Chapter 10

Mutagenesis in Plant Breeding for Disease and Pest Resistance

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Additional information is available at the end of the chapter

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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.

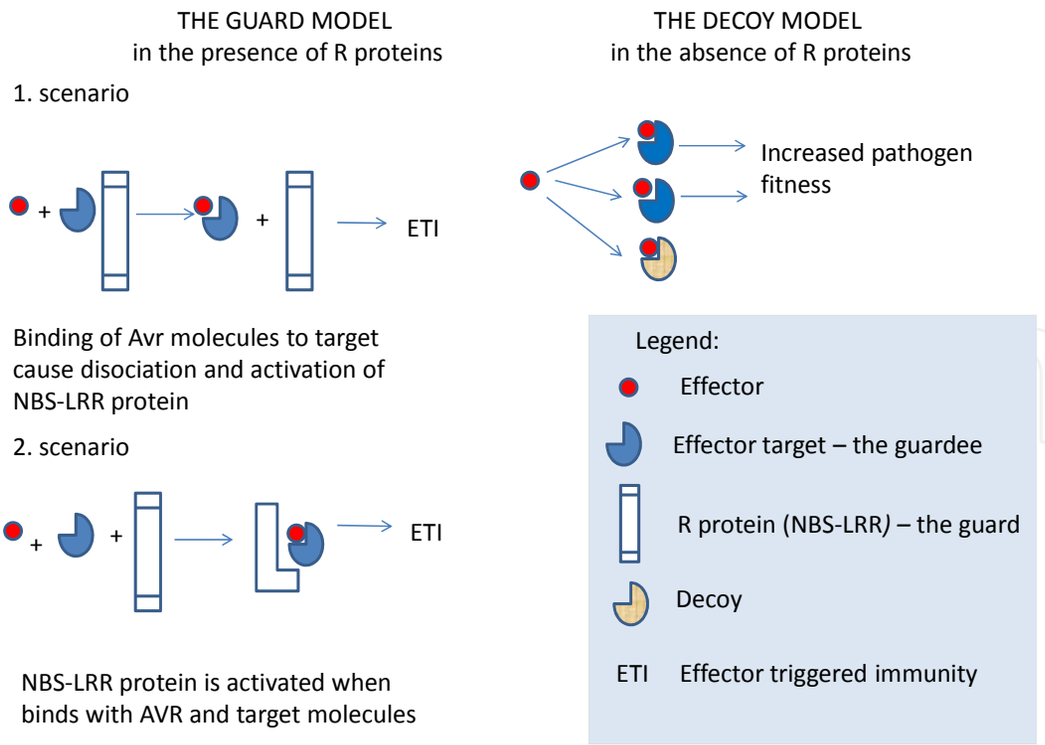

Figure 1. Schematic presentation of Guard and Decoy model

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.

Although most of *R* genes are dominantly inherited, there are recessive genes that confer race non-specific resistance such as *Mlo* gene at barley against *Erysiphe graminis* (Buschges et al., 1997), *RRS-e* gene at Arabidopsis against different races of *Ralstonia solanacearum* (Deslandes et al., 2002) and *Xa5* at rice against *Xanthomonas oryzae* pv. *oryzae* (Iyer & McCouch, 2004).

3. Breeding for disease and pest resistance

Commonly used methods for improving elite cultivars for disease and pest resistance combine traditional breeding methods (hybridization, selection, and introduction), alternative methods (tissue culture) and mutagenesis using forward and reverse genetic techniques (Figure 2). The induction of mutations for crop improvement is termed mutation breeding. To identify genes and its function two main approaches are employed: forward and reverse genetic techniques. The term forward genetics is used for identifying (cloning) gene, while the term reverse genetics is used to reveal gene function by analyzing phenotypic effects of a gene with known sequence. With the establishment divison for Agriculture & Biotechnology at the International Atomic Energy Agency (IAEA) and the Food and Agriculture Organization, more than 2000 varieties have been released that derived from either direct mutant or crosses between mutants (Ahloowalia et al., 2004). Most of these varieties are improved for increased yield and enhanced quality (improved processing quality, increased stress tolerance,...). Improved characteristics have been released in more than 175 varieties and plant species.

