Strategies for developing bacterial disease resistant plants

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INTRODUCTION

Bacterial diseases are of high economic importance in many crop plant species including different vegetable species, fruit trees. Conventional breeding efforts have not resulted in the generation of varieties resistant to Bacterial diseases due to the lack of known resistance traits. Resistance traits known to be present in wild species often cannot be used for classical breeding programmes as these are genetically too distant from today's cultivars. So, no valuable resistance or tolerance traits are known. Furthermore, chemical plant protection is not as evolved as for other pathogens. Actually, the most prominent means of prevention of bacterial infections are phytosanitary practices and highly developed harvesting techniques minimizing wounding as well as storage conditions reducing temperature and humidity.

More recently, transgenic plants have been produced that are resistant to a wide variety of bacterial diseases. These forms of resistance follow a number of chemical strategies, including the use of genes for bacterial toxin tolerance, antimicrobial peptides, and other defense related proteins that tend to act as bactericidal compounds. This paper reviews the various potential strategies for developing bacterial disease resistant plants through genetic engineering. Rice diseases are among the most significant limiting factors threatening food productivity. Genetic engineering provides promising strategies to develop and improve disease resistance in rice (Huijuan Zhang et al., 2009).

One approach to control bacterial disease is to improve a plants' defense against a particular pathogen. This has been made possible by genetic engineering by using genes found in fungi, insects, animals and other plants. Antimicrobial proteins, peptides, and lysozymes that naturally occur in insects (Jaynes et al., 1987), plants (Broekaert et al., 1997), animals (Vunnam et al., 1997), and humans (Mitra and Zhang, 1994; Nakajima et al., 1997) are now a potential source of plant resistance. Antimicrobial peptides exert an important role in plant defence and their structure/activity relationship against pathogens is widely described. Although the most striking feature of these antimicrobial peptides is their molecular diversity, they share some common features, such as a relatively low molecular weight, and the presence of a variable number of cysteines residues that contribute to stabilize conserved scaffolds through disulphide bond formation, and can be assigned to different structural classes. Peptides from different classes in some cases act synergistically against pathogens when produced by the same tissue, and contribute to extending defence to a wider range of microbes. In this
review we briefly describe the structure of some of the main plant antimicrobial peptide classes: thionins, defensins, lipid transfer proteins, cyclotides and snakins, and how they are reported to contribute to the plant protection. In many cases these antimicrobial peptides show a wider activity spectrum than that suggested by their name, exerting an action also against predatory insects and revealing useful antiviral activities (Padovan et al., 2010).

**Expression of antimicrobial proteins:**

Antimicrobial peptides (AMPs) with \(-\)helical structures are ubiquitous and found in many organisms. AMPs have been isolated from frogs, insects, and mammalian phagocytic vacuoles (Biggins and Sansom, 1999; Tossi et al., 2000). AMPs are selective for prokaryotic membranes over eukaryotic membrane due to the predominantly negatively charged phospholipids in the outer leaflet of the prokaryotic membrane (Biggin and Sansom, 1999; Tossi et al., 2000). Such preference is considered a regulatory function in target selectivity.

Antibacterial peptides have a broad range of antibacterial properties that makes them highly toxic for expression in *Escherichia coli*. For prepare an antiserum to detect these peptides, we developed a cecropin B mutant with a green fluorescent protein fusion partner resulting in high expression of a 37 kDa fusion peptide in E. coli with a yield of 7.9 mg/l culture medium after purification on Ni-IDA resin. Guinea pigs when immunized with the fusion peptide produced a specific antiserum which titers in excess of 1:25,600 (Fuxian Yu et al., 2011)

**Magainins:**

Magainin is a defense peptide secreted from the skin of the African clawed frog (*Xenopus laevis*), first discovered by Zasloff (1987). Magainins and their analogs have been studied as a broad-spectrum topical agent, a systemic antibiotic, a wound-healing stimulant, and an anticancer agent (Jacob and Zasloff, 1994). However, only magainin analogs (MSI-99 and Myp30) have recently been transferred into plants against bacteria. Li et al. (2001) have reported disease resistance, to both a fungal and a bacterial pathogen, conferred by expression of a magainin analog, Myp30, in transgenic tobacco (*Nicotiana tabacum* var. Petit Havana). Another analog MSI-99, when expressed in tobacco via chloroplast transformation conferred both *in vitro* and in planta resistance to plant pathogenic bacteria and fungi (De Gray et al., 2001).

