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REVIEW

Genetic Engineering for Disease Resistance in Rice (*Oryza sativa* L.) Using Antimicrobial Peptides

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Abstract

Pathogen attack is a serious problem in rice, which is one of the most economically important crops worldwide. Plant genes with disease resistance have been extensively analyzed. Antimicrobial peptides from a variety of organisms are known to inhibit the growth of pathogens. Antimicrobial peptides are usually small, cationic, and amphipathic and have open-chain forms with disulfide bonds leading to rigid and compact structures. A gene family of plant defensins (AFP) is conserved in several plant species, including those of the Brassicaceae, and does not appear to be toxic to mammalian and plant cells. Rice plants do not contain these peptides. AFP1 homologs in 8 Brassicaceae vegetables have been identified, and their structural differences have been determined. AFP1 gene variants from *Brassica oleracea* and *B. campestris* conferred an effective resistance to both rice blast and bacterial leaf blight. The results of *in vitro* and *in vivo* analyses suggest that plant defensins have the potential to enhance broad-spectrum disease resistance in rice through genetic engineering. Modification of signal peptides and mature peptides could contribute to the improvement of broad disease resistance in crop plants, including rice.

Discipline: Biotechnology
Additional key words: rice blast, bacterial leaf blight, plant defensin, pathogenesis-related (PR) protein

Introduction

Environmental stresses exert a critical influence on crop yields[6]. Pathogen attacks are sometimes the most devastating biotic stresses. The enhancement of disease resistance in crops has contributed significantly to increasing the productivity of crops and decreasing the application of pesticides, which can adversely affect human health and the environment. Genetic engineering has provided a new strategy, based on recent advances in cellular and molecular biology, for improving disease resistance. Cloning of several disease resistance genes, some of which exhibit induced or constitutive expression, is providing insights into their function and on how they protect plants against pathogen attacks[12].

Rice (*Oryza sativa* L.) is one of the most important crops worldwide and is grown mainly in Asia. In a

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humid, temperate climate, fungal rice blast caused by *Magnaporthe grisea* is the most serious problem for crop yield. Indeed, the proportion of rice production damaged by rice blast has been expanding in Japan. Bacterial leaf blight caused by *Xanthomonas oryzae* is a serious disease of rice in subtropical and tropical countries. Despite the progress achieved in rice breeding and the sophistication of the rice cultivation systems, rice cultivation and food production have experienced critical damage from these 2 major diseases.

Genes for disease resistance

Plant-pathogen interactions are sometimes controlled by specific interactions between avirulence genes of pathogens and gene-for-gene disease resistance (R) genes of plants. Their common motifs reveal that most of the R genes encode proteins with a nucleotide-binding site and a leucine-rich repeat (LRR) domain. The LRR domain plays an important role in determining the R gene's specificity for pathogens[7]. Xa21, a receptor-like protein kinase gene with an LRR domain, was cloned from a wild rice species and genetically engineered into japonica rice to confer an enhanced resistance to *X. oryzae*[21]. Another approach for the analysis of disease resistance genes includes the involvement of the GTP-binding protein (G protein). The signal transduction pathway of the molecular switch in defense signaling, which includes the G protein, has been analyzed by using transgenic rice[17].

Most of the multicellular organisms produce a complex of multiple peptides within their tissues in response to pathogen attacks. Small antimicrobial peptides play an important role as part of the natural defense systems of plants against infectious microorganisms. Genes encoding antimicrobial peptides are widely conserved among multicellular organisms, including invertebrates and vertebrates. The peptides can recognize a broad range of microbes. Over 500 antimicrobial peptides have been identified[24]. Many authors have reported the enhancement of disease resistance by transgenic approaches, as demonstrated in tobacco[4], potato[18], and rice[9]. It is also possible to utilize antimicrobial peptides for therapeutic and herbicidal uses. In contrast to the situation with conventional antibiotics, which microbes can readily circumvent, the acquisition of resistance to antimicrobial peptides is unlikely[24].

