Chapter 4
Breeding for Biotic Stress Resistance/Tolerance in Plants

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Contents

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Abstract The long-term goal of crop improvement for biotic stress tolerance in plants is a traditional objective of breeders. Plants must continuously defend themselves against attacks from bacteria, viruses, fungi, invertebrates, and even other plants. This chapter will therefore summarize the benefits and drawbacks of resistance versus chemical protection. Attempts will be made to provide a description on the effective genetic and molecular mechanisms that plants have developed to recognize and respond to infection by a number of pathogens and pests, such as non-host resistance, constitutive barriers and race-specific resistance, including recent advances in elucidating the structure and molecular mechanisms used by plants to cope with pathogens and pest attacks. This chapter also covers the most relevant problems in breeding for resistance to parasites and will include aspects related to specificity of defense mechanisms, specificity of parasitic ability, inheritance of resistance, gene-for-gene interaction, and durability of resistance. Major considerations in breeding for resistance to parasites, conventional sources of resistance and possible alternatives, namely mutation breeding, genetic manipulations, tissue cultures, and molecular interventions to develop plants resistant to pests and pathogens will also be dealt.

Keywords Defense mechanisms • Genetic basis of resistance • Signal transduction network • Pathogenesis related proteins • Transgenic plants

1 Introduction

Most of the problems facing agriculture in the twenty-first century relate to the growing world population, which is expected to stabilize at around 10–12 billion during the next 70 years (Heszky 2008). The almost doubled population will require a more than proportional increase in food production. During the last decade, world grain yield increased around 0.5% per year, which is three-fold lower than the population growth rate in the same period. The main task for breeders and agronomists will therefore be to increase yields while reducing the use of chemicals. The difficulties of this mission are due to: (i) the limited possibilities of expanding the cropped land area; (ii) the environmental legislation which limits the use of chemicals for disease control; (iii) climate change and the/predicted worsening of biotic and abiotic stresses; (iv) the reduced source of useful traits from crops wild relatives (Cook 2000).

Beacuse more than 42% of the potential world crop yield is lost owing to biotic stresses (15% attributable to insects, 13% to weeds, and 13% to other pathogens), a reduction in this incidence will be one of the more important possibilities for improving plant production (Pimentel 1997). Cook (2000) divided these possibilities as follows: (i) improvement of plant material (breeding for tolerance/resistance); (ii) improvement of root health (e.g. field rotation, soil tillage, soil-borne diseases control); (iii) improvement of irrigation practices (optimal water quality and availability); (iv) protection against airborne hazards (foliar diseases etc.). In this context, the development of tolerant plants to biotic stresses is therefore an important objective of plant breeding strategies with relevant implications for both farmers and the seed and agrochemical industries. In fact genetic resistance has several obvious advantages over the use of chemical pesticides or other methods for parasite control. These include nominal genetic permanency, negligible cost once cultivars are developed, and quite high efficiency. The major downside of genetic resistance to biotic stresses is the fact that selection pressure is placed on parasites populations to develop means of overcoming the resistance, thus practically limiting the time of effectiveness (Table 4.1). In this chapter the genetic, biochemical and molecular mechanisms by which plants defend themselves against attack from pathogens will be examined. In addition breeding approaches towards their improvement will be described.

Table 4.1 Overview of potential and actual losses attributable to fungal and bacterial pathogens, viruses, animals pests and weeds as well as the efficacy of the applied pest control operations in maize, wheat, rice, barley, potatoes, soybean, sugar beet, and cotton

	Pests and pathogens				
	Fungi and bacteria	Viruses	Animal pests	Weeds	Total
Loss potential (%)[a]	14.9	3.1	17.6	31.8	67.4
Actual losses (%)[a]	9.9	2.7	10.1	9.4	32.0
Efficacy (%)[b]	33.8	12.9	42.4	70.6	52.5

Source: Modified from Oerke and Dehne (2004)

[a]As percentage of attainable yields

[b]As percentage of loss potential prevented

2 Fungal and Bacterial Diseases

A plant pathogen is defined as an organism that for a part or all of its life cycle grows inside a plant; this has a detrimental effect on the plant growth and development and ultimately on yield. Several reviews have been published in this field to which the readers are referred for a more in-depth description (e.g. Hammond-Kosack and Jones 2000; Dickinson 2003). The main findings emerging from these studies indicate that pathogens have evolved specialist ways to invade plants: (i) some penetrate the plant surface directly using mechanical pressure or enzymatic attack; (ii) others pass through natural plant openings, for example, stomata or lenticels; (iii) many take advantage of existing wounds. Once inside the plant, three main colonization strategies are deployed by pathogens to use the host plant as substrate for their growth and development: (i) biotrophic organisms ensure the plant cell remains alive; (ii) hemibiotrophic organisms initially keep host plant cells alive, but then kill them at later stages of the infection; (iii) necrotrophic organisms first kill plant cells and then metabolize their contents. In this respect, pathogenesis is the term used to describe the sequence of processes from host and pathogen contact (infection, colonization and plant pathogen reproduction) to the development of the complete syndrome. A pathogen strain that causes disease is termed virulent and its success can be attributed to several factors such as : (i) rapid and high rate of reproduction during the main growing season for plants; (ii) a very efficient dispersal mechanism and long-term survival capacity; (iii) high capacity to generate genetic diversity through haploidy and subsequent sexual reproduction.

2.1 Plant Defense Mechanisms

An overview of forms of plant resistance, defined on the basis of innate and acquired resistance, and their mechanisms of response to pathogens is given in Table 4.2. According to Kiraly et al. (2007) innate resistance is exhibited by the plant in two forms: non-specific (nonhost or general) resistance, which is effective against several pathogenic species or several strains (races, biotypes, pathovars) of a single pathogen, and specific resistance. In the latter case, one plant cultivar (variety) can resist infection by one or a few pathogenic strains.

Although plants are in continual contact with potential pathogens, a successful infection is rare. The ability of a particular plant species to prevent successful colonization by a given pathogen species is referred to as nonhost resistance. The molecular basis of nonhost resistance is poorly understood, but presumably relies on both constitutive barriers and inducible responses that involve a large array of proteins and other organic molecules produced, respectively, prior to infection or during pathogen attack (cf. Jones and Dangl 2006; Ferreira et al. 2007). This is in contrast to the vertebrate immune system, in which specialized cells devoted to defense are rapidly mobilized to the infection site, where they either kill the invading organisms or limit their diffusion.

Table 4.2 Overview of forms of plant resistance

Resistance phenomenon	Mechanism
1. *Innate resistance*	
1.1. Non specific, general resistance	
Non-host resistance	HR; ROS; BAX inhibitor-1; PEN genes
Basal resistance against bacteria	Flagellin/FLS2 interaction; ROS; antimicrobial compounds
Race non-specific mlo resistance and quantitative resistance to fungi	Cell wall thickening; Antimicrobial compounds; ROS
Resistance to necrosis-inducing stresses	High antioxidant capacity
1.2. Specific resistance (cultivar/pathogenic race specificity)	
Extreme resistance-symptomless gene-for-gene resistance	Unknown
Rx-resistance against viruses without HR	
Symptomless reaction to rust pathogens, no visible HR	
Gene-for-gene resistance	ROS; Phytoalexins; Phenol oxidation; Stress proteins
R-gene ↔ Avr-gene interaction associated with the hypertensive response (HR)	
Resistance to pathogen toxins	Enzymatic detoxification; Lack of toxin recept
Gene silencing	Recognition and decomposition of foreign RNAs with ribonucleases
2. *Acquired resistance*	
After a primary infection and acquired resistance develops against a second infection "Stress memory"	Accumulation of SA; Stimulated antioxidants; Gene silencing; Rhizobacterial induction

Source: Modified from Kiraly et al. (2007)

The typical preformed, constitutive defenses are morphological, structural, and chemical barriers. An example of morphological barrier is the height of lips of stomatal guard cells (Hoch et al. 1987). Certain fungal rust pathogens possess specific detection mechanisms that sense the height of stomatal guard cell lips encountered on susceptible plants. Moreover, waxes, cutin, suberin, lignin, cellulose, callose, and cell wall proteins act as structural barriers that are rapidly reinforced upon the pathogen infection process (Punja 2001). Plants also constitutively produce a variety of secondary metabolites (e.g. phenolics, saponins, terpenoids, steroids and glucosinalates), and antifungal proteins, many of which act as antimicrobial compounds during defense (see Dangl and Jones 2001, for a review). These compounds may be present in their biologically active forms or stored as inactive precursors that are converted to their active forms by host enzymes in response to pathogen attack or tissue damage.

Plants employ two modes of their innate immune system to contrast pathogen infections (see Tsuda and Katagiri 2010, for a recent review). The first mode of immunity is referred to as pattern-triggered immunity (PTI) that is triggered by

molecular patterns common to many microbial types. The second mode is triggered by recognition of pathogen effectors and is called as effector-triggered immunity (ETI). At least some cases of PTI and ETI extensively share downstream signaling machinery, that is, PTI and ETI appear to be mediated by an integrated signaling network. However, activated immune responses in ETI are more prolonged and robust than those in PTI. Furthermore, the previous cited authors have reported that network analysis has also revealed that synergistic relationships among the signaling sectors are evident in PTI, which may amplify the signal, whereas compensatory relationships among the sectors dominate in ETI, explaining the robustness of ETI against genetic and pathogenic perturbations. Thus, plants seem to use a common signaling network that differs in PTI and ETI.

There is evidence that induced or acquired resistance includes the hypersensitive response (HR), a form of programmed plant cell death, cell-wall strengthening, and the expression of various defense-related *R* genes (R, resistance; Staskawicz et al. 1995) that mediated recognition of pathogen effectors. The *R* genes activate a series of defense signaling cascades and pathogenesis-related (*PR*) gene expression to generate systemic acquired resistance (SAR); this primes the plant for resistance against a broad spectrum of pathogens (Durrant and Dong 2004; Dangl and Jones 2001). This multicomponent response requires a substantial commitment of cellular resources, including extensive genetic reprogramming and metabolic re-allocation. Thus, defenses are kept under tight genetic control and are activated only if the plant detects a prospective invader.

2.2 Genetic Basis of Resistance

Genetic analysis of disease resistance in plants began over 100 years ago when Biffin (1905) reported that resistance in wheat to stripe rust (*Puccinia striiformis*) was inherited as a single recessive Mendelian trait. Since this initial work, many genes conferring resistance to pathogens in crop plants have been characterized, and the genetic basis of pathogenicity (virulence/avirulence) has been studied in many plant pathogens. This knowledge culminated in the development of the gene-for-gene hypothesis by Flor (1971) based on genetic studies of the interaction between flax and the flax rust pathogen, which has provided a framework for much if not all of the work on disease resistance in the years since. In genetic terms, resistance is generally defined by the mode of inheritance, with broad distinctions between oligogenic (controlled by one or few genes of major effect) and polygenic (controlled by many genes of low individual phenotypic effect) resistance.

2.2.1 Qualitative Resistance

Evidence made it clear that many cases of resistance were inherited in a simple way. Most characterized resistance genes are dominant in action; for example the *Hm1*

gene of maize conferring resistance to *Cochliobolus carbonum* race 1, a causal agent of northern leaf spot of maize, secretes an HST known as HC-toxin, which interferes with a histone deacetylase (HD) altering host gene expression (Brosch et al. 1995). Indeed, resistant (insensitive to HC-toxin) maize lines contain a dominant resistance gene *Hm1*, which encodes for a NADPH-dependent reductase whose function is to reduce (detoxify) the HC-toxin that the fungus produces to cause disease in susceptible maize (Johal and Briggs 1992). However, some recessive resistance genes have proven important sources of durable resistance – e.g. gene *Sr2* conferring resistance to stem rust in wheat (McIntosh et al. 1995); gene *mlo* for mildew resistance in barley (Jørgensen 1994). The barley *Mlo* gene has been cloned and encodes a transmembrane protein that is a negative regulator of cell death and powered mildew resistance (Büschges et al. 1997). Notably, further research showed that functional *Mlo* genes also exist in *Arabidopsis* (Consonni et al. 2006). Thus, *mlo*-mediated non-specific resistance to powdered mildew might be a more widespread phenomenon among plants than previously hypothesized. There are also many examples of resistance genes that display partial dominance (gene dosage dependence; e.g. the resistance gene *Lr9* in wheat to *Puccinia recondita*; Samborski 1963).

Dominance or recessiveness of resistance genes is, however, not absolute and can even be governed by the attribute used to measure the disease phenotype (Johnson 1992), genetic background, pathogen isolate or environment. Examples of oligogenic resistance are known in which additive and non-additive interaction occurs between genes at separate loci. The genes *Lr13* and *Lr34* in wheat interact in an additive manner to confer resistance to leaf rust, not only with each other, but also with other genes for resistance to leaf rust (Kolmer 1992). Non-additive gene interaction occurs when two genes in the host are only effective when present together. In such cases, the genes in the host are referred to as complementary. Baker (1966) demonstrated complementary action of the genes *Pc3* and *Pc4* conferring resistance to *Puccinia coronata* in the oat cultivar Bond.

Resistance to bacterial infections is not well developed as virus and fungal resistance, partly because bacterial diseases are a main problem only in crop plants like potato, rice, and some fruit trees. Similarly to fungal diseases the most effective type of protection is genetic resistance, which is based on single dominant or semidominant genes. Different classes of *R* genes cloned from various plant species were characterized and tested for their ability in conferring resistance against bacterial pathogens. For example, among these, a map-based cloned *Xa21* gene from rice, gave resistance to bacterial blight , a serious disease in rice caused by *Xantomonas oryzae. Xa21* specify a receptor- like kinase formed bi LRRs in the putative domain and a serine-threonine kinase in the putative intracellular domain pv. *oryzae,* after transferring this gene from a wild rice species to a cultivated indica variety (Wang et al. 1996). Moreover, the last cited authors found on the broad-spectrum resistance of transgenic rice with the *Xa21* gene against 29 diverse isolates, suggesting that a single cloned gene is sufficient to confer multi-isolate resistance. In the same way, the resistance gene *Bs2* from pepper was transferred to tomato, which then had resistance to bacterial disease (Tai et al. 1999). Infiltration of different maize lines

with a variety of bacterial pathogens of maize, rice and sorghum has permitted to identify a maize gene, *Rxo1*, which conditions a strong HR to the non-host bacterial pathogen *X. o.* pv. *Oryzicola* (Zhao et al. 2004). The same locus carries a gene (designated *Rba1*) controlling resistance to the maize and sorghum bacterial stripe pathogen *Burkholderia andropogonis*. It was surprising that the same locus controlled resistance to two of only four bacterial pathovars tested. This suggests that this locus may condition defense reactions to other bacterial pathogens.

