Chittaranjan Kole *Editor*

Genomic Designing for Abiotic Stress Resistant Fruit Crops

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Dedicated to

Dr. K. L. Chadha

Padma Shri Awardee, former Deputy Director General (Horticulture), Indian Council of Agricultural Research and Founder President of the Indian Academy of Horticultural Sciences

With regards and gratitude for his generous appreciations of my scientific contributions and service to the global academic community, and his constant support and encouragement during my professional journey!

Preface

Crop production is drastically affected due to external or environmental stresses. The biotic stresses cause significant yield losses in the range of 31–42% together with 6–20% loss during the post-harvest stage. The abiotic stresses also aggravate the situation with crop damage in the range of 6–20%. Understanding the mechanisms of interaction of plants with the biotic stresses caused by insects, bacteria, fungi, viruses, and oomycetes, etc., and abiotic stresses due to heat, cold, drought, flooding, submergence, salinity, acidity, etc., is critical to develop resilient crop varieties. Global warming and climate change are also causing emergence of new diseases and insects together with newer biotypes, and physiological races of the causal agents in one hand and aggravating the abiotic stress problems with additional extremes and unpredictability. Development of crop varieties resistant and/or adaptive to these stresses is highly important. The future mission of crop improvement should, therefore, lay emphasis on the development of crop varieties with optimum genome plasticity by possessing resistance or tolerance to multiple biotic and abiotic stresses simultaneously. A moderate estimation of world population by 2050 is about 9.3 billion that would necessitate an increase of crop production by about 70%. On the other hand, the additional losses due to climate change and global warming somewhere in the range of 10 to 15% should be minimized. Therefore, increase in the crop yield as well as minimization of its loss should be practiced simultaneously focusing both on 'adaptation' and 'mitigation'.

Traditional plant breeding practiced in the last century contributed a lot to the science of crop genetic improvement. Classical plant breeding methods including selection, hybridization, polyploidy, and mutation effectively catered to the basic F^5 needs—food, feed, fiber, fuel and furniture. The advent of molecular breeding and genetic engineering in the latter part of that century complimented classical breeding that addressed the increasing needs of the world. The twenty-first century came with a gift to the geneticists and plant breeders with the strategy of genome sequencing in Arabidopsis and rice followed by the tools of genomics-aided breeding. More recently, another revolutionary technique, genome or gene editing, became available for genetic correction of crop genomes! The travel from 'plant breeding' based on visual or perceivable selection to 'molecular breeding' assisted by linked markers to

'transgenic breeding' using genetic transformation with alien genes to 'genomics-aided breeding' facilitated by known gene sequences has now arrived at the age of 'genetic rectification' employing genome or gene editing.

Knowledge on the advanced genetic and genomic crop improvement strategies including molecular breeding, transgenics, genomic-assisted breeding and the recently emerged genome editing for developing resistant, tolerant and/or adaptive crop varieties is useful to students, faculties and scientists in the public and private universities and organizations. Whole genome sequencing of most of the major crop plants followed by genotyping-by-sequencing has facilitated identification of exactly the genes conferring resistance, tolerance or adaptability leading to gene discovery, allele mining and shuttle breeding which is turn opened up the scope for 'designing' or 'tailoring' crop genomes with resistance/tolerance to biotic and abiotic stresses.

To my mind, the mission of agriculture in this century is FHNEE security meaning food, health, nutrition, energy and environment security. Hence, genome designing of crops should focus on breeding of varieties with higher yields and improved qualities of the five basic F^5 utilities; nutritional and neutraceutical compounds; and other industrially and aesthetically important products, and possibility of multiple utilities. For this purpose of 'precise' breeding employment of the genetic and genomic techniques individually or in combination as and when required, will play a crucial role.

The chapters of the 12 volumes of this twin book series entitled, "Genomic Designing for Biotic Stress Resistant Crops" and "Genomic Designing for Abiotic Stress Resistant Crops", will deliberate on different types of biotic and abiotic stresses and their effects on and interaction with crop plants; will enumerate the available genetic diversity with regard to biotic or abiotic stress resistance among cultivars; illuminate on the potential gene pools for utilization in interspecific gene transfer; will brief on the classical genetics of stress resistance and traditional breeding for transferring them to their cultivated counterparts; will discuss on molecular mapping of genes and QTLs underlying stress resistance and their marker-assisted introgression into elite crop varieties; will enunciate different emerging genomics-aided techniques including genomic selection, allele mining, gene discovery and gene pyramiding for developing smart crop varieties with genetic potential to produce F^5 of higher quantity and quality; and also will elaborate the case studies on genome editing focusing on specific genes. Most of these chapters will discuss on the success stories of genetic engineering in the relevant crops specifically for generating crops with resistance and/or adaptability to diseases, insects and abiotic stresses.