3.1. Classical breeding

The most effective approach to prevent disease outbreak is to cultivate resistant varieties. Transferring genes through conventional transfer process may be hampered by the vertifolia effect that refers to the loss of horizontal resistance during the breeding for vertical resistance (Van der Plank, 1963). A frequent problem associated with *R* genes is their short-term efficacy. Disease resistance of genetically uniform lines with single source of resistance is often defeated by new pathogen races when cultivated large-scale and long-term. This was the case with rice carrying only *Xa4* gene against bacterial blight across several Asian countries (reviewed in Kameswara Rao et al., 2002). Planting a mixture of cultivars would reduce the disease incidence, but intensive mechanization of crop production and modern markets demand uniform crops.

3.1.1. Map-based cloning

Map-based cloning is an approach to identify *R* gene and determine the sequence of a gene using molecular markers. We distinguish two different types of mapping: i) genetic mapping based on the classical techniques using pedigree or breeding of recombinant phenotypes and ii) physical mapping based on the use of biotechnological techniques (genetic fingerprinting) to determine the order and spacing between markers or genes. Linkage map is a genetic map presenting genes in lineage order and distance in between in

centimorgans (cM). Mapping Quantitative Trait Loci (QTLs) is effective approach for studying plant disease resistance. The first step in map-based cloning is to place molecular markers that lie near a gene of interest and co-segregate with proposed gene without recombination. It has been shown that soybean cist nematode resistance, rice blast resistance and black mold resistance in tomato, grey leaf spot and common rust in maize are under the control of QTL (Wang et al., 1994, Robert et al., 2001; Concibido et al., 2004; Danson et al., 2008). The second step is to clone the gene by chromosome walking and sequencing the gene. Determination of QTLs is important for studying epistatic interactions and race specifity. More than 35 QTLs in rice were found near *R* genes for resistance to blast (Ballini et al., 2008; Fukuoka & Okuno, 1997; Tabien et al., 2000, 2002). Identification of markers linked to QTL facilitates the targeting of recessive alleles, which can be masked by epistasis in the specific environment (Joshi & Nayak, 2010).

