Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2021) **19**, pp. 1495–1510 doi: 10.1111/pbi.13605

Review

Novel and emerging biotechnological crop protection approaches

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Received 18 November 2020;
revised 1 April 2021;
accepted 13 April 2021.
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Keywords: new plant breeding techniques, cisgenesis, genome editing, RNA interference, disease resilience, abiotic stress, DNA methylation, epigenetics.

Summary

Traditional breeding or genetically modified organisms (GMOs) have for a long time been the sole approaches to effectively cope with biotic and abiotic stresses and implement the quality traits of crops. However, emerging diseases as well as unpredictable climate changes affecting agriculture over the entire globe force scientists to find alternative solutions required to quickly overcome seasonal crises. In this review, we first focus on cisgenesis and genome editing as challenging biotechnological approaches for breeding crops more tolerant to biotic and abiotic stresses. In addition, we take into consideration a toolbox of new techniques based on applications of RNA interference and epigenome modifications, which can be adopted for improving plant resilience. Recent advances in these biotechnological applications are mainly reported for non-model plants and woody crops in particular. Indeed, the characterization of RNAi machinery in plants is fundamental to transform available information into biologically or biotechnologically applicable knowledge. Finally, here we discuss how these innovative and environmentally friendly techniques combined with traditional breeding can sustain a modern agriculture and be of potential contribution to climate change mitigation.

Introduction

Increasing plant resilience against biotic or abiotic stress and improvement of quality traits to make crops more productive as well as nutritious are focal targets in plant breeding programmes. Opposing pressure comes from the increasing virulence of a large number of pests and diseases, caused by insects, fungi, bacteria, viruses and nematodes (Gimenez *et al.*, 2018), and legislation limiting the use of agrochemicals (Directive 335 2009/128/EC and Regulation (EC) No 1107/2009 of the European Parliament and of the Council). On the other hand, climate changes expand abiotic stress conditions forcing plant breeders to select genotypes resistant to water and thermal stresses to cope with the modification of rainfall patterns and rise in temperatures (Mohanta *et al.*, 2017b; Porter *et al.*, 2014). These unfavourable constraints are leading to insufficient yield and a strong decrease in quality features (Ebi and Loladze, 2019).

The development of genetically improved varieties of crop plants has long been taking advantage of crossings and mutagenesis to obtain plants with better characteristics in terms of yield and quality features, as well as improved stress resilience traits (Dempewolf *et al.*, 2017). Since the 1920s, when introgression of the desired traits from the available germplasm has not been possible, mutagenesis through radiation or chemical agents has been used. Over the last century, genetic engineering and biotechnologies have broadened the toolbox of geneticists and breeders with new instruments and approaches, leading to the creation of genetically modified organisms (GMOs) (Lusser *et al.*, 2012). The potential of this approach to obtain improved disease resistance, abiotic stress resistance and nutritionally improved genetically modified crops have been widely demonstrated and discussed, together with the limitations and the concerns associated with the use of GMOs (Kumar *et al.*, 2020; Low *et al.*, 2018; Sabbadini *et al.*, 2021; Van Esse *et al.*, 2020).

Thanks to these techniques, the gene pool potentially available to plant breeders has considerably increased, allowing the isolation and transferring of genes to crops from sexually incompatible plant species as well as from other organisms (Carrière *et al.*, 2015). Although in 2018 GM crops covered 191.7 million hectares with remarkable benefits (Brookes and Barfoot, 2016; Change, 2018), their use is still associated with strong public concern, which is related to putative risks for human health and environment contamination (Carzoli *et al.*, 2018; Frewer *et al.*, 2011). Insertion in the crop genome of genes isolated from

genetically distant and/or unrelated organisms (transgenes), which usually includes selectable markers (e.g. resistance to antibiotics), is one of the most criticized aspects by citizens. Over the years, to overcome GM crop limitations, many techniques have been developed up to the latest new plant breeding techniques (NPBTs, e.g. genome editing).

In the last 15 years, next-generation sequencing (NGS) technologies fostered a major advancement in crop genomics and contributed to the public availability of many reference crop genomes (Jaillon *et al*., 2007; Linsmith *et al*., 2019; Sato *et al*., 2012; Verde *et al*., 2017; Xu *et al*., 2011). Moreover, high-throughput re-sequencing of hundreds of genotypes allowed researchers to describe the allele diversity of both domesticated and wild plant populations (Morrell *et al*., 2012). In this context, the increased data availability on genome structures deepened the comprehension of plant domestication history, the identification of genes responsible for traits of agrochemical interest and gene functions, promoting the development of NPBTs for overcoming the major GMO laborious and costly regulatory evaluation processes and public concerns. Actually, NPBTs allow a single gene to be transferred, mimicking sexually compatible crosses (cisgenesis) and precise modification of specific DNA sequences (genome editing).

In this review, we summarize the main features, advantages and challenges of various biotechnological approaches, providing examples of applications for the amelioration of plant traits to better cope with biotic and abiotic stresses. The common thread is to describe the recent biotechnological advancements which allow crop traits to be precisely modified and overcome the restrictions imposed on genetically modified products. Therefore, we focused our discussion on cisgenesis and genome editing as the more known techniques, but we also addressed our attention on latest innovative crop breeding technologies, such as RNA interference and epigenome editing. Emphasis is given to non-model plants, such as woody crops, for which the application of biotechnological approaches is not as easy as for herbaceous model plants.

Cisgenesis: approaches and potentials in plant protection

The idea of cisgenesis was first proposed by Shouten in 2006. In its widely accepted definition, the results of cisgenic approaches are crops modified with genes isolated exclusively from sexually compatible plants, including gene introns and regulative regions, such as promoters and terminators, in their sense orientation (Schouten *et al*., 2006a; Schouten *et al*., 2006b).

Cisgenic strategies

Cisgenic plants may resemble plants derived from traditional breeding and share the same genetic pool with them, since genes of interest are isolated from a species that could be used for traditional crosses and transferred, preserving its 'native' form. One of the main drawbacks of gene introgression in a crop genome by classical crosses is that a large number of undesirable associated genes are transmitted along with the gene(s) of interest to the next generation, often negatively influencing many agronomic traits, related to product quality and yield. This phenomenon, defined as linkage drag, is common in introgression breeding, and marker-assisted selection (MAS) is often adopted to reduce the amount of undesired genes (Hospital, 2005). The use of MAS-complex schemes slows down new cultivar release, which can require decades in the case of woody plants that have long juvenile phases. Cisgenesis allows the linkage drag issue to be overcome by transferring only the desired gene(s) in a single step, preserving all the quality traits selected in the élite cultivars.

The limit of cisgenesis is its suitability only to monogenic traits, although it could also be applied to oligogenic characters: indeed, the technical complexity of the procedure is directly correlated with the number of genes to be transferred. On the other hand, cisgenic plants display greater public and farmers positive consensus compared to transgenic ones (Delwaide *et al*., 2015; Rousselière and Rousseliere, 2017; De Steur *et al*., 2019).

Detailed methods and strategies with an interesting success rate for the development of cisgenic plants have been comprehensively reviewed by several authors over the last decade (Cardi, 2016; Espinoza *et al*., 2013; Holme *et al*., 2013; Schaart *et al*., 2011) so these approaches are quite mature for a wide use.

