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原告準備書面　（１４）

――証人尋問に関する原告意見の補足（その２）――

２０１７年　４月１０日

東京地方裁判所民事第３８部Ｂ１係　　御中

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本書面は、「平八重氏と園田氏は本研究プロジェクトの屋内実験において実験を実施したか否か」の論点に関して、今般提出の被告準備書面（１０）に対する反論と前回期日における裁判所の釈明の検討を踏まえて、平八重氏と園田氏の証人尋問のもう１つの意義を提案し、その実施を求めたものである。

目　　次

１、甲１１の実験３（抗菌活性実験）にあたり、「平八重氏と園田氏は川田氏に実験材料を渡し、実験方法・評価方法等を指導・伝授しただけ」という被告主張（準備書面（１０）４頁以下）について

被告は、「平八重氏と園田氏は川田氏に実験材料を渡し、実験方法・評価方法等を指導・伝授しただけ」という従前の主張に関連して、今回提出の準備書面（１０）で、甲１１の論文２３１頁左段３行目以下記載の実験３（以下、本抗菌活性実験という）の内容を初めて具体的に示した。

しかし、原告は、既に原告準備書面（１３）６頁４行目～７頁で、「本抗菌活性実験は微生物の取り扱い経験が少しでもある研究者であればだれでもなし得る（それこそ学部生でも容易に行える）程度の実験にすぎない」という被告主張が誤りである理由を３点指摘したが、これに対しては今回提出の被告準備書面（１０）でも何の釈明も反論もない。従って、今回の被告主張によっても、「平八重氏と園田氏は川田氏に実験材料を渡し、実験方法・評価方法等を指導･伝授しただけ」という肝心の事実は依然証明されていない。要点をくり返すと、次の問題点が依然解明されていない。

①．カビの拡散・コンタミネーション（混入）防止

いもち病菌のようなカビにおいては、研究室内の大腸菌の培養液やプレートにカビが混入すると容易なことでは取り除けず、カビの拡散・コンタミネーション（混入）防止は重要課題である。そのためにはシャーレに塗りつけ

る際の道具の使い方や塗布の手順、あとしまつなどがことこまかに決められている。このような作業手順は通常、熟練の実験者が行うものであり、仮に初心者に行わせる場合でも熟練者がつきっきりで指導することが必要となる。

この点、本抗菌活性実験でカビの素人の川田氏はどうしたのか。

②. 実験結果（抗菌活性）の評価方法

菌糸を伸ばして生育し、胞子を作るカビに対する抗菌活性の評価方法としては、大腸菌のようにコロニーの数をかぞえたり、溶菌した部分の半径を測定したりする一般的な客観評価が使えないため、その評価は困難である。そのため、ここは熟練の実験者によりどのように正確に評価するのか手腕が問われる局面であって、学部生の到底なし得ることではない。

この点、本抗菌活性実験でカビの素人の川田氏はどうしたのか。

さらに、今回の準備書面（１０）の被告主張に対しても次の問題点がある。第１に、被告は《提供した再生菌をさらに継代培養することで生物活性が低下したり、遺伝変異が発生したりする》（５頁１２～１３行目）と主張する。まさしくその通りであり、その結果、再現性のよい検定試験ができなくなる。しかし、いもち病菌の生物活性が低下したり、遺伝変異が起こって正常な生育をしなくなった状況であるかどうかを判断することは、カビの取り扱いに慣れていない素人にとって容易なことではない。

この点、本抗菌活性実験でカビの素人の川田氏はどうしたのか。

尤も、被告は、この問題に対処するため、《毎年２～３回ほど、凍結乾燥保存株からいもち病菌を再生して、川田氏に提供した。川田氏にいもち病（菌）を提供する際の担当者は上記のとおり平八重氏・園田氏である》（５頁１３～１６行目）と主張している。しかし、実験に従事していないとされる平八重氏・園田氏は一体どのようにして、いもち病菌の状況を知り得たのだろうか。なぜなら、いもち病菌の状況も確認しないまま、単に機械的に４ヶ月や半年ごとにいもち病菌を提供するような杜撰なことを研究者は通常やらない

からである。実際に菌の様子を見て、「そろそろ新しい菌を起こして更新した方がよい」という判断がするのが研究者の通例である。しかし、この観察と判断はカビの素人の川田氏単独では不可能である。この点、川田氏はどうしたのか。

第２に、被告は、《川田氏に対して植物病理学の教科書や多数の論文の中から標準的な論文を選んで提示することで、主に病原菌の培養法に関する手法を伝えた》（５頁下から６～４行目）と主張する。しかし、上述の通り、カビの拡散・コンタミネーション（混入）防止などの取扱いは熟練の実験者にして初めてよくなし得ることであり、他方、そのような熟練技は通常、研究者の間の口伝や肩越しの指導によって伝えられるものであって、教科書や標準的な論文の類には記述されていない。もしカビのこうした取扱いまで丁寧に記載されている「教科書や標準的な論文」を川田氏に提供したというのであれば、それを実際に示すことができるはずであるが、被告準備書面（１０）では全く示されていない。

