Potential Candidate Genes for Improving Rice Disease Resistance

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Abstract Diseases caused by fungal and bacterial pathogens like *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* are responsible for considerable yield loss. Up to now, in rice, the modification of the expression of more than 60 genes from diverse origins has shown beneficial effects with respect to disease resistance. In this paper, we review this large set of data to identify the best genes and strategies to achieve disease resistance by transgenic approaches. Altered expression of genes involved in signal transduction and transcription may lead to many unwanted side effects, like lesion mimic phenotypes. Moreover, modification of resistance to abiotic stress has been neglected and should be carefully examined in the future. Genes like resistance genes and pathogenesis-related genes can confer broad spectrum and high levels of resistance to several pathogens. Preformed expression of defense is often observed but does not necessarily lead to detrimental effects. Although examples of gene pyramiding are scarce, they suggest that this is a very promising strategy. More field evaluation of the transgenic plants is required to draw final conclusions on the usefulness of these genes for improving disease resistance.

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Introduction

To meet the increasing demand on the world food supply, we will have to produce up to 40% more rice by 2030 (Khush 2005). This will have to be on a reduced sowing area due to urbanization and increasing environmental pollution. For example, the sowing area in China decreased by eight million hectares between 1996 and 2007 (China Statistical Bureau; http://www.stats.gov.cn/english/statisticaldata/yearlydata/). Improvement of yield per plant is not the only way to achieve this goal; reduction of losses by biotic and abiotic stress is also a solution. According to FAO estimates, diseases, insects, and weeds cause as much as 25% yield losses annually in cereal crops (Khush 2005). In particular, fungal diseases can cause important losses (between 1% and 10%) regionally (Savary et al. 2000). In China alone, it is estimated that one million hectares are lost annually because of blast disease (Khush et al. 2009).

Fungal and bacterial pathogens represent a permanent threat on rice cultivation. Between 1987 and 1996, fungicides represented up to 20% and 30% of the culture costs in China ($46 million) and Japan ($461 million) respectively, whereas they represent approximately 10% of the costs in Europe and the USA (http://beta.irri.org/solutions/). Thus, a strong effort has been invested in improving disease resistance. These efforts could now benefit from the

knowledge gained on mechanisms underlying disease resistance.

Since the initial definition of the plant resistance (*R*) genes by Flor (1942), many *R* genes have been identified. The vast majority of the known *R* genes is composed of proteins carrying nucleotide-binding sites and leucine-rich repeat motifs (NBS-LRR; Jones and Dangl 2006). Most *R* genes recognize pathogen effectors developed by pathogens to inhibit defense, although there are a few exceptions (e.g., Lee et al. 2009). Some of these effectors thus correspond to the initial definition by Flor of the avirulence gene. Depending on the presence/absence of these *R* genes and of the matching avirulence product, the interaction will be incompatible (plant is resistant) or compatible (plant is susceptible).

Many *R* genes have been identified in rice and most code for *NBS-LRR* genes (Ballini et al. 2008; White and Yang 2009). After recognition mediated by the *R* gene, signal transduction occurs and requires regulators such as MAP kinases (Mishra et al. 2006). Finally, transcription factors like WKRYs activate a deep transcriptional reprogramming of the cell (Eulgem 2005), leading to the activation of defense responses per se. These include production of antimicrobial secondary metabolites (phytoalexins like momilactones in rice; Peters 2006), pathogenesis-related (PR) proteins (e.g., chitinases, glucanases, PBZ1 in rice; Jwa et al. 2006; van Loon et al. 2006), cell wall strengthening (Hückelhoven 2007), and programmed cell death, leading to hypersensitive response (HR; Greenberg and Yao 2004). The genes that act downstream of the disease resistance pathway are collectively called defense genes.

Most of the elements involved (receptors, regulators, transcription factors, and defense genes) are well conserved across species. For example, the *NPR1* gene is a central regulator in both monocots and dicots. The *NPR1* gene was successfully used in several plant species like *Arabidopsis* (Cao et al. 1998), rice (Chern et al. 2001), tomato (Ekengren et al. 2003), and wheat (Makandar et al. 2006). Similarly, the *mlo* gene functions in barley (Peterhänsel and Lahaye 2005), tomato (Bai et al. 2008), and *Arabidopsis* (Consonni et al. 2006). These observations are critical for biotechnology approaches since they suggest that one gene from one species can provide an interesting trait in another.

Preformed defense systems likely play a role in basal resistance in limiting the growth of a normally virulent pathogen. Preformed or constitutive defense systems involve cuticle (Skamnioti and Gurr 2007) and cell wall (Juge 2006) strengthening. In rice, like in other plants, silicon accumulation plays direct and indirect roles in basal resistance (Ma and Yamaji 2006). Antimicrobial molecules, called phytoanticipins, can accumulate before infection (Morrissey and Osbourn 1999). Increasing information from *Arabidopsis* and other plants indicates that the overproduction of PR proteins confers resistance, that mutations in regulator genes