Since its initial application, several strategies have been conceived for cisgenesis (Figure 1), by considering the differences in transformation and regeneration efficiency and length of the breeding cycle, which depend on the selected plant species. The simplest approach consists of the use of vectors where only the gene of interest is cloned in the T-DNA region, transferred to plants through *Agrobacterium*-mediated transformation and then selected by PCR analysis (Figure 1a) (Basso *et al*., 2020; De Vetten *et al*., 2003). Another similar strategy exploits minimal gene cassettes, made just by promoter, coding sequence and terminator, which are introduced into the plant genome by biolistic transformation (Figure 1b), thus avoiding partial or complete backbone integrations (Low *et al*., 2018; Vidal *et al*., 2006). Nevertheless, these systems require long and expensive PCR screenings and are suitable only for species with a high transformation efficiency (Low *et al*., 2018; Malnoy *et al*., 2010; Petri *et al*., 2011; Vidal *et al*., 2006). In species where transformation is recalcitrant, the transformation with cisgenic reporter genes or co-transformation with selectable marker genes could greatly simplify the recovery of transformed plants. For example, Myb transcription factors involved in the regulation of anthocyanin biosynthesis were tested in apple (Krens *et al*., 2015) and grapevine (Li *et al*., 2011) as selectable markers for cisgenic plants. The use of exogenous or endogenous reporter genes has been already successfully applied in herbaceous species (Basso *et al*., 2020). However, the possibility of using such reporters is confined to those cases where tissue coloration does not interfere with selection for other traits of interest. In seed propagated crops (e.g. wheat, barley, rice and tomato), it is possible to use a co-transformation strategy (Figure 1c), crossing them with the parental or original variety and hence exploiting segregation of the selectable marker in the progeny, obtaining plants with the cisgene but without the selectable marker (Holme *et al*., 2012a).

For vegetative propagated species with poor transformation efficiencies, a novel developed approach relies on the excision of unwanted DNA sequences after the selection of transformed plants through recombination systems (Figure 1d). In 1991, Dale and Ow used the bacteriophage P1 Cre/lox recombinase/sites for marker excision in tobacco plants (Dale and Ow, 1991). Since then, other alternative systems from *Zygosaccharomyces rouxii* (R/Rs) and *Saccharomyces cerevisiae* (FLP/frt) have been tested (Lyznik *et al*., 1993; Schaart *et al*., 2011). In all these systems, the recombinase expression is usually controlled by chemical or heat shock inducible promoters to avoid a premature excision of the

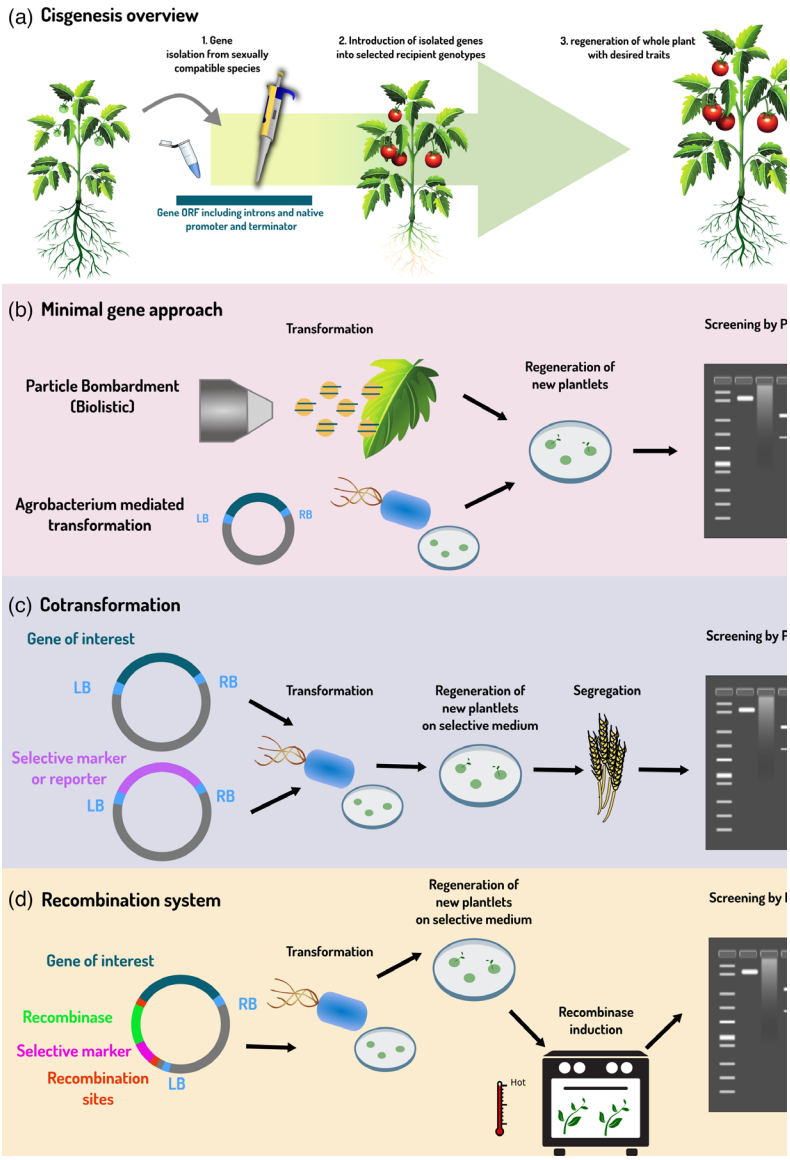

Figure 1 (a) Overview of cisgenic strategies from gene selection to plant phenotyping; (b) minimal gene approach, only the gene of interest is cloned in the T-DNA region; (c) co-transformation strategy, the selective marker and gene of interest are introduced by independent transformation events, segregation of the genes allows the selection of cisgenic plants in F1 progeny; (d) excision of unwanted DNA sequences through recombination systems: chemical or physical stimulation induce the excision of DNA fragments flanked by the recombination sites.

selectable markers (Figure 1d) (Dalla Costa *et al*., 2016; Schaart *et al*., 2011).

Stress-tolerant cisgenic crops

Cisgenic approaches were adopted in potato, apple, grapevine, melon, wheat, barley, poplar, rice and strawberry (Benjamin *et al*., 2009; Dhekney *et al*., 2011; Gadaleta *et al*., 2008a; Han *et al*., 2011; Haverkort *et al*., 2016; Holme *et al*., 2012a; Krens *et al*., 2015; Maltseva *et al*., 2018; Tamang, 2018). In most cases, the aim was to increase pathogen resistance, although some studies were focused on quality trait improvement.

Haverkort and colleagues pursued a marker-free approach to obtain four cisgenic late blight (*Phytophthora infestans*)-resistant *potato varieties, by transferring from one to three resistance genes (Haverkort et al*., 2016). In addition, cisgenic apple varieties were developed by introducing the apple scab (*Venturia inaequalis*) resistance gene *Rvi6* in the susceptible cultivar 'Gala' (Schaart *et al*., 2011). In the same work, the authors achieved the removal of the selectable marker gene by inducing the *recombinase R* with dexamethasone. The obtained cisgenic plants were tested in field conditions for three years and showed a stable resistant phenotype (Krens *et al*., 2015). Interestingly, the effectiveness of the same recombinase system was recently also tested in banana, inducing the excision of the green fluorescent protein, used as reporter gene (Kleidon *et al*., 2019).

Several pathogen resistance genes (PR1 variants, *VvTL1*, *VvAlb1*, homologues of *VvAMP1* and *VvAMP2*/defensin, and an orthologue of Snakin-1) have been isolated from species sexually compatible with *Vitis vinifera* and overexpressed in transgenic lines, which are now under evaluation in field conditions (Gray *et al*., 2014). In grapevine, methods using a heat shock controlled FLP/frt recombination system for selectable marker excision have also been reported (Dalla Costa *et al*., 2016; Dalla Costa *et al*., 2020).

Transgenic lines of melon have been developed overexpressing the glyoxylate aminotransferase *At1* and *At2* genes, conferring resistance to *Pseudoperonospora cubensis*, which causes downy mildew in cucurbits (Benjamin *et al*., 2009). Since the resistance is given by the increased transcription level of these genes, it remains to be assessed whether such an increase can be obtained in cisgenic lines.

In durum wheat, biolistic co-transformation with minimal gene cassettes was used to develop cisgenic lines expressing *1Dy10* HMW glutenin gene, isolated from bread wheat and associated to an improved baking quality. Homozygous cisgenic lines were obtained by segregation at the 4th generation (Gadaleta *et al*., 2008b; Gadaleta *et al*., 2008c). Moreover, cisgenic lines of wheat carrying a class I chitinase gene displayed partial resistance to fungal pathogens (Maltseva *et al*., 2018). Holme *et al*. (2012b) used a barley phytase gene (*HvPAPhy_a*) and the co-transformation strategy to test cisgenic feasibility in barley, obtaining lines with increased phytase activity (Holme *et al*., 2012a).

