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Review

Engineered pathogenesis related and antimicrobial proteins weaponry against *Phytopthora infestans* in potato plant: A review

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***Phytopthora infestans* (Mont.) de Bary, causal organism of late blight disease is referred to as the most destructive specific pathogen of potato (*Solanum tuberosum* L.). Casualties usually go beyond mere plant destruction, due to its flaring ability to also demolish scientific concerted efforts in establishing novel combat techniques. With high capacity to overcome control measures, it stands at par, and can simply be referred to as a potato-scientist tormentor. This retrospective work examines the role of pathogenesis related proteins and antimicrobial proteins in transgenic potato vis-à-vis *P. infestans* and prospect for associative introduction and overexpression of synergistically resistance conferring genes as a critical step for developing viable transgenic potatoes. The exploitation of enhanced pathogen-inducible promotor overexpressing key pathogenesis related proteins (PR) exemplified by PR-5 (especially osmotin and thaumatin-like (TL) proteins), PR-12 (defensins such as alfAFP (alfalfa antifungal peptide), *Nicotiania megalosiphon* defensins (NmDef02)), PR-13 (the thionins); and antimicrobial encoders such as StEREBP1 (*Solanum tuberosum* ethylene responsive element binding proteins), HEWL (hen egg white lysozyme), CAP (cationic antimicrobial peptide), Barnase cytotoxic protein and oxidative burst through glucose oxidase (GO) have all been incorporated in transgenic potato with variable successes. Apparently, engineering potato with an array of 'transgenes construct' of selected pathogenesis related proteins and antimicrobial proteins may provide a chance to terminate both the dissemination of *P. infestans* and consequent emergence of new strains, terminating the concept of a new agrochemical for *P. infestans* referred to as 'oomicides'.**

Key words: Pathogenesis related proteins, osmotin, thaumatin-like (TL) protein, alfalfa antifungal peptide (alfAFP), *Nicotiania megalosiphon* defensins (NmDef02), cationic antimicrobial peptide (CAP), hen egg white lysozyme (HEWL), glucose oxidase (GO).

INTRODUCTION

Natural selection ensured the survival and multiplication of the most viable species. Modernization in genetic engineering followed by classical and modern biotechnology paved the way for selectable artificial evolution, with direct benefits. Today, plants with genomes acquiring single or multiple incorporated genes from a different species are referred to as 'transgenic'; if introgression is through *in vitro* recombinant DNA technology (RDT). Precisely, genetically modified organism (GMO) comprises those created by natural recombination processes or natural selection; whereas transgenic organisms emerge solely from *in vitro* RDT.

The main aims of creating transgenic plants were varied and diversified. The first was obtaining a net gain in productivity, indispensible to sustain 11 billion people as projected as the world population (Moneret, 1998) in 2050. Achievability was through the creation of plant species resistant to biotic and abiotic challenging factors. The second main aim was based on improving the

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nutritional content, especially in protein, high carotene (vitamin A), high tocopherol (vitamin E) and other micro and macro nutrients for most food amongst which potatoes, rice, wheat and tomato topped the list (Prabhu, 2010).