There are obviously a number of reviews and books on the individual aspects of plant molecular breeding, genetic engineering and genomics-aided breeding on crops or on agro-economic traits which includes the 100-plus books edited by me. However, there is no comprehensive reviews or books available that has coverage on crop commodity groups including cereals and millets, oilseeds, pulses, fruits and nuts, vegetables and technical or industrial crops, and modern strategies in single volumes with precise focuses on biotic and abiotic stresses. The present volumes will fill this gap with deliberations on about 120 important crops or their groups.

This volume on "Genomic Designing for Abiotic Stress Resistant Fruit Crops" includes seven chapters focused on apple, banana, citrus, grapevine, almond, cherries, and berries contributed by 36 scientists from 9 countries including India, Iran, Italy, Lithuania, Morocco, Spain, Tunisia, UK, and USA. I remain immensely thankful for their highly useful contributions.

I am indebted to my wife Phullara who as always has assisted me directly in editing these books and indirectly through maintaining an academic ambience to pursue my efforts for science and society pleasantly and peacefully.

New Delhi, India Chittaranjan Kole

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Abbreviations

2-DE	Two-dimensional electrophoresis
6mA	N6-methadenine
ABA	Abscisic acid
ABF	ABRE binding factor
ABRE	ABA-responsive element
ADH	Alcohol dehydrogenase
AF	Annual-fruiting
AFLP	Amplified fragment length polymorphism
AFP	Antifreeze protein (from Antarctic fish)
AGPase	ADP-glucose pyrophosphorylase
ALA	5-aminolevulinic acid
AMFs	Arbuscular mycorrhizal fungi
AP	*Apetala* gene
AP2/EREBP	AP2/ethylene-responsive
APEDA	Agricultural and Processed Food Products Export Development Authority
APX	Ascorbate peroxidase
AQP	Aquaporin
ARF	*A*uxin response factor
ASR	Abscisic acid-stress and ripening-inducer
ATAF	Arabidopsis transcription activation factor
ATG8f	*A*utophagy-related protein 8f
BAC	Bacterial artificial chromosome
BSA	Bulk segregant analyisis
bZIP	Basic leucine zipper
CAGR	Compound annual growth rate
CAPS	Cleaved amplified polymorphic sequences
Cas9	CRISPR-associated protein 9
CAT	Catalase
CBD	Convention on Biological Diversity
CBF	C-repeat binding factor

CCS	Copper chaperone for superoxide dismutase
cDNA	Complementary DNA
CDPK	Calcium-dependent protein kinase
CEBAS	Centro Edafología y Biología Aplicada del Segura
CG	Candidate gene
CIB	Cold-induced gene
CIRAD	Centre de cooperation International en Recherché Agronomique pour le Développement
CMT	Chromomethylase
COR	Cold responsive
CQD	Carbon quantum dot
CR	Chilling requirement
CRISPR	Clustered regularly interspaced short palindromic repeats
CRT	C-repeat
CS	Cabernet sauvignon
CSIC	Consejo Superior de Investigaciones Científicas
CUC	Cup-shaped cotyledon
DAM	Dormancy associated MADS-box
DEG	Differentially expressed gene
DHAR	Dehydroascorbate reductase
DHN	Dehydrin
DI	Deficit irrigation
DM	Downey mildew
DNMT	DNA methyltransferase
DRE	Drought-responsive element
DRE(B)	Drought-responsive element binding
DREB	Dehydration responsive element binding protein
DRM	Domain-rearranged methyltransferase
DSB	Double-strand break
EBI	European Bioinformatics Institute
ECe	Electrical conductivity of equivalent
ECs	Electrical conductivity for soil
ECw	Electrical conductivity for water
EMS	Ethylmethyl sulphonate
EPA	Environmental Protection Agency
ERD	Early response to dehydration
ERF	Ethylene response factor
ESP	Exchangeable sodium percentage
EST	Expressed sequence tag
ET	Evapotranspiration
F1	First filial generation
F3H	Flavanone 3-hydroxylase
FAO	Food and Agriculture Organization
FAOSTAT	FAO Corporate Statistical Database
FDA	Food and Drug Administration

