CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Rice blast: strategies and challenges for improving genetic resistance

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Crop Breeding and Applied Biotechnology
21(S): e387721S9, 2021
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332021v21Sa22

Abstract: *Rice blast, caused by the fungus Pyricularia oryzae L., is considered one of the main threats to world rice production. The development of resistant cultivars is one of the best and sustainable control alternatives. Plant breeding efforts have been accelerated by genetic mapping (linkage and associative) and marker assisted selection. On the other hand, genomic editing techniques, such as meganucleases (MNs), Zinc-finger nucleases (ZFNs), Transcription Activator–like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindrome Repeats/CRISPR-associated protein 9 (CRISPR/Cas9), can be used to promote specific genetic modifications. Likewise, transgenics can also be used to manipulate specific genes. In this sense, this work aims to characterize rice blast and elucidate available biotechnological alternatives to accelerate the development of improved rice cultivars resistant to rice blast.*

Keywords*: Abiotic stress, biotechnology tools, Oryza sativa L., Pyricularia oryzae L.*

INTRODUCTION

Rice (*Oryza sativa* L.) has a high social and economic importance, playing a major role in the world production of cereals, serving approximately three billion people around the world, with a total production of ca. 756.5 million tons of husked grains (Filippi et al. 2009, SOSBAI 2018). Breeders have intensified efforts to develop superior cultivars, in view of the main challenges for cultivation, which are often aggravated by climate change, as is the case with plant diseases (Hirabayashi 2013). Rice cultivation has a range of diseases that have a significant impact on yield. In Brazil, diseases of fungal origin are the majority due to the predominant irrigated production system (Prabhu et al. 1995). Blast, caused by the fungus *Pyricularia oryzae* L., is among the most important diseases that affect rice (Miah et al. 2013, Srivastava et al. 2017).

Phytopathogens such as *P. oryzae*, are constantly evolving and are therefore considered a threat to food security. Among the alternatives to control plant diseases, genetic resistance is considered the most efficient and sustainable, which is due both to its economic and environmental advantages (Bonman 1992). However, the durability of blast resistance is a major challenge for plant breeders and pathologists due to the high variability of *P. oryzae* (Li et al. 2009, Devi et al. 2015, Maciel and Danelli et al. 2018).

Conventional breeding techniques for biotic factor resistance can have their efficiency increased if complemented by biotechnology approaches

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Received: 22 June 2021
Accepted: 27 June 2021
Published: 06 July 2021

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(Miah et al. 2013, Ashkani et al. 2015). Marker-assisted selection, for example, has long been used as a strategy for improving germplasm, screening, selection and developing new blast resistant cultivars in rice (Miah et al. 2013, Ashkani et al. 2015).

Another strategy is genetic mapping, which makes it possible to observe the physical association between genes or genome fragments and phenotypic variations, accelerating the identification of genotypes with favorable alleles and durable resistance (Desta and Ortiz 2014). Likewise, association or linkage disequilibrium mapping, also known as genome wide association, is another approach that is effective when applied to complex traits, providing valuable information when choosing strategies to increase the resistance to blast (Korinsak et al. 2019).

Genome edition is also a powerful tool that allows the introduction of precise changes in a cultivar (Andolfo et al. 2016). Directed nucleases are used, such as Meganucleases (MNs), Zinc-Finger Nucleases (ZFNs), Transcription Activator–like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9) is a tool that allows the introduction of precise modifications in specific genes or sequences (Kim et al. 1996, Christian et al. 2010, Ashkani et al. 2016, Andolfo et al. 2016) and can be used to improve tolerance to blast in rice (Wang et al. 2016). Similarly, transgenics is another approach with a potential impact to assist breeding for blast resistance (Liu et al. 2012, Helliwell et al. 2013).

Strategies such as the above mentioned can be used to solve problems that have the potential to affect world food security. Thus, in this article the importance of rice blast, the major biotechnological tools available to assist improvement and advances in blast durable resistance research will be addressed.

PERSPECTIVES REGARDING BLAST IMPACT ON RICE

The causal agent of rice blast has been referred to by different names over the years. Its asexual phase was named *Pyricularia grisea* by Saccardo in 1880, later the name *Pyricularia oryzae* was established by Cavara in 1892. In its sexual phase, the pathogen was first named *Magnaporthe grisea* (Hebert) Barr in 1970, but soon was changed to *Magnaporthe oryzae* (Couch and Kohn 2002, Zhang et al. 2016). However, recent studies point to *Pyricularia oryzae* Cavara, both for the asexual and for the sexual phase, as the correct way to name the causative agent of rice blast, due to its pathogenicity and ecological and evolutionary traits (Moreira et al. 2015, Zhang et al. 2016). It is recommended that the synonym *Magnaporthe oryzae* be mentioned in publications such as "*Pyricularia oryzae* (syn. *Magnaporthe oryzae*)" (Zhang et al. 2016). In its asexual (anamorphic) phase, which is usually found in the field, *P. oryzae* presents spores called conidia, characterized by its pear-shaped, obclave shape. Conidia often have two septa, with a circular base and a thin apex, slightly dark or hyaline in color, and having a small hilum at the base with which it attaches to the conidiophore (Couch and Kohn 2002). Its sexual phase (teleomorphic) is not observed naturally but can be performed by pairing compatible individuals in vitro (Moreira et al. 2015).