Late blight disease is the most important devastator of potatoes in the world (Edwards, 1956; Song et al., 2003). The gravity of its destruction is traced back from the Great Irish potato famine in the 19th century resulting in over a million Irish starved to death and many more emigrating (Edwards, 1956; Kamoun, 2001). Costs of losses and crop protection against the late blight are estimated at US$3.25 billion per annum worldwide (Fry and Goodwin, 1997b). Furthermore, Germany during the Second World War went on famine since all copper supplies were commandeered for war efforts; hence copper fungicides for potato production was not available, *Phytopthora infestans* largely damaged the crops (Carefoot and Sprout, 1967). Into the 21st century, no concrete solution to either curb the spread or eradicate the pathogen has been found despite continued efforts of scientist. Worst still, resistance to chemical fungicide further complicate the issue, giving rise to the idea of an eventual 'oomicides' creation (Gover, 2001); which is very illusive**.** The emergence of mating types (A1 and A2) of *P. infestans*, undergoing sexual reproduction provides an insight into the pathogenicity of the organism to colonize new terrain (Drenth et al., 1994); this implies production of recombinant strains. The pathogen population in Europe is now highly diverse and there is more evidence of sexual reproduction in several European countries (Drenth et al., 1994; Flier et al., 2003). The incidence of this pathogen has been controlled notably by agronomic practices that include crop rotation, agrochemicals (Fry and Goodwin, 1997b; Shattock, 2002) and by breeding wild species that contain resistance conferring genes (Lara et al. 2006). The use of agrochemicals poses many dangers that include harmful effects on the ecosystem, increased farmer production cost; on the other hand, breeding programs are necessarily time consuming. Moreover, re-emergence of new strains, slow time consuming breeding method and the ploidy nature of potato, led to the introduction of RDT for improvement against biotic and abiotic stress factors. This paper aims to expose the most successful advancement of genetic engineering, in solving the invincibility of *P. infestans* through transgenic potatoes. Bringing out the implications of pathogenesis related proteins and some antimicrobial proteins to the spotlight, usually involved in local resistance and systemic acquired resistance (SAR) responses in conventional plants.

Pathogenesis related proteins (PR) as weaponry for transgenic potatoes against *P. infestans*

The operational definition of PRs is that of polypeptides with relatively low molecular weights (of 10 to 40 Kilo Dalton (KDa)) that accumulate extracellular infected plant tissue, exhibit high resistance to proteolytic degradation, and often, but not always, possess extreme isoelectric points (Van Loon, 1985). Since their discovery in 1970, 17 families has been identified and reclassified based on amino acids sequences, serological relationship and/or enzymatic or biological activity (Van Loon et al., 2006).

One of the most exploitable pathogenesis related proteins used in transgenic potatoes is osmotin, a 24 KDa protein. This PR-5 family member had been a hotspot for biotechnologist, since it was suspected to play an important role in enhancing the level of resistance to secondary challenges by pathogens, a phenomenon referred to as SAR. The first evidence that osmotin and other PR-5 family had antifungal properties came from the works of Vigers et al. (1991); indicating the N-terminal sequence of zeamatin, an antifungal protein from corn, was very similar to osmotin. *In vitro* assays, demonstrated that osmotin had antifungal activity against a variety of fungi, including *P. infestans*, *Candida albicans, Neurospora crassa, and Trichoderma reesei* (Woloshuk et al., 1991; Vigers et al., 1992). Stress con-ditions such as NaCl, desiccation, ethylene, wounding, abscisic acid, tobacco mosaic virus, fungi, and UV light were tipped to be inducers of this protein (LaRosa et al., 1992). The large spectrum of cues, both abiotic and biotic gave, good indications that; osmotin gene could always be activated under field conditions. The constitutive expression of osmotin led to enhancement of potato resistance to late blight (Liu et al., 1993); its role in resistance consist of causing sporangia lysis of *P. infestans* (Woloshuk et al., 1991), plant protection against osmotic stress (Kononowicz et al., 1992) and freezing tolerance (Hon et al., 1995). It was previously unveiled that, at high concentration, it causes the lysis of hyphae tips of fungi (Vigers et al., 1991). A more conclusive antifungal potential of osmotin was shown by Abada et al. (1996).

They concluded that osmotin induces spore lysis, inhibit spore germination or reduce its viability in seven fungal species that exhibited some degree of sensitivity in hyphal growth inhibition tests. These broad spectral mechanisms of action validated osmotin gene, as potential sentinel candidate against *P. infestans* in transgenic potatoes. Overexpression of PR-5 (or thaumatin-like (TL) proteins) in potato delayed development of disease symptoms of *P. infestans* (Liu et al., 1994*) in vitro*, whereas trials in transgenic potato plants overexpressing antisense PR-5 did not exhibit any higher susceptibility (Zhu et al., 1996). Curiously, a basic PR-5 has been identified on the cell wall of *P. infestans* (Jeun and Buchenauer, 2001), implying this pathogen is equally armed and definitely ruling out the use of PR-5 thaumatin-like proteins and osmotin 'single-transgene-construct' (STC) in genetically engineered potatoes since both belongs to the PR-5 family.

