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Principles and Practices of OMICS and Genome Editing for Crop Improvement

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Chapter 1
Principles and Practices of Genome Editing in Crop Plants

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1.1 Introduction

The total world population is expected to increase up to 10 billion at the end of 2050. As the land area and existing resources are reducing, the need of food will be be increased to 27% in 2050. This is therefore a big challenge to solve this problem (Hunter et al. 2017). Therefore, it is an urgent need of time to increase food production for the rapidly growing world population. In earlier times, the crops were improved through conventional methods (Rasheed et al. 2020a, b, c, d), which were not very convenient and time-saving (El-Mounadi et al. 2020). Due to lack of knowledge about gene numbers, mutagenesis, and genetic mapping, people have selected the crops over many generations for release of new cultivars (Li et al. 2013).

Several techniques of plant breeding have been developed to speed up the process of genome editing (GE) and to attain the goal of high-yielding cultivars. GE via programmable endonucleases is newly developed technique of genetic engineering in crops. Endonucleases are used to bring the breaks in the targeted regions of DNA. The DNA repairing process is then initiated by homology direct repair (HDR), and non-homologous end joining (NHEJ). These factors act for completion of the process of insertions and deletions in breaking regions (Symington and Gautier 2011). Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are currently being used for genome editing. The newly emerged tool is CRISPR/Cas9 which is now a leading technique in genome editing (Bao et al. 2019; Shah et al. 2018). ZFNs are a kind of chimeric proteins made up of an artificial zinc finger genetic material mandatory area and a cleavage area, and they can be used to edit DNA to bring certain modifications (Cathomen and Joung 2008). ZFNs are being used in many crops like rice, maize, and *Arabidopsis* (Gallego-Bartolomé et al. 2019).

Unlike ZFNs, the TALENs are structure enzymes that contain a transcription activator-like effector attached to an enzymatic area of endonuclease (Christian et al. 2010). The attaching area of DNA in TALENs monomers in turn consists of essentially repeated area that regulates the binding of DNA and specificity of host. This CRD made by tandem recurrences of amino acids remains, and they attach with nucleotides' targeted sequence which permits the targeted design and enhances the number of sites to be targeted by ZFNs GE tools (Moscou and Bogdanove 2009). The TALENs GE tools have been used in variety of crops like potato (Clasen et al. 2016), rice (Shan et al. 2015), soybean (Du et al. 2016), and wheat (Liang et al.

2014). Genome editing consists of several types including TALENs, ZFNs, MNs, and CRISPR/Cas9 (Fig. 1.1). The types of genome editing are presented in Fig. 1.1.

CRISPR is a third-generation genome editing tool which was first used in 2013 and now considered as a potent editing tool (Shan et al. 2013). CRISPR/Cas9 is a type 11 structure and has the following three components, sgRNA, Cas9 proteins, and trans-activating crRNAs, and a precursor (Bhaya et al. 2011). Cas9 protein consists of HNH domain and nuclease domain and responsible for the maturation of ceRNA and DNA cleavage (Bhaya et al. 2011). The second part is tracrRNA which is a small trans-encoded RNA composed of arrangement which is almost complementary to the pre-crRNA repeats and permits the formation of duplex of RNA vital for crRNA growth and DNA cleavage (Horvath and Barrangou 2010). Pre-crRNAs are copied from CRISPR locus, which contains a recurrent repeat-spacer group. The recurrences are characteristically matching in stretch and order within a CRISPR loci but differs prominently among dissimilar locus. Most repeat orders show palindromes that can form hairpin-formed secondary assemblies (Horvath and Barrangou 2010). The insertions are resulting from entering viral genetic material and can monitor Cas9 to slice an entering protospacer, the structure in the external genome from which spacers are formed on following attack by viruses (Bhaya et al. 2011).

Since it was first discovered, Cas9 has been widely used in a large number of crops, like rice (Jiang et al. 2013), *Arabidopsis* (Feng et al. 2014), potato (Wang et al. 2015), apple (Nishitani et al. 2016), and cotton (Chen et al. 2017). In this chapter we have summarized the recent advancements on the use of genome manipulation in crops, their applications, their challenges, and their future prospective. CRISPR/Cas9 lead to the improvement of important crop traits like quality improvement, development of high-yielding varieties for growing population of world, climate-resilient cultivars to cope with environmental fluctuations, disease resistance to prevent yield lose, and domestication of wild crops to bring novel traits in cultivated crops as shown in Fig. 1.2. The role of CRISPR/Cas9-based genome editing in crops is also shown in Fig. 1.2.

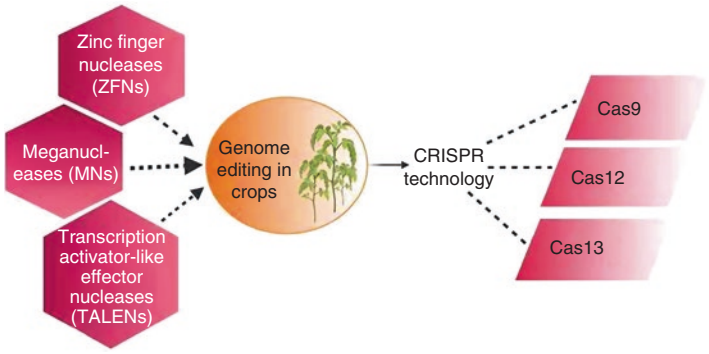

Fig. 1.1 Illustration of the types of genome editing and different types of proteins used in CRISPR-based genome editing system

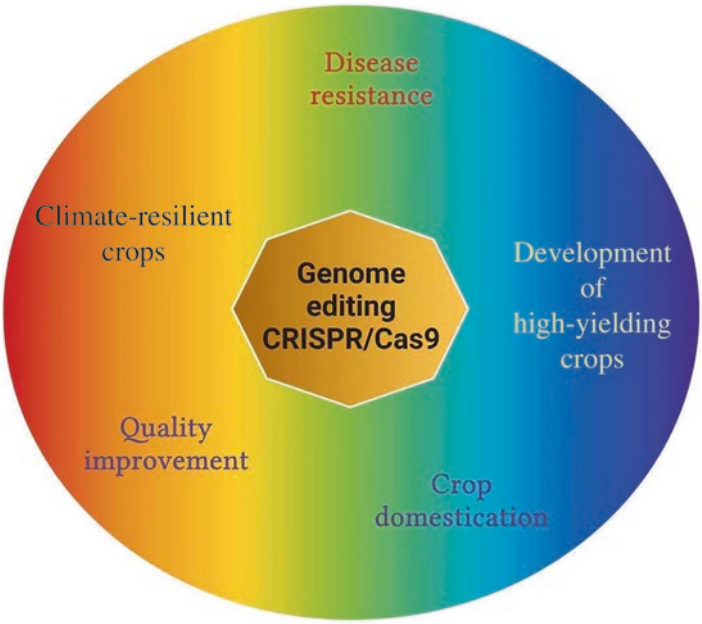

Fig. 1.2 A graphical overview of the applications of genome editing in crop improvement, including quality improvement, development of high-yielding varieties, climate-resilient cultivars, diseases resistance, and crop domestication

1.2 CRISPR/Cas9 Revolution

Nothing was same before CRISPR introduction. CRISPR protein Cas9 in not only a protein, but it is also a big protein family like other protein families. The latest research has shown much more about new CRISPR systems with advantages and disadvantages (Zess and Begemann 2021).

1.2.1 SpCas9 Protein

Due to engineering of new Cas9 protein, the compatibility, specificity, and functionality of PAM have been broadened (Karvelis et al. 2017). The Cas9 protein requires 5-NGG PAM, while N is a nucleotide, and its genome can be changed, mainly in AT-rich species. Due to editing of Cas9 proteins resulted in development of many new variants having four PAM preferences has been completed (Kleinstiver et al. 2015; Nishimasu et al. 2018). These variants have been used in many crops like rice and *Arabidopsis* (Niu et al. 2020; Zhong et al. 2019). The rest of the SpCas9 modifications comprise of nuclease domain mutant, which result in new practical features. Silencing of a single nuclease area resulted in an enzyme that only creates a

single-strand break, called nCas9, while deactivation of dual areas yields a non-functional Cas9 protein called dCas9 (Jiang and Doudna 2017).

1.2.2 Cas12

Cas12 also called Cpf1, from *Prevotella* and *Francisella*, is a class 2-type enzyme which has been broadly utilized in GE (Zess and Begemann 2021). The working principle of Cas12 is same like Cas9 which attaches with sgRNA to bring breaks in double-stranded DNA. Cas12 needs T-rich sequence of PAM when compared to G-rich PAM sequence of Cas9. The number of orthologs of Cas12 currently under use includes, *Acidaminococcus* (AsCas12a) and pathogen *Lachnospiraceae bacterium* (LbCas12) (Zetsche et al. 2015). In many biological systems, the orthologs of Cas12 are found to be more specific than the orthologs of Cas9 (Tang et al. 2017; Zhong et al. 2018). Frequent progress in the use of Cas12a for genome alteration will place this enzyme as substitute to Cas9.

1.2.3 Cas13

Cas13 also called as C2c2 possesses an RNase activity and aims to bring breakdown in RNA (Abudayyeh et al. 2016). Cas13a needs a protospacer bordering a place to bring a break in RNA molecule. Wonderfully, Cas13a also shows a non-specific RNase action which slices collateral RNA succeeding early attachment to the targeted RNA in virus and bacteria (Abudayyeh et al. 2016; Gootenberg et al. 2017). Analysis and development of additional orthologs and alternatives of Cas13a in crop plants will enhance extra RNA-targeting techniques for transcriptional guideline, RNA-based manipulation, useful research, pathogen discovery, and plant diseases (Zhang et al. 2019).

Hence, a conventional way of gene transformation is not reliable and time-saving, so CRISPR/Cas9-based GE technique is now widely used for crop improvement, which involved genomic DNA, construction of sgRNA, cloning using vector, then transformation via *Agrobacterium* method, then regeneration of plants/mutants, screening of mutants, and DNA extraction for sequence analysis (Fig. 1.3).

1.3 CRISPR/Cas9 uses to Improve the Diversity of Plants

When CRISPR was adopted as a novel GE technique, CRISPR has brought together a wide range of scientific community and quickly applied for gene editing. The first use of CRISPR/Cas9 in two widely used food crops, wheat and rice, was reported

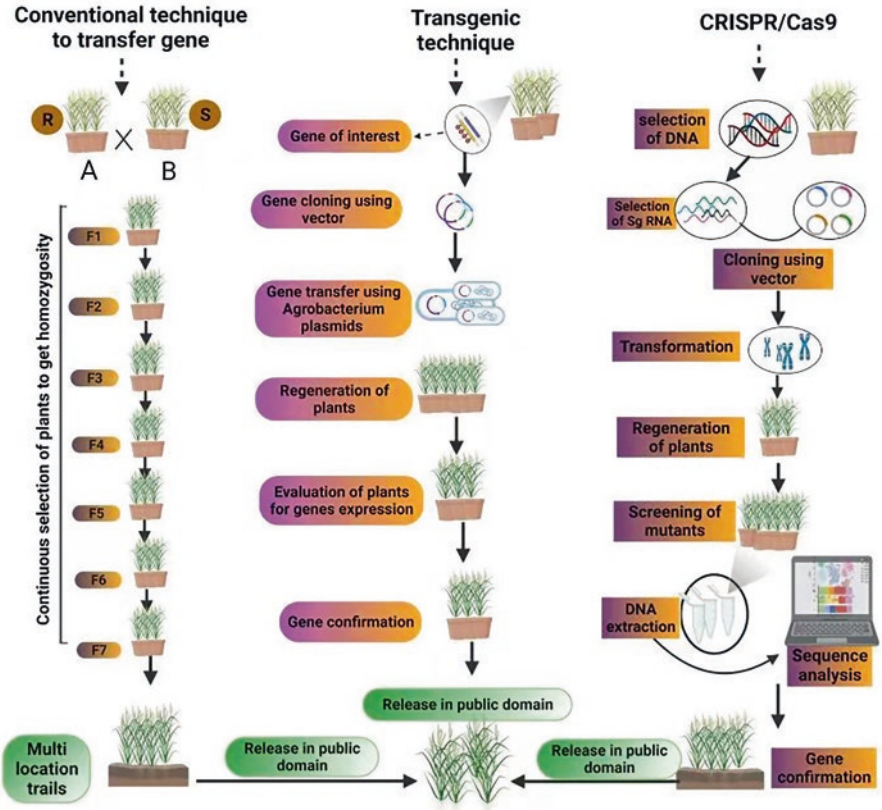

Fig. 1.3 The comparison of genome editing techniques including conventional, transgenic, and CRISPR/Cas9. CRISPR/Cas9 is a novel and the most reliable genome editing technique. The steps involved selection of DNA, sgRNA, cloning, transformation, regeneration, screening, DNA extraction, and sequence analysis

by Shan et al. (2013). After the initial success, CRISPR/Cas9 has been used in all agriculturally important crops like soybean, cotton, and potato (Li et al. 2021a).

1.3.1 The Use of CRISPR/Cas9 for Yield and Quality Improvement in Crops

Yield and quality improvement is the ultimate goal of any crop improvement program (Rasheed et al. 2021a, b, c). Yield and quality can be improved by improving plant resistance to several environmental stresses. Crop yield is a polygenic trait, and it is often hard to find the genes which are controlling this feature (Li et al. 2021a). Using modern techniques such as high-throughout sequencing, we can examine crop molecular architecture and identify genes that are related to crop

yield. The negative regulator genes are negatively affecting the crop yield and quality and provide the best opportunity for CRISPR/Cas9 to induce the targeted mutations to improve yield (Pyott et al. 2016).

Gn1a and *dep1* are the two negative controller genes connected to the traits like, number of grains/panicle, seed size, as well as grain size of rice crop (Ashikari et al. 2005). Cytokinin oxidase is synthesized by gene *Gn1a*. Due to the reduced expression level of *Gn1a*, the number of reproductive organs has been increased which ultimately increased the grain number (Ashikari et al. 2005). These three genes were successfully knocked out through CRISPR/Cas9 (Li et al. 2016). The mutant's plants exhibited dense panicles, enhanced grain number, and large grain size. The grain width 2 (*gw2*) adversely controls the weight of grain (Li 2014; Xu et al. 2016). These genes were edited and rice grain weight increased significantly, and knockouts of two or more genes produced a higher crop yield as well as larger grains (Xu et al. 2016). CRISPR/Cas9 also successfully mutated gene, *OsPAO5* which resulted in an increase in grain weight, number of grains, and yield capability in rice crop (Lv et al. 2021). The switchgrass is a biofuel crop, and CRISPR-based 9 editing of teosinte branched 1 *tb1*, the plant biomass, and plant tiller density were improved by gene expression (Lotfi and Rezaei 2020).

Direct knockout of *Bna MAXI* alleles in rapeseed resulted in development of plants with more branches having semi-dwarf stature. As a result, these plants yielded more than wild counterparts (Zheng et al. 2020). Four *GW2* homeologs, *TaGW2*, *A1*, *B1*, and *D1*, in wheat are knocked out by CRISPR/Cas9 which result in the increase in length and width of grain and 1000 GW (Zhang et al. 2018c). Wu et al. (2020) targeted the early heading date gene *Edhi* in rice using CRISPR/Cas9, and rice mutants with both in-frame shifts and frame shifts were generated. All of these mutants showed the high-yielding potential and longer vegetative growth period. The *TaSBElla* gene in spring and winter wheat cultivars was knocked out and mediated by CRISPR/Cas9 technique (Li et al. 2021a). Results showed that all edited plants showed higher concentration of starch, protein, and amylose compared to wild types (Li et al. 2021a). Sun et al. (2017) knocked out the starch-branching enzyme genes, *SBE1* and *SBEIIb*, and got transgene-free rice showing high amylose content. By using CRISPR/Cas9 gene manipulation technology, Zhang et al. (2018a) successfully targeted the *Waxy* gene in rice genotypes. Due to knockout of genes, the elite rice cultivars showed lower content of amylose without affecting other desirable traits. There are several kinds of oil crops having different features. Usually oleic oil has a good quality due to good-quality fats (Zhou et al. 2020). The *fad2* gene is responsible for good-quality oil production. Thus *fad2* gene is a potential target for CRISPR/Cas9 to modify the quality of oil in many oil species. The *Camelina sativa* plants have high content of oleic acid which was improved from 16% to 50% using CRISPR/Cas9 gene-edited tool (Jiang et al. 2017).

The results showed that CRISPR/Cas9 based editing of *fad2* in plants showed lower content of less favorable fatty acids and linolenic acid. At the same time, the *fad2* gene was also knocked out in other crops like tobacco (Tian et al. 2020), cotton (Chen et al. 2021), soybean (Al Amin et al. 2019), as well as peanut (Yuan et al. 2019). CRISPR/Cas9-targeted mutagenesis of gene *fad2* resulted in enhanced oleic

acid contents in tobacco (Tian et al. 2020). Numerous plant oils comprise of important quantities of long-chain fatty acids which are unwanted for many diverse goals. By using CRISPR/Cas9, Ozseyhan et al. (2018) successfully edited the fatty acid elongase 1, *FAE1*, gene in *C. sativa*. Their outcomes presented that C20–C24 very long-chain fatty acid *VLCFAs* were decreased to below 2% of the overall fatty acids in the wild genotypes (Ozseyhan et al. 2018). The amino acid GABA is a non-proteinogenic amino acid which possesses beneficial effects on human health. By increasing the GABA content, the health benefits also increased. Its synthesis is controlled by glutamic acid decarboxylase (GAD). By using CRISPR/Cas9, *GIGAD3* in tomato plants is targeted, and the resulted tomato plants showed more content of GABA in fruits (Nonaka et al. 2017).

CRISPR/Cas9 successfully edited the genes linked with GABA production, containing *GABA-TP2* and *GABA-TP3*. The resulted tomato plants had 19-fold higher content of GABA than wild plants (Li et al. 2018d). Hunziker et al. (2020) merged the CRISPR/Cas9 technology with Mark-AID to edit the *SlDET1* and *SlCYC-B* genes to modify carotenoid synthesis. Banana plants enriched with β-carotene were obtained by Kaur et al. (2020) which showed six folds compared to wild types. CRISPR/Cas9 also used to improve the storage life of crops. For example as fruits are developed with good flavor, they turned into soft and problematic for long-term storage. Yu et al. (2017) used CRISPR/Cas9 to get both mutagenesis of *ALC* gene as well as replacement in tomatoes. The CRISPR-modified tomato revealed improved storage life and long shelf life without disturbing other characters like plant height and fruit inflexibility (Yu et al. 2017). Likewise, Li et al. (2018c) achieved tomato plants with changed fruit maturing in tomatoes. All these information would be valuable for future research. A list of crop improved for their yield quality using CRISPR/Cas9 is shown in Table 1.1.

1.3.2 The Use of CRISPR/Cas9 to Develop Disease-Resistant Cultivars

The CRISPR/Cas9 gene manipulation technology is now used for the development of disease-resistant cultivars (Wheatley and Yang 2021). Development of disease-resistant cultivars (Zaidi et al. 2020) is a promising approach for a sustainable agriculture (Pickar-Oliver and Gersbach 2019). Gene *MLO* also called mildew resistance locus is originally identified in barley. CRISPR/Cas9 mutagenesis of *MLO* in tomato (Nekrasov et al. 2017) and wheat (Wang et al. 2014) enhanced the resistance to mildew by decreasing its penetration into host. Likewise in *Arabidopsis* gene *EDRI* is a negative controller of defense signaling system, and CRISPR-based mutation of *TaEDRI* genes enhanced the plant resistance to mildew in wheat crop (Zhang et al. 2017). Likewise, the targeted mutagenesis of *DMR6* also enhanced the crop resistance against powdery mildew diseases in tomato (de Toledo Thomazella et al. 2016) (Table 1.2). CRISPR/Cas9 also brought the resistance to potyviruses by mutation in translation initiation factor *eIF4E* of eukaryotic organisms like cucumber (Chandrasekaran et al. 2016), cassava (Gomez et al. 2019), and *Arabidopsis*.

Table 1.1 List of genes targeted by CRISPR/Cas9 to improve yield and quality in different crops

Crop	Gene	Trait	Technique	References
Wheat	*TaSBEIIa*	High amylose content	CRISPR/Cas9	Li et al. (2021b)
Rice	GS3	High yielding	CRISPR/Cas9	Huang et al. (2021)
Rice	*P450*	High yielding	CRISPR/Cas9	Usman et al. (2020)
Groundcherry	*ClV1*	Size of fruit	CRISPR/Cas9	Lemmon et al. (2018)
Rice	*OsBADH2*	High yielding	CRISPR/Cas9	Usman et al. (2020)
Wheat	*TaGW2*	Grain weight	CRISPR/Cas9	Zhang et al. (2018c)
Tomato	*ALC*	Long shelf life	CRISPR/Cas9	Yu et al. (2017)
Faba bean	*VfTT8*	White flower color	CRISPR/Cas9	Gutierrez et al. (2020)
Rice	*Waxy*	Amylose content	CRISPR/Cas9	Zhang et al. (2018a)
Tomato	*GABA-TP3*	GABA content	CRISPR/Cas9	Li et al. (2018d)
Soybean	*Fad2*	Oleic acid content	CRISPR/Cas9	Al Amin et al. (2019)
Maize	*ZmiIPK*	Low contents of phytic acid	CRISPR/Cas9	Liang et al. (2014)
Potato	*St16DOX*	Low steroidal content	CRISPR/Cas9	Nakayasu et al. (2018)
Grape	*IdnDH*	Low tartaric acid	CRISPR/Cas9	Ren et al. (2016)
Rapeseed	*BnTT2*	High fatty acid	CRISPR/Cas9	Xie et al. (2020)
Flax	*FAD2*	High oleic acid	CRISPR/Cas9	Jiang et al. (2017)
Sorghum	*Wholek1Cgenefamily*	Protein	CRISPR/Cas9	Li et al. (2018a)
Banana	*PDS*	Albino phenotype	CRISPR/Cas9	Kaur et al. (2018)
Apple	*TEF1*	Early flowering	CRISPR/Cas9	Charrier et al. (2019)
Cotton	*GhMYB 25-like*	Albino phenotype	CRISPR/Cas9	Li et al. (2017)
Ipomoea nil	*CCD*	Flower color	CRISPR/Cas9	Watanabe et al. (2018)
Rice	*TMS5*	Higher yield	CRISPR/Cas9	Zhou et al. (2016)

(continued)

Table 1.1 (continued)

Crop	Gene	Trait	Technique	References
Barley	*HvPM19*	Short stature genotypes	CRISPR/ Cas9	Lawrenson et al. (2015)
Orange	*CsLOB1*	Tolerance to citrus canker	CRISPR/ Cas9	Peng et al. (2017)
Mung bean	*Receptor-like kinase gene*	Nitrogen fixation	CRISPR/ Cas9	Ji et al. (2019)
Cucumber	*eLF4E*	Resistance to *Papaya ring spot mosaic virus*	CRISPR/ Cas9	Chandrasekaran et al. (2016)
Lettuce	*LsBIN2*	Biallelic mutations	CRISPR/ Cas9	Woo et al. (2015)
Petunia	*F3H*	Flower color	CRISPR/ Cas9	Yu et al. (2021)

The GE of *TALE* gene within the promotor of *CsLOBI* in citrus (Peng et al. 2017) and *OsSWETII* in rice (Oliva et al. 2019) also conferred resistance to multiple ecotypes of *Xanthomonas* pathogen. Gene *TaNFLXI* is a wheat transcription factor which is responsible for limiting yield, and potential of crop is mutated by a CRISPR/Cas9 tool to enhance resistance against mycotoxin fungi (Brauer et al. 2020). In tobacco two genes, *NtPDS,* and *NtPDR6* which are accountable for pleiotropic drug resistance were modified by using CRISPR/Cas9 which resulted in increased resistance in crop. Results showed that plants showed 87.5% and 81.5 8 of mutation rate in *NtPRD6* and *NtPD*, but no effects were observed near off-targeted regions (Gao et al. 2015). In the same way in Arabidopsis, the knockout of *4E* gene resulted in the increase of resistance to *Turnip mosaic virus*, but it did not influence plant vigor (Pyott et al. 2016).

Several studies showed that CRISPR/Cas9 is very beneficial in increasing resistance to several diseases, like resistance to *tomato yellow leaf curl virus* (TYLCV) as well as *bean yellow dwarf virus* (BeYDV) (Ji et al. 2015; Oliva et al. 2019). Ethylene-dependent path in rice crop has been changed by CRISPR/Cas9 followed by mutation of the *OsEFF922* gene. The resulting mutants showed resistance to *Magnaporthe oryzae* (Wang et al. 2016). Such CRISPR/Cas9 uses together specify that it is a vital tool of GE and an important contributor to enhance plant disease resistance. A list of disease-resistant genes in crops is shown in Table 1.2.

1.3.3 Crop Domestication

Crop domestication is an important goal for a modern agricultural system. This is an alternate goal of the future agriculture (Lemmon et al. 2018; National Academies of Sciences and Medicine 2019). This process involves research in genetics, botany, and other fields (Larson et al. 2014). Domestication involves modification of the

Table 1.2 The use of CRISPRR/Cas9 for the development of disease-resistant cultivars

Crop	Gene	Trait	Technique	References
Tomato	*SlMlo1*	Resistance to powdery mildew	CRISPR/Cas9	Pramanik et al. (2021)
Sweet basil	*ObDMR6*	Resistance to downy mildew	CRISPR/Cas9	Hasley et al. (2021)
Tomato	*DMR6*	Resistance to powdery mildew	CRISPR/Cas9	de Toledo Thomazella et al. (2016)
Rice	*OsSWEET11*	Resistance to *Xanthomonas oryzae*	CRISPR/Cas9	Oliva et al. (2019)
Banana	*Viral genome*	Resistance to streak virus	CRISPR/Cas9	Tripathi et al. (2019)
Cucumber	*eIF4E*	*Cucumber vein yellowing virus*	CRISPR/Cas9	Chandrasekaran et al. (2016)
Banana	*DMR6*	Resistance to bacterial diseases	CRISPR/Cas9	Tripathi et al. (2021)
Citrus	*CsLOB1*	Resistance to *Xanthomonas citri*	CRISPR/Cas9	Peng et al. (2017)
Tomato	*SlMapk3*	Resistance to *Botrytis cinerea*	CRISPR/Cas9	Zhang et al. (2018b)
Apple	*DIPM-2*	Fire blight disease resistance	CRISPR/Cas9	Malnoy et al. (2016)
Chickpea	*RVE7*	Drought tolerance	CRISPR/Cas9	Badhan et al. (2021)
Grape	*VvSWEET4*	*Botrytis cinerea*	CRISPR/Cas9	Breia et al. (2020)
Brassica	*BrSWEET1a*	*P. brassicae*	CRISPR/Cas9	Li et al. (2018b)
Arabidopsis	*AtSWEET11*	Resistance to clubroot disease	CRISPR/Cas9	Li et al. (2018b)
Soybean	*NBS-LRR*	Novel disease resistance	CRISPR/Cas9	Nagy et al. (2021)
Cotton	*T7EI*	Resistance against cotton leaf curl virus	CRISPR/Cas9	Mubarik et al. (2021)
Banana	*DMR6*	Resistance to powdery mildew	CRISPR/Cas9	Low et al. (2020)
Grape	*LOB1*	Resistance to canker	CRISPR/Cas9	Jia et al. (2021)
Tomato	*MLO ol-2*	Resistance to powdery mildew	CRISPR/Cas9	Prajapati and Nain (2021)
Potato	*S-genes*	Resistance to *Phytophthora*	CRISPR/Cas9	Andersson et al. (2017)
Barley	*MORC6a*	Resistance to *Blumeria graminis*	CRISPR/Cas9	Galli et al. (2021)
Cotton	*CP and rep sequences*	TYLCV	CRISPR/Cas9	Tashkandi et al. (2018)
Cotton	*Gh14–3-3d*	Cotton *Verticillium* wilt	CRISPR/Cas9	Zhang et al. (2018d)

existing traits of wild species to the desired one (Denham et al. 2020; Kantar et al. 2017). The best examples of these characters comprise loss of pod shattering, loss of seed dormancy, seed dehiscence, removal of anti-nutritional composites, modifications in growth pattern, and flower as well as seed color. Understanding of genetics of these traits would help us to transfer these traits to the cultivated ones through several breeding techniques (Denham et al. 2020; Iqbal et al. 2020). It has become obvious in current years that understanding the nature of the multiplicity of procedures controlling domestication process is necessary to understand the origin of crop domestication (Buzdin et al. 2021).

1.4 Limitations of CRISPR/Cas9-Based Genome Editing System

The large size of CRISPR/Cas9 systems hinders its efficiency of editing, and it cannot be packed into viral carriers to transfer into somatic cells (Manghwar et al. 2019). The small size of CRISPR/Cas9 makes it suitable for efficient genome editing in crops. Cas9 needs a 5′-NGG-3′PAM directly neighboring to a 20-nt DNA target arrangement where it only identifies the NGG PAM location, and this can hinder its efficiency paralleled with novel CRISPR/Cas alternatives. Thus, the Cas9 alternate has further target competence, large DNA speciality, low off-target effects, and wide PAM compatibility. The Cas9 system is not able to bring target mutations in all regions compared to other variants (Zhang et al. 2015). However, we are hopeful that newly developed Cas variants will have more efficiency to bring the desired mutations (Hua et al. 2019). CRISPR/Cas9 brings changes at the basic loci which are related, but not same in homology to mark locations. The use of *a Agrobacterium*-mediated system of transformation used in CRISR/Cas9 is time-consuming and costly. Thus, the application of tissue culture-free genome alteration methods suggests potential developments to its effectiveness. Problems still exist for commercialization of non-transgenic cultivars developed by CRISPR/Cas9 in numerous countries, mainly because of the development prices and limitations forced by regulatory organizations.

1.5 Regulatory Concerns of GE

GE is a promising tool of breeding to develop novel genotypes both in the public and private sectors (Gleim et al. 2020). The editing of genes and use of modified crops created an environment of uncertainty in many countries, which have been cleared over the last years. In recent years, the main countries in genome editing like the USA, Brazil, Australia, and Argentina agreed that all crops which have no foreign DNA element would be considered as traditionally developed crop cultivars,

while at the same time, the European Union (EU) decided that all crops used for genome editing would go under the same regulatory measures like GM crops (Bhowmik et al. 2021).

The USA state secretary announced that novel crop breeding tools such as GE which are not pests or which are not developed using pests would not under go any extra regulatory measures. Following this announcement other nations like Brazil, Argentina, and Australia determined that if gene editing does not involve any foreign element, that crop would be considered as a traditionally developed crop. The EU Court of Justice in 2018 announced that all gene editing technologies would undergo the same regulations like other crops in the EU. The influence of this presiding on gene editing research share in the EU was instant. At the end of 2018, minor plant breeding companies were revealing identical investment policies to those of the large multinationals and would no longer be capitalizing in GE research within the EU (https://resource.wur.nl/en/show/Innovation-in-a-bind-European-ruling-on-CRISPR-Cas-has-major-consequences.htm).

In Canada in 2018, a survey conducted in private and public breeding firms founded that more public breeders were practicing GE as paralleled with private crop breeders, 33% to 31% (Gleim et al. 2020). More public breeders were sympathetic of reviewing PNT guidelines as they relate to the use of gene editing technologies compared to private breeders (46% to 31%), respectively.

1.6 Concluding Remarks and Future Research Directions

The latest genome editing techniques has brought a remarkable benefit to improve crop genetics and breeding. The CRISPR/Cas9-mediated genome editing technique has emerged as the most powerful tool to bring targeted mutagenesis in crop beyond biological boundaries. CRISPR/Cas9 is now being used in a variety of crops due to its simplicity, versatility, and robustness. Due to targeted mutagenesis, insertions, deletions, frameshift mutations, gene knockout, and multiple gene editing at the same time, the use of CRISPR/Cas9 is now helping the researchers to improve crop traits like yield, quality, disease resistance, and domestication. The CRISPR/Cas9 genome editing technique has a great potential to bring any desirable change in plant genome. The use of CRISPR/Cas9 to develop high-yielding and disease crop cultivars will be the most promising way to solve the issues raised from a conventional way of crop breeding. The main challenge for crop researchers is to identify the wild types of plants and bring them under targeted mutagenesis as they possess a large number of desirable alleles that can be used to improve all kinds of traits in crops. It is a time-saving and cost-effective technique, and it allows the development of non-transgenic crops and increases the chances of its acceptance in all countries.

The CRISPR/Cas9 technique not only improved the genome editing in crops, but also many important traits like quality (Shufen et al. 2019), yield (Hussain et al. 2018), and resistance to biotic (Yin and Qiu 2019) and abiotic stresses (Zafar et al. 2020). CRISPR/Cas9 based genome editing techniques has also solved the issue of

male sterility in crops (Barman et al. 2019). CRISPR-based genome editing tools have the capability to mutate the multiple genes at one time (Xu et al. 2019). Hence, mutate multiple genes at one time will be a primary goal for plant breeder to improve any trait in cultivars. CRISPR-based genome editing is limited to several crops. In the future due to more application of CRISPR/Cas9, the present issues would be easily solved, and this will help scientists fulfill the global food supply demand. This technique will also lead to the development of environmentally sustainable crops. Researchers can easily knock out the negative regulators of important traits using CRISPR/Cas9. The improvement of multiple traits in cultivars could bring revolution in agricultural science. As more and more research is going on, integration of functional genomics, next generation sequencing (NGS), and other techniques will lead to the development of crop varieties with improved traits in the future. All these informations would be very helpful to understand the basis of GE and its utilization in crop breeding programs.

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Conflict of Interest Authors declare no conflict of interest.

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Chapter 2
The Revolution of Omics Technology in Plant Science

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2.1 Introduction

Plants are exposed to many different environmental stressors during their life cycle. Depending on the species or genotype, these biotic and abiotic stress conditions can hinder plant growth and development and lead to yield penalties. Tolerance or resistance mechanisms have been studied extensively for years to characterize individual genes, proteins, and metabolites involved in these mechanisms. The development of DNA sequencing approaches facilitated the characterization of genomic regions leading to the whole-genome sequencing (WGS) of different species. Advancements in nucleic acid sequencing accelerated the WGS studies. At present, more than 1,000 plant genome assemblies are accessible in GenBank, even though many of them have low quality. WGS approach was extended to the sequencing of RNAs, proteins, and metabolites. Therefore, a new scientific discipline was required to study the genes, transcripts, proteins, and metabolites holistically.

The Greek terms "ome" and "omics" are expressions derived from the suffix -ome which implies "whole," "all," or "complete." Genome, transcriptome, proteome, and metabolome are the expressions generated by adding the suffix with the terms of the gene, transcript, protein, and metabolite, respectively. Genomics, transcriptomics, proteomics, and metabolomics/lipidomics are the areas of studies that are referred to as omics. As the collective and high-throughput analyses, omics technologies integrated through robust systems biology, bioinformatics, and computational tools aim to study the mechanism, interaction, and function of cell populations, tissues, organs, and the whole organism at the molecular level (Nalbantoglu and Karadag 2019).

The approach toward omics studies has evolved since next-generation sequencing (NGS) technologies are generated. The outputs of next-generation sequencing brought about brand-new approaches to gene regulation and the data on crop genomes. It serves a potential to be used in plant breeding within metagenomic and agrigenomic researches. Gene regulation mechanisms, genes taking part in the plant defense system against pathogens, and abiotic stress factors in the whole plant or at a cellular scale can be revealed via RNA sequencing. The genotypes of lots of single-nucleotide polymorphisms (SNPs) are also determined with the methods developed within NGS. Additionally, molecular markers required for investigating genetic relationships among breeding materials, detailed genetic mapping of targeted genes, and genome-wide associations are developed with the methods called genotyping-by-sequencing (GBS) and whole-genome resequencing. Determination of the genotypes of the required genetic materials enables improving the selection of individuals that resist abiotic stressors and increases the efficiency in agriculture (Vlk and Řepková 2017).

Nowadays, the omics terminology is adapted to other fields of study, including ionomics that deals with ionic changes, methylomics studying the methylation changes in nucleic acids, and toxicogenomics. Here in this chapter, we first describe the evolution of sequencing techniques and give examples of each omics technology in plant science.

2.2 First-Generation Sequencing

The sequencing technologies that give rise to decoding and sequencing the genomes of the organisms are based on the discovery of the DNA, which is a double-helix structure consisting of bases (adenine, thymine, cytosine, and guanine). The first laboratory methods used in the interpretation of the DNA sequences in terms of the letters of A, T, C, G, and N representing an ambiguity were generated by Sanger et al. from Cambridge University in 1977 and Maxam et al. from Harvard University in 1980 (Kchouk et al. 2017).

The first-generation sequencing technique was further improved by the Maxam-Gilbert method, which enables sequencing the DNA with chemical degradation of the fragments at specific bases with reagents such as formic acid, dimethyl sulfate, and hydrazine (Maxam et al. 1977). In this method, the strands of the DNA fragments are denatured, and the phosphate groups at the 5' ends of the denatured DNA strands are removed with phosphatase to identify the fragments on the gel after the radioactive isotopes of phosphorus. The radioactively labeled DNA fragments are exposed to chemical reactions in four different tubes in the presence of distinct base-specific chemical reagents. Each of the reagents results in base modification, removal of the base, and phosphodiester cleavage of the DNA strand at that site. Guanine cleavage is induced by DMS + piperidine, while the cleavage of guanine and adenine requires DMS + formic acid + piperidine. Hydrazine piperidine causes cytosine cleavage, and sodium chloride + hydrazine piperidine facilitates the cleavage of cytosine and thymine. At the end of the reactions in the four distinct tubes, labeled fragments with various sizes are separated by electrophoresis (Saraswathy and Ramalingam 2011). The polyacrylamide gel contains urea which prevents the formation of secondary structures in the single-stranded DNA. Then, the DNA sequence is determined by using autoradiography. This sequencing method does not involve DNA cloning. On the other hand, the development of the Sanger sequencing method is more applicable compared to the Maxam-Gilbert method due to its greater simplicity, higher accuracy, and lower radioactivity (Kulski 2016).

As mentioned above, Sanger sequencing developed by Frederick Sanger in 1977 is expressed as the chain termination, dideoxynucleotide, or the sequencing by synthesis method in which one strand of the DNA is used to identify the sequence (Kchouk et al. 2017). In this technique, dideoxynucleotides (ddNTPs) are used which are the analogs of the monomers of the DNA molecules, deoxyribonucleotides (dNTPs), lacking 3′ hydroxyl groups required for the extension of the DNA strands (Heather 2015). The integration of the ddNTPs to the elongating DNA prevents the process to be terminated successfully as the subsequent base cannot be incorporated into the strand. Thus, the DNA fragments with different sizes and the ddNTP molecules at their ends as the analogs of the related bases are obtained. Chain termination reactions are conducted in four different tubes. Each tube contains a different type of ddNTP and the common reaction components including dNTP mix, template DNA, radiolabeled primer, and DNA polymerase. Radioactive isotopes of the phosphorus (^{32}P or ^{33}P) enable identifying the DNA sequence. The

tubes contain a small percentage of ddNTP (about 1%). The polyacrylamide gel with urea is also used, and the DNA sequence is determined in autoradiography (Sanger et al. 1977). The bands of the DNA fragments separated regarding their sizes on the gel slab are displayed with an imaging system, either of X-ray or UV light. The Sanger sequencing was firstly used to sequence the *phiX174* genome (5374 bp) and the bacteriophage λ genome (48501 bp). The speed and accuracy of the sequencing were improved with the automatic sequencing machine based on capillary electrophoresis developed by Applied Biosystems in 1995. The genetic materials of varying plant species such as *Arabidopsis* (The Arabidopsis Genome Initiative 2000), rice (Goff et al. 2002), and soybean (Schmutz et al. 2010) and human genome were also sequenced with Sanger sequencing. The Sanger sequencing has been used for three decades and is still preferred in single or low-throughput DNA sequencing. On the other hand, Sanger sequencing is considered to be time-consuming and expensive. The limited analysis speed also reduces the efficiency besides the inability to decode the complex genomes with the Sanger sequencing (Kchouk et al. 2017).

2.3 Next-Generation Sequencing

Following the domination of Sanger sequencing for 30 years, NGS was developed as a high-throughput DNA sequencing technology considered within the second- and third-generation sequencing methods (Kulski 2016). By this method, a high number of simultaneous sequencing reactions become feasible, and the cost of sequencing is lowered due to the developments in detection systems, microfluidics, and integrating the sequencing reactions to minimized dimensions (Türktaş et al. 2015; Kulski 2016). Increased scalability and speed of generating data paved the way for advanced studies on biological systems besides the decrease in time for obtaining gigabase-sized sequences from years to days or hours via NGS (Noman et al. 2017).

NGS enables carrying out studies on genetic approaches in plant breeding and biotechnology, evolution, discovering genetic markers, gene expression profiling via mRNA sequencing, and de novo draft genome sequences within the relevant method of NGS applications such as WGS, exome sequencing (exome-seq), RNA sequencing (RNA-seq), and methylation sequencing (methyl-seq) (Türktaş et al. 2015; Low et al. 2019). The NGS platforms with 99% accuracy rates may also detect nucleotides with errors. Although the current NGS methods are highly accurate, they are still prone to errors. Even the accuracies of more than 99% may accumulate hundreds of thousands of errors in the sequencing of large genomes since NGS platforms generate high amounts of output. The number of times a nucleotide is sequenced is referred to as "coverage" or "depth" (Sims et al. 2014). Coverage may also be used to refer to the percentage of target bases that have been sequenced a specific number of times. Coverage varies depending on the type of NGS and the research application. More coverage tends to be used when in search for a variant

that is less common (<1%) in a sample. For example, whole-genome sequencing generally requires approximately 30x coverage as this will detect 98% of heterozygous single-nucleotide variants identified in a microarray. The coverage can be calculated by the Lander-Waterman equation (Sims et al. 2014).

2.4 Second-Generation Sequencing

To overcome the limitations of the first-generation sequencing tools that were used for three decades such as Sanger sequencing, brand new sequencing methods were developed (Kchouk et al. 2017). Second-generation sequencing methods enable sequencing multiple DNA fragments simultaneously that facilitate assembly and determination of complex genomic regions, methylation detection, and gene isoform detection (Muhammad et al. 2019).

Millions of short fragments are read in parallel, the speed of the sequencing process is increased, electrophoresis is not required for detecting the output and the cost is reduced within the second-generation sequencing methods (Kchouk et al. 2017). Template libraries of randomly fragmented DNA or complementary DNA (cDNA) obtained from reverse transcription are generated with shotgun sequencing by ligating the linker or adapter sequences with the DNA molecules rather than performing cloning via a host cell (Kulski 2016). In second-generation sequencing, the read length of these technologies is shorter than the first generation; therefore, amplification is necessary for signal detection (Kang et al. 2019). A solid surface or beads are used in the library amplification process in the presence of miniaturized emulsion droplets or arrays, while the nucleotides to be sequenced are detected via luminescence or changes in electrical charge (Kulski 2016). These sequencing methods are classified in two, namely, sequencing by ligation (SBL) and sequencing by synthesis (SBS), and the sequencing platforms used are Roche/454 established in 2005, Illumina/Solexa in 2006, and the ABI/SOLiD (Sequencing by Oligonucleotide Ligation and Detection) in 2007 (Kchouk et al. 2017; Meera et al. 2019).

2.5 Pyrosequencing Technology

Pyrosequencing also known as 454 technology was the first second-generation technology developed in 2005. In this technology, the main principle is to determine the base with chemical luminescence. The pyrosequencing method is different from the Sanger sequencing since the nucleotide incorporation is performed in the presence of DNA polymerase, ATP sulfurylase, luciferase, and apyrase enzymes which are kinetically well-balanced (Ramon et al. 2003). PCR amplification and pyrosequencing of the query DNA fragments are utilized to carry out real-time sequencing (Rothberg et al. 2008). In the pyrosequencing method, adapter molecules provide

the DNA molecules that have been previously fragmented to bind the agarose beads after attaching the DNA fragments. The agarose beads with DNA fragments are mixed with Taq polymerase and buffer solution before being introduced to an oil-water emulsion to induce emulsion PCR (emPCR). The DNA fragments are then amplified in the presence of dNTP and adapters considered as primers (Saraswathy and Ramalingam 2011). The nucleotides are formed and tested in terms of their inclusion in a DNA template which occurs by the release of pyrophosphate (PPi) proportional to the amount of the nucleotides (Ramon et al. 2003). ATP sulfurylase is the enzyme that uses pyrophosphate in ATP synthesis by converting it to ATP in the presence of adenosine 5' phosphosulfate. Production of oxyluciferin from luciferin is facilitated by luciferase driven by ATP. Light emission from the oxyluciferin formed previously providing chemical luminescence takes place as a result. The number of nucleotides is associated with the amount of light emitted providing the determination of the base sequence. The emitted light is illustrated with peaks having heights proportional to the number of nucleotides in a program after it is spotted with a charge-coupled device (CCD) camera. As the apyrase enzyme degrades the excess ATP and dNTP, another pyrosequencing cycle initiates with the integration of the subsequent dNTP, and the complementary strand of the DNA is constructed. A cyclic nucleotide dispensation order (NDO) is utilized to decode an unknown sequence with pyrosequencing. In this method, one of the dNTPs is recruited to the DNA template where the rest of the dNTPs are degraded in the presence of apyrase after each cycle of dNTP dispensation. Non-cyclic NDOs are also generated with the order of nucleotide dispensation and the heights of the peaks in the program in case the DNA sequence is known (Ramon et al. 2003).

Besides the disadvantages such as high cost and low accuracy of reading, the 454 technology can read long sequences (around 700 bp). In addition, the sequences are expected to be smaller than the outputs of the other second-generation sequencing methods, and homopolymers would be sequenced with lower accuracy (Saraswathy and Ramalingam 2011).

2.6 Illumina Technology

After developing Illumina sequencing in 2006, Solexa commercialized it as Illumina/Solexa Genome Analyzer. The platforms developed by this company, namely, MiSeq, NextSeq 500, and HiSeq 2500, can put forward 15 Gb, 120 Gb, and 1000 Gb of sequencing data in each run while their maximum read lengths are 2×300 bp, 2×150 bp, and 2×125 bp, respectively. In addition, the NovaSeq 6000 System is declared to present output up to 6 Tb and 20B reads in less than 2 days. It is also claimed that the Illumina sequencing technology has been used in generating more than 90% of the sequencing data of the world as being the most remarkable technology in the NGS market (Krishna et al. 2019).

As being a sequencing by synthesis (SBS)-based technology, cluster generation involves the fragmentation of DNA molecules and ligation of the fragments with

short adapter oligo at both ends. This aids connection and amplification of fragments on a flow cell where sequencing reactions take place. There are microfluidic channels on the flow cell called lanes. Oligonucleotide sequences are attached in each lane and are complementary to the adapters. These complementary oligos form a cluster that is called polony since the appearance of each PCR-amplified DNA fragment looks like a bacterial colony (Turcatti et al. 2008). The flow cell surface that is used for the immobilization of the templates for sequencing enables increased stability of DNA and accessibility of enzymes to the DNA. It also reduces the nonspecific binding of the fluorescently labeled nucleotides. One thousand copies of a template with a diameter of one micron or less are generated within solid-phase amplification. Single-molecule cluster densities reaching the order of 10 million per square centimeter are obtained by different methods including photolithography and mechanical spotting.

In Illumina technology, the PCR amplification of the DNA fragments is performed using the adapter sequence as a primer, and each type of dNTP is labeled with different types of fluorescent labels. In each sequencing cycle, only a single-labeled dNTP is introduced to the nucleic acid chain, and thus each type of dNTP signal helps the detection of base calling, while the signal length helps the identification of the number of the attached dNTPs. Fluorescently labeled nucleotides with a reversible terminator are used in Illumina sequencing. Therefore, the polymerization terminates in the presence of the nucleotide label. The fluorescent label is screened to determine the base after each dNTP incorporation. The dye is then removed from the 3' end by the enzymes for the subsequent nucleotide to be incorporated, and the next cycle begins. Even though the sequences generated after the process are short, large data can be generated accurately and fast (Turcatti et al. 2008). As a technology displaying an error rate below 1%, Illumina sequencing is claimed to be one of the most accurate NGS technologies. The incorporation bias is reduced by the natural competition in the presence of reversible terminator-bound dNTPs that are single, separate molecules. In each cycle, the measurements of the intensities of the signals induce the base calls which are the reasons behind significantly reduced raw error rates compared to the alternative technologies. Imaging the clusters on the flow cell surface is the most time-consuming step of the process besides the nucleotide incorporation phase facilitated by the enzymes. The substitution of a nucleotide located in a specified position in the genome which is named as single-nucleotide substitution is the error taking place most frequently (Turcatti et al. 2008).

Within the resequencing approaches, the sequences are allowed to be aligned to a reference in the Illumina data collection software. The full range of data collection, processing, and analysis modules to streamline collection and analysis of data with minimal user intervention is enabled with this software that was generated with the help of leading researchers. The open format of the software with simple application program interfaces also provides accessing data at various stages of processing and analysis.

2.7 Ion Torrent Technology

Ion Torrent technology is based on an SBS process similar to Illumina technology. DNA fragments are amplified by an emulsion PCR (emPCR) on beads that are washed over a picowell plate, and each nucleotide is added later on to release pyrophosphate (Heather 2015). Each ion chip contains a liquid flow chamber which helps the influx and efflux of nucleotides (Merriman et al. 2012). A complementary metal-oxide-semiconductor technology is used to detect the difference in pH caused by the release of protons (H^+ ions) during polymerization (Rothberg et al. 2011). The bottom of each chip is covered with millions of pH microsensors. The pH change is not specific to nucleotide types, and each type of dNTP is released in a fixed order. According to the measurement of pH change, the sequence is determined (Merriman et al. 2012). This technology allows for very rapid sequencing during the actual detection phase (Glen et al. 2011). The error rate of the Ion Torrent technology is higher than the Illumina since the indels are the major error in this technology. This technology cannot detect homopolymer sequences of identical nucleotide stretch such as TTTTTT due to the loss of signal as multiple matching dNTPs incorporate (Loman et al. 2012). If the DNA template has a homopolymeric region, pH change should be proportional to the attached nucleotide number. Instead, as the attached nucleotides increase in a homopolymer, the expected pH change decreases gradually. In addition, the lengths of the sequence read obtained in one experiment of Ion Torrent are various rather than being the same. The sequence reads from both ends of a fragment cannot be obtained with the current generation of Torrent devices (Lahens et al. 2017).

2.8 Third-Generation Sequencing

Several biological limitations such as assembly and determination of complex genomic regions, gene isoform detection, and methylation detection are not eliminated by the second-generation sequencing technologies because of the short read lengths, even though they present developments outstripping Sanger sequencing (Rhoads and Au 2015). Third-generation sequencing is presented as a promising technology to eliminate the mentioned limitations. The length of the read is also improved to tens of thousands of bases from tens of bases per read within the third-generation sequencing approaches, besides the decrease in time required for sequencing from days to hours and elimination of sequencing biases resulting from the PCR amplification process (Lu et al. 2016).

Unlike second-generation sequencing, third-generation sequencing technologies do not require the sample amplification step and can sequence a single DNA molecule. Also, they may produce more than 10 Kb reads and thus produce highly precise de novo assemblies and contiguous genome reconstruction even at the regions of high content of repetitive elements. These technologies include Pacific

Biosciences, Helicos System (Helicos single-molecule sequencing), and Oxford Nanopore Technologies (ONT). The first commercial NGS implementation was the Helicos System that utilized single-molecule fluorescent sequencing. However, the Helicos Biosciences company filed for bankruptcy in 2012.

2.9 Pacific Biosciences Technology

Pacific Biosciences developed the PacBio RS II sequencer and the single-molecule, real-time (SMRT) sequencing system based on the properties of zero-mode waveguides (Schade et al. 2010). PacBio sequencing enables closing the gaps in reference assemblies and determination of structural variation in genomes with the highly contiguous *de novo* assemblies. The mutations that are related to the diseases can be spotted, and extended repetitive regions are sequenced by using relatively long reads. Additionally, isoforms of genes, novel genes, and isoforms of annotated genes can be determined with PacBio transcriptome sequencing as the whole transcripts and relatively long fragments are sequenced. Base modifications such as methylation can also be spotted with PacBio sequencing. Moreover, cost-effective and scalable hybrid sequencing strategies are generated to utilize short reads in relation to long reads (Rhoads et al. 2015).

SMRT sequencing is a method carried out in cells with 150,000 ultra-microwells at a zeptoliter scale. Each well contains a DNA polymerase molecule stabilized at the bottom with a nanostructure including a biotin-streptavidin system called zero-mode waveguides (ZMWs) (Kulski 2016). DNA chains pass through the DNA polymerase, and complementary binding nucleotides promote the detection of the sequences via the signals from fluorescence labels that are attached to the end phosphate groups, which are generated by them (Rhoads et al. 2015). Long reads with high accuracy are obtained with a circulating structure (SMRTbell) constructed by the adaptors. In this technology, first, the sequencing templates are annealed. The complex consisting of template-primer-polymerase is immobilized to the 150,000 ZMWs. After the labeled nucleotides interact with the polymerase, the end phosphate group is cleaved, and the fluorescent signal is detected simultaneously and recorded with a CCD camera. Because the wavelength of the visible light is more than the diameter of a ZMW, the light reflected through the glass bottom reaches the bottom 30 nm of the ZMW. Therefore, the reduction in background noise and the detection of a recruited nucleotide are facilitated with the detection volume.

As the nucleotides are integrated and detected simultaneously rather than the second-generation technologies in which the nucleotides are added in order, the sequencing is completed faster. The accuracy of sequencing 900 bp read length has increased from 99.3% to 99.9% by circularizing the template and sequencing it multiple times by using SMRTbell (Travers et al. 2010; Koren et al. 2013). The drawbacks of SMRT are the high cost, the need for the high amount of DNA samples, and the error rate of 10–15%, which is mostly caused by indels.

2.10 Oxford Nanopore Technology

The third-generation sequencing technology developed in 2005 by Oxford Nanopore Technologies Ltd. enables simultaneous analysis of native DNA or RNA sequences at any length in fully scalable formats from pocket to population scale. It uses a nanometer-level channel in a membrane, and it determines the base sequence by the potential difference changing between the membranes passing through a single-stranded DNA (ssDNA). In this technology, the leader and the hairpin adapters are used. Each adapter is ligated to one end of the double-stranded DNA (dsDNA). The leader adapter is denoted as the Y adapter since it has a Y-shaped structure, while the hairpin adapter is called the HP adapter. Sequencing starts at the single-stranded 5' end of the Y adapter, followed by the template strand, then the HP adapter, and the complementary strand. Helicase enzyme translocates along dsDNA to ssDNA, and the hairpin protein makes each base of ssDNA pass through the nanopore at a constant rate, and so base calling may then be performed. Each type of dNTP causes different electrical potential changes that are read, and base sequences are determined (Branton et al. 2010). In this technology, sample preparation is minimal, and long read lengths can be generated in the Kb range compared to the second-generation sequencing technologies. Also, amplification and ligation steps are not required before sequencing. However, the optimization of the speed of DNA translocation through the nanopore should be needed to obtain the accurate measurement of the ionic current changes and to decrease the high error rates of base calling (Stoddart et al. 2009). Thus, the current error rates (roughly around 98%) are very high; therefore it cannot compete with existing sequencing technologies. Moreover, the low depth of coverage obtained with this technology is a possible barrier to accurate eukaryotic genome sequencing at the moment.

2.11 Genomics

Shotgun sequencing was used for some of the early plant genomes including *Arabidopsis*, soybean, poplar, and papaya (Michael and Van Buren, 2015). The sequence and genetic structure of plant genomes are determined with an extensive sequencing method called whole-genome sequencing (WGS). In early sequencing projects that focus on WGS, the genomes of strawberry (Shulaev et al. 2011) and wheat (Brenchley et al. 2012) were randomly fragmented, and elements with varying sizes are obtained. The reads obtained from the sequencing process were assembled with bioinformatic tools after the bacterial artificial chromosome (BAC)-end sequencing is performed. De novo projects also utilize WGS besides the resequencing attempts. Preparing a draft of unknown plant genomes is managed with the whole DNA or mRNA de novo sequencing even though the process is time-consuming (Türktaş et al. 2015). Despite the possibility of determining locations of

the contigs or scaffolds with low accuracy, and missing several genes while generating draft genomes, the presence of genome information enables analyses with high throughputs and characterization of genes (Sarethy and Saharan 2021). Later, WGS approach was used to generate the draft genomes of einkorn (Ling et al. 2013), wheat, and *A. tauschii* (Jia et al. 2013). Moreover, resequencing is considered to be useful in transcriptome profiling and detecting SNPs to generate molecular markers. For instance, WGS enabled the construction of the reference genome of potato and discovering SNPs to compare a homozygous doubled-monoploid line with its heterozygous diploid line (The Potato Genome Sequencing Consortium 2011). WGS of many different crop and vegetable species has been completed in the last decade. Although the second-generation sequencing resulted in many lower-quality assemblies, a massive extension WGS of different plant species, especially of the crops, leads to a revolution in plant genomics.

One thousand one hundred twenty-two plant genome assemblies are deposited in GenBank, representing 631 land plant species. The advancements in the long-read sequencing markedly improved the NGS data quality; therefore the number of plant genome assemblies has increased dramatically in the past 20 years. Almost 60% of the plant genome assemblies have been sequenced in the last 3 years alone. Model plants and some crops were the first species whose genomes were fully sequenced. But now, any plant species can be sequenced due to a steady decline in sequencing costs.

The exons considered to be the coding region for the protein synthesis in the genome of an organism are called the exome. Even though they involve the total of the sequences inducing the generation of proteins taking part in phenotypic regulation, they are insufficient to decode fully the mechanisms behind the gene regulation. To enlighten the molecular background of the diseases and phenotypic traits, exome sequencing was introduced as an essential genetic tool. Exome sequencing helps with the identification of genes (whole exome, genes responsible for a disease, or class of genes), determination of phenotypic traits, identification of exome SNPs, and further computational and statistical applications to identify the signals of diseases (Hashmi et al. 2015).

WGS of different populations of the same plant species showed a high degree of genomic variation within the species; therefore it was obvious that single reference genomes no longer can represent the diversity within a species. This observation led to the advancement of the pan-genome concept, which was first developed in bacteria in 2015 (Tettelin et al. 2005). Pan-genomes can distinguish the primary genes that are present in all individuals and variable genes that are found in some individuals but absent in others. Hence, it symbolizes the genomic diversity within the species. Pan-genomes can be curated by three different methods, each with its benefits and disadvantages over the others (Bayer et al. 2020). The first pan-genome study in plants was a comparison of WGS of wild soybean relatives (Li et al. 2014). Another study in rice compared the genomes of three accessions (Schatz et al. 2014). At present, more than 8,000 studies reported pan-genome comparisons in plants (Bayer et al. 2020). These studies have impacts on understanding the biological

significance of genotypic variances at loci linked with tolerance and resistance, developmental processes, and yield enhancement.

The transposable elements in the plant genome are high in copy number since their segmental or tandem duplication takes place frequently. Therefore, an extended amount of repetitive elements is found in plant genomes. Autopolyploid or allopolyploid character of the genome or the age of ploidization affects the progress of the sequencing as ploidy is considered a challenge. To eliminate the obstacles caused by the complexity of the genome, library sequencing of fragmented genome elements is executed by using restriction enzymes or obtaining the sequences without using enzymes (Vlk and Řepková 2017). Variations or significant polymorphisms in the genome are considered to be useful in pre-breeding attempts with resequencing projects. The reference genomes of the desired plant are also intended to be generated within various projects. They are considered as providing information about the structure and function of the genome and the genome assembly patterns of the related species together with molecular markers and candidate genes that can be used in further studies (Vlk and Řepková 2017).

Epigenetic changes such as chromatin modifications, transposable element inactivation, paramutation, transgene silencing, and co-suppression are investigated with the sequencing approaches in detail in various plant species. The changes in gene expression and chromatin-based expressional responses generated against environmental stimuli prove the importance of epigenetic studies in plants (Köhler and Springer 2017). Traditional methods used in epigenetic studies involve bisulfite conversion, methylation-sensitive restriction enzymes, and antibodies specific to 5-methylcytosine. Microarray-based methods were also started to be combined with these methods to carry out a genome-wide analysis of DNA methylation (Buck and Lieb 2004). Recently, NGS technologies paved the way for epigenetic studies (Vlk and Řepková 2017). Therefore, the studies of applied epigenetics cause new opportunities for crop improvement. It has been suggested that varietal selection of crops is associated with variability caused by epigenetic mechanisms (Rodríguez López and Wilkinson 2015; Crisp et al. 2016; Fortes and Gallusci 2017; Gallusci et al. 2017). The potential to develop crop performance and energy use efficiency was shown in *Brassica napus* via an epigenetic selection of isogenic lines (Hauben et al. 2009). Organ-specific epigenetic modifications were determined in maize by Illumina sequencing technology (Wang et al. 2009a). The expression levels of genes are regulated by epigenetic mechanisms in response to plant development and biotic and abiotic stresses, and this affects the phenotype of plants (Kumar 2018).

DNA methylation, histone modifications, and small RNA molecules are the major epigenetic mechanisms affecting the expression levels of genes (Rodríguez López and Wilkinson 2015). DNA methylation is an important chromatin modification that can be inherited in animals and plants. It has been recently suggested that methylation of the promoter and the gene coding region has different effects on gene expression (Wang et al. 2015a, b). The methylation of the promoter region of a gene is related to the repression of transcription (Kass et al. 1997). On the other hand, the methylation of the gene coding region is found with an intermediate expression level in plants. It was shown that it can be involved in reducing erroneous

transcription by reducing intron retention by single-cell transcriptome sequencing data from *Arabidopsis* root quiescent center cells (Horvath et al. 2019). Furthermore, it can enhance the gene expression in certain gene families (Dubin et al. 2015; Anastasiadi et al. 2018). DNA methylation which targets cytosines in varying sequence patterns such as CG, CHG, and CHH can be revealed efficiently with NGS after treating the DNA with sodium bisulfite. Even though mostly the transposons are methylated as being primary targets for epigenetic silencing, the relation between the transposon polymorphism and DNA methylation variation is not easily described because they are highly repetitive and result in large insertion/deletion polymorphisms in the genome. The connection between transposon methylation and transposon insertions was studied using whole-genome bisulfite sequencing data sets by Daron and Slotkin (2017). Also, bisulfite conversion and Illumina sequencing were used together for the identification of the methylated genomic regions in tomato, and it was suggested the ripening of tomato fruits was under the control of epigenetic regulation along with hormonal control (Zhong et al. 2013).

2.12 Functional Genomics

Biological investigations were focused on genes and proteins in vitro during the early 1990s. However, as technologies improved and evolved, the approach shifted to research on different molecular aspects, viz., structural genomics, transcriptomics analysis, proteomics, and metabolomics. For instance, a multidisciplinary approach involving integrative analysis is crucial to study the complexity of plant-microorganism interactions (Sarethy and Saharan 2021).

2.13 Transcriptomics

The complete set of transcripts in a cell, and their quantity, for a specific developmental stage or condition, is called transcriptome. It is essential for understanding the functional elements of the genome and the molecular regulations of cells and tissues and also for revealing disease and development. The ultimate goals of transcriptomics are to determine all species of transcript such as mRNAs, small RNAs, and non-coding RNAs for revealing the transcriptional structure of genes and the changes in expression levels of each transcript during development and under different conditions (Wang et al. 2009). Several technologies have been developed for transcriptomics, including hybridization- or sequence-based approaches. Commercial high-density oligo microarrays and custom-made microarrays with fluorescently labeled cDNA are the important techniques used in hybridization-based approaches. Furthermore, specialized microarrays have been used for some specific purposes such as the detection of spliced isoforms.

They are high-throughput, relatively inexpensive, and high sensitivity by lowering the detection threshold of the transcriptional level of the less represented genes of a mixture, thus facilitating the analysis of thousands of genes in the same reaction (Kerr et al. 2000). However, they have several limitations such as existing knowledge about genome sequence, high background levels due to cross-hybridization, and complicated normalization methods. Microarrays have been widely used to produce global expression profiles under abiotic stresses in plant species (Kayıhan and Eyidoğan 2019). For instance, the AtH1 *Arabidopsis* GeneChip from Affymetrix has been employed to study transcriptome changes in *Arabidopsis* under salt stress. Accordingly, approximately 35% of the genome (~8000 genes) exhibited expression changes under salt or other abiotic stresses (El Ouakfaoui and Miki 2005). Changes in gene expression caused by drought stress by using microarrays have been suggested by several research groups. For the first time in the literature, Ozturk et al. (2002) found that genes encoding jasmonate-responsive, late embryogenesis abundant, and ABA-responsive proteins were upregulated in barley seedlings exposed to drought. Also, it was found by microarray that changes in the expressions of 300 genes were revealed in spring and winter wheat under cold stress (Gulik et al. 2005). Furthermore, microarray technology provided comprehensive data for K^+ deficiency in plants, and this showed a more integrative point of view considering all aspects of K^+ management in plants (Kayıhan and Eyidoğan 2019). Kayihan et al. (2017) and Öz et al. (2009) performed the microarray experiments in wheat and barley cultivars exposed to excess boron, respectively. They suggest that WRKY transcription factors, genes related to jasmonate biosynthesis, glutathione S transferase, and NIP4;1 can have a role in boron tolerance mechanisms in cereals. Also, global gene expression analyses were performed in *Arabidopsis thaliana* exposed to high B and low B conditions (Kasajima and Fujiwara 2007). They identified novel high B-induced genes including heat shock protein and multidrug and toxic compound extrusion (MATE) family transporter genes. On the other hand, microarrays have been widely used for transgenic plants such as maize, canola, cotton, tomato, and soybean events (Leimanis et al. 2006; Xu et al. 2007; Schmidt et al. 2008; Zhou 2008; Kim 2010; Feng 2013).

The transcript levels of genes depending on their changes under different conditions are important information, and they can reflect the functions and transcriptional regulation relationships of genes. Modern omics technologies play an important role in better understanding gene expression. The best approach for the characterization of candidate transcripts that are responsible for many biological functions is transcriptome study. NGS technology provides us a powerful tool to reveal the transcriptional landscape of investigated tissue(s) at special developmental stage(s) because it can easily obtain transcriptome data from different plant tissue(s) and developmental stage(s). RNA-seq approach that uses NGS techniques is used for analysis and study of the entire transcriptome, and this approach provides an insight on the expression level of transcripts. Genes expressed within a defined period of time from a particular tissue or cell can be found by RNA-seq. There are some universal steps for this approach. RNA fragments are converted to a cDNA library by reverse transcriptase, and from both ends of cDNA fragments, cDNA

library fragments are ligated to adapter molecules. Then adaptor attached library fragments are sequenced. Through cDNA sequencing, transcriptomes are studied deeply and efficiently. For plant transcriptomes, Illumina technology has generally better coverage. Reference genome and de novo assembling are two types of the assembly methods. For large NGS data of complex genomes without a reference genome, de novo assembly is useful (Wang et al. 2009). De novo transcriptomes are provided by some bioinformatic tools such as TRAPID (Van Bel et al. 2003) and Trinity (Brain et al. 2013). RNA-seq data is used for the development of molecular markers (Trick et al. 2009) and gene characterization (Dassanayake et al. 2009).

RNA-seq has successfully assisted in identifying several genes responsible for biotic and abiotic stress responses in various plant species. A large number of genes related to developmental stages were identified by RNA-seq in cucumber via 454 pyrosequencing (Ando et al. 2012). A combination of microarray and Roche technology was used to identify genes that were linked to the quality of cotton fibers (Nigam et al. 2014). To find genes associated with drought tolerance, RNA-seq analysis was performed in *Populus euphratica* Oliv. grown in arid or semi-arid regions using the Roche 454-GS FLX System (Tang et al. 2012). Likewise, sequencing red clover with Illumina technology discovered genes related to drought tolerance and determined the increase in metabolites such as pinitol, proline, and malate in leaves (Yates et al. 2014). The transcriptome of soybean (Fan et al. 2012), cotton (Xu et al. 2013a, b), and halophyte grass (Yamamoto et al. 2015) was sequenced to explore a molecular mechanism of salt tolerance in these plants. In addition, a whole-genome study was performed in soybean using Illumina technology, which examines the function of the plant-specific family of NAC transcription factors during development and dehydration stress (Le et al. 2011). Ion Torrent technology has been used in transcriptome analysis of finger millet, a hardy grain known for its tolerance to salinity, drought, and disease (Rahman et al. 2014). Transcriptome profiling of jatropha roots was carried out to elucidate molecular reactions to waterlogging (Juntawong et al. 2014). As the third generation, Pacific Biosciences' SMRT technology was used to investigate the interaction of *Xanthomonas oryzae* pv. *oryzicola* and its host, *Oryza sativa* L., by whole-genome sequencing of the pathogen and RNA-seq of the host under attack (Wilkins et al. 2015). Illumina sequencing was used to obtain responsible herbicide resistance genes for *Lolium rigidum* Gaudin (Gaines et al. 2014) and for copper tolerance (Wang et al. 2015a, b). In sweet potatoes (*Ipomoea batatas* L.), biotic stress resistance analyses of catalase genes were performed using NGS technologies, and it was found that a positive response to IbCAT2 may play an important role in stress responses (Yong et al. 2017). In tomatoes, an abiotic stress tolerance identification study was conducted to understand the plant responses and genetic regulatory networks involved in abiotic stress responses (Chaudhary et al. 2019). In plants, RNA-seq technology has been used to determine the patterns of differentially expressed genes between hybrids and their parents to understand the genetic basis of heterosis (Zhai et al. 2013; Hansey et al. 2012; Sexane et al. 2014). Accordingly, gene expression for allopolyploid heterosis was predominant in the emerging hexaploid wheat dominance (Swanson-Wagner et al. 2006), but over-dominance was the key element for

nicotine biosynthesis in tobacco (Tian et al. 2018). Dominance and over-dominance effects were shown by heterotic genes in connection with ear development earlier in maize inforescence (Ding et al. 2014). In the chrysanthemum, two characteristics of flowering – the initial flowering time and the flowering duration – are regulated by the presence of two pairs of main genes (Zhang et al. 2011).

MicroRNAs (miRNAs) are the key regulators at the post-transcriptional level in eukaryotic organisms. They regulate the expression levels of genes in response to development and various stress responses in plants. They are complementary with the target mRNAs and are highly conserved. Up till now, several technical approaches have been used to identify and verify the miRNAs. These are in silico prediction based on conserved sequences, to create miRNA libraries and to follow this with cloning and sequencing and finally the sequencing of miRNAs. In silico prediction was applied in rice (Bonnet et al. 2004). Cadmium-responsive miRNAs and their target genes in *Raphanus sativus* L. roots were identified by Illumina sequencing technology (Xu et al. 2013a, b). Also, circular RNAs were identified by transcriptome analysis by means of SMRT technology by Pacific Biosciences, and it was found that they had an important role in the function of miRNA and transcriptional control (Lu et al. 2015). On the other hand, long non-coding RNAs (lncRNAs), which are longer than 200 bp and do not encode any protein product, are another important regulatory mechanism associated with gene silencing, flowering time regulation, and abiotic stress responses (Wang et al. 2014; Zhang et al. 2014). These molecules were identified in crops, such as wheat (Xin et al. 2011), rape mustard (Yu et al. 2013), apple (Celton et al. 2014), and poplar (Shuai et al. 2014) by tiling array, EST analyses, and RNA-seq.

2.14 Proteomics

Proteomics is one of the growing fields of biological research with an immersive positive impact on plant science. Proteomics is a term that refers to the comprehensive identification and quantitative study of protein expression in an organism, cell, tissue, or organelle at a certain time and under specific conditions (Tan and Chen et al. 2011). Understanding proteome profiles provides a direct connection between genomic and transcriptomic regulation and phenotype. Since the first plant proteomic study in maize (Touzet et al. 1996), exponential progress has been made in different crop species although the full potential of plant proteomics has yet to be realized. Recent advances in new or improved technologies, protocols, or workflows have opened up new possibilities for high-throughput proteome analysis and reduced protein assessment errors.

Two-dimensional polyacrylamide gel electrophoresis and differential in-gel electrophoresis (DIGE) have been used in many early proteomic studies to separate the proteins. However, its resolution is not enough to ensure reproducibility and sensitivity (Rabilloud et al. 2010). Therefore, chromatographic

separation followed by mass spectrometry (MS) is now routinely employed in proteomic studies. There are some deviations of chromatographic separation techniques such as high-pressure liquid chromatography (HPLC) and gas chromatography (GC). After proper separation of protein mixtures, they can be identified by single or double MS systems. Sometimes samples can be ionized by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) before identification in MS. This technique uses a laser energy-absorbing matrix to create ions from large molecules with minimal fragmentation (Jurinke et al. 2004).

Genomics and proteomics have developed separately into two different disciplines, thus limiting the cross talk between scientists in the two fields, limiting the integration of useful information from both fields into a single data modality. However, depending on the encoded genomic variants, mutations, or post-transcriptional modifications at the nucleotide level, the final expressed sequence of a protein may vary. NGS can be used to capture and correctly decipher these variants. Single-nucleotide polymorphisms (SNPs) and small insertion-deletion (INDELs) can be characterized using NGS, and these sequence variants can be easily translated in silico into different proteoforms that can be added to existing protein databases (Hernandez et al. 2014). As a result of the merging of genomics and proteomics, a new field known as proteogenomics has emerged (Jaffe et al. 2004; Nesvizhskii et al. 2014; Low et al. 2016; Sheynkman et al. 2016; Ruggles et al. 2017). The expression of a gene, for example, can be determined at the level of mRNAs and proteins in each allelic form using proteogenomics (Wingo et al. 2017). Exon-exon splice junctions, on the other hand, allow for the analysis of alternatively spliced proteomes. Moreover, proteogenomics has been increasingly used to understand the adaptive diversification of plant species and populations (Voelckel et al. 2017).

Tens of studies have been completed on proteomic analysis of various plant species under different developmental stages, abiotic or biotic stress conditions, at different tissues, organs, and cells (Reviewed by Tan et al. 2017; Mustafa and Komatsu et al. 2021; Smythers et al. 2021). Recently the Arabidopsis PeptideAtlas (www.peptideatlas.org/builds/arabidopsis/) was released to solve critical questions about the *Arabidopsis thaliana* proteome (van Wijk et al. 2021). It includes around 0.5 million unique peptides and 17,858 unique proteins at the highest confidence level.

2.15 Metabolomics

Metabolomics is the large-scale study of small molecules, also known as metabolites, in cells, biofluids, tissues, or organisms. The metabolome refers to these small molecules and their interactions within a biological system. Metabolomics is a powerful approach because, unlike other omics approaches, metabolites and their concentrations directly reflect the underlying biochemical activity and state of cells and tissues. As a result, metabolomics is the most accurate representation

of the phenotype. Advancements in chromatographic separation and MS allowed for unbiased, high-throughput screening and characterization of the metabolites to study the metabolic pathways and phytochemicals to complement the other omics approaches (Lee et al. 2012; Kang et al. 2013; Kin et al. 2013; Lee et al. 2015). Because of the metabolome complexity, functional characterization of metabolites is a challenging strategy in plants (Chen et al. 2013; Lee et al. 2019). Moreover, plants within the same family generally produce the same or similar metabolites since the metabolic pathways are highly conserved in plant families, which make it easier to study the metabolites in the same family in different species (Ntie-Kang et al. 2013). Plant metabolomics studies can explain the spatio-temporal differences of some essential metabolites in different plant species, which are affected by environmental factors together with genetic determinants (Lee and Lee et al. 2015; Son et al. 2016). In general, genetic factors, nutritional status, and geo-climatic conditions all influence the chemical composition of different plant parts (Dias et al. 2016).

Currently, MS or nuclear magnetic resonance (NMR) spectrometry is used in many metabolomics studies. Some studies use gas chromatography (GC)-MS for the separation and analysis of volatile compounds. However, studying all metabolites is a big challenge since the combination of multiple metabolomics methods is required for this purpose. Many metabolomics studies have been completed in different crop species (Reviewed by Kumar et al. 2017; Sharma et al. 2018; Fernandez et al. 2020). Recent efforts in plant metabolomics science focus on natural variations of metabolites (Reviewed by Sun et al. 2021). These efforts determined the type of natural variations reflecting the metabolomics changes in a given plant family or taxon (Hu et al. 2014; Kusano et al. 2015; Albrecht et al. 2016; Zhen et al. 2016; Yang et al. 2018a, b; Fang et al. 2019). Later, these natural variations were used to select for the genotypes with superior metabolic profiles (e.g., Zhen et al. 2016) and link a specific metabolite or metabolic pathway to a genomic region via the identification of metabolite QTLs (mQTLs) (Chen et al. 2018a, b; Shi et al. 2020; Jamaloddin et al. 2021) or metabolome-based GWAS (mGWAS) (Luo 2015; Fang and Lou 2019; Chen et al. 2020; Wei et al. 2021).

Similar studies have recently been employed in the determination of ionic changes in different plant species (Yang et al. 2018a, b; Pita-Barbosa et al. 2019; Ali et al. 2021; Singh et al. 2022). Comparative metabolomics and ionomics studies revealed the evolutionary divergence of metabolic pathways and how they are conserved in some species or genotype for enhancing the adaptation to a specific condition (Dos Santos et al. 2017; Mawalagedera et al. 2019; Deng et al. 2020; Rastogi et al. 2020). We are now at the beginning of a new phase in plant metabolism research, in which integrative genomics and metabolomics approaches are used (Rai et al. 2017). The supremacy of genomics and transcriptomics should be integrated with metabolomics and proteomics studies to identify novel genes controlling the metabolism.

2.16 Multi-omics

Transcriptomics, proteomics, and metabolomics studies can represent the overall changes in transcripts, proteins, and metabolites, respectively (Aizat et al. 2018); however, a more diverse overall approach is needed to combine and compare large data sets to understand the complex biological systems such as the interactome. Multi-omics data generation and acquisition have become an essential part of modern molecular biology and biotechnology to study the biological pathways under different conditions because of recent advancements in NGS, proteomics, and metabolomics technologies as well as computational and statistical tools (Fondi and Liò 2015; Fabregat et al. 2018). Advancements in systems biology, the computational and mathematical analysis, and modeling of complex biological systems led to a more accurate understanding of complex biological systems. Systematic multi-omics integration (MOI) is essential for systems biology in plant science. MOI in plants has been a difficult task since the genomes of many non-model plant species are not well-annotated, the metabolic processes are diverse, and the interactome is massive (Jamil et al. 2020).

The earliest examples of MOI studies were very successful to demonstrate the power of the integrative omics approach to identify potential candidate genes, proteins, or metabolites for further functional characterization. For example, correlation analysis of transcriptomic and metabolomic data from the potato tubes led to the identification of novel transcript-metabolite pairs that can be further characterized in the future (Urbanczyk-Wochniak et al. 2003). In another study, transcriptomic and metabolomic data were integrated to understand the interactions of sulfur and nitrogen metabolisms and the involvement of secondary metabolites in *Arabidopsis thaliana* (Hirai et al. 2004). Since then, the MOI has been extensively used by plant scientists for functional characterization of unknown genes and to understand the behavior of complex systems under different conditions. Several different online software have been developed to integrate multi-omics data, such as MapMan (Thimm et al. 2004), and reviewed by Fondi and Lio (2015). The systems biology approach has been integrated extensively in different plant species (Rai et al. 2019).

In contrast to these advancements, some hurdles have slowed the utilization of the systems biology approach, particularly in crop species. These include the incomplete transcriptome, proteome, and metabolome data sets or their total unavailability. Current software is not designed to integrate different omics data sets to describe the phenome. Machine learning and artificial intelligence should yet to be incorporated into this software. The metabolome or ionome can be easily influenced by the environmental changes so that the extensive data generated by metabolomics and ionomics may not be readily integrated with the date of transcriptomics and genomics. Therefore, the results obtained at the levels of transcriptome and genome may not be fully reflected at the metabolome or even phenotype (do Amaral and Souza 2017). Therefore, there are lots of complex and dynamic processes working in parallel in the cell.

2.17 Single-Cell Technologies

The sequencing of a single-cell genome or transcriptome to obtain genomic, transcriptome, or other multi-omics information to show cell population distinctions and cellular evolutionary relationships is referred to as single-cell sequencing technologies (Wen et al. 2018). The molecular insight into tissue and/or time point/developmental groupings using bulk techniques, which average over many cells, has been gained. However, the inherent biases introduced by averaging over different cell populations limit these approaches. Bulk averaging can, in some cases, lead to qualitatively wrong conclusions, a phenomenon known as Simpson's dilemma (Trapnell et al. 2015). Single-cell technologies have the advantages of detecting heterogeneity among individual cells, distinguishing a small number of cells, and outlining cell maps when compared to standard sequencing technology (Wen et al. 2018). Single-cell genomic approaches offer a potent set of tools for identifying cellular heterogeneity, as well as the formation and differentiation of cell types in complex tissues.

Due to its expensive cost, early single-cell sequencing was not widely used (Wen et al. 2018). High-throughput single-cell transcriptomics has become an accessible and powerful tool for unbiased profiling of complex and heterogeneous systems, thanks to recent improvements in cost and throughput (Klein et al. 2015; Zilionis et al. 2017; Macosko et al. 2015) and the availability of fully commercialized workflows (Zheng et al. 2017). These data sets can be utilized in concert with novel computational approaches to uncover cell types and states (Shekhar et al. 2016; Villani et al. 2017a, b), recreate developmental pathways, make destiny decisions (Trapnell et al. 2014; Welch et al. 2016), and spatially model complex tissues (Satija et al. 2015; Achim et al. 2015).

The emergence of omics techniques has quickly revolutionized our perspectives on plant biology, thanks to the advancement of sequencing technologies. The cellular diversity inside a tissue or organism, on the other hand, is far more complex than can be assessed using bulk analysis, which can only produce population-averaged results (Gawad et al. 2016). As sequencing technologies advanced, allowing smaller and smaller samples, eventually allowing single-cell analysis, the traditional consensus from bulk-based omics studies was questioned (Shapiro et al. 2013). Characterizing the single-cell genome is of significant interest because each cell undergoes a unique chain of DNA synthesis and damage repair events. In a single-cell sequencing-based investigation, there are numerous basic processes. The first step is the preparation of a cell lysate. Plant cell isolation and lysis, unlike animal models, are hampered by the natural cell wall, requiring the use of specific techniques. Single-cell whole-genome amplification (WGA) must be performed once the plant cell lysate has been generated. Single-cell genomics and epigenomic technologies are both based on single-cell WGA; however, single-cell epigenomics is more diverse due to the addition of sample preprocessing procedures for capturing various epigenomic features such as bisulfite conversion for DNA methylation (Smallwood et al. 2014) and proximity DNA ligation for chromatin conformation

(Nagano et al. 2013). Single-cell sequencing technologies have been used to investigate the cell heterogeneity that underlies several bulk omics characteristics, such as genomic variation, DNA methylation, and chromatin accessibility, in a variety of animal models (Huang et al. 2015; Kelsey et al. 2017). In recent years, they have been advanced greatly in terms of sensitivity and throughput. These developments have made it possible to profile cell-specific genomic variants and epigenomic characteristics in plant models for the first time, and they hold a great promise for answering a wide range of plant biological problems at the single-cell level (Stuart and Satija et al. 2019). Recently, multiple experimental protocols, including the Assay for Transposase-Accessible Chromatin with High-Throughput Sequencing (ATAC-seq) (Buenrostro et al. 2015), single-cell combinatorial indexing ATAC-seq (sci-ATAC-seq) (Cusanovich et al. 2015), single-cell transposome hypersensitivity site sequencing (scTHS-seq) (Lake et al. 2018), plate-based scATAC-seq protocol (Chen et al. 2018a, b), and droplet-based single-cell combinatorial indexing ATAC-seq (dsci-ATAC-seq) (Lareau et al. 2019), have been developed to profile genome-wide chromatin accessibility in single cells. Very recently, the use of single-nucleus RNA sequencing (sNucRNA-seq) and single-nucleus assay for transposase-accessible chromatin sequencing (sNucATAC-seq) technologies on *Arabidopsis* roots was reported, and it was suggested that the differential chromatin accessibility is a critical mechanism to regulate gene activity at the cell-type level (Farmer et al. 2021). Furthermore, single-cell resolution maps of open chromatin in the *Arabidopsis* root to address the issue of tissue heterogeneity and to detect likely endoreduplication events were provided by single-cell ATAC-seq (Dorrity et al. 2021).

2.18 Single-Cell Transcriptomics

Differential gene expression is largely responsible for the development of multiple cell types and cell-specific functions in multicellular organisms. The transcriptome of individual cells is frequently profiled using single-cell RNA sequencing (scRNA-seq). scRNA-seq (single-cell RNA sequencing) is a next-generation sequencing technology that generates gene expression data from thousands of single cells. This large data collection can be used to answer questions like how many different cell kinds are present in a sample and how common each cell type is. The recent development of single-cell RNA sequencing (scRNA-seq) has deepened our understanding of the cell as a functional unit, revealing new populations of cells with distinct gene expression profiles previously hidden within gene expression analyses performed on bulk cells and providing new insights based on gene expression profiles of hundreds to hundreds of thousands of individual cells (Ziegenhain et al. 2017; Macosko et al. 2015).

Single-cell RNA sequencing has been particularly useful in gaining insight into tissue cellular heterogeneity and identifying previously unknown cell types (Artegiani et al. 2017; Villani et al. 2017a, b; Glass et al. 2017). Single-cell technologies can also be used to identify subpopulations within a known cell type by

looking for differences in gene expression patterns within the cell population (Artegiani et al. 2017; Shalet and Satija et al. 2013). Furthermore, these technologies can effectively isolate the signal from rare cell populations, which would otherwise be lost in the output of RNA sequencing on a bulk cell population (Shalet and Satija et al. 2014; Grün et al. 2015; Mahata et al. 2014; Torre et al. 2018). Besides that, the technology can be used to infer potentially useful markers for cell types that lack known markers, such as cell surface proteins. Because single-cell sequencing is driven by cell clustering based on differentially expressed genes, the genes that drive the clustering can be studied as potential unique markers for the cell population of interest (Artegiani et al. 2017; Zhao and Gao et al. 2017). Finally, single-cell sequencing can be used to investigate cell lineage and differentiation regulation. A population of stem cells, for example, can be induced to differentiate, and single-cell sequencing at various time points can provide "snapshots" of the differentiation process. The trajectories that cell flows to reach each terminally differentiated state, as well as the key genes that are differentially regulated at each branch point, can then be inferred using these snapshots (Artegiani et al. 2017; Treutlein et al. 2014; Trapnell et al. 2014; Qiu et al. 2017).

Biological tissue samples are frequently used as an input material for single-cell experiments. In the first phase, a single-cell suspension is created by digesting the tissue in a process known as single-cell dissociation. Cells must be isolated to profile the mRNA in each one separately. Depending on the experimental protocol, single-cell isolation is done differently. Droplet-based approaches focus on catching each cell in its microfluidic droplet, whereas plate-based methods separate cells into wells on a plate. Multiple cells can be captured together (doublets or multiplets), non-viable cells can be captured, or no cell can be captured at all (empty droplets/wells) in both circumstances. Droplet-based approaches rely on a low concentration flow of input cells to manage doublet rates; hence empty droplets are typical. Each well or droplet includes the chemicals required to break down cell membranes and perform library construct. The process of capturing intracellular mRNA, reverse-transcribed to cDNA molecules and amplified, is known as library construction. The mRNA from each cell can be labeled with a well- or droplet-specific cellular barcode, while the cells go through this process in isolation. Moreover, captured molecules are labeled with a unique molecular identifier (UMI) in many experimental protocols. To enhance the probability of being measured, cellular cDNA is amplified before sequencing.

Cellular cDNA libraries are labeled with cellular barcodes and, depending on the protocol, UMIs after library formation. For sequencing, these libraries are pooled together (multiplexed). Read data is generated by sequencing and is subjected to quality control, grouping based on assigned barcodes (demultiplexing), and alignment in reading processing pipelines. Read data can be further demultiplexed for UMI-based methods to produce counts of captured mRNA molecules (count data). However, analyzing and utilizing the large amounts of data created by single-cell RNA sequencing research is difficult and requires knowledge of the experimental and computational pathways that go from the preparation of input cells to the production of interpretable data. Single-cell gene expression analysis was previously

limited to a few select transcripts from a few individual cells. Modern single-cell sequencing platforms like as Fluidigm C1, Drop-Seq, Chromium 10X, SCI-Seq, and many others have been developed during the past decade thanks to high-throughput sequencing and high-yield cell separation approaches. At any given time, these technologies can define the transcriptional profile of hundreds to thousands of single cells. All rely on the use of DNA barcodes to label mRNA molecules during reverse transcription and/or later processes, allowing the transcripts to be indexed back to their individual cells of origin. Despite the fact that each technique has its own manner of separating cells and labeling mRNA molecules, they all use the same computational pipelines to represent transcriptional profiles.

