# Contribution of Omics and Systems Biology to Plant Biotechnology

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**Abstract**

The development of modern genetic engineering approaches and high throughput technologies in biological research, besides the holistic view of systems biology, have triggered the progress of biotechnology to address plant productivity and stress adaptation. Indeed, plant biotechnology has the potential to overcome many problems we currently face that impair our agriculture, such as diseases and pests, environmental pressures, or climate change. The system biology field encompasses the identification of the general principles and patterns found in living systems, by studying the molecular diversity and integrate this knowledge in complex models of regulatory networks. The "omics," which comprises but not limited to genomic, transcriptomic, proteomic, epigenomic, and metabolomic studies in entire plants, allow a better understanding of plant system biology and further contribute to biotechnology development. In this chapter, we provided an overview on omic studies for the searching and identification of metabolites and proteins employed by microorganisms to develop biotechnological products. Moreover, we present an overview of the central aspects of small RNA as regulators of gene expression connecting system networks and the potential application into plant biotechnology.

**Keywords**

Plant biotechnology · Plant-microbe interaction · Effectors · Small RNAs · Omics

## 10.1 Introduction

### 10.1.1 Plants Have Shaped Human Life History on Earth

The energy of sunlight converted by algae and plants to carbohydrates and other organic molecules is fundamental for life in our planet. Our society has developed along with the improvement of our capacity to cultivate and store plants as a main source of food through agriculture. Climate change, diseases and pests have reduced the sources of energy and, besides suitable agricultural-land area, represent the main obstacles for the optimal production and yield in agriculture nowadays. Furthermore, the population

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growth rate is rapidly increasing, which raises concerns about food security in a near future. To balance the equation, many people are trusting on the development of plant biotechnology.

Indeed, plant biotechnology have the potential to overcome many problems we currently face that impair our agriculture, such as diseases and pests, environmental pressures or climate change, to cite a few examples. However, it is not yet known if the rate of plant biotechnology development will cope with the always-growing needs for food. Besides increasing plant productivity and resistance against biotic and abiotic stresses, plant biotechnology is also crucial to the development of the much needed second and third generation biofuels.

In this scenario, "omics" and plant system biology emerges as fundamental knowledge to understand, not only the physiology of a single plant, but also to extrapolate this information to more complex natural and anthropogenic ecosystems, which in turn have the potential to accelerate the development of plant biotechnology. Besides the biotechnological products that have arisen from genetic manipulation of organisms, such as genetic modified organisms (GMO), antibiotics and vaccines, the modern biotechnology provides advances in the study of omics, and consequently to the system biology field. This emerging field, which is closely related to synthetic biology, encompasses the identification of the general principles and patterns found in living and engineered systems, along with the study of the molecular diversity of living organisms, to finally, integrate this knowledge in complex models of the regulatory networks (Breitling 2010).

Several new methods for DNA sequencing known as "next-generation" or "second-generation" sequencing were developed around the year 2000, and expand enormously the genomic information available nowadays, which comprises hundreds of organisms. Together with transcriptomics, proteomics, epigenomics, and metabolomics studies that are now facilitated by high-throughput methodologies and bioinformatics analyses, the enormous growth in omics studies now makes systems biology expand in biological research.

In the next sections, it will be discussed the characterization of metabolites and proteins employed by plant-associated beneficial microorganisms, and also plant susceptibility genes that are targeted by pathogen effectors, in order to develop biotechnological products. Additionally, the posttranscriptional regulatory role of small RNAs, representing another layer of gene expression regulation, is presented. Finally, our perspectives for the contribution of omics and systems biology to advance plant biotechnology are further discussed.

## 10.2 Development

### 10.2.1 Plant–Microbe Interaction: Effectors, Omics and Strategies for Plant Breeding

Plants are in constant interaction with microbes in the environment. The nature of those relationships might range from no obvious interactions (not compatible), beneficial (mutualistic) to harmful (pathogenic), which also can be influenced by changes in environmental conditions. In almost all cases, microbes utilize effectors to modulate host physiology aiming to establish successful colonization. In this topic we will discuss the effector-based strategies employed by both beneficial and pathogenic microbes, the omic tools to identify effectors and plant targets, and the biotechnological approaches to engineer plants with higher productivity and resistance.

### 10.2.2 Effectors from Beneficial Microorganisms

Mutualistic microbes, which provide essential biochemical products/processes to host plants, are mainly associated with the root system and usually referred to as plant growth-promoting bacteria (PGPB) and fungi (PGPF) (Pieterse et al. 2014). These beneficial organisms can improve plant growth and development by using both direct and indirect mechanisms. Probably

the most well-known beneficial relationship between plants and microorganisms is the interactions of Rhizobium and other nitrogen-fixing bacteria with plants colonized by these bacteria.

Direct mechanisms, such as nitrogen fixation, phosphorous solubilization and production of growth-promoting compounds as plant regulators (auxin, cytokinin, gibberellin), refer to a directly induction of plant growth and development by microbe-associated molecules (Olanrewaju et al. 2017). The production of auxins by beneficial microbes has been greatly explored due to the numerous positive effects that this versatile hormone can cause, for instance by regulating cell division and cell enlargement to provide growth of roots, stem and leaves (Vanneste and Friml 2009). The indole-3-acetic acid (IAA) produced by the plant-associated microorganisms can stimulate root development if the plant IAA concentration is insufficient, or causes contrary effect to inhibit root growth in cases where the concentration of the hormone is optimal (Spaepen et al. 2007). In *Triticum aestivum*, the IAA content produced by strains belonging mainly to *Bacillus* and *Pseudomonas* species increased the number of tillers, the spike length and seed weight, demonstrating the potential of this hormone to increase plant growth and yield (Ali et al. 2009). Cytokinin is also produced by soil microorganisms capable to work as a plant growth regulator (PGR) (Arkhipova et al. 2007). Cytokinins content can cause beneficial effects on plant growth and yield, by acting in a lot of biological processes, including cell division, cell enlargement, tissue expansion, stomatal opening and shoot growth (Weyens et al. 2009). For example, treatment of *Platycladus orientalis* (oriental thuja) seedling with cytokinin produced by *Bacillus subtilis* increased drought stress tolerance thus improving plant health (Liu et al. 2013).