Defensins (PR-12 family), like thionins (PR-13 family) are cysteine rich-PR proteins (of about 15 kDa) which distinctively confer resistance with a unique mechanism of action; consisting of pores formation within cell membrane resulting in membrane disruption and consequently leading to cell death. Many defensins exhibit antimicrobial activity (Thevissen et al., 2007), while some are apparently void of antifungal and antibacterial potentials (Liu et al., 2006).

A well studied defensins is Rs-AFP2 (*Raphanus sativus* antifungal protein-2), exploited in tomatoes. As a consequence of its activities, Gao et al. (2000) using a gene for cysteine-rich defensins from alfalfa seeds alfAFP (alfalfa antifungal peptide)-gene regulated by a ^{35}S promoter in transgenic potato, revealed an imposing resistance against *Verticillium dahliae*, *Alternaria solani* and *Fusarium culmorum* but not to *P. infestans*. This lack of activity suggested that, defense gene expressions are pathogen specific.

This further strengthens the need for broad synergistic introgression of transgenes, to constitutively express defense proteins in a given transgenic plant if broad viable resistance is the main aim of RDT. Previously, *Nicotiania megalosiphon* was shown to be highly resistant to *Peronospora hyoscymi* f.sp *tabacina* and oomycetes which causes foliar diseases in tobacco (Borrás-Hidalgo et al., 2010). In similar operational approach of RDT, Portieles et al. (2010) reported that engineered transgenic potato and tobacco over-expressing *N. megalosiphon* defensins (NmDef02) gene successfully conferred high-level resistance both under greenhouse and field conditions against *P. infestans* and *P. hyoscymi* f.sp *tabacina*.

An exceptional breakthrough in improving transgenic potato broad-spectrum resistance to *P. infestans* was initiated in 2007. Lee et al. (2007) identified *Solanum tuberosum* L. ethylene responsive element binding proteins (StEREBP1) to be cold-inducible, playing important regulatory functions in plant development, as well as environmental stress and defense responses (Song et al., 2003).

With the continuous effort to improve potato resistance to phytopathogens, especially oomycetes, transgenic potato lines overexpressing StEREBP1 gene from potato (*S. tuberosum* L.) generated by *Agrobacterium tumefaciens*-mediated transformation was shown to exhibit intense resistance to *P. infestans* (Seok-Jun et al., 2009).

Reported absence of observed symptoms on leaves of this transgenic potato prior to infection with *P. infestans* was glaring indications of resistance conferred by introgressed gene. This case suggested that, enhance resistance to the phytopathogens could be due to up-regulated expression of stress-response genes encoding pathogenesis related proteins; that may have been induced by overexpression of StERESP1 (Seok-Jun et al., 2009).

Some engineered antimicrobial proteins (AP) against *Phytopthora infestans* in transgenic potatoes

The quest to eradicate *P. infestans* had prompted the introgression of animal genes in potatoes. Such introgressed animal genes and genes products are known to interact intracellularly, but their activities in an alien cellular environment of transgenic potato can not be predicted precisely in terms of allergenicity and toxicity (Sharma, 2010), raising concerns of consumer safety. The use of hen egg white lysozyme (HEWL) gene, tested both in transgenic potatoes and tobacco, elaborating a certain degree of resistance to several bacteria and chitin fungi such as *Botrytis cinerea*, *Verticillium alboatrium* and *R. solani* (Trudel et al., 1995; reviewed in Grover (2003)) was an obvious demonstration on the enormous power of rDNDt in resolving the late blight disease palaver.