Antimicrobial peptides

Antimicrobial peptides are usually small, cationic, and amphipathic and have open-chain forms. The amphipathic structure with an α-helix and an anti-parallel β-sheet is highly conserved, and cationic hydrophobic residues are organized as segregated patches, resulting in a structure that is capable of forming ion channels through membrane bilayers[1]. Most of the antimicrobial peptides contain cysteine residues, which are likely to form disulfide bonds, leading to a rigid and compact structure. Antimicrobial peptides from plants harbor these structural features with 3 or 4 disulfide bonds, such as thionin from barley (*Hordeum vulgare*)[19] and plant defensin from radish (*Raphanus sativus*)[23].

Generally, mature, active antimicrobial peptides with a net positive charge are associated with the outer leaflet of cell membranes of targeted organisms via hydrophobic interactions. The cytoplasmic membranes of bacteria are composed of negatively charged phospholipids in the outer leaflet of the bilayer. In contrast, plasma membranes of plants and animals are composed of lipids without net charge in the outer leaflet and with a negative charge in the inner leaflet[15]. The state of the negative charge on the outer membrane of multicellular organisms is highly related to the specificity of antimicrobial peptides for the membrane target. The plasma membranes of animals consist of phospholipids with cholesterol, whose role is either to stabilize the lipid bilayer or to interact with the peptides[15]. Bacterial membranes do not contain cholesterol. The Shai–Matsuzaki–Huang (SMH) model describes the activity of most antimicrobial peptides[24]. Antimicrobial peptides operating via the SMH mechanism kill microbes generally at micromolar or nanomolar concentrations.

Plant defensins

Various types of antimicrobial peptides have been identified in plants, including thionins[2], maize zeamatin[14], coffee circulin[22], and wheat puroindoline[13]. Plant defensins (PDFs) from radish (Rs-AFP1, 2, 3, 4), which share structural features with insect and mammalian defensins (which have 3 disulfide bonds), are small, cysteine-rich peptides consisting of 45–54 amino acids with 4 disulfide bonds[3]. They are conserved in several plant species, including members of the Brassicaceae, and inhibit the growth of a broad range of microbes, but do not appear to be toxic to mammalian and plant cells. Rice plants do not contain these peptides. The arrangement of known plant defensins reveals the existence of cysteine-stabilized antiparallel β-sheets and an α-helix as the 3-dimensional structure. Thirteen putative PDF genes have been identified in *Arabidopsis thaliana*, one of which (*PDF1.2*) is widely used as a marker gene induced by jasmonic acid and ethylene[23]. Known plant defensins are divided into 3 groups based on their mode of action. The first group causes depolarization of the microbial membrane, slowing microbial growth without inducing morphological changes. The second group, such as Sia2 from sorghum (*Sorghum bicolor*), inhibits the activity of α-amylase instead of showing a direct antimicrobial effect. In the third group, Rs-AFP1 and Rs-AFP2 from radish are potent antifungal proteins, causing morphological distortions of the fungal hyphae, resulting in hyperbranched fungal structures[8]. A gene family from Brassicaceae vegetables consisting of the AFP1 homologs of radish has been identified, and structural differences have been determined[10]. The amino acid sequences of this family differ in the signal peptides rather than in the region of mature peptides. Signal peptides induce post-translational modifications, such as proteolytic processing, glycosylation, carboxy-terminal amidation, amino acid isomerization, and halogenation[24]. These structural differences may be useful for understanding the antimicrobial mechanisms of peptide antibiotics and may lead to the analysis of peptide kinetics. The degree of genetic diversity in the AFP gene family is consistent with the classification based on taxonomy. A series of substitution variants of AFP1 genes from *B. campestris* were prepared by site-directed mutagenesis (Fig. 1) and their antimicrobial activity against a gram-negative bacterium, *Escherichia coli* was assessed *in vitro*. The antimicrobial potency was dramatically influenced by minor amino acid substitutions (Fig. 2), which is consistent with the results of the analysis of *in vitro* antimicrobial activity using Rs-AFP1 substitution variants[5].

