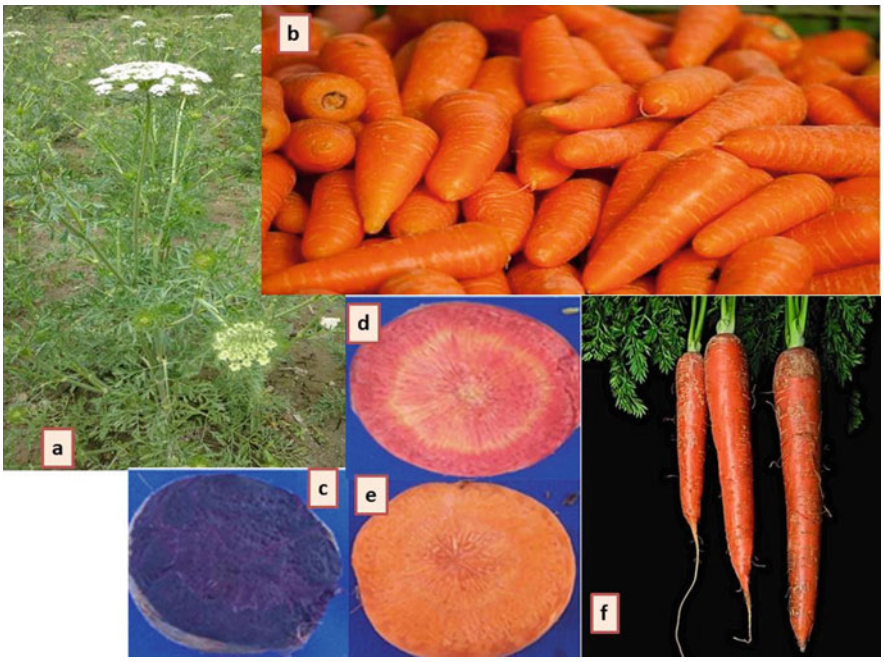

Fig. 15.1 (**a**) Carrot plant in flowering (**b**) orange-coloured carrots (**c**) cross-sectional view of black carrot (**d**) cross-sectional view of red carrot (**e**) cross-sectional view of orange carrot (**f**) red carrot roots

(Ellison et al. 2018). Carotenoids synthesized in plant kingdom play a critical role in various aspects of growth and development. They have the role to play in the photosynthetic process as light harvesting accessory pigments where they absorb light and drive the process of photosynthesis (Hashimoto et al. 2016; Zulfiqar et al. 2021). Isoprenoid molecules manifest diversity of colours, viz., red, orange and yellow in carrots and other crops (Sun et al. 2018). Specifically, there is strong evidence of efficacy of β-carotene in protection from blindness, xerophthalmia and premature death (Sun et al. 2018). These are vital health nurturing metabolites though not produced by animals and humans hence meant to be obtained through dietary supplementation.

In the plant kingdom, the Apiaceae family is one of the most important families harbouring of about 3700 species and 454 genera globally (Parveen et al. 2019). The carrot (*Daucus carota* subsp. *carota* L.) is one of the important members of this plant family and is grown as root vegetable crop globally (Ghemeray et al. 2021). Carrot roots are essential component of healthy human diet as they are enriched with provitamin A (Ellison et al. 2018). Different-coloured carrots (Fig. 15.1) are rich source of plant pigments, like carotene, lycopene, anthocyanins and mineral elements (Fe, Ca, P, Mg) (Ghemeray et al. 2021; Iorizzo et al. 2016). The accumulation of carotenoids in carrot taproot is not essentially to offer a credit for natural

Table 15.1 Colour classes of carrot due to carotenoids

Colour class of carrot	Major carotenoids	Minor carotenoids
Yellow	Lutein	Small amounts of zeaxanthin, α- and β-carotene
Orange	α- and β-carotene	Small amounts of phytoene, lutein, ζ-Carotene (zeta-carotene), and lycopene
Red	Lycopene	Small amount of α- and β-carotene, lutein

selection but has importance in terms of domestication (Iorizzo et al. 2016). However, trace amount of carotenoid is available in Queen Anne's Lace (Wild carrot) or carrot ancestor, while there is abundance of carotenoid content in majority of cultivated carrots globally. Different-coloured carrots have different types of carotenoids (Table 15.1).

Dark orange carrot genotypes may contain up to 500 ppm of total carotenoid content on fresh weight basis (Simon et al. 1989). The domestication of carrot has been documented in Afghanistan around 900 AD (Baranski et al. 2012). It is well evident that originally purple- or yellow-coloured carrots were first domesticated (Banga 1963; Stolarczyk and Janick 2011; Iorizzo et al. 2016), and later on the spontaneous mutation and selection led to the evolution of orange-coloured carrots from yellow carrots. However, in the Indian sub-continent the earliest introductions were of red or purple carrots, while orange carrots were introduced much later probably during British period. Based upon the root pigmentation, the purple/black carrots rich in anthocyanins concentration are categorized under Eastern/Asiatic carrots (also comprises some of yellow carrots), while orange-, red- and white-rooted carrots are described as Western carrots (Rong et al. 2014). The world market today is dominated by orange carrots, but the red-coloured carrot market is also growing significantly in Asia. Breeding of carrot in the past decades has substantially enhanced the α- and β-carotene, lutein and lycopene content in orange, yellow and red carrots, respectively. Hence, much progress has been made in elucidating the molecular mechanism and regulation of carotenoids in carrot.

15.2 Understanding Carotenoid Biosynthetic Pathway in Carrot

The prime focus of molecular evolutionary experiments in colourful carrots is to reveal the cause of genetic variation in its genome. The biosynthesis pathway of carotenoids, member of pigmented terpenoids, has been well studied and extensively reviewed across the species with respect to genes and enzymes regulating it (Fig. 15.2) (Ellison et al. 2018; Cao et al. 2021; Sandmann 2022; Yuan et al. 2022; Gupta and Hirschberg 2022). In carrot, 44 and 24 genes have been characterized in isoprenoid biosynthetic pathway (Iorizzo et al. 2016) and carotenoid biosynthetic pathway, respectively (Iorizzo et al. 2016; Ellison et al. 2018), with numerous paralogous genes in a number of different pathway genes. These gene duplication events led to the evolution of particular functions of different tissues,

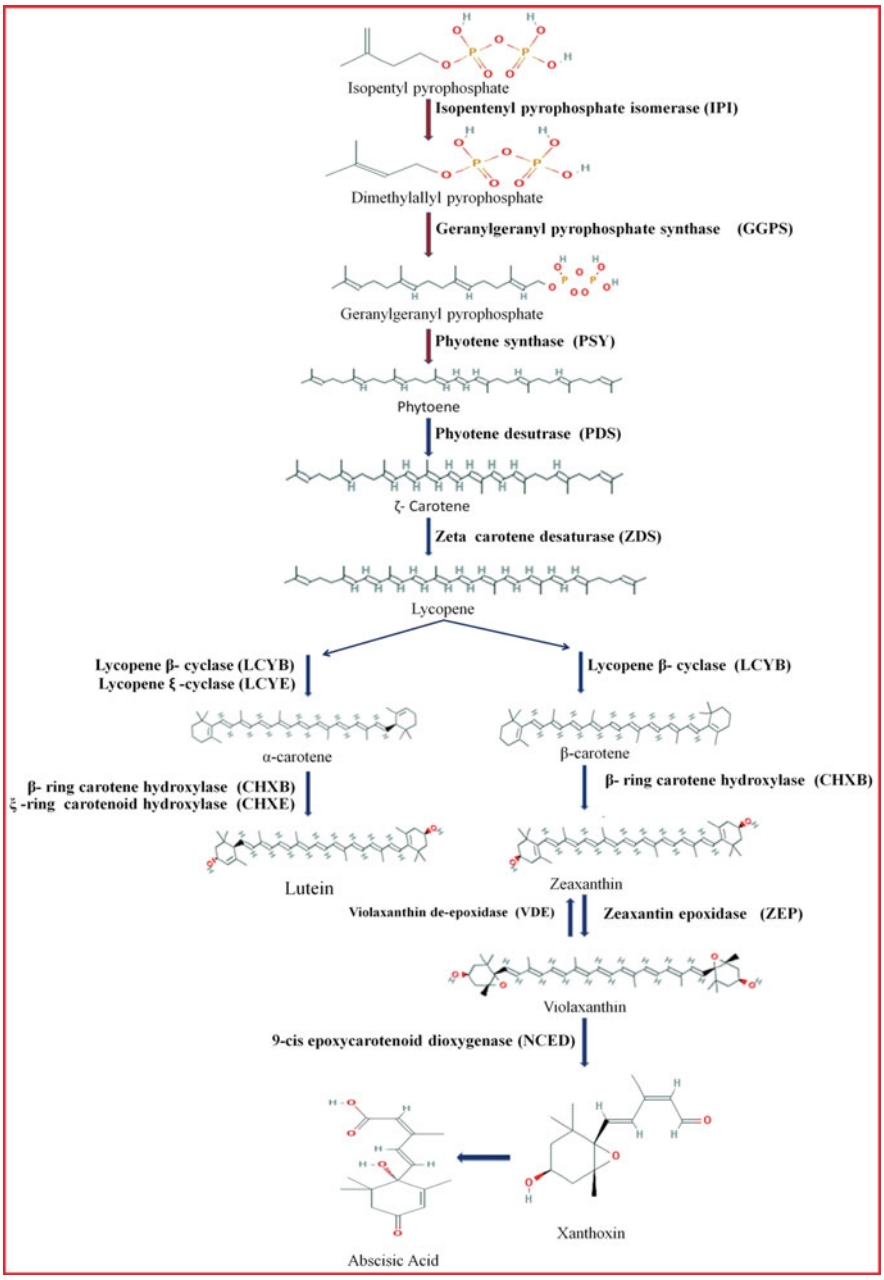

Fig. 15.2 Carotenoid biosynthesis pathway

plastids or cross-talk mechanisms of pathways (Iorizzo et al. 2016). The genes regulating upstream enzymes evolved slowly as compared to genes encoded by downstream enzymes owing to lack of regressive selection (Clotault et al. 2012). Hence, it is well evident that rigorous artificial selection was imposed in the genes governing carotenoid biosynthesis for developing different-coloured carrots. Extensive molecular analysis has been carried out for the different processes linked with biosynthesis of carotenoids (e.g. condensation, oxygen desaturation, isomerization, cyclization, oxygenation and hydroxylation). These widely distributed pigmented carotenoids are produced by general isoprenoid biosynthetic pathway (Sun et al. 2018). The condensation of isopentenyl diphosphate (IPP), a C-5 compound, and its isomer DMAPP (dimethylallyl diphosphate) leads to generation of carotenoids. The geranylgeranyl diphosphae (GGPP), a C-20 carbon compound, is a precursor of carotenoid and condensation of two GGPP compounds through interaction with phytoene synthase (PSY) results into the development of first carotenoid product, that is colourless phytoene (Clotault et al. 2012; Sun et al. 2018). The phytoene generation through the condensation of PYS is an important rate-limiting step in the biosynthesis pathway of carotenoids. Subsequently, the first coloured product 'lycopene' is generated by the steps of desaturation and isomerization by PDS (phytoene desaturase), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO), respectively. After this, the lycopene undergoes the process of cyclization by lycopene ε-cyclase (LycE) or lycopene β-cyclase (LycB) enzymes, which leads to the generation of orange-coloured products α-carotene and β-carotene. Subsequently, lutein and zeaxanthin are synthesized by hydroxylation of α-carotene and β-carotene by enzymes β-ring carotene hydroxylase (CHXB), ε-ring carotene hydroxylase (CHXE) and β-ring carotene hydroxylase (CHXB), respectively. Then, transformation of zeaxanthin to violaxanthin occurs via epoxidation and de-epoxidation by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), respectively. Afterwards, the core carotenoids biosynthesis pathway is concluded by transformation of violaxanthin into neoxanthin by the enzyme neoxanthin synthase (NXS). Eventually, xanthoxin is generated by deoxygenation process of neoxanthin and violaxanthin by 9-cis-epoxycarotenoid dioxygenase (NCED). The other class of enzymes, like carotenoid cleavage dioxygenases (CCDs), leads to oxidative cleavage of carotenoids, which yields apocarotenoid (phytohormones, volatile compounds and signalling molecules) (Sun et al. 2018). The carotenoid biosynthesis pathway provides effective analysis of pathway position on gene evolution, since it includes more than ten enzymes acting at various locations and two metabolic nodes (Fig. 15.1) (Clotault et al. 2012). The nucleotide variation in the genes encoding structural enzymes of carotenoids biosynthesis pathway has intended variability in pigmentation of different plant parts. In carrot also cultivars exhibiting different-coloured storage roots have been evolved depicting variable concentration of different pigments. The basic mechanisms liable for differences in carotenoids concentration in different cultivars have also been explored, such as role of mutations altering amino acid sequences of some carotenogenic enzymes that have been reported (Clotault et al. 2008). For instance, in case of watermelon the allelic sequence variation in *LCYB* was observed in red-

and yellow-fleshed cultivars (Bang et al. 2007), and in tomato the alternation in expression of carotenoids biosynthesis genes resulted in different patterns of carotenoids in orange-fruited *Delta* and *Beta* mutants (Ronen et al. 2000).