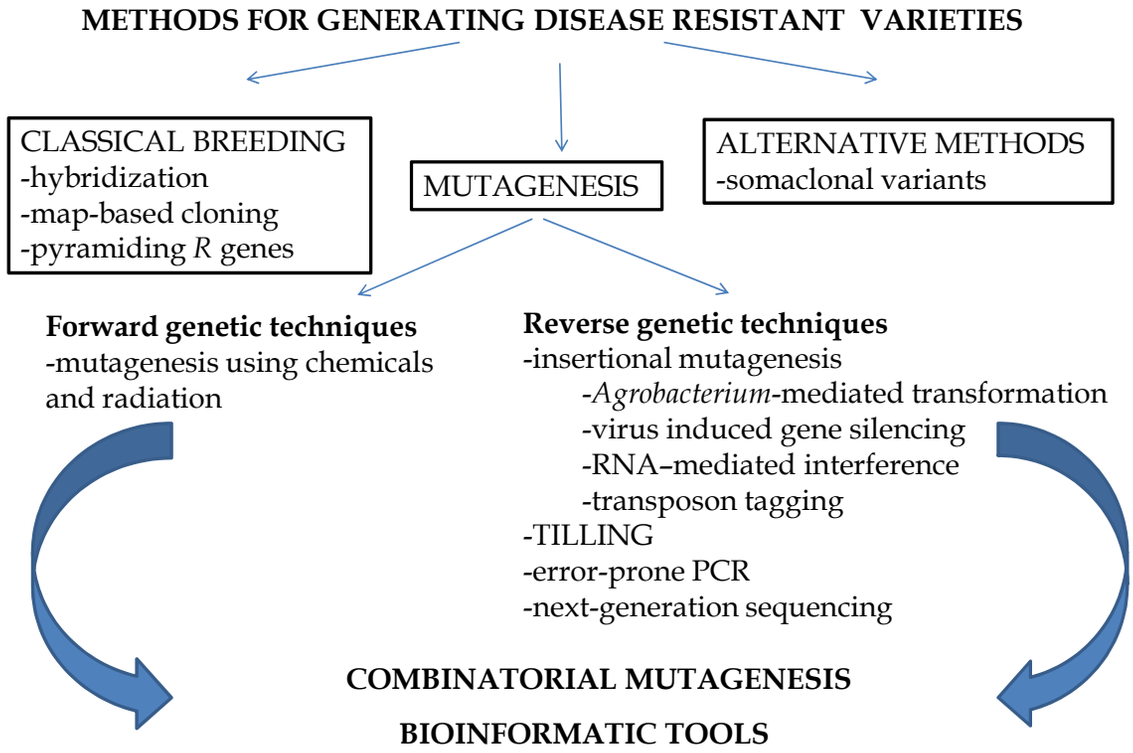

Figure 2. Schematic presentation of most commonly used methods for pest and disease resistance breeding in crops

3.1.2. Pyramiding R genes by marker assisted selection (MAS)

In order to avoid breakdown of resistance conferred by single *R* gene, pyramiding multiple *R* genes in genetically uniform lines presents an alternative. The idea of pyramiding *R* genes into crops is to construct sufficiently large pools of *R* genes that correspond to all avirulence genes in pathogen populations of specific regions. The probability of pathogen to break

resistance to two or even more genes is much lower than to single gene. Advantages of pyramiding genes in single genotype are: i) more effective control of insect resistant to single toxin that may be controlled by a second toxin, ii) lower probability of evolving resistance to two independent actions through selection of one toxin, and iii) a single effector cannot break resistance to binding to immunologically different targets (Gahan et al. 2005). The problem of introducing several genes by classical breeding is the transfer of undesirable traits that need to be removed by backcrossing. Gene pyramiding by classical breeding is also difficult due to the dominance and epistatic effects (Singh et al., 2001), but the identification of molecular markers linked to resistance genes or loci ease the identification of desired plants. The selection of desirable phenotype by molecular markers is termed marker assisted selection (MAS). MAS-based gene pyramiding is an analogue approach to classical breeding but less time consuming and relying on the use of molecular markers that speed up the selection procedures. Using sequence tagged sites (STS) markers, MAS based gene pyramiding and marker-aided backcrossing procedures several genes have been successfully transferred in elite rice cultivars (Huang et al. 1997; Singh et al. 2001). In common wheat, three leaf rust resistance genes *Lr41*, *Lr42*, *Lr43* were successfully pyramided as well (Cox et al. 1993).

3.2. Mutagenesis

The discovery of x-rays inducing mutations in *Drosophila melanogaster* presents the beginning of mutation breeding in plants. The term mutagenesis applies to methods used for the induction of random or site directed mutations in plant DNA to create new valuable traits in well-adopted cultivars. According to the FAO/IAEA database there are 320 cultivars with improved disease resistance using mutagenic agents that were obtained as direct mutant or derived from hybridization with mutant or from progeny (for example by self-fertilization). Induced mutations have been used to improve economically important crops such as wheat, barley, rice, cotton, peanut, banana etc. Disease and pest resistance in commercial crops was improved mostly in cereals (rice, barley, maize, wheat) and legumes (bean, green pea).