Cisgenesis has also been applied in rice, to overcome one of the most diffuse and devastating pathogens (*Magnaporthe grisea*), by using a co-transformation strategy to introduce rice blast disease resistance gene *Pi9* into elite rice cultivars (Tamang, 2018).

In addition to stress resistance, cisgenesis is also an effective approach for modifying other crop traits as it has been demonstrated in poplar. Genes from *Populus trichocarpa* (*PtGA20ox7*, *PtGA2ox2*, *PtRGL1_2*) involved in gibberellin metabolism were transformed in *Populus tremula* × *alba*, showing that negative gibberellic acid regulators determined a slower growth (*PtGA2ox2*) and longer xylem fibres (*PtRGL1_2*), while the positive regulator determined an increased growth rate (*PtGA20ox7*). However, the poplar plants obtained still contained the positive selectable marker and cannot be considered as cisgenic (Han *et al*., 2011).

Intragenic plants, as in the case of cisgenesis, possess only genetic material deriving from sexually compatible species, but the inserted gene is the result of a genetic element isolated from different species (e.g. a gene promoter from one species and a coding sequence from another, both sexually compatible) (Holme *et al*., 2013). An interesting example of this approach comes from the overexpression of cisgenic polygalacturonase inhibitor protein (*FaPGIP*) in strawberry which conferred resistance to grey mould (*Botrytis cinerea*). The overexpression was achieved by cloning the *FaPGIP* coding sequence under the promoter of the strawberry expansin-2 gene and for this reason should be referred to as intragenic (Schaart, 2004).

Genome editing

Genome editing introduces changes in specific target DNA sequences without altering other regions (including the target flanking regions) and with the potential to avoid introduction of foreign DNA. The genome editing is performed using endonucleases which are able to recognize specific DNA sequences. Once the target sequence is recognized, the endonuclease introduces a double-strand DNA (dsDNA) break (DSB) and induces subsequent activation of the DNA repair pathway (Manghwar *et al*., 2019). This result can be achieved by exploiting three different classes of enzymes: zinc-finger nucleases (ZFNs), transcription activator-like effectors nucleases (TALENs) and Cas proteins (Zhang *et al*., 2017). Strong efforts have been made by numerous researchers all over the world to improve the Cas-mediated genome editing technology, which became the most used and efficient tool to edit target genomes (Xie and Yang, 2013). The ability of genome editing techniques to help breeders in improving plant resistance against biotic and abiotic stresses is only in its infancy, but some examples are already available and a concise overview of the steps involved in the development of edited plants is presented in Figure 2.

Focus on CRISPR-Cas: a brief overview

The clustered regulatory interspaced short palindromic repeats (CRISPR)-Cas systems, discovered as conserved mechanisms against viral invasions in bacteria, require three distinct components: a protein with nuclease activity (e.g. Cas9, Cas12 and Cas13), a single guide RNA (sgRNA) necessary to guide the Cas protein on target sites and a protospacer adjacent motif (PAM) and a short sequence upstream of the complementary DNA strand acting as tag of the target site (Figure 3a) (Doudna and Charpentier, 2014). The sgRNA-Cas complex scans the genomic DNA looking for the complementary sequence, and once identified, the Cas protein induces a dsDNA cleavage at a specific position that is determined by the Cas type (Jiang and Doudna, 2017). After DNA cleavage, there are two major pathways of DNA repair in plants: homologous recombination (HR) and non-homologous end joining (NHEJ), the latter being the most commonly used (Ran *et al*., 2013; Schwartz, 2005). These two repair mechanisms are the basis for exploiting the Cas in NPBTs.

The CRISPR-Cas system shows very versatile features to produce knockout mutants, to insert a DNA fragment using a donor vector through the HR system, to base edit a target sequence (e.g. substitutions of C to T and/or A to G etc.), to induce mutation in regulatory sequences and modify the epigenome (Vats *et al*., 2019). Nevertheless, if multiple genes that are closely related have to be targeted (e.g. gene family members, multiple alleles of the same gene), two different strategies are available: (i) multiple guide RNAs under the control of a same promoter (polycistronic construct) or multiple guides under the control of their own specific promoter (Cermak *et al*., 2017; Tang *et al*., 2016; Xing *et al*., 2014) and (ii) one or a few sgRNAs capable of driving the Cas protein on different genes (Yu *et al*., 2018).

Initial steps through a wide use of CRISPR/Cas system

The first reported genome editing application using CRISPR/Cas systems in plant was achieved in 2013 using two model organisms: *Arabidopsis thaliana* and *Nicotiana benthamiana* and easily observable reporter genes (Li *et al*., 2013; Mao *et al*., 2013). Over the years, more progress has been made, with several reports in different herbaceous plant species (e.g. tomato, rice, soybean and wheat) up to the application in woody species (e.g. citrus, apple and grape) (Ghogare *et al*., 2020). Furthermore, different laboratories are committed in developing new delivery methods for plant systems. Indeed, classically the DNA sequences encoding for Cas and sgRNA(s) have to be delivered into the host plant genome, and to date, different methods have been tested: *Agrobacterium*-mediated transformation, nanoparticle platforms, biolistic transformation and protoplast transfection (Ahmad and Amiji, 2018; Kalinina *et al*., 2020). Even though *Agrobacterium*-mediated transformation is widely used in plants, this method requires integration of T-DNA into the host genome together with selectable marker genes (Dalla Costa *et al*., 2016; Duensing *et al*., 2018). Actually, the integration of selectable markers is an important legislative issue as it can be stably transferred to sexually compatible species and also to other organisms, without reproduction or human intervention, as a consequence of horizontal gene transfer (HGT) (Keese, 2008; Soda *et al*., 2017). Conversely, protoplast transient transformation and regeneration approach allows the direct delivery of ribonucleoproteins (RNPs) in plant tissues without introducing foreign DNA and GM plant creation (Baltes *et al*., 2015; Bruetschy, 2019; Cermak *et al*.,

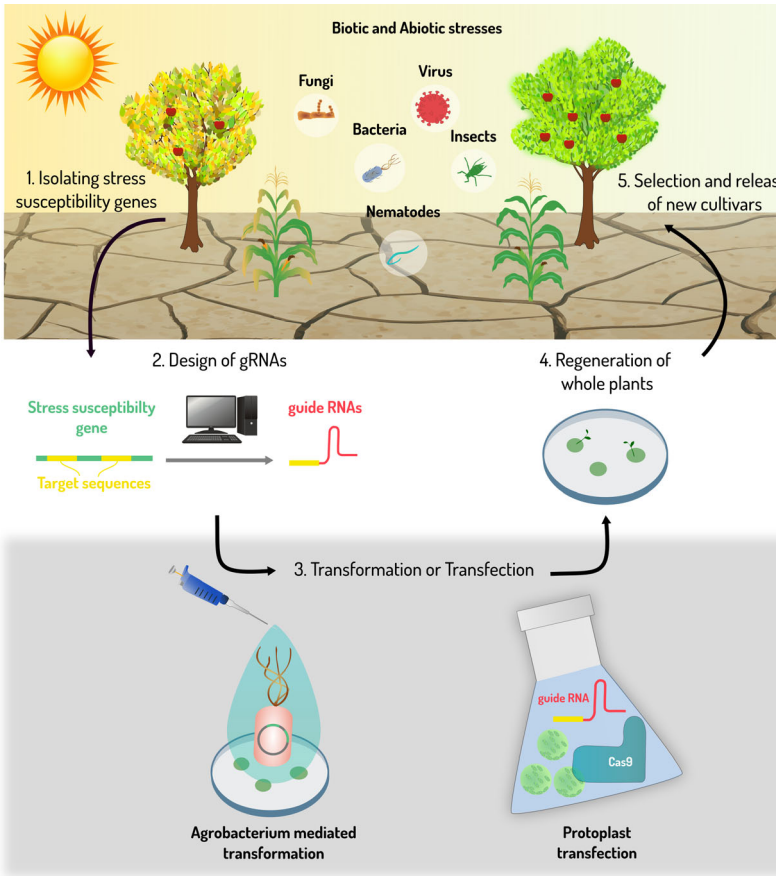

Figure 2 Workflow for the development of genome edited stress-resistant crops: (1) susceptibility genes are isolated and characterized by genetic and functional genomics studies; (2) informatics-aided design of gRNAs for increased specificity and off-target minimization; (3) Agrobacterium tumefaciens-mediated transformation of plant tissue cultures or ribonucleoprotein protoplast transfection. (4) Regeneration and selection of transformed plants; (5) testing and selection of transformed lines, release of new varieties.