2.2.2 Quantitative Resistance and QTLs

Quantitative resistance, in contrast to qualitative resistance, is generally considered as partial resistance in a particular cultivar (Young 1996). This type of disease resistance is controlled by multiple loci, referred to as polygenes or quantitative trait loci (QTLs), and does not comply with simple Mendelian inheritance. Examples of such polygenetically inherited resistance are the partial resistance in potato to *Phytopthora infestans*, in maize to *Puccinia sorghi*, and in barley to *Puccinia hordei* (Parlevliet and Zadoks 1977).

Although genetically complex forms of disease resistance are still poorly understood, an effective strategy for studying complex and polygenic forms of disease resistance is known as QTL mapping, which is based on the use of DNA markers (see Young 1996, for a review). With QTL mapping, the roles of specific loci in genetically complex traits can be described; this has also permitted insight to be gained into fundamental questions that have puzzled researchers in the field of plant pathology for decades. Although results of QTL mapping indicate that it is generally not the case, there are examples of several (>10) QTLs involved in quantitative resistance; however, it is much more common to find only three to five loci: frequently, 1 or 2 QTLs predominate.

QTL mapping may also help to determine whether individual QTLs are race-specific or not, and when there is an indication of specificity, the degree to which partial resistance differs between races. For example, quantitative resistance to *P. infestans* in potato was initially described as race-nonspecific (Van der Plank 1982). Dissecting the contributions of individual QTLs, it was clearly demonstrated that loci show distinctly different resistance effects against different pathogen races (Leonards-Schippers et al. 1994). Indeed, only 5 of the 11 statistically significant genomic regions showed no specificity against just two races tested, while the others were significant against just one. Moreover, genetic mapping with DNA markers makes it possible to ask whether homologous resistance genes exist in related plant taxa and may help to test the hypothesis that QTLs are simply variants of qualitative resistance loci that have been (partially) overcome by their respective pathogen. For instance, in rice blast, 3 of the QTLs mapped to the same marker intervals as previously identified qualitative blast resistance genes. It is conceivable that these QTLs represent allelic variants of the known qualitative resistance genes, though only more precise mapping and gene cloning can resolve this definitely. In potato late blight, 1 QTL coincided in location with a dominant, race-specific gene known as *R1*,

as well as *Rx2,* a gene for resistance to potato virus (Leonards-Schippers et al. 1994). Moreover, a second late blight QTL mapped to the same region as *Rx1,* a second major resistance gene for potato virus X. Further progress in QTL mapping technology will include the molecular cloning of the underlying genes, including those that confer partial resistance. For example, major genes such as resistance to *Pseudomonas syringae* in tomato and *Arabidopsis* have been isolated and cloned based on map position, while others have been isolated through transposon-tagging (cf. Young 1996). More recently, Hu et al. (2008), to isolate disease resistance QTLs, have established a candidate gene approach that integrates linkage map, expression profile and functional complementation analyses. This strategy has proven applicable in rice for identifying the genes underlying minor resistance QTLs in bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* and fungal blast caused by *Magnaporthe grisea* systems and it may also help to shed light on disease resistance QTLs of other cereals. The results also suggest that a single minor QTL can be used in rice improvement by modulating the expression of the gene underlying the QTL. Pyramiding 2 or 3 minor QTL genes, whose expression can be managed and that function in different defense signal transduction pathways, may allow the breeding of cultivars that are highly resistant to bacterial blight and blast.

2.3 Plant R Gene-Mediated Disease Resistance

As mentioned above, plants do not have the benefit of a circulating antibody system, so plant cells autonomously maintain constant vigilance against pathogens by expressing vast arrays of *R* genes. These genes have been genetically defined in interactions with all major classes of plant pathogens including fungi, bacteria, and viruses.

In the classic gene-for-gene model – also known as receptor-ligand model – of host pathogen interactions, *R* gene products recognize pathogen elicitor, encoded by avirulence (*Avr*) genes (Fig. 4.1). Resistance gene-mediated resistance is a host-specific defense and can only be activated when both *R* gene and corresponding *Avr* gene are present (Staskawicz et al. 1995); the absence of either component results in disease, which is typically associated with damage and a reduction in yield of the host plant.

Because gene-for-gene are operative in defense response to fungal, bacterial and virus pathogens, and because the host defense responses are similar irrespective of the type of pathogen, common recognition and signal recognition transduction mechanisms are postulated to underlie the gene-for-gene relationship. A single model of pathogen response will therefore be given herein.

Currently, there are two alternative mechanisms to explain this model: direct and indirect interaction (Fig. 4.2). Direct interaction suggests that the pathogen *Avr* effectors interact with plant R proteins directly to trigger *R* gene-mediated resistance signaling. For example, the rice *R* gene *Pi-ta* was initially shown to directly interact with *AVR-Pita* from *Magnaporthe grisea* but no interaction between

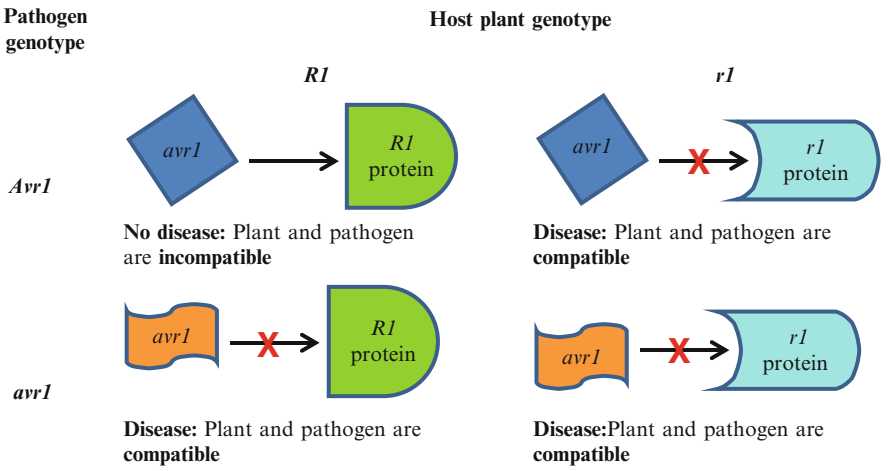

Fig. 4.1 Flor's gene-for-gene model. For resistance (incompatibility) to occur, complementary pairs of dominant genes, one in the host and one in the pathogen, are required. An alteration or loss of the plant resistance gene (*R* changing to *r*) or of the pathogen avirulence gene (*Avr* changing to *avr*) leads to disease (compatibility) (Modified from Hammond-Kosack and Jones 2000)

AVR-Pita and its susceptible allele *Pi-ta* was observed (Jia et al. 2000). In addition, a direct interaction was recently observed between the flax *L* alleles and corresponding flax rust *Avr* genes, which provides evidence for direct, allele-specific interaction between R proteins and diverse Avr proteins (Dodds et al. 2006). Conversely, most studied data prefer the indirect model also called "guard" hypothesis (Jones and Dangl 2006). In this model, R proteins act as "guardees" to monitor the variation/modification of host proteins after coupling with the corresponding Avr effectors. Moreover, evidence suggests that plants possibly employ alternate mechanisms to prevent pathogens from invading in different plant-pathogen interaction systems, which maintain a good balance between different R proteins and Avr effectors to coordinate the conflicts of interaction between different *R* genes and varied Avr effectors in host-pathogen co-evolution (Van der Hoorn et al. 2002). In these two models, R proteins may detect Avr effectors with conserved structure through direct interaction, or could indirectly recognize diverse unrelated pathogen factors after Avr proteins couple with their virulence targets (Chisholm et al. 2006). However, when and how R proteins detect diverse Avr effectors directly or indirectly is unclear and requires in-depth analysis.

More recently, Hann et al. (2010), by reviewing published results on bacterial virulence effectors and their activities, indicated that the major virulence strategy for plant pathogenic bacteria is a deployment of effector molecules within the host cytoplasm. As summarized by these authors (i) each bacterial strain possesses a set of 20–30 effectors which are redundant and interchangeable, and interact promiscuously with host targets, (ii) bacterial effectors have weak, somewhat nonspecific interactions with host molecules, targeting conserved protein domains or molecular

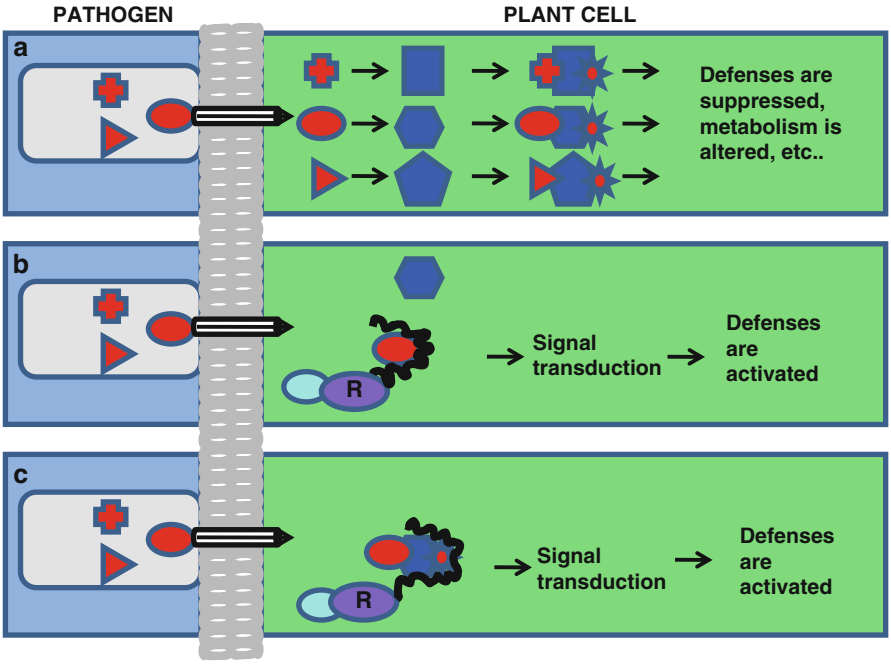

Fig. 4.2 Interactions between pathogen Avr proteins (*red*) and plant R proteins (*blue*). Once inside the plant cell, pathogen Avr proteins target host proteins that control defense responses, metabolism or other plant process that affect pathogen virulence. Panel (**a**): the plant cell does not express an R protein that is capable of recognizing any virulence protein, plant defenses are not activated, disease results from the collective action of the virulence proteins. Panel (**b**): classic receptor-elicitor hypothesis, in which an R protein (*purple*) directly binds a virulence protein, so a complex signal transduction network is activated, which in turn triggers plant defense responses. Panel (**c**): guard hypothesis in which an R protein (*guard*) detects a modified host protein (*guardee*, *red star*), eventually as a complex with the "attacking" virulence protein (Modified from McDowell and Woffenden 2003)

structures, (iii) these structures have been coopted by the plant defense machinery as accessory proteins or baits in NB-LRR complexes, and (iv) the link between pathogenicity and immunity apparently lies in the molecular (enzymatic) activities of each effector. Although, the direction of evolution of host immune components with respect to effectors is unclear, some authors have suggested a positive-negative selection model in which positive selection for effectors is balanced with strong negative selection for specific effectors by NB-LRR complexes. The clearest example for such a positive-negative selection scenario is presented by AvrPto and AvrPtoB, which suppress PRR receptor kinases and are recognised by the Prf NB-LRR complex in tomato (Zipfel and Rathjen 2008). Thus, the two levels of pathogen perception may interact to slow pathogen evolution, which is important when recognition specificities are innate and cannot be acquired.

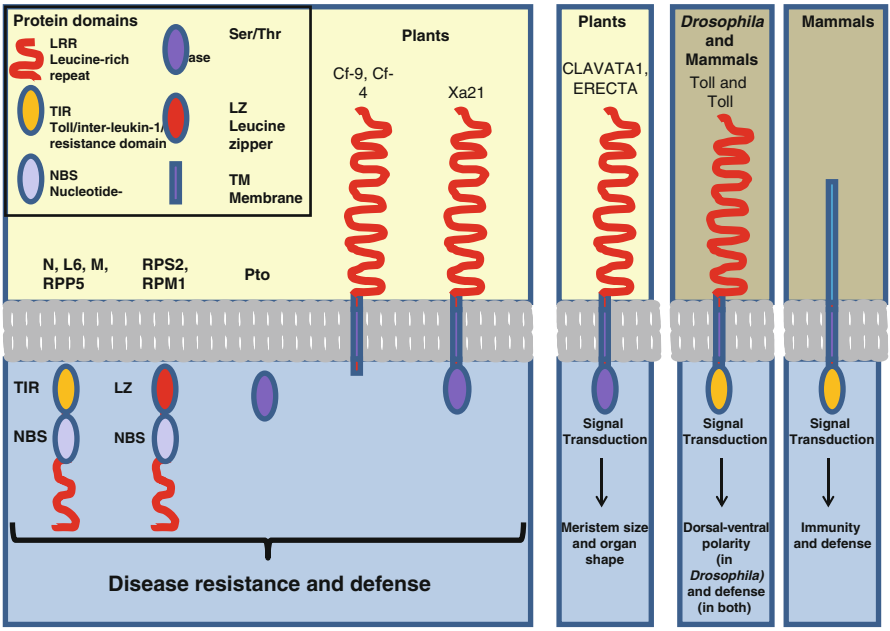

Fig. 4.3 Schematic diagram illustrating several plant resistance proteins and various plant LRR-containing proteins synthesized during the defense response. For comparison are included structurally related proteins that are involved in various aspects of plant development as well as other eukaryotic proteins that coordinate development and the induction of the immune response in animals (Modified from Hammond-Kosack and Jones 2000)

2.3.1 Structure of *R* Genes

The strong phenotypes and natural variability at *R* loci have been exploited by molecular geneticists to clone the *R* genes and investigate their molecular modes of action. To date, over 70 *R* genes have been cloned and some of them have been well characterized (for reviews see Jones and Dangl 2006; Shan et al. 2004; Toyoda et al. 2002). Notably, these studies have not only provided a large body of information on the structure, function and evolution of *R* genes that control resistance to diverse pathogens, but also have generated useful genetic materials to engineer novel resistant cultivars. In addition, some critical defense signaling components, such as NDR1, EDS1, RAR1, and SGT1, have been identified in *R* gene-mediated resistance signaling, which provide important clues to understanding the mechanism of *R* gene-mediated defense signaling (Lin et al. 2007; reviewed by McDowell and Woffenden 2003; Rathjen and Moffett 2003).