negatively regulating disease resistance can increase defense gene expression (*e.g.* Noutoshi et al. 2005; Petersen et al. 2000), or that over-expression of regulator genes acting positively on disease resistance can increase defense gene expression (Hammond-Kosack and Parker 2003).
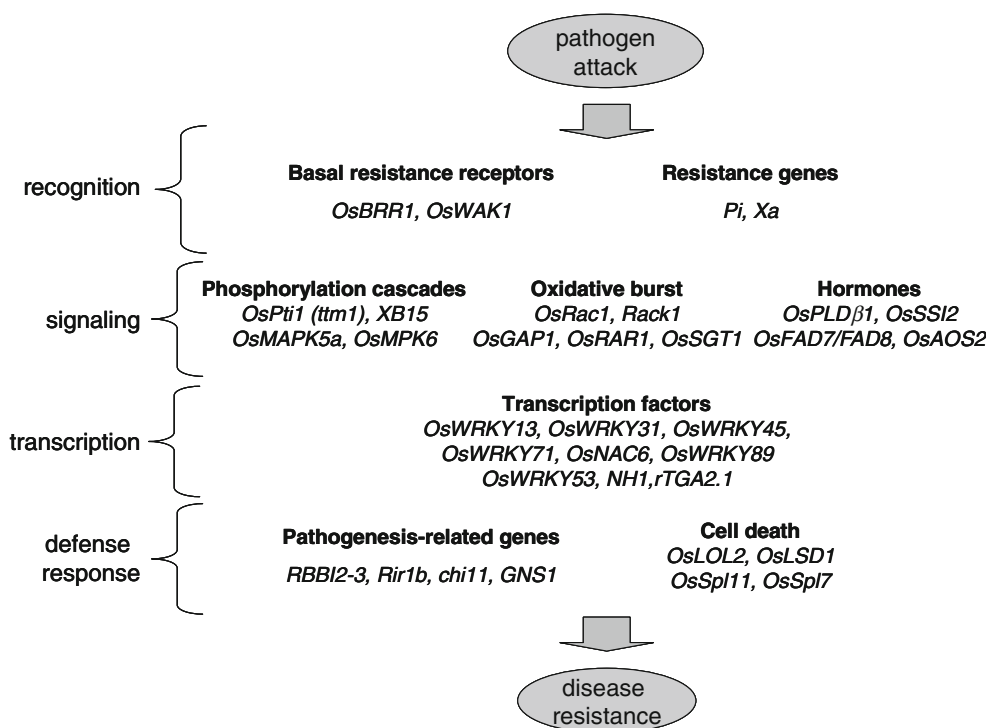
More than 60 genes have now been over-expressed or mutated in rice for fundamental or applied research. Molecular and biological studies of these transgenic plants allow a better understanding of signaling pathways, cross talks, and pathogen specificities. These experiments lead to the putative disease resistance pathway shown in Fig. 1. This figure illustrates that genes in the three major steps of the disease resistance pathway are known in rice, opening the possibility to assess the usefulness of each category of gene.

Rice stable transformation via *Agrobacterium tumefaciens* has become a routine technique in laboratories (Toki et al. 2006). Nowadays, even if *japonica* is frequently used because it is highly amenable to transformation, *indica* transformation efficiency has sufficiently improved to allow its use in functional validation experiments and field trials in the appropriate environments.

A tool kit for breeders for marker-assisted selection was recently described (Ballini et al. 2009). Here, we extensively review the cases of improvement of disease resistance by over-expression of transgenes. Rice blast caused by the fungus *Magnaporthe oryzae* and bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* are the most serious and widespread diseases in rice production. Therefore, we focused this paper on these two pathogens. We did not review the abundant literature on strategies to increase insect resistance (see for example Ye et al. 2009 and references therein). We provide guidelines for selecting transgenic strategies for fungal and bacterial disease improvement, for selecting genes, and analyzing their effects. General elements on the different possible strategies to improve disease resistance in plants were previously described (Campbell et al. 2002) and are not presented here.

Rice genes improving disease resistance

Sixty rice genes for which improvement of disease resistance was shown were presented in Tables 1, 2, 3, and 4. We selected a gene only if the corresponding paper provided data concerning in vivo resistance and some information on the spectrum of pathogens that were tested. These genes were separated into three major groups: 41 rice genes (Table 1 is a selection of 24 the most characteristic genes, the remaining genes are in Electronic supplementary materials (ESM), Supplemental Table 2), five genes from plants other than rice (Table 2), and nine genes from organisms that are not plants (Table 3). Finally, we also review five cases of gene pyramiding (Table 4). Figure 1 provides a few examples of

Fig. 1 Position in the putative disease resistance signaling pathway of the rice genes that were used in plants to improve disease resistance. The genes listed here are the ones for which the demonstration of usefulness was made *in planta*. Details on these genes are provided in Table 1.The position of the *OsDR8*, *OsDR10*, *pi21*, *spl18*, and *OsSBP* genes is not shown. This schematic view illustrates the three steps leading to pathogen resistance: recognition via general (e.g., *OsWAK1*) and specific receptors (*Pi* and *Xa* genes), signal transduction, transcription, and defense response, involving pathogenesis-related genes and cell death. The *OsPLDβ*, *OsFAD7/FAD8*, and *OsAOS2* genes are involved in the jasmonic acid pathway and *OsSSI2* in the salicylic acid pathway. The *OsLOL2*, *OsLSD1*, *OsSpl11*, and *OsSpl7*-genes are negative regulators of cell death, and mutants for these genes display spontaneous lesions resembling disease.

where the rice genes are likely acting. Although the genes tested were not all precisely positioned on the disease resistance pathway, they can be tentatively attributed to four major steps in this pathway: recognition, signaling, transcription, and defense response.

Major resistance genes for *Xanthomonas* resistance (*Xa* genes) and blast resistance (*Pi* genes) were recently reviewed by White and Yang (2009) and Ballini et al (2008), respectively. We selected only a few examples (*Pi9* and *Xa21*) to illustrate the use of these *R* genes in transgenics. Other genes coding for receptor-like proteins likely involved in basal resistance such as *OsWAK1* and *OsBRR1* were also included in our study. These two genes may have broad-spectrum effects since at least for blast disease, they were not identified as major resistance genes.

Genes with a role in signaling and transcription factors represent the majority of the cases (30 out of 41 genes) found in the literature. Although most of the transgenics built in these reports were initially obtained for fundamental research, they also provide information on their potential for protecting plants from disease. Eight of these genes (*SPL11*, *XB15*, *OsPLDB1*, *OsRac1*, *SPL18*, *PACK1*, *OsGAP1*, and *OsDR8*) were originally found in rice,

whereas the other genes were found by homology to genes in other models (ex *NH1* in *Arabidopsis* and *OsPti1* in tomato).

Out of the nine transcription factors identified in the literature, six are *WRKYs*. Their detailed effects on disease resistance were recently reviewed (Pandey and Somssich 2009). *WRKYs* are also known to be involved in abiotic stress response and development. The challenge in using *WRKY* genes in transgenic is to identify genes that have a positive effect on resistance and no or little detrimental effects on abiotic stress and/or development.

Although 14 *PR* protein families are known in rice (Van Loon et al. 2006), only *PR* genes from a few families have been over-expressed.