FLS	Flavonol synthase
FPP	Farnesyl diphosphate
GA	Gibberellic acid
GBS	Genotyping by sequencing
GES	Geraniol synthase
GM	Genetically modified
GMO	Genetically modified organism
GolS	Galactinol synthase1
GP	Guaiacol peroxidase
GPCR	G protein-coupled receptors
GS	Genomic selection
GTPases	Guanosine triphosphatase
GWA	Genome-wide association
GWAS	Genome-wide association study/ studies
HK	Histidine kinase
HR	Heat requirement
HR	Homologous recombination
HS	Heat stress
Hsf	Heat shock factor
HSF	Heat shock transcription factor
HsfA2	Heat stress factor A2
HSP	Heat shock protein
HT	High tolerance
ICE1	Inducer of CBF expression
ICE1	Little elongation complex subunit 1
IGG	Intergovernmental Group
InDel	Insertion/deletion
INIBAP	International Network for the Improvement of Banana and Plantain
IPCC	Intergovernmental Panel on Climate Change
IPGRI	International Plant Genetic Resources Institute
ISSR	Inter-simple-sequence repeat
ITC	Indian Tobacco Company
JA	Jasmonic acid
K+/Na+	Potassium/sodium ratio
KASP	Kompetitive allele amplification
Lb	Late blooming
LD	Linkage disequilibrium
LEA	Late embryogenesis abundant protein
LG	Linkage group
LOD	Logarithm of odds
LT	Low temperature
LT	Low tolerance
LTR(E/B)	Low-temperature-responsive (element/binding)
Ma	*Musa acuminata*
MABCB	Marker-assisted backcross breeding

MANT	Methyl anthranilate
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MAS	Marker-assisted selection
MASS	Marker-assisted seedling selection
Mb	*Musa balbisiana*
MDA	Malondialdehyde
miRNA	MicroRNA
MSAP	Methylation sensitive amplified polymorphism
MT	Medium tolerance
MTP	Metal tolerance protein
MYB	Myeloblastosis
MYC	Myelocytomatosis
NAC042	NAC domain-containing protein 42
NAC68	NAC domain-containing protein 68
NAM	No apical meristem
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NHB	National Horticultural Board
NHEJ	Non-homologous end-joining
NIP	Nodulin-like plasma membrane intrinsic protein
NIR	Near-infrared spectrometry
NMU	N-nitroso-methyl urethane
NOS	Nitric oxide synthase
NP	Nanoparticle
NPBT	New plant breeding techniques
NPC	Nuclear pore complex
NR	Nitrate reductase
NRCB	National Research Center for Banana
NSP	Nanoscale particle
O·−2	Superoxide radical
OE	Overexpression
OIV	International Organization of Vine and Wine
PA	Proanthocyanidin
PAC	P1-derived artificial chromosome
PCD	Programmed cell death
PDS	Phytoene desaturase
PGIP	Polygalacturonase-inhibiting protein
PIP	Plasma membrane intrinsic protein
PIP1; 1	Aquaporin PIP1-1
PIP1; 2	Aquaporin PIP1-2
PIR	Protein information resource
PM	Powdery mildew
PN	Photosynthetic rate

POD	Peroxidase
PP2C	C-type protein phosphatase
PRD	partial root drying
PRP	Plant proline-rich protein
PS I/II	Photosystem I and II
PSY	Phytoene synthase
PTM	Post-tranlational modification
Put	Putrescine
QD	Quarentadias (Name of a banana variety in Portuguese language)
QD	Quantum dot
qPCR	Quantitative PCR
QTL	Quantitative trait locus
QTLs	Quantitative trait loci
RAD	Restriction site associated DNA
RAPD	Random amplified polymorphic DNA
RdDM	RNA-directed DNA methylation
RDI	Regulated deficit irrigation
REMAP	Retrotransposon-microsatellite amplified polymorphism
RFLP	Restriction fragment length polymorphism
RIP	Ribosome inactivating protein
RLK	Receptor-like kinass
RNAi	RNA interference
RNA-Seq	RNA Sequencing
RNP	Ribonucleoproteins
ROP	Repressor of primer protein
ROP	Repressor of primer protein
ROS	Reactive oxygen species
RT-PCR	Real time–PCR
SAP	Switch-activating protein
SAR	Sodium absorption ratio
SCAR	Sequence-characterized amplified region
sgRNA	Single guide RNA
sHSF	Small heat stress transcription factor
sHSP	Small heat shock protein
SIB	Swiss Institute of Bioinformatics
SiNP	Silicon nanoparticle
SIP	Small intrinsic protein
siRNA	Small interfering RNA
SLAF-seq	Specific length amplified fragment sequencing
SNP	Single nucleotide polymorphism
SnRK2	SNF1-related protein kinases2
SOD	Superoxide dismutase
SPL	Squamosa promoter binding protein-like
SRAP	Sequence-related amplification polymorphism
SSN	Site-specific nuclease