以上の通り、被告の主張する実験３に対する「平八重氏と園田氏の関与」は「限りなく実施に近い関与」ではないかという疑いが強まるばかりであり、その解明のためには彼らに直接尋ねるほかない。

２、甲１１の注２１の論文から平八重氏らの前任者が病害抵抗性検定実験を実施したことが示されたこと

前回期日において、裁判所より被告に対し、甲１１の論文の注２１の論文（組換え稲を栽培して、抗菌活性を調べる実験に関する論文）に、実験の情報がもう少し詳しく載っていないかと問い合わせがあったが、被告より何の回答がなかったので、代わりに原告より検討した結果を回答する。

本書面別紙の論文が注２１の論文である。その２番目の執筆者が平八重氏・園田氏の前任者の中島敏彦（甲３１の１頁に肩書きとして「病害研究室長」

と記載）氏である。中島氏は、本研究プロジェクト[1]の中で開発された「複数の病気に強い遺伝子組み換えイネ」について、２００１年９月特許出願した際の発明者の１人である（甲２７の１頁目の発明者の表示参照）。ところで、別紙１の論文４枚目の図３及び４はいもち病と白葉枯病に対する抵抗性検定実験の結果を示す写真であるが、これらの写真は甲２７の特許出願書類１１頁記載の図１及び２の写真と同一である。従って、これらのいもち病と白葉枯病に対する抵抗性検定実験は別紙論文の２番目の執筆者であり、甲２７の特許発明の発明者であり、植物病理学が専門の中島氏が実施したとしか考えられない。

しかも、被告は、本研究プロジェクトの２００５～２００６年の屋外実験では、平八重氏が病害抵抗性検定の実験を実施し、実験ノートを作成したことを自認している（甲４９補充理由説明書１頁・被告準備書面（４）８頁４）。

以上から、一方で、屋内実験のうち、平八重氏・園田氏が着任する２００３年４月[2]までは、前任者の中島氏が抗菌活性や病害抵抗性検定の実験を実施し、他方で、２００５～２００６年の屋外実験では平八重氏が病害抵抗性検定の実験を実施しているのに、なにゆえ、２００３年４月以降の屋内実験だけ平八重氏・園田氏はカビの素人の川田氏にこれらの実験を委ねたのか、その合理的な説明がつかない。

３、平八重氏・園田氏の証人尋問の意義

以上の検討により、従前にも増して「平八重氏と園田氏は川田氏に実験材料を渡し、実験方法・評価方法等を指導・伝授しただけ」という被告主張の疑わしさは深まった。従って、今回の被告準備書面（１０）により一層明確となった上記被告主張の疑問点・不明点の解明のために、平八重氏・園田氏の

1　被告が行った、ディフェンシン遺伝子を導入した遺伝子組み換え稲の開発及び栽培の研究プロジェクトのこと（原告準備書面(1)３頁３行目）。

2 今回の被告準備書面（１０）５頁２～３行目参照。

証人尋問は不可欠と考える。

ただし、両名の証人尋問にはもう１つ別な意義がある。それは、被告も指摘する通り、平八重氏・園田氏は本研究プロジェクト以外に「植物の耐病性と病原菌の病原性に関連する研究」[3]の実験を実施していたことである。それゆえ、原告は両名の証人尋問において、両名の２００３年の着任以後、本研究プロジェクト以外で実施した「植物の耐病性と病原菌の病原性に関連する研究」の実験で作成した実験ノートの作成・利用・保存状況について確認したい。その証言によって、これらの実験ノートの法人文書性の有無を導くことができる。もとより本訴ではこれらの実験ノートは審理の対象外であるが、しかし同一人物の同時期の同種の実験で作成した実験ノートの法人文書性の有無は、本研究プロジェクトで平八重氏・園田氏により作成された実験ノートの法人文書性を判断する上で、極めて有力な手がかりになるからである。

４、結語

以上の通り、被告準備書面(１０)によっても、平八重氏・園田氏が本研究プロジェクトの屋内実験において抗菌活性実験を実施していないという主張の問題点は何ひとつ解決しておらず、むしろ裁判所の釈明による甲１１の注２１の論文から、平八重氏・園田氏が抗菌活性実験を実施している可能性が一層高まった。そこで、第１に、同準備書面(１０)により明確なとなった疑問点、不明点の解明のために、第２に、平八重氏・園田氏の着任以後、本研究プロジェクト以外で両名が実施した同種の実験で作成した実験ノートの作成・利用・保存状況を確認するため、速やかに両名の証人尋問を実施すべきである。

以　上

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[3] 同書面同頁下から１０～９行目。

別紙

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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