Other plant genes improving rice disease resistance

A few genes from plants other than rice have been tested in rice (Table 2). Not surprisingly, the central regulatory gene *NPR1* (Cao et al. 1998) belongs to this group of genes. Three *PR* genes are also found, suggesting a good potential for this large category of genes. However, more than 50

Table 1 Rice Genes Potentially Useful for Improving Rice Disease Resistance

Gene Name	Annotation	Type	Transgenic plant Genotype	Japonica/Indica	Expression	Type	Disease reduction M. oryzae	X. oryzae pv oryzae	Other pathogens or pes	Isolate tested M. oryzae	X. oryzae pv oryzae	Enhanced defense expression Before infection	After infection	Side effects	References	Accession	QTL
OsBRR1	LRR-RLK	Receptor	Nipponbare	Japonica	Constitutive	OX	80%	nd	nd	3	nd	nd	nd	None	Peng et al. 2009	Os03g12730	Yes
Pi9	NBS-LRR	Receptor	TP309	Japonica	From own promoter	OX	Yes	nd	nd	21	nd	nd	nd	nd	Qu et al. 2006	Os06g17920	Yes
OsWAK1	Protein kinase	Receptor	Aichiasahi	Japonica	Constitutive	OX	50%	nd	nd	nd	nd	nd	nd	None	Li et al. 2009	Os11g46900	Yes
OsMAPK5a /BIMK1/ OsMP K5	MAP kinase	Signaling	Nipponbare	Japonica	Constitutive	RNAi	85%	nd	85% (Burkholderia glumae)	nd	nd	+	nd	Susceptible to abiotic stress	Xiong and Yang 2003; Song and Goodman 2002	Os03g17700	Yes
OsSpl11	U-boX/ Armadillo Repeat Protein Endowed with E3 Ubiquitin Ligase activity	Signaling	IR64	Indica	Constitutive	KO	90%	85%	nd	10	4	+	nd	Lesion mimic	Zeng et al. 2004; Yin et al. 2000	Os12g38210	Yes
OsPLD β 1	Phospholipase D	Signaling	Nipponbare	Japonica	Constitutive	RNAi	90%	70%	nd	nd	nd	+	nd	Lesion mimic	Yamaguchi et al. 2009	Os10g38060	Yes
OsSSI2	Steroyl Acyl Carrier FA Desaturase	Signaling	Nipponbare	Japonica	Constitutive	RNAi and KO	95%	90%	nd	nd	nd	+	nd	Lesion mimic	Jiang et al. 2009	Os01g69080	Yes
OsRac1	rac-like GTP-binding protein 2	Signaling	Kinmaze	Japonica	Constitutive	Active form	20%	46%	nd	nd	nd	+	+	Lesion mimic	Kawasaki et al. 1999; Ono et al. 2001; Thao et al. 2007	Os01g12900	Yes
NH1	Regulatory protein	Signaling	TP309	Japonica	Constitutive	OX	No effect	95%	Susceptibility to white-backed planthopper	nd	nd	+	nd	Lesion mimic	Yuan et al. 2007; Chern et al. 2005	Os01g09800	Yes
OsAOS2	ALLENE OXIDE SYNTHASE (P450 74A2)	signaling	Nipponbare	Japonica	Inducible	OX	85%	nd	nd	nd	nd	+	+	nd	Mei et al. 2006	Os03g12500	Yes
OsGH3.1	Indole-3-acetic acid-amido synthetase	signaling	Bomba	Japonica	Constitutive	OX	51%	nd	nd	nd	nd	+	nd	Reduced height	Domingo et al. 2009	Os01g57610	Yes
OsGAP1	GTPase-activating protein	signaling	Aichi asahi	Japonica	Constitutive	OX	nd	55%	nd	nd	3	+	nd	nd	Cheung et al. 2008	Os02g22130	Yes
OsDR8	Thiazole biosynthetic enzyme 1-1	signaling	Minghui63	Indica	Constitutive	RNAi	Yes	350% increase	nd	nd	nd	-	-	nd	Wang et al. 2006	Os07g34570	no

Table 1 (continued)

Gene			Transgenic plant				Disease reduction			Isolate tested		Enhanced defense expression		Side effects	References	Accession	QTL
Name	Annotation	Type	Genotype	Japonica/ Indica	Expression	Type	M. oryzae	X. oryzae pv oryzae	Other pathogens or pes	M. oryzae	X. oryzae pv oryzae	Before infection	After infection				
OsWRKY13	WRKY TF protein	TF	Minghui63	Indica	Constitutive	OX	Yes	Yes	nd	nd	nd	+	nd	Susceptible to abiotic stress	Qiu et al. 2007; Qiu et al. 2009a, b;	Os01g54600	Yes
OsWRKY31	WRKY TF protein	TF	Zhonghua 17	Japonica	Constitutive	OX	67%	nd	nd	nd	nd	+	nd	Root growth affected	Zhang et al. 2008	Os06g30860	Yes
OsWRKY45	WRKY TF protein	TF	Nipponbare	Japonica	Constitutive	OX	100%	nd	nd	nd	nd	+	nd	Reduced height	Shimono et al. 2007	Os05g25770	Yes
OsWRKY71	WRKY TF protein	TF	Nipponbare	Japonica	Constitutive	OX	nd	71%	nd	nd	nd	+	nd	nd	Liu et al. 2007	Os02g08440	Yes
OsWRKY53	WRKY TF protein	TF	Nipponbare	Japonica	Constitutive	OX	Yes	nd	nd	nd	nd	+	nd	None	Chujo et al. 2007	Os05g27730	Yes
OsNAC6	NAC domain-containing protein 48	TF	Nipponbare	Japonica	Constitutive and stress-inducible	OX	Yes	nd	nd	nd	nd	nd	nd	Reduced height/ improved abiotic resistance	Nakashima et al. 2007	Os01g66120	Yes
RBBI2-3	Serine proteinase inhibitor	PR	TP309	Japonica	Constitutive	OX	100%	nd	nd	5	nd	nd	nd	None	Qu et al. 2003	Os01g03360	Yes
GNS1	1,3;1,4-beta glucanase	PR	Nipponbare	Japonica	Constitutive	OX	Yes	nd	nd	nd	nd	+	+	Lesion mimic	Nishizawa et al. 2003	Os05g31140	Yes
OsDR10	Unknown	other	Minghui63	indica	Constitutive	RNAi	nd	95%	nd	nd	+	+	+	None	Xiao et al. 2009	Os08g05960	Yes
pi21	Proline-rich protein	other	Aichiasahi	Japonica	Constitutive	RNAi	90%	no effect	nd	10	na	No change	nd	None	Fukuoka et al. 2009	Os04g32850	Yes
OsSBP	Selenium-binding protein homologue	other	Kinmaze	Japonica	Constitutive	OX	50%	50%	nd	nd	nd	+	+	nd	Sawada et al. 2004	Os01g68770	Yes