Spontaneous mutations occur at low frequencies, one in a million per gene. If two independent mutations are necessary in recessive alleles to obtain resistant phenotype, the frequency lowers to 10^{-18} per nucleotide (Gressel & Levy, 2006). Mutagenesis is used to accelerate spontaneous mutations in driven evolution. Using chemical mutagen (EMS) in *Arabidopsis* about ten mutations per gene were recorded among 192 genes in 3,000 M_2 plants examined (Greene et al., 2003) with an average of 720 mutations in single M_2 plant (Till et al., 2003). For the improvement of disease resistance, the induction of spontaneous mutations is applied by different mutagenesis approaches: virus induced gene silencing, RNA-mediated interference, *Agrobacterium*-mediated insertional mutagenesis, radiation and chemical mutagenesis and with combined approaches such as Targeting Induced Local Lesions in Genome (TILLING). For the identification of mutants, different methods have been developed through years, that include: i) high resolution melting techniques (HRM), ii) protein truncation test that detect mutants from the terminatioin of mRNA translation, iii) single-strand conformation

polymorphism (SSCP) for the detection of frameshift mutations, nonsense and missense mutations, iv) Southern hybridization for detecting large mutations (deletion, insertions, rearrangements), v) denaturing gradient gel electrophoresis (DGGE), vi) DNA microarray, vii) single and multiparallel DNA sequencing, viii) TILLING for the detection of mutations in large exon-rich amplicons and ix) PCR based detection technique. Novel sequencing approaches based on Sequence Candidate Amplicons in Multiple Parallel Reactions are now most commonly used in genomic analyses of gene expression and regulation modes, including the production of genetic maps. The new generation machines (Illumina Genome Analyser, ABI SOLiD, Roche 454) are capable of producing millions of DNA sequences in a single run. The advantage of multiparallel sequencing using pooling strategy is the identification of rare mutations that are distinguishable from background sequencing errors.

3.2.1. Induced mutagenesis by chemical or physical mutagens

Most mutagenic populations are generated by treating seeds with radiation or chemical mutagens. Physical mutagens are X-rays, Gamma rays, alpha particles, UV and radioactive decays. Irradiation usually cause large mutations (large-scale deletions of DNA), while chemical mutagens usually cause point mutations. Fast neutrons are high-energy thermal neutrons produced by nuclear fission. They induce broad range of deletions and chromosomal changes and are often accompanied by gamma radiation. The major fast neutron bombardment technique is Delete-a-gene, a knockouts gene system for plants (Li et al., 2002; Li & Zhang, 2002; Rogers et al., 2009). Delete-a-gene combines fast neutron radiation of seeds and identification of mutants by PCR using two specific primers for targeted locus and shortened PCR extension time to suppress the amplification of a wild type gene. Delete-a-gene is used as alternative method to insertional methods in cases when we do not have well characterized transposons or when the genes are placed in tandem duplication and we cannot inactivate them at the same time in order to observe mutant phenotype (Li et al. 2002). It can be applied to all plants since no transformation and tissue culture is needed. The carbon ions with high linear energy transfer (LET) has been proven very successful for the induction of base substitutions or small deletions/insertions in *Arabidopsis* that can be determined by single nucleotide polymorphism (SNP) detection system which is beneficial for forward genetics and plant breeding (Kazama et al., 2011).

Mutations induced by chemical mutagens are point mutations and are less damaging (not lethal) than large rearrangements.. The advantage of chemical mutagenesis is that is can provide loss- and gain– of - function of genes. There are various chemical mutagens used for generating variability, such as sodium azide, ethyl methanesulphonate (EMS), methyl methanesulphonate (MMS), hydrogen fluoride (HF), diethyl sulphate, hydroxylamine and N-methyl-N-nitrosourea (MNU). Most commonly used mutagen in creating TILLING populations in maize, rice, *Brassica* sp., pea, barley, wheat, soya and cucumber is ethyl methanesulphonate (EMS) (reviewed in Kurowska et al. 2011).

Induced mutagenesis by chemical or radiation mutagens have advantages over insertional methods, since mutagens introduce random changes throughout genome and can generate

variety of mutations within a single plant. Comparing to other methods, it is applicable to all crops and it does not demand the establishment of species-specific protocols for transformation and regeneration.