SSR	Simple sequence repeat
SWEETs	Sugars will eventually be exported transporters
TA	Tartaric acid
TAC	Transformation-competent artificial chromosome
TALE	Transcription activator-like effector
TALEN	Transcription activator-like effector nuclease
TE	Transposable element
TF	Transcription factor
TGE	Targeted genome editing
TIP	Tonoplast intrinsic protein
TK	Traditional knowledge
TSS	Total soluble sugars
UNDESA	United Nations Department of Economic and Social Affairs
UniProtKB	UniProt Knowledgebase
UPOV	International Union for the Protection of New Varieties of Plants
USDA	United States Department of Agriculture
VLT	Very low tolerance
VNTRS	Variable number of tandem repeats
Vv	*Vitis vinifera*
WBF	World Banana Forum
WGS	Whole-genome shotgun
WL	Waterlogging
WT	Wild type
YAC	Yeast artificial chromosomes
ZFN	Zinc-finger nuclease
ZIP	Zipper protein

Chapter 1
Genomic Approaches to Improve Abiotic Stress Tolerance in Apple (*Malus* × *domestica*)

Madhushree Dutta, Rajesh Kumar Singh, and Gaurav Zinta

Abstract Apple ranks third in global fruit consumption owing to its high nutritional properties, specifically antioxidant and mineral constituents. Apples are widely grown in temperate regions of the world. Apple is a perennial woody fruit tree with high commercial value. In recent years, the cultivation and production of apple is declined due to abiotic stresses associated with climate change. Heat, cold, salinity and drought are the major stresses which affect apple productivity. Apple genetic resources available can be exploited to breed varieties resistant against diverse abiotic stresses, which can help expand the cultivation area of apples. Also, the mechanisms underlying abiotic stress tolerance need to be clarified. Although, molecular markers and modern plant breeding techniques have helped in the identification and characterization of genes involved in stress resistance. However, genetic manipulation and molecular breeding approaches can pave ways for the development of stress resistant apple cultivars.

Keywords Abiotic stress · Breeding · Genome editing · Molecular markers · QTLs

1.1 Introduction

Fruits are an essential source of vitamins, minerals, antioxidants, and fibers, which form an integral part of a healthy human diet (Amao 2018). Fruit is a fleshy and mature part of the plant ovary that can be sweet (apples, oranges and strawberries) or non-sweet in its raw state (Mintah et al. 2012). Apple is one of the most favored fruits with economic and cultural significance that is widely grown over the temperate regions of globe (Spengler 2019). Over the last decades, many efforts have been made to improve apple yield and productivity, which has led to cheaper and year-round availability of apple (Sharma et al. 2014). The global annual production of apples