Examination of the predicted R protein sequences, based on protein domains that are described in Fig. 4.3, reveals the existence of shared sequence motifs that can be grouped into several superfamilies. The large majority of genes cloned so far belong to the nucleotide-binding site (NBS), leucine-rich repeat (LRR), a motif with homology

to the cytoplasmic domains of the *Drosophila* Toll protein and the mammalian interleukin-1 receptor (TIR), a coiled-coil (CC) or leucine zipper (LZ) structure, transmembrane domain (TM), and protein kinase domain (PK). According to these features, at least four principal classes are distinguished among most *R* genes as follows: NBS-LRR, Receptor-like kinase (RLK), LRR-TM and TM-CC (Fig. 4.3). The NBS-LRR genes represent the largest class of *R* genes, and encode proteins with a variable N-terminal domain of approximately 200 amino acids (aa), connected by a predicted NBS domain of approximately 300 aa and a more variable tandem array of approximately 10–40 short LRR motifs. The NBS-LRR genes are further categorized into three subgroups based on the motif within their N-terminus: TIR group, CC or LZ group and non-motif group. Furthermore, studies regarding the NB-LRR signaling pathway have been recently summarized by Eitas and Dangl (2010). The main findings reviewed by these workers indicate that (i) two NB-LRRs can function together to mediate disease resistance against pathogen isolates, (ii) the NB-LRR protein fragments that are sufficient to initiate defense signaling, and (iii) importantly, distinct fragments of different NB-LRRs are sufficient for function. Finally, the cited authors described that accessory proteins (e.g. Pto) and highly related Pto-like kinases have a significant role in regulating the function and down-streaming host genes in NB-LRR signaling.

As more *R* genes have been cloned in recent years, new motifs or structures have been uncovered in R proteins. *RRS1-R* from *Arabidopsis*, conferring resistance to *Ralstonia solanacearum* strain GMI1000 with a type III effector PopP2, which belongs to the YopJ/AvrRxv protein family encodes a typical TIR-NBS-LRR protein, but containing a transcriptional factor WRKY domain in its C-terminus (Deslandes et al. 2002). The WRKY domain is highly conserved and is composed of a region of about 60 aa containing a heptapeptide WRKYGQK in its N-terminus and a zinc-finger motif, which plays a crucial role in regulating plant defense responses (Journot-Catalino et al. 2006).

Recently, the plant resistance gene *Pi-d2*, conferring gene-for-gene resistance to the Chinese rice blast strain, ZB15, encodes a novel type of receptor-like kinase R protein with a predicted extracellular domain of a bulb-type mannose specific binding lectin (B-lectin) and an intracellular serine-threonine kinase domain (Chen et al. 2006). Among all isolated *R* genes, three novel genes do not belong to any of the four types: *Xa5* encoding a TFIIA transcription factor (Jiang et al. 2006), *Xa13* with homologous to nodulin MtN3, and *Xa27* without any hits in the available protein database, *R* genes encode putative receptors that respond to the products of '*Avr* genes' expressed by the pathogen during infection.

2.3.2 Evolution Mechanism of *R* Genes

In plants many *R* genes are located in clusters that comprise several copies of homologous *R* gene sequences arising from a single gene family (simple clusters) or colocalized *R* gene sequences derived from two or more unrelated families (complex clusters), and may also contain unrelated single genes interspersed between the

homologs (reviewed in Friedman and Baker 2007). *R* clusters range in size from two tandem paralogs to large complexes spanning several megabases. The largest *R* clusters characterized to date include the maize *Rp1* cluster (~1–52 homologs per haplotype; Smith et al. 2004), the lettuce *Dm3* (aka RGC2) cluster (~12–32 homologs per haplotype), and the potato major late blight resistance (MLB) cluster (~45 homologs per haplotype; Kuang et al. 2005).

Genic and intergenic sequence repeats within *R* clusters, generated by duplications and transposon insertions, provide a structural environment that permits mispairing during recombination, giving rise to unequal crossovers and interlocus gene conversions (McDowell and Simon 2006). Intergenic unequal crossover has the potential to place *R* genes in new structural contexts that may alter expression, whereas intragenic mispairing generates chimeric genes that may encode novel functions. Both types of unequal recombination will also result in altered gene copy number within the cluster (gene duplication on one chromosome and loss on the other) according to the number of genes present in the region between the mispaired recombination sites.

Sequence exchanges (unequal crossovers and/or gene conversions) have been reported in several *R* clusters (Kuang et al. 2005) and are associated with genic diversity, characterized by sequence shuffling and chimeric genes, and haplotypic diversity, characterized by a variable number of *R* homologs within the cluster and a general loss of syntenic/orthologous relationships between haplotypes. Furthermore, unequal recombination, at the *Rp1* cluster and at the *Cf4/9* cluster, has been shown to generate novel *R* haplotypes with resistance specificities that differ from either parent. Interestingly, similar clustering phenomena are seen at (a) virulence loci in multiple, evolutionarily distinct pathogen genomes (Dodds et al. 2006). This accumulated evidence indicates that *R* clusters facilitate rapid evolution via recombinatorial mispairings, generating novel *R* gene sequences that may encode altered specificities or have altered expression patterns.

2.3.3 Signal Transduction Network

The most relevant features of the defense condition indicate that the activation of defense responses in plants is initiated by host recognition of pathogen-encoded molecules called elicitors (e.g., microbial proteins, small peptides and oligosaccharides). A simplified model for signal transduction in plant defense provided by Yang et al. (1997) is given in Fig. 4.4. According to this model the interaction of pathogen elicitors with host receptors (many of which may be encoded by *R* genes) likely activates a signal transduction cascade that involves oxidative burst i.e. reactive oxygen species (ROS), calcium fluxes, ion channel fluxes, G-proteins, nitrogen oxide production, and dephosphorilation of unknown pathogens. Subsequent transcriptional and/or post-translational activation of transcription factors eventually leads to the induction of plant defense genes (Zhu et al. 1996). In addition to eliciting primary defense responses, pathogen signals may be amplified through the generation of secondary plant signal molecules such as salicylic acid (SA) (Durner et al. 1997).

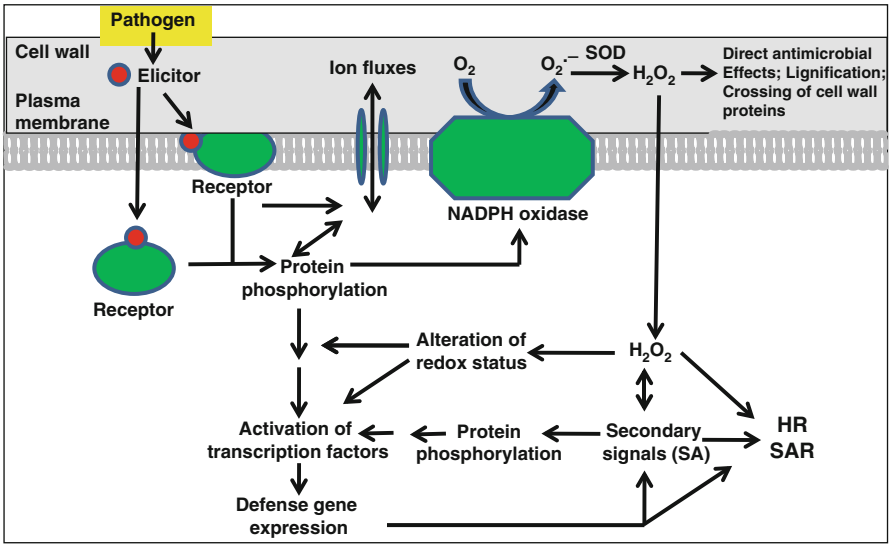

Fig. 4.4 Simplified model for signal transduction in plant defense responses. Host recognition of pathogen elicitors initiates early signaling events such as protein phosphorylation/dephosphorylation, ion fluxes and oxidative burst. Subsequent transcriptional and/or posttranslational activation of transcription factors leads to induction of plant defense genes and biosynthesis of endogenous secondary signals such as SA. Additionally, the activated NADPH oxidase complex generates reactive oxygen species (ROS) such as $O_2^{\cdot-}$ and H_2O_2 that alter the redox status of plant cells and affect defense signaling. SA, ROS, as well as defense genes, all contribute to the development of HR and SAR during plant-pathogen interactions. SOD, superoxide dismutase (Modified from Yang et al. 1997)

Moreover, advances in induced defense signaling research revealed regulators of induced systemic resistance and suggest a model in which jasmonic acid (JA)-related transcription factors play a central role in establishing the primed state for enhanced resistance (reviewed in Van der Ent et al. 2009).

Both primary pathogen elicitors and secondary endogenous signals may activate a diverse array of plant protectant and defense genes, whose products include glutathione S-transferases , peroxidases, cell wall proteins, proteinase inhibitors, hydrolytic enzymes (e.g., chitinases and β~-1,3-glucanases), pathogenesis-related (PR) proteins – PR proteins are host-encoded, abundant proteins induced by pathogens and many of them have antimicrobial activity *in vitro* or when overexpressed in transgenic plants, and phytoalexin – phytoalexins are low-molecular-weight, antimicrobial compounds (e.g., phenylpropanoids, terpenoids), whose synthesis is induced following pathogen infection, plus biosynthetic enzymes, such as phenylalanine ammonia lyase and chalcone synthase (reviewed in Yang et al. 1997). Notably, more recently Clay et al. (2009) have identified a metabolic pathway for glucosinates, previously identified as important in avoiding damage by herbivores, as a component of plant defense response against microbial pathogens.

Table 4.3 Families of pathogenesis-related proteins

Family	Type member	Biochemical properties	Molecular mass range (kDa)
PR-1	Tobacco PR-1a	Unknown	15–17
PR-2	Tobacco PR-2	B-1,3-glucanase	30–41
PR-3	Tobacco P,Q	Chitinase class I, II, IV, VI, VII	35–46
PR-4	Tobacco R	Chitin-binding proteins	13–14
PR-5	Tobacco S	Thaumatin-like	16–26
PR-6	Tomato inhibitor I	Proteinase inhibitor	8–22
PR-7	Tomato P69	Endoproteinase	69
PR-8	Cucumber chitinase	Chitinase class III	30–35
PR-9	Tobacco "lignin forming peroxidase"	Peroxidase (POC)	50–70
PR-10	Parsley "PR-1"	"Ribonuclease-like"	18–19
PR-11	Tobacco class V chitinase	Chitinase class V	40
PR-12	Radish Rs-AFP3	Defensins	5
PR-13	Arabidopsis THI-2.1	Thionons	5–7
PR-14	Barley LTP4	Lipid Transfer Proteins	9
PR-15	Barley OxOa (germin)	Oxalate oxidases	22–25
PR-16	Barley OxOLP	Oxalate oxidase-like protein	100 (hexamer)
PR-17	TobaccoPRp27	Unknown	Unknown

Source: Modified from Van Loon et al. (2006)

2.3.4 Pathogenesis Related Proteins (PR)

The concept of PR was introduced in 1980 to designate any protein encoded by the host plant but induced only in pathological or related situations (Antoniw et al. 1980), including viral, fungal or bacterial infections, parasitic attack by nematodes. The main criterion for inclusion among the PR is that the protein concerned is newly expressed upon infection, although not necessarily in all pathological conditions (Van Loon 1999). The term PR-like protein was proposed to accommodate proteins that are present in healthy plants, being induced essentially in a developmentally controlled, tissue-specific manner, and that are not synthesized in response to pathogen infection or related stresses. The distinction between PR proteins and PR-like proteins became blurred by the discovery of specific PR proteins in healthy tissues and the induction of PR-like proteins upon pathogen attack. Recently Van Loon et al. (2006) introduced the general term "inducible defense-related proteins" to include proteins that are mostly non-detectable in healthy tissues and for which induction at the protein level has been demonstrated after pathogen infection. The PR proteins encompass several different groups of structurally and functionally unrelated proteins, which have been grouped into protein families according to coding sequence similarities, serological relationships, and/or enzymatic or biological activities (Tarchevsky 2001; Ferreira et al. 2007). Seventeen classes are now considered, numbered in the order in which they were discovered, from PR-1 to PR-17 (Table 4.3; Van Loon et al. 2006); members of several of these families were demonstrated to have damaging actions on the structures of the parasite in *in vitro*

bioassays, thus exhibiting antifungal activity and supporting a possible role for these proteins in plant defense. PR-1 and PR-5 (thaumatin-like proteins and osmotins), are thought to create transmembrane pores and have therefore been termed permatins; PR-2 (β-1,3-glucanases) and PR-3, 4, 8 and 11 (chitinases), which attack β-1,3-glucans and chitin respectively, components of the cell walls in most higher fungi (Honeé 1999). PR-6 proteins (proteinase inhibitors) may target nematodes, whereas the PR-7 protein (an endoproteinase) may be involved in microbial cell wall dissolution (Jordá et al. 2000). The PR-9 family may act in cell wall reinforcement by catalyzing lignifications, leading to enhanced resistance against multiple pathogens (Passardi et al. 2004). Some members of the PR-10 family exhibit a weak ribonuclease activity, suggesting a role in defense against viruses (Park et al. 2004). Members of the PR-12 (defensins), PR-13 (thionins) and PR-14 (lipid transfer proteins) families display antibacterial and antifungal activities (Epple et al. 1997; García-Olmedo et al. 1995; Lay and Anderson 2005). PR-15 (oxalate oxidases) and PR-16 (oxalate oxidase-like proteins) proteins generate hydrogen peroxide that may be toxic to attackers or stimulate plant defense responses (Hu et al. 2003). PR-17 proteins, as yet uncharacterized, have been detected in infected tobacco, wheat and barley (Christensen et al. 2002).

2.4 Breeding Strategies

It is a common notion that if a new character is added to a breeding program, either gains in other characters will suffer (for example yield potential), or the program will have to be expanded by a factor which is dependent on the selection rate. The breeder therefore has to consider whether breeding for disease or pest resistance is economically sustainable. This decision depends mainly on the frequency and extent of disease in the area where the crop is to be grown and on the economic damage caused by the parasite. Moreover, the breeder should identify which type of defense mechanism is most suitable for introduction into the crop. He may choose major-gene resistance with complete expression. Advantages of this type of resistance are: (i) the simple inheritance, which is of course very desirable in a breeding program; (ii) the normally complete protection of the crop from the parasite. A risk in choosing complete major-gene resistance is that this type of resistance may turn out to be transitory. There are, however, cases where major-gene resistance has been durable.