OX over-expresser, *KO* knockout mutant

Table 2 Plant (Non-rice) Genes Potentially Useful for Improving Rice Disease Resistance

Gene				Transgenic plant				Disease reduction			Isolate tested		Enhanced defense expression		Side effects	References
Name	Annotation	Origin	Type	Genotype	Japonica/ Indica	Expression	Type	M. oryzae	X. oryzae pv oryzae	Other pathogens or pest	M. oryzae	X. oryzae pv oryzae	Before infection	After infection		
Rxo1	NBS-LRR	Maize	Receptor	Kitaake	Japonica	Constitutive	OX	nd	nd	X. oryzae pv oryzicola	nd	nd	nd	nd	nd	Zhao et al. 2005
AtNRP1	Transcription	Arabidopsis thaliana	Signaling	TP309	Japonica	Constitutive	OX	86%	Yes	increased susceptibility to RYMV	nd	nd	+	+	Lesion mimic susceptible to abiotic stress	Fitzgerald et al. 2004; Quilis et al. 2008
Dm-AMP1	Defensin	Dahlia merckii	PR	Pusa Basmati 1	Indica	Constitutive	OX	Indica	Constitutive	OX	45%	nd	68% (R. solani)	nd	nd	no change
PRms	PR protein	Zea mays	PR	Senia	Japonica	Constitutive	OX	95%	nd	F. verticillioides, H. oryzae and E. chrysanthemi	nd	nd	+	+	nd	Gomez-Ariza et al. 2007
Rs-AFP2	defensin	Raphanus sativus	PR	Pusa Basmati 1	Indica	Constitutive	OX	77%	nd	45% (R. solani)	nd	nd	No change	No change	None	Jha et al. 2009

OX over-expresser, *KO* knockout mutant

Table 3 Non-plant Genes Potentially Useful for Improving Rice Disease Resistance

Gene		transgenic plant						Disease reduction			Isolate tested		Enhanced defense expression		Side effects	References
Name	Annotation	Origin	Function	Genotype	Japonica/ Indica	Expression	Type	M. oryzae	X. oryzae pv oryzae	Other pathogens or pest	M. oryzae	X. oryzae pv oryzae	Before infection	After infection		
Cecropin A	Cecropin A	Hyalophora cecropia	Antimicrobial	Senia	Japonica	Constitutive	OX	87%	nd	nd	nd	nd	No change	No change	Fertility affected	Coca et al. 2006
AFP	Antifungal	Aspergillus giganteus	Antimicrobial	Senia	Japonica	Constitutive	OX	45%	nd	nd	nd	nd	nd	nd	None	Coca et al. 2004
cht42	Endochitinase	Trichoderma virens	Antimicrobial	Pusa Basmati1	Indica	Constitutive	OX	nd	nd	62% (R. solani)	nd	nd	nd	nd	None	Shah et al. 2009
ChiC	Family 19 chitinase	Streptomyces griseus	Antimicrobial	Nipponbare	Japonica	Constitutive	OX	85%	nd	nd	nd	nd	nd	nd	None	Itoh et al. 2003
lactoferrin	Lactoferrin	Human	Antimicrobial	nd	nd	Constitutive	OX	No effect	No effect	Burkholderia (Pseudomonas) plantarii	nd	nd	nd	nd	nd	Takase et al. 2005
hrf1	Hairpins (protein elicitor)	Xanthomonas oryzae pv. oryzae	Elicitor	R109	Japonica	Constitutive	OX	56%	nd	nd	nd	nd	+	nd	None	Shao et al. 2008
avrXa27	Transcription factor	Xanthomonas oryzae pv. oryzae	Elicitor	Nipponbare	Japonica	Inducible	OX	No effect	38%	35% (Xoc), no effect on E. chrysanthemi	nd	nd	+	nd	Delay of Flowering and early senescene	Tian and Yin 2009
flagellin	Flagellin	Acidovorax Avenae	Elicitor	Koshihikari	Japonica	Constitutive	OX	Yes	nd	nd	nd	nd	+	nd	Lesion mimic	Takakura et al. 2008
pemG1	Unknown	Magnaporthe grisea	Elicitor	Nipponbare	Japonica	Constitutive	OX	35%	nd	nd	nd	nd	+	nd	nd	Qiu et al. 2009a, b

OX over-expresser, KO knockout mutant

Table 4 Pyramiding for Improving Rice Disease Resistance

Gene				Transgenic plant				Disease reduction			Isolate tested		Enhanced defense expression		Side effects	References
Name	Annotation	Origin	Function	Genotype	Japonica/ Indica	Expression	Type	M. oryzae	X. oryzae pv oryzae	Other pathogens or pest	M. oryzae	X. oryzae pv oryzae	Before infection	After infection		
tlp, chi, Xa21	Thaumatin-like protein, chitinase and Receptor like kinase	*Oryza sativa*	PR and receptor	ASD16, ADT38, IR72, IR64, White Ponni,	Indica	Constitutive	OX	nd	90%	95% (*R. solani*)	nd	nd	nd	nd	nd	Maruthasalam et al. 2007
Xa21, chi, Bt	Receptor like kinase, Chitinase and Bt toxin	*Oryza sativa* and *Bacillus thriengensis*	PR and receptor	IR72	Indica	Constitutive	OX	nd	90%	63% (*R. solani*) and yellow stem borer	nd	nd	nd	nd	None	Datta et al. 2002
RCH10, RAC22, Glc, RIP	RCH10 (basic chitinase), RAC22 (acidic chitinase), β-Glucanase and BRibosome Inactivating protein	*Oryza sativa*	PR	9311	Indica	Constitutive	OX	Yes	nd	60-100% reduction of kernel smut (*T. barclayana*); also resistance to *U. virens*	nd	nd	nd	nd	None	Zhu et al. 2007
RIP, chi	Ribosome inactivating protein and chitinase	*Oryza sativa* and Maize	PR	Kenfong	nd	Constitutive	OX	no effect	nd	25% (*R. solani*), no effect on *B. oryzae*	nd	nd	nd	nd	nd	Kim et al. 2003
PINA, PINB	Structural proteins	*Triticum aestivum*	other	M202	Japonica	Constitutive	OX	53%	nd	22% (*R. solani*)	nd	nd	nd	nd	nd	Krishnamurthy et al. 2001