**Cecropins:**

Cecropins are antibacterial lytic peptides native to the hemolymph of *Hyalophora cecropia*, the giant silk moth. These peptides interact with the outer phosphor lipid membranes of both Gram-negative and Gram-positive bacteria and modify them by forming a large number of transient ion channels (Durell et al., 1992). Native (Cecropin B), mutant (SB37, MB39) and synthetic (Shiva-1, D4E1) cecropins are active *in vitro* against a wide range of plant pathogenic bacteria including *Erwinia carotovora*, *E. amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas campestris* whereas they exert no toxicity at bactericidal concentration to cultured cells or protoplasts of several plant species (Kaduno-Okuda et al., 1995; Nordeen et al., 1992; Rajasekaran et al., 2001). Therefore, cecropins are considered
as potential candidates to protect plants against bacterial pathogens. Transgenic tobacco plants expressing cecropins have increased resistance to \textit{P. syringae pv. tabaci}, the cause of tobacco wildfire (Huang \textit{et al.}, 1997). Synthetic lytic peptide analogs, Shiva-1 and SB-37, produced from trans genes in potato plants reduce bacterial infection caused by \textit{Erwinia carotovora} subsp. \textit{atroseptica} in transgenic potato plants (Arce \textit{et al.}, 1999). Transgenic apple expressing the SB-37 lytic peptide analog showed increased resistance to \textit{E. amylovora}, pathogen for fire blight, in field tests (Norelli \textit{et al.}, 1998). Recently, the expression of the D4E1 in poplar has resulted resistance to \textit{Agrobacterium tumefaciens} and \textit{Xanthomonas populi} (Mentag \textit{et al.}, 2003). The cationic lytic peptide cecropin B (CB), isolated from the giant silk moth (\textit{Hyalophora cecropia}), has been shown to effectively eliminate Gram-negative and some Gram-positive bacteria (Pey-Shyan Jan \textit{et al.}, 2010).

\textbf{Attacins:}

Attacins are another group of antibacterial proteins produced by \textit{Hyalophora cecropia} pupae (Hultmark \textit{et al.}, 1983). Genetic transformation with genes that code for antimicrobial peptides has been an important strategy used to control bacterial diseases in fruit crops, including apples, pears, and citrus. Asian citrus canker (ACC) caused by \textit{Xanthomonas citri} subsp. \textit{citri} Schaad \textit{et al.} (Xcc) is a very destructive disease, which affects the citrus industry in most citrus-producing areas of the world. Here, we report the production of genetically transformed Natal, Pera, and Valencia sweet orange cultivars (\textit{Citrus sinensis} L. Osbeck) with the insect-derived attacin A (attA) gene (Suane \textit{et al.}, 2009). The mechanisms of antibacterial activity of this protein are to inhibit the synthesis of the outer membrane protein in gram negative bacteria (Carlsson \textit{et al.}, 1998). Attacin expressed in transgenic potato enhanced its resistance to bacterial infection by \textit{E. carotovora} subsp. \textit{atroseptica} (Arce \textit{et al.}, 1999). Transgenic pear and apple expressing attacin genes have significantly enhanced resistance to \textit{E. amylovora} in \textit{in vitro} and greenhouse (Norelli \textit{et al.}, 1994; Reynoird \textit{et al.}, 1999; Ko \textit{et al.}, 2000). In field tests, reduction of fire blight disease has been observed in transgenic apples expressing attacin genes (Norelli \textit{et al.}, 1999). Transgenic apple expressing attacin targeted to the intercellular space, where \textit{E. amylovora} multiplies before infection, has significantly reduced fire blight, even in apple plants with low attacin production levels (Ko \textit{et al.}, 2000).