The next step in a breeding program for resistance is the identification of an appropriate source of resistance. The genotypic variation within the genotypes of the crop and often also within related species should be investigated. Source for resistance may be found in taxonomic groups that are more or less distantly related to the crop, such as the cultivar itself, commercial cultivars, landraces, wild progenitors, related species and genera. The potential sources of resistance indicated here are listed in the order in which complications for the breeder increase. The main problems are: (i) failure to secure crosses between the crop and the donor species,

(ii) sterility of the interspecific or intergeneric hybrid, and (iii) poor intrachromosomal recombination (Harlan and De Wet 1971). Many generations of backcrossing are usually needed to remove undesirable traits introduced together with the resistance.

2.4.1 Conventional Methods

In many cases, a single *R* gene can provide complete resistance to one or more strains of a particular pathogen, when transferred to a previously susceptible plant of the same species. For this reason, *R* genes have been used in conventional resistance breeding programs for decades (Austin et al. 2002). *R* gene-mediated resistance has several attractive features for disease control. When induced in a timely manner, the concerted responses can efficiently block pathogen growth with light collateral damage to the plant. No input is required from the farmer and there are no adverse environmental effects. Unfortunately, *R* genes are often quickly defeated by co-evolving pathogens. In this context, it is worth noting that durable resistance is defined as "resistance that remains effective when a cultivar is grown widely in environments favoring disease development" (reviewed by Michelmore 2003). The concept of durable resistance makes no assumptions concerning the mechanisms or genetic control of resistance, and has proved a very useful concept in disease resistance breeding. Although it is now easier to identify and deploy useful *R* genes, the problem of durability remains. Many *R* genes lack durability because they can be defeated by a single loss-of-function mutation in the corresponding *Avr* gene (thereby rendering the pathogen 'invisible'). Because individual *Avr* genes often make only incremental contributions to virulence, pathogens can afford to alter or discard an *Avr* gene with little or no fitness penalty (Leach et al. 2001).

Traditional breeding strategies have used *R* genes 'one at a time' in crop monocultures. Such homogeneous host populations exert strong selection for mutation of the relevant *Avr* gene, and then become extremely vulnerable to the emergent pathogen. As an alternative to single-gene deployment, multiple *R* genes ('pyramids') can be bred into individual plant lines (Pink 2002). In principle, these pyramids require the pathogen to accumulate mutations in multiple *Avr* genes to escape detection. This is unlikely to occur if the mutations have a strong cumulative effect on virulence. Another approach is to grow a mixture of lines, each expressing a different *R* gene(s), in the same plot. A susceptible line can be included in the mixture, to reduce the selection pressure for mutations in *Avr* genes (Mundt 2002). A multiline protocol was tested in a study, with striking success (Zhu et al. 2000). Pyramiding and multiline deployment have not been widely used, owing to the time required for breeding assortments of *R* genes into elite cultivars. However, these strategies will become much more practical as the approaches described earlier are further developed. Furthermore, many *R* genes recognize only a limited number of pathogen strains and therefore do not provide broad-spectrum resistance. Furthermore, introgression of *R* genes into elite cultivars by conventional breeding is a lengthy process. However, recent molecular-level insights into the function of R proteins and downstream signal transduction pathways might provide strategies to remedy these deficiencies.

2.4.2 Applications of Marker-Assisted Breeding

Though polygenically inherited forms of resistance is nearly always durable (Parlevliet 1979) this type of inheritance is more difficult to handle in breeding programs. In particular, backcross programs to introduce polygenes from wild relatives of the crop are heavy or not easily managed. In many agricultural crops a low level of infection is acceptable, and partial resistance may be combined with other control measures, such as the application of pesticides. In the specific case of disease resistance, marker-assisted breeding may have a special role. In this respect, pyramiding several major resistance genes into a valuable genetic background is simplified via the use of marker-based selection (Song et al. 1995). Studies have indicated that pyramiding resistance QTLs can achieve the same level or even a higher level of resistance than that conferred by an *R* gene (Castro et al. 2003a, b; Richardson et al. 2006). This should be especially helpful when screening for one resistance gene interferes with the ability to screen for another, a frequent occurrence in disease resistance breeding. Rather than screen sequentially for the inheritance of single resistance (or simultaneously through progeny screens), individuals that have retained all of the genes of interest can be selected based on DNA marker genotype.

Similarly, gene deployment can be speeded-up via the use of marker assisted breeding. This approach, in which cultivars with complementary sets of resistance genes with differing race-specificities are grown by farmers, aims at achieving durable disease protection. In theory, the capacity to pyramid or deploy genes of interest is not restricted to major, single locus resistance genes. With QTL mapping, partial resistance loci can be treated as Mendelian factors and manipulated just like any major gene. This includes resistance alleles that apparently come from otherwise susceptible parents (Wang et al. 2006), providing the potential for selecting transgressively resistant genotypes. Consequently, QTLs from diverse donors can be quickly introduced into a desirable genetic background or deployed in a set of cultivars. Information about the race-specific (or race-nonspecific) nature of partial resistance loci can obviously play a key role in this process.

2.4.3 Alternative Possibilities for Resistance

It is worth noting that alternative possibilities of obtaining resistance have been exploited. These include mutation breeding and transgenic technologies.

Mutation Breeding

A mutagenic treatment may convert a susceptible genotype into a resistant one. If the mutation is a point mutation, the resistant mutant will be identical to the original cultivar, except for its resistance. Usually, however, there are undesirable side-effects of the mutagenic treatment. Several other genes may also have undergone changes,

or the mutation for resistance has undesirable pleiotropic effects. As a consequence, the selection of a resistant mutant should be followed by further breeding efforts (i.e. backcrossing) to produce a commercially acceptable cultivar.

Transgenic Prospects

Currently, there are no fungus-resistant transgenic crops on the market. However, a number of reports have shown promising results in field trials. One example is a potato line that is resistant to late blight (Song et al. 2003). Late blight, caused by the oomycete *Phytophthora infestans*, is infamous as the cause of the Irish potato famine in the nineteenth century and still today causes serious crop losses around the world. The gene that was introduced into the potato line was called *RB* and came from a wild Mexican potato species called *Solanum bulbocastanum*.

There are also prospects for transgenic use of single *R* genes that have previously been proven durable. For example, the pepper gene *Bs2* has provided long-standing resistance against bacterial spot disease, caused by the bacterium *Xanthomonas campestris*. *Bs2* has been cloned from pepper and shown to encode a NB-LRR protein (Tai et al. 1999). *X. campestris* is also a significant pathogen of tomato and a pepper *Bs2* transgene works effectively in tomato against *X. campestris*. Recently cloned *R* genes with potential use against fungal pathogens include the barley *Rpg1* gene and the tomato *Ve1* and *Ve2* genes (Kawchuk et al. 2001). *Rpg1* has provided remarkably durable resistance to stem rust for decades, while *Ve1* and *Ve2* target *Verticillium* species that cause wilt in many different crops. The *Ve* genes can provide resistance to different *Verticillium* species and are functional in potato when expressed as transgenes. The *Rpg1* and *Ve* genes are also interesting from a basic research standpoint because they have novel structural features that distinguish them from previously characterized *R* genes. Additionally, it will be particularly interesting to determine whether these genes can be used as prototypes to identify additional *R* genes by sequence similarity. Additional useful genes might be unearthed through investigations of so-called ‘non-host resistance’ (Heath 2000). This term refers to interactions in which all varieties of a plant species are resistant to all strains of a particular pathogen species (as opposed to intraspecific variability, which is observed for *R* gene-mediated resistance). Because non-host resistance is not genetically variable, this trait has not been amenable to classical genetic analyses. However, experimental tools now available in model plants (e.g. large-scale forward and reverse genetic screens) have made non-host resistance more accessible to genetic dissection. For example, *Arabidopsis* and tobacco are uniformly resistant to many microbes that plague crops (e.g. *P. infestans*, which caused the Irish potato famine) (Kamoun 2001). Moreover, it is worth noting that certain signal transduction components are used in *R* gene resistance and for some non-host resistances (Peart et al. 2002). Thus, it might be possible to identify effective resistance genes against crop pathogens from model species and transfer them to crops. It will be of great interest to determine whether non-host resistance results from natural pyramiding of *R* genes, and/or from use of *R* genes that recognize virulence factors that

are essential for the pathogen to cause disease. Note that non-host resistance might result from several mechanisms (Heath 2000) and it is possible that genetic dissection of non-host resistance will provide unanticipated tools for engineered resistance.

Efforts to transfer *R* genes from model species to crops, or between distantly related crops, could be hampered by a phenomenon termed 'restricted taxonomic functionality' (RTF) (Tai et al. 1999). For example, *Bs2* and several *R* genes from tomato can function as transgenes within related species from the same family (Hulbert et al. 2001) (e.g. tobacco, potato and pepper, which belong to the *Solanaceae*). However, *Bs2* does not function in *Arabidopsis*, nor does the *Arabidopsis RPS2* resistance gene function in tomato (Tai et al. 1999). The molecular basis of RTF is unknown but might reflect an inability of the R protein to interact with signal transduction components that have diverged in the heterologous host (Ellis et al. 2000). It remains to be seen whether RTF is a general attribute of *R* genes. A recent report suggests that it will indeed be possible to transfer certain *R* genes between distantly related species: the *Arabidopsis RPW8* gene provides broad-spectrum resistance to powdery mildew in *Arabidopsis* and in tobacco (Xiao et al. 2003). A solution to the RTF problem might be developed as we gain a deeper understanding of *R* gene signaling.

In bacterial the tomato disease resistance gene *Pto* gives race-specific resistance to *Pseudomonas syringa*e pv. Tomato carrying the *avrPto* gene, by overexpressing *Pto* race- non specifical resistance in transgenic tomatoes exhibited a superior tolerance (Tang et al. 1999). Similarly to fungal resistance, overexpression of PR proteins or transfer of PR protein genes, such as the barley lipid transfer protein (LTP2; Molina and García-Olmedo 1997), from other sources has led to increase resistance against bacterial infection. In several plant species, bifunctional enzymes with lysozyme activity have been detected which are hyphotised to be involved in defense bacteria. After transfer of the bacteriophage T4 lysozyme gene, transgenic potatoes had reduced susceptibility toward *Erwinia carotovora atroseptica* infection (Duering et al. 1993). Transfer of the human lysozyme gene resulted to increase resistance against both fungal and bacterial diseases (Nakajima et al. 1997)

In several studies, transgenic plants expressing cereal ribosomal inactivating proteins (RIPs) were used to test defense properties attributed to this group of proteins (reviewed in Balconi et al. 2010). Research in our laboratory showed that transgenic tobacco plants, expressing the maize RIP *b-32* gene driven by the *wun 1* promoter, had increased protection against infection from the soil-borne fungal pathogen *Rhizoctonia solani*. Similarly, other research with wheat transgenic lines, indicated that maize RIP b-32 protein was effective, as anti-fungal protein, in reducing Fusarium head blight (FHB) symptoms. To further explore the antifungal activity of the maize RIP b-32, transgenic maize plants have been developed containing the *b-32* coding sequence driven by a constitutive *35SCaMV* promoter. In this study four homozygous independent maize transgenic lines, with differential ectopic expression of RIP b-32, were evaluated, in comparison with plants expressing RIP *b-32* only in the endosperm, for response to *Fusarium verticillioides* colonization by leaf tissue bioassays. The identification of progenies with a differential RIP b-32

expression in the leaves was useful for setting up pathogenicity experiments. Transgenic progenies expressing RIP b-32 in leaf tissues resulted as less susceptible than the negative control when evaluated for response to *F. verticillioides* attack, showing significantly reduced colony diameter around the inoculated leaves; a good correlation between the RIP b-32 content in the leaves and the level of resistance to Fusarium attack was observed. Collectively, these results confirm that the incorporation of maize RIP *b-32* gene and the ectopical expression of RIP b-32 protein, appears to be an effective and reliable tool in crop disease management programs.

3 Viral Diseases

Viruses are among the most important kinds of plant pathogens causing severe economic losses in many crops. Genetic resistance is one of the different systems to protect crops from virus infection, including also the control of biotic vectors, the use of virus-free plant materials, and practices for avoiding the transmission. If available, the genetic resistances are still considered the most effective mean for avoiding the viral diseases. The study of virus resistance genes implies several questions regarding the molecular mechanisms involved in the plant-virus interaction. Resistance to viral diseases has been divided, similarly for other pathogens, into two principal families: non-host and host-resistance. Host resistance to plant viruses has been more investigated and considers the case where all genotypes within a plant species show resistance or fail to be infected by a particular virus. More than 80% of reported viral resistances is monogenic. The remainder shows quantitative inheritance. About half of monogenic resistances show dominant inheritance. In most but not all cases, dominance has been reported as complete (Fraser 1986). Furthermore, a third important family of host resistance has been identified, initially in studies involving TMV, i.e. SAR. This response can be activated in many plant species by diverse pathogens that cause necrotic cell death (Ross 1961), resulting in diminished susceptibility to later pathogen attack. As SAR has recently been reviewed (Durrant and Dong 2004), this topic is not discussed further here. Virus-induced gene silencing, another induced defense mechanism to virus disease, has also been reviewed recently (Baulcombe 2004).

3.1 Genetic Basis of Virus Resistance

Plants contain many (>200) resistance genes (*R*) that confer resistance to different strains of viruses. The largest class of *R* genes encodes a NB-LRR type of protein. So far all *R* genes that have been isolated conferring resistance to viruses belong to this class. It is tempting to assume that the *R* gene products directly or indirectly interact with other (host or virus-encoded) factors, but this still needs to be demonstrated. Approximately 30% of the *R virus* genes have been tagged with molecular genetic markers that can be exploited for indirect selection via genotype, for locating *R* genes in plant genomes, and for gene isolation. Relatively few QTL for plant viral resistance have been tagged or genetically mapped.

Considerable progresses were also made in the study of *R* gene structure and in the explanation of mechanisms of resistance and viral evolution. The advent of molecular methods has demonstrated that *R* genes may represent different loci with shared or independent evolutionary history, or different alleles at the same locus. There are cases where resistance alleles at two or more loci are required to observe the resistant response. A well-known example is the *bc-u* system in *Phaseolus vulgaris* for resistance to a wide array of BCMV pathotypes. Resistance is observed only when the *bc-u* locus is homozygous recessive and one or more pathotype-specific genes, *bc-1*, *bc-2*, and *bc-3*, are also homozygous at one or more of three additional loci (Drijfhout 1978). In *Capsicum*, for example, full resistance is observed to another potyvirus, *Pepper veinal mottle virus*, only when the resistance alleles *pvr1*2 (formerly *pvr2*2) and *pvr6* are homozygous (Caranta et al. 1996).

