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Review

Rabbit defensin (NP-1) genetic engineering of plant

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Defensin is a small, cysteine-rich, cationic peptides family with antimicrobial and cytotoxic properties. Among all known defensins, neutrophil peptide-1 (NP-1), which is expressed mainly in rabbit neutrophils, has a broad resistance spectrum to pathogens such as *Treponema pallidum*, many Gram-positive bacteria, Gram-negative bacteria, fungi, viruses. Besides, it has the same striking inhibition or toxic effect on some nausea and tumor cells. Due to the broad antibacterial spectrum and special mechanism of microbial inhibition, rabbit defensin has been transformed into some plants and expressed via genetic engineering. And it plays an important role in genetic engineering of anti-disease plants and plants species' improvement. This article reviewed and discussed the advantages and research progress of the rabbit defensin genetic engineering of plant in recent years, and also focuses on the existing problems and new strategies in this area.

Key words: Rabbit defensin (NP-1), structure, bioactivity, genetic engineering of plant.

INTRODUCTION

Research on disease resistance plant, especially in the field of disease resistance transgenic plants obtained, has attracted much attention and got some achievements in recent years. However, there are still some defects left. On one hand, the spectrum antimicrobial of many disease resistance genes is not broad enough, which could be used to design for just one kind of sickness and resist only one or other relative disease(s); on the other hand, it is not easy to identify the genes that resist some pathogenic bacteria. A kind of broad-spectrum antimicrobial peptides which involves in the essentially ancient natural defense activity has been isolated in a range of organisms including mammals, birds, invertebrates, plants and recently in the ebony-cup fungus. Regarding the fundamental roles in both innate and adaptive immunity, toxic mechanism of defensin is very special compared with traditional antibiotics. Microbicidal and cytotoxic properties of defensins are most likely a consequence of their ability to insert into biological membranes and to generate pores depending on electrostatic interaction, which causes the death of the target cell because of the imbalance of ion exchange inside or outside the cell. Right now, defensins that scientists have identified are more than 300 kinds. Among them is neutrophil peptide-1(NP-1) from rabbit neutrophils, which has the broadest antimicrobial. It has striking inhibition or toxic effect on the *Treponema pallidum*, many Gram-positive bacteria, Gram-negative bacteria, fungi, viruses; besides, it has the same effect on some nausea and tumor cells. Since the rabbit defensin embraces many bioactivities and makes target microbes hard to generate disease resistance mutations, it has a bright future in research of disease resistance plant application as a new antibiotic.

THE STRUCTURE AND BIOACTIVITY OF RABBIT NEUTROPHILE PEPTIDE 1

The structure of rabbit neutrophile peptide 1

Defensin typically consists of 28 to 54 amino acids, with relative molecular mass of 4000 to 6000, which is full of Arg and disulfide linkage among its 6 cysteines. Based on source, molecular structure and express sites, we separate defensins into 4 classes: Sect defensins, plant defensins, α-defensins and β-defensins (Han et al., 2004; Liu et al., 2005). There are a lot of cationic antimicrobial peptides with small molecular mass in the rabbit neutrophils, which are from the same gene family, grouped into α-defensin (Zeya and Spitznagel 1968, 1963). α-Defensin is thought to have the following structural characteristics:

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Table 1. Antimicrobial and cytotoxic spectrum of rabbit defensin (NP-1).

Bacteria		Fungi	Virus	Malignant cell	Helicoid
Gram positive	Gram negative				
Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	Herpes simplex virus	Mouse tumor cells	*Treponea Pallidun*
Staphylococcus	*Salmonella typhimurium*	*Candida spp*	Vesicular stomatitis virus		
Streptococcus agalactiae	*Serratia marcescens*	*Cryptococcus neoformans*	Influenza virus (A / WSN)		
Streptococcus pneumoniae	*Haemophilus influenzae*	*Coccidioides immitis*	HIV		
Streptococcus faecalis	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*			
Bacillus subtilis	*Actinobacills actinomycetemcomitans*	*Rhizoctonia solani*			
Helicobacter pylori	*Neisseria gonorrhoeae*	*verticillium wilt*			
		Fusarium spp.			