15.3 Factors Influencing Carotenoid Profile in Carrot

The multiple variables affect the carotenoid content in different-coloured carrots, such as genotype, agro-climatic region, temperature, edaphic factors, light intensity, agronomic and post-harvest factors (Bozalan and Karadeniz 2011; Karabacak and Karabacak 2019; Ghemeray et al. 2021). Based on the results of numerous studies it is well evident that marked variation is present in carrot carotenoids across the genotypes (Nicolle et al. 2004; Perrin et al. 2017; Soleti et al. 2020; Ghemeray et al. 2021). Thus, we can say that carotenoid content is genetically governed trait. The content of carotenoid also varies in different parts of carrot and in different-coloured carrots as well. Perrin et al. (2017) reported that α- and β-carotene are main compounds in orange carrots, lutein in yellow carrots, lycopene in red carrots and no presence of carotenoid in white carrot genotypes. Albeit carotenoid components in carrots are under the control of hereditary factors, the difference in carotenoid content as a result of growing same variety under varying ecological conditions indicates influence of environmental factors in total carotenoid accumulation (Karabacak and Karabacak 2019). In this context, Ghemeray et al. (2021) carried out the nutritional profiling of petaloid cytoplasmic male sterile (pt-CMS) lines of temperate carrot and reported that major genes controlled the carotenoid content in carrot roots which indicates about oligogenic nature of carotenoids. A recent study conducted under the control and water stress conditions for carotenoid content and gene expression analysis for 13 carotenoid biosynthesis pathway genes involving five different carrot genotypes with contrasting root colour revealed higher content of carotenoid in phloem than xylem in orange and purple genotypes (Perrin et al. 2017). However, no significant variation was recorded in xylem and phloem of red carrot with respect to carotenoid content. These results depicted the importance of structural aspect of carrot roots for accumulation of carotenoids in connection with levels of gene expression.

Different research studies have clearly indicated the effect of climatic factors on carotenoid content of carrot roots. The longer growing season leads to more accumulation of carotenoid content and varies accordingly with the modification of climatic factors (Karabacak and Karabacak 2019). Marked differences have also been recorded with respect to phenolics, total sugars, β-carotene, lycopene and dry matter content in carrots grown under varying locations or agro-climatic conditions (Yusuf et al. 2021). The colour development in carrot root is regulated by genotype, season of growing, fertilization, temperature and root development stage. The temperature of 15.5–21.1 °C is considered best for colour development in carrot root, while the temperature below 10 °C and beyond 30 °C results in poor colour development (Saha et al. 2016).

It is evident that carotenoids play a protective role in higher plants. They have the role to play in photosynthesis process, light harvesting and chlorophyll protection from photo-oxidation and act as photoprotectors under excessive light situations (Young 1991). For this purpose the major amount of chloroplast carotenoid is present in the thylakoid membranes (Sun et al. 2018). The carotenoid production is stimulated during photomorphogenesis by the light which increases the expression of PSY gene in carotenoid biosynthesis pathway (Arias et al. 2022). The carrot *DcPAR1* (*PHYTOCHROME RAPIDLY REGULATED1*) gene expression is more in underground taproot than those grown in the light, which indicated the role of *DcPAR1* in carotenoid accumulation in underground carrot taproot (Arias et al. 2022). The total carotenoid content is positively impacted in majority of the cases with light, but when coloured carrot roots are illuminated, there is reduction of total carotenoid level.

At a certain root weight of carrot, the higher soil moisture content results in lower level of carotenoid content in carrot roots (Banga 1963). The nitrogen fertilization has positive correlation with β-carotene content in carrots which increases with increase in nitrogen rate to some extent (Hochmuth et al. 1999). Further, the storage time and temperatures also helpful in enhancing provitamin A carotenoid in carrot (Hammaz et al. 2021). Thus, the edaphic, climatic, agronomic, post-harvest, biotic and abiotic stress factors must be considered while describing the nutritional profile of carrots for human health.

The difference in accumulation of carotenoids in different genotypes is associated with production of different colour carrots. The carotenoid degrading enzymes also play a key role in carrot colour variation. The major enzymes leading to carotenoid degradation in plants are carotenoid cleavage dioxygenases (CCDs) and 9-cis-epoxycarotenoid dioxygenase (Li et al. 2021). The genome sequencing of carrot has led to the annotation of seven *CCD* genes in the genome of this nutritious root crop (Iorizzo et al. 2016). In this context, recently, Li et al. (2021) reported the higher expression level of *DcCCD4* gene in white rooted carrots as compared to orange rooted cultivar. The overexpression of this *DcCCD4* gene in orange-rooted carrots led to the reduction of α- and β-carotene content of orange carrots. The loss of function of *DcCCD4* gene in mutant carrots resulted in yellow colour and accumulation of β-carotene content. The functional analysis also supported the role of *DcCCD4* gene in breakdown of α- and β-carotene content.

Nowadays, the trend of cultivating carrots in greenhouses and having off-season production is gaining popularity. In this context, the elucidation of phenomenon behind carotenoid metabolism under CO_2-enriched conditions, like greenhouses, is of utmost importance. The saturation point of carrots for CO_2 can be as high as 1819 μmol mol^{-1}. The enrichment with CO_2 has been reported to enhance carotenoid content in carrots besides increasing biomass (Song et al. 2021). To elucidate the molecular mechanism behind this phenomenon, Song et al. (2021) performed the RNA-seq-based differential gene expression (DGE) analysis and determined 20 genes regulating carotenoids among 482 DGEs. These genes were regulating carotenoid content in carrot either through carotenoid biosynthesis or by regulating photosystem membrane proteins.

15.4 Genetics and Mapping of Carotenoids

Extensive studies regarding colour genetics of carrot and mapping of genes or QTLs controlling them have elucidated the colour behaviour of carrot. In the beginning, the conventional intercrossing approaches revealed that white colour of carrot root is dominant to orange and yellow colour (Vilmorin 1859; Emsweller et al. 1935). Then, yellow root colour of carrot was put under monogenic control over the complete dominance for orange colour (Lamprecht and Svensson 1950). Further, intercrossing of orange and red carrots revealed the dominant nature of orange colour to red colour (Katsumata et al. 1966). The gene characterized by Laferriere and Gabelman (1968) which controls white colour over yellow root colour was named as gene '*Y*' by Kust (1970). Then as depicted in the white × orange crosses, the additional one or two dominant genes reducing xylem colour were named as *Y1* and *Y2*. The genes enhancing phloem colour were named as *O* and *IO* by Kust (1970). Further, the segregation pattern of three major genes controlling root colour was demonstrated in red × yellow intercrossing by Buishand and Gabelman (1980) as carotenoid synthesis inhibitor 'Y2', lycopene synthesis stimulator 'L' and 'Á1' having similar behaviour to IO gene characterized by Kust (1970). Numerous research reports have documented the role of carotenoid biosynthesis pathway genes in the genetic control of carotenoid content (Paran and van der Knaap 2007; Iorizzo et al. 2016). According to the crop species, all the genes involved in carotenoid biosynthesis may regulate genetic basis of carotenoid content. The heritability of carotenoid content in carrot has been reported to vary from 28 to 98% depending upon the investigated carotenoid component in the respective genetic background (Santos and Simon 2006; Ghemeray et al. 2021). In a recent report, the polymorphism in *CYP97A3* (carotene hydroxylase) gene, which governs the accumulation of α-carotene content, was determined (Arango et al. 2014; Jourdan et al. 2015). The association study of diverse carrot roots following the genome sequence of *CYP97A3* determined the frame shift mutation in this gene in the orange colour carrots, which revealed the molecular mechanism of high accumulation of α-carotene content in these orange roots (Arango et al. 2014). On the other hand, the study conducted by Goldman and Breitbach (1996) reported the occurrence of new spontaneous mutant in orange carrot controlled by a recessive gene '*rp*', which results in 90% reduction of α- and β-carotene content in storage taproot and enhances the phytoene content. Simon (1992) studied the population of three orange × yellow crosses and documented the monogenic control of *Ý2* gene.

In a population of yellow × orange cross developed by Bradeen and Simon (1998), the *Y2* gene was mapped using AFLP markers which were converted to PCR-based markers to stimulate phenotyping of genes determining carotenoid content. Later on, following this study, the AFLP markers were used to develop first genetic map of *Y2* gene governing orange colour (Vivek and Simon 1999). For further identification of more numbers of carotenoid genes on the genetic map of carrot, the QTL mapping study was carried out by Santos and Simon (2002) by using two mapping populations and employed AFLP markers. They concluded that 8 QTLs are linked with α-carotene content governing orange colour and there

QTLs were linked with α- and β-carotene content. One QTL for β-carotene content was found common in both the mapping populations under study. In an another experiment, a mapping population developed by crossing a wild, white colour carrot root (called as QAL) × B493, a dark orange colour carrot, was used to determine the link between carotenoid biosynthetic genes and colour governed by them (Just et al. 2007, 2009). In this study, a total of 22 putative genes encoding carotenoid enzymes were mapped assuming them as candidate genes for carotenoid content. The carrot genome sequencing (Iorizzo et al. 2016) facilitated the candidate gene mapping for Y gene. The sequencing advances led to the identification of a candidate gene '*DCAR_032551*' governing Y locus based on sequence polymorphism and differential gene expression (DGE) analysis (Iorizzo et al. 2016). As determined in previous studies involving the population derived from B493 × QAL (Just et al. 2009), Y2 gene is another segregating gene in addition to Y gene. By integrating fine mapping and transcriptome analysis techniques in the population derived from the same cross which is homozygous for y and segregating for Y2 gene, the mapping of Y2 was confirmed to a region of 659 kb comprising 72 genes (Ellison et al. 2017). In this experiment, at 40 and 80 days after planting, the transcriptome analysis was performed for differential gene expression analysis. The study reported differential gene expression of numerous carotenoid biosynthetic pathway genes in yy_2y_2 (orange) roots, while not such expression was reported in yellow (yyY_2Y_2) roots. Recently, in a genome-wide association study comprising 154 wild types and 520 cultivated type carrots from geographically diverse regions, a new gene governing carotenoid accumulation in carrot roots was identified (Ellison et al. 2018). Based on GBS (genotyping by sequencing) approach, genomic signatures of domestication were investigated by studying this large collection of carrot germplasm (Ellison et al. 2018). On the chromosome number 3, a 143-kb region was found to be linked with carotene availability. This region accumulated the carotenoid beyond MEP or carotenoid biosynthetic genes, rather it comprised the '*Or'* gene. This *Or* gene has been reported to be important factor for carotenoid accumulation in an array of crop plants, like cauliflower, sweet potato, Arabidopsis (Li et al. 2012; Welsch et al. 2019; Tran et al. 2017) and carrots, as well as via chromoplast development which acts as a sink for accumulation of these pigmented terpenoids. Interestingly, the frequent allelic variation was reported in cultivated carrots from the place of origin, Central Asia, as compared to the carrots from Europe with respect to *Or* gene (Iorizzo et al. 2013). In the genetic background of orange carrot (yyy_2y_2), the homozygous wild-type (Or_w) alleles of *Or* results yellow colour in root, while in heterozygous conditions it yields light orange colour. The plants homozygous for cultivated allele (Or_c) in the background of orange carrot with genotype $Or_cOr_cyyy_2y_2$ produce yellow colour storage roots. These results indicate fixation of *Or* gene for Or_c in the European carrots; on the contrary the allelic variation at *Or* gene was reported to play a role in domestication of early carrots in Central Asia region. Ellison et al. (2018) provided the evidence for role of genomic regions harbouring *Or* gene in carotenoid accumulation in carrot based on GWAS (genome-wide association study). The allelic variation in the carotenoid biosynthetic pathway genes does not fully support the hypothesis of higher accumulation of

carotenoid in carrot taproot. A nonsynonymous mutation transmitting together with carotenoid accumulation was determined by analysing sequence-based variation at *Or* locus. The absence of this mutation in all the wild-type samples and conservation in all the orange-coloured domesticated samples indicated the role of *Or* gene in carotenoid accumulation in domesticated carrots. The further analysis by Ellison et al. (2018) reported that the Western domesticated carrots aligned largely in a single genetic group, albeit there was variation at phenotypic level among the different market classes.

The GWAS and quantitative trait loci (QTL) analyses have been performed by different research groups for the carotenoid content and root colour of carrot using different populations (Santos and Simon 2002; Just et al. 2007, 2009; Jourdan et al. 2015). Recently, by integrating double-digest restriction site-associated DNA sequencing (ddRAD-seq) and SNP genotyping, QTLs for root colour and carotenoid content were identified in Japanese orange carrots (Shibaya et al. 2022). Two F_2 mapping populations developed by intercrossing the orange carrot genotype, Fs001 × Fs002 and Fs002 × Fs003, respectively, were used for mapping QTLs governing carotenoids. The genome-wide single nucleotide polymorphism (SNP)-based association study was conducted to determine genomic regions and DNA markers in carrot genome controlling root colour and carotenoid content. The QTL analysis and association studies determined two putative candidate genes: *Or* gene for determining visual colour, α- and β-carotene contents and *CYC-B* (chromoplast-specific lycopene β-cyclase) gene for determining ratio of β/α carotene. These findings are important for selecting bright orange colour carrot roots in a large orange root population, especially in the countries, like Japan, where bright orange colour carrot is a major breeding objective.

