Chapter 10

Mutagenesis in Plant Breeding for Disease and Pest Resistance

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1. Introduction

Food production and food security faces several challenges such as climate change and expanding human growth, the competition of food and non-food uses, and decreasing area of arable land. The role of plant breeding in providing sustainable food production is to enable stable yields with lower inputs of fertilizers, energy and water use, to produce safe and quality food and to meet the demand of a projected raise in human population and livestock production. World population is projected to reach 10 billion by 2100 (United Nations, 2011) with the trend of changing diet towards higher quality food. Mutagenesis could be one of the solution to challenges facing the agriculture. Mutation breeding has substantially contributed the countries' economies and to conservation of biodiversity by stopping gene erosion. Improvement of crop production regarding pest and disease management is one of the main goals in agricultural breeding. Pathogens cause huge yield losses in the agriculture every year with large economic losses and damage to ecosystems. Disease outbreaks pose threats to global food security causing global yield loss of 16% (Oerke, 2006). Actual losses due to pests (weeds, animal pests and pathogens) range from 26-29% for sugar beet, barley, soybean, wheat and cotton, to 31-40% for maize, potato and rice (Oerke, 2006). The actual loss is referring to the losses sustained despite protection measures applied. Plant parasitic nematodes cause crop losses up to 125 US dollars annually (Chitwood, 2003). The constant challenge in plant breeding is to deal with the overcome disease and pest resistance and the development of new aggressive strains of pathogens such as fungi *Puccinia striiformis*, a causal agent of wheat yellow rust. The advances in molecular technology and in recent findings in cloning of disease resistance (*R*) genes allow the improvement of crop disease resistance by applying traditional breeding, genomic approaches, transgenic deployment and mutagenesis tools for enhancing disease and pest resistance. Using radiation breeding, traits for yield, quality, taste and disease and pest resistance have been improved in cereals, legumes, cotton, peppermint, sunflowers, peanut,

grapefruit, sesame, banana and cassava. Basic scientific research has substantially benefited from mutagenesis. Using *in vitro* mutagenesis, a considerable progress in understanding the evolution of molecular mechanisms of resistance was achieved.

2. Disease and pest resistance in plants

Plants encounter numerous beneficial and harmful organisms (pathogens) in the environment and use different strategies and mechanisms to cope with in order to survive and reproduce successfully. Basal resistance is referring to the constitutive defence provided by pre-existing physical and chemical barriers in order to disable penetration of pathogen to the host-cell. Another aspect of basal resistance is the recognition of microbial surfaces by cell surface receptors that trigger immune response and offer broad-spectrum resistance. This non-specific resistance is called pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) (Jones & Dangl, 2006). There is an evidence of structural similarity of cell-surface receptors, usually receptor-like kinases, between plants and animals (Nurenberger et al., 2004). The term PAMP is referring to small conserved molecules secreted on the surface of a class of microbes. In bacteria, well characterized PAMPs are: i) flagellin, which is a major structural protein essential for bacteria motility (Ramos et al., 2004), ii) lipopolysaccharides (LPS), a component of the cell wall of Gram-negative bacteria, and iii) peptidoglycan (PGN), a polymer forming the cell wall common to all bacteria (Akira & Takeda, 2004; Janeway & Medzhitov, 2002). In fungi, well characterized PAMPs are chitins, mannans and proteins (Cohn et al., 2001; Holt et al., 2003; Parker, 2003). PTI immune system exist in all higher plants (Boller & He, 2009). For example, homologues of *Arabidopsis FLS2* gene, coding for LRR receptor-like kinase, were found in all sequenced plants. Apart from structural conservation of *FLS2* gene there is proven functionality between different species. Rice *FLS2* gene is functional in *Arabidopsis fls2* mutant, thus suggesting conservation of associated signalling pathways (Takai et al., 2008). During the co-evolution of interplay between successful plant defence and pathogen attack, plant evolved rapid defence responses, involving programmed cell death during hypersensitive response. The response is mediated through R proteins that are either directly involved in the recognition of pathogen effectors or act as a guardian for the modification of plant proteins. Higher level of defence is able to detect specific pathogen effectors and is referred to effector-triggered immunity (ETI). Recent advances in understanding plant immunity suggest that basal resistance and race-specific resistance (ETI) evolve simultaneously as an answer to selection pressure on both actors. Natural selection drives the pathogen to avoid resistance either by evolving the existent effector gene or by acquiring additional effectors. This new effector put the selection pressure on host plant to evolve new *R* gene alleles. The co-evolution of plant defence and pathogen attacks are the result of constant selection pressure that occur across spatial and temporal scales (Ravensdale et al., 2011). In PTI immunity system there is an evidence of molecular evolutionary conservation in structure and functions across kingdoms borders (Medzhitov & Janeway, 1997; Imler & Hoffmann; 2001), however the evidence of existence of ETI in animals is missing. ETI enables the detection of pathogen-specific effectors by