One type of *R* gene cluster contains a set of genes, showing similar inheritance and resistance phenotypes that control very closely related viral genotypes. A notable example of this pattern occurs in *Pisum sativum* where recessive resistance has been mapped to two *R* gene clusters on linkage groups II and VI. This type of *R* gene cluster occurs widely in monocots and dicots. For example, the wheat *Bdv1* allele conferring resistance to *Barley yellow dwarf virus* (BYDV) is linked to fungal *R* genes *Lr34* and *Yr18* (Singh 1993). A comprehensive genome-wide analysis of *R* gene clusters and their distribution within a series of crop genomes linked by comparative genetic mapping has been published for the *Solanaceae* (Grube et al. 2000). This study clearly demonstrated that *R* gene clusters often occur at homologous positions in related genomic regions, even in genera that diverged tens of millions of years ago. These clusters may therefore consist of evolutionarily related sequences that diverged to control very different pathogen groups.

The typical R-gene-mediated responses include host-cell death (HR) like that occurring in fungi and bacteria and will not here repeated. The induction of this response is preceded by a specific recognition of the virus, and in many cases this is based on matching (dominant) gene products of the plant (produced from dominant resistance genes, *R* genes) and the virus (avirulence genes). Dominant resistance is frequently associated with the HR response (Fraser 1986), possibly due to the frequent use of HRas a diagnostic indicator for field resistance by plant breeders. HR, induced by specific recognition of the virus, localizes virus spread by rapid programmed cell death surrounding the infection site, which results in visible necrotic local lesions. HR-mediated resistance is a common resistance mechanism for viruses and for other plant pathogens. Because the extent of visible HR may be affected by gene dosage (Collmer et al. 2000), genetic background, environmental conditions such as temperature, and viral genotype, etc., schemes that classify or name virus *R* genes based on presence or absence of HR may obscure genetic relationships. Over the past 10 years significant advances have been made in the understanding of the molecular basis of the HR-mediated resistance. More than 40 plant *R* genes showing monogenic dominant inheritance have been cloned (reviewed in Kuang et al. 2005). Several of these confer resistance to plant viruses (Martin et al. 2003).

Few resistance genes have proved exceptionally durable. Genetic resistance often fails because a resistance-breaking (RB) pathogen genotype increases in frequency.

Based upon data obtained predominantly from plant resistance to fungi, polygenic resistance is often presumed to be more durable than monogenic resistance. The analysis of polygenic resistance traits tends to be much more complex than monogenic or oligogenic traits, so researchers often focus on monogenic resistance because it can be studied and utilized more readily.

3.2 Resistance Mechanisms

The natural resistance mechanisms underlying virus resistance in plants have been largely treated in several reviews (Goldbach et al. 2003) and will be here briefly summarized. The main finding emerging from these studies indicates that the genetic material of viruses may be either DNA or RNA, and may be single- or double-stranded. Approximately 77% of characterized plant viruses possess a single plus-(messenger) sense strand of RNA. Infection of plant tissue requires damage to the cell wall and/or plasma membrane which, for insect-borne viruses, is achieved by the penetration by the insect stylet during feeding. Once inside the cell, the virus particle is uncoated to release its nucleic acid, and for at least some plus-stranded RNA viruses, such as tobacco mosaic virus (TMV), uncoating is achieved by cytoplasmic ribosomes which also translate the RNA. Plant virus nucleic acids are not integrated into the host genome. Common translational products amongst most, if not all, viruses, include coat protein, one of more proteins involved in the replication process, and factors involved in the systemic transmission of the virus away from the site of infection. The genomes of cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV) contain seven and five open reading frames (ORFs), respectively, which function in the replication and movement of the viral DNA, symptom development, and encapsidation. Genome replication for positive-strand RNA viruses occurs in the cytoplasm, apparently utilizing the translation apparatus of the host. Plant virus movement proteins (MPs), in association with various components of the cytoskeleton of the host cell, facilitate transport of nucleoprotein complexes or virus particles into adjacent cells by way of modified plasmodesmata, channels between plant cells. The processes controlling long-distance transport of virus particles or viral nucleic acids within the phloem are distinct from those controlling movement between mesophyll cells. Once inside the phloem, a rapid movement of virus particles has been documented; for some viruses (e.g. TMV), the coat protein (CP) is necessary for this process; however, for other viruses the CP protein may not be involved.

3.3 Breeding for Viral Diseases

3.3.1 Conventional Strategies

One of the most important durable successes of plant breeding for virus resistance was the development of sugar beet with the source of resistance to rhizomania (Fig. 4.5). The disease is caused by the virus BNYVV (Beet Necrotic Yellow Vein Virus)

Fig. 4.5 Section of sugar beet root infected by rhizomania

transmitted by the fungus *Polymyxa betae*. Rhizomania is widespread in many Europeans countries and available data indicate a spread on 60% of total sugar beet cultivated area. Damage to the sucrose production can cause up to a 80–100% yield loss (Biancardi et al. 2002). Three forms of virus have been classified (A, B and P) according to the structure of RNA.

The rhizomania symptoms are evident especially on the roots as: (i) excessive proliferation of the rootlets assuming a beard-like appearance around the tap root; (ii) constrictions of the root tip leading to a wineglass shape; (iii) necrotic rings in the root tip section. Diseased beets, if analyzed, show low sugar content, processing quality, etc. Immunoenzymatic tests (ELISA) performed on the roots can easily quantify the infection.

A and B types there are often associated, while the P is always alone and has been localized only near Phitivier (France). The use of ELISA test for the determination of virus content in the storage root has significantly contributed to the selection of resistant varieties. These genotypes have allowed survival of the sugar beet crop in many cultivated areas. After the discovery of the first resistant materials of

Italian origin, derived from sugar beet progenitor *Beta maritima*, with multigenic resistance, defined "type Alba", more efficient sources of monogenic resistance were introduced, also these derived from *Beta maritima*, allowing optimal productive performance also in infected soils. The sugar beet cultivars with the "Rizor" source of resistance, developed in 1985 by De Biaggi, was the first variety showing an optimum level resistance in heavily infected fields. Later, after some years, the source "Holly" has been isolated on materials of USDA's origin. These two sources of resistance have a good heritability and few cycles of selection are sufficient to improve the resistance trait. Resistance such as "Holly" is classified as monogenic, like that of type "Rizor". The two resistant traits have been mapped very close on several genetic maps. Other sources of resistance have been found recently in these wild beets also belonging to the *Beta vulgaris* L. ssp. *maritima* (L.) Arcang, that is the ancestor of the cultivated beets. Among these, the source named "WB42", developed at the USDA in Salinas (California, USA), is stirring a considerable interest. Studies are still in progress to determine the relationship between the two major sources of resistance (Holly and Rizor).

Genotypes carrying the monogenic sources of resistance frequently exhibit different levels of expression, probably due to the presence of minor genes interacting with the major allele in heterozygous individuals. The resistant varieties used today, when tested in severe disease conditions applied in greenhouses, display no more than 80% resistant plants. Improvement of this percentage should allow better sugar yield even in severely diseased fields. Since the resistance in commercial varieties is usually transmitted by the pollinators, this goal should be possible using varieties in which all plants carry the genes of resistance at least in heterozygous conditions. This result is becoming possible by: (i) using resistant pollinators and seed-bearers; (ii) analyzing with molecular markers for rhizomania resistance genes all pollinating and/or seed-bearing beets employed in seed production; and (iii) discarding the recessive and, when possible, the heterozygous plants. In addition, further sugar yield improvements should be possible combining in the same variety the different sources of resistance. This would be essential where the known sources of resistance appear to be overcome by suspected mutations of BNYVV, or in presence of the more pathogenic strains of the virus (Liu and Lewellen 2007; Panella and Lewellen 2007).

3.3.2 Transgenic Strategies

A knowledge of the molecular biology of aspects of virus function has led to the proposal of three general strategies for plant protection against viruses using genetic engineering techniques: (1) modified cross-protection; (2) the use of satellite nucleic acids; and (3) the use of anti-sense RNA.

Transgenic crop varieties have been successfully deployed to control viral diseases. One of the classical examples is the success with the genetically engineered papaya, which virtually rescued the papaya industry in Hawaii from the threat of the dreaded ring spot disease (Yeh et al. 1998). The transgenic approach would be more appropriate in

situations where sufficient levels of resistance to the virus are not available in the related germplasm or the resistance is difficult to transfer by normal crossing techniques because of either reproductive isolation or linkage with other undesirable traits. The production of virus-tolerant transgenic plants has been based on several approaches which follows into two categories: protein-mediated and RNA-mediated protection (reviewed by Prins et al. 2008). In most instances, a gene coding for the complete viral protein or part of a viral protein has been introduced into the crop by transformation.

One strategy used to obtain virus-resistant plants is to transfer genes from the pathogen itself into the plant (pathogen-derived resistance). The most widely used approach is to express the virus coat protein in transgenic plants. In theory, the expression of viral genes disrupts viral infection or symptom development. The first virus-resistant variety to be grown was papaya ringspot virus (PRSV)-resistant papaya (Ferreira et al. 2002; Gonsalves 1998). The GM variety contains a gene that encodes a PRSV coat protein, a strategy that mimics the phenomenon of cross protection. In true cross protection, infection by a mild strain of a virus induces resistance to subsequent infection by a more virulent strain (reviewed in Culver 2002). This approach has been extended to other plants, for example rice (Hayakawa et al. 1992), plum tree (Ravelonandro et al. 1997), tomato (Kaniewski et al. 1999), and peanut (Magbanua et al. 2000). Field trials have also been performed, in the USA, with coat protein-mediated virus-resistant wheat, soybean, sugarcane, sugar beet, cucumber, sweet potato, grapefruit, pineapple, and papaya (USDA 2002).

Another form of pathogen-derived resistance is the use of viral replicase genes (or RNA-dependent RNA polymerase genes), which presumably act by post-transcriptional gene silencing. This technique has been used to confer resistance to potato leafroll virus in potato, to barley yellow dwarf virus in oats, cucumber mosaic virus in tomato, rice tungro, spherical virus in rice, and wheat streak mosaic virus in wheat (Koev et al. 1998; Gal-On et al. 1998; Huet et al. 1999; Sivamani et al. 2000). Because different degrees of virus resistance have been obtained with coat protein-mediated resistance, attempts have been made to ameliorate resistance against cucumber mosaic virus via satellite RNA, especially in tomato (Stommel et al. 1998). This approach has caused controversy, however, because a single-point mutation in the satellite RNA can transform it into a harmful necrogenic form (Tepfer 1993). To protect plants against more than one virus, RIPs, have been expressed in transgenic plants. RIPs are strong inhibitors of protein synthesis and, depending on the plant species from which they originate, they have different levels of toxicity against different hosts. Poke weed antiviral protein (PAP) confers resistance to PVX and PVY in transgenic potatoes and PAPII confers resistance to TMV, PVX, and fungal infections in tobacco (Balconi et al. 2010).

On a more experimental scale are approaches to achieve virus resistance by using antibodies against the virus coat protein. Such antibodies can neutralize virus infection, presumably by interacting with newly synthesized coat protein and disrupting viral particle formation Xiao et al. 2000). Similar to RIPs, broad-spectrum antibodies might be used to protect plants against a wider range of viruses, as has been demonstrated for poty viruses.

Notably, virus-resistant transgenic crops, which offer numerous benefits to growers and consumers, need to be deployed safely after due assessment of safety considerations. However, risk assessment studies need to be realistic to provide valuable assistance to regulatory authorities for the safe and timely release of such crops (Fuchs and Gonsalves 2007).

4 Insect Diseases

Crop losses due to insects and nematodes, estimated at 10–20% for major crops, are a significant factor in limiting crop yields. To overcome this problem modern agriculture uses a wide range of insecticides and nematocides to control pest damage. However, chemical control of pests, in addition to being expensive, frequently results in negative environmental effects. The development of insect- and nematode-resistant plants is therefore an important objective of plant breeding strategies with relevant implications for both farmers and the seed and agrochemical industries. In this section attention will mainly be given to plant response to insects, indicating some specific examples related to nematodes.

4.1 Nature of Plant Resistant Mechanisms

According to Maxwell and Jennings (1980) insect resistance is defined as "those heritable characteristics possessed by the plant which influence the ultimate degree of damage done by insects". Resistance is relative and is measured by using susceptible cultivars of some species as controls. Additionally, host-plant resistance may be the result of a series of interactions between insects and plants which influence the selection of plants as hosts and the effects of plants on insect survival and multiplication. Within this context three mechanisms of plant resistance have been described: (i) non-preference (or antixenosis), (ii) antibiosis and (iii) tolerance (Painter 1958). Tolerance differs from non-preference and antibiosis in its mechanism: non-preference and antibiosis require an active insect response or lack of response. However, tolerance is more subject to variation as a result of environmental conditions than non-preference and antibiosis. The age or size and general vigor of the plant and size of the insect-resistant population also strongly influence the degree of tolerance.

In their long association with pests and pathogens, plants have evolved an impressive arsenal of defensive tools. In this respect, natural pest resistance mechanisms occurring in higher plants have been classified into preformed and inducible resistance mechanisms and throughout the last century agricultural pest control has attempted to harness these mechanisms wherever possible (reviewed in Howe and Jander 2008). Furthermore, plant traits conferring resistance to insect pests may also be classified according to the manner in which they are regulated. Some traits are expressed constitutively under the control of hard-wired develop-

mental programs, irrespective of the insect threat level. For example, reproductive tissues typically accumulate large amounts of defensive proteins and metabolites. In contrast to these preformed barriers, herbivore-challenged plants mount defense responses at the site of tissue damage and, in many cases, systemically in undamaged tissues. Moreover, to induce defensive traits, plants can minimize the fitness consequences of tissue loss by activating physiological processes, such as sequestration of sugars in below-ground tissues, which allow plants to better tolerate insect damage.