15.4.1 Fate of Genome Editing in Carrot Carotenoids

The genome editing tools especially CRISPR/Cas9 system have revolutionized plant breeding research in post-genomic world. Recent studies have indicated that altering carotenoid biosynthetic pathway may cause change in expression of genes regulating cell wall composition (Oleszkiewicz et al. 2021). The key gene, which is a rate-limiting step for carotenoid biosynthesis, is *PSY* (phytoene synthase) gene, which plays species- and organ-specific roles in carotenoid content including its paralogous genes. To determine the importance of two paralogues of *PSY*, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated protein CRISPR/Cas9-based genome editing system were employed to induce mutations in *psy1* and *psy2* paralogous genes (Oleszkiewicz et al. 2021) of carotenoid rich carrot. The results depicted the critical role of *psy2* gene in carotenoid synthesis based on combination of gene sequencing, gene expression and carotenoid content analysis approach. The inhibition of carotenoid biosynthesis is also linked with cell wall remodelling. Likewise, the same approach employed to purple-coloured callus from purple carrot by targeting carrot flavanone-3-hydroxylase (*F3H*) gene in anthocyanin biosynthesis of carrot roots (Klimek-Chodacka et al. 2018). The knockout of this

anthocyanin biosynthetic gene, *F3H,* caused discolouration of calli indicating the key role of *F3H* in anthocyanin biosynthesis. The complete sequencing of carrot genome (Iorizzo et al. 2016) has illuminated the promising application of CRISPR/Cas9 and other genome editing tools for accelerating basic and translational research in this globally important root vegetable crop.

15.5 Conclusion and Future Perspectives

The pigmented terpenoids, 'carotenoids', accumulate in large amount in domesticated carrots and are major quality attributes for health, hence a prime breeding goal of carrot breeders. In the post-genomic era, much progress has been made in deciphering these complicated processes via state-of-the-art tools. Numerous genes, SNPs associated with carotenoid components and root colour have been documented in the post-genomic world. Recently, the advent of high-throughput sequencing technology has accelerated the carrot research. The Nobel prize-awarded CRISPR/Cas9-based genome editing technology has been implicated in carrot carotenoid research. The allelic variation at carotenoid biosynthetic pathway genes, photomorphogenesis and plastid development is responsible for diverse root colours in carrot. The carrot genome sequencing further accelerated the research on determining genomic regions carrying useful genes beyond carotenoid biosynthetic pathway. The sequencing platforms have facilitated the development of genomic resources for further improvement of this root crop. Further availability of genome sequence data with respect to different populations or genotypes may enhance research programmes on understanding carrot evolution, elucidating complicated carotenoid accumulation pathways and other essential aspects in carrot. The wide utility of transcriptome dataset has been investigated in carrot referring to differential gene expression analysis for carotenoid synthesis and accumulation in taproot. For instance, based on transcriptome analysis, *DcPSY1* gene was reported to be a critical factor for carotenoid accumulation in carrot leaves. Likewise, the differential behaviour of carrot leaves and roots for carotene and xanthophyll levels may be attributed to *DcLBCY*, *DcLECY* and *DcZEP1* genes.

In the post-genomic era numerous transcriptome sequence datasets have been developed in carrot. Further investigations on the micro-RNA (miRNA) research may be helpful for improving different agronomic and quality traits in carrot. In the post-genomic world, the integration of novel tools and omics sciences (genomics, transcriptomics, proteomics and metabolomics) will pave the way for enhancing carrot breeding programme for different traits, including carotenoids via mining carrot functional genes. Improving genome editing systems, like CRISPR/Cas9, prime editing, base editing, etc., for speeding up carrot research should be the focus of future studies.

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Advances in Potato Breeding for Abiotic Stress Tolerance

16

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Abstract

Potato is the third important food crop for global food security. To breed new varieties with stress tolerance, tuber yield and quality are a base for sustainable development of the potato industry. With climate and environmental change, the breeding of abiotic stress tolerance potato varieties has become a research hotspot. This paper summarizes the evaluation methods, genetics, and molecular mechanism of potato tolerance to abiotic stress, including salt and alkali, drought, cold, and heavy metal. The progress of transgenic breeding of potato under abiotic stress was introduced. Finally, the prospect of potato abiotic stresses breeding was put forward.

Keywords

Stress tolerance · Salt and alkali · Drought · Cold · Heavy metal

16.1 Research on Salt and Alkali-Tolerant Breeding of Potato

16.1.1 Overview

Soil salinization is a global issue. The increase of saline alkali land area has become the main factor restricting agricultural development. The low content of organic matter, exhausted soil fertility and poor physical and chemical properties in saline

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_16

alkali soil restrict the normal growth and development of plants and seriously reduce the yield of grain crops. It is also the fourth most important crop with high grain and high adaptability in the world, second only to wheat and potato. Potato is a medium salt-tolerant crop, but the potato varieties popularized at this stage have poor salt tolerance. Salt and alkali stress affects the growth and development of potato leaves, stems, roots, and tubers and seriously inhibits the yield of potato. Therefore, it is of great significance to explore the salt and alkali resistance mechanism of potato and improve the salt and alkali tolerance of potato.

Saline alkali stress inhibits the growth of plant roots, thus limiting the growth and development of plants and then affecting the yield of crops (An et al. 2021). When potato is subjected to saline alkali stress, its morphological characteristics, such as root length, plant height, leaf length, and leaf width, are inhibited, and the degree of inhibition is more obvious with the increase of soil salinization (Liu et al. 2011; Ji et al. 2021). In addition, salt alkali stress affects the yield and quality of potato. The quality indexes of potato mainly include dry matter content, starch content, protein content, reducing sugar content, vitamin C content, etc. (Li et al. 2020a, b). Yao et al. (2020) set five NaCl concentration stress treatments for potatoes. The results showed that with the gradual increase of NaCl concentration, potato yield gradually decreased, and the contents of dry matter, starch, and protein decreased, resulting in the decline of potato quality. When potato tissue culture seedlings were treated with $NaHCO_3$, potato leaves wilted and plants withered under alkali stress, and could not grow normally (Kang et al. 2021). For different potato varieties, there are also some differences in salt and alkali tolerance (Chen et al. 2018; Zhao and Bei 2007).

16.2 Salt and Alkali Resistance Evaluation

When the total salt content in the medium reached 0.1%, the plants with the worst salt and alkali-tolerant potato clones (144-4, 3-2, 65) began to die. However, the plants with strong salt and alkali-tolerant potato clones (I, 131, 31) could survive even if the total salt content reached 0.4%. For the plants with strong salt and alkali tolerance, the pH value of culture medium gradually decreased in the process of culture and reached the range suitable for plant growth when sampling. It is suggested that salt-tolerant potato can secrete a large amount of H^+ into the soil (Wang et al. 1997). The cluster analysis by using Ward deviation square sum method is divided 130 varieties into eight groups, among which Bintje, Amisk, Onaway, BelRus, Tobique, and Sierra had the strongest salt resistance, while Mainechip had the worst salt tolerance (Khrais et al. 1998). There were significant differences in salt tolerance of callus among different potato varieties. The maximum tolerance concentrations of E 1 and Dongnong 303 were greater than those of Xiabodi and Favorita (Li et al. 2004). The stems and leaves of 12 potato varieties were used as explants for callus induction to screen salt-tolerant callus. The results showed that the callus varieties with strong tolerance were Bashu 10, Jizhangshu 5, and 1867 (Zhang et al. 2005) (Table 16.1).

Table 16.1 Salt-tolerant potato varieties

Variety name	Salt and alkali resistance	Reference
144-4, 3-2, 65	Weak	Wang et al. (1997)
I, 131, 31	Strong	
Bintje, Amisk, Onaway, BelRus, Tobique, Sierra	Strong	Khrais et al. (1998)
Mainechip	Weak	
E 1, Dongnong 303	Strong	Li et al. (2004)
Xiabodi	Weak	
Bashu 10, Jizhangshu 5, 1867	Strong	Zhang et al. (2005)
1533, 2191, V1-1	Weak	

16.3 Physiological Response to Saline Alkali Stress

16.3.1 Affecting Endogenous Hormones

When plants are subjected to saline alkali stress, a series of responses will occur to endogenous hormones in plants (Yu et al. 2020). Under salt stress, the content of auxin in leaves of potato tissue culture seedlings will decrease, while the content of auxin and abscisic acid in roots will increase with the extension of stress time (Wang et al. 2020). Under alkaline stress, the contents of abscisic acid and brassinolide in potato increased with the increase of alkali concentration, but the content of gibberellin decreased gradually (Kang 2021).

16.3.2 Interference Ion Steady State

Saline alkali stress will increase the content of Na^+ in plants, while higher concentration of Na^+ will affect the absorption of K^+, resulting in the increase of Na^+/K^+ ratio in plants. Li et al. (2010) showed that the K^+/Na^+ ratio in potato virus-free seedlings decreased under different concentrations of mixed salt stress. Kang (2021) showed that under the condition of alkali stress of different concentrations of $NaHCO_3$, the Na^+ content of different potato varieties increased with the increase of alkali concentration, the K^+ content decreased with the increase of alkali concentration, and the content ratio of sodium and potassium ions in roots, stems, and leaves decreased with the increase of alkali stress concentration.

16.3.3 Causing Osmotic Stress

Under salt stress, osmotic substances are very important to maintain plant salt tolerance (Singh et al. 2015). As an osmotic protective agent, proline can regulate

the osmotic balance in cells. Sun et al. (2009) showed that after mixed salt stress treatment, the proline content of potato leaves increased with the increase of salt concentration, and the content of soluble sugar also increased gradually. The same results were obtained after alkali stress treatment with different concentrations of $NaHCO_3$ (Kang 2021).

16.3.4 Weakening Photosynthesis

Chlorophyll is an important substance for plant photosynthesis. Salt alkali stress will reduce the content of chlorophyll and affect crop yield (Fang et al. 2021). Ma (2014) showed that with the gradual increase of salt concentration, the chlorophyll content of salt-tolerant and salt-sensitive strains decreased, which affected the photosynthesis of plants. When potato plants were subjected to saline alkali stress, the contents of chlorophyll a and chlorophyll b among different varieties decreased (Hu et al. 2020).

16.3.5 Leading to oxidative stress

When potato plants are subjected to saline alkali stress, the content of malondialdehyde (MDA) increases with the increase of saline alkali concentration (Li 2016). Charfeddine et al. (2019) introduced the *StERF94* gene into potato plants and obtained overexpression transgenic lines. The research showed that the activity of superoxide dismutase (SOD) in the plants was high, which could reduce the damage of reactive oxygen species (ROS)-mediated membrane system caused by salt stress. Zhang (2010) and Li et al. (2020a, b) studied several potato varieties/lines with different salt sensitivities. When the stress time gradually increased, the activities of peroxidase (POD) and SOD showed a gradual downward trend, while with the increase of salt stress time, the activity of catalase (CAT) first decreased and then increased. Zhao et al. (2014) compared the morphological and physiological characters of diploid potato with different salt tolerances under alkali stress. The results showed that whether at the concentration of 5 mmol/L $NaHCO_3$ or 10 mmol/L $NaHCO_3$, the relative value of SOD activity from high to low was salt-tolerant group > medium salt-tolerant group > salt-sensitive group, and the relative value of POD activity from high to low was salt-tolerant group > medium salt-tolerant group > salt-sensitive group.

16.4 Salt Tolerance Gene

ABF is a bZIP transcription factor. The results of the plantlets of transgenic potato with *GhABF2* showed that the biomass of *GhABF2* transgenic materials T1 and T2 can be significantly increased compared with the control. The transgenic plants had significant salt stress resistance (Pei et al. 2015). Studies have shown that *ABF4* can improve the salt tolerance of *Arabidopsis* (Pan 2020). Noelia et al. (2018)

overexpressed the *ABF4* gene in *Arabidopsis thaliana* in potato. The results showed that under normal conditions, there was no significant differences in various physiological indexes between transgenic potato and wild type, but the tuber yield and tuber quality of transgenic potato were higher than those of wild type. After salt stress treatment, the relative water content, proline content, and chlorophyll content of transgenic potato were significantly higher than those of wild type, indicating that abf4 overexpression plants had enhanced salt tolerance. Overexpression of *StDREB2* gene in potato plants can improve potato salt tolerance by participating in ABA hormone signal and proline synthesis (Bouaziz et al. 2012). In addition, the researchers also found that overexpression of *StDREB1* can also significantly improve the salt tolerance of transgenic potato (Bouaziz et al. 2013).

The salt and alkali tolerance of potato can be improved by increasing the activity of antioxidant enzymes, increasing the content of proline, and reducing the content of MDA through an appropriate amount of exogenous brassinolide (Hu et al. 2016). Zhou et al. (2018) overexpressed *DWF4* gene in potato to obtain transgenic potato plants. After stress treatment, the physiological indexes, such as soluble sugar content, soluble protein content, and antioxidant enzyme activity of *StDWF4* transgenic potato lines, are higher than those of normal plants, indicating that overexpression of *StDWF4* can improve the salt tolerance of potato.

Wu et al. (2019) studied the clonal generation plants of potato transformed with *HaBADH* (betaine aldehyde dehydrogenase) and showed that with the gradual increase of salt concentration, compared with normal plants, the growth, weight, number, proline, and MDA contents of *HaBADH* transgenic plants were significantly higher than those of non-transgenic materials. The results showed that the expression of *HaBADH* gene under salt stress improved salt tolerance of potato transgenic lines. *HAL1* (Luo 2007) and *BADH* (Li et al. 2007) were transferred into potato and the salt resistance of potato plants was significantly enhanced.

Chen et al. (2013) transferred the S-adenosyl-L-methionine synthetase (*SAMS*) gene into potato for the first time. Comparing seven potato lines (SM13, SM33, SM4, SM22, SM30, SM40, and SM27) with non-transgenic "Atlantic" varieties, the yields of four transgenic lines SM22, SM4, SM33, and SM13 were higher than those of non-transgenic plants, indicating that the transfer of *GsSAMS* into potato can improve its alkaline tolerance. *NHX* gene encodes a Na^+/H^+ antiporter. The study of potato lines with *AtNHX1* and non-transgenic "Gannongshu 2" showed that the introduction of *AtNHX1* had a significant effect on the salt tolerance of potato plants in the field (Li et al. 2017a) (Table 16.2).