Indirect mechanisms, such as production of antibiotics, quorum quenching and induced systemic resistance (ISR), refer to an indirectly induction of plant growth and development by the inhibition of pathogens attack (Olanrewaju et al. 2017). Bacteria from the genera *Pseudomonas* and *Bacillus* have been shown to produce a large variety of effectors with antimicrobial properties, such as ecomycins, 2,4 Diacetyl Phloroglucinol (DAPG), Phenazine-1-carboxylic acid (PCA), subtilin, TasA, and sublancin (Goswami et al. 2016). Beneficial microbes can also inhibit infection of phytopathogenic bacteria by disrupting their communication (Olanrewaju et al. 2017). In response to fluctuations in cell population density, quorum-sensing bacteria synthesize extracellular signaling molecules, called autoinducers, which triggers gene expression regulations in proximal bacterial cells. By using quorum sensing, bacteria can regulate a diverse array of physiological activities, such as biofilm formation and virulence, in a coordinated action within bacterial population (Miller and Bassler 2001). Some beneficial PGPBs produce lactonase enzymes that degrade pathogen-produced autoinducer, thus disrupting quorum sensing and preventing bacterial pathogens from inhibiting plant growth (Olanrewaju et al. 2017).

Indirect promotion of plant growth by beneficial microbes can also be achieved by triggering the ISR, a plant priming for defense against subsequent attacks from a broad spectrum of pathogens and herbivores. Induced resistance is triggered not locally at the site of contact with the mutualistic microbe but also systemically in plant parts that were not exposed to the inducer. Both PGPB and PGPF in the rhizosphere have been described to stimulate plant health by triggering the plant immune system (Pieterse et al. 2014). For instance, pioneer studies reported that plants with root system colonized by a PGPB strain of *Pseudomonas fluorescens* had a higher production of antimicrobial phytoalexins and enhanced resistance to the pathogen *Fusarium oxysporum* (Van Peer et al. 1991).

Similarly, the colonization of cucumber roots by *Pseudomonas* and *Serratia* PGPB strains resulted in reduced anthracnose disease symptoms caused by *Colletotrichum orbiculare* (Wei et al. 1991). Since then, numerous studies had reported the ability of plant growth-promoting microbes to induce ISR and enhance plant health (Pieterse et al. 2014). Many microbial effectors responsible for the onset of ISR have been described. Examples from PGPB include antibi-

otics, homoserine lactones, iron-regulated siderophores, lipopolysaccharides-containing cell wall and flagella. Volatiles such as 2R,3R-butanediol and C13 synthesized by *B. subtilis* and *Paenibacillus polymyxa*, respectively, also elicit ISR. ISR-inducing effectors from PGPF include enzymatic proteins, such as xylanases and celulases (Pieterse et al. 2014).

Besides effectors that induce systemic plant defenses, beneficial microbes might also deliver effectors that suppress local plant defenses to help the establishment of mutualistic interactions with the host. Some effectors, employed by PGPB and PGPF to overcome plant immune responses, have been described, e.g., the SP7 from *Rhizophagus intraradices* (Kloppholz et al. 2011). Suppression of plant defenses is a mechanism also typically exerted by effectors from pathogenic microbes to achieve successful infection, and it will be addressed in the following subtopic.

### 10.2.3 Effectors of Plant-Pathogens

During the co-evolution of plants and pathogens, plants have developed a multilayered immune system to self-protect while adapted pathogens acquired mechanisms to overcome its defenses. At the cell surface, plants carry pattern recognition receptors (PRRs) to recognize conserved molecules associated to pathogens/microbes (pathogen/microbe-associated molecular patterns—PAMPs/MAMPs) and elicit the so-called pattern-triggered immunity (PTI). To counteract PTI, specialized pathogens deliver effector proteins that suppress the plant defense signaling and induce an effector-triggered susceptibility (ETS). As a counter-counter-defense strategy, plants have evolved proteins coded by resistance genes (R genes) to sense the effectors or their effects in plant cells, triggering the effector-triggered immunity (ETI) (Fig. 10.1) (Jones and Dangl 2006).

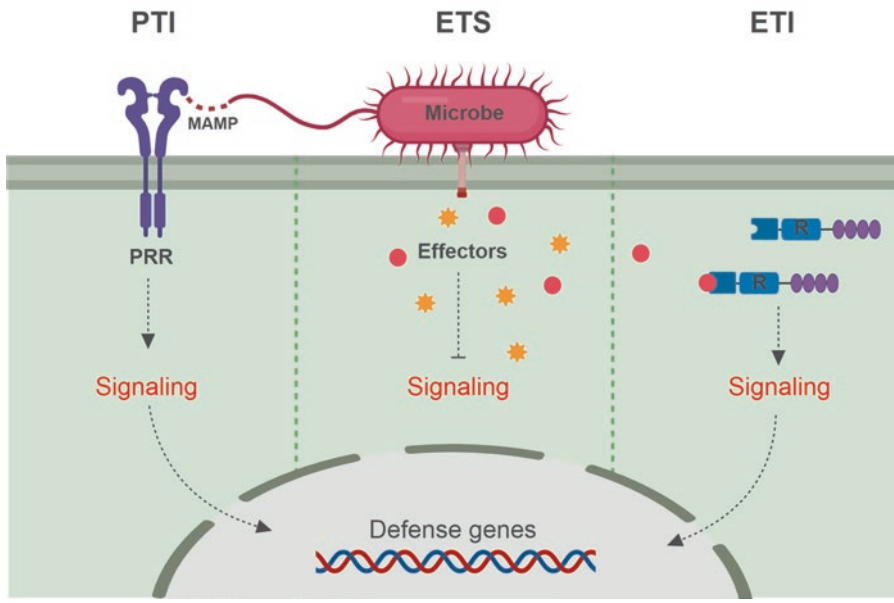


**Fig. 10.1** Multilayered plant immune system. Plants carry pattern recognition receptors (PRRs) that recognize pathogen/microbe—associated molecular patterns (PAMPs/MAMPs) and elicit the pattern-triggered immunity (PTI). Adapted pathogens have acquired effector proteins that are delivered in the host cell to suppress PTI, inducing an effector-triggered susceptibility (ETS). As a counter-counter-defense strategy, plants have acquired resistance (R) proteins that recognize the effectors or their effects in plant cells, triggering the effector-triggered immunity (ETI)