Optimism at the horizon for eradicating late blight disease came from four synthetic cationic peptides pep6, pep7, pep11 and pep20 having *in vitro* inhibitory activities against *P. infestans* and *A. solani* (Ali and Reddy, 2000); however, lack of *in vivo* bioassay, leaves doubts on their antifungal potential against late blight causal. A chimaeric gene is a gene constructed by combining coding sequence from one gene with the regulatory sequence of another gene. This ability to create synthetic recombinant and combinatorial variant proteins encoders offers an opportunity to rDNAt to engineer resistance to a range of phytopathogens simultaneously (Dhekney et al., 2007). This concept was exploited in transgenic potatoes. Engineered chimaera cationic antimicrobial peptide (CAP), composed of cecropin-A at the N-terminus and modified melittin-C terminus; constitutively expressed in transgenic potatoes and has been a powerful source of resistance to *Phytophthora cactorum, Erwinia carotovora* and *Fusarium solani*, but not *P. infestans* [reviewed in Grover (2003) and Osusky et al. (2004)]. Chimaeric genes (or synthetic genes) were also introduced into two potato (*S. tuberosum L.*) cultivars, 'Desiree' and 'Russet Burbank' characterized by stable introgression and expression as confirmed by reverse transcription (RT)-PCR and with the recovery of biologically active peptide. Yield and morphology of transgenic 'Desiree plants and tubers was unaffected (Haggag, 2008). Barnase (a cytotoxic protein having RNase activity) is naturally present in *Bacillus amyloliguefaciens.* It's introgression in potatoes led to severe inhibition of *P. infestans* spores (Strittmatter et al., 1995; reviewed in Grover (2003)). Once again, the absence of field studies created a big vacuum on the applicability due to the harsh legislation regulating transgenic field trials.

Expression of gene product which functions by releasing elicitors that regulate plant defenses are highly envisaged in transgenic potatoes. This elicitor includes hydrogen peroxides (H_2O_2), salicyclic acids (SA) and ethylene (C_2H_4). Oxidative burst giving rise to hydrogen peroxide (H_2O_2) generally induced PR, phytoalexins,

other relative SAR agents and hypersensitive reactions, conferring broader microbial inhibition. rDNAt exploits the ability of glucose oxidase (GO) hydrogen peroxide and gluconic acid producing potentials. In this sphere, *Aspergillus niger* GO gene incorporation into potato proved to reduce considerably the vulnerability of the plant to *P. infestans, E. carotovora* subspecies *carotovora* and *Verticillim dahlia* (Wu et al., 1995). In this trend, the expression of tobacco catalase, an enzyme with SA-binding activity, in transgenic potato enhanced tolerance to *P. infestans* (Yu et al., 1999).

A resistance gene (R-genes) product acts by mediating rapid localized cell death or hypersensitive responses (Rowland et al., 2005). RB-gene is a race independent resistant gene to late blight (Naess, 2000), discovered in wild species of potato called *Solanum bulbocastanum,* mapped to chromosome 8 (Jones and Simko, 2005). The resistant RB-gene was subsequently cloned and found to belong to the largest class of *R*-genes, encoding proteins with a nucleotide binding site and leucine-rich repeats (Song et al., 2003; van der Vossen et al., 2003). Revelation of RB-gene from the wild type *S. bulbocastanum Dunal* subsp*. bulbocastanum* through traditional breeding instilled hope within breeders. This new optimism was short-lived by gene-drag in this ploidy plant. However, Jones and Simko (2005) and Lara et al. (2006) revealed that, transgenic potato lines containing the RB-gene exhibited strong late blight resistance, compared to backcrossed progenies derived from breeding programs; or those derived from somatic hybrids between *S. tuberosum* and *S. bulbocastanum.*

The performance of transgenic potato was further evaluated by Halterman et al. (2008) using late blight resistance gene RB. This RB-transgenic potato exhibited a strong foliar resistance under greenhouse conditions to *P. infestans,* but with no noticeable tuber resistance to the phytopathogen. Moreover, Halterman et al. (2008) noted that all RB transgenic lines exhibited an increase in resistance to *P. infestans* compared with their non-transgenic counterparts. Hence, RDT approach seems logical in this heterozygous and vegetatively propagated crop (Park et al. 2009).