The pathogen that causes blast is hemibiotrophic, a survival mode in which the fungus starts in a biotrophic stage during which the plant's immune system is suppressed and then passes to a necrotrophic stage in which it promotes cell death (Fernandez and Orth 2018).

Impact on the rice crop since blast first appearances

The first records of blast occurrence date from the year 1600 and were found in China and Japan, where it was first described as "rice fever" (Bedendo and Prabhu 2005). In Brazil, the first diagnosis of rice blast occurred in 1912 in São Paulo and in Rio Grande do Sul in 1918 (Prabhu and Filippi 2006). Since then, the disease has been found in virtually all regions where rice is grown on a commercial scale. Losses are variable depending on the cultivar and environmental conditions, reaching 100% under favorable conditions and susceptible cultivars (Agbowuro et al. 2020).

Rice blast has never been fully eradicated, but it is possible to significantly reduce the damage caused by the disease through integrated crop management (Prabhu and Filippi 2006). The continuous use of fungicides to its control is a potential danger to health and the environment and can lead to the emergence of resistant races (Srivastava et al. 2017, Maciel and Danelli 2018). Therefore, it is recommended to use resistant cultivars and good crop management practices (Filippi et al. 2009).

In the southern region of Brazil there are irrigated rice cultivars available with different levels of blast resistance (Ogoschi et al. 2018). It is recommended to change cultivars that have resistance every three or four years to avoid the strong pressure of selection of virulent races of the pathogen (Maciel and Danelli 2018). In southern Brazil there are blast resistant irrigated rice cultivars available, such as IRGA 423, IRGA 424 and IRGA 424 CL, IRGA 426, IRGA 431 CL, SCSBRS Tio Taka, SCS122 Miura and BRS 7 "Taim" (Ogoschi et al. 2018). One of the technical recommendations for the crop is to change the cultivars that have resistance every three or four years to avoid an increase in the selection pressure of virulent races of the pathogen (Ogoschi et al. 2018). The breakdown of resistance, after a few years, occurs due to increased exposure and high genetic variability of the pathogen (Bonman 1992, Devi et al. 2015, Li et al. 2019).

How the pathogen acts in the plant

Microorganisms are in constant evolution and can frequently develop strategies to overcome plant defenses in order to become pathogens (Vale et al. 2001, Fernandez and Orth 2018). Rice plant infection begins with *P. oryzae* spore adhering to the hydrophobic surface of the leaf. The pathogen recognizes the cuticle constituents and, only then, induces spore germination and formation of specialized structures for penetration (Martin-Urdiroz et al. 2016). It is the germ tube that recognizes the surface of the leaf on which a specialized cell, the appressorium, is formed, which allows the fungus to penetrate the host plant tissues through mechanical force and enzymatic activity (Martin-Urdiroz et al. 2016, Yan and Talbot 2016, Fernandez and Orth 2018). The appressorium is present in an apical spore compartment that is released after conidium hydration (Yan and Talbot 2016, Fernandez and Orth 2018). This interaction occurs within epidermal and mesophilic cells and results in tissue colonization and the formation of lesions that become apparent after 72 hours (Prabhu and Filippi 2006, Martin-Urdiroz et al. 2016). In resistant cultivars, this process is mostly inhibited (Fernandez and Orth 2018). The plant colonization depends on the environmental and climatic conditions favorable to the pathogen. Leaf moisture and temperature around 25 to 28 ºC favor the germination of the conidia and the beginning of the infection (Filippi et al. 2009). Sporulation is increased with air humidity above 90%, and cloudiness, excess nitrogen and late sowing, also favor the establishment of the pathogen (Prabhu and Filippi 2006).

Symptoms occur in the form of lesions throughout the shoots, including leaves, leaf sheath, neck, panicles, pedicels and seeds, however, there is still no consensus on effects on the roots (Ribot et al. 2008). Small brown necrotic spots appear on the leaves and evolve to an elliptical shape with a brown margin and a gray or whitish center (Agbowuro et al. 2020). Over time, these lesions increase in size in the direction of the veins and, in more advanced cases, present a yellowish halo circling the lesion until the tissue dies (Agbowuro et al. 2020).

The main consequence of the severity of the disease is the reduction in grain yield caused by the direct effect of blocking the passage of nutrients, causing poor grain formation, or even in the sterility of the panicle (Prabhu et al. 1995). In the vegetative phase it affects the plant's stature and the number of tillers, consequently impacting productivity (Ribot et al. 2008). The greatest losses in rice grain yield are associated with neck blast (Filippi et al. 2009).

Plant defenses against the pathogen

During their life cycle, cultivated plants are constantly challenged by a wide variety of microorganisms. To survive, plants must be able to detect pathogens and activate their defense responses (Maciel and Danelli 2018). The plant defenses are controlled by major and minor resistance genes (*R*), which are responsible for activating a signaling cascade in the hosts (Srivastava et al. 2017, Maciel and Danelli 2018). These operate through a classical gene-to-gene interaction, resistance to conditioning to a single corresponding dominant avirulence (AVR) gene in a particular pathogen strain (Srivastava et al 2017, Maciel and Danelli 2018). In other words, the plant hypersensitivity response is caused by the effect of the AVR gene present in the pathogen together with the effect of the R gene on the host. Both genes need to be present for resistance to occur (Srivastava et al. 2017, Maciel and Danelli 2018). Qualitative or complete resistance is controlled by one or few genes which tend to be highly effective but are vulnerable to attack by different races of the pathogen. Quantitative or partial resistance, on the other hand, is governed by many genes, located in QTLs (Young 1996, Srivastava et al. 2017, Maciel and Danelli 2018).