Most R genes encode members of a family of nucleotide-binding leucine-rich repeat (NLR) receptors that recognize specific pathogen effectors. The first described R gene, Pto, was identified more than 20 years ago in tomato conferring resistance to strains of *Pseudomonas syringae* carrying specific effectors (former called as avirulence genes) (Martin et al. 1993; Scofield et al. 1996). Since then, several R genes have been identified in distinct plant species, as promoters of resistance to all kinds of pathogens. Classic examples include the tobacco gene N that confers resistance to tobacco mosaic virus (TMV) (Whitham et al. 1994), the Arabidopsis RPS2 and RPM1 that recognize effectors from *P. syringae* (Bent et al. 1994; Grant et al. 1995), and the tomato Cf-2 and Cf-9 that promotes resistance to *Cladosporium fulvum* (Jones et al. 1994; Dixon et al. 1996). When pathogens attempt to overcome plant defenses by delivering effector molecules, R genes-encoded proteins might recognize either the effectors itself or plant affected proteins, triggering a signaling cascade that culminate in the plant resistance.

Along with suppression of plant defenses, pathogen effectors might exploit the so-called plant susceptibility genes (S genes) that facilitates the infection process or supports compatibility with a pathogen (Zaidi et al. 2018). Proteins coded by S genes might assist pathogen in several steps of the establishment of a compatible interaction such as host recognition, penetration, proliferation and spread. The best-known example of an S gene is the *Mildew resistance locus O* (*Mlo*) that encodes a membrane-associated protein required for powdery mildew fungal penetration of host epidermal cells. Besides *Mlo*, the rice *SWEET* genes were identified as susceptibility genes to bacterial blight (Zhou et al. 2015). The associated pathogen, *Xanthomonas oryzae*, encodes transcription activator-like (TAL) effectors that recognize specific regions (effector binding elements, EBE) in the promoter of the *SWEET* genes and induce their expression (Zhou et al. 2015). Because *SWEET* genes encode sugar transporters, they likely promote susceptibility to bacterial blight by triggering sugar release to the apoplast and thus providing nutrient to the pathogen (Blanvillain-Baufumé et al. 2016). In fact, several S genes targeted by *Xanthomonas* spp. TAL effectors have been identified (Hutin et al. 2015). Another S gene recently characterized is the citrus LOB1, which support host susceptibility to citrus canker disease, caused by *Xanthomonas citri* subsp *citri* (Hu et al. 2014). Like *X. oryzae*, *X. citri* also uses its TAL effectors to bind EBEs in the promoter of LOB1 and induce its expression (Hu et al. 2014). Even though its biological role remains to be determined, induction of LOB1 using custom-designed TAL effectors leads to similar citrus canker symptoms (Zhang et al. 2017), highlighting its central role in the development of the disease.

### 10.2.4 Omics as Tools to Identify Microbe Effectors and Plant Targets

The increasing advances in omics technologies are boosting the discovery of microbial effectors in a rapid and efficient manner. Next-generation sequencing technologies are used to sequence microbe genomes, allowing in silico prediction of effectors. Putative effectors can be predicted from sequence datasets by detecting features associated to secreted proteins, such as the presence of a signal peptide, the absence of transmembrane and membrane anchorage domains, and small sequence size/length (Dalio et al. 2017). Genome sets from different strains of the same microbe can be compared by searching for core effectors, known to be important for the microbe colonization and, hence, less subjected to mutations that could help them to escape from introduced sources of plant resistance (Dangl et al. 2013). Following such strategies, sets of effectors of several microorganisms have been disclosed with high efficiency (Vleeshouwers and Oliver 2014). Further proteomic and transcriptomic data, from microbe upon contact with plant signals, help to select secreted proteins potentially involved in the host–microbe interaction.

Once the most promising effector candidates are selected, their biological activity can be vali-

dated by transient or stable gene expression in plants (Dalio et al. 2017). The set of transcriptomic, proteomic, metabolomic, and phenomic data from transformed plants compared to wild type shall settle the status of the predicted protein as a true effector and can contribute to the elucidation of plant modifications imposed by effector activity. Further approaches to validate effector function is knocking out or knocking down the effector gene-by-gene editing or silencing (Dalio et al. 2017). In such cases, the obtainment of omics data from both microbe with the disrupted gene and colonized test-plant are also useful to demonstrate that the function of the effector is compromised.

Subsequently, the identification of effectors has facilitated the discovery of corresponding plant target genes. "Effectoromics" studies have been successful in identifying a growing list of effectors and their corresponding R and S genes (Dangl et al. 2013; Vleeshouwers and Oliver 2014). By using the functionally validated effector, plants can be screened for proteins that directly interact with the effector. For instance, candidate targets for effector manipulation can be elucidated using yeast two-hybrid screening, which has been applied at genomic scale, or pull-down assays followed by proteomics identification of interacting proteins (Dalio et al. 2017). Irrespective if those or other approaches are employed, the searching for targeted R or S genes directed by effector-based screens provide higher throughput and more straightforward phenotypes than pathogen-based screens (Dalio et al. 2017; Vleeshouwers and Oliver 2014). Similar strategy could be employed to identify plant targets from beneficial microbe effectors.