4.2 Genetic Bases in Imparting Insect Resistance

At least 30 major or single genes for insect resistance have been tagged or mapped in various crops (e.g. maize, rice, wheat, tomato, mung bean, apple), conferring resistance to species from 5 orders: *Homoptera*, *Hemiptera*, *Diptera*, *Lepidoptera* and *Coleoptera* (reviewed in Yencho et al. 2000). Each gene is known to confer resistance to only one insect species or to closely related species within the same genus. The *Mi* gene from tomato provides an interesting example because it was originally identified as a dominant gene for resistance to a root-knot nematode, *Meloidogyne incognita*. Further studies have shown that it is located at the same locus as that previously known as *Meu-1*, which provides resistance to some isolates of the potato aphid (*Macrosiphum euphorbiae)* and to the silverleaf whitefly *(Bemisia tabaci*) (Nombela et al. 2003). Interestingly, *Mi* is one of the few examples of genes for insect resistance cloned from a plant; it is a member of the nucleotide-binding, leucine-rich (MBS-LRR) repeat family of resistance genes, many members of which have been found to confer isolate-specific resistance to viruses, bacteria, fungi, and nematodes (Hammond-Kosack and Parker 2003). Another NBS-LRR protein, encoded by the melon *Vat* gene, confers increased resistance to both *Aphis gossypii* (cotton aphid) and the transmission of plant viruses by this aphid species (Dogimont et al. 2007). By analogy to plant defense against pathogens, these findings suggest a gene-for-gene interaction between the plant and the insect. However, the presumed avirulence proteins in aphid saliva have not yet been identified. Similarly, research on nematodes has identified at least 15 genes conferring resistance to nematodes in various crop species (reviewed in Jung et al. 1998). For example 2 genes for nematodes resistance have been cloned on the basis of their chromosomal position and identified by genetic complementation. The first was $Hs1^{pro\text{-}1}$ from sugar beet that confers resistance to the beet cyst nematode (*Heterodera schachtii*). The second was *Mi-1* which is responsible for the hypersensitive reaction of tomato roots after infection with *Meloidogyne* spp.

4.3 Gene Mapping and Molecular Markers

Molecular markers have been used to map the above-cited genes for insect resistance in most of the major crop species and to map QTLs for resistance to 11 species of insects from 3 orders (*Homoptera*, *Lepidoptera* and *Coleoptera*) in 6 plant species (reviewed in Yencho et al. 2000). Traits evaluated include direct measures of insect fitness or behavior (e.g. larval weight, population growth, ovipositional preference); plant damage (e.g. scores on scales of 1–9, tunnel length, leaf area defoliated); plant morphology (e.g. trichomes, leaf toughness); and plant chemical content or enzyme activity (e.g. acyl sugars, maysin, polyphenol oxidase). For any single trait scored, the number of QTLs identified varies from 1 to 10, and the percentage of variation explained by any single QTL varies from 1.3% to 58%. More recently, Pfals et al. (2007), by mapping in *Arabidopsis thaliana* QTLs for resistance agents of 2 cruciferan specialist lepidopteron herbivores (*Pieris Brassicae* and *Plutella xilostella*), identified 6 QTLs for resistance against *Pieris* herbivory and found only a weak QTL for *Plutella* resistance. Similarly Omo-Ikerodah et al. (2008), in genetic mapping QTLs affecting resistance to flower bud thrips (*Megalurotrhips sjostedti*) in cowpea, found association between 23 DNA markers and resistance to flower bud thrips. QTLs with effects on resistance were identified in five linkage groups which accounted for 77.5% of the phenotypic variation for resistance. Moreover, molecular markers can greatly speed up the identification of new resistant genes. This aspect is well documented for the Hessian fly (Hf), *Mayetiola destructor* (Say) (*Diptera:Cecidomyiidae*), one of the most destructive pests of wheat worldwide. To date, 31 major *Hf*-resistance genes (named *H1* through *H31*) have been identified from wheat and its relatives (Williams et al. 2003), but distinguishing new genes is difficult by traditional phenotypic differentiation with biotypes. Liu et al. (2005), using molecular markers, have identified a new gene or a new allele of an *H* gene, (tentatively named *Hdic*) on the short arm of wheat chromosome 1A, which confers a high level of resistance to Hf. of a known *H* gene on chromosome 1A. The broad spectrum of resistance conferred by the *Hdic* gene makes it valuable for developing Hf resistant wheat cultivars. Selection for nematode resistance has a long tradition in potato breeding; both polygenic and monogenic types of resistance have been mapped with molecular markers (cf. Jung et al. 1998). These include the *Gro1*, genic resistance to all pathotypes of the root cyst nematode . *Globodera rostochiensis*. Similarly, in soybean cyst nematode different types of resistance to *Heterodera glycine* have been mapped with molecular markers such as the *Rhg1* and the *Rhg4* loci. In barley, the nematode resistance loci *Ha1 and Ha2* have been mapped to chromosome 2*,* while a new gene*, Ha4* has been mapped to chromosome 5. In *Triticeae*, two loci*, Cre1* and *Cre3*, have been mapped.

In addition to their utility as selectable markers to facilitate breeding efforts, molecular markers can be employed to increase our understanding of the mechanisms of plant resistance to insects. By mapping QTLs encoding for specific plant physical and/or biochemical attributes associated with insect resistance, and comparing the locations of these QTLs with those identified for the phenotypic expression of resistance to a pest

species, valuable insights can be obtained into the nature of resistance. Often, these insights have both basic and applied implications that can be used to develop insect-resistant crops more efficiently. The advantages of these techniques is well illustrated by researches carried out by Byrne et al. (1998). These authors have used molecular markers and QTL mapping techniques to unravel the genetic mechanisms of resistance in maize to the corn earworm (CEW), *Helicoverpa zea*, larvae which cause considerable direct yield loss as well as development of kernel-rotting fungi. Moreover, significant negative correlations were reported between maysin concentrations of fresh silks and growth of CEW larvae in dried-silk bioassays (Wiseman et al. 1996). Because C-glycosyl flavones are synthesized via a branch of the well characterized flavonoid biosynthetic pathway, Byrne et al. (1998) hypothesized that loci of that pathway would explain a large portion of the quantitative variation in maysin concentration, and by extension, resistance to CEW. These loci were proposed as "candidate genes" in a series of QTL analyses. (A candidate gene is one that is hypothesized to affect expression of the trait of interest, either *a priori* based on knowledge of trait biology, or *a posteriori*, guided by similar locations of QTLs and genes of known function).

4.4 Direct Defense Responses

Upon attacks by insects, individual plants rely on a matrix-like variety of defense mechanisms, involving physical barriers (leaf toughness and trichomes), toxic or anti-nutritive secondary metabolites, synthesis of defensive proteins, volatile attractants and extrafloral nectars, and/or recruitment of predators and parasitoids, as well as the reallocation of resources upon attack. Additionally, a plant's defense arsenal depends on various genetic, ontogenetic, and environmental factors, which together modulate the complex defensive phenotype and outcome of the interaction.

Although it is known that plants change their primary and secondary metabolism in leaves to resist and tolerate aboveground attack, there is little awareness of the role of roots in these processes (reviewed in Erb et al. 2009). This is surprising given that plant roots are responsible for the synthesis of plant toxins, play an active role in environmental sensing and defense signaling, and serve as dynamic storage organs to allow re-growth. Studying roots is therefore essential for a better understanding of resistance and tolerance to leaf-feeding insects and pathogens. Indeed roots are increasingly recognized to synthesize secondary metabolites implicated in leaf defenses. However, the active role of roots in plant resistance against leaf herbivory implies shoot-root communication. A model of a defensive shoot-root-shoot loop in plant defense reaction has recently been provided by Erb et al. (2009) to which readers are referred for details.

4.4.1 Defensive Metabolites

A remarkably diverse array of over 200,000 low-mass natural products, known as secondary metabolites, are produced by plants. These include alkaloids, furanocoumarins, tannins, saponins, glucosinolates, cyanogenic glycosides, phenolics and benzoaxinoids. This rich diversity results in part from an evolutionary process driven by selection for improving chemical defense against microbial and herbivorous predation. For instance, several terpenoids, the most metabolically diverse class of plant secondary metabolites (>40,000 known structures), play a role in plant defense (Aharoni et al. 2005). The alkaloids, widely distributed secondary metabolites that are best known for their metabolic effects in mammals likely evolved as a defense against insect herbivory.

Benzoxazinoids are also secondary metabolites that are effective in defense and allelopathy. They are abundant in grasses, including the major agricultural crops, i.e. maize and wheat, and other Gramineae, and are synthesized in seedlings and stored as glucosides (glcs) in the vacuole (see Frey et al. 2009, for a recent review). A specific glycosidase, located in the chloroplast, catalyzes the formation of the toxic aglucon when a cell is damaged and disintegrates. DIBOA [2,4-dihydroxy-2H-1,4-benzoxazin-3-one and its C-7-methoxy] derivative DIMBOA are the prevalent representatives of benzoxazinoids in plants. Figure 4.6 gives a schematic representation of the benzoxazinoid biosynthetic pathway in maize as provided by Frey et al. (2009). It has also been shown that DIMBOA is an enzyme inhibitor of α-chymotrypsin, aphid cholinesterase and plasma membrane H^+-ATPase. The correlation between DIMBOA content and protection against insect feeding damage was especially investigated, indicating that DIMBOA can act as a feeding deterrent and reduce the viability of insect larvae, with practical application in developing maize plants with improved insect resistance, as previously suggested by Klun et al. (1970).

Plants also contain significant quantities of various polyphenolic acids, as well as their glycosides and esters. These compounds are implicated in two defense mechanisms: the phenolic fortification of cell walls and the deterrent effect of fiber content (Bergvinson et al. 1995). Free phenols, mainly 4-coumaric and ferulic acid, were implicated as factors contributing to resistance of maize against ECB and the maize weevil (*Sitophilus zeamais*), and recently, to pink stalk borer (*Sesamia nonagrioides*; Santiago et al. 2006). Notably, the transgenic expression of wheat oxalate oxidase in maize significantly increased the phenolic concentrations, mainly ferulic acid. Field testing showed that the transgenic maize exhibited more resistance to ECB than its non-transgenic counterpart. It was suggested that transgenic oxalate oxidase elicits defense responses by generation of H_2O_2 and activating jasmonic acid signaling (Mao et al. 2007).

In addition to possible synergistic effects, metabolic diversity in toxin production by individual plants can also provide defense against multiple herbivores with different feeding styles or resistance mechanisms. Recent work on glucosinolates demonstrates how natural selection for a diverse profile of secondary metabolites can provide defensive specificity. Glucosinolates are found almost exclusively in Brassicales (Hansen et al. 2008); nearly 40 different glucosinolates have been found in *A. thaliana*, and more than 100 breakdown products are likely formed after activation by the enzyme myrosinase. Experiments with four insect herbivores showed

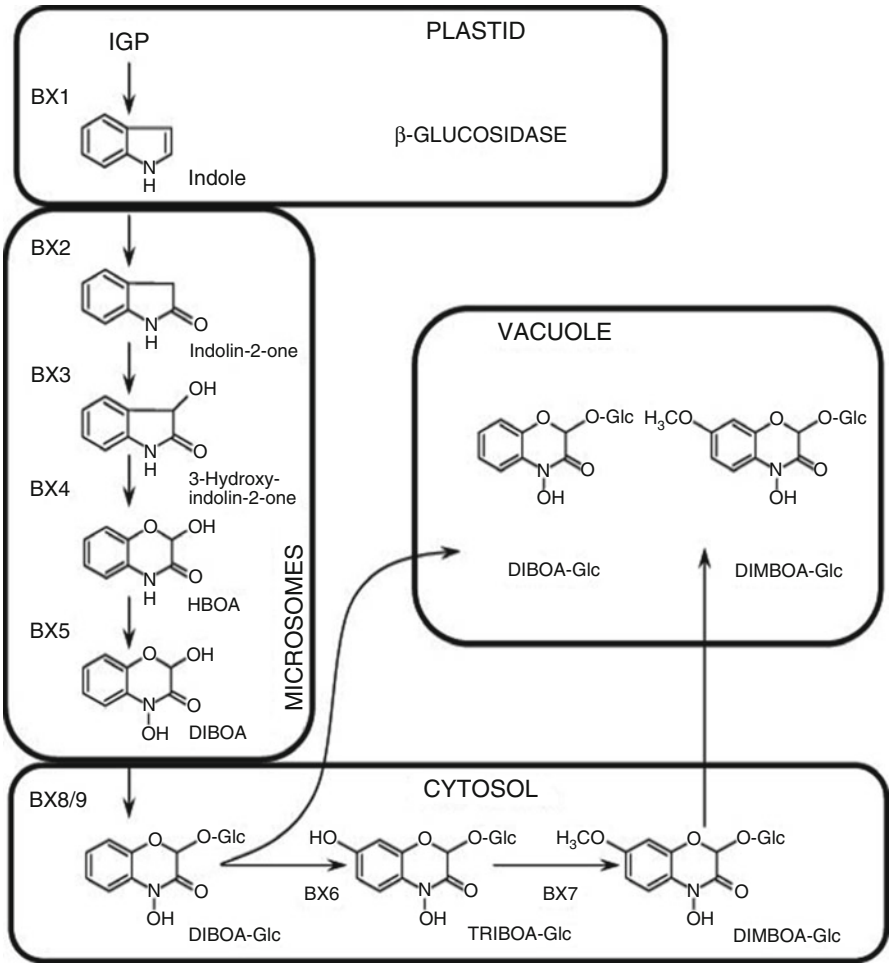

Fig. 4.6 Enzymes and intermediates of benzoxazinoid biosynthesis in maize. In this scheme the BX1 is encoded by the Bx1 gene, a homolog of the Trp synthase a-subunit, catalyses the formation of indole in the first specific pathway. The introduction of four oxygen atoms into the indole moiety that yield DIBOA is catalysed by four cytocrome P450 monooxygenases, termed BX2 to BX5. DIBOA-glc is the substrate of the dioxygenase Benzoxazinless6 (Bx6) and the produced 2,4,7-trihydroxy-2H-1,4-benzoxazin-3-(4H)-one-glc is metabolized by the methyltransferase Bx7 to yield DIMBOA-glc. The enzymatic function of BX1-BX5 is indicated. DIMBOA and DIBOA are accepted as substrates by BX8, while DIMBOA is the preferred substrate of BX9. DIMBOA-glucoside is the predominant benzoxazinoid glucoside in young maize plants. IGP; indole-3-glycerolphosphate, TRIBOA-Glc; TRIBOA-glucoside (Adapted from Frey et al. 2009)

that tryptophan-derived indole and methionine-derived aliphatic glucosinolates have differing effects on Hemiptera and Lepidoptera (Mewis et al. 2005). Indole glucosinolates, which break down in the absence of the activating enzyme myrosinase (Barth and Jander 2006), provide a better defense against *Myzus persicae* than do the more stable aliphatic glucosinolates (Kim and Jander 2007). Almost all genes

required for the production of glucosinolates, a diverse class of metabolites found in the model plant *A. thaliana* and other *Cruciferae*, have been identified (Halkier and Gershenzon 2006). As an example of how such knowledge of biochemical pathways can be applied to change plant immunity to herbivory, *A. thaliana* was engineered with three enzymes from grain sorghum to produce the cyanogenic glycoside dhurrin, thereby enhancing resistance to yellow-striped flea beetle *(Phyllotreta nemorum;* Tattersall et al. 2001).