3.2.2. Agrobacterium-mediated transformation

Plant transformation technologies employ physical incorporation of foreign DNA into the host genome by different approaches, directly or indirectly. The indirect methods include transformations using *Agrobacteria tumefaciens* or *Agrobacteria rhizogenes*, while direct approaches include protoplast transformation and biolistic or microprojectile bombardment. *Agrobacterium* mediated insertional mutagenesis rely on a natural process of transferring T-DNA as a short segment of *Agrobacterium* plasmid to plant genome when infected by the *Agrobacterium*. The main transgenic crops improved for disease and pest resistance are soybean, maize, rapeseed, cotton, wheat, potato and rice (GMO Compass, 2012). Most of the transgenic research has been focused on virus resistance. In the past, it was believed that monocots are not amenable to *Agrobacterium* mediated transformation, but a successful transformation of wheat (Cheng et al., 1997), maize (Ishida et al. 1996) and barley (Tingay et al., 1997) was reported. Nevertheless, the transformation efficiency in monocots is still unsatisfactory. *Agrobacterium*-mediated transformation is fast and efficient method for introducing genetic material into the host cell and is preferable to many other insertional methods, since it introduce single copies of gene construct using highly efficient vectors that enhance virulence gene expression. However, some crops express hypersenstitive response during inoculation. Alternative transformation methods that exclude tissue culture steps are called *in planta* transformation that allow circumvent the transformation constraints in some monocots. *In planta* transformation, transgenes are delivered into apical meristem of differentiated seed embryo in the form of naked DNA or from *Agrobacterium*. Transgene is injected into the floral tissue of a plant using a needleless-injection device. Once the tissue is transformed, it is removed from the plant and regenerated separately *in vitro*. It has been successfully applied in mulberry, cotton, soybean, and rice (reviewed in Keshamma, 2008).

With sequencing plant genomes, such as *Arabidopsis*, rice and poplar, many genes were identified but their function and localization needs to be proven experimentally *in vivo*. A modified version of *Agrobacterium*-based transformation is Fast Agro-mediated Seedling Transformation (FAST). It offers a transient transformation assay that can take a week, starting from sowing seeds to protein analysis (Li et al. 2009). The advantages of these methods are in addition to time saving also minimal handling with seedlings, high transformation efficiency and big potential for automated high-throughput analysis. This system was applied in *Arabidopsis* to examine the biochemical activity of gene product; it's localization as well as protein-protein interactions. The limitations are in non-expression in different tissues and the need for biological compatible species. It may not be useful for studying disease resistance gene functionality since the co-cultivation with *Agrobacterium* could induce host disease resistance defences. Necrotic responses have been reported in several crops. The defense reaction in grapes was triggered by elevated levels of auxin

produced by wild-type T-DNA (Deng et al. 1995), while in tomato, resistance responses were triggered by flagellin (Felix et al. 1999).

3.2.3. Insertional mutagenesis using transposon

Transposon mutagenesis is used when plants are not susceptible to *Agrobacterium*-mediated transformation. Transposons are mobile elements able to move within genome and exist in several copies within the wild-type genome. In order to distinguish novel insertion events from wild type transposon, foreign sequences are introduced into transposon construct. Alternatively, transposon is transferred between different species. Comparing to T-DNA insertional mutagenesis, transposon insertion is more unstable, so different systems are developed to generate more stable transposon insertions (Twyman and Kohli, 2003). The most common is two-component transposon system. One component consists of Activator (Ac) mobile element that includes its own transposase for mobility within the genome, while the second component is lacking the transposase gene, named Dissociation (Ds) element. For the incorporation into host cell, both components are necessary but Ac element can be eliminated by further crossing in order to disable Ds element to move. A transposon tagging is a method of cloning genes whose function is not known. The first step is to identify mutant plants with changed phenotype for a specific trait because of insertion of transposon, truncation and inactivation of a gene. The genomic library is then generated from selected mutants and screened for transposable element. Any clone containing the element will also contain the mutated gene adjacent to the transposon that can be further sequenced and analysed. By transposon tagging, the first cloned *R* genes were isolated from maize, *Hm1* gene that conferrs resistance fungal pathogen *Cochliobolus carbonum* (Johal & Briggs, 1992), *Cf-9* gene from tomato against fungus *Cladosporium fulvum* (Jones et al., 1994) and *N* gene from tobacco conferring resistance to tobacco mosaic virus (TMV) (Dinesh-Kumar et al., 1995). Targeted tranposon tagging is a choice when we target single gene, while for isolating a group of genes a modified method, non-targeting transposon tagging, is an alternative (Gierl & Saedler, 1992).