Single-cell gene expression analyses have not been widely used in plants to date, owing to the presence of the plant cell wall, which makes it difficult to separate and acquire individual cells. Although there is recognition of the potential benefit of large-scale single-cell transcriptome studies in plants, single-cell gene expression studies in plants have so far been limited to a small number of cells (Lieckfeldt et al. 2008; Brennecke et al. 2013; Efroni et al. 2015; Frank and Scanlon 2015; Efroni and Birnbaum 2016; Libault et al. 2017). Several groups have recently used single-cell transcriptomics to plants with high throughput (Denyer et al. 2019; Efroni et al. 2015; Efroni et al. 2016; Jean-Baptiste et al. 2019; Kubo et al. 2019; Nelms et al. 2019; Ryu et al. 2019; Shulse et al. 2019; Zhang et al. 2019). Plant studies using single-cell RNA-seq have primarily focused on the well-studied and understood *Arabidopsis* root system (Denyer et al. 2019; Jean-Baptiste et al. 2019; Ryu et al. 2019; Shulse et al. 2019; Zhang et al. 2019). The *Arabidopsis* root, in particular, is a useful organ for scRNA-seq because it has a relatively small number of cells and cell types, and methods for isolating individual cells by protoplasting are available (Birnbaum et al. 2005; Bruex et al. 2012; Efroni et al. 2015; Li et al. 2016). Even in this highly tractable and well-understood system with many known marker genes and cell types, these landmark studies revealed a slew of new and more robust cell-type marker genes and begun to characterize the transition states that give rise to developmental trajectories (Denyer et al. 2019; Jean-Baptiste et al. 2019; Ryu et al. 2019; Shulse et al. 2019; Zhang et al. 2019). Recently, Qing et al. (2020) performed the scRNA-seq on root tips of two agronomically important rice cultivars and identified more than 20,000 single cells. Using integration analysis of two cultivars, most of the major cell types were identified, and novel cell-type-specific marker genes for both cultivars were characterized. In addition, they found well-conserved cell types between the two rice cultivars associated with specific regulatory programs, including phytohormone signaling, biosynthesis, and response. To identify the effects of tissue heterogeneity, Dorrity et al. (2021) applied scATAC-seq and scRNA-seq to *Arabidopsis* roots separately. They identified thousands of differentially accessible sites using scATAC-seq results and the entirety of a cell's regulatory landscape and its transcriptome using scRNA-seq. To define the endoreduplication, cell division, and developmental progression, they integrated the scATAC-seq and scRNA-seq data and characterized cell type-specific motif enrichments of transcription factor family analysis and linked the expression of family members to changing accessibility at specific loci, resolving direct and indirect effects that shape expression (Dorrity et al. 2021).

2.19 Conclusion

The omics technologies have been tremendously developed since their first-time introduction in plant science, which was flowed by exponential studies in different plant taxa. At present, at least one research study on plant omics is published every day. These omics studies have generated extensive data such that the pace of software development to analyze this much of data cannot meet the demand. In the past, individual studies of genomics, transcriptomics, or metabolomics were enough to make a judgment about the plant species or genotypes. However, now the focus has shifted from generating high-throughput biological data sets to the integration of these data sets to derive biological meaning out of it. These data sets are valuable for future efforts in establishing models that can describe plant adaptation, cultivation, and production. Novel approaches such as artificial intelligence and machine learning will be required in the near future to get the most out of these data sets and predict the future scenarios, especially under ongoing climate crises.

In the near future, plant biologists will focus on understanding the interactome of different metabolisms in the plant and how these interactions are affecting the phenome. They will utilize integrated omics technologies together with genome editing and speed breeding. Identification of novel genomic, proteomic, or metabolomic markers will be very useful in screening different plant genotypes, wild relatives, or breeding lines to find and develop new cultivars highly adapted to the changing climate with higher yields and nutritional quality.

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Chapter 3
Multi-omics Approaches for Strategic Improvements of Crops Under Changing Climatic Conditions

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3.1 Introduction

Variations in climatic conditions generally disturb the agro-ecosystems and food supply chain (Hasegawa et al. 2018). As agriculture significantly depends on the prevailing climatic conditions, thus crops often undergo external environmental and/or abiotic stresses due to climate variations (Hussain et al. 2018). Crop plants may suffer individual and concurrent effects of many abiotic stresses, such as salinity, chilling, drought, waterlogging, heavy metals, and temperature fluxes, which extensively impede the growth and development and reduce agricultural productivity over the globe (Ashraf et al. 2015; Raza et al. 2020; Ashraf et al. 2017a,b; Anjum et al. 2017). Moreover, global warming due to emissions of greenhouse gases also resulted in an increase in temperature and drought conditions that also threaten crop productivity, e.g., 20–30% yield loss was noticed in two major cereals, i.e., wheat and maize (Wang et al. 2019). Previously, traditional plant breeding techniques have produced several widely used high-yielding crop cultivars throughout the world for decades; however, requirements of longer time span on a variety of development and breeding cycles are the major drawbacks regarding implementation of such techniques. In addition, it is a roadblock in plant breeders' ability to respond quickly to rising food supply demands under changing climate scenarios (Lenaerts et al. 2019).

In the current era, the focus of plant breeding is to develop high-yielding crop varieties for the traits under consideration with minimal interventions during crop growth period. This has been accomplished by adopting modern biotechnological techniques which helped introduce genes with novel properties by breaking the inter-species and inter-genus barriers (Moose and Mumm 2008; Shiferaw et al. 2013). This all aims to furnish crops at a faster rate with the properties which their ancestors possessed to tolerate adverse environmental conditions. This does not mean that conventional breeding is ruled out from the agricultural system due to its inappropriateness. Actually, the current century is based on techniques to realize maximum output from the existing agricultural system due to huge food demand for rapidly increasing population from the existing set of agricultural land (Moose and Mumm 2008; Voss-Fels et al. 2019). The said task that increased production from limited land area with better adaptability for adverse environmental conditions could not be accomplished by depending merely upon conventional breeding. Although conventional breeding techniques are classical, most promising with least controversies associated with them, but, the time required to get the objective, blindness to ascertain the intrusion of target trait and transfer of only a fraction of genetic variations among crossing species, hindered its efficacy (Swamy and Sarla 2008). These factors majorly paved the way in the adoption of modern plant breeding and multi-omics approaches to address the debate of "sustainable agricultural intensification" by advocating ever-increasing global food demand under climate change conditions (Tester and Langridge 2010; Cheema 2018). The general impacts of abiotic stresses due to changing climate and importance of multi-omics approaches for crop improvement have been depicted in Fig. 3.1.

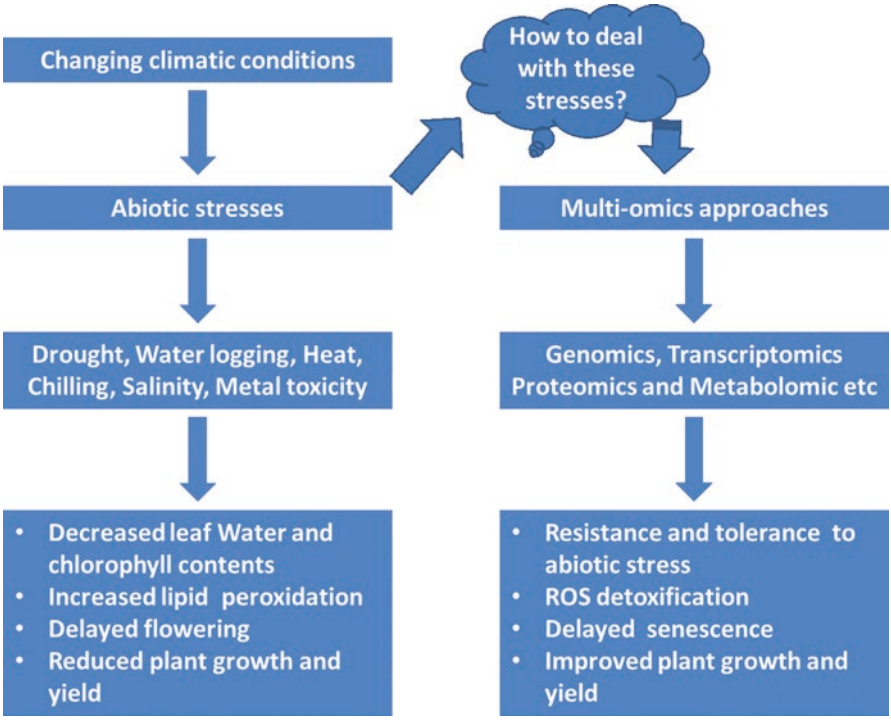

Fig. 3.1 Impacts of abiotic stresses on crop plants due to changing climate and role of multi-omics approaches in crop improvement

3.2 Impacts of Changing Climate on Food Crops

Crop production is vulnerable to climate change, which linked to an increase in carbon dioxide levels, shifting rainfall patterns, and rising temperatures (Mo et al. 2017). Enhancing agricultural output in one way while also addressing climate change risks on the other war is a difficult task. As a result, we need to pay greater attention to adaptation and mitigation. Climate change has substantial impacts on food crops; therefore, changes in policies, as well as the assistance of national adaptation funds and other resources, are required to alleviate negative influences of climate change on crop plants especially food crops. The growth responses, yield variability, and crop performance of vegetables, cereals, and pulses have been discussed below:

3.2.1 Vegetables

Vegetables are the best source of micronutrients and provide more revenue to smallholder farmers than staple crops (Rojas et al. 2013). Among vegetables tomato, cabbage, onion, hot pepper, and eggplant are widely cultivated (Julkowska and Testerink 2015). In Asia, the highest yields are generally obtained in the east region, where the temperature is mostly temperate or sub-temperate. Because most vegetables prefer lower temperatures, thus the production is the lowest in hot and humid lowlands of Southeast Asia (Ali 2000). Average tomato yields in sub-Saharan Africa (excluding South Africa) and tropical Asia are only approximately 10–12 MT/hectare, well below those in temperate areas. The intensity of environmental stress placed on vegetable crops will be influenced by climate change. For instance, rising temperatures, decreasing irrigation water supply, floods, and salt will be key stumbling blocks to maintaining and growing vegetable output. Temperature fluctuations hider vegetable growth and productivity, e.g., high temperatures are common in the tropics during the growing season, and as the climate changes, crops in this region would be susceptible to high-temperature stress. Temperatures are rising, according to climatic trends in tomato-growing areas, and the intensity and regularity of above-optimal temperature events will rise in the future decades (Tao et al. 2008). Temperature, alone or in combination with other environmental variables, has a significant impact on tomato vegetative and reproductive activities (Makeen et al. 2021). Plants' metabolic processes that are essential for regular cell and photosynthetic activity are disrupted by high temperatures (Ergin et al. 2021). High temperatures can impair tomato output by reducing fruit set and producing smaller, lower-quality fruits (Solankey et al. 2021). Moreover, high-temperature stress shortens the time of photo-assimilation, triggers the developmental stages in determinate vegetables, and hampers fruit-set of fruiting vegetables (Bisbis et al. 2018).

3.2.2 Cereals

3.2.2.1 Maize

Temperatures are predicted to increase 1.5–4 °C globally during the next century (Vescio et al. 2021). Temperatures that are higher than the optimum for plant development are thought to be harmful for successful crop production (Battisti and Naylor 2009). Warmer temperatures and greater vapor pressure deficit (VPD) reduced yield of maize by 8.5 ± 1.4% and 12.9 ± 1.8%, respectively, as compared to the normal conditions, while applying both variables at the same time reduced yield by 24.2 ± 2.8%. Higher CO_2 concentrations, on the other hand, increased maize production by 17.2 ± 3.5%. When increasing CO_2 levels were combined with warmer temperatures or greater VPD, this rise in yield reduced, and the combination of the three climatic impacts (Temp, VPD, and CO_2) largely cancelled each

other out, resulting in a lesser drop in yield of −4 ± 3.4% (Resop et al. 2016). The onset of drought conditions impaired the normal metabolism, photosystems, and chlorophyll pigments in maize (Efeoğlu et al. 2009). Drought conditions hampered the early establishment of maize, whereas seed priming with Zn and Se enhanced germination and antioxidant activities (Nawaz et al. 2021). Moreover, Dong et al. (2021) reported that heat tolerance in long-season than short-season maize lines is subjected to more kernel formation by sustaining comparatively stable anthesis-silking interval. Based on meteorological and phenological data for 30 years, i.e., 1981–2010, it was observed that the vegetative growth interval has extended in spring-planted and inter-cropping maize and shortened in the summer season, whereas reproductive period has extended in spring- and summer-planted maize (Liu et al. 2021a, b). Overall, quantification of the changes in crop phenological development stages owing to climate change and anthropogenic management activities may assist to design promising climate change adaptation strategies in crop plants.

3.2.2.2 Rice

Rice, a staple crop in Asia, nourishes roughly 557 million people and is critical for maintaining food security, promoting rural employment, and generating export money (Xu et al. 2012; Le 2016). Rice yield growth in Asia declined at the start of the twenty-first century, and it is unable to keep up with the regional rapid population increase, resulting in high demands, supply shortages, and high prices. Rice is typically very susceptible to climatic fluctuations; thus recent trends of climate change would have serious impacts on its growth and productivity (Maruyama et al. 2014a, b; Kong et al. 2017). Future climate change is unknown, which would exacerbate the food security issues in Asia and other vulnerable regions (Palazzo et al. 2017; Carpena 2019).

Climatic change is responsible for a third of worldwide rice, maize, wheat, and soybean production variability, i.e., 32–39% (Ray et al. 2015), whereas the effects on rice yield in various locations have been estimated using external factors that include temperature, CO_2 fertilization, rainfall, and other climatic aspects. Without accounting for CO_2 fertilization effects, predictions using an "InfoCrop rice model" under the A1b, A2, B1, and B2 scenarios suggested that rice yields in India would decline by 4% in 2020, 7% in 2050, and 10% in 2080 (Soora et al. 2013). Rice yields in China will reduce by 6.1 to 18.6%, 13.5 to 31.9%, and 23.6 to 40.2%, respectively, if world temperatures rise by 1, 2, or 3°C (Tao et al. 2008). Under the representative concentration route (RCP) 8.5, Cambodia would see the greatest loss in rice yields in Southeast Asia (about 45% in the 2080s) (Chun et al. 2016). Climate change would have a detrimental influence on rice production. Other researches, on the other hand, show that climate change has a favorable influence on rice production. For example, according to the A2a, A2b, A2c, and B2a scenarios, rice yields in Bangladesh would improve by 7.58%, 8.31%, 4.57%, and 1.67%, respectively.

Variations in rice output in major rice-producing nations such as Bangladesh, China, India, and Myanmar (BCIM) were observed as a result of climate change. The BCIM countries have been identified as the world's most climate-vulnerable areas because they account for 60.63% of world rice production and 59.74% of global rice consumption; these four nations play critical roles in the global rice trade pattern. A study conducted in Nepal reported that climate change has a significant impact on rice production, with an increase in severe precipitation having a negative impact. According to the extreme climate condition model, a 1% increase in days with extreme rainfall variation (i.e., three standard deviations above or below the long-term average) reduces rice output by 0.28% or 5.34 kg per family (Baldos et al. 2019). However, there is little indication that the rise in severe temperature days has had a similar impact. The average climate condition model, on the other hand, shows that a rise in long-term average monsoon temperature has a considerable negative influence on rice production. They further reported that a 1 °C rise in average summer temperature reduces rice output by 0.48% (4183 kg) per season; nevertheless, with increasing average temperatures, this impact diminishes. However, no evidence was found about the linkage of rise in average monsoon precipitation with increased rice output (Janes et al. 2019).

3.2.2.3 Wheat

Climate change may cause prolonged dry spells or more intense heat waves in some areas, thus severely reducing agricultural productivity by inducing water and temperature stress, especially during critical crop growth stages (Rounsevell et al. 1999). Dry land areas, such as dry and semi-arid parts of South Asia, are particularly susceptible to climate change, especially given their current water scarcities and higher temperatures approaching tolerance limits (Liu et al. 2019). Land degradation and restricted water supplies have hampered current agricultural output and put the food security of most of the countries in this region in danger.

Monsoon patterns, driven by monsoon lows and depressions in the Arabian Sea and the Bay of Bengal, dominate the weather system in Swat (Pakistan). The average annual temperature in Chitral district is 16 °C, and this area receives 451 mm of total annual rainfall, with more than 60% of that falling during the wheat growing season (October–April). The average annual temperature in Swat district is 19 °C and it receives 966 mm of rain annually, with 41% of that falling during the wheat growing season (October–March). These two districts were chosen to enable a comparison of the effects of climate change on wheat production at various elevations and in different weather systems. Wheat cultivated in the highlands and foothills is generally utilized for both food and fodder. Wheat is cultivated for food up to a height of around 1500 meters. On the other hand, wheat is used as fodder above this temperature since lower temperatures prevent the crop from ripening into edible grain (Hussain 2003). For temperature rises of 1.5 °C and 3.0 °C, yields in Chitral district (Pakistan) are anticipated to increase by 14% and 23%, respectively (Hussain and Mudasser 2007). Furthermore, direct and indirect impacts of climate change on

wheat productivity were assessed using crop models by Daloz et al. (2021) and reported that the direct impacts (via fluctuations in temperature and precipitation) caused –1 to –8% wheat yield loss, whereas indirect impacts (via changes in water availability) led to –4% to –36% yield loss in wheat. Moreover, climate change conditions were found to substantially affect the phenology and yield of wheat, while these effects can be minimized by some agronomic strategies by modifying sowing times and irrigation scheduling (Azmat et al. 2021). Rise in temperature could enhance water deficit and the chances of drought in winter wheat (Zhang et al. 2021a, b). Hence, the uncertainty and complexity of climate change, it is important to modify agronomic, crop management, and breeding strategies to induce the crop tolerance and resilience in food crops to cope against deleterious effects of climate change.

3.2.3 Pulses

Climate change has been one of the most widely debated topics in recent decades, owing to its direct influence on food supply and security. Changes in the statistical distribution of weather patterns have a significant influence on agricultural crops, especially rain-fed crops such as pulses (Gorim and Vandenberg 2017). Pulse crops suffer from forced maturity and reduced yields as a result of very high temperatures throughout the growing season, particularly during flowering and pod development. Drought, flood, excessive salinity, soil acidification, waterlogging, and other abiotic stressors are made easier by variations in rainfall patterns connected with humidity and their associated consequences (Basu et al. 2009). The impacts of abiotic stresses induced by changing climatic conditions on vegetables, cereals, and pulses have been presented in Table 3.1.

3.3 Conventional Breeding vs Novel Approaches for Crop Improvement

3.3.1 Modern Breeding Approaches for Crop Improvement

Crop breeding has been boosted by the addition of mutagenesis techniques which further expanded on a larger scale with the emergence of recombinant DNA technologies, both contributed in bio-diversification of crop plants to a great extent (Sammina et al. 2021; Wang et al. 2012). Genetic alteration of crop plant for one or multiple genes by recombinant DNA technology relies on *Agrobacterium*-mediated transformation. This technique which remained in use since the 1990s has marked several success stories in the production of major food and oil crops with improved resistance to biotic and abiotic environmental conditions. These crops include

Table 3.1 Impacts of different abiotic stresses on vegetables, cereals, and pulses owing to changing climatic conditions on food crops

Crop type	Crop name	Experiment type	Stress type	Impact on crop	References
Vegetables	Potato (*Solanum tuberosum*)	Field	Drought	Drought negatively affected the primary vegetative growth and tuber formation in potato	Rojas and Pino et al. (2013)
	Tomato (*Lycopersicum esculentus*)	Field	Salinity	Salinity reduced shoot and leaf growth and causes premature death of leaves	Julkowska and Testerink (2015)
	Brinjal (*Solanum melongena*)	Botanical garden of University of Agriculture, Faisalabad (UAF)	Salinity	Salinity reduced plant growth and net carbon dioxide assimilation rate	Shahbaz et al. (2013)
	Cauliflower (*Brassica oleracea*)	Field	Low temperature	Low temperature increased yield and number of curds	Kalisz et al. (2014)
	Radish (*Raphanus sativus*)	Field	Heat	Reduced yield	Gao et al. (2019)
	Pumpkin (*Cucurbita pepo*)	Field	Salinity	Decreased chlorophyll content and root and shoot biomass	Akram et al. (2017)
	Beetroot (*Beta vulgaris*)	Field	Drought	Reduced plant growth, height, and leaf area	Hosseini et al. (2019)
	Spinach (*Spinacia oleracea*)	Lab	Salinity	Reduced water uptake efficiency, total phenolic contents, and seed vigor	Ibrahim et al. (2019)
	Lettuce (*Lactuca sativa*)	Field	Salinity	Reduced growth, relative leaf water contents, stomatal conductance, and nutrient elements	Yildirim and Correia (2015)
	Cucumber (*Cucumis sativus*)	Lab	Drought	Reduced plant growth and productivity	Akula and Ravishankar (2011)

Crop type	Crop name	Experiment type	Stress type	Impact on crop	References
Cereals	Barley (*Hordeum vulgare*)	Field	Salinity and drought	Reduced grain size and yield loss	Korhonen et al. (2019)
	Wheat (*Triticum aestivum*)	Field	Drought	Reduced grain quality and yield loss	Liu and He et al. (2019)
	Rice (*Oryza sativa*)	Field	Salinity and drought	Increased sucrose content in leaf and root tissues	Mathan et al. (2021)
	Chia seeds (*Salvia hispanica*)	Field	Salinity	Reduced carbohydrate content and lipid peroxidation	Younis et al. (2021)
	Oats (*Avena sativa*)	Field	Drought	Reduced leaf area and grain yield	Zhou and Yarra (2021)
	Sorghum (*Sorghum bicolor*)	Field	Heat	Increased production of flavonoids and decreased the microbe interactions	Cloutier et al. (2021)
	Maize (*Zea mays*)	Field	Frost	Decreased primary and lateral root growth	Vescio and Abenavoli et al. (2021)
	Rye (*Secale cereale*)	Field	Drought	Yield loss	Godoy et al. (2021)
	Corn (*Zea mays*)	Green house	High temperature	Reduced plant growth	Amrutha et al. (2021)

(continued)

Table 3.1 (continued)

Crop type	Crop name	Experiment type	Stress type	Impact on crop	References
Pulses	Lentil (*Lens culinaris*)	Field	Drought	Yield loss, delayed flowering, and reduced plant growth	Gorim and Vandenberg (2017)
	Chickpea (*Cicer arietinum*)	Field	Salinity	Decreased leaf water and chlorophyll contents and increased lipid peroxidation	Kaur (2014)
	Mung bean (*Vigna radiata*)	Field	High temperature and waterlogging	Yield loss, reduced the number of flowers and production of pods	Singh et al. (2011)
	Peas (*Pisum sativum*)	Field	Drought and frost	Decreased yield and net profit ratio	Kumar et al. (2019)
	Faba beans (*Vicia faba*)	Field	Low and high temperature	Damaged reproductive organs	Zhang et al. (2018)
	Cowpeas (*Vigna unguiculata*)	Field	Drought	Reduced growth rate and produced immature pods	Junior et al. (2021)
	Kidney beans (*Phaseolus vulgaris*)	Field	Salinity	Decreased fresh and dry weight of seedlings and reduced biomass	Osnato et al. (2021)
	Black mung bean (*Vigna mungo*)	Green house	Heavy metals	Yield loss and decreased shoot and root length	Ahmad et al. (2021)
	Dry peas (*Pisum sativum*)	Field	Salinity	Higher accumulation of Na^+ in shoots and roots	Ahmad and Ali et al. (2021)
	Broad beans (*Vicia faba*)	Field	Drought	Reduced plant height and the number of pods per plant	Ucar et al. (2021)

wheat, rice, maize, soybean, cotton, rapeseed, sunflower, etc., grown worldwide as major transgenic crops with improved yield and least use of agrochemicals (Young et al. 2019). However, besides the immense contribution of transgene technology in crop improvement, in the recent decade, attention is paid to precise editing of plant genome. Introduction of novel genome sequencing, genotyping, and genome editing tools has revolutionized plant breeding worldwide. These methods allow for targeted and precise DNA alteration/modification in a short time and are considered to have a greater impact on plant genetics and breeding program (Chen et al. 2019; Li et al. 2019; Young et al. 2019). These novel technologies include next-generation sequencing (NGS) (Varshney et al. 2009), high-throughput marker genotyping technology (Kumar et al. 2012; Mammadov et al. 2012, Chen et al. 2015; Singh et al. 2016), and most recently cutting-edge technique called clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) (Mao et al. 2013; Zhang et al. 2016; Lee et al. 2019). All these technologies are based on detection of genetic variation within a gene pool.

3.3.2 Genome Applications by Next-Generation Sequencing (NGS) Technology

Next-generation sequencing (NGS) in the current era is meant for a platform which provides nucleotide information of millions of sequence reads in a cost-effective manner within a short time (Varshney et al. 2009). Three major approaches are currently being used under NGS which includes applied biosystems such as SOLiD, genome sequencer FLX (Roche/454), and Illumina genome analyzer each having their own mechanism, benefits, and drawbacks (Gupta 2008; Mardis 2008; Shendure and Ji 2008). All three approaches are in use with differential impacts and effects based on objective, target species, and resource availability. The common among all is to generate nucleotide sequence of millions of reads within a short time with a variable potential. The genome sequence obtained has revolutionized the field of molecular marker development and marker-assisted selection (MAS) in plant breeding programs. Several success stories have been marked in multiple plant species, e.g., soybean (Hyten et al. 2008), maize (Barbazuk et al. 2007), sorghum (Paterson et al. 2009), rice (Goff et al. 2002), potato (Diambra 2011), apple (Velasco et al. 2010), etc. Apart from its role in rapid molecular marker development, other applications of NGS include association and comparative mapping, de novo sequencing, polymorphism detection, whole-genome analysis, transcriptome expression analysis, evolutionary biology, and population genetics (Varshney et al. 2009).

Decreasing the cost of sequencing with the passage of time is promoting its use for the crops having large genome size, polyploids, and underutilized crops which otherwise are not being paid attention before. The NGS data obtained could be manipulated in various ways by the use of data analysis tools for various applications. These applications could be structural/functional genomics, DNA-protein

interaction, polygenetics, meta-genomics, and single-cell genomics, and the knowledge obtained from these studies could be utilized to decipher the complex network of genes with various biological functions, like never before. In the future, NGS technology has the potential to boost the crop improvement programs to its next level with more number of sequenced plant genomes (Kumar et al. 2012; Mammadov et al. 2012; Chen et al. 2015; Singh et al. 2016).

3.3.3 High-Throughput Marker Genotyping Technology Role in Crop Improvement

Genome sequencing paved the way to the development of molecular markers in high-throughput manner as per the sequencing data obtained and analyzed by the use of genome analysis tools. The success of marker development also depends upon its cost-effectiveness and high-throughput development with enhanced accuracy within a short period of time. Unlike before where few reads were obtained and analyzed for marker presence, NGS made it possible to develop thousands of molecular markers within a short time. Molecular markers exhibit a variety of basic and applied applications in crop improvement programs. These applications associated with successful and presence of abundant identifiable molecular markers include linkage map development, quantitative trait loci (QTLs) mapping in population, genome finger printing, marker-assisted selection (MAS), genetic diversity, and evolutionary analysis (Zalapa et al. 2012). Several types of molecular markers have been developed which include AFLP, SSR, ESTs, SNP, etc., each having variable merits, de-merits, uses, and benefits. A large number of SSRs (simple sequence repeats) and EST (expressed sequence tags) SSRs were developed in multiple crops by using NGS successfully (Kumari et al. 2013; Chen et al. 2015; Hao et al. 2015; Singh et al. 2016).

In addition, the SNP (single-nucleotide polymorphism) are found the most promising and innovative marker as it has the potential to detect the variation at the single-nucleotide level among the species (Kumar et al. 2012; Mammadov et al. 2012). For genotyping of this most promising marker, two assays are mostly used, i.e., (1) illumine golden gate assay and (2) whole-genome genotyping infinium assay (Rostoks et al. 2006; Hyten et al. 2008; Akhunov et al. 2009; Hyten et al. 2009). Both assays have different base mechanisms to get activated and the processes of development which is called high-throughput marker genotyping. This high-throughput marker genotyping is particularly important for diversity, characterization, and genome/association/comparative mapping (Rostoks et al. 2006; Hyten et al. 2009).

3.3.4 Genome Editing by CRISPR/Cas9 Approach

CRISPR/Cas9 is a nature-occurred gene editing tool and a high-throughput cutting-edge genomics technique having extensive applications in gene transformation, knockout mutation, monitoring of gene expression, and its regulation at the genomic and epigenomic level and in drug discovery (Bortesi et al. 2016; Fiaz et al. 2021). With the addition of CRISPR/Cas9 in the field of molecular biology, it is possible to manipulate or edit an organism's genome in a precise and specific way, leading toward precision breeding. CRISPR/Cas9 is categorized into two main classes and six types: class I (types I, III, IV) and class II (types II, V, VI), with type II being the most widely used system in genome editing (Zhang et al. 2016; Lee et al. 2019). This system is discovered in bacterial and archaeal immune system in which CRISPR loci contained a cluster of consecutive repeats separated by spacer sequence. Other parts of the functional system include a Cas9 encoding operon and transcription setup. In response to immune system activation, these specific repeats get incorporated into bacterial genome by the activity of foreign invader or any viral genome (Jinek et al. 2012; Wang et al. 2012; Zhang et al. 2014). After infection, bacteria produce the same DNA which gets back incorporated into an invader virus along with one nuclease, called "Cas." "Cas" then eventually acts as internal molecular scissors and cuts the invader/virus DNA into pieces. Based on its mechanism of action, it is also known as acquired immune defense system in prokaryotes against viruses (Zhang et al. 2014).

In the recent decade, the CRISPR/Cas9 system has two major targets with respect to crop breeding. Firstly, to find the efficient delivery method to enhance the potential of this system by eliminating off-target effects. Secondly, tracing and modification of key genes controlling growth and development in plants. This happens with the aid of genome sequencing, which is helpful in recognizing and locating specific sequences within the genome, which could be altered by the use of genome editors (Li et al. 2017). In a short period of time, CRISPR/Cas9 has a promising achievement in crop improvement.

Under natural conditions, plants are exposed to various biotic and abiotic stresses simultaneously, which results in reduction of yield and crop quality (Pandey et al. 2017; Ashraf et al. 2020; Hussain et al. 2020). Many of these stresses are under quantitative gene action, and it's very difficult to figure out the exact mechanism of action and control. A remarkable achievement had been attained by creating resistant crop cultivars against diseases through knockout mutants by the use of the CRISPR/Cas9 system. Few success stories include *Potyvirus* in *Arabidopsis*, causing *Turnip mosaic virus* (TuMV) which is a major issue controlled by eIF(iso)4E; thus the knockout mutants using CRISPR/Cas9 have been generated which showed resistance to TuMV (Porter and Grills 2016). Likewise in watermelon, Clpsk1 gene editing showed enhanced resistance against *Fusarium oxysporum* f. sp. *niveum* (Zhang et al. 2020a, b), as well as Os8n3 and OsERF922 mutations exhibited resistance to *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *Magnaporthe oryzae*, respectively (Wang et al. 2016; Kim et al. 2019). Other success examples include genes in

tomato for SiMlo (Nekrasov et al. 2017) and SlJAZ2 (Ortigosa et al. 2019) and mlo in wheat (Diambra 2011; Hückelhoven and Panstruga 2011; Wang et al. 2014). All given genes are effective in imparting resistance against bacterial and fungal diseases which are located and manipulated by the use of CRISPR/Cas9 and resistance created against the pathogen successfully.

Common abiotic stresses which a plant copes with under natural conditions include drought, heat, salinity, and environmental pollution. Lots of structural and regulatory genes are found associated with a single stress condition and not a single gene which plays a dominant role in stress tolerance against any of these abiotic stress conditions (Zhang 2015; Zafar et al. 2020). Recently, ARGOS8 gene regulating ethylene production in maize is manipulated by the use of CRISPR/Cas9 and as a result enhanced drought tolerance observed in mutant plants under field conditions (Shi et al. 2017). Likewise in tomato knockout mutant for the gene SIAGAMOUS-like6 allowed the plant to tolerate heat stress successfully (Klap et al. 2017). Water use efficiency in tomato enhanced under drought and salinity stress in knockout mutant of ARF transcription factor (Bouzroud et al. 2020). The "G protein" mutants gs3 and dep1 in rice enhanced tolerance against salt stress (Cui et al. 2020), while ppa6 to alkaline stress positively (Wang, Wang et al. 2019). Thus, CRISPR/Cas9 is not only useful in creating knockout mutants but also useful in activation and regulation of gene expression (Meng et al. 2015; Wang et al. 2017; Paixão et al. 2019; Peng et al. 2020). Overall, to cope with fluctuating adverse environmental conditions is a complicated task and controlled by multiple genes or gene network with different magnitude of effects. In the current era, plant breeders had made a remarkable progress in a very short time by the use of the CRISPR/Cas9 approach which otherwise was nearly impossible. The quickly developed and adopted CRISPR/Cas9 approach provided a perfect tool to realize yield increments with improved tolerance to adverse environmental conditions. Current multiple omics, including whole-genome sequencing (DNA, RNA), further aid into the success stories in CRISPR/Cas9-based crop improvement.

3.4 Omics Approaches for Crop Improvement Under Climate Change Scenario

Plant molecular biology is concerned with the study of biological processes, their genetic regulation, and interactions with the environment (Yadav et al. 2020). In recent years, advances in omics technologies like genomics have made molecular biology research more efficient. As a result, the linkages between molecular machineries may be examined in great detail, allowing researchers to incorporate genes, proteins, and a wide range of other key regulatory components into their research (Muthamilarasan et al. 2019). The term "omics" refers to this large-scale research. The genomic, transcriptomic, proteomic, and metabolomic analyses are all important aspects of omics (Subramanian et al. 2020). These omics methods are often

utilized in many agricultural and plant research areas. As technology develops, omics techniques have advanced dramatically in the previous decade (Anders et al. 2021). All these aspects of omics are discussed below:

3.4.1 Genomic Approach

In genomics, genes and whole genomes are analyzed in depth. It is now possible to read an organism's whole genome using high-throughput sequencing practices such as next-generation sequencing (NGS) (Tanaka et al. 2020). The stress-related genes may now be predicted with the use of genome-wide association studies, or GWAS, and next-generation sequencing (NGS) (Saidi and Hajibarat 2020). The use of genomics techniques has grown in relevance for the discovery of genes involved in the tolerance of multiple abiotic stresses. Researchers have made great progress in the genomics era in scanning the genomes of several crop species during the last few years (Brozynska et al. 2016).

Furthermore, genomics is critical in identifying genetic diversity that underpins improved performance and breeding efficiency, therefore contributing to crop species' genetic improvement. Quantitative trait locus (QTL) mapping is an outstanding mode to explore the genetically complex systems for abiotic stress tolerance in plants (Shen et al. 2018). Through QTL mapping, specific resistance loci were studied which indicates the presence of genes involved a development process of the plant during stress conditions (Adhikari et al. 2019). For instance, "B73" and "Mo17," the two inbred maize lines commonly used in American maize germplasm, have varying levels of resistance to low temperatures during the seedling stage. In addition, seedling reaction to low-temperature exposure varied greatly among different inbred maize lines. Quantitative trait loci (QTLs) were identified to study this variation from the recombinant line population of maize. Two QTLs with several candidate genes were identified which also involved in hormone response and abiotic stress response (Qiu et al. 2020).

Different types of markers are involved in genome-wide analysis of the desired traits and use them in crop improvement during the breeding process as RFLP (restriction fragment length polymorphism), RAPD (rapid amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), SAMPL (selectively amplified microsatellite polymorphic microsatellite loci), SRAP (sequence-related amplified polymorphism), and TRAP (target region amplification polymorphism) (Singh et al. 2021; Soltabayeva et al. 2021). However, with the introduction of next-generation sequencing (NGS) for large-scale genotyping and genomics-assisted breeding, SNPs have been the most preferred markers (Thabet et al. 2021).

Recently, Liu et al. (2020) identified 26 QTLs from rice cultivars of Vietnam genotyped with SNP markers and found that several genes present in QTLs were involved in salinity stress tolerance and also in signal transduction and hormonal response. These QTLs are further used in the breeding process for crop improvement. Alkali-tolerant gene loci were studied in two varieties of japonica rice under

alkali treatment. Four candidate genes were identified on chromosomes 2 and 3 using next-generation sequencing technology (Sun et al. 2021). Furthermore, 302 wild landraces and modified soybean accessions were sequenced, and 230 selective sweeps and 162 copy number variations were found. By studying genome-wide correlations between novel sequences and domestication or improvement characteristics, 13 previously unidentified loci for agronomic variables were identified which include oil content, plant height, and pubescence shape. Using quantitative trait locus (QTL) data, 230 areas were studied, from which 96 correspond to previously discovered oil QTLs and 21 contain genes involved in fatty acid production (Zhou et al. 2015).

3.4.2 Transcriptomic Approach

It is possible to discover genes that respond to stress through the use of genome-wide transcriptome analysis methods. Through plant breeding and/or gene editing methods, these genes might serve as prospective targets for improving drought stress tolerance (Dudziak et al. 2019). Transcriptional responses to abiotic stress have been studied using a variety of techniques, including expressed sequence tags (ESTs), superSAGE, and microarrays. These findings show that abiotic stress alters the expression patterns of numerous genes involved in diverse biological processes (Alonge et al. 2020). It is possible to quantify transcriptome alterations underlying developmental transitions and stress responses using RNA sequencing at the genome scale (Ding et al. 2020).

It is via ESTs that new gene structures, such as expression systems, gene maps, and cDNA sequencing initiatives, may be discovered and built upon. The ESTs have long been regarded as the ideal method for revealing sequence-related information under adverse environmental circumstances (Khazaei et al. 2020). It has been shown that SAGE is a powerful tool for studying gene expression. The SAGE technique generates a tag that identifies a gene transcriptome product. SAGE is regarded as the most cost-effective and high-throughput method for mRNA isolation, cloning, and sequencing (Kumar et al. 2015). The "tag" used in the SAGE method is a short nucleotide sequence with a pointed head-to-head specific restriction enzyme. In other words, SAGE represents digitally expressed gene expression. Several SAGE applications are found in plants, such as pathogen-host interactions, plant responses to a variety of abiotic stressors, transcriptome profiling, and the metabolism of numerous hazardous chemicals (Jain et al. 2021).

To identify salt-responsive genes, a variety of methods are being used including cDNA microarrays, serial gene expression analysis, suppression subtractive hybridization, differential display RT-PCR, and complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) (Bajwa and Khan 2021). The cDNA-AFLP's specificity and sensitivity allow it to differentiate between homologous sequences and find even the low-expression genes. However, because of the great sensitivity of this technique, even uncommon transcripts can be identified (Kamyab et al. 2021).

In addition, this is a less labor-intensive and very effective mRNA fingerprinting technique for identifying genes that exhibit differential expression in stressed plants/crops (Zhang et al. 2020a, b).

Gene expression may be observed using cDNA microarrays, which are an effective tool for evaluating the possible roles of many genes. Microarray cDNA studies of gene expression in different plants under a variety of abiotic factors have been well published (ul Qamar et al. 2020). RNA-sequencing is a new technology that makes use of and provides a new perspective on the transcriptome sequence by allowing total access to transcripts. As a result, RNA-seq may be used to replace a variety of approaches for quantifying transcripts, with the added benefit of increased sensitivity and the capacity to distinguish between related gene paralogs that differ by only a few nucleotides (Xu et al. 2021).

The thermotolerant germplasm of rice, SDWG005, was identified, and their anther structures that are involved in thermotolerance were studied. By transcriptome analysis, 3559 differentially expressed genes (DEGs) were identified and divided into categories according to their expressions. The major gene categories involved in thermoregulation include transcription factors and protein and nucleic acid metabolism-related genes (Liu et al. 2020). Differentially expressed genes (DEGs) were also identified from the wheat cultivar, TW004, under salt stress. Expression analysis of DEGs revealed that they are mostly involved in metabolic pathways that are related to salt stress. Different types of transcription factors involved in salt stress tolerance were also identified, such as MYB (Deng et al. 2020).

3.4.3 Proteomic and Metabolomic Approaches

In proteomics, the proteome in a complicated biological system under a particular circumstance is studied in detail. Plant proteomics offers a unique chance to study the process and production of various proteins in agricultural plants under distinct environmental conditions. Because of this, it's possible to better understand gene regulation and metabolic pathways within plant species because it offers more information about the proteins' interactions with one another (Jorrin-Novo 2020).

Because of the activation of stress-responsive pathways in response to biotic and non-biotic stressors, the plant proteome changes significantly. Heat-shock proteins, abundant proteins in late embryogenesis, kinases and phosphatases, redox enzymes, secondary metabolism enzymes, osmolyte biosynthetic enzymes, photosynthesis, and enzymatic reactive oxygen species (ROS) scavenger proteins are proteins that are known to play a function in the abiotic stress response (Kosová et al. 2018).

Salt-induced proteome changes were studied in barely landraces using the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) proteomic study. Results revealed that under salt stress, the accumulation of 11 salt-responsive proteins have differed between the two accessions, while 43 proteins were genotype-specific (Jardak et al. 2021). Proteomes of anthers of two soybean cultivars, heat-resistant and heat-sensitive, were studied using the iTRAQ approach. Results

showed that most of the proteins related to heat tolerance are involved in carbohydrate and energy metabolism, protein synthesis and degradation, nitrogen assimilation, and ROS detoxification (Li et al. 2020).

In *Arabidopsis*, drought tolerance is controlled by jasmonate ZIM-domain protein 7 (JAZ7) utilizing TMT-based quantitative proteomics and targeted metabolomics (Meng et al. 2019). *Arabidopsis* AT-Hook-Like10 phosphorylation needed for stress growth control is elucidated using more sophisticated proteomics techniques, such as quantitative phosphoproteomics (Wong et al. 2019). To understand the function of genes in eukaryotic organisms, including plants, RNA silencing has proven to be a novel and potentially useful reverse genetics tool (Rajam 2020).

Metabolomics is a systematic research in which all the metabolites of a crop plant are identified and quantified. A plant's biochemical response to climate change may be described using metabolomics, which allows us to understand the interconnections between different components in a biological system. Other functional genomics techniques, such as genomes, proteomics, and transcriptomics, can be utilized in combination with metabolomics (Razzaq et al. 2019).

Endogenous plant component proteomics and metabolomics study is becoming increasingly popular, and a strong technique like MS imaging is being used frequently to conduct this research. For protein analysis, mass spectrometry methods like matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have been frequently employed (Agarrwal and Nair 2020).

Primary and secondary metabolites make up the majority of the plant's metabolome. The metabolic profiling of primary and secondary metabolites offers substantial information on the biochemical processes that take place during plant metabolism (Sung et al. 2015). Some plant secondary and primary metabolites are related to metabolic processes that are quite complicated. These metabolites may be effectively identified, measured, and studied using contemporary metabolomics methods such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectroscopy (LCMS), and non-destructive nuclear magnetic resonance (NMR) spectroscopy (Che-Othman et al. 2020).

As none of the single technique or instrument can be utilized to examine all of a metabolome's metabolites, many distinct technologies are necessary for comprehensive coverage. The techniques involved in metabolomics study include mass spectrometry (MS) (Yadav et al. 2019), non-destructive nuclear magnetic resonance (NMR) spectroscopy (Cuperlovic-Culf et al. 2019), high-performance thin-layer chromatography (HPTLC) (D'Amelia et al. 2018), gas chromatography-mass spectrometry (GC-MS) (Chang et al. 2019), liquid chromatography-mass spectrometry (LC-MS) (Zhou et al. 2019), direct infusion mass spectrometry (DIMS), ultra-performance liquid chromatography (UPLC), and high-resolution mass spectrometry (HRMS) (Thomason et al. 2018).

Bernardo et al. (2019) studied the drought-induced changes in wheat secondary metabolism. By employing metabolomics, mycorrhizal inoculation was initiated in two wheat varieties at various phases of drought. According to the findings, arbuscular mycorrhizal fungi (AMF) colonization in a water-stressed environment modulates several molecules, most of which are sugars and lipids. Integrated transcriptome

and metabolic profile analysis were used to determine the response of sunflower under drought stress. The findings indicated that increasing the expression level of photosynthesis in sunflower plants delays senescence by affecting many key metabolic pathways and candidate genes (Moschen et al. 2017).

3.4.4 Phenomic Approach

Breeding operations rely heavily on genetic and phenotypic connections. The study of a plant's phenotypic or genotypic expression under certain environmental conditions is known as plant phenomics (Deery and Jones 2021). Due to the intricate biosynthetic mechanisms that regulate various plants' abiotic stress resistance, phenotyping remains a difficult challenge under abiotic stress circumstances. The importance of phenotyping has emerged in the postgenomic age since crop improvement techniques such as GWAS, GS, MAS, and QTL mapping largely rely on high-throughput phenotyping (HTP) in crops (Ndlovu 2020). Modern phenomics methods make use of hyperspectral/multispectral cameras to collect hundreds of reflectance data points in a variety of settings and phases of crop development using discrete narrow bands. Agronomic characteristics may now be rapidly and precisely gathered using phenotyping technology (Atkinson et al. 2018). Furthermore, utilizing highly heritable secondary phenotypes closely related to the selection phenotypes, the main goal of a high-throughput phenotype (HTP) is to reduce the cost of data per plot and enhance the accuracy of the crop-growing season forecast (Matias et al. 2020). The proper mapping of genes/QTLs/alleles responsible for the trait of interest requires precise phenotyping of germplasms. Phenotyping using traditional methods is time-consuming and labor expensive (Bohra et al. 2021). Phenomics has proven successful in selecting field crops that can withstand abiotic stressors, such as drought (Jangra et al. 2021).

High-throughput, precise, and automated measurements of phenotypic information such as plant growth, architecture, and composition are all part of the process. This new technology has allowed for the collection and analysis of a huge amount of phenotyping data, which has previously been a barrier to functional genomics research and agricultural breeding (Pazhamala et al. 2021).

3.4.5 Ionomic Approach

Ionomics is the study of a plant's whole mineral nutrient and traces element makeup. Additionally, the field of ionomics studies the molecular mechanisms that underlie plant elemental composition (Du et al. 2020). There have been several applications of ionomics in plant biology, ranging from studying plant growth and stress biology to meeting human food needs (Shakoor et al. 2016). To find out how metabolic, genetic, developmental, and environmental factors impact the elemental

composition of target tissues and organs, ionome research looks at the entire organism's chemical makeup (Pita-Barbosa et al. 2019).

Ionomics relies on mutant screening and natural variation to uncover genes and alleles that are crucial for elemental accumulation and variations in the ionomes of different genotypes (Hindt et al. 2017). Ionomics-based biomarkers in stress biology can help identify whether or not a plant has achieved a specific biochemical or physiological state under a variety of unfavorable environmental conditions. As a result, they can aid in the screening of plants that are more vulnerable to biotic and abiotic stressors, which is impossible with current high-throughput techniques. Ionomics has also identified genes that govern natural variation in the plant ionome and are important for stress tolerance (Ali et al. 2021).

The physiological and ionomic differences between Cd-tolerant and Cd-sensitive maize genotypes will be investigated using ionomic and physiological studies. Cd-tolerant cultivars had considerably greater proline, phenolics, and antioxidant accumulation, as well as enhanced rhizosphere and root cell sap absorption and translocation of N, P, K, Ca2+, Mg, Zn, and Fe when compared to Cd-sensitive cultivars. These nutrients are crucial in enhancing plant physiological, biochemical, and molecular responses to stress (Abbas et al. 2021). The role of silicon nanoparticles (SiNPs) in rice toxicity reduction was investigated. They observed that molecular priming with SiNPs decreased F accumulation and increased the amount of non-enzymatic antioxidants in rice grains, such as glutathione, flavonoids, anthocyanins, and phenols, leading to improved injuries and yield (Banerjee et al. 2021). A comparison of gluten-containing and gluten-free foods was studied. Ionomic analysis revealed that gluten-free foods contain a high amount of arsenic and mercury than the gluten-containing foods (Punshon and Jackson 2018).

Ionomics study reveals the susceptibility and resistance characteristics of two olive cultivars to discover target genes for the treatment of olive disease (D'Attoma et al. 2019). By increasing the content of non-enzymatic antioxidants in *T. aestivum* L., 60 mg/kg K substantially decreased the harmful effects of Cd (Yasin et al. 2018). *Malus halliana* was subjected to saline-alkali stress, which generated an ion imbalance and Na+ toxicity, and was then analyzed at the molecular level. An ionomics study demonstrated the importance of Ca2+-mediated signaling for maintaining Na+/K+ balance and decreasing stress-induced damage (Jia et al. 2020).

3.5 Abiotic Stress Tolerance in Crops: Integrating Omics Methods

Genomics is the study of the complex biological activities of a cell's genetic material, the genome, using high-throughput technology. Functional genomics is critical for determining and proving the functions and roles of all genes discovered on the genome in cellular metabolism. Genomic analysis is widely used in agriculture to discover single-nucleotide polymorphisms (SNPs) using a comparative genomics method. The ultimate goal is to find SNPs or quantitative trait loci (QTLs)

associated with a certain phenotype. SNPs are relatively prevalent in genome, however, and normally do not generate a discernible phenotype because of significant environmental stress on plants. This is particularly essential in the case of abiotic stress studies that need the monitoring of the wanted phenotypic expression, the tolerance of abiotic stress, to prevent changes in the intended trait under various environmental circumstances due to genotype x environment (G x E) interaction. As a result, genome-wide association studies (GWAS) and genotyping-by-sequencing (GBS) techniques are frequently used to find SNPs linked to abiotic stress tolerance in field crops (He et al. 2014; Unamba et al. 2015; Kang et al. 2016). For numerous field crops, both GWAS and GBS have been used to discover SNPs or QTLs that may be linked to tolerance to a variety of abiotic stressors (Table 3.2). More study is needed to enhance field crop tolerance to abiotic stressors, and more research is needed to assure agriculture's long-term viability.

Transcriptomic is the result that all RNA molecules from the genome are sequenced, profiled, and counted, enabling comprehensive and/or comparative analysis in quantitative terms of gene expression. Gene expression or transcription, on the other hand, varies depending on the stage of development, tissue, and even cells within the same tissue. Transcriptomics, therefore, shows the metabolic activity of the plant at a specific moment in time, development stage, and tissues or on the environmental circumstances. Microarray platforms or next-generation sequencing (NGS) platforms are often used for transcriptomic research, and both provide high-throughput data for all transcripts depending on the available genome sequence in NGS and the sequence depth in microarrays (Unamba et al. 2015). Most work on transcriptome of abiotic stress tolerance reacts to a particular treatment, such as drought, salinity, cold, or heat. However, as previously indicated, the climate change models predict numerous stressors to impact field crop output. Thus, transcriptomic alterations following combination therapies became the principal technique in the study of transcriptomes. The knowledge of changes in gene expression patterns when they are primed is another perspective that may provide insights into the important components to increase the tolerance of the plant toward abiotic stress situations. Both of these approaches are required to get new insights into transcriptome experiments in order to improve field plant abiotic stress tolerance.

Proteomics uses high-performance technologies to identify and investigate proteins, potential post-translational modifications, structures, activities, and interactions with other proteins or cellular components in order to better understand cellular metabolism (Table 3.2). Proteomics research focused on using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to examine the reactivity and/or tolerance of agricultural crops to abiotic stress, as well as the identification and characterization of the protein spots produced. In 2D-PAGE, the first dimension separates proteins based on their isoelectric point (pI), whereas the second dimension separates proteins based on their molecular weight (MW). In theory, no two proteins in a cell can have the same pI or MW, allowing for high-resolution separation with each spot on the gel representing a different protein. Interactions with other proteins, DNA, or RNA can be investigated using yeast two-hybrid systems or protein microarrays, as well as structural studies using nuclear magnetic resonance

Table 3.2 Abiotic stress tolerance in different crops under multi-omics approaches

Omics approach	Crops	Abiotic stress tolerance	References
Genomics	Soybean	Drought	Kaler et al. (2017)
		Salinity	Zeng et al. (2017)
	Rice	Salinity	Naveed et al. (2018)
		Drought	Guo et al. (2018)
	Wheat	Drought	Mwadzingeni et al. (2017)
		Heat	ElBasyoni et al. (2017)
	Cotton	Drought	Batayeva et al. (2018)
		Salinity	Cai et al. (2017)
Transcriptomics	Rice	Metal toxicity	Song et al. (2014)
		Cold, salt	Ganguly et al. (2012)
		Cold	Guan et al. (2019)
	Cotton	Cold, salt, drought	Imran et al. (2020)
		Drought	Hasan et al. (2019)
		Cold	Cheng et al. (2020)
	Soybean	Drought	Xu et al. (2018)
		Aluminum	You et al. (2011)
		Cold	Min et al. (2020)
	Wheat	Cold	Díaz et al. (2019)
		Drought	Ma et al. (2017)
		Water deficit	Reddy et al. (2014)
Proteomics	Cotton	Salinity	Peng et al. (2018)
		Drought	Xiang et al. (2018)
		Chilling	Cheng et al. (2010)
	Soybean	Heat, water	Katam et al. (2020)
		Drought	Hossain and Komatsu (2014)
		Drought, salinity	Yan et al. (2021)
	Wheat	Salt	Zhu et al. (2021)
		Abiotic stress	Soriano et al. (2021)
		Drought	Lin et al. (2021)
	Rice	Salinity	Damaris et al. (2016)
		Chilling	Ji et al. (2017)
Metabolomics	Soybean	Drought	Silvente et al. (2012)
		Heat	Chebrolu et al. (2016)
		Salinity	Lu et al. (2013)
		Chilling	Maruyama et al. (2014a, b)
	Rice	Salinity, drought	Fumagalli et al. (2009)
		Heat	Wada et al. (2020)
		Abiotic	Saia et al. (2019)
	Cotton	Salinity	Liu et al. (2021a, b)
		Drought	Zhang et al. (2021a, b)
		Drought	Saia and Fragasso et al. (2019)
	Wheat	Water	Bernardo and Carletti et al. (2019)
		Abiotic	Guo et al. (2020)
		Nitrogen	Zhang et al. (2017)

(NMR) or X-ray crystallography and subcellular localization using fluorescent proteins like EGFP or X-ray tomography (Moreno-Risueno et al. 2010). Nonetheless, proteomics has been widely utilized to better understand how many field crops, including rice, wheat, and maize, respond to abiotic stress situations (Table 3.2). Proteomics, rather than genomes and transcriptomics, appears to be more promising in terms of giving relevant background information for understanding and creating abiotic stress-tolerant field crops, and technical advances will definitely be necessary to attain these aims.

Metabolomics is the study of all metabolic byproducts, hormones, signaling molecules, and secondary metabolites present in a biological sample using methods such as mass spectroscopy (MS), gas chromatography-MS (GC-MS), liquid chromatography-MS (LC-MS), capillary electrophoresis-MS (CE-MS), and Fourier transform infrared spectroscopy (FTIR) (Oliver et al. 1998; Hong et al. 2016; Tian et al. 2016; Samota et al. 2017). Understanding complex phenotypic responses of plants to changes in environmental conditions requires metabolomic analysis. Metabolomics is perhaps the most difficult of the omics techniques, because the metabolome varies greatly depending on developmental stage and growth factors (Fuhrer and Zamboni 2015). As a result, it is reasonable to state that metabolomics has not been employed efficiently in investigations of field crop abiotic stress tolerance. Despite the fact that it does not yield definitive results like genomes, transcriptomics, or even proteomics, metabolomics provides a consistent metabolic quantitative trait locus (mQTL) that seems to be used as an indicator for breeding experiments to enhance agricultural crop tolerance to abiotic stress (Mochida and Shinozaki 2011). The mQTL can be used as metabolite markers in response to abiotic stress conditions, in addition to phenotypic studies such as GWAS, if the change in metabolite concentration influences the phenotypic change.

The field of omics technologies has broadened as a result of the introduction of high-performance technologies in many study fields. To integrate data from genomics, transcriptomics, proteomics, and metabolomics studies, systems biology techniques are employed (Fig. 3.2). The primary challenge is the integration of data from diverse omics techniques to abiotic stress with different plants and/or genotypes.

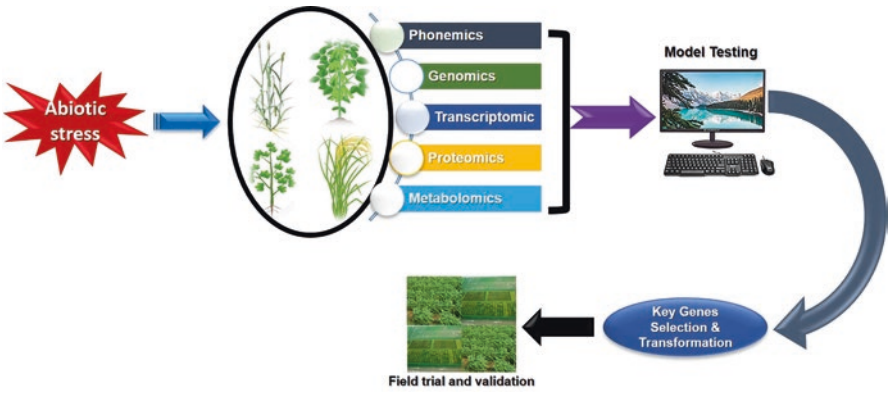

Fig. 3.2 Schematic diagram showing integration of different omics approaches for abiotic stress tolerance in crops

3.6 Conclusion and Future Perspectives

Climate change affects crop growth cycles, phenological development, crop productivities, and food quality. Variations in temperature, CO_2 levels, and rainfall patterns have substantial impacts on agricultural crops; thus it could be a threat to future food security. Studying growth and yield responses of crop plants to different abiotic stresses owing to climate change is important in order to develop climate-resilient crops. Development and integration of modern genetics into classical breeding and development of new plant types and resistant cultivars through genetic manipulation are now being focused to meet the future food demands to reduce food security risk. Multi-omics approaches, i.e., genomics, proteomics, transcriptomics, phenomics, and ionomics, are playing a crucial role to identify a genetic basis of crop performance and improvement as well as stress tolerance mechanisms in crop plants under various environmental stresses. In the future, integration and combination of multi-omics approaches would be helpful to identify potential candidate genes and their pathways to develop models to mark agronomically important traits to enhance crop performance under changing climatic scenario through precision breeding.

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Chapter 4
The Applications of Genomics and Transcriptomics Approaches for Biotic Stress Tolerance in Crops

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4.1 Introduction

One of the major challenges faced by the twenty-first-century agriculture relates to the ever-increasing human population worldwide and the subsequent food requirements. Presently, 820 million people among the global population of 7.3 billion experience chronic hunger; with the population expected to reach 9 billion by 2050, the problem of food security may get even more exacerbated. In order to meet the global food needs by 2050, at least 70% rise in food production has been predicted. Furthermore, there is a need to double the food production in the developing countries of Asia and Africa, where the future population increase is predicted to be more concentrated (Sharma et al. 2017). Owing to the fact that mankind is primarily relied upon plant-based diets, enhancing crop productivity in a sustainable manner is the need of the hour. Crop productivity is often hindered by several factors including both biotic and abiotic stresses. In addition, plants, in its natural habitats, are subjected to a wide array of stresses throughout their life cycle. This comprises both the abiotic (physicochemical) and the biotic stresses. The major abiotic stress factors include drought, cold, heat, UV radiation and high salinity, while biotic stress includes the damages caused by other living organisms, viz. pests and pathogens. Biotic stress is regarded as one of the important causes of global crop losses accounting for about 35% of the total food production (Bainsla and Meena 2016).

In order to cope with the stresses, plants have evolved a robust defence mechanism. Plant responses to biotic stress are highly complex and involve the regulation and expression of a wide array of resistance-related genes and changes at physiological, biochemical, and molecular levels culminating in the tolerance and/or resistance to the biotic stressors (Boyko and Kovalchuk 2008; Li et al. 2019). Yet, the degree of tolerance to various biotic stressors varies widely between different plants. On the counterpart, the pests and pathogens can quickly acquire new virulence factors, overcoming the host resistance. Hence, it is highly essential to improve host plant defence against biotic stressors in order to prevent huge crop yield losses and attain sustainable food production.

Understanding the host plant response to biotic stress and the underlying aspects would pave the way for breeding crop varieties with improved biotic stress tolerance. Breeding for biotic stress tolerance is an indispensable part of most crop improvement programmes. Conventional breeding strategies to develop stress-tolerant crops are now being synergized by various biotechnological tools including molecular markers and omics-based approaches. Recently, the application of genomic and transcriptomic tools has paved the way for better understanding of stress tolerance in model and non-model crops. Over the past decade, there has been an explosion of publicly available *omics* resources that could provide an in-depth understanding of molecular aspects in plant biology research, including the stress tolerance studies. Further, to completely exploit the often heterogeneous and expanding data, bioinformatics tools are continuously developed and upgraded to deal with state-of-the-art sequencing technologies. Analytical methods in bioinformatics are inseparable components in extracting the wealth of data in genome and transcriptome analysis, evolutionary studies, comparative genomics and

genome-wide association studies. Also, transcriptomic datasets have a predictive potential for tissue-specific or condition-specific classification and gene or biomarker identification. With the increasingly accessible multi-omics datasets, the challenge lies in the aptitude to combinatorically analyse the data. In this chapter, we will discuss the various biotic stresses and their responses by plants and how the high-throughput multi-omics approaches can impact studies on biotic stress tolerance in economically important crops. We will also cover the scope of emerging artificial intelligence-based methods in stress biology.

4.2 Biotic Stress

4.2.1 Biotic Stress: General Concepts

The term biotic stress refers to the damage caused to plants by other living entities, including weeds, birds, insect pests, mites, nematodes and pathogenic microorganisms such as bacteria, virus, fungi, etc. Among the biotic stressors, most damage to plants is caused by the pathogens and insect pests. Bacterial and fungal pathogens are capable of inducing a number of symptoms including galls, blights, soft rots, vascular wilts, leaf spots and cankers. Viral pathogens can cause local lesions as well as systemic damage to the plants that is manifested as stunting and chlorosis, among other symptoms. Nematode parasites attack various plant parts and produce symptoms related to nutrient deficiency, viz. stunting and wilting (Schumann and D'Arcy 2006; Doughari 2015; Gimenez et al. 2018). Weeds compete for sunlight, water, nutrients and space with crops. Furthermore, they serve as a breeding ground for insects and pathogens that attack crop plants. The virulence factors of these pathogenic microorganisms may vary widely and include proteins, intra- or extracellular toxins and/or RNA that attacks the plant's immune system. Insect pests and mites are the other major biotic stressors that attack plants. Insect pests can be either monophagous (feeding on a single host) or polyphagous. Most insect pests are surface feeders impairing foliage through chewing (*Plutella xylostella*, a pest of cruciferous crops), piercing or sucking (mealy bugs), while some are subsurface feeders which cause desiccation damage by attacking roots (grubs) (Clark and Kenna 2010). Besides, many insects are viral vectors too; for instance, the brown planthopper, *Nilaparvata lugens* (Stål), transmits ragged stunt and grassy stunt virus.

4.2.2 The Impact of Biotic Stress in Crop Production and Productivity

Biotic stresses have a significant impact on the availability and safety of plants for human and animal consumption. They are serious stumbling blocks to global food production combined with post-harvest spoilage and quality deterioration, with an

Table 4.1 Yield losses due to biotic stress

Biotic stress	Causative agent	Crop	Yield loss	References
Fungus	Blast (*Magnaporthe oryzae*)	Rice	10–30%	Wilson and Talbot (2009)
Bacteria	Wilt (*Ralstonia solanacearum*)	Potato	50%	Mukherjee and Dasgupta (1989)
Virus	Cassava mosaic virus	Tapioca	70%	Fargette and Fauquet (1988)
Viroid	Potato spindle tuber viroid	Potato	59%	Singh and Kaur (2014)
Phytoplasma	Root wilt	Coconut	35–80%	Ramjegathesh et al. (2012)
Insect	Fall armyworm (*Spodoptera frugiperda*)	Maize	15–73%	Day et al. (2017)
Nematode	Rice root-knot nematode (*M. graminicola*)	Rice	15–30%	Khan and Ahamad (2020)
Weeds	*Striga asiatica*	Sorghum	5 to 90%	Obilana and Ramaiah (1992)

estimated yield loss of over 40% resulting in huge economic losses (Oerke 2006; Savary et al. 2019). The extent and severity of biotic stress damages vary from crop to crop and location to location. For instance, among crops, global attainable yield loss due to pests ranged from 50% in wheat to greater than 80% in cotton (Oerke 2006). The impact of various biotic agents in terms of crop loss is depicted in Table 4.1.

It is noteworthy that diseases can cause an annual estimated loss of 10–16% of the global harvest to the tune of US$220 billion (Strange and Scott 2005; Oerke 2006). On the other hand, an estimated 18–20% of annual crop production at a value of $ 470 billion has been reportedly destroyed by arthropod pests globally, with a greater proportion of the losses in the developing countries. On the global scale, the projected yield loss by plant parasitic nematodes is 12.3%, amounting to $157 billion dollars. Weeds account for an annual financial loss of 3.3 billion, USD 11 billion and USD 33 billion in Australia, India and the USA, respectively (Llewellyn et al. 2016; Gharde et al. 2018; Pimentel et al. 2005). Therefore, a key aspect of food security is timely and effective management of aforementioned biotic stresses which often disproportionately impact the most vulnerable and health-disparate populations locally and globally.

4.2.3 Mechanism of Plant Defence Responses to Biotic Stress

Biotic stress is known to co-exist since the evolution of plants, and during these years, plants have developed a sophisticated defence system to overcome the deleterious effects of biotic stress. Plant defence to biotic stress is highly dynamic and includes the constitutive and induced responses (Fig. 4.1). Once a plant senses any

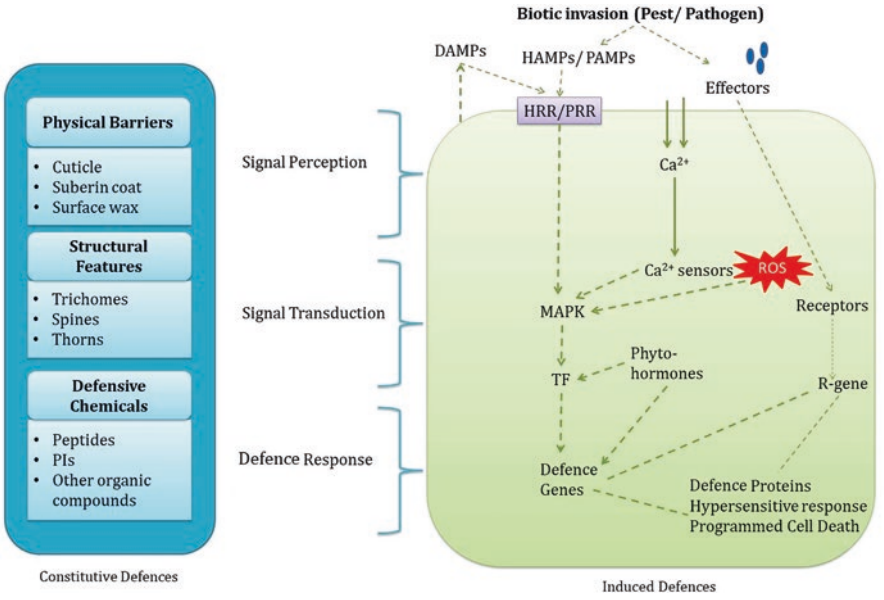

Fig. 4.1 Interplay of various plant defence mechanisms

kind of biotic invasion, a series of cellular and molecular processes are triggered to surmount detrimental effects on its growth and survival.

Plant constitutive defence includes various physical barriers, including cuticle, suberin coat and surface waxes, which help block the entry of various pathogens including fungi and bacteria and structural features including trichomes, spines and thorns to hinder or deter insect pests. These physical barriers serve as passive front-line defence against biotic stress. Also, plants are capable of producing a wide range of defensive chemicals which provide protection against herbivory and microbial invasion. Some of these chemicals are constitutively produced by the plants and include peptides, proteins, protease inhibitors and other organic compounds called secondary metabolites with anti-nutritional or toxic properties (Taiz and Zeiger 2006; Santamaria et al. 2013).

Other mechanisms of defence that are induced upon an attack by a herbivore or microbial pathogen are referred to as induced defence, and it includes the local response (response primarily produced at the site of infestation) and the systemic response (response produced in the distal undamaged tissue of the plant) (Santamaria et al. 2013). Induction of plant response often requires appropriate receptors that perceive the biotic invasion and in turn triggers the signal transduction pathways leading to the expression of appropriate genes involved in defence response. The biotic defence response in plants is known to be triggered by two levels of pathogen recognition. The first level of response, referred to as the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), involves the plant pattern recognition receptors (PRRs) that recognize the pathogen-associated molecular

patterns (PAMPs), which are molecular signatures of the pathogen. PTI is also triggered when the PRRs recognize the damage-associated molecular patterns (DAMPs), which are molecules produced by damaged plant cells or those plant cells undergoing pathogen invasion (Gimenez et al. 2018; Iqbal et al. 2021; Rato et al. 2021). In case of insect herbivores, PRRs recognize the conserved herbivore-associated elicitors (HAEs), herbivore-associated molecular patterns (HAMPs) or herbivore effectors. In the more robust second level, specific effectors from pathogens and herbivores (Avr proteins) are recognized by the plant resistance (R) proteins (Dangl and McDowell 2006). This type of defence is called the effector-triggered immunity (ETI). Most R gene products have a specific domain with conserved nucleotide-binding site (NBS) and also a leucine-rich repeat (LRR) involved in the recognition of pathogen effectors (Dodds and Rathjen 2010).

Initial events that occur upon the sensing of biotic stress by plants include ion imbalances leading to variations in the membrane potentials. Precisely, PTI and ETI initially trigger the activation of membrane channels with subsequent enhancement in the cytosolic calcium (Ca^{2+}) concentrations. Further, the Ca^{2+} sensors in the cells which are Ca^{2+}-binding proteins sense the Ca^{2+} ions modulating the downstream cascade which includes several transcriptional activation events generating specific defence responses. Calmodulin (CaM), CaM-like proteins (CMLs) and Ca^{2+}-dependent kinases (CDPKs) are among the well-identified Ca^{2+} sensors (Santamaria et al. 2013; Iqbal et al. 2021). The generation of reactive oxygen species (ROS) and the activation of mitogen-activated protein kinases (MAPKs) are among the other early responses triggered by PTI and ETI (Muthamilarasan and Prasad 2013). Activated MAPKs modulate the downstream responses which includes phosphorylation of a number of transcription factors that regulate the expression of pathogenesis-related (PR) proteins as well as the phytohormones, viz. salicylic acid (SA), jasmonic acid (JA) and ethylene (Nishad et al. 2020). Abscisic acid (ABA), auxin, brassinosteroids (BRs), cytokinins (CK), gibberellic acid, etc. are among the other hormonal molecules involved in the regulation of plant immune response. ETI shares downstream signalling cascades with PTI; however the former stimulates hypersensitive responses (HRs) in the infected cells and often results in programmed cell death (PCD), thus eliciting a long-lasting response (Mur et al. 2008; Rato et al. 2021).

Phytohormones including SA, JA and ET are components of downstream signalling cascades induced by PTI and ETI and play important roles in regulating the defence responses to biotic agents including pathogens. SA is involved in stimulating the more robust defence systemic acquired resistance (SAR), often part of R gene downstream response, which triggers the expression of PR genes, generating systemic resistance against a broad spectrum of pathogens. The phytohormone pathways including those of SA, JA and ET often interact and elicit plant responses against a number of pathogens (Glazebrook 2005; Iqbal et al. 2021).

Owing to their diverse feeding habits, plant responses to insect herbivores are highly complex and include both direct and indirect responses. Direct responses involve repairing the tissue damage caused by the herbivores and activation of defence mechanisms for preventing future attacks. The indirect response includes

the release of volatile organics that attract the natural enemies of the biotic agents especially herbivores, thereby strengthening the defence. These volatiles also serve as signals to trigger defence in the neighbouring plants still not encountered with the pest or pathogen (Alba et al. 2012). Responses to insect pests often involve activation of local or systemic responses that triggers signalling pathways involving JA, systemin and oligogalacturonoids (OGAs). among others (Fürstenberg-Hägg et al. 2013; Gimenez et al. 2018). Among the phytohormones, JA plays a pivotal role in both direct and indirect responses to herbivores; plant cells transduce JA signals into the activation of transcriptional regulation of defence genes, with many dependent on the MAP kinase pathway. SA and ET are among the other hormones involved in the expression of plant defences against herbivores (Santamaria et al. 2013). Plants also exhibit indirect response to herbivore attack by producing an array of volatiles from the lipoxygenase (LOX) and terpenoid signalling pathways that repel the herbivore or attract its natural enemies (Iqbal et al. 2021). Furthermore, a number defensive proteins that interfere with insect digestion and development including protein/enzyme inhibitors, lectins, chitinase and polyphenol oxidases are produced as a response against herbivore attack (Fürstenberg-Hägg et al. 2013).

These resistance strategies help the plants combat various biotic stressors. The term 'biotic stress tolerance' refers to the ability of the host to compensate in part for the fitness costs brought about by the biotic stressors, i.e. the ability of the plants to survive and reproduce under biotic stress (Redondo-Gomez 2013). Despite the robust resistance strategies evolved by plants to overcome biotic stress, economic losses due to the damage caused by biotic stressors are still high. Therefore, development of biotic stress-tolerant crops is one of the major aspects of crop improvement programmes.

4.3 Management of Biotic Stress

4.3.1 Challenges and Opportunities

To ensure food security and safety, there is an urgent and inevitable demand of effective management and control of biotic stresses in crops in the light of ever-increasing global population. Major management strategies that are being followed are farming practices, application of chemical and biological agents and breeding of resistant varieties. However, effectiveness of these strategies is confronted by multiple challenges like evolution of novel variants of pathogens/pests, wide host range and narrow genetic base of the cultivated varieties in crops. Despite their effectiveness, agrochemicals can be a threat to human health, food safety and the environment. Hence, exploitation of biological control methods coupled with breeding of resistant varieties offer cost-effective, eco-friendly and sustainable strategy to combat biotic stresses.

4.3.2 Breeding for Resistant Varieties

Over the years, various pursuits have been made to develop new cultivars that are resistant to several biotic stresses by conventional breeding. However, breeders have faced significant challenges in the process, like a longer breeding cycle, laborious selection process and linkage drag. Moreover, it could fetch only temporary success due to fast-evolving pests and pathogens, narrow genetic base and scarcity of durable resistance sources leading to the breakdown of resistance in the field (Mgonja et al. 2016). Abiotic stresses, such as drought, also increase the likelihood of resistance breakdown (Gupta et al. 2017). Hence, there is a need of adopting strategies for the development of durable resistance in crops, which is a key to sustainable management of biotic stresses. Gene staking/pyramiding of multiple R genes or allele of a major R gene can provide an excellent strategy for the attainment of broad-spectrum, dynamic and durable resistance against different strains of pest and pathogens. Therefore, exploring new resistant genes and exploiting intrinsic genetic defence resources are the imperative tasks both in practice and theory. Recent breakthroughs in molecular genetics and biotechnology have bestowed novel tools and technologies for breeders to develop resistant varieties through a precise and durable strategy known as molecular breeding. Over the past decades, we have seen the successful use of advanced molecular and genomic tools like molecular markers, expressed sequence tags (ESTs), microarrays, RNA-Seq and genetic transformation and genome editing methods to explore the genetic basis of stress tolerance and eventually to develop improved crop cultivars.

4.4 Omics Technologies: A Paradigm Shift in Breeding Approaches

Recent advances in next-generation sequencing technologies have shed light on the genomic sequences, genes and pathways involved in the mechanism of stress response as well as tolerance. Fast and memory-efficient computational tools have become the need of the hour to perform complex operations on genomes and transcriptomes. Bioinformatics tools play a major role in stress management through computational algorithms to extract meaningful knowledge from high volume of data generated through state-of-the-art sequencing methods. The opportunities in integration of omics data (also known as multi-omics/integromics/panomics) are plentiful in this era of affordable data generation technologies.

Forthcoming of omics resources in combination with efficient computational algorithms has shed light on novel plant resistance genes in the past years. Analysis of resistance gene datasets and databases has aided in facilitating the development of diagnostic tests and breeding strategies. Omics data warrants a greener revolution, according to the guidelines outlined by the Food and Agriculture Organization (FAO) (Pérez-de-Castro et al. 2012).

4.4.1 Generations of Sequencers

The evolution of sequencing technology began in 1977 when Frederick Sanger developed the chain termination method which relied on DNA polymerase. This method was used in sequencing the complete genome of bacteriophage PhiX174. A chemical-based dideoxy method was developed by Maxam and Gilbert in 1977 that cut DNA at specific bases. The chain termination method and dideoxy technique constituted the first generation of sequencing technology and could produce read lengths less than 1 kb. Following the successful efforts using first-generation technologies, cheaper and faster alternatives were sought that ushered to the next generation which attempted to parallelize sequencing reactions. Roche 454 Pyrosequencing, Illumina (sequencing by synthesis) and SOLiD (sequencing by ligation) come under second-generation sequencers. The third-generation single-molecule real-time (SMRT) sequencers attempted to reduce the cost of sequencing. Pacific Biosciences, Helicos sequencing and Oxford Nanopore Technologies are pioneers in SMRT which produced long reads. Long-read sequencing can provide gapless assembly of sequences with minimal loss of information. Highly efficient computational algorithms are being developed for accurate interrogation and extraction of meaningful pattern from the genomic data to answer the questions of biological context. The richness of the information derived from sequencers is ever increasing, and the cost is declining rapidly indicating a biological data boom in the near future.

4.4.2 Genomics

Plant genomes are characterized by their large size, gene composition, number of repetitive sequences and polyploid nature indicating that all plant genomes are not equally sequencable. Genomic approaches have been infiltrating into almost every aspect of plant science. Genomics act as a bridge in between the genotype and phenotype of plants. Out of the 39,100 plant species, only around 600 complete plant genome assemblies are available (Kersey 2019). Among plant genomes, *Arabidopsis thaliana* remains the gold standard with well-annotated five chromosomes and organellar genomes with only a gap of 161 bp. The largest plant genome assembly is that of sugar pine (Stevens et al. 2016). Several genome projects such as 10KP Project (Twyford 2018) and Earth BioGenome Project (Lewin et al. 2018) are working towards sequencing of plant genomes. Genome sequencing is an inevitable component of plant research that can help uncover pathways and genes in precision agriculture practices.

4.4.3 Transcriptomics

Transcriptome is the small percentage of genome which gets transcribed (Frith et al. 2005). In other words, it is the link between the genome and function of a gene. The earliest attempts to sequence the transcriptome began in the 1990s. Microarray and RNA-Seq are two contemporary technologies to characterize the transcriptome. Microarrays use predetermined capture sequences called probes, while RNA-Seq uses high-throughput sequencing to quantify the sequences. Gene regulation may be well understood by measuring the expression at different time points, conditions or tissues.

Quantification of RNA molecules is vital in understanding the condition-specific behaviour of plants. A cDNA library is prepared from replicated samples after enrichment of mRNAs by rRNA depletion/polyA selection/RNA capture methods. This is followed by fragmentation of cDNA of various lengths. In the presence of longer fragments, paired end sequencing may be considered. Appropriate sequencing technologies are applied to the prepared samples. Transcriptomic studies are inevitable to identify which genes are turned on or off in a particular condition.

The avalanche of data generated from genomic studies remains obsolete until proper data mining and inference algorithms are applied to deduce meaningful patterns from them. Despite the evolving knowledge base in plant stress, the underlying factors contributing to the response mechanisms have not been fully understood. Some of the challenges of exploring expression datasets are as follows:

(a) Extracting genes that contribute to a particular condition: In experimental conditions where we have controls and samples treated under biotic stress conditions, the aim is to identify genes that are activated or inactivated during the treatment. Tools to infer differential gene expression pattern on count data are also developed. This includes popular R-based tools such as DeSeq2, edgeR and limma.
(b) Identify relationships between genes: A group of genes whose expression pattern is similar across all samples under similar conditions are co-expressed genes. It is hypothesized and proven that genes that display similar expression pattern belong to a common biological pathway. The most commonly used similarity metrices to compare the expression of a pair of genes include Pearson correlation, Euclidian distance and mutual information, but are not limited to these. Clustering algorithms extract groups of similar genes from transcriptomic data.
(c) Infer causal relationships between genes: Causal inference assumes that a change in expression values will subsequently bring about a change in another gene, if they have common regulators. There is a time lag between the direct targets of regulated and the downstream targets. Causal inference takes into consideration the strength and directionality of the response genes.
(d) Identify expression patterns between regulator and target: This type of pattern identification problems considers the Boolean expression concept of the target and regulator. Transcriptional regulators of plants include DNA-binding tran-

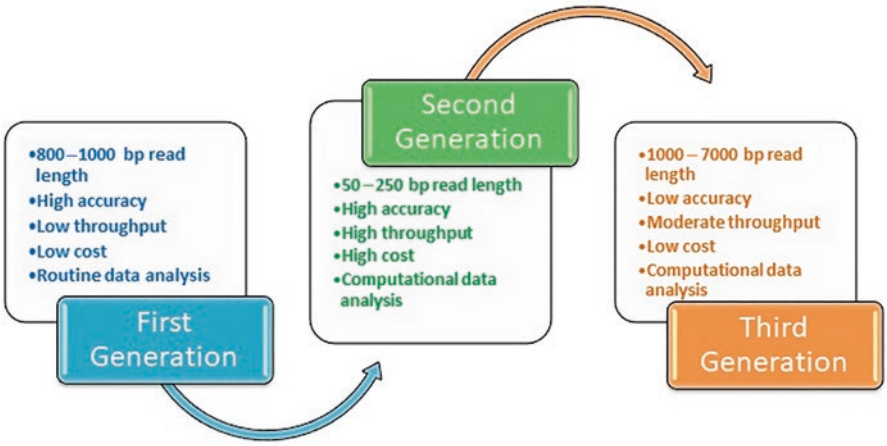

Fig. 4.2 Evolution and features of sequencing technologies

scription factors that act as activators or repressors and co-factors that interact with DNA-binding TFs that activate or co-repress RNA synthesis (Moore et al. 2011). The regulator gene may be assigned the states *on*/*off* for the expressions active/inactive or high/low. The expression pattern is then propagated to the target genes of the network as Boolean states. Hence in response to a stress stimulus, the plant immunity is rapidly activated, and hundreds of downstream genes are massively reprogrammed.

(e) Explore the dynamic nature of transcriptional network: To explore the dynamicity of a network, ordinary differential equations are used to model the changes in gene expression either by interpolation or extrapolation (Fig. 4.2).

4.4.4 Bioinformatics Tools for Transcriptomics

In order to harness the complete potential of hidden information in transcriptomic and genomic datasets, several computational tools have been developed (Table 4.2).

4.4.5 Artificial Intelligence-Based Prediction Methods

Artificial intelligence methods are increasingly used in predicting complex biological phenomena using high-quality training data. Prediction methods are highly useful in bringing field-scale observations to genome-scale data. Machine learning methods encompass data-driven predictions and are widely used in analysing big data in agricultural research.

Table 4.2 Computational tools equipped to process raw sequence data to provide biologically meaningful insights

Tool	Hypothesis	References
FASTQC	Quality control checks on raw sequence data	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Cutadapt	Adapter trimming	Martin (2011)
Trim galore!	Adapter trimming and quality control	https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
PRINSEQ	Sequence-based statistics	Schmieder and Edwards (2011)
UCHIME	Detection of chimeric sequences	Edgar et al. (2011)
SVASeq	Batch effect correction	Leek (2014)
Bowtie	Short-read aligner	Langmead et al. (2009)
BWA	Short-read aligner	Li and Durbin (2009)
HISAT	Spliced aligner	Kim et al. (2015)
STAR	De novo aligner for long reads	Dobin et al. (2013)
featuresCount	Read quantifier	Liao et al. (2014)
Kallisto	Calculate read abundance	Bray et al. (2016)
DeSeq2	Geometric normalization strategy	Love et al. (2014)
edgeR	Trimmed mean of M- values is computed as the weighted mean of log ratios between this test and the reference	Robinson et al. (2010)
Limma	Quantile normalization method and uses weighted mean of log ratio-based method	Smyth (2005)

Transcriptome analysis reveals differentially expressed genes (DEGs) under various environmental conditions. A machine learning-based gene classification approach has been devised using DEGs in biotic and abiotic stress conditions in rice (Shaik and Ramakrishna 2014). Gene prioritization using machine learning has been performed in *Arabidopsis* and rice using sequence annotation and protein interaction network information (Lin et al. 2019). Time series analysis of multiple stress types was predicted using neural networks in *Arabidopsis*. The analysis method named StressGenePred utilizes two prediction models, namely, biomarker model and stress-type model (Kang et al. 2019).

Development of a model that can explain and quantify stress will have a significant impact in scientific crop research, crop improvement and plant breeding. Application of deep learning in agriculture has led to the identification of biotic stress in apple (AlexNet, GoogLeNet, VGGNet-16, ResNet-20 algorithms), banana (LeNet), cassava (Inception-v3, ImageNet), olive (LeNet), cucumber (CNN), tomato (AlexNet, ZFNet, VGG-16, GoogLeNet, ResNet-50, ResNet-101, ResNetXt-101, Faster RCNN, R-FCN, SSD), maize (CNN), wheat (VGG-FCN, VGG-CNN) and radish (VGG-A, CNN). However, integration of image data along with multi-omics datasets will allow better feature selection in the models.

4.4.6 Data Integration and Knowledge Discovery

Recent advances in bioinformatics resources including software, databases and web servers have brought a major change in plant stress research. In the last few decades, massive data emerging from stress-associated studies in plants require appropriate management and analysis. These resources enable better interpretation of data generated through several experiments and prove useful for stress biology community. With a rapid progress in sequencing technologies producing high-throughput massive data, it is essential to integrate the information in public secondary databases of relevant themes.

Data integration can be considered as an important aspect of bioinformatics. The comprehensive understanding of genome functionalities requires a multidimensional perspective of several omics layers. The ultimate aim of any data integration platform should be to extract meaningful knowledge from multiple datasets like genomics, proteomics and metabolomics to incorporate data that can be hardly extracted from a single experimental source. Mapping the relationships among various components of the transcriptome such as methylome and non-coding RNAs to gene expression is integral to strengthen the assumptions derived from genomic and transcriptomic studies. Integration of multi-omics data requires the experimental approaches to be considerably unbiased and how each dataset is generated. Several databases have been developed to integrate the available information regarding abiotic stress in one single platform. The list of biotic stress gene databases is summarized in Table 4.3.

Table 4.3 Online resources for accessing stress-related data derived from genomic and transcriptomic methods

Resources	Description	References
Plant stress gene database	Stress-related genes across 11 species	http://ccbb.jnu.ac.in/stressgenes/
The Arabidopsis stress responsive gene database	Stress-related genes in *Arabidopsis thaliana*	Borkotoky et al. (2013)
Arabidopsis stress responsive transcription factor database	Stress-related transcription factors	Shameer et al. (2009)
STIFDB2	Stress-responsive genes in *Arabidopsis thaliana* and *Oryza sativa*	Naika et al. (2013)
Plant stress protein database	Manually curated stress protein from 134 species	Anil Kumar et al. (2014)
Plant PhysioSpace	Compare response to stress in various plant species	Esfahani et al. (2020)
Plant stress RNA-Seq nexus	Expression profiles of coding and non-coding stress-related transcripts	Li et al. (2018)

4.5 Recent Achievements and Applications of Omics Tools for Biotic Stress Tolerance

4.5.1 Application of Genomics in Biotic Stress Tolerance

Genome-based approaches are highly useful in detecting genetic variation which enhances breeding efficiency and thus contributes to crop improvement. Genomic approaches also facilitate to decipher the unexplored genetic mechanisms of stress tolerance and enable the development of stress-tolerant crop varieties. Structural and functional genomic aspects have been widely employed in plant biotic stress tolerance studies since the past few decades. Construction of physical and genetic maps to identify traits of interest is dealt with structural genomics, while functional genomics involves the study of gene functions with regard to the trait of interest.