Many defensive compounds are potentially toxic to the plants that produce them. Therefore, the storage of relatively benign precursors that are activated by herbivory is a recurring theme in plant biology. For instance, all three defensive systems mentioned in the previous paragraph include compounds that are sequestered in plants, but not activated until the onset of herbivory. DIBOA is stored as inactive DIBOA-glucoside, glucosinolates are enzymatically activated to produce toxic breakdown products, and the respiratory inhibitor hydrogen cyanide is released from cyanogenic glycosides during herbivory attack.

4.4.2 Defensive Proteins

Insect feeding triggers the expression of plant defensive proteins that exert direct effects on the attackers. The best known plant proteins supposedly involved in defense mechanisms are lectins, ribosome-inactivating proteins (RIPs), inhibitors of proteolytic enzymes, chitinases, and glycohydrolases (reviewed in Carlini and Grossi-de-Sà 2002).

Protease inhibitors (PIs), which impair various mechanistic classes of digestive proteases in the insect midgut, have been thoroughly studied for their role in the active defense response (Ryan 1990). Inhibition of gut proteases by PIs results in amino acid deficiencies that negatively affect the growth and development of the herbivore (Lison et al. 2006; Zavala et al. 2004). The effectiveness of PIs as a defense is often thwarted by the insect's adaptive ability to express digestive proteases that are insensitive to the host plant complement of PIs or that inactivate PIs (e.g. Bayes et al. 2005; Rivard et al. 2004). PIs are synthesized and stored in seeds and tubers of plants and the expression of some *PI* genes is induced in response to mechanical wounding or insect damage. For instance, local and systemic induction of expression of *MPI*, a maize protease inhibitor gene, efficiently inhibits elastase and chymotrypsin-like activities from the larval midgut of *Spodoptera littoralis* (Cordero et al. 1994); this suggests that *MPI* is a factor of maize insect resistance. Similarly, strains of tropical maize germplasm were found to exhibit resistance to Lepidoptera. In these strains, larval feeding led to the induction of a unique cysteine proteinase, *Mir1-CP*; proteinase accumulation was detected at the feeding site, localized predominantly in the phloem of minor and intermediate veins and was correlated with a significant reduction in larval growth (Lopez et al. 2007).

The plant's defensive protein arsenal also includes enzymes that disrupt insect digestive physiology and other aspects of food consumption. Members of the cysteine protease family of enzymes, for example, disrupt the chitin-rich peritrophic membrane that protects the gut epithelium (Konno et al. 2004; Mohan et al. 2006).

Plant lectins and chitinases may also target carbohydrate containing components of the insect gut (Lawrence and Novak 2006; Peumans and Vandamme 1995). Oxidative enzymes such as polyphenol oxidase (PPO) and lipoxygenase (LOX) covalently modify dietary protein through the production of reactive *o*-quinones and lipid peroxides, respectively (Wang and Constabel 2004). Because catalysis by O_2-dependent enzymes is limited by low oxygen levels in the foregut and midgut of some insect species (Thipyapong et al. 1997), an alternative possibility is that PPO and LOX act rapidly (i.e., within seconds) during tissue mastication by insect mouthparts.

The discovery of novel defensive proteins can be facilitated by proteomic analysis of gut content and feces (frass) of insect herbivores. This approach is based on the premise that defensive proteins are relatively resistant to gut proteases and, as a consequence, are highly enriched during passage of the food bolus through the insect. Application of this procedure to the tomato-reared *Manduca sexta* larvae led to the identification of isoforms of arginase and threonine deaminase , which degrade the essential amino acids arginine and threonine, respectively, in the lepidopteran midgut (Chen et al. 2005).

4.4.3 Volatile Defenses

Plants synthesize and emit blends of volatile organic compounds (e.g. terpenoids, green leafy volatiles, and ethylene) in response to damage from herbivorous insects (reviewed in Unsicker et al. 2009). The induced volatiles are proposed to serve a variety of physiological and ecological functions, including the attraction of natural enemies of herbivores, which is termed "indirect defense". Advances in plant biotechnology have allowed investigators to manipulate plant volatile emissions and demonstrate their defensive function in laboratory studies with model plants (Schnee et al. 2006; Kappers et al. 2005). The specificity of this interaction has recently been proved by Degenhart et al. (2009), by restoring the emission of a specific belowground signal emitted by insect-damaged maize roots. According to these authors, the sesquiterpene (E)-β-caryophyllene is highly attractive to the entomopathogenic nematode *Heterorhabditis megidis.* It was shown that (E)-β-caryophyllene is emitted by ancestral maize and European lines, but most American varieties have lost this ability and do not attract the nematode, which is therefore much less effective as a control agent of the larvae of the western corn rootworm, *Diabrotica virgifera virgifera*, a serious root pest in maize cultivation. To restore nematode attractions, a non-producing maize line was transformed with a *caryophyllene-synthase* gene from oregano, resulting in constitutive emissions of (E)-β-caryophyllene. In rootworm infested field plots, in which they released nematodes, transformed plants received significantly less root damage and had 60% fewer adult beetles emerge than isogenic lines. This demonstration that plant volatile emissions can be manipulated to enhance the effectiveness of biological control agents opens the way for a novel ecologically sound pest control strategy.

4.4.4 Signal Transduction Pathways

There is relatively little information about the signal transduction pathways that connect insect-specific elicitors to the plant defense responses they generate. Evidence indicates that the calcium ion (Ca^{2+}) is involved as a second messenger in many plant signaling pathways, including responses to herbivory (Maffei et al. 2007). Transient increases in cytosolic Ca^{2+} levels activate calmodulin and other calcium-sensing proteins that subsequently promote downstream signaling events, including protein phosphorylation and transcriptional responses. Although no complete mitogen-activated protein kinase (MAPK) signaling cascades (Pitzschke et al. 2009) leading to insect resistance has been identified, there is evidence that such pathways play a role in some plant-insect interactions. In tomato, *Mi-1* mediated resistance was attenuated when expression of certain MAPKs and MAPK kinases was reduced by virus-induced gene silencing (VIGS) (Li et al. 2006). VIGS studies in tomato also showed that at least three MAPKs are required for systemin-mediated defense responses to *Manduca sexta* (tobacco hornworm) (Kandoth et al. 2007).

Many inducible defenses are expressed rapidly (i.e., within hours) in undamaged leaves of herbivore-challenged plants. This systemic response, which has been reported in a wide range of plant species, provides effective resistance to future insect attacks (Karban and Baldwin 1997). Since the discovery of this phenomenon more than 35 years ago (Green and Ryan 1972), research effort has been devoted to the identification of systemic wound signals and the underlying mechanisms by which they are produced, transported, and perceived. In this respect, it was found that systemin, which is a strong peptide elicitor of PI expression in *Solanum lycopersicum*, appears to enhance systemic defenses by amplifying jasmonate synthesis in damaged leaves (Schilmiller and Howe 2005).

The plant hormone jasmonic acid (JA) and related signaling compounds (collectively referred to as jasmonates) appear to be ubiquitous signals for tissue injury and for the subsequent activation of defense responses to many, if not most, insect herbivores (Howe and Jander 2008).

Recent studies with *Nicotiana attenuata* indicate that fatty-acid amino acid conjugates (FACs) in oral secretions of *M. sexta* elicit rapid activation of MAPK activity and defense-related genes in undamaged areas of the attacked leaf (Wu et al. 2007). FAC binding to a hypothetical receptor was proposed to generate a rapidly acting, short-distance mobile signal that triggers MAPK cascades in the damaged leaf. This intraleaf systemic response is followed by the production of a second mobile signal (e.g., jasmonate) that initiates PI expression in distal undamaged leaves. These findings are consistent with the idea that multiple intercellular signals, acting over a range of distances, mediate the complex spatiotemporal responses of plants to herbivory. The fact that both *S. lycopersicum* systemin and FACs positively regulate jasmonate synthesis via a MAPK cascade (Kandoth et al. 2007) suggests that parallel signaling pathways initiated at the plant-insect interface may converge on the jasmonate pathway. In this context, evidence has been provided in the past few years to indicate that the jasmonate family of signaling compounds is involved in endogenous regulation of plant resistance to insects.

4.4.5 Breeding Strategies for Improving Plant Pest Resistance

Although there have been many notable successes in conventional breeding for improved plant resistance to insects, the breeding process is often slow and laborious, and sufficient levels of resistance have not been achieved for some pests. However, recent progress in plant transformation technologies has made it possible to produce new genetically modified cultivars with improved resistance to insect pests by genetic engineering. In addition, with advances in biotechnology, breeding of horizontal resistance, whereby resistance is based on many genes, along with genetically enhanced sustainable pest resistance with fusion genes, offer new strategies in improving plant insect resistance (Wan 2006). Genomic tools are enabling significant progress in the understanding of nematode diseases (reviewed in Bellafiore and Briggs 2010). Genome-wide expression profiling of infected plants has revealed genes that respond to infection and functional tests show they can mediate the interaction with nematodes. Several candidate effectors from nematodes have been identified and functional tests using RNAi have supported their putative roles in pathogenesis. These will increase the possibility to design novel approaches to developing crops resistant to nematode injuries.

Marker Assisted Selection

Once a major gene or QTL has been identified and mapped, marker assisted selection (MAS) and/or mapbased gene cloning can be initiated. Particularly, MAS offers the opportunity of combining different genes for a given pathosystem in a single genotype (gene pyramiding). A prerequisite for gene pyramiding is that loci are not allelic. Moreover, it would be wise to determine if the resistance genes targeted for introgression are indeed potentially durable. In choosing the resistant parent(s) for a mapping population, or choosing among existing mapping populations for a study of insect resistance, knowledge of the mechanisms of resistance involved or prior observation of the durability of a resistant cultivar in the field or in selection experiments can identify cultivars that may be sources of promising major genes or QTLs (Alam and Cohen 1998). The results of a QTL analysis itself will indicate whether the insect resistance in the resistant parent of the mapping population indeed has a polygenic basis. Insight into whether the QTLs influence multiple resistance factors acting on multiple targets within the pest can be gained by analyzing the mapping population for a series of carefully chosen traits.

Selective breeding for QTLs conferring a particular modality of insect resistance (antibiosis, antixenosis), or tolerance (Painter 1958), is another approach to achieving more durable varietal resistance. In this respect, Alam and Cohen (1998), in a QTL analysis of six traits associated with rice resistance to the brown planthopper, found a total of seven QTLs, one of which was predominantly associated with antixenosis and a second with tolerance. Most of the other QTL analyses of insect resistance conducted to date have scored chemical or morphological antibi-

otic resistance factors, or plant damage ratings under free-choice conditions, the results of which can be influenced by all three resistance modalities (see Yencho et al. 2000, for a review).

Transgenic Plants for Pest Control

Insect-resistant transgenic crops for enhancing insect pest control is currently one of the most important successes of plant biotechnology: more than 30 million hectares are planted worldwide with crops expressing *Bacillus thuringiensis* (Bt) δ-endotoxins (James 2009).

Bt is a soil bacterium that makes crystalline inclusions (Cry proteins) during sporulation (De Maagd et al. 2001; Bravo et al. 2007). These crystals dissolve in the alkaline environment of insect midguts and release protoxin molecules (65–140 kDa) that are processed by midgut proteases to yield active insecticidal proteins (60–70 kDa). These proteins interfere with the ion channel pumps and ultimately lead to the death of insect larva that ingests the crystal. They are quite specific in their host range (determined by ligand-receptor interaction) and this property has been exploited in the development of transgenics tolerant of specific groups of insect pests. The Bt δ-endotoxins are now known to constitute a family of related proteins for which 140 genes have been characterized for Lepidopterans, Coleopterans and Dipterans, and are not toxic to other organisms (Crickmore et al. 1998). Hence, they are safe insecticides and offer an interesting alternative to chemical control agents.

Cry-1 encoding genes have been introduced into several crop species such as maize, rice, cotton, tomato, potato and tobacco, with resistance target insect pests (Hilder and Boulter 1999), the modified varieties are generally referred to as Bt varieties. Transgenic Bt varieties are in several ways better than Bt spray formulation. In Bt transgenic plants, the protein is expressed in all tissues at all times, while the effectiveness of the sprays would be affected by a lack of uniform coverage and instability of the Bt protein, especially on exposure to sunlight.

As an example, several events of transgenic Bt maize have been developed over the past decade and there are currently varieties registered able to control lepidopteran and coleopteran species including the corn borer complex (European Corn Borer- ECB), southwestern corn borer – *Diatraea grandiosella* – and sugarcane borer – *Diatraea saccharalis*-, corn earworm (ECB), fall earmyworm (*Spodoptera frugiperda*), black cutworm (*Agrotis ipsilon*), and Worm Corn Root (WCR) complex (Figs. 4.7 and 4.8; Western, Northern and Mexican rootworms; *Diabrotica virgifera virgifera* LeConte). These insects can cause significant economic damage to maize production and all these transgenic varieties have provided a more effective control than insecticides, with lower cost than traditional insecticide applications and fewer logistical, health, and environmental concerns (Head and Ward 2009). Furthermore, this technology reduces the risk associated with lepidopteran pests like the European corn borer by improving yield stability. The use of multiple Bt proteins in a single product offers the potential for an extended spectrum of pest control and reduced risk of resistance evolving in the target pests.

Fig. 4.7 Damage inflicted by WCR larval feeding on transgenic (MON863) and conventional maize hybrids: the *left* root is a conventional hybrid and has been severely damaged while the root on the *right* side of the frame is protected by event MON863 (Modified from Vaughn et al. 2005)

The benefits of using Bt crops depend on many factors, most obviously the nature or the major insect pests in the area (not all are controlled by Bt) and the insect pressure in a given season (Christou et al. 2006). However, there are concerns regarding the use of Bt transgenic crops, the two major ones being: the effect on non-target organisms, and the possibility of the target insects developing resistance to the Bt protein. In this respect, several studies showed that the effect of maize pollen from Bt crops was negligible on non-target insects, including butterflies, under field conditions (Hodgson 1999). Moreover, though Bt crops have been widely cultivated since 1995, there has been no instance of a pest developing resistance (Ferry et al. 2006). However, given the experience of the diamondback moth having developed resistance to Bt sprays, the development of resistance in the insects cannot be discounted. As a proactive measure, several strategies for insect resistance management have been developed as a package for the cultivation of Bt crops. These strategies include refugia (growing a non-Bt crop on a small proportion of the area along with the Bt transgenic crop), gene pyramiding, and a high dosage of the protein in the plant to prevent any insects escaping from the Bt field (Christou et al. 2006; Ferry et al. 2006).