3.2.4. Insertional mutagenesis using RNAi silencing

The phenomena of RNA silencing were discovered as a side effect during the plant transformation, in which the transgene and homologous endogene were silenced after the successful transformation. RNA silencing is a natural mechanism of wild *R* gene regulations that can be exploited in molecular breeding. This regulatory mechanism provides defence systems by destroying foreign nucleic acids of different nature. In *Arabidopsis*, *RPP5* locus contains structurally unrelated genes combining *RPP4* gene that confers resistance to downy mildew *Peronspora parasitica* and *SNC1* gene against multiple pathogens (van der Biezen et al., 2002). Small RNAs are generated from *RPP5* locus that could be a gradient form for generation of double-stranded small RNAs involved in RNA silencing (Nakano et al. 2006; Kasschau et al., 2007). It has been shown that RNA silencing may reduce fitness costs for expressing multiple *R* genes in the absence of pathogen and offers the possibility to express broad-spectrum resistance (Eckardt, 2007). Disadvantage of using RNA silencing is that it

has very variable success in different crops and its time consuming due to the vector construction and transformation of a plant. One of the first commercial outcome using RNA silencing was transgenic papaya with resistance to Papaya ringspot virus (Fuchs and Gonsalves, 2007). Destroying RNAs of viruses can also be achieved by using artificial short RNAs called miRNA. Apart from conferring resistance to viruses, miRNA has broader application for resistance to other pathogens as well. RNA silencing was induced in tobacco plants transformed with constructs against root-knot nematode gene that showed effective resistance (Fairbairn et al., 2007). Baum et al. (2007) identified 14 genes at western corn rootworm larvae that are destroyed by the dsRNA. Transforming maize with dsRNA genes gave protection similar to *Bacillus thuringiensis* transgene. Example of miRNA contributing to bacteria resistance is miRNA from *Arabidopsis* against *P. syringae* (Navarro et al., 2006). It has also been shown that *Arabidopsis* gene silencing is involved in specific resistance to funghi of the *Verticillium* genus (Ellendorff et al., 2009).

3.2.5. Virus-induced gene silencing (VIGS)

Virus-induced gene silencing is based on cloning 200-1300 bp long plant gene cDNA in RNA of a virus and incorporates it into plant genome by *Agrobacterium*-mediated transformation. It is applicable in monocot and dicot species. The advantage is that several homologues genes are targeted by single construct, but the phenotype is transient and mutations are not inherited.

In plants, manifestation of a pathway is termed as post-transcriptional gene silencing (VIGS). VIGS was used to unravel tobacco genes involved in *N* gene mediated defence pathway conferring resistance to tobacco mosaic virus (TMV). Three genes *Rar1*, *EDS1* and *NPR1*/*NIM1* were recognised to play an important role in signalling pathway aginst TMV, since the infection with TMV occurs in the presence of *N* gene if these genes are silenced (Liu et al., 2004). In *Arabidopsis*, silencing genes *rar1*, *hsp90* and *ndr1* in functional analysis of RPS2-dependent resistance demonstrated their involvement in expressing disease resistance caused by *P. syringae* (Cai et al., 2006*).* Using VIGS, the role of Hsp90 was proven in *I-2*-mediated resistance pathway against fungus *Fusarium oxysporum* in tomato (de la Fuente van Bentem et al., 2005) and *Mla13*-mediated resistance against fungus *Blumeria graminis* in barley (Hein et al., 2005). Although VIGS has been employed in important findings, the main disadvantage is the inability to employ vectors in certain varieties due to the natural resistance against those vectors.