2017). Recently, the *Agrobacterium*-mediated transformation was compared with the RNPs delivery through PEG-mediated protoplast transfection approaches in apple and grapevine (Osakabe *et al*., 2018). Although the biolistic method allows the production of transgene-free plants, it displays huge limitations in woody plants (Osakabe *et al*., 2018) due to restraint in obtaining the embryogenic tissue, which is then able to regenerate the edited plant (Altpeter *et al*., 2005).

CRISPR technology as a valuable tool to improve crop protection

One of the main tools to enhance plant resistance against fungal and bacterial pathogens relies on targeting susceptible genes (S genes) (Pavan *et al*., 2009) as proven in *Theobroma cacao* and several other species (Fister *et al*., 2018; Langner *et al*., 2018). Susceptibility gene distinctiveness relies on the fact that they are genes that critically facilitate the compatibility between the plant and the pathogen. They are essential for their interaction, especially in the case of biotrophic pathogens. Therefore, mutation or loss of an S gene can limit the ability of the pathogen to cause disease (van Schie and Takken, 2014). An interesting example was given by Paula de Toledo Thomazella et al. (2016), who introduced a mutation in *Solanum lycopersicum DMR6* gene lowering tomato susceptibility not only to downy mildew but also to *Pseudomonas syringae*, *Phytophthora capsici* and *Xanthomonas* spp. (Paula de Toledo Thomazella *et al*., 2016). A similar approach was used in apple (*Malus domestica*) to achieve resistance against *Erwinia amylovora* (Pessina *et al*., 2016). Pompili *et al*. (2020) used the Cas9 system to produce an *MdDIPM4* knockout mutant enhancing plant resistance against the fire blight pathogen. A novelty introduced by this approach is an inducible recombination system (FLP/frt) able to remove almost all the T-DNA insertions after confirming the editing event. CRISPR technology was latterly applied to rice in order to obtain bacterial blight-resistant varieties: Cas9-mediated genome editing to introduce mutation in one or multiple susceptible genes, belonging to the sugar transporters SWEET family, was successfully achieved in recent works (Oliva *et al*., 2019; Zeng *et al*., 2020). Finally, another interesting application of CRISPR to counteract biotic stress was provided in tomato. By targeting a microRNA (miRNA), it was demonstrated the possibility to enhance plant immunity against *Fusarium oxysporum* f. sp. *Lycopersici*, the causal agent of tomato wilt disease, enhancing the basal expression of nucleotide-binding site leucine-rich repeat (NBS-LRR) protein (Gao *et al*., 2020).

As for fungal and bacterial pathogens, the CRISPR technology can provide a strategy to generate plants with virus resistance. For instance, it is possible to both directly target viral replication, by producing GMO plants expressing constitutive Cas protein and gRNA(s) that target viral sequences (Baltes *et al*., 2015; Ji *et al*., 2015) or to generate virus-resistant cultivars through modification of plant genes (Kalinina *et al*., 2020 and references therein).

Beyond biotic stresses, and despite a limited number of papers, abiotic stresses such as water deficit, high temperature and soil salinity can also be tackled by editing plant genes involved in stress response (Joshi *et al*., 2020; Nguyen *et al*., 2018; Zafar *et al*., 2020). An interesting example was reported in a work where the *OST2/AHA1* locus (which regulates stomata response to abscisic acid) was edited to obtain Arabidopsis with increased

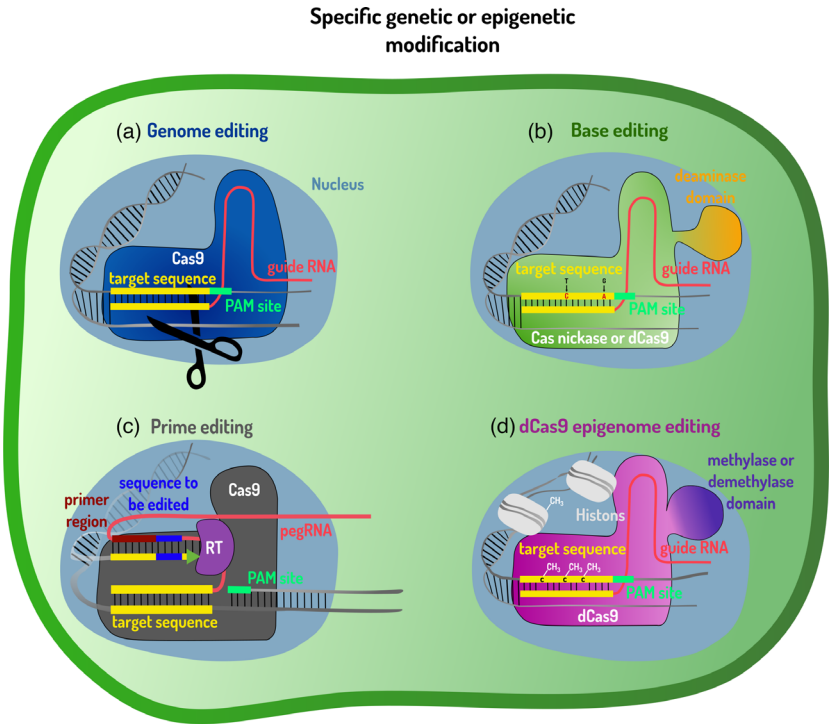

Figure 3 Highly specific genetic and epigenetic modifications by CRISPR-Cas technology: 3a-genome editing; 3b-base editing; 3c-prime editing; 3d-epigenome editing.

stomatal responses upon drought and a consequent lower water loss rate (Osakabe and Osakabe, 2017). In parallel, if not directly applied to achieve drought-resistant crops, CRISPR technology can be exploited to study the function of gene(s) along complex regulatory mechanisms. This was the case of non-expressor of pathogenesis-related gene 1 (NPR1), a special receptor of salicylic acid (SA), considered as an integral part in systemic acquired resistance (SAR) (Wu *et al.*, 2012). Cas9 was used to obtain NPR1 tomato mutants, which showed reduced drought tolerance, demonstrating that, despite its involvement in biotic stress responses, NPR1 is also involved in abiotic stress resilience (Li *et al.*, 2019). More recently, the CRISPR activation (CRISPRa) system (Brocken *et al.*, 2018) (based on an inactivated version of the nuclease known as dead Cas9 – see next paragraph for more information – fused with a transcription activator) targeting the promoter of ABA-responsive element-binding proteins (AREB) was used to study stress-related responses and enhance the drought tolerance in *Arabidopsis* (Roca Paixão *et al.*, 2019).

New frontiers in CRISPR/Cas application

Although genome editing has been widely used for editing specific plant genes, several studies relied on the improvement of its efficiency, versatility and specificity (Gleditzsch *et al.*, 2019). Indeed, despite many theoretical advantages and potential applications, the genome editing techniques still present one major drawback: Cas proteins can recognize PAM sites in non-target sequences and thus induce DSBs in these sequences, leading to undesirable phenotypes. To mitigate the off-target activities, different bioinformatic approaches were developed and used for computational prediction of Cas activity on specific genomes (Bae *et al.*, 2014; Lin and Wong, 2018; Liu *et al.*, 2020a). Moreover, development of Cas variants with improved specificity, such as Cas12a and b (Ming *et al.*, 2020; Schindele and Puchta, 2020), eSpCas9 (Slaymaker *et al.*, 2016), HiFi-Cas9 (Kleinstiver *et al.*, 2016) and HypaCas9 (Ikeda *et al.*, 2019), tried to mitigate the off-target activity and these variants have already been applied in plant genome editing strategies.