In rice both qualitative and quantitative blast resistance genes have been reported (reviewed in Srivastava et al. 2017). For a better result in the control of the disease, the incorporation of genes of both effects in the development of new resistant varieties can be a great alternative (Miah et al. 2013). The complexity of the inheritance of resistance

is related to its durability and is determined by a test in which different plant genotypes are tested against different pathogen races. According to Van der Plank, horizontal resistance (uniform, race-non-specific) is stable, on the other hand, vertical resistance (differential, race-specific) is unstable (Parlevliet and Zadoks 1977).

GENETIC RESISTANCE OF THE RICE PLANT AGAINST BLAST CAUSING PATHOGEN

Rice (*Oryza sativa* L.) was the first plant species of agricultural importance to have its genome completely sequenced and is considered a model for research among plants of the same family (Arumuganathan and Earle 1991). The species genome comprises approximately 430 million base pairs, with an estimated 46 to 56 thousand genes for the indica subspecies, and 32 to 50 thousand genes for the japonica subspecies (Goff et al. 2002, Yu et al. 2002, IRGSP 2005, The RAP 2007). This information enables numerous advances in understanding the molecular mechanisms that govern genetic resistance to diseases that affect the crop, such as blast.

The genome of *P. oryzae* encodes hundreds of effectors that lead to blast resistance, some of which are recognized by intracellular immune receptors belonging to the nucleotide-binding, leucine-rich repeat (NLR) (Stein et al. 2018, De la Concepcion et al. 2021). Plants have developed a set of NLRs in order to neutralize virulence factors, effector molecules secreted by pathogens (De la Concepcion et al. 2021). After detecting pathogens, NLRs trigger the activation of immune responses capable of interrupting the spread of the pathogen. The architecture of plant NLRs consists of integrated non-canonical domains. These integrated domains mimic effector-directed host proteins and serve as baits for the detection of pathogens (Stein et al. 2018, De la Concepcion et al. 2021). NLR receptors harboring integrated domains are responsible for some of the best characterized resistance genes against rice blast (De la Concepcion et al. 2021). However, this mechanism ends up driving the evolution of new effector variants that escape immunological detection. For this reason, the use of NLR information in reference genomes represents a great opportunity for crop improvement programs. In a study using 13 reference genomes covering the *Oryza* species tree, 5.408 NLR genes were identified, being 535 in *O. sativa* vg. indica (Stein et al. 2018). Also in the same study, the sequencing of seven wild relatives of agricultural species enriches the collection of new haplotypes and resistance loci, including the *Pi-ta2* locus, which in combination with *Pi-ta* provides resistance with broad specificity for *P. oryzae*, information that is critical in strategies such as gene pyramiding (Stein et al. 2018).

QTLs associated to blast resistance

Approximately 350 Quantitative Trait Loci (QTL) are known to be associated with rice resistance, and there are 85 described resistance loci (reviewed in Ashkani et al. 2016, and Srivastava et al. 2017). The first QTLs associated with blast resistance were mapped by Wang et al. (1994), and since then, the search for QTLs has been increasing, which can be explained by the fact that partial resistance implies a more durable resistance (Ashkani et al. 2016). Multiple QTLs would mean many sources of partial resistance and could reduce the spread of the pathogen and keep a low selection pressure in the *P. oryzae* population, keeping a durable resistance (Sharma et al. 2012, Maciel and Danelli 2018). QTLs are used for the identification of resistance genes and also for the development of markers related to these genes. For blast resistance in rice, QTLs effective against several races of *P. oryzae* have been identified, and most are associated with qualitative genes (Srivastava et al. 2017). Several QTLs were used for gene pyramiding (Sharma et al. 2012).

Genes associated to blast resistance in rice

Currently, approximately 100 genes of resistance (*R*) to rice blast are known, of these 51% are from *indica* genotypes, 45% from *japonica* genotypes and 4% from wild species of rice (Sharma et al. 2012) (Table 1). The identified *R* genes have broad nomenclature and, often, the same resistance gene can have different names (Koide et al. 2009).

The advances in molecular technologies and the sequencing of the rice genome have made it possible to clone and characterize blast resistance genes, namely *Pib*, *Pita*, *Pik-h*, *Pi9*, *Pi2*, *Piz-t*, *Pid2*, *Pi36*, *Pi37*, *Pik-m*, *Pit*, *Pi5*, *Pid3*, *Pi21*, *Pb1*, *Pish*, *Pik*, *Pik-p*, *Pia*, *NLS1*, *Pi25* and *Pi54rh*, allow understanding the resistance spectrum and use in several breeding programs (Liu et al. 2010). The *Pi-1 (t), Pi2, Pi9, Pi20 (t), Pi27 (t), Pi39 (t), Pi40 (t)* and *Pikh* genes confer a broad spectrum of resistance, whereas the *Pia, Pib, Pii, Pi- km, Pi-t, Pi12 (t)* and *Pi19 (t)* provide resistance to specific races of the pathogen (Koide et al. 2009). The largest class of *R* genes encodes NBS-LRR (nucleotide binding-leucine rich repeats) protein class. The C-terminal LRR participates in protein–protein interactions of *R* and *Avr* genes (Takken and Tameling 2009).