3.2.6. Targeting induced local lesions in genome

Targeting Induced Local Lesions in Genome (TILLING) was developed as an alternative to insertional mutagenesis. The strategy was described by McCallum et al. (2000), who describes three main steps: i) treatment of seeds with mutagen and development of M_1 and M_2 generation and creation of DNAs pools of M2 plants, ii) detection of mutations (PCR amplification of specific fragments, heteroduplex formation and identification of heteroduplex using DHPLC, cleavage by specific endonucleases, high-throughput

sequencing, identification of mutant plants and determination of mutations) and iii) analysis of mutant phenotype (Kurowska et al., 2011). TILLING is a non-transgenic strategy for providing large spectrum of mutations (point mutations, small insertion, truncation and deletions) that can be applied when the sequence of gene is known and the methodology of detection of SNPs has been developed (Colbert et al., 2001). Advantages of TILLING over T-DNA insertions are in smaller population needed for the saturation mutagenesis (5,000 M_1 plants for TILLING compared to 360,000 M_1 plants for T-DNA mutagenesis) due to higher frequency of mutations (Østergaard & Yanofsky, 2004; Alonso & Ecker, 2006). This method provides allelic series of mutants, including knockouts. There is no need to have fully sequenced genome of the studied species, the sequences can be retrieved from gene databases (GenBank) and homologs identified by the BLAST search. Nevertheless, the search for evolutionary distant species should be done for amino acid rather than nucleotide queries. Bioinformatics analyses are necessary during all steps in TILLING strategy, from the determination of a gene to the determination of allele impact on protein function (Kurowska et al. 2011). TILLING is used mainly for basic research, the potential for commercial purposes still need to be established.

Since the invention of the method, many modifications have been developed such as Eco-TILLING (Comai et al., 2004) and individual TILLING (iTILLING) (Bush & Krysan, 2010). The difference between Tilling and iTILLING is that in Tilling DNA is polled from M_2 plants, while in iTILLING, DNA is isolated from pooled seeds collected in bulks of M_1 plants, which is cheaper and less time consuming. In iTILLING the detection of mutations is based on high-resolution melt-curve analysis of PCR products to reveal mutations of interest. Eco-TILLING is efficient method for cataloguing natural polymorphisms (SNPs, small insertions and deletions) in wild populations. It is cost effective because only one individual per haplotype is sequenced and it is applicable to all species. Eco-TILLING is used for identification of resistance genes to novel diseases and in discovering disease resistance gene variation. Allelic variants of *eIF4E* and *eIF(iso)4E* genes in *Capsicum* species that are involved in elimination of RNA viruses were identified as valuable source for resistance to RNA viruses (Ibiza & Nuez, 2010). Using Eco-TILLING different allele variants were examined in barley at *Mlo* and *mla* genes that are involved in resistance to powdery mildew (Mejlhede et al., 2006). The powdery mildew resistance gene *mlo* is a single copy gene that encodes protein involved in cell wall process. Using EcoTILLING it was possible to identified 11 *mlo* mutants. The *Mla* region combines several classes of genes with defence responses. More than 20 alleles of *Mla* locus have been identified from wild barley in Israel (Jahoor & Fischbeck 1987 & 1993, Kintzios et al., 1995).

3.2.7. Error-prone PCR

Error-prone PCR is a method for generating mutants in order to analyse the relationship between gene sequence, structure and function of protein (Pritchar et al. 2005). It uses imperfect PCR to enhance natural error rate of polymerase to generate beneficial mutations in directed evolutional experiment. Imperfect PCR reaction conditions reduce the fidelity of *Taq* polymerase to generate randomized nucleotide sequences, which is called gene

shuffling. This method was used to study protein interaction of RIN4 and RPS2 association in *Arabidopsis* conferring resistance to *P. syringae* (Day et al., (2005). Association of RIN4 and RPS2 was previously confirmed *in planta* (Axtell & Staskawicz, 2003). Day et al. (2005) identified two distinct regions in RIN4 protein as key determinants for RPS2 regulation in *Arabidopsis*.