OX over-expresser, *KO* knockout mutant

genes have now been identified in different plant species as regulating disease resistance (Hammond-Kosack and Parker 2003). Although some of these genes may not work in rice for biological reasons, the important conservation of the disease resistance pathways between plants should allow transfer from one plant to another. There is therefore a large potential in this category of genes.

Transferring one gene from one species to another may lead to unexpected effects. This was the case when the *Arabidopsis NPR1* gene was transferred to rice (Fitzgerald et al. 2004). The rice plants over-expressing *AtNPR1* also displayed an environmentally regulated and heritable lesion mimic phenotype (see below).

Moreover, a recent report on *OsWRKY45* demonstrates that over-expressing (in *japonica* rice) a *japonica* allele of the gene confers increased susceptibility to bacterial blight, whereas over-expressing an *indica* allele of the gene confers increased resistance to bacterial blight. In contrast, both alleles conferred increased resistance to blast disease (Tao et al. 2009). This finding suggests that one should be careful when transferring one gene from one background to another, even within the *Oryza sativa* species.

Non-plant genes improving rice disease resistance

Table 3 summarizes available data on non-plant genes. Two types of genes were used. One group of genes is represented by proteins having known antimicrobial effects. Most of the genes reported are from fungi, one is an insect gene (Cecropin A), and one a human gene (lactoferrin). Side effects could be expected in these cases. For example, Cecropin A is a known anticancer molecule (Mader and Hoskin 2006). Moreover, the acceptance of plants for human food containing a human protein is likely to be very low.

A second group of genes like avirulence genes are known to activate plant defense responses. In this case, one expects that constitutive or inducible activation of defenses will increase disease resistance. When avirulence genes are used (e.g., *avrXa27*), pathogen-specific effects are expected. For this reason, most of the cases deal with avirulence genes that are broadly distributed (e.g., flagellin).

There is also a risk that such plants may show unwanted effects because such genes have a strong potential in inducing defense responses. For this reason, it is recommended to use pathogen-inducible promoters. However, such promoters are often either leaky or controlled by developmental signals (Van Loon et al. 2006). Thus, the genes that activate plant defense responses are unlikely to be a good strategy to increase disease resistance.

We excluded from this review 19 rice genes for several reasons (ESM, Supplemental Table 1). First, we excluded genes that have only been tested in heterologous systems. Second, we did not include rice genes for which only negative effects on disease resistance were reported in rice. This is for example the case of the over-expression of the *OsMT2b* gene that confers enhanced susceptibility to blast (Wong et al. 2004). Finally, there were genes that had only been tested in rice cell cultures but not in entire rice plants. There is thus no *in planta* demonstration of the usefulness of these genes. These genes should be tested by stable transformation in rice.

Disease protection quantitative effects

Where possible, we quantitatively evaluated the level of resistance. The increase of resistance was measured as the percentage of disease reduction as compared to the presented wild-type controls. It is noteworthy that in most cases, the best control was missing. This control consists in the nullizygous plant, i.e., a non-transgenic sibling originating from a hemizygous plant. In most cases, the control shown is a transformed plant with the empty vector. Since rice transformation induces many somaclonal mutations, such a control is inappropriate. In other cases (e.g., *NH1*; Chern et al. 2001), several transgenics are presented, highlighting the fact that different insertion events can show large differences. Moreover, the disease protection values were obtained from data obtained on a few plants. Only field evaluation in natural conditions could give a real idea of the effectiveness of these genes. With these caveats in mind, effects smaller than 50% should be considered as weak. For bacterial blight disease, the same criterion was always used (length of lesions), whereas for blast disease resistance, many criteria were used (lesion number, diseased area, etc.). In all cases, the value presented is the most favorable.

Independently of the pathogen, the majority of the reported cases show disease reduction higher than 50%. For blast, two genes (*RBBI2-3* and *WRKY45*; Table 1) showed the best effects with a complete absence of symptoms. Disease reduction higher than 90% was obtained with four other rice genes (Table 1) and the *PRm* gene from maize (Table 2).

For bacterial blight, the best levels of resistance (higher than 95% reduction of disease) were achieved with two rice genes (*OsDR10* and *NH1*; Table 1) and with two cases of pyramiding (Table 4).

For sheath blight caused by *Rhizoctonia solani*, the best level was achieved with pyramiding three genes (thaumatin-like protein, chitinase, and *Xa21*; Table 4). In this case, the data also indicate that this strong effect is only due to the combined action of the thaumatin-like and chitinase proteins, independently of the *Xa21* gene (Maruthasalam et

al. 2007). Some excellent levels of protection were also shown for kernel smut (caused by *Tilletia barclayana*; Zhu et al. 2007) by pyramiding (Table 4).

Pyramiding was a good strategy to increase quantitative effects of individual transgenes. For example, while the single chitinase and thaumatin-like genes each conferred about 80% increase of resistance toward sheath blight, the combination of the two genes showed a reduction of 95% of disease.

These different examples of gene pyramiding clearly demonstrate that it is a good strategy to quantitatively improve resistance. From the quantitative point of view, it is noteworthy that all categories of genes (except *R* genes) provide, on average, the same levels of resistance.