Beside the improved Cas variants, different authors have been focusing on the implementation of dead Cas9 (dCas9) (a Cas9 where both the nuclease domains have been inactivated) that could be used for several purposes. The simplest one is the ability to interfere with transcription via steric blockage of polymerase without performing endonuclease activity (Brocken *et al.*, 2018). Furthermore, the dCas9 system can be engineered by linking it to a transcription activator or repressor. These systems can be applied to species that lack a controllable expression system or to study the overexpression or down-regulation of target genes, without changing the genome context or introducing a transgene (Mohanta *et al.*, 2017a; Moradpour *et al.*, 2020).

The CRISPR-Cas system has also been engineered to perform base editing. Base editing is the ability to directly manipulate DNA sequences enabling the conversion of one base pair to another without performing a DSBs (Anzalone *et al.*, 2019; Yang *et al.*, 2019). A few years ago, Shimatani *et al.* (2017) used CRISPR-Cas9 fused to *Petromyzon marinus* cytidine deaminase *(PmCDA1)* and gRNAs to introduce point mutations in the acetolactate synthase (ALS) gene of rice and tomato, obtaining herbicide resistance (Shimatani *et al.*, 2017). Recently, base editing has been improved thanks to the development of prime editing, which is more efficient than the classic base editing (Anzalone *et al.*, 2019; Yang *et al.*, 2019). Differently from the classic dCas9, in prime editing only one nuclease domain is inactivated, generating a DNA nickase enzyme. The latter, combined with a retrotranscriptase enzyme (RT) and a prime editing guide RNA (called pegRNA), can produce both transition and transversion mutations, extending the possibility of common base editing (Figure 3b-c) (Anzalone *et al.*, 2019). In a recent article, plant prime editing (PPE) was tested in rice and wheat, giving the first proof of concept in plants. The authors chose six different genes and by evaluating

the single base editing efficiencies, confirmed the ability of PPE to produce all kinds of base substitutions (Lin *et al*., 2020).

Lastly, it is worth noting that a new class of CRISPR-Cas systems specifically targets RNA instead of DNA (Abudayyeh *et al*., 2017) and has been successfully used in plants to induce interference towards RNA viruses (Lotterhos *et al*., 2018). Added to this RNA targeting ability of the Cas13, a dCas13 conjugated to a deaminase was also suitable for RNA editing converting A to G and hence obtaining a system that can be used to edit full-length transcripts with pathogenic mutations (Cox *et al*., 2017). The rapid development of such a powerful and innovative techniques is the basis to achieve increased crop yields, resilient crops to both biotic and abiotic stress and to address consumer's concerns on GMOs approaches as well as nutritional needs (Kumar *et al*., 2020).

Towards new GMO-free approaches: exogenous dsRNA application for crop protection

Small RNAs (sRNAs) and RNA interference (RNAi) have emerged as modulators of gene expression in plant immune responses, pathogen virulence and communications in plant–microbe interactions. Since the RNAi machinery discovery, many efforts have been made to improve its applicability in plant protection (Cagliari *et al*., 2019; Dalakouras *et al*., 2020). In plants, RNAi is well known as a conserved regulatory strategy playing key roles in endogenous transcript regulation as well as viral defence, resulting in the post-transcriptional down-regulation of the target RNA sequence(s). The RNAi machinery is triggered by double-stranded RNA (dsRNA) molecules that, once produced in the cell, are processed by RNase III DICER-LIKE endonucleases and cleaved into 21-24 nt short interfering RNAs (siRNAs) (Liu *et al*., 2020b). After cleavage, one of the two siRNA strands associates to ARGONAUTE (AGOs) proteins to form RNA-induced silencing complexes (RISCs) (Meister, 2013; Poulsen *et al*., 2013). Consequently, these RISCs specifically interact with transcripts on sequenced-based complementarity, resulting in mRNA cleavage or translational repression, in a process known as post-transcriptional gene silencing (PTGS) (Figure 4) (Kim, 2008; Mi *et al*., 2008). Additionally, siRNAs can promote the deposition of repressive chromatin marks in target genomic DNA sequences triggering transcriptional gene silencing (TGS). In plants and invertebrates, siRNAs also have an important function in plant host–pathogen interactions: in the case of viral infections, siRNAs are produced in infected cells directly by processing dsRNA molecules derived from the viral genome itself. Interestingly, there is evidence that siRNAs, once produced in a specific cell, are able to move via plasmodesmata reaching the surrounding cells and, through the vascular system, up to distal parts of the plant, inducing the systemic silencing. Both siRNA short-distance and long-distance transport mechanisms to the whole plant have been documented and are still under scrutiny (Ham and Lucas, 2017).

Natural cross-kingdom RNAi and its biotechnological application

The RNAi processes are also pivotal in triggering plant immunity against pests and pathogens, modulating their development and virulence. There are lines of evidence supporting the observation that sRNAs can be exchanged bidirectionally among the interacting partners (e.g. plant–fungi) inducing gene silencing in each other and leading to a mechanism named as cross-kingdom RNAi (Cai *et al*., 2018b; Ma *et al*., 2020; Wang *et al*., 2016a). The latter is mediated by exosome-like extracellular vesicles able to deliver sRNAs into the interacting organisms, as recently demonstrated in *Arabidopsis–B. cinerea* pathosystem (Cai *et al*., 2018a). In particular, it was demonstrated that plant-delivered sRNAs can down-regulate the production of pathogen effectors, whereas *Botrytis* is able to deliver sRNAs, which turn off plant defences. All this evidence indicates that cross-kingdom RNAi can be utilized to control plant diseases caused by pathogens, including fungi, viruses and pests, such as nematodes and insects and foster the application of RNAi strategy to counteract crop pathogens.

Indeed, beside the fascinating mechanisms of siRNA production and translocation in plants, RNAi also represents a promising sustainable and environmentally friendly tool that can be used against crop pests and pathogens and might represent a good alternative to the application of chemicals. So far, in plants, RNAi has been largely used in functional genomic studies or for inducing resistance against insects in transgenic plants (e.g. in maize against *Diabrotica virgifera virgifera*; Fishilevich *et al*., 2016). *Agrobacterium*-mediated transformation has been applied to express pathogen/pest gene-targeting sRNAs or dsRNA against a selected target. This procedure named as host-induced gene silencing, HIGS, has led to the production of GM crop varieties, not commercialized in Europe (Baulcombe, 2015; Dalakouras *et al*., 2020 and references therein). Alternatively, a virus-induced gene silencing (VIGS) approach can be applied to express designed pathogen-targeting RNAs in plant tissue and circumvent the generation of GMOs (Dommes *et al*., 2019; Lee *et al*., 2012). Indeed, a recent report demonstrated the potentiality of VIGS as a tool for transiently targeting diverse regulatory circuits within a plant and indirectly affecting important agronomic traits, without incorporating transgenic modifications (Torti *et al*., 2021). However, VIGS relies on the use of virus expression vectors, which are themselves pathogenic to the plant and currently the development of a low or non-pathogenic virus expression vector is a major obstacle to the application of VIGS in crops.