This effector-rationalized approach (Dangl et al. 2013) was used to search for the source of *Phytophthora infestans* resistance in the potato "Sarpo Mira," one of the few cultivars reported to retain field resistance to late blight for several years (Rietman et al. 2012). A collection of core effectors was predicted from *P. infestans* genome and expressed in potato leaves. The induced resistance response to specific effectors, whose corresponding R genes were mostly known, enabled the dissection of R genes that confers late blight resistance in "Sarpo Mira" genotype (Rietman et al. 2012). Similar strategies can be used to provide breeding programs with R genes for deployment in susceptible genotypes. A different approach relied on the TAL effectors from *Xanthomonas* species, which binds EBE regions in the promoter of S genes, inducing their expression and facilitating pathogen infection. Using the knowledge on EBE regions, an engineered R gene was produced by adding EBE regions to the promoter of *Xa27* gene and deployed in rice (Hummel et al. 2012). The synthetic R gene was successfully activated by TAL effectors, conferring rice resistance to both bacterial blight and bacterial leaf streak (Hummel et al. 2012). Besides deploying engineered R genes, S genes targeted by TAL effectors have been edited to generate resistant genotypes. Using an effector-rationalized approach, the discovery of *Xanthomonas* TAL binding sites combined with transcriptomic data has led to the discovery of several S genes for different *Xanthomonas*/host interaction (Hutin et al. 2015). The identified S genes are greatly increasing the knowledge on *Xanthomonas*-causing diseases and have now been used as targets for gene editing to confer resistance to such diseases (Hutin et al. 2015; Li et al. 2012).

### 10.2.5 Biotechnology Approaches for Genetic Engineering Plants to Improve Productivity and Disease Resistance

Crops have been selected for higher yield and disease resistance throughout the history of agriculture (Table 10.1). Traditional breeding methods allowed the introgression of interesting traits well before the comprehension of the molecular mechanisms involved in plant–microbe interactions. Currently, the elucidation of effector-targeted plant genes is used by breeding programs to develop crop varieties with higher levels of resistance and productivity. Combined with conventional time-consuming breeding techniques, technologies based on genetic engineering (Fig. 10.2) have been used to improve and speed up the process of developing high-yield and durable disease-resistant crop varieties (Table 10.1).

**Table 10.1** Historical scientific events that have developed the modern biotechnology

| Years | Scientist/pioneer/discoverer | Innovative events |
|---|---|---|
| 8500 BC | Southwest Asians | Emergence of plant and animal domestication |
| 1675 | Anton Van Leeuwenhoek | Discovery of microorganisms by "The Father of Microbiology" |
| 1862–1885 | Louis Pasteur | Discoveries of the principles of vaccination, microbial fermentation, and pasteurization |
| 1865 | Gregor Mendel | Establishment of the principles of genetics and theories of heredity by "The Father of Genetics" |
| 1919 | Károly Ereky | Creation of the term biotechnology |
| 1928 | Ludwig von Bertalanffy | Proposition of the general systems theory, one of the precursors of systems biology |
| 1929 | Alexander Fleming | Purification of penicillin from the fungus *Penicillium notatum* |
| 1930 | George Beadle and Edward Tatum | Confirmation that genes direct the production of proteins |
| 1944 | Oswald Avery | Identification of DNA as the material of which genes and chromosomes are made and transmit the genetic information |
| 1953 | Francis Crick, Maurice Wilkins, and James Watson | Revelation of the structure of DNA molecule |
| 1961 | François Jacob and Jacques Monod | Elucidation of the control of enzyme expression levels as the result of regulation of DNA transcription |
| 1967 | Har Gobing Khorana and Marshall Niremberg | Elucidation of the genetic code |
| 1972 | Paul Berg | Development of recombinant DNA techniques—"the emergence of genetic engineering" |
| 1976 | Walter Fiers | Sequencing of the first complete genome of bacteriophage |
| 1976 | Herbert Boyer and Robert Swanson | Establishment of the first biotechnology company, the Genentech |
| 1977 | Frederick Sanger | Determination of the first DNA sequence |
| 1978 | Werner Arber, Daniel Nathans, and Hamilton Smith | Isolation of restriction enzymes from bacteria |
| 1982 | Richard Palmiter | Generation of the first genetic modified organism (GMO) |
| 1985 | Kary Banks Mullis | Development of the polymerase chain reaction (PCR) technique |
| 1986 | Thomas H. Roderick | Creation of the term genomics |
| 1986 | USA and France | Establishment of the first field trials of transgenic tobacco resistant to herbicide |
| 1994 | USA | Approval of the first GMO to be commercially available, a transgenic tomato |
| 1998 | Washington Uni and Sanger Institute | Sequencing of the first complete animal genome, of the *Caenorhabditis elegans* |
| 1998 | Craig Mello and Andrew Fire | Elucidation of the mechanism of RNA interference (RNAi) in animals |
| 1998 | Peter Waterhouse and Ming-Bo Wang | Discovery that the double-stranded RNA (dsRNA) induces the RNAi in plants |
| 2000s | Roche, ABI, and Solexa/Illumina technologies | Development of high throughput-sequencing technologies |
| 2000s | Several | Emergence of modern systems biology approaches, "the age of systems" |
| 2000 | Arabidopsis Genome Initiative | Sequencing of the first complete plant genome, of the *Arabidopsis thaliana* |
| 2002 | Koichi Tanaka, John Fenn, and Kurt Wüthrich | Recognition for the development of identification and structure analyses for proteomics |
| 2008 | Solexa/Illumina technologies | Development of RNA-seq for modern transcriptomic studies |
| 2012 | Jennifer Doudna and Emmanuelle Charpentier | Development of a precise gene editing technology using CRISPR-Cas9 |
| 2017 | David Liu and Feng Zhang | Development of a flexible RNA base editing technology using CRISPR-Cas13 |