In rice, the addition of broad-spectrum resistance *R* genes is able to confer resistance to different strains of *P. oryzae* (Skamnioti and Gurr 2009). This strategy was successfully applied with the broad-spectrum *Pi2* gene that conferred resistance to 455 *P. oryzae* isolates from different regions of the Philippines and to most of the 792 isolates from 13 important rice growing regions in China (Chen et al. 1996). In another study, the first cloned broad-spectrum gene,

Table 1. List of genes manipulated for rice blast resistance

Gene name	Function	Manipulation transgenic	Effects	Reference
OsPi-d2	*R* gene	Overexpression	Resistance to neck blast incidence	Chen et al. (2010)
MoHrip1	Elicitor gene	Overexpression	High resistance against blast	Wang et al. (2017)
OsWRKY53	*R* gene	Overexpression	High resistance against blast	Chujo et al. (2014)
OsGF14b	Induces expression of jasmonic acid (JA)	Overexpression	Resistance to neck blast incidence	Liu et al. (2016)
WRKY45	Induces expression of salicylic acid (SA)	Overexpression	High resistance against blast	Shimono et al. (2007)
CYP71Z18	-	Overexpression	High resistance against blast	Shen et al. (2019)
MoSDT1	Effector protein	Overexpression	High resistance against blast	Wang et al. (2019)
Pi54	*R* gene	Overexpression	High resistance against blast	Singh et al. (2020)
OsCPK4	Calcium-dependent	Overexpression	High resistance against blast	Bundó et al. (2016)
RACK1A	Receptor for activated C-kinase 1A	Overexpression	High resistance against blast	Nakashima et al. (2008)
OsCDR1	*R* gene	Overexpression	High resistance against blast	Prasad et al. (2009)
OsWRKY13	Regulating defense-related genes in salicylate-and jasmonate-dependent signaling	Overexpression	High resistance against blast	Qiu et al. (2007)
GH3-2	-	Overexpression	High resistance against blast	Fu et al. (2011)
OsGH3.1	Component of the hormonal mechanism regulating	Overexpression	High resistance against blast	Domingo et al. (2009)
OsNAC6	Transcription factor	Overexpression	High resistance against blast	Nakashima et al. (2007)
OsSBP	Homologue of mammalian Selenium-binding proteins	Overexpression	High resistance against blast	Sawada et al. (2004)
OsRacB	Allene oxide synthase gene	Overexpression	High resistance against blast	Jung et al. (2006)
OsAOS2	Allene oxide synthase gene increases the endogenous jasmonic acid level	Overexpression	High resistance against blast	Mei et al. (2006)
OsSERK1	Regulates somatic embryogenesis	Overexpression	High resistance against blast	Hu et al. (2005)
OsOxi1	Regulates basal disease resistance	Overexpression	High resistance against blast	Matsui et al. (2010)
Gns1	Stress-inducible β-glucanase	Overexpression	High resistance against blast	Nishizawa et al. (2003)
Rir1b	Defense-related	Overexpression	High resistance against blast	Schaffrath et al. (2000)
OsWAK1	Wall-associated receptor-like protein kinase gene	Overexpression	High resistance against blast	Li et al. (2009)
OsSYP71	Oxidative stress and rice blast response gene	Overexpression	High resistance against blast	Bao et al. (2012)
BSR1	Putative receptor-like cytoplasmic kinase gene	Overexpression	High resistance against blast	Dubouzet et al. (2011)
OsACS2	Key enzyme of ethylene biosynthesis	Overexpression	High resistance against blast	Helliwell et al. (2013)

Pi9, conferred resistance to 43 blast-causing pathogen isolates from 13 countries (Qu et al. 2006). In addition to having proven resistance, this strategy is considered friendlier to the environment and more economical to control the disease in the crop. Regarding durability, broad spectrum genes in native rice were widely used in introgressions, showing durable resistance (Deng et al. 2006). Still, the other genes must be evaluated for durability of resistance. For this reason, much research is underway to further characterize broad-spectrum R genes (Skamnioti and Gurr 2009). After the characterization of the resistance spectrum of the genes, the develop of broad spectrum and durable resistance to rice blast can be achieved using gene pyramiding (Sharma et al. 2012).

The role of R genes in rice blast resistance has been widely studied. An RNA gel blot analysis of *Pib* family members (*Pib, PibH8, HPibH8-1* and *HPibH8-2*) revealed that their expression is tightly regulated by environmental signals such as temperature, light, water availability and chemical treatments such as jasmonic acid, salicylic acid, ethylene and probenazol (Wang et al. 2001). In another study, the relationship between blast resistance and the expression of a key gene in jasmonic acid biosynthesis was explored (Mei et al. 2006). Even though many R genes were identified and even cloned, it is a difficult task to establish what are the real factors that make a resistance effective and lasting (Sharma et al. 2012, Maciel and Danelli 2018). The problem lies in the fact that one would need to use multiple genes for resistance to address more than one race of the pathogen, which is often limited due to epistatic interactions between genes (Maciel and Danelli 2018).