β-flake structure linked by 3 disulfide bond with lack of α-helix domain; the linking way of 3 disulfide bond is Cys1-Cys6, Cys2 - Cys4, Cys3 - Cys5, in which Cys-1 links N-terminal while Cys-6 links C-terminal Cys and forms large molecular ring. In all of the α-defensins, rabbit NP-1 has the broadest spectrum antimicrobe and displays strong antibacterial activity (Ganz and Sflsted, 1990; Ganz and Lehrer, 1994; Han et al., 2005). Neutrophil peptide-1(NP-1) consists of 33 amino acids and 3 intramolecular disulfide bonds, and the primary structure is: VVCACRR-ALCLPRERRAGFCRIRGRIHPLCRCE. Its senior structure contains 3 pairs of intramolecular disulfide bonds. There are 6 similar Cys, 10 Arg that account for one third in 33 amino acids residues of mature peptide, while there is only one negative charge amino acids-glu. The function of Cys is to form disulfide bond and make the mature peptide folds correctly in space so that it could play a normal biological role; Arg could take the whole defensin with positive charge to attach to the surface of microorganisms (Liu et al. 2000).

Biological activity

Being one of the antibacterial peptide, rabbit defensin (NP-1) is a short peptide, full of Arg with small molercular mass and positive charge, which is mainly expressed in rabbit neutrophils. Antibacterial test *in vitro* demonstrated that rabbit defensins (NP-1) has a strong killing effect on these pathogenic micro-organisms such as Gram-positive bacteria, Gram-negative bacteria, fungi, viruses, even mycoplasma, chlamydia, spirochetes, and some malignant cells (such as tumor cells and HIV) in micromoler concentrations. The MIC of rabbit NP-1 for Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomo aeruginosa*) is 1.5 to 2.6 μg/ml, and its MIC for Gram-positive bacteria (Listeria monocytogene) is 2.1 μg/ml, while its MIC for *Candida albicans* is 5.4 μg/ml, suggesting that its antibacterial spectrum is broad (Periathamby et al., 2000). In addition, experiment *in vitro* has indicated that the antibacterial activity of NP-1 is 5 to 10 fold stronger than HNP-1, and the obvious difference should be attributed to their molecular net charge: there are 9 net positive charges in the molecule of NP-1, while there are only 3 in the molecule of HNP-1 (Hoffmann and Hertru 1992). At the same time, NP-1 also has strong antifungal activity. From the experiment we can observe that rabbit defensin with very low concentration could eliminate fungal *C. albicans* in only several minutes (Ganz and Lehrer, 1995). Many defensins, such as rabbit defensins (NP1-2) have been identified to have a killing effect on virus. Their inhibition to virus is direct and relative. The extent of their inhibition to virus depends on defensin concentration, and tightness of intramolecular disulfide bonds; besides, the antivirus effect can also be influenced by many factors such as time, PH, temperature (Li et al., 2001). According to experiment data, rabbit defensin has a significant toxic effect on mouse tumor cells, especially on helicobacter pylori and HIV (Li et al., 2001, 2005) (Table 1). Rabbit defensin NP-1 could not only resist pathogenic microorganism but also have immuno-regulation effect. Befus et al. (1999) isolated defensin from rabbit neutrophils and discovered that it can induce mast cells to degranulate and release histamine.

THE STUDY OF TRANSFERRING RABBIT DEFENSIN TO PLANTS

The study of transferring rabbit defensin (NP-1) to lower plants

The unicellular eukaryotic green alga chlorella may be of

great potential interest in biotechnology as a good candidate bioreactor. For example, it can be photo-autotrophy and be cultured easily, rapidly and inexpensively. In addition, chlorella is rich in nutrients without endotoxin. It can be administered orally, which renders the purification of recombinant protein relatively easy. Therefore, chlorella is used as a bioreactor for the production of rabbit defensin protein, which has a good application prospects by means of genetic engineering. Wang et al. (2001) transferred the gene encoding mature rabbit neutrophil peptide-1 (NP-1) into chlorella cell, and confirmed that it had integrated into chlorellla genome by molecular studies and *in vitro* anti-microbial tests. This strongly demonstrated the expression of NP-1, biologically active in transgenic chlorella cells. This study was also firstly reported by Wang (Wang et al., 2001) who produced rabbit defensin for large-scale by means of genetic engineering, which has laid the foundation for industrial produce for new antibiotics- rabbit defensin. Based on the experiment of obtaining chlorella for stable expression of NP-1, scientists have optimized a heterotrophic medium for transgenic chlorella, by using the optimal medium; cell density of the transgenic chlorella in flasks and 5 L bioreactor are 3.39 and 3.17 fold, respectively. That is what can be obtained in the mixotrophic Knop medium while the expression capabilities of NP-1 remain unchanged (Han et al., 2006). Thus, the study offered a major breakthrough to high yield and stable production of rabbit defensin by transgenic chlorella.