Molecular markers, viz. restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), selectively amplified microsatellite polymorphic locus (SAMPL), sequence-related amplified polymorphism (SRAP), target region amplification polymorphism (TRAP) and SNPs, are among the genomic tools widely applied for tagging and mapping the genes of interest and their further utilization in targeted breeding. With the advancements in NGS and the high-throughput sequence analysis platforms being freely available, SNPs became the preferred markers for large-scale genotyping and genomics-assisted breeding (Muthamilarasan et al. 2019). SNPs, which are the single nucleotide variations in the genome of an organism, enable survey of a large number of sites in a given DNA sequence and have been extensively deployed in plant stress tolerance studies, among others. Array-based genotyping and genotyping by sequencing (GBS) are the two most important techniques for SNP detection (Kaur et al. 2021).

The various genome-wide markers are further utilized in genetic mapping. Quantitative trait locus (QTL) mapping and association mapping are important tools to understand the genetic basis of phenotypic variation (Kloth et al. 2012). Owing to higher mapping resolution, association mapping has gathered momentum among breeders for genetic mapping studies. Candidate genome association mapping (identification of SNPs in candidate genes responsible for the phenotypic variation) and genome-wide association (GWA) mapping (genome-wide analysis of SNPs and its correlation with various complex traits) are the two broad categories of association studies. GWA mapping has emerged as an important genomic tool that is useful to understand multiple traits in crops including those related to single or multiple stresses (Challa and Neelapu 2018). GWAS also provides insights on structural variants (SVs) that are pivotal in regulating several crop traits. Hence, GWAS is being widely applied in the tolerance of crops to various stresses. The association between genetic variation and phenotypic trait of interest, viz. biotic stress tolerance, can be further exploited for marker-assisted selection (MAS) in breeding programmes. Further, the enhancement in hybrid breeding has been achieved through MAS with GBS. GBS enables the discovery of polymorphisms

and simultaneously obtains the genotypic information across the whole population of interest, especially for high diversity species. This process employs restriction enzyme-based complexity reduction coupled with DNA-barcoded adapters to generate multiplex libraries of samples for NGS (Elshire et al. 2011).

Functional genomics encompasses the functional characterization of genes of interest, for instance, stress-responsive genes. With the recent developments in sequencing technologies, high-quality genome sequences of a wide array of crops were made available. For example, the PacBio sequencing platform with long single-molecule reads, viz. >5 kb, provides better assembly of genomes with reduced gaps. Genome sequences of crops provide access to genes, thereby enabling isolation of genes for functional characterization (Muthamilarasan et al. 2019). Also comparative genomics enables the identification of mutants related to various traits in crops including disease resistance. The past few studies involving application of genomic approaches for deciphering biotic stress tolerance in plants are described here.

Application of GWAS could accomplish the identification of genomic regions for seedling resistance as well as adult plant resistance (APR) against yellow rust of wheat (Rosewarne et al. 2013; Zegeye et al. 2014; Jighly et al. 2015). Similarly, in wheat pre-breeding lines derived from various exotic crosses, GWAS opened the avenue for identification of 14 SNP markers and 7 haplotypes associated with yellow rust which can be promising targets for marker-assisted selection (Ledesma-Ramírez et al. 2019). GWAS can also be successfully employed to understand tritrophic interactions which allow plants to attract potential natural enemies against infestation by insect pests. Recently, GWAS involving 146 maize genotypes was conducted to gain insights on the plant trait of emitting herbivore-induced plant volatiles (HIPVs) to attract the natural enemy, *Cotesia sesamiae*, upon exposure to the stem borer, *Chilo partellus* eggs. The study identified 101 marker trait associations, many of which are adjacent to defence -related genes, viz. genes involved in the JA-defence pathway, terpene biosynthesis, benzoxazinone synthesis and known resistance genes (maize insect resistance 1), thus revealing the maize genomic regions associated with indirect defence (Tamiru et al. 2020). Further, Manivannan et al. (2021) could discover SNP markers and constructed linkage map from F5 population of pepper with contrasting parents for powdery mildew resistance, viz. AR1 (PM resistant) and TF68 (PM sensitive) by high-throughput GBS.

The advent of pathogenomics could significantly unravel genome evolution and mechanism of pathogenesis which can pave the way for the sustainable deployment of broad-spectrum and durable resistance to fast-evolving pathogens. For instance, Firrao et al. (2018) could reveal genomic structural variations affecting virulence clonal expansion of *Pseudomonas syringae* pv. *actinidiae* (Psa) biovar 3 in Europe through the analysis of Illumina sequencing datasets of 11 European and 1 non-European Psa genomes. In a similar manner, the genome-wide analysis of the plant pathogenic bacteria *Ralstonia solanacearum* was performed by Cho et al. (2019). Similarly, the study conducted by Vleeshouwers et al. (2008) demonstrated that *Phytophthora* effector genomics (effector-omics) greatly accelerates discovery and

functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes.

Comparative genome sequencing between highly resistant (Tetep and Tadukan) and susceptible (HR-12 and Co-39) *indica* rice cultivars against *Magnaporthe oryzae* could identify prominent blast-resistant genes *Pi-ta* and *Pi54* which can be further exploited in marker-assisted breeding programme (Mahesh et al. 2016). To decipher the changes in gene expression in cucumber after aphid attack and the candidate genes that enhance resistance to the pest, the whole genome of aphid-resistant cucumber cultivar was analysed through digital gene expression (DGE). The studies identified several target genes involved in aphid resistance in cucumber, viz. genes encoding LRR proteins (Liang et al. 2015).

4.5.2 Application of Transcriptomics in Biotic Stress Tolerance

The transcriptome reveals the expression levels of genes in an organism and hence can provide meaningful insights on plant tolerance to stress conditions (Wang et al. 2020). Transcriptomic analyses have been widely used to study biotic stress tolerance in plants. Monitoring of the dynamics of SA, JA and ET signalling in *A. thaliana* after infestation by a set of pathogens and pests revealed that the kinetics of phytohormone signalling varies greatly with each host-attacker combination. Each of the host-attacker combination signal signatures was translated into a wide array of transcriptional alterations which involved the overexpression of stress-related genes. The transcriptomic profiles induced by pathogens and pests with different modes of infestation can overlap with each other (De Vos et al. 2005).

Transcriptomics approaches can be broadly classified into three, based on their functional applicability, and are described as follows and summarized in Table 4.4.

4.5.2.1 Identification of Disease-Resistant Gene, Susceptibility Genes and Defence Regulatory Genes

Transcriptomic tools enable better understanding of mechanism under pinning molecular and physiological basis of plant-pest interactions. It provides pristine knowledge on resistance mechanism of R gene and defence regulatory network in plants. It helps in exploring potential novel R genes that can be used for developing resistant varieties through conventional and/or molecular breeding. The genes upregulated in resistant varieties can be targeted for improvement through molecular breeding and transgenic approaches. On the other hand, genes that are upregulated in susceptibility reaction against disease could be potential targets for silencing by T-DNA insertion, transposon tagging and RNAi and genome editing. The study of expression profiles of host and pathogen enables identification of virulence genes in the pathogen and effector target sites in the host cells (Westermann et al. 2012;

Table 4.4 Transcriptomic studies on biotic stress-induced responses in some economically important crops

Crops	Biotic agent	Comparative transcriptome strategy	Findings	References
Pathogen				
Rice	Bacterial leaf blight	Resistant vs susceptible	Upregulation of genes encoding peroxidase, phytosulfokinase, RLKs, serine/threonine kinase, TFs (WRKY, NAC, MYB, bZIP, AP2/ERF, etc.) and phytohormones in resistant line	Tariq et al. (2018)
Rice	Blast (*Magnaporthe oryzae*)	Resistant vs susceptible	Higher expression of genes involved in PTI such as brassinolide-insensitive 1, flagellin-sensitive 2 and elongation factor Tu receptor, ethylene (ET) biosynthesis and signalling leading to partial resistance *Pi21*-RNAi line	Zhang et al. (2018)
Wheat	Stripe rust (*Puccinia striiformis* f. sp. *tritici*)	Inoculated vs uninoculated	Identified genes regulating the mechanisms of lignification, reactive oxygen species and sugar, respectively, are involved in adult plant resistance	Hao et al. (2016)
Wheat	Powdery mildew	Resistant vs susceptible	Developed molecular markers linked to powdery mildew resistance gene Pm4b in $F_{2:3}$ mapping population	Wu et al. (2018)
Wheat	Stripe rust (*Puccinia striiformis* f. sp. *tritici*)	Resistant vs susceptible	Constructed a high-density genetic map of wheat and localized the stripe rust resistance gene *Yr15* to a 0.77 cM interval	Ramirez-Gonzalez et al. (2015)
Wheat	Stripe rust (*Puccinia striiformis* f. sp. *tritici*)	Resistant vs susceptible	*YrMM58* and *YrHY1* for resistance to stripe rust were mapped in the distal ~16 Mb region on chromosome 2AS	Wang et al. (2018)

(continued)

Table 4.4 (continued)

Crops	Biotic agent	Comparative transcriptome strategy	Findings	References
Rice	Bacterial leaf blight (*Xanthomonas oryzae* pv. *Oryzae*)	Wild vs cultivated	Identified genes involved in phytohormone signalling, ubiquitin-mediated proteolysis and phenylpropanoid biosynthesis and resistance genes	Cheng et al. (2016)
Rice	Bacterial leaf blight (*Xanthomonas oryzae* pv. *Oryzae*)	Virulent vs susceptible	Identified 1235 DEGs at various time points	Wang et al. (2019)
Sorghum	Grain mould (fungal complex)	Resistant vs susceptible	Increased expression of SbLYK5 SbDFN7.1 and SbDFN7.2 in resistant gene	Nida et al. (2021)
Maize	*Colletotrichum graminicola*	Inoculated vs uninoculated	Defence signalling genes involved in the accumulation of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signalling pathways and chromatin modification against pathogen	de Jesus Miranda et al. (2017)
Tomato	*Tomato spotted wilt virus* (TSWV)	Resistant vs susceptible	Identification of the role of PR-5 in Sw-7-mediated resistance	Padmanabhan et al. (2019)
Tomato	Bacterial wilt (*Ralstonia solanacearum*)	Silicon (treated vs untreated)	Upregulation of genes involved in PTI, oxidation resistance, water-deficit stress tolerance and differential expression of multiple hormone-related genes	Jiang et al. (2019)
Potato	Late blight of potato (LBP)	Ethylene (treated vs untreated)	Multiple signalling pathways including ET, salicylic acid, jasmonic acid, abscisic acid, auxin, cytokinin and gibberellin were induced involved in the response to exogenous ET	Yang et al. (2020)

(continued)

Table 4.4 (continued)

Crops	Biotic agent	Comparative transcriptome strategy	Findings	References
Canola (*B. napus*)	*Sclerotinia sclerotiorum*	Resistant vs susceptible	Elucidated the role of peroxisome-related pathway cell wall degradation and detoxification of host metabolites in pathogenesis	Chittem et al. (2020)
Coffee	Rust (*Hemileia vastatrix*)	Resistant vs susceptible	Unfolded the role of prehaustorial resistance in resistant line	Florez et al. (2017)
Cotton	Cotton leaf curl disease (*cotton leaf curl virus*)	Wild vs cultivated	Identified multiple genes involved in disease resistance and pathogen defence	Naqvi et al. (2017)
Cabbage	Clubroot (*Plasmodiophora brassicae*)	Virulent and avirulent in resistant line	Robust activation of ETI in avirulent-resistant reaction	Fu et al. (2019)
Chinese cabbage	Downy mildew (*Pseudoperonospora cubensis*)	Resistant and susceptible	Expression of more cis- and trans-functional long non-coding RNAs in the resistant lines Identified and functionally characterised candidate resistance-related long non-coding RNA, MSTRG.19915	Zhang et al. (2021)
Ginger	Bacterial wilt (*Ralstonia solanacearum*)		Identified nine miRNA/miRTGs as the candidate pairs involved in disease resistance	Snigdha and Prasath (2021)
Sugarcane	Ratoon stunting (*Leifsonia xyli* subsp. *xyli*)	Inoculated vs uninoculated	Downregulation of gibberellin response via increased DELLA activity and downregulation of GID1 proteins, coupled with inhibition of photosynthetic processes	Zhu et al. (2021)
Insect pests				
Cotton	Aphid and whitefly	Infested vs noninfested	Suppression of genes involved in phytohormone-mediated plant defence response, viz. cationic peroxidase 3, lipoxygenase I, TGA2 and non-specific lipase	Dubey et al. (2013)

(continued)

Table 4.4 (continued)

Crops	Biotic agent	Comparative transcriptome strategy	Findings	References
Cotton	Whitefly	Resistant vs susceptible	Upregulation of genes encoding protein kinases, transcription factors, metabolite synthesis and phytohormone signalling WRKY40 and copper transport protein were identified as the hub genes in host defence	Li et al. (2016)
Rice	Leaffolder and BPH	Resistant to leaffolder vs moderately resistant to BPH	Activation of genes involved in JA biosynthesis and regulation of shikimate and phenylpropanoid pathways in response to leaffolder and induction of salicylic acid-responsive genes and cellular signalling cascades to BPH	Li et al. (2021)
Wheat	Aphid (*Schizaphis graminum*)	At various post-infestation times	Upregulation of salicylic acid-mediated pathway and MAPK-WRKY pathway of the antioxidant enzyme peroxidase and superoxide dismutase	Zhang et al. (2020a)
Pepper (*Capsicum annum*)	Spider mites	Resistant vs susceptible	Mite infestation induces both JA and SA signalling and pathogen-related defence responses including WRKY transcription factors	Zhang et al. (2020b)
Tomato	*Tuta absoluta*	Resistant vs susceptible	Activation or repression of key transcription factors regulating important defence genes such as those involved in cuticle formation, synthesis of anti-nutritive enzymes, chemical toxins and various secondary metabolites	D'Esposito et al. (2021)

(continued)

Table 4.4 (continued)

Crops	Biotic agent	Comparative transcriptome strategy	Findings	References
Tomato and eggplant	*Tuta absoluta*	Tomato vs eggplant	Tomato is prone to the pest infestation than eggplant. Genes associated with salicylic acid, PR1b1, NPR1, NPR3, MAPKs and ANP1 families mediate the immunity of eggplant	Chen et al. (2021)
Nematodes				
Cotton	Southern root-knot nematode (*Meloidogyne incognita*)	Resistant vs susceptible	3789 differentially expressed genes out of which a large number of significant genes were downregulated in the susceptible genotypes. The defence response, detoxification and callose deposition genes in the qMi-C11 and qMi-C14 loci were upregulated	Kumar et al. (2019a, b)
Chickpeas	*Pratylenchus thornei*	Resistant vs susceptible	Members of WRKY and bZIP family of transcription factors were uniquely expressed in the resistant genotypes	Channale et al. (2021)
Rice	*Meloidogyne incognita* and *Meloidogyne graminicola*	Inoculated and uninoculated	2062 and 1386 differentially expressed genes (DEGs) were identified in response to infection by *Meloidogyne graminicola*	Zhou et al. (2020)
Wheat	*Heterodera avenae*	Inoculated and uninoculated	Defence-related genes were downregulated in biotic stress pathways upon infection	Chen et al. (2017)
Pepper	*Meloidogyne incognita*	Resistant vs susceptible	Identified differentially expressed genes on chromosome P9	Hu et al. (2020)
Sweet potato	*Meloidogyne incognita*	Resistant vs susceptible	Determined the significant role of peroxidase genes	Sung et al. (2019)

Boyd et al. 2013), which will further help understand the supplanting of plant resistance by new physiological races of the pathogen.

Capitalizing the knowledge on molecular mechanisms in plant-pest interaction unravels the defence gene networks in pathogen recognition, signal transduction, transcription and defence-related proteins. Exploitation of such signalling networks that regulate innate defence mechanisms may be one among the several strategies for broad-spectrum and durable plant resistance, either by overexpression of positive regulators or silencing of regulators involved in innate immunity. Yet another strategy is by modifying the R gene through choosing appropriate promoters or altering the domains involved in effector recognition. Ultimately, the aforesaid downstream application of transcriptomics may be utilized for achieving sustainable and durable resistance.

4.5.2.2 Identification of Long Non-coding RNAs and MicroRNAs

MicroRNAs have been identified in many crops which regulate plant-pest interaction, either through silencing genes or by blocking mRNA translation. Expression patterns can open avenues on their stress adaptation functions. Several researchers reported that pathogen attacks in plants induce numerous miRNAs and later control and contribute towards the reprogramming of gene expression (Ramachandran et al. 2020; Zhang et al. 2018; Xin et al. 2010; Hunt et al. 2019; Fahim et al. 2012; Kis et al. 2016; Sun et al. 2016). The role of miRNA regulatory network in guarding ginger against bacterial wilt caused by *Ralstonia solanacearum* was elucidated by analysing the transcriptome of resistant and susceptible lines using 'psRNATarget' server and identified nine miRNA/miRTGs which are the key candidate pairs in response to *R. solanacearum* infection in ginger (Snigdha and Prasath 2021). Similarly, differential expression of miRNAs was observed in wheat and barley after infection with powdery mildew (Xin et al. 2010; Hunt et al. 2019). Much research has been carried out to overexpress significant miRNAs to enhance disease resistance in susceptible plants. Overexpressing miR 319b in rice was found to be a positive regulator of the rice defence response against the blast disease (Zhang et al. 2018). The same strategic approach has also been successfully adopted against viral infection in economically important crops such as wheat, maize, tomato and grape wine (Fahim et al. 2012; Kis et al. 2016; Sun et al. 2016).

In addition to miRNAs, long non-coding RNAs are mainstream regulators in plant defence against biotic stress. The study carried out by Zhang et al. (2021) on comparative gene profiling of two resistant lines (T12–19 and 12–85) and one susceptible line (91–112) of Chinese cabbage against downy mildew could reveal increased expression of more *cis*- and *trans*-functional long non-coding RNAs in the resistant lines. Furthermore, they identified and functionally characterised candidate resistance-related long non-coding RNA, MSTRG.19915, which is a long non-coding natural antisense transcript of a MAPK gene, BrMAPK15.

4.5.2.3 Identification of Functional Markers

Lack of tightly linked marker is the major hurdle that prevents precise identification of resistant gene in marker-assisted selection practices. Bulked segregant analysis-RNA-Seq (BSR-Seq) is a new genetic mapping strategy that combines the power of bulked segregant analysis (BSA) (Michelmore et al. 1991) and the ease of RNA-Seq technique that can resolve the issue. For instance, Wu et al. (2018) could develop molecular markers linked to powdery mildew resistance Gene Pm4b in $F_{2:3}$ mapping population of wheat by coupling SNP discovery from transcriptome sequencing data with BSR-Seq. Similarly, Ramirez-Gonzalez et al. (2015) constructed a high-density genetic map of wheat and localized the stripe rust (caused by *Puccinia striiformis* West.) resistance gene *Yr15* to a 0.77 cM interval. In the same manner, *YrMM58* and *YrHY1* for resistance to stripe rust were also mapped in the distal ~16 Mb region on chromosome 2AS (Wang et al. 2017). These studies have demonstrated that BSR-Seq is highly effective in identifying SNP markers for fine-mapping and even cloning target genes, especially in the less polymorphic regions of the plant genome.

4.6 Conclusion

Biotic stress is one of the major limiting factors for production and productivity of food crops. A paradigm shift from conventional breeding into molecular breeding and now the genomics-assisted breeding has bestowed new opportunities in developing biotic stress-tolerant crops. Genome- and transcriptome-scale studies have been assisting not only to unravel the complex mechanisms underlying biotic stress tolerance but also to identify novel and valuable genic resources that can be exploited in breeding programmes. Further, the advent of genome editing techniques has ameliorated targeted breeding strategies in terms of simplicity, accuracy and power. Therefore high-throughput multi-*omics* strategies have opened new avenues in crop protection and improvement. However, memory-efficient computational tools to handle the big genomic data are still in its infancy. Hence, hand-in-hand development of both sequencing technology and bioinformatics analysis is the need of the hour.

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Chapter 5
Role of Metabolomics and Next-Generation Sequencing for Sustainable Crop Production

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5.1 Introduction

In recent times, a huge advancement has been made in "omics" including transcriptomics, genomics, metabolomics, proteomics, phenomics, and epigenomics (Yang et al. 2012). These "omics" approaches have heightened data precision and speed up the selection process for breeding programs, which is important to ensure global food security (Kumar et al. 2017). Recent studies have witnessed the notable contribution of genomics and phenomics in producing higher genetic seeds and improving crop growth performance through the breeding system (Langridge and Fleury 2011; Wang et al. 2017a; Xavier et al. 2017). Nevertheless, the omics has a great selection potential of key traits and allows us to improve plant performance by developing new strategies. Among all omics approaches, metabolomics is a multifaceted approach and received very less attention in plant selection and trait mapping, particularly in crop science.

Metabolites are essential components owing to their effect on root architecture and plant biomass (Turner et al. 2016). To date, metabolomics has arisen as a major breakthrough, which facilitates accurate metabolite profiling of plants, microbes, and animals (Takáč et al. 2017; van Dam and Bouwmeester 2016). Metabolomics is capable of making precise and efficient detection of a wide range of metabolites from any extract (Wen et al. 2015). In another context, metabolomics can offer metabolite analysis even at the cellular level, for instance, tiny organic compounds involved in regulating cellular events therefore represent the entire cell physiological state. With the rapid advancement in metabolomics, investigation of metabolite transgenic/mutant lines holds a potential to identify underlying genetic or metabolic network. In addition, metabolomics integrated with functional characterization of gene allow us to investigate how a candidate gene or genes impact metabolic pathway and reveal regulation among linked pathways, which are otherwise difficult to accomplish through conventional approaches like microarray (Kusano and Saito 2012). Integrated-omics approaches permit researchers to screen out potential traits for improving crop yield, quality, and nutritional values. Furthermore, omics studies may extend to other regulatory processes at posttranscriptional and posttranslational modification and epigenetic regulation. Consequently, interactome studies aimed to explore molecular interaction among biomolecules which expand our knowledge on genotype-phenotype association (Anguraj Vadivel 2015; Vidal 2011).

Metabolomics approaches are applied in a range of crop species irrespective of successful transgenic system with a great potential to assist screening of key traits (Daygon and Fitzgerald 2013; Simó et al. 2014). With the advent of integrated metabolomics, the accessibility to whole genome, genotyping assays, and genetic

variants was prime breakthroughs to efficiently incorporate metabolomics in the crop breeding system (Zivy et al. 2015). The major tools that witnessed considerable improvement in metabolomics analysis are nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Lei et al. 2011; Matsuda et al. 2010).

Next-generation sequencing (NGS) revolution agriculture sciences through Illumina sequencing, high-throughput marker, applied biosystems SOLiD technologies (Lyu et al. 2020). The availability of high-quality genome of plants holds an evident ability for targeted research toward crop improvement. Among other NGS technologies, transcriptomics offers the understanding of functional mechanisms that the genome encodes (Imadi et al. 2015). In the past decades, agricultural scientists have relied on NGS technologies that enable them to identify high-resolution interaction of targeted traits and gene variants which resulted to a significant rise in the scope of transcriptomics toward improving agricultural systems (Tian et al. 2011). NGS platforms (NGSPs), serial analysis of gene expression (SAGE), microarrays, and massively parallel signature sequencing (MPSS) are representative approaches employed for genome-wide associated and high-throughput analysis (Costa et al. 2010; Mardis 2008). NGS-based techniques also offer DNA methylation study of single-base mutation among various plant species such as rice and *Arabidopsis* (Wang et al. 2011). These approaches dissect in-depth knowledge about DNA methylation and regulation. In this chapter we focus on the role of metabolomics and NGS approaches in improving crop traits, for better understanding of gene functioning and for the development of sustainable agricultural system.

5.2 Role of NGS in Agricultural Crop Production

The rise of genomics revolutionizes plant breeding, facilitates better understanding of plant genomes, and dissects the genetics of pivotal agronomic traits (Table 5.1). Further, the recent breakthroughs in genome editing approaches, particularly CRISPR/Cas9, an efficient gateway to accurate genome alteration in the target genome (Scheben and Edwards 2018), offer a robust transfer of knowledge from the lab to the field. The genes with beneficial agronomic traits may be specifically transformed via genome editing, thus allowing better phenotypic manipulation.

Genetic diversity is one of the key requisites in breeding programs. However, crop domestication and evolution have considerably decreased genetic diversity, causing the loss of numerous loci associated with important agronomic traits (Gross and Olsen 2010; McCouch 2004). The availability of draft genome sequences of numerous accessions of a plant species facilitated genome dissection using genome-scale research. Recently, genome-wide associated studies (GWAS) coupled with a penal of several accessions from diverse crops have been proven to be an invaluable resource for studying dynamics of genomic variation during selective breeding and crop domestication (Zhou et al. 2015; Varshney et al. 2017a). Comparative genome studies by Morrell et al. (2012) emphasize the significance of genomics that "the future of agricultural development will focus on comparisons of individual plant

Table 5.1 Application of NGS-based approaches in phenotypic traits of agricultural crops

Crops	SNPs	Approaches	Phenotypic traits	References
Oryza sativa	16,345	GWAS	Days to heading and culm length	Ogawa et al. (2018)
O. sativa (IR64)	7152	Joint linkage analysis	Days to heading	Fragoso et al. (2017)
Triticum aestivum	17,267	Interval QTL mapping	Powdery mildew	Stadlmeier et al. (2018)
T. aestivum (Berkut)	800,000			Jordan et al. (2018)
T. aestivum (Asassa)	13,000	GWAS	Phenological traits and plant height	Kidane et al. (2019)
Sorghum bicolor	79,728	GWAS	Height of the plant	Ongom and Ejeta (2018)
S. bicolor (RTx430)	90,000	Joint linkage analysis	Heading time and plant height	Bouchet et al. (2017)
Pisum sativum (Cameor)	13,204	GWAS	Seed yield components, seed composition, plant phenology, and plant morphology	Tayeh et al. (2015)
Vigna unguiculata	51,128	Interval QTL mapping	Heading time, growth habit, seed size, maturity, photoperiod	Huynh et al. (2018)
Vicia faba	156	Association mapping	Morphological traits, shoot water, fatty acid synthesis	Sallam and Martsch (2015)
Hordeum vulgare (Barke)	27,000	GWAS	Glossy spike, glossy sheath, and black hull color	Nice et al. (2016)
	5398	GWAS	Yield-related traits	Sharma et al. (2018)
Glycine max (IA3023)	5303	GWAS	Stability of grain yield	Xavier et al. (2018)
Zea mays	54,234	Linkage mapping and association mapping	Days to pollen shed, plant height, ear height, and grain yield	Dell'Acqua et al. (2015)
Z. mays (B73)	1106	Joint linkage analysis and GWAS	Northern leaf blight	Poland et al. (2011)
	1106	Joint linkage analysis and GWAS	Kernel composition	Cook et al. (2012)
	–	GWAS	Leaf traits	Tian et al. (2011)

genomes." The accessibility to enormous, sequenced genomes offers to identify core and essential genes in crop speciation that stepped genomics into pangenomic analysis.

Plant genomics has slow transition from individual genome assemblies toward multiple genomes, representing the entire genetic repertoire of a species or family

(Bayer et al. 2020; Golicz et al. 2020) and exploring the complete genomic variation (Fuentes et al. 2019; Golicz et al. 2016; Varshney et al. 2017b). It became more clear that an individual reference genome sequence was insufficient to reflect the wide range of genomic diversities within species, prompting growth and development, and adaptation (Golicz et al. 2020).

5.2.1 Genomics and Pangenomes for Agriculture

Global food security is a prime challenge with the present world's population (Tomlinson 2013), increased urbanization, and reduced agricultural land (Satterthwaite et al. 2010). Global warming, notably varying patterns of rainfall, temperature extremes, CO_2 levels, as well as ozone, poses threats to agriculture through salinity and drought, which restrict both water and agricultural land use (Godfray et al. 2010). Therefore, to ensure global food security, climate-resilient crop able to adapt to varying environments needs to be developed for sustainable growth and development. Advancements in conventional crop breeding boosted the crop yields between 1960 and 2015; instead, yield attributes to the green revolution are diminishing for the main crops (Grassini et al. 2013) to meet the demand in the foreseeable future (Ray et al. 2013). Therefore, developing novel crop varieties that are sustainable and can produce high yields and adapt to distinct environmental stresses is a key requisite for improving agricultural productivity to food security.

NGS facilitates the more readily accessible to certain crop traits at a very low cost, thereby improving the use of genomics for crop development (Yuan et al. 2017). Considerable sequencing exertions have been made in many plants which serve as a foundation for crop development in breeding programs (Badouin et al. 2017; Springer et al. 2018; Varshney et al. 2017b). Besides, resequencing efforts of wild and cultivated cultivars have gotten insights into the genetic diversity (Varshney et al. 2017a; Wang et al. 2018) that exists in the species in terms of InDels and single nucleotide polymorphisms (SNPs) facilitating marker breeding. However, the bottleneck of the studies is the failure to represent the whole genetic diversity of a particular species (Saxena et al. 2014; Springer et al. 2018).

Pangenome studies revealed approximately 50% of the genes in species functioning as accessory genes (Golicz et al. 2020). In plants, pangenome studies in *Brassica oleracea* L. revealed that about 20% of the overall genes functioned related to major agronomic traits, including flowering, metabolism of glucosinolate, disease resistance, and biosynthesis of vitamin, are in variable regions (Golicz et al. 2016). Later on, Montenegro et al. (2017) detected several thousands of novel genes and variable genes accounting for about 40% of the total gene in wheat. Presently, pangenomes have been assembled for different species including soybean (Liu et al. 2020), tomato (Alonge et al. 2020; Gao et al. 2019), sesame (Yu et al. 2019), rice (Zhao et al. 2018), pigeon pea (Zhao et al. 2020), barley (Jayakodi et al. 2020), apple (Sun et al. 2020), and others.

Pangenome increases read mapping accuracy and variant calling (Garrison et al. 2018; Kim et al. 2019; Tian et al. 2020). Plant accessory (variable) genes are over-represented in signaling, disease resistance, and abiotic stress response activities (Hurgobin et al. 2018; Wang et al. 2018). The use of the pangenome reference will also enable the inclusion of variations other than SNPs in GWAS. Several studies showed that structural variations in association analyses might aid in identifying causative variants (Fuentes et al. 2019) with integrated phenotypic plasticity and environmental adaptability presenting exciting opportunity for crop development (Gage et al. 2019). For instance, the oilseed rape genomic variation permitted to identify missing quantitative trait loci (QTLs) linked with disease resistance. Therefore, the genomic selection in the future will involve the integration of pangenomes to obtain all potential associations.

5.2.2 Agri-genomics for Sustainable Agricultural Production

Current agricultural production systems are under enormous pressure to double their output to feed the world's ever-growing population. Climate change exacerbates the global food production system, and global crop yields fall with an increase in global mean temperature (Varshney et al. 2020). Importantly, this fall in yield further declines with even decreasing land resources and water systems while fulfilling ecosystem preservation requirements (Ronald 2014). The selection of crops with desirable traits time consuming and labors in breeding programs poses a roadblock to plant breeders responding more quickly to rising food supply demands (Lenaerts et al. 2019). Recent advancements in genomic technology have strengthened breeders' toolbox (Bohra et al. 2020; Varshney et al. 2019). When genomic technologies are integrated into novel approaches like rapid generation turnover, gene editing, haplotype-based breeding, and genomic selection, the pace of genetic gains in breeding programs was expected to rise to accomplish sustainable food production.

Conventional QTL mapping approaches have poor throughput, are labor-intensive and time-consuming, and have an inadequate genetic resolution. The availability of a whole genome sequencing has considerably aided trait analysis and gene identification in plants (Jaganathan et al. 2020). Over the last decade, a set of trait mapping techniques, including SNP ratio mapping (SRM), SHOREmap, MutMap, next-generation mapping (NGM), and QTL-seq, have emerged (Bohra 2013; Zhang et al. 2019).

The release of genome-wide markers like SNPs/CNVs from whole genome resequencing initiatives has considerably aided GWAS to identify phenotypic variation associated with the smallest feasible genomic regions. For instance, WGRS-based GWAS in rice (Huang et al. 2010), sesame (Wei et al. 2015), soybean (Zhou et al. 2015), pigeon pea (Varshney et al. 2017a), chickpea (Varshney et al. 2019), and cotton (Ma et al. 2018) identified highly resolved marker-trait associations (MTAs) associated with economically important

traits particularly plant domestication. A GWAS of rice landraces for 500 accessions revealed 80 MTAs for 14 distinct aspects related to grain production and quality, physiology, and drought stress (Huang et al. 2010). GWAS of a core sample of 419 cotton lines allows for fine dissection of fiber-related characteristics and the flowering time trait in cotton (Ma et al. 2018). WGRS-based GWAS on 302 sequenced in legumes, particularly soybean genotypes effectively identify novel QTLs or MTAs affecting a variety of domestication-related traits (Zhou et al. 2015). Similarly, another WGRS-SNP study on 106 accession soybeans under salinity delineates 328 SNPs associated with leaf chlorophyll content, while 401 SNPs account for leaf scorch score (Patil et al. 2016). Likewise, GWAS of 234 lines in soybean revealed the genetic architecture of salinity resistance, with significant MTAs for chlorophyll content ratio, leaf scorch score, leaf chloride content, and leaf sodium concentration (Do et al. 2019).

Gene editing methods that encompass a range of sophisticated techniques for directly generating new alleles are the most efficient approach without transgenics (Zhang et al. 2018). CRISPR (clustered regularly interspaced short palindromic repeat) with the CRISPR/Cas system is the simplest gene editing system widely used in crop gene editing techniques and applications (Chen et al. 2019; Zhang et al. 2018). CRISPR/Cas9 causes a double-strand break in the DNA and depends on the cell's endogenous DNA repair mechanisms to re-join the broken strands. While most DNA repair processes are fundamentally correct, they are mistake-prone, and these faults result in the formation of new alleles. CRISPR/Cas9 for biallelic editing and Mendelian inheritance of these changes was initially described in *Arabidopsis* and crop plants such as rice (Zhang et al. 2014) and tomato (Brooks et al. 2014). Gene editing integrated with genomics is an excellent tool for gene discovery frequently in model species. Wang et al. (2014) demonstrated that wheat powdery mildew resistance is developed by simultaneous editing of three homeoalleles.

When genomic methods are employed for gene identification, increasing the recombination events around the target haplotype can be time-consuming. As a result, identifying the actual causal gene among many possible candidates can be time-consuming. Classical transgenics have proven beneficial and instructive, but they are inaccurate due to location impact and gene dosage where the transgene inserts into the host genome. The availability of gene editing methods provides significant benefits in finding candidate genes and genetic connections to unravel gene activity in the knowledge of QTL areas. The edit(s) can be performed in the actual gene; thus there are no position or dose consequences. Gene expression can be completely knocked out, which was previously impossible utilizing RNAi methods, which generally resulted in a decrease in gene expression but rarely in zero expression (Eamens et al. 2008). Also this implies that editing candidate genes allows for the precise identification of single-gene activity. Another advantage is the ability to target several candidate genes in a single experiment.

5.2.3 Application of Next-Generation Sequencing in Plant Breeding

Recent sequencing initiatives have put forth an enormous effort to sequence increasingly complicated genomes. Plant genomes have a high concentration of repetitive elements, with tandem or segmental duplication. Ploidy is also a sequencing barrier, and findings are affected by various factors, including the genome's autopolyploid or allopolyploid status or the age of the polyploidization event. For a long time, genome complexity has been a concern. It needed to be minimized using sequencing library with the genome partial representation employing restriction enzymes without enzyme digestion (Ray and Satya 2014). Numerous studies sought to create species representative genome in question. A representative genome is a valuable resource to investigate genome function and guide the assembly of the genome in adjacent species. Furthermore, the full accessibility to genome sequences allows for mining a huge number of candidate genes. Resequencing schemes are appropriate to pre-breeding events since they aim to detect genetic variants and infer information on beneficial polymorphisms.

Transcriptome sequencing (RNA-seq) is a novel approach for measuring and transcriptome mapping that uses newly developed deep-sequencing technologies. This method involves turning RNA molecules into cDNA library containing fragments along with adaptors, sequencing of these fragments, and then aligning the resultant sequence against representative genome or de novo assembly (Wang et al. 2009). RNA-seq is a technique for obtaining sequence-expressed data in a given sample/tissue over a set period, even for those species without genome availability (Novaes et al. 2008). An attractive way to study significant and complex genomes and de novo assembly using NGS data is a better option. Roche technology has been used effectively to sequence several non-model plants, including comparative transcript sequencing that resulted from two olive trees at the stage of fruit development (Alagna et al. 2009) and also used in gene characterization (Dassanayake et al. 2009) and molecular marker development (Trick et al. 2009). TRAPID and TrinotateWeb (http://trinotate.github.io) are two user-friendly interfaces to discover candidate genes from de novo assembly (Van Bel et al. 2013). Kamei et al. (2016) created a platform that allows plant breeders, even those having no bioinformatics experience, to use any crop to examine de novo transcriptome assembly from a very complex genome.

NGS and RNA-seq technologies allow gene expression analysis, an essential tool for molecular breeders, and identify gene interest that imparts defense mechanisms under biotic or environmental stresses. It has been reported that *Puccinia striiformis* is a pathogen that causes wheat damage, while its RNA-seq discovered key genes encode effector proteins, which may improve tolerance pathogen (Garnica et al. 2013). Using Roche 454, many genes related to developmental stages were discovered in cucumber transcriptome analysis (Ando et al. 2012). Tang et al. (2013) used Roche's 454-GS FLX System in an RNA-seq investigation of *Populus*

euphratica Oliv. and identified genes relevant for drought resistance grown under semiarid/dry regions.

Similarly, a transcriptome of *Trifolium pratense* sequenced using Illumina technology and genes accountable in drought resistance were identified. Upon drought stress, malate, proline, and pinitol metabolites were found in leaves with high concentrations (Yates et al. 2014). Salinity is also becoming a severe issue; numerous reports are dedicated to identifying a plant's molecular mechanism resistant to salt stress. A similar process has been discovered in cotton (Xu et al. 2013). Furthermore, in *Lolium rigidum*, Illumina technology was utilized to discover genes responsive to herbicide resistance (Gaines et al. 2014). In this regard to examine the role of the NAC transcription factors during dehydration and development of soybean, Illumina technology was utilized (Le et al. 2011).

In addition, transcriptome study of finger millet was analyzed using Ion Torrent, and finger millet is a tough crop known for its tolerance against disease, salt, and drought (Rahman et al. 2014). This technology was later used for the transcriptome profiling of *Jatropha curcas* to unravel molecular responses to waterlogging (Juntawong et al. 2014). Pacific Biosciences SMRT technology was also used to investigate the relationship between the bacterial pathogen *Xanthomonas oryzae* pv. *oryzicola* and its host (rice) utilizing pathogen genome sequencing and attacked host RNA sequencing (Wilkins et al. 2015).

There are many different molecular markers, but the most common are single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSR). Initially, identification of molecular markers was limited to *Arabidopsis* and rice species. Even in species with no reference genomes, such as common bean (Cortés et al. 2011) and wheat (Trebbi et al. 2011), molecular markers have been gradually discovered. Nonetheless, mining for molecular markers in other important species is hampered due to the error of sequencing techniques caused by an inadequate reference genome, the presence of repetitive sequences, or sequencing errors. As a result, Azam et al. (2012) developed coverage-based consensus calling (CbCC), a new method for finding SNPs in *Cicer arietinum*. It comprises four publicly accessible local alignment tools: Maq, BowTie, NovoAlign, and SOAP2.

NGS provides many methods capable of making genome-wide SNP identification and genotyping in one step. Genotyping techniques employ restriction enzymes to capture a genome's reduced representation (Miller et al. 2007). Genotyping by sequencing (Poland and Rife 2012) is a novel method for sequencing multiplexed samples that is both fast and robust. It combines the discovery of genome-wide molecular markers with genotyping (Davey et al. 2011). Desired characteristics can also be introduced into plants that do not naturally express them via genetic engineering techniques. However, genome editing of site-specific nucleases is a very sophisticated method for accurate and effective engineering of the genome, and it assures to transform crop improvement in applied research (Andersen et al. 2015). It involves insertion, deletion, or replacement of a segment of DNA at specific places using designed nucleases that cause clear double-strand breaks (DSBs) and activate DNA repair mechanisms. Although with significant limitations, all the nucleases listed above have been employed to introduce specific mutations in plants.

A recurring challenge in all situations is the practical and functional transport of all reagents to the cells or organisms' level under study.

The CRISPR/CRISPR-associated protein 9 (Cas9) tool seems to overcome the shortcomings of the previous methods (Fichtner et al. 2014). Targetable nucleases have been successfully used for *Arabidopsis*, rice, soybean, tobacco, barley, maize, cabbage, and grass utilizing various strategies such as protoplasts, *Agrobacterium* T-DNA plasmids, embryonic callus, and subsequent plant regeneration (Araki and Ishii 2015; Jiang et al. 2013; Zhang et al. 2010). Mutations may be generated highly using targetable nucleases and known mutations transmitted across cultivars without disturbing their beneficial genetic background. Although genome editing approaches are still in their early stages and are not commonly used, their advantages in robustness, speed, and precision over traditional mutagenesis are undeniable (Osakabe and Osakabe 2015). Genome-editing using artificial nucleases, with precise gene expression measurements, can speed up plant breeding by allowing for the same and predictable modification of genomes (Andersen et al. 2015) and restoring lost characteristics through reverse breeding (Carroll 2012).

5.3 Metabolomics Approaches to Improve Crop Production

Omics improved our understanding of the behavior of organisms at genetics to the environment level (Valdés et al. 2013). Plant secondary metabolites are considered as non-essential natural products but key components for plant adaptation and protection to environmental stresses (Yang et al. 2018). Metabolomics emerge as a powerful tool to dissect GMCs and allow to unveil the influences of genetic application. In this context, it is challenging to the faces of metabolomics due to the diversity of metabolites in plant compared to other organisms (Dixon et al. 2006). In the past decades, metabolomics has been extensively used in breeding programs for various crop species due to its potential for selection of superior traits (Kumar et al. 2021). Metabolomics tools, such as non-destructive liquid chromatography mass-spectroscopy (LC-MS), nuclear magnetic resonance spectroscopy (NMR), high-performance liquid chromatography (HPLC), high-resolution mass spectrometry (HRMS), direct flow injection (DFI), gas chromatography-mass spectrometry (GC-MS), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), and ultra-performance liquid chromatography (UPLC), have accelerated metabolic profiling. Among these LC-MS, NMR-based GC-MS, and CE-MS approaches have been increasingly used in metabolomics (Razzaq et al. 2019).

Integration of modern metabolomics with next-generation sequencing technology, whole genome sequencing, mGWAS, and mQTLs provides an efficient and robust opportunity in sustainable agriculture. Moreover, in the last decade, a plethora of plant genomes has been sequenced, and integrated metabolomics to next-generation sequencing technology enable the comparative genome analysis in a number of species to identify hundreds of genes and pathways. For instance, different types of cucurbitacin in different species of Cucurbitaceae include cucurbitacin

B, C, and D from melon, cucumber, and watermelon, respectively (Shang et al. 2014). A comparative genome analysis identified a few highly conserved genes such as acyltransferases and cytochrome P450 involved in cucurbitacin biosynthesis (Zhou et al. 2016). Introduction of a wide range of metabolites profiling tools in plant physiology and genetics largely enhancing our technical capacities in these research frontiers will be critical for future metabiotic engineering approaches.

5.3.1 Association Analysis Between Metabolites and Trait

Metabolomics is a very powerful approach for several agronomic goals including sustainability/crop resilience, food quality, safety, and fortification and the shelf life fruits (Jacobs et al. 2021). Crop breeding is largely based on selection and development of crop varieties with desirable traits either through conventional breeding approaches or using modern genetic technologies. Integration of genetic approaches with metabolomics enables breeders or researchers to solve key agronomic issues based on their performance under a wide range of environments (Langridge and Fleury 2011). Next-generation sequencing approaches identify a number of metabolic quantitative trait loci (mQTLs) and metabolic genome-wide association study (mGWAS) to analyze the nature of qualitative and quantitative traits in crops. Metabolomics identifies a plethora of secondary metabolites and their correlation with each other and important agronomic traits. It could lead to the development of more rational models to link genotype-phenotype or pathway with yield or specific metabolite or quality-associated traits (Carreno-Quintero et al. 2013). Therefore, the application of both targeted and untargeted metabolomics assists in spatial-temporal metabolic profiling of developing plants. These spatial-temporal metabolic signatures are capable of identifying a set of key biomarker metabolites reliable to produce crops with desirable genetic and developmental traits. For instance, various metabolic approaches have been applied to study rice tillering with 83% metabolic variation (Tarpley et al. 2005). Phytochemical metabolomics profiling of soybean identifies a set of flavonoid kaempferol glycosides during vegetative to reproductive-phase transition (Song et al. 2014). Even through metabolomics has certain limitations, it has a potential to revolutionize plant science and crop breeding and improvement.

Plants produce a range of metabolites at a specific stage of development, in specific organs, and in response to various environmental stimuli (Roldan et al. 2014). Therefore, these metabolites have plant-/organ-specific functions (Dong et al. 2015). For instance, phenol amides, a secondary metabolite ubiquitous in plants, play a pivotal role in a wide range of biological processes involving defense and development (Park et al. 2009). Comprehensive mGWAS profiling of rice revealed two spermidine hydroxycinnamoyltransferases for natural variation of levels of spermidine conjugates (Dong et al. 2015). In *Arabidopsis*, sphingolipids are required for male reproductive development (Feldman et al. 2015). Anthocyanins, a class of flavanols, accumulate in hypocotyls, while few alkaloids pile up in roots/radicals,

and some phenolic compounds and flavanols build up in cotyledons (Roldan et al. 2014). Gong et al. (2013) identify 900 mQTLs in rice metabolome showing distinct accumulation patterns and regulating complex genetic and developmental roles. Moreover, several mQTLs were identified in germinating seed and leaf. Similarly, tissue-specific accumulation of metabolite is pivotal for adaptation and survival of species. Metabolic profiling of 76 tomato introgression lines revealed 30 mQTLs regulating seed metabolism (Gong et al. 2013). Interpreting the effects of genetic improvement on plant primary metabolism will provide useful insights for genetic manipulation and improve our underlying understanding of growth and development of the plant (Wen et al. 2015).

Evaluating the biochemical and genetics of plant metabolism in genetically diverse populations may facilitate the identification of common genomic regions to establish morphological and metabolic traits and determine physiological linkage (Schauer et al. 2006). Combined metabolic studies revealed a considerable variation in levels of lignin precursors and few primary metabolites in *Arabidopsis* and maize (Lisec et al. 2008; Riedelsheimer et al. 2012). The study in potatoes demonstrated the colocalization of starch- and cold sweetening-related traits with loci for metabolites (Carreno-Quintero et al. 2012). These approaches were further placed by evaluating correlation networks between metabolites and specific traits. For example, Toubiana et al. (2012) demonstrated tomato metabolites are influenced by developmental stages. While in maize, correlation between different genetic determinants of metabolic features across tissues was revealed (Wen et al. 2015). The importance of metabolomics in crop breeding becomes extensive, will deepen or broaden our understanding, and provide insights in crop domestication.

5.3.2 Metabolomics to Evaluate GM Crops

Since 1983, genetically modified crops (GMCs) have been developed owing to various beneficial agronomic traits including fast ripening, high feed value, enhanced antioxidant capacity, tolerance to herbicides, and value-added micronutrient contents (Lönnerdal 2003). Currently, there is no evidence of a biological concern on the availability of GMCs. Applications of metabolomics have been increasingly used in analyzing GMCs to broaden our understanding on the nature or composition of GMCs with those produced through conventional approaches (Simó et al. 2014). Currently, mass spectrometry-based metabolomics approached are being extensively used to evaluate MGCs for un-/intended influences. The complexity of plant biological system restricts the development of a suitable tool for global metabolomics. Therefore, a combination of various analytical approaches such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS) is routinely employed (Stewart and Shepherd 2013). The plant has a plethora of complex and diverse metabolites and poses limitations in the global metabolomics.

Other included metabolites with low abundance are difficult to measure but still critical for plant safety or toxicity.

The development of crops with improved phenotype characteristics, particularly quality of fruit and grains as well as yields and productivity, is of utmost importance to breeders and farmers. Traditional breeding programs allow the development of varieties with desirable characteristics such as yield but for complex traits like quality and composition are achieved only through genetic approaches (Breseghello and Coelho 2013). The genetic modification allowed to achieve desirable traits through modification of a single or two genes. However, more recently, integration of omics in crop breeding and genetics discloses the unintended effects of GMCs to the global food safety analytical technologies (Yang et al. 2021). Rice is a global staple food owing to nutritional values and can influence the quality of human life (Cantrell and Reeves 2002). Comparative metabolomics assisted in identifying the desired or undesired metabolites in almost all agricultural crops such as rice, tomato, maize, corn, and wheat (Simó et al. 2014). The results also demonstrate that certain environmental factors exert influences on metabolic profile of plants than genetic modification (Zhou et al. 2009). In this context, the metabolic studies comparing GMCs with non-GMCs are frequently combined with parallel studies under different growth conditions, different ecological environment, and over several generations to assess the intended or unintended effects of genetic manipulation (Zhou et al. 2009). If the metabolic variation falls in the range of natural variations between GMC and non-GMC counterpart parent lines, it has been considered at the safe metabolic level (Harrigan et al. 2010). Recently, detection of any undesirable metabolite caused by genetic modification in GMCs seems to be more powerful than the desirable metabolite (Harrigan et al. 2010). Nonetheless, for GM food safety, the applications of undesirable metabolites in GMCs have not been approved and require counter validation within the global regulatory framework. Metabolomics need to integrate with more sophisticated, efficient, and robust metabolic profiling approaches for detection of as many secondary metabolites as possible.

5.3.3 Metabolomics-Guided Elucidation of Plant Stress Responses

Plants being sessile encounter a variety of environmental stresses throughout their life cycle and have evolved on a variety of adaptive strategies at the transcriptional and posttranscriptional level (Verslues et al. 2006). Efforts have been placed in evaluating the metabolic responses to different stresses and could lead to the development of stress-resistant crops in metabolism-assisted breeding programs. There is an urgent need for metabolomics integration in genomics for crop improvement and with high environment resiliency. The quantitative and qualitative metabolite screening in response to various environmental perturbations identifies

stress-tolerant plant and reveals the biochemical and genetic mechanisms governing plant responses to stresses (Hong et al. 2016).

Environmental stresses arise from conditions that are unfavorable for the optimal growth and development of organisms (Bueno and Lopes (2020) Abiotic stresses are produced by inappropriate levels of physical components of the environment and cause injury through unique mechanisms that result in specific responses (Kaplan et al., 2004). Thus, it is important to improve the traits of available crops for the next generation of crops with high productivity and resilience to climate change (Lankadurai et al. 2013). Although plants are unceasingly affected by abiotic stresses such as drought, temperatures, and salinity, metabolomics has brought ways to elucidate the response-to-stress mechanisms and create resistance strategies in affected plants (Michaletti et al. 2018). Stress causes innumerable transformations in plant metabolisms, such as disturbances in enzyme activities, high requirement for various metabolites, and high levels of reactive oxygen species (Nadarajah 2020). Stress influences each species in different ways and even under some circumstances can be positive to obtain a desired crop response. There is an abundance of plant response mechanisms against stresses as stated by Fahad et al. (2017). Metabolomics is important in the analysis of stress biology in plants and also has the potential to elucidate the mechanisms for tolerance to abiotic stress in plants (Razzaq et al. 2019).

5.3.4 Metabolomics for Improvement of Fruits

Fruit is a complex organ that varies greatly in color, size, and shape and undergoes a series of coordinated growth and development processes. Fruit quality such as nutritional and organoleptic aspects is closely related to their biochemical composition like metabolites. Crop domesticated, genetically manipulated, and further influenced by various environmental stresses tuned fruit biochemical properties. The red ripe fruit's biochemical composition results from complex metabolic changes (Allwood et al. 2021). Moreover, biochemical changes occur during fruit ripening, after its harvest, storage, and transportation (Fortes et al. 2017). Tomato fruits accumulate more secondary metabolites is of great agronomic interest placed it as an ideal model in fruit metabolomics (Stevens et al. 2018). Over a decade, integrated application of genomics and metabolomics in transgenic crops revealed different genomic regions associated with metabolic traits (Hanhineva et al. 2008). Mass spectroscopy and NMR are predominated approaches within plant fruit metabolomics. The proton nuclear magnetic resonance (^{1}H NMR) spectroscopy is more appropriate to evaluate bulk metabolites. To analyze the metabolites importance to fruit including volatile organic compounds and nonvolatile metabolites after derivatization, GC-MS and, while LC-MS is appropriate for polar and nonpolar compounds. Therefore, for fruit metabolite analysis, integration of multiple analytical techniques is commonly applied to address metabolic reprofiling during fruit

set, onset of ripening, postharvest, and other effects induced by different environmental stresses, including both biotic and abiotic (Fig. 5.1).

Fruit has a complex tissue structure, and metabolic studies at fruit set and fruit development revealed several sensitive metabolites (Ru et al. 2017). A study of grapes using stenospermocarpy highlighted regulation of hormone or sugar-mediated pathways (Domingos et al. 2016) or regulatory genes for specialized metabolism (Mounet et al. 2009). These findings expended the understanding of the underlying molecular events during fruit set and inflorescence. In the past, breeding programs are focused on selection of agronomic traits including fruit size, yield, and shelf life. More recently, comparative metabolomics approaches were used to explore genetic and natural variation in different crops that contribute to nutraceutical quality and fruit flavor (Allwood et al. 2021). Integrated metabolomics revealed a molecular basis of fruit involving fruit coloration. In *Ficus carica*, cyanidin 3-O-malonylhexoside accumulates in purple-peeled cultivar (Wang et al. 2017b). Five anthocyanins are identified as candidates involved in flesh coloration (Ying et al. 2019). In *Actinidia arguta*, a study focuses on seven anthocyanin biosynthesis genes associated with pigmentation and elucidates the role of pigmentation development in fleshy fruit at the transcriptional level (Li et al. 2018).

Fruit shelf life is critical for a commercial fruit. Key metabolite analysis shows that sugars including sucrose, myo-inositol, galactinol, raffinose, trehalose, and sorbitol play a remarkable role and negatively affect postharvest physiology (Uarrota et al. 2019). Utilization of metabolomics in postharvest stages in the fruit volatilome demonstrates the reduced supply of electrons from TCA (Araújo et al. 2011). However, targeted approaches revealed plenty of branched-chain fatty acids, amino acids, and ethanol fermentation (Tietel et al. 2011). Malate influences fruit shelf life (Centeno et al. 2011), and a study in tomato revealed a correlation between malate and loss of fruit quality, in postharvest shelf life pomelo (Sun et al. 2013).

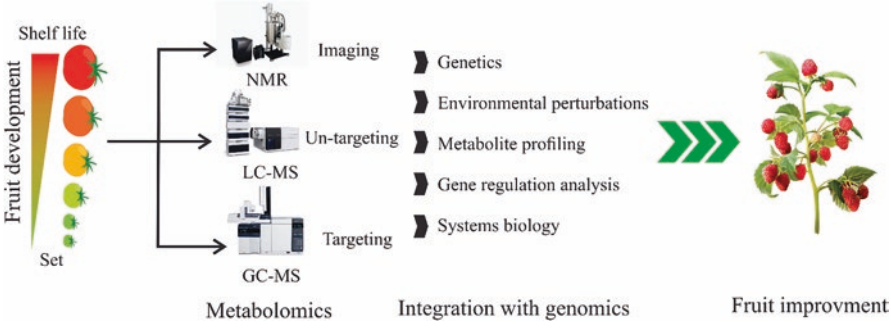

Fig. 5.1 Overview of integration of genomics and metabolomics approaches to fruit improvement. These approaches are performed at various stages of fruit development and at postharvest aided with one or several analytical techniques

5.4 Future Perspectives

Undoubtedly, plant metabolomics has offered us the advantage of precise screening of desirable traits and their underlying metabolic pathway toward improving key attributes of plants. In this context, next-generation sequencing has provided a tremendous and cost-effective support to interpret the metabolic traits. The newly emerged avenues such as EWAS, GS, and GWAS permit proficient metabolite profiling and metabolomics-assisted breeding. Among all "omics" approaches, transcriptomics is the most diverse field that offers deep insights in regulating gene expression and functional characterization. Taken together, transcriptomic sequencing shed light on the selection of gene and their functionality. NGS technologies are improving, and their application is rapidly expanding in scientific disciplines. Plant biology gained a lot from genomics, including plant breeding and evolutionary studies. In comparative genomics, the accessibility of whole genome sequence of the number of plants enables understanding of genetic, evolutionary, and developmental processes which create a diversity of agricultural plants. Therefore, we anticipate that the integration of next-generation sequencing and metabolomics greatly improves plant breeding programs to develop superior plants with better agronomic traits and fulfills the challenge of food security in the twenty-first century.

5.5 Conclusion

Next-generation sequencing technology has been evolved to the fourth generation of sequencing technology and paved the path from whole genome sequencing to resequencing, metagenomics, and methylome. Thus, the application of NGS in the agricultural sector is pivotal in breeding programs. On the other hand, metabolomics differed from nuclei acid sequencing approaches. Altering the metabolic profile of crops will impact the taste, texture, bioactivity, nutrient composition, shelf life, and functionality. In combination with genomics, the exploration of the molecular network of the plants helps understand its biochemical networking and finally results in the functional development of a system in relation to the genotype. The integration of NGS with metabolomics will help develop a powerful tool for investigating phenotype-genotype and prediction of metabolism in non-model plants.

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Chapter 6
Effect of Climate Change on Abiotic Stress Response Gene Networks in *Arabidopsis thaliana*

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6.1 Introduction

Climate change threatens future food security, and, in turn, societal welfare since changing climatic conditions has been changing agricultural production and is expected to worsen in coming decades. Climate change decreases crop yield by intensifying the environmental stress duration or strength on plants. Environmental stresses such as drought, high/low temperature, salinity, and flooding put extensive pressure on plant growth and development (Fahad et al. 2021a).

Plants respond to environmental stress conditions in some conserved regulatory networks, whereas there are minute unique differences between the stresses (Fahad et al. 2021b). These global stress response pathways are essential to determine the stress tolerance level of a species. Among global stress response pathways, generally, genes coding for antioxidants and/or reactive oxygen species (ROS)-scavenging enzymes stand out. Additionally, there is a vast majority of different proteins, including transporters, aquaporins, heat shock proteins, freeze proteins, and secondary metabolites, integrated into stress response pathways. Taken together, all these responses act in synchrony to develop a tolerance response in the organism. Integration of different stress response pathways is a difficult task and needs precise control. The pathways are controlled at transcriptional, post-transcriptional, translational, and post-translational levels. Transcriptional control is performed with the help of transcription factors (TFs) that bind to the cis-elements at the promoter regions of the genes. TFs can induce or suppress the transcription, thereby affecting the mRNA abundance of target genes. This suggests that TFs are the most important regulators in stress response pathways. According to one report, there are more than 1533 TFs in the *Arabidopsis* genome (Riechmann et al. 2000), whereas this number is over 1789 according to another report (Guo et al. 2005). Transcription factors are organized into 49 families and constitute 6% of *Arabidopsis* genes (Riechmann 2008).

In addition to a high number of transcription factors encoded in the *Arabidopsis* genome, they can be induced or repressed by other TFs or other ways of stress signaling such as divalent metal binding, phosphorylation, or receptor–protein interactions. Moreover, the same transcription factor can induce a downstream gene while repressing another one. A TF gene can also be induced or suppressed by different stress conditions. Therefore, stress signaling triggers a massive network of genes to be activated and leads to the interaction of their products to develop the proper tolerance response (Song et al. 2016). This suggests that the understanding of abiotic stress response gene networks is essential to develop food crops resilient to climate change.

Recent bioinformatics analyses have identified a core gene network that is essential in abiotic stress tolerance in plants. These genes include various TFs with essential roles in regulating the downstream tolerance mechanisms. However, a more detailed analysis is required to understand the core transcriptional regulatory network of stress-responsive genes in the model plant *Arabidopsis thaliana*. Here, we explain the identification of a core transcriptional regulatory network of stress-responsive genes in *Arabidopsis* by bioinformatic analyses under several abiotic

stress conditions. These TFs are grouped in different families with previously known functions in stress tolerance in plants while some families need further studies to investigate the potential roles in the network.

6.2 Bioinformatic Analyses

To identify the gene networks affected by different abiotic stresses, extensive bioinformatic studies have already been done in *Arabidopsis thaliana* (Shinozaki et al. 2003; Fujita et al. 2006; Seki et al. 2007; Shinozaki and Yamaguchi-Shinozaki 2007; Tran et al. 2007; Nakashima et al. 2009; Urano et al. 2010; Nakashima et al. 2014), rice (Cooper et al. 2003; Yun et al. 2010; Seo et al. 2011; Zhang et al. 2012; Sharma et al. 2013), and wheat (Tardif et al. 2007). Here, we analyzed the differentially expressed genes in *A. thaliana* from a total of eight abiotic stress conditions that were deposited under the AtGenExpress global stress expression dataset (accession numbers: TAIR-ME00325, TAIR-ME00327, TAIR-ME00328, TAIR-ME00329, TAIR-ME00338, TAIR-ME00339, TAIR-ME00340, TAIR-ME00345) (Kilian et al. 2007; Goda et al. 2008; Wanke et al. 2009). First, differentially expressed gene lists were developed from individual stress microarray data by GeneSpring GX software (Agilent), then the list of overlapping genes that are differentially expressed in all stress conditions was generated. In all stress conditions, 11 or 9 genes were upregulated or downregulated, respectively. To understand the stress regulatory networks that function in all stress conditions, transcription factors or proteins that show putative DNA-binding function have been screened out from the list of all differentially expressed genes. Eleven genes in nine transcription factor families were found to be upregulated, whereas eight genes in eight transcription factor families were downregulated under all stress conditions (Tables 6.1 and 6.2). These genes are defined as the core gene cluster differentially expressed in various abiotic stresses, and the details about their known functions are provided in the following sections.

To elucidate the potential functions of the core gene cluster, network interactions of these core genes were identified via Atted II (Obayashi et al. 2017) and String software (Szklarczyk et al. 2016). According to these analyses, the co-expressed network of genes included very well-known abiotic stress response genes, such as late embryogenesis-abundant proteins, aquaporins, kinases, phosphatases, and chaperons. The extended gene networks included several transcription factors that are co-expressed with the core transcription factor gene clusters, suggesting that the abiotic stress response and tolerance is a complex phenomenon that integrates different signaling pathways (Fig. 6.1). Upregulated core genes were grouped in three gene clusters. The biggest of the three, the first cluster, included *SAP12*, *SCL11*, *WRKY25*, *CPDK32*, *EDF2*/*RAV2*, *RD26,* and *ZAT6*. The other two clusters were centered separately around *MYB44* and *NDB2*. Downregulated core genes, on the other hand, were separated into four groups. *BZIP61*, *BBX27*, *HAT1,* and *MYB30* were clustered in the largest group of clusters, whereas *MYB59* and *DEWAX* generated separate clusters of their own. *SOS3* and *ARR5* were clustered together.

Table 6.1 Core gene cluster upregulated in various abiotic stresses

AGI	Gene abbreviation	Gene name	Family name
AT3G21890	*BBX31*	*B-BOX DOMAIN PROTEIN31*	C2C2-CO-like
AT3G57530	*CPDK32*	*CALCIUM-DEPENDENT PROTEIN KINASE32*	EF-hand-containing protein
AT4G05020	*NDB2*	*NAD(P)H DEHYDROGENASEB2*	
AT5G67300	*MYB44*	*MYB DOMAIN PROTEIN44*	MYB
AT2G30250	*WRKY25*	*WRKY DNA-BINDING PROTEIN25*	WRKY
AT4G27410	*RD26 / NAC72*	*RESPONSIVE TO DESICCATION26 / NAC DOMAIN-CONTAINING PROTEIN72*	NAC
AT3G28210	*SAP12*	*STRESS-ASSOCIATED PROTEIN12*	C2H2
AT5G04340	*ZAT6*	*ZINC FINGER OF ARABİDOPSİS THALIANA6*	
AT4G36990	*HSF4/TBF1/ HSFB1*	*HEAT SHOCK FACTOR4 / TL1-BINDING TRANSCRIPTION FACTOR1 / HEAT SHOCK FACTORB1*	Heat shock factor
AT5G59450	*SCL11*	*SCARECROW-LIKE11*	GRAS
AT1G68840	*TEM2/EDF2/ RAV2*	*TEMPRANILLO2 / ETHYLENE RESPONSE DNA BINDING FACTOR2 / RELATED TO ABI3/VP1 2*	B3 DNA binding

Table 6.2 Core gene cluster downregulated in various abiotic stresses

AGI	Gene abbreviation	Gene name	Family name
AT1G34760	*GRF11*	*GENERAL REGULATORY FACTOR11*	14-3-3
AT1G68190	*BBX27*	*B-BOX DOMAIN PROTEIN27*	C2C2-CO-like
AT3G48100	*ARR5*	*ARABİDOPSİS THALIANA RESPONSE REGULATOR2*	aRR-B
AT3G58120	*BZIP61*	*BASIC LEUCINE ZIPPER*	bZIP
AT4G17460	*HAT1*	*HOMEOBOX-LEUCINE ZIPPER PROTEIN HAT1*	EaR repressome
AT5G24270	*CBL4/SOS3*	*CALCINEURIN B-LIKE PROTEIN4 / SALT OVERLY SENSITIVE3*	EF-hand-containing protein
AT5G61590	*DEWAX*	*DECREASE WAX BIOSYNTHESIS*	AP2/ERF
AT5G59780	*MYB59*	*MYB DOMAIN PROTEIN59*	MYB
AT3G28910	*MYB30*	*MYB DOMAIN PROTEIN30*	

Interestingly, *GRF11* was not clustered with any other genes, suggesting that the gene requires more attention in understanding its functions in general stress response networks.

Cluster analysis of proteins that are identified in our core gene network indicated a large group of proteins interacting with each other (Fig. 6.2). The proteins in the network included the majority of the transcription factors affected by all abiotic stresses. Only some proteins, including HAT1, BZIP61, ARR5, BBX31, MYB59, DEWAX, and SCL11, were not identified in this network. Interestingly, some other

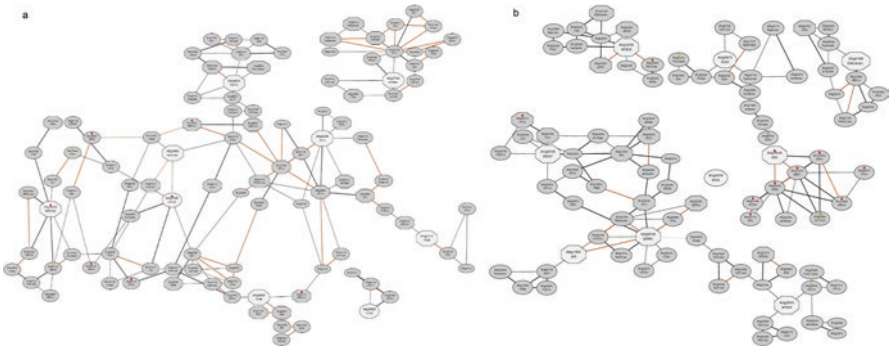

Fig. 6.1 Abiotic stress response gene networks in *Arabidopsis thaliana*. (**a**) Co-expression gene network of upregulated core genes (**b**) Co-expression gene network of downregulated core genes. The gene networks were drawn with Atted II software (Obayashi et al. 2017)

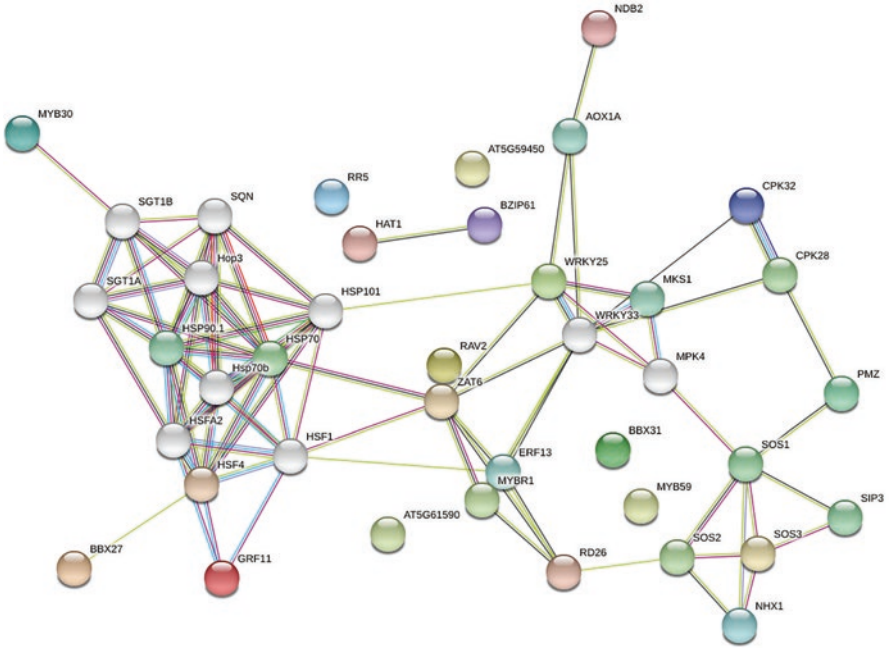

Fig. 6.2 Abiotic stress response protein networks in *Arabidopsis thaliana*. The networks were drawn with String software (Szklarczyk et al. 2016)

stress-related proteins were also clustered in this network, including heat shock proteins and salinity tolerance pathway proteins. Protein cluster analysis represented significantly more interactions than expected (with a protein–protein interaction enrichment p-value of 2.22×10^{-16}), suggesting that the interactions in the protein network are theoretically significant. Therefore, further wet laboratory

experiments are needed for their verification. Blast2GO software (Conesa et al. 2005) was used to determine gene ontologies (GOs) that the protein network is representing (Fig. 6.3). As can be seen in the figure, the majority of the GOs were related to abiotic stress responses and hormonal signaling, indicating the necessity

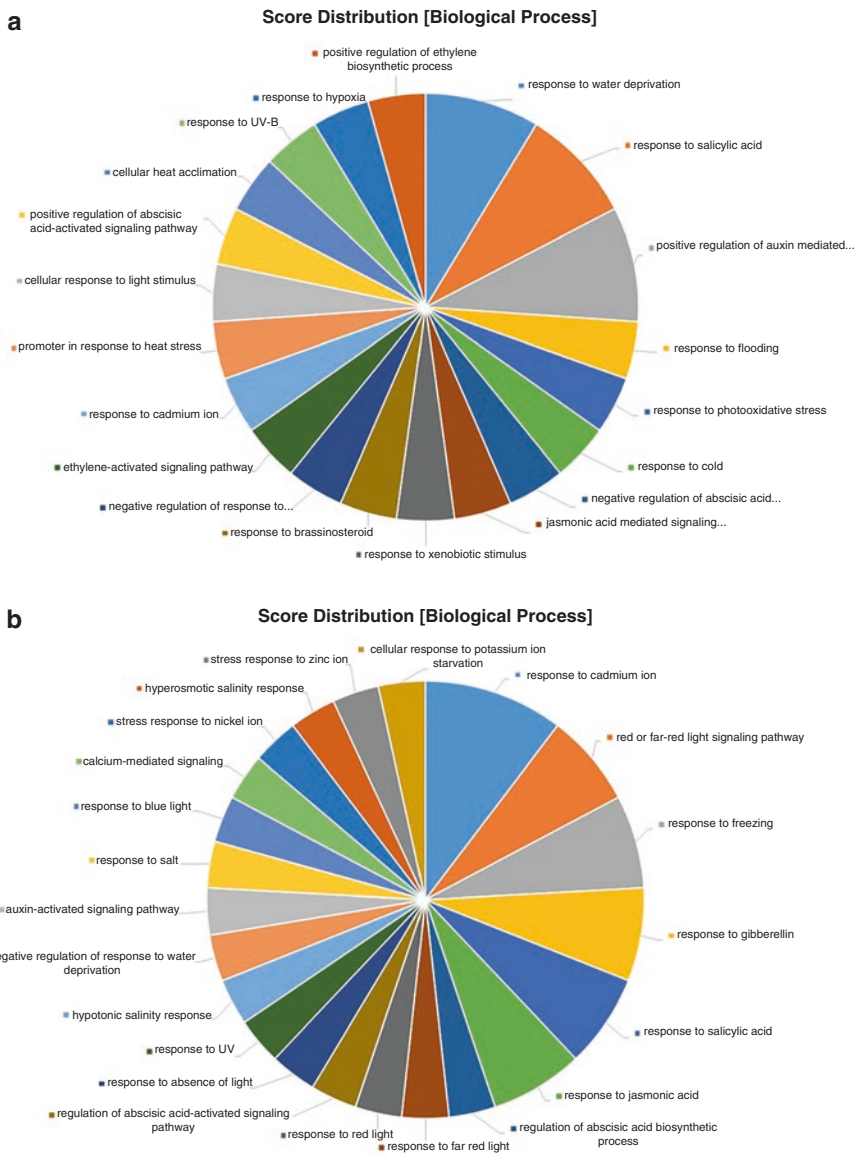

Fig. 6.3 Gene ontology analysis according to the biological processes. (**a**) GO analysis of upregulated genes. (**b**) GO analysis of downregulated genes. The analysis was done with Blast2GO (Conesa et al. 2005)

of hormonal control on abiotic stress tolerance signaling. To confirm the Blast2GO analysis results, GO enrichment analysis was performed by using String software (Table 6.3). Accordingly, the top 10 biological processes GO terms enriched in abiotic stress response protein networks were related with environmental or chemical stress responses, further indicating the identified list of transcription factor genes and proteins that are functionally involved in stress response signaling networks.

Finally, four entities were shared between the networks identified in Atted II and String software, except for the transcription factors identified in our core gene list (Table 6.4). Since Atted II only compares the co-expressed genes whereas String compares any potential protein–protein interactions (even including the text mining), the entities that are identified in the intersection of both software specifically indicate the most striking genes in the entire dataset. Among these genes, *DREB26* and *WRKY33* have been shown to function in abiotic stress signaling and tolerance (Jiang and Deyholos 2009; Kazama et al. 2013). EXORDIUM (EXO) was identified as a probable intermediary protein that functions in brassinosteroid-promoted leaf and root growth (Schröder et al. 2009). Its mutants show several morphological deficiencies. However, its potential functions under abiotic stress tolerance have not been studied yet. Finally, CALCIUM-DEPENDENT PROTEIN KINASE28 (CPK28) was identified as a functional negative regulator of the BIK1 innate immune response pathway via regulating cytosolic calcium (Ca^{2+}) levels (Monaghan et al. 2015). It contains a 14-3-3-binding motif and was shown to phosphorylate 14-3-3 proteins (Swatek et al. 2014) as well as itself (Bender et al. 2017) for regulation of calcium signaling via the Ca^{2+}-calmodulin pathway. Although no reports prove the integration of CPK28 in abiotic stress response signaling networks, through the Ca^{2+}-calmodulin pathway and activation of 14-3-3 proteins, CPK28 shows a high potential to have an important function in stress signaling in the cells.

Table 6.3 Functional enrichments in abiotic stress response protein networks

GO term	Description	Gene count		False discovery rate
		Observed	Background	
GO:0009628	Response to abiotic stimulus	23	1699	1.51e-15
GO:0050896	Response to stimulus	33	5064	1.51e-15
GO:0006950	Response to stress	26	2932	2.60e-14
GO:1901700	Response to oxygen-containing compound	19	1398	1.00e-12
GO:0042493	Response to drug	14	533	1.16e-12
GO:0042221	Response to chemical	23	2654	4.73e-12
GO:0009408	Response to heat	10	184	8.44e-12
GO:0009266	Response to temperature stimulus	12	505	1.23e-10
GO:0010200	Response to chitin	8	113	1.44e-10
GO:0046677	Response to antibiotic	9	253	1.84e-09

Table 6.4 Genes identified by Atted II and String software in abiotic stress response network

AGI	Gene abbreviation	Gene name
AT1G21910	DREB26	*DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN26*
AT2G38470	WRKY33	*WRKY DNA-BINDING PROTEIN33*
AT4G08950	EXO	*EXORDIUM*
AT5G66210	CPK28	*CALCIUM-DEPENDENT PROTEIN KINASE28*

6.3 Basic Region/Leucine Zipper Family

The *Arabidopsis* genome includes 77 members of the basic region/leucine zipper family (bZIP) (Riechmann et al. 2000; Corrêa et al. 2008). Genetic and molecular studies of these AtbZIP factors demonstrated that they have key functions on diverse biological processes such as abiotic stress, biotic stress, light signaling, phytohormone response, seed maturation, and other developmental processes (Dröge-Laser et al. 2018). During the evolution of plants, early recruitment of bZIP TFs may contribute to this diversity, which is in contrast to apparently more bounded functions of the plant-specific R2R3-MYB and WRKY TFs (Eulgem et al. 2000). The ABFs or AREBs are bZIP TFs regulating ABA-dependent gene expression by binding to the ABRE motifs. ABA and abiotic stresses such as drought, cold, or salinity activate gene expression through cis-elements that consist of the ABA-responsive elements (ABRE) in vegetative tissues. Based on the in vitro and yeast assays, ABA-responsive element-binding proteins (AREBs) and ABRE-binding factors (ABFs) can bind to different ABRE-containing promoters (Choi et al. 2000). ABA and relevant stresses therefore possibly activate both transcriptional and post-translational regulation of several groups of bZIPs (Jakoby et al. 2002). One of the members of the bZIP transcription factor family, *AtbZIP61*, was downregulated under relevant stress conditions in our bioinformatic analyses. The bZIP34 and bZIP61 are two putative *Arabidopsis* E group bZIP transcription factors that can form heterodimers through their N-terminal regions for transcriptional activation. AtbZIP34 and AtbZIP61 have eight close homologs in *Arabidopsis,* and they contain a proline residue in the third heptad of the zipper region, which is a common property for physiological responses in plants to various abiotic stress (Kaur and Asthir 2015). In *Arabidopsis* mutants of *bZIP34* and *bZIP61*, heterodimer formation was not observed by yeast two-hybrid assay or electrophoretic mobility shift assay (EMSA), and interestingly mutated forms of AtbZIP34m and AtbZIP61m, where the proline residue was replaced by an alanine residue in the zipper region, could form a homodimer and bind G-box element instead of ABRE (Shen et al. 2007). Both bZIPs are highly expressed in the pollen and interact with bZIP18, which is required for proper pollen development (Gibalová et al. 2017). AtbZIP34 was shown to be necessary for correct pollen wall development (Gibalová et al. 2009). Interestingly, there are no previous studies on the potential roles of these two bZIPs under abiotic stress tolerance.

6.4 Homeodomain Leucine Zipper Family

The homeodomain leucine zipper (HD-ZIP) family of TFs is special to the plants, indicating their roles in growth and developmental processes unique for plants. The HD-ZIP family includes four subfamilies containing leucine zipper domain (ZIP) adjacent to a homeodomain (HD), which can form homo- and heterodimers (Ariel et al. 2007). HD-ZIP II subfamily can be discriminated from HD-Zip I subfamily proteins by containing a conserved "CPSCE" motif, which is present downstream of the leucine zipper (Chew et al. 2013). *HOMEOBOX-LEUCINE ZIPPER PROTEIN1* (*HAT1*) encoding an EaR repressome family protein was found to be downregulated in relevant stress conditions based on our study. HAT1 and its close homologs belong to the class of HD-ZIP II transcription factors, with their roles as a repressor binding to promoters of their target genes, and are involved in plant development and abiotic and biotic stress responses (Tan et al. 2018). The HD-ZIP II family has many important roles in biological processes such as meristem determination, gynoecium and fruit development, auxin and other hormone responses, shade avoidance, and leaf polarity (Harris et al. 2011). Each of these roles suggests that this family is directly or indirectly connected to the environmental stress signaling (Zhang et al. 2014). Tan et al. (2018) demonstrated HAT1 and HAT3 as the important regulators of ABA signaling under drought stress since they suppressed ABA signaling and drought responses.

6.5 Zinc Finger Protein Family

Zinc finger proteins (ZFPs) possess one or more zinc finger(s) that bond zinc ion(s) by histidine (His) and cysteine (Cys) residues, playing several important regulatory roles in plant development and growth, stress responses, and phytohormone responses through four ZFP subfamilies, C_2H_2, CCCH, C_3HC_4, and C_4 (Li et al. 2013). The cysteine2/histidine2-type zinc finger proteins are one of the largest TF families, and some of its members have essential roles in abiotic and biotic stress responses. In our analysis, two members of the C_2H_2 transcription factor family have been identified among the core stress-related transcription factors. Both *SAP12* and *ZAT6* were upregulated under relevant stress conditions. STRESS-ASSOCIATED PROTEIN12 (SAP12) is a member of the stress-associated protein (SAP) family that is considered a novel regulator of abiotic stress responses (Ströher et al. 2009; Giri et al. 2013). Its expression is highly induced under several abiotic stress conditions, resulting in the accumulation of reactive oxygen species (ROS) (Gadjev et al. 2006). Similar to our study, the *SAP12* transcript level was highly increased with respect to the redox potential under salt and cold stresses (Ströher et al. 2009). Cysteine2/histidine2-type transcription factor *ZINC FINGER of ARABİDOPSİS THALIANA6* (*AtZAT6*) was transcriptionally induced under dehydration, cold, salt, osmotic stresses, as well as hydrogen peroxide (H_2O_2) treatment

and pathogen infection (Mito et al. 2011; Liu et al. 2013; Shi et al. 2018a). Exogenous melatonin application-enhanced freezing tolerance is largely relieved in *AtZAT6* knock-down mutant, but was improved in *AtZAT6*-overexpressing plants (Shi and Chan 2014). It was shown that *AtZAT6* overexpression lines could tolerate the cold more efficiently than the nontransgenic plants, and the enhanced tolerance was due to the upregulation of *CBF* genes. Additionally, ZAT6 was shown to have roles in increased cadmium (Cd) tolerance (Chen et al. 2016). It was shown that ZAT6 positively regulates the expression of genes involved in glutathione production such as *GSH1, GSH2, PCS1,* and *PCS2* under Cd treatment.

6.6 B3 Domain Family

Members of the plant-specific B3 transcription factor superfamily contain a B3 domain, and the superfamily is divided into four subfamilies: LAV (LEAFY COTYLEDON2 [LEC2]-ABSCISIC ACID INSENSITIVE3 [ABI3]-VAL), ARF (AUXIN RESPONSE FACTOR), REM (REPRODUCTIVE MERISTEM), and RAV (Related to ABI3/VP1), encoding 118 genes in *Arabidopsis* (Swaminathan et al. 2008). ABI3 binds with bZIP TF, ABI5, to regulate the seed and seedling development and growth (Finkelstein et al. 2005; Lumba et al. 2014). RAV superfamily in *Arabidopsis* includes 13 members. Six members of the RAV superfamily consist of the DNA-binding domain and AP2 domain. Due to the AP2 domain, they are classified in AP2/ERF transcription factors. One of the members of the B3 transcription factor family, TEMPRANILLO2/*ETHYLENE RESPONSE DNA BINDING FACTOR2/RELATED TO ABI3/VP1 2* (*TEM2/EDF2/RAV2*), was upregulated under relevant stress conditions in our study. RAV2 is required for the upregulation of genes involved in stress response pathways in various species (Li et al. 2011a, b). Fu et al. (2014) investigated the potential functions of three homologous proteins, RAV1, RAV1L, and RAV2, and prove that these three RAV transcription factors have functions in both growth and abiotic stress responses in *Arabidopsis*. They performed their analysis with the mutants (*rav1*, *rav1l,* and *rav2*) and overexpression lines (*35S-RAV1OE*, *35S-RAV1LOE,* and *35S-RAV2OE*) of these three RAV transcription factors. When drought stress was applied to the mutants and wild type, they both showed a similar pattern. However, water loss of the *35S-RAV1OE* and *35S-RAV2OE* transgenic plants was significantly higher than that of wild type. Under salt stress conditions, no differences were observed in seed germination of *rav* mutants in comparison with the wild type. *RAV* overexpression lines showed decreased seed germination rates compared with the wild type. These results indicate that RAVs negatively regulate drought and salinity tolerance in *Arabidopsis*. When *AtRAV1* and *AtRAV2* were overexpressed in cotton, it resulted in longer cotton fibers under drought in the field (Mittal et al. 2015). The overexpression lines showed delayed flowering and retained bolls at higher nodes. RAV2 was also identified to interact physically with a trihelix transcription factor GT-4 in salt tolerance (Wang et al. 2014).

6.7 APETALA2/ETHYLENE-RESPONSIVE FACTOR Family

APETALA2/ETHYLENE-RESPONSIVE FACTOR (AP2/ERF) family of transcription factors from several plant species have demonstrated that this TF family is involved in abiotic stress responses (Mizoi et al. 2012). Many of the transcription factors in AP2 and ERF/DREBP subfamilies are mostly involved in ethylene- and ABA-related responses (Zhu et al. 2010). DREB1/CBF and DREB2 subgroups have key roles in the acquisition of stress tolerance by controlling gene transcription sets via DRE/CRT sequences in stress-inducible gene promoters (Agarwal et al. 2006). By crosstalking with each other, AP2/ERF family transcription factors mostly regulate physiological, biochemical, and developmental responses to several environmental stress factors (Mizoi et al. 2012). *DECREASE WAX BIOSYNTHESIS* (*DEWAX*) encoding an AP2/ERF transcription factor (*ERF107*) was found to be downregulated in our study under relevant stress conditions. DEWAX is known as a negative transcriptional regulator that can repress the expression of genes included in cuticular wax biosynthesis in *Arabidopsis* (Go et al. 2014; Suh et al. 2014). It is specifically expressed in the epidermis and upregulated at dark. DEWAX is involved in resistance to *Botrytis cinerea* in *A. thaliana* and *Camelina sativa* by physically binding to the promoters and inducing the expression of genes involved in biotic stress tolerance such as *PRX37, IGMT1,* and *PDF1.2a* (Ju et al. 2017). Under abiotic stress conditions, there are no specific studies about the functions of DEWAX; therefore, there is a gap in the literature.

6.8 Myeloblastosis Family

Myeloblastosis (MYB) superfamily is the most abundant group of transcription factors in plants. In total, 198 genes in the *MYB* superfamily were discovered in the *Arabidopsis* genome. Among these, 5 are *R1R2R3-MYB,* 126 are *R2R3-MYB*, 64 are *MYB*-related, and 3 are atypical *MYB* genes (Cominelli and Tonelli 2009). Based on many studies about the MYB family, most of its members showed significant regulation by abiotic and biotic stresses (Persak and Pitzschke 2014). There are also some studies related to the hormonal regulation of MYB TFs (Roy et al. 2016). Based on our study, three members of the MYB TF family were included in the core gene set. *MYB DOMAIN PROTEIN59* (*MYB59*) and *MYB DOMAIN PROTEIN30* (*MYB30*) were downregulated, whereas *MYB DOMAIN PROTEIN44* (*MYB44*) was upregulated under stress conditions. MYB59 is identified as a negative regulator of nutrient stress signaling in plants. It is overaccumulated under Cd and regulates the cell cycle progression and root elongation. Moreover, it is a repressor of Ca^{2+} homeostasis and signaling under Ca^{2+} deficiency, thus regulating stress responses (Fasani et al. 2019). MYB59 also maintains the balance of K^+ and NO_3^- distribution between roots and shoots by regulating the transcription of the nitrate transporter

NRT1.5/NPF7.3 under low K^+ stress (Du et al. 2019). Liao et al. (2017) have demonstrated that transcription factor MYB30 can bind and regulate *ANNEXIN* (*ANN)* genes and promote their expression at a functional level. *ANNs* have controls on oxidative and heat stress responses. Moreover, MYB30 participates in ABA responses via SUMO ligase SIZ1-mediated sumoylation, and the stability of MYB30 is disrupted by a RING-type ubiquitin E3 ligase RHA2b in ABA signaling (Zheng et al. 2018). According to a recent study, *MYB30* is highly upregulated under ROS treatment and controls a gene network, leading to the blockage of root cell elongation via hydrogen peroxide production (Mabuchi et al. 2018). *MYB44* overexpression lines showed elevated tolerance to salt stress compared with wild type (Persak and Pitzschke 2013). Moreover, AtMYB44 was shown to suppress the gene transcription of type 2C protein phosphatases (*PP2C*s) involved in ABA signaling such as *ABI1, ABI2*, and *HAI1* (Nguyen et al. 2019a, b) or late embryogenesis-abundant protein *LEA4-5* (Nguyen et al. 2019a, b) through histone H3 acetylation (H3ac) and methylation (H3K4me3) around transcription start site under salinity and osmotic stresses.