\textbf{Lysozymes:}

Lysozymes are a ubiquitous family of enzymes that occur in many tissues and secretions of humans, animals, as well as in plants, bacteria and phage. The lysozyme attacks the murein layer of bacterial peptidoglycan resulting in cell wall weakening and eventually leading to lysis of both Gramnegative and Gram-positive bacteria. Hen egg-white lysozyme (HEWL), T4 lysozyme (T4L), T7 lysozyme (Huang \textit{et al.}, 1994), human and bovine lysozyme genes have been cloned and transferred to enhance plant bacterial or fungal resistance. The lysozyme genes have been used to confer resistance against plant pathogenic bacteria in transgenic tobacco plants (Trudel \textit{et al.}, 1995). T4L, from the T4-bacteriophage, also has been reported to enhance resistance of transgenic potato against \textit{E. carotovora}, which causes bacterial soft rot (Düring \textit{et al.}, 1993).
Transgenic apple plants with the T4L gene showed significant resistance to fire blight infection (Ko, 1999). Human lysozyme transgenes have conferred disease resistance in tobacco through inhibition of fungal and bacterial growth, suggesting the possible use of the human lysozyme gene for controlling plant disease (Nakajima et al., 1997). There is evidence of efficacy of bovine lysozyme isozyme c2 (BVLZ) transgene against a variety of Xanthomonas campestris strains in both monocotyledon and dicotyledon crops including tomato, tobacco, rice and potato (Mirkov and Fitzmaurice, 1995). Since this bactericidal transgene has been shown to function in monocot and has clear efficacy against at least several strains of X. campestris, its usefulness as a transgene for resistance to X. campestris in Musa has a high probability of success. Lysozymes play a key role in the innate immune system of vertebrates and invertebrates by hydrolyzing peptidoglycan, a vital component of the bacterial cell wall. Gram-negative bacteria produce various types of lysozyme inhibitors that allow them to survive the bactericidal action of lysozyme when their outer membrane is permeabilized. So far, three lysozyme inhibitor families have been described (Leysen et al., 2011).

**Thionins:**

Thionins are plant antimicrobial proteins which are able to inhibit a broad range of pathogenic bacteria in vitro (Molina et al., 1993). Carmona et al. (1993) reported the expression of alpha-thionin gene from barley in transgenic tobacco confers enhanced resistance to two pathovars of P. syringae. Unfortunately, most thionins can be toxic to animal and plant cells and thus may not be ideal for developing transgenic plants (Reimann-Philipp et al., 1989).

**Expression of plant defense genes:**

Plants have their own networks of defense against plant pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack. Recombinant DNA technology allows the enhancement of inherent plant responses against a pathogen by either using single dominant resistance genes not normally present in the susceptible plant (Keen, 1999) or by choosing plant genes that intensify or trigger the expressions of existing defense mechanisms (Bent and Yu, 1999; Rommens and Kishore, 2000). The inheritance of resistance against bacterial leaf blight diseases has been extensively studied (Song and Goodman, 2001). Major genes or loci conditioning resistance against this disease have been identified and some of these resistance genes or loci have been widely used in breeding programs (Song and Goodman, 2001). During the last decade, many disease resistance (R) genes, e.g. Xa1, Xa3/Xa26, xa5, Xa21 and Xa27 for leaf blight resistance, have been characterized at molecular and genetic level (Song et al., 1995; Yoshimura et al., 1998, Sun et al., 2004; Xiang et al., 2006; Gu et al., 2005; Iyer and McCouch, 2004;). These cloned R genes are very useful novel resources for improving blast and leaf blight resistance by means of genetic engineering. Most of the cloned R genes confer high level of race-specific resistance in a gene-for-gene manner and thus the resistance is effective against one or a few related races or strains of the pathogens. However, transgenic rice plants overexpressing Xa21 showed resistance to 29 isolates of X. oryzae pv. Orzyae from eight countries (Wang et al., 1996).
Pathosystem-specific plant resistance (R) genes have been cloned from several plant species (Bent, 1996). These include R genes that mediate resistance to bacterial, fungal, viral, and nematode pathogens. Many of these R gene products share structural motifs, which indicate that disease resistance to diverse pathogens may operate through similar pathways. The Bs2 resistance gene of pepper specifically recognizes and confers resistance to strains of X. campestris pv. vesicatoria that contain the corresponding bacterial avirulence gene, avrBs2 (Tai et al., 1999). Transgenic tomato plants expressing the pepper Bs2 gene suppress the growth of Xcv. The Bs2 gene is a member of the nucleotide binding site–leucine-rich repeat (NBS-LRR) class of R genes. The Xa1 gene in rice confers resistance to Japanese race 1 of X. oryzae pv. oryzae, the causal pathogen of bacterial blight (Yoshimura et al., 1998). Xa1 is a member of the NBS-LRR class of plant disease resistance genes. The rice Xa21 gene confers resistance to X. oryzae pv. oryzae race 6 (Song et al., 1995). Fifty transgenic rice plants carrying the cloned Xa21 gene display high levels of resistance to the pathogen. The sequence of the predicted protein carries both a leucine-rich repeat motif and a serine/threonine kinase-like domain. The Pto gene is another class of R genes, encoding a serine/threonine protein kinase that confers resistance in tomato to P. syringae pv. tomato strains that express the type III effector protein AvrPto. (Martin et al., 1993; Kim et al., 2002). Overexpression of Pto in tomato under control of the cauliflower mosaic virus (CaMV) 35S promoter has been shown to activate defense responses in the absence of pathogen inoculation. Pto-overexpressing plants show resistance not only to P. syringae pv. tomato but also to X. campestris pv. vesicatoria and to the fungal pathogen Cladosporium fulvum (Mysore et al. 2003). Therefore, Pto genes are considered as potential candidates to protect plants against pathogens. Effectiveness of the bacterial blight resistance genes Xa4, xa5, and Xa7, all in a common genetic background, was evaluated at different developmental stages by measuring lesion length and bacterial numbers after inoculation with the bacterial pathogen, Xanthomonas oryzae pv. Oryzae (Kimberly et al., 2008).