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has doubled from 41 million tons in 1990 to 86 million tons in 2018 (FAO 2020). Apple is the third largest produced fruit after bananas and watermelon (FAO 2020). Interestingly, whole fruit is edible (except the seeds), which is a key source of market consumables like jam, jelly, tea and wine (Spengler 2019). Apart from nutritional value, they contain excellent immune boosters including active phytochemicals that are beneficial for humans (Boyer et al. 2004). Such active bio-ingredients are mostly found in the pulp and peel of apples. The list of such bioactive substances comprises polyphenols, polysaccharides, plant sterols, pentacyclic triterpenes, and organic acids with a significant difference in concentration in pulp and peel (Patocka et al. 2020). In the current scenario, there is still a great prospect for developing and utilizing bioactive substances in apples. The consumption of apple and its extracts rich in phytochemicals has been linked to reduced risk of cancer, cardiovascular disease, diabetes, and many other chronic diseases, including asthma. Mainly polyphenols govern such health benefits through antioxidant and anti-inflammatory activities and by modifying biomarkers in different cell signalling pathways (Patocka et al. 2020). The adverse climatic factors negatively affect apple productivity and quality. For instance, late spring frosts cause more severe crop loss than those of low winter temperatures. The flower bud and young fruit stages exposed to late spring frosts are vulnerable to low temperature (Aygün and Şan 2005; Tomasz et al. 2008). Environmental conditions also promote the development of apple scab and powdery mildew, which are some of the well-known fungal diseases affecting commercially important cultivars (Sansavini et al. 2004). Such infections cause significant fruit yield and quality losses (Blagov 2011). Additionally, climate plays an essential role in determining the overall crop production at a global scale by influencing the yield and quality traits. The global and regional scale climate change factors jointly pose a severe threat to sustainable apple crop production (Tharaga et al. 2021). The predictions for an increase in temperature are made from 1 to 6 °C by the year 2100, while CO_2 concentration might increase to 850 ppm (Collins et al. 2013; IPCC 2009). Such conditions will inevitably affect apple production along with associated changes such as more frequent drought episodes and decline in the net chilling hours. Presently, the traditional apple farming is under stress owing to these climatic aberrations (Basannagari and Kala 2013). It has been observed that insufficient winter chilling units have caused a dramatic decline in apple production (Singh et al. 2016). The indicators of climate change at mid-hills and low hills are apple scab and pest attack, respectively (Singh et al. 2016). The traditional apple breeding practices takes years for commercial variety release. The development of new cultivars by conventional breeding is also constrained by a narrow gene pool, self-incompatibility, inbreeding depression, longer juvenile period and larger plant size (Brown and Maloney 2003). Together, such limitations of breeding techniques combined with biotic and abiotic stresses have limited apple production and the situation has become alarming for farmers and plant scientists. Thus, improving stress resilience and nutritional value of apple is of high importance in the face of global climate change.

1.2 Abiotic Stresses

Plants are exposed to different abiotic and biotic stresses in their natural habitat, and to endure stressful conditions various signaling pathways are initiated. Here we discuss the impact of abiotic stresses and underlying molecular regulators in apple (Fig. 1.1).

1.2.1 Heat Stress

Heat stress is one of the most ominous abiotic factors which limit productivity and quality, thereby incurring huge economic losses. It has been reported earlier how high temperature affects the morphological, anatomical, physiological and biochemical changes in the plant system. The success in overcoming heat stress is limited owing to poor knowledge of heat stress effects during critical stages of fruit development in crops. There is an urgent need to improve heat stress tolerance by using breeding and biotechnological approaches (Sharma et al. 2020). Generally, heat stress alters the several biological processes in the plant system. Photosynthetic apparatus is vulnerable to damage at high temperatures leading to a reduced photosynthetic rate (Wang et al. 2018). However, plants on encountering such adverse conditions undergo a series of damaging events like protein misfolding, denaturation, and production of radicals like reactive oxygen species (ROS; Li et al. 2018). Such misfolded or truncated proteins are highly toxic for the cellular processes. On encountering such misfolded proteins, cells trigger cytoplasmic protein response or unfolded protein

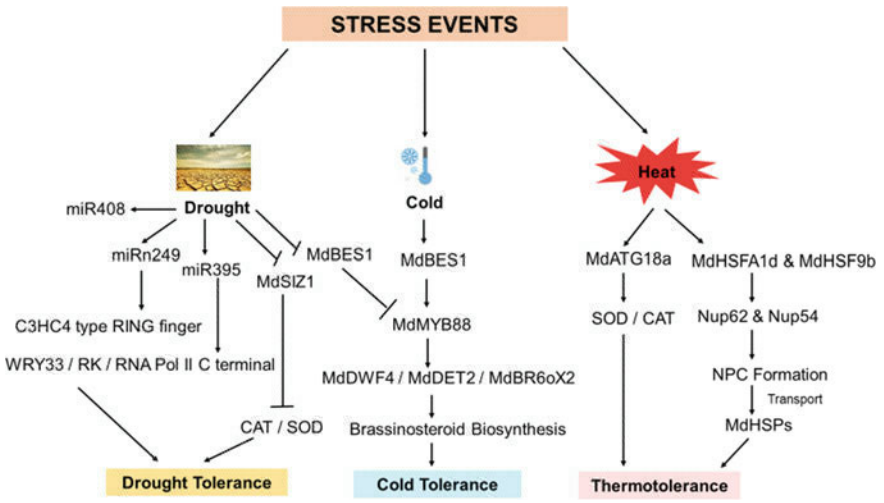

Fig. 1.1 Molecular regulators involved in different abiotic stress responses in apple