The new frontier of RNAi for crop protection

GMO-free RNAi strategies, based on exogenous dsRNA/siRNA direct applications on plants (Dubrovina and Kiselev, 2019) are among the new approaches developed to overcome plant transformation and its limitations. Some examples of plant endogene modulation by exogenous dsRNAs application are available in the literature. In Arabidopsis, dsRNAs mixed with nanoparticles were adsorbed by plant roots and triggered RNAi against *SHOOT MERISTEMLESS* (*SSTM*) and *WEREWOLF* (*WER*) genes, which are involved in apical meristem and root epidermis regulation (Jiang *et al*., 2014). In another work, the authors suppressed the expression of a *MYB1* gene using crude bacterial extract containing dsRNAs (Lau *et al*., 2015). These studies confirmed the activation of RNAi in plants by dsRNAs adsorption through different tissues and by root soaking in a solution of dsRNAs (Dalakouras *et al*., 2018; Dalakouras *et al*., 2016; Li *et al*., 2015). These results also suggest that dsRNAs direct application could represent an effective disease-control strategy against fungal pathogens in crops. Several articles have indeed reported that the exogenous application *in vitro* or *in vivo* of synthesized long dsRNAs (through bacteria-mediated biosynthesis), hairpin RNAs (hpRNAs) or siRNAs can down-regulate the expression of pest essential genes, thus controlling harmful insects, fungal and viral pathogens. The RNA molecules were successfully applied by using several methods, such as high- or low-pressure spraying (spray induced gene silencing, SIGS), trunk injection, petiole

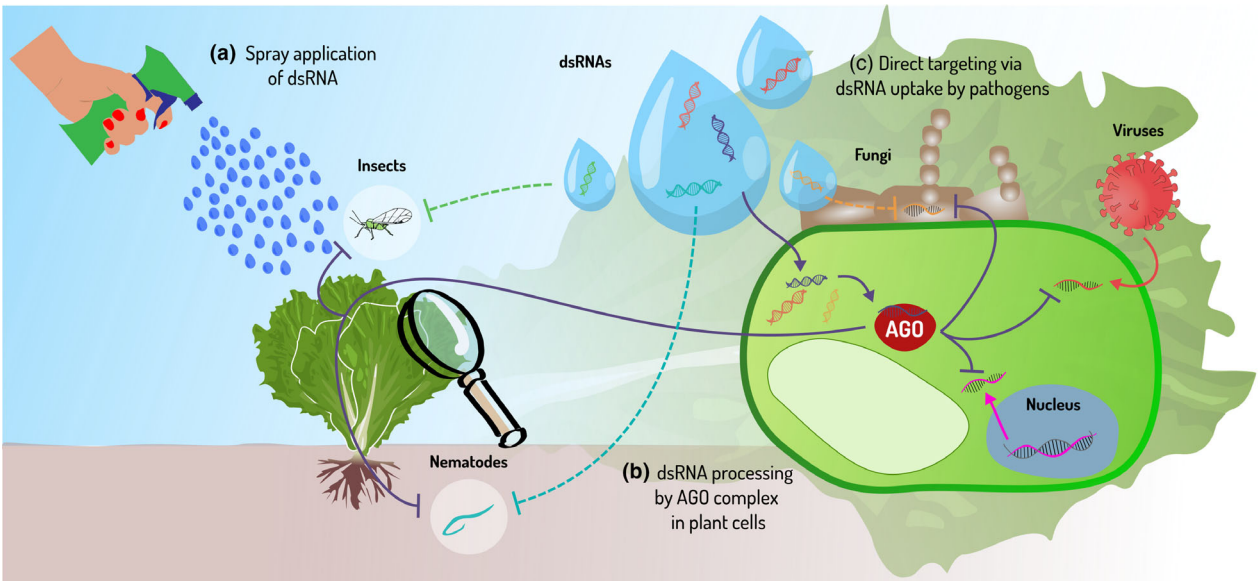

Figure 4 dsRNAs applications in crop protection: (a) dsRNA are sprayed on plants in field conditions; (b) dsRNAs penetrate the plant cells and after being processed by DICER-like nucleases associated with Argonaute protein (AGO) inducing post-transcriptional gene silencing towards pathogens or endogenous genes, continuous lines (━); (c) dsRNA directly enters pathogen cells silencing one or more essential genes, dotted lines (---).

absorption, soil/root drenching or mechanical inoculation and delivered naked or loaded into carriers (e.g. clay nanosheet, nanoparticles, proteins) to facilitate their uptake and survivability in plant tissues up to 7–8 weeks (Dalakouras *et al*., 2020; Dubrovina and Kiselev, 2019; Mitter *et al*., 2017). In the past few years, reports on plant-mediated delivery of dsRNAs against insects demonstrated the lowering of biological activity and/or increased mortality of aphids, whiteflies, mites and marmorated sting bugs in tomato and bean crops (Ghosh *et al*., 2018; Gogoi *et al*., 2017). In addition, dsRNAs microinjection in *Euscelidius variegatus*, a natural vector for phytoplasmas, has recently been reported (Abbà *et al*., 2019). In this respect, Dalakouras *et al*. (2018) provided very useful information to improve the plant-mediated dsRNAs efficacy against insects, suggesting the delivery of intact dsRNA, by using specific methods (e.g. petiole adsorption or trunk injection) to avoid the activation of plant RNA processing mechanisms. Indeed, the intact dsRNAs can be translocated by xylem vessels to plant distal tissues, picked up by insects and processed into siRNAs by their own RNAi system, resulting in a more effective response.

Exogenously delivered dsRNAs have been successfully applied in several fungal–plant pathosystems. As for insects, also in fungi, intact dsRNAs are proved to be more efficient in controlling pathogen development. This was first demonstrated by Koch *et al*. (2016), in which spraying dsRNAs on barley leaves achieved control of *Fusarium graminearum*. In addition, SIGS was effective against several fungal pathogens such as *Sclerotinia sclerotiorum* in *Brassica napus* (McLoughlin *et al*., 2018), *Fusarium asiaticum* in wheat coleoptiles (Song *et al*., 2018b) and *Botrytis cinerea* in several plants (Wang *et al*., 2016a) including grapevine, in both natural and post-harvest condition (Nerva *et al*., 2020).

The exogenous dsRNAs applications for plant gene regulation still require further investigation and development, especially as concerns the necessity to unveil cell regulatory aspects, which are still largely ignored. In detail, some reports showed that the majority of plant endo-genes display a low RNAi susceptibility, depending on the presence of introns, well known to suppress the RNA silencing processes (Christie *et al*., 2011). Similarly, it is worth noting that several technological developments are still needed to achieve the wide diffusion of dsRNAs as protective molecules in crops. First of all, formulations with nanoparticles and/or other synthetic carriers are needed to slow down the rapid dsRNAs degradation, which is a major hurdle in the practical application of SIGS. Secondly, new delivery strategies such as the high-pressure spraying or brush-mediated leaf applications (Dalakouras *et al*., 2018; Dalakouras *et al*., 2016) need to be implemented for effective field applications. Finally, a specific science-based risk assessment procedure for exogenous application of dsRNA have to implemented since the actual evaluation of plant protection products (PPP) is not appropriate to establish the environmental fate and the risk associated to the field application of such products (Mezzetti *et al*., 2020).

Challenges for exogenous dsRNAs application in crop protection

In addition to the above-mentioned formulation issues, it is worth noting that the application of dsRNAs as bio-based pesticides requires a good knowledge of the target organisms. In fact, differences in dsRNAs susceptibility among different organisms and even among genera belonging to the same family have been reported. Specifically, concentrations, length of dsRNA molecules, uptake and recognition pattern by the RNAi machinery can influence the efficacy of the applied treatments.

The total amount of sprayed/supplied dsRNA is one the most variable factors among different reports: effective concentrations from pmol to mg per treated organism were reported (Das and Sherif, 2020 and references therein). This might be one of the most important limiting factors for field applications and implementation, because the amount of dsRNAs/treatment would affect the price per treatment, discouraging their application in

case of high costs. Encapsulation methods would probably reduce this problem protecting from degradation and/or facilitating the entrance of dsRNAs into the target tissues (Dalakouras *et al*., 2020). Together with the concentration, other parameters which show discrepancy in the literature are the optimum length of dsRNAs: lengths from 21 bp to more than 1kb were analysed in several works. In this case, all reports highlighted that dsRNAs within a size from 150 bp to 500 bp are the most efficient in inducing the activation of the RNAi pathway (Das and Sherif, 2020; He *et al*., 2020; He *et al*., 2020; Höfle *et al*., 2020). These results are explained by the nature of RNAi pathway, which requires sequences long enough to be recognized by the molecular machinery but which also need to pass through the cell membrane (and in case of plants and fungi the cell wall) which works as a molecular sieve.