16.5 Methods of Improving Salt and Alkaline Tolerance of Potato

16.5.1 Using Plant Growth-Promoting Rhizobacteria (PGPR)

PGPR can alleviate the negative effects of saline alkali stress on plants, improve plant growth, and improve the salt tolerance of various crops. It has important application value in the field of agricultural development (Kumar et al. 2019;

Table 16.2 Functional genes expressed in potato

Donor plant	Gene	Effect	Reference
Gossypium herbaceum	*ABF2*	Increase proline, activities of SOD and POD	Pei et al. (2015)
Arabidopsis thaliana	*ABF4*	Induce the tuberization	Pan (2020); Noelia et al. (2018)
Solanum tuberosum	*DREB2; DREB1*	Promote proline synthesis	Bouaziz et al. (2012); Bouaziz et al. (2013)
Solanum tuberosum	*DWF4*	Increase proline content	Zhou et al. (2018)
Haloxylon ammodendron	*BADH*	Increase proline content	Wu et al. (2019)
Saccharomyces cerevisiae	*HAL1*	Decrease Na^+/K^+ ratio	Luo (2007)
Solanum tuberosum	*BADH*	Increase proline content	Li et al. (2007)
Glycine soja	*SAMS*	Improve the chlorophyll content and relative water retention rate of leaf	Chen et al. (2013)
Arabidopsis thaliana	*NHX1*	Increase proline and SOD activity	Li et al. (2017a)

Etesami and Maheshwari 2018). It was found that the symbiosis of plant rhizosphere growth-promoting bacteria with potato can improve the growth ability of potato plants and improve the quality of potato (Lu 2020; Li 2020). Bacillus is a common plant rhizosphere growth-promoting bacterium. When Bacillus and potato coexist under saline alkali stress, Bacillus enhances the salt alkali tolerance of potato by promoting the production of potato rhizosphere auxin, maintaining ion homeostasis and regulating the activity of antioxidant enzymes, so as to improve the biomass, number of tubers per plant, and tuber yield of potato plant (Tahir et al. 2019).

16.5.2 Application of Exogenous Substances

By applying exogenous regulatory substances, the ability of plants to resist salt and alkali can be improved, so as to improve plant salt tolerance (Su et al. 2021; Gao et al. 2017; Jiang et al. 2020). Faried et al. (2017) showed that salicylic acid can significantly improve the activity of antioxidant enzymes, such as SOD, CAT, and POD, regulate the content of proline and phenol, remove ROS, improve the utilization rate of potassium, and reduce the content of sodium in potato leaves, so as to endow potato with salt tolerance. Under saline alkali stress, jasmonic acid can reduce the effect of NaCl on photosynthetic pigments, maintain the osmotic substances of cells, and enhance their salt tolerance (Efimova et al. 2019). The application of exogenous phosphatidylserine can reduce the K^+ outflow caused by salt stress and regulate the H^+-ATPase activity on the membrane to delay leaf senescence and induce the improvement of salt tolerance of potato (Yu et al. 2019). Ca^{2+} is not only

an important nutrient element but also an important second messenger. When plants are stressed, Ca^{2+} can improve the stress resistance of plants (García Bossi et al. 2020). The application of exogenous calcium ion can increase the chlorophyll content and enhance the photosynthesis of potato. Secondly, it can also improve the activity of antioxidant enzymes and maintain the stability of membrane, so as to improve the salt tolerance of potato (Wei 2014).

16.6 Research on Drought-Tolerant Breeding of Potato

16.6.1 Overview

Potato is a typical temperate climate crop. It likes the growth conditions of low temperature and cold. It is susceptible to water shortage and lacks effective drought tolerance mechanism (Qin et al. 2019). At the same time, with the rise of global temperature, the drought in potato planting areas is becoming more and more serious, and the water shortage is serious. About potato grows, resulting in serious yield reduction (Wang et al. 2016; Xu et al. 2011).

Under drought stress, the growth of plant height, main stem and root system, leaf number, leaf area, and yield are basically inhibited (Yang et al. 2016; Yao et al. 2013). Zhang et al. (2016) believed that the number of tubers per plant, weight of tubers per plant, and biomass of potato were greatly reduced by drought. Some scholars believe that potato plants will encounter drought stress when the soil water potential drops to −25 kPa or the soil relative field water capacity is less than 50%, and are sensitive to drought in all growth stages (Schafleitner et al. 2007). Especially in the tuber expansion stage, if drought stress is encountered, the yield will be affected, and serious drought can lead to a significant decline in yield. At the same time, it will cause a series of adverse reactions and reduce the quality of potatoes, such as appearance distortion, abnormal tissues and organs, metabolic disorder, and hollow potatoes (Liu et al. 2019; Qin et al. 2018). Wang et al. (2016) showed that the plant height stress index was inversely proportional to the drought resistance index, and reached a significant level of 1%. The drought resistance index will decrease with plant height.

16.7 Drought Tolerance Evaluation

16.7.1 Morphological Yield Index

Identification of drought resistance of potato by plant morphology is a widely used research method at home and abroad. The morphological characteristics of potato, such as root tension, root length, root weight, root shoot ratio, plant height, stem diameter, biomass, fresh weight, leaf weight, stomatal subsidence, and leaf area, are related to drought resistance. The research on drought-resistant morphological characteristics of potato at home and abroad mainly focuses on the root system of

the plant. Root system is the organ of potato to receive soil water signal and absorb soil water. Under drought stress, root absorption and root activity decreased, resulting in nutrient imbalance (Xie and Zheng 2015). Du et al. (2012) believed that varieties with strong drought resistance have developed roots and high yield under drought conditions. At the same time, root tension is significantly proportional to root weight, root number, and root length, which is an important index to measure the degree of root development.

Under drought stress, potato yield index is more intuitive. The drought resistance is mainly reflected in the yield, which can be used as an important index to screen the drought-resistant varieties of potato. Chionoy's drought resistance coefficient method, Fish's drought sensitivity index (SI) method and Hu Fushun's drought resistance index (DI) method are its traditional methods (Xie and Zheng 2015). Gu et al. (2013) considered that the change degree of potato tuber yield under water stress is the evaluation index of drought resistance, and the drought resistance index directly shows the sensitivity of plants to drought.

16.7.2 Physiological and Biochemical Indexes

The physiological indexes of plant drought resistance evaluation include soil water content, leaf water content, photosynthetic rate, water potential, respiratory rate, dry matter stress index, osmotic regulation ability, water stress index, water holding capacity of isolated leaves, etc. Du et al. (2012) showed that the canopy coverage was directly proportional to the yield, reaching a significant level of 5%, which can be used as an important physiological index for drought resistance evaluation. The results showed that 80% soil water content was the best for potato plant growth, 60% soil water content was mild drought stress, 40% soil water content was moderate drought stress, and 20% soil water content was severe drought stress. Therefore, soil water content can be used as one of the physiological indexes to evaluate potato drought resistance (Yin and Xiao 2017; Jiao et al. 2011). When potato plants suffer from drought stress and water deficit, photosynthesis will be weakened and photosynthetic rate will be reduced, resulting in crop yield reduction (Yang et al. 2019b). Therefore, photosynthetic rate is an important index for drought resistance evaluation of potato (Li and Tong 2018). The water holding capacity of leaves is an embodiment of potato drought resistance. Wang et al. (2016) showed that the varieties with lower drought resistance have stronger water loss capacity, which is equivalent to the lower water retention capacity. The drought resistance coefficient decreased with the increase of water loss. There was a negative correlation between water loss and drought resistance coefficient, which reached a significant level of 1%, and the potato yield decreased.

Biochemical indexes of plant drought resistance include soluble sugar content, proline content, betaine content, MDA content, ATPase activity, vitamin C content, SOD activity, POD activity, CAT activity, etc. Under drought stress and water shortage conditions, potato plants will accumulate a large amount of proline, and there are differences between varieties and stress time (Li and Tong 2018). Ding

et al. (2013) studied that under drought stress, the proline content of different varieties of potato increased by 1.01~5.40 times, the MDA content increased by 1.10~1.91 times, and the free proline content and MDA content of potato leaves showed an upward trend, which can be used as the biochemical index of potato drought resistance evaluation. Soluble sugar is an important osmotic regulator. In many plants, soluble sugar content will almost always increase under drought conditions (Yang et al. 2016). However, Yang (2016) studied six potato varieties, such as "Atlantic," and the result showed that there was a negative correlation between soluble sugar content and stress time. Therefore, soluble sugar as an index of drought resistance needs to be further studied.

16.7.3 Comprehensive Index (Membership Function Method)

The comprehensive index method can accurately evaluate the drought resistance of potato. The membership function method is to accumulate the membership values of all drought resistance indexes of each variety, calculate its average, and compare among varieties, so as to evaluate its drought resistance capacity (Du et al. 2012). Wang (2014) showed that MDA content is an important index for potato drought resistance evaluation by using the principal component analysis method to analyze the physiological and yield indexes of potato. The membership function method was used to evaluate the drought resistance of potato, and the evaluation results are almost the same as the practice.

16.8 Physiological Response to Drought Stress

Under drought stress, physiological and metabolic indexes of potato, such as soil water content, free proline content, soluble sugar content, chlorophyll content, root pulling force, ATP content, leaf water potential, SOD activity, and MDA content, will change, reflecting the stress status of potato (Yin et al. 2017; Fan et al. 2006). In general, the low concentration of proline indicates the low degree of stress (Ren et al. 2011). Yin and Xiao (2017) believed that drought stress leads to a large increase in proline, soluble sugar and soluble protein in potato leaves, and a decrease in osmotic pressure, which promotes the adaptability of potato plants to arid environment and their drought resistance. Zhao et al. (2018) analyzed the relative amount of photosynthetic characteristic indexes of potato plants under drought stress for 15 days and blank treatment. The results showed that drought stress affected the photosynthetic characteristic indexes of potato plants to a great extent, resulting in the decrease of net photosynthetic rate, transpiration rate, air pore conductivity, and intercellular CO_2 concentration of potato plants. Gu et al. (2013) studied the partial and energy metabolism indexes of potato leaves under drought stress and found that under stress, the soluble protein content in leaves showed an increasing trend, the leaf area index showed a decreasing trend, and the soluble protein content would increase with the decrease of water supply. The water loss rate of leaves increased and the

drought resistance decreased. On the contrary, the leaf water loss rate decreased and the drought resistance increased (Du et al. 2012). Ding et al. (2011) showed that the drought resistance of the strain was positively proportional to the soluble sugar content of leaves and reached a significant level of 1%, and inversely proportional to the water loss rate and water content of leaves in vitro and reached a significant level of 5%. Liang et al. (2018) believed that drought stress caused a large accumulation of MDA in potato cells. The deepening of drought stress will enhance the activity of POD, so as to eliminate hydrogen peroxide in time to resist adversity.

16.9 Drought Tolerance Gene

With the rapid development of molecular biology, the research on plant drought resistance is also deepening, and many drought resistance genes have been found. By 2014, more than 100 drought-related genes had been found in nearly 500 plants, of which 68 genes were found in *Arabidopsis* and major crops (Li 2018; Blum 2014), while few drought-related genes were found in potatoes, only WRKY1, DREB1, and SnRK2 (Wang et al. 2017). When potato is under drought stress, many adverse reactions will occur. This stage is an important stage of signal recognition. After being stimulated by the outside world, it can be regulated into transcription factors by signal transduction. After being regulated, the regulatory genes of transcription factors complete the drought resistance process through metabolism (Li 2018; Wang and Guo 2017). At present, many domestic and foreign scholars gradually study the molecular mechanism of potato drought resistance and focus on using modern molecular biology technology and biochemical technology to enhance the drought resistance of potato. Pei et al. (2019) analyzed the transgenic *GhABF2* of potato test tube seedlings under drought stress compared with the wild type. The results showed that the transgenic potato with *GhABF2* gene could significantly increase the biomass and show strong drought resistance. Li et al. (2014) believed that the SOD activity of *P5CS* transgenic plants was enhanced under drought stress and salt stress, thus reducing the probability of stress. Yang et al. (2019a) transferred the *Haloxylon ammodendron* NAC family gene hanac1 into potato and found that it improved the drought resistance of potato by participating in a variety of hormone synthesis and signal transduction and regulating the expression of genes related to downstream stress response (Table 16.3).

Table 16.3 Functional genes expressed in potato

Donor plant	Gene	Effect	Reference
Gossypium herbaceum	*ABF2*	Increase proline, activities of SOD and POD	Pei et al. (2019)
Solanum tuberosum	*P5CS*	enhance SOD activity	Li et al. (2014)
Haloxylon ammodendron	*NAC1*	promote hormone synthesis	Yang et al. (2019a)

16.10 Cold-Tolerant Breeding of Potato

16.10.1 Overview

Potatoes can be planted from 69° north latitude to 50° south latitude and from sea level to 4000 meters above sea level (Hijmans 2003). Potato tubers are rich in protein, vitamins, and dietary fiber. They have high nutritional value and play an important role in solving regional food shortage and eradicating world poverty. Potato has been cultivated in China for 400 years. At present, the planting area is six million hectares and the total output is more than 120 million tons. It has become the largest producer in the world (FAOSTAT 2017). As a cool but not cold-tolerant crop, most potato varieties are not resistant to low temperature and frost. China's potato ecological planting areas include the first cropping area in the north, the second cropping area in the Central Plains, the single and double cropping mixed cropping area in the southwest, and the winter cropping area in the south. The first cropping area in the north is vulnerable to early autumn frost and sudden drop in humidity, the second cropping area in the Central Plains is vulnerable to early spring frost and late autumn frost, the single and double cropping mixed cropping area in the southwest is vulnerable to late spring cold, and the winter cropping area in the south is vulnerable to low temperature. Low-temperature frost seriously threatens the development of China's potato industry. For example, in 2008, snow and ice disasters in southern China affected an area of about 409,300 hm^2 of potatoes, causing economic losses of up to one billion yuan (Kou et al. 2015). In 2016, the low-temperature and cold wave in Poyang Lake area of Jiangxi Province caused the freezing death rate of potato seedlings to be 75~95%, and the yield was reduced by 40%, resulting in serious economic losses (Zhang et al. 2016). In 2018, the low-temperature and cold wave in Yulin, Guangxi province led to the failure of local potato production to meet the commercial potato standard (more than 100 g), resulting in serious economic losses (Zhu et al. 2020).