response in the endoplasmic reticulum, which stimulates the occurrence of autophagy (Deng et al. 2011). Autophagy is an evolutionarily conserved pathway that degrades unwanted cytoplasmic constituents and facilitates the circulation of cellular proteins. Its role in nutrient cycling in plants is well documented, which underpins plant tolerance to various biotic and abiotic stresses (Wittenberg et al. 2018). Studies show that autophagy plays a crucial role in basal thermotolerance in apples. It was found that *MdATG18a* improved thermotolerance by enhancing autophagic activity, protecting chloroplasts, maintaining higher levels of photosynthesis, scavenging toxic ROS, and inducing heat shock protein (HSP) expression in apple (Huo et al. 2020). In addition to this, global warming has also affected apple production by causing them to flower earlier, making them susceptible to freezing injury (Zhang et al. 2021). Nuclear pore complexes (NPCs) are central channels controlling nucleocytoplasmic transport by regulating plant development and stress responses. Recently, the components of NPC were studied, and it was found that they are a well-characterized nucleoporins group, *MdNup62*, interacts with *MdNup54*, forming the central NPC channel (Zhang et al. 2021). This interaction of nucleoporins is further associated with *MdHSFs* to regulate flowering and heat resistance in apples (Zhang et al. 2021). Additional analyses in heat stress demonstrated that plant proline-rich proteins (PRPs) are characterized cell wall proteins activated during stress events. Nine PRP genes were studied amongst which, *MdPRP6* positively regulated heat stress tolerance in transgenic plants (Zhang et al. 2021). The *MdPRP6* overexpressing plants showed comparatively lesser oxidative damage and higher photosynthetic capacity suggesting the role of PRP proteins in the apple in abiotic stress. Such findings will aid the future characterization and mechanism of PRP proteins in apples on accounting adverse environments.

1.2.2 Drought Stress

Drought is another major limiting factor for crop productivity (Cabello and Chan 2012). Since climates are warming and water is limited, drought has become a global concern threatening future crop production (Zhao and Running 2010). Naturally, plants have developed several molecular and physiological mechanisms to cope with stress. Drought triggers key regulatory genes that in turn regulate physiological processes such as stomatal closure (Taiz and Zeiger 2002) and the detoxification of reactive oxygen species (ROS) (Sun et al. 2018; Chen et al. 2019). Likewise, heat and drought conditions also generate ROS leading to membrane damage and oxidative stress (Tsugane et al. 1999). The role of autophagy in combatting such stress conditions is also reported. It has been found that overexpression of *MdATG18a* in apple plants enhances their tolerance to drought stress, probably because of greater autophagosome production. Those processes are known to help degrade aggregated protein and limit oxidation damage (Sun et al. 2018). Another important agricultural technique to improve drought resistance is to inoculate plants with arbuscular mycorrhizal fungi (AMFs; Chitarra et al. 2016). AMFs are well-known symbionts with most terrestrial plants and play a crucial role in adaptation to various stresses

(Huang et al. 2020). The role of AMFs on the drought resistance of plants is very complex, which involves many metabolic pathways (Wu and Xia 2006). It facilitates drought adaptation by uptake of plant nutrients and water uptake and transport, increased plant osmotic regulation, induced hormone signalling responses, improved gas exchange capacity and water use efficiency, and enhanced antioxidant capacity (Yang et al. 2014).

Furthermore, MAPK signalling genes were upregulated during drought, suggesting their important role in facilitating the interaction between AMF and apple trees, leading to drought tolerance in apples (Huang et al. 2020). Plant transcription factors (TFs) also play a key role in regulating stress responses (Century et al. 2008). Amongst such, the homeodomain–leucine zipper (HD-Zip) family inevitably regulates drought responses (Yang et al. 2018). The apple HD-Zip gene *MdHB-7* led to significant endogenous abscisic acid (ABA) accumulation which further caused ROS detoxification and stomatal closure in response to drought, whereas RNA interference (RNAi) lines of this gene had the opposite effect (Zhao et al. 2020). Furthermore, ethylene response factors (ERFs) affect anthocyanin biosynthesis. A well-characterized ERF protein MdERF38 is involved in drought stress-induced anthocyanin biosynthesis. Molecular experiments showed the interaction between two partners ERF protein (MdERF38) and a positive modulator of anthocyanin (MdMYB1), promoting drought resistance (Jian-Ping An et al. 2020). *MdMYB88* and *MdMYB124* are the positive regulators of drought tolerance (Li et al. 2020). In apple, 42 apple specific miRNAs are present, out of which miR156, miRn249, miR408, miR395 are the positive regulators of drought stress tolerance (Li et al. 2020).