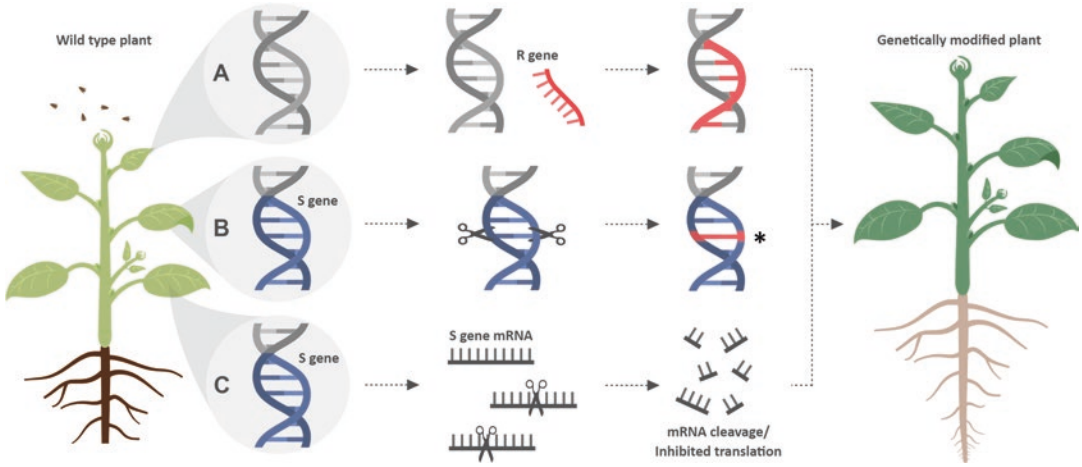


**Fig. 10.2** Genetic engineering approaches to generate plants with improved disease resistance. (**a**) Transgeny to introduce genes of interest in wild type plants, including resistance (R) genes. (**b**) Gene editing to disrupt native genes such as susceptibility (S) genes. Sire-directed gene editing can be achieved using tools such as CRISPR/Cas9 system, that introduce double-stranded DNA breaks and triggers an error-prone DNA repair pathway, resulting in indels mutations (*). (**c**) Gene silencing to disrupt the function of undesirable genes (e.g., S genes). RNA interference can promote the cleavage or translation inhibition of mRNAs, knocking down the expression of the target gene

Transgenic approaches have been used to introduce genes of interest in plant species, including dominant R genes for disease resistance (Dangl et al. 2013). For instance, transgenic tomatoes with field-level resistance to bacterial spot disease were produced by transferring the R gene *Bs2* from pepper (Horvath et al. 2012). Similarly, the gene *RB* from potato wild relatives was introduced in the cultivated potato by transgeny and generated increased resistance to late blight (Halterman et al. 2008). Showing that R genes from non-hosts can effectively promote plant resistance, the maize R gene *Rxo1* was used to generate a transgenic rice, conferring resistance to bacterial streak (Zhao et al. 2005). The downside of using dominant R genes to generate plant resistance is that they usually present a short life in the field due to the adaptative potential of the corresponding pathogen effectors (Dangl et al. 2013). On the one hand, stacking multiple R genes simultaneously should provide more durable resistance since multiple effector genes would have to suffer mutation to evade resistance (Dangl et al. 2013). On the other hand, enhancing plant resistance by disrupting S genes rather than expressing R genes is an attractive approach.

Along with the introduction of foreign genes by conventional transgeny, the disruption of native gene functions might be achieved by gene silencing or editing. Gene silencing can be activated by the presence of double-stranded RNAs (dsRNA) and results in the cleavage or translation inhibition of RNAs. Briefly, dsRNA triggers their own cleavage by Dicer nucleases, producing small interfering RNAs (siRNA), which in turn are recruited by RNA-induced silencing complexes (RISC) that target RNAs with sequence homology to the incorporated siRNA (Kamthan et al. 2015). Transgenic plants with constructs designed to produce siRNA contain in the dsRNA a sequence to target gene, a technology known as RNA interference (RNAi). With the employment of engineered siRNA, RNAi can be used to manipulate gene expression and suppress undesirable traits such as susceptibility to pathogens.

The RNAi system have been applied as a strategy to control plant insects (Galdeano et al. 2017; Mao et al. 2007), viruses (Fuentes et al. 2016; Niu et al. 2006) and other attackers by directly targeting the pathogen/herbivore genes. Another approach is the use of RNAi to target plant S

genes. Such strategy was used to silence the potato *SYR1* gene, resulting in reduced formation of papillae components in response to infection with *P. infestans* and increased resistance to late blight (Eschen-Lippold et al. 2012). In another example, RNAi was employed to silence *SSI2* (suppressor of salicylate insensitivity of npr1-5) gene in rice, a negative regulator of plant defenses, conferring resistance to fungal blast and bacterial leaf blight diseases (Jiang et al. 2009). We will discuss more about sRNAs in the next chapter section.

Besides gene silencing, an increasing approach within molecular plant breeding is the use of site-directed genome editing. One of the most revolutionary tools within gene editing techniques is the CRISPR-Cas9 system. With CRISPR/Cas9 tool, double-stranded DNA breaks can be introduced at specific genome regions by a site-specific nuclease, leading to the activation of DNA repair pathways. In the absence of a repair template, the non-homologous end-joining (NHEJ) pathway repairs the DNA in an error-prone process that often causes insertions or deletions around the DNA breaks, generating mutated alleles (Zaidi et al. 2018). Though recently developed, CRISPR/Cas9 system has already been applied in several economically important crops such as rice (Jiang et al. 2013), maize (Char et al. 2017), tomato (Brooks et al. 2014), and sweet orange (Jia and Wang 2014). CRISPR quickly became successful due to its high simplicity, efficiency, specificity and versatility (Bortesi and Fischer 2015; Zaidi et al. 2018). The major advantage of the gene editing, however, is the possibility to generate genetically modified cultivars that lack transgenes in the final line and thus can be exempted from GMO legislation and are more likely to be accepted by the public. Without transgenes or other foreign DNA sequences, some genome-edited plants using CRISPR already evaded regulation by USDA and are reaching market in record time (Waltz 2018).