BREEDING FOR BLAST RESISTANCE

Conventional breeding

Conventional breeding is mainly based on the phenotypic selection of varieties or lines in selected locations (Ashkani et al. 2015), a process highly influenced by environmental interactions and the complexity of resistance inheritance. In this case, the breeder should consider the genotype of the plant, the race or races of the pathogen and whether the resistance is qualitative or quantitative (Wang et al. 2017).

In the case of qualitative resistance, backcrossing is the most widely used breeding method. Improvement methods for quantitative resistance do not differ from those used in other agronomic traits with the same genetic inheritance. For self-cross species, such as rice, the most used selection methods are genealogical (Pedigree), population (Bulk), recurrent selection and mutation breeding (Wang et al. 2017, Srivastava et al. 2017). As an alternative to the addition of only one gene, quantitative pyramiding strategy of R genes with different resistance spectra tends to have a better outcome (Skamnioti and Gurr 2009). However, backcrossing and relying on phenotypic assays only can sometimes be a cumbersome task. Despite its huge contribution to breeding programs, Conventional breeding takes longer when the nature of the resistance is quantitative, it requires crossings, many generations of self-crossing, and many plants to test the resistance. For this reason, the use of molecular biology techniques has become a great option to assist in the process of releasing new cultivars (Miah et al. 2013, Ashkani et al. 2015).

Molecular breeding

With advances in plant genomics, breeders have a range of biotechnological tools, as well as fundamental information about the molecular biology involved in disease resistance and the virulence of pathogens (Ashkani et al. 2015). In the rice crop, understanding and applying molecular biology is important to accelerate the development of cultivars resistant to blast (Miah et al. 2013, Ashkani et al. 2015, Srivastava et al. 2017). To slow the pathogen's evolution, it is necessary to think about research in a strategic way, aiming to fill gaps in the existing knowledge in the improvement and molecular genetics of blast resistance (Ashkani et al. 2015).

BIOTECHNOLOGICAL TOOLS IN BREEDING FOR RESISTANCE

Marker assisted selection (MAS)

Molecular markers started in the 80's, with RFLPs, followed by many variations of fragment detection (Botstein et al. 1980, Mohan et al. 1997, Shanti et al. 2010). The application of molecular markers in plant breeding is intended to assist breeders by allowing indirect selection (Shanti et al. 2010). Some of the main advantages of these are the reliability of the information, the saving of time and space, the possibility of early selection and the pattern of Mendelian inheritance

(Mohan et al. 1997). Some of the main advantages of these are information reliability, time and space savings, the possibility of early selection and the Mendelian inheritance pattern (Mohan et al. 1997, Heffner et al. 2010). Objectively, molecular markers can be defined as genetic loci that can be easily identified and quantified in a population and are linked to one or more genes (Mohan et al. 1997). With the use of markers, it is possible to carry out an indirect selection, which is carried out based on a molecular analysis, as they are involved in the genetic control of traits that enable the distinction between contrasting individuals (Mohan et al. 1997, Heffner et al. 2010). They can be identified through use of techniques of Electrophoresis, Hybridization, Restriction Enzymes, Polymerase Chain Reaction (PCR), Sequencing, or even by combining some of these techniques (Ashkani et al. 2015). The available markers are classified into dominant and codominant, according to the allelic information provided. Initially, the use was predominant of Restriction Fragment Length Polymorphism (RFLP) (Botstein et al. 1980), codominant, and Randomly Amplified Polymorphic DNA (RAPD) (Williams et al. 1990), dominant markers. Subsequently, codominant Simple Sequence Repeats (SSR) markers (Akkaya et al. 1992, Ashkani et al. 2015) were developed. Markers generated by analysis of Amplified Fragment Length Polymorphisms (AFLP) and Retrotransposon-Microsatellite Amplified Polymorphism (REMAP) markers (Kalendar et al. 1999) were also widely used. The most commonly used markers today are Single Nucleotide Polymorphism (SNP), which are considered to be highly efficient (Korinsak et al. 2019). In this case, a large number of markers that are spread across the genome are analyzed simultaneously in automated systems, increasing the probability that regions associated with traits of interest are in strong linkage disequilibrium (Miah et al. 2013).

The use of a marker for indirect selection of a trait of interest is called marker assisted selection (MAS) (Das et al. 2017). MAS integrates molecular genetics with phenotypic selection and is based on the presence of a marker linked to a region of the genome that controls a certain characteristic to perform the selection (Ashkani et al. 2015), and in some specific cases, the markers may be part of the gene of interest. In plant breeding programs for resistance to diseases such as blast, which use MAS as an auxiliary tool, it is necessary to continuously characterize the genetic variability of pathogens and the host, the introduction and characterization of new sources of resistance, and the identification of new molecular markers linked to resistance alleles (Ashkani et al. 2015).

Some successful examples of MAS for blast resistance in rice cultivars were summarized by Tanweer et al. (2015). In addition, MAS selection for rice blast resistance was used to screen for resistance genes *Pi-b*, *Pi-k*, *Pi-i*, *Pi-z* and *Pi-ta*, as well as for the pyramiding of genes using *Pi-ta*, aiming for broad spectrum resistance (Biswal et al. 2017). Recently, a study using the quantitative resistance gene *pi21* was introduced in strains of rice *indica* and *japonica* with the aid of marker-assisted backcrossing. All lines showed resistance to 11 blast isolates in leaves, both at field and greenhouse conditions (Angeles-Shim et al. 2020).