6.9 WRKY Family

WRKY transcription factor family is one of the largest families that have comprehensive biological functions in plant biotic and abiotic stress responses, nutrient deprivation, hormone-controlled processes, seed and trichome development, senescence, and embryogenesis (Rushton et al. 2010). WRKYs can act as transcriptional activators or repressors in various homo- and heterodimer combinations (Ülker and Somssich 2004; Eulgem and Somssich 2007; Rushton et al. 2010; Agarwal et al. 2011). One of the WRKY members, *WRKY25*, was upregulated under all stress conditions in our analysis. In addition to our study, Castillo et al. (2018) studied high levels of nitric oxide (NO) combined with stress-triggered responses and development, analyzing early changes in the transcriptome for the identification of transcription factors involved in NO sensing. In that study, WRKY25 was found hypersensitive in ABA signaling in NO-triggered responses. Moreover, Zheng et al. (2007) have investigated the function of WRKY25 in plant defense responses against the bacterial pathogen *Pseudomonas syringae*. WRKY25 was found as a negative regulator of salicylic acid (SA)-mediated defense mechanism against *P. syringae.* According to Kilian et al. (2007), *WRKY25* was negatively regulated by heat and salt stresses. In contrast to Kilian et al. (2007), Jiang and Deyholos (2009) have shown that the transcript abundance of *WRKY25* and its close relative *WRKY33* were increased under salt stress. Additionally, the expression level of *WRKY25* was also induced by heat stress (Li et al. 2009, 2011a, b; Zhou et al. 2015).

6.10 NAM, ATAF, and CUC Families

One of the largest TF families, the plant-specific NAM, ATAF, and CUC transcription factor (NAC) family, has vital roles in plant growth, development, and abiotic plant responses (Nakashima et al. 2012; Nuruzzaman et al. 2013). Many studies reported that lots of the stress-responsive NAC factors have been used to develop stress-tolerant crops by genetic engineering (Nuruzzaman et al. 2013). There are more than a hundred NAC proteins included in *Arabidopsis* and rice (Olsen et al. 2005). Based on our study, one of the members of the NAC family belonging to the ATAF subfamily, *RD26/NAC72,* was positively regulated under all relevant stress conditions. Similar to our work, *RD26* was markedly upregulated by salt, drought, and abscisic acid (ABA) treatments (Chung et al. 2014; Fujita et al. 2004; Tran et al. 2004; Takasaki et al. 2015). However, Huang et al. (2018) showed that the transcript level of *RD26* was negatively regulated by sodium chloride (NaCl), but slightly increased under NaCl + ABA treatment. In addition, RD26 has been shown as a key regulator of starch degradation and the accumulation of mono- and disaccharides by directly enhancing the expression of *AMY1*, *SFP1,* and *SWEET15* that are involved in carbohydrate metabolism and transport under the control of dark-induced senescence (Kamranfar et al. 2018). Interestingly, heterologous expression of AtRD26 homolog in *Eutrema salsugineum*, NAC1, in *Arabidopsis* inhibited the vegetative growth of *Arabidopsis*, and the overexpression lines were more tolerant to salt and oxidative stresses than the wild type (Liu et al. 2018).

6.11 Heat Shock Factor Family

Heat shock transcription factors (HSFs) act as regulators of genes induced by thermal stress, coding heat shock proteins. Most eukaryotes have 1–3 heat shock factors; however, plants have more than 20 heat shock factors, classified as classes A, B, and C (Baniwal et al. 2004). HSFs are essential for plants to respond to adverse abiotic stress conditions by regulating stress-responsive genes (Nover et al. 2001; Klaus-Dieter 2012). In our analysis, we found that one of the HSFs, *HSF4/TBF1/HSFB1* (*ARABİDOPSİS THALIANA HEAT SHOCK FACTOR4, TL1-BINDING TRANSCRIPTION FACTOR1, ARABİDOPSİS THALIANA HEAT SHOCK FACTOR B1*), is upregulated under adverse stress conditions. Normally, *HSF4* is shown to respond to heat stress (Charng et al. 2007; Tunc-Ozdemir et al. 2013; Weng et al. 2014), but its transcript level also increases under several biotic and abiotic stress conditions (Nover et al. 2001; Klaus-Dieter 2012). Genome-wide expression profiling showed that HSF4 has an essential role in the growth to defense transition (Pajerowska-Mukhtar et al. 2012). Additionally, *HSF4* expression is tightly regulated at both the transcriptional and translational levels. In vitro and in vivo experiments indicate that HSF4/TBF1 binds to TL1 cis-element (translocon 1; *GAAGAAGAA*), which is in the promoter regions of NPR1-dependent ER-resident

genes through the involvement of an unknown TF, regulating the genes associated with pathogen infection and salicylic acid (Wang et al. 2005; Pajerowska-Mukhtar et al. 2012). Additionally, HSFB1 and HSFB2b suppress the response of heat shock under nonheat stress conditions (Ikeda et al. 2011). *HsfB1* and *HsfB2b* are the necessary heat stress-inducible heat shock protein genes to acquire thermotolerance, and they are activated by histone chaperone ASF1 under heat stress conditions (Weng et al. 2014). Moreover, HSF4/TBF1 is associated with the unfolded protein response (UPR) (Nagashima et al. 2014).

6.12 GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA), and SCARECROW (SCR) Families

GRAS proteins are plant-specific proteins, which are found in higher angiosperms (Cenci and Rouard 2017). Thirty-three GRAS proteins are present in the *Arabidopsis* genome, and they are divided into eight groups according to their amino acid sequence similarities (Lee et al. 2008). Its name is derived after the three initially identified members GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GA (RGA), and SCARECROW (SCR) (Pysh et al. 1999). GRAS proteins have key roles in very diverse processes, including plant growth and development, signal transduction, meristem maintenance, and development (Hirsch and Oldroyd 2009; Sun et al. 2012). They are especially very critical for gibberellin and mycorrhizal signaling (Xue et al. 2015). GRAS proteins include several conserved regions at their N-terminus, two leucine-rich areas (LHRI and LHRII) flanking VHIID motifs, PFYRE and SAW (Pysh et al. 1999; Bolle 2004). Based on our study, one of the GRAS proteins *SCL11 (SCARECROW-LIKE11)* was upregulated under all relevant stress conditions. In addition to our study, Ma et al. (2006) investigated that *SCL11* was extensively upregulated under salt stress. However, the biological roles of SCL11 are largely unknown; therefore, it requires further studies.

6.13 C2C2-CO-Like Family

The *Arabidopsis* genome consists of 30 members of the C2C2-CO-like transcription factor family containing an atypical domain composition. These proteins have one response regulator receiver domain and two motifs that suggest a role in transcriptional regulation: an acidic domain and a basic motif within the CONSTANS family of transcription factors (Griffiths et al. 2003). Saibo et al. (2008) investigated that the C2C2-CO-like gene family was primarily involved in drought stress responses in *Arabidopsis* (Mun et al. 2017). Two of the members of the C2C2-CO-like transcription factor family were determined in our bioinformatic analyses.

B-BOX DOMAIN PROTEIN31/MICROPROTEIN1A (*BBX31/MIP1A*) was upregulated, whereas *B-BOX DOMAIN PROTEIN27* (*BBX27*) was downregulated under relevant stress conditions. In addition to our study, Gangappa and Botto (2014) investigated that BBX31 negatively regulated photomorphogenesis under visible light. It induced photomorphogenesis under UV-B and increased tolerance to high doses of UV-B radiation. BBX31 and HY5 oppositely and independently have control on seedlings to adapt to varying light intensities (Gangappa and Botto 2014). In another research, BBX31 was identified as a key signaling factor in visible and UV-B light signal transduction in *Arabidopsis* (Heng et al. 2019). *BBX31* expression is promoted by UV-B radiation in a fluorescence-dependent manner. HY5 can directly bind to the promoter of *BBX31* and increase its transcript levels (Heng et al. 2019). Gain-of-function and loss-of-function mutants of *BBX31* showed that it plays a negative role in photomorphogenesis under white light; however, it acts as a positive regulator of UV-B signaling. Genetic interaction studies investigated that BBX31 promotes photomorphogenesis apart from HY5. There was no evidence for direct BBX31-HY5 interaction, and they primarily induced different sets of genes in white light. Under the high intensity of UV-B radiation, BBX31 induced the accumulation of UV-protective flavonoids and phenolic compounds, resulting in tolerance to UV-B radiation by regulating genes involved in photoprotection. Under UV-B radiation, overexpression of BBX31 promoted transcript level of *HY5* in a UV RESISTANCE LOCUS8-dependent manner, indicating that BBX31 may regulate *HY5* transcription. Under low intensities of white light, BBX31 also controlled primary root elongation. Multiple primary and secondary metabolites were identified in *35S-BBX31* by GC-MS- and HPLC-based metabolite profiling, which may suggest their involvement in UV-B tolerance in plants (Yadav et al. 2019). *BBX27* was downregulated in our study; however, there is no study about its control under abiotic stress conditions in the literature. Therefore, further studies need to be conducted to understand the functions of members of the C2C2-CO-like family in plants under environmental stress conditions.

6.14 Type B *Arabidopsis thaliana* Response Regulator Family

There are 15 members of the aRR-B family in the *Arabidopsis* genome. Type B *Arabidopsis thaliana* response regulators (ARRs) are transcription factors having functions in the final step of signaling systems (Mason et al. 2005). ARR family members function as DNA-binding transcriptional regulators, whose activities are mostly seen as regulated by phosphorylation/dephosphorylation. The major subfamily of type B ARRs was investigated, resulting particularly in high expression in regions where cytokinin plays a key role (Yoshinori et al. 2004). Pavlů et al. (2018) studied that cytokinin is a plant hormone having major roles not only in plant growth and development processes but also in stress responses. Expression patterns of abiotic stress-related genes were overlapped with those of cytokinin metabolism and signaling genes (Pavlů et al. 2018). Based on our analysis, one of the members of

the aRR-B family, *RESPONSE REGULATOR5/ARABIDOPSIS THALIANA RESPONSE REGULATOR2/INDUCED BY CYTOKININ6* (*ARR5/ATRR2/IBC6*), was downregulated under relevant stress conditions; however, this transcription factor has not been characterized yet, and further research is needed to determine the function of ARR5 under abiotic stress conditions.

6.15 14-3-3 Family

The 14-3-3 family of proteins are not classified as transcription factors; however, they are phosphoserine-binding proteins that have key functions on targets via direct protein–protein interactions and play essential roles in metabolic pathways (De Lille et al. 2001). The 14-3-3s have important features in generating proton gradients through the plasma membrane and promoting H^+-ATPase in the plasma membrane (Jahn et al. 1997). Due to this specific feature of the 14-3-3 proteins, their genes can be regulated by several biotic and abiotic stresses such as low temperature (Jarillo et al. 1994), pathogen attack (Brandt and Neve 1992), salt stress (Xu and Shi 2006), hypoxia (De Vetten and Ferl 1995), and drought (Porcel et al. 2006). During plant growth and development, various members of the 14-3-3 family are involved in multiple stress and signaling pathways, suggesting their roles in crosstalk between abiotic and biotic stresses (Cao et al. 2007). In the *Arabidopsis* genome, 15 members of the 14-3-3 protein family are present (Rosenquist et al. 2001). This family is also called general regulatory factors (GRFs) in *A. thaliana* because of their potential roles in a wide range of cellular processes (Rooney and Ferl 1995). Based on our analysis, the transcript level of one member of the GRF protein family *GRF11* (*GENERAL REGULATORY FACTOR11*) was upregulated under all relevant stress conditions. In addition to our study, *GRF11* is frequently reported to be induced by Fe deficiency (Jian et al. 2013). There is no more study about GRF11 in the literature. Therefore, further studies need to be conducted under abiotic stress conditions to determine the functions of this protein in signal transduction.

6.16 Other Regulatory Proteins: Calcium Sensor Family

Ca^{2+} sensors are known as Ca^{2+}-binding proteins, and most of the Ca^{2+}-binding proteins consist of EF-hand motifs having a conserved helix–loop–helix structure that can bind a single Ca^{2+} ion (Day et al. 2002). The family of Ca^{2+} sensors includes calmodulin (CaM), calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBL). Most of the Ca^{2+}-binding proteins have been studied to involve in the transduction of signals related to biotic and abiotic stress (Wang et al. 2013). Based on our study, three members of EF-hand-containing protein family, namely, CALCINEURIN B-LIKE PROTEIN4/*SALT OVERLY SENSITIVE3* (CBL4/SOS3), *CALCIUM-DEPENDENT PROTEIN KINASE32* (*CDPK32*), and

NAD(P)H DEHYDROGENASEB2 (NDB2), were differentially expressed under relevant stress conditions. SOS3 protein is mainly known to be associated with salinity responses (Gong et al. 2004). Salt stress responses in plants are being controlled mainly by the proteins involved in the salt overly sensitive (SOS) pathway, which have responses to ion homeostasis and salt tolerance in plants (Ji et al. 2013). *SOS1*, *SOS2*, and *SOS3* loci were first discovered by forward genetic screens through salt-hypersensitive growth. The calcium sensor *SOS3* promotes the kinase *SOS2*, which positively regulates *SOS1* under salt stress conditions (Ma et al. 2014). SOS1 is a Na^+/H^+ antiporter that transports Na^+ out of cells under salt stress (Shi et al. 2000; Qiu et al. 2002). High-affinity K^+ TRANSPORTER1 (HKT1) can limit the root-to-shoot sodium transportation and has been proven to be involved in the salt tolerance with SOS pathway in *A. thaliana* (Uozumi et al. 2000). Distinct physiological roles for HKT1 and SOS3 have been shown to protect plants against salinity (Horie et al. 2006). Salinity tolerance mechanisms have been studied a lot; therefore, the details of SOS3 functions are not detailed here. Recently, proteins that induce or suppress the SOS pathway have been shown (Yang et al. 2019a, b), suggesting that there are still missing steps in the salinity tolerance mechanisms. Another member, *CDPK32*, has been shown as the critical component of Ca^{2+} homeostasis based on the overexpression and mutant studies (Zhou et al. 2014). AtCPK32 has autophosphorylation activity and can phosphorylate ABF4 in vitro, thereby regulating the ABA-responsive gene expression via ABF4 (Shi et al. 2018a). Nitrate triggers a unique Ca^{2+}–CPK signaling via CPK32 and its orthologs in *Arabidopsis* (Liu et al. 2017). The third member, NDB2, has been shown to regulate hormones such as auxin (through alternative oxidase *AOX1a*) and play important roles in salt and oxidative stresses (Elhafez et al. 2006). NDB2 and AOX1a cluster together as they are members of an alternative respiratory pathway in mitochondria (Van Aken et al. 2009). Together, these proteins can form a complete respiratory chain, dissipating energy as heat and not contributing to ATP synthesis (Clifton et al. 2005). The relation of *NBD2* with hormones suggests that it can also get involved in plant growth and development (Smith et al. 2011).

6.17 Conclusion and Future Prospects

As the climatic changes will lead to extensive environmental stress conditions in the future, sessile plants are expected to be affected the most. This eventually causes a drastic decrease in crop yield and food production. Technological advances in molecular plant breeding, hybrid breeding, plant genetic engineering, genomics, and genome editing have been deployed in the development of new crop species that can tolerate environmental stress conditions. Even though the genetics of abiotic stress tolerance mechanisms have been studied in the last 50 years, we now start to understand the real meaning of those mechanisms and the network of genes regulating them with the help of bioinformatics and systems biology. As transcription factors are located in the heart of abiotic stress signaling networks, our approach here

detailed a core cluster of 20 differentially regulated transcription factors in various abiotic stresses. Important functions of some of these transcription factors have already been demonstrated under different abiotic stresses. However, more extensive research is required to understand their roles in stress tolerance and regulation of downstream gene products. Since transcription factors can target several genes involved in various metabolic pathways, genetic engineering or molecular breeding of plants by using one or multiple of these identified transcription factors may eventually lead to the development of crop species with higher levels of stress tolerance.

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Chapter 7
The Application of Databases and Bioinformatics for the Genome Editing of Crops

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7.1 Introduction

Humans take 66% of their daily energy requirements from different staple crops, like wheat, rice, and corn (Ahmad et al. 2015a). With the increasing world population, food demand is also increasing, which puts pressure on plant breeders around the globe to produce high-yielding crops (Hu et al. 2018). In addition to this, water scarcity, climate change, and limited agricultural land are posing more challenges to agriculture (Iglesias et al. 2011; Mancosu et al. 2015; Schneider and Asch 2020). However, genomics is helping to modify genetic components for increasing crop productivity (Kole et al. 2015). Genome-editing techniques have proven useful in the improvement of crop yields, the resistance against stresses, and enhancing the nutritional contents of crops, as shown in Figs. 7.1 and 7.2. Crop genome sequences are needed for identifying agronomically important variations in the genomes of crop plants. In the last decade, the cost of DNA sequencing has decreased rapidly, which has led to the production of genomic data in enormous amounts, which provides substantial opportunities for crop breeders (Edwards et al. 2013).

Advancements in bioinformatics tools have provided fast procedures for analyzing large genomes of plants (Ahmad et al. 2021b). Third-generation technologies that can produce >10-kb-long sequence reads have been developed in recent years and have made crop genome sequencing easier. Today, genomic sequences of approximately 260 plants can be found in GenBank, which includes almost all major crops. For a long period of time, crop breeding has primarily relied on crossing and phenotypic selection, which can produce better genotypes by recombination of genetic elements. The genome sequences of plants help in identifying all genes, variants, and genetic pathways responsible for beneficial agronomic traits, and the modifications that emerged throughout the breeding process can be analyzed on a genotypic level. The readily available genomic data sets support breeders in mapping the quantitative trait loci. New and better crops can be developed after the identification of different genomic variations produced during breeding in crop species, followed by a selection of the best variants for desired traits (Dwivedi et al. 2017; Mousavi-Derazmahalleh et al. 2019).

Genomic selection (GS) is an approach that connects different variants present in a whole-genome sequence to avoid repeated phenotyping in the breeding process. Advanced bioinformatics tools are crucial for analyzing, processing, and gaining functional insights from big genomic data sets of plants (Moore et al. 2010; Batley and Edwards 2016). Variant identification, sequence alignment, and genome assembly are some standard bioinformatics approaches used while analyzing sequence data. The algorithms required for performing these analyses are nontrivial, and different computational tools use unique parameters. Sequence alignment tools

developed for aligning short sequences did not perform well when used for long sequences (Hwang et al. 2015; Sedlazeck et al. 2018).

The other challenge is to reduce impact of preferences of using various tools during assembly of genome sequences (Ong et al. 2016). Therefore, choosing the right tool for analyzing the data set is crucial. There are many tools available for genome assembly and processing and for identifying variants, but selecting an appropriate analysis method and tool is another challenge (Grierson et al. 2011). The existing databases for crops, like Gramene (https://www.gramene.org), GrainGenes (https://wheat.pw.usda.gov/GG3/), and Wheat Information System (http://www.wheatis.org), would help in storing an enormous amount of data and allowing breeders to access the genetic information easily (Scheben et al. 2018; Tello-Ruiz et al. 2018). Bioinformatics and genomics play a substantial role in enhancing the yield of modified crop varieties. The integrative approaches that simultaneously use genomics, phenotyping, and bioinformatics can assist breeders in dealing with big data sets and identifying targets (Evans et al. 2013; Ahmad et al. 2021b). In this book chapter, we have tried to explain the application of databases and bioinformatics in developing improved crops. Furthermore, we have integrated the role of computational tools in the genome editing of crop plants.

7.2 Integrated Crop Databases

With the establishment of new "omics" technologies and third-generation sequencing, an enormous amount of data is being produced and is becoming available to scientists for investigating various crop traits at gene and population levels (Pathak et al. 2018; Khandagale et al. 2020). Crop genomic databases are divided into two categories: crop-specific databases and general databases. Crop-specific databases are limited to some specific crops, while general databases contain information on various crop species. General databases such as European Molecular Biology Laboratory (https://www.ebi.ac.uk/), GenBank (https://www.ncbi.nlm.nih.gov/genbank/), Phytozome (https://phytozome-next.jgi.doe.gov/), and Plant Genomic Database (https://www.plantgdb.org) play significant roles in storing genomic data; however, they provide very less or no phenotypic information (Nakaya et al. 2017; Wei et al. 2018; Madeira et al. 2019; Sayers et al. 2019). It is a major challenge for plant scientists and breeders to link genotypic information to phenotype because phenotypes depend on genomics, environmental factors, and epigenomics (Alonso et al. 2019; Rey et al. 2020). However, some crop databases, like GrainGenes (https://wheat.pw.usda.gov/GG3/), integrate the marker and gene expression data with genomic information, but there is a need to develop such repositories further (Ahmad et al. 2021b). Creating an integrative database by merging different annotated genomes, phenomes, interaction networks, and gene functions is quite difficult because the relevant data sets are dispersed in many formats in various databases that have dissimilar coverage and quality. Complex plant genomic databases can be combined by the intelligent mining of big crop databases that would allow easy

gene discovery for crop improvement (Hassani-Pak and Rawlings 2017). The various databases for important crop species are presented in Table 7.1. A web-based artificially intelligent search tool known as KnetMiner (https://knetminer.com) has been developed that integrates biological data present in different repositories. KnetMiner has the ability to discover unique connections among morphological traits and genes (Hassani-Pak and Rawlings 2017). In the KnetMiner approach, the four major steps involved are as follows: (i) integrating diverse biological data sets into a knowledge graph, (ii) improving the knowledge graph by mining the literature, (iii) identifying links among evidence nodes and genes, and (iv) visualizing the integrated data by applying an algorithm selected based on the evidence obtained. Integrative databases can be made for important crops, such as corn, wheat, barley, etc., by using the KnetMiner approach, which provides useful insights into indirect associations between biological processes and distant traits. A single information system for rice and wheat is also in its development stage, which would help scientists in improving these crops (Yuan et al. 2017; Scheben et al. 2018). The identification of genes associated with various phenotypic traits and the understanding of complex traits can be achieved through advances in data mining approaches and by developing integrative crop databases, which are useful for improving crops.

Table 7.1 Important crop-specific databases and their web links

Name of database	Plant species	Online web link
The Arabidopsis information resource (TAIR)	*Arabidopsis thaliana*	https://www.arabidopsis.org
Phytozome V13	Hosts genome of about 261 spp.	www.phytozome.net/
Sunflower genome database (SGD)	*Helianthus annuus*	https://www.sunflowergenome.org
MaizeGDB 2018	*Zea maize*	https://www.maizegdb.org
Brapa_1.0	*Brassica rapa*	https://plants.ensembl.org/Brassica_rapa/Info/Index
CottonFGD	*Gossipium spp.*	https://cottonfgd.org
OryzaGenome	*Oryza spp.*	http://viewer.shigen.info/oryzagenome21detail/index.xhtml
TOMATOOMICS	*Solanum lycopersicum*	http://bioinf.mind.meiji.ac.jp/tomatomics/
WGVD	*Triticum aestivum*	http://animal.nwsuaf.edu.cn/code/index.php/Wheat
BarleyVarDB	*Hordeum vulagre*	http://146.118.64.11/BarleyVar
GmGDB	*Glycine max*	http://www.plantgdb.org/GmGDB/
GrainGenes	*Avena sativa* and *Triticum aestivum*	https://wheat.pw.usda.gov/GG3/
Chickpea transcriptome database (CTDB)	*Cicer atretinum*	http://www.nipgr.res.in/ctdb.html
European cultivated potato database	*Solanum tuberosa*	https://www.europotato.org

7.3 CRISPR/Cas9-Based Genome Editing Databases

The domestication of crops and the improvement of crop genomics have always been the target of plant breeders. However, with the development of modern technologies for crop improvement have been shifted from selection of superiority phenotypic traits to targeted genome editing assemblies. Previously used genome-editing techniques (i.e., "transcriptional activator-like effector nuclease" (TALEN) and "zinc finger nuclease" (ZFN)) were designed with the help of DNA-binding proteins to break double-stranded DNAs. But these methods were not populated as it was difficult to design a specific protein for each targeted gene. Since the development of the first-ever genome-edited plant by homing endonucleases and zinc finger nucleases, a lot of development have been made in genome-editing techniques. Recent advances in genome editing include the development of a new technique called CRISPR/Cas9, which is like a bacterial II immune system and can target foreign nucleic acids (Farooq et al. 2018). Currently, CRISPR/Cas9 is being extensively used for miRNA editing, fluorescent imaging, bi-allelic mutation, epigenetic control, multiplex genome editing, and controlling gene expression (Farooq et al. 2018). Due to the widespread use of CRISPR/Cas9 in plant genome editing, various databases have been developed that contain different information about the designing of gRNAs, CRISPR-mediated mutants, DNA/RNA plugs, base editing, etc. Some important CRISPR databases are presented in Table 7.2.

Table 7.2 Different databases used for genome editing in crops by using CRISPR/Cas9 system

Name of database	Web link	Function	References
CRISPR-PLANT	https://www.genome.arizona.edu/crispr/	Used for designing and constructing sgRNAs for plant genome editing	Lei et al. (2014)
Cas-database	http://www.rgenome.net/cas-database/	Tool to construct gRNA library for Cas9	Park et al. (2016)
CRISPR-P 2.0	http://cbi.hzau.edu.cn/crispr/	Synthesis of plant sgRNA for CRISPR Cas9 system	Liu et al. (2017)
CRISPR-P 2.0	http://crispr.hzau.edu.cn/CRISPR2/	Designs sgRNAs with minimum off-target results with high efficiency	Liu et al. (2017)
Cpf1-database	http://www.rgenome.net/cpf1-database/	Constructs genome-wide gRNA libraries for gene knockout studies	Park and Bae (2018)
Synergizing CRISPR	https://github.com/Alexzsx/CRISPR	Tool designed to determine CRISPR Cas9 off-target activities	Zhang et al. (2019)
GuidePro	https://github.com/MDhewei/GuidePro	Predicts sgRNA in CRISPR/Cas9 protein knockouts	He et al. (2021)

continued

Name of database	Web link	Function	References
PAVOOC	https://pavooc.me	Homology modeling, template generation, and visualization of amino acids in 3D	Schaefer et al. (2019)
Plant genome editing database (PGED)	http://plantcrispr.org	Tool established to provide information regarding plant mutants developed by CRISPR/Cas9	Zheng et al. (2019)
Cas-OFFinder	http://www.rgenome.net/cas-offinder/	Detects potential off-target sites of Cas9 RNA-guided endonucleases	Bae et al. (2014)
Cas-designer	http://rgenome.net/cas-designer/	Online tool to identify CRISPR-Cas9 sites	Park et al. (2015)
CRISPOR 4.99	http://crispor.tefor.net	Detection of off-target sites and primer designing for gRNAs in plants	Concordet and Haeussler (2018)
CRISPETA	http://crispeta.crg.eu	Causes genome deletion with paired gRNAs	Pulido-Quetglas et al. (2017)
CRISPR-ERA	http://CRISPR-ERA.stanford.edu	Web tool to design sgRNAs for CRISPR/Cas9	Liu and Wang (2021)
CHOPCHOP v3	https://chopchop.cbu.uib.no	CRISPR tool designed to identify CRISPR Cas single-guided RNAs	Labun et al. (2019)
CRISPR-P 2.0	http://crispr.hzau.edu.cn/CRISPR2/	Design and construct specific gRNAs for CRISPR-Cas9-mediated genome editing in plants	Liu et al. (2017)

7.3.1 Cas-Database

Cas-Database is an online tool for designing genome-wide gRNA libraries for Cas9 nucleases from *Streptococcus pyogenes* (SpCas9). Cas-Database's user-friendly online interface enables users to determine optimum target sequences by simply altering the filtering variables. It also offers a strong method for selecting several best possible target sequences present in multiple genes at the same time to construct a genome-wide library (Park et al. 2016).

7.3.2 Cpf1-Database

Cpf1-Database is a web-based tool that can select guide RNAs (gRNAs) from all potential genome-wide target sites in the most appropriate ways. This database is designed for gRNA library and works on LbCpf1 and AsCpf1 nuclease on genome scale, having recognition sequences "5′-TTTN-3′" on 5′ ends of the targeted sites (Park and Bae 2018).

7.3.3 CRISPR-Local

CRISPR-Local is a large and easy-to-use tool that helps in creating sgRNAs in different plants and other organisms. The genetic difference among individuals of various genetic backgrounds can be understood by this tool because it is based on high sgRNAs approach. The sgRNA design can be improved by CRISPR-Local in the following ways: (i) designing of appropriate sgRNAs for nonreference crops; (ii) screening of sgRNAs that can target several genes at the same time; (iii) no need to make multiple submissions and repeat calculations, which helps in conserving computational resources; and (iv) use of both graphical user interface (GUI) and command-line modes in diverse applications (Sun et al. 2019).

7.3.4 MultiGuideScan

MultiGuideScan is a modified version of GuideScan, which was not much populated because it was computationally expensive to design CRISPR gRNA libraries from large genomic data. For this reason, Li et al. (2019) developed this new web tool, which was more efficient in the construction of gRNA libraries as it implements the different processes of GuideScan. MultiGuideScan can construct RNA libraries from large genomic data with 9–12 times higher speed.

7.3.5 Synergizing CRISPR

This tool determines various ensemble learning approaches to predict the CRISPR/Cas9 system's off-target effects. The suggested ensemble learning technique works to maximize off-target prediction capabilities by combining various CRISPR off-target prediction tools. Integration of the best prediction models may lead towards better performance than using the individual tools. It may help to understand the impact of enhanced prediction skills on chromatin annotations and evolutionary conservation (Zhang et al. 2019).

7.3.6 GudePro

GuidePro is a two-layer ensemble predictor that allows numerous variables to be used to prefer sgRNAs in protein knockouts. GuidePro surpasses the current techniques and consistently outperforms the existing methods for predicting phenotypes caused by protein loss-of-function, indicating its resilience in selecting sgRNAs in diverse CRISPR/Cas9 knockout applications (He et al. 2021).

7.3.7 PAVOOC

A web-based CRISPR sgRNA design tool that prioritizes the most effective sgRNA candidates using state-of-the-art machine learning models is known as "prediction and visualization of on- and off-targets for CRISPR" (PAVOOC). Unlike other tools, it maps sgRRNAs to functional domains and protein structures and may visualize cut sites on corresponding crystal structures. PAVOOC also allows the creation of homology-directed repair templates for genome editing experiments as well as a 3D visualization of amino acids that are mutated (Schaefer et al. 2019).

7.3.8 SWISS

This database describes the editing of genome persuaded through single system. This simultaneous and wide-editing induced by a single system (SWISS) tool provides multiple genome-editing tools for plants. By using paired sgRNAs, SWISS can produce insertions/deletions in addition to base editing (Li et al. 2020).

7.3.9 CRISPOR 4.99

CRISPOR aims to provide a complete solution, including cloning, guide RNA selection, expression analysis, and primer designing, for assessing the activity of guide RNA and finding possible off-targets.

7.3.10 CHOPCHOP V3

This genome editing database works for about 200 genomes. Its major functions include identifying sgRNAs and supporting RNA accessibility predictions and alternative transcript isoforms. Furthermore, this database plays a role in CRISPR repression or activation and the enrichment of long red target loci as well as predicts Cas9 repair outcomes.

Improvement of Crop Genome Assemblies by Third-Generation Sequencing
Since the prices for next-generation sequencing (NGS) have become low, whole genome sequencing and resequencing are becoming important for many plant-related studies. Although, there are major limitations of NGS, like: vague alignment of repetitive elements that may inherent biases that produces highly fragmented genome assemblies (Sedlazeck et al. 2018). The advent of third-generation sequencings, such as Oxford Nanopore Technology sequencing (ONT) and the real-time sequencing of Pacific Biosciences (PacBio), has allowed the sequencing of long

reads with more accurate and continuous genome assemblies (Pérez-de-Castro et al. 2012; Li et al. 2018). Third-generation sequencing techniques help in generating high-quality de novo assemblies of genomes by spanning reads of complex genomic sites, like regions with more repetitive sequences necessary for understanding different structural variants. Furthermore, genome annotation can be improved by a third-generation sequencing technique known as isoform sequencing, which produces full-length sequenced transcripts, allowing a precise study of splice sites, alternatively spliced regions, and exons (Li et al. 2018). A well-annotated and completely assembled genome can allow plant breeders to identify the genes responsible for beneficial agronomic traits, designs genome-wide molecular markers, determine the location of genes, and describe the functions of genes. Long-range mapping techniques are useful for the mapping of long reads on chromosomes with great accuracy, which is possible for nonmodel crops, too (Jiao and Schneeberger 2017).

To identify structural variants and generate high-quality platforms, BioNano Genomics (https://bionanogenomics.com) is a new optical mapping platform that can perform labeling of big DNA sequences (>250 kb) at low cost. For instance, optical mapping and PacBio sequencing have assembled the genome of *Oropetium thomaeum*, which is a drought-tolerant species of grass, and a contig with the size of 2.4 Mb with 99.5% genome coverage was obtained (Vanburen et al. 2015). A high-resolution chromosome of wheat was also mapped with contigs having N50 of 1.3 Mb by using optical mapping data (Staňková et al. 2016). The repetitive regions can be identified by long-read sequencing with high accuracy. The repetitive sequences cannot be mapped through short mapping sequencing because these techniques wrongly assess their content. The improvement in the assembly of highly complicated crop genomes is the most beneficial application of third-generation sequencing. Genome size, repetitive sequences, ploidy, and available funds are some important parameters for selecting a genome sequencing technique for a crop. Illumina short-read sequencing, ONT, and PacBio are some recently developed sequencing techniques that can be used alone or in combination with other mapping technologies, like BioNano. The cost of these techniques varies from country to country, but with the passage of time, the cost of third-generation sequencing is decreasing. As such, its application can be broadened to include the improvement of crops.

Role of Integrative Genomics in Crop Improvement and Trait Discovery

Integrative genomics provides useful insights for identifying economically important traits for improving crops. The following strategies could be used for integrative genomics.

Identification of Quantitative Trait Loci

The association between phenotype and genotype could be estimated by an analysis of quantitative trait loci (QTL) (Ahmad et al. 2015a, b). With the increased number of studies on QTL in plants, the identification of high-quality candidate loci and the integration of information obtained from various QTL studies have become very difficult (Ahmad et al. 2018). In this scenario, a meta analysis tool is needed, which

can combine the outcomes of different studies and completely uses existing sources for predicting the exact location of QTL in genomes (MacKay et al. 2009; Mace and Jordan 2011). There are some bioinformatics tools available that can perform a meta analysis of QTL. For example, MetaQTL is a consensus model and statistics-based computational source that could precisely etimate the exact location and function of QTL; also, here, the confidence interval of QTL is reduced (Veyrieras et al. 2007; Ahmad et al. 2018).

Some other bioinformatics tools, like RASQUAL (https://bio.tools/rasqual) and solQTL (http://solgenomics.net/qtl/), also help in the visualization and linking of QTL data with various genome databases (Tecle et al. 2010; Kumasaka et al. 2016). A meta analysis of QTL was done on wheat, soyabean, cotton, and maize crops to map associated characteristics to mitigate biotic and abiotic stresses in these crops (Liu et al. 2012; Said et al. 2013). The QTL studies have helped plant breeders improve the yields and variety development in important food crops in previous years. QTL mapping is one of the most powerful and effective tools for establishing a linkage between genotypes and phenotypes in plants. The complete genome can be scanned, and rare alleles can be identified by using limited genetic markers with the help of QTL mapping, given that it is a hypothesis-driven approach. When a gene of interest segregates from a crop population, this approach proves useful. However, there is a major disadvantage of QTL mapping; that is, due to its low resolution, it is difficult to distinguish between physically adjacent and pleiotropic genes. Only the allelic diversity of the parent population can be assessed. This problem can, however, be resolved with the help of genome-wide association studies (GWASs), which determine the genomic regions with diverse traits in unrelated crop populations (Hu et al. 2018).

Genome-Wide Association Study and Determination of Breeding Targets

A genome-wide association study (GWAS), in contrast to QTL mapping, depends on natural populations and provides a good resolution for the identification of multiple events in crops, like recombination and natural variations linked with different phenotypes (Ali et al. 2019). In crop species, the links between phenotype and genotype have been explored by detecting linkage imbalance to achieve high-resolution mapping in GWAS (Huang and Han 2014). GWAS can detect a relatively wider range of genomic traits as compared to QTL mapping analysis. When the aim is to identify specific candidate genes which could be integrated in crops directly for improvement, then genome wide association studies uses more likely same technique as of QTL mapping (Korte and Farlow 2013). GWAS has been carried out in various food and oilseed crops, such as wheat, maize, rice, canola, soybean, and sunflower (Huang et al. 2010; Tian et al. 2011; Sonah et al. 2015; Gacek et al. 2017; Ahmad et al. 2021a, b). In *Oryza sativa*, 3.6 million single nucleotide polymorphisms (SNPs) have been identified in a genome-wide study that can explain 36% of phenotypic variants by using identified loci, which allow the further discovery of alleles and genes with beneficial traits for improving crops (Huang et al. 2010). Through GWAS, it has been revealed that variations in liguleless genes can interfere with a leaf's structure and turn leaves in an upright direction in maize (Tian et al.

2011). The advancements in bioinformatics tools offer more opportunities to perform GWAS with more ease. For example, a bioinformatics tool known as PLINK (https://zzz.bwh.harvard.edu/plink/) is widely used for GWAS and employs standard regression analysis to establish the linkage between the genotypes and phenotypes of crops (Purcell et al. 2007). But standard regression cannot provide enough sensitivity for GWAS to identify rare variants (Ma et al. 2014a). Another common GWAS tool is Trait Analysis by association, Evolution and Linkage (TASSEL), which is based on a linear model that incorporates family and population structures; it can also describe population effects, unlike PLINK (Bradbury et al. 2007). The Genome Association and Prediction Integrated Tool (GAPIT) (https://zzlab.net/GAPIT/) is another important bioinformatics tool that is based on a linear model that can efficiently handle big data sets (Lipka et al. 2012).

Forward and Reverse Genetic Approaches for the Screening of Desired Traits
Forward genetic screening is a plant-breeding tool that can identify and characterize genes depending on the known phenotype (Jankowicz-cieslak and Till 2015), while reverse genetic screening identifies the change in phenotype due to modifications in a particular gene or regulatory region (Hou et al. 2020; Jo et al. 2021). In forward genetics, the targeted phenotype that segregates from a crop population can be initially monitored with QTL analyses. However, the reverse genetic approach is used to determine the phenotype associated with known genes (having mutation or strating point of a transformed pathway) that have been identified through QTL mapping or GWAS. Both forward genetic and reverse genetic approaches are important to determine the functional variations associated with different agronomic traits, like improved nutritional quality, improved yield, and biotic and abiotic resistance (Ahmad et al. 2015b). Molecular markers and gene cloning can be enormously improved through forward genetic screening. The forward genetic approach only allows the screening of a selective coding region and excludes intergenic sequences, rather than analyzing whole-genome sequences and identifying multiple sequences that have no association to phenotype development. It is reported that only 20 Mb out of 389 Mb of rice genome can efficiently recover induced mutations (Henry et al. 2014).

Crop breeding and functional genomics can be studied using a reverse genetic approach. Targeting Induced Local Lesions in Genomes (TILLING), a molecular biology technique based on reverse genetics, is beneficial for inducing mutations conventionally and nonconventionally (inducing mutations by high-throughput techniques). TILLING is helpful in recovering mutation from any genomic region and determining the novel phenotypes in crops. This approach was used to induce homozygous mutations in a gene responsible for wax synthesis in wheat crops (Slade et al. 2005). These both approaches can also be used in combination, for example, in Lotus, after applying technique of TILLING (a reverse genetics approach), the alleles were recovered by forward genetics in 275 cultivars and it can reduce the cost up to tenfold in comparison with whole genome sequencing (Hu et al. 2018).

Selection of C Is Regulatory Elements for Crop Editing

DNA sequences that regulate gene expression are termed cis-regulatory elements (CREs). In the promoter regions of genes, CREs act as enhancers and silencers to interact with their corresponding transcription factor proteins, i.e. activators and repressors, respectively. Surprisingly, almost half of crop variants have been produced through mutations in CREs (Meyer and Purugganan 2013). The majority of mutations necessary for the domestication of crops are often present in CREs (Swinnen et al. 2016). It has been reported that reducing the repression of grain width genes by creating mutations in rice CREs can produce slender rice without decreasing its yield (Wang et al. 2015). When breeding, targeting CREs can be more beneficial when the goal is to enhance or reduce the gene expression instead of knocking out the gene completely. CREs are present in chromatin and support protein binding, but they are not expressed, like genes, which makes them difficult to identify and characterize. Techniques like ATAC-se, Plant PAN, PLACE, PlantCare, DNase-I hypersensitivity mapping, and ChIP-seq are some important methods that help predict CREs in genomes (Johnson et al. 2007; Buenrostro et al. 2016). The genome-wide identification, validation, and functional assessment of CREs can be achieved by combining genome editing and chromatin detection approaches. In addition to this, many bioinformatics approaches, like the analysis of sequence conservation of various promoter sequences, can be used for CRE detection in crops. With the help of these approaches, it has become much easier to identify CREs in plants and animals (Velde et al. 2014). As the high-throughput techniques have been generating an enormous amount of data linked with CREs, it has become difficult to target specific CREs for breeders. Plant cis-acting regulatory element database is an integrated database that provides comprehensive information about CREs and their functions in plants (Waqas et al. 2019). However, the functions of CREs in regulatory pathways are unknown, and their effects on gene regulation can be determined by experimentation only.

Application of Machine Learning in Crop Editing

Machine learning (ML) can allow different algorithms to interpret big data sets by making patterns. The data sets produced by sequencing and photo imaging can be easily interpreted by using different analytical approaches through ML in lesser time. It can help plant breeders in developing the relationship between the phenotypes and genotypes of crops.

Crop Phenotyping

Crop phenotyping is used to measure structural and functional characteristics at cell and organism levels and is mandatory for crop improvement and GWAS (Walter et al. 2015). With the advancements in sequencing techniques and genomic research, the demand for crop phenotyping has increased because it helps in understanding complex genomic data. Conventional crop phenotyping is often considered a bottleneck approach because it is time- consuming, labor intensive, applicable to limited data sets only, and error-prone (Liu et al. 2014). Technological advances like machine learning (ML), automatic sensors, and high-throughput imaging have laid the foundation for the development of robotic high-throughput crop phenotyping to

overcome the disadvantages associated with traditional crop phenotyping. The phenotypical features within and across crop populations can be generated with the help of this new technique.

High-throughput crop phenotyping primarily consists of four steps: (i) detection via automatic sensors or high-troughput imaging techniques, (ii) classification of phenotypical data, (iii) quantification of important features, and (iv) providing specific predictions based on algorithms or models (Singh et al. 2016). ML-based crop phenotyping is mainly used for identifying stress-associated phenotypes and monitoring various plant diseases. It has been stated that the severity of iron deficiency was assesed in soybean using a real-time ML-based crop phenotyping techinque (Naik et al. 2017). The real-time severity of iron deficiency in soybean was determined by developing a model with the help of collected phenotypic data through ML algorithms, multi-class support vector machines and linear discriminant analysis. Another study has revealed the severity of stressses due to nutrient deficiency, bacteria and fungi in *Glycine max* with the help of a deep ML based crop phenotyping method (Ghosal et al. 2018). Although ML has been successfully implemented in phenotyping and genomics, it is still facing several challenges. The modeling based on ML involves only big data sets to construct a model and develop an algorithm because small data sets may lead towards staistically non-significant predictions (Ubbens and Stavness 2017). Crop phenotyping is an expensive and time-consuming approach because it uses big data sets, and most of the crops require a long time to complete their growth cycle. Thus, high-throughput ML is limited to commercial companies and big research institutes only (Heckmann et al. 2017). The cost of production and applying machine learning should be minimized to widen its use in farms for crop improvement.

Functional Annotation and Crop Genomics

Machine learning (ML) plays an important role in numerous areas of plant genomics, including the identification of SNPs in polyploid crops, genome assembly, and the iterative interpretation of gene regulatory pathways. ML can be beneficial for improving the genome assemblies of polyploid crops that have complicated genome redundancies (Ma et al. 2014b). Complete genome assembly and annotation are bases for the identification of genetic modifications and the determination of the structure and function of genes, which are crucial steps in a crop trait discovery pipeline. It is very difficult to assemble a highly redundant genome by conventional genome assembly approaches that are based on linear algorithms. It has been reported that a high-quality genome assembly of *Triticum aestivum* was generated with the help of ML (Brenchley et al. 2012). Portcullis is an RNA-seq mapping tool that is based on ML, which can differentiate between natural and artificial splice sites, and it was proved helpful for annotating the genome of wheat (Mapleson et al. 2018). Unknown candidate genes can be identified by establishing a relationship between genes and cis-regulatory elements for improving crops. A regulatory network constructed on the basis only of the coexpression of genes cannot provide complete information about gene regulation (Hecker et al. 2009). Therefore, gene regulatory networks have been constructed by using ML approaches that can

incorporate data on different regulatory signals simultaneously. SNPs are important genetic modifications of plant genomes (Hu et al. 2018). False-positive SNPs can be filtered with the help of a tool known as SNP-ML, which uses tree bagging and neural networks and is based on machine learning. This can determine SNPs in strawberry, cotton, and peanut with 98% accuracy (Luo et al. 2019).

Role of Databases and Bioinformatics Tools in Crop Editing

Information on the characteristics of crops can be accessed, transferred, and retrieved with the help of bioinformatics. It uses different computational approaches to answer different biological questions. The storage, retrieval, and analysis of various forms of biological data (nucleic acids, protein sequences, functions, structures, and regulatory pathways) come under the domain of bioinformatics (Libault et al. 2017). The different computational tools used in genome editing are presented in Fig. 7.1. Nucleic acid and protein databases are a prerequisite for collecting data in bioinformatics. The data stored in these databases should be managed by creating a system based on defined roles, known as a database management system.

Biological data are stored in two types of databases: (i) sequence databases, which store the sequence information of nucleic acids and proteins, and (ii) structure databases, which store information on protein structures. There are three famous databases that store the sequence information of nucleic acids: EMBL (https://www.embl.de), DDBJ (https://www.ddbj.nig.ac.jp/index-e.html), and NCBI (https://www.ncbi.nlm.nih.gov/). Important databases for storing and retrieving protein information are Protein Information Resource (https://proteininformationresource.org/), SWISS PROT (https://www.expasy.org/resources/uniprotkb-swiss-prot), and Martinsried Institute for Protein Sequences (http://www.pnfsoftware.com/mips-decompiler). Two other databases—Molecular Modeling Database (https://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml) and Protein Data Bank (https://www.rcsb.org/)—are used to predict the structure of proteins (Table 7.3).

Bioinformatics tools or programs help plant breeders analyze data with great ease. The Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) is one of the most extensively used bioinformatics platforms. BLAST is a time-efficient approach that identifies similar patterns among different sequences, but its sensitivity varies. BLAST needs a query sequence for searching against databases that have multiple sequences related to a query sequence. There are different variants of BLAST available that are used for the comparison of protein and gene sequences with public databases. These are (i) BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), which uses nucleotide query sequences and returns the most related sequences from databases; (ii) BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), which uses protein query sequences and returns the most related protein sequences from databases; (iii) PSI-BLAST (https://www.ebi.ac.uk/Tools/sss/psiblast/), which is used for finding distant relatives of protein sequences; (iv) BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), which uses all the six open reading frames of a nucleotide query

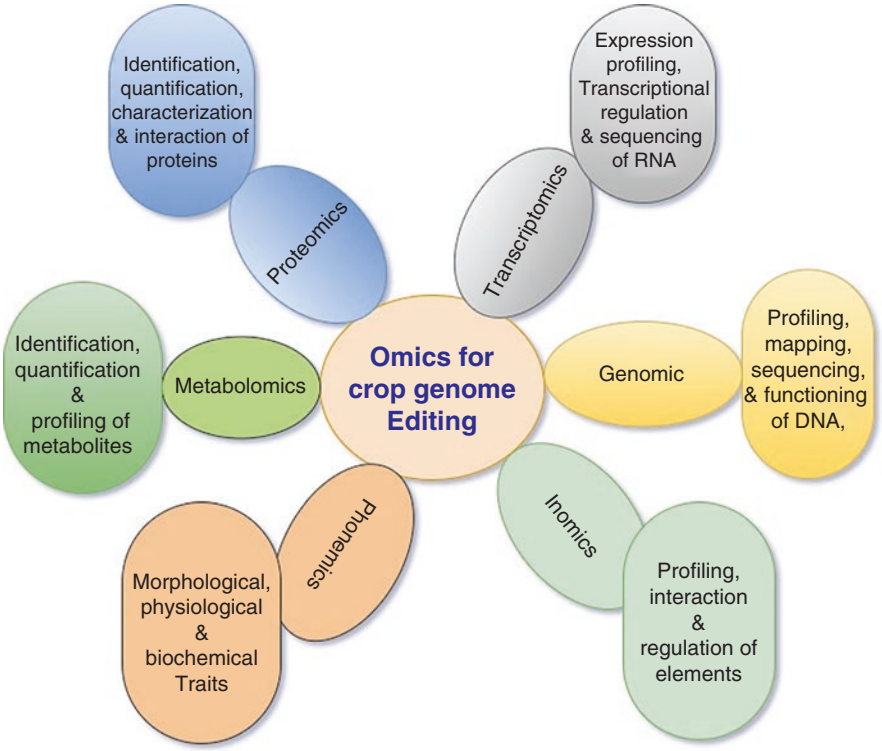

Fig. 7.1 Various omics approaches integrated into the genome editing of crops

sequence to compare it against a protein database; (v) tBLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastx&PAGE_TYPE=BlastSearch&BAST_SPEC=&LINK_LOC=blasttab), which is used for determining the distant relatives of nucleotide sequences; (vi) tBLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), which is used to compare protein query sequences against the six open reading frames of nucleotide sequences; (vii) Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?ORGANISM=1235996&INPUTSEQUENCE=JX869059.1&LINK_LOC=nuccore), which is used to design the primers of a specific sequence; and (viii) MegaBLAST (https://www.ultimateears.com/en-us/wireless.speakers/megablast.html), which is used to compare large numbers of query sequences by using command-line BLAST. Out of these all, the variants BLASTn and BLASTp are most widely used because they directly compare the sequence and do not require the translation of sequences. All variants of BLAST are used for comparing sequences, DNA mapping, establishing phylogeny, locating domains, and identifying species.

Cn3D (https://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml) is a powerful stand-alone tool for analyzing the 3D structures of proteins. It displays

Fig. 7.2 Prime objectives for the genome editing of crops plants

alignments, structures, and sequences simultaneously. Another important tool is Taxonomy Browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi), which enables plant breeders to identify phylogenetic trees by using common names, names of phenotypically similar crops, and partial taxonomic names (Mohnot 2020). Here, we have mentioned the most important tools available at the National Center for Biotechnology Information (NCBI) and the different bioinformatics databases and their applications in plant breeding.

Table 7.3 Databases for structural and functional characterization of plant proteins

Database	Functions	Web link
Protein data Bank (PDB)	Determines the structure of large biological molecules, i.e. proteins, nucleic acids, etc.	https://www.rcsb.org/
Universal protein resource (UniProt)	Database for providing functional information about protein sequences	https://www.uniprot.org
Protein data Bank Japan (PDBJ)	Provides an archive of macromolecular structures	https://pdbj.org
Structure integration with function, taxonomy and sequences (SIFTS)	Determines structure-based annotations for proteins	http://pdbe.org/sifts/
Cn3D	Analyzes the 3D structure of proteins	https://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml
Protein information resource (PIR)	Stores and retrieves protein information	https://proteininformationresource.org/
Swiss-Prot	Provides structural domains, functions, and posttranscriptional modifications of proteins	https://www.expasy.org/resources/uniprotkb-swiss-prot/
Martinsried Institute for Protein Sequences (MIPS)	Protein sequence, homology modeling, and yeast genome	http://www.pnfsoftware.com/mips-decompiler/
Molecular modeling database (MMDB)	Modeling and 3D sturcture of macromolecules	https://www.ncbi.nlm.nih.gov/Structure/MMDB/docs/mmdb_help.html
Expasy	Determines the physio-chemical characteristics of proteins	https://www.expasy.org
RCSB protein data Bank	Determines the 3D structures of biomolecules	https://www.rcsb.org
STRING	Used to find interactions among proteins	https://string-db.org

7.4 Conclusion

Plant breeders face substantial challenges in analyzing crop data obtained through high-throughput techniques, and it is necessary to understand and analyze these data for improving crop production. These problems can be solved by applying novel breeding methods and using bioinformatics tools. This process can be accelerated by genetic screening, such as metal QTL mapping and forward and reverse genetics approaches. Furthermore, genome-editing techniques have accelerated the breeding process through the manipulation of targeted genes. Computational tools have helped develop various databases, which are important sources of plant genomic and proteomic information. Machine learning has improved crop phenotyping, genome annotation, and crop genomics, which help in the discovery of beneficial crop traits. These techniques may be helpful in meeting the food demand of the rapidly increasing world population.

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Chapter 8
Omics Technology: Revolution in Plant Biology

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Contents

8.1 Introduction

In the age of rapid developments in the field of science and technology, there is a great shift from macro- to micro- or even nanolevel developments. Twenty-first century is considered the century of nanodevelopments, where millions of nanotechnologies, such as omics, have emerged and replaced the old ones. Omics are among those revolutionary technologies that are helpful in understanding, altering,

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mapping, and shaping the genetic constitution of an individual, including individual plants. Of the approximately 391,000 plant species available on earth, 94% are flowering plants and have significant importance for human needs, such as food, shelter, health, etc., as well as for the whole ecological system. To understand plant genetics with respect to its adaptation to environmental adversaries, a prominent area of research for crop improvement is plant genomics. For a better exploitation of plants, it is imperative to better characterize plants genotypically and phenotypically; that is, we need new advanced tools. Advancements in RNA or DNA sequencing techniques revolutionized transcriptomics and genomics. Similarly, analytical techniques such as HPLC, LC-MS, GC-MS, and MALDI-TOFF helped in the development of metabolomics, proteomics, and lipidomics. Various researchers are of the view that if all the techniques for prebreeding are combined, we will have extensive and elaborated information to explain phenotypic unique variations in genetics (Scossa et al. 2021).

There is constant pressure to increase the production of major crops, like maize, rice, wheat, and cotton. Moreover, climate change causes a further decrease in productivity (Voss-Fels et al. 2019). There is an urgent need to develop high-yielding and resistant cultivars for greater production with lesser inputs using new techniques such as omics, which may help in feeding nine billion people in 2050 (Godfray et al. 2010a, b). Technologies like proteomics, genomics, and transcriptomics are helpful in the identification of relevant genes, proteins, and molecules, which play important roles in plant stress tolerance (Soni et al. 2015). The different disciplines of omics are listed in Fig. 8.1.

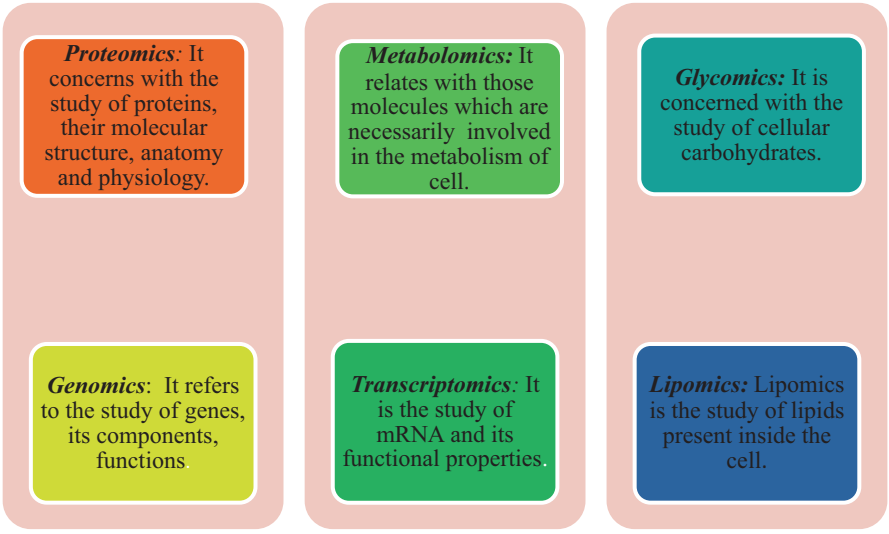

Fig. 8.1 The different omics disciplines

Table 8.1 Advance operations of omics in plant sciences

Transcriptomics	miRNomics	Epigenomics	Genomics
Gene expression analysis	Expression analysis	Targeted bisulfite sequencing	Targeted sequencing
Noncoding RNA analysis	miRNA identification	CHIP-seq	Whole-exome sequencing
Gene fusion detection	miRNA editing	MeDIP-seq methylCap-seq	Whole-genome sequencing
mRNA splice analysis		Whole-genome bisulfite sequence	
RNA editing			

Metabolomics techniques enlighten us about the depth of complex metabolic mechanisms, the diverseness of biological processes involved in plant growth and development, and stress resistance against biotic or abiotic stresses. A few metabolites were routinely analyzed in plants using spectrophotometric techniques. However, GC-MS and LC-MS helped in analyzing small to large metabolites present in low or high quantities. Furthermore, associated bioinformatics tools helped in the exploration of complex metabolic networks and complicated biosynthetic pathways. Large data sets or reference databases have already been developed for different plant species for bioinformatics analysis. Recent research has shown significant advancements in the field of metabolomics, which have opened the doors for more improvements in plants for commercial cultivation (Shalini et al. 2018). The advance applications of omics in plant sciences are listed in Table 8.1.

The cryopreservation of plants is a complex process for the preservation of quality donor parent materials, which usually involves preculture, preconditioning, cryopreservation, cryoprotection, rewarming, and regrowth (Kaczmarczyk et al. 2012; Reed 2008). Cryobionomics gives us information about cryoinjuries on stored germplasm, cell signaling during this process, the mechanism of regrowth, as well as the implications of genetic constancy in stored material. Research on omics will dig into the whole cryopreservation mechanism and help in tackling the issue of the recalcitrance of germplasm. Omics, in general, have unlimited application not only in plant divisions but also in all divisions of living beings (Carpentier et al. 2005; Vertommen et al. 2007). The different kinds of omics are listed in Fig. 8.2.

This chapter significantly reveals how important omics disciplines are due to their revolutionary contribution in the genome of plant sciences and their helpful application in food safety, security, sustainability, and the welfare of mankind.

8.2 Genomics as a Revolutionary Discipline

Genome is the complete set of genetic information of an individual's DNA. Genomics is a discipline concerned with the internal structure of genomes, the functional properties and mapping of genomes, and the editing of genomes. Genomics works on the

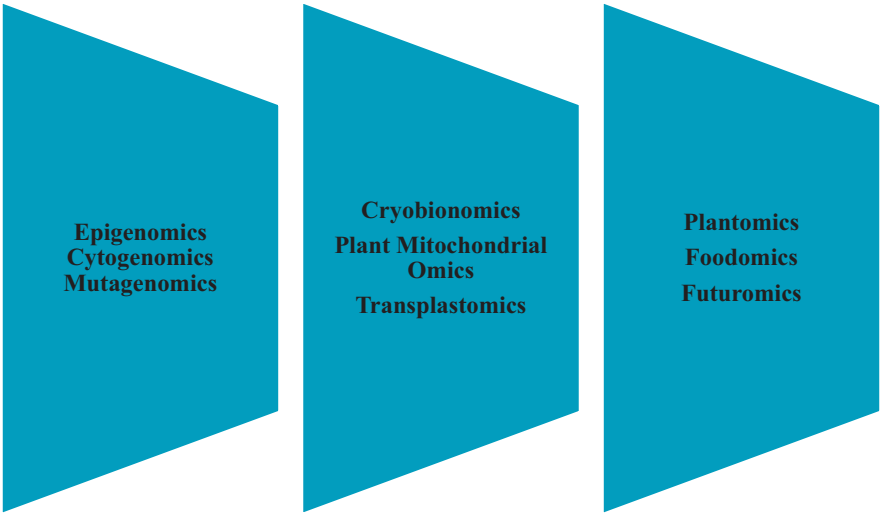

Fig. 8.2 A list of the different omics disciplines

phenomena of quantification and combined characterization of an individual's genes, their interaction and effects on an individual ("**WHO definitions of genetics and genomics**") (Geschwind and Flint 2015). Genomics is considered as one of the developing, emerging, evolutionary and revolutionary discipline which consists of various sophisticated tools and techniques that can be used in different disciplines especially in plants to program the internal mechanisms of an organism. It has been used in different areas of agriculture such as plant protection, plant pathology, plant genetics, plant breeding, molecular genetics, biotechnology, animals, livestock, biochemistry and many more (Bustamante et al. 2011).

Accurate and appropriate methodologies for marker assisted selection and molecular breeding, provision of approaches based on knowledge to plant biotechnology are provided by genomics, in order to accelerate the synthesis of novel plant cultivars. Functional gene products are recognized by the studies of gene expression that provides phenotypes and this data can be used for the crop improvement process. The specific addition of genes inside the plant can give rise to desirable phenotype rapidly as compared to conventional plant breeding methods (Lander 1996).

The interrelationship of genes of an individual is very much necessary for continuity of bodily activities. Important economical traits are incorporated into agricultural crops through the advanced genomic technologies, thus helps in designing of crops of desirable characteristics which fulfill the food demands of people. Molecular level analysis, genomic sequencing of plants are being exploited by researchers in different areas of plant sciences (Shalini et al. 2018).

Advanced genomic technologies have paved the way forward to produce qualitative foods along with different beneficial objectives such as high grain yield of rice and drought tolerant cultivars in maize, grain quality in wheat, etc. (Ashikari et al. 2005), as well as shelf life longevity in banana cultivars (Mehrotra and Goyal 2013).

SNPs and SSR which are the discoveries of genome sequencing technologies are being provided to improve qualitative traits in economical crops (Salgotra et al. 2014). Marker-assisted selection is also used to select progeny with desirable traits. Mutation breeding is also used to produce desirable crops having certain targeted economic characters by exposure to mutagenic reagents (Fleming 1983).

The prior identification of desirable traits in an individual has become easy through the application of molecular markers. The genetic diversity can be amended and accessed of the economical characters of crop plants by the help of such DNA markers (Collard and Mackill 2008). Generations of genetical as well as physical mapping have been done through the appropriate use of these DNA markers. They are also use to recognized areas which are needed for the crop environmental adaptation against different harsh circumstances (Varshney et al. 2009). The process of cosegregation is needed to create genetic maps which represent the position of markers in the group of linkage. Greater marker densities of genetic maps have been provided by next-generation sequencing (NGS) technologies. The mapping technology of quantitative trait loci (QTL) has been replaced by these enhanced maps technology. As molecular tool of characterization, the association maps are more accurate and detailed (Perez-de-Castro et al. 2012).

The transition of gene study from a single hereditary unit to a complete genome is a revolutionary step of genomics. It is helpful in understanding of gene interrelationships inside the genome. Such genomic modernization and technology would helpful in exploitation of evolutionary hierarchy of edible crops and reorganization of their ancestry that from where these present modern-day crops have been evolved. These genomic tools are equipping scientists to produce genetically engineered crops which provide sustainability of the food system and equal distribution of qualitative food among the global population. Furthermore, these genomic studies performs better to explore and understand the functional properties of genes in order to exploit better-off hereditary information for sustainable use(Shalini et al. 2018).The whole mechanisms of Genome sequence are as sculptured in Fig. 8.3.

8.2.1 Proteomics

The large-scale study of proteins is known as proteomics. Proteins comprise 50% dry weight of the cell, and it is an important part of an individual and consider as the main hub of various bodily activities. Meanwhile, the complete set of proteins produced by an organism is considered a proteome. Proteomics concern the handling of proteins at the molecular level (Anderson 1998; Blackstock and Weir 1999).

Identification and characterization of complete set of proteins' received from a genome can be done through the applications of proteomics (Wilkins et al. 1996). Amino acids are the building blocks of proteins. The determination of the concentration of amino acid sequences and different post-translational changings can be determined through proteomics (Barbier-Brygoo and Joyard 2004). In order to maintain the structure and the necessary regulatory mechanisms of proteins, its code

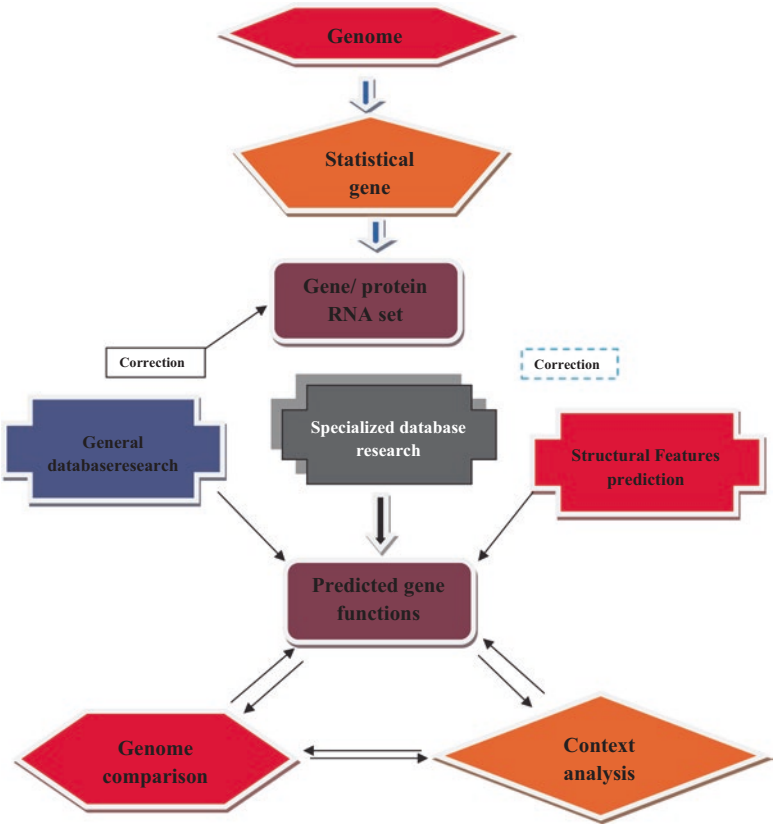

Fig. 8.3 Flowchart indicating the mechanism of genome sequence

of the genome is needed (Whitelegge 2002). Proteomics explains us the complex life processes and functional responses of a cell against environmental issues. The balancing of homeostasis inside the cells and networks of cell signaling for structural adjustment of cell are the main functions of proteins and these are all can be appropriately controlled by proteomics(Renaut et al. 2006).

The protein needed for MS traits has been identified for the hybrid selection and detection of male sterility traits in the hybrid by the help of proteomics (Yu et al. 2002). For the development of transgenic crops, proteomics can be applied (Gong and Wang 2013). To increase the photosynthetic mechanisms and abiotic stress tolerance of plants, certain efforts have been made in this regard. C4 plants are more workable relative to energy conversion because they contain two kinds of chloroplasts in their cell. In order to recognize the proteins responsible for the process of light fixation, proteomic study was conducted for both C3 and C4 plants respectively(Zhao et al. 2013).

The diversified functions of bio-molecules inside the body, their roles and interrelationship in metabolism of cell can be determined through advanced technology

of proteomics and bioinformatics. The information of omics has been updated and advanced through the involvement of proteomics applications, and it provides in-depth of various biological mechanisms that take place inside the living organisms. The interaction between proteins and post-translational modifications can be known by the applications of proteomics. Probably, the development of protein-based biosynthetic fungicide can be synthesized through the techniques of proteomics. Simultaneously, such protein based biosynthetic herbicides and pesticides as well fertilizers would be developed by utilizing the proteomics technology(Shalini et al. 2018). The flowchart proteomics protocol is as shown in Fig. 8.4.

8.2.2 Transcriptomics

The gene sequences which are expressed in a specific cell at a specific period are provided by the applications of transcriptomics. The nature of transcriptome is dynamic because the cell's transcriptome continuously changes due to different internal conditions. The function of genes, level of transcription and processes of molecules can be understood by the study of transcriptome (Borevitz and Chory 2004). Enzymes are the major players of metabolic activities. Analysis of expression of genes help in identification of particular gene controlling specific character, thus helpful in identification of main enzymes of that metabolic network (Poole et al. 2007).

In order to study variation in transcriptome information during growth, stresses, development, seed germination, the technology of microarray has been utilized successfully (Poole et al. 2007). For the successful improvement of plant cultivars

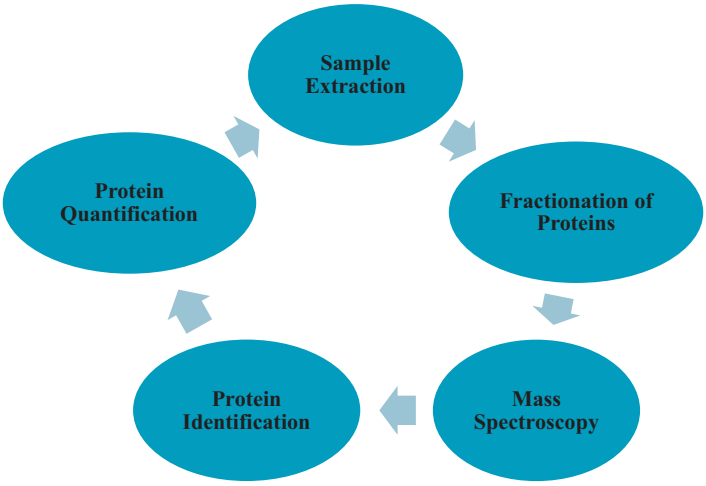

Fig. 8.4 Flowchart indicating proteomics protocol

incorporation of QTLs specific to resistance against biotic and abiotic stresses and grain development have been mapped on plant genomes (Saha et al. 2010). For the purpose of refinement of economical plants, functional tools like mutagenesis, epigenetics and RNA interference can be applied to gene silencing. For the recognition of chromosomal region related with expression of agricultural crop traits, bulk segregate analysis technique of genome mapping is used efficiently. For the development of disease resistant pigeon pea, this BSA technique was used successfully (Wei et al. 2009).

Significant data about genes involved in specific metabolic process is given by the gene expression analysis of mutant varieties. Generation of expression profile information depends on developmental stage of an individual, physiological circumstance and cell types can be developed through the application of transcriptomics. For comparing the relative DNA expression sequences of different species can be done by the evaluation of various information sets simultaneously. The relationship between plants and their wild relatives can be revealed through the analysis and assessment of molecular and expression diversity of an organism (Shalini et al. 2018).

8.2.3 Metabolomics

Living body is full of chemical reactions. The food we eat, the water we drink, they all have their own mechanisms in body to run bodily activities. The scientific study of these chemicals and their by-products, smaller molecules and metabolites is known as metabolomics. Basically, it is the study of unique finger print characteristics associated with it or the patterns of finger print metabolites that we leave behind (Daviss 2005).

The linkage between genotypes and phenotypes is known through the study of metabolomics. The major difference between proteomics and metabolomics is that metabolomics find out metabolically active expression of proteins, recognize biochemical processes and different metabolites roles that comes after result. The internal and environmental circumstances are subjected to dynamic metabolome. It involves continuous observation of associated changes caused in metabolic pathways; such changes are due to abiotic and biotic stress respectively. This dynamic monitoring helps in the development of improved cultivars and provides foundation mechanism to know about the biology of systems (Aliferis and Jabaji 2011). The manipulations occur in the metabolome proves helpful in the discovery of novel pesticides by the information comes from the studies of distinction of the pesticides action mode consequently. By the observation of manipulations of metabolite sequences, it will be helpful and easy to cause changes in plant health and qualitative changes in its nutrition simultaneously (Dixon et al. 2006). The profiling of metabolic pathways is helpful by providing data about what activities are going on inside the cell. For instance, during seedling stage, identification of activities inside the seed (Dunn and Ellis 2005).

The sign of similarity in composition between traditional and manipulated plants can be provided by metabolomics and it can find out the unwanted changes caused in the whole composition of metabolite. Mass spectrometry and nuclear magnetic resonance are the two analytical tools used in metabolic profiling. These techniques can be utilized in herbicides to find out their metabolic responses, examine and observe metabolic regulations and manipulations occur in metabolites in relation to environmental and genetic conditions respectively (Aliferis and Jabaji 2011).

An insight study of metabolites' and their appropriate analysis is helpful in decline the utilization of pesticide and synthesis of new pesticides, enhancing nutritional content, and provides better nourishment to the main traits of interest. Metabolomics possess a great potential to study the small molecular patterns specifically associated with different molecules to alter the genetic adjustment of plants (Idle and Gonzalez 2007).

8.3 Application of Foodomics in the Safety, Security, and Sustainability of Food

Foodomics is an emerging discipline which combines omics technologies of advanced level to make better health and consumer's well-being. It integrates tools like food chemistry and biology of food to utilize technology in a proper way (Cifuentes 2009). Foodomics and its processing mechanisms are as listed in Table 8.2.

It aims to focus on provision of better qualitative food along with a sufficient quantity to fulfill the requirements of rapidly growing global population. As it is already mentioned that world population is about to reach 9 billion in 2050 and now breeders, scientists, researchers, and food scientists are on the way to overcome the scarcity of food all over the world. In this whole scenario, advanced technologies like omics have been proved helpful to revolutionize the world with the efficient quantity of food for safety, security and sustainability of food. Foodomics was officially emerged as a discipline in the first international conference held in 2009 at Cesena, Italy (García-Cañas et al. 2012). Due to high throughput analysis

Table 8.2 Foodomics and its processing mechanisms

Foodomics			
Bioactivity	Safety	Quality	Traceability
Microbial growth	Assessment and evaluation of food	Assesses and evaluates the quality and life of food	Identification of sources
Identify pathogens	Transgenic food	Food	Food
Adaptation of pathogens	Contaminants	New food	Ingredients
Impacts of molecules on health	New ingredients	Processing	Plant breeding and crop improvement

requirement of foodomics, it is still bounded in sense of research and developmental progress. Foodomics based upon four major disciplines of omics namely (Balkir et al. 2020):

(a) **Genomics:** It concerns the detailed examination of genomic structures (Brennan et al. 2020).
(b) **Transcriptomics:** It uses different tools of analysis, including microanalysis, to explain genomic sets and recognizes differences among various conditions and individuals (Dong and Chen 2013).
(c) **Proteomics:** It concerned with the structure and physiological functions of protein. it is the large-scale study of the proteome (set of proteins) present in organisms. (Graves and Haystead 2002).
(d) **Metabolomics:** It is particularly concerns with the comprehensive study of metabolites in a biological system and their effects cellular behavior(Clish 2015).

All these abovementioned disciplines have been previously discussed in depth. The Foodomics utilizes the applications of these four major areas in order to design and synthesis food of its own choice that meets the demands of global community. Foodomics has various advantages for the welfare of human beings. It provides easy data access to scientists to arrange food analysis and its effects on health living organisms. It is a forward step towards progress and growth of food and technology to better understand them. It also leads to nutrional genomics which is the further extention of foodomics and helpful to analyze the interaction among genetics, food and human health studies. Foodomics is really an emerging field whose applications are using to check food effects on human, plants and animals, in order to cure the diseases genetically and on time. It ensures safer provision of food and paves the way for sustainable food development (Capozzi and Bordoni 2013). The overlapping of the different omics disciplines in foodomics is shown in Fig. 8.5.

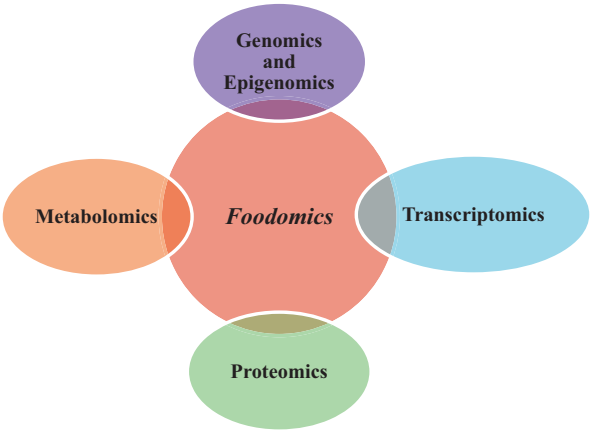

Fig. 8.5 Shows the overlapping of different omics disciplines in foodomics

8.3.1 Cryobionomics

As an emerging, evolutionary and developing discipline, cryobionomics provides an ultimate solution to preserve germplasm stock better-off. The process of cryopreservation of in vitro germplasm material for the purpose of long-term conservation results in the exposition of tissues to physical, physiological and chemical stresses resulting cryo-injury. However, the impacts of cryo-injury on the genome are rare; any DNA polymorphisms' accumulation would not be induced by cryopreservation itself, but the consequence of complete cryo-protection culture regeneration mechanism. It is desirable to evaluate the complete genetics of plants surviving cryogenic storage to find out either they are 'true to type' after cryopreservation. It can be approached at the phenotypic, biochemical, cytological, histological, and molecular level (Kaviani 2021) (Kaviani 2021) (Kaviani 2021).

To obtain a sophisticated picture of the basic biological mechanisms that consist of cryopreservation of whole systems, scientists are now capable to work on the same sample through the complete access of omics technologies (Morrison et al. 2006). Generally, high-throughput tools of omics such as genomics, transcriptomics, proteomics, metabolomics, and all other omics technologies can be applied to the same sample, following a detailed examination into biomolecular manipulations which combine the plant materials cryopreservation. Contemporary research in plant cryopreservation is now advocated by omics technologies that prepare a new knowledge foundation that will help in growth to resolve some of the more complex cryobiological issues (Basu 2008; Carpentier et al. 2005, 2007, 2008a, b; Volk 2010).

This whole process requires standard operating procedures to achieve a profound recovery that takes decades of collaborative examination using docile model systems to understand the complexity of the species and specific genotype responses to cryopreservation (Johnston et al. 2007, 2009, 2010). Necessary advancements have been marked in the last decade (Normah et al. 2013; Reed 2008), but there remained some instances where cryopreservation is restricted by scarcity of process where its utilize is highly desirable for the valued material preservation (Häggman et al. 2008). As progress in cryopreservation growth towards recalcitrant non model individuals, an increasing number of hurdles have been stumbled on with low levels of post-storage survival (Reed et al. 2013).

It is an evolving phenomenon that deals with the two aspects of cryopreservation viz. the linkage present between genetic stability and cryo-injury and secondly, the functionality and behavior of plant species in relation to their re-introduction in natural habitats environments and atmospheres (Harding 2004). The applications of omics disciplines in cryobionomics are as shown in Fig. 8.6.

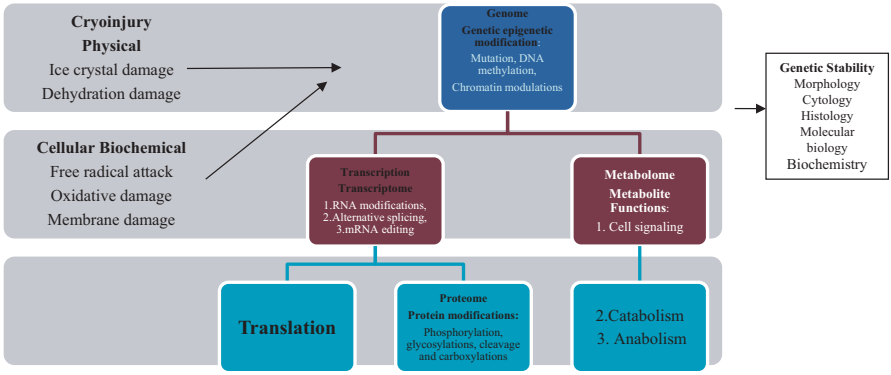

Fig. 8.6 Indicates the application of omics disciplines in cryobionomics

8.4 Conclusion and Future Perspectives

The science of omics contains great potential to alter the genetic make-up of plants, to achieve desirable objectives in plants, vegetables and crops respectively. It aims to bring such productive changes inside the genes, DNA, RNA, macro and micro-molecules at the highest level. The genuine issue which the world is facing today is the shortage of qualitative and healthy food, continuous change in climatic patterns and environmental stress. The Food and Agriculture Organization is working on strategies to overcome this barrier; fulfill the needs of the population all over the world, with the objective of producing novel cultivars with higher yield, less pesticide consumption, and higher thermo tolerance; and develop sustainable food production practices. Other national and international organizations are also working on formulating sustainable food production policies to ensure food and health safety among the masses. The recent inventions of modern molecular and nanotechnologies have overcome various issues related to the safety and sustainability of food. Their revolutionary contribution to plant production can never be ignored. Disciplines like omics can give us a genetic map to yield possibly the deepest-red apple easily. Its scientific results are beyond imagination, if one reveals its mysterious phenomena.

The wide-ranging dump of omics tools are increasing the taste, quality, and nutritional composition of major food crops, enhancing agricultural yield for food, feed, and fiber, playing a vital role in plant protection, and consequently impacting agricultural economics. Through the appropriate utilization of genomics, proteomics, transcriptomics, and metabolomics, the predictability, uniformity and consistency in plant breeding have been improved, reduction in time occurs and expenses of producing best qualitative major edible crops which possess resistance against biotic and abiotic stresses, but still possess high nutrition. Omics has given depth to the molecular processes of insect resistance to pesticides, and the tolerability in plants towards herbicides for better management of a pest. Linking genes to characters provides extra scientific assurance leading to improved varieties and lines and understanding the processes of insect and weed resistance. Omics

provides a systems' biological approach in order to understand the complex interrelationships between proteins, genes and metabolites within the resulted phenotype. This integrated approach depends heavily upon chemical analytical methodologies, computational analysis, bioinformatics and various biological disciplines, leading to plant protection and trait improvement. The application omics technologies in plant science is shown in Fig. 8.7.

Omics can be applied in agriculture research expansion in different sectors like health and food sector, energy division, animal feed, and special preparation of chemicals while it can be helpful in enhancement, preservation and remediation of the environment. Technologies of the omics are focusing on important target characters along with accuracy. It can enhance traits of nutrition of food for the advantage of a consumer, such as tomato which contains high lycopene concentration, fruits which contain delayed trait of ripening, as well as yield along with potential antioxidant capacities. Omics potential targets, focus and outcomes are as shown in Table 8.3.

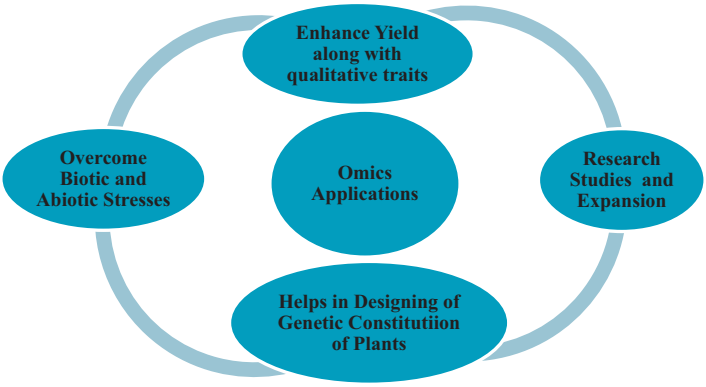

Fig. 8.7 Some applications of omics technology in plant sciences

Table 8.3 Omics potential targets, focus, and outcomes

Approach	Target molecule	Focus	Potental influence and outcomes
Metagenomics	DNA	Whole community taxonomic and functional analysis	Novel organisms, processes, and bioactive compunds
Metabolomics	Small molecules (metabolomics/ metabolites)	Elucidating composition, diversity, and richness of metabolomes	Characterization of novel metabolomes (metabolites)
Proteomics	Amino acids, peptides, and protein molecules	Expressed protein composition and quantification	Highlights the differences between protein translation and degradation in food waste
Transcriptomics	RNA (total RNA, rRNA, or mRNA)	Elucidating the composition, diversity, and richness of transcriptomes (expressed genes)	Gene expression profiling, identification, and characterization uncovers novel RNA species

Abiotic stresses affect plant growth as plants are the earth's basic producers and potential of yield in an individual. Stress conditions at certain times, plant species adjust per se to temporarily adaption to the present status by manipulating the expression design of metabolites, proteins, genes. In order to recognize such manipulations, many techniques such as transcriptomics, genomics, metabolomics, genomics and glycomics have been designed to appropriately tackle the genetic constitution of plants and their succession in adaptability under stressed conditions. Hence, there are uncountable applications of omics are available which can be utilized to take various benefits in plants, to create food sustainability and safety for the welfare of human beings.

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Chapter 9
Nanobiotechnology and Its Applications in Plant System Biology

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9.1 Background

Materials with a dimension less than 100 nm and a high surface-to-volume ratio are called nanomaterials due to their small size. They have unique physicochemical properties like increased reactivity and a small surface area with a typical surface structure. They are highly reactive because of their small size, accumulation of nanoparticles, stability, surface structure, shape, and chemical composition (Wang et al. 2016). Nanomaterials, in addition to their unique physicochemical features, are highly receptive to surface conjugation, allowing them to be produced as adaptable platforms with a wide range of applications in plant science (Machado et al. 2020; Hu et al. 2020). Nanotechnology has showed great promise in agriculture: it can increase plant stress tolerance by scavenging reactive oxygen species (ROS) with nanozymes (nanomaterials that imitate antioxidant enzyme functions) (Gao et al. 2007; Pirmohamed et al. 2010). Cerium oxide enhances tolerance against abiotic stresses (heat, cold, salinity, and drought) in plants (Liu et al. 2021b; Wu et al. 2018; Djanaguiraman et al. 2018; Wu et al. 2017b). Nanobiotechnology also enables the targeted and controlled release of agrochemicals (Zhang et al. 2020b; Santana et al. 2020); stress detection at early stages (Giraldo et al. 2019; Kwak et al. 2017) by using carbon nanotubes for sensing Ca^{2+}, H_2O_2, and NO (Wu et al. 2020); successful delivery of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) in non-model plant species (Demirer et al. 2019; Kwak et al. 2019); as well as stress tolerance by seed priming (nanopriming) with nanoparticles (Rizwan et al. 2019; Mahakham et al. 2017).

By 2050, the world's population is expected to reach 9 billion, and feeding such a large number is a great challenge. Scientists have estimated that Agriculture production must grow by 60% from 2005–2007 levels to feed a population of nearly 9 billion people by 2050 (Van Ittersum et al. 2016). Many efforts have been made through plant breeding, cultivation practices, and farm management to reduce the gap between demand and supply of food, but it is still an emerging problem that can only be solved by modern techniques, like nano-enabled agriculture, to mitigate food shortage. An emerging field called nano-enabled agriculture has the potential to increase plant tolerance to biotic and abiotic challenges, as well as plant breeding and agriculture. By 2050, it is expected that plant nanobiotechnology will address food shortage and have great importance in sustainable agriculture.