Disease protection qualitative effects

As pathogen populations are quite complex, it is important to have an evaluation of the spectrum of action of the tested genes. Unfortunately, this information is lacking in most cases. For 27/60 genes, only one isolate of one pathogen was tested. Clear information on the spectrum of resistance was provided for only 11 genes. For blast resistance, nine genes were shown to provide resistance to more than one isolate, including *RBBI2-3* and *Pi21* that also show elevated quantitative effects (Table 1). For blight resistance, five genes showed relatively broad-spectrum resistance, including *OsDR10* plants that have high levels of resistance. The *SPL11* gene was the only one for which broad-spectrum resistance was shown against both pathogens. However, these plants suffer from strong developmental side effects (see below).

There are 15 genes for which both blast and blight disease resistance have been evaluated (Tables 1, 2, and 3). All genes protecting against blast, except the *pi21* gene, were shown to also confer resistance to blight. This is a very promising observation showing that the genes used for one pathogen can be used for another. It is noteworthy that the *NH1* gene, a key disease pathway regulator, does not seem efficient against blast disease, whereas it confers good levels against blight (Chern et al. 2005; Yuan et al. 2007).

Quite surprisingly, the expression of the *avrXa27* gene, in the presence of the *Xa27* gene, did not confer resistance to blast. This may be due to the lack of induction of the *avrXa27* gene under the control of the inducible *PR1* promoter. However, the *PR1* gene is known to be induced by *M. oryzae* (Mitsuhara et al. 2008). Alternatively, it is likely that the events triggered by the *Xa27* gene are not efficient against *M. oryzae*.

There is so far no report of simultaneous improvement of resistance against blast, blight, and sheath blight diseases.

This may be simply due to the absence of data rather than to biological reasons since there are genes conferring enhanced resistance to all combinations of two of these pathogens.

Expression of defense in transgenics

For some plant genes encoding proteins involved in disease resistance (Tables 1 and 2) and encoding avirulence products (Table 3), one can expect perturbation of the events downstream of the pathway, like *PR* gene expression and phytoalexin production. These perturbations can occur either before and/or after infection. Defense gene expression, often using the *PR1* and *PBZ1* genes as markers, was evaluated for a large number of cases (37/55).

Elevated defense gene expression before infection was found in 80% of the cases, and these levels could be extremely high. For example, *PBZ1* and a peroxidase gene were found to be expressed 40 times higher in over-expressor plants as compared to wild type in the case of the *WRKY53* gene (Chujo et al. 2007). Some upstream signaling events were measured in a few cases. The signaling molecule jasmonic acid was found to be less present in plants over-expressing the *WRKY13* gene (Qiu et al. 2007). Similarly, reactive oxygen species were detected in several cases (e. g., *OsPLDβ1*, Yamaguchi et al. 2009; flagellin, Takakura et al. 2008). In one puzzling case, *OsDR8*, many PRs were down-regulated before (and after) infection, although the plants showed slightly enhanced resistance to blast and a strong increase of susceptibility to bacterial blight (Wang et al. 2006; Table 1). Available micro-array data on over-expressor plants further indicate that defense responses are often widely constitutively over-expressed. For example, 84 genes annotated as responsive to abiotic stress are up-regulated in plants over-expressing OsGH3.1 (Domingo et al. 2009). Similarly, more than 430 genes involved in secondary metabolite synthesis are up-regulated in plants over-expressing *OsWRKY13*, leading to increased production of the phytoalexin momilactone (Qiu et al. 2009a, b).

An enhanced defense response after infection was observed in eight cases. This was rather expected for most of them since they encode positive regulators of defense. This was less expected for the *GNS1* gene (Nishizawa et al. 2003) since this gene encodes a classical glucanase. It is possible that enhanced degradation of the cell wall of *M. oryzae* by the additional glucanase activity led to the release of molecules that in turn over-induced the defense system. No change of defense induction was observed for five genes (Cecropin A, Dm-AMP1, rTGA2.1, OsMPK6 and Rs-AFP2; Tables 1, 2, and 3) in spite of increased resistance.

Side effects

For 19 cases, there was no specific mention of side effects. No major visible side effects were clearly found for 13 genes. It is noteworthy that for six of these 32 genes for which there are likely little or no visible side effects (*OsAOS2*, *Rack1*, *OsWRKY71*, *OsWRKY53*, *OsGAP1*, and *OsSBP*; Table 1), enhanced defense expression before infection was measured. This suggests that it is possible to increase preformed defense and disease resistance with no obvious side effect. This seems particularly clear for the plants over-expressing *OsWRKY53* (Chujo et al. 2007). In this case, more than 200 genes are up-regulated as indicated by micro-array analysis, and one third of these genes, besides genes related to defense, are related to metabolism, transport, proteolysis, and signal transduction.

However, there are 28 cases for which detrimental side effects were reported. Side effects were found with all kinds of genes except receptor genes, although this may be due to an absence of data. Genes involved in signaling and transcription factors appeared to display frequent secondary effects, with 82% and 70% of the observed cases, respectively. Although the number of reported cases is scarce, avirulence genes also produced secondary effects (Table 3).

There were eight rice genes (*OsMPK6*, *OsPti1*, *XB15*, *OsPLDβ1*, *OsSSI2*, *OsRac1*, *NH1*, and *GNS1*; Table 1) and one bacterial gene (flagellin; Table 3) where an unexpected lesion mimic phenotype was observed. Lesion mimic plants are well known for their enhanced resistance (Lorrain et al. 2003), such as the *spl7*, *spl11*, and *spl18* rice mutants (Table 1). This enhanced resistance often correlates with constitutive expression of defense genes (Lorrain et al. 2003). This is also true for the 11 cases found here showing a lesion mimic phenotype, as constitutive expression of defense markers was found. Thus, in these cases, it is likely that constitutive activation of defense pathway(s) leads to the uncontrolled and constitutive triggering of cell death. Pathogen-inducible over-expression of these genes could reduce this side effect while maintaining high levels of resistance after pathogen infection.

Developmental phenotypes were also often observed (13 cases). In many cases, the plants showed a reduced height that often correlated with enhanced expression of *PR* genes before infection (Tables 1 and 2). For the genes that are well known to be involved in plant development, increase of disease resistance may in fact be a side effect. This may be true for the *GH3.1* and *GH3.8* genes that both affect auxin metabolism. In the case of *WRKY31*, a root phenotype was found (Zhang et al. 2008), further indicating that some side effects may have been missed when looking at aerial parts of the plant only.