protein receptors coded by *R* genes in every single cell in contrast to invertebrate animals that have circulating system, which constitutes to important distinction between plant and animal innate immune systems (Ausubel, 2005). The major players in expressing ETI are plant *R* and pathogen *Avr* genes. Unlike PTI, which is expressed in all plants of a given species, ETI is often expressed in some but not all genotypes within a plant species against pathogen race specific effectors. Although ETI response is fast and effective, plant can also detect pathogens through basal immune system.

2.1. *R* genes

For most proteins coded by *R* genes there are characteristic, conserved, structural domains. In general, we can divide R proteins according to the mode of resistance, to race-specific and race-non-specific. According to structural motif, they can be divided into five classes (Hammond-Kosack & Parker, 2003). In the first class, there are serin/threonin kinases such as *Pto* gene at tomato conferring resistance to bacteria *Pseudomonas syringae*. All other R proteins, combined in four classes, have leucine rich repeat domain and are distinguished by the localization of these domains. R proteins of second class are transmembrane receptors with extracellular LRR domain (*Cf* gene family in tomato), while R proteins of third class have extracellular LRR domain connected to kinase domain (*Xa21* gene at rice). *R* genes belonging to the fourth and fifth group code for intracellular proteins with NBS and LRR domain. LLR domain is important for ligand binding and the recognition of pathogen effectors (Young, 2000). The C- and N-terminal end of LRR domain are proposed to have distinct functions, the C-terminal end is responsible for the ligand recognition and important for determining R-Avr specifity, while N-terminal end is responsible for activation of further signal transduction (Inohara & Nunez, 2003; Tanabe et al., 2004; Chen et al., 2004). Structural similarities between NBS-LRR proteins of different species and taxa confirm the conservation of basic mechanism of defence against pathogens during the evolution and diversification (Moffet et al., 2002). Although R proteins share similar structure at the amino acid level, they clearly differentiate at the nucleotide level. For example, the level of amino acid hop (*Humulus lupulus* L.) RGA sequences compared to cloned *R* genes of evolutionary distant plants such as *Arabidopsis* is mainly restricted to the presumed functional domain (Kozjak et al., 2009).

2.2. Interplay between plant defence and pathogen attack

There are few models describing the interaction between pathogen avirulence (*Avr)* molecules called effectors and *R* proteins that are differing in the mode of action (direct or indirect).

2.2.1. Gene-for-gene

Gene for gene concept is based on direct physical interaction between plant *R* gene and corresponding pathogen avirulence *Avr* gene (Flor, 1955). Examples of such interactions have been described in tomato, where *Pto* interacts with *AvrPto* gene product of

Pseudomonas syringae (Scofield et al. 1996), in rice-rice blast pathosystem, where *Pi-ta* interacts with Avr-Pita (Jia et al., 2000) and in *Arabidopsis*, where RRS1 protein interacts with *Avr-PopP2* gene product of *Ralstonia solanacearum* (Bernoux et al. 2008).

2.2.2. Guard hypothesis

Alternatively, the guard hypothesis is based on the assumption that R proteins act as guards on host target proteins (guardee) and are a part of protein complex. This hypothesis predicts R proteins to be part of surveillance machinery and suggests indirect interaction between R proteins and corresponding *Avr* gene products. R proteins are activated by the modifications of host targets of corresponding pathogen effector (van der Biezen & Jones, 1998; Dangl & Jones, 2001). Two scenarios are proposed for indirect interactions (Figure 1). The Guard model was proposed to explain how the single *R* gene product perceives multiple effectors (Jones & Dangl, 2006) (Figure 1). The first experimental evidence shown for RPM1-mediated disease resistance to *P. syringae* revealed that RPM1 signalling cascade is activated by a protein component RIN4 which also needs to be activated by the phosphorylation in the presence of *AvrB* or *AvrRpm1* (Mackey et al., 2002, 2003). In the absence of effectors, RPM1 is negatively regulated by the RIN4 and stays in inactive form (Mackey et al. 2003). Axtell & Staskawicz (2003) demonstrated that RIN4 has a dual role and acts as a negative regulator of RPS2 activation conferring resistance to *P. syringae* expressing AvrRpt2. In contrast to RIN4 phosphorylation, for the activation of RPM1 signalling pathway, RPS2 activity requires the AvrRpt2-mediated disappearance of RIN4.