The other important parameters, which represent the most limiting factors at the moment, are the uptake mechanisms of dsRNAs into cells and, once entered, the recognition of specific pattern/sequences by the target RNAi machinery. The dsRNAs uptake mechanism was first described in *C. elegans*, with the description of systemic RNAi defective (SID) proteins, which are involved in the acquisition and transportation of dsRNAs and the derived siRNA along the nematode body (Hinas *et al*., 2012; Winston *et al*., 2002; Winston *et al*., 2007). Several SID-like proteins were described in insects with not uniform results: in some insects, these proteins are crucial for the activation of a strong RNAi response, whereas in some other cases they seem to be unnecessary (Wytinck *et al*., 2020a and references therein). Another mechanism which has been proposed as one of the preferred routes of entry for dsRNAs is the clathrin-mediated endocytosis. Both in insects and in fungi, it has been demonstrated that endocytosis facilitated the uptake of dsRNAs (Pinheiro *et al*., 2018; Wang *et al*., 2016b; Wytinck *et al*., 2020b) but further studies are needed to clarify the mechanism in more details. Information about adsorption and transportation is fundamental also to understand the onset of resistance mechanisms in pest and pathogens, as already reported for *D. virgifera*, which showed a reduced dsRNAs uptake with an increased resistance to the treatment in just 11 generations (Khajuria *et al*., 2018). Additionally, one of the most important, but poorly understood, factors is the recognition of the dsRNAs by the RNAi pathway of the target organism. In this respect, contrasting results have been reported for fungi and insects. In case of fungi, application of dsRNAs to the plant, that will process them into siRNAs, and which are then adsorbed by the fungus, resulted the most effective strategy (Nerva *et al*., 2020; Song *et al*., 2018a; Wang *et al*., 2016b). These results are consistent with the inability of fungi to activate a secondary siRNA amplification mechanism and the exploitation of the plant machinery to enhance the gene silencing treatment effectiveness. In contrast to fungi, insects display a puzzling variety of responses, which are not always linked to evolutive features and show differences among genera of the same family. For example, as recently reviewed (Dalakouras *et al*., 2020), Coleoptera order is the most susceptible to RNAi, whereas lepidopterans and hemipterans seem recalcitrant to RNAi due to either impaired dsRNAs uptake or to the production of nucleases in their saliva. For this reason, GMO approaches relaying on the expression of dsRNAs in chloroplasts, which do not process them into siRNA, displayed a stronger efficacy (Bally *et al*., 2018). Apart from the preference of siRNAs or intact dsRNA delivery treatments, there is also a lack of information about the recognition of preferred nucleotide residues on the dsRNA for their processing into siRNAs by dicer-like enzymes (DCL). Particularly, DCL sequence evolution characteristics appear to be species-dependent (Arraes *et al*., 2020; Guan *et al*., 2018) and can lead to the generation of siRNAs with species-dependent length distribution among different insects (Santos *et al*., 2019). Taken together, these data suggest that for an optimal exploitation of dsRNAs as sustainable plant protection strategies, data on formulations (intended as dsRNAs size and concentration) uptake mechanisms and features of RNAi machinery of target pests/pathogens need to be implemented.

Epigenetic signatures and modifications to improve crop resilience against biotic and abiotic stresses

Both PTGS and TGS are involved in plant immunity and specifically in the control of viral virulence through RNA silencing. However, plants use gene silencing mechanisms and, in particular, the RNA-dependent DNA Methylation pathway (RdDM) for regulation of their own gene expression and the transcriptional repression of transposable elements (TEs).

In plants, chromatin can be modified at the level of DNA sequence by DNA methylation at CG, CHG and CHH (H = A, T or C) contexts through distinct pathways. While METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) are plant enzymes responsible for the maintenance of CG and CHG methylation, respectively, after DNA replication, CHH methylation is established *de novo* through two pathways. Plant RNA-dependent DNA methylation pathway (RdDM) involves the biogenesis of small interfering RNAs. ARGONAUTE (AGO) family members target 24-nt siRNAs to corresponding genomic loci, which in turn are methylated in CHH and CHG context *via* DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2). DRM2 is responsible for *de novo* DNA methylation of transposons located within euchromatic regions (Yaari *et al*., 2019). A second pathway requires CHROMOMETHYLASE 2 (CMT2) through interaction with DECREASE IN DNA METHYLATION1 (DDM1) in histone H1-enriched chromatic regions (Zemach *et al*., 2013). A family of bifunctional methyl-cytosine glycosylases-apurinic/apyrimidinic lyase actively removes DNA methylation, through a base excision repair mechanism (Penterman *et al*., 2007). DNA methylation may affect gene expression, regulate imprinting and activate transposable elements (TEs) and TE-associated genes, particularly in response to environmental cues (Law and Jacobsen, 2010).

Numerous studies indicate that DNA methylation plays a part in the pathogen-induced immune system and can strongly influence the resistance response in different plant species, as recently reviewed in Tirnaz and Batley (2019). Among these studies, interestingly it has been reported in rice that the epigenetic regulation of PigmS, a gene involved in resistance to rice blast caused by the fungal pathogen *Pyricularia oryzae*, affects plant resistance and indirectly yield. A genome-wide methylation analysis demonstrated that the PigmS promoter region contains two tandem miniature transposons MITE1 and MITE2 that are repressed by DNA methylation. Indeed, CHH methylation levels at MITE1 and MITE2 and in particular RdDM-mediated silencing of the MITE-nested PigmS promoter control PigmS expression and consequently resistance to rice blast (Deng *et al*., 2017). Intriguingly, this work on rice highlights the need for a thorough characterization of the RdDM epigenetic pathway and DNA methylation pathway in crops. The double aim of studying the

involvement of these pathways in plant–pathogen interactions can be to clarify how they regulate the expression of resistance genes and what genes are activated in crops, when exogenous double-stranded RNAs are introduced in the plant cell. Answering these questions might pave the way for new strategies for both crop protection management and breeding programmes for plant resistance, which can incorporate DNA methylation as a new source of variation.

In the plant cell, along with DNA methylation, other chromatin marks can arrange various chromatin states that epigenetically determine specific transcriptional outputs, thus influencing both biotic and abiotic plant stress response (Pecinka *et al*., 2020). Nucleosome association to DNA is influenced by many kinds of reversible covalent post-translational modifications (PTMs e.g. acetylation, methylation, phosphorylation, ubiquitination and many others) of the histone tails, in particular of histone H3 and H4 that are enriched in lysine (K) and arginine (R). In addition to PTMs and the positioning of nucleosomes, DNA accessibility is also affected by the incorporation of histone variants (H2A.Z, H2A.X, H3.1, H3.3) which have different specialized properties and can replace canonical core histones in the nucleosome. The histone code hypothesis postulates that deposition, removal and recognition of each PTM to histones requires specialized enzymes defined as writers, erasers and readers, respectively (Jenuwein, 2001). Although there is some evidence that histone modifiers and chromatin remodelers can affect the expression of genes involved in the plant immune response, this evidence is limited to a few plant species, such as Arabidopsis and rice (Ramirez-Prado *et al*., 2018). Histone deacetylases (HDACs), acetyltransferases (HATs), methylases, demethylases and ubiquitinases can act as positive and negative regulators in plant resistance to different stressors. In a recent work, the authors have studied the interactions between the bacterium *Pseudomonas piscium*, from the wheat head microbiome, and the plant pathogenic fungus *Fusarium graminearum*. They have observed that phenazine-1-carboxamide, a compound secreted by the bacteria, influences the activity of a fungal histone acetyltransferase, leading to deregulation of histone acetylation suppression of fungal growth, virulence and mycotoxin biosynthesis. This study highlights a novel mechanism of epigenetic regulation in antagonistic bacterial–fungal interaction that might be potentially useful in crop protection (Chen *et al*., 2018).