In the context of developing disease resistance, S genes are promising targets for gene editing, since their mutation can limit the ability of a pathogen to cause disease. By using gene editing, the S gene *LOB1* was successfully modified in grapefruit, generating plants without symptoms of *Xanthomonas citri* bacterial infection (Jia et al. 2017). Likewise, CRISPR/Cas9 was used to edit the rice S gene *SWEET13*, resulting in resistance to bacterial blight (Zhou et al. 2015). Regarding fungal pathogens, gene editing disabled multiple homeoalleles of *MLO* gene in wheat, conferring heritable broad-spectrum resistance to powdery mildew (Wang et al. 2014). Resistance to potyviruses was obtained in *Arabidopsis* (Pyott et al. 2016) and cucumber (Chandrasekaran et al. 2016) by disrupting the S gene *eIF4E* (*eukaryotic translation initiation factor E*), which codes for a protein essential to the viral infection cycle. The CRISPR/Cas9 system was also employed in tomato to inactivate *DMR6* (*downy mildew resistance 6*), an S gene involved in the homeostasis of the defense hormone salicylic acid, generating plants with high levels of resistance to a wide variety of pathogens (Thomazella et al. 2016). The results obtained so far using CRISPR technology have proven that mutation on S genes can generate plant resistance to several diseases. Ongoing studies are focusing in obtaining final lines that do not contain foreign DNA to facilitate consumer acceptance. The use of genome editing to mutate S genes is emerging as a revolutionary approach to provide a transgene-free, long term, and efficient control measure of plant diseases.

Apart from breeding strategies to obtain genetically engineered plant, another biotechnology strategy used to improve plant health is the use of heterologous expression systems to produce molecules of interest. This approach can be used to large-scale production of effectors from mutualistic microbes that stimulate plant growth or disease resistance. Heterologous expression of microbe-associated quorum quenching molecules have been explored aiming disruption of biofilm-forming phytopathogenic bacteria (Kalia 2015). For instance, the *aiiA* gene coding for lactonase effectors from distinct *Bacillus* species was engineered into *Lysobacter enzymogenes* and *E. coli*, resulting in reduced virulence of *Pectobacterium carotovorum* on Chinese cabbage (Qian et al. 2010) and attenuated soft rot symptoms of *Erwinia carotovora* in potato,

respectively (Pan et al. 2008). Similar strategy of recombinant protein systems can be used to synthesize other effectors from beneficial microbes in a commercial scale to increase plant health such as growth-promoting hormones, hydrolytic enzymes, siderophores, or antibiotics.

The improved identification of microbe effectors using omics technologies is providing valuable resources for plant breeding programs. Knowledge of microbe effectors and their target plant genes can be applied in combination with biotechnology techniques to speed up the development of plant varieties with higher productivity and durable disease resistance.

### 10.2.6 Gene Expression Regulation by Small Noncoding RNAs

The comprehension of system biology depends on a wide data collection, integration and analysis of biological molecules, focusing on interactions and emerging properties. In this context, the small noncoding-RNAs (sRNAs) has appeared, in the last couple of decades, as active and essential regulatory molecules for protein-coding gene expression, influencing several interconnected biochemical pathways. Therefore, the identification of sRNAs and characterization of their interactive network, including the discovery of sRNA target genes and associated biochemical pathways is crucial for the application of systems biology to plant biotechnology.

In this section, we provide an overview of the central aspects of endogenous sRNAs, mostly microRNAs (miRNAs), function during plant development and the evolutionary history of *MIRNA* genes. MiRNAs have been shown to act as posttranscriptional regulators, directing several essential processes in the plant, and miRNA-based technology is also a target for plant engineering to achieve high yields, quality and stress resistance. The applications of sRNAs e miRNAs research on plant biotechnology and the importance to incorporate these regulatory molecules into systems biology are further discussed.

#### 10.2.6.1 Biological Roles of Plant miRNAs

Expansion of the miRNA regulatory system is associated with requirements for additional endogenous control of genomic information (Mattick 2004). The remarkable and constant expansion of miRNAome coincides with the major morphological innovations present in the animal bilaterians, vertebrates, and placental mammals, where many tissue- and organ-specific miRNA/target regulatory associations could have been fundamental to the emergence of complex bodies. This is reflected in the strong correlation between the number of *MIR* families contained in an organism and its position in the hierarchy of the animal kingdom (Hertel et al. 2006; Sempere et al. 2006). Moreover, there is a correlation between the number of target genes regulated by a miRNA and the age of a *MIR* gene. In animals, the number of targets of an individual miRNA also appears to increase over evolutionary time, with the more phylogenetically ancient miRNAs having more target genes than young miRNAs (Brennecke et al. 2005).

In plants, analyses of the miRNAome of common ancestors have suggested that only a few *MIR* genes are highly conserved across the entire kingdom (Cuperus et al. 2011; Nozawa et al. 2010; Ma et al. 2010). The sRNAs derived from conserved *MIR* families represent the most abundant miRNAs in a particular miRNAome, as a result of a moderate to high levels of *MIR* gene expression (Axtell 2008; Cuperus et al. 2011). These conserved miRNAs are usually derived from multi-gene *MIR* families, containing identical or highly similar mature miRNA sequences (Jones-Rhoades 2012). A high level of functional redundancy is noticed among members of the same *MIR* family (Sieber et al. 2007; Allen et al. 2007). However, the expansion of *MIR* gene families, combined with the occurrence of mutations outside the mature miRNA sequence, would provide diversification of the spatiotemporal expression in different *MIR* family members (Li and Mao 2007).