Previously, plant biotechnology was not much focused on by researchers; rather, they emphasized nanosensors, nanotoxicity, and nanoparticles in agricultural production (Zhao et al. 2020; Xin et al. 2020; Zhang et al. 2020a; Acharya et al. 2019). Nanomaterials' biosafety concern might be substantially handled with adequate management and design (Gilbertson et al. 2020; Adisa et al. 2019). The use of nanomaterials in sustainable agriculture is being focused on in the current chapter. The

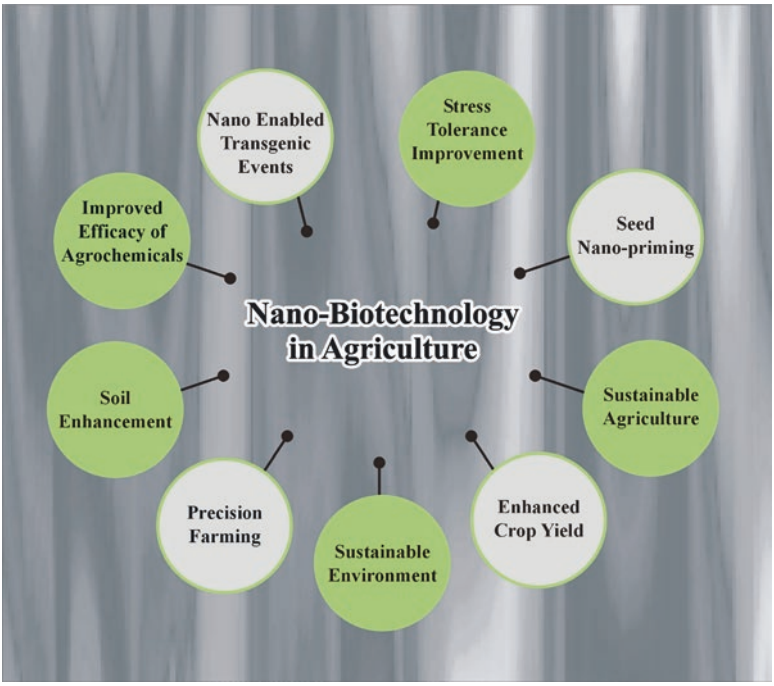

Fig. 9.1 Role of nanobiotechnology in various aspects of agriculture

major objective of this chapter is to encourage researchers specializing in plant biotechnology to further work on nano-enabled agriculture. Figure 9.1 illustrates the dynamic role of nanobiotechnology in various aspects of agriculture to improve the current agricultural production system.

9.2 Plant Stress Tolerance by Nanoparticles

9.2.1 Nano vs Bulk

Many researchers have recently worked on nano- and bulk materials in plants for agricultural production. By comparing the technique of using nanofertilizers and nanopesticides with conventional approaches, it was proved that nanomaterials are 20–30% more efficient (Dietz and Herth 2011). Therefore, we emphasize the higher efficacy of using nanomaterials as compared to conventional methods. Nanomaterials offer features like catalytic ROS (reactive oxygen species) scavenging ability and self-fluorescence that bulk or commercial equivalents lack due to changes in physical and chemical properties at the nanoscale level. It was reported that CeO_2 nanoparticles are ROS scavengers, and they have a wide application in plant sciences, industries, and medical research (Kah et al. 2018). CeO_2 nanoparticles

feature a significant number of surface oxygen vacancies that alternate between two oxidation states Ce^{3+} and Ce^{4+} to offer substantial ROS scavenging capacity, in contrast to bulk cerium oxide (Walkey et al. 2015). ROS is catalyzed; the dangling bonds in Ca^{3+} efficiently scavenge ROS, while lattice strain increases surface oxygen vacancies by redox reactions (Boghossian et al. 2013). Another newly developed nanoparticle is Mn_3O_4, which has a greater in vivo ROS scavenging ability because it exists in two oxidation states, Mn^{2+} and Mn^{3+}, at a ratio of 1:2 (Yao et al. 2018). Salinity stress tolerance in crop plants (*Brassica napus*) was observed after the application of cerium oxide. The plants treated with cerium oxide had 48% more fresh biomass than the control plants (Rossi et al. 2016).

In two other studies, polyacrylic acid coated cerium oxide nanoparticles improved salt tolerance in Arabidopsis (50 mg L^1, 10.0 nm, –17.0 mV, foliar spray) and cotton (100 mg L^1, 8.0 nm, –15.3 mV, foliar spray), with plants under salinity stress showing 18% and 40% increases in biomass, respectively, compared to no-nanoparticle controls (Wu et al. 2018; Liu et al. 2021b). The mechanism was described as nanoceria scavenging of ROS. Thus, it enabled the modulation of channels and transporters for K^+ retention in the mesophyll (KOR gene expression with downregulation) and the exclusion ability of N^+ from the shoot (HKT1 gene expression with upregulation) (Liu et al. 2021b; Wu et al. 2018). Salt tolerance in cucumber was induced by foliar application (1 mg $plant^{-1}$, 226.4 nm, –7.7 mV) of Mn_3O_4. A 19% increase in the fresh biomass of cucumber was observed as compared to the control (Lu et al. 2020). Sorghum plants were treated with cerium oxide nanoparticles and were recorded to have drought stress tolerance. CeO_2 nanoparticle application also enhanced the temperature and UV and high light tolerance in *Arabidopsis thaliana*.

Carbon-based nanoparticles are another example of nanomaterials being used in the improvement of crop production. Carbon quantum dots (CD) have a wide application in agriculture due to their various physical and chemical properties, *i.e.*, superficial synthesis, lower toxicity, higher stability, adjustable surface functions, higher water stability, strong photoluminescence, and biocompatibility. Drought resistance has been induced in peanut plants by applying carbon nanodots through leaf infiltration (Su et al. 2018); however, its mechanism is still unclear. The application of multiwalled carbon nanotubes increased salinity stress tolerance in *Brassica napus*. The nanotubes were added into Skoog medium and Murashige (Zhao et al. 2019). Hydroponically applying carbon nanotubes also induced salinity stress tolerance in broccoli (Martínez-Ballesta et al. 2016). Carbon nanotubes promoted nitric oxide (a gas-signaling molecule) and the transduction of aquaporins. Silicon nanoparticle is another nanomaterial used to increase stress tolerance in crop plants. The soil application of silicon nanoparticles increased fresh biomass (up to 27%) and chlorophyll content (up to 17%) in barely grown plants under drought stress, so recovery from drought stress was observed in these plants (Martínez-Ballesta et al. 2016). ZnO, Fe nanoparticles, and TiO_2 nanoparticles have also played a crucial role in improving plant tolerance against abiotic stresses (Sun et al. 2020; Abdel Latef et al. 2018). Drought tolerance was also observed in maize through the application of ZnO nanoparticles in soil. Plants treated with ZnO (100 mg L^{-1}, 37.7 nm, 14 mV) had a 75% increase in proline and an 18% decrease in H_2O_2 (Sun et al. 2020). A list of nanoparticles, their concentration, and their effective performance against certain abiotic stress are provided in Table 9.1.

Table 9.1 Nanoparticles, their concentrations, plant species, application, and the type of abiotic stress

Sr #.	Nanoparticle	Concentration	Species	Treatment	Stress	Reference
1	Ag	25, 50, 75, and 100 mg/L	*Triticum aestivum*	Potting soil	Heat stress	Iqbal et al. (2019)
2	Ag	0, 2, 5, and 10 mM	*Triticum aestivum*	Seed priming	Salinity stress	Mohamed et al. (2017)
3	Ag	0, 10, 20, 30, and 40 µg mL−1	*Trigonella foenum-graecum*	Petri dish exposure	Salinity stress	Hojjat and Kamyab (2017)
4	Ag	1 mg/L	*Triticum aestivum*	Seed priming	Salinity stress	Abou-Zeid and Ismail (2018)
5	Al2O3	50 ppm	*Glycine max L. cv.*	Petri dish exposure	Flooding stress	Mustafa and Komatsu (2016)
6	CeO	500 mg/L	*Gossypium hirsutum L.*	Seed priming	Salinity stress	An et al. (2020)
7	Chitosan NPs	0, 30, 60, and 90 ppm	*Hordeum vulgare L.*	Foliar application	Drought stress	Behboudi et al. (2018)
8	Chitosan-PVA and CuNPs	50, 100, and 150 mg/L	*Solanum lycopersicum L.*	Nutrient solution	Saline stress	Hernández-Hernández et al. (2018)
9	CNTs and graphene	50 and 200µg/ml	*Catharanthus roseus*	Murashige and Skoog medium	Salinity stress	McGehee et al. (2019)
10	CNTs and graphene	50 and 200µg/ml	*Gossypium hirsutum*	Seed priming	Drought stress	Pandey et al. (2019)
11	Cu	3.333, 4.444, and 5.556 mg/L	*Zea mays*	Plants priming	Drought stress	Van Nguyen et al. (2021)
12	Fe	0, 25, 50, and 100 mg/kg	*Triticum aestivum*	Potting soil	Cadmium and drought stress	Adrees et al. (2020)
13	Fe2O3	0, 10, 20, and 30 µM	*Mentha piperita L.*	Hoagland solution	Salinity	Askary et al. (2017)
14	Fe3O3	0, 30, 60, and 90 ppm	*Dracocephalum moldavica L.*	Foliar application	Salinity stress	Moradbeygi et al. (2020)
15	Fe3O4	0.8 ppm	*Fragaria × ananassa*	Murashige and Skoog	Drought stress	Steinfeld et al. (2015)
16	$FeSO_4$	2 g/L	*Helianthus annuus*	Foliar spray	Salinity stress	Torabian et al. (2017)
17	Mn	0.1, 0.5, and 1 mg/L	*Capsicum annuum L.*	Nanopriming	Salinity stress	Ye et al. (2020)
18	MWCNT	10, 30, 50, 100, and 200 mg/L	*Dodonaeaviscosa L.*	Nanopriming	Drought stress	Yusefi-Tanha et al. (2020)

(continued)

Table 9.1 (continued)

Sr #.	Nanoparticle	Concentration	Species	Treatment	Stress	Reference
19	Poly(acrylic)-CeO	~50 mg/L	*Arabidopsis thaliana*	Leaf infiltration	Multiple stress	Wu et al. (2017a)
20	Se	10 mg/L	*Sorghum bicolor (L.) Moench*	Foliar and water spray	Heat stress	Djanaguiraman et al. (2018)
21	Se	0, 1, 4, 8, and 12 $_{\mu}$M	*Lycopersicum esculentum*	Hydroponic solution	High and low temperature stress	Haghighi and Pessarakli (2013)
22	Si	10 $_{\mu}$M	*Triticum aestivum*	Nutrient solution	UV-B stress	Sedghi et al. (2013)
23	SiO_2	0.5, 1, 2, and 3 mM	*Solanum lycopersicum L.*	Exposure *in vitro*	Salinity stress	Almutairi (2016)
24	SiO_2	50 and 100 mg/L	*Strawberry*	Exposure in nutrient sol	Salinity stress	Avestan et al. (2019)
25	SiO_2	0, 200, 400, and 600 mg/L	*Musa acuminata "Grand Nain"*	*In vitro*	Salinity and water deficit	Mahmoud et al. (2020)
26	TiO_2	0, 10, 100, and 500 mg/L	*Linum usitatissimum*	Leaf treatment	Drought	Aghdam et al. (2016)
27	TiO_2	0.01, 0.02, and 0.03%	*Triticum aestivum L. cv. "Pishtaz"*	Spraying by backpack sprayer	Drought	Jaberzadeh et al. (2013)
28	TiO_2	0, 2, 5, and 10 ppm	*Cicer arietinum L.*	Amended soil	Cold stress	Mohammadi et al. (2013)
29	TiO_2	500, 1,000, and 2,000 mg/kg	*Triticum aestivum*	Amended soil	Drought stress	Faraji and Sepehri (2020)
30	Yttrium doped	100, 160, 200, and 400 mg per plant	*Brassica napus*	Nutrient solution	Drought	Palmqvist et al. (2017)
31	ZnO	0, 0.5, and 1 g/L	*Glycine max*	Petri dish exposure	Drought	Sedghi et al. (2013)
32	ZnO	10 mg/L	*Abelmoschus esculentus L.*	Foliar application	Salinity stress	Alabdallah and Alzahrani (2020)
33	ZnO and Si	50, 100, 150 mg/L; ZnO NPs and 150, 300 mg/L; SiNPs	*Mangifera indica L.*	Foliar spray	Salinity stress	Elsheery et al. (2020)

Additionally, diseases and insects can impact the quality and harvest of crops. There are concerns about the health and environmental impacts of pesticides and an increase in fungal and insect resistance (Damalas and Eleftherohorinos 2011). Despite this, novel tactics for biotic stress defense that are both environmentally benign and effective are still required. The use of nanobiotechnology in the

production of insecticides (Jameel et al. 2020), fungicides (Ma et al. 2020), and herbicides (Cao et al. 2017) has the potential to improve their effectiveness. For example, *Spodoptera litura* larvae fed with a mixture of thiamethoxam (10–90 mg L^{-1}) and ZnO nanoparticles (TEM size 30 nm) died at a 27% greater rate than the larvae fed in the control. Plants have been exposed to a wide range of nanoparticles to determine whether they can improve their biotic stress tolerance, including Ag- and Cu-based nanomaterials. Ag-based nanoparticles are effective in inhibiting diseases as well as nematodes (Mishra et al. 2014; Ali et al. 2015). Disease control and pest activity inhibition are two applications for copper-based nanoparticles that are commonly applied (Cumplido-Nájera et al. 2019; Borgatta et al. 2018; Ayoub et al. 2018). A 58% reduction in the progression of *Fusarium oxysporum* infection in watermelon was demonstrated by $Cu_3(PO_4)23H_2O$ nanoparticles (10 mg L^{-1}, 151 nm) because of their smaller size, distinct structure, and faster initial release of copper ions (Borgatta et al. 2018). Carbon nanotubes (Wang et al. 2014), carbon dots (Li et al. 2020), Si-based nanoparticles (Buchman et al. 2019), MgO nanoparticles (Huang et al. 2018; Cai et al. 2018), TiO_2 nanoparticles (Paret et al. 2013), and CeO_2 nanoparticles (Adisa et al. 2018) are examples of nanomaterials that have shown the capacity to prevent plant diseases. Many researchers have applied nanoparticles to reduce biotic stress on plants (Servin et al. 2015).

Crop losses resulting from diseases, insects, and weeds amount to more than $2000 billion every year globally (Popp et al. 2013). Applied fungicides for disease management cost more than $600 million per year in the United States (González-Fernández et al. 2010). *Pyricularia oryzae* is a pathogen that causes rice blast, reducing rice production by as much as 80% in Thailand (Kongcharoen et al. 2020; Srivastava et al. 2017). The fungi can quickly evolve and become resistant to present fungicides if they are applied to resistant rice varieties. Therefore, the use of nanoparticles to combat rice blasts is worth studying in the future.

Drought stress is a substantial hindrance to the production of agricultural crops in semi-arid regions. Crop yields are increased by using environmentally safe nanoparticles. Farmers in water-stressed areas may benefit from drought resilience, which can help them maintain or enhance their revenue. The use of nanotechnology to strengthen drought tolerance has been demonstrated in several plant species (Sun et al. 2021; Taran et al. 2017). Drought tolerance in sorghum increased with the use of cerium oxide nanoparticles (nanoceria). Salt tolerance in cotton was improved by seed priming using polyacrylic-acid-coated nanoceria (An et al. 2020). Making polyacrylic acid-coated cerium oxide nanoparticles for nano-priming cotton seed for sowing a one-hectare area costs less than $30, while foliar spraying a one-hectare area costs more than $100. If output were to grow, chemical expenses would be reduced as a result. Personnel, equipment, and utilities (such as water, gas, and electricity) are all factors that contribute to the cost of manufacturing a commercial product.

9.2.2 Dispersion of Nanomaterials

Heavy metal nanoparticles, such as Cd^{2+} quantum dots (Li et al. 2018), cerium oxide nanoparticles, and Ag nanoparticles, may constitute a threat to human health and the environment. The use of cerium oxide nanoparticles to improve plant stress tolerance has raised concerns about their biosafety, despite cerium being the most abundant rare-earth element in the soil (Tan and Chi-Lung 1970). Nanoparticles applied to plants can also reduce their ability to respond to stress (Tan et al. 2017). Therefore, aggregated nanoparticles have a smaller surface area and less cellular absorption than scattered ones (Spicer et al. 2018). As a result, the use of appropriate nanomaterials will be required for the development of nano-enabled agriculture. One technique for increasing the longevity of nanomaterials is surface conjugation, which is shown to limit heavy metal leakage (Sharifi et al. 2012). Gold nanoparticles with diameters ranging from 4 to 22 nm displayed the least intracellular degradation (Balfourier et al. 2020), with the smallest particles degrading the most rapidly. It would be good to increase the dispersion quality of nanoparticles to prevent agglomeration after application to support consistent biological activity and the application of nanomaterials in agriculture (Kobayashi et al. 2014). The dispersion of Cu—the combination of copper and chitosan—improves chitosan nanomaterial compared to their bulk equivalents (Saharan et al. 2015). Cu–chitosan nanomaterials boost tomato fresh weight (16%) and seedling length (18%) while simultaneously decreasing harmful fungus mycelial development and spore germination (Saharan et al. 2015).

Nanomaterials that are free of heavy metals and are highly dispersible in water should be considered. These molecules should be used in sustainable agriculture, which can be accomplished by using nanotechnology. It seems that employing nanoparticles derived from plant nutrients would be beneficial in preliminary studies. For example, manganese is vital for plant health and is typically found in agricultural fertilizers due to its high concentration. Mn_3O_4 nanoparticles, a novel type of nanoenzyme, can scavenge ROS. Utilizing Mn_3O_4 nanoparticles in cucumber cultivation enhanced salt resistance in cucumber (Lu et al. 2020).

9.3 Stress Detection and Early Exposure by Nanoparticles

9.3.1 Nanoparticle Sensors

Sessile crop plants have evolved erudite stress-tolerance systems. Stress sensing and signaling are two very important systems. The oxidant H_2O_2 has been shown to operate as a signaling molecule in plants (Mittler 2017; Gilroy et al. 2016). Other signaling molecules involved in plant stress responses include sugars and gaseous molecules like acetic acid esters, acetic acid, ethylene, methyl salicylate, jasmonic acid, abscisic acid, hydrogen sulfide, carbon monoxide, and nitric oxide. The

signals were transported from the root to the shoot in plants subjected to salt stress mediated by Ca^{2+} signaling events (Choi et al. 2014). In response to various environmental stresses, the patterns of these signaling molecules can change. Plants produce different Ca^{2+} signals when exposed to salinity and drought stress (Shabala et al. 2015). It is still possible to detect several signaling chemicals, including Ca^{2+} (Toyota et al. 2018), H_2O_2 (Nietzel et al. 2019), glucose (Zhu et al. 2017), and sucrose (Chaudhuri et al. 2011) in nonmodel plant species without becoming invasive plants. Several technologies, including ratiometric quantum dot sensors, DNA aptamers coated on single-walled carbon nanotubes (SWCNTs), AT15-coated carbon nanotubes for nitrogen oxide detection (Giraldo et al. 2014), and nanoneedle transistor-based Ca^{2+} sensors are effective for monitoring signaling molecules in nonmodel plant species. Glucose concentration in the leaf of *Arabidopsis* plants was evaluated using the TGA–QD method after 60 minutes of incubation. QD and boronic acid are used to determine whether the glucose level in the leaf has changed. In addition, they created QD nanosensors (11.3-nm leaf infiltration) to use in environmental monitoring. These nanosensors have demonstrated excellent performance in conjunction with other sensors that monitor temperature, humidity, and even stomatal activity (Di Giacomo et al. 2015; Oren et al. 2017; Koman et al. 2017). Researchers developed an electrical conductometric sensor that measures the delay between single stomata opening and closing in real time.

Nanosensors can be useful tools in the plant research field since they can fix some fundamental complications. Still, there is not much information about how plants detect the presence of Na^+ in their surroundings (Jiang et al. 2019; Wu 2018). Through Na^+-specific nanosensors, the researchers were able to track Na^+ transit in plants at both granular and temporal scales. A similar problem exists with hydroxyl radicals (Mittler 2017), the most destructive ROS. This is owing to a lack of a reliable tool for researching their biological activities in stressed plants. Because fluorescent dyes such as hydroxyphenyl fluorescein do not detect hydroxyl radicals only (Setsukinai et al. 2003), also present visualization approaches rely on them to detect changes in plant development and defense. Their inclusion hampers this research in these luminous dyes. Developing hydroxyl radical-specific nanosensors will allow scientists to better understand how hydroxyl radicals function within plants.

9.3.2 Plants Sensors for Initial Stress Recognition

Early detection of stress may aid in the reduction of agricultural losses. Modern approaches for detecting chlorophyll fluorescence in leaves, evaluating morphological changes in plants, and monitoring water status employ remote sensing or hyperspectral imaging. However, these features only represent plant performance after the stress has been established (Giraldo et al. 2019). Therefore, the early detection of stress signals should be carefully observed and investigated. It is possible to improve remote sensing by using high-resolution nanosensors that monitor stress-signaling molecules to identify crop stress. It was also proposed in the case of

transforming plants into smart plants using nanosensors (Giraldo et al. 2019). Nanosensors can detect and indicate the presence of analytes by quenching or changing the fluorescence of the light emitted by the sensor (Rong et al. 2019; Lew et al. 2020; Kwak et al. 2017). The detection of chemical signals by agricultural equipment is made possible by nanosensors, which can detect and respond to stress-signaling molecules such as H_2O_2, glucose, and NO, and convert them to optical or radio waves. Agricultural management and the early identification of plant stress could be made more efficient and effective in the future using this type of technology. Recent advancements in the noninvasive real-time in vivo detection of glucose H_2O_2 in stressed plants by a QD system (Lew et al. 2020) illustrate the agricultural potential of nano-enabled smart plants. Using nanosensors is possible to detect stress in plants at an earlier stage and develop smart plant sensors.

Making the smart plant sensor a reality will necessitate further effort, particularly in the field of agriculture. A common occurrence in the field is a combination of high temperatures and drought (Suzuki et al. 2014). It is possible for chemical signaling to become more complex when many stresses are present at the same time. In this case, the employment of nanosensors in response to specific signaling molecules can decode signals with a higher resolution. It is possible to improve nanosensor sensitivity, selectivity, and accuracy to get a higher-decoded signal resolution in field conditions. Therefore, an important study objective should be to create a database of chemical signaling molecule alterations that respond to stress, such as Ca^{2+} and ROS scavengers. An organic field-effect transistor for detecting carbon monoxide, a signaling molecule in plants, was constructed using zinc oxide nanoparticles as the active medium (Narayana et al. 2020).

9.4 Agrochemicals Based on Nanoparticles

9.4.1 Nanofertilizers and Nanopesticides

Commercial fertilizers are expected to account for 30% and 50% of total crop yields. However, 40–90% of agrochemicals are lost to the environment each year. Even though plants have high nutrient utilization efficiency for essential nitrogen (N), phosphorus (P), and potassium (K) components, there is still room for improvement in terms of agrochemical efficacy in plants (Adisa et al. 2019). This is because plant nutrient utilization efficiency for essential N, P, and K components is between 30–35%, 18–20%, and 35–40%, respectively (Adisa et al. 2019). Plant performance can be improved by using engineered nanomaterials. There are two types of engineered nanomaterials (ENMs) that deliver one or more nutrients directly to plants and boost plant performance. “Engineered nanomaterials” are “any pesticide formulation or product that comprises designed nanoparticles as active components and demonstrates biocidal action.” Nanoagrochemicals are expected to perform better in agricultural production compared to conventional agrochemicals. Nanoagrochemicals are projected to boost efficacy by 20–30% as compared to conventional ones (Kah et al. 2018). Foliar Mn nanoparticle treatment increased rootlet

number, root and shoot length, and biomass by 40–70% (Pradhan et al. 2013). Nanoparticles encapsulating common agrochemicals like dsRNA, siRNA, ascorbic acid, and abscisic acid might be used to release the nanoparticles in a controlled and targeted manner. Several recent studies have added to the body of knowledge about nanopesticides and nanofertilizers (Mikula et al. 2020; Dimkpa and Bindraban 2017).

It is possible to control the release and dispersion of nanoagrochemicals by altering their surface properties. In experiments involving the controlled release of nanoformulations (37% of the studies), the most frequently used trigger is pH (Camara et al. 2019). Using the GA3-HMSN/Fe_3O_4 combination, the release of the growth promoter GA3 (gibberellic acid 3) at pH greater than 5 or less than 4 resulted in a 44% increase in cabbage growth. Temperature- and pH-responsive nanopolymers were developed to administer and release agrochemicals into tomato plants (Zhang et al. 2020b). Three days after foliar spraying tomato with the star polymer PAA50-b-PNIPAm450, up to 43% of the star polymer was translocated to other plant compartments, including the roots (Zhang et al. 2020b). Stimuli including light, temperature, enzymes, and the redox state are all used to produce a response. To improve the surface functionalization of nanoagrochemicals, aptamers, which are oligonucleotide or peptide molecules that bind precisely to specific targets, have been proposed for use. It was discovered that beta-cyclodextrin-conjugated quantum dots coupled to a shorter chloroplast transit peptide were significantly more effective than random peptides or controls that did not contain any peptides. A better understanding of the uptake and fate of customized nanofertilizers or nanopesticides and their impacts on plants and the surrounding environment may pave the way for their application in sustainable agriculture.

Until now, nanofertilizers and nanopesticides are still not commonly used in agriculture due to concerns about biosafety, ambiguities about their long-term environmental impact, and the likelihood of interspecies transfer. However, these concerns have been addressed. A lack of information about the long-term effects of nanoproduct accumulation in the environment on ecological systems and a lag in the development of legislation and regulations governing their usage are also contributing to the current situation. Still, more research work is needed to understand the long-term environmental destiny and accumulation of nanomaterials. It is necessary to develop policies and laws governing the use of nanoparticles in agricultural production. Farmers and the general public may be more accepting of nanotechnology-enabled farming if they are taught about the benefits of the technology and the most effective ways to use it.

9.4.2 Targeting the Chloroplast to Synchronize Plant Performance

Nanomaterials can be used to aid the regulated release and transport of agrochemicals to specific organelles, organs, or tissues in plants and the transport of agrochemicals to specific organelles, organs, or tissues in animals, and this can help reduce agrochemical waste by increasing the efficiency of the process. Nanoparticles

are transported in a regulated manner in animal cells (Anselmo and Mitragotri 2016), suggesting that the distribution of nanoproducts to plants might be tailored. Methyl viologen and ascorbic acid have been successfully delivered into chloroplasts to manage their redox state by employing quantum dots coated with beta-cyclodextrin and truncated guidance peptides (Santana et al. 2020) to regulate the redox status of the chloroplasts. This work indicated fine-tuning in the activities of cell organelles while using fewer agrochemicals in their experiments. To obtain superior chloroplast colocalization, the results imply that QDs alone are insufficient and that an optimal nanoplatform design is required.

However, it has taken an extremely long period and high cost to get to the high efficiency of targeted distribution in plants. It has been achieved principally through a combination of nanomaterials and peptide-guiding molecules (Zhu et al. 2012; Santana et al. 2020). The discovery of novel technologies for the targeted administration of nanoparticles will enable a wider range of agricultural applications for this methodology. Changing the size and charge of nanoparticles allow for the infiltration of cell compartments such as the apoplast in plants and the epidermis and guard cells in leaves (Hu et al. 2020). This method has certain advantages and also limitations. Future nanoparticle delivery systems may be capable of efficiently delivering nanoparticles to plant tissues or organs and specific cell types and compartments through the engineering of nanomaterials with controllable features, such as size, charge, shape, and hydrophobicity/hydrophilicity, among other characteristics.

It is necessary to develop methods for dispersing nanomaterials into certain cell organelles, along with developing ways for dispersing nanomaterials to specific plant tissues such as the apical meristem of a shoot or organs such as flowers. Neutral and negatively charged nanoparticles are more effective at migrating from the roots to the shoot compared to positively charged CeO_2 nanoparticles, which are demonstrated to attach to and primarily reside on roots (Spielman-Sun et al. 2019). Therefore, an in-depth examination of the nanomaterials transported into plant tissues and organs, cells, and organelles is required. Furthermore, the development and management of nanoparticle mobility in plants should be carried out within the intended application of the studied materials.

Abiotic stress causes a decline in photosynthetic activity in all plants, regardless of the type of stress experienced (Foyer and Shigeoka 2011). This mechanism is related to an increase in ROS in plants when subjected to abiotic stress. An excess of ROS can induce oxidative damage to proteins, lipids, and cell structures and programmed cell death (Liu et al. 2021a; Das and Roychoudhury 2014). Plant biologists and breeders are working on producing more efficient plants against scavenging reactive oxygen species to increase their abiotic stress tolerance. Genetic transformation is restricted to model plants or species that are easily altered. Thus, plants must be protected from abiotic stress, and their photosynthetic efficiency must be increased. A scalable and universal approach is needed to achieve these goals. This technique may use ROS-scavenging nanoparticles, such as cerium oxide nanoparticles and manganese oxide nanoparticles, to reduce the formation of reactive oxygen species. When plants are treated with nanoceria, the improved ROS

scavenging capacity of leaf mesophyll cells increased the carbon assimilation rate. Plants treated with SWCNT had higher ROS scavenging capacities and higher electron transport rates than control plants (Giraldo et al. 2014). Other ROS-scavenging nanomaterials may aid in protecting chloroplasts from oxidative stress and increasing photosynthesis in plants. By utilizing a newly developed targeted delivery method, it may be possible to preserve chloroplasts from stress and convert them into a "chloroplast factory," allowing for a wider range of applications. This newly developed technique may have applications in the pharmaceutical and bioenergy industries and the field of plant photosynthesis research.

9.5 Transgenic Events Assisted by Nanoparticles

9.5.1 Nanomaterials as Delivery Platform

Agrobacterium tumefaciens or gene gun bombardment are the two most prevalent methods of transformation to produce transgenic plants. These two approaches target a small number of genetically sensitive plant species or cause harm to the plants (Landry and Mitter 2019; Yu et al. 2017). Other than model species, callus cultivation (Altpeter et al. 2016) is inefficient and labor-intensive. Nanobiotechnology has recently demonstrated a considerable promise in creating transgenic wild-type plants, and it has the potential to be used in a much wider range of plant species than model plants. Single-walled carbon nanotubes have the potential to transport functional genetic elements into chloroplasts and nuclei (Demirer et al. 2019; Kwak et al. 2019). Wide properties have been shown to allow carbon nanotubes to enter plant cells (Wong et al. 2016; Chaudhuri et al. 2011). Variations in pH across cell organelles should trigger the release of plasmid loads. These studies advocate employing nanoparticles rather than *Agrobacterium* or gene cannon bombardment to provide functional genetic resources to plants (Kwak et al. 2019).

The use of positively charged carbon dots (2.0–10.0 nm in diameter) to transport siRNA to plants resulted in a reduction in GFP (green fluorescent protein) expression in plants. For the first time, carbon dots were used to deliver siRNA to plants to silence genes. GFP was precisely delivered to tobacco chloroplasts by using peptide/pDNA complexes (Thagun et al. 2019). They employed nanomaterials to make transgenic plants, which included nonmodel and model species. Nanomaterials can also be used as scaffolding to transfer unstable molecules such as RNA and RNA polymerase (e.g., siRNA or dsRNA). After loading dsRNA onto a clay nanosheet, which had a mean diameter of 45 nm and side dimensions ranging from 20–80 nm (d-value = 0.82), the stability of the loaded dsRNA significantly improved for 20 days (Mitter et al. 2017). A siRNA delivery system based on DNA nanostructures was used to enhance the growth of tobacco (Zhang et al. 2019).

Researchers and farmers may favor carbon nanotubes over other nanomaterials because they are less expensive or more biocompatible (Mohanta et al. 2019). It is

possible that increasing the number of nanoparticles for supplying functional genetic resources may improve adoption. A study of *Arabidopsis* plants found that polyethylenimine (PEI)-coated gold nanoparticles effectively delivered siRNA to the NPR1 gene (Lei et al. 2020). Carbon dots built on PEI can be used to carry DNA or RNA molecules. It is feasible under theoretical conditions to use positively charged nanomaterials such as carbon dots or silica nanoparticles to transport negatively charged functional genetic components into plant cells. The fact that nanoparticles are associated with biosafety issues suggests that this could be a cost-effective method to conserve costs. The availability of nanomaterials will impact the frequency by which this nano-enabled transgenic approach will be employed.

9.6 Nano-Enabled-CRISPR-Cas Complex

As an added benefit to the construction of transgenic plants, nanomaterials may be employed to provide a platform for organelle-specific CRISPR-Cas genome editing, which would otherwise be impossible. Although tissue culture is now widely employed in plant breeding, it is still crucial in the process. It is still restricted to a few numbers of plant species, genotypes, and organs. It was demonstrated that nanoparticles might be used to deliver the CRISPR-Cas9 system (Wei et al. 2020). However, there have been no reports of plant-based nano-enabled CRISPR-Cas genome editing that have been published. One of the most likely causes is barriers within the plants' cell wall (Albersheim et al. 2011). Recently, it was discovered that a virus was carrying the ultra-compact genome editor CRISPR Cas, which was previously thought to be inactive. A minimally functional CRISPR-Cas system comprises the Cas protein (70 kDa, about 3 nm Rmin (Erickson 2009) and a CRISPR array (Pausch et al. 2020)). Due to the ability to manipulate the size of the complex, it may be possible to deliver nanoparticle CRISPR-Cas complexes to plants more efficiently and precisely using this technology. The complex's size can be adjusted to pass more easily through the plant's cell membrane. As a result, nanoparticles may prove to be a useful vector for the CRISPR-Cas system, targeting certain organelles or plant regions. The CRISPR-Cas system may be supplied to the chloroplast using nanoparticles directed by a chloroplast transit peptide, transforming it into a plant factory (Santana et al. 2020). Nanotechnology can be utilized to enable CRISPR-Cas gene editing in plants (Demirer et al. 2021). Details on the application of nanomaterials for CRISPR genome editing in transportation, species independence, germline transformation, and gene editing efficiency have been discussed.

9.7 Seed Nanopriming

Drought, salt, and heat are all factors that impact seedling growth, with the most noticeable influence occurring during the germination stage of most crops. The germination, establishment, and adaptation of plants to a range of conditions are all assisted by seed vigor. The rate of seed germination and the uniformity of seed germination can be improved by utilizing a range of approaches and strategies. A range of techniques is available for priming seeds, including the use of a salt solution, an osmotic solution with a low-water potential, bioactive chemical combinations, solid matrix priming, and chemo-priming. As the stressed green gram (*Vigna radiata* (L.) Wilczek) variety Pusa Ratna was halo primed with 35 mmol L^{-1} NaCl, the fresh weight increased by 47% (under salt stress) and 28% (under drought stress) compared to unprimed controls under stress (Jisha and Puthur 2014).

It is a novel method of seed priming that, compared to conventional priming strategies, can significantly improve crop development and performance, particularly in adverse conditions such as drought, salty environment, and heat. Priming seeds with nanoparticles has the potential to improve crop development and performance significantly. When cotton seeds were primed with cerium oxide nanoparticles (2nm, 51.7 mV, 500 mg L^{-1}, nanopriming) and grown under salt stress (200 mmol L^{-1} NaCl), fresh seedling weight increased by 41% (An et al. 2020), compared to a water-primed control. Many crop species have been shown to benefit from nanopriming, including wheat, Fe_2O_3 nanoparticles (Sundaria et al. 2019), ZnO nanoparticles (Rizwan et al. 2019), rice, silver nanoparticles (Mahakham et al. 2017), sorghum (Maswada et al. 2018), broad bean (Younis et al. 2019), cotton, cerium oxide nanoparticles, onion (An et al. 2020), and gold nanoparticles (Acharya et al. 2019). In comparison to an untreated control, onion seed nanopriming with gold nanoparticles (93.6 nm, –8.5 mV, 5.4 mg L^{1}, nanopriming) resulted in a 69 percent increase in emergence percentage and a 24 percent increase in mean yield. Gilbertson et al. stated that nanoZn/ZnO is one of the most promising seed-coating options based on the increase in seed germination and the environmental effect of the embodied energy (Gilbertson et al. 2020).

Despite the promising findings obtained by seed nanopriming, additional research is required to comprehend the fundamental principles fully. Nanoceria priming can improve crop salinity stress resistance, which modulates the plant's reactive oxygen species (ROS) and ion homeostasis signaling pathways (An et al. 2020). Many additional seed nanopriming methods have been identified or proposed. There are four techniques for reducing electrolyte leakage: using ZnO nanoparticles (Rizwan et al. 2019), Fe_2O_3 nanoparticles (Maswada et al. 2018), or silver nanoparticles (Younis et al. 2019) or a combination of the three methods. The first goal is to reduce lipid peroxidation, and the second is to improve the amount of water in plants and the effectiveness of photosynthesis and respiration.

What methods of seed nanopriming persist when a single nanomaterial is applied to a range of plant species, and how does this affect the experiment's outcome? What processes, such as changed redox state or seed dormancy, can be used to explain the phenomenon of seed nanopriming? The uptake, distribution, and fate of nanoparticles and their interactions with seeds should all be investigated further by looking at the mechanisms that cause these events to occur in the first place. When it comes to nanomaterial uptake, the size of the hole pores in the seed coat matters. A concern has been expressed regarding differences across plant species in the spread of nanomaterials. Is there a relationship between the distribution pattern of nanomaterials in seeds and their biological effects? What is the relationship between this and the scavenging of reactive oxygen species (ROS) mediated by nanomaterials or epigenetic changes? Is there a critical stage or position in the seed nanopriming process critical for nanomaterials' biological impacts? Combining seed nanopriming with seed coating technologies may be worth investigating to ensure that efficacy and performance are not degraded over time. The nano-enabled seed coating technique used in the agrochemical business is only vaguely known by the general public (Acharya et al. 2019).

9.8 Light Harvesting by Nanoparticles

Photosynthesis is largely dependent on the availability of visible light. Plants do not make good use of most natural light sources. nIR light is absorbed mostly by chlorophylls in plants, whereas UV light causes chlorophylls to deteriorate (Antonaru et al. 2020). Even though the ozone layer prevents UV-C (100–280 nm), UV-A (315–400 nm) and UV-B (280–315 nm) are still able to reach the Earth (Stapleton 1992). It is difficult to see through the bottom leaves of plants in high-density cropping systems because of limited visible light. Visible light is diminished during overcast or rainy days. Sustainable agriculture may benefit from developing new photosynthesis technologies that allow plants to utilize a greater proportion of lost light resources.

Although cyanobacteria contain chlorophyll d and f, which absorb near-infrared light (Airs et al. 2014), cyanobacteria could only utilize light with wavelengths up to approximately 750 nm. Chlorophyll d and f are pigments that can be introduced into higher plants to escape the photochemical red limit of the light spectrum. nIR light has a wavelength limit of 800 nm and cannot be used. The use of nanomaterials to transform ultraviolet and near-infrared radiation into visible light for plant photosynthesis may pique the interest of scientists, farmers, and even the industrial sector. By fine-tuning and complementing plant photosynthesis, this revolutionary technology can potentially enhance food production by as much as 50% considerably.

The upconversion and downconversion of nanoparticles are two types of nanoparticles commonly employed in biophotonics and nanomedicine to convert near-infrared and ultraviolet light to visible light (Loo et al. 2019). It has been demonstrated that upconversion nanoparticles (UCNPs) doped with Yb, Nd, and Er

can convert light stimulated between 808 and 980 nm to visible light between 510 and 570 nm (Wiesholler et al. 2019). When downconversion nanoparticles (DNCP), such as $bNaYF_4:Gd^{3+}$ and Tb^{3+}, were encapsulated in PEI, they converted ultraviolet light with a wavelength of 273 nm into visible light (between 480 and 630 nm in wavelength) (Malik et al. 2019). Shoot length and dry weight were increased in rice by 19% and 64%, respectively, when CD 1:0.2 was used as a converter for converting UV radiation to PAR (photosynthetically active radiation). These UCNP and DCNP nanoparticles can be sprayed on the surface of leaves or injected directly into cells to assist plants in maintaining photosynthesis during periods of low light, such as shadow or continuous cloudy days. Because of advances in nanotechnology, this approach to photosynthetic light amplification may be helpful for agriculture and allied industries, such as biofuel manufacturing. Nanomaterials can be used to transform near-infrared and ultraviolet energy into visible light in plants.

9.9 Capturing More Electrons

Photosynthesis eliminates more than 120 billion tons of CO_2 from the atmosphere each year in terrestrial ecosystems. Photosynthesis in green plants is based on the harvesting of light and the passage of captured electrons into the electron transport chain, both critical processes. Reactive oxygen species can form when an excessive number of electrons are removed (Foyer 2018). On overcast and rainy days, especially when plants are shaded, they might not capture enough electrons to complete the light response. New tactics that allow plants to gather more electrons in low-light conditions may considerably boost plant photosynthesis and output. Plants may be able to collect more electrons with the assistance of nanomaterials. A structure is formed by a combination of organic and metallic components.

Nanomaterials are capable of both absorbing and transmitting electromagnetic radiation. The excited state can be transmitted to gold nanoparticles that have been triggered by light (Robatjazi et al. 2015). Thus, photosynthesis could be enhanced in low-light conditions by incorporating light-capturing nanomaterials into the chloroplasts. Research on innovative light-harvesting nanomaterials and their tailored distribution to chloroplasts should be carried out in the future. Scientists have proposed the use of nanomaterials to fine-tune plant photosynthesis under low-light conditions. These materials have the potential to trap more electrons.

9.10 Future Perspectives

We discussed the potential applications of plant nanobiotechnology in modern and sustainable farming practices. Plant nanobiotechnology could improve stress tolerance, sensing and early detection, pesticide targeted delivery and controlled release, nonmodel crop species transgenic events, and seed nanopriming. Heavy metals

should be avoided in agricultural nanomaterials, and their dispersibility should be as high as possible. As previously asserted, more research into the biological effects of nanoenzymes, such as Mn_3O_4 nanoparticles, on stressed plants is required. It is critical to keep looking into the mechanisms that influence nanoparticle absorption, dispersion, and fate, as well as their interactions with seeds. With the help of nanomaterials, plants can be converted into chloroplast factories, enhancing their functionality. In addition, nanomaterials can transform ultraviolet and near-infrared light into visible light, enabling more electrons to be retrieved for photosynthesis when employed in low-light settings. According to the researchers, understanding how nanoparticles help plants cope with stress should make it easier to develop nanomaterials specifically useful in agricultural applications. Legislation and regulatory restrictions may help reduce the biosafety risks associated with the use of nanoparticles in agriculture and assuage public worries about nanomaterials. Nanotechnology has the potential to have a substantial impact on agriculture.

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Chapter 10
Advances in Integrated High-Throughput and Phenomics Application in Plants and Agriculture

Muhammad Waseem, Iffat Shaheen, and Mehtab Muhammad Aslam

Contents

10.1 Introduction

Since the new millennium, emerging next-generation sequencing (NGS) technology has assisted researchers in measuring intractable and complex traits in biological data acquisition (Furbank et al. 2019). Among these technologies, crop functional genomics and whole-genome sequencing (Li et al. 2018) allowing in the acquisition of genome-wide association studies (GWAS) and quantitative trait locus (QTL) mapping

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(Xiao et al. 2017) on the large-scale phenotypic and genetic architecture of complex traits (Shi et al. 2019; Wang et al. 2019) have stepped into the high-throughput and big-data era. For instance, plant genome annotation revealed 26500 loci for *Arabidopsis* to 41000 genes in rice. Recently, the poplar genome was revealed to encode 45000 genes, and more than 40000 genes regulate multidimensional physiological and biological processes in Medicago and Lotus (Sterck et al. 2007). However, a genome size of 950 MB encoding about 35,000 genes in the tomato revolution our understanding of tomato biology (Barone et al. 2008). In the last two decades, thousands of genes in tomato (Barone et al. 2008), rice (Yao et al. 2018), and *Arabidopsis* (Bouché and Bouchez 2001) were functionally characterized through various traditional phenotypic techniques. The challenge is to attach the functions of these enormous numbers of genes restricting functional genomics studies and crop breeding (Deery et al. 2016).

The term "phenome" was first characterized by Davis (1949): approach to systematically explore the comprehensive set of extragenic, non-autoreproductive portions of the cell and represented the set of phenotypes, either cytoplasmic or nuclear. Later on, phenomics was described as a complex interaction of an organism's genotype and phenotype (Houle et al. 2010). Conventional crop phenotyping is laborious, tedious, intensive, and potentially injurious to plants (Chen et al. 2014). Recently, plant phenomics has been growing and advancing rapidly in the last decades includes the set of approaches used to precisely assess individual cells, tissue, leaf, or plant to the large scale, i.e., ecosystem (Fiorani and Schurr 2013). In addition, crop phenomics is the inter/intradisciplinary study of high-throughput phenotyping platforms for accurate acquisition and an organism-wide scale analysis of phenotypes in crop development (Fig. 10.1) (Zhao et al. 2019). In recent years, next-generation genotyping and phenotyping have been advantageous over traditional breeding approaches due to the accuracy of these methods and their robust capability to accelerate crop breeding (Pasala and Pandey 2020).

Advanced sophisticated sensors, vision-guided robotics, automation technology, and machine learning, with applications in harvesting, quality assessment, sorting, screening, and packaging, have been extensively implemented in the agri-production industry to promote efficiency (Ruiz-Garcia et al. 2009) and for breeders to have a breakthrough in making rapid genetic progress (Furbank et al. 2019). The integration of genomics and phenomics can accelerate genetics gain in breeding programs and identify new traits in diverse plant germplasms that help breed populations through the crossing and artificial genomics (Bortesi and Fischer 2015). However, these approaches should apply at the early stages of plant development. To search for how and why to measure the whole genome and whole-plant phenotypes has been extensively explored. Over the last few decades, the answer to the former has been examined in detail. Recent achievements in high-throughput technologies allow us to conclude how and why to measure organism-level phenotype in the coming decades (Houle et al. 2010). For instance, RICE 2020 has been initiated to systematically and functionally characterize all protein transcripts and gene transcripts in rice by 2020 (Zhang et al. 2008). This chapter tries to cover the significant advances in applying integrated high-throughput and phenomics approaches in genetics studies. Finally, we discuss the challenges in agri-phenomics and specify our standpoint on phenomics-related studies.

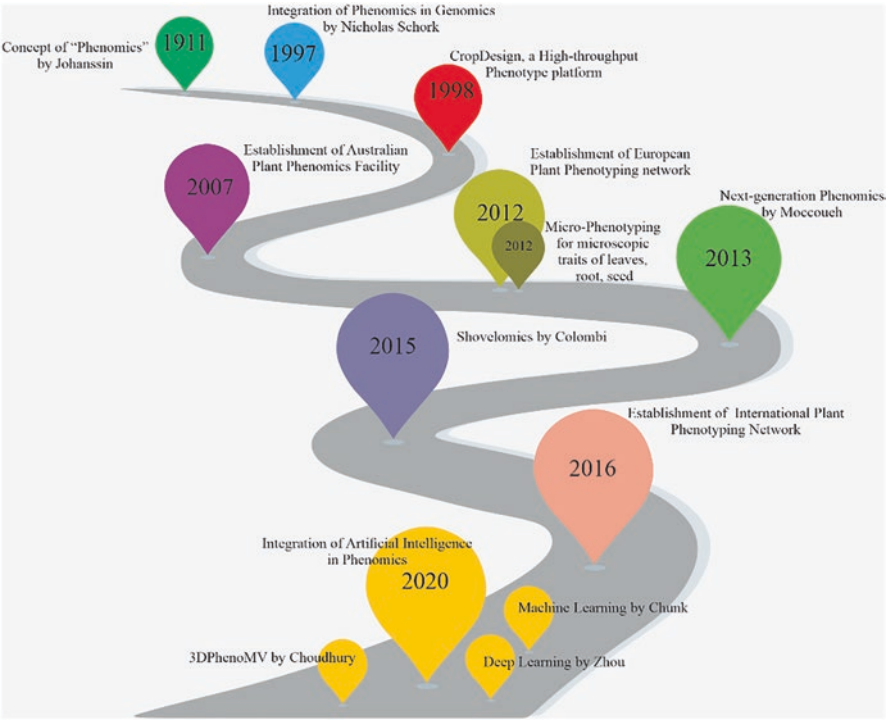

Fig. 10.1 Timeline of significant achievements in the deployment of phenomics approaches, including genomics, phenotyping, machine learning, and artificial intelligence

10.2 Phenomics

The plant being sessile organisms interact with multiple environmental stress across the life cycle and developed multiple avoidance strategies (Lymperopoulos et al. 2018). Conventional phenomics approaches focus on crop productivity and yield, while advanced sensing approaches enable plant scientists to record the environmental history of plants, together with their dynamic responses (Pratap et al. 2019). Robust phenomics is vital to plant breeding due to its fundamental basis for developing new varieties. However, advancement in phenomics including robotics, image processing, and deep learning enables non-destructive monitoring of plants development and function to extract valuable information (Hickey et al. 2019). The ongoing challenges with new generation phenomics are data handling and continuous contribution by computational technologies critical to maintaining rapid advancements in accelerated breeding programs (Tester and Langridge 2010).

10.2.1 Phenomics for Crop Microphenotypic Traits

Phenotyping at organ, tissue, and cellular levels requires complex procedures, and several automated high-throughput and large-scale phenotyping platforms have been developed for screening and assessing *Arabidopsis*, rice, and maize under controlled environmental conditions or in the field (Table 10.1) (Kaul, Koo et al. 2000). Phonoscope, a large-scale high-throughput phenotyping platform, monitors the plant growth rates of more than 700+ plants (Tisné et al. 2013). Renovator, accurate quantification for genotypic variation in natural genetic population using growth and photosynthesis as a phenotypic indicator of plant performance (Flood et al. 2016), utilizes a monochrome camera on the moving rail system platform with 1440 plants carrying capacity. The renovator can collect leaf area and light-use efficiency. Instead, PlantScreen with automatic weighing and watering moving conveyor belts transported plants from growing chamber to RGB imaging cabinets, chlorophyll fluorescence, and dark acclimation chamber (Awlia, Nigro et al. 2016). Plant root traits play a critical role in nutrient acquisition and the transport of water from the soil to the aerial parts of plants. Microimaging and sensing have improved our understanding of root anatomy and functions. Wu et al. (2011) developed a low-cost computer-aided 3-D visualization and quantitative analysis technique based on classical paraffin embedding serial sections and microtome techniques. In 2012, a high-throughput, high-resolution phenotyping platform, RootSlice, aided with laser and 3-D visualization was introduced to analyze (Burton, Williams et al. 2012) and quantify root anatomy with semi-auto RootScan (Chimungu, Loades et al. 2015). RootAnalyzer, automatic versatile root tissues, and root cells image phenotypic analysis tools. RootAnalyzer has more than 90% accuracy and improves image segmentation efficiency in quantifying the properties of tissues (Chopin, Laga et al. 2015). Walter et al. (2007) introduced an automated imaging pipeline GROWSCREEN to acquire the dynamics of seedling growth acclimation such as relative growth rate, total leaf area, and root area. Moreover, GROWSCREEN FLUORO allowed the simultaneous phenotyping of chlorophyll fluorescence in rosette plants (Jansen, Gilmer et al. 2009). The mapping power of GROWSCREEN for GWAS or QTL analyses reduced due to relatively limited carrying capacity in of platform in combination with micro-environmental heterogeneity.

TraitMill by CropDesign (Belgium) yield-related morphometric traits measure exclusive bioinformatics tools and a high-throughput phenotyping platform (Reuzeau et al. 2010). Computer-controlled Scanalyzer3D platform accelerating plant phenomics aided with automated watering and weighing, a conveyor with a capacity of 2400 plants, and RGB imaging stations to estimate plant biomass using RGB images (Virlet et al. 2016). In addition, the salinity tolerance of chickpea can be assessed by Scanalyzer3D (Hairmansis, Berger et al. 2014). In addition, rice (Hairmansis, Berger et al. 2014), and nutrient starvation in crops, in field diseases monitoring, and more physiological features including growth date. In recent years, an array of algorithms and tools has been developed (Table 10.1). However, there is

Table 10.1 Phenomics tools deployed for microphenotyping

Plant organ	Platform	Parameters	References
Canopy	LabVIEW	Growth parameters	Bai et al. (2016) and Zhang et al (2017)
Leaf	PHENOPSIS		Granier et al. (2006)
	WIWAM		Clauw et al. (2015)
	GROWSCREEN	Leaf discs	Nagel et al. (2012)
	LemnaTec	Growth and yield	Neumann et al. (2015)
	Phenodyn/Phenoarch	Leaf elongation	Sadok et al. (2007)
	Integrated Analysis Platform	Leaf orientation	Klukas et al. (2014)
	LAMINA	Leaf parameters	Bylesjö et al. (2008)
	Leaf Analyser	Leaf architecture	Weight et al. (2008)
	Phenovator	Photosynthesis	Flood et al. (2016)
	LeasyScan	Canopy traits	Vadez et al. (2015)
Root	Shovelomics	Root growth parameters	Burridge et al. (2016)
	Self-construction		Bucksch et al. (2014)
	LemnaGrid		Guo et al. (2017)
Shoot	PlantScreen™	Imaging/nonimaging chlorophyll fluorescence and plant growth parameters	Humplík et al. (2015)
	Rosette Tracker	Leaf area, perimeter diameter	De Vylder et al. (2012)
	PHENOSCOPE	Vegetative growth and homogeneity	Tisné et al. (2013)
Whole plant	TraitMill	Growth and yield parameters	Reuzeau et al. (2010)
	PlantScan		Sirault et al. (2013)
	HRPF		Yang et al. (2012)
	GlyPh	Growth and soil water content estimation	Pereyra-Irujo et al. (2012)
	BreedVision	Growth and genetic parameters	Busemeyer et al. (2013)
	OloPhen	Leaf area, growth, and survival rate	De Diego et al. (2017)

still a need to simplify complex phenotypic procedures at cellular and tissue levels. The introduction of advanced imaging techniques will accelerate microscopic phenotyping and assist in advanced phenotyping studies, particularly on specific cell phenotypes and crop organ characteristics.

10.2.2 High-Throughput Plant Phenotyping Platforms in a Controllable Environment

In crops, the breeding and selection of desirable traits is vital for sustainable agriculture, global food security, and the growing global demand for fiber, feed, and fuel (Dungey et al. 2018). High-throughput plant phenotyping with non-destructively image approaches facilitate efficient screening of plants based on their morphological and physiological traits, may assist in increasing productivity, shorten the crop cycle, improve plant efficiency in the environment, and help in linking phenomics to genomics (Li et al. 2014). However, the application of high-throughput plant phenotyping in agriculture is still in its infancy. It needs to have the accuracy and efficiency to assess the growth and morphological traits of plants, such as growth patterns, development rate, plant aerial architecture, root architecture system, and plant biomass. These features are fundamental to understand function-structure of plant in assesing biotic and bitoic responses for sustainable management of crops.

Global warming has posed a risk to global food demand as it has impacted agricultural productivity in the past few decades. Despite the negative influences of climate change, however, the selection and breeding of environmental resistance crops are needed today, but they should be done without compromising the quality and quantity of crops (Langstroff et al. 2021). Controlled environment phenotyping (CEP) is a nondestructive approach for exploring plant behaviors, which enable breeders to search for genotypes capable of coping with future environments (Xue et al. 2019). The primary problems in upcoming plant breeding programs are the lack of infrastructure and the diversity of users (Carpentier et al. 2019). Currently, a practical approach that is being used for searching scientific opportunities is bibliometric science mapping, which is done by analyzing scientific publications (Van Raan 2004). This method has been applied for analyzing phenomics. Under such a scenario, there is a need to develop dedicated tools, infrastructures, and resources for phenotyping genomics resources. The production of such high-throughput plant phenotyping resources could only be possible through a public-private partnership.

Luckily few initiatives arose to integrate fully controlled environment facilities climate-specific locations between laboratory-based work and "real-world" scenarios (Carpentier et al. 2019; Costa et al. 2019). For instance, the projects EPPN (http://www.plant-phenotyping-network.eu/), the COST Action FA1306 (http://www.cost.eu/COST_Actions/fa/FA1306), and EPPN2020 (https://eppn2020.plant-phenotyping.eu/). Similarly, European Infrastructure for Pan-Phenomics and simulation for global food security, the ESFRI-project EMPHASIS, jointly launched synergistic pan-European excellence in phylogenomics for developing relevant approaches and shared infrastructures. German Plant Phenotyping Network (DPPN) provides a robust phenotypic portfolio and shares productive and efficient infrastructure. On a global scale, China, USA, and Canada national phenotyping efforts include Asia-Pacific Plant Phenotyping Conference (APPP, www.APPP-con.org), North American Plant Phenotyping Network (NAPPN, https://www.plantphenotyping.org/), and the International Plant Phenotyping Network (IPPN; https://www.

plant-phenotyping.org/) develops integrated approaches beyond the national and regional perspectives.

High-throughput plant phenotyping can be integrated into greenhouses which allow crop phenotyping at the whole-population level and observe natural-variation in GWAS citrus (Minamikawa et al. 2017), rice (Crowell et al. 2016; Rebolledo et al. 2016; Yang et al. 2014), maize (Gage et al. 2018; Wang et al. 2019), wheat (Beyer et al. 2019; Rasheed et al. 2014), barley (Bergsträsser et al. 2015; Neumann et al. 2017), soybean (Bergsträsser et al. 2015). Besides acting as a shield from light, rain, and extreme temperatures, greenhouses provide a straightforward environment conducive for plant nutrient, salinity, and drought studies (Neumann et al. 2015). During drought, identification, and selection of precisely multitude drought heritable traits beneficial to characterize a phenotype (Chen et al. 2014). Global warming impacts agriculture productivity at global scale proxies drought indices to account and predict drought severity (Mukherjee et al. 2018) owing to few crop species spatiotemporal adaptations with varying productivity. Temperatures have drastic effects on crop yield and productivity (Zhao et al. 2017); for example, an elevated temperature decreases crop yields in maize up to 90% (Hatfield and Prueger 2015). Control environment integrated with high-throughput phenotyping enables identification of QTL and GWAS necessary for repeated phenotyping to ultimate phenotype (Muraya et al. 2017). The ecosphere is highly sensitive to temperature changes and differentially affects plant growth at different altitudes (Rosenzweig et al. 2014), influencing plant adaptation and productivity. Flowering in plant is controlled by daily temperature fluctuation, day length, light intensity, and seasonal cues to help understand the dynamic genetic components to plant adaptation (Li et al. 2010). In general, intercepted light can increase total plant biomass (60%) (Poorter et al. 2016). Dynamic environmental components are integrated with high throughput approaches to explore genetic variations in field or controlled environments.

10.3 Application of Machine Learning in Phenomics

Integration of artificial intelligence in interdisciplinary fields has been grown exponentially in the last decade. Artificial intelligence applications such as deep learning, sensors, and machine learning successfully enable high-throughput phenotyping of plant traits into non-invasive imaging approaches (Nabwire et al. 2021). The accuracy and efficiency of data collection and analysis Improve through deep learning and machine learning for vigorous image analysis and influential study of phenotypes. Conventional breeding approaches of phenotyping are destructive with sufficient resolution and require crop harvesting at specific plant growth stages (Furbank and Tester 2011). The plant breeding programs are significantly lagging behind genomics, slow, time consuming, and require repeated experiments to validate certain traits pivotal for crop improvement (Fahlgren et al. 2015). The non-invasive high-throughput imaging approaches enable phenotype visualization at a

cellular scale. The imaging techniques such as chlorophyll fluorescence (Zarco-Tejada et al. 2009), thermography (Oerke et al. 2006), spectroscopic imaging (Montes et al. 2006), and digital imaging (Jensen et al. 2007) carry a large amount of extractable data to support biological interpretations of plant growth (Walter et al. 2010). Hight-throughput artificial intelligence architecture applied in phenotyping are listed in Table 10.2.

Currently, high-throughput approaches like growth chambers (Bai et al. 2016), imaging sensors (Chaerle and Van Der Straeten 2000), data acquisition, and statistical software are employed for data collection, management, and interpretation at laboratory and field levels. Integration of these techniques into artificial intelligence in the form of machine learning (Kruse et al. 2014) and computer vision (Casanova et al. 2014) attribute to the non-invasion aspect of phenomics (Montes et al. 2007). Artificial intelligence applications are expanding with a public-private partnership in developing and disseminating these phenomic approaches that address the challenges of costly infrastructure and proprietary data formats. Thus far, computer vision, deep learning, and machine learning have been applied in phenomics. Since 1970, various machine learning models, such as Bayesian networks, support vector machines, and perceptron, have been developed, but none have proved to be the best

Table 10.2 Integration of artificial intelligence tools in plant phenomics

Model	Sensor	Crop	Trait	Reference
Machine learning	RGB/NIR	*Macrotyloma uniflorum*	Plant height, shoot length, flower percentage and pods, pod length, seeds per pod	Amal et al. (2020)
		Brassica napus, *Camelina sativa, Fabaceae, Cicer arietinum*	Flowering detection UAV	Obidiegwu et al. (2015)
		Zea mays	Identifying growth rate	Dutta et al. (2016)
	Scanner	*Vicia faba*	Root system architecture	Mula et al. (2016)
	RGB, IR, HS	*Beta vulgaris*	Water, nutrient stress	Mula et al. (2016)
		Glycine max	Canopy wilting	Howarth et al. (2011)
	3D laser scanning	*Cicer arietinum*	Evapotranspiration	Leport et al. (2011)
Deep learning	RGB/ multispectral	*Glycine max*	Plant yield estimation	Waring and Cleary (1967)
		Zea mays	Water stress	De Bei et al. (2011)
		Triticum	Root system architecture	Zakaluk and Ranjan (2008)
	HS	*Zea mays*	Relative water content	Patanè et al. (2016)

as they all have certain limitations (Roscher et al. 2020). Later on, neural networks integrated with data collection and information processing infrastructure enable machine learning to determine best-fitting models (Roscher et al. 2020). One of the advantages of phytogenomic machine learning is their ability to simultaneously processing massive amounts of data in combination with other related features (Roscher et al. 2020) assist in the identification and classification of plant traits, including disease or pest detection (Wetterich et al.), floral transition (Wetterich et al.), and seeds classification (Sabanci et al. 2017).

Deep learning is a subset of machine learning instead of several complex high throughput sensors with a wide range of phenotype applications (LeCun et al. 2015). However, the data collected through this versatile tool contained high variability making its application more complicated but providing more reliable prediction (Singh et al. 2018). Deep learning in computer vision-based phenomics ensures the more reliable processing of phenotypic images involving multilayered approaches network, each performing its operation in succession, improving prediction and discrimination ability (Pound et al. 2017) by a process called transfer learning. Table 10.2 lists some deep learning phenomics approaches that have been used for plant morphology and stress identification. However, applying machine learning and deep learning subsets of artificial intelligence enables plant scientists robust identification, classification, and detection of environmental variability influences on plant growth, development, and other related physiological parameters.

10.4 High-Throughput Phenomics Enhances Phytogenetics

High-throughput phenomics has been widely used in remote sensing, root phenomics, deep learning for plant stress, and vision sensing technologies in disease and pest detection (Atkinson et al. 2019; Mahlein et al. 2018). However, applying phenotyping technologies and genome sequencing is still limited in genetics and crop breeding studies both in the field and within the lab. A plethora of studies have identified QTL in many plant species, but still sveral issues need to be resolved. Among them is how to charctarizes dymanic QTLs for complex traits at mutiple grwoth stage or at different species level or districtive tarit measurements including root architechture, biomass allocation, and nutrient assessment. Identification and efficient functional characterization of potential QTLs. Integrating various genomics approaches with systemic and synthetic molecular biology approaches will significantly facilitate future breeding programs.

The regulation of the size of maize shoot apical meristems (SAMs) is correlated with flowering. High throughput analysis enables integration of SAM morphological traits with GWAS and QTL, demonstrated their contribution to SAM development (Leiboff et al. 2015). High-throughput non-destructively micro-CT-RGB phenotyping and genomics enable large-scale assessment of rice tiller traits, tiller growth, and plant traits nine growth stages. Among these traits, 402 significantly influence grain yield, vigor-related traits and yield (Wu et al. 2019). However, such

integration of genomics and phenomics is beneficial in crop breeding programs required for high yields and compact planting. Genome selection is another robust genotype-phenotype approach that involves statistical modeling and genome-wide markers. It allows efficient and accurate markers to be identified, but associated phenotype prediction is still a bottleneck in crop breeding (Taylor 2014). The process of photosynthesis and transpiration in leaves (Wang et al. 2015) depends on the number of leaves inm plants as well as the leaf size, shape, and greenness (Wang et al. 2011). The genetics study of rice and maize leaves by high-throughput leaf scoring revealed nine loci associated with leaf traits in 533 rice accessions at thee growth stages. In maize, QTL mapping of 22 leaf traits of a RIL population at 16 growth stages predited leaf tarits (leaf angle and length) being an indicator of yield (Yang et al. 2015).

Integration of high throughput phenomics with large-scale GWAS or QTLs expanded our understanding of crop developmental dynamics and emerged as a tool for plant genomics, gene expression, and characterization. The root system architecture is a promising trait for nutrients and water acquisition from the soil. Dissecting the root genetic will be helpful in increased nutrient and water acquisition from the soil. Two genetic studies on root traits were performed on rice and *Brassica* (Courtois et al. 2013; Shi et al. 2013). They conducted a GWAS of 15 root traits using vision sensors and detected associations between deep root number and mass. In *Brassica napus*, 38 QTLs were predictive indicators under phosphorus variability. Shi et al. (2013) used an agar-based high-throughput root phenotyping system to identify QTLs associated with phosphate variability correlated with *Brassica napus* root architectural traits.

Environmental factors, including both abiotic and biotic factors, can produce a variety of phenotypic effects. The rapid development of non-destructive high-throughput plant phenotyping approaches has been popularized in a plethora of crop populations to reveal the genetics of complex quantitative traits to various environmental factors (Yang et al. 2020), such as phosphate deficiency tolerance of *Brassica napus* (Shi et al. 2013), drought response of wheat (Parent et al. 2015), salinity tolerance of rice (Al-Tamimi et al. 2016), and drought resistance of rice (Guo et al. 2018). Most of these studies have focused on external responses, such as the morphology, biomass, and greenness-related traits. The internal response of plants to drought is mainly unknown. Wu et al. (2021) develop a non-destructively image-based traits (i-traits) approach to plant responses to drought. The i-traits are high-throughput image analysis pipelines aided by RGB optical sensors, X-ray computed tomography and hyperspectral imaging. In maize, i-traits identified 4322 drought-responsive loci encoding 1529 QTLs, including 15 QTLs containing potential markers for drought tolerance breeding in maize. Combining crop genetics information with genotype-phenotype approaches revolutionizes researchers' understanding of complex traits and reinforces the new era of crop breeding.

10.5 Conclusion and Future Perspective

Crop breeding evolved from conventional approaches to phenotypes-genotype aided breeding through advancement in next-generation sequencing of crops. Plant breeding enters the next era of phenomics which enables breeders accurate sampling to phenotype various traits. In the last few decades, phenomics has entered a new period of advancements as it integrated machine learning, deep learning, and artificial intelligence to predict the phenotypic characteristics of different crop populations. These approaches inherent data from various sources tend to accelerate crop breeding programs.

Considerable efforts have been placed in agriculture and global food security to maintain sustainable crop growth and productivity. Sustainable agriculture and crop production by integrating genetic tools lead to the advancement in phenomics, but demand for financial investments diminishes the development of agriculture. Phenotypic studies focus on aerial plant parts and underground plant parts; however, the integrated role of aerial-phenomics to underground phenomics influenced, but various biotic and abiotic stresses need to consider. For instance, the primary root of plant roots determines the plant's capacity to store water or other essential plant nutrients in the stem. Integrated phenotype, the ratio of root length to stem height, could be related to crop yield. For such integrated phenotypes, algorithms to compute plant imagery are required to be developed.

Furthermore, for early detection of environmental stresses, including water, drought, salinity, temperature, etc, effort should be placed to investigate the phenotypes for characterizing the propagating stress and classified them into different stages such as moderate, extreme, or exceptional. Similarly, controlling the root growth angel may contribute to the speed of recovery.

The advancement of phenomics in "big data" enables the plant science community to establish new theories in plant phenotypic approaches to integrate artificial and collaborative research at global levels. Crop phenotypic information should focus on developing tools that comprehensively integrate multi-tudinal scale emphasize on pheno-envir-genotype and physiological parameters to systematically and complete phenomic information. The critical problem of functional phenomics is its development and application in phenotyping. The introduction of new methodologies integrated with artificial intelligence and machine learning help minimize environmental challenges. These throughput approaches collect digital features efficiently. These features' precise and robust interpretation dig out critical quantitative and qualitative phenotypic traits for functional genomics. High throughput approaches also facilitate the integration of multi-tudinal phenotypic information for big-data development, management, shareability, and globality in crop genopheno-envirotype analysis and utilization. In short, for the future of phenomics, we urgently need synergism at the global level. Search for novel tools and methodologies offer powerful tools to dissect the processes in plant growth, development, and producing high-yielding and climate-resilient crops.

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Chapter 11
Understanding Abiotic Stress Tolerance in Plants by Proteomic Approach

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Contents

11.1 Introduction

Plants inhabit constantly fluctuating environments that can be stress-inducing and challenging for the growth and development of plants. These ecological nuisances include biotic stresses such as pathogenic invasion and herbivore attacks, and abiotic stresses such as extreme temperature (high and low), drought, salinity, depletion of nutrients, and accumulation of extra salts and toxic metals (Fahad et al. 2013; Hesham and Fahad 2020). Water deficiency, excess salt, and high temperature are the main abiotic stresses and pose a universal threat to plant population. These

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environmental stresses have a catastrophic effect on the geographic location of plants and restrict plant yield (food crops) and nutritional security of crop plants (Fedoroff et al. 2010; Mantri et al. 2012). The way a plant disposes itself to these stresses and develops adaptation to the severity of these stress signals is of prime significance. The approach to establish stress tolerance in plants is the current main focus of agricultural researches (Zhu 2016; Fahad et al. 2021a, b, c, d, e). A number of methods have been exploited previously to induce abiotic stress tolerance in crops. Among them, traditional plant-breeding methods have negligible success rate due to the involvement of multiple genes in stress tolerance. As discussed, stress tolerance is a multigene phenomenon, and it is quite exhausting to understand the mechanisms for advanced breeding programs. Recently, many molecular approaches have been considered to uncover the mechanism with which plants sense stress signals and respond to them. The biological advancement has made it possible to fully understand abiotic stress tolerance in plants using throughput sequencing and functional genomics (Mantri et al. 2012). The exploitation of potential genes that can safeguard and maintain the functions of cells to develop plants with abiotic stress tolerance is the ultimate point of future research in agronomy and agricultural science (Valliyodan and Nguyen 2006). In the following sections, we will evaluate the abiotic stresses, plant responses, and approaches in detail to understand the overall mechanism with specific interest in proteomic approaches to understand the role of protein in abiotic stress tolerance in the plant kingdom.

11.2 Abiotic Stress Tolerance in Plants

Environmental stresses pose a threatening pressure to the survival of plants and have been associated with the influence on the physiology, morphology, biochemistry, and molecular biology of plants (Fahad et al. 2014a, b, 2020). The main abiotic stresses posing a serious threat to plant population include extreme temperatures, drought, salinity, depletion of nutrients, and accumulation of extra salts and toxic metals (Zhu 2016). Abiotic stress is completely different from biotic stresses as it is facilitated by nonliving factors, whereas abiotic stresses are established by the living components of the ecosystem, such as virus, bacteria, fungi, insects, and weeds (Atif et al. 2021; Saleem et al. 2020a, b, c). Abiotic stresses in the form of water deficit, waterlogging, harsh temperatures (extreme hot and cold, frost), saline condition, and mineral toxicity adversely influence the rates of growth and development, productivity, crop quality, and other kinetics (Mahmood Ul et al. 2021; Mohammad I. Al-Wabel et al. 2020a, b)). In the future, it is anticipated that limited water availability will further increase abiotic stresses. Among these stresses, cold is one of the proven stresses that could affect crop productivity via interrupting crop quality and postharvest maintenance. To survive the severity of coldness, plants have developed tolerance against freezing/chilling using an effective phenomenon known as cold acclimation. Salt is on the same page when it comes to threatening global agriculture fraternity by depreciating crop production in salt-abundant regions. Two

fundamental effects of salt on crops are mediated by osmotic stress and ion toxicity. Under salt stress, the osmotic pressure beneath soil surpasses the osmotic pressure in plant cells due to salt abundance, thereby limiting plant's ability to acquire water and important minerals. Due to continuous fluctuation in climate, there is a significant increase in global temperature and atmospheric CO_2. The occurrence of rainfall is not consistent globally due to variable climate, which is leading toward drought conditions. Plants restrict their shoot growth and metabolic activities as a result of experiencing water deficit. The global deterioration is also heating up the environment that affects agricultural yields along the growth of crops. The rate of seed germination, photosynthesis, and productivity is worsening when crop plants are confronted with heat stress. Other stresses that are being added to the environment are toxins (toxic metals) resulting from the activities of chemical fertilizers, industries, and use of waste water for irrigation purposes (Gull et al. 2019).

11.2.1 Drought Tolerance in Plants

Drought-induced stress is one of the most common and lethal stresses all over the world. Drought has resulted in loss of large amounts of crop productivity (Ashraf 2010). The application of genomics, transcriptomics, and proteomics has identified the stimulation and regulation of many transcripts and proteins associated with stress tolerance, which are included in two separate groups. One group is lined with cascade signaling and transcriptional regulation, while the other group consists of members involved in the protection of membranes. These members provide their services as osmoprotectants, antioxidants, and scavengers of reactive oxygen species (ROS). The subjection of plant to water-deficient conditions during development can trigger certain physiological and development-related processes. The changes in the physiology and biochemistry upon exposure to drought stress include loss of turgidity and variation in fluidity of membranes, along with composition, fluctuation in solute concentration, ad interactions between proteins–proteins and protein–lipids. Decline in photosynthesis, production of organic acids, and changes in sugar metabolism are some of the physiological and biochemical ways of plants to respond. The assessment of gene expression using cDNA technology has nourished our knowledge about the gene hub that is functional in response to abiotic stresses (Valliyodan and Nguyen 2006). Multiple sequences have been identified in *Arabidopsis*, mainly grouped into two classes: responsive to dehydration (rd) and early response to dehydration (erd) genes (Shinozaki and Yamaguchi-Shinozaki 1996). At least four autonomous regulatory systems are involved in gene expression in response to water-limiting stress. Two of them are abscisic acid dependent (ABA), while the remaining two are abscisic acid independent (Shinozaki and Yamaguchi-Shinozaki 2000). A dehydration-responsive element/C repeat (DRE/CRT), a cis-acting element, is invested in abscisic-independent regulatory system. This responsive element also coordinates in cold- and high-salt-induced expression system. Often overexpressing DRE/CRT-binding protein DREB1/CBF in the

genetically modified plant *Arabidopsis*, more than 40 stress-sensitive genes were identified. The level of expression of these genes led to the induction of tolerance in *Arabidopsis* against cold, salt, and water scarcity (Seki et al. 2001; Fowler and Thomashow 2002; Maruyama et al. 2004).

There are small-sized molecules that show no toxicity to cells at molar concentration and provide stability to proteins and cell membrane under stress conditions. These neutral molecules are called osmoprotectants and coordinate the cellular functions to withstand under severe effects of the stress (Yancey 1994). Several main crops do not possess the ability to produce these molecules in response to abiotic stress. Hence, it has been postulated that introducing the pathways essential for the formation of osmoprotectants can be a good effort to enhance stress-bearing ability in plants (Rathinasabapathi 2000). Another product known as mannitol, which is produced during photosynthesis in algae and higher groups of plants, accelerates tolerance in water-limiting conditions with the help of osmotic regulation. By manipulation of *mannitol dehydrogenase* (*mtlD*) gene into wheat, a significant amount of tolerance has been generated under water stress condition (Valliyodan and Nguyen 2006). The shielding of enzymes and membranes from ROS is mediated by D-ononitol and myo-inositol in cytoplasm of cell. In genetically modified tobacco plants, the overexpression of *inositol methyl transferase* gene (*IMT1*) isolated from rice plant has resulted in the enhanced tolerance for drought and salt by the production of methylated form of inositol known as D-ononitol (Sheveleva et al. 1997).

Abiotic stresses interrupt the physiological and biochemical pathways, leading to a sharp decline in plant productivity (Fahad et al. 2016a, b, c, d, 2019a, b). The response system of plants varies depending on various genotypes (Wang et al. 2018). The genomic sequences of plants are triggered by transcription factors in conjugation with other transcription-binding sites (Shinwari et al. 2020). These transcription factors bind to the cis-acting elements in upstream of all gene promoters (Ciarmiello et al. 2011). Furthermore, the transcription factors enhance or restrict the functionality of DNA polymerase for gene expression (Riechmann et al. 2000). The transcription factors stimulate genes associated with stress and elevate drought tolerance response. A group of basic amino acids participate to provide resistance in plants to abiotic stresses (Annunziato 2008). In model plant, for example, *Arabidopsis*, there has been the identification of 1533 transcription factors grouped into about 34 families (Riechmann et al. 2000). In recent years, the tolerance response of *Betula platyphylla* (birch) has been evaluated. In this study, 2917 genes linked to stress tolerance were identified using RNA-sequencing approach. Among them, some drought-responsive transcription factor families, ethylene responsive factor, and myeloblastosis oncogene were reported in maximum amount. Moreover, BpERF2 and BpMYB102 transcriptional factors were associated to tolerance in response to water-deficit stress. These two transcriptional factors further triggered a cascade of other stress-related genes and also boosted drought tolerance in the plant (Wen et al. 2019). Sakuma et al. (2002) explained some types of DREB transcription factors in model plant. They predicted that DREB1A and DREB2A attach to a certain six-nucleotide sequence of DRE and enhance drought-associated

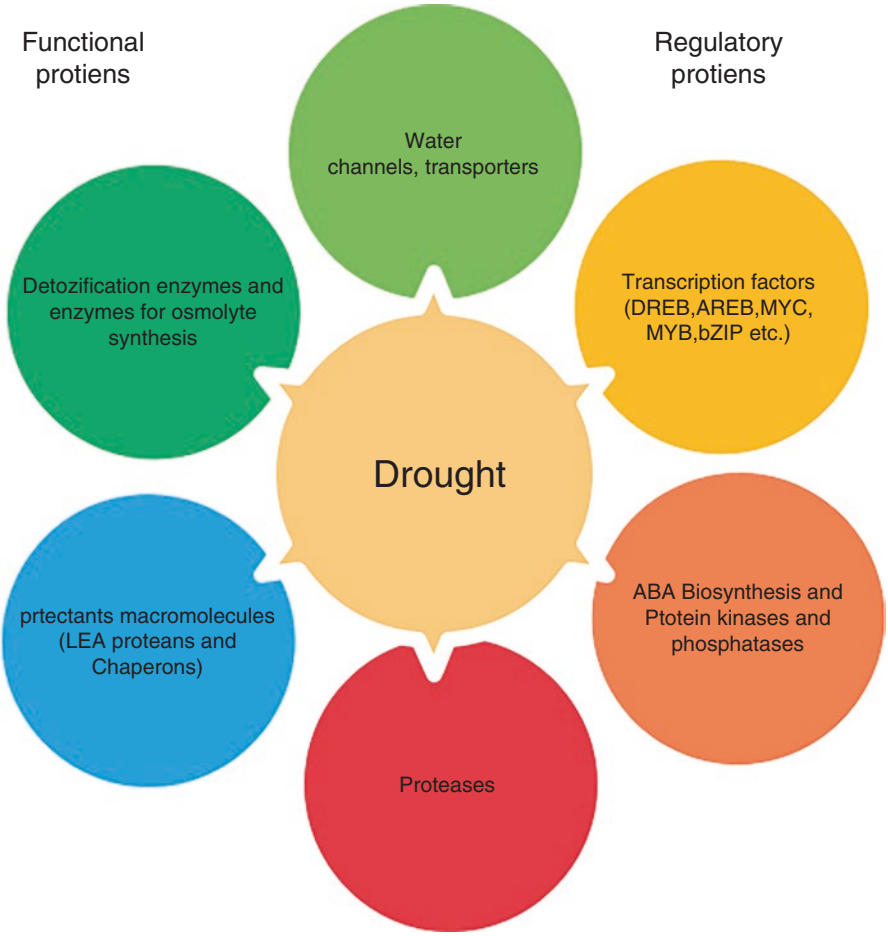

Fig. 11.1 The functions of drought stress-inducible genes in stress tolerance and response. Two types of proteins are produced in response to drought stress. The first group includes function proteins that probably function in stress tolerance, and the second group includes regulatory proteins that function in signal transduction and gene expression of other abiotic stress-related genes. (Shinozaki and Yamaguchi-Shinozaki 2007)

tolerance in *Arabidopsis*. The two main groups of proteins, along with other factors that responded to drought, can be seen in Fig. 11.1.

Studies have proposed in the past that the amount of calcium in cytoplasm elevated in response to certain triggers such as cold, drought, and salt. This calcium influx results in the signal transduction, which is probably linked to a cascade of protein phosphorylation/dephosphorylation. It has been reported that most of the calcium-triggered protein phosphorylation is primarily carried out by members of calcium-dependent kinases (CDPKs) in plants. The gene expression of many CDPK members is also involved in mediating stress signals across some plant species. It is

also worth mentioning that a number of transport proteins such as aquaporins and ion channels, which are active during stress regulation, are controlled by CDOKs. The overexpression of *OsCDPK7*, a gene encoding CDPK in rice, increased the stimulation of some stress-responsive genes in reaction to drought/salinity (Saijo et al. 2000).

Aquaporins are trans-membrane proteins mainly responsible for the homeostatic regulation of water in cells. Many researchers have focused on the aquaporins as potential candidates for the integration of drought-tolerance ability into breeding programs (Zargar et al. 2017). The advancement in sequencing technology has made the transcriptome profiling less expensive but effective. The information from the transcriptomes and genomes of hundreds of plants will be supportive to apprehend the phylogenetic distribution and expression of aquaporins in a wide range of species. Some studies proposed that an exposure to dehydration can increase the transcript level of aquaporins, leading to higher water permeability in membrane, mediating water transport, while others assumed that in response to water deficit plants reduce their membrane water permeability to escape the loss of water by down-expression of some aquaporins. The aquaporins located in root tissues also help to avoid stress condition related to water deficiency. The aquaporins in plants' roots are highly regulated and are associated with water-limiting conditions and morphological characterization of the plant. The overexpression of aquaporins is not always a good mechanism in plants. When tobacco plant overexpresses *AtPIP1;2*, the plant gets wilted in drought stress compared to control plants. Moreover, the prominent aquaporin *e HvPIP2;1* when overexpressed in rice resulted in a faster rate of leaf transpiration and decrease in efficiency of water usage (Zargar et al. 2017).

11.2.2 High-Temperature Tolerance in Plants

Global warming has devastating effects on plants due to the lethal effects of higher temperature on the growth and development of plants. The extremity in climatic conditions has resulted in the less productivity and higher chances of famine. According to some reports, India is the main producer of wheat by contributing to 15% of total wheat production, but it is predicted that climatic variation will transpose this production rate into temperature-stressed and negligible seasonal duration. A rise in temperature by 3–4°C could lead to reduction of productivity by 15–35% in Asia and Africa and by 25–35% in the Middle East(Ortiz et al. 2008). In tropical and subtropical regions, the temperature during the growing season may surpass the maximum value of the highest temperature recorded till date (Battisti and Naylor 2009; Varshney et al. 2011). Latin America is supposed to experience blows in temperatures and declines in precipitation all over the continent, particularly in Central America and the Caribbean. In European countries, Southern and Central regions will face increase in temperature, with Spain, Portugal, and Italy suffering the most. Interestingly, the fluctuating climate will significantly enlarge the agricultural

production in northern countries, where agriculture is limited due to low temperature (Lotze-Campen and Schellnhuber 2009).

Among all abiotic stresses, the heat stress has an autonomous mechanism of action on the physiology and metabolic pathways of plant cells. The heat-related stress is accompanied by drought and salt stress, but it is obligatory to uncover the independent route of action and biological effects of extreme temperature so that the combined effect of multiple stresses could be apprehended. The vulnerability of plant toward high temperature is dependent on the stage of development and hot conditions that influence the plant in both vegetative and reproductive stages. The affects are also linked to species and genotypes along with interspecific and intraspecific alterations (Barnabás et al. 2008; Sakata and Higashitani 2008). Multiple physiological scratches have been reported in plants upon experiencing rise in temperatures, such as slashing of leaves/stems, abscission and senescence in leaves, and retarded growth in root and shoot and distorted fruits, which ultimately resulted in restricted plant yield (Vollenweider and Günthardt-Goerg 2005). Furthermore, heat stress decreases plant development through changing net shoot assimilation rates and total dry weight of the plant (Wahid et al. 2007).

Plants experiencing higher degree of heat, at least 5°C above their normal growth temperature, demonstrate a unique set of properties of cellular and metabolic responses in order to neutralize the lethal conditions of higher temperatures (Guy 1999). These alterations include modifications in cellular organizations (organelles and cytoskeleton) and membrane activities (Weis and Berry 1988), reinforced by decline in the production of normal protein and incline in the expression of heat shock proteins (Bray 2000). Additionally, the production of phytohormones such as abscisic acid, antioxidant compounds, and certain molecules with protective nature was also reported (Maestri et al. 2002).

One of the proteins that were produced in response to high temperature is heat shock protein (HSP). Recently, the rise in temperature as a result of global warming is the subject of great concern as it has deteriorated the agricultural production in all regions of the world, putting billions of dollars at potential risks (Mittler et al. 2012). Heat stress causes substantial damage to the plants (crops) by affecting protein production, inhibiting main enzymes, and destroying membrane structures. Heat shock instigates oxidative stress, which suppresses plant development and limits yield and nutritional quality. Therefore, plants have acquired molecular chaperons to fold and unfold proteins, and stabilize the assembly of multiprotein complexes and regulation of cell cycle and cellular signaling; the transportation of essential proteins to cellular compartments is also the route of response by plants to ensure protection against the imposing stress or programmed cell death (apoptosis) (Lindquist and Craig 1988).

HSPs, heat shock transcription factor, and molecular chaperons are mandatory for the survival of plants and maintenance of the protein stability under heat shock. High intensity of heat develops heat shock response, which safeguards the plant from cellular injuries and ensures the rehabilitation of cellular and physiological functionalities. These effects also establish a significant level of thermostability in plants. Higher heat exposure also incites the plants to produce ROS and oxidative pressure (Khan and Shahwar 2020). The plants adjust themselves to afford

high-temperature stress by adopting certain modifications in plants such as the production of various metabolites like antioxidants, osmoprotectants, and heat shock proteins by changing their metabolic pathways. Further, heat shock also modifies the regulation of genes encoding factors that include osmoprotectants (glycine betaine, proline, trehalose, etc.), phytohormones (gibberellic acids, abscisic acid, brassinosteroids, jasmonic acids, salicylic acid, etc.), detoxifying enzymes, signaling molecules (e.g., nitric oxide), transporters, regulatory proteins, free radical scavengers, polyamines (spermidine, putrescine, and spermine), and protectants in trace elements (silicon, selenium, etc.). These factors mediate signal transduction and transcriptional regulations that have been reported to counteract stress associated with high temperature.

HSPs are stress-associated protein family in cells of almost all organisms synthesized in response to stressful conditions. HSPs are common to both prokaryotes and eukaryotes. Due to the presence of higher conservative sequences, it serves prominent roles in cells. It has been demonstrated that HSPs work in folding, assembling, translocating, and degrading of proteins. There are about five main families of HSPs in plants and animals on the basis of molecular sizes and weights. Some HSPs are localized into cytoplasm and can be found in organelles such as mitochondria, chloroplast, endoplasmic reticulum, and nucleus (Khan and Shahwar 2020). The classification of HSPs on the basis of their cellular location and functions is given in Table 11.1 (Kregel 2002).

Table 11.1 Classification of heat shock proteins on the basis of their cellular location and function

HSP family/ classification	Cellular location/site	Function/role
HSP 20 (sHSPs)	Cytosol. Endoplasmic reticulum, Mitochondria, Chloroplast	Preventing aggregation, co-chaperons
HSP 27 (sHSPs)	Cytosol, nucleus	Microfilament, antipoptotic, stabilization
HSP 60	Mitochondria	Prevents aggregation of denatured protein, refold proteins, proapoptotic
HSP 70 family	Nucleus, cytosol	Antipoptotic
HSP 72 (HSP 70)	Nucleus, cytosol	Molecular chaperons
HSP 72 (Hsc 70)	Mitochondria	Molecular chaperons
HSP 75 (Mhsp 70)	Endoplasmic reticulum (ER)	Molecular chaperons, cytoprotection
HSP 78 (GRP 78)	Cytosol, nucleus, endoplasmic reticulum (ER)	Translocation of protein, regulation of steroid hormone receptors
HSP 100/104	Cytosol	Folding of protein

11.2.3 Low-Temperature Tolerance in Plants

Low temperature, known as temperature from subzero to extremely chilling conditions, is part of natural cycle and inflicts an environmental lockdown on plant's abilities, particularly in cold climates (Janmohammadi et al. 2015). Low temperatures could induce stress in plants via desiccation of cells and tissues when water inside the cells gets frozen (Beck et al. 2004). Low-temperature stress tolerance in plants is a multigenic phenomenon consisting of a network of inducible genes. These genes are related to the synthesis of three types of proteins: structural proteins, regulatory proteins, and osmoprotectants (Breton et al. 2003). While studying low-temperature shock, much of the attention has been given to identify low-temperature-induced proteins and the transcriptional regulation of genes that are responsible for the synthesis of these proteins. Plant response to low-temperature stress is a multifactorial process that is connected to many factors like the stage of development, duration of exposure, intensity of the corresponding stress, thermal rates, and the sites of ice formation where it is produced intracellularly or extracellularly. Low-temperature-tolerant species have adopted different mechanisms to cope with the low-temperature stress. Plants growing over winter can elevate their capacity to afford low temperature, but not chilling temperature, a phenomenon known as cold acclimation (Thomashow 1999). However, it is inappropriate to suggest that even hardy plants possess low temperature/freeing tolerance in all developmental stages. Certain hardy species postpone the transformation of plant from vegetative to reproductive phase until they experience low but nonchilling temperatures, a mechanism known as vernalization that induces the plants to over winter as seedlings (Amasino 2004).