When potato plants suffer from low-temperature freezing injury, they will show that the stems are paralyzed and collapsed, and the leaves are dark green and watery. The leaves lose photosynthetic capacity and stop growing, which will seriously affect the potato yield (Li and Jin 2007). Low-temperature stress is accompanied by the changes of cell membrane structure and lipid components, mainly manifested in the changes of unsaturated fatty acid content and lipid peroxidation in plant cells. The lower the content of unsaturated fatty acids, the more prone the cell membrane to the low-temperature phase transition (Theocharis et al. 2012). Lipid peroxidation induced by low-temperature stress decreased the fluidity of the cell membrane and impaired the function of the cell membrane. Freezing injury will cause freezing between or within plant cells, directly destroy the integrity of the cell membrane, deform cells, and lose cell function. Low temperature induced photoinhibition in plants, resulting in the closure of plant stomata, blocking the source of carbon dioxide, resulting in the decline of photosynthetic rate and the inhibition of photosynthetic function.

16.10.2 Cold Resistance Evaluation

16.10.2.1 Identification Method

Vega and Bamberg (1995) first used the field natural frost method to divide the cold resistance of potato into seven grades: Grade 0 represents no damage; Grades 1 to 6 represent slight injury to top leaves, freezing death of a few top leaves, freezing death of most top leaves, freezing death of all top leaves and petioles, and freezing death of all leaves and stems, respectively. The field natural frost method depends on the natural climate environment in the open field, which can directly and accurately reflect the freezing damage of plants under natural freezing conditions (Lin et al. 2020).

Electrolyte leakage method is the most reliable identification method at present by measuring plant cell membrane conductivity and calculating half lethal temperature (LT50) by the logistic equation. It has the advantage of more accurate than physiological and biochemical index determination method (Jiao et al. 2019). The physiological and biochemical index determination method is to directly reflect the cold resistance of plants through the determination of physiological and biochemical indexes, such as soluble sugar, soluble protein (SP), proline, MDA, antioxidant enzyme SOD, CAT, POD under low-temperature stress, and simple and easy to operate. This method has been widely used to identify the cold resistance of pomegranate, kiwi fruit, potato, and wheat (Soloklui et al. 2018; Wan et al. 2001; Pan 2016; Li et al. 2019).

16.10.2.2 Cold-Tolerant Potato Varieties

There are great differences in cold resistance among different potato varieties. The selection of cold-resistant potato varieties is of great significance for the cultivation of potato resistance to low temperature and frost. Potato varieties with strong cold resistance should be selected as far as possible during planting. Li (2008) evaluated 26 materials by using the field natural frost method and selected 13 frost-resistant materials, such as 03079-444, 03079-435, and 03079-343, and 3 frost-sensitive materials, such as Zhongshu 3, 03079-322, and 03088-344. Zhao (2013) treated 108 wild species test tube seedling materials from *Solanum chomatophilum*, *Solanum acaule,* and *Solanum paucisectum* at −3.5 °C for 24 h and screened 27 cold-resistant materials, such as *Solanum acaule,* and 69 low-temperature-sensitive materials, such as *Solanum demissum*. Tu et al. (2015) identified the cold resistance of 40 potato materials by using the electrolyte leakage method and the direct evaluation system of cold resistance identification at seedling stage, and screened and obtained 21 potato materials with strong cold resistance, such as *Solanum acaule*, *Solanum paucisectum,* and *Solanum albicans*. Li et al. (2016) identified 65 potato materials by conductivity measurement combined with logistic equation and selected Zhengshu 6, Guinongshu 1, and 4 progeny lines (0712801, 0917-8056, D540 and 0719017) as cold-resistant materials. Pan (2016) identified the cold resistance of 54 potato materials by electrolyte leakage method and selected 5 cold-resistant materials, such as av9, gs393, and 8033 (4) and 8033 (9) and 21 × 11、41 × 25 copies of non-cold-resistant materials, such as 5. Using the

method of physiological index determination, it was found that on the fifth day after low-temperature stress, the activities of SP, CAT, SOD, and POD in the leaves of Guinongshu 1 seedlings were significantly higher than those of Favorita, showing strong cold resistance (Deng et al. 2017). The cold resistance of different winter potato varieties in Guangxi was compared. The chlorophyll, relative water content, SP, MDA content, and antioxidant enzyme activity of Lishu 6, Favorita, and Xingjia 2 were measured under 4 °C low-temperature stress. The results of cold resistance were as follows: Lishu 6 > Xingjia 2 > Favorita (Li et al. 2017a, b). Wei et al. (2017) used conductivity leakage method and natural frost method to identify the cold resistance of 116 potato varieties and screened four cold-resistant materials Jinshu 2, Kexin 2, Zhengshu 5 and Zhengshu 6. Yang and Guo (2017) analyzed the changes of leaf relative conductivity, MDA, SOD, POD, and SP contents of 10 potato varieties subjected to low-temperature stress at seedling stage, and comprehensively evaluated the cold resistance by combining principal component analysis, membership function method, and cluster analysis. The order of cold resistance is Lishu 6 > Zhongshu 20 > dianshu 701 > jizhangshu 12 > Qingshu 9 > cooperative 88 > Zhongshu 18 > Diantongshu 1 > normal university 6 > Xuanshu 2. 103 potato materials were identified by conductivity method, field natural frost method, membership function method and cluster analysis method, and 16 cold-resistant materials, such as V9, bs214, gs393, Goutou yam, and Linshu 3 and 21 frost-sensitive materials, such as anti-10, Longshu 5, and sten-1 were obtained (Ding et al. 2019b) (Table 16.4).

Table 16.4 Cold-tolerant potato varieties

Variety name	Cold resistance	Reference
03079-444, 03079-435, 03079-343	Cold-resistant	Li (2008)
Zhongshu 3, 03079-322, 03088-344	Sensitive	
Solanum acaule	Cold-resistant	Zhao (2013)
Solanum demissum	Sensitive	
Solanum acaule, Solanum paucissectum, Solanum albicans	Cold-resistant	Tu et al. (2015)
Zhengshu 6, Guinongshu 1 and four progeny lines (0712801, 0917-8056, D540 and 0719017)	Cold-resistant	Li et al. (2016)
AV9, GS393, 8033(4)	Cold-resistant	Pan (2016)
8033(9), 21 × 11, 41 × 5	Sensitive	
Guinongshu 1	Cold-resistant	Deng et al. (2017)
Favorita	Sensitive	
Lishu 6, Xingjia 2	Cold-resistant	Li et al. (2017b)
Favorita	Sensitive	
Jinshu 2, Kexin 2, Zhengshu 5 and Zhengshu 6	Cold-resistant	Wei et al. (2017)
Lishu 6 > Zhongshu 20 > Dianshu 701	Cold-resistant	
Diantongshu 1 > Normal University 6 > Xuanshu 2	Sensitive	
V9, bs214, gs393, Goutou yam, Linshu 3	Cold-resistant	Ding et al. (2019b)
Anti-10, Longshu 5, sten-1	Sensitive	

16.10.2.3 Physiological Response to Low-Temperature Stress

The cold tolerance of potato seedlings can be improved by spraying growth substances, cross breeding, and chemical mutation. López-Delgado et al. (2018) found that salicylic acid (SA) and H_2O_2 can mediate the tolerance of potato to low-temperature stress, induce the enhancement of cat enzyme activity, and improve the cold tolerance of potato. Li et al. (2018a) tested osmoregulatory substances and antioxidant enzyme activities by exogenous spraying 0.5 mmol L^{-1}sa on potato seedlings under low-temperature stress. The results showed that exogenous spraying SA could reduce cell membrane damage and resist low-temperature damage by regulating potato osmoregulation and antioxidant capacity. Spraying potato seedlings with three growth substances at concentrations of 10 mg L^{-1} abscisic acid (ABA), 0.5 mmol L^{-1} spermidine (SPD) and 0.01 mg L^{-1} brassinolide (BR) can improve the low-temperature tolerance of potato (Wang et al. 2018). Ding et al. (2019a) found that the cold resistance of potato seedlings was the strongest after spraying 3 mg L^{-1} ds twice. Huang et al. (2019) mutated potato Favorita callus with ethyl methanesulfonate (EMS), added L-hydroxyproline (L-HYP) for culture and screening, and obtained potato mutants mutated by EMS with cold resistance. Through the determination of physiological indexes of potato plants, more accurate cold resistance resources can be obtained, but most of the potatoes with strong cold resistance are diploid wild species, which cannot be directly used in potato production. Spraying growth substances or chemical mutagenesis through exogenous sources has a positive effect on improving the freezing resistance of potato seedlings. At the same time, it will also lead to a series of problems, such as slow growth of potato, short plant, environmental damage, and waste of resources, which will restrict the development of the potato industry.

16.10.2.4 Cold Tolerance Gene

With the continuous development of molecular biology technology, a series of progress has been made in the research of potato cold resistance-related genes. The damage of low-temperature stress to plants mainly includes destroying the cell membrane and affecting photosynthesis and physiological metabolism. The up-regulation of stearoyl-acyl carrier protein desaturase *(SAD)* gene expression after potato cold acclimation at low temperature can enhance potato cold stress tolerance (Amiri et al. 2010). Li (2013) isolated the key dehydrogenase gene sad in the pathway of unsaturated fatty acid synthesis from three wild potato species by using transcriptome sequencing technology and successfully overexpressed the sad gene in the cultivated species Zhongshu 8, which improved the cold resistance of Zhongshu 8. Studies have shown that diploid wild species *Solanum commersonii* has strong cold resistance. Through transcriptome sequencing, it was found that the expression of 855 genes was up-regulated after cold acclimation, mainly including cold resistance genes, such as CBF3, E3 ubiquitin ligase (HOS1), CBF-regulated transcription factor (ICE1), and sumo E3 ligase (small ubiquitin-like modifier E3 ligase, SIZ1) (Aversano et al. 2015). *Arabidopsis thaliana* cold responsive-element binding factor 3 (*AtCBF3*) was overexpressed in potato to enhance the antioxidant effect and low-temperature tolerance of potato (Dou et al. 2015). Protoplast sugar

and cell wall invertase (CWI) significantly affect the cold resistance of potato mainly by inducing ABA (Deryabin et al. 2016). Transcriptome and metabolome analysis showed that potato spermidine decarboxylase gene (*ADC1*) was highly expressed under low-temperature treatment. Overexpression of *ADC1* resulted in an increase in putrescine content and significantly improved the freezing resistance of potato (Kou et al. 2018). Li et al. (2018a, b) overexpressed *Solanum tuberosum* CBF1 (*StCBF1*) and *Solanum commersonii* CBF1 (*ScCBF1*) genes in *Arabidopsis*. Compared with the control, the overexpressed plants of the two genes had stronger low-temperature tolerance, and the effect of *ScCBF1* was more significant than *StCBF1*. Overexpression of CBF1 (*SpCBF1*) gene of *Solanum pinnatisectum* increased the expression of cold-responsive genes (COR) in potato, increased SOD activity and SS content, and improved the cold resistance of potato (Zhu et al. 2018). Xie et al. (2019) found that stumirna390 isolated from potato material gs393 was induced by low-temperature stress and inhibited the expression of its target gene *ScLRRK1* (lrr-rlk). Che et al. (2020) found that overexpression of potato *StSOD1* gene (*Solanum tuberosum* superoxide dismutase, *StSOD1*) can regulate antioxidant enzyme activity in potato and improve cold resistance. Overexpression of amylase inhibitor gene (*Solanum berthaultii* amylase inhibitor, *SbAI*) can reduce potato starch synthesis to resist short-term low-temperature stress (Slugina et al. 2020). Although a series of potato cold resistance genes have been reported, the molecular mechanism of potato cold resistance is not completely clear and needs further research (Table 16.5).