In addition to the *R* genes, another promising strategy in the search for durable resistance is the introduction of quantitative resistance controlled by QTLs with the aid of MAS. For that, markers that flank up to three QTLs are used, which should explain a large proportion of the phenotypic variation (Ashkani et al. 2015). The application of *R* and QTLs genes in rice breeding programs is considered a strategic way to control blast, due to its effectiveness, economy and low environmental risk (Biswal et al. 2017). Increasingly, breeders are seeking the help of MAS for the development of new commercial rice cultivars to complement conventional breeding (Prabhu and Filippi 2006).

GENETIC MAPPING

Linkage mapping

Procedures using biotechnological tools, such as the mapping of genes of economic importance, based on genetic maps, have been shown to be important complements in breeding programs for a wide range of plant species (Ashkani et al. 2015). Among the most important applications of genetic maps is the location of genes that control complex inheritance traits, such as disease resistance. In genetic mapping, an analysis is performed that determines the number of molecular markers linked to genetic loci that control quantitative traits (QTLs), as well as to loci that control qualitative traits (Nordborg and Weigel 2008, Raboin et al. 2016, Korinsak et al. 2019).

Techniques based on the use of molecular markers allow the study of regions that influence the expression of traits of interest, as well as their respective loci, facilitating the selection of superior genotypes (Desta and Ortiz 2014). One of the strategies is linkage mapping, using populations properly constructed for mapping, which allows identifying the

position of the QTL through linkage maps, in addition to estimating its effects (Desta and Ortiz 2014, Xu et al. 2017). For the formation of a population, it is required to select contrasting parents, which show clear differences for one or more traits (Bered et al. 1997).

When it comes to blast, knowing the chromosomal locations of resistance genes in a genetic map is essential for manipulating these genes in rice breeding programs. Information like this is useful when selecting contrasting parents for the construction of populations, often composed of NILs. These NILs can be used to evaluate the individual performance of resistance genes and the characterization of the pathogen population and also for mapping clones and pyramiding of genes (Nunes et al. 2007). Even though this method proves to be effective, there are some limitations that must be considered, such as the restricted number of alleles, and thus, low genetic variability (populations from the crossing of only two parents), the low resolution of the map and the high demand for time and resources for obtaining populations (Desta and Ortiz 2014, Xu et al. 2017). However, through the previously mentioned approach, linkage maps have made it possible to identify numerous QTLs and their positions in the genome of the studied individuals (as described in section 3.1), as well as to estimate their genetic effects, showing themselves as a viable alternative for the genetic improvement of blast resistance.

Association mapping

Association or linkage desequilibrium mapping, also known as genome-wide association studies (GWAS) is a model that aims to detect the statistical association between genotypic and phenotypic values (Raboin et al. 2016, Xu et al. 2017, Korinsak 2019). These studies have made it possible to evaluate the germplasm available in order to explore the genetic variability in a plant population of a crop for use in breeding programs (Korinsak 2019). The technique is based on the concept of linkage desequilibrium (DL), which refers to the non-random association of alleles between different loci (Oraguzie and Wilcox 2007, Xu et al. 2017). Analyzes performed with a large number of markers spread throughout the genome increase the probability that regions associated with traits of interest are in strong linkage imbalance with the markers (Oraguzie and Wilcox 2007). GWAS becomes more efficient in species that have been sequenced, such as rice, because in this way it is possible to analyze an unlimited number of traits in genetically identical material and different environments (Oraguzie and Wilcox 2007, Raboin et al. 2016). In recent years, many loci have been identified in several studies with the aid of GWAS (Toledo et al. 2008). In a study of GWAS with indica rice, 366 varieties were selected to compose the population that was inoculated with 16 isolates of *Pyricularia oryzae*, which resulted in the identification of 30 loci associated with resistance to the disease (Wang et al. 2014). Another study with GWAS used a population of 1,495 hybrid rice varieties to assess resistance to blast and 38 other agronomic traits and identified four loci associated with resistance (Huang et al. 2015). Other GWAS for blast resistance in rice were performed (Lin et al. 2018, Li et al. 2019, Lu et al. 2019, Volante et al. 2020, Frontini et al. 2021).

Genome editing

Variability is essential for the breeding process. When the genetic variability is absent from the germplasm or hard to transfer, mutation-inducing approaches can be used (Zhu et al. 2017, Viana et al. 2019). These methods are being aided by editing technologies, which make it possible to precisely manipulate specific sequences in the genome, that is, it allows the insertion, deletion or substitution of nucleotides in specific genes or sequences (Viana et al. 2019, Ijaz and Ul Haq 2020). Editing strategies use Sequence-specific nucleases (SSNs), which promote the induction of double-strand DNA breaks (DSBs) in specific locations within the genome in a mediated way. These breaks are solved with the help of cell repair mechanisms (homologous recombination - HR and non-homologous end joining - NHEJ) (Zhu et al. 2017, Viana et al. 2019). Among the first techniques involving nucleases there were the Meganucleases (MNs) and Zinc finger nucleases (ZFNs) (Zhu et al. 2017). Soon after, Transcription activator-like effector nucleases (TALENs) were developed. More recently, a new genomic editing technology, called Clustered regularly interspaced short palindromic repeat/CRISPR-associated protein (CRISPR/Cas9) has been developed (Viana et al. 2019).