The study of transferring rabbit defensins (NP-1) to higher plants

Monocotyledon

The effects of several commonly used promoters and enhancers on GUS transient expression in maize Ubil promoter were compared, and the most efficient promoter was selected to construct a vector carrying the rabbit defensin (NP-1) gene; and then transferred into immature embryos of wheat via particle bombardment. The integration of gene into wheat genome was confirmed by PCR and Southern blot (Guo et al., 1999). The pollen-tube pathway was used by Zhou et al. (2007) to hybridize rabbit defensin (NP-1) gene into wheat. The integrated NP-1 gene has been detected in the genome of these transgenic plants by PCR and PCR-Southern, and it was a stable genetic to T1 generation. The results of disease resistance and insect resistance evaluation showed that these plants had got higher resistance to powdery mildew, leaf rust and stripe rust, but the resistance to aphid had not been improved, which revealed that NP-1gene had been expressed in wheat genome. Thus, the ability of rabbit defensin antimicrobial has been fully confirmed. Nevertheless, insect-resistant capacity needs further study.

Scientists have transferred the NP-1gene into Lily scale by agrobacterium-mediated transformation and verified that rabbit defensin NP-1gene had been integrated in lily by antibiotic selection and PCR analysis (Li et al., 1999). Rabbit defensin gene (NP-1) was first introduced into embryonic callus cultures of maize hybrid lines by particle bombardment and the regenerated transgenic plants were obtained; PCR and Southern blotting analyses confirmed that NP-1 gene was integrated into the maize plants genome; in addition, disease resistance to northern corn leaf blight was greatly improved in transgenic plants (Zhang et al., 2003).

Dicotyledon

Defensin gene (NP-1) has been transferred into tobacco, the model plant, firstly in the study. Gene integration was confirmed by Southern hybridization and Northern hybridization. *In vitro* anti-microbial tests demonstrated that the transgenic plants enhanced resistance to the bacterial wilt pathogen in tobacco (Fu et al., 1998). By application of DNA recombination method, a vector (pBin35SGAFP-NP1) containing two anti-disease genes (NP-1 and GAFP) was constructed and transferred into tobacco using leaf-disk method mediated by *Agrobacterium tumefaciens*. The results of PCR and PCR-Southern determination indicated that NP-1 and GAFP genes were both integrated into tobacco genome. *In vitro* testing of transgenic plants to virus, bacterium and fungal showed that they had the resistance to the growth of *A. tumefaciens*, *Trichoderma reesei* and *Sphaeropsis sapinea*, which appeared that NP-1and GAFP genes were expressed efficiently in transgenic tobacco and these transgenic plants had disease-resistant ability to pathogens (Chen et al., 2005).

NP-1gene that was stably integrated in part of the recovered chrysanthemum genome was confirmed by Southern blot analysis, which established and paved the way for pathogen resistance breeding for the flower via genetic engineering (Fu et al., 1998). Transformation of multi-genes related to disease resistance into plants is an efficient measure for both improving disease resistance and extending disease resistant spectrum. GAFP and NP-1 genes were constructed into one vecter (pBin35SGAFP-NP1) and transferred into *Dianthus caryophyllus* via agrobacterium-mediated method; *in vitro* antibiosis assay of the transgenic plants extraction of *Trichoderma* suggested that these transgenic plants have the ability of disease-resistance and the two genes can express efficiently in transgenic carnation (He et al., 2007).

Currently, only a few disease-resistance genetic engineering about poplar have been reported. NP-1 genes have been transferred into poplar plants through agrobacterium-mediated transformation and integrated into the poplar genome detected by PCR amplication and

Southern analysis. Antimicrobial activity test showed that the extract of transgenic plants inhibited the growth of *Bacillus subtilis* and agrobacterium LB4404 (Zhao et al., 1999). The bivalent resistance gene (NP-1 and GAFP) was integrated into poplar genome, and resistance tests showed that the transgenic poplars inhibited the activity of bacteria *E. coli* and fungus *Trichoderma reesei* (Chen et al. 2002).