Acclimatization to low-temperature stress is triggered through severe changes in expression of genes that further alter the composition of transcripts, proteins, and metabolites (Thomashow 1999; Chinnusamy et al. 2007). Moreover, some low-temperature-induced protein sequences pass through various types of post-translational modifications such as phosphorylation, ubiquitination, N-glycosylation, SUMOylation, and lipid alterations. These modifications determine the specific aspects of proteins' functionality, such as subcellular localization, stability, and the affinity to associate with other proteins. Hence, it is mandatory to investigate the proteome since, unlike transcript, the proteins are the direct indicators in plant stress response. In the recent decade, efforts have been made to understand the low-stress signaling and regulatory processes underlying cold acclimatization by using proteomics strategies (Weckwerth et al. 2008). In the following sections, the proteome of different crops plants is discussed.

11.2.3.1 Analysis of Wheat Proteome Under Low Temperature

In cold regions, the winter wheat is the most crucial part of agriculture and frost-induced injury during winter and early spring can be disastrous for crops. Plant breeders have sensed the need to introduce wheat cultivars with significant resistance toward winter, but they have negligible success in the development of cultivars that propose enhanced tolerance to freezing conditions. In winter crops, the complete expression of low-temperature-tolerant DNA sequences happens during the vegetative phase. It looks like that developmental genes tend to control genes influencing the transcription and translation of low-temperature-induced genes, a phenomenon recognized as developmental regulation of low-temperature-responsive genes. During cold climate, the wheat vernalization serves a primary role in the acquisition of low-temperature tolerance via inhibition of early transition into reproductive phase prior to the ending of chilling stress (von Zitzewitz et al. 2005).

Low-temperature stress predominantly influences protein synthesis. The participation of RNA-binding proteins in cold acclimation has been apprehended in plants with the help of proteomics (Kosová et al. 2011). A class of RNA-binding proteins known as glycine-rich RNA-binding proteins (abundant in glycine at C-terminal) has been explored in the mediation of low-temperature stress response and can be used as an effective marker for cold tolerance. The synthesis of free and proteinogenic amino acids can be influenced as a result of cold exposure. An enhanced level of several proteins responsible for the biosynthesis of methionine has been reported following cold shocks. Furthermore, the cysteine synthase has been announced as cold stress-responsive protein. In addition to the role of CS in the biosynthesis of cysteine, the protein is also a pacemaker biocatalyst that catalyzes the formation of glutathione. The overgeneration of CS may result in the elevated synthesis of glutathione, which is considered to be associated with tolerance to low temperature (Janmohammadi et al. 2015). Proteome investigation of winter wheat crow after prolonged exposure to cold climate displays a decline in some enzymes catalyzing the biosynthesis of UDP-glucose. Hence, it was presupposed that this may result in the decreased synthesis of cellulose in plants and ultimately could lead to retardation in growth. However, reports on the biochemistry/physiology have shown that low-temperature shocks at the first hour elevated the catalytic activities of b-fucosidase and b-glucosidase, which could in turn intensify hemicellulose turnover with the synthesis of biologically active oligosaccharides that could induce cold tolerance (Zabotin et al. 2009).

11.2.3.2 Analysis of Barley Proteome Under Low Temperature

Barley (*Hordeum vulgare* L.) is one of the widely grown cereal crops in the world, supplementing as a staple food in 1/3 of global population (FAO and Foods 2008). Barley is the most tolerant *Triticeae* species to water stress and salinity. In winter, barley resistance to cold stress is essential for successful overwintering. However, unlike wheat, the proteome reaction to low-temperature stress in barley is less

understood. The cold acclimatization process in barley has been investigated based on genomic and transcriptomic. Janská et al. (2011) investigated the translational reaction of barley to periodic low temperature. Their results explored that synthesis of nucleation substances in leaves and crow was downregulated during cold acclimation, while the genes encoding major antifreeze components such as chitinases, glucanases, thaumatin-like proteins, and ice recrystallization inhibition proteins were upregulated. These proteins belong to the class of proteins in plants that enable plants to withstand subzero conditions.

Interestingly, the proteome analysis of barley plants resistant to frost-uncovered (which suffered prolonged cold acclimatization) level of protein variability was higher in leaves as compared to crown and various chloroplast-associated proteins affected, pointing to the tendency to prioritize the protection of photosynthetic machinery (Hlaváčková et al. 2013). This result concluded the presence of unique approaches toward responding to shivering stress in crowns and leaves. Out of all the proteins that exhibited variability at high level, the authors picked out the 33-kDa oxygen-evolving protein of photosystem II and the AAA ATPase in leaves, or the HSP70 and "enhanced disease susceptibility 1" protein in crowns. These findings further stated that these specific proteins might be involved in frost-resistant winter barley, serving as the most distinguished low-temperature-responsive proteins. According to these outcomes, the process of water oxidation during food and energy production in leaves and defense responses in roots is the most forthcoming response to chilling shock. It is the indication of the presence of a complicated connection between chloroplast and cold acclimatization. Those genes that are responsible for chloroplast-localized proteins were also overexpressed, giving green signals about the involvement of plastid-attached proteins in cold tolerance (Svensson et al. 2006). These exemplifications clearly supported the possible assumptions about the response of plants toward decline in temperature of the surrounding and how the plants adapt to extreme climates by regulating its important activities and recruitment of potentially stress-tolerant proteins.

11.2.3.3 Analysis of Rice Proteome Under Low Temperature

Rice (*Oryza sativa* L.) is one of the most consumed cereals and source of sustainable food production in the world. The molecular mechanism underlying cold resistance has been at the center of attention for many decades due to the vulnerability of rice to cold conditions. The cold-induced proteome investigation of the plasma membrane in roots of rice shows that most of the cold-associated proteins were involved in energy generation, cellular signaling, protein formation, cell growth/division, and defense networks (Hashimoto et al. 2009). Furthermore, it has been claimed that variation in membrane permeability in response to chilling temperature resulted in elevated level of cytosolic cations with calcium-dependent membrane-binding proteins such as annexins, which mediate the function of signal processing to low-temperature stress.

Reports of rice seedlings when exposed to short-period low-temperature stress explored that there is a network of low-temperature-responsive proteins (regulatory and functional proteins) serving a potential role in chilling stress tolerance (Yan et al. 2006). Moreover, it has been revealed that a number of proteins become denature in the midst of cold stress. For instance, the injury to enzymes such as glycine dehydrogenase and RuBisCO activase can result in interrupted photorespiration. This mechanism is involved in photosynthetic metabolism and is associated with the recovery of RuBP (ribulose 1,5 bisphosphate) in the Benson–Calvin cycle (Janmohammadi et al. 2015). Cold-responsive proteins are not only restricted to protein metabolism, but are also functional in the biosynthesis of components in the cell wall, free radical-scavenging process, energy generation, and provision of metabolite needed for cold resistance in plants (Cui et al. 2005). In the same study, exposure to cold stress for a short time led to a substantial rise in the level of UDPglucose pyrophosphorylase (UGPase) and sucrose synthase 1; enzymes contribute to Krebs cycle, giving rise to the idea that freeing stress influences the metabolic pathways of carbohydrates. In the meantime, the production of ROS hunting enzymes has been reported as one of the responsive elements during chilling stress. However, the transcriptome of the low-temperature-resistant rice unveiled that, among other genes with antioxidant potential, glutathione peroxidase (GPX) and glutathione S-transferase (GST) were overexpressed in response to low-temperature shock (Zhao et al. 2015). This result may indicate that an integrated regulation of ROS-related enzymes at transcriptional and post-transcriptional levels is most probably active in advancing cold stress tolerance in plants.

An approach to evaluate less-abundant proteins has been used by fractionating these polypeptides with polyethylene glycol (PEG). Using this method, an elevated level of new proteins that included cysteine proteinase, thioredoxin peroxidase, a RING zinc finger protein-like, drought-inducible late embryogenesis abundant protein, and fibrillin-like, was identified in leaves of rice against low-temperature stress (Lee et al. 2007). Neilson et al. (2011) also identified extra-low-abundant proteins such as histones (H2A.3, H2B.9, H3.2, and H4) and vitamin B synthetic proteins mediating response to chilling stress.

Studies in the recent decade have strongly concentrated on the regulatory role of post-transcriptional and post-translational modification of proteins in the low-temperature responses, and hence, there is great fondness in investigating such aspects with proteomic approaches (Miura and Furumoto 2013). In order to investigate the individual mechanism mediating chilling stress, it is believed to be imperative that foundations of proteomic investigation should include comparative analysis of different cultivars of rice.

11.2.4 Heavy Metal Tolerance in Plants

Anthropogenic actions have caused great distress to life on earth. A number of human activities have disrupted the biodiversity around the world with their unplanned and reckless activities. In the same sense, these advanced creatures have

contaminated the region with heavy metals, disturbing both the living environment and conditions for plants, animals, and humans. The emergence of such problems is of great concern to the public and governing bodies, and a demand to restore the natural balance is required. The toxicity of contaminants might cause the killing of plants installed for phytoremediation by disrupting different metabolic processes. However, plants survive the severity of metal-induced stress by developing a strategy of tolerance to sustain and survive in contaminated soil. Various studies have focused on the molecular mechanisms involved in the induction of heavy metal tolerance in plants with the help of genomics and proteomics (Macnair et al. 2020).

Plants counteract the environmental contaminants by activating certain genes that are responsible for the synthesis of protein involved in heavy metal stress tolerance. The ions of heavy metals affect the homeostatic regulation of cellular proteins by intervening the folding and trigger aggregation of non-native proteins, affecting the endoplasmic reticulum (ER) and resulting in disturbed cellular activities. However, plants have adopted various survival extinct and enhanced tolerance systems that are used to neutralize heavy metals (Hasan et al. 2017). In cells, the heavy metal ions induce oxidative stress by producing ROS species, which advances DNA deterioration and interrupts the DNA repair strategy of the cell. Moreover, they also handicap membrane integrity, nutrient maintenance, and protein activity (Tamás et al. 2014). When plants are exposed to high concentrations of heavy metals, the cells adopt a complex mechanism of storage and detoxification. This includes the chelation of ions (from heavy metals) with phytochelatins and metallothioneins in the cytoplasm trafficking and sequestration into the vacuole by vacuolar transporters (Zhao and Chengcai 2011).

To escape the destructive effects of heavy metals, plants produce minor cysteine-rich oligomers known as phytochelatins at the start of metal-induced stress. It has been noted that the most peculiar factor in metal-inflicted stress in plants in the synthesis of phytochelatins. Moreover, it is also an established fact that the biosynthesis of the phytochelatins is modulated at post-translational event by metal ions in a variety of plant species. However, the upregulation of phytochelatin synthase gene does not ensure increase in metal stress tolerance in plants (Hasan et al. 2017).

Another important group of proteins associated with metal stress forbearance in plants is intracellular cysteine-rich major metal-binding proteins known as metallothioneins that are recruited by the cells for immobilization, sequestration, and detoxification of metal ions (Capdevila and Atrian 2011). The presence of metallothioneins has been reported in plants since the last few decades; however, the exact physiological roles of these proteins have not yet been fully apprehended (Liu et al. 2015). The anticipated functions of these metallothionein proteins include (a) the homeostatic regulation of essential transition metals, (b) sequestering of lethal heavy metal, and (c) the shielding of cells against oxidative pressure by metal stress (Hossain et al. 2012).

The growth of plants in soil contaminated with heavy metals drives the plants to adjust their physiology by developing metal stress tolerance. Plants overcome these heavy metal stresses through various ways such as synthesis of metal-attaching compounds, metal accumulation in vacuoles, glands-modulated excretions, changes in membrane conformity, and, most importantly, the formation of stress-responsive

proteins, including the abovementioned proteins and HSPs (Neumann et al. 1994; Hasan et al. 2017).

11.2.5 Salinity Stress Tolerance in Plants

Each plant has a unique ability of salt tolerance ranging from glycophytes (salt-sensitive species) such as *Arabidopsis* to halophytes (salt-tolerant species) such as Atriplex species (saltbush). Although cereal crops have been claimed as glycophytes, different crops have a separate level of salt tolerance and coping mechanism. For instance, rice shows more glycophtytic characters compared to wheat and barley (Munns and Tester 2008). There can also be considerable genetic variation for salt tolerance within closer species, and this fact can be used to develop salt-tolerant crop breeds (Roy et al. 2011). The collective approaches of functional genomics such as genomics, proteomics, transcriptomics, metabolomics, and ionomics have been exploited to study and analyze abiotic stress tolerance in plants. Investigations of proteins response in plants to saline stress have been carried out in many important species, including cereals like maize (*Zea mays),* rice (*Oryza sativa*), barley (*Hordeum vulgare*), and wheat (*Triticum durum* and *Triticum aestivum*) (Zhang et al. 2012). Proteomic studies of single-cell proteins have been successfully used in mammalian systems where cells can be cultured to enhance the starting materials (Schirle et al. 2003; Diks and Peppelenbosch 2004). However, there are limited examples of single-cell proteins in plants, particularly in cereals, due to the inconvenience in obtaining sufficient materials. Such proteomic evaluation has been demonstrated in guard cell of *Arabidopsis*, trichomes, soybean (*Glycine max*), and tobacco (*Nicotiana tabacum*) trichomes. Proteomic studies of plant tissues in response to salt stress have highlighted rice anthers, rice plasma membranes, and wheat root seedlings (Shelden and Roessner 2013). *Physcomitrella patens* plant shows prominence due to its significant role in the study of plant systematics and evolutionary biology. The high-salt-resilient property makes this plant a unique model to evaluate molecular processes involved in salinity stress response. Proteomic analysis of *P. patens* upon exposure to high-salinity conditions showed that 16 protein spots were downregulated while 49 spots out 65 proteins spots were upregulated. These proteins were related to a diverse set of functions such as energy and metabolic activities, protein biosynthesis, protein degradation, defense cellular behaviors, mitotic division, and signal transduction and transportation. Mainly the overexpressed proteins were involved in defense potentials, protein folding, and homeostatic balancing of ions. Among them, HSP70 is expected to protect from denaturation and degradation due to injury caused by salinity, signally proteins, and phototropin involved in the regulation of H+/ATPase homeostasis and ROS-scavenging proteins, making a potent antioxidative system to safeguard cells from damaged caused by oxidative stress postsalinity exposure in *P. patens* (Wang et al. 2008).

Enhancement in proteomic technology in the term of protein separation and detection and mass spectrometry-based protein analysis has a substantial role in

studying plants reflexing under extreme saline conditions (Joseph and Jini 2010). While multiple research groups are active in identifying salt-responsive proteins in plants, a study by Salekdeh et al. (2002) recovered many salt-responsive proteins such as ABA and stress-responsive proteins, ascorbate peroxidase, and several more in the root proteome of both salt-sensitive and salt-tolerant rice genotypes. Most of the proteins were regulated by the salt level in a direct manner (Nohzadeh et al. 2007). The activities of these proteins were seen in photosynthetic activity, metabolic homeostasis, photorespiration, signal transduction, antioxidation, regulation of ions channels, and protein folding. In plants, salt shock proteins (SSPs) are accumulated in response to salt stress that can result in the synthesis of other soluble proteins and bolster the activities of many enzymes (Joseph and Jini 2010). The variation in genomics content (DNA/RNA) of plants upon experiencing saline stress could be accountable for the expression of specified salt shock proteins (SSPs) with molecular weights of 15.28 and 72 kDa in salt-tolerant genotypes, whereas they were entirely absent in vulnerable genotypes (Gomathi and Vasantha,2006). One way to encounter salinity stress is with induction of molecular chaperons. It is interesting in nature that drought, salt, and heat stresses are often unanimous in their effects. Heat shock proteins are a group of proteins consisting of conserved protein families such as HSP100, HSP90, HSP70, HSP60, and other small heat shock proteins (Joseph and Jini 2010)

11.3 Conclusions

The main abiotic stresses in plants and protein-producing mechanisms in plants to tolerate these unfavorable signals were discussed. There are other abiotic stresses evolving with fluctuation in climate that have devastating effects on the plants, particularly crops, and need utmost scientific attention. We should investigate the crops in different geographic regions around the world to understand the significance of abiotic stresses in variable regimes. Moreover, it is also mandatory to use proteomic strategies along with genomics to identify versatile proteins that can provide a grasping view of stress and tolerance interaction in plants so that the main cash crops can be exploited with these potential tolerant proteins to make a balance between agriculture and economy.

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Chapter 12
Novel Nanotechnology-Based Vector Delivery in CRISPR System for Transgene-Free Editing

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12.1 Introduction

CRISPR (clustered regularly short palindromic repeats)/Cas (CRISPR-associated proteins) is an acquired and advanced phage immune system discovered in many arches and bacteria to protect them against invasive genetic materials such as nucleic acid (Ahmar et al. 2020; Barrangou et al. 2007; Fineran and Charpentier 2012; Horvath and Barrangou 2010; Wiedenheft et al. 2012). Since its first application in 2013, it was first introduced in mammalian cells. CRISPR mechanism is based on RNA-guided programmed nuclease, which has altered much more functions than just editing of genome (Cong et al. 2013; Mali et al. 2013). The CRISPR/Cas technology is based on functionalized target sequences, core components, and multiple subunits that have been characterized into three classes and their subtypes (Gasiunas and Siksnys 2013; Makarova et al. 2011a, b). The Type I CRISPR systems consisted of CRISPR-associated complex for antiviral defense (CASCADE) structure that comprised various subunits of Cas proteins that bind and form complexes with CRISPR RNA (crRNA) to initiate and signal the desired target loci. However, in Type III, crRNAs are integrated into various subunits complex called Csm or Cmr to signal, bind, and cleave the invaded RNA. On the contrary, in Type II systems, the Cas9 protein is the only prerequisite for the integration of DNA (Brouns et al. 2008; Garneau et al. 2010; Hale et al. 2009; Hsu et al. 2014). The Type II Cas system from specie *Streptococcus pyogenes* has a broad-term application in biomedical research due to the processive features such as high efficiency, specificity, rapid, inexpensive, simplicity as well as great versatility (Bikard et al. 2014; Cho et al. 2013; Hwang et al. 2013; Nekrasov et al. 2013; Wu et al. 2015). This system consists of two functional components: guide RNA (gRNA) and a DNA endonuclease (Cas9), which were engineered by the fusion of tracrRNA and a crRNA into a single RNA molecule. Usually, gRNA is easily switched by a synthetic single-guide RNA (sgRNA). Target site recognition begins in the presence of protospacer-adjacent motif (PAM) that is immediately located at (50-NGG) site, where the Cas9 signals the sgRNA to be positioned to the targeted site to initiate and unwind the site-specific double-stranded DNA breaks (DSBs); in these cases, the two cellular repair pathways, homology-directed repair (HDR) and nonhomologous end joining (NHEJ), can ensure to initiate with the potential alterations or error-prone insertions/deletions (indel) products (Garneau et al. 2010; Gasiunas et al. 2012; Jinek et al. 2012; Sapranauskas et al. 2011). Furthermore, in recent years, CRISPR interference (CRISPRi) technology has been developed that utilized deactivated dead Cas9 (dCas9) protein showing absence or no endonuclease activity, thus actively regulating the genes in a well-defined manner (Gilbert et al. 2013; Larson et al. 2013; Qi et al. 2013). In addition, the Cas9 nickases (HNHH840A or RuvCD10A), which cleave only a single strand other than both strands of the target site, have become effective for genome editing mechanism (Guilinger et al. 2014; Ran et al. 2013a). Due to its high versatility, this mechanism is much appropriate for the editing of the genome with less off-target effects in any model organism (Kleinstiver et al. 2016; Shen et al. 2014). More recently, an alternate technology based on CRISPR-nuclease

Cpf1 has been efficiently developed that showed various advantages as well as disadvantages of each Cas9 system (Zetsche et al. 2015, 2017).

Presently, the delivery approaches of the CRISPR/Cas system are primarily focused on physical strategies (transformation, electroporation, microinjection, and so on) via viral and/or nonviral vectors (Adeno-associated virus (AAV), lentivirus, adenovirus), etc. (Niu et al. 2014; Xue et al. 2014; Yu et al. 2017). In the above-mentioned platforms, the physical and viral vectors have been subjected to be achieved through powerful delivering components of CRISPR/Cas9 systems. Despite high editing efficiencies, most of the physical delivery methods are only applicable for in vitro but not fit for in vivo applications (Chen et al. 2017). For viral vectors, the potential apprehensions are the limited DNA packaging capacity, limited scale-up production rate as well as clinical therapeutics such as carcinogenesis and immunogenicity (Chen and Gonçalves 2016). Nonetheless, the current delivery strategy for nonviral-based nanoparticles showed its significant considerations to overcome the limitations of safety concerns (Mintzer and Simanek 2009; Pack et al. 2005).

The emergence of material sciences and nanotechnology has offered tunable and significant aspects that hold potent applications in the field of genome editing (Yin et al. 2017). However, lipid and polymeric-based nanoparticles (NPs) offered encapsulation of large size genetic payloads and favor high efficiency and immunogenicity response (Liu et al. 2018a, b). However, gold-based nanoparticles (AuNPs) have triggered the delivering approach of ribonucleoprotein (RNP) in vitro and in vivo applications in mice (Lee et al. 2017). Up to date, previous pieces of literature focused on nonviral NPs-based delivery in the CRISPR system, which aims to enhance the delivery efficiencies, mitigate the off-target effects, and recognize Cas9 protein on the target sites (Dever et al. 2016; Yin et al. 2016). This chapter exclusively elaborates the recent barriers for the delivery of the CRISPR/Cas9 system with great emphasis on the potential development of vector delivery in CRISPR/Cas9 based on nanomaterials for transgene-free genome editing applications.

12.2 Limitations and Challenges of CRISPR/Cas9-Based Genome Editing

So many technical pitfalls need to be addressed before CRISPR/Cas genome editing system can be efficiently utilized for clinical use. Based on a genetic basis, three consecutive limitations are subjected to be addressed. First, signaling and targeting the desired site actively, accurately, and efficiently of both cleavage and repair machinery to mitigate the chances of off-target possibility. Second, consider switching repair pathways such as HDR- or-NHEJ that handicamp on various experimental designs. Third, the system requires to be more specific to trigger therapeutics applications in diagnosing multiple diseases. However, due to the high probability of infrequent recombination efficiency, there are various shortcomings to achieve

potential applications, as presented in Fig. 12.1 (Wang et al. 2013; Weber et al. 2015; Yang et al. 2013; Yu et al. 2015). Therefore, the gene-based delivery vehicle of CRISPR components governs the integration of foreign genes into the desired genomic site, and the resultant leads to poor immune feedback. However, various viral vectors are functionally utilized for in vitro delivery vehicles of CRISPR-based reagents, and the frequency of activation mutagenesis leads to the generation of proto-oncogenes due to the insertion of viral genes into the desired target genome, which results in the development of tumorigenesis (Yin et al. 2016). Recently, in vivo delivery of nano-based vectors in the CRISPR system could resolve these concerns to overcome these pitfalls for transgene-free editing.

12.3 Modes of CRISPR/Cas9 Delivery Approaches

Usually, sgRNA can be integrated into the vector plasmids (pX459, pX330, etc.) that consist of sgRNA complex or can be achieved through in vitro transcription. In contrast, the desired template single-stranded oligodeoxynucleotide (ssODN) required for the gene correction through HDR-mediated pathway can be constructed in plasmids or achieved through in vitro mechanism.

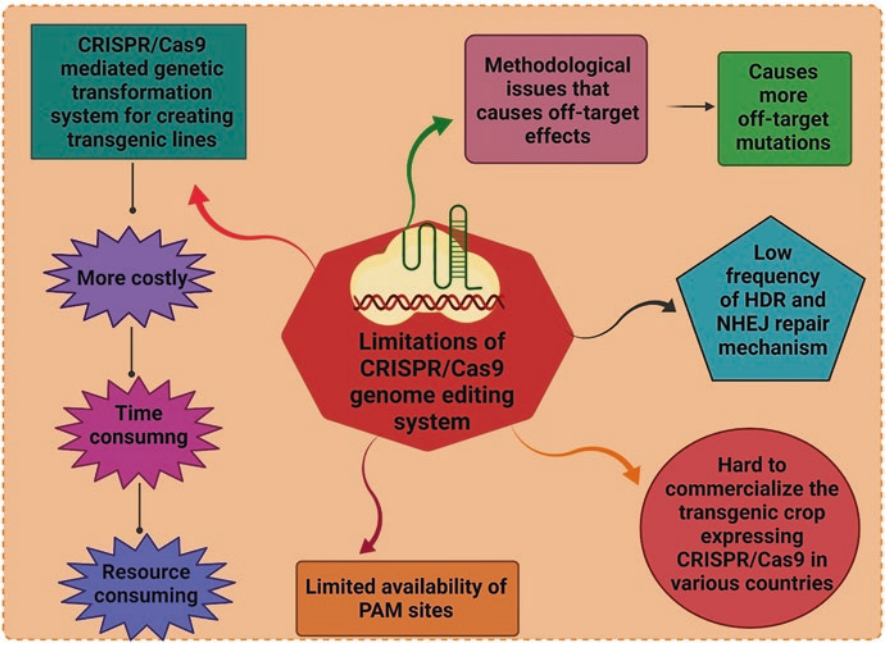

Fig. 12.1 Limitations and challenges of CRISPR/Cas9 genome editing system

Multiple CRISPR/Cas9 cargoes having different characteristic features can be integrated into various genetic payloads, such as DNA, mRNA, sgRNA, protein, and plasmids, respectively. Each of these genetic repertories encapsulated with NPs for vector delivering system showed pros and cons to fulfill the possessive challenges as summarized in Table 12.1.

The most forthcoming strategy is to integrate the Cas9 cassette into the sgRNA scaffold efficiently. Principally, this procedure triggers immediate gene editing as there are no chances of transcriptional and translational control. It also progressively offers transient gene expression with less toxicity and mitigates off-target effects. Moreover, the positively charged Cas9 protein, the negatively charged sgRNA, as well as large-sized Cas9 protein (~160 kD) can inhibit the direct and

Table 12.1 Various forms of CRISPR/Cas9 repertories and NPs for vector delivery system

CRISPR/Cas9 repertories	Nano-based delivering platforms	Advantages	Disadvantages
Cas9 protein, and sgRNA	Lipid NPs	Low toxicity, minimum off-target effects, poor stability, less inflammatory response (Chen et al. 2020) Commercially utilized cationic transfection lipids that form stables complexes with genetic payloads such as DNA, RNA, mRNA, and proteins, respectively (Chen et al. 2020) Significant scale-up and manufacturing efficiency (Evers et al. 2018)	High cost, low transfection efficiency, high encapsulation risk, rapidly degraded from the nucleases. Poor stability, endotoxin effect that leads to cellular toxicity (Li et al. 2018)
Cas9 mRNA and sgRNA	Polymeric NPs	A novel form of PACE-based cationic polymers showed higher transfection capacity (Wahane et al. 2020) Provide a broad-term application based on biocompatibility, biodegradability, temperature, light-sensitive, pH, and low immunological response, respectively (Wahane et al. 2020)	Difficulty in manufacturing large-scale efficiency, cellular toxicity, rapidly escape from the endosomal membrane during DNA repair mechanism (Wahane et al. 2020)
CRISPR/Cas9 plasmid	Gold NPs	High delivering, editing efficiency, and reduced off-target effects (Lee et al. 2017) The modified form of the CRISPR-gold RNP vector proposed a low toxic effect (Mout and Rotello 2017) Handy to maintain by the context of charge and size distribution (Chen et al. 2019)	Existence of cationic charge governs the speedy escape from the endosomal barrier (Deng et al. 2019)

efficient delivery of Cas9/sgRNA ribonucleoprotein (RNPs) cassettes (Subburaj et al. 2016; Zuris et al. 2015). On the contrary, the suitable and handy option is the interaction of Cas9/mRNA with sgRNA (Shen et al. 2014). Furthermore, the third choice is the encapsulation of plasmid-based CRISPR/Cas9 complexes (Ran et al. 2013b). Interestingly, this is an attractive delivering strategy due to its cost-effectiveness, efficiency, and simplicity. The Cas9 and sgRNA complexes, as well as the desired HDR template, can be easily integrated into the same plasmid, which reveals higher stability than mRNA and protein. However, the larger plasmid size (>7 kb), as well as Cas9 protein (~4.5 kb) potentially, enhances the limitations of delivery cargoes and the mechanism of CRISPR/Cas9 genetic payloads (Ran et al. 2015).

12.4 Recent Nano-Based Vector Delivery Modes for the CRISPR System

12.4.1 Viral and Nonviral Delivery Modes for the CRISPR System

The systematic CRISPR/Cas genome editing technique has rapidly expanded significant concerns in the area of biomedical research, especially for the treatment of genetic disorders and cancer therapeutics, respectively. The efficient delivery approaches showed a pivotal role in the applications of the CRISPR/Cas9 genome editing system. In recent years, both viral and nonviral vector approaches have been studied well for sgRNA delivery systems. Conventionally, the viral vectors consisting of lentivirus (Chakraborty et al. 2014) and adeno-associated virus (Long et al. 2016) have tremendously limited the capacity for the delivery of CRISPR/Cas components because of the generation of undesired mutations ratios, high off-target effects, integrational mutagenesis (Schumann et al. 2015), immunogenicity, limited packaging capacity, as well as carcinogenesis probability (Kay 2011). In contrast to viral vectors, the deliveries of nonviral vectors of the CRISPR/Cas system through nanoparticles (NPs) may significantly address various challenges recently. These recent challenges include safety issues (Schmidt and Grimm 2015), huge packaging of genetic payloads (Chamberlain et al. 2016), constructed protocols (Li et al. 2015), low cost, and robust scale-up production (Ramamoorth and Narvekar 2015), which may tremendously address the above-mentioned challenges. Furthermore, the nonviral vectors can be constructed to deliver the genetic cargoes to the desired cell lines. The delivery system can be widely categorized into lipids, polymers, and gold NPs, respectively, as demonstrated in Fig. 12.2. The delivery of nonviral vectors based on CRISPR systems in the form of DNA, mRNA, protein, and Cas9 plasmid together with sgRNA has governed efficient delivering strategies in CRISPR/Cas systems for the applications of genome editing.

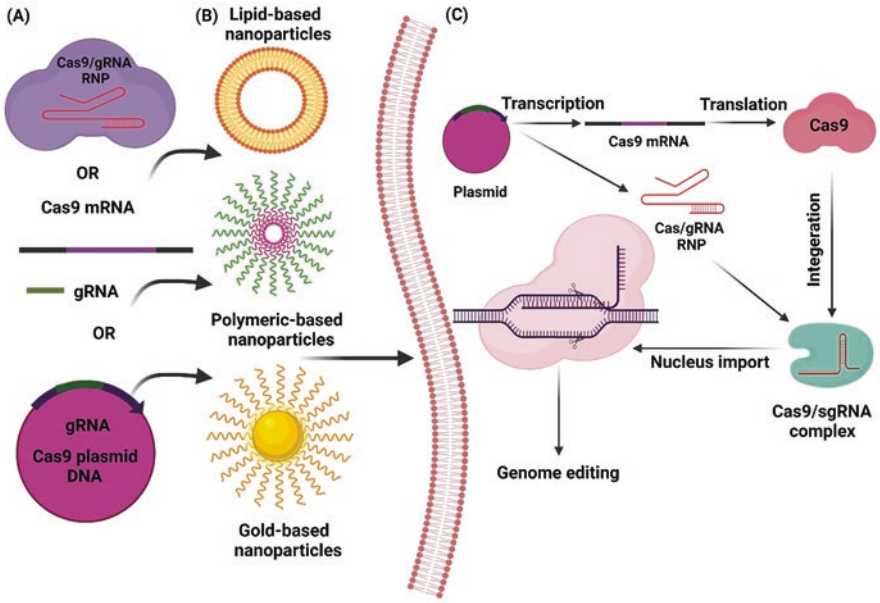

Fig. 12.2 Illustration of various NPs for the delivery of sgRNA and CRISPR/Cas system (**a**) Different strategies of CRISPR/Cas system. (**b**) Different types of NPs-based delivery systems for sgRNA and CRISPR/Cas system. (**c**) Genome editing of nanoparticles-based delivered Cas9/sgRNA plasmid DNA complex

12.4.2 Nonviral Delivery Modes of CRISPR/Cas9 System

12.4.2.1 Lipid NPs

Lipids hold the most remarkable and promising material for the potential delivery of nonviral-based genome editing systems (Mohammadinejad et al. 2020). The chemistry of various lipids is amphiphilic that possesses hydrophilic head and hydrophobic tails for the gene delivery system. More precisely, cationic lipid complexes progressively showed a helpful characteristic and have been considered adequate due to possessing charge-charge interaction with negatively charged DNA/RNP complexes. Previous studies literature proposed that these lipid-based complexes turn to decrease the chances of genetic cargoes by the degradation of nucleases (Möller et al. 2016). The ability of the most arsenal lipid-based nanoparticles (LNPs) is being the most versatile system for the delivery of genetic payloads and any other LNPs that have been an effective gene therapy for clinical researches (Felgner et al. 1987).

Appropriately, anionic charges present on DNA, mRNA, and gRNA are electrostatically attached with cationic charged lipids complexes to formulate LNPs (Cong et al. 2013). The lipid bilayer is not only helpful for the barrier of genetic payloads across the cell membrane barrier, but also hinders the genetic payloads from the

degradation of enzymatic, RNases, and immunological feedback (Liu et al. 2018b). Next, the context of commercially accessible lipids is a deep-rooted engineered delivery approach that was generated for CRISPR gRNA, mRNA, plasmids, and Cas9/sgRNA/RNPs complexes like RNAiMAX and lipofectamine that are utilized in combination with various cell lines for the treatment of gene therapeutics or gene knock-in/out in model organisms (Mout et al. 2017).

The positively charged Cas9 protein hampers the valuable characteristics of the commercially engineered cationic lipids owing to poor stability, high toxic effect, less inflammatory response, less transfection efficiency, and poor target delivery to the target sites. Though the engineered cationic lipids are used as a carrier in the CRISPR system, there are still some boundaries that hinder their further applications (Whitehead et al. 2009). Therefore, extensive alterations have been navigated for the modification of the above-mentioned pitfalls.

Engineered Cas9-sgRNA RNPs with Cas9 proteins forming complexes have been proposed to achieve 80% gene modification with the potential delivery achieved by LNPs in human cell lines (Zuris et al. 2015). Modified liposomes, such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol (Ch), and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), showed a great enhancement in cellular uptake efficiency and also modified pharmacokinetic distribution in vivo (Onuki et al. 2016). Engineered solid-LNPs can enhance the sustainability and storage stability for a wide range of applications for the lyophilization process (Leung et al. 2014). Previously reported literature ameliorated that the chemically modified lipids can progressively expand transfection efficiency and mitigate the toxic nature of cationic lipids (Kish et al. 2007) by structural modification of the ammonium groups via DOTAP (Wang et al. 2018), DC-Chol (Cardarelli et al. 2012), DOPE (Wang et al. 2018), as well as DOTMA (Lotti 2017), respectively. The first-generation liposomal-based vectors constructed through DOTMA were employed for the in vitro delivery of plasmid DNA. Moreover, DOTMA-based constructions mainly triggered cellular toxicity with the activation of immunological response owing to the presence of cationic charge. Therefore, DOTMA-based constructions cannot be applicable for in vivo studies. Additionally, LNPs are somehow distinct from liposomes due to the rapid integration with ionizable lipids and rapidly interact with genetic payloads such as nucleic acids and do not possess aqueous core components. Wheeler and his correspondence for the first time generated LNPs by utilizing stabilized plasmid particles (SPLPs) by following a detergent dialysis technique. Various synthetic lipids such as dioleoyl-ammonium chloride (DODAC) act as cationic lipids with a helper lipid 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), and polyethylene glycol (PEG) was utilized for the encapsulation of plasmid cytomegalovirus chloramphenicol transference (pCMVCAT). The results showed that produced small particles own 70 nm is size to achieved encapsulation efficacy up to 70% (Wahane et al. 2020). Recently, LNPs are fabricated with different techniques such as microfluidic hydrodynamic focusing (MHF), T-junction mixing, and staggered herringbone mixing (SHM) consisting of ethanol that acts as a controller phase for the rapid solubilization of lipids (Evers et al. 2018). Furthermore, many lipids complexes have been synthesized to drive the efficient delivery of

mRNA, gRNA, plasmids, and Cas9-sgRNA RNPs complexes for various in vitro and in vivo applications (Chen et al. 2020).

12.4.2.2 Polymeric NPs

Polymeric vector is an extensively utilized nonviral strategy for the delivery of CRISPR cargoes which aims to drive the significance for the functionalization and chemical diversity for the CRISPR delivery applications with strong biodegradability, biocompatibility, and low immunological response. Recently reported studies concerning polymeric-based NPs, viz., polyethyleneimine (Givens et al. 2018), poly (L-lysine) (Spoelstra et al. 2021), chitosan (Qiao et al. 2019), and polyamidoamine (PAMAM) (Givens et al. 2018), have captivated a tremendous consideration for efficient delivery of genome editing. Usually, polymeric vectors can be constructed from chemical subunits, viz., chitosan and PLGA, with a wide range of applications. Like lipid carriers, polymeric carriers can diffuse across the cell membrane and defend the genetics cargoes from degradation pathways and immunological responses (Zou et al. 2016). Furthermore, owing to the flexible configuration of polymeric carriers, many polymeric carriers possess specific applications for a variety of in vitro and in vivo approaches such as: discharging of intracellular microenvironments, specificity for in vivo targeting of receptors across the cell membrane, and encapsulation of various genetic payloads (Chen et al. 2015). To overcome the toxicity issue, many scientists revealed that either formulation of cationic polymeric vectors by itself or with other lipids and polymers could mitigate the chances of toxicity (Zhang et al. 2019). Therefore, to overcome the pitfalls as mentioned above, next-generation cationic-based polymeric platforms such as poly[(2-dimethylamino) ethyl methacrylate] (pDMAEMA), biodegradable poly (B-amino ester) (PBAE) polymers, and PAMAM dendrimers were synthesized. Owing to consisting of tertiary amine groups, the synthesized pDMAEMA and PBAE polymers also assist in escaping from the endosomal layer and have proven to be of higher transfection efficacy (Wahane et al. 2020).

Scientists also confirmed that polymer-based NPs are prone to deliver CRISPR-mediated cargoes with superior editing efficiency for a desired target antimicrobial in contrast to lipids (Kang et al. 2017). Polymeric carriers were encapsulated with Cas9/sgRNA networks that facilitated delivery effectively into the human genome with low toxicity and higher editing efficiency (Sun et al. 2015). Previously reported studies suggested that PEI 25 kDa (BPEI-25K) is an efficient nonviral vehicle for in vitro CRISPR genome editing systems (Ryu et al. 2018). Subsequently, the amine-terminated polyamidation (PAMAM) dendrimer cationic polymer significantly triggered the potential delivery of intracellular cytosolic Cas9 proteins and achieved high genome editing in multiple cell lines (Liu et al. 2019). Therefore, researchers enhance the rate of transfection efficiency and reduce the chances of nontarget binding sites by synthesizing new and promising synthetic poly (amino-co-ester) (PACE)-based cationic polymers for the potential delivery of genetic payloads. The newly developed mechanism demonstrates a reduced cytotoxicity effect

in contrast to other forms of cationic-based polymers. The high molecular weight PACE system navigates higher transfection capacity owing to the formation of DNA systems as well as narrowing the range of genetic materials (Wahane et al. 2020).

12.4.2.3 Gold NPs

Gold nanoparticles (AuNPs) are extensively utilized for the CRISPR-mediated RNP delivery. The gRNA, Cas9 proteins, and AuNPs are co-encapsulated into synthesized NPs (Wang et al. 2018). Distinct from lipids, viruses, and polymer vectors, AuNPs are facile concerning charge distribution and size (Chen et al. 2019). In genome editing, the synthesized AuNPs demonstrated ~90% delivery efficacy and ~30% gene-editing potentiality for CRISPR/RNP-mediated strategy in multiple cell lines (Mout et al. 2017). Previously reported literature on mice assumed that AuNPs are attributed for both HDR in vivo and CRISPR gene editing through in vivo delivery of donor DNA and CRISPR RNP-mediated approach with reduced off-target effects and high editing efficiency in many cell lines (Lee et al. 2017). Scientists confirmed that the CRISPR-Au-RNP complex vector delivery system could edit various genes in the brains of multiple mice and showed no toxic effect (Mout and Rotello 2017). The CRISPR-Au complex was synthesized by AuNPs and co-assembled with poly(*N*-(*N*-(2-aminoethyl)-2-aminoethyl) aspartamide) (PAsp(DET), glutathione, donor DNA, and Cas9 RNP complexes (PAsp(DET)) that increase in vitro and in vivo endosomal escape in DNA repair mechanism. This research revealed a benchmark study for the efficient nonviral delivery for HDR in treating genetic disorders (Deng et al. 2019).

12.5 Critical Challenges for Nonviral Delivery of CRISPR System

Compared with other efficient delivering approaches, the native CRISPR/Cas systems circumvent stringent obstacles for in vivo applications. The first challenge is the active integration of genetic payloads in vector delivery due to possessing large size and presence of various charge characteristics of donor DNA, mRNA, and Cas9 protein, respectively (Subburaj et al. 2016). Hence, cationic polymers and lipids are more likely prone to encapsulate negatively charged proteins that cannot be feasible to deliver Cas9 proteins. Like other types of protein, large size (~4.5 kb) of DNA and Cas9/mRNA also triggers hindrance for the potential encapsulation application. Although the novel and newly generated Cas9 system isolated from the specie *Staphylococcus aureus* strain (SaCas9) possesses 1 kb in size that is shorter than previously developed SpCas9, the average size of genetic payloads is still too large for genome editing applications (Kleinstiver et al. 2015). Furthermore, CRISPR tools integrated into nonviral vectors must be stable during the extracellular and

intracellular transport until they reach the desired target sites. A nonviral delivery platform consisting of CRISPR/Cas9 tools at first meets extracellular degradation components such as RNases, proteases, DNases in the blood, activation of cytokines, signaling of immune cells, and phagocytosis by macrophages (Li et al. 1999). Second, the effective signaling of the CRISPR/Cas9 tools to recognize the target-specific site and remove the extravasation out from the bloodstream to mitigate the off-target effects and enhance the efficiency of gene editing in the untargeted locus. Furthermore, cell-penetrating peptides or target-specific ligands can be chemically assembled on the surface of NPs and easily trafficked and reached to the membrane of the target sites to attain desired gene editing (Cabral et al. 2011). After the NPs are attached to the desired cell lines, the third obstacle rapidly escapes the NPs from the endosomal barrier (pH ~ 5.0) to circumvent the degradation from the endosomal effect (Harush-Frenkel et al. 2007). Subsequently, the system based on CRISPR/Cas9-plasmid-DNA complexes must ameliorate many challenges as compared to CRISPR/Cas9 RNP or mRNA complexes because the ability of plasmid DNA needs to cross nucleus membranes to transcribe the nucleus likewise, in the same manner actively, get entry to the cytoplasm to translate the Cas9 proteins and efficiently edit the desired target genome site (Chen et al. 2020).

12.6 Conclusion

CRISPR/Cas9 genome editing system is one of the most robust editing tools, which potentially simplifies previous gene manipulation strategies. Recently, this system has been extensively applied for vector delivery to achieve transgene-free gene-editing applications. Besides this, the modification of the vector delivery system is also one of the serious concerns. The delivery through viral vectors showed various issues of their own such as high cost and restriction in packaging efficiency. Therefore, the construction of nonviral vectors is an efficient and safe delivery approach with significant applications. The nonviral vectors showed hindrance to trigger the valuable advantages for the CRISPR/Cas9 genetic cargoes that include large-sized packaging stability, high retention time, and preventing safekeeping from the degradation through enzymes during transportation (Wan et al. 2019). Though the synthesized lipids, polymerics, and gold nanoparticles have demonstrated rapid progression for all delivery approaches of CRISPR-mediated payloads, for the targeted delivery of various gene-editing components, the applications of novel NPs substantially investigated more to achieve tunable desired translation for genome editing. Overall, despite these obstacles, the novel and rapid achievements in the field of gene editing and tailored nano-based vector delivery will significantly facilitate the shortcomings to accelerate the clinical translation of CRISPR-based transgene-free editing shortly in the near future.

Conflict of Interest The authors declared that they have no conflict of interest.

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Chapter 13
The Mechanisms of Genome Editing Technologies in Crop Plants

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13.1 Introduction

Global crop productivity is facing unprecedented threats due to climate change. By 2050, the world population would exceed 9.5 billion which demands an increase of 60% in the productivity of staple crops (Tilman et al. 2011). This task is difficult considering the productivity of cereal crops must be accomplished under adverse conditions. Due to the millennia of evolution by breeding, the genomes of the most important cereal crops are fixed, which limits the potential for improving many traits in agriculture (Chen et al. 2019). Traditional breeding strategies such as cross-breeding, mutational breeding, and transgenic breeding are key approaches for improving crop characteristics. However, these strategies cannot cope up with the increasing demand for food productivity (Chen et al. 2019). For example, several generations of plants and subsequently many years are required to introduce desirable alleles and increase variability via crossbreeding (Scheben et al. 2017). This is followed by a selection of progenies with the most desired agronomic traits. Mutational breeding via chemical mutants or physical irradiance introduces random mutations throughout the genome and increases the genetic variability (Pacher and Puchta 2017). Over 3000 commercial crop varieties have been produced through mutagenesis-based breeding (Oladosu et al. 2016). However, screening for a large number of mutants is a time- and labor-consuming process, particularly for polyploid crops (Hua et al. 2019). Transgenic breeding offers great potential as it permits the insertion of exogenous genes into high-yielding cultivars. However, the commercialization of transgenics raises concern among the general public and is further restricted by lengthy and expensive regulatory assessment processes (Prado et al. 2014). Hence, innovations in crop breeding technology are necessary to meet the rising food demand of the ever-growing world population (Chen et al. 2019). In the past two decades, the genomes of various plant species have been sequenced together with several vital food crops. The successive step is to utilize these genomic data to breed stress-tolerant crops/plants and develop high-yielding cultivars. The development of a customizable, precise, and highly efficient system could greatly facilitate the quest for crop trait improvement.

13.2 Genome Editing

Genome editing (GE) refers to a technique that modifies the target DNA sequence by insertion/deletion (INDELS) or base substitution (Manghwar et al. 2019). GE efforts are based on the generation of target-specific double-stranded breaks, induced by sequence-specific nucleases (SSNs) (Doudna and Charpentier 2014). The ensuing DSBs are then repaired by DNA repair mechanisms. Eukaryotes have two major mechanisms to repair these breaks which could otherwise be lethal. These include error-prone nonhomologous end joining (NHEJ) and the donor-dependent homology-directed repair pathway (HDR) (Wyman and Kanaar 2006).

The NHEJ frequently results in INDELS, and if occurs in the coding region, can effectively knock out genes. Alternatively, when DSBs create overhangs, NHEJ can facilitate the targeted incorporation of desired dsDNA template (Cristea et al. 2013). When a template is available with homology to right and left arm of the targeted sequence, the damaged DNA can be repaired by HDR. The HDR has been exploited to achieve precise gene modification or gene insertion (Bortesi and Fischer 2015). However, the NHEJ is by far the most dominant mechanism of DNA repair in many organisms including plants, and the frequency of DNA modification or integration through HDR remains much lower than random integration.

13.2.1 Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are a collection of sequence-specific restriction enzymes capable of cleaving any significant length of a DNA molecule (Zhang et al. 2010; Osakabe et al. 2010). ZFNs are synthetic nuclease designed by fusing zinc finger-specific DNA-binding domain of Cys_2-His_2 with the nonspecific cleavage domain of *Fok*I (*Flavobacterium okeanokoites* I) (Curtin et al. 2011). The binding of two ZFN monomers to their target DNA sequence causes its digestion. In the target region of DNA, these two monomers flank a 5–6 bp long nucleotide sequence. When *Fok*I dimerizes, it inserts custom DSBs in the spacer region, which is surrounded by two binding sites made up of zinc fingers (Fig. 13.1) (Puchta and Fauser 2013; Curtin et al. 2012). The cell's endogenous DNA repair mechanisms correct these breaks by triggering either the NHEJ which is error-prone or the HR. The absence of HR resorts the cell to adapt NHEJ for repair mechanism. It processes the broken ends to rejoin them directly, which results in frameshift mutation either by loss or the addition of a nucleotide in the gene (Qi et al. 2013).

Despite their intricate structure, ZFNs have already been employed in various plant species to engineer gene editing, such as *Arabidopsis*, *Nicotiana tabacum* (tobacco), etc. (Zhang et al. 2010; Townsend et al. 2009). Crops have also been subjected to ZFN-based gene modifications, like *Glycine max* (soybean), *Zea mays* (maize), and *Brassica napus* (canola) (Mushtaq et al. 2019). As ZFNs are complex to create and difficult to multiplex, they have had limited impact in enhancing crop disease resistance by altering host plant genes involved in disease development (Jaganathan et al. 2018). Chen et al. 2014, published a report according to which artificial zinc finger proteins (AZPs) were engineered to target a conserved sequence motif of begomoviruses to enhance disease resistance in crop plants using ZFN technology. Multiple resistance to begomoviruses, such as *Tomato yellow leaf curl China virus* (TYLCCNV) and *Tobacco curly shoot virus* (TbCSV), was attained by targeting a conserved region in pathogenic DNA (Chen et al. 2014).

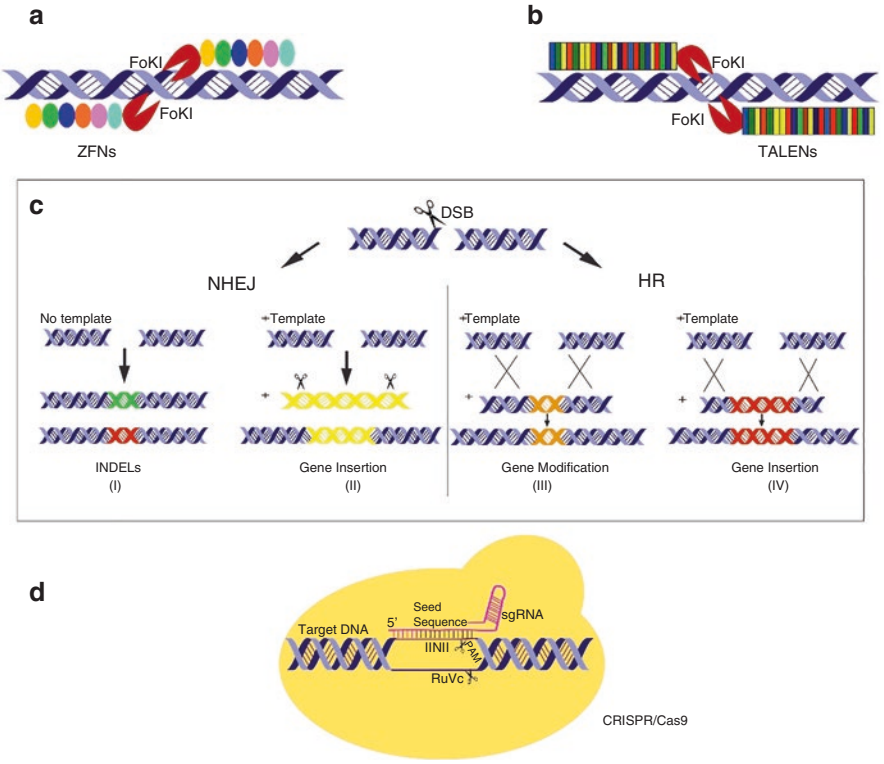

Fig. 13.1 The mechanism of GE and DNA repair system. Both ZFNs (**a**) and TALENS (**b**) use FokI endonuclease to induce DSBs at the target site. Since FokI functions as a dimer, when either ZFNs or TALENS bind to the target site and bring sequence-independent FokI endonucleases close to the target sequence, the cleavage occurs. (**d**) CRISPR-Cas9 system utilizes two components, the sgRNA that guides the CRISPR-Cas complex to the target site and Cas9 endonuclease that induces the DSBs. The prerequisite for CRISPR-Cas-mediated genome editing is the presence of a PAM sequence. After DSBs are induced by any genome editing tool, the cellular DNA repair system is activated (**c**). This could be NHEJ or HDR. NHEJ frequently induces blunt DSBs that result in INDELS (i) and might lead to loss of gene activity. However, in circumstances where NHEJ creates overhangs, targeted gene insertion might be achieved if a template is provided (ii). HDR, though a less dominant repair system, can be used to precisely modify/insert the targeted gene if a sequence homologous to the left and right flanking side is provided (iii, iv)

13.2.2 Transcription Activator-Like Effector Nucleases (TALENs)

TALENs are a group of transcription factors that are naturally produced by the *Xanthomonas oryzae* (Xanthomonas), a phytopathogen (Doyle et al. 2013). They stimulate the gene expression by binding to its promoter region. TALENs' peculiar structural properties entail a DNA-binding repeat that oversees specificity of DNA-binding by governing repeat to base-pair-binding correspondence (Deng et al.

2012). Manipulation of the quality and quantity of these repeats may allow TALENs to attach to any DNA sequence (Li et al. 2012).

Structural foundations of the TALE-DNA-binding domain consist of two factors. Repeat variable di-residue (RVD), which is a TALEN repeat sequence and promotes the stabilized contact, and the amino acid (Chandrasekaran et al. 2016) which allows specific binding capacity (Deng et al. 2012). TALEs can be used as DNA-binding modules in the construction of synthetic transcriptional and epigenetic regulators because of their DNA-binding specificities. For TALEs, many engineering platforms have been created. Furthermore, other than *Xanthomonas*, researchers have found another group of TALE-like proteins (RTLs) from the bacterium *Ralstonia solanacearum*. It shows similarity in structure but the difference in repeats, along with the high number of RVDs which recognize the specificity of repeating sequences (Mushtaq et al. 2019).

Deciphered code of RTL attachment to DNA explained that RVDs act as a major source for TALEN-based gene editing. Various RVD like binding formations have been observed, according to the standard TAL-binding RVD code (specified using single-letter amino acid code) like HD to cytosine, NS to any nucleotide, NG to thymine etc. . These additional specific bindings have given TAL-based engineering a wide range of alternatives and opportunities (Li L. et al. 2012). However, TALEN-based genome engineering is time-taking and costly due to the necessity of having a distinctive protein and two TAL monomers to concurrently bind the DNA strands, for each target (Joung and Sander 2013). Despite these difficulties, several businesses have selected TALEN-mediated genome editing because of its accuracy.

Although both ZFNs and TALENS were successfully demonstrated in plants, difficulties in vector construction, complexities of protein engineering, and frequent off-target cleavage limited their widespread adoption. A major revelation came from the study of Jinek et al. (2012), who reported a facile genome engineering technology; the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system could be used to edit the genome in a programmable way. Unlike ZFNs and TALENS which require substantial protein engineering steps to target each novel site, the CRISPR-Cas9 system is dependent on single guide RNA (sgRNA) for base-pairing activities. Thus, to target each novel site within the genome, only the 20 bp sequence at the 5′ end of sgRNA has to be changed (Jinek et al. 2012). CRISPR as an efficient and versatile system has since then revolutionized life sciences owing to its capability to generate targeted, predictable, and stable genetic changes in living organisms.

13.3 The CRISPR-Cas System: From Bacterial Origin to Genome Editing Tool

Ishino *et al*. (1987) first reported the presence of a unique sequence of DNA fragments in the genome of *E. coli*. These fragments contained repeats of DNA interspaced with short variable sequences. Later it was reported that the CRISPR arrays

are present in the genome of the number of bacteria and archaea (Mojica et al. 2000). The study by Mojica et al. (2005) revealed that the variable sequences (spacers) between DNA fragments were acquired from the pathogenic viruses and plasmids, which invaded the bacterium. This laid a clue for scientists that the CRISPR system could be responsible for adaptive immunity in prokaryotes (Pourcel et al. 2005). It was later reported that the addition or deletion of particular spacers could alter the phage-resistant phenotype of bacterium, further strengthening the notion that CRISPR might be involved in defense mechanism (Bolotin et al. 2005; Barrangou et al. 2007). Brouns et al. (2008) exhibited in a study that a complex of Cas proteins cut the CRISPR RNA precursor (pre-crRNA) within the repeats into mature crRNA molecules. Later, it was reported that base-pairing with pre-crRNA's repeat regions in the presence of RNAase III, Cas9, and *Streptococcus pyogenes*' trans-noncoding small RNA can drive the maturation process of crRNAs (Deltcheva et al. 2011). These mature crRNAs then guide a complex of Cas protein to cleave the target viral DNA through sequence-specific base-pairing. Garneau et al. (2010) reported the DNA cleavage ability of the CRISPR/Cas system in *Streptococcus thermophilus*.

Makarova et al. (2011) divided adaptive immunity process governed by CRISPR into three stages, namely, adaptation, expression, and interference. Adaptation involves the identification and introduction of a spacer sequence extracted from target DNA into the CRISPR array as a new spacer. During the expression stage, pre-crRNA is transcribed from the CRISPR locus and then pre-crRNA is matured into crRNA with the help of tracrRNA, RNAase III, and Cas9. Finally, in the interference stage, Cas proteins are guided by matured crRNAs to degrade the target sequences complementary to crRNA (Jackson et al. 2017). The proto-spacer adjacent-motif (PAM), a short stretch of di- or tri- nucleotides, is a prerequisite to complete the cleavage of target DNA. PAMs are present immediately after proto-spacers and their position involves them in the important task of recognition of specific proto-spacers (Mojica et al. 2009). Within this overall theme, two classes of the CRISPR-Cas system have been proposed, class 1 and class 2 (Makarova et al. 2011). These class systems are classified on the basis of the number of multifunctional proteins involved in the pre-crRNA processing and interference. Both single and multiple multifunctional proteins are used in the CRISPR system and are classified as class I and II, respectively (Koonin et al. 2017). These classes are further divided into different systems; Class I groups down to type I, III, and IV, while class II groups as type II, V, and VI of systems (Ishino et al. 2018). Based on operon organization and Cas proteins, the CRISPR system can be further divided into different subtypes (Zhang et al. 2019). The class 2 type II CRISPR system from *S.pyogenes* has become an attractive choice due to its relatively simpler design (Zhang et al. 2019).

13.3.1 *The Mechanistic Features of* Sp*Cas9*

Jinek et al. (2012) reported that the class 2 type II CRISPR system from *S.pyogenes* could be utilized in a programmable manner to edit the genomes of living organisms. After repurposing the CRISPR-Cas9 as a GE tool, the system was effectively reduced from three to two components; a single guide RNA (sgRNA) (which was formed by fusing the crRNA-tracrRNA) and SpCas9. The 20-base pair (bp) sequence present at the 5′ end of sgRNA binds to the target DNA sequence via Watson-Crick base-pairing and guides the CRISPR ribonucleoprotein (RNP) complex to target the DNA site adjacent to PAM. Recognition of PAM triggers DNA melting, enabling the crRNA strand invasion which forms the so-called R-loop (Doudna and Charpentier 2014). The PAM sequence for SpCas9 is 'NGG'. However, it has been reported that SpCas9 also recognizes and cleaves the target sites with noncanonical PAMs in mammalian cells (Leenay et al. 2016) and rice (Meng et al. 2018). The Cas9 cleaves the DNA strand that is complementary to the 20-nucleotide sgRNA through the HNH domain, while the RuvC-like domain cleaves the DNA strand opposite to the complementary strand (Jinek et al. 2012; Gasiunas et al. 2012). This triggers the cellular DNA repair system which repairs the damaged strand through NHEJ or HDR (Fig. 13.1) (Doudna and Charpentier 2014). Mutating either catalytic domain (D10A of RuvC-like domain and H840A of HNH, correspondingly) produces a DNA nickase (nCas9), while mutating both catalytic domains produces a catalytic dead Cas9 (dCas9) null mutant. The dCas9 can still bind to and deliver functional cargo to the programmed site (Brezgin et al. 2019).

13.3.2 *Variants and Orthologs SpCas9 Systems*

PAM selection limits the targeting scope of SpCas9. Therefore, Cas9 has been engineered with a thoughtful design and evolved to obtain variants having broader PAM requirements. For example, Cas9 has been engineered to recognize a broader range of PAM sequences such as SpCas9 VQR (NGA), SpCas9 EQR (NGAG), SpCas9 VRER (NGCG) (Kleinstiver et al. 2015), SpCas9 D1135E (NGCG) (Cong et al. 2013), SpCas9 QQR1 (NAAG) (Anders et al. 2016), SpCas9-NG (NG) (Nishimasu et al. 2018), iSpy-macCas9 (NAA) (Jakimo et al. 2018), SpCas9-HF1 (NGG) (Kleinstiver et al. 2015), SpCas9 (K855A) (NGG), eSpCas9 (1.0) (NGG), and eSpCas9 (1.1) (NGG) (Slaymaker et al. 2016) to expand targetable sites and enhance Cas9 specificity. Additionally, several Cas9 orthologs from other bacterial species have been surveyed to broaden the PAM target range. For example, *Staphylococcus aureus* Cas9 (NNGRRT) (Nishimasu et al. 2015), *Francisella novicida* Cas9 (NGG) (Hirano et al. 2016), *Neisseria meningitides* Cas9 (NNNNGATT) (Lee et al. 2016), *Streptococcus thermophilus* 1 Cas9 (NNAGAAW) (Kleinstiver et al. 2015), *Streptococcus thermophilus* 3 Cas9 (NGGNG) (Müller et al. 2016), and Campylobacter jejuni Cas9 (NNNNACAC and NNNRYAC) (Kim et al. 2017) have also been developed as a GE tool.

13.3.3 The CRISPR-Cpf1 System: An Advanced System for Genome Editing

Various CRISPR nucleases have been engineered as GE tools in recent years, to overcome the limitations of CRISPR-Cas9 systems. A new group of CRISPR nucleases from Prevotella and Francisella, named Cpf1(Cas12a), has been discovered by Zhang and his research group at MIT and Board Institute (Zetsche et al. 2015). Cpf1 is an RNA-guided endonuclease with a length of 1200–1500 amino acids and is classified as a class 2 type V CRISPR nuclease (Manghwar et al. 2019). The Cpf1 nucleases from *Francisella novicida* (FnCpf1), *Acidaminococcus* spp (AsCpf1), and *Lachnospiraceae bacterium* (LbCpf1) have been used as a GE tool in the range of plant species (Zhang et al. 2019) (and references therein). Recently, Cpf1 has emerged as an alternative for Cas9 due to its unique features. First, the Cpf1 requires only crRNA of ~42 nucleotides (as compared to Cas9 which requires ~100 nucleotide sgRNA) making Cpf1 crRNA easy to synthesize, multiplex, and engineer than SpCas9 (Yamano et al. 2016; Manghwar et al. 2019). Second, Cpf1 targets T-rich PAM sequences unlike G-rich PAM sequences recognized by SpCas9 which broadens its target range (Safari et al. 2019). For example, *Prevotella* and *Francisella* 1 (Cpf1) rely on T-rich PAM sequence (5′-TTTN-3′ or 5′-TTTV-3′) at the 5 ′ end of proto-spacer sequence. Third, Cpf1 produces staggered dsDNA breaks (creating 5 or 8 nucleotides 5′ overhang initiating at 18 nucleotides 3′ of the PAM) facilitating HDR-mediated gene insertion/modification and NHEJ-mediated gene insertion (Moon et al. 2018; Safari et al. 2019). Finally, Cpf1 possesses RNAase activity in addition to DNA nuclease activity, enabling Cpf1 to process a CRISPR system for multilevel GE (Yamano et al. 2016). Cpf1 relies on a RuvC-like endonuclease domain along with a Nuc domain for DNA cleavage (Yamano et al. 2016). Cpf1 lacks the HNH domain and may include a single active site in the RuvC domain (Jeon et al. 2018). However, mutating the catalytic domain of Cpf1 abolishes the DNA cleavage ability of both strands (Yamano et al. 2016). This means that there is no Cpf1 nickase currently available (Zhang et al. 2019).

13.3.4 Variants and Orthologs of Cpf1 Systems

To expand the utility of the Cpf1 nuclease as a GE tool, several orthologs from diverse bacterial species have been surveyed (Yamano et al. 2016). These orthologues include *Moraxella bovoculi* Cas12a (MbCas12a), Mb2Cas12a, Mb3Cas12a, *Pseudobutyrivibrio ruminis* Cas12a (PrCas12a), *Pseudobutyrivibrio xylanivorans* Cas12a (PxCas12a), *Lachnospira pectinoschiza* Cas12a (LpCas12a), *Btyrivibrio* sp. Cas12a (BsCas12a), *Lachnospiraceae bacterium* Cas12a (LbCas12a), and *Thiomicrospira* sp. Cas12a (TsCas12a). These orthologues show editing activity at loosened or shorter PAM sites and could be utilized for plant GE (Yamano et al. 2016; Tóth et al. 2018; Zetsche et al. 2017; Teng et al. 2019).

13.3.5 Base Editing

Single base changes are responsible for the variation of many important traits among the elite crop plants (Henikoff and Comai 2003). However, either HDR or NHEJ is not efficient in triggering the single nucleotide substitutions in plants (Zhang et al. 2018). Therefore, innovations to introduce precise point mutations in crop plants are needed. Base editing (BE) introduces nucleotide changes in the genome with great precision and reproducible manner (Manghwar et al. 2019). Importantly, BE is independent of DSBs, donor template, and either NHEJ or HDR (Zhang et al. 2019). BE as an alternative to NHEJ and HDR has emerged as a powerful tool that has been utilized to improve many traits among plants (reviewed by Bharat et al. 2020). Two types of BEs are routinely used in molecular biology experiments. These include cytosine base editors (CBEs) and adenine base editors (ABEs) (Bharat et al. 2020). For CBE, the dCas9 is fused with an enzyme, cytidine deaminase, that is involved in the conversion of cytosine to uracil (C-U) by deaminating cytosine under the window of nontargeted protein (Lu and Zhu 2017; Li et al. 2018b). The uracil is recognized as thymine in the next round of DNA replication, thereby resulting in C-G to T-A single base substitution (Komor et al. 2016). A uracil glycosylase inhibitor (UGI) is usually added to the expression construct to block the base excision repair pathway that might convert uracil back to cytosine (Zhong *et al*., 2019). Similarly, in ABE, either nCas9 or dCas9 are fused to adenosine deaminase which catalyzes the deamination of adenosine causing adenosine to inosine substitution (A-I). During DNA replication, inosine is recognized as guanine (G). As a result, ABE allows A-T to G-C base swaps (Nishida et al. 2016).

13.4 Applications of Genome Editing Techniques

Various abiotic stresses are the major constraints to agricultural production such as drought, salinity, heavy metals toxicity, heat, flooding, etc. Biotic factors like bacterial, fungal, and insects attacks also affect the global crop yield. Molecular approaches such as TALENs, ZFNs, CRISPR-Cas, etc. have been used for genome editing in plants to make them stress-resilient (Voytas 2013; Mahfouz et al. 2014). However, the CRISPR-Cas technology provides an unprecedented opportunity to target the genomic DNA sequence with great efficiency and precision at a low cost. Since CRISPR-Cas was repurposed as a genome editing tool, it has been extensively utilized by researchers to target desired genes and increase plant stress resilience and agricultural productivity among others (Manghwar et al. 2019). In the following section, we will emphasize and discuss the various applications of CRISPR-Cas systems to enhance plant abiotic stress resistance. (Table 13.1.)

Table 13.1 Application of CRISPR-Cas system for targeted mutagenesis of various traits of crop plants

Application	Plant	Target Gene	Function of gene	Insertion Method of Cas9 System	Genome editing/ repair mechanism	Mutation type	Promoter for Cas9	Promoter for sgRNA	Mutant screening method	Resulting phenotype/trait	References
Yield	Soya bean (*Glycine max* (L.) Merr.)	GmFT2a	Integrator in photoperiod flowering pathway, encoding florigen	Agrobacterium-mediated transformation	NHEJ	Site-directed INDELS mutations	2 × 35S	AtU6	Sequencing Analysis	Lagged flowering time during both short and long-day conditions	Cai et al. (2018)
Yield	Rice (*Oryza sativa* L.)	TMS5	Encodes the endonuclease RNase Z in AnS-1 and determines the TGMS traits	″	NHEJ	Single nucleotide insertions, deletions, and substitutions	ZmUbi	OsU6a, OsU3	Sanger Sequencing	TGMS achieved along with greater yield with stronger off-springs	Zhou et al. (2016)
Quality	Potato (*Solanum tuberosum*)	GBSS (Granule-Bound Starch Synthase)	Encodes granule-bound starch synthase, responsible for the synthesis of amylose	PEG-mediated transfection	NHEJ	Allelic mutations, INDELS mutations	CaMV 35S	AtU6, StU6	High-Resolution Fragment Analysis (HRFA)	Fall in amylose content and rise in the amylopectin	Andersson et al. (2017)
Quality	Tomato (*Solanum lycopersicum* L.)	SIGAD2, SIGAD3	Encode glutamate decarboxylase	Agrobacterium-mediated transformation	NHEJ	INDELS	ZmUbi	AtU6-26	Sanger Sequencing and GABase assay method	Increase of γ-Aminobutyric acid (GABA)	Nonaka et al. (2017)

Table 13.1 (continued)

Application	Plant	Target Gene	Function of gene	Insertion Method of Cas9 System	Genome editing/ repair mechanism	Mutation type	Promoter for Cas9	Promoter for sgRNA	Mutant screening method	Resulting phenotype/trait	References
Abiotic Stress	Arabidopsis (*Arabidopsis thaliana*)	CBF1 and CBF1/ CBF2	Responsible for cold stress response	″	NHEJ	INDELS (Insertions)	2 × 35S	AtU6-26	mRNA sequencing analysis (Illumina HiSeq) and qRT-PCR	Increased tolerance against cold stress	Jia et al. (2016)
Abiotic Stress	Maize (*Zea mays* L.)	ARGOS8	Negative regulator of ethylene responses	Particle bombardment	HR	Insertion mutations	ZmUbi	ZmU6	qPCR assay, mRNA sequencing analysis (Illumina HiSeq), Immunoblot analysis	Increased grain yield under drought stress	Shi et al. (2017)
Biotic Stress	Citrus (*Citrus paradise* Macf.)	CsLOB1	Promotes citrus canker susceptibility and formation of an erumpent pustule	Agrobacterium-mediated epicotyl transformation method	NHEJ	Insertion, deletions, and substitutions	35S	CaMV 35S	Next-Generation sequencing analysis (Illumina HiSeq)	Mutants resulted in canker-resisted citrus	Jia et al. (2017) and Peng et al. (2017)
Biotic Stress	Cucumber (*Cucumis sativus* L.)	eIF4E	Translation initiation factor; plays a major role in the *Potyviridae* life cycle	Agrobacterium-mediated transformation	NHEJ	Small deletions and single nucleotide polymorphisms (SNPs)	35S	AtU6	PCR and RNA sequencing analysis	Broad virus resistance against infection of *Papaya ringspot mosaic virus-W* (PRSV-W)	Chandrasekaran et al. (2016)

13.4.1 Improvement of Yield Traits

Precise genome editing with the help of engineered Cas9 protein has been successfully implemented in some model plants. Soya bean being an important legume crop requires more yield to fulfill the needs of living beings. Its photoperiodic sensitivity limits its geographical range of cultivation, as it is a short-day plant (Wang et al. 2016; Xu et al. 2013). To increase its yield by introducing it in regional and domestic areas, molecular biologists studied the function of its genes that control the flowering time and expression in soybean. GmFT2a is a target gene that encodes florigen. With the help of the CRISPR-Cas9 system, GmFT2a's targeted mutagenesis has been induced in the soybean, which knocked out the endogenous gene. The resulting generation showed delayed flowering time and passed on the mutation to the next-generation stably (Cai et al. 2018). Rice is a staple food of the majority of the world population. Thermo-sensitive genetic male sterility (TGMS) line has been used majorly by researchers for hybrid breeding, to improve its yield (Zhou et al. 2012). The engineered mutations via the CRISPR/Cas9 system were induced in the TGMS5 to gain 11 new hybrid lines in 1 year. It demonstrates the potential of CRISPR/Cas9 in producing high-yield crops (Zhou et al. 2016).

13.4.2 Enhanced Produce Quality

Improvement of qualitative traits with the help of CRISPR/Cas9 is another breakthrough application of the CRISPR-mediated GE system. Andersson et al. 2017, disrupted the GBSS gene in potato (Solanum tuberosum), which encodes a granule-bound starch synthase. Observations were made that the GBSS enzyme only lost its activity in the lines experiencing mutations in all four of its alleles. These varieties showed a rise in the amylopectin ratio and a fall in amylose content. Recently, genes in tomato (*Solanum lycopersicum*), i.e., SIGAD2, SIGAD3, which encode glutamate decarboxylase, have been modified with the CRISPR/Cas9 technology. These modifications allowed more accumulation of *γ-Aminobutyric acid* (GABA), which is a health-beneficial compound. (Nonaka et al. 2017; Li et al. 2018a, b)

13.4.3 Gene Editing for Plant Abiotic Stress Resilience

Abiotic stresses are responsible for the reduction of crop yield worldwide (Haider et al. 2021). To study the stress response of plants against abiotic stresses, CRISPR/Cas9 has been also employed for the development of mutants. In Arabidopsis thaliana, the role of C-repeat binding factors (CBFs) against cold stress has been determined by CRISPR/Cas9 system. Damage of CBF1 and CBF2 in cbf3, a mutant produced by T-DNA insertion, results in the production of mutants of CBF1,2,3.

The resulted mutants showed great tolerance towards cold stress (Jia et al. 2016). Designing stress-tolerant crops with the help of specific gene editing through CRISPR/Cas9 is another important milestone. Shi et al. (2017) produced a drought-tolerant maize crop, by using a similar approach. The expression of *the ARGOS8* gene, which encodes for negative regulation of ethylene responses, was enhanced by switching native promoter with *GOS2* promoter with the help of Homologous Recombination (HR). Improved grain quality had been exhibited by these variants under drought stress conditions. These examples prove the potential of CRISPR/Cas9 as a sophisticated tool to engineer plants to gain resilience against various biotic and abiotic stresses.

13.4.4 Engineering Plant Biotic Stress Resistance

Enhanced disease resistance against pathogens in plants by CRISPR/Cas9 technique has been observed. *Citrus paradisi*, when infected with bacterial pathogen *Xanthomonas citri* (citrus canker causing agent), experiences the expression of the *CsLOB1* gene, which increases the plant's susceptibility towards canker disease. CRISPR/Cas9 disrupted the expression of *CsLOB1* and produced canker-resistant varieties (Hu et al. 2014; Jia et al. 2017). The eukaryotic translation initiation factors (eIF) in cucumber (*Cucumis sativus*) hold recessive-resistant properties against viral attacks. CRISPR/Cas9 system mutated the eIF4E and eIF(iso)4E to produce virus-resistant cucumber varieties (Chandrasekaran et al. 2016). Novel findings explain that CRISPR/Cas9 can also disrupt the viral genome and can produce *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV)-resistant species as well (Abdullah et al. 2017).

13.5 Future Perspectives

Various GE techniques offer a ground for developing strategies to combat the destructive effects of environmental stresses on plants. CRISPR/Cas9 system-based genome editing has revolutionized the field of genetic engineering in past few years. Its diagnostic applications and precise genome alteration make it a potential technique for gene editing. Technological advancements are being used to create knock-outs along with knock-ins, as well as to activate and inhibit gene expression. Many improvements have already been made in a short amount of time because of their usefulness in a practical system. DNA-free genome editing, Cas9's various variants, precision in base editing and multigene targeting methods, and enhancing frequency of HDR are some of the practical uses of CRISPR/Cas's system. Bioinformatic tools relative to this technique have also improved and have resulted in the rising use of the CRISPR/Cas system. However, educating the public masses about transgene crops is crucial. It is necessary for sustainable crop growth to bring laboratory

research into the field. In this scenario, establishing rules to ensure the distinction between genetically engineered plants with or without foreign DNA is crucial to exempt the latter from any limitations. Genetically engineered plants with mutations close to natural variations have benefits over plants with novel mutations, allowing them to have public access in countries with strict rules against transgenic crops. In this way, precise genome editing techniques appear to be promising. Because of the lack of well-defined regulatory regulations, the distance between the lab and the field is always growing. Because gene editing can result in natural genome alterations, and the outcome of genome editing done by 'traditional' means and naturally happening genome editing cannot be differentiated in some circumstances, specific control of the technology proves unjustified. Another viewpoint is that, in any event, the legislator should focus on the precise qualities of the end product rather than the manufacturing technique to detect the potential danger. Aside from these difficulties, considerable efforts are needed to enhance transformation procedures, which are not yet optimal for numerous crop species and have been identified as a constraint for efficiently exploring recent advancements. CRISPR/Cas-based GE is still restricted only to advanced molecular biology labs that are primarily interested in fundamental biological questions. Crop breeders, on the other hand, still have a long way to go in terms of using technological advancements in crop development initiatives. On a pay-per-use basis, both public and independent facilities offering facilities for construct generation, genetic transformation, and analysis of GE plants will be a turning point in moving advances from the experimental labs to the crop field by supplying breeders with highly efficient GE tools. The material presented here will aid in understanding current technical advancements, knowledge gaps, and technological adaption issues that are necessary for the effective use of genome editing technologies for crop development.

13.6 Conclusion

In the past 5 years, GE techniques have flourished and revolutionized the disciplines of plant crop improvement and functional genomics. Due to its simplicity, ease of usage, flexibility, and acceptable off-target consequences, CRISPR/Cas9 has become the most promising method. The genome editing technique has a lot of potential for creating crop varieties with increased disease resistance, higher yield and quality, and unique agronomic characteristics that will benefit farmers and consumers. The technique has been utilized for targeted mutagenesis in a variety of models and food crops with great success. CRISPR–Cpf1 has recently been utilized as a novel and alternative technique for engineering plant genome editing, overcoming some of the constraints of the CRISPR/Cas9 system, such as the necessity for a PAM site, thereby expanding the scope of genome editing in crops. Nonetheless, a rising number of case studies show that CRISPR/Cas9 is an effective and widely utilized technique that can accelerate both scientific and applied agricultural research.

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Chapter 14
Genomic Region Analysis and Genome Editing for Grain Quality Improvement in Cereals

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14.1 Introduction

Cereals are the principal stable crops for global population and thus are the primary source of micronutrients. However, cereals have a low level of micronutrients, and majority of them are lost during processing. Around two billion people mainly in Africa, South Asia, and Latin America suffer from malnutrition or nutrient deficiency which is responsible for the death of around 24,000 people daily all over the globe (Majumder et al. 2019). To achieve nutrient security, fortification of food, especially cereals, is necessary owing to the primary source of energy and nutrition in human diet. The availability of biofortified staple food crops would be a sustainable approach to provide a nutritious diet to people having limited access to variable dietary resources.

The principal aim of the breeders is to improve the yield potential of the cereals. Improving grain nutritional quality of cereals to attain nutritional security seems to be a newer area for breeders. Conventional and molecular approaches have enabled the breeder to develop cereals with higher micronutrients. QTLs for various micronutrients, identified in rice, maize, wheat, barley, and pearl millet, have been introgressed to develop improved versions of these cereals (Mahender et al. 2016; Govindaraj et al. 2019; Gaikwad et al. 2020; Saini et al. 2020; Prasanna et al. 2020; Swamy et al. 2021). HarvestPlus, a program under CGIAR (Consultative Group on International Agricultural Research), is working on development of biofortified crops in low- and middle-income countries. Under this program, cereals like maize, wheat, rice, and pearl millet along with other important crops have been biofortified (Bouis and Saltzman 2017). The biofortified crops developed under this program are being planted by over 8.5 million farmers across Africa, Asia, and Latin America (https://www.harvestplus.org). With the combined efforts of HarvestPlus and the institutions working with it, over 300 varieties of biofortified crops have been released for commercial cultivation in 40 developing nations using conventional and molecular breeding. A successful biofortified cereal in addition to enhanced nutritional value must be high yielding and acceptable to stakeholders.

In some cases, germplasm lacks the genetic variability in the desired trait for biofortification. Therefore, genetic modification (GM) of crops is a possible way to overcome the problem. GM technology involves introduction of the desired trait from the novel source (Yadav et al. 2018). With the low acceptability of GM crops, the ongoing agricultural practices are struggling to meet the nutrient security of the increasing global population (Rani et al. 2021; Jangra et al. 2021a, b). GE has emerged as a potential tool to overcome the challenges associated with the current crop improvement technologies. GE-assisted breeding has been successfully employed to modify the trait of interest in various crop plants without introduction of any foreign gene. In a very short time, GE had a great impact on crop improvement with its high precision and efficient genetic modification (Chen et al. 2019). The era of GE began with the introduction of double-stranded breaks (DSBs) at specific sites using endonucleases like zinc figure nucleases (Kim et al. 1996), transcription activator-like effector nucleases (TALENs) (Christian et al. 2010), and

clustered regularly interspaced short palindromic repeats (CRISPR)-associated 9 (Cas9) (Jinek et al. 2012). Since its development, GE has been employed to improve various crop plants (Park et al. 2019). Recently, there is an exponential increase in the utilization of GE for crop improvement. In this chapter, we will be focusing on the advancements made in the field of grain quality improvement in cereals using genomic regions/QTLs and GE.

14.2 Genomic Regions/QTLs for Cereal Grain Quality Improvement

The primary requirement for breeding biofortified crops is to look into the available germplasm to identify genomic regions with higher micronutrient content. The wild relatives of the crop plants are a rich source of various micronutrients and therefore can be utilized in the breeding program. The quality traits are polygenic and are controlled by several genes. Therefore, improvement of these traits through conventional breeding is quite difficult (Jangra et al. 2017; Jangra et al. 2018). The advancement in molecular marker technology has gained the interest of plant breeders to improve crop plants against complex traits. Molecular markers can be employed to identify the exact location of the genomic region/ QTL, determining the trait for nutritional quality. Once identified, these QTLs can be introgressed to elite cultivars/ varieties. QTL mapping based on biparental mapping populations is found to be less significant. However, genome wide-association mapping studies (GWAS) utilized the diverse germplasm which offers a large number of variations. The extent of linkage disequilibrium determines the marker-trait association. The identified markers linked to the target trait can be utilized in the breeding program to improve the crop plants. The overview of crop improvement based on genomic regions/ QTLs is denoted in Fig. 14.1.

14.3 QTLs for pro-Vitamin A

Deficiency of vitamin A is responsible for irreversible loss of vision and is one of the serious health issues in developing nations. It has been reported that over 30% of children and 19 million pregnant women are facing the problem of vitamin A deficiency in developing nations (Duo et al. 2021). Two alleles, viz. *β-carotene hydroxylase 1* (*crtRB1*) and *lycopene epsilon cyclase* (*lcyE*), have been identified which favors pro-vitamin A biosynthesis in maize (Muthusamy et al. 2015). This has gained the interest of researchers to identify QTLs related to pro-vitamin A content in maize. Maize is an important cereal and consumed across the globe. Several QTLs for vitamin A content have been identified in maize (Table 14.1) (Babu et al. 2013; Azmach et al. 2013).