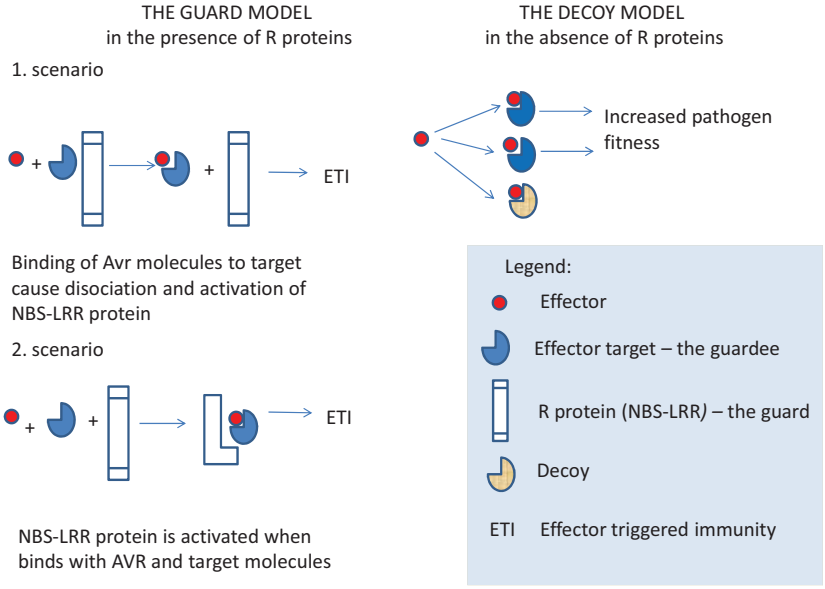

Figure 1. Schematic presentation of Guard and Decoy model

2.2.3. The Decoy model

The physical nature of the R-Avr interaction has big impact on the evolution of these proteins (Ravensdale et al., 2011). Effector target and plant guardee are under opposing selection pressures. First, in the absence of *R* gene product, the binding affinity of guardee should decrease in order to avoid detection and modification of a guardee. Just opposite, in the presence of functional *R* gene product, the selection pressure is put on guardee to enhance pathogen detection by improved interactions (van der Hoorn & Kamoun, 2008). This opposite pressure lead to unstable situation that could be released by the host protein that mimics the effector target without contributing to pathogen fitness. This host protein is termed as a "decoy" and is specialized in attracting effector. Difference between the Decoy and Guard models is that in the Decoy model, the pathogen fitness does not benefit from the absence of R protein (van der Hoorn & Kamoun, 2008) (Figure 1).

The Decoy model was proposed just recently and has to be experimentally proven, however few well-studied effector-perception mechanisms support this model. Tomato Pto interacts with avrPto to trigger the resistance to *P. syringae*, with the associated NB-LRR Prf protein that is necessary to trigger further defences. Prf protein acts as a guard on Pto. In addition to Pto, AvrPto binds to different receptor kinase targets, including FLS2 in *Arabidopsis* and LeFLS2 in tomato to block plant immune responses. AvrPto contributes to virulence on tomato even in the absence of Pto (Chang et al. 2000) but not on Arabidopsis lacking FLS2 (Xiang et al., 2008). On *fls* mutants, AvrPto no longer contributes to virulence (Xaing et al., 2008). It has been proposed that Pto competes with FLS2 for AvrPto binding (Zhou & Chai, 2008; Zipfel & Rathjen 2008). In this case, Pto acts as a decoy. Since AvrPto inhibits multiple kinases, Pto could evolve by mimicking one of them by losing some of the structural properties or by duplication and subsequent divergent evolution (Tian et al., 2007, van der Hoorn & Kamoun, 2008). Both of the models, Guard and Decoy, are not necessarily excluding each other since "guardee" may evolve into the "decoy".