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
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*

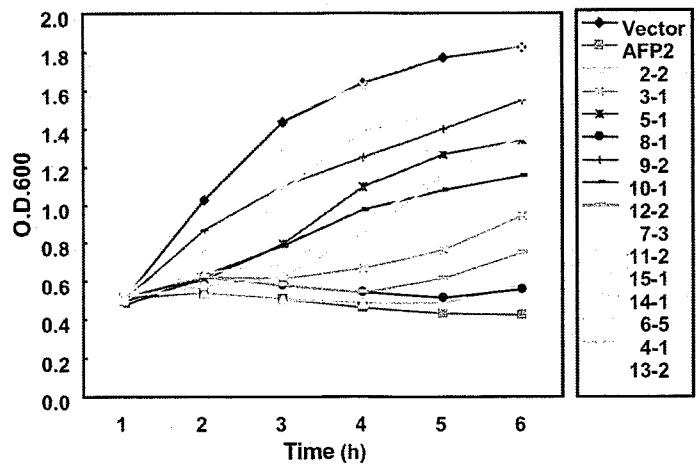

Fig. 2. Antimicrobial activity against *E. coli* of defensin peptides from *B. campestris* with single amino acid substitutions

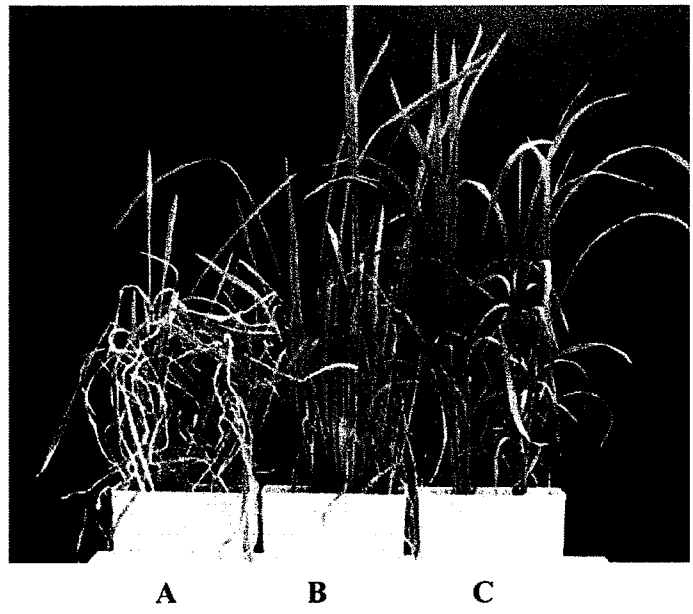

Fig. 3. Resistance to rice blast disease in transformant by introduction of defensin gene
A: Non-transformant. B: Transformant. C: Non-transformant, not infected.

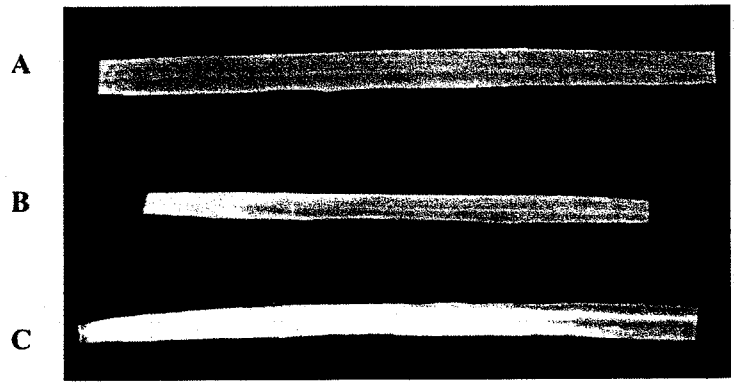

Fig. 4. Resistance to bacterial leaf blight in transformants by introduction of defensin gene
A: Transformant by introduction of modified defensin gene.
B: Transformant by introduction of native defensin gene. C: Non-transformant.

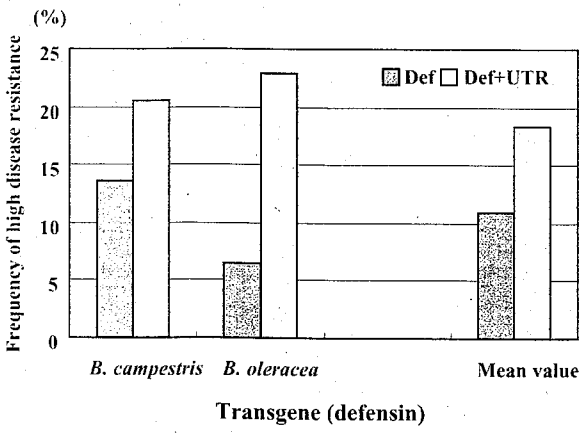

Fig. 5. Effect of addition of 3′ UTR region to introduced defensin gene

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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