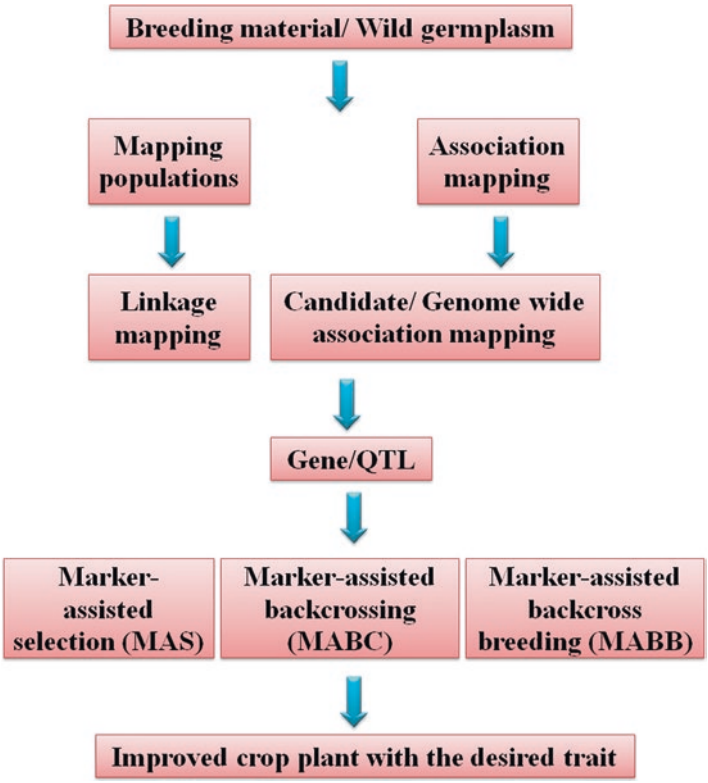

Fig. 14.1 Schematic representation of QTL-mediated improvement of crop plants

14.4 QTLs for Iron (Fe) and Zinc (Zn) Content

Cereals consumed as food are deficient in various micronutrients including Fe and Zn (White and Broadley 2005). As per WHO reports, 30% of the global population are suffering from anemia. To overcome this problem, there is a need to develop cereals with higher Fe and Zn content. In the past few years, several mapping populations have been developed to identify QTLs associated with Fe and Zn content (Table 14.1). In wheat, QTLs for Fe and Zn content have been reported by several authors (Hao et al. 2014; Srinivasa et al. 2014; Tiwari et al. 2016; Crespo-Herrera et al. 2016; Velu et al. 2017, 2018; Gorafi et al. 2018; Liu et al. 2019). Similarly, in barley, QTLs for Fe and Zn were identified on chromosomes 6 and 2 (Mamo et al. 2014; Sadeghzadeh et al. 2015). In rice, several QTLs for Fe and Zn have been mapped on almost all chromosomes. Recently, two QTLs for Fe ($qFe_{9.1}$ and $qFe_{12.1}$) and four QTLs for Zn ($qZn_{1.1}$, $qZn_{5.1}$, $qZn_{9.1}$, and $qZn_{12.1}$) content were identified in double haploid rice (Calayugan et al. 2020). Novel QTLs, qFe3.3 and qFe7.3 for Fe content and qZn2.2, qZn8.3, and qZn12.3 for Zn content, were identified using association mapping (Pradhan

Table 14.1 List of genomic regions/ QTLs for grain quality in cereals

Trait	Genomic region/QTLs	Chromosome	Crop	References
Vitamin A	*LcyE5′TE, LcyE3′Indel, rtRB1–3′T,*	10	Maize	Babu et al. (2013)
	PSY1, lcyE, crtRB1	10		Azmach et al. (2013)
Fe and Zn content	*QGzncpk.cimmyt_2BL*	2BL	Wheat	
	QZn.bhu_2B, QZn.bhu_6A, and QFe.bhu_3B	2B, 6A, 3B		Srinivasa et al. (2014)
	QZn.bhu_2B, QFe.bhu_2B	2B		Tiwari et al. (2016)
	QZn.Across_4BS, QFe. Across7DS	4BS, 7DS		Crespo-Herrera et al. (2016)
	QGzn.ada_1B, QGzn.sar_1B, QGFe.ada_2B	1B, 2B		Velu et al. (2017)
	QGFe.iari-2A, QGFe.iari-5A, QGFe.iari-7A and QGFe.iari-7B, QGZn.iari-2A, QGZn.iari-4A, QGZn.iari-5A, QGZn.iari-7A and QGZn.iari-7B	2A, 4A, 5A, 7A, 7B		Krishnappa et al. (2017)
	QZn 2A, QZn7B	2A, 7B		Velu et al. (2018)
	qFes1, qfes2, qZns1, qZns2	4D, 2D, 5D, 1D		Gorafi et al. (2018)
	QGZn.co-5A, QGZn.co-7A, QGFe.co-3B.1, QGFe.co-5A.2	5A, 7A, 3B		Liu et al. (2019)
	QZn.caas-1DS, QZn.caas-2AS, QZn.caas-3BS, QZn.caas-4DS, QZn.caas-6AS, QZn.caas-6DL, QZn.caas-7BL, QFe.caas-3BL, QFe.caas-4DS, QFe.caas-6AS, QFe.caas-7BL	1DS, 2AS, 3BS, 4DS, 6AS, 6DL, 7BL, 3BL		Wang et al. (2021a, b)
	Zn-qtl-6H_ SCRI_RS_10655	6HL	Barley	Mamo et al. (2014)
	QTL.Zn	2HS, 2HL		Sadeghzadeh et al. (2015)
	Fe-2H-84.74, Fe-2H-139.62, Fe-4H-67.9, Fe-1H-54.5, Fe-1H-57.85, Fe-1H-90.04, Fe-2H-84.74, Fe-4H-53.87, E-4H-54.95, Fe-6H-102.03, Fe-7H-17.62, Zn-2H-87.34, Zn-1H-21.97, Zn-2H-148.16, Zn-2H-40.12, Zn-2H-86.84	2H, 4H, 1H, 4H, 6H, 7H		Gyawali et al. (2017)
	qFe_2, qZn_5	2, 5	Rice	Zhang et al. (2014)
	$qFe_{1.2}$ *(gene OsYSL1),* $qFe_{5.1}$ *(gene OsZIP6),* $qFe_{7.2}$ *(gene OsZIP8)*	1, 5, 7		Agarwal et al. (2014)
	qFe_6, qZn_8	6, 8		Xu et al. (2015)

(continued)

Table 14.1 (continued)

Trait	Genomic region/QTLs	Chromosome	Crop	References
	$qFe_{10.1}$, $qZn_{6.2}$, $qZn_{7.1}$	10, 6, 7		Descalsota et al. (2018)
	$qFe_{1.2}$, $qFe_{11.1}$, $qZn_{2.1}$, $qZn_{3.2}$, $qFe_{3.2}$, $qFe_{4.1}$, $qZn_{5.1}$, $qZn_{12.1}$	1, 11, 2, 3, 4, 5, 12		Swamy et al. (2018)
	$qFe_{1.1}$ $qFe_{1.2}$, $qZn_{1.1}$, $qFe_{6.1}$, $qZn_{6.1}$, $qFe_{6.2}$, $qZn_{6.2}$	1, 6		Dixit et al. (2019)
	QTL.Fe.$_9$, QTL.Zn.$_4$	9, 4		Islam et al. (2020)
	$qFe_{3.3}$, $qFe_{7.3}$, $qZn_{2.2}$, $qZn_{8.3}$, $qZn_{12.3}$	3, 8, 12		Pradhan et al. (2020)
	$qFe_{9.1}$, $qFe_{12.1}$, $qZn_{1.1}$, $qZn_{5.1}$, $qZn_{9.1}$, $qZn_{12.1}$	9, 12, 1, 5		Calayugan et al. (2020)
	qFe_7, qZn_7	7		Jeong et al. (2020)
	qZPR.$_{1.1}$, qZPR.$_{11.1}$	1, 11		Suman et al. (2021)
	Fe, Zn	LG 3, 5, 7	Pearl millet	Kumar et al. (2016)
	Fe, Zn	LG 3, 5, 7		Anuradha et al. (2017)
	qFe1/54 and qZn1/54	LG 1, 7		Kumar et al. (2018)
	PglZIP, PglNRAMP, PglFER (gene families)	LG 7		Mahendrakar et al. (2020)
	Fe, Zn	Pgl01, Pgl02, Pgl04, Pgl05, Pgl06 (2), Pgl07		Pujar et al. (2020)
	QFe2.1, QFe2.1, QFe3.1, QFe5.1, QFe7.1, QZn2.1, QZn3.1, QZn3.2, QZn6.1,	LG 1, 2, 3, 5, 6, 7		Singhal et al. (2021)
Amino acids and GPC	*QPro.mgb-4B, QPro.mgb-5A, QPro.mgb-6A.1, QPro.mgb.6A.2, QPro.mgb.6B, QPro.mgb-7A, QPro.mgb-7B*	4B, 5A, 6A, 6B, 7A, 7B	Wheat	Blanco et al. (2002)
	GPC	2AS, 6AS and 7BL		Blanco et al. (2006)
	QGpc.sdau-3B, QGpc.sdau-5A, QGpc.sdau-6A	3B, 5A, 6A		Sun et al. (2008)
	QGpc\tgw.WL-1D, QGpc.WL-2A, QGpc\yld.WL-2B, QGpc.WL-3B, QGpc.WL-4A, QGpc\yld.WL-4A, QGpc.WL-5B, QGpc.WL-5D, QGpc.WL-6B, QGpc.WL-7A	1D, 2A, 2B, 3B, 4A, 5B, 5D, 6B, 7A		Wang et al. (2012)
	QGPC.bhu_1A	1A		Tiwari et al. (2016)
	GPC, Protein yield	1A, 1B, 2A, 2D, 3A-1, 3A-2, 3B, 3D-2, 4A, 4B, 5A, 5B, 5D, 6A, 7A-1, 7B		Mahjourimajd et al. (2016)

(continued)

Table 14.1 (continued)

Trait	Genomic region/QTLs	Chromosome	Crop	References
	QGpc.sdau-1D, QGpc.sdau-2D, QGpc.sdau-4A, QGpc.sdau-1A, QGpc.sdau-2A.2, QGpc.sdau-4B, QGpc.sdau-5D, QGpc.sdau-7A, QGpc.sdau-2A.1,	1A, 1D, 2A, 2D, 4A, 4B, 5D, 7A		Sun et al. (2016)
	QGpc.2B-yume	2B		Terasawa et al. (2016)
	QGpc.uhw-4B, QGpc.uhw-5A.1, QGpc.uhw-6B, QGpc.uhw-7B.2	4B, 5A, 6B, 7B		Fatiukha et al. (2020)
	QGpc-1B-2, QGpc-4B-1.4	1B, 4B		Guo et al. (2020)
	QGPC.cib-4A	4A		Li et al. (2020)
	PC	6, 7	Rice	Tan et al. (2001)
	pro1	1		Aluko et al. (2004)
	qCP-12	12		Zhang et al. (2008)
	qPC11.2	11		Qin et al. (2009)
	qPC-3, qPC-4, qPC-5, qPC-6, qPC-10	3, 4, 5, 6, 10		Yu et al. (2009)
	qAa1, qAa7, qAa9	1, 7, 9		Zhong et al. (2011)
	qPro-2, qPro-10	2, 10		Yun et al. (2014)
	qPro-2	2		Lee et al. (2014)
	qPC1	1		Peng et al. (2014)
	qPC-1	1		Yang et al. (2015)
	qPC6.2	6		Kinoshita et al. (2017)
	qGPC1.1, qSGPC2.1, qSGPC7.1	1, 2, 7		Chattopadhyay et al. (2019)
	qPC3.1, qPC5.1, qPC9.1	3, 5, 9		Pradhan et al. (2019)
	QTL.pro.1	1		Islam et al. (2020)
	qAAC6.1, qAAC7.1, qPC1.2	1, 6, 7		Jang et al. (2020)
	qGPC1–1	1		Wu et al. (2020)
	PC	6H	Barley	See et al. (2002)
	GPC	2, 6		Mickelson et al. (2003)
	GPC	2H, 4H, 5H 7H		Emebiri et al. (2003)
	Qgpc1H, Qgpc2H, Qgpc4H, Qgpc5Ha, Qgpc5Hb, Qgpc5Hc, Qgpc7H	1H, 2H, 4H, 5H, 7H		Emebiri et al. (2005)
	Qcp2a, Qcp3a, Qcp5a, Qcp6a, Qcp7a, Qcp7b	2H, 3H, 5H, 6H, 7H		Abdel-Haleem et al. (2010)
	QGpc.ZgSc-2H.1, QGpc. ZgSc-2H.2, QGpc.ZgSc-2H.3, QGpc.ZgSc-4H.1, QGpc. ZgSc-4H.2, QGpc.ZgSc-4H.3, QGpc.ZgSc-5H.3, QGpc. ZgSc-5H.1, QGpc.ZgSc-5H.2, QGpc.ZiSc-7H.1, QGpc. ZiSc-7H.2, QGpc.ZiSc-7H.3	2H, 4H, 5H, 7H		Fan et al. (2017)

et al. 2020). Pearl millet is a nutrient-rich cereal; QTLs/ candidate genes for Fe and Zn content were identified on linkage group (LG) 3 and 5 (Kumar et al. 2016), LG 3, 5, and 7 (Anuradha et al. 2017), LG 1 and 7 (Kumar et al. 2018), LG 7 (Mahendrakar et al. 2020), Pgl01, Pgl02, Pgl04, Pgl05, Pgl06 (2), and Pgl07 (Pujar et al. 2020), and LG 2 and 3 (Singhal et al. 2021).

14.5 QTLs for Amino Acids and Grain Protein Content

Grain protein content (GPC) of cereals is an important component of human diet and is the determining factor of nutritional quality of plant-based diet. In the past few years, several researchers have mapped QTLs for GPC in both durum and hexaploid wheat (Table 14.1) (Blanco et al. 2002, 2006; Mahjourimajd et al. 2016; Sun et al. 2016; Fatiukha et al. 2020). In rice, QTLs for GPC have been mapped on all the chromosomes. However, most of them are located on chromosomes no 1, 2, 6, 7, 10, and 11 (Tan et al. 2001; Aluko et al. 2004; Zhang et al. 2008; Qin et al. 2009; Yu et al. 2009; Zhong et al. 2011; Lee et al. 2014; Yun et al. 2014; Yang et al. 2015; Chattopadhyay et al. 2019; Pradhan et al. 2019; Jang et al. 2020; Wu et al. 2020). Similarly, in the case of barley, QTLs for GPC have been reported on all seven chromosomes by several researchers. It has been found that seven consensus QTLs are present on chromosomes 2H, 4H, 5H, 6H, and 7H (See et al. 2002; Mickelson et al. 2003; Emebiri et al. 2003, 2005; Abdel-Haleem et al. 2010; Fan et al. 2017).

14.6 Commercial Varieties with Improved Nutritional Value

Once the QTLs get identified, marker-assisted breeding (MAB) can be employed to develop improved versions of commercial hybrids/ cultivars. This started with the development of improved version of commercial pearl millet hybrid against downy mildew (Hash et al. 2006). Thereafter, marker-assisted selection (MAS) has been widely adopted to improve cereals like rice (Improved Pusa Basmati 1, Improved Samba Mahsuri, Swarna sub1, IR64 sub1, PRR78/IRBB60, Pusa 6A, and Improved Pusa RH10), wheat, and maize. Various commercial hybrids/ cultivars of rice have been improved against bacterial blight, submergence tolerance, rice blast, drought tolerance, and several other traits (Kottapalli et al. 2010; Reddy et al. 2009; Singh et al. 2011; Das et al. 2017). Similarly, wheat varieties (Patwin, Expresso, Lassik, Farnum, Westmore, and AGS2026) have been improved against various biotic and abiotic stresses using MAS (Gupta et al. 2010). Several attempts have been made to improve the nutritional value of cereals using MAS. One of the success stories in cereals is development of quality protein maize (QPM) (Vivek et al. 2008). Marker-assisted backcrossing (MABC) was utilized to introgress the Opaque 2 allele on chromosome 2 and led to the

release of 'Vivek-QPM-9' in 2008 (Gupta et al. 2013). The improved hybrid possessed 41% more tryptophan and 30% more lysine than the original hybrid (Vivek Hybrid 9). Later on in the year 2017, improved versions of three popular hybrids, viz. Pusa HM-4, Pusa HM-8, and Pusa HM-9, were released for commercial cultivation in India (Hossain et al. 2018). MABC has been utilized to develop biofortified rice with higher Zn content. With the efforts of BRRI (Bangladesh Rice Research Institute), five high Zn content varieties, viz., BRRI dhan62, BRRI dhan64, BRRI dhan72, BRRI dhan74, and BRRI dhan84, have been released for commercial cultivation. Similarly, high-Zn varieties, DRR Dhan45 and Chhattisgarh Zinc Rice-1 in India, NSIC Rc 460 in Indonesia, and Nutri Zn in Philippines, have been commercialized (Calayugan et al. 2021). In 2017, two Fe and Zn biofortified varieties, WB 02 and HPBW 01, have also been released for commercial cultivation by the Indian Institute of Wheat and Barley Research (Yadava et al. 2020). In the same year, two Fe and Zn biofortified hybrids of pearl millet, viz. AHB 1200 and HHB 299, were released with the combined efforts of CCS Haryana Agricultural University, Hisar and ICRISAT, Hyderabad (Yadava et al. 2020).

Over the past decade, the MAB has been widely adopted to develop improved cereals. However, the global population is rising at an alarming rate and is projected to touch the mark of 9 billion by 2050 than its present level 7.53 billion (Priti et al. 2018). To keep pace with the rising population, researchers need to develop healthier and high-yielding cereals. The present, modern, and conventional agricultural practices are not capable of meeting such high demands. There is a need for newer technology that is fast with higher precision rate. GE is one such technology which can be utilized to its full potential to meet the global demands.

14.7 Genome Editing (GE)

The existing modern breeding technologies need to be supplemented with advanced techniques like GE to develop nutrient-rich cereals. The emergence of GE technology has revolutionized the cereal improvement program with its superior precision rate and speed (Matres et al. 2021). GE allows site-specific modification of DNA. This offers significant advantage over GM technology that mostly relied on random integration of introgressed DNA. Since the development of GE in 2010, it has been consolidated into three major platforms, viz., ZNFs, TALENs, and CRISPR-Cas. These use designed nucleases to introduce targeted DSBs. These breaks are repaired in an error-prone pathway and mutations are introduced within the target gene. These genetic changes have been utilized for crop improvement (Christou et al. 2021). The utilization of the three above-mentioned GE platforms for grain quality improvement in cereals is discussed below.

14.8 Zinc Finger Nucleases (ZFNs)

In recent years, new advances empowering targeted alteration of plant genomes have been made possible and new genome editing technologies have been developed including ZFNs (Bibikova et al. 2002). ZFNs are a class of designed restriction enzymes that proves to be a powerful means for GE. ZFNs are chimeric proteins that consist of an N-terminal DNA-binding domain and a nonspecific DNA cleavage domain. Each designed zinc finger (ZF) identifies a specific 3-bp DNA sequence by binding and a FokI nuclease. FokI space dimerization is profoundly a basic requirement for ZFN enzymatic action (Kim et al. 1996). FokI belongs to the type IIS class of restriction endonucleases. The capacity of these designed nucleases to make targeted double-stranded breaks at assigned locations throughout the genome has enabled the editing of genes with great precision. A couple of Zinc finger arrays (30 amino acid residues) exhibit ties to respective sequences and get aligned in contrary style with one another resulting in a particular configuration (Petolino 2015). The binding sites of two Zinc finger arrays are 18–24 bp in length separated by 5–8 bp. This space is important for creating DSB in the target sequence. The DSBs introduced by these endonucleases are followed by error-prone nonhomologous end joining (NHEJ) repair results in deletions or insertions, base substitutions, or incorporation of exogenous donor sequences at the ZFN cleavage site. If this damaged repair is in the coding region of a gene, it can interrupt the reading frame resulting in an inactive gene creating new genetic variations either by addition or deletion. The mechanism of action of ZFNs is represented in Fig. 14.2. ZFNs were first reported in Arabidopsis (Wright et al. 2005; Llyod et al. 2005) and tobacco (Cai et al. 2008; Maeder et al. 2008; Townsend et al. 2009), and later on, effectively used in different plants such as soybean (Curtin et al. 2011) and rice (Cantos et al. 2014; Jung et al. 2018).

One of the most significant examples of GE for grain quality improvement by ZFNs targeted the *IPK1* gene which encodes inositol-1,3,4,5,6-pentakiphosphate 2–kinase, which is an important enzyme in phytate biosynthesis in maize seeds (Shukla et al. 2009), resulting in herbicide tolerance and altered inositol phosphate profiles in the developing seeds. Starch biosynthesis pathway was targeted in rice with engineered ZFNs which can effectively cleave and induce mutations at SSIVa locus. This modification resulted in dwarfism and reduced starch content (Jung et al. 2018).

The field of GE has been revolutionized by Zinc finger nucleases, showing the ability to utilize genomic sites of concern and opened the entryways for both essential and applied research. As for productivity, ZFNs give an advantage over different tools, extraordinary explicitness, and insignificant nontarget impacts and present challenges are focused on further improving plan and conveyance just as expanding their utilization in different crops. The advances in ZFN-based GE give huge freedom to focus on any DNA sequence in the genome. However, there are a few intrinsic detriments that have confined their wider scope of utilization, for example, costly and tedious cycles for enhanced assembly of the ZF domains and off-target effects (Eid and Mahfouz 2016; Mushtaq et al. 2019).

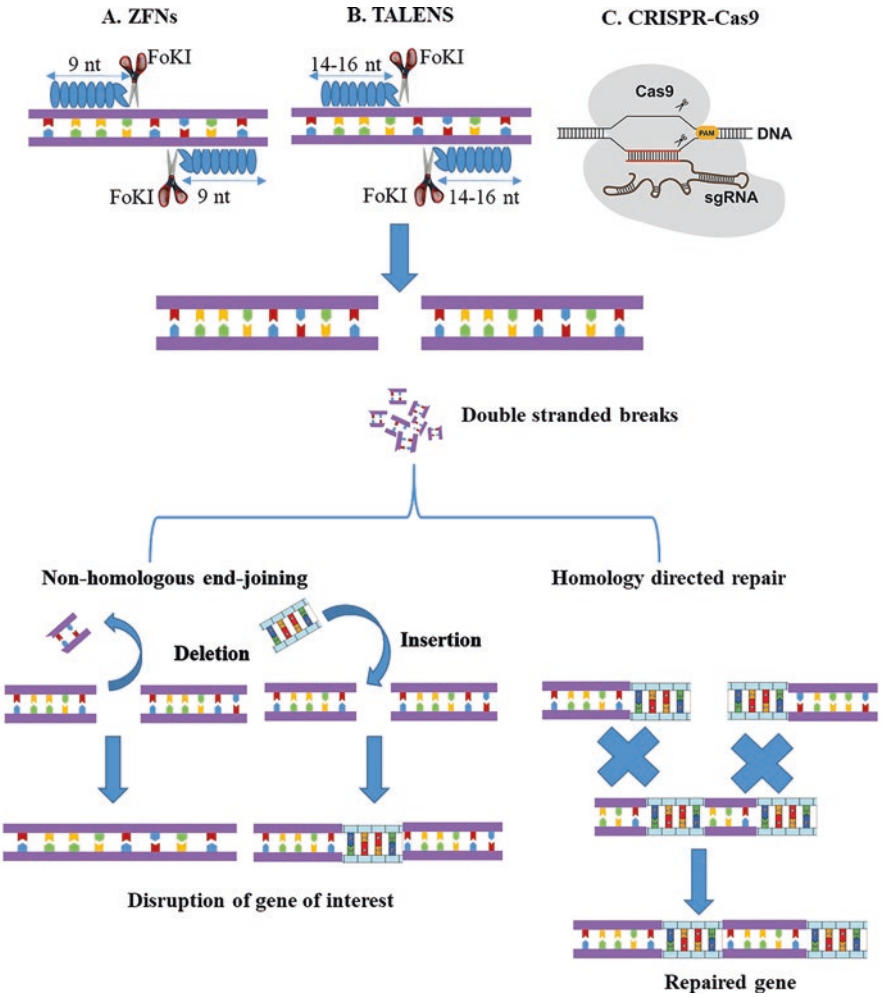

Fig. 14.2 An overview of nonhomologous end joining (NHEJ) and homology-directed repair (HDR)-mediated genome editing

14.9 TALENs

Precise genetic editing or modification has remained the fundamental goal for researchers engaged in the fields of molecular breeding and agricultural biotechnology. Engineered nucleases are dynamic tools for precise in vivo genetic modifications in genomes (Bogdanove and Voytas 2011). TALENs promptly turn up as an alternative to ZFNs, by introducing targeted DSBs for GE, and provide a novel and excellent route for crop improvement. TALENs and ZFNs have similar features of containing FokI nuclease domain combined with customizable DNA-binding

domain (Fig. 14.2). This DNA-binding domain is comprised of highly conserved repeats which are derived from transcription activator-like effector proteins (TALEs), which are fused with nonspecific cleavage domain of FokI endonucleases. TALEs are Type III effector proteins that were discovered in plant pathogenic bacteria *Xanthomonas* spp. from rice and cotton (Boch and Bonas 2010). Similar to ZFNs, TALENs facilitate specific GE via induction of DSB in a specific target sequence of genome, followed by nonhomologous end joining (NHEJ) (Moehle et al. 2007) or homology-directed repair (HDR) (Rémy et al. 2010). The emergence of engineered nucleases has revolutionized the field of genetic engineering and TALENs use these engineered nucleases to introduce specific additions and deletions in targeted genes. TALEN assembly comprises central DNA-binding domain, highly conserved acidic transcription activation domain at C terminal, secretion, and translocation at N terminal and nuclear localization signal (NLS). In central DNA-binding domain, 33–35 amino acids tandem repeats are present which recognizes one nucleotide in the target sequence (Li et al. 2011). The specificity of TALEN mainly depends on amino acids of polymorphic nature located at 12 and 13 positions, also called repeat variable di-residue (RVD). Many RVDs have been reported; some major ones are NI (Asn ile), HD (His Asp), NN (Asn Asn), and NG (Asn Gly), which recognize nucleotides adenine (A), cytosine (C), guanine (G), thymine (T), respectively. These tandem repeats are followed by a sequence of 20 amino acids, which are called half repeats.

TALENs have been used widely for crop improvement for engineering disease resistance and increasing shelf life (Haun et al. 2014; Wang et al. 2014; Wendt et al. 2013; Lor et al. 2014). TALENs have also been used for grain quality improvement in cereal crops being utmost priority for sustainable agriculture (Table 14.2). In a study by Ma et al. (2015), the storage tolerance of rice was increased by knocking

Table 14.2 Overview of various crop plants improved for grain quality using GE

Sr. No.	Crop	Gene	Trait	References
ZFNs				
1.	Maize	*IPK1*	Phytic acid	Shukla et al. (2009)
2.	Rice	*SSIVa* locus	Starch content	Jung et al. (2018)
TALENS				
1.	Rice	*LOX3*	Storage	Ma et al. (2015)
2.	Rice	*OsBADH2*	Fragrance	Shan et al. (2015)
CRISPR-Cas				
1.	Maize	*ZmIPK1*	Phytic acid	Sun et al. (2007)
2.	Maize	*ZmIPK1*	Phytic acid	Liang et al. (2014)
3.	Maize	*ZmMADS47*	Protein content	Qi et al. (2016)
4.	Maize	*Wx1*	Starch content	Waltz (2016)

(continued)

Table 14.2 (continued)

Sr. No.	Crop	Gene	Trait	References
5.	Maize	*Wx1*	Starch content	Qi et al. (2020)
6.	Maize	*Wx1*	Starch content	Gao et al. (2020)
7.	Maize	*BADH2*	Fragrance	Wang et al. (2021a, b)
8.	Rice	*OsFAD2–1*	RBO	Abe et al. (2018)
9.	Rice	*OsFAD2*	RBO	Bahariah et al. (2021)
10.	Rice	*BADH2*	Fragrance	Shao et al. (2017)
11.	Rice	*BADH2*	Fragrance	Fuhua et al. (2018)
12.	Rice	*BADH2*	Fragrance	Usman et al. (2020)
13.	Rice	*BADH2*	Fragrance	Ashokkumar et al. (2020)
14.	Rice	*Osor*	Vitamin A	Endo et al. (2019)
15.	Rice	*Osor*	Vitamin A	Dong et al. (2020)
16.	Rice	*Wx*	Amylose content	Zhang et al. (2018a, b)
17.	Rice	*Wx*	Amylose content	Yunyan et al. (2019)
18.	Rice	*Wx*	Amylose content	Li et al. (2020a)
19.	Rice	*SBEI* and *SBEII*	Amylose content	Sun et al. (2017)
20.	Rice	*SBEII*	Amylose content	Baysal et al. (2020)
21.	Rice	*Rc*	Proanthocyanidins and anthocyanins	Zhu et al. (2019)
22.	Wheat	α-Gliadin	Gluten content	Sánchez-León et al. (2018)
23.	Wheat	α-And γ-gliadin	Gluten content	Jouanin et al. (2019)
24.	Wheat	α-Gliadin	Gluten content	Sánchez-León et al. (2018)
25.	Wheat	*Pinb, waxy,* and *DA1*	Grain hardness, starch quality, and kernel size	Zhang et al. (2018a, b)
26.	Wheat	α-Amylase/trypsin inhibitors	Protein quality	Camerlengo et al. (2020)
27.	Wheat	*TaSBEIIa*	Amylose content	Li et al. (2020b)
28.	Wheat	*Pinb, waxy, ppo* and *psy*	Grain hardness, starch quality, and dough color	Zhang et al. (2021)
29.	Barley	*HvPAPhy_a*	Phytic acid	Holme et al. (2017)
30.	Barley	*HvITPK1*	Phytic acid	Vlčko and Ohnoutková (2020)
31.	Barley	D-hordein	Glutenins	Yang et al. (2020)
32.	Sorghum	*k1C*	Kafirins	Li et al. (2018)

out *LOX3* gene. Fragrant rice is favored over non-fragrant rice all over the world. In fragrant rice, more than a hundred volatile compounds are found, one of which is 2-acetyl-1-pyrroline (2AP). Its quantity is higher in fragrant rice than non-fragrant rice. Shan et al. (2015) employed TALENs to disrupt *OsBADH2* gene which inhibited the synthesis of 2AP. The study showed that the 2AP content of non-fragrant rice increased from 0 to 0.35–0.75 mg/kg, which was almost similar to the positive control variety with mutation in *BADH2* gene.

14.10 CRISPR/ Cas9

The combination of research and technology in developing improved genotypes of crop plants has led to the basis of modern agriculture. Though nowadays traditional breeding is much faster than 50 years back, it is not able to cope with the increasing food demand with the global climate change making the situation more challenging. As far as crop improvement is dependent on conventional breeding, i.e., exploitation of natural germplasm variation and introgressing the desired trait in target crops, time and resources will always limit the crop improvement (Jangra et al. 2017, 2019a, b). These limitations can be overcome by exploiting CRISPR technologies and crop improvement can be accelerated at a rate that was not possible earlier. CRISPR in agriculture can be considered as a novel breeding method that is much faster, predictable, and cheaper and the results are identical to conventional breeding (Gao et al. 2018). Since its first report in 2012, this technology has revolutionized research in life sciences.

Initially, CRISPR was identified as repeats (Ishino et al. 1987) and was later characterized in 1990s. The term CRISPR was coined by Jansen et al. (2002). It is a bacterial and archaeal defense mechanism that provides immunity against bacteriophages through RNA-programmed DNA cleavage. The Cas9 system is composed of a cascade of different proteins generally classified into two classes according to the structure, 6 type and 19 subtype (Shmakov et al. 2017). The composition of effector nucleases determines the level of variation among the classes. The class I effectors comprise of a complex of several proteins with different functions; however, the class II effector comprises a multi-domain single protein (Makarova et al. 2015). The most used CRISPR is type II-A CRISPR/Cas9 system, and due to its high efficiency in producing double-stranded breaks, the spCas9 is derived from *Streptococcus pyogenesis*. Some restrictions like proto-spacer adjacent motif (PAM) were showed by spCas9. PAM is NGG (N-Any nucleotide, G-Guanine), making its application difficult in sequences having higher AT and also prone to produce off-target effects. These limitations have been overcome by high fidelity variants of Cas9 with mutations that prevent nonspecific interactions between DNA and nuclease domains leading to reduced off-target effects (Kleinstiver et al. 2016). Expression, interference, and adaptation are the three stages of which the CRISPR/Cas9 system is comprised. In the expression of CRISPR array, sequences that are homologous to the target sequence (proto-spacers) get transcribed into pre-CRISPR

RNA (pre-crRNA). Homologous bonds with trans activating crRNA (tracrRNA) are formed by these pre-crRNAs. After the formation of pre-crRNA/tracrRNA complex, the Cas9 protein gets attached and RNase III cut the long pre-cdRNAs into separate crRNA/tracrRNA complexes (gRNA). Interference starts with guiding of Cas9 complex by crRNA/tracrRNA to target sequence and gRNA binds to target sequence after PAM. As the PAM sequence is not present in CRISPR array, PAM allows the discrimination between self/nonself. The target sequence, unwound as Cas9, is equipped with helicase and nuclease activity and the cuts are produced by the RuvC and HNN domain of Cas9, leading to DSB in the target sequence. NHEJ or HDR repairs the DSB and the repaired sequence is transcribed and adapted into the genome as described in Fig. 14.2 (Jackson et al. 2017). CSISPR/Cas9 offers several advantages over other GE technologies being simple and cost-effective. Also, the Cas9 system is readily available making it a highly valuable GE tool. The multi-target approach of this technique could be utilized to target multiple genes simultaneously. The off-target effects of the Cas9 could be reduced by mutating RuvC domain.

CRISPR allows researchers to perform gene knockout, DNA-free gene editing, gene insertions or knock-ins, and transient gene silencing. In the case of gene knockout/ gene silencing, CRISPR utilizes a single guide RNA (sgRNA) to initiate double-stranded breaks at the target site using Cas9 endonuclease. The repairing of these breaks through NHEJ mechanism (error-prone) results in genomic deletions or insertions, leading to permanent silencing of target gene. The DNA vector-free CRISPR-based GE requires only RNA or protein components. This DNA-free editing eliminates the possibility of unwanted genetic alterations that may be caused due to integrating plasmid DNA or random vector integration at the cut site. The double-stranded breaks induced through CRISPR can be utilized for creating gene 'knock-ins' through homology-mediated repair. The gene codon can be altered by the precise addition of donor template. In earlier studies, it has been found that precise insertions can be made by CRISPR-Cas9 system with the help of single-stranded DNA (Cong et al. 2013). Transient gene silencing or suppression of transcription can be done with modified Cas9 which is unable to cut DNA. The promoter region is targeted by the modified Cas9 and the transcriptional and gene expression activity is hampered. This can also be utilized for transient activation or upregulation of target genes (Ishino et al. 2018).

An efficient CRISPR-Cas delivery system is the only prerequisite for the application of this technology in crop improvement. The transformation of major crop plants is generally confined to a few genotypes per species, which are not probably the elite cultivars. Therefore, the development of user-friendly and robust CRISPR delivery system for commercial varieties is essential. A recent study showed that the transformation efficiency of cereals can be improved with the help of morphogenic regulators (Lowe et al. 2016). The transformation of recalcitrant elite cultivars of wheat and corn can be improved by haploid-inducer-mediated GE approach (Kelliher et al. 2019; Wang et al. 2019). The time-taking process and labor-intensive process of plant regeneration through tissue culture after transformation could be avoided by

administrating the CRISPR components to shoot apical meristem, pollen, or flowering tissues (Hickey et al. 2019). This tissue culture-free technique has been recently employed to develop gene-edited plants by de novo meristem induction (Maher et al. 2020). Though in eukaryotes NHEJ is the major mechanism involved in DNA repair in crops, many desired traits can be attained by specific substitution or insertion of DNA segments. A novel method of base substitution is provided by base editing; however, it is currently restricted to A-G or C-T substitutions (Komor et al. 2016; Gaudelli et al. 2017). In a recent development, a ground-breaking genome editor called 'prime editing' has been developed that delivers genetic information directly into specific DNA sites providing a powerful tool to expand the scope and capabilities of GE (Anzalone et al. 2019). In prime editing, the Cas9 is engineered to function as nickase combined with reverse transcriptase, and the sgRNA is replaced with pegRNA (prime editing guide RNA), which comprises both a sgRNA for target site identification and RNA template for determining the DNA sequences that are to be integrated at the target site (Anzalone et al. 2019). Similar applications may follow in crop plants in the not-so-distant future.

Since the recognition of CRISPR as cutting-edge technology, it has gained the interest of researchers and industries to improve major crop plants. In a very short time since its first application in plants, it has been utilized to improve various traits like tolerance against biotic and abiotic stresses, quality, nutritional value (Table 14.2), and yield (Arora and Narula 2017; Jaganathan et al. 2018; Gao et al. 2018; Wang et al. 2019; Ahmad et al. 2020; Zaidi et al. 2020; Zhang et al. 2020; Zhang et al. 2021). At present, CRISPR/Cas has become a major biotechnology tool to introgress the desired trait.

14.11 CRISPR/Cas for Grain Quality Improvement in Cereals

CRISPR/Cas has been widely employed to edit quality-related genes in the case of cereals to enhance their nutritional value. Maize, rice, wheat, barley, oats, rye, and sorghum are the principal cereal crops across the globe. In the following section, an overview of CRISPR-Cas9-based improvement of grain nutrient quality in cereals is summarized in Table 14.2.

14.12 Maize (*Zea Mays*)

Maize is the most cultivated cereal all across the globe. It is widely used for human consumption, animal feed, and biofuel production. Phytic acid (inositol 1, 2, 3, 4, 5, 6-hexakisphosphate) is an anti-nutritional compound present in maize and reduces

the assimilation of minerals after human and animal consumption (Feil 2001). To overcome this problem, the gene (Inositol phosphatase kinase 1, *ZmIPK1*) encoding for phytic acid production was knocked out using CRISPR-Cas9. The study showed that over 50% of the IPK1 open reading frames were interrupted in leaves and seeds (Sun et al. 2007). Similarly, ZmIPK1 was targeted, knocked out by mutagenesis induced by specifically designed gsRNA (Liang et al. 2014).

Zeins are the most abundant storage protein in maize and are deficient in two essential amino acids (lysine and tryptophan), contributing to poor nutrient quality. Opaque 2 (O2), a basic leucine zipper protein-based transcription factor, regulates the synthesis of zeins in maize (Schmidt et al. 1990). It has been reported that this problem can be overcome by altering the zeins production, which allows production of other proteins with higher lysine and tryptophan content. CRISPR technology has been employed to target *ZmMADS47* gene encoding a MADS-box protein, an interacting partner of O2. A reduction of 12.5% in zeins content was recorded in MADS/CAS9–21 lines (Qi et al. 2016).

Starch (amylose and amylopectin) content is another important target trait. Generally, the starch content in normal maize is around 70%, of which 75% is amylopectin and 25% is amylose. Waxy maize that has high amylopectin content was first discovered over 100 years ago in China. The high starch content makes waxy corn an ideal product for implementing CRISPR-Cas to overcome the challenges associated with conventional breeding. CRISPR-Cas technology has been utilized to alter the waxy gene (*Wx1*), which encodes granule-bound starch synthase responsible for amylose production in endosperm. The alteration can lead to accumulation of high amylopectin content in endosperm, making it suitable for various industries like processed foods, adhesives, and high-gloss paper. For commercialization, CRISPR-Cas-based editing of *WX1* has been applied to elite commercial cultivars and crossbreed as CRISPR-waxy hybrids (Waltz 2016). Waxy maize was presented among first CRISPR-edited crops that can be cultivated and sold free from USDA regulations and will be available in the market by DuPont Pioneer in the coming years after field trials (Waltz 2018). In another study, the *Wx1* gene has been targeted to develop waxy maize employing CRISPR-Cas9 (Qi et al. 2020). Field trials of CRISPR-edited waxy corn hybrids of 12 elite inbred lines indicated that these were agronomically superior to the introgressed line and produced on an average 5.5 bushels per acre higher yield (Gao et al. 2020).

Recently, aromatic maize has been developed using CRISPR-Cas9. Two maize betaine aldehyde dehydrogenase 2 (BADH2) homologs, ZmBADH2a and ZmBADH2b, were identified in maize. Zmbadh2a and zmbadh2b single mutants and the zmbadh2a-zmbadh2b double mutant were developed by CRISPR/Cas in four inbred lines. The double mutants accumulated 0.028 and 0.723 mg/kg 2-acetyl-1-pyrroline (2AP) in fresh and dried kernels, respectively (Wang et al. 2021a, b).

14.13 Rice (*Oryza Sativa*)

Rice is consumed by more than 3.5 billion people across the globe and accounts for around 20% of the global dietary supply (Fiaz et al. 2019; Ku and Ha 2020). It has been estimated that there should be a 40% increase in production by 2030 to meet the global rice demand (Khush 2005). In addition, with the improvement in people's living standards, the demand for rice with higher nutritional quality is expected to increase. Therefore, newer technologies like CRISPR can be utilized to improve the nutritional value of rice at a much faster rate than conventional breeding. Rice bran oil (RBO) is widely used in Asian countries and the major components of RBO are monounsaturated oleic acid (37–52%) followed by 13–22% of linoleic acid (polyunsaturated) and 27–40% palmitic acid (saturated) (Taira et al. 1988). The presence of oleic acid in RBO makes it good for health which has increased the demands of rice bran oil. Further, increasing the oleic acid in rice can add to the health benefits. In plants, the conversion of oleic acid to linoleic acid is catalyzed by an enzyme fatty acid desaturase 2 (FAD2). In rice, three functional *FAD2* genes are present, of which *OsFAD2–1* is highly expressed in seeds. Thus, by altering this gene, the oleic acid content in RBO can be elevated. The disruption of *OsFAD2–1* gene by targeted mutagenesis utilizing CRISPR led to two-fold increase in oleic acid content, thereby increasing the quality of RBO (Abe et al. 2018). In another study, CRISPR-Cas9 was utilized to knock out *FAD2* gene using two sgRNA. The knocked-out plants showed higher oleic acid content as compared to wild-type plants (Bahariah et al. 2021).

The demand for fragrant rice, particularly Indian Basmati, is gaining worldwide due to the presence of a characteristic fragrance in its grains. This fragrance is due to the presence of defective *OsBAD2* which favors the production of 2AP, one of the most abundant components of various volatile compounds responsible for fragrance (Zafar et al. 2020). Fragrance gene *BADH2* of Zhonghua 11 rice was edited using CRISPR/Cas9. An additional base (T) was introduced in the first exon of BADH2, leading to higher 2AP content in edited rice (Shao et al. 2017). CRISPR-Cas9 was applied to alter the *BADH2* gene in Zhengdao 19, a rice variety suited for direct sowing. The 2AP content in field planted T_0 was found to be increased from 0.003 μg/g (in the wild type) to 1.259 ± 0.072 μg/g for T_0 mutants. In greenhouse-planted T_1, it increased from 0.002 μg/g (in the wild type) to 0.537 ± 0.111 μg/g for T_1 mutants (Fuhua et al. 2018). A significant increase in grain yield and fragrance (2AP) was observed in CRISPR-edited rice. The mutants exhibited 2AP levels ranging from 0.72–0.78 mg/kg while 2AP was absent in wild type (Usman et al. 2020). CRISPR was used to introduce aroma in elite rice variety ASD16 by creating novel alleles of *OsBADH2* gene. SgRNA-mediated mutations were introduced in the seventh exon of *OsBADH2* gene. Novel aromatic comparatives, viz. pyrrolidine, pyridine, pyrazine, pyridazine, and pyrozole, were detected in the comparative volatile profiling of T_1 progenies grains (Ashokkumar et al. 2020).

Rice, one of the staple crops worldwide, is known to be deficient in vitamin A. Golden rice was developed to overcome this deficiency by enhancing the β-Carotene content in the endosperm (Paine et al. 2005). CRISPR-Cas has also been

utilized to improve the vitamin A content in rice. An ortholog of *Orange* (*Or*) gene in cauliflower, the *Osor* gene in rice was targeted using CRISPR-Cas9. Accumulation of orange color in the callus showed the enhanced β-carotene level in edited rice (Endo et al. 2019). A 5.2 kb carotenoid biosynthesis cassette at two genomic safe harbors was introduced in rice. An enhancedβ-carotene level was detected in seeds with no change in yield. Whole-genome sequencing revealed that no off-target mutations were created in Cas9 engineered plants (Dong et al. 2020).

Soft rice with 7–10% amylose content is quite popular in south China. The amylose content is determined by *Waxy* (*Wx*) gene. CRISPR-Cas9 has been recently applied to alter this gene to produce softer versions of elite cultivars. A loss of function in *Wx* gene of two widely cultivated elite japonica varieties, Xiushui134 (XS134) and Wuyunjing 7 (9522), was introduced by CRISPR-Cas9. A two-fold increase in gel consistency (GC) and a marked reduction in gelatinization temperature (GT) for CRISPR-waxy seeds were observed as compared to wild (Zhang et al. 2018a, b). The *Wx* gene of two elite rice cultivars, Huaidao 5 (HD5) and Suken 118 (SK118), was targeted to develop soft rice. The amylose content in the edited lines was around 2.6%–3.2% (Yunyan et al. 2019). Three elite rice cultivars, viz. Suijing 18 (SJ18), Songjing 2 (SJ2), and Longqingdao 3 (LQD3), were targeted for CRISPR-based editing of *Waxy* gene. The edited lines showed a significant reduction in the amylose content (Li et al. 2020a). An elite indica variety TianFengB was targeted to improve the cooking quality by reducing the amylose content using CRISPR-Cas9. The edited lines showed a significant reduction in amylose content. The study showed that in some of the edited lines the amylose content was similar to glutinous rice (Zeng et al. 2020). Cereal grains with higher starch content are known to be a good source of resistant starch (Jiang et al. 2010). Resistant starch is a form of nondigestible starch and is not absorbed in the body and protects from various noninfectious diseases (Regina et al. 2006). Keeping in mind the health benefits of resistant starch, CRISPR has been utilized to increase the starch content in rice. The calli derived from *japonica* cv. Kitaake was targeted for introduction of CRISPR-mediated mutagenesis in starch branching enzymes (SBE, SBEI, and SBEIIb). The *SBEI* and *SBEII* mutants showed a significant increase in amylose content and resistant starch by 25.0 and 9.8%, respectively (Sun et al. 2017). In another study, CRISPR-Cas9-mediated mutagenesis in *SBEII* resulted in increased amylose and resistant starch content from 19.6 to 27.4% and from 0.2 to 17.2%, respectively (Baysal et al. 2020).

Proanthocyanidins and anthocyanins are the major health-promoting nutrients present in rice. The red color in rice is governed by two recessive complementary genes, *Rc* and *Rd*. The wild species *Oryza rufipogon* has RcRd genotype which is responsible for red pericarp. However, a 14-bp frame-shift deletion in the seventh exon of *Rc* gene results in white phenotype in most of the cultivated rice varieties. Recently, this frame-shift mutation in recessive Rc was revered with the help of CRISPR-mediated editing, resulting in the conversion of white cultivar to red cultivar. The mutants showed high accumulation of proanthocyanidins and anthocyanins than the wild type. No significant difference in other agronomic traits was observed in the mutants as compared to wild type (Zhu et al. 2019).

14.14 Wheat

Wheat is an economically important cereal that supplies 20% of the calorie intake to over 60% of the global population. Presently, it is cultivated on around 220 million hectares with an annual production of 700–750 million tons and used in a wide range of products. The continued economic development has led to increased demand for premium quality wheat with improved grain quality. Grain quality is a mutagenic trait and the hexaploid genome of around 16 Gb with around 85% repetitive elements makes it more complex (Zhang et al. 2021). The advent of CRISPR-Cas9 has allowed researchers to create novel allelic variations to improve wheat grain quality. Recently, a web-based tool has been developed to design sgRNA for GE in wheat (Cram et al. 2019).

Celiac disease (CD) is the most common disease associated with wheat. It is an autoimmune disease prevalent in around 1–2% of the global population (Jouanin et al. 2020). Among food intolerances, CD is relatively well-understood from the standpoint of human immunity (Tye-Din et al. 2010). Gluten-free diets (GF), which exclude all wheat products, are the only way to prevent CD and this is very difficult as wheat gluten is present in almost every product. In the case of wheat, the gene family responsible for this autoimmune disease is α-gliadin, of which 33-mer is most immunogenic. The coding region of 33-mer was targeted using CRISPR and 21 mutant lines were developed. A total of 35 different genes were mutated and the immunoreactivity was reduced by 85%. These GE lines could be utilized in making low-gluten foodstuffs (Sánchez-León et al. 2018). In another study, both α- and γ-gliadins were targeted using CRISPR-Cas9. The T_1 generation showed altered gliadins production (Jouanin et al. 2019). Two genes related to grain quality, *pinb* (grain hardness) and *waxy* (starch quality), and one for kernel size *DA1* were mutated using CRISPR. Three mutant lines were generated and a mutation efficiency of 54.17% was recorded (Zhang et al. 2018a, b). Two subunits of α-amylase/trypsin inhibitors (ATI), viz. WTAI-CM3 and WTAI-CM16, were targeted to reduce allergen proteins in durum wheat using CRISPR (Camerlengo et al. 2020). The resistant starch content of modern wheat varieties is quite low. Targeted mutagenesis of *TaSBEIIa* using CRISPR was employed to increase amylose content in winter wheat *cv.* Zhegmai 7698 (ZM) and spring wheat *cv.* Bobwhite. Flour quality analysis showed that the triple-null lines possessed significantly increased amylose content (Li et al. 2020b). Four-grain quality genes, viz. *pinb* (grain hardness), *waxy* (starch quality), *ppo,* and *psy* (dough color), were targeted using CRISPR-Cas9. The mutants showed a significant reduction in expression of all four genes (Zhang et al. 2021).

14.15 Barley (*Hordeum Vulgare*)

Barley is one of the first crops to be domesticated, a major constituent of the brewing industry and primarily used as feed and food. Like other cereals, the presence of phytic acid is a major drawback associated with barley (Cosgrove 1980).

CRISPR-Cas9-mediated mutations in the promoter of the barley phytase gene *HvPAPhy_a* showed reduced mature grain phytase activity (Holme et al. 2017). In another study, *HvITPK1* gene responsible for phytic acid production in barley was mutated. The mutants contained altered levels of phosphate in the mature grains, ranging from 65% to 174% of the wild-type content (Vlčko and Ohnoutková 2020). D-hordein component of barley storage protein was mutated using CRISPR. Barley grains without D-hordein protein in T_2 seeds showed a decrease in the prolamines and an increase in the glutenins. Further, there was increase in starch, amylose, and β-glucan content (Yang et al. 2020).

14.16 Sorghum (*Sorghum Bicolor*)

Sorghum, a drought-tolerant crop, is a major staple food and feed crop in semiarid regions where cultivation of other cereals is not possible. Sorghum is deficient in essential amino acids like lysine and its protein is difficult to digest (Aboubacar et al. 2001). The reason behind the nondigestibility is the presence of prolamins, known as kafirins, which account for 70% of the total seed protein (Hamaker et al. 1995). Kafirins mainly comprise α-kafirins encoded by gene family *k1C*. The gene family *k1C* was targeted using CRISPR. A reduced T1 and T2 α-kafirin and increased grain protein digestibility and lysine content were observed in T_2 generation seeds (Li et al. 2018).

14.17 Conclusion and Future Prospects

Over the past several decades, conventional and molecular breeding has made significant contributions to agriculture. They utilize the variations in the available germplasm to develop improved versions. However, the improvement through these technologies is limited due to nonavailability of diverse germplasm. Moreover, these methods are time- and labor-intensive. GE, an advanced biotechnological tool, can be utilized to overcome the limitations. GE opens new avenues for researchers in the field of plant sciences to modify the target trait to interest, leading to a prodigious success in plant biotechnology. The use of genome editing has allowed efficient, rapid, specific, and targeted editing for increasing yield and nutritional value in cereals and other horticultural and fruit crops. These technologies add an edge to the existing genetic engineering struggles in attaining food security and combating malnutrition with the increasing global population. As GE crops are not likely to undergo strict regulations as in the case of GM crops (Yadav et al. 2018), they have come up as the first choice of researchers to develop improved crop plants. Despite several advancements in the field of GE, several challenges are associated with GE in cereals. Some of the major challenges are development of efficient transformation protocol, off-target effects, multiplexing, requirement of specific promoters,

and complex design (Ansari et al. 2020). There are also certain restrictions and regulations for application of GE to crops (Zhang et al. 2020). All these challenges must be resolved to utilize GE to its full potential. The development of crop plants with enhanced nutritional value using GE promises that everyone gets a healthier diet.

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Chapter 15
Genome Editing for Nutrient Use Efficiency in Crops

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15.1 Introduction

The rapid growth in the world population increases the demand for crop production, whereas the available arable lands are diminishing due to human activities. In addition to these two mostly encountering problems in agriculture, climate change and changes in soil structure/texture also affect crop production and yield at different extents. Other environment-related external factors and farmers' extensive use of irrigation and fertilizers make a significant part of the problem in agriculture such that irresponsible use of water and fertilizers pollutes the environment and therefore negatively affects the environment and crop production (Khan et al. 2018). To adapt the plants in these unfavorable conditions, the improvement of plant characteristics has taken the attention of plant breeders in the last century. The improvement of the plant is conducted by two main steps: creating a variation and selecting superior individuals. As sessile organisms, plants obtain their nutrients from the soil via their root systems. Absorbed nutrients are later translocated to other parts of the plant such as leaves and stem. Plants harness various pathways to uptake, translocate, and accumulate essential minerals and nutrients in their bodies (Brown et al. 2021). These pathways include a diverse mixture of mineral transporter, transcription factors, and regulator. Numerous genes involved in these pathways have already been characterized; however, our knowledge to enhance nutrient uptake and accumulation in crops is still limited.

Plants need different nutrients for growth and development. They are not always able to reach these nutrients in nature easily, therefore they evolved different ways to cope with the environmental extremities based on their needs. This adaptation enabled plants to develop mechanisms to avoid any type of danger or extremity or scarcity, and ultimately, plants shaped their internal processes according to the external supplies (Maqbool et al. 2020). Nutrient use efficiency (NUE) is, basically, the uptake of available nutrients in the environment and their allocation for the production of certain biomass in plants (Nieves-Cordones et al. 2020). These processes are controlled by multiple genes, therefore they are highly complex. Moreover, the complete structure of these mechanisms has not been illuminated yet. On the other hand, the knowledge about NUE gives a chance for improvement of use efficiency of each nutrient type one by one. Thus, according to the environmental conditions and plant needs, the genome of the crop can be edited by genome editing techniques for specific nutrients, which will be discussed later (Korotkova et al. 2019; Lu and Zhu 2017; Faiz et al. 2021).

Past several years, new techniques have been developed to edit certain genome parts of plants. The main idea behind genome editing is the modification of a DNA strand by inducing double-stranded DNA breaks. Nowadays, it is possible to create double-stranded DNA breaks on purpose at a specific region and insert or delete genes of interest (Wada et al. 2020). For this purpose, Zing-Finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease 9 (CRISPS/Cas9) system are the most commonly used techniques (Kamburova et al. 2017). ZFN is an artificial endonuclease, which contains Zing-Finger Proteins (ZFP) and the Fok1 enzyme. The ZFP part recognizes the target region in the genome and the Fok1 enzyme cleavages the double-strand DNA and creates a break in the genome. The defined technique is used for 'knock-out' or 'gene insertion' purposes (Urnov et al. 2010). Another technology used for genome editing is TALENs. The working principle of TALENs is almost the same with ZNF with small changes. The TALE proteins are used as recognition domain instead of ZFP and the Fok1 enzyme is used as a restriction enzyme in the TALENs system, like ZNF (Jankele and Svoboda 2014). CRISPR/Cas is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements (Horvath and Barrangou 2010). It has been used in genome editing in many different organisms to cause mutations in precise locations. This genome editing system enabled genome editing in a much simpler and cheaper way in plants. It produces double-strand breaks (DSBs) (Nasti and Voytas 2021). DSB repair is followed by NHEJ (nonhomologous end joining) or HDR (homology-dependent repair) pathways. NHEJ can cause insertion/deletion, whereas HDR can cause the introduction of a DNA template (Dangol et al. 2019; Nasti and Voytas 2021). The targeting of DNA occurs at PAM (protospacer adjacent motifs) sites and the most used PAM sequence is NGG in terms of Cas9 derived from *Streptococcus pyogenes*. Other Cas9 variants have also been utilized in plants to recognize the relaxed PAM sites (Ren et al. 2021). T-rich PAMs are recognized in another genome editing system known as CRISPR/Cas12a (previously CRISPR/cpf1) (Zhang et al. 2018; Zhang and Qi 2021). Other genome editing technologies have also been used in plants such as using deamination of cytosine and adenine via deaminases to create base excision repair (Nasti and Voytas 2021). Base editing can be performed via conversion of a single base to the other, such as CBE (cytosine base-editing) mediated by CRISPR/Cas9 technology causing cytosine deamination converting CG to TA. Similarly, ADE (adenosine base-editing) can convert AT to GC (Bharat et al. 2020). The ploidy level of plants can also make functional genomics studies quite challenging and the CRISPR/Cas9 system can be used to select the best single guide RNA (sgRNA) candidates in targeting multiple homoeoalleles (Sattar et al. 2019). Using various gene editing and CRISPR technologies, several plant species have been studied under different environmental conditions (Zhang et al. 2018). This technology is currently the simplest, most versatile, and precise method of genetic manipulation and is therefore causing a buzz in the crop breeding sector. In this chapter, the use of genome editing techniques on nutrient use efficiency and its implications in biofortification is discussed.

15.2 Transgenic Approach for Enhancement of Nutrient Use Efficiency

Nutrient use efficiency (NUE) encompasses a combination of efficiently acquiring nutrients from the soil followed by nutrient redistribution, assimilation, and utilization efficiency (i.e., high amount of dry matter production per unit nutrient uptake). All these processes are coordinated and mediated through a complex network of genes (Giehl and von Wiren 2014). The biological function and the working model of these sets of genes were studied within the framework of functional genomics. Transgenic approaches are considered to be an effective tool to study and improve various aspects of NUE in different crops together with plant transformation methodology. It includes gene-targeted or site-directed mutagenesis (i.e., T-DNA or transposon-tagging to generate loss-of-function mutations) and gene overexpression studies in transgenic plants (i.e., insertion of random transcriptional enhancers into the genome or expression of transgenes under the control of strong promoters) (Abdeeva et al. 2012; Ichikawa et al. 2006).

NUE can be improved through biotechnological intervention with an attempt to decipher the molecular basis of plant responses to macro- and micronutrients and by the identification of nutrient-responsive genes. One of the promising approaches is to engineer the overexpression of genes encoding NUE to enhance the nutrient assimilation efficiency and nutrient utilization efficiency of crops (Good et al. 2007). Wan et al. (2017) showed that key processes such as nutrient uptake, redistribution, assimilation, signaling, and storage are mediated by several transporters, regulatory elements, and transcription factors (TF). Transporters showed differences depending upon the location and affinities and were helpful in nutrient homeostasis and assimilation. Moreover, regulatory elements and TFs modulate the expression of genes involved in nutrient signaling that impacts NUE and ultimately improves crop productivity.

15.3 Improvement of Macronutrients Use Efficiency in Crops by Transgenic Approach

15.3.1 Nitrogen

Nitrogen (N) is a key limiting nutrient required for the growth and development of staple crops. Excessive N application in the form of nitrogenous fertilizers maximizes crop yields, but causes ecological imbalance. Plants uptake N in the form of nitrate (NO_3^-) and ammonium (NH_4^+). The obvious candidate genes responsible for N uptake, assimilation, and regulation include nitrate and ammonium transporters, glutamine and glutamate synthase genes, such as alanine aminotransferase (*AlaAT*), nitrate reductase, and transcription factors, such as Dof1, etc. (Kurai et al. 2011). In plants, nitrate transporters are of two types: nitrate transporter 1/peptide transporter

(NRT1/PTR also referred to as NPF family) and nitrate transporter 2 (NRT2 family). NPF family can transport broad substrates and generally possess low affinity except for AtNPF6.3 or NRT1.1 (dual affinity NO_3^- transporter) (Chiba et al. 2015). NRT2, on the other hand, possesses high affinity and may require a partner protein like NRT3 (NAR2) to mediate NO_3^- transport at a relatively low concentration (Feng et al. 2011). NRT1.1 serves as a nitrate sensor and aids in the gene regulation of *NRT2.1* in plants (Ho et al. 2009). AMT/MEP/Rh transporters comprise a family of ammonium transporters. The activity of NH_4^+ transporters is important in the regulation of nitrogen use efficiency in NH_4^+ preferred rice crop (Khademi et al. 2004).

An overexpression of ammonium transporter *OsAMT1.1* in rice (*cv.* Kaybonnet) enhanced NH_4^+ uptake in the plant and significantly improved N assimilation in the roots and shoots ultimately causing a rise in grain yield (Ranathunge et al. 2014), but the expression of *AMT* transporter is genotype-dependent (Kumar et al. 2006). Although NH_4^+ transporters are crucial for rice, NO_3^- uptake may also improve nitrogen use efficiency. Overexpression of *OsNRT2.1* with a NO_3^--inducible promoter of *OsNAR2.1* increases the rice grain yield by 25% and 21% as compared to the wild-type plants at the N application rates of 180 and 300 kg ha^{-1}, respectively. A 40% enhancement in agronomic NUE, recovery efficiency, and nitrogen uptake was also observed by overexpression of *OsNRT2.3b* in rice (Chen et al. 2016; Fan et al. 2016). The expression of *NRT2* genes for enhancing NUE in crops is subjected to the use of suitable promoters. Overexpression of *OsNRT2.1* with a maize *ubiquitin* promoter (*pUbi*) significantly inhibited grain yield and N utilization efficiency in maize (Chen et al. 2016). N metabolism in potato has been improved owing to the key roles of nitrate transporters such as *StNRT2.4*, *StNRT2.5,* and *StNRT2.7*, glutamine synthetase, glutamate dehydrogenase, and carbonic anhydrase. Genetic manipulation of these transporter activities using tissue-specific or constitutive promoters improved NUE in potato (Zhang et al. 2020a). NPF family (nitrate transporter 1/peptide transporter) constitutes promising sources of genes for improving NUE in crops. Rice and Arabidopsis consist of at least 84 and 53 *NRT-1/PRT* genes, respectively (Zhao et al. 2015). Tsay et al. (2007) found 7 *NRT2* genes in Arabidopsis. Among them, *AtNPF8.2/AtPTR5* encodes a high-affinity dipeptide transporter and its overexpression causes an improvement in N uptake and shoot growth (Komarova et al. 2008). The uptake of N and its translocation within the plants in nitrate form has been carried out by *AtNRT1.1-1.9*. Likewise, the expression of *NRT1.1b* has been extensively studied in rice with major contributions in N uptake and root to shoot ratio in *indica* species compared to *japonica* species (Hu et al. 2015). In transgenic rice, elevated expression of *OsPTR9* under the control of cauliflower mosaic virus (*CaMV*) *35S* promoter followed by *Agrobacterium*-mediated transformation increased the ammonium uptake, enhanced lateral root formation, and finally improved grain yield. Alternatively, downregulation studies with T-DNA insertion lines (04Z11AH79, *OsPTR*) and *OsPTR9*-RNAi approach produced opposite results (Fang et al. 2013). The overexpression of *OsPTR*9 is known to influence glutamine synthetase (GS) activity, which helps in efficient remobilization of amino acids from senescing leaves towards the filling of grains in cereals (Tabuchi et al. 2007).

N utilization and remobilization efficiency in rice, canola, wheat, and sugarcane have been efficiently enhanced due to the upregulation of genes involved in glycolysis and the TCA cycle. Alanine aminotransferase (*AlaAT*) gene from *Hordeum vulgare* performs a key role in such circumstances, converting glutamate to pyruvate and then to alanine and α-ketoglutarate. Alanine is the main source of amino acid during the conditions of hypoxia (Miyashita et al. 2007). A root-specific *btg26* promoter is used for the overexpression of *HvAlaAT* in canola lines, which resulted in higher above-ground biomass and seed yield (Good et al. 2007). An elevated shoot and root biomass has been observed in transgenic rice lines overexpressing *HvAlaAT* driven by rice *antiquitin* promoter (*OsAnt1*) (Selvaraj et al. 2017). The same promoter (*OsAnt1*::*HvAlaAT*) was used for the transformation of sugarcane and wheat plants displaying enhanced NUE and higher biomass production compared to the untransformed plants (Snyman et al. 2015; Peña et al. 2017). The roots are primary sites of *AlaAT* expression perturbing pyruvate levels and affecting the TCA cycle, causing an increase in energy production and hence promote N uptake followed by assimilation. Overexpression of *HvAlaAT* in transgenic plants resulted in the upregulation of genes involved in ethylene production. The biosynthesis of ethylene involves ATP and thus generates higher biomass. Higher levels of glycine production and reactive oxygen species (ROS) detoxification were some other factors of enhanced NUE and biomass production as a result of *AlaAT* overexpression in plants (Tiong et al. 2021). Other N metabolizing and assimilation enzymes like glutamine synthetase (GS) and glutamate synthase (GOGAT) enhance N remobilization into biomass mainly through the degradation of proteins (Rubisco, PEPc, and GS) which act as storage reservoirs containing glutamate, glutamine, aspartate, and asparagine (Brauer and Shelp 2010; McAllister et al. 2012). Evidence from the overexpression studies of *GS1/GOGAT* in dicots (tobacco, pea, and alfalfa) (Ortega et al. 2001; Oliveira et al. 2002; Fei et al. 2003) and monocots (rice, wheat, and maize) (Habash et al. 2001; Martin et al. 2006; Cai et al. 2009a, b) displayed a potential for improvement of usage index and uptake efficiency in dicots and uptake efficiency and utilization efficiency in monocots.

Transcription factors are regulatory elements vital for efficient signaling, remobilization, and utilization of key nutrients in plants. N assimilation in crops can be enhanced by transcriptional regulators or TFs such as NAC, DEP1, MADS25, NAP, Dof1, NLP7, bZIP, and HY5. Overexpression of *NAC2-5A* in wheat enhances root nitrate influx rate, accumulation of N in aerial parts, N HI (harvest index), and grain yield (He et al. 2015). In rice, DEP1 enhanced NH_4^+ uptake and assimilation (Sun et al. 2014), while MADS25 played a critical role in mediating NO_3^- uptake through primary and lateral root development and increased the expression levels of nitrate transporter genes and accumulation of nitrate in rice (Yu et al. 2015). Maize Dof1 (ZmDof1) is a potent plant-specific TF that holds the capacity to enhance N assimilation and modulates the gene expression of the C-N skeleton network involved in the biosynthesis of amino acids such as glutamine (Glu). Under a low N environment, an overexpression of *ZmDof1* in transgenic Arabidopsis and potato increases carbon flow towards N assimilation with induced expression of phosphoenolpyruvate carboxylase (*PEPC*) genes (Yanagisawa et al. 2004). Net photosynthetic rate,

root biomass, and root to shoot ratio were increased due to overexpression of *Dof1* under N-deficient conditions in rice (Kurai et al. 2011). Transgenic tobacco plants overexpressing *NLP7* (Nin-like protein 7) influence nitrate signaling, transporter, and assimilation genes, and ultimately, enhance NUE in both N-rich and deficient conditions (Yu et al. 2016). Nitrate reductase is a key enzyme known to influence the assimilation of nitrate in plants. In Arabidopsis, bZIP HY5 (LONG HYPOCOTYL5) was identified as the regulator and activator of nitrate reductase encoding gene *NIA2,* a key enzyme, and the gene for N assimilation in plants (Jonassen et al. 2009). *MdHY5* gene was cloned and overexpressed in apple; the resulting transgenic apple calli showed enhanced anthocyanin accumulation along with improvement in nitrate contents and nitrate reductase activity. *MdHY5* positively regulates the expression of *MdNIA2* and *MdNRT2.1*, *MdNRT2.4*, and *MdNRT2.7* genes, which is in accordance with observations in *Arabidopsis.* Thus, nitrogen assimilation and NUE in apple are controlled by the *MdHY5* gene (An et al. 2017; Jonassen et al. 2009).

15.3.2 Phosphorous

Phosphorous (P) is another crucial macronutrient that is absorbed by the plants in the form of orthophosphate ions, i.e., HPO_4^{-2} and $H_2PO_4^{-}$ (forms of inorganic phosphate (Pi) in aqueous solution). In acidic soils, P forms low solubility molecules with aluminum (Al) and iron (Fe), while the reaction of P with calcium (Ca) and magnesium (Mg) forms sparingly soluble compounds of phosphate in alkaline soils (Bar-Yosef 1991). P availability depends upon a very narrow range of neutral soil pH. Approximately 80% of applied P becomes immobile in the soil and unavailable to the plants due to adsorption, precipitation, and transformation to organic forms (Holford 1997). Various strategies are displayed by the plants to improve the acquisition of inorganic phosphate (Pi) in P-deficient soils, including remodeling of root morphology, the release of carboxylates in the rhizosphere, and induction of high-affinity Pi transporters (Hammond et al. 2004).

Phosphorous use efficiency (PUE) largely depends on the acquisition and remobilization of Pi within the plants. It relies on activating the expression of phosphorous transporter genes. Phosphate transporters are classified into PHT1, PHT2, PHT3, and PHT4 families (Liu et al. 2011). Rice genome contains 13 *PHT1* genes. In rice, overexpression of *OsPHT1.1* is responsible for increasing Pi in the shoots and the number of tillers (Seo et al. 2008). *Agrobacterium*-mediated cotyledonary node transformation method was used to develop transgenic soybean lines expressing rice phosphate transporter gene (*OsPT6 or PHT1.6*) using *CaMV 35S* promoter (Yan et al. 2014). The results suggested that overexpression of *PHT1.6* enhances the accumulation of P in roots, stems, and leaves under Pi-deficient conditions. Transformed soybean plants showed better growth and development and produced a greater number of seeds and pods compared to the untransformed plants mainly because they regulate the transfer of Pi at the junction of root nodules along with controlling expression in the vascular

bundles of both juvenile and mature nodules. Phosphorous transporters (PTs) serve to improve the acquisition and translocation of Pi in the plants during the periods of Pi starvation. In another study, 41% Pi accumulation was noted in the leaves of transgenic rice plants overexpressing tobacco PT, i.e., *NtPT1* under Pi stress conditions (Park et al. 2010). In wheat, the high-affinity *TaPT2* gene correlates with external Pi concentration and showed sensitivity to low Pi conditions as the overexpression enhanced photosynthetic efficiency, dry mass, and content of P in the plant (Guo et al. 2014). The acquisition of Pi into the cell is an energy-dependent process mediated by H^+-ATPases. They pump protons outside the cell creating a concentration gradient with an influx of Pi within the cell via H^+-Pi cotransporters. In addition to PHT acting as Pi cotransporters, another gene, *PHO1*, belonging to PHO (phosphate permease) family was identified in Arabidopsis. Expression patterns of this gene in Arabidopsis suggest that it is involved in the loading of Pi to the xylem, root epidermal, and cortical cells (Hamburger et al. 2002). Transgenic rice lines overexpressing *OsPHT1;8* enhanced the acquisition of *Pi* from the soil by 3 to 4 folds; hence, PUE was enhanced (Jia et al. 2011). Stefanovic et al. (2011) revealed that overexpression of *AtPHO1* in *Arabidopsis* improved shoot *Pi* uptake by 2 to 3 folds.

Apart from proton secretions and homeostasis mechanisms involving cotransporters, plants have adapted other strategies to deal with immobilized and unavailable Pi within the soils. The mobilization of both organic and inorganic P can be achieved through the release of organic anions and phosphatase enzymes into the soil. Engineering plants to produce more organic acids such as overproduction of citrate results in more biomass and fruit weight. For instance, transgenic tobacco plants overexpressing *Pseudomonas aeruginosa* citrate synthase (*CSb*) yielded 25% to 35% more dry weight in comparison to non-transformed plants (Lopez-Bucio et al. 2000; Iqrar et al. 2020). Transgenic *CSb* lines accumulated more P within the tissues due to citrate exudation, which causes rhizosphere acidification resulting in more P solubilization. Furthermore, phosphorous was removed from sparingly soluble calcium phosphate complexes as it is converted to calcium citrate. This free Pi is readily available for uptake by the plants. In barley and tobacco, increasing malate exudation through overexpression of malate dehydrogenase (*MDH*) and wheat malate transporter (*TaALMT1*) genes from mycorrhizal fungi (*Penicillium oxalicum*) enhanced the P uptake, biomass, and tolerance to P deficiency (Lu et al. 2012). As 80% of organic P within the soil is present in the form of phytic acid, overexpression of genes encoding phytases (*PHY1* and *PhyA*) and purple acid phosphatases (*GmACP1*, *LaSAP2*, *PAP1*, *PAP3*, *PAP15*) contribute to Pi uptake and higher enzyme activity and enhance PUE and more biomass production in soybean, white lupin, *Medicago trunculata,* common bean, and Arabidopsis (Wang et al. 2009; Wasaki et al. 2009; Liang et al. 2010a, b; Ma et al. 2013; Zhang et al. 2014).

TFs such as MYB2P-1 (Rice), WRKY45 (Arabidopsis), BnPHR1 (*Brassica napus*), and TaPHR1 (wheat) increase root growth and dry matter partitioning owing to improved PUE. The majority of these molecular regulatory components improved PUE under low or deficient P environments since sufficient conditions may lead to excessive Pi accumulation and growth retardation (Zhou et al. 2008; Dai et al. 2012; Wang et al. 2014).

15.3.3 Potassium

Potassium (K) use efficiency (KUE) depends upon the uptake and redistribution of K^+ regulated by a complex network of genes encoding K^+ channels and transporters. Engineering KUE in plants relies on manipulating the expression of K^+ transporters and channels. The genes include three families, namely tandem-pore K^+ (TPK), shaker, and K^+ inward rectifier-like family (K_{ir}-like channels), and encode for K^+ channel proteins in plants. K^+ transporters belong to different families including KUP/HAK/KT (K^+ transporter/ high-affinity K^+ transporter/K^+ uptake permease), HKT (high-affinity K^+ transporter), CHX (cation:proton antiporter), and NHX (Na^+/H^+ antiporter) (Wang and Wu 2013; He et al. 2017).

Approximately 71 K^+ channels and transporters have been reported in the model plant *Arabidopsis thaliana* (Wang and Wu 2010). Arabidopsis acquired K^+ through the roots through inward rectifier potassium channel AtAKT1. It is sensitive to low K^+ stimulus in the rhizosphere and activated by the protein kinase AtCIPK23. *AtCIPK23* overexpression in Arabidopsis improved tolerance to low K^+ by enhancing its uptake from the rhizosphere (Xu et al. 2006). A similar response of this channel protein was noticed in potato (Wang et al. 2011). The overexpression of *AtCIPK23* in transgenic tobacco plants enhanced primary root length, dry biomass, K^+ content, and overall growth parameters in comparison to wild-type tobacco (Xue et al. 2016). AtCIPK23 regulates one of the largest groups of transporters (i.e., KUP/HAK/KT) in plants. K_{ir} channels belong to TPK (tandem-pore K^+) family based on evolutionary events, i.e., duplication and partial deletion (Gomez-Porras et al. 2012). TPK family is involved in maintaining cellular K^+ homeostasis due to their presence in the vacuolar membrane. For instance, rice lines overexpressing *OsTPKb* enhance uptake of K^+ in the roots and shoots and also provide tolerance against stress conditions (low K^+ or water stress) mainly due to the increase of cytoplasm: vacuole K^+ ratio (Ahmad et al. 2016).

Overexpression of *AtHAK5* transporter in Arabidopsis at low edaphic K^+ levels enhanced the uptake and absorption of K^+ ions within the plant (Nieves-Cordones et al. 2010). The expression of this significant transporter was regulated by four transcription factors (TFs), namely JLO (Jagged Lateral Organs), DDF2 (Dwarf and Delayed Flowering 2), bHLH121 (basic Helix-Loop-Helix 121), and Transcription factor II A Gamma chain (TFII-A) (Hong et al. 2013). These TFs were upregulated due to overexpression in the plant under salt and K deficiency stress conditions, enabling an increase in root growth as compared to the control. In rice, the overexpression of *OsHAK5* not only improved K^+ acquisition from the rhizosphere, but also enhanced its transport to the aerial parts, ultimately boosting up the K^+/Na^+ concentration in the shoots (Yang et al. 2014). *ApKUP3* gene from alligator weed (*Alternanthera philoxeroides*) overexpressed in rice showed an improved drought tolerance, K^+ nutrition, and resulted in better net K^+ influx and accumulation in rice plants due to an increase in net chlorophyll content, photosynthesis rate, stomatal conductance, and antioxidant activities (Song et al. 2014). Studies about K^+ homeostasis and recirculation within the model plant Arabidopsis showed that knockout of

AtCHX14 and overexpression of *AtCHX13* enhanced K^+ uptake (Zhao et al. 2015). The former mediates low-affinity K^+ efflux, while the latter was involved in relatively high-affinity K^+ efflux. These cation: proton antiporters were mainly expressed in the vascular tissues of the plant.

15.3.4 Sulfur

Sulfur (S) is taken up from the soils in the form of sulfate (SO_4^{-2}). It is stored in the plant vacuoles. Roots are the major sites of reduction and assimilation, while transportation of sulfate to the leaves was carried out through the xylem. Remobilization of sulfate to the roots was done through the phloem (Hartmann et al. 2000). Sulfate transporters (SULTRs) control the process of transport and mobilization of sulfate within the plant body (Hawkesford and De Kok 2006). Sulfur utilization efficiency (SUE) in plants is divided into (i) efficient transporters (transporters involved in sulfur uptake and assimilation) (ii) mobilizable sinks and reserves (the use of stored or accumulated S efficiently) (Iqrar et al. 2020).

There are 12 members of sulfate transporters organized in 4 groups, i.e., SULTR1 to SULTR4 in rice (monocots) and Arabidopsis (dicots) (Takahashi et al. 2012). Sulfate acquisition from the rhizosphere in the roots is done by *SULTR1;1* and *SULTR1;2*. The studies conducted by Rae and Smith (2002) and Yoshimoto et al. (2002) demonstrated the expression of these genes in root epidermal cells, root hairs, and cortical cells. *SULTR1;3* is involved in the mobilization of sulfate within phloem (Yoshimoto et al. 2003). An overexpression of soybean *GmSULTR1;2b* in transgenic tobacco plants resulted in improved biomass, seed weight, and accumulation of organic matter. Transcriptome analysis of the tobacco leaves overexpressing *GmSULTR1;2b* revealed differential expression of 227 genes involving transporter gene families like *NRAMP* and *OPT* (Ding et al. 2016). In yeast, the coexpression of both *SULTR3;5* and *SULTR2;1* increased S uptake (Kataoka et al. 2004). Overexpression of an S-assimilation gene *PaAPR* from bacteria in *Arabidopsis* and maize resulted in the enhancement of S-containing compounds such as cysteine, glutathione, and sulfite (Tsakraklides et al. 2002). Group 3 StSULTR3 members constitute the largest cluster of S transporters in potato (Vatansever et al. 2016). In Arabidopsis, the overexpression of *SULTR3;1* under the control of *CaMV 35S* promoter confirmed that it was involved in sulfate transport within the chloroplast (Cao et al. 2013; Nikolić and Tomašević 2020). Apart from *SULTR*, the genes involved in sulfur metabolism include sulfurylases (*APS*), adenosine 5′-triphosphate (*ATP*), sulfite reductase (*SiR*), and 5-adenylylsulfate reductase (*APR*) (Cui et al. 2020). An overexpression of *ZmSiR* in Arabidopsis plants showed tolerance against cold, oxidative, and SO_2 stress through the modulation of sulfite reduction and scavenging of H_2O_2 via glutathione (GSH)-dependent pathway (Xia et al. 2018). *APS4* transcript was downregulated by overexpression of *mir395* (Liang et al. 2010a; b). These overexpression plants depicted higher accumulation of sulfate, but encounter S deficiency symptoms due to impaired sulfate

translocation transporters such as *SULTR2;1*. Taken together, these findings suggest that S transport involves complex players regulated at the transcriptional, posttranscriptional, and posttranslational levels.

Cysteine formation is the major step for assimilation of reduced S into organic compounds (Kortt et al. 1991). Sunflower seed albumin (SSA) was isolated and characterized from sunflower seeds. It is expressed from the gene *sfa8*. SSA is rich in sulfur-containing amino acids, i.e., methionine and cysteine. In grain legumes, the overexpression of *sfa8* improved the nutritional increment of the feed so that a significant improvement in weight and wool quality was observed in the sheep fed with transgenic *Lupinus angustifolius* overexpressing *sfa8* in the seeds. The nutritional gains were due to higher methionine (16%) and cysteine (8%) accumulation within the seeds (Molvig et al. 1997). In rice, however, an expression of the chimeric gene encoding *SSA* under the control of wheat glutenin promoter did not show a significant enhancement in total S seed content or either of the S-rich amino acids. It may be due to the transcriptional and posttranscriptional mechanisms that result in the replacement of endogenous proteins with little or no S-rich amino acids (Hagan et al. 2003). Tomato contains sulfur transporter gene (*LeST1.1*), the constitutive expression of which in transgenic *Brassica juncea* resulted in enhanced S status, ATP sulfurylase activity, chlorophyll content, protein content, and higher biomass as compared to the non-transgenic plants. This gene encodes HAST (high-affinity sulfate transporter) and is thus involved in S uptake and assimilation particularly under S inefficient conditions (Abdin et al. 2011).

15.4 Improvement of Micronutrient Use Efficiency in Crops by Transgenic Approach

15.4.1 Iron

Iron (Fe) homeostasis is tightly regulated in plants as both deficiency and excess can result in stunted plant growth. Ferrous (Fe^{+2}) iron is relatively soluble but oxidized to form Fe^{+3} (ferric) in the soil. The availability of iron in ferric form largely relies upon the soil pH. Fe represents the third most important limiting nutrient in calcareous-alkaline soils for the growth and development of plants, after N and P (Marschner 1995). Plants usually adopt two distinct strategies for the uptake, solubilization and mobilization of Fe. The strategy I is utilized mainly by dicots such as peas, tomato, and Arabidopsis, which is based on soil acidification and reduction of Fe^{+3} to Fe^{+2}.

The genes involved in strategy I belong to the family of proteins including *AHA* (H^+-ATPase), *FRO* (Ferric chelate reductase/oxidase), and *IRT* (iron-regulated transporter) (Palmgren 2001; Vert et al. 2002; Waters et al. 2002). These protein families carry vital genes (*FRO1*, *FRO2*, *FRO6*, *IRT1,* and *IRT2*), which are induced by Fe-deficient conditions. *bHLH* transcription factors are involved in the iron

uptake as shown by the overexpression of the *MdbHLH*104 gene in apple. It enhanced the AHA activity causing Fe solubilization in the rhizosphere (Zhao et al. 2016). Plasma membrane (PM)-localized AHA provides an electrochemical gradient within the rhizosphere due to the release of protons which results in the soil acidification and uptake of mineral nutrients. In *Arabidopsis*, PM AHAs are encoded by 11 *AHA* genes, and the *AHA2* gene is mainly involved in rhizosphere acidification, resulting in Fe solubility and uptake (Santi and Schmidt 2009; Zhao et al. 2016). *FRO1* and *FRO2* are involved in catalyzing the reduction reaction of Fe^{+3} in pea and *Arabidopsis*, respectively (Waters et al. 2002). The conversion of ferric iron (Fe^{+3}) to ferrous (Fe^{+2}) is the rate-limiting step for high-affinity iron uptake as shown in the *CaMV 35S-FRO2* transgenic Arabidopsis lines grown under Fe deficiency (Connolly et al. 2003). Interestingly, the posttranscriptional regulation studies also showed that it hampers the accumulation of potentially toxic amounts of iron in the plants, meaning that no elevated levels of iron were detected due to FRO2 activity under Fe-deficient conditions. Li et al. (2011) demonstrated that the overexpression of the Arabidopsis *AtFRO6* gene in tobacco increased the ferric chelate reductase activity rendering the plant tolerant against IDC (iron deficiency chlorosis). The reduced iron in ferrous form (Fe^{+2}) serves as a substrate for the IRT1 transporter to be taken up from the root epidermal cells. *IRT1* loss-of-function mutants were found to be chlorotic under normal growth conditions and cannot be complemented by the overexpression of *IRT2* (Vert et al. 2002). It simplifies that *IRT2* had a distinct function from *IRT1,* controlled at transcriptional and posttranscriptional levels. Both these transporters belong to the ZIP (Zinc-regulated and iron-regulated transporter like-protein) family, which is not specific only to the Fe uptake, but also involved in mediating the uptake of other critical divalent elements such as zinc (Zn^{+2}), manganese (Mn^{+2}), and cadmium (Cd^{+2}) (Maser et al. 2001). An overexpressing *IRT1* line revealed protein accumulation only under Fe-deficient conditions, suggesting it regulates Fe homeostasis in plants (Barberon et al. 2011). In rice, *OsIRT1* is involved in the direct uptake of Fe^{+2} under anaerobic conditions, and its overexpression depicted low FRO enzyme activity (Ishimaru et al. 2006). This observation explains the additional diverse functional role of *IRT1* for transporting Fe in monocots.

Strategy II features the release of chelating agents like mugineic acids (MAs) or phytosiderophores in the rhizosphere which are natural Fe^{+3} chelators enhancing the solubilization, uptake, and mobilization of Fe^{+3}. It is adopted by plants belonging to the Poaceae family (wheat, rice, and maize) (Forieri and Hell 2014). These plants using the chelation strategy enhance Fe uptake due to the expression of certain enzymes including NAS (nicotianamine synthase), NAAT (nicotianamine aminotransferase), TOM (transporter of mugineic acids), YS (Yellow stripe), and YSL (Yellow stripe-like). Nicotianamine (NA) synthesized by NAS is the precursor for MAs. *NAS* gene cloning was done from rice, barley, and Arabidopsis and expression analysis revealed that it was induced under Fe-deficient conditions (Nakanishi et al. 2000; Forieri and Hell 2014). Masuda et al. (2009) demonstrated that the barley *NICOTIANAMINE SYNTHASE1* (*HvNAS1*) gene under the control of *CaMV 35S* promoter improves NA and DMA (2′-deoxymugineic acid) concentrations, thereby increasing both Fe and Zn levels in the rice grains. NA production had a positive

impact on the internal translocation of Fe towards the grain. DMA functions as both internal transporter of Fe^{+3} and a metal chelator. The chelated MA-Fe^{+3} complexes are up by the roots via *YS1* and *YSL1* transporters. *yellow stripe1 (ys1)* mutants depict IDC and fail to uptake phytosiderophores attached to Fe^{+3} (von Wiren et al. 1994). *YSL1* shares homology with *YS1*, which was identified in crops other than maize including Arabidopsis (*AtYSL1*) and rice (*OsYSL2*). Loss-of-function *AtYSL1* mutant and *OsYSL2* overexpressing lines showed that YSL transporters are involved in long-distance Fe transport through phloem and aid in Fe loading to the seed and grains (Koike et al. 2004; Jean et al. 2005). Studies on rice and barley revealed a family of transporters of phytosiderophores (i.e., TOM) responsible for the secretion of MAs in the rhizosphere (Nozoye et al. 2011). In rice, the overexpression of *OsIRO2*, which controls key genes involved in strategy II Fe^{+3} uptake like *OsNAAT1*, *OsDMAS1*, *OsNAS1*, *OsNAS2*, *OsYSL15*, and *TOM1,* resulted in enhanced MA secretion and showed tolerance to IDC in calcareous soils (Ogo et al. 2011; Masuda et al. 2019). The overexpression of *NRAMP* (natural resistance-associated macrophage protein) transporters such as *NRAMP1, NRAMP2*, and *NRAMP4* in both non-graminaceous and graminaceous plants enhanced Fe uptake and its accumulation in developing seeds. These transporters can also mediate the transport of other divalent metal elements such as Zn^{+2}, Mn^{+2}, nickel (Ni^{+2}), and Cd^{+2} (Cailliatte et al. 2010; Forieri and Hell 2014).

15.4.2 Zinc

Zinc is deficient in the plants if the concentration drops below 15 to 20 µg/g dry weight, leading to stunted growth, early senescence, and chlorotic older leaves (Marschner 1995). The factors affecting Zn uptake by the plants include soil pH and composition and soil phosphate (P) amounts. The suggested mechanism of Zn uptake from the rhizosphere showed that it is taken up in the form of Zn-DMA complexes and Zn^{2+} ions (Kawakami and Bhullar 2018). *ZIP* transporters were involved in Zn^{2+} uptake as shown in a study conducted by Ramesh et al. (2003). The study depicted an upregulation of *OsZIP1* in root exodermis under Zn-deficient environment, suggesting its role in Zn uptake. A study by Arnold et al. (2010) confirmed the uptake of Zn^{2+} in the form of the Zn2+-DMA complex. On the other hand, Zn^{2+}-PS is the primary form of Zn^{2+} uptake in maize and barley (Kawakami and Bhullar 2018). Zn^{+2} is uptaken and transported via apoplastic and symplastic pathways up to the xylem vessels (Moreira et al. 2018).

Several members of the ZIP family are thought to be involved in Zn uptake. For instance, overexpression of *OsZIP4*, *OsZIP5*, and *OsZIP8* in rice enhanced the content of Zn^{2+} in the roots (Ishimaru et al. 2005). Zn in edible plant parts can be increased and Zn deficiency in plants can be engineered by targeting the transporters of plasma membrane and vacuole. ZIP family proteins (ZRT, IRT-like protein) constitute a group of plasma membrane transporters to transport iron

and zinc into the cytosol through the plasma membrane (Maser et al. 2001). Additionally, other transporters such as ZAT and MHX transport Zn^{2+} through the vacuolar membranes (Ramesh et al. 2004). An overexpression of *AtZIP1* from Arabidopsis in *Hordeum vulgare* using *Ubiquitin* promoter showed increased Zn uptake in comparison to non-transgenic lines (Ramesh et al. 2004). Xylem unloading and translocation to shoots were improved in *ZIP* overexpressing plants. *AtZIP1* is also involved in Mn^{2+}/Zn^{2+} homeostasis, and mainly, the movement of Mn^{2+} out of the vacuole to the cytosol and the xylem parenchyma. Functional heterologous expression of 6 Arabidopsis *ZIP* genes (*ZIP1*, *ZIP2*, *ZIP3*, *ZIP7*, *ZIP11*, and *ZIP12*) complemented the yeast Zn uptake deficient mutants, *zrt1* and *zrt2* (Milner et al. 2013). Overexpression of *OsZIP4* in transgenic rice under the control of *CaMV 35S* promoter increased the Zn accumulation mainly in the roots (Ishimaru et al. 2007). Zinc Arabidopsis transporter (*ZAT*) belongs to a large transporter family called CDF (cation diffusion facilitator). These are characterized and identified in plants including barley, Arabidopsis, and poplar for their roles in vacuolar Zn^{2+} transport (Yang and Chu 2011; Ajeesh Krishna et al. 2017). MHX (Mg^{+2} /H^{+} exchanger) belongs to the CAX (cation exchanger) family responsible for the mobilization of Mg^{2+} and Zn^{2+} into the organelles. An overexpression of *AtMHX1* improved Zn^{2+} transport in transgenic tobacco lines (Shaul et al. 1999). Similarly, *NcTZN1* and *AhHMA4* genes from *Neurospora crassa* and *Arabidopsis halleri* were overexpressed in tobacco-depicted-enhanced Zn^{2+} acquisition and accumulation under Zn-deficient conditions (Barabasz et al. 2010; Dixit et al. 2010).

15.4.3 Manganese

Manganese (Mn) is an essential micronutrient that plants use as a cofactor for enzymes (almost 35 known so far) and is involved in redox reactions during photosynthesis (Hänsch and Mendel 2009; Williams and Pittman 2010; Sun et al. 2019). The uptake of Mn^{2+} contributes to plant biomass and yield (Schmidt et al. 2016). Mn^{2+} is prevalent in acidic soils and plants increase the acidity of the soil to increase the concentration of available Mn^{2+} under deficient conditions. The symptoms of Mn-deficient plants are mostly reflected in plant development particularly in root architecture (Wei Yang et al. 2008; White and Neugebauer 2021). Mild symptom under deficit-Mn is chlorosis in leaves (Schmidt et al. 2016).