2.2.4. Co-evolution of plant resistance and pathogen virulence

The co-evolution of host-pathogen interaction is driven by different factors, such as environmental conditions, population size and pathogen dispersal mechanisms that put the selective pressure on each other across space and time. Plant defences against pathogen attacks are dynamic processes that involve regulation of many defence components on the cellular level. NBS-LRR genes take a part in network with other components of signal transduction, since most proteins act as a complex with other components. During the defence, multiple organelles are included in the recognition and signalling mechanisms. The intracellular trafficking of pathogen effectors, mRNA and R proteins between the cytoplasm and nucleus is crucial for successful immune responses (Deslandes & Rivas, 2011). There has been evidence that effectors modulate transcriptional machinery by activation or repression suggesting the involvement of defence associated loci through changes of chromatin (van der Burg & Takken, 2009). The co-evolution of other components is prerequisite for optimal functioning, which is seen as different quantitative characteristics among species (Jones &

Dangl, 2006). This is the case of *Bs2* gene from pepper carrying resistance against bacteria *Xanthomonas sp.*, which is functional in many species within the *Solanaceae* family but not outside the family. Similarly, in *Arabidopsis* some traits may not be relevant to non-brassicaceous plants. Diversity for the virulence (or specialization) and the host resistance is dependent on the reproductive strategies of the host (out crossing or inbreed) and geographical distribution. Host populations can represent distinct groups regarding disease resistance. Ravensdale et al. (2011) analysed host resistance in flax against flax rust resistance and found that resistance structure within populations varied from nearly monomorphic to highly polymorphic, having at least 18 different resistance phenotypes. He concludes that temporal and spatial variation of disease resistance between populations puts stronger selection pressure or drift on the evolution of resistance than on the gene flow. The ZIGZAG model, proposed by Jones & Dangl (2006), illustrates the quantitative output of the plant immune system that can be presented in four phases. In phase 1, plants detect pathogen effectors or PAMPs and trigger PAMP triggered immunity (PTI). In phase 2, pathogen interfere with PTI, in phase 3, an effector is recognized by R protein activating effector triggered immunity (ETI) and in phase 4, pathogen evolve new effectors to suppress ETI thus putting the selection pressure on new R protein alleles in plants.

2.2.4.1. Development and evolution of R genes

R genes develop by different natural mutagenesis mechanisms such as: i) recombination, ii) tandem or segmental duplication gene events, iii) unequal crossing-over, iv) point mutation and v) selection pressure from the environment (Meyers et al. 2005). *R* genes and analogs of *R* genes (RGAs) have strong tendency for clustering in plants (Meyers et al., 1998; Gebhardt and Valkonen, 2001; Mutlu et al., 2006; Di Gaspero et al,. 2007). NBS-LRR genes are unevenly distributed and usually organised in clusters including pseudogenes (Meyers *et al*. 1999). Pseudogenes are assumed to be the source of higher variation than in coding genes and offer a potential reservoir for the *R* gene evolution, so the polymorphism detected in non-coding area of genome is rather expected (Calenge et al., 2005). Recombination is often in closely related and physically close *R* genes (Meyers et al., 2003; Baumgarten et al., 2003) however, in *R* gene cluster of soybean and lettuce a phenomena of suppressed recombination was observed (Kanazin et al. 1996; Meyers et al., 1998). Genome analyses of *Arabidopsis* shows translocation events of NBS-LRR genes by genomic duplications at distant, probably random locations in the genome, these mutations are called ectopic mutations (Meyers et al., 2003; Baumgarten et al., 2003; Leister, 2004). At some loci gene families expand by tandem duplications, doubled sequences are accumulating mutations, which increase the complexity of *R* gene sequences. Comparative sequence analyses of different plant species of *Arabidopsis* (Meyers et al., 2003), tomato (Seah et al., 2007), wild potato (Kuang et al., 2005), wheat (Wicker et al., 2007), rice (Dai et al., 2010), soybean (Innes et al., 2008) and common bean (David et al., 2009) suggest that *R* gene follow different evolution path. Assuming that *R* genes evolve as response to selection pressure of pathogens and changing environment, Kuang et al. (2004; 2005) proposed two evolutionary categories: type I, include genes of frequent sequence exchange among paralogs and type II include slowly evolving genes with the accumulation of single amino acid substitutions.