Table 16.5 Functional genes expressed in potato

Donor plant	Gene	Effect	Reference
Synechocystis sp. PCC6803	*SAD*	Increase unsaturated fatty acid concentration in lipids	Amiri et al. (2010)
Solanum commersonii	*SAD*	Increase linoleic acid content	Li (2013)
Solanum commersonii	*CBF3, HOS1, ICE1, SIZ1*		Aversano et al. (2015)
Arabidopsis thaliana	*CBF3*	Improve photosynthesis and antioxidant defense	Dou et al. (2015)
	CWI	Induce ABA synthesis	Deryabin et al. (2016)
Solanum tuberosum	*ADC1*	Increase putrescine content	Kou et al. (2018)
Solanum commersonii	*CBF1*	Promote the expression of *COR*	Li et al. (2018b)
Solanum pinnatisectum	*CBF1*	Increase the expression of COR, SOD activity, and SS content	Zhu et al. (2018)
Solanum tuberosum	*LRRK1*	Target gene of ScmiR390-5p	Xie et al. (2019)
Solanum tuberosum	*SOD1*	Regulate the activity of antioxidant enzyme	Che et al. (2020)
Solanum berthaultii	*AI*	Regulate starch synthesis negatively	Slugina et al. (2020)

16.11 Heavy Metal Tolerance Breeding of Potato

16.11.1 Overview

As the fourth food crop, potatoes (*Solanum tuberosum* L.) are grown worldwide from sea level to over 4000 m elevation (Tabaldi et al. 2009a). Plant species differ in their aluminum (Al) tolerance and potato is inherently tolerant to Al (Little 1988). Potato crops grow well at pH 5.0–6.5, but yield decreases in the soils with a pH value below 5.0 (Castro 1983). In Southern China, most potatoes are planted in red and yellow soil or winter postharvest rice field. The pH value of winter rice field prolonged exposure to the sun quickly drops below 5.0. At that moment, Al ion mainly exists in the form of highly toxic trivalent cation. Al toxicity has become a vital factor affecting potato production. Potato showed a strong preference for cadmium absorption. The accumulation of heavy metals in potatoes was detected by planting potatoes in composted soil with municipal solid waste (Topcuoglu and Onal 2012), and potatoes were more enriched than wheat and barley. The ability of the genus is stronger (Casova et al. 2009). The accumulation of heavy metal cadmium in potato tubers is as high as 0.04–0.20 mg/kg (Fan et al. 2009), and the accumulation law in plants is stem > leaf > fruit > root (Fan et al. 2011). According to the standard, except for fruits, the cadmium content in roots, stems, and leaves of potatoes almost all exceeds the national food safety limit, which has a high ecological risk (Fu et al. 2014).

With the increase of Al concentration (1–10 mg/kg), Mg concentration in the root and stem apex of potato was declined. Low concentration of Al could improve Mg contents in potato varieties Acer and Gleditsia, but was the opposite at high Al concentration. When Al concentration in the solution was increased to 10 mg/kg, Fe accumulated in potato root (Xiao and Wang 2006). The absorption and distribution of Zn, Fe, Mn, and Cu were affected by excessive Al accumulation in roots and shoot of potato clones. With the increase of Al levels, the concentrations of Zn, Mn, and Fe in root decreased linearly in Macaca and SMIC148-A, but increased linearly in *Solanum microdontum* (Tabaldi et al. 2009b). Meanwhile, the competition of combining site on the root surface between Al and Cu resulted in Cu accumulation in potato root (Xiao and Wang 2006). There was a quadratic relationship in root Cu content among three potato clones (Tabaldi et al. 2009b). Al over 100 mg L^{-1} decreased the concentrations of chlorophyll and carotenoid in the Al-sensitive potato clones (Tabaldi et al. 2007). Characterized by the production of ROS and RNS, oxidative stress is a toxic mechanism in the Al-sensitive Macaca clone (Tabaldi et al. 2009a). Al toxicity not only depends on Al availability but growth condition and the clone used. The increasing H_2O_2 concentration of root and shoot was dependent on the concentration and distribution of Al (Tabaldi et al. 2009a). $A1^{3+}$ accelerated the death of potato cells and produced much H_2O_2, but $Al(OH)_3$ caused higher H_2O_2 accumulation and did not cause significant death of potato cells. There was dual influence of dissolution rate of Al in soil and its form change on the resistance of potato tuber during soft rot bacteria infection (Shi et al. 2008). Lipid peroxidation was an early symptom induced by Al in the Al-sensitive Macaca clone. Protein

oxidation was also observed in roots. Under Al treatment, CAT and APX activity in Macaca roots firstly were decreased and then increased. 200 mg L^{-1} Al lowered NPSH and AsA concentration in Macaca roots. In potato plants, the cellular redox status was sensitive to Al injury (Tabaldi et al. 2009a). Al interferes with root acid phosphatase (APase) activity in potato clones. The Apase activity *in vivo* cannot be used to screen Al adaptation of potato clones, because it depends on Al availability, plant organ, growth conditions, and genetic background (Tabaldi et al. 2011).

16.11.2 Evaluation of Heavy Metal Resistance

Lee (1971b) founded that Al tolerance of four potato varieties decreased in the order: Netted Gem > Sedago > Katahdin > GreenMountain. Tabaldi et al. (2007) evaluated Al tolerance of four potato clones. Based on relative root growth and Al content in roots, the screening results arranged from strong to weak as SMIC148-A > *Solanum microdontum* > Dakota Rose>Macaca. 200 mg L^{-1} Al significantly lowered relative root growth in Macaca clone, which did not depend on exposure time. Al content in Macaca roots increased linearly in a dose-dependent manner. Therefore, Macaca was considered Al-sensitive potato clones, whereas SMIC148-A was considered to be Al tolerant. Al depressed plant growth and number and yield of tubers for both potato varieties Netted Gem and Sebago. Al treatment lowered the yield of knobby tubers. 20 ppm Al decreased small tuber yield and increased the dose-dependent yield of larger tubers and the specific gravity of the tubers for both potato varieties (Lee 1971a). Therefore, Al accumulation in potato tissues affects the growth and plant yield. The yield per plant, leaf dry weight, stem dry weight, tuber dry weight, and root dry weight were declined under Cd stress. For both varieties, Cd toxicity to different organs ordered as follows: root > tuber > leaf > stem. The resistance of Kexin 1 to Cd was better than that of Favorita (Bai et al. 2012) (Table 16.6).

16.11.3 Physiological Response to Heavy Metal Stress

Through the proliferation of deep roots, the root of SMIC148-A potato clone was growth into the higher Al level soil zones (Tabaldi et al. 2009a). Al avoidance

Table 16.6 Heavy metal-tolerant potato varieties

Variety name	Heavy metal resistance	Reference
Netted Gem>Sedago>Katahdin>GreenMountain	Al	Lee (1971b)
SMIC148-A > *Solanum microdontum* > Dakota Rose>Macaca	Al	Tabaldi et al. (2007)
Kexin 1	Cd-resistant	Bai et al. (2012)
Favorita	Cd-sensitive	

responses differed because of the distinct Al sensitivity in potato clones. Al avoidance was more obvious for the Al-sensitive clone, whereas SMIC148-A had a stronger antioxidant response to Al stress (Tabaldi 2008). The alteration of nutrient element distribution in potato root can improve Al tolerance of potato. Al tolerance among certain potato varieties may be related to the absorptive ability of plant roots to Mg and K (Lee 1971b), which are in accordance with the results of Liu and Jiang (1995). The alteration of shoot Cu concentration mainly occurred in Al-sensitive clones. The higher concentrations of Zn, Fe, and Mn in the roots were associated with Al tolerance in *Solanum microdontum* (Tabaldi et al. 2009b). Potato roots accumulate more Al and more micronutrients could not be transported to the shoot. So antioxidant systems in roots efficiently scavenged the side effect of Al stress (Tabaldi et al. 2007).

In terms of potato, the enhancement of antioxidant capacity has been identified as an Al-tolerant mechanism. CAT activity in SMIC148-A roots was increased, but APX activity was decreased with the increase of treatment time. Higher level non-protein thiol groups (NPSH) and ascorbic acid (AsA) in root occurred at 120 h. Al supplies could not alter H_2O_2 concentration in root and shoot in Al-tolerant potato clones (Tabaldi et al. 2007). The transgenic potato expressing tomato Cu-Zn SOD enhanced oxidative stress defense (Perl et al. 1993). With the cadmium concentration increased, the activities of SOD and CAT in the two cultivars of potato leaves increased firstly and then decreased. The content of MDA and proline in the two cultivars of potato leaves increased with the cadmium concentration increased (Zhou et al. 2012).

16.11.4 Heavy Metal Tolerance Gene

Compared with the wild type, the transgenic potato mutant overexpressing PME was more sensitive to Al (Schmohl et al. 2000). Zimmermann et al. (2004) reported that *Solanum tuberosum* purple acid phosphatase (*StPAP1*) was expressed more lowly in young leaves, stolons, and flowers. In contrast, P starvation induced the higher expression of *StPAP2* and *StPAP3* in roots and stem. Due to precipitation of Al phosphates, the relative Al avoidance was associated with the response of local P sources to Al-induced internal P shortage. The gene could be induced by heavy metals Pb^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+}, and the *StERF10* expression level varied with different times and different tissues. Among the five heavy metal stresses, *StERF10* gene was the most sensitive to Ni^{2+} stress and it was speculated that *StERF10* might be involved in the regulation of abiotic stress response in potatoes (Meng et al. 2020a). NAC is one of the unique transcription factor families in plants. The expression of *StNAC2* gene was inhibited under high concentration stress and *StNAC2* gene could involve in cadmium stress (Meng et al. 2020b). Potatoes had certain tolerance under low concentration of Pb or Cd, and their injuries of active oxygen were alleviated through increasing the activity of antioxidant enzymes and decreasing the membrane lipid peroxidation, but the resistance gradually reduced along with increase of the stress (Li and Liu 2014). As an important antioxidant in

Table 16.7 Functional genes expressed in potato

Donor plant	Gene	Effect	Reference
Solanum tuberosum	*PME*	Promote Al accumulation	Schmohl et al. (2000)
Solanum tuberosum	*PAP1, PAP2, PAP3*	Precipitate Al phosphates	Zimmermann et al. (2004)
Solanum tuberosum	*ERF10*	Transcription regulation	Meng et al. (2020a)
Solanum tuberosum	*NAC2*	Transcription regulation	Meng et al. (2020b)
Solanum tuberosum	*GS*	Remove reactive oxygen radicals	Tian et al. (2020)
Solanum tuberosum	*HMA*	Transport metal ions	He et al. (2020)
Solanum tuberosum	*ABC*	Heavy metal detoxification	He et al. (2021)

plants, glutathione (GSH) can effectively remove the oxidative damage. Glutathione synthetase (GS) is the key enzyme of GSH biosynthesis (Hasanuzzaman et al. 2017). *StGS* is a cadmium-responsive gene, because it is differently expressed in various organs of potato plants in response to cadmium stress (Tian et al. 2020). Genes containing a heavy metal associated (HMA) domain are required for the spatiotemporal transportation of metal ions that bind with various enzymes and co-factors within the cell. 36 gene members in the *StHMA* family were identified and divided into six subfamilies by phylogenetic analysis (He et al. 2020). ATP-binding cassette (ABC) transporter proteins without transmembrane domains may play important roles in heavy metal detoxification. A new approach was established to evaluate ABC transporter family functions using microRNA targeted inhibition (He et al. 2021) (Table 16.7).

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Genomics-Assisted Breeding for Abiotic Stress in *Pisum* Crop

17

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Abstract

Present scenario of global warming has impact on the overall progress of mankind from agriculture sector to health sector. Abiotic stress due to climate change has declined crop yield and production worldwide. This decline in crop production has impact on agriculture sector which finally affected the human being by scarcity of food supply. According to the survey conducted, by the end of 2050 global population is expected to cross 9.7 billion. The increased population will create pressure on the present limited natural resources to produce more food. For feeding the increasing population there is need to produce almost 50 per cent more food, feed, and bio-fuel. Breeders across the world are indulged in developing smart crops by utilizing both conventional and modern breeding techniques. The most important breeding approach is identification of novel quantitative trait loci/candidate gene which can be used further for improvement of breeding program of a particular crop. Pisum is one of the most important vegetable crops consumed globally. Like in other crops, QTL mapping, gene mapping, and association mapping for various traits have been studied in *Pisum* crop. Here, in this chapter, we will discuss the importance of different mapping in

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S. Singh et al. (eds.), *Smart Plant Breeding for Vegetable Crops in Post-genomics Era*, https://doi.org/10.1007/978-981-19-5367-5_17

vegetable crops and provide a detail of identified QTLs/candidate genes for various traits in *Pisum*.

Keywords

Pea · QTL mapping · Association mapping · GWAS · Germplasm · Abiotic stress

17.1 Introduction

Pea (*Pisum sativum* L.) is an annual herbaceous leguminous plant having a one-year life cycle. Peas are a cool-season crop that are to be planted from starting winter to early summer, depending on the climate. When the soil temperature reaches to 10 °C, seeds can be planted and grow best at temperatures between 13 and 18 °C. Peas do not do well in the heat of summer and in lowland tropical regions, but grow well in cooler high altitude tropical climates (Oplinger et al. 1991). Being one of the members of the Leguminosae family its key ecological benefit is in helping to build low-input farming systems by fixing atmospheric nitrogen and also by reducing the requirement for external inputs. Legumes are considered as the third most diverse flowering plant family, having nearly 650 genera and 18,000 species (Lewis et al. 2005). In terms of economic importance, after Poaceae (grass family), legumes are the second most important family of crop plant which accounts for 27% of global crop production (Graham and Vance 2003). The second most extensively grown grain legume in the world is the dry pea, first being the common bean, with primary production in temperate regions and global production of 10.4 million tons in 2009 (FAO 2012). Pea (*Pisum sativum* L.) is one among the world's oldest domesticated crops (Ambrose 1995). Its area of origin and initial domestication lies in the Mediterranean, primarily in the Middle East. In the Middle East and Europe toward the end of the last Ice Age, peas together with vetches, vetchlings, and chickpeas were part of the daily food of hunter–gatherers. At high frequencies remains of these legumes occur in sites dating from the tenth and ninth millennia BC (Zohary and Hopf 2000). Grain legumes were important crops during the "agricultural revolution," which made it easier to create permanent settlements. Following that, over centuries of selection and breeding, many of pea varieties were generated and these are now preserved in various germplasm repositories all around the world (Smýkal et al. 2011). The genus *Pisum* contains the wild species *P. fulvum* found in Jordan, Syria, Lebanon and Israel; the cultivated species *P. abyssinicum* from Yemen and Ethiopia, which was likely domesticated independently of *P. sativum*; and a large and loose aggregate of both wild (*P. sativum* subsp. *elatius*) and cultivated forms that comprise the species *P. sativum* in a wider sense. Pea is mostly cultivated in temperate climates on well-drained and productive soils. China is the largest producer of vegetable peas (10.60 Mt, FAOSTAT) followed by India (4 Mt). Canada is the main producer of dry peas (3.85 Mt) followed by China (1.6 Mt), the Russian Federation (1.35 Mt), USA (0.71 Mt), India (0.60 Mt), France (0.50 Mt), and Ethiopia (0.40 Mt). To meet worldwide demand for pea consumption and to increase

Table 17.1 Compositional data for peas (*Pisum sativum* L.)