DISCUSSION

Under-, normal- and over-expressions are the three expressive modes of ‘transgenes-construct’ so far exploited by biotechnologist in transgenic plants depending on the cis-regulatory elements used for controlling the transgenes. Most success stories in engineered genes are stringently through transgenes-construct over-expression. For most if not all, biotechnologists are switching by choice towards overexpressing ‘transgenes-construct’ to confer broad-spectrum plant defense, mediated by pathogenesis related proteins and other antimicrobial proteins. However, outcome from successful defense enhancement due to introgression greatly varies, depending mostly on the targeted phytopathogenic agent susceptibility and the mode of expression of ‘transgenes-construct’. Generally, introgression of traits from wild relatives of potato has been difficult, and had mostly been restricted to disease resistance traits (Hermsen, 1994). In an attempt to explain some of the failures seen so far in transgenic potatoes, the most noticeable one is the orientational use of some key amino acids. Transgenes (of PR or AP) in potato genome expression products requires tryptophan, which is also a building block for plant cell wall lignifications; the first line of plant defense. Nicholson and Hammerschidt (1992) showed a decrease in polyphenolic compounds, such as lignin, in transgenic potato tubers due to redirection of tryptophan in transgenic plants through expression of tryptophan decarboxylase; which in turns renders tissues more susceptible to *P. infestans*.

The quality and tenor of rDNAt and the resistance it confers to transgenic potato is so far from satisfaction. In most experiment, STC has been used with shortcomings. From our investigation the following reasons can be suggested to explain the invincibility of *P. infestans* regarding the already created transgenic potato:

1) Antimicrobial proteins- and PR-phytopathogens interactions are specific - that is, gene-for-gene model response. Hence, STC narrowly confer resistance to a particular biotic factor, not a broad-spectrum resistance.
2) *P. infestans* encodes some of the introgressed STC proteins, as in the case of PR-5 TL proteins (Jeun and Buchenauer, 2001). Meaning the pathogen is immune to some of the STC proteins.
3) Misclassification of *P. infestans* as 'fungus' actually placed vital research on wrong track (Govers F (2001). This revelation compromised most previous work geared at eradicating late blight disease.
4) rDNAt method of gene insertion in any recipient DNA is rather a random process which might lead to: a) silencing of the inserted STC itself or surrounding genes at the site of insertion (Azahaguvel et al. 2006); b) genetic instabilities in other genes of interest and c) finally activating some evolutionary silenced genes (Sharma, 2010).
5) Finally, lack of field trials and *in vivo* assays probably due to restrictions pose a challenge to the deployment of field trials, and *in vivo* assays in some countries generally slows down the usefulness of rDNAt in the field of transgenic potatoes.

Of late, a disturbing sequel tormenting first evidence that *P. infestans* can defeat an R-protein through inhibition of recognition of the corresponding effectors protein by Halterman and Chen *(*2010) is a landmark message to all stakeholders, advocating the use and deployment of the new options transgenic potatoes offers. Halterman and Chen (2010) reports deeply compromise future breeding of RB varieties for the

search of broad-spectrum resistance, whereas RDT report from findings of Portieles et al. (2010) gives hope in conferring broad-spectrum resistance against *P. infestans*. Moreover, the scarcity of resistance traits in potatoes or their exhaustive usage in broad-spectrum resistance breeding is an indication; mobilization of genes from other sources through recombinant DNA technology is indispensible. A new approach of using an array of PR and AP encoding genes in 'chimeric-transgenes-construct' is the way forward in conferring a sustainable and durable resistance to potato plants. This biotechnological concept not only improves yield to feed more people, it reduce inputs in terms of chemical fungicide application on the part of the farmers; but also, consumers benefit from nutritionally enriched plants free from chemical residues.

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