Micro-array data are available for five genes (*OsPLDβ1*, *OsSSI2*, *OsGH3.1*, *OsWRKY13*, and *OsNAC6*; Table 1),

providing further information on the molecular events that are modified in the corresponding plants. In *Osssi2* knockout plants, almost 300 genes are up-regulated, among which a large set of genes related to metabolism (71), transcription factors (32), kinases (28), and defense (16; Jiang et al. 2009). Quite surprisingly, plants over-expressing the *WRKY13* gene, besides over-expressing genes of the flavonoid pathway, also displayed up-regulation of a large set of genes involved in amino acid (475 genes) and nitrogen (162) metabolism (Qiu et al. 2009a, b). Thus, despite this major transcriptional reprogramming, these plants do not seem to have developmental defects (Qiu et al. 2007). In the case of the *OsGH3.1* gene, the micro-array data are consistent with the developmental phenotype. Almost 500 genes related to growth and cellular morphology are down-regulated in the plants over-expressing the *OsGH3.1* gene (Domingo et al. 2009). Also consistent with the improved tolerance to dehydratation and high-salt concentrations, genes related to response to abiotic stress are differentially regulated in the mutant.

There are four reports for which response to abiotic stress was also indicated. This confirms a well-known observation that biotic and abiotic stresses are strongly connected. There were three genes for which there is an antagonism between disease and abiotic stress resistance (*OsMAPK5a*, *OsWRKY13*, and *AtNPR1*; Table 1). In these cases, the impact on abiotic stress could not be predicted and the gain for disease resistance is counterbalanced with the loss of resistance to abiotic stress. The available data do not clearly indicate whether there are cases where biotic stress resistance is improved without affecting abiotic stress resistance. However, there are several examples (Table 1) where abiotic stress resistance does not seem affected. Thus, at this stage, one should carefully examine effects on abiotic stress resistance when manipulating genes involved in biotic stress resistance.

The complex case of the *OsNAC6* gene demonstrates that it will probably be difficult to use this category of genes to improve stress tolerance (Nakashima et al. 2007). Plants constitutively over-expressing *OsNAC6* are more resistant to *M. oryzae* and abiotic stresses but have abnormal plant growth. To try to circumvent this side effect, the *OsNAC6* gene was over-expressed under stress-inducible promoters. The resulting plants did not show the initial developmental defect, were resistant to abiotic stress, but were no more resistant to *M. oryzae*.

Transgene strategies

The vast majority (75%; for details, see Tables 1, 2 and 3 as well as ESM, Supplemental Tables 1 and 2) of the cases of transgenic plants were made in the *japonica* subspecies. This is a consequence of the fact that *japonica* rice is easier

to transform than the *indica* type. Nipponbare was the *japonica* cultivar used in most cases (16 genes), reflecting the fact that this is the original genotype adopted by the rice community for genomic studies.

We only found three examples where inducible promoters were used. In the case of the avirulence gene *avrXa27*, this was necessary to avoid massive HR cell death triggered by the recognition of the product of the avirulence gene and the corresponding major resistance gene *Xa27* (Tian and Yin 2009). In the case of the *OsNAC6* transcription factor, this was needed to avoid side effects (growth retardation). Moreover, the permanent activation of some disease signaling pathways caused by the use of a constitutive promoter has a metabolic cost that could be measured in terms of carbohydrate storage losses (see below). In one case of pyramiding, several promoters were used, limiting potential silencing issues (Kim et al. 2003). It is likely that inducible promoters will be required in the future to limit side effects (see below) and yield penalty. Finding such specific promoters is one of the major challenges in this field, and current experiments on whole transcriptome analysis under different biotic and abiotic stresses will provide numerous candidate promoters for cloning.

The vast majority of the favorable effects (46 out of 60) on disease resistance were obtained by over-expressing positive regulators. In the case of the *OsRac1* gene, a dominant constitutively active form of the protein was used (Thao et al. 2007). For genes that are negative regulators, two main approaches were taken: silencing using RNAi and insertion mutants. In one case, a dominant negative allele was introduced (*rTGA2.1*; Fitzgerald et al. 2004).

We only found a few cases (Table 4) of gene pyramiding. The pyramiding was obtained either by crossing individual lines (e.g., Datta et al. 2002) or by co-bombardment of separate transgenes (e.g., Kim et al. 2003). We found no example of several transgenes borne on one single T-DNA, indicating that transferring several transgenes in one single transformation event is not a common strategy.

Finally, it should be noted that the general issues relative to GMOs apply for the plants described in this review. For acceptance of these products, marker-free plants will be required. RNAi plants are also likely to be more easily accepted as no new protein is produced, although co-silencing may also lead to unwanted side effects.

Conclusions and recommendations for future transgenic approaches

Examples of transgenic rice plants expressing genes related to disease resistance have tremendously increased in the past decade. With the publication of the first case of improvement of resistance by over-expressing the *Rir1b* gene (Schaffrath et al. 2000), more than 60 other cases are now available (Table 1). Besides providing information on the disease regulation networks in rice, they also represent a solid sample of examples for the evaluation of transgenic strategies. The key elements coming from this review are listed in Box 1. Based on the review of these 60 cases, several recommendations can be made.

The 60 genes over-expressed or silenced in rice
- Plant genes
41 rice genes and 5 genes from other plants
5 receptors, 22 signalisation, 9 transcription, 7 PR, 3 other
- Genes from micro-organisms
4 elicitors and 5 with antimicrobial properties

Transgenic strategy
- Promoters
58 cases of constitutive and 3 cases of inducible promoters
- Type of mutant
46 over-expresser constructs, 14 knock-out mutants
- Gene pyramiding
5 cases involving receptor and PR genes

Side effects
- 80% plants show modified defense expression before infection
- 28 genes induce negative side effects
including 14 lesion mimic phenotypes, 13 developmental defects

Best genes on quantitative and qualitative criteria
pi21, PRm, RBBI2-3, OsDR10, OsDBP, defensins

If examples of gene pyramiding (Table 4) are excluded, this represents the individual examination of the potential of 56 genes (Tables 1, 2, and 3). When excluding genes for which detrimental side effects were observed, only half (28) of the genes remain.