The development of multicellular organisms depends on complex regulatory networks that

integrate endogenous and environmental signals. The signaling effectors in this process include phytohormones, peptides, transcription factors, and sRNAs, which are globally interconnected over long and short distances within the plant, acting in a spatiotemporal manner (Sparks et al. 2013).

Phytohormones are important mediators throughout plant development, perceiving and transmitting the internal and external cues, and whose signaling pathways are under constant cross-talk mechanisms to adjust the responses (Vanstraelen and Benková 2012). A close relationship between miRNAs and phytohormones has been seen in several studies, showing intersections in their pathways and feedback mechanisms where *MIR* genes respond to hormones which in turn regulate several genes involved in hormonal signaling pathways (Liu and Chen 2009; Liu et al. 2009; Curaba et al. 2014). Tissue- or stage-specific miRNA accumulation often plays a central role affecting, directly or indirectly, the expression of genes to adjust the transcriptome in accordance with the development requirements, in a highly dynamic regulatory network (Válóczi et al. 2006; Meng et al. 2011). The fine-tuning regulation of plant development by miRNA has been revealed from the characterization of several plant mutants, either impaired in steps of the miRNA biogenesis, displaying pleiotropic developmental defects, or impaired in particular *MIR* genes and targets, leading to more specific developmental defects (Mallory and Vaucheret 2006). However, pleiotropic defects have been also observed, mostly in cases where a miRNA has several targets.

During seed development, miR160 and miR167 regulation of the auxin-related transcription factors, *ARF17* and *ARF6/8*, affect embryo development, seed production and germination rates (Mallory et al. 2005; Todesco et al. 2010). The gibberellin (GA)- and abscisic acid (ABA)-regulated transcription factors *MYELOBLASTOSIS* (*MYB*) GAMYB-like genes *MYB33/65* are regulated by miR159, affecting seed size and fertility (Allen et al. 2007). In the early stages of embryogenesis, miR165/166 and miR394 seem to be essential for stem cell differentiation and shoot apical meristem (SAM) maintenance. MiR165/166 regulates the *HD-ZIPIII* transcription factors to define the vascular cell types in the roots and maintain cell pluripotency in the SAM, via association with AGO10 (Carlsbecker et al. 2010; Zhu et al. 2011) whereas miR394 is required for stem cell differentiation and targets an F-Box encoding gene *LCR* (Knauer et al. 2013; Litholdo et al. 2016). Although these studies did not show any phytohormone relationship with these miRNAs regulation, hormones, such as auxin and cytokinin could be contributing to this cell differentiation processes (Knauer et al. 2013; Leibfried et al. 2005).

During leaf development, miR165/166 and miR394 also play an important role. MiR165/166, in conjunction with miR390/ta-siRNAs, determine the abaxial-adaxial leaf polarity (Nogueira et al. 2007), and miR394 influences leaf shape and curvature, which is suggested to involve auxin signaling (Song et al. 2012). The miR393 regulation of the auxin receptors *TIR1* and *AUXIN SIGNALING F-BOX* (*AFB*s) genes also mediates some auxin-related aspects of leaf development (Si-Ammour et al. 2011). Moreover, miR164 and miR319 are important regulators during leaf initiation, growth and differentiation, by targeting *CUC1*/*CUC2* and *TCP* transcription factors genes, respectively (Pulido and Laufs 2010).

During the plant life cycle, miR156 and miR172 are the main players regulating the transition from juvenile to adult vegetative phase, and from the vegetative to reproductive phase (Spanudakis and Jackson 2014). Both miRNAs regulate transcription factors, including 11 genes encoding SPL protein regulated by miR156, and six *AP2*-like genes regulated by miR172. Interestingly, both miRNAs show opposite expression pattern during phase changes, mediated by integrated and coordinated transcriptional activation of their pathways (Wu et al. 2009). Additionally, miR159, miR319, and miR390 also regulate flowering time, implying that GA and auxin might coordinate the regulation of these miRNAs.

During root development, the regulation of *HD-ZIPIII* genes by miR165/166 modulates lateral root initiation, vascular tissue differentiation and nitrogen-fixing nodule development (Boualem et al. 2008; Carlsbecker et al. 2010; Miyashima et al. 2011). Another auxin-dependent process in the roots involves miR160 regulation of the transcription factors *ARF10/ARF16*, and the miR390-triggered production of ta-siRNAs, targeting *ARF4* (Wang et al. 2005; Yoon et al. 2010). Moreover, miR828-triggered ta-siRNAs target members of *MYB* transcription factors, playing a role in root hair patterning and anthocyanin production (Luo et al. 2012; Xia et al. 2012).

Interestingly, the complex network of interactions between miRNAs and hormonal signaling pathways integrates plant development and stress response signals. For instance, the miR393 regulation of the F-Box genes *TIR1* and *AFB2* mediates the auxin-dependent root development in response to ABA-related drought stress (Chen et al. 2012). Moreover, the metabolism of some inorganic nutrients depends on the gene regulation mediated by mobile miRNAs, such as miR395, miR398, and miR399, which are responsive to starvation of sulfur, copper/zinc, and phosphate, respectively (Kawashima et al. 2009; Yamasaki et al. 2007; Bari et al. 2006). All the *MIR* genes exemplified in this section are conserved among several evolutionary distant plant species, demonstrating the crucial roles of these conserved miRNAs in fundamental and ubiquitous aspects of plant development. However, non-conserved *MIR*s has also been uncovered, such as miR824 that is only found in Brassicaceae yet plays a role in development. It regulates the conserved transcription factor *AGAMOUS-LIKE16* (*AGL16*), which is important for normal stomata development (Kutter et al. 2007). This suggests that non-conserved miRNAs can emerge and acquire developmental functions in a restricted number of species.