Genomic editing technologies can accelerate the process of plant breeding, allowing the creation of cultivars that are resistant to pathogens by modifying loci involved in the plant's defense system (Yin and Qiu 2019). In particular, the CRISPR/Cas9 technique has so far shown the greatest promise to address emerging challenges in agriculture (Haque et al. 2018). Compared to other genome editing tools, CRISPR/Cas9 is easier, more economical, accurate and highly efficient because it allows the expression of several genes (multiplex) to be modulated (Molinari et al. 2020).

MNs

Meganucleases (MNs) are highly specific enzymes that recognize and cleave specific sequences, from 11 to 40 bp, inducing homologous recombination in different types of cells (Daboussi et al. 2015, Viana et al. 2019). Recombination induced by MNs generates DSBs in specific target DNA sequences, which will be repaired by HR using the donor DNA or by NHEJ (Daboussi et al. 2015). The understanding and use of this tool came after many studies involving the LAGLIDADG family of proteins (Arnould et al. 2011). Among the functionalities of LAGLIDADG proteins are their role as RNA maturases, involved in the splicing of their own intron, and as highly specific endonucleases, which are able to recognize and cleave the exon-exon junction sequence in which their intron resides. The understanding of MNs from the LAGLIDADG family and their interaction with DNA allowed the exploration of a wide range of biotechnological applications, including the modification of MNs for use in gene editing (Arnould et al. 2011). However, this is an underused tool due to its limitations, such as recognizing few DNA sequences and the possibility of errors occurring due to insertions or deletions at the cleavage site (Daboussi et al. 2015, Majid et al. al. 2017, Yadav et al. 2019, Yin and Qiu 2019).

This tool has been successfully used in many species, such as Arabidopsis, cotton and corn (Daboussi et al. 2015, Zhu et al. 2017, Viana et al. 2019). In a study in transgenic cotton, MNs developed for the cleavage of a specific target DNA sequence adjacent to a locus responsible for insect resistance were used. MNs induced DBS in embryogenic callus cells, in the presence of exogenous DNA containing resistance to two herbicides. It was possible to observe that approximately 2% of the events were shown to contain the correct insertion and to transfer the characteristics of both insect and herbicide resistance to subsequent generations (D'Halluin et al. 2013). However, even though there are no reports of the application of MN in rice crops, this may be an available alternative (Daboussi et al. 2015, Zhu et al. 2017, Viana et al. 2019).

ZFNs

Zinc finger nucleases (ZFNs) are composed of the modified restriction enzyme *Fok* I (*Flavobacterium okeanokoites*) associated with zinc finger amino acid sequences (Osakabe and Osakabe 2015, Zhu et al. 2017, Ijaz and Ul Haq 2020). *Fok* I has two domains, an N-terminal DNA-binding domain and a C-terminal domain with non-specific DNA cleavage activity. Zinc-finger amino acid sequences are capable of recognizing specific sequences of 3 or 4 nucleotides (Kim et al. 1996, Qi 2015, Zhu et al. 2017). Fusion of zinc finger motifs with the *Fok* I cleavage domain forms ZFNs, which are used for genome editing by introducing DSBs into specific genomic DNA sites (Qi 2015, Zhu et al. 2017). Thus, using repair mechanisms, target genes can be disrupted by NHEJ-induced mutations, or edited via HR if a homologous/donor DNA is provided (Qi 2015, Zhu et al. 2017, Ijaz and Ul Haq 2020).

The ZFNs genome editing tool has been successfully employed in species such as rice, maize and Arabidopsis (Cantos et al. 2014, Yin et al. 2019, Ijaz and Ul Haq 2020). In rice, ZFNs were used to induce DBS in the sequence containing the SSIVa gene in order to understand its involvement in starch synthesis and its other functionalities (Jung et al. 2018). This study demonstrated that disruption of the SSIVa gene had no effect on other genes related to starch synthesis. Thus, ZFNs proved to be efficient in cleaving and stimulating mutations in the SSIVa locus and this can be used for other characters of interest, such as disease resistance (Jung et al. 2018).

TALEN

Transcription activator-type artificial effector nuclease (TALEN) has emerged as a plant genome editing tool that offers an alternative to Zinc-finger nucleases (ZFNs) (Christian et al. 2010, Daboussi et al. 2015, Hilscher et al. 2017). TALE are proteins, or transcription factors, that occur naturally in bacteria of the *Xanthomonas* genus. When the bacterium infects the plant, it secretes proteins into the cytoplasm of the host's cells, which bind to DNA and activate the expression of target genes, in this case, genes that are favorable to infection by the bacterium (Hilscher et al. 2017). These transcription factors recognize specific sequences as they are composed of blocks of repeated 34 amino acids, with each block recognizing a base pair (Christian et al. 2010, Hilscher et al. 2017). The specificity of TALE is determined by two hypervariable amino acids (VDRs), located at position 12 and 13 of the block (Hilscher et al. 2017). VDRs can be manipulated to generate proteins that are programmed to bind to DNA and perform targeted editing at specific points in the genome. The nuclease-independent sequence *Fok* I acts as a site-specific nuclease and, through fusion with the DNA recognition domain of TALE, creates TALEN that recognize and cleave target DNA sequences (Ma and Liu

2015, Viana et al. 2019). Similar to what happens in ZFNs, TALEN promotes DSBs for genome editing (Chen et al. 2014, Hilscher et al. 2017). DSBs induce activation by NHEJ, which result in insertions or deletions, and can also be repaired by HR when a homologous DNA is introduced, generating specific alterations (Christian et al. 2010, Chen et al. 2014, Daboussi et al. 2015, Zhu et al. 2017).