The over-expression or silencing of genes involved in signaling and transcription often leads to negative side effects (e.g., lesion mimics, impaired plant growth; Tables 1, 2, and 3). Thus, one should be careful when using genes involved in signaling and transcription for transgenic strategies.

Alternatively, inducible promoters should be used to reduce the risk and the extent of side effects. This was done, with some success, with the *OsNAC6* gene (Nakashima et al. 2007).

Surprisingly, despite the large number of known secreted proteins from pathogens, only a few were used (e.g., *pemG1*; Table 3). However, pathogen genes coding for avirulence products may not be the best candidates for transgenic approaches as they often trigger secondary effects.

With the example of impaired root development observed for the *OsWRKY31* gene (Zhang et al. 2008), one should carefully characterize transgenic plants. An extensive phenotyping with respect to abiotic stress tolerance and

development seems to be required to detect subtle side effects. Available data still suggest that it should be possible to improve resistance to biotic stress without negatively affecting abiotic stress resistance.

When applying quantitative requirements for the 28 genes not associated with side effects, a few genes clearly stand out as favorable, depending on the pathogen. For blast disease only, the *pi21*, *PRm*, and *RBB12-3* genes are amongst the best (disease reduction >90%). For bacterial blight disease only, *OsDR10* alone provides more than 95% reduction of symptoms. For sheath blight only, the best gene so far seems to be the *Dm-AMP1* gene (Table 1). When applying qualitative and quantitative requirements for the 28 genes without side effects, only a few genes providing resistance to several pathogens can be found. For combined blast and bacterial blight disease resistance, the *OsSBP* gene is the only one providing increased resistance, although the level of this resistance is rather low (50%). Defensins (*Dm-AMP1* and *Rs-AFP2*) seem to confer good levels of resistance to both blast and sheath blight. These seven genes are amongst the best genes so far for use in transgenic approaches.

R genes and *PR* genes seem to be a good class of genes to use for transgenic approaches. Plants over-expressing these genes display very little side effects but strong (but likely not durable) protection effects for *R* genes as well as for *PR* genes. Thus, very beneficial and straightforward effects will likely be obtained in future transgenic plants over-expressing *R* genes and *PR* genes. Defensins seem to have particularly interesting effects (Table 2). While ignored for a long time in rice, defensins seem to exist in rice (Silverstein et al. 2007). Along with the eight classes of *PR* genes that have not yet been over-expressed in rice, we also suggest using defensins and other small peptides in future transgenic approaches.

It is obvious that missing data limit the possible analysis. It is likely that many genes show protection effects against several pathogens and multiple isolates of one pathogen. One recommendation would be, for the future, that several pathogens and isolates should be tested.

Although we did not review them here, there are several examples of successful improvement of viral resistance by transgenic approach. Expression of the *RF2a* and *RF2b* genes provided RTBV resistance (Dai et al. 2008). There are also several cases of improvement of RYMV resistance by a transgenic approach (Pinto et al. 1999; Kouassi et al. 2006). In all these cases, no side effects were observed, indicating that except for potential durability issues, these strategies are efficient.

We identified 13 negative regulators for which a beneficial effect was obtained in knockout plants (Table 1). As in the case of the *pi21* gene where good alleles exist in nature, one can expect to find interesting alleles of negative regulators by

mutational approaches. With this respect, the Tilling technology (Till et al. 2007) opens the possibility of finding agronomically interesting alleles of negative regulators.

It was previously shown with a limited subset of 35 rice genes involved in disease resistance that these genes tend to co-localize with blast disease quantitative trait loci (QTLs). These genes were found in 72% of the cases in genome areas containing QTLs, and this was significantly different from randomly selected genes (Vergne et al. 2008). In a similar way, the rice genes presented in this updated list of genes involved in disease resistance were tested for co-localization with blast QTLs. For the 41 genes for which favorable disease resistance effects could be produced in transgenic rice (Table 1 and ESM, Supplemental Table 2), 90% were found in blast QTLs. This further supports the idea that disease regulators probably explain many disease resistance QTLs. Thus, the genes of Table 1 and ESM, Supplemental Table 2 can be used as markers for marker-assisted selection.

There are as of yet only a very limited number of examples of gene pyramiding (Table 4). However, these examples are very promising as the corresponding plants show high levels of resistance against several pathogens, and when this was measured, no side effect was observed. Mixed planting of transgenic lines should also be efficient for controlling disease epidemics as shown using wild-type mixtures for blast resistance (Zhu et al. 2000).

It is important to note that the final grain yield has not yet been tested in most cases (e.g., *Xa21*; Tu et al. 2000). Only two cases of transgenic plants listed here were tested in the field (*hrf1*plants, Table 3; *Xa21*, *chitinase* and *Bt* toxin plants, Table 4). Although more than 285 GM field trials across the world were performed between 1990 and 2008 (http://www.gmo-compass.org/eng/database/plants/64.rice.html), it is difficult to establish how many assays are planted for rice. Thus, most of the results discussed here need to be validated in the field.

Finally, the question of durability of these transgenic plants needs to be addressed. Resistance in transgenic plants over-expressing *R* genes is likely to be overcome by the corresponding pathogen. We can make this conclusion based on the many natural situations where newly introgressed *R* genes did not last more than 1 to 2 years in the case of blast (Correa-Victoria et al. 2004). In contrast, rice plants over-expressing *R* genes for which the corresponding avirulence product is not highly polymorphic could be more durable, as pathogens are less likely to harbor mutations circumventing recognition. We have no knowledge on the durability of resistance conferred by genes like *PR* genes and regulators. These genes could be more durable than genes for which the corresponding protein is in close contact with the pathogen. In this case, the development by the pathogen of counter-defense systems

could be more difficult. However, the recent finding that many effectors inhibit plant pathogen defense may lead to poor durability of these solutions. Establishing the durability of the transgenic plants resistant to disease represents a major scientific challenge for the coming decades.

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