Constituent	Concentration (%)[a]
Protein	21·2–32·9
Starch	36·9–49·0
Resistant starch	2·1–6·3
Amylose	20·7–33·7
Total dietary fiber	14–26
Insoluble fiber	10–15
Soluble fiber	2–9

[a] Values are expressed on a moisture-free basis except for amylose, which is expressed on a starch basis

pea production output, pea breeding initiatives around the world have produced significant gains in yield, disease resistance, plant architecture, and lodging tolerance. Pea breeding must also focus on both crop output and seed quality (Duc et al. 2015). Peas have been associated to a variety of health benefits, apart from other basic dietary requirements, as shown in Table 17.1.

Over the past few years some important achievements were obtained in pea cultivars through conventional breeding and are listed below:

- Approximately 2% of yield gains per year have been achieved (Warkentin et al. 2015).
- Improvement of lodging resistance through deployment of the *afila* gene for semi-leafless type (Kujala 1953; Goldenberg 1965).
- Resistance for powdery mildew based on the single recessive gene *er-1* (Harland 1948) has been widely deployed.
- Through pyramiding of genes partial resistance to the ascochyta blight complex has been achieved with minor effects (Kraft et al. 1998).
- Through backcrossing resistance to pea weevil (*Bruchus pisorum* L.) that was identified in the secondary gene pool (*P. fulvum*) (Clement et al. 2002) was transferred into cultivated pea.
- In Europe and north-west USA to avoid late season drought and heat stress such cultivars have been developed and deployed that adapted to winter sowing giving the potential for better yields because of a longer growing season, higher biomass production, and earlier maturity (Hanocq et al. 2009).

Promising lines, that are resistant to diverse biotic and abiotic stresses, have been found and are being evaluated and introgressed in various research programs. In France and USA research has led to the identification and introgression of useful variation for resistance to *Aphanomyces* root rot; partial resistance controlled by several quantitative trait loci (QTLs) (Pilet-Nayel et al. 2002, 2005; McGee et al. 2012). Stress tolerance has been improved and identified in landrace accessions for toxicity to boron (Bagheri et al. 1994), salinity (Leonforte et al. 2013a), iron deficiency (Kabir et al. 2012), and for heat tolerance during flowering (Petkova et al. 2008).

17.2 Genomics-Assisted Breeding in Crops

For industry, crops are the prime source of food and raw materials. Crop yields and world food consumption still have a great significance. Among the reasons of significant losses in production, plant diseases, insects, and adverse environmental conditions are the major causes, along with a rapidly increasing global population this results in catastrophic food shortages all over the world. Genomics-assisted breeding (GAB) plays a pivotal role in cultivar development by efficient utilization of genomic resources and exploiting germplasm variability. GAB has increased breeding efficiency to a great level and has long been the standard in breeding operations (Xu and Crouch 2008). Among few of the GAB strategies that have been developed are marker-assisted backcrossing or QTLs, enrichment of favorable alleles in early generations, and selection for quantitative traits using markers at multiple loci, short breeding cycle, and high selection efficiency (Hospital et al. 1992; Eathington et al. 2007; Gupta et al. 2010). In GAB, breeders start out with a large population of only genotypically characterized offspring, and then only use a selected subset for more expensive phenotypic evaluation (Cooper et al. 2014). GAB is particularly beneficial for improving complex traits because of its high precision, direct improvement, quick breeding cycle, and high selection efficiency.

GAB has become possible only because more and more interesting genomic datasets become available, and this has become a tool for speeding up the breeding cycle for crop improvement. GAB has advanced breeding methods across a broad spectrum of agricultural species by establishing more than 130 publicly produced cultivars of various crops over the last 15 years (Vogel 2014). Among the remarkable crop products bacterial blight and blast in rice and rust in wheat are the improved cultivars with enhanced resistance levels against significant diseases supplied by GAB and are being used in a range of breeding projects. The most important abiotic stress attributes are tolerance to submergence, salt, and drought and all have been improved through GAB.

In improving crop breeding efficiency and yielding superior varieties GAB methods have made a significant contribution. Large-scale germplasm sequencing projects, fast-growing capacity, and affordability of DNA sequencing have motivated the opening of exciting avenues for mining haplotypes for breeding applications and also have the capability of enhancing genetic gains. Inspite of the fact that pulses have long been regarded as orphan crops, recent advances in pulse genomics, such as the discovery of genome-wide genetic markers, high-throughput genotyping and sequencing platforms, high-density genetic linkage/QTL maps, and, most importantly, the availability of whole-genome sequence, are noteworthy. With the genome sequence in hand, there is a lot of potential for applying genome-wide approaches for trait mapping and selecting favorable genotypes via genomic selection. Because pulses have such a high agricultural importance, extensive research has been done to improve them through conventional breeding, resulting in the development and distribution of multiple high-yielding cultivars. (Gaur et al. 2012; Pérez de la Vega et al. 2011; Saxena 2008; Singh 2005; Torres et al. 2011). With respect to productivity, however, appreciable gains have not been materialized so far

in any of the major pulse crops. The productivity of major pulse crops remains dismally low, around 1000 kg/ha, and large gap exists between their potential and actual yields (FAOSTAT 2011; Varshney et al. 2013). Integrating genomic techniques with traditional breeding approaches holds the key to speed up crop development advances. One possible reason is that the international research community has paid little attention to pulse crops. As a result, there has been a scarcity of genomic tools needed to start GAB on a broader scale (Varshney et al. 2009). The availability of plant genome information has generated many next-generation sequencing-based approaches for allele mining and candidate genes identification. At present, high-throughput trait-associated markers, cost-effective genotyping approaches, and precise phenotyping platforms will facilitate the rapid deployment of GAB. Newly developed genetic and genomics tools will enhance conventional breeding and evaluation processes. GAB's ultimate goal is to uncover the best allele (or haplotype) combinations, optimal gene networks, and specific genomic locations to aid crop improvement (Xu et al. 2012). It is believed that GAB has the potential to speed up the development of novel plant varieties and modern agriculture.

17.3 Germplasm Selection and Enhancement

Crop germplasm banks are essential for maintaining genetic variety that would otherwise be lost as a result of prior domestication and current breeding efforts aimed at generating homogeneous or uniform crops. "Germplasm enhancement" refers to the early stage of sustainable plant breeding, where a valuable character is identified, its genetic diversity is "captured," and those genes are converted into a "usable" form (Peloquin et al. 1989). Germplasm enhancement should be regarded as a long-term activity because exotic germplasm seldom has an immediate use without selection for local adaptation and enhanced yield potential. Public rather than private breeders have been more proactive in adopting germplasm enhancement because many generations are needed to produce substantial progress without any guarantee of success. Thus, germplasm enhancement programs are independent of the local crop genetic base until they become sources of parental material for the conventional breeding pool. Germplasm enhancement broadens the genetic base of breeding materials, either in specific genome regions (introgression) or more generally (incorporation); however, the procedures vary depending on the crop biology (Simmonds 1993). By placing genes into a usable form in conventional breeding programs, germplasm enhancement has become an important technique for genetic improvement of breeding populations through gene introgression or incorporation of wild and landrace genetic resources into respective crop breeding pools (Ortiz 1999).

The most widespread application of this approach has been in resistance breeding with genetic resources of wild species (Lenné and Wood 1991). The levels of resistance to pests and diseases available in the primary gene pool are sometimes low or only a limited number of resistance sources have been incorporated into elite materials by plant breeders. Germplasm enhancement with wild species has seldom

resulted in direct cultivar release, but many parents with "wild" genes have become available.

17.4 Why GWAS (Genome-Wide Association Mapping)?

Genome-wide mapping facilitates the target gene mapping and cloning in crops. The availability of high-density SNP markers has paved the path for genome-wide association study (GWAS), an approach using natural populations. GWAS has the potential to overcome various limitations of traditional linkage mapping and provide powerful complementary strategy for dissecting complex features. GWAS gives insights into the genetic architecture of complex phenotypes in maize by combining high-throughput phenotypic and genotypic data, which is especially important considering the rapid decay of linkage disequilibrium in maize (Yan et al. 2011). A total of 26 loci were identified to be associated with oil concentration in maize kernels through GWAS (Li et al. 2013), and this dataset can now be directly used to facilitate marker-based breeding for oil quantity and quality GWAS has become a powerful tool for QTL mapping in plants because a broad range of genetic resources may be accessed for marker trait association without any limitation on marker availability. The whole genome association study (WGA study, or WGAS) is a study that looks at many common genetic variants in different individuals to discover if any variant is linked to a trait. GWASs typically look for connections between single-nucleotide polymorphisms (SNPs) and features, such as serious diseases. The GWAS is a useful method for understanding the genetic basis of complex phenotypes by utilizing naturally occurring genetic variability (Korte and Farlow 2013). GWAS has been used to identify markers linked with desirable features in a wide range of crops, and gives higher mapping precision than traditional bi-parental populations for detecting relationships between molecular markers and traits of interest (Liu et al. 2016; Cui et al. 2017; Xu et al. 2017). GWAS requires an assessment of the population structure of the diversity panel to determine the genetic relatedness of individuals and minimize detection of false associations (Korte and Farlow 2013; Sul et al. 2016) and is dependent on the use of an adequately large number of markers. Recent advances in NGS platforms and SNP genotyping provide additional tools to characterize genetic diversity at a high resolution and allow breeders to select for useful diversity to develop new varieties. GWAS has been successfully used to uncover alleles associated with traits in a variety of crop species, including legumes (Mourad et al. 2018). GWAS has already been used to find SNP markers linked to various essential field pea breeding features. The natural diversity of pea accessions selected in the 23 pea breeding programs across the world was used to identify trait-linked SNP markers, which could potentially be used for MAS in pea breeding programs.

17.5 QTL Mapping for Abiotic Stress in *Pisum*

Abiotic stress is one of the severe stresses of the environment that threats to the growth and production of any plant worldwide. During the past decade, several studies have been published on molecular mapping of abiotic stress resistance. QTL mapping is statistical procedure used to distinguish the complex plant traits into their components (Ahmad et al. 2015a, b; Yao et al. 2016). It controls the heritable variations in crop plants (Collins et al. 2008). It is also helpful to learn the genetic architecture of plants to improve them for desirable traits during the course of their evaluation (Bo et al. 2015). This approach also dissects the physiological and genetic elements affecting source sink relationship under abiotic stress (Welcker et al. 2007). In the field of agriculture, evolutionary biology, and medicines, QTL mapping is being intensively used to find the precise location of the interested regions/genes. Functional genomics is an important tool to find the correlation between phenotype and genome of an organism subjected to diverse environmental conditions (Soda et al. 2015). To identify the abiotic stress-resistant QTLs, a lot of work has been done by plant biologists, but identified QTLs proved unstable across different environmental conditions due to their complex inheritance mechanism of abiotic stress tolerance. For QTL analysis, two basic things are required, (1) two or more strains of genetically different organism and (2) availability of molecular markers (SSRs, SNPs, RFLPs, etc.) to distinguish between these strains. The identification of genomic regions controlling frost tolerance has initially been completed for cultivated species through the assessment of mapping populations and QTL mapping. In pea, QTL mapping studies for frost damages have been conducted in multiple field environments as well as in controlled conditions.

QTLs for water-deficient conditions have been studied in almost all cereals, regarding the factors controlling drought stress. QTLs have been mapped for a wide range of agronomic traits in pea, including biotic and abiotic stresses. QTLs for incomplete and complete resistance have been detected for the most important diseases affecting pea (Rubiales et al. 2009) and for tolerance to abiotic stress, such as winter frost and frost damage (Lejeune-Henaut et al. 2008; Dumont et al. 2009). The genetics and heritability of drought tolerance in pea are analyzed and the main zones of the genome controlling water stress tolerance have been identified. QTLs responsible for the genetic control of yield-related traits, seed protein content, aerial and root architecture, and biotic and abiotic stress resistance have been detected under multiple environmental conditions and located on different maps. In addition to QTL mapping analyses in bi-parental populations, association analyses have emerged as a complementary approach to dissect quantitative traits in pea by exploiting natural genetic diversity and ancestral recombination events characterizing germplasm collections. Pea (*Pisum sativum* L.) is an important grain legume whose yield stability and production is constrained by drought stress in most environments.