1.2.3 Cold Stress

Premature fruit drop is one of the major concerns during cold stress. During the early development, apple fruits are exposed to abnormal cold conditions. Studies indicate apple trees with early developing fruits are subjected to abscission owing to ABA production. Abscission induction causes upregulation of ABA biosynthesis (*MdNCED1*) and metabolism (*MdCYP707A*) genes, and ethylene biosynthesis (*MdACS1*) and receptor (*MdETR2*) genes in the pedicel (Lee et al. 2021). Once the ABA in the pedicel spreads to adjacent organs, increasing ABA orchestrates cold response (Lee et al. 2021). Cold tolerance in apples is mediated by the ethylene biosynthesis gene *MdERF1B,* which upregulates the expression of the cold-responsive gene *MdCBF1* in apple seedlings (Wang et al. 2021). Moreover, another positive regulator, *MdCIbHLH1*, functions upstream of CBF-dependent pathways facilitating the binding of *MdERF1B* to target gene promoter and increasing transcription rate (Wang et al. 2021). In a nutshell, upregulation of such key genes resulted in enhanced ethylene biosynthesis production leading to cold tolerance in apple cultivars (Wang et al. 2021).

1.2.4 Salinity Stress

Salt stress has the ability to hamper plant growth and development (Yang and Guo 2018). Approximately one-fourth of the global cultivated land area is salinized, and such conditions are going to worsen more in the upcoming years due to rapid climatic changes (Zhu 2016). The perennial woody apple trees are non-halophyte (Flowers et al. 2010). However, salt damages in apple-producing zones are contributed mainly by improper fertilization and irrigation that has adversely affected the overall stature of the plant. Due to such secondary salt damage, considerable economic losses are caused. Hence, developing salt-tolerant varieties is the need of the hour. The role of microRNAs in salt resistance is reported (Kumar et al. 2018). These small non-coding RNAs function by inhibiting or degrading the complementary mRNAs (Catalanotto et al. 2016). The first identified miRNA in plants is miR156 that targets and regulates the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors (Cardon et al. 1997). The miR156/SPL regulatory modulate the *MdWRKY100* transcription factor in salt-tolerant pathways (Jiang et al. 2017; Ma et al. 2020).

1.2.5 Nutrient Stress

The apple farming system is more sustainable owing to lower nutrient removal in the yield, high nutrient recycling and retention in the system (Wu et al. 2008). The nutrient uptake and removal by apple trees (through harvest and pruning) is comparatively lower. Apple trees have lower rooting densities making them inefficient in using nitrogen (Neilsen and Neilsen 2002). The growth of apple production is reliant on nutrient accumulation over multiple seasons (Wünsche and Lakso 2000). Annually only 50% of total uptake N is retained leading to yield of 90 t ha^{-1} (Neilsen et al. 2001). On the other hand, water loss beneath the root zone during the coolest months were greater than scheduled irrigation application (Neilsen and Neilsen 2002). Thus, in the irrigation systems if water is added to meet plant demand, then water and N movement through root zone can be reduced.

1.2.6 Role of MdCAX Proteins in Abiotic Stress Tolerance

Calcium plays an important role in maintaining homeostasis for plant survival and growth. Besides, studies on many fruits have shown their role in regulating fruit development, quality, ripening, and preventing it from adverse climatic alterations (Michailidis et al. 2020). CaCA (Ca^{2+}/cation antiporter) superfamily comprises several subgroups of exchangers, including Ca^{2+}/H^{+} exchangers (CAXs) (Taneja et al. 2016). The role of CAX proteins is widely explored in preventing excessive

accumulation of Ca^{2+} in the cytosol by promoting its efflux into vacuoles (Pittman et al. 2016). Interestingly, such CAX proteins are recently characterized in apple cultivars playing a significant role in building acquired tolerance against various abiotic stresses. Based on protein studies conducted by Yeast 2Hybrid, it was revealed CAX proteins interact with multiple stress key regulators like *SOS2*, *CXIP1*, *MHX*, *NRAMP3*, and *MTP8* (Mao et al. 2021).