Transporters are known to enable Mn^{2+} uptake through root and translocate Mn^{2+} to other parts of the plants (Gao et al. 2018). ZIP family members, MTP/CDF family transporters, and NRAMPs are the main Mn^{2+} transporters, and they have a role in Mn translocation, detoxification, sequestrating, and partitioning (Delhaize et al. 2003; Milner et al. 2013). Therefore, scientists target these transporters to understand the Mn^{2+} pathway in plants. Transgenic *irt1* Arabidopsis plants showed limited Mn^{2+} uptake (Vert et al. 2002). NRAMPs are functioning in the Mn pathway

such that their single (*nramp1* and *nramp2*) and double (*nramp3nramp4*) mutants showed retarded growth in Arabidopsis due to failure in Mn^{2+} uptake (Cailliatte et al. 2009, 2010). The overexpression of cell number regulator (*CNR*) in rice enhanced Mn^{2+} and Cd^{2+} translocation and could promote Mn-biofortification in plants (Qiao et al. 2019).

15.4.4 Magnesium

Magnesium (Mg) is a vital micronutrient required for many cellular processes such as photosynthesis, protein synthesis, and cell wall structure and has a role as a cofactor for enzymatic reactions (Guo 2017; Cakmak and Yazici 2010; Hermans et al. 2013). Plants in the field generally do not experience Mg^{2+} deficiency because soil already contains high concentrations of it (Guo 2017). N, P, K fertilization, however, can limit Mg^{2+} uptake by plants so that plants in the field may not uptake adequate Mg^{2+} from the soil due to antagonistic effect of other cationic ions (Farhat et al. 2016). Calcareous soils also prevent Mg^{2+} uptake by plants (Cakmak and Kirkby 2008). Plants take Mg^{+2} via roots and translocate to shoots in apoplastic and symplastic ways (Alcock et al. 2017). Plants suffering Mg^{2+} deficiency show chlorosis and increased ROS and malondialdehyde production (Cakmak and Marschner 1992; Tewari et al. 2004). The photosynthetic capacity of plants decreases when plants do not take needed Mg^{2+} (Farhat et al. 2016). Mg-deficient plants have problems with root growth and gametophyte development (Cakmak et al. 1994; Chen et al. 2009) as well as sucrose transport (Farhat et al. 2016). Mg deficiency can further cause other nutrient deficiencies in plants, therefore it has a big role in defining plant biomass.

MRS2/MGT-type Mg^{2+} transporter family is responsible for low- and high-affinity Mg^{2+} transport in plants and the overexpression of one of its member, *MGT*, in tobacco and Arabidopsis ensures normal growth compared to non-transgenic lines under Mg-deficiency (Deng et al. 2006; Mao et al. 2014; Li et al. 2017; Liu et al. 2019). Current knowledge can explain the Mg pathway comprehensively; however, transgenic studies are very less and existing information is only restricted to MRS2/MGT-type Mg^{2+} transporter family members.

15.4.5 Boron

Boron (B) is an important micronutrient that is involved in plant developmental processes with a structural role in membrane/cell wall (by generating cis-diol complexes) and has a critical role to define final plant biomass (Brdar-Jokanović 2020; He et al. 2021). Plants take boron from the soil through their roots as boric acid. Although many soils are deficient in boron, it accumulates more in saline

soils (Brdar-Jokanović 2020). Current management strategies focus to replace deficient B by applying fertilizer; however, it should be done very carefully since the excess B can lead to toxic effects in plants (Brdar-Jokanović 2020). Under deficient B conditions, meristematic regions in root cells are severely affected and plants become more sensitive to drought and other abiotic stresses (Snowball and Robson 1983). B deficiency can cause failure in seed formation and yield can be negatively affected so plants may not generate any biomass or will have very low biomass (Rerkasem et al. 1993). Besides breeding strategies that target the development of boron efficient crops, some transgenic studies have been also accomplished. These strategies are based on manipulating two main transporters responsible for B uptake in plants: NIP5;1 (NOD26-LIKE MAJOR INTRINSIC PROTEIN5;1) and BOR1 (Takano et al. 2002; He et al. 2021). The overexpression of *BOR1* in Arabidopsis or *NIP5;1* in Arabidopsis and *Brassica napus* could be able to compensate B and these transgenic plants were less likely to show deficiency symptoms under B deficiency (Miwa et al. 2006; Kato et al. 2009; He et al. 2021). Additionally, *nip3;1* mutant in rice had deficient-like symptoms (Hanaoka et al. 2014).

15.4.6 Calcium

Calcium (Ca) is an essential nutrient that mainly has a structural and regulatory role in plants. Ca^{2+} deficiency is not common; however, if plants survive its deficiency, they can show mild or severe symptoms (Simon 1978; Hepler 2005). The deficiency can be visible during the vegetative reproductive stage or after harvest. Fruits may crack easily (almost over 30% cracking in Cape Gooseberry) under low or limited Ca^{2+} concentrations (Álvarez-Herrera et al. 2019). This cracking can result in significant losses in yield and fruit production. Necrosis is a common symptom in young leaves of vegetables like lettuce, cauliflower, artichoke, and cereal (Saure 1998; Rosen 1990; Francois et al. 1991; Koik and Smith 2010; de Freitas et al. 2016). Ca has a key role in abiotic and biotic stress tolerance. Therefore, its deficiency can make plants more susceptible to different stress factors (Dayod et al. 2010).

The prominent role of Ca^{2+} in plants and humans has well-gained importance for transgenic studies. Unlike other micronutrients, Ca^{2+} biofortification using transgenic approaches has plenty of implementation in different crops (Dayod et al. 2010). The overexpression of calcium proton/exchanger (*CAX*) using constitutive *CaMV 35S* promoter has enhanced Ca^{2+} accumulation in tomato, potato, and tobacco (Hirschi 1999; Park et al. 2005; Chung et al. 2010). Ca^{2+} accumulation was as high as 100% in tomato fruit and tobacco root (Hirschi 1999; Park et al. 2005). Transgenic potato tubers accumulated three times more Ca^{2+} than non-transgenic plants (Park et al. 2005).

15.5 Genome Editing Approach for the Enhancement of Nutrient Use Efficiency in Crops

As mentioned above, the nutrient use efficiency of crops can be improved by transgenic technologies. In addition to conventional genetic engineering technologies, genome editing technologies can also be employed to develop crop plants with better NUE. Using CRISPR/Cas9 technology, crops can be improved to develop into elite qualities via knocking out negative regulators through the NHEJ mechanism (Korotkova et al. 2019). Genome editing of plants can be used to scrutinize the role of the genes with high efficacy and specificity, introducing targeted indels or substitution (targeted mutagenesis). With respect to TALENs and ZFNs, their intricate engineering and off-target DNA cleavage, which can arise from domain interactions, have led scientists to move to CRISPR/Cas9 system for their lower cost, simple foundation in designing targets, multiplexing, higher efficiency, and applications in trait discovery and improvement. The different types and variants of CRISPR, including Cas9 orthologues with varied PAM specificities, have led to flexibility in sgRNA design as well as multiple gene targeting (Kumar et al. 2019; Zhan et al. 2021). In addition, ZFNs further confer cytotoxic effects and can re-cleave a site that has been repaired (Papaioannou et al. 2012). Several CRISPR gene-edited crops have been generated to improve or alter nutritional levels in food crops, such as generating sweet orange, sweeter strawberries, attractive/appetizing mushrooms, non-browning apples, low phytic acid-containing maize, improved aroma in rice grains, increased oleic acid in soybean, etc. Of course, these new genome-edited crop lines show a very high potential to solve the malnutrition and/or undernutrition problems in the world. For instance, the further potential of gene-editing technology in highly promising superfood rice-bean (*Vigna umbellate*) (contains high nutritional values for human health) needs to be explored to treat malnutrition in the world (Kaul et al. 2020). For these reasons, genome editing techniques are also used for the improvement of NUE of crops with several promising studies (Table 15.1) as discussed in this section.

15.6 Improvement of Macronutrient Use Efficiency in Crops by Genome Editing Approach

15.6.1 Nitrogen

Several genes have been chosen to conduct gene manipulation in cereal plants to improve nitrogen use efficiency such as ammonium/nitrate transporters involved in pathways of nitrogen metabolism, transcription factors as well as assimilation genes (Tiwari et al. 2020). A study conducted by Li et al. (2020) examined data for meta-analysis from several published papers in determining nitrogen use efficiency to elucidate the reasons behind ameliorated nitrogen use efficiency in wheat, rice, and

Table 15.1 Examples of genome editing for nutrient use efficiency in crops

Gene	Crop	Technology	Nutrient	Inference	Reference
MIR396ef	rice	CRISPR/Cas9	nitrogen	Knockouts exhibited ameliorated grain size and panicle branching. Under nitrogen deficiency, more gains in grain size and biomass in above-ground parts	Zhang et al. (2020b)
NRT1.1B	rice	base-editing system	nitrogen	Improved nitrogen use efficiency	Lu and Zhu (2017)
MYB61	rice	CRISPR/Cas9	nitrogen	*myb61* mutants showed decreased nitrogen use efficiency. *grf4* mutants repressed *MYB61*.	Gao et al. (2020)
OsNramp5	rice	CRISPR/Cas9	cadmium	Low cadmium accumulation	Tang et al. (2017)
OsIRO3	rice	CRISPR/Cas9	iron	*OsIRO3* is imperative for rice survival under iron deficiency	Wang et al. (2020)
FEP1	*Arabidopsis*	CRISPR/Cas9	iron	Suggested role of FEP1 in iron homeostasis for iron uptake	Hirayama et al. (2018)
OsVIT2	rice	CRISPR/Cas9	iron	*vit2* mutants showed increased iron allocation to rice grains/leaf blades	Che et al. (2021)
IMA genes	*Arabidopsis*	CRISPR/Cas9	iron	Silencing caused absence of iron uptake	Grillet et al. (2018)
BnaA9.WRKY47	*Brassica napus*	CRISPR/Cas9	boron	BnaWRKYs has role in plant adaptation under boron deficiency	Feng et al. (2020)
OsZIP9	rice	CRISPR/Cas9	Zinc/iron	Knockouts showed that zinc uptake was reduced, and iron accumulation increased in shoots under zinc deficiency	Huang et al. (2020)

maize after gene manipulations. Apart from the rise in nitrogen uptake efficiency, other factors such as shoot biomass, yield, etc. were increased upon genetic transformation, specifically improved nitrogen use efficiency and yield via transporter genes; however, the study noted a plunge in nitrogen use efficiency of grains and shoots (Li et al. 2020). NUE differences in various studies were not due to methods used such as overexpression/ectopic expression, but mainly occurred due to the type of crop and experimentation, and NUE was not related to overexpression levels of utilized genes (Li et al. 2020). In recent years, CRISPR/Cas9 technology and base editing have been seen as promising tools to improve plant nitrogen use efficiency (Tiwari et al. 2020).

In a study conducted by Zhang et al. (2020b), *miR396* (represses growth-regulating factor) overexpression was found to increase the yield and branching of

panicles in rice. At the same time, knockouts using CRISPR/Cas9 for *MIR396ef* were found to improve grain size and branching of panicles. Under deficiency of nitrogen, the knockouts exhibited further increment in grain size and increased biomass of plant parts above the ground. The study concluded that miR39ef can be targeted for developing varieties that would need reduced nitrogen fertilizers (Zhang et al. 2020b).

Lu and Zhu (2017) demonstrated one instance of base editing in rice performed with a gene affecting NUE. The genes of rice such as *SLR1* (encodes DELLA protein) and *NRT1.1B* (responsible for encoding a nitrogen transporter) were chosen to be edited via the base-editing system. C/T replacement in rice for *NRT1.1B* gene caused amelioration of the NUE, whereas substitution of amino acid in DELLA protein near the motif of TVHYNP could decrease the height of the plant. After the transformation of rice calli, it was observed that the base-editing efficiency of *SLR1* was higher than *NRT1.1B* (Lu and Zhu 2017).

CRISPR/Cas9 technology can also be used to determine the function of a gene, such as in a study conducted by Gao et al. (2020). In this study, it was observed that at depleted nitrogen status, *MYB61* is responsible for improving NUE as well as the production of grains. In their study, they generated mutants of *myb61* (1-bp insertion that generated a stop codon) using the CRISPR/Cas9 system. The *myb61* mutants exhibited a decline in NUE. Further, to show that MYB61 (transcriptional regulator in the biosynthesis of cellulose) along with GRF4 (GROWTH-REGULATING FACTOR4) could have the same regulatory pathway, they generated (loss-of-function) *grf4-1* and *grf4-2* mutants using CRISPR/Cas9 technology. At the same time, it was observed that in *grf4* mutants, *MYB61* was highly repressed. It was concluded from their study that under low nitrogen status, the *indica* allele of *MYB61* in association with *indica GRF4* as well as *MYB61* ameliorated NUE (Gao et al. 2020). Such a study using CRISPR/Cas9 technology can verify the candidate genes that can be suitable in improving nutrient use efficiency in crop plants.

15.6.2 Phosphorous

A study conducted by Lee et al. (2019) showed decreased lateral root growth in rice mutants obtained by targeting the *OsACS* (1-aminocyclopropane-1-carboxylic acid (ACC) synthase) gene that is responsible for rate-limiting ethylene biosynthesis under phosphate-limited conditions, suggesting the role of this gene in phosphate uptake. It is inevitable for the plant to initiate elongation of lateral roots to detect the phosphorous-deficient condition and to uptake maximum phosphorous from the rhizosphere (Lee et al. 2019). Inhibitor(s) or repressor(s) of the *OsACS* gene can be edited to explore their role in the phosphorous uptake mechanism. Another study conducted by Qin et al. (2019) with the use of CRISPR/Cas9 technology in rice generated mutants of *OsDOF15* (DNA-binding with one finger proteins), showing that OsDOF15 interacts with the *OsACS1* gene promoter and positively regulates

the development of rice primary roots. However, this study did not study the nutrient uptake efficiency under nutrient-deficient conditions, which can be explored in future studies given its interaction with *OsACS1*.

15.6.3 Potassium and Phosphorous

The use of the CRISPR/Cas9 system has been performed by Mohammed (2018) to study nutrient use efficiency in rice by knocking out sodium transporters (*OsHKT1;1* and *OsHKT1;4*) in a bid to find its potential role in potassium use efficiency, and *OsHA1* (H^+-ATPase) was knocked out in an expectation to improve uptake of phosphorous. Manipulation of the regulatory domain via CRISPR technology was aimed to elevate H^+-ATPase activity. Approximately 80% of T_0 plants stopped showing wild-type allele of *OsHA1*, with nearly 80% (33) of the tested plants (42) exhibiting biallelic or homozygous mutations. The majority of the mutations were either deletion or insertion of 1 bp. Chimeric plants were also observed in the study. The study observed various truncated sizes of OsHA1 protein with expectations of changes that would influence enzyme activities differently. The authors believe that this would improve the phosphorous uptake. It is also yet to be determined further by genotyping for the two candidates, *OsHKT;1* and *OsHKT;4,* in double knockouts (Karunarathne et al. 2020). In a study conducted by Mao et al. (2018), *OsPRX2* (localization of which is chloroplast) was knocked out using CRISPR/Cas9 technology that led to detrimental effects in the opening of stomata as well as the phenotype of the leaf (causing chlorosis) under the condition of potassium deficiency. The results from the overexpressed *OsPRX2* further corroborated its potential role in the improvement of plants' tolerance to potassium-deficient conditions (Mao et al. 2018).

15.7 Improvement of Micronutrient Use Efficiency in Crops by Genome Editing Approach

15.7.1 Iron

Using the floral dip method in Arabidopsis, Cui et al. (2018) used CRISPR/Cas9 technology to transform Arabidopsis to target the first exon of *bHLH18*, *bHLH19,* and *bHLH20* using different sgRNA combinations and constructs. The indels generated in targeted sites formed frameshifts generating premature termination of translation. The mutants were grown under conditions such as without iron, without iron, and with jasmonic acid, with iron, and finally with iron and with jasmonic acid. Iron deficiency tolerance was observed increasingly through single, double, and triple mutants, respectively, under the condition of without iron and with jasmonic acid. Not much variation was seen in the case of two triple and

quadruple mutants. There were no apparent variations for the conditions of without iron, with iron, and with iron + jasmonic acid, in terms of single, double, triple, and quadruple mutants as compared to the wild types. Triple/quadruple mutant lines showed a greater effect on phenotype as well as FIT accumulation as compared to single/double mutants (Cui et al. 2018). Cui et al. (2018) showed that jasmonic acid in Arabidopsis has a role in negatively regulating the uptake of iron and homeostasis. Jasmonic acid has been shown to repress *FIT* and some *bHLH* genes (transcription factors). The study suggests that jasmonic acid can increase sensitivity to deficiency of iron in plants.

In a study conducted by Wang et al. (2020), it was found that OsIRO3 protein (a bHLH protein, functioning as a repressor in iron homeostasis regulation) was imperative at the time of iron deficiency for homeostasis of iron. Using the CRISPR/Cas9 system, Wang et al. (2020) developed *osiro3* mutants which showed reduced iron deficiency response gene induction in shoots; however, the response genes related to iron deficiency were induced in the roots. The study highlighted the importance of *OsIRO3* in rice plants' survival under the conditions of iron deficiency (Wang et al. 2020). Another study performed by Cai et al. (2021) aimed to scrutinize the crosstalk between signaling networks of copper and iron homeostasis. Using the CRISPR/Cas9 system, Cai et al. (2021) generated *bhlh4x* mutants by knocking out the *bHLH39* gene. The study reported that when copper was applied to mutants of *bhlh4x* and *fit-2* under iron deficiency, plant growth was improved (Cai et al. 2021). Further studies can be performed by regulating a set of *bHLH* genes in improving Fe or other nutrient use efficiency in crop plants.

Hirayama et al. (2018) found that the Arabidopsis *ahg2-1* mutant (*aba hypersensitive germination2-1*; a mutant with ABA hypersensitivity) upregulated genes responsive to the deficiency of iron (including *bHLH38* and *bHLH39, IRT1*, *FRO2*) in RNA sequencing analysis, but *FER1* (gene related to iron overload response) was downregulated. Furthermore, the study suggested the possibility of disturbance of iron metabolism and homeostasis in the case of *ahg2-1* mutants. Further analysis on this mutant is recommended by this study for its role in the metabolism of iron via plant hormones generated by the stress conditions (Hirayama et al. 2018). CRISPR/Cas9-generated *fep1* mutants (*FEP1*: *FE-UPTAKE-INDUCING PEPTIDE1*) exhibited weakened root-to-shoot iron translocation (Hirayama et al. 2018). The study suggested a role of FEP1 in the homeostasis of iron in Arabidopsis for iron uptake (Hirayama et al. 2018). The role of *FEP1* and *AHG2* can be further explored for their roles in iron uptake efficiency in crop plants, with further emphasis on CRISPR/Cas9-generated *ahg2* in future studies.

Che et al. (2021) aimed to understand the mechanisms in iron loading to rice grains via the *OsVIT2* gene (vacuolar iron transporter). CRISPR/Cas9-generated *vit2* mutants exhibited ameliorated iron allocation to rice grains as well as leaf blades, with abundant iron accumulation in grain endosperm and embryos with no effect in polished rice yield. The study found that the CRISPR/Cas9-generated *vit2* had a role in the biofortification of iron in rice. To help with the deficiency of micronutrients in crop plants, biofortification is crucial. This research has an important

aspect in providing iron nutrients via rice grains for humans, especially to cure the problem of anemia caused by the deficiency of iron in humans, as the rice contains less amount of iron which is decreased further in the polished rice (Ansari et al. 2020; Che et al. 2021).

IMA1 and *IMA2* genes were silenced by Grillet et al. (2018) using microRNA constructs to elucidate its role in iron uptake, as well as silencing of all 8 *IMA* genes (IMA: IRON MAN, highly diversified peptides responsible for iron uptake in angiosperms) using CRISPR/Cas9 system in Arabidopsis. microRNA-based knock-down plants showed characteristics similar to the wild type. Silencing *IMA1* and *IMA2* via artificially constructed microRNA generated silenced plants with a decreased level of expression of these genes; however, these plants grew in size similar to that of the controls as well as the health of the silenced plants was similar to that of the wild types. Further, these silenced plants did not affect the induction of iron deficiency. On the other hand, CRISPR/Cas9-generated silencing caused the absence of iron uptake as well as the presence of a huge amount of chlorosis. *IMA* overexpression in Arabidopsis demonstrated genes responsible for iron uptake being induced in roots, leading to more iron accumulation in Arabidopsis seeds and other parts. Their study elucidated the role of IMA in tuning nutrient uptake via soil (Grillet et al. 2018).

15.7.2 Zinc

Lilay et al. (2021) showed that bZIP19 and bZIP23 transcription factors (regulators in response to zinc-deficient condition) have a role in sensing zinc via binding of the motif of zinc sensor to Zn^{2+} ion. Deleting/modifying such a motif can lead to the accumulation of zinc in seeds and other plant parts (Lilay et al. 2021). Their study suggests the implication of the CRISPR/Cas9 system in modulating F-bZIP homologs to ameliorate the uptake of zinc in crop seeds. Huang et al. (2020) used CRISPR/Cas9 technology in knocking out *OsZIP9* in elucidating its role in zinc transport. Its knockout mutation resulted in reduced uptake of zinc absolutely under the condition of zinc deficiency, in line with Inaba et al. (2015) who demonstrated the role of bZIP19 in the expression of *ZIP9* under the condition of zinc deficiency (Inaba et al. 2015; Huang et al. 2020). The knockout mutants of *OsZIP9* under the condition of decreased zinc content exhibited elevated accumulation of iron in shoots. The authors point that the few genes associated with uptake of iron in roots might have been induced due to zinc deficiency in mutants. The study reported that the shoot iron accumulation of *OsZIP9* knockouts did not change when grown in soil. It was suggested by the study that other transporters need to be characterized (such as those which function at higher concentrations of zinc). To elucidate the role in zinc uptake, genes such as *OsZIP1* can also be explored since *OsZIP9* knockout did not result in full abortion of zinc uptake under the condition of zinc deficiency (Huang et al. 2020).

15.7.3 Boron

Feng et al. (2020) found that 51 BnaWRKY transcription factors in *Brassica napus* are involved in response to boron deficiency. Knockout studies using CRISPR/Cas9 system in *B. napus* of *BnaA9.WRKY47* gene demonstrated ameliorated sensitivity to boron deficiency, with reduced boron in mutants as compared to the wild type, while its overexpression improved the plant's adaptation to boron deficiency as well as an increase in boron content as compared to wild-type plants. This study demonstrated the role of BnaWRKYs under boron deficiency and the role of BnaA9.WRKY47 in plant's adaptation to boron-deficient condition by upregulation of *BnaA3.NIP5;1* gene to improve boron uptake efficiency (Feng et al. 2020).

15.7.4 Cadmium

The CRISPR/Cas9 technology can also be used in decreasing the toxic metal concentrations in plants by reducing their uptake from the rhizosphere as demonstrated by Tang et al. (2017). To minimize the health risks of consuming a high concentration of cadmium in rice grains, Tang et al. (2017) developed low cadmium accumulated lines of rice using CRISPR/Cas9 technology by targeting *OsNramp5*, a metal transporter gene responsible for cadmium uptake in rice roots. The study showed that the *osnramp5* mutants in hydroponic culture exhibited a strong decrease in the root and shoot cadmium concentrations. This resulted in absence of impaired growth in the *osnramp5* mutants even under the elevated concentration of cadmium. In the field trials at the paddy fields contaminated with cadmium, grains of *osnramp5* mutants accumulated cadmium lower than 0.05 mg/kg. In the case of the wild-type plant, the cadmium concentration in the grain was as high as 2.90 mg/kg. The study reported that the yield change in the mutants was not compromised as compared to the wild types.

15.8 Improvement of Other Compounds' Use Efficiency in Crops by Genome Editing Approach

15.8.1 Phytic Acid

The study performed by Liang et al. (2014) described the use of both TALENs and CRISPR/Cas9 systems in maize to target *ZmMRP4*, *ZmIPK*, and *ZmIPK1A* involved in phytic acid biosynthesis to minimize the seed phytic acid content. Phytic acid comprises 75% phosphorus in maize seeds which is an undesired compound causing the digestive problem in some animals, and at the same time,

can pollute the environment (Liang et al. 2014). The study targeted four different genes using five TALENs. These genes were *ZmMRP4*, *ZmIPK*, *ZmIPK1A,* and *ZmPDS*. The efficiency of this gene-editing at the protoplast level was reported to be as much as 23.1%. In terms of the somatic mutations obtained in transgenics, the efficiency was between 13.3% and 39.1%. To target the *ZmIPK* gene in the protoplasts, the construction of two gRNAs was performed. The mutation efficiency of gRNA1 was 16.4% and that of gRNA2 was 19.1%. The study saw a closer targeting efficiency in the protoplast study of *Zea mays* of 13.1% in the CRISPR system as compared to 9.1% in TALENs (Liang et al. 2014). Karunarathne et al. (2020) point the potentiality of this study in ameliorating nutrient value in maize (Karunarathne et al. 2020).

15.8.2 Amylose/Amylopectin

Sticky rice or glutinous rice that has negligible or little amylose can be used in brewing. Yunyan et al. (2019) developed two elite varieties of rice using CRISPR/Cas9 technology to knock out the *Wx* gene in Suken 118 (SK118) and Huaidao 5 (HD5). The study received *Wx* gene deletion greater than 200 bp in the six homozygous T_0 plants. Further, they have also reported several homozygous T_1 plants which were also transgene-free. The phenotype of these seeds was opaque and white just like the sticky rice with an amylose content of 2.6-3.2% with no compromise in rice quality (Yunyan et al. 2019). Ma et al. (2015) knocked out the *OsWaxy* gene (responsible for amylase biosynthesis) in rice, which reduced amylose content as much as 2.6% in mutated rice seeds from 14.6% amylose content (cut seeds stained with I_2-KI solution, 1%), similar to glutinous natural rice varieties. In this study, the authors used the Cas9 gene that was codon-optimized for plants. Biallelic and homozygous were major mutagenesis types with 85.4% efficiency obtained after several target sites were aimed (46) in rice (Ma et al. 2015). The CRISPR construct targeted three different sites in the *OsWaxy* gene. Seeds obtained from the T_1 generation of T_0 plants were chosen for analysis which had up to two targeted mutations in this gene. The transformation was performed in the rice cultivar of japonica named Taichung 65 (T65) (Ma et al. 2015).

Amylose-rich crops in association with resistant starch can have health impacts on humans. In a study performed by Sun et al. (2017), rice *SBEI* and *SBEIIb* were targeted. The mutagenesis obtained in this targeted gene editing ranged from 26.7% to 40% with biallelic as well as homozygous mutations. The mutants of *sbeII* showed much elevated debranched amylopectin, higher amylose (25%), and resistant starch (9.8%). In the case of *sbeI* mutants, there was not much variation with the wild type (Sun et al. 2017; Ansari et al. 2020).

15.9 Conclusion

Genome editing has been utilized in many different aspects of crop biotechnology from yield enhancement to stress tolerance. As expected, its implications will be tremendous in the biofortification of plants (Fig. 15.1). As compared to earlier genome editing tools, CRISPR/Cas-based technology shows high promise due to its several advantages. More importantly, this technology is continuously being improved so that the disadvantages such as off-targets cease away. With the advancement of this technology, now it is easier to manipulate multiple genes from the same family or function in the same pathway simultaneously. Also, new Cas variants show high potential for their usage in base-editing and overexpression studies. We can use these technological advancements in developing new cultivars with enhanced nutrient uptake, root system architecture, and root colonization of beneficial microorganisms such as bacteria and fungi. We can also design plants that efficiently translocate and accumulate the nutrients in their green and/or edible tissues. Therefore, we expect to see new crop lines with higher nutrient use efficiency, biofortified with nutrients and vitamins. Moreover, we can now precisely design new varieties that are biofortified with essential minerals, and at the same time, devoid of toxic or unhealthy organic/inorganic materials.

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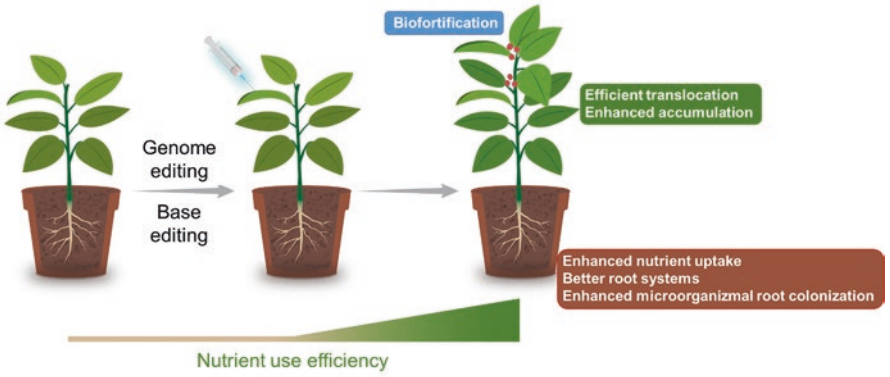

Fig. 15.1 Future implications of CRISPR/Cas-based genome editing in NUE in crops

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Chapter 16
Aromatic Plants as New Candidates in Phytoremediation-OMICS Technology

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16.1 Introduction

The industrial revolution has led to significant improvements in people's socioeconomic conditions, and it has also received a by-product that is environmental contamination and pollution. Environmental pollution is a major cause of concern for global health, as its accumulation of contaminants in complex compartments presents a significant risk for the development of several diseases in humans (Manisalidis et al. 2020). The amount of soil, water, and air pollutants has risen to thresholds that are unhealthy for present generations. Environmental contamination with heavy metals has enhanced well beyond the recommended limit, which is harmful to all life forms. Pollution from heavy metals are undoubtedly a significant environmental concern as metal ions affect the environment due to various nonbiodegradable natural environment (Ali et al. 2019). In particular, any metal (or metalloid) species may also be perceived as the "contaminant" until it appears undesired or in a form or concentration that causes a deleterious impact on humans or the environment. As a consequence, a few soil pollution-based major issues have been evolving which include disorders, the decline in soil fertility and efficiency, biodiversity loss, and natural resource loss. Both anthropogenic and natural sources release heavy metals into soil properties. There are growing public health and environmental concerns due to the noxious effects of these metals, as well as a corresponding need for increased awareness to remedy the polluted heavy metal environment. Accordingly, the removal or reduction of accumulation of heavy metals is imperative to reduce or eliminate environmental contamination and the likelihood of food web uptake. The effective protection and restoration of heavy metal-contaminated soil ecosystems require their characterization and remediation. Remediation methods that are used to clean up contaminated soils of heavy metal(loid) may be in situ; ex situ, on-site; off-site which may include biological, physical, and chemical techniques. All such technologies are used in combined effects with each other to remediate a contaminated site more cost-effectively and effectively (Cristaldi et al. 2017). Phytoremediation is among the most promising ways of rectifying and restoring the natural state of soil deemed harmful to the environment and health and uses microorganisms/plants for detoxification or removal of heavy metals and trace elements from the soil.

16.2 Medicinal and Aromatic Plant Species

The medicinal and aromatic plants (MAPs) are widespread and predominantly gathered for human health care from natural environments. The World Health Organization (WHO) reports that conventional or traditional medicines derived from medicinal plants are still the keystone of about 75–80% of the global population for primary care, particularly in developing countries, owing to better cultural appropriateness, compliance with the human system, and fewer complications. India has a vast heritage of medicinal plants and stands on the well-recorded and quite well-practiced awareness of traditional medicine for the treatment (more than 8000 species). However, India has an abundant tradition of Ayurvedic, Siddha, and Unani medicinal plant usage. The worldwide distribution of medicinal and aromatic plants includes South and South-East Asia, America, Europe, Africa, and Australia. The ethnomedicine industry in India employs more than 7500 plant species, which is half of the native plant species in the nation (Shiva 2011). China contains approximately 6000 species that are used for their medicinal properties (Shan-an and Zhong-ming 1991). Brazil, the South American region's largest country, emerges in unique topographical conditions as well as extensive flora comprising medicinal and aromatic plants. In Brazil, over 48,576 plant species are used for medicinal purpose (Flora do Brasil 2019). Despite the large Brazilian biodiversity (20–22% total worldwide), it includes two of the hottest hotspots; Mata Atlântica or Atlantic rainforest (19,355 species) and Cerrado (12,669 species) (Forzza et al. 2012). In Cerrado (Central Brazilian Savanna), 220 species produce medicinal values and a wide range of native fruits are consumed regularly by the local population and sold in urban centers.

16.3 Aromatic Plants for a Phytoremediation

Aromatic plants are more stress-tolerant and perennial in a natural environment; however, with low input, these crops provide significant importance. Aromatic plants for the phytoremediation of contaminated heavy metal sites were recognized from families – Asteraceae, Poaceae, Geraniaceae, and Lamiaceae. Tropical aromatic grasses produce large biomass and are extensively cultivated for the production of essential oil of high value, such as, Palmarosa (*Cymbopogon martinii* (Roxb.) Watson), Vetiver (*Chrysopogon zizanioides* (L.) Nash), Lemongrass, and Citronella (*Cymbopogon winterianus* Jowitt ex Bor). Such aromatic grasses provide tremendous potential for heavy metal-polluted areas to be phytoremediation. Aromatic grasses have been demonstrated to become more effective targets for the phytoremediation of a variety of contaminants from wasteland, and in addition, impact from environmental to socioeconomic significance (Verma et al. 2014; Pandey and

Singh 2015; Pandey et al. 2019). Many widely planted aromatic types of grass correspond to the Vetiver grass and lemongrass. On the other hand, the recent endorsement of aromatic grasses for phytoremediation is because of its economic benefit, adaptability, effectiveness for remediation, and marginal pollution of marketable goods (Table 16.1). The importance of OMICS technologies and their impact and applicability of aromatic plants contribute to phytoremediation, which has been discussed in detail in this chapter.

16.3.1 Cymbopogon

Cymbopogon (Nees ex Steud.) has a huge proportion of odoriferous organisms throughout the family of grasses (*Poaceae*) around 50 other species, but it is categorized as aromatic plant with essential oils (70%–90% citrol). Within the grass family, Lemongrass is widely used as a medicinal herb. Lemongrass oil is among the most valuable essential oils broadly used in perfumes, cosmetics, and flavors. Aromatic grasses are metal accumulators or metal stress-tolerant species that are therefore effective for the phytoremediation of metal soils (Verma et al. 2014; Pandey and Singh 2015). *Cymbopogon citratus* (D.C.) Stapf., commonly referred to as lemongrass, is a metal plant tolerant to adverse environmental conditions (Das and Maiti 2009). Gautam et al.(2017) evaluated the research study utilizing lemongrass grown in different RM (Red mug) treatment options in soil modified with livestock manure to ascertain the physicochemical characteristics of the soil under various soil treatment conditions, to evaluate the likelihood for phytoremediation with lemongrass, as well as to evaluate the effects of metals mostly on the productivity of crop production under various soil treatment options. From these studies, the authors observed that Lemongrass acted as potential phytostabilizer of Fe, Mn, and Cu, whereas Al, Zn, Cd, Pb, Ni, Cr, and As from roots to shoot were found to be efficiently translocated cultivation and could be used to phytoremediation of RM-contaminated sites. Moreover, Jha and Kumar (2017) reported *Cymbopogon flexuosus'* applicability to remove hexavalent chromium and arsenic from aqueous media. The authors (Lee et al. 2014) studied the potential of *Cymbopogon citratus* (*C. citratus*) as an effective alternative bio sorbent for removing nickel (Ni^{2+}) from aqueous solution. In another study, Lermen et al. (2015) observed that *arbuscular mycorrhizal fungi* (AMF) *Rhizophagus clarus* are inoculated in the lemongrass uptake of heavy metal such as Pb (100 kg^{-1} soil) and its metabolites. However, without AMF inoculation, the substantial changes were not ascertained in the production of essential oil content in lemongrass, the values being estimated 0.5% distinct of the Pb levels together with the soil. Aromatic grasses are metal accumulators or metal stress-tolerant plants and are useful for the phytoremediation of metallic soils.

The species *C. flexuosus* has been evaluated for its ability to phytoremediation and its potential role in stabilization of chromium (Cr^{+6}) contaminated soil. Lemongrasses displayed distinguishing properties of proline accumulation in the

Table 16.1 Essential oils of aromatic plants

Common names	Scientific names	Main components of essential oil	References
Lemon grass	*Cymbopogon citratus*	Citral α, citral β, nerol geraniol, citronellal, terpinolene, geranyl acetate, Myrecene and terpinol methylheptenone	Patra et al. (2018a)
Geranium	*Pelargonium*	citronellol, geraniol	Pohlit et al. (2011)
peppermint	*Mentha Piperita* L.	Menthyl acetate, cineole, limonene, beta-pinene and beta-caryophyllene	Pandey et al. (2020)
Spearmint	*Mentha spicata* L.	Carvone, limonene, cineole pinene *cis*-dihydrocarvone, and dihydrocarveol	Chrysargyris et al. (2019)
Chamomile	*Matricaria chamomilla* L.	terpenoids, flavonoids, and lactones, including matricin and apigenin.	Mann and Staba (1986)
Palmarosa	*Cymbopogon martini*	Geraniol, geranyl acetate, and linalool	Rao (2001)
Clary Sage	*Salvia sclarea* L.	Linalool, linalyl acetate geraniol, geranyl acetate, terpineol, nerol, neryl acetate, and sclareol	Hayet et al. (2007), Džamić et al. (2008)
Culinary sage, common garden sage, or garden sage	*Salvia officinalis* L.	Thujone, camphor, pinene, humulene, camphene, pinene, limonene, bornyl acetate, linalool, and cineol	Angelova et al. (2016)
Genus	*Lavandula* L.	Major component linalyl acetate, linalool, tannins and caryophyllene, with lesser amounts of sesquiterpenoids, perillyl alcohols, esters, oxides, ketones, cineole, camphor, beta-ocimene, limonene, caproic acid, and caryophyllene oxide	Baptista et al. (2015)
Spike lavender	*Lavandula latifolia Medik*	Cineole, linalool, and camphor	Afonso and Franco (2013)
Fernleaf lavender	*Lavandula multifida* L.	Linalool, camphene, linalyl acetate, thujene, bornyl acetate, caryophellene, nerol, and terpinolene	
Green lavender or white lavender	*Lavandula viridis*	Pinene, camphene, cineol, linalool, camphor, borneol	
species of tree in the family Fagaceae	Lithocarpus gracilis	Carvacrol, ρ-cimene, and thymol	Matos (2007)
Oregano	*O. vulgare*	Monoterpenoids and monoterpenes and monoterpene hydrocarbons, carvacrol, oxygenated monoterpenes, fenchyl alcohol, terpineol and thymol	Teixeira et al. (2013)
Oregano	*Origanum heracleoticum L.*	Carvacrol, thymol, and phellandrene	Zheljazkov et al. (2008)

shoot and root under high levels of soil present in chromium using metal stress tolerance capacity (Patra et al. 2018a). Subsequently, the research with manure in the farmyard has proven its efficacy by boosting the growth of *C. citratus* holding a high ratio of root: shoot. These can be applied effectively in the area for the regeneration of fly ash dumps without even any possibility of pollution of groundwater. Furthermore, stress-resistant, effective biomass production and rapidly expanding species of plants are promoted for the restoration of fly ash dump sites, which will help to establish rapid natural vegetation (Maitti and Prasad 2017). Consequently, fly ash with soil modification can provide correction of these limitations and an effective mixture to promote plant growth with reduced metal toxicity risk(Verma et al. 2014). Low levels of fly ash can only be used to modify the soil for a short period; however, persistent using fly ash can cause significant contamination of the soil by raising the load of toxic metals (Panda et al. 2018). In addition, lemongrass may be an appropriate species to develop as a cover crop in low and alkaline soils that can sustain steep slopes and reintegrate mining and processing disposal sites or wasteland (Pandey and Singh 2015). Additionally, Gautam et al. (2017) conducted an assessment of the impact of different concentrations of red mud (5 and 10%) in soil adjusted with sewage sludge on the material and chemical properties of lemongrass-derived essential oil. Furthermore, the content of Fe, Zn, Cu, Cd, Ni, and Pb in the above-ground parts of plants increased, whereas Mn was observed under WHO allowable limits for edible herbs. Chemically induced phytoextraction was proposed due to the obvious lower growth efficiency and low biomass production. Elevated biomass and high growth crops are used in this methodology to retrieve substantial amounts of heavy metals whose strength in the soil increased by chelating agents such as EDTA, DTPA, and CA generally increasing the solubility, absorption of metals, and stimulated growth into the lemongrass plants. Patra et al. (2018b) evaluated the analysis and indicated enhanced bioavailability of Chromium (50 mg/kg) bio to chelator and metal ion (Fe, Mg and Zn) applications by reducing the dependence of heavy metals contamination in contaminated soil.

16.3.2 Vetiver Grass

Vetiver (*Crysopogon zizanioides*) is a plant that is a species of the Poaceae family. Since it grows well in sandy loam soil, it is a perennial plant. The word "vetiver" is a Tamil word ((வெட்டிவேர்)) describing vetti" (to cut/dug up) and "ver" (root), "root that is dug up." The vetiver has been in use in India since ancient times. Vetiver is predominantly produced in tropical countries. Old Tamil literature mentions vetiver use for medical applications. The plant is cultivated because of its aromatic root system, an origin of essential oil widely recognized as vetiver oil. Also, it is defined as "Tranquility Oil" and used in aromatherapy and perfumery. However, few variations with vetiver prevalent in northern and southern India were observed.

Along with its notable characteristics, including a tremendous and profound root system and sensitivity to drastic weather differences like continuous drought, flooding, frost, and heatwaves, vetiver grass is especially appropriate for phytoremedial implementations and also for processing biosorption. It can allow high soil salinity, alkalinity, acidity, and elevated concentrations of heavy metals such as Al, Mn, Mg, As, Cr, Hg, and Cu and the pesticides and herbicides in soils. Vetiver is a $C_{4,}$ whereas a four-carbon chemical called the first product is Oxaloacetic acid (OAA), a persistent organism that grows in tropical climates. This species is best able to continue providing facility as a model to the ecosystem and due to the various unique characteristics that have a significant opportunity for carbon sequestration along with remediation potential. Vetiver has applications worldwide to overcome numerous environmental problems such as soil erosion, soil remediation, and water pollution. Suelee et al. (2017) stated which vetiver grass (VG) is used in polluted water suitably for heavy metals. The removal efficiency of heavy metals such as Cu, Fe, Mn, Pb, and Zn in water was assessed in this method by different root lengths and densities from VG. Likewise, metal uptakes in vetiver root were usually higher than in shoot. The plant is a strong heavy metal accumulator and the root portions of the plant acquired a large proportion of metals than the shoot, suggesting that VG is a strong phytostabilizer (Banerjee et al. 2016); also, they exhibited increased metal concentrations improving catalase, guaiacol peroxidase, and glutathione activities. Environmental contamination by antibiotics such as Ciprofloxacin (CIP) and tetracycline (TC) are broadspectrum antibiotics, not just to disturb the ecosystems and raise a hazard to public health through accelerating the development of multiantibiotic-resistant bacteria. Since the abundance of pharmaceutical products is increasingly released into the environment, research is underway to investigate different methods for their remediation. Datta et al. (2013) recommended to remove and degrade TC from aqueous solutions and soil, utilizing vetiver grass as an agent for phytoremediation under hydroponic conditions within 40 days. The high root to shoot translocation factors (TF) in higher TC treatments (10 and 15 mg L^{-1}) demonstrate the great possibility of vetiver grass. Constructed wetlands (CW) are widely recognized as a promising technology among phytoremediation techniques with the best efficient wastewater treatment technology. The current study has shown that wetlands built from integrated vertical flow have a significant potential for natural products, nutrient-dissolved particles (nitrogen, phosphorous), and pathogen removal owing to their special redox-reducing environment, microbial growth, and community dispersion. Some evidence suggests that *Vetiveria zizanioides* is used effectively inbuilt wetlands for waste management, has quite a great potential to a reduction of chemical oxygen demand (COD) (80.65%), Total Kjeldahl Nitrogen (TKN) (91.44%), total suspended solids (TSS) (98.34%), free and saline ammonia (98.95%), but also has a considerable effect on the water quality improvement (Suelee et al. 2017; Badejo et al. 2018).

16.3.3 Basil (Ocimum Species)

The genus Ocimum, a member of the family of Lamiaceae, features prominently among many of the herbs with therapeutic potential. Tulsi (The queen of herbs), also widely recognized as "holy plant" or "elixir of life", is native to the Indian continent and is highly respected for its medicinal properties and within Ayurvedic and Siddha medical systems. Among some of the distinct Ocimum species, *Ocimum basilicum* L. "Basil/Sweet basil" (common name in Tamil language, Naitulasi), *Ocimum sanctum* L. or *Ocimum tenuiflorum* L. "Holy basil" (common name in Tamil language, Tulasi) (Samy et al. 2008) also known as Tulsi in India, and *Ocimum gratissimum* L. Basil plants with 22–88% oestragole may be used efficiently for aromatherapy. Additionally, phenolic compounds including rosemarinic and cynamic acids flavonoids and essential oils and saponins anthocyanins were among the major sweet basil biochemicals (Filip et al. 2017).

Heavy metals like Cd, Cu, and As can prevent the germination of seeds at even small concentrations. However, contaminated soils with Cd and Pb had a major impact on the growth of sweet basil. Fattahi et al. (2019) suggested the amount of essential oil (EO) yield generated under heavy metal stresses and those EO components with a high correlation with the quantity of contaminations of the Cd and Pb. Likewise, Siddiqui et al.(2013) reported that an increase in essential oil content could be directly linked to a decrease in primary metabolites in addition to the impacts of heavy metal stress. Soil bioaugmentation with heavy metal phytoremediation bacteria is a promising approach to cleaning contaminated soil. Prapagdee and Khonsue (2015) described that the highest percentage of cadmium removal was found in the soil that was used to grow *O. gratissimum L* treated with EDTA followed by an inoculated plant with *Arthrobacter sp.* Kunwar et al. (Kunwar et al. 2015) studied that heavy metal addition such as Cu, Cd, and Pb leads to an enhanced level of linalool and EO composition. This important relation demonstrates the ability to provide *O.basilicum* with EO quality through the implementation of opportunities for improvement of these metals. In a recent study, *O.basilicum* demonstrated its feasibility for the phytoremediation of soil contaminated with Cd, which was further strengthened when different varieties of fertilizers (Potassium nano-chelate) were supplied to the plants (Zahedifar et al. 2016). In another experiment, Chand et al. (2015) reported that root accumulation of Cd and Pb acceptance by *O. basilicum* significantly increased at 20 t ha^{-1} sludge use. Nevertheless, Cr accumulation increased with a sludge dose of up to 50 t ha^{-1}. The yield of basil essential oil (*Ocimum basilicum*) decreases with maximum dosages of assessment of sludge. Recently, Dinu et al.(2020) observed that basil plants were exposed to a combination of heavy metals that could significantly decrease the mobility of metals from soil to plants. The existence of other metals in contaminated soil had a stimulating effect on the growth of plants evaluated by the amount of vegetable biomass.

16.3.4 Geranium

The genus Pelargonium belonging to the family Geraniaceae consists of approximately 270 species, of which only 10 species are used for geranium oil production; commercially grown rose-scented geranium in various parts of the world such as Egypt, Brazil, China, Russia, India, and Mideast, European countries. Currently, it grows all over the globe and is grown in herb gardens in a variety of nations, mostly for its mosquito repelling properties (Pohit et al. 2011). Geranium crop was grown in the environment that would allow a high rate of photosynthesis as well since oil yields are derived by herb yield and oil content. Rose-scented geranium, *Pelargonium sepulchre L.*, is an aromatic plant. Geranium essential oil (GEO) is retrieved from geranium leaves and is considered to be essential both for fragrance and cosmetic formulations as well as for the food industry. Other benefits of essential geranium oil, which has become more prominent, usually involve dysentery treatment, inflammation, haemorrhoids, menstrual disorders, and cancer (Dimayuga 2004; Tajkarimi et al. 2010). Besides this, similar botanical terms are also in use, including certain *Pelargonium graveolens* and *Pelargonium. roseum*. Recently, Chand et al. (2016) identified that response to the stress of heavy metals demonstrated that geranium is most sensitive to chromium accompanied by Cd, Ni, and Pb in terms of its yield of EO and the concentration of heavy metals in plant parts at its increased soil concentration. Arshad et al**.** (2016) investigated the potential of Pb as a relation to organic species in roots and the binding with cysteine and glutathione indicates the possible drawback of phytochelatins and metallothiones, which can also play an essential part in metal accumulation and plant tolerance.

16.3.5 Mentha Genus

Mentha Piperita belongs to the *Lamiaceae* family, a popular herb called peppermint, that can be used in many aspects, including leaf extract oil and leaf water (Pandey et al. 2020). There are approximately different mentha species native to India, which include *M. arvensa, M. piper, M. speck, M. waters, M. sylvestris, and M. citrus. M.Piperita* (pepper mint) is a major crop-producing essential oil that contains menthol, menthofuran, menthone, 1,8 cineole, isomenthone, etc. Chelating agents are used to significantly boost nutrient and heavy metal bioavailability for plant growth through the roots. Recently, Khair et al. (2020) proposed that *M. Piperita* could be a potential candidate for Ni-contaminated wastewater and soil phytoextraction technique once integrated with citric acid as suitable chelating agents. In addition, higher concentrations of Ni and accumulation according to CA application in plants and all parts (stem, root and leaves) were evaluated. Amirmoradi et al. (2012) reported that with a medium range of Cd and Pb concentrations, the essential oil percentage was not affected. Hasanpour et al. (2019) reported that *Mentha aquatica L* roots have a much greater capacity to uptake and accumulate Cd

than shoot. In another study, Cu addition extended in commercial cultivation (both in soil and soilless production systems) as a process of enhancing the content of active substances in spearmint plants (*Mentha spicata L*) essential oil without altering the essential oil yield (Chrysargyris et al. 2019)**.**

*16.3.6 Chamomile (*Matricaria sps*)*

Chamomile (*Matricaria chamomilla* L.) is a very well-known Asteraceae family of medicinal plants often referred to as "star among medicinal plant species." The flower *Chamomilla* contains 0.2 to 1.9% of the blue essential oil, which is used in a variety of ways (Mann and Staba 1986). Chamomile contains various classes of bioactive components that have been extracted and used as medicinal products and cosmetic products. Chamomile's most well-known active molecules involve luteolin, apigenin, α-bisabolol, quercetin, matricin, and α-bisabololol oxides A and B. Chamomile also contains a high proportion of coumarins, hydroxycoumarins, and terpenoids and is also one of the richest food sources of antioxidants (Bigagli et al. 2017). Typical applications include acne diagnosis, ulcers, digestive disorders, wounds, stomach ache, gastroenteritis, skin rashes, and rheumatoid arthritis (Sanjay et al. 2010). According to Stancheva et al. (2014), chamomile grown from heavy metals-contaminated soils effectively accumulated cadmium, lead, and zinc, with significantly higher potential for cleaning polluted sites, without even a decrease in essential oil quality and yield. Phytoavailability evaluation of cadmium and lead in contaminated soil and accumulation by *chamomilla matricaria*. Throughout the case of cadmium, which were greater similarities among its presence of various plant parts (flowers, stems and roots) and also between the content of cadmium in chamomile sections and all soil derivatives. This demonstrates that Cd is dispersed on the soils in forms easily extractable, both with acetic acid and EDTA, which would provide a reasonable estimate of possible cadmium mobility from soil to plant and of the danger for cadmium pollution, respectively (Voyslavov et al. 2013).

*16.3.7 Rosemary (*Rosmarinus Officinalis**)**

Rosmarinus officinalis, popularly called rosemary, belonged to the family Leguminosae and is native and very widespread in the Mediterranean basin native herb with an extreme pinewood indicative of aromatic odor and is recognized for a wide range of applications and biological properties (Oliveira et al. 2019). The chemical properties of rosemary EO with various chemotypes are quite variable depending on the specific percentages of 1,8-cineol (12%), camphor (14.5%), α-pinene (8.5%), borneol (10.5%), and camphene (7%) (Satyal et al. 2017). Bozdogan et al. (Bozdogan Sert et al. 2019) showed that *R. officinalis* is a useful tool for an estimate of the concentration of pollution associated with traffic in urban

areas. In addition, this plant could be capable of accumulating Al, Cd, Cr, Cu, Fe, Mn, Pb, and Zn in both leaves and stems. (Abbaslou et al. 2018) demonstrated that rosemary was evaluated and resulted in an endeavor to remediate mine tailing sites with significant concentrations of Cd, Pb, Cu, and Zn. Research has also shown that roots are more capable of utilizing and extracting elements from contaminated soils, and also that leaves may be better included as herbal medicine for rosemary plants. Nevertheless, plants adsorbed metals by phytoextraction and phytostabilization, where root phytostabilization was more effective.

16.3.8 Cymbopogon Martini

Cymbopogon martini is a perennial aromatic herb of the Poaceae family that produces essential oil in its flowering shoot. The essential oil (Commonly known in India as rosha grass oil) is effective in geraniol, which is used to make high-grade fragrances of cosmetic aromas and pharmaceutical products (Rao 2001). Generally, phytoremediation of sewage sludge is known to contain heavy metal by greater proportion biomass and essential oil-producing palmarosa aromatic crop. Palmarosa acts as an excellent plant for phytoextraction, as well as the essential oil derived from the plant had no risk of contamination by heavy metals. Expanding the aromatic grass in soil modified by sewage sludge could provide a cost-effective, long-term, and environmentally sustainable solution to minimize the risk of pollution from heavy metals (Singh et al. 2020). Likewise, Palmarosa was found to be an acceptable product to the phytostabilization of metals when grown in tannery sludge mixed soil. Overall, tannery sludge increased crop yield, and accumulation of metals existed in roots with a meager translocation to shoots. That also implies this plant‘s suitability as a good phytostabilizer (Pandey et al. 2015).

16.3.9 Sage

Salvia sclarea L., Lamiaceae, (clary sage) are 60–100 cm tall with large hairy leaves and small blue, white, or purple flowers. The plant is native to countries in southern Europe and northern Africa, including Italy, France, and Morocco, being grown in these countries to obtain essential oils and other compounds used in perfumery (Hayet et al. 2007; Džamić et al. 2008). Its inflorescences present in the fresh state 0.15% to 0.20% essential oil. Sage oil is obtained by steam distillation because the distillation of water reduces the oil yield and the content of the esters in it. The analysis of essential oils obtained from *S. sclarea* inflorescences and leaves has been reported in the literature(Yadav et al. 2010). The essential oil of *S. sclarea* has a low viscosity, transparent, colorless to pale yellow, liquid with a characteristic smell of similar inflorescences lavender, bergamot, and amber and has a long-lasting effect, besides having a strong fixation, being used in perfume and cosmetics

industry. The major constituents present in the essential oil of *S. sclarea* leaves in vivo were, in decreasing order, germacrene D (51%), E-karyophylene (12%), γ-gurjunene (10%), α-copaene (6%), and δ-element (3%). In the seedling leaves in vitro, the two major sesquiterpenes were germacrene D (15%) and E-karyophylene (3%). The predominance of germacrene D compared to other constituents of the leaves of this species had already been observed by other authors (Carrubba et al. 2002). *Salvia sclarea* is a plant species that is tolerant of heavy metals and can be attributed as hyperaccumulators of Pb and accumulators of Cd and Zn, having its successful use when used in phytoremediation of soils contaminated with heavy metals. The amounts of Pb, Cu, and Cd in the essential oil of *S. sclarea* grown in contaminated soils were less than the maximum acceptable concentrations accepted, which allows its use in the perfumery and cosmetics, and tobacco industries (Angelova et al. 2016). The essential oil of *Salvia officinalis* L. contains composition of oxygenated monoterpenes, monoterpenic hydrocarbons, and sesquiterpene hydrocarbons. *Salvia officinalis* L. demonstrated growth in soils enriched with lead, cadmium, and zinc; the tolerance of these metals reflects the accumulative and translocating capacity of PB, Cd, and Zn (Angelova et al. 2016).

16.3.10 Lavandula

The genus *Lavandula* L. belongs to the Lamiaceae family whose plants present themselves as aromatic subshrubs that can measure between 20 and 170 cm in height depending on the species. The species-genus are characterized by the presence of a chalice (8-)13(−15)-nervous, whose upper dentition presents, normally, an apical appendage, and by a bilabiated corolla with coloration between pale purple and blue-violet, whose upper lip is bilobed and the lower lip is trilobed (Figueiredo et al. 2014). The genus *Lavandula* L. comprises about 39 species, some of which are used for centuries, not only because of their interest in perfumery, food, and cosmetics, but also due to biological activity and, consequently, action therapy (Baptista et al. 2015). Essential oils from *Lavandula* L. have therefore been used traditionally for various purposes. There are descriptions related to a potential activity antispasmodic in intestinal and uterine smooth muscle, antifungal, and antibacterial which, for times, has shown efficacy even against strains resistant to conventional drugs. The genus *Lavandula* L. has a complex taxonomy, with designations scientific knowledge, which sometimes makes it difficult to analyze existing scientific information about these plants. There are five species of *Lavandula* L., namely *Lavandula latifolia* Medik., *Lavandula multifida* L., *Lavandula pedunculata* (Mill.) Cav., *Lavandul stoechas* subsp. *Luisieri* (Roseira), and *Lavandula viridis* L'Hér; also exist are cultivated species, namely *Lavandula dentata* L., for their value ornamental, and *Lavandula angustifolia* Mill. and some hybrid species due to their industrial interests (Afonso and Franco 2013). Heavy metals accumulate in different

ways in vegetative organs of lavender species. Lavender accumulates heavy metals through the root system, leaving low retention in the roots. In addition, the movement of metals and their accumulation more readily in the above-ground parts (stems, leaves, and petals). Generally, lavender species are highly tolerant of heavy metals; therefore, they are Pb hyperaccumulators and Cd and Zn accumulators and can be successfully used in the phytoremediation of soils polluted by these heavy metals (Angelova et al. 2015). In addition, lavender plant species (*Lavandula dentata* L) have phytomanagement capability in soils at maximum Cr concentration levels; cost-effective biochar and citrus peel waste were incorporated as soil modifications owing to their higher capacity to respond quickly directly or indirectly with heavy metals in the soil atmosphere, consequently lowering Cr uptake bioavailability (Ye et al. 2020).

16.3.11 Lippia

The genus *Lippia*, the second largest in the Verbenaceae family, has approximately 200 species of herbs, shrubs, and small trees, whose largest centers of dispersion are found in countries in South and Central America, as well as in territories in tropical Africa. The main centers of specific diversity of the *Lippia* genus are in Mexico and Brazil (Pascual et al. 2001). *Lippia gracilis* S. is an aromatic plant endemic to northeastern Brazil, typical of semiarid vegetation, popularly known as "Alecrim-da-chapada" or "Alecrim-serrote". It is found predominantly in the states of Bahia, Sergipe, and Piauí. It presents itself as a shrub approximately 2.5 cm high, well-branched, with small leaves and white flowers, both very odorous (Sairam and Tyagi 2004; Oliveira et al. 2007).

L. gracilis leaves are rich in essential oil with significant antimicrobial activity against fungi and bacteria. In addition, they are widely used in throat and mouth infections, vaginal problems, acne treatment, white cloths, impigens, dandruff, burns, and wounds (Matos 2007). Several results are referring to the chemical composition of *Lippia* species which is known widely for the presence of volatile oils. However, other minor compounds such as flavonoids, iridoids, and naphthoquinones are also often cited. The major essential oil components were analyzed by Gas Chromatography coupled with Mass Spectrometry (GC-MS); it is clear that limonene (1), β-karyophylene (2), *p*-cymene (3), camphor (4), linalool (5), α-pinene (6) and thymol (7) are the components that appear most frequently (Gomes et al. 2011). *L. gracilis* was able to accumulate Pb extracted from the soil and stored in the vegetable, without a change in oil production, signaling the possibility of being used for program remediation of areas contaminated by Pb using the phytoextraction technique. The content of *L. gracilis* essential oil was not influenced by exposure to Pb (Brandão 2016).

16.3.12 Origanum

Origanum vulgare L. (OV), also known as "oregano" or "marjoram", is an aromatic plant commonly used as culinary herb of the Lamiaceae family and is popular across Asia, Europe and Northern Africa. There are other origanum species, such as, *O. microphyllum*, *O. ramonese*, *O. heracleoticum,* and *O. scabrum*. *O. Vulgare* is used as folk medicine and to treat respiratory problems, dyspepsia, menstrual cramps, muscular dystrophy, and urinary infections. OV essential oil (EO) mainly consists of monoterpenoids and monoterpenes (53.8%) and monoterpene hydrocarbons (26.4%), carvacrol (14.5%) oxygenated monoterpenes, β-fenchyl alcohol (12.8%), δ-terpineol (7.5%), and thymol (12.6%)(Teixeira et al. 2013). Accordingly, OVEO showed antispasmodic, anti-inflammatory, antibacterial, diaphoretic, carminative, and analgesic functions (Faleiro et al. 2005; Souza et al. 2007; Tommasi et al. 2009). Phytoremediation effectiveness depends on the metal bioaccumulation efficiency of the specified plant species which is related to the soil contamination reduction. Currently, Levizou and coworkers investigated Cr phytoremediation of *Origanum vulgare* (Levizou et al. 2019). The oregano roots showed the highest identified Cr (VI) (4300 mg kg^{-1}) bioaccumulation value for soil-checked plant species. In some medicinal plants grown in heavily metal-contaminated soil, the bioavailability factor (BF) raised with decreased cadmium concentrations in soils. Zheljazkov et al. (2008) detected cadmium, lead, and copper mainly in roots, while large quantities of manganese and zinc were detected in *Origanum heracleoticum* L. and other plant leaves and it could be cultivated on soils contaminated with heavy metal without contamination of the commercial product, essential oil (Table 16.2).

16.4 Metabolomics Research Within Analytical Technologies in Phytoremediation

Genetic studies through genomics and metagenomics and the further achievements acquired by proteomics have offered a great contribution to the understanding of various kinds of diseases and its processes. However, biological processes function via a complicated network of connections between genes, RNA, proteins, enzymes, and metabolites, which may also describe this complex interaction network as an interactom (Dunn 2011). Furthermore, environmental factors may alter the interaction and subsequent biological processes of living organisms (Fig. 16.1), as it is observed for human metabolic diseases such as type 2 diabetes mellitus and the development of atherosclerosis.

Furthermore, genome and proteome research is just an aspect of the picture and ultimately provides an ideal pathway to creating novel therapies and monitoring impacts for both pathogenesis and environmental activities. The urge to comprehend this complex interaction network led to the creation of biological systems of novel research techniques, such as metabolomics, that investigate endogenous and

Table 16.2 The phytoremediation potential of different aromatic plants against various elements

Aromatic plants	Trace elements studied	Results	References
Lemongrass	Fe, Mn, Cu, Al, Zn, Cd, Pb, Ni, Cr, and As	Lemongrass acted as a potential phytostabilizer, whereas Al, Zn, Cd, Pb, Ni, Cr, and As from roots to shoot were found to be efficiently translocated and could be used to phytoremediation of RM contaminated sites.	Gautam and Agrawal (2017)
lemongrass	Pb	Aromatic grasses are metal accumulators or metal stress-tolerant plants and are useful for the phytoremediation of metallic soils	Lermen et al. (2015)
Lemongrass	Cr^{+6}	Lemongrasses displayed distinguishing properties of proline accumulation in the shoot and root under high levels of soil present in chromium using metal stress tolerance capacity	Patra et al. (2018b)
Lemongrass	Fe, Zn, Cu, Cd, Ni, Pb, and Mn	Other elements in above-ground parts of plants increased, whereas Mn was observed under WHO-allowable limits for edible herbs. Chemically induced phytoextraction was proposed due to the obvious lower growth efficiency and low biomass production	Gautam and Agrawal (2017)
Lemongrass	Cr, Fe, Mg, and Zn	Enhanced bio-availability of chromium and metal ion applications by reducing the dependence of heavy metals contamination in contaminated soil	Patra et al. (2018a)
Vetiver grass	Al, Mn, Mg, As, Cr, Hg, and Cu	Vetiver has applications worldwide to overcome numerous environmental problems such as soil erosion, soil remediation, and water pollution	Truong and H BHMEC (2001)
Vetiver grass	Cu, Fe, Mn, Pb, Zn	The plant is a strong heavy metal accumulator, and the root portions of the plant acquired a large proportion of metals than the shoot, suggesting that Vetiver grass is a strong phytostabilizer	Suelee et al. (2017)
Vetiver grass (*Vetiveria zizanioides* L. Nash)	SO_4 , Cl, Na, K, Mg, Ca	Assessed the potential efficiency of vetiver system phytoremediation in Mine wastewater for innovative water treatment and salinity reduction	Keshtkar et al. (2016)
	Cl, Na, PO_4	A new and novel phytoremediation technique is used for wastewater treatment	Maharjan and Pradhanang (2017)
	Cr $_{(5\ ppm)}$ (87%), Cr $_{(10\ ppm)}$ (51%)	Indicated vetiver grass' phytoextraction of chromium and hyperaccumulator capability for other heavy metals	Masinire et al. (2020)
	NH_3, NO_2, NO_3, NH_4, PO_4	Compared the efficacy of various vetiver planting densities in reducing nitrogen and phosphorus from tilapia wastewater in aquaponic recirculating systems	Effendi et al. (2020)
	NO_3, Cl, PO_4, K.	Utilizing *V. zizanioides* as a phytoremediator in tofu wastewater treatment with zeliac	Seroja et al. (2018)

(continued)

Table 16.2 (continued)

Aromatic plants	Trace elements studied	Results	References
Sweet basil	Cd and Pb	The amount of essential oil (EO) yield generated under heavy metal stresses, and those EO components with a high correlation with the quantity of contaminations of the Cd and Pb	Fattahi et al. (2019)
Basil	Cu, Cd, Pb	Heavy metal addition such as Cu, Cd, and Pb leading to an enhanced level of linalool and EO composition.	Kunwar et al. (2015)
Basil	Cd	*O. basilicum* demonstrated its feasibility for the phytoremediation of soil contaminated with Cd	Zahedifar et al. (2016)
Basil	Cd, Pb,Cr	Root accumulation of Cd and Pb acceptance by *O. basilicum* significantly increased at 20 t ha^{-1} sludge use. Nevertheless, Cr accumulation increased with a sludge dose of up to 50 t ha^{-1}	Chand et al. (2015)
Pelargonium	Cr, Cd, Ni, and Pb	The stress of heavy metals demonstrated that geranium is most sensitive to chromium accompanied by Cd, Ni, and Pb in terms of its yield of EO and the concentration of heavy metals in plant parts at its increased soil concentration	Chand et al. (2016)
Pelargonium	Pb	The potential of Pb was a relation to organic species in roots, and the binding with cysteine and glutathione indicates the possible drawback of phytochelatins and metallothiones, which can also play an essential part in metal accumulation and plant tolerance	Arshad et al. (2016)
Peppermint	Ni	*M. piperita* could be a potential candidate for Ni-contaminated wastewater and soil phytoextraction technique once integrated with citric acid as suitable chelating agents	Khair et al. (2020)
Mentha genus	Cd, Pb	Medium range of Cd and Pb concentrations, and the essential oil percentage was not affected	Amirmoradi et al. (2012)
Water mint	Cd	*Mentha aquatica L* roots have a much greater capacity to uptake and accumulation Cd than shoot	Hasanpour et al. (2019)
Spearmint	Cu	Cu addition extended in commercial cultivation (both in soil and soilless production systems) as a process of enhancing the content of active substances in spearmint plants (*Mentha spicata L*) essential oil without altering the essential oil yield	Chrysargyris et al. (2019)
Chamomile	Cd, Pb, Zn	Chamomile grown from heavy metals contaminated soils effectively accumulated cadmium, lead, and zinc, with significantly higher potential for cleaning polluted sites, without even a decrease in essential oil quality and yield	Stancheva et al. (2014)

(continued)

Table 16.2 (continued)

Aromatic plants	Trace elements studied	Results	References
Chamomile	Cd ,Pb	Cadmium has greater similarities among its presence of various plant parts (flowers, stems, and roots). And it is distributed in the soils in forms readily extractable with both acetic acid and EDTA and that both extraction procedures would give a reliable estimate for potential cadmium mobility from the soil to the plant and for respective risk assessment for cadmium pollution	Voyslavov et al. (2013)
Rosemary	Al, Cd, Cr, Cu, Fe, Mn, Pb, and Zn	*R. officinalis* is a useful tool for an estimate of the concentration of pollution associated with traffic in urban areas. In addition, this plant could be capable of accumulating Al, Cd, Cr, Cu, Fe, Mn, Pb, and Zn in both leaves and stems	Bozdogan Sert et al. (2019)
Rosemary	Cd, Pb, Cu, and Zn.	Roots are more capable of utilizing and extracting elements from contaminated soils, and also that leaves may be better included as herbal medicine for rosemary plants. Nevertheless, plants adsorbed metals by phytoextraction and phytostabilization, where root phytostabilization was more effective	Abbaslou et al. (2018)
Europe sage or Clary sage	Pb, Cd, Cu, and Zn	*Salvia sclarea* is a plant species that is tolerant of heavy metals and can be attributed as hyperaccumulators of Pb and accumulators of Cd and Zn, having its successful use when used in phytoremediation of soils contaminated with heavy metals	Angelova et al. (2016)
Garden sage	Pb, Cd, and Zn	*Salvia officinalis* L. demonstrated growth in soils enriched with lead, cadmium, and zinc; the tolerance of these metals reflects the accumulative and translocating capacity of PB, Cd, and Zn	Angelova et al. (2016)
Lavender	Pb, Cd and Zn	Lavender species are highly tolerant of heavy metals; therefore, they are Pb hyperaccumulators and Cd and Zn accumulators and can be successfully used in the phytoremediation of soils polluted by these heavy metals	Angelova et al. (2015)
French lavender	Cr	Lavender plant species are phytomanagement capability in soils at maximum Cr concentration levels, cost-effective biochar and citrus peel waste were incorporated as soil modifications owing to its higher capacity to respond quickly directly or indirectly with heavy metals in the soil atmosphere, consequently lowering Cr uptake bioavailability	Ye et al. (2020)

(continued)

Table 16.2 (continued)

Aromatic plants	Trace elements studied	Results	References
Slender Bottle-daisy	Pb	*L. gracilis* was able to accumulate Pb extracted from the soil and stored in the vegetable, without a change in oil production, signaling the possibility of being used for program remediation of areas contaminated by Pb using the phytoextraction technique	Brandão (2016)
Oregano	Cr	The oregano roots showed the highest identified Cr (VI) (4300 mg kg^{-1}) bioaccumulation value for soil-checked plant species	Levizou et al. (2019)
Oregano	Cd, Pb, Cu, Mn, and Zn	Cd, Pb, and Cu were accumulated mainly in roots, while large quantities of Mn and zinc were detected in *Origanum heracleoticum* L. and other plant leaves, and it could be cultivated on soils contaminated with heavy metal without contamination of the commercial product, essential oil	Zheljazkov et al. (2008)
Lavender	Cr	Phytoremediation performance can be limited by low plant production and low lavender species growth rate in these increasingly Cr-contaminated soils	Ali et al. (2013)

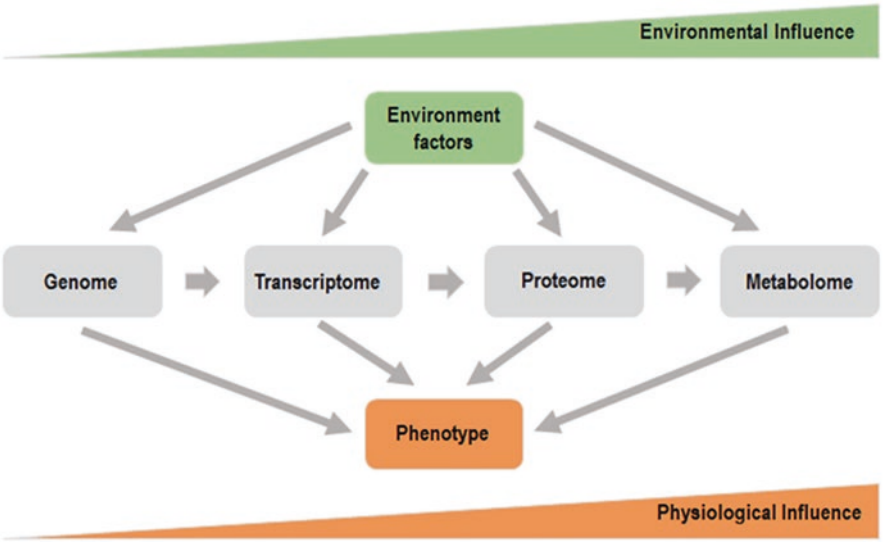

Fig. 16.1 The representation of the interactome (interaction between genome, transcriptome, proteome, and metabolome with the environment in a given biological system). (Figure adapted from Tan et al. 2016)

external metabolites. The objective of the survey consists of quantitative and qualitative information on the biological system investigated via its metabolites and is often employed as a supplementary method in the fields of genomics, transcriptomics, and proteomics, because the measurement of these metabolites may be precise and sensitive (Tan et al. 2016).

The emphasis of the metabolome research on the metabolome typically defines the metabolome benefit of the entire collection of metabolites in a biological sample which are the end result of the expression of the gene where changes in the metabolome occur quickly at timescales of seconds or minutes, precisely representing the status of the biological process at a particular stage (Tan et al. 2016). Metabolites are small compounds that undergo chemical transformation throughout metabolism and, as a result, they serve as a functional readout of cellular state. Unlike genes and proteins, their activities serve as direct markers of biochemical activity and hence are simpler to connect with the phenotype. In this respect, metabolite (or metabolomics) profiling has now become a strong technique extensively used in clinical, agricultural, and environmental research and applications (Patti et al. 2012). Metabolomics has become a new area in biology promising to clarify the functional analysis of genes with unknown functions. Although the ideas of unbiased and unbiased cellular metabolite analysis have grown in parallel with hypothesis-led approach, methodological methods in the life sciences have drastically altered (Villas-Bôas and Bruheim 2007).

Currently, metabolomics approaches and technologies are of interest in a range of fields such as animal and human nutrition (Wang and Hu 2018; Di Renzo et al. 2019), medicinal prognosis and diagnosis of metabolic syndromes (Karczewski and Snyder 2018; Taware et al. 2020), toxicology (Viant et al. 2019), drug discovery (Wishart 2016), bioremediation (Singh 2006; Singh et al. 2008), (Desai et al. 2010) and phytoremediation (Zhang et al. 2019; Hedgespeth and Nichols 2019). Recent advances in plant physiology, biochemistry, and their ecology can be a good and significant terrain for metabolomics study, due to the fact that plants produce a huge number of metabolites than microorganisms and animals could, and the most important point is that plants secondary to metabolites are generally a response to the stress caused to the environment. Comprehending the metabolome alterations and the changes they bring to plant physiology is an important step to see how environmental stress is affecting the plant ecology. Thus, environmental metabolomics is a promising field and has plenty of space to apply its technology with enough power to understand plant behavior towards environmental and ecological factors.

The development of the analytical methods for metabolomical investigations, notably the mass spectrometry, enabled the fast screening concurrently of thousands of metabolites from any complicated biological sample and provided extremely valuable information about the metabolism of sampled live organisms. Because metabolites are the intermediates of biological processes, they serve a major role in enabling many distinct paths in the living cell. Consequently, the metabolite level in the cell or tissue provides integral cell function information and yet determines the cell or tissue's phenotype in respect to genetic and environmental changes (Villas-Bôas and Bruheim 2007).

However, the mass spectrometry does not stand alone in this scenario, and the use of already well-stabilized analytical technologies for chemical separation, like liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE), and nuclear magnetic resonance (NMR) for imaging metabolomics screening works, coupled to different kinds of mass spectrometers; in an association that works just fine for an accurate refinement in metabolomic studies when it is needed. Recent advances in devices, techniques, and software allow an integrated study of cellular and fluid metabolites without bias (Patti et al. 2012). The uses of such technologies have shown wide changes to unanticipated metabolites, which caused unforeseen changes in the metabolism-related disturbance paths, where many of these identified compounds are not included in the metabolite reposits and databases, indicating how inadequate the concept of metabolism has been till now (Baker 2011). To perform an accurate and reliable metabolomics analysis, it is fundamental to choose the best analytical(s) platform(s) possible, able to extract the maximum information from the biological samples and the experimental design, so in this way, will be possible to answer the experimental question which is the focus of the study. Then, for that we have to follow a set of essential rules stabilized to define, in order to construct a metabolomics line of work, as follows: (1) Define the experimental design; (2) A clear biological question defined; (3) Sample type and size; (4) The analytical platform to be used; (5) Confounding factors; (6) Sampling and the sample extraction(s) method(s); (7) Data analysis scenario(s); (8) Randomization; and (9) Validation methods.

The analytical technologies that surround the metabolomics studies provide two different lines of analysis, wherein some instances, it may be of interest to perform a targeted approach where it is possible to examine a predefined set of metabolites, and the possibility to measure the levels of each metabolite on the biological sample. Normally, targeted analysis is employed when the focus is one or more pathways of interest or even a specific metabolite that is part of the pathway of interest. Target analysis is usually motivated by particular biochemical concerns or hypotheses which are highly useful for drug metabolism pharmacokinetic investigations, for example (Patti et al. 2012). Advances in mass spectrometry and NMR offer advantages in performing targeted analysis due to their high specificity and quantitative reproducibility even in large-scale metabolomics studies. On the other hand, an untargeted metabolomics approach possesses a global extent workflow for simultaneously detecting as many metabolites as possible from any sample without bias, metabolomics profiling (or fingerprinting), which can be related to the global picture of the cellular metabolism. Untargeted approaches have been employed massively in metabolomics studies today due to their huge power of analysis, and this is because it can be performed using either NMR or newly mass spectrometry technologies (Time-of-Flight (TOF) and/or Triple Quadrupoles (QqQ)). The use of LC/MS for an untargeted approach has been preferred for several studies nowadays, enabling the detection of thousands of peaks routinely even in high throughput studies (Nandania et al. 2018). Although targeted metabolomic findings do not show large file sizes, untarget metabolomics data sets have gigabyte file size per sample

in view of modern mass spectrometry devices with high resolution, making manual examination of thousands of peaks an impracticable and tedious task.

Albeit the handling in LC/MS instruments works with the concept of exact mass, minor issues usually occur, like the small deviations in retention time among samples as a consequence to column degradation, sample carryover, and variations on mobile phase pH and room temperature fluctuations. Recent progress in the past decade has been realized to surpass these obstacles in interpreting untargeted data sets, and now this task has become routine with the development of metabolomics softwares such as MetAlign, NOREVA, MZMine, and XCMS (Smith et al. 2006; Katajamaa et al. 2006; Lommen 2009; Yang et al. 2020). Despite the available analyzing software suites used for the untargeted approach being intuitive and complete to analyze gigabytes of the data, the metabolite identification is still an obstacle and a time-intensive process that represents a rate-limiting step of the untargeted metabolomics workflow. To identify the feature of interest, public databases like Human Metabolome Database (HMDB) (Wishart 2009) and METLIN (Smith et al. 2005) are freely available, yet the match in the database indicates just a potential metabolite assignment that must be verified by comparing certain data set characteristics such as retention time and the model compound MS/MS fragmentation pattern data, after ionization method of data acquisition information (if the analysis was taken on positive or negative ionization mode).

Once knowing the approach to be employed in the metabolomics study and the experimental design once set up, the next is choosing the analytical instrument(s), which must have some characteristics for analyzing extracted metabolites, whereas its reliability, high sensitivity, robustness, and the capacity to screen a large number of samples are essential features required for this purpose. Considerable advances in the technology of the instruments were realized so far, offering good choices for multiplatform analysis of complex biological samples, being that the most preferred instruments used for metabolomic analysis are the NMR spectroscopy, GC/MS, and LC/MS. Once an entire metabolome can be analyzed inside a single platform owing to a large dynamic and chemical range of biological low molecular-weight chemicals in biological mixtures (Patti et al. 2012). GC/MS is the most powerful and established analytical platform for analysis of very complex samples matrices such as those plant extracts, and this popularity is partly attributed to the possibility to detect a large number of molecules from a profiling metabolomics analysis, such as sugar-sugar alcohols, amino acids and organic acids, and others (Jorge et al. 2016; Beale et al. 2018).

Environmental conditions such as biotic and abiotic stress induce plants to produce specific classes of metabolites as polyols (mannitol and sorbitol), amino acids (glycine betaine), and carbohydrates (sucrose, trehalose, and fructan). Metabolites as proline and ectoine may be involved whether the plant is under high salt concentration, drought, and desiccation stresses, as osmolytes and osmoprotectant (Baharum and Azizan 2018). Fields such as plant physiology and plant biochemistry have increased their interests in metabolomics studies, where applications of metabolomics in plant studies and its response to environmental stress have become increasingly common. Among the several kinds of abiotics stresses that threaten

plant growth worldwide (drought, temperature, salt, flooding, nutrient deficiency, heavy metals excess, and the lack of a few chemical elements), oxidative stress is probably the major limiting factor for plant growth. Its process occurs by overproduction of reactive oxygen species (ROS), for example, hydrogen peroxide (H_2O_2), superoxide (O^{2-}), and the single oxygen ($^1O_2$). We may assert here that oxidative stress can be caused by jointly several other abiotic stresses, like high sunlight, low temperature, drought, and salted environment. This procedure is caused by the constant ROS production by the plant cells as a by-product of aerobic metabolism even under nonstress conditions, and notably during photosynthesis, in mitochondria and peroxisomes in different biochemical processes, like photosynthesis or fatty acids b-oxidation. However, the cell antioxidant system is able to detoxify ROS via the ascorbate glutathione (GSH) cycle, maintaining in this way the redox status of the cells (Jorge et al. 2016; Ghatak et al. 2018).

Investigating metabolic variations under abiotic stresses may detect a different class of metabolites that could permit restoration or treatment of plant conditions to its homeostasis by normalizing the metabolic modifications caused by abiotic stresses. Many analytical instruments and tools, like NMR, LC/MS, and GC/MS, that work in synergy with metabolomics are employed to elucidate stress tolerance in plants; also, TOF-MS and QqQ for mass detecting technologies for example also provide the detection of hundreds or even thousands of metabolites from various types of samples or treatments (Ma et al. 2018; Guo et al. 2018). The conjunction between post-genomics tools with metabolomics approaches offers new possibilities to study plant physiology and ecology, taking into consideration their metabolism and their complex metabolic networks, where metabolomics is still very young, but with an extensive potential to grow in a complementary way with the other omics sciences (genomics, transcriptomics, and proteomics). The forthcoming tools and applications for metabolomics, like identifying markers, may predict the nature and scale of abiotic/biotic stress, or even assist crop improvement programs with more resistant engineering species with better varieties. Moreover, metabolomics profiling of genome-edited plants that use the CRISPR/Cas9 system can be applied to understand the changes in the environment through the plant metabolome for example (Razzaq et al. 2019).

Lastly, metabolomics is the ideal platform that can be employed to identify and quantify small molecules which are present in plant complex samples and transformed into the data set information acquired through high precision instruments, like the mass spectrometry coupled to any chromatography system, and leading this information to the understanding of the environmental stimuli responsible to the living organism physiological and biochemical alterations. The finality of these approaches is their application to accelerate and improve plant adaptation strategies towards adverse environments as well as leading it to the next generation of crops more tolerant to environmental stresses worldwide (Ghatak et al. 2018). A promising use of the metabolomics tools would offer an improvement in the plant biology research nowadays, reminding its important role in monitoring soil, plants, and environmental characteristics and lacks, making also the procedure of phytoremediation more accessible to be employed by the governments and agriculture. At long

last, plant metabolomics is a field with an extensive demand for data mining, processing, and data evaluation, where its integration with even more precision bioinformatics tools could result in an excellent scenario for helping elucidate a comprehensive view of plant metabolome (Razzaq et al. 2019).

16.5 Advantages and Limitations of Phytoremediation

The advantages of phytoremediation (i.e., green technology and low costs) have led to high public acceptance; generally, these methods are considered esthetically pleasing and environmentally friendly management strategies for the remediation of contaminated soils (Ali et al. 2013). Phytoremediation is associated with effective, cost-efficient, and environmentally friendly rehabilitation strategies with using plants and correlated soil microorganisms to accumulate pollutants that are present in various contaminated environments. In addition, the technology's wide applicability enables to use it at sites not commonly remediated by any other methods. The benefits of using phytoremediation include application in situ, passive, 'green', solar-driven technology, ease of application, and suitability of a wide variety of metals, organic substances, and radionuclides. It also provides the benefit of eliminating secondary air or water-borne waste that can contribute to possible sources of human and environmental exposure. Furthermore, phytoremediation is used as an economically viable way to clean up pollutants in wastewater and sediments. It has prospective adaptability in treating a wide variety of potentially dangerous environmental contaminants. The main limitation on the clean-up of metal-contaminated soils is the long period required to adequately remediate the sites. Furthermore, the treatment is usually applied to soils within a few meters from both the surface and groundwater; the soil surface at the site can need to be changed to prevent erosion or flooding; contaminants can also enter the food chain by animals /insects that consume contaminated plant material (Mahar et al. 2016); the treatment is usually applicable to soils in a few meters of the surface and groundwater; climate or hydrological conditions will also limit the growth rate of plants that could be used, and soil modifications might be mandated. The remediation productivity can also be significantly affected by site characteristics including certain soil characteristics, combined contamination, and climatic conditions(Mendez and Maier 2008). Despite temperature conditions, vetiver needs to improve grass-based phytoremediation strategies around the nations specifically hot and cold countries. Generally, the aromatic plant of vetiver roots grows out leaves of soil that are sensitive to soil erosion and rippling due to being broad and reaches the ground deeply. Hence, in order to avoid unregulated cultivation, adequate vigilance is required. However, the low yield of plants as well as the low growth rate of lavender species under such heavily contaminated soil may limit its phytoremediation. Interestingly, phytoremediation performance can be limited by low plant production and low lavender species growth rate in these increasingly Cr-contaminated soils. The study of effectiveness and comparison with emerging technologies is notably still needed to

assess the different proportions of phytoremediation over the remediation industry as well as the potential for marketing. In order to enhance the phytoremediation of polluted areas, the targeted plants should be perpetual to nature and sensitive of environmental and strategic value, and harvestable. However, the environmental factors, particularly low plant productivity especially implanting to planting, in other words planting season and growth stages, together with bioavailability and bioaccessibility of heavy metal ions present in the soil, will prolong the removal time (Ali et al. 2013). Although phytoremediation is a rather cost-effective approach for cleaning up contaminated land, even more, validation verification field conditions and cost comparisons were also required to enable effective task cost estimates and site-specific process feasibility.

16.6 Prospects and Conclusion

The rise in the heavy industry associated with global growth during the last decade has accelerated the release into the ecosystem of chemical materials. The plants have been documented very frequently for generating different endophyte microbial communities which can be very successful in improving the efficiency of extracting contaminants from plant species. In addition, the use of aromatic plants requires additional benefits like biodiversity resources for both farmers and society than traditional methods used throughout phytoremediation. Research nowadays should concentrate on heavy metals-induced signal transduction pathways as they can be used through signal components that could really help to better understand metal homeostasis; certain environmental stresses will also be exhibited. Advancements including the use of chelating agents, transgenic, and soil modifications may also be effective ways of overcoming the limitations (Zahedifar et al. 2016; Patra et al. 2018b). A new era of "integrative-omics" has enabled the development and effective implementation of smart remediation methods. However, effective statistical methods and bioinformatics tools are still required to understand and handle the enormous data produced by these "-omics" techniques. Recent advances in microbial biodegradation and/or biotransformation have a huge effect on our attempts to keep industrialized society clean. In general, techniques to improve the efficiency of phytoremediation include practical challenges in large-scale applications, predominantly resulting from increased costs and potential environmental risks like leaching, which hinder the commercial deployment of these methods (Mahar et al. 2016). The comprehensive and detailed investigations are needed to optimize processes that understand the problems of plant–pollutant interactions and means of proper disposal with minimal environmental damage. In addition, the use of advanced molecular methods and the production of transgenic plants with intensified phytoremediation performance are growing wide acceptance; genetic modification is indeed considered to play an increasing role in strengthening the applicability of phytoremediation developments.

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