This technique has been successfully used in several crops such as maize, barley, wheat and rice (Ijaz and Ul Haq 2020). In rice, the TALEN technique was successfully applied to generate plants resistant to bacterial spot caused by *Xanthomonas oryzae* pv. oryzae. The plants were edited in the promoter region of a gene that encodes a sucrose efflux transporter (OsSWEET14), which acts on the survival and virulence of the pathogen in the plant. The gene was silenced to generate resistance to the pathogen (Li et al. 2012). It was also shown that a modification in the TALEN technique can improve up to 100% the efficiency of replacement of specific genes for resistance to the fungus *Pyricularia oryzae*. The technique using modified TALENs, which was called Platinum-Fungal TALENs (PtFg TALENs), showed great potential for applications in filamentous fungi, such as the one causing blast (Arazoe et al. 2015).

CRISPR/Cas9

The application of CRISPR/Cas9 as a genomic editing tool emerged from fundamental discoveries about the immune system in bacteria against invasion of foreign DNA (Wiedenheft et al. 2012). Two simple components are needed to use the CRISPR/Cas9 technique. The first component is sgRNA, in which crRNA is associated with tracrRNA to make a hybrid sequence called single guided RNA (sgRNA), which stimulates/guides the second component, the enzyme Cas9, to a specific sequence in the genome. This enzyme cleaves the sequence in the genome whose correspondence is present in the sgRNA (Langner et al. 2018, Viana et al. 2019). This break will be repaired by HR (if a donor/homologous DNA is provided) or by NHEJ (if no homologous DNA is provided).

The development of the CRISPR/Cas9 technique has opened up a wide range of applications and should also be explored in improving plant resistance to pathogens (Langner et al. 2018, Viana et al. 2019). In rice, a specific mutation in the *OsERF922* gene using the CRISPR / Cas9 technique was effective in increasing blast resistance. This is due to the fact that, in culture, plants that express the *OsERF922* gene involved in the ethylene route, show a reduction in the expression of defense-related genes and increased susceptibility to *Pyricularia oryzae* (Wang et al. 2016).

Transgenics

Using this technology, breeders can precisely manipulate the gene that encodes a trait of interest by inserting genes from unrelated species or by silencing specific genes (Miah et al. 2013). For the control of pathogens, it is possible to transfer *R* genes involved in the recognition of the pathogen and transfer genes from the pathogen that, when expressed, activate the plant's defense system (Miah et al. 2013, Biswal et al. 2017). Transgenics involving *R* genes from sexually incompatible plants have been seen as alternatives for obtaining durable resistance to pathogens (Toenniessen et al. 2003, Maciel and Danelli 2018). In rice, some transgenic strategies for breeding aimed at blast resistance have been developed. Homozygous transgenic rice lines that harbor the *Pi-d2* gene showed high resistance to neck blast incidence (Chen et al. 2010). In another study, transgenic rice plants were developed using two elicitor genes (*MoHrip1* and *MoHrip2*) from *Pyricularia oryzae*, and these plants showed high resistance against blast and higher tolerance to water stress (Wang et al. 2017). Similarly, rice overexpressing the effector gene *MoSDT1* showed improved blast resistance (Wang et al. 2019). In addition, the overexpression of calcium-dependent protein kinase (*OsCPK4*), involved in calcium influx upon pathogen recognition in plant, confer more resistance to blast (Bundó and Coca 2015). In another study Chandran et al. (2019) verified that transgenic rice overexpressing Growth Regulating Factor genes (*OsGRF6*, *OsGRF7*, *OsGRF8*, and *OsGRF9*) exhibited enhanced resistance to blast, but showed different alteration of growth. In another study, the association between blast resistance and overexpression of the transcription factor WRKY45 was explored by Shimono et al. (2007).

FINAL CONSIDERATIONS

Rice blast, caused by the fungus *Pyricularia oryzae*, is an example of the destructive potential of plant pathogens, and has the ability to affect world food security. Improvement aimed at resistance to diseases has been one of the main objectives in plant breeding programs and, in rice, the work on developing blast resistant cultivars has been going on for several years. There are several plant breeding methods available for the development of resistant cultivars and it is up to breeders to find the best ways to deal with this problem.

Conventional breeding is difficult to keep up with the evolutionary potential of the pathogen and, consequently, there is a constant deficit in commercial blast resistant rice. Molecular biology techniques, such as genetic mapping and marker-assisted selection, have been used for some years to improve germplasm and develop new cultivars. More recent techniques, such as transgenics and genomic editing, can be applied to carry out specific genetic modifications and have great potential for the development of plants resistant to the disease.

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