**Expression of monoclonal antibodies and derived fragments specific for pathogenicity factors**

The expression of monoclonal antibodies in transgenic plants as a new tool for achieving resistance has already been proved successful in antiviral strategies (Tavladoraki et al 1993, Weber et al., 1994). Disease development after viral infection could be reduced by expression of suitable inhibiting antibodies. We are pursuing this approach to develop a specific resistance to Erwinia spp. in transgenic potato. The potential of generating highly specific or cross-reacting monoclonal antibodies allows designing different types of strategies in terms of specificity. A detailed knowledge about the molecular interactions and the role of pathogenicity factors is a prerequisite for the design of a promising strategy. On the other hand, as mentioned above, bacterial pathogens share some common features which might be exploited here. Availability of efficiently inhibiting antibodies will be essential for the success of this approach. We have chosen to generate inhibiting antibodies directed against the secreted pectolytic enzymes of E. c. and Erwinia chrysanthemi. Monoclonal antibodies or derived fragments (Fab fragments, single-chain antibodies) can be used in this context. Full-size antibodies as
well as singlechain antibodies have been shown to be secreted to the intercellular spaces of transgenic plants (Diiring K, 1994, Ma JKC et al., 1995, Van Engelen et al., 1995) where bacterial pathogenicity factors such as pectolytic enzymes are present. Production of monoclonal antibodies using the classical hybridoma technology is highly laborious and requires excellent technical skills. The phage display methodology developed in recent years (Griffiths et al., 1994, Nissim et al., 1994, Vaughan et al., 1997) can most probably soon be used in most molecular biology laboratories. This provides the technical means for selecting antibodies without the need of a monoclonal antibody laboratory and will be necessary for a potential application of the plantibody strategy for application purposes.

CONCLUSIONS

The range of potential strategies for genetically engineered resistance in crops has expanded dramatically during the past few years. The general antibacterial approaches, such as expression of lysozyme, cecropin or glucose oxidase, will easily be applicable to other economically important species. Other approaches, such as the use of antibody-based specific inhibitors of pathogenicity factors, can be designed either to be specific for one bacterial strain or to inhibit several species producing highly related pathogenicity factors. Given the diversity of strategies that pathogens use and their ability to rapidly adapt, it would be rash to predict the development of a magic bullet for durable, broad-spectrum resistance. However, it is reasonable to expect a forthcoming array of sophisticated weapons that will provide effective protection in certain contexts, when judiciously integrated with other control measures.

REFERENCES


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