The majority of *MIR* loci identified in a specific miRNAome have been found to be young, non-conserved microRNAs (Jones-Rhoades 2012; Axtell 2013). It has been assumed that most of the recently evolved *MIR* genes are short-lived, imprecisely processed, and functionally irrelevant. This is mostly due to the lack of identified and/or validated target genes and therefore some of the non-conserved miRNAs are likely to be under neutral selective pressure (Axtell 2008; Jones-Rhoades 2012). However, these assumptions could be the result of restricted spatiotemporal expression pattern of young *MIRs* or their expression being activated only under a particular stress condition. Moreover, it has been suggested that recently evolved miRNAs could have a distinct mode of interaction with their target genes or even in their mode of targeting, which might prevent the identification of the targets using the usual rules and approaches (Axtell 2008, Cuperus et al. 2011).

### 10.2.7 Recent Applications of Omics and Small RNA Research in Plant Biotechnology

The development of modern genetic engineering approaches and high-throughput technologies in biological research, besides the holistic view of systems biology, have triggered the progress of biotechnology to address plant productivity and stress adaptation (Table 10.1). The introduction of transgenes into plants has been widely and efficiently used for crop breeding, generating genetically modified organism with desired traits. Currently, the available omics information for selection of specific characteristics for breeding has offered a range of opportunities. Due to omics-scale molecular analysis and elucidation of genetic information and interactive networks, the modern biotechnology has the potential to target any traits for breeding, by interfering in one or multiple genes and/or networks.

Small RNA research has many potential applications in the plant biotechnology, aiming to increase food production, disease and pest controls, and to overcome the consequences of climate change (Zhou and Luo 2013; Kamthan et al. 2015; Zhang and Wang 2015, 2016; Liu et al. 2017). Several miRNAs may target multiple genes at a same time and it has been shown that manipulating a single *MIR* gene can significantly interfere in intricated gene networks, to provide an appro-

priate strategy for crop improvement. For instance, *MIR156*—the sRNA miR156 targets transcription factors-encoding genes, namely SQUAMOSA-promoter binding like proteins (SPL) (Schwab et al. 2005; Wang et al. 2008; Yamaguchi et al. 2009; Lal et al. 2011; Kim et al. 2012), and an increase by more than 100% in plant biomass is observed by overexpressing *MIR156* in different plant species, including Arabidopsis, rice, tomato, and switchgrass (Schwab et al. 2005; Fu et al. 2012; Xie et al. 2012).

MiRNAs also play an important role in plant responses to biotic and abiotic stresses (Ku et al. 2015; Litholdo et al. 2017), and accordingly, the manipulation of miRNAs to increase plant defenses has been applied to several plants, including agricultural crop species (Djami-Tchatchou et al. 2017). The first *MIR* gene revealed to play a role in plant stress responses was the *MIR393*—miR393 regulates the auxin signaling transcription factors and the overexpression of this miRNA leads to inhibition of bacterial growth (Navarro et al. 2006). Transgenic plants overexpressing miR7696 and miR396 also confers enhanced resistance to rice blast infection and cyst nematode infection in Arabidopsis, respectively (Campo et al. 2013; Hewezi et al. 2008). For abiotic stresses, the increased abundance of miR169 in transgenic tomato plants enhanced drought tolerance, by regulating target genes involved in stomatal opening, transpiration rate, and therefore, leaf water loss (Zhang et al. 2011). MiR319 has been shown to confer resistance to different environmental conditions, such as cold, salt and drought stress—transgenic rice and creeping bentgrass plants, overexpressing miR319, showed respectively increased tolerance to these conditions (Yang et al. 2013; Zhou et al. 2013).

Besides the manipulation of single miRNA/target genes module, to generate transgenic plants, the miRNA-mediated gene silencing serves also as a biotechnological tool and is currently applied in plant science, to generate mutants of theoretically any gene of interest. Individual genes can be silenced by introducing into plants engineered RNA silencing expression constructs, such as artificial miRNAs to target and inactivate endogenous gene expression (Molesini et al. 2012). This approach can disrupt the production of a specific unwanted compound, for example the caffeine to deliver a decaffeinated coffee plant. Conversely, the expression of endogenous small RNAs can be altered by suppression or overexpression of the mature sRNA sequence to alter plant development and protection (Djami-Tchatchou et al. 2017). The deregulation of specific plant miRNAs, and consequently the target gene(s), can aim numerous purposes, such as an increase in plant biomass, tolerance to biotic and abiotic stresses, fruit maturation control, and production of compounds of interest (Molesini et al. 2012; Sunkar et al. 2012; Zhang 2015).

## 10.3 Concluding remarks

With this chapter, we provided an overview on omic studies for the searching and identification of metabolites and proteins employed by microorganisms to develop biotechnological products. Additionally, we present an overview of the central aspects of small RNA as regulators of gene expression connecting system networks and the potential application into plant biotechnology. First used to generate virus resistance, several other RNAi strategies have been used for trans-kingdom gene regulation. Double-stranded RNA (dsRNA) produced by plants to target pathogen endogenous gene and reduced virulence has been one of the most successful approach to control insects, nematodes, and more recently, fungi. Host-induced gene silencing (HIGS) by the generation of transgenic plants carrying pathogen-targeting constructs, and spraying dsRNA solution in target organisms are experimentally validated in biotechnology approaches.

The omics, which comprises but not limited to genomic, transcriptomic, proteomic, epigenomic, and metabolomic studies in entire plants, allow a better understanding of plant biology and contribute further to biotechnology development. Recent methodological advances are enabling biological analyses of single-cells to provide opportunities to enhance our understanding of plant biology as a system (Libault et al. 2017).

During the last decade, the discovery of regulatory small RNAs altered the perception that only protein-coding genes are the players in gene regulatory network, since sRNAs emerged as central players in the transcriptional and posttranscriptional gene expression. The change in paradigm altered the way system biology is comprehended and how we can use this regulatory mechanism to improve biotechnology toolbox.

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