1.3 Traditional Breeding Methods

Traditional breeding is one of the main strategies used to improve agronomic traits. Furthermore, such breeding techniques are usually a long-term and expensive process requiring many resources to meet the end. To complicate such events more, sexual breeding involved in the conventional breeding process is not always feasible because cultivars involved at times are incompatible, sterile, or polyembryonic (Talon and Gmitter 2008). However, after successful breeding, even repeated backcrosses are required to recover elite characters of improved cultivars, causing further delay in the breeding program. Such processes are even longer in the case of rootstock breeding (25 years and more). New plant breeding techniques (NPBTs) can overcome such limitations in traditional breeding to obtain improved organoleptic traits and resistance to biotic and abiotic stress. For carrying out such activities, thorough knowledge about the target gene is essential to imply techniques such as genome editing and cisgenesis (Salonia et al. 2020).

1.4 Molecular Breeding

1.4.1 Molecular Markers

It is essential to have proper genetic analysis and marker-assisted selection (MAS) for efficient plant breeding. MAS is used to fish out specific traits or quantitative trait loci (QTLs) by use of genetic markers, usually named as named marker-trait association and marker-locus-trait association, respectively (Ru et al. 2015). Studies on genetic markers like isozymes, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPD), amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs) are conducted to provide insights into modern breeding ways (Pereira-Lorenzo et al. 2009). On the other hand, marker-assisted seedling selection (MASS), uses DNA markers to produce genetically improved cultivars from biparental crosses (Ru et al. 2015). MASS can also have additional advantages, e.g. identifying individuals with multiple disease resistance alleles, marking out phenotypic variance caused by environmental factors, and further revealing unexpected outcomes in context to genotype and environmental

interactions (Ru et al. 2015). Although such DNA markers proved to be promising in generating improved seedling selection, especially for traits with low heritability, still less work is reported for rosaceous fruit trees. Recently, single nucleotide polymorphisms (SNPs) have been preferred for their ability to genotype at a large-scale concomitant with release 8 K, 20 K, and 450 K SNP arrays for apple using the Infinium II or Axiom system (Chagné et al. 2012a; Bianco et al. 2014, 2016a, b). Furthermore, whole-genome sequences are reported for 'Golden Delicious' (Velasco et al. 2010) and the doubled haploid 'GDDH13' (Daccord et al. 2017). Thus, such improvised tools have made the breeding process efficient by screening out target genes involved in the development of valuable traits.

1.4.2 Quantitative Trait Loci (QTLs)

QTL mapping is usually conducted in a biparental population to uncover the markers associated with the trait of interest. QTLs for various traits of interest have been identified using standard protocols. Such traits include assessment of malic acid content, flesh firmness and softening, harvest time, and polyphenol and sugar components (Liebhard et al. 2003; Kenis et al. 2008; Costa et al. 2010; Longhi et al. 2012; Chagné et al. 2012b; Kunihisa et al. 2014). The genetic variations determining the flesh firmness and crispness was identified using genetic markers based on QTL. Such developmental studies depicted the role of ethylene response factors, MdERF2 and MdERF3 binding to the MdACS1 promoter and oppositely regulate its transcription during post-harvest ripening in apple (Li et al. 2016). Additionally, two potential QTLs identified as *Ma* and *Ma3* on linkage group (LG) 16 and LG8 are key players in regulating acidity in apples. The research was done using pedigree-based QTL mapping software, FlexQTL (Verma et al. 2019). However, such QTL studies on apple acidity put forth few loopholes owing to the bi-allelic nature of the QTL models used in QTL mapping. Surprisingly, multiple Q alleles are found, each with a different effect contributing to software inability to uncover QTL genotype estimates for parents with correct designated alleles (Verma et al. 2019). Other challenges with QTLs include limited crossover of markers, need for a larger population of related individuals and a lower rate of recombination events (Costa 2015; Soto-Cerda and Cloutier, 2012).

1.5 Genome-Wide Association Studies (GWAS)

The genome of cultivated apples is the base for generating many tools to navigate the genetic analysis to its best (Velasco et al. 2010). Domesticated apple has thousands of genes as compared to other crops to be used for genome-wide functional studies. Many of such genes are capable of providing resistance to abiotic stress, enhancing flavor and agronomical traits (Pereira-Lorenzo et al. 2009). The genomic selection