Iglesias-García et al., 2015 studied the genetics of drought adaptation in pea and identify the genomic regions controlling the trait. Toward this objective, it was assessed that drought symptoms and relative water content in soil (RWCS) and

leaves (RWCL) along a time course of water stress on a pea Recombinant Inbred Line (RIL) population from two parents known to segregate for drought adaptation. Drought adaptation in this population was a quantitative trait. QTL analysis using composite interval mapping (CIM) and multiple interval mapping (MIM) allowed us to identify ten QTLs associated with the traits explaining individually from 9 to 33% of the phenotypic variation depending on the variable assessed and altogether from 20 to 57%. A set of reproducible markers linked to these QTLs (A6, AA175, AC74, AD57, AB141, AB64, *Psblox2*, PsAAP2_SNP4, and DipeptIV_SNP1) were identified. These markers can be used to select the individuals harboring the desired QTLs in pea breeding programs for drought adaptation. This study identified ten QTLs from pea which are related to different aspects of drought stress. The knowledge of the genetic system controlling tolerance to drought in accession P665 and cv. Messire and molecular markers linked to the genes controlling the trait would facilitate drought tolerance gene transfer into elite pea cultivars in MAS schemes.

17.6 Importance and Future Perspectives of QTL Mapping in Various Crops

Mapping has the potential not only to identify and map QTL but also to identify causal polymorphisms within a gene that are responsible for the difference between two phenotypes (Palaisa et al. 2003). Association Mapping has the potential to identify more and superior alleles and to provide detailed marker data in a large number of lines which could be of immediate application in breeding (Yu and Buckler 2006).

A. Genes underlying the QTLs linked to drought tolerance associated traits have been cloned in sorghum, rice, wheat, and maize (Pinto et al. 2010). Cloning of these genes ultimately help in understanding drought mechanism as well as function markers development based on genes of interest, so they can be directly used in breeding programs for drought tolerance improvement.
B. The epigenetic control of genes expressed under water-stressed conditions due to histone modification or DNA methylation has been reported in some plant species, including rice (Rivero et al. 2009). So accurate and precise identification of epi-QTLs associated with drought tolerance must be done in order to understand role of epigenetics on quantitative traits associated with drought stress in different plant species.
C. High-throughput genotyping can be done through GBS and SNP chips more frequently in recent years which facilitated molecular markers identification associated with or in close vicinity with targeted QTL which will lead to candidate gene identification underlying desired QTLs.

17.7 GWAS for Abiotic Stress in *Pisum*

Genetic improvement of pea for heat and drought resistance (abiotic stress) is a promising approach to stabilize yield under environmental stresses. Pea germplasm has a wide range of diversity in morpho-anatomical, biochemical, and physiological characteristics (Ouafi et al. 2016). Tafesse et al. 2020 conducted a GWAS analysis in which 32 MTAs and 48 candidate genes for attributes linked with pea heat responsiveness were discovered. These findings are expected to contribute to a better understanding of the genetic loci that govern these features. The identified candidate genes are involved in various biological functions and require further functional validation. The detected MTAs and candidate genes should be useful for marker-assisted selection for heat-tolerant pea varieties. In pea, association and linkage mapping has been employed to uncover the genetic bases of several traits, including agronomic and seed quality traits (Tafesse et al. 2019), disease resistance (Sudheesh et al. 2015), seed mineral concentrations (Diapari et al. 2015), seed lipid content (Ahmad et al. 2015a, b), salinity tolerance (Leonforte2013), and frost tolerance (Klein et al. 2014).

GWAS has been conducted in many plant species to dissect complex quantitative traits, including winter survival and frost tolerance. High-throughput genotyping resources now available in pea (Tayeh et al. 2015a, b) have also allowed to carry out GWAS in order to dissect the genetic determinism of resistance to *Aphanomyces euteiches*, plant architecture, and frost tolerance. Desgroux et al. (2018) studied associations for resistance *to A. euteiches* in 175 pea lines using a high-density SNP genotyping consisting of 13.2 K SNPs from the developed GenoPea Infinium BeadChip (Tayeh et al. 2015a, b). Several markers significantly associated with resistance to *A. euteiches* harboring relevant putative candidate genes were identified. Significantly associated markers also allowed to refine the confidence interval of QTLs previously detected in bi-parental populations. Using the same SNP resource and a collection of 266 pea accessions, including the 175 former lines, Desgroux et al. (2016) also identified genomic intervals significantly associated with plant architecture and resistance to *A. euteiches*, of which 8 were overlapping for both traits. In a different genetic background composed by a set of 672 pea accessions genotyped with 267 simple sequence repeat (SSR) markers, Liu et al. (2017) detected 7 SSRs significantly associated with frost tolerance of which one was located on LG VI and was shown to colocalize with a gene involved in the metabolism of glycoproteins in response to chilling stress.

Beji et al. carried out study on GWAS which enabled to confirm QTLs significantly associated with frost tolerance in pea, such as WFD 3.2, WFD 5.1, and WFD 6.1. It also allowed to identify one region on LG II, which has not been detected yet and provided significant associations for two regions on LGI and LG VII that were formerly detected in only one environment. The results showed that GWAS is an effective strategy to identify markers precisely defining frost tolerance loci, which can be useful to breed for antagonistic traits as it is for the frost tolerance and Tri loci on LG V which are in linkage disequilibrium and in a repulsion phase. The results also highlight that GWAS enables to find new sources of frost tolerance within

collections of pea genetic resources. Finally, the present GWA study also brought to light the presence of CBF transcriptions factors as potential genetic determinants of the frost tolerance locus on LG VI, with one C-repeat/dehydration-responsive element binding factors (CBF)-annotated marker being in high linkage disequlibirum (LD) with significant flowering duration (FD)-associated markers of the locus and six additional CBF/dehydration-responsive element-binding (DREB)-annotated genes mapped at the vicinity. As 12 tandemly duplicated CBF genes were already found to be relevant candidates underlying the orthologous frost tolerance QTL on *Medicago truncatula* chromosome 6, the hypothesis of a similar genomic organization in pea deserves to be tested.

Genetic improvement of pea for stress resistance is a potential strategy for developing cultivars that will grow and yield well under stress. Pea germplasm possesses a wide spectrum of morpho-anatomical, biochemical, and physiological properties that may be linked to stress resistance traits (Bueckert et al. 2015; Tafesse et al. 2019; Jiang et al. 2015). The GWAS has become a useful method for examining genomic areas that control multiple phenotypes using naturally occurring genetic variability acquired over numerous generations. (Korte and Farlow 2013; Sun et al. 2016; Gali et al. 2019). Linkage disequilibrium, the association of alleles at different loci, is the foundation of association mapping that provides a greater mapping resolution of QTLs (Abdurakhmonov and Abdukarimov 2008). The emergence of high-throughput next-generation sequencing (NGS) technologies at an affordable price has made use of genome-wide SNPs ideal for genetic diversity studies and linkage disequilibrium estimation in numerous crops, including pea (Elshire et al. 2011). GWAS on pea has already discovered numerous markers and candidate genes linked to agronomic and seed quality features, disease resistance, root architecture, critical minerals, and other agronomic variables of relevance. GWAS was also conducted to identify SNP markers associated with six heat and drought adaptive traits (lamina wax, petiole wax, stem thickness, flowering duration, NDVI, and NPCI) using 135 genetically diverse pea accessions.

Tafesse et al. (2019) indicated that a significant marker detected for a given trait would be more trustworthy if the marker has great reproducibility and would be found in multiple trials. Therefore, for the six traits we examined, the SNP markers declared significant were consistent in at least two of the three environments for lamina and petiole wax, and three of the five environments for the remaining four traits. The detected markers could be used for marker-assisted selection of these traits in the breeding effort of developing stress-resistant pea cultivars. A total of four SNPs (Chr1LG6_277526227, Chr4LG4_209093982, Chr6LG2_384797968, and Chr7LG7_128419954) for lamina wax and three SNPs (Chr4LG4_16602920, Chr7LG7_346970562, and Uscaffold03717_87257) for petiole wax were detected on different chromosomes and the non-chromosomal scaffold. This is the first report evaluating waxes in pea by GWAS. Abiotic stresses triggered increased wax concentrations as a stress resistance response (Shaheenuzzamn et al. 2021). For example, as a drought avoidance mechanism in pea, epicuticular wax reduced residual transpiration and thus minimized water loss so that tissue water status was maintained in the present GWAS; we observed significant phenotypic variation in six stress-adaptive traits (lamina wax, petiole wax, stem thickness, flowering

duration, normalized difference vegetation index (NDVI), and normalized pigment and chlorophyll index (NPCI)) among 135 pea accessions in multi-environment tests. Further, 15 SNPs were identified significantly ($p \leq 0.0005$) associated with the six stress-adaptive traits. These results are believed to advance the knowledge of the genetic bases governing these traits. The detected SNPs should be useful for marker-assisted selection for breeders in developing stress-resistant pea cultivars. Gawłoska et al. (2020) in a study of a pea bi-parental mapping population reported several QTLs in pea associated with stem mechanical properties, including stem diameter and wall thickness, traits strongly relating to lodging resistance. Both genotype and environment had significant effects on stem thickness. Stem thickness ranged from 2.42 mm to 4.81 mm in the 135 accessions and 5 environments. Heat and drought stress reduced the stem thickness by 15%. In addition to lodging, stem thickness was also reported to be associated with disease resistance and seed yield (Porter et al. 2009; Smitchger et al. 2020) (Table 17.2).

17.8 Genomic Selection

Genomic selection (GS) is a breeding tool which is used to capture diversity of the genome on the basis of information from a large number of markers distributed across that genome and is further used to design novel breeding programs. In GS, prediction models are developed on the basis of genotypic and phenotypic data of training population and further genomic breeding values (genomic estimated breeding values, GEBVs) are predicted for all the individuals of breeding population (Meuwissen et al. 2001). Many factors influence the efficiency of GS for a crop which include number of QTLs controlling the target trait(s), Genotype X Environment interaction, phenotyping accuracy, allelic diversity and linkage disequilibrium, breeding methodology, inbreeding, size of population, and the type and number of markers used. Genomic selection is being applied in pea also but only very few instances are available in Table 17.3. But, the recent publication of reference genome of pea is expected to accelerate the marker-assisted selection vis-à-vis various crop improvement programs.

17.9 Conclusion

There is great potential for discovery and use of existing genetic variation preserved in germplasm, which can be efficiently identified and introduced into current pea cultivars. The integration of molecular methods and applied plant breeding toward the common objective of increased yield and high-quality pea seed production for many end-uses is rapidly approaching. The availability of plant genome information has generated many next-generation sequencing-based approaches for candidate genes identification. At present, high-throughput trait-associated markers, cost-effective genotyping approaches, and precise phenotyping platforms will facilitate the rapid deployment of GAB. Newly developed genetic and genomics tools will enhance conventional breeding and evaluation processes. In the coming years, it is

Table 17.2 Enlisted various QTLs/SNPs identified in pea (*Pisum sativum*)

S. No.	Bi-parental cross/ diverse germplasm	Approach	QTL/ SNP	Linked trait	Reference
1	China (JI1491) × Caméor	QTL mapping	161	Internode length, branching type, hilum color, thousand seed weight, harvest index, and seed protein content.	Klein et al. (2014)
	Champagne × Terese	QTL mapping	25 QTL	Concentration of sugars, electrolyte leakage, osmotic pressure, and activity of RuBisCO	Dumont et al. (2009)
2	Diverse Germplasm	GWAS	62 SNPs	Frost damage (FD) loci	Beji et al. (2020)
3	Kaspa × Parafield	QTL mapping	705 SNPs	Salt Tolerance	Leonforte et al. (2013a, b)
4	Wt11238 × Wt3557	QTL mapping	37 QTLs	Seed weight, Seed yield and seed protein content	Krajewski et al. (2012)
5	Baccara × PI180693 (178F8-derived RILs) Baccara × 552 (178F9-derived RILs)	QTL mapping	135 QTLs	Aphanomyces root rot resistance.	Hamon et al. (2011)
6	Champagne (frost tolerant) × Térèse (frost sensitive) and China (frost tolerant) × Caméor (frost sensitive)	QTLs	4QTLs	Cold Stress	Beji et al. (2020)
7	CDC Centennial × CDC Sage	QTLs	10 QTLs	five for flowering traits and five for yield component traits	Huang et al. (2017)
8	Diverse Germplasm	QTLs	06 QTLs	Frost Stress	Lejeune-Henaut et al. (2008)
9	Diverse Germplasm	GWAS	15 MTAs	Heat and drought	Tafesse et al. (2021)
10	Bi-parental populations	GWAS	62 SNPs	Frost Tolerance	Beji et al. (2020)
11	Diverse Germplasm	GWAS	32 MTAs	Heat Stress	Tafesse et al. (2021)
12	Diverse Germplasm	GWAS	16 SNPs	Frost Tolerance	Liu et al. (2017)

Table 17.3 Genomic selection in Pea

S. No.	Target traits	Reference
1	Grain yield and inter environment prediction	Annicchiarico et al. (2018)
2	Ascochyta blight resistance	Carpenter et al. (2018)
3	Seed yield and yield-related components	Bari et al. (2021)

believed that the extensive implementation of MAS and GS either alone or in combination will help improve plant breeding at genomic level. The whole-genome and transcriptome sequences from a large group of accessions should enhance significant advances in pea breeding in the next few years and foster the use of more diverse genetic resources for pea improvement. The genome sequence released will further advance the pea genomic breeding revolution.

Furthermore, the availability of improved molecular tools and the adoption of standard phenotypic evaluation methods will allow for considerable and rapid implementation of genomic-based pea breeding. This may now be conceivable, with the development of large-scale automated and digitalized formats that make it possible to correlate phenotype and genotype on a whole genome basis, a procedure that Mendel pioneered 150 years ago.

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