Transgenic rice

Transgenic rice plants expressing genes for pathogenesis-related (PR) protein, for example thionin[9], chitinase[16], and puroindoline[13], show a high level of resistance to rice blast or bacterial leaf blight. Plants that constitutively expressed a plant defensin gene from *B. oleracea* or *B. campestris* were tested for resistance to rice blast and bacterial leaf blight[10]. As shown in Fig. 3, transgenic plants with a highly enhanced resistance to rice blast and without phenotypic abnormality were successfully obtained. Progeny testing revealed that the progenies were also highly resistant. Among the transgenic plants with high resistance to rice blast, estimation of the resistance to bacterial leaf blight was carried out. The infected leaves of the transgenic plants were highly resistant to bacterial leaf blight. When defensin genes of *B. oleracea* and *B. campestris* that were modified to substitute a single amino acid at each position were individually introduced into rice, some conferred a much higher resistance than the wild-type defensin genes (Fig. 4). These results indicate that the plant defensins from *B. oleracea* and *B. campestris* conferred an effective resistance to both rice blast and bacterial leaf blight, and that the modification of the defensin genes led to an increase in the broad disease resistance spectrum.

The effect of the 3′-flanking region on the plant defensin activity was examined in transgenic rice plants. Accurate integration of each transgene into the genomic DNA can be estimated by PCR[11]. When genes containing the 3′-flanking region were introduced, the frequency of transgenic plants with a high level of disease resistance increased, compared with the introduction of plant

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AFP2	Q	K	L	C	Q	R	P	S	G	T	W	S	G	V	C	G	N	N	N	A	C	K	N	Q	C	I	R	L	E	K	A	R	H	G	S	C	N	Y	V	F	P	A	H	K	C	I	C	Y	F	P	C
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Fig. 1. Site-directed mutagenesis of AFP2 genes from *B. campestris*

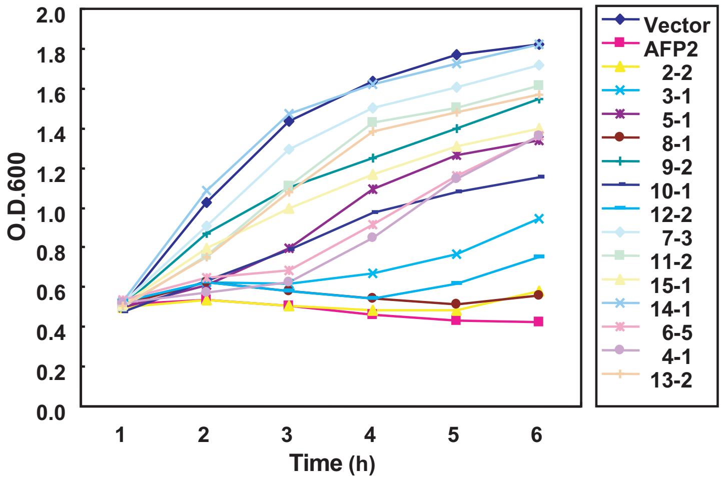

Fig. 2. Antimicrobial activity against *E. coli* of defensin peptides from *B. campestris* with single amino acid substitutions

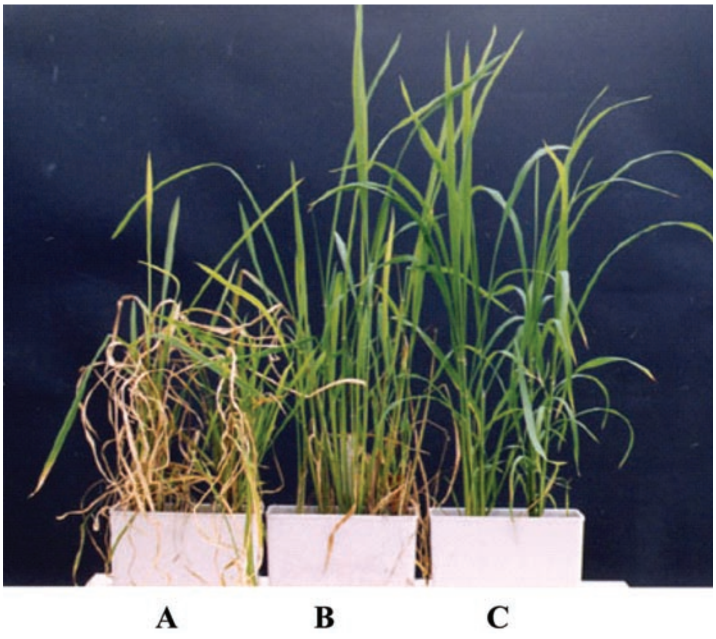

Fig. 3. Resistance to rice blast disease in transformant by introduction of defensin gene

A: Non-transformant. B: Transformant. C: Non-transformant, not infected.