3.2.8. Alternative methods

There are also unconventional ways of producing mutants. Plant tissue culture can be used as a source for generating variability in regenerants called somaclonal variability. It can be of genetic or epigenetic nature. Genetic variability is caused by mutations or other changes in DNA (changes in ploidy, structural changes in DNA, activation of transposon and chimera rearregement) and is heritable, while epigenetic variability is caused by temporary phenotypic changes (rejuvenation). Seven wheat cultivars having some degree of resistance to *Bipolaris sorokiniana*, *Magnaporthe grisea* or *Xanthomonas campestris* pv. *undulosa* (Xcu) provide somaclonal variation for disease resistance (Mehta & Angra, 2000). The stability of somaclonal variants must be examined through several generations in order to distinguish from epigenetic changes, which is the reason for lower utility.

4. Conclusions

One of the main goals of future agriculture is to achieve durable and broad-spectrum resistance. *R* genes do not always provide durable resistance due to the evolution of pathogen that break host ETI immune system (Jones and Dangl, 2006). Mutagenesis enables the identification of wild *R* genes or the creation of novel *R* genes. Induced mutagenesis offers many benefits to agriculture, especially when there is no reliable source of resistance found in the nature that makes it impossible to introduce to susceptible cultivars by hybridization. Understanding defence responses offer the possibility to introduce new combinations of alleles from ancestral varieties into modern crop. Genetic characteristics of pea (*Pisum sativum* L.) recessive *er1* gene show similarities to *Arabidopsis*, barley and tomato resistance to powdery mildew, which is caused by the loss-of-function of MLO gene family members. An *er1* resistance line was produced by the induction of mutagenesis using alkylating diethyl sulphate that carry a single nucleotide polymorphism in the PsMLO and lead to the premature termination of translation (Pavan et al., 2011). Genetic variation in basal resistance of ancestral plants can be exploited to provide more durable disease protection, which was proven successful with introduction of *WKS1* gene from ancestral wheat *Triticum turgidum* to commercial wheat variety (Fu et al., 2009). *WKS1* gene confers partial and temperature-dependent resistance to stripe rust *Puccinia striiformis* (Fu et al., 2009). In barley, *Mlo* locus comprises different recessive alleles that confer resistance to broad spectrum of fungal pathogen causing powdery mildew and offer durable resistance.

Although mutagenesis is valuable toll to researcher, mutants produced by genetic transformation (GMOs) cause some public concerns, especially in Europe. On the other

hand, mutagenized plants are much more acceptable to consumers, breeders, environmentalist and governments. New findings of trans-generation memory of mutants, improved either by mutagenesis or transformation, open the debate if should mutagenized crops also be considered as GMO. Each mutation or transgenic event cause a stress to the cell or organism and lead to altered expression of genes. Stress event is memorized through several generations that can be explained by epigenetic modifications, although the changes are decreasing with each new generation (Molinier et al. 2006). The environmental factors lead to changes in physiology and in genome flexibility that can be transferred to next, untreated generations. Batista et al. (2008) summarized genes whose expression was altered by microarray analysis into six groups: 1) genes involved in signalling pathways and stress, 2) genes involved in regulatory pathways as second messengers, 3) gene involved in reactive oxygen species (ROS) network; 4) genes implicated in protein modification, 5) genes encoding transcription factors and 6) genes encoding for retrotransposons. They pointed out that similar phenotype does not necessarily mean similar expression profile.

Future assignments of mutation breeding are:

- to speed the use of mutations and the release of commercial varieties,
- to establish public mutant gene banks,
- to maintain free access of mutant varieties to global agriculture,
- to apply next generation sequencing techniques for evaluation of wild genetic variation of entire genomes of a population,
- to improve bioinformatics tools. The distinction between true SNPs and sequencing errors still remain problem that can be solved by programmers,
- to improve combined traits such as tolerance to abiotic and biotic stress. *R* genes play part of signalling pathway involved in many metabolic processes, so the change in disease resistance may affect other traits as well,
- to introduce mutants in organic breeding and
- to transfer findings from basic research of plant disease resistance mechanisms to other organisms and research fields, as is the use of RNA silencing in human chemotherapy.

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