Disease is one of the important factors which lead to decrease of agricultural production. Spraying of chemical and biological pesticides is a main preventive method, but the disadvantages of using these two pesticides are obvious. For instance, the former may cause environmental pollution while the latter costs too much. So, enhancing disease resistance of plants by genetic engineering is an effective way. In the study on improving antiviral ability of tomato, NP-l gene was constructed into a plant expression vector and transgenic plants containing this gene were obtained through agrobacterium-mediated transformation. The transgenic plants were analyzed by PCR, Southern hybridization, Northern dot blot hybridization and *in vitro* microbicidical. The results showed that NP-1gene was transformed into tomato because these transgenic tomatoes showed resistance to pathogen *Fusarium oxysporum in vitro* (Zhang et al., 2000). The work that transferred rabbit defensin(NP-1) into cotton began in 2000, and the transgenic plants have been obtained through pollen tube, which has been identified as resistant to Verticillium wilt in cotton (Hao et al. 2000).

Recently, the microbial disease has become an important factor in yield and quality on XinJiang cantaloup. In order to improve the capacity of its anti-pathogen hazards, cotyledons of XinJiang cantaloupes were transformed with *A. tumefaciens* strains (LBA4404), harboring expression vector pBIC-35S NP-1. The transgenic plant which was selected by Kanamycin was verified by PCR and RT-PCR analysis, and at the same time, the total protein was extracted to carry out the bacterium restraining experiment *in vitro* (Zhang, 2004). In our country, the improvement of the yield and quality of flax was hampered by diseases. Based on *A. tumefaciens* mediated, scientists have built genetic transformation system and successfully gained transgenic flax plants, identified by PCR and PCR-Southern blotting, too. In addition, scientists inoculate pathogen with flax plants through draw line and spray methods to measure the ability of resistance disease. The conclusion is that the genetic flax improves the ability of resistance to Fusarium wilt (Yuan, 2005). Rapeseed is an important oilseed plant in china. However, this crop has been suffering from *Sclerotinia sclerotiorum*. Introducing resistant genes into rapeseed varieties by transformation technology is one of the most important ways to resolve rapeseed disease. They begin with establishing highly effective regeneration systems, then introducing rabbit defensin NP-1 gene into rapeseed via Agrobacterium, and ultimately obtaining the Kan-resistant plantlets (Li, 2007).

According to statistics, there are a lot of transferred rabbit defensin plants, example tomato, lily, wheat, poplar, cotton, cantaloup, corn, carnation, peanuts, flax, *Dianthus caryophyllus* and other plants (Table 2).

THE EXTANT PROBLEMS AND PREDICTION

As antibiotics are extensively used, the resistance rate of bacterium has an incremental tendency coupled with a common occurrence of diverse diseases resulting from drug-fast bacteria. These diseases have high mortality, especially in children, the elderly and immunosuppressed patients. The common drug-fast bacterium consists of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *K. pneumoniae*, *E. coli* and so on, all of which greatly inhibit the choice of extant antibiotics. In the light of the special role of rabbit defensin mechanism making the target microorganism difficult to produce resistance and having a broad highly efficient spectrum bactericidal, defensin has become a hot point of anti-infection research (a variety of infections caused by bacterium).

In the research of rabbit defensin transgenic plant, some of them have been detected existing defensin, some of which have been able to express biology active defensin protein. There are still many problems deeply in the defensin gene engineering. Firstly, natural rabbit defensin has a very broad anti-infection spectrum while the defensin produced by transgenic plants usually has a poor activity in anti-micor. How to enhance the stability and biologic activity, compound stronger bactericidal activities, broader spectrum bactericidal of defensin in the plant are still requiring deep research. Secondly, the main purpose of the current defensin transgenic plant engineering research is to cultivate new type of pest and disease resistance plants. Based on the biologic function, we expected more to clone defensin gene which human body needs or transfers into edible plant species such as vegetables and fruit trees, so that we can uptake defensin by direct consumption to prevent and treat disease instead of extraction and purification. Thirdly, how can we improve transformation efficiency and expression in transgenic plants? Once a highly efficiency transformation system is established and the transformation efficiency is improved and gene silencing problem is solved, defensin would be expected to be expressed in a large scale by the means of genetic engineering. We can not only find new types of antibacterial, antivirus even antitumor drugs, but also can we apply it in food preservation to ensure the security of food? With further research, rabbit defensin will greatly benefit mankind in the near future.