Fig. 4. Resistance to bacterial leaf blight in transformants by introduction of defensin gene

A: Transformant by introduction of modified defensin gene.
B: Transformant by introduction of native defensin gene. C: Non-transformant.

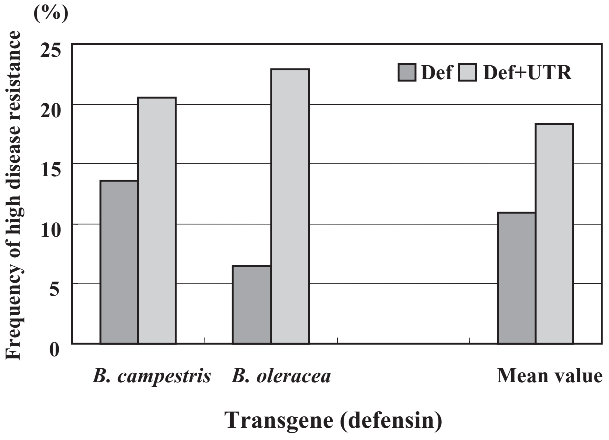

Fig. 5. Effect of addition of 3′ UTR region to introduced defensin gene

Def: Open reading frame (ORF) of defensin gene.
Def+UTR: Fragment of ORF and 3′ untranslated region (UTR) of defensin gene.

defensin genes without the 3′-flanking region (Fig. 5). These findings suggest that the addition of the 3′-flanking region is correlated with the stable expression of the mature mRNA of the plant defensin genes.

Broad spectrum of disease resistance

Antimicrobial peptides have the potential to enhance the broad-spectrum disease resistance of plants by genetic engineering. Indeed, transgenic rice expressing wheat puroindolines showed an enhanced resistance to rice blast and sheath blight[13], and oat thionin in rice was effective in the control of bacterial diseases such as bacterial leaf blight and the disease caused by *Burkholderia plantarii*[9]. Plant defensin genes from *B. oleracea* and *B. campestris* have successfully conferred a resistance to rice blast and bacterial leaf blight in transgenic rice[10]. Based on *in vitro* analysis, many antimicrobial peptides, including plant defensins, inhibited the growth of a broad range of plant pathogenic fungi and some bacteria. The results suggest that these peptides could confer a much broader disease resistance in rice than had so far been observed. Because most pathogens display a variable host range and cultivar specificity, whether the effect of disease resistance is expanded, regardless of the difference in the race of targeted microbes, is another important factor in estimating the broad spectrum activity of each antimicrobial peptide. Estimation of the degree of resistance to each race of rice blast and bacterial leaf blight by the introduction of defensin genes from *B. oleracea* or *B. campestris*, including amino acid variants, into rice is the next target. Increasing the antimicrobial activity could contribute to the improvement of broad disease resistance through the modification of signal peptides and mature peptides. For example, when the signal peptide of rice chitinase was fused with the coding sequence for the mature cecropin B peptide, a much higher antimicrobial activity was obtained than when the native signal region of the corresponding gene was used for the development of transgenic rice[20]. This result suggests that the signal peptide is associated with the level of transcription of the inserted gene. Thus, diversity of signal peptides of plant defensins from Brassicaceae species may contribute to the improvement of broad disease resistance to critical pathogen attacks by genetic engineering. Since genetic engineering using antimicrobial peptides is becoming a powerful tool for the introduction of new traits into plants, many studies are in progress to improve plant breeding for disease resistance by transgenic approaches.

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