Genome editing tools for epigenome modification

Genome-wide mapping of epigenomic marks and epigenetic target identification are currently two major efforts in many important crops. In the future, it is desirable that these efforts will offer breeders new application to increase and manipulate epigenomic variability, for selecting novel crop varieties more resilient to biotic and abiotic stresses. In recent years, different techniques have been developed to modify the epigenome globally or at target sites. In crops, gene silencing and variation in DNA methylation profiles could be achieved by inducing siRNA expression, because DNA methylation-deficient mutants, which would be useful to alter the methylome, have not been identified in all crops, suggesting that they might be lethal (Kawakatsu and Ecker, 2019). At specific genome sites, fusions of epigenome-modifying enzymes to programmable DNA-binding proteins can achieve targeted DNA methylation and diverse histone modifications (Mendenhall *et al*., 2013; Rivenbark *et al*., 2012). Particularly, the genome editing tool CRISPR/deadCas9 can be fused to epigenetic-state-modifying enzymes and targeted to genes or cis-regulatory elements (CREs) to modulate plant gene expression. A complete set of plant epigenetic editing tools can be generated by fusing CRISPR-dCas9 system to target modifying enzymes for applications in plant breeding for crop protection. The so-called epigenome editing can be used to re-write an epigenetic mark modifying the endogenous gene expression level of one or several genes (Hilton *et al*., 2015; Miglani *et al*., 2020; Figure 3d). An example of such an approach was given in Arabidopsis using a dCas9 linked to the histone acetyltransferase *AtHAT1* to improve the transcription of *AREB1*, a gene involved in abscisic acid (ABA) perception (Miglani *et al*., 2020; Roca Paixão *et al*., 2019). The epigenome-edited plant showed enhanced drought resilience and chlorophyll content when compared to controls.

The use of genome editing tools that modify the epigenome at the recombination sites has been proposed as a possible application for manipulating the rate and positions of crossing over (CO), to increase the genetic and epigenetic variation accessible to breeders. In Arabidopsis, the disruption of histone 3 di-methylation on lysine 9 (H3K9me2) and non-CG DNA methylation pathways increases meiotic recombination in proximity to the centromeres (Underwood *et al*., 2018). Although the results obtained in a model species suggest that manipulation of epigenetic marks can allow CO position and frequency to be expanded, further studies are needed to determine the effectiveness of similar approaches in different plant species. Strategies for controlling recombination represent novel potential tools to both reveal unexplored epigenetic diversity and control its inheritance, since they have the potential to reduce the time for breeding novel more resilient crops.

Beyond the limits

A main factor limiting the success of NPBTs is plant regeneration after *in vitro* manipulation, particularly for woody plants, being sometimes a cultivar-dependent process. Although the key pathways and molecules have recently been unveiled (Sugimoto *et al*., 2019), the mechanism of regeneration is not fully understood, and technical issues are still present. Improvements of the regeneration efficiency have been obtained by crop transformation with morphogenic regulators (e.g. *Baby boom* and *Wuschel* genes) which can induce a more efficient meristem differentiation in recalcitrant species (Lowe *et al*., 2016; Maher *et al*., 2020; Yavuz *et al*., 2020). Despite the great potential of such approach, the fact that gene sequences of morphogenic regulators are protected by patents from private companies (Lowe *et al*., 2016; Maher *et al*., 2020) might limit the application of this technological innovation. Hence, it is fundamental to achieve higher regeneration efficiency, opening the way to the minimal gene approach even in recalcitrant woody plant species.

Another limiting factor is the low number of available genes involved in the resistance response with an identified function. Indeed, the identification of resistance genes from landraces and wild crop relatives and their functional genetic validation represents the first steps towards the development of new cisgenic varieties. The importance of these steps was recently reported in several herbaceous and woody plants. In wheat, for example, several genes conferring partial resistance to stem rust have been cloned, including *SR35* (Saintenac *et al*., 2013), *SR33* (Periyannan *et al*., 2013), *SR50* (Mago *et al*., 2015), *SR60* (Chen *et al*., 2020) and *SR55/LR67* (Moore *et al*., 2015). For woody plants, resistance genes *Rpv1* and *Run1* conferring resistance to *Plasmopara viticola* and *Erysiphe necator* have been identified in the wild grapevine

relative *Muscadinia rotundifolia* (Feechan *et al*., 2013) and are good candidates on which several research groups are working. In spite of this, the number of genes with a known function is still limited. In parallel, more information on promoters, transcriptional terminators and regulatory elements to control the transcription efficiency has to be addressed because of the high impact on gene of interest expression levels and consequently on the final phenotype (Basso *et al*., 2020; Low *et al*., 2018).

With respect to the CRISPR/Cas DNA editing, RNA editing using Cas13 has the advantage that it is not stable but reversible. This could enable a delicate temporal control over the editing process when editing RNA; both edited and non-edited transcripts can be present simultaneously in the cells, which could enable fine-tuning of the edited transcript amount, whereas DNA editing affects all transcripts. Furthermore, in addition to classic gene knockout mediated by CRISPR/Cas systems, new approaches were developed to target microRNA genes (MIR) instead of protein-coding ones. By fine-tuning specific MIR genes, the up- or down-regulation of derived miRNAs and target mRNAs can be achieved, for controlling either crop different biological responses or phenotypes and, consequently, specific agronomic traits (Basso *et al*., 2020 and references therein). Similarly, an approach called gene editing-induced gene silencing (Kuscu *et al*., 2017) can be applied to target redundant non-coding RNA sequences that are involved in miRNA/siRNA biogenesis. Once modified, the new RNA molecule will target new sequences, which could be endogenous plant sequences (leading to transcript down-regulation) or pathogen vital genes. Contrary to traditional gene editing techniques, gene editing-induced gene silencing could be used to indirectly target pathogenic genes by redirecting the silencing activity of the endogenous RNA interference (RNAi) pathway, supporting a more sustainable crop protection (Zotti *et al*., 2018).

Concluding remarks and future prospects

The NPBTs Era displays the potential to revolutionize the agricultural research field (Pandey *et al*., 2019). Indeed, recent applications and literature data available to date represent only the tip of the iceberg of further discoveries that may change molecular biology. Just as an example, through the combination of DNA and RNA editing systems, the cellular transcriptome can now be manipulated on the transcriptional and post-transcriptional level simultaneously, allowing delicate, and also reversible fine-tuning of gene expression (Schindele *et al*., 2018).

Taking them singularly, they all still present limitations. Pros and cons can be found both in fine-tuning each application as well as their application in a wide range of species. For instance, looking at cisgenic strategies, these have been developed and tested for woody and herbaceous crops, but their application still seems far from fulfilling their potential. The lack of efficient tissue culture and regeneration protocols for many crops hinders the range of possible applications. In addition, the identification of candidate genes involved in abiotic and biotic stresses still represents an important limit. For this reason, all NPBTs could greatly benefit from functional genomics, metabolomic and proteomic studies.

Nevertheless, a wide range of different techniques are becoming mature for substituting GMO approaches and supporting traditional breeding, with a realistic possibility of being largely accepted by the international community. Several NPBTs, making small modifications to plant own DNA without introducing foreign genes, do not leave any trace of their application in the improved phenotype. Despite the high impact of such techniques, and because the genome modifications introduced by genome editing are indistinguishable from those introduced by spontaneous mutations or conventional breeding (Bortesi and Fischer, 2015), to date the debate about considering organisms obtained by NPBTs as non-GMO is still open (Purnhagen *et al*., 2018).

Although NPBTs are powerful tools for basic research and more precise crop improvement, further knowledge, such as the comprehension of the genetic bases of important crop traits, has to be produced for efficiently transferring these tools from the laboratory to the field. Indeed, NPBTs can pave the way for further understanding of plant–pathogen interaction and different facets of climate change adaptation and for exploiting them for improving food security and nutrition quality.

Conflict of interest

No conflict of interest declared.

Funding

This work was funded by the Mipaaf in the framework of the BIOTECH-VITECH and DIBIO-BIOPRIME projects, the Veneto Region FSE project ESCinterfere (project number 2105-0061-1463-2019 – CUP C94E19000770008) and BIRD 2020 funded by the University of Padova.

Vectors attributions

Some elements present in the figures were obtained from www.Vecteezy.com.

Authors' contribution

GG, LM, LN and WC wrote the introduction. GG wrote the cisgenesis sections. LM and LN wrote the genome editing paragraphs. WC and LN wrote the RNAi strategies sections. SV wrote the epigenetic paragraphs. MFC, CB, GDL and RV commented on the first draft and critically reviewed the final manuscript.

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