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Table 2. Rabbit defensin (NP-1) genetic engineering of plant.

Host	Method	Explant	Detection	Bioactivity	Selection	Plasmid vector	Reference
Tobacco	A.T.	Leaf	Southern, Northern, Resistance tests	Anti-bacterial wilt	Km	pBIC-35SNP-1	Fu et al., 1998
	Leaf-disk method	Leaf	PCR	Not detected	Km	pX6-GFP-NP1	Wang, 2006
	Leaf-disk method	Leaf	PCR, PCR-Southern, Resistance tests	*Trichoderma Reesei*, *Sphaeropsis sapinea*	Km	pBin-35SGAFP-NP1	Chen et al., 2005
Poplar	A.T.	Leaf	-	Not detected	Km	pX6-GFP-NP1	Wang, 2006
	A.T.	Leaf	PCR, PCR-Southen	*E. coli*, *Trichoderma reesei*	Km	pBin-35SGAFP-NP1	Chen et al., 2002
Tomato	A.T.	Leaf	PCR, Southern, Northern, Resistance tests *in vitro*	*Fusarium oxysporum*	Km	pBin-35S NP-1	Zhang et al., 2000
Poplar (*Prunus tomentosa*)	A.T.	Leaf	PCR, Southern, Resistance tests *in vitro*	*Bacillus subtilis*, *Agrobacterium* LB4404	Km	pBIC-35S NP-1	Zhao et al., 1999
Chlorella	Electroporation	Algal	PCR, Southern, Northern, Resistance tests *in vitro*	*Bacillus subtilis*, *E. coli*, *Fusarium* spp.	G418	PbinU NP-1	Wang et al., 2001
	Protoplast transformation method	Protoplast	PCR, RT-PCR	*E. coli*	G418 NO_3-	Pbin-NR-U-NP1	An et al., 2008
Wheat	Pb	Embryo	PCR, Southern	Not detected	Bast	pUNP1-BAR	Guo et al., 1999
	Pollen-tube Pathway	Pollen-tube	PCR, PCR-Southern, Resistance tests *in vitro*	Powdery mildew, leaf rust, stripe rust	Bast	pBIC-35S NP-1	Zhou et al., 2007
	The pollen-tube pathway	Pollen-tube	PCR	Not detected	Basta	pBI35S-gafp-NP1-bar	Huang et al., 2004

Table 2. Continue.

Chrysanthemum	A.T.	Leaf stem	Southern	Not detected	Km	pBIC-35SNP-1	Fu et al., 1998
Corn	Pb	Embryogenic callus	PCR, Southern	*Helminthosporium turcicum*	Amp	pAct1NP-1hygr	Zhang et al., 2003
Xinjing cotton	The Pollen-tube Pathway	Leaf	Antibiotic selection	Not detected	Km	PBI35SNP-1	Zhang et al., 2000
Colored cotton	A.T.	Hypocotyl Cotyledon	Antibiotic selection	Not detected	Km	pBIC-35SNP	Zhang et al., 2004
Lily	A.T.	Scale	PCR	Not detected	Km	pBIC-35S NP-1 PbiNP1	Li et al., 1999
Flax	A.T.	Hypocotyl	PCR, Southern, Resistance tests *in vitro*	Fusarium Wllt	Km	pBIC-35S NP-1	Yuan, 2005
XinJiang Cantaloupe	A.T.	Leaf	PCR, Resistance tests *in vitro*	*Bacillus subtilis, Staphylococcus aureus*, *E. coli*	Km	pBIC-35S NP-1	Zhang, 2005
Rapeseed	A.T.	Petiole	Antibiotic selection	Not detected	Km	pBin35S NP-1	Li, 2007
Dianthus caryophyllus	A.T.	Leaf	PCR, Resistance tests *in vitro*	Trichoderma	Km	pBin35SGAFP-NP1	He et al., 2007
Peanut	A.T.	Hypocotyl	PCR	Not detected	Hyg	PSC1300-NP1	Cheng, 2008

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