As alternatives to the Bt *Cry* genes, several candidate genes have been used to develop insect-resistant transgenic plants, such as protease inhibitors (Xu et al. 1996), α-amylase inhibitors (Ishimoto et al. 1996), vegetative insecticidal proteins from Bt (Estruch et al. 1996), cholesterol oxidases (Corbin et al. 1994), and toxins from predators such as mites and scorpions (Barton and Miller 1991). Transgenic tobacco plants expressing chitinase, one of the most important enzymes implicated in insect integument, have shown increased resistance to lepidopteron insects (Ding et al. 1998). Studies on rice (reviewed in Deka and Barthakur 2010) show that some of these candidates appear promising and provide an effective alternative to the Bt

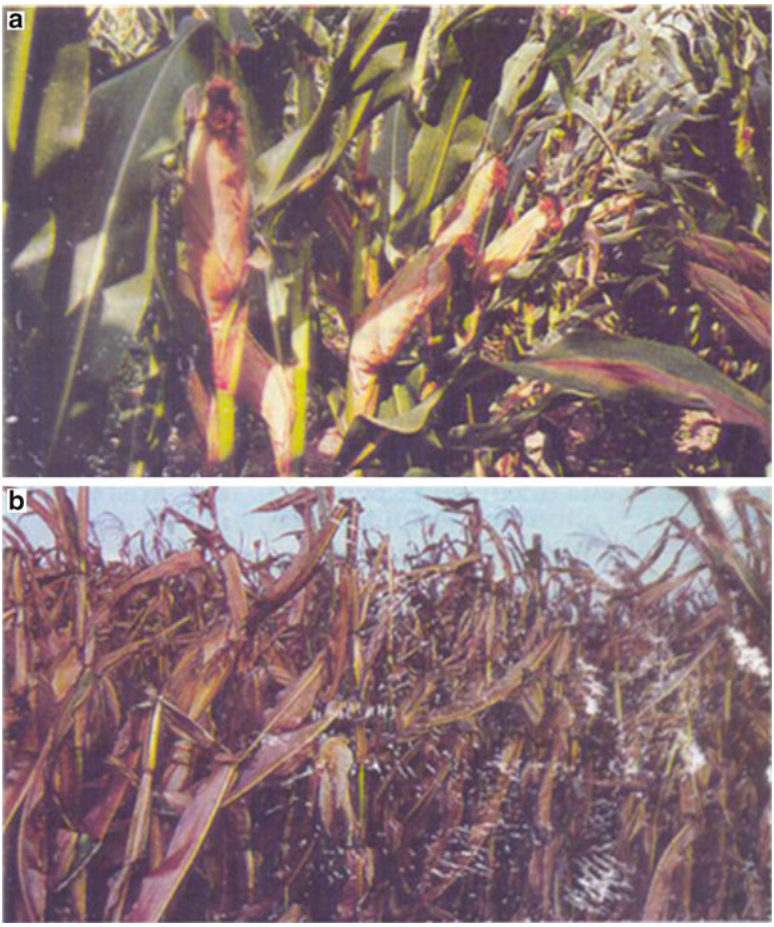

Fig.4.8 Comparison of Bt (**a**) and conventional (**b**) maize hybrids in field trials in Italy (Courtesy CRA-MAC, Bergamo, Italy)

approach. In a transgenic assay in tobacco, enhanced resistance to *Helicoverpa zea* was generated by constitutive expression of the maize ribosome inactivating protein, named RIP- b-32, suggesting that this RIP plays a role in resistance to maize-feeding insects (Dowd et al. 2003). Characteristics of b-32, the developmentally regulated expression and synthesis of a non-toxic precursor, are reminiscent of the concept of phytoanticipine in the chemical defense strategy.

A further interesting solution to the development of insect-resistant plants was provided by Baum et al. (2007) for the control of coleopteran insect pests. Through RNA interference (RNAi) technology, they demonstrated that ingestion of double stranded (ds)RNA supplied in an artificial diet triggers RNAi in several coleopteran species, most notably WCR, which may result in larval stunting and mortality. Interestingly, transgenic maize plants engineered to express WCR dsRNAs show a

significant reduction in WCR feeding damage in a growth chamber assay, suggesting that the RNAi pathway can be exploited to control insect pests via *in planta* expression of a dsRNA.

A molecular strategy for establishing nematode resistance in plant species has also been proposed with the development of artificial resistance. This can be achieved by introducing effector genes into the host plant that have a nematocidal impact. Such transgenes can encode enzymatic inhibitors that block physiological processes within the nematodes (e.g. PIs toxins) or degrading enzymes (e.g. collagenases, chitinases), toxic compounds that are ingested (cytotoxins), compounds that bind molecules (e.g. lectins, monoclonal antibodies), enzymes that interact with the nematodes, and substances that cause the breakdown of specific feeding structures (cytotoxins). More recently, RNAi was used to evaluate the role of the 16D10 secretory peptide of *Meloidogyne incognita*, which apparently interacts with the root Scarecrow protein. Expression of dsRNA in *Arabidopsis* to silence the *16D10* gene of infecting nematodes confers resistance to four *Meloidogyne* spp. (*M. incognita, M. javanica, M. arenaria*, and *M. hapla*; Huang et al. 2006). Moreover, RNAi in soybean has been used to target essential genes of *H. glycine*, causing a reduction in the number of females developing on transgenic roots (Klink et al. 2009). Despite this positive finding, results from RNAi experiments should be taken with caution: exposure to dsRNA per se is capable of causing aberrant phenotypes in both cyst and root knot nematodes (Dalzell et al. 2009). To address this problem, a novel design strategy to generate 21 bp siRNAs has been successfully applied to the potato cyst nematode, *G. pallida,* and to the root knot nematode, *M. incognita* (Dalzell et al. 2009).

5 Herbicide Tolerance

Farmers must control weeds that compete with their crops for water, nutrients and sunlight. Depending on the crop and location, weeds can decrease crop yields by 35%–100%. A number of options are available to farmers for minimizing the impact of weeds on crop productivity; one of these is the application of herbicides to the weeds. Indeed, effective weed control is a prerequisite in any crop production system if high yields and good quality are to be achieved, and herbicides have revolutionized weed control in many cropping systems and play an important role in modern agriculture. They provide economical weed control and increase the efficiency of crop production. A number of new herbicides combine high weed killing potency with low- or no-environmental persistence. However, the very effective broad spectrum herbicides available also lack selectivity, thus limiting their use in some cropping operations. On the other hand, the continuous use of the few available selective herbicides is speeding up the development of herbicide resistance in weeds; hence making effective control difficult to achieve in some crops.

5.1 Mode-of-Action and Metabolism

A large amount of knowledge exists on the mechanisms of herbicide mode-of-action and metabolism; these have frequently been described by several authors (e.g. Mazur and Falco 1989; Powles and Shaner 2001) so will not be repeated herein. Briefly, herbicides generally function by disrupting unique and essential processes in plants e.g. photosynthesis, mitosis, pigment biosynthesis or essential amino acid biosynthesis. This in turn has permitted a number of herbicide-tolerant target enzymes naturally existing in different plant species and microorganisms to be identified, as well as a number of herbicide-modifying enzymes leading to herbicide-tolerant organisms. Among the input traits offered to farmers, herbicide resistance has been the most widely adopted.

5.2 Breeding Strategies

Both crops and weeds share essential biochemical processes. Consequently, selectivity is mostly based on differential herbicide uptake between weeds and crops, controlled timing and site of application or rapid detoxification of the herbicide by the crop plants. Reliance on these natural selection processes limits the effective use of potent herbicides; hence mechanisms to impart better herbicide selectivity in crops need to be investigated.

Two approaches can be exploited. The first is the design of specific chemicals with broad selectivity for crops. This approach, however, is expensive and the products thereof may be uneconomical for use by growers, not to mention that it may also increase the already growing chemical load to the environment. Moreover, it has become increasingly difficult to discover new herbicides and even harder to come up with one that has a novel mode of action (Gressel 2002; Tan et al. 2005). The second and more popular approach to crop herbicide selectivity is the development of crop cultivars with tolerance to the already existing effective broad-spectrum herbicides so as to expand the crop options in which they can be used. Two methods can be used to develop crops with resistance to herbicides.

5.3 Conventional Methods

Conventional plant breeding utilizing strains that are known to be tolerant to specific herbicides is one approach that could confer resistance on susceptible crops from closely related species. However, this approach has limitations in that naturally herbicide resistant plants are found more among weed species than in crops. In addition, conventional plant breeding takes a long time to produce a single useful genotype.

5.4 Biotechnology Techniques

A faster approach is the use of biotechnology techniques such as *in vitro* cell culture, mutagenesis and selection in physiologically inhibitory concentrations of herbicides (also referred to as brute force selection) or genetic transformation of already existing crop cultivars with genes that confer resistance to herbicides.

5.4.1 Cell Culture and Selection

A number of mutant enzymes have been identified from plant cells in cultures. A trait of agronomic interest that may be expressed by cultured cells is herbicide sensitivity. Herbicides that interfere with basic metabolic activities are expected to inhibit growth of cultured cells as well as of the whole plant. In such instances, herbicide-tolerant mutants can be selected by culturing cells in the presence of a herbicide concentration that is toxic to normal cells, favoring subsequent identification of the herbicide-tolerant target enzyme.

Using cell culture techniques, BASF Inc. produced a maize hybrid that is resistant to the sulfonylurea herbicide, sethoxidim. In their analysis, a mutant cell line (named S2) was identified following continuous culture of maize embryo tissues under high sethoxidim selection pressure. Plants regenerated from this somaclonal mutant line were found to contain a form of the enzyme, acetolactate synthase (ALS, target of sulfonylureas/imidazolinones), which was insensitive to the herbicide. This resistance was subsequently transferred to the commercial hybrid (DK404SR) by backcrossing the S2 line with both of its parental lines. Further investigations showed that the sethoxidim tolerance was inherited as a single partially dominant allele. Similarly, Zambrano et al. (2003) selected a glyphosate-tolerant sugar cane cell line in liquid medium containing 0.8 mM glyphosate and regenerated plants that could tolerate up to five-fold the concentration of glyphosate that killed plants from unselected cells. Cell culture under lethal concentrations of certain herbicides also results in gene amplification in surviving cells that leads to resistance through the overproduction of enzymes targeted by herbicides. For example, a petunia cell line with resistance to glyphosate was selected in this manner and plants regenerated from it survived lethal levels of glyphosate (Steinrucken and Amrhein 1986). This resistance was found to be due to amplification of the gene encoding 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase that caused its overproduction in the cells. Similarly, Caretto et al. (1994) selected carrot cells and subsequently regenerated plants that were resistant to the sulfonylurea herbicide, chlorsulfuron. Resistance in these plants was due to amplification of the *ALS* gene. *In vitro* development of phosphinothricin (PPT) resistant rice has also been reported by inducing plantlet regeneration in explants collected from 7-day old seedlings on medium supplemented with sublethal doses of PPT (Toldi et al. 2000). Other *in vitro* cell selection studies have developed resistance to paraquat in tomato cells (Thomas and Pratt 1982), resistance to glyphosate in carrot and groundnut cells

(e.g. Jain et al. 1999) and resistance to a protoporphyrinogen oxidase (PPO) inhibitor in soybean cells (Warabi et al. 2001); however, no viable plant regeneration was reported in these studies.

5.4.2 Mutagenesis

Chemical or physical mutagenesis of seed, microspores or pollen followed by selection under herbicide selection pressure has also been used to develop crop resistance to herbicides. The most common mutagen used is ethyl methanesulfonate (EMS), which is efficient at producing chloroplast mutants (McCabe et al. 1990). In this strategy, seeds or pollen are treated with EMS then grown either *in vitro* or *in vivo* in the presence of a herbicide. Surviving plants are selected and grown to maturity to provide seed that is used for further screening with herbicides. Using this method, Sandhu et al. (2002) developed 21 rice lines that were resistant to glyphosate. Ashfaq-Farooqui et al. (1997) produced atrazine resistant *Solanum melongena* plants by mutagenizing seeds followed by germination and *in vitro* regeneration of plants from the resultant seedling cotyledons. Similarly, Mourad et al. (1993) isolated, by screening seedlings of M2 populations from EMS-treated seeds, a triazolopyrimidine (herbicide) resistant mutant. The resistance was found to be due to a single, dominant, nuclear gene mutation that encodes the ALS enzyme. ALS activity in enzyme extracts from the mutant was about 1,000-fold less sensitive to inhibition by triazolopyrimidine than in extracts from wild-type plants.

Ultra-violet (UV) or EMS treated microspores or pollen can be grown *in vitro* into haploid plantlets whose chromosome number can be doubled to create instant inbred lines bearing a specific herbicide tolerance trait. This method was applied by Ahmad et al. (1991) using microspore UV mutagenesis and haploid culture to develop canola plants that were resistant to chlorsulfuron. Syngenta Seeds Inc. produced the EXP19101T line of imazethapyr-resistant maize using pollen mutagenesis. In that work, EMS mutagenized pollen was used to fertilize the parent line, UE95, progeny plants were screened for tolerance to lethal doses of imazethapyr and resistant ones selected. Tolerance in these plants was found to be the result of a single nucleotide substitution within the ALS encoding gene, which gave a single amino acid change (Ser_{621} to Asn_{621}) in the sequence of the enzyme. This change prevents the binding of the herbicide to the enzyme active site, thus maintaining normal enzyme function. More recently, Venkataiah et al. (2005) reported the production of atrazine-resistant pepper (*Capsicum annuum*) plants regenerated from 3-week-old seedling cotyledons obtained from EMS treated seeds. They also noted maternal inheritance of the atrazine resistance trait. Finally, BASF (Ludwigshafen, Germany) markets non-transgenic CLEARFIELD® imidazolinone-resistant canola, wheat, sunflowers, maize, lentils, and rice, while DuPont (Wilmington, DE) markets non-transgenic STS® soybeans with tolerance to sulfonylurea herbicides. These crops all contain mutagenized versions of the ALS, which are not inhibited by imidazolinone and/or sulfonylurea herbicides (Devine and Preston 2000). Herbicides that inhibit ALS are considered low or very low use-rate herbicides with a good spectrum of weed control and are likely to remain an important part of weed resistance management programs.

5.4.3 Genetic Transformation

Herbicide tolerance is the most common trait in commercial transgenic crops, being part of 82% of all transgenic crops in 2009 (James 2009). Transgenesis for herbicide selectivity involves the identification of a herbicide resistance gene from a plant or microorganism, its isolation and manipulation for efficient plant expression (if it is of microbial origin) and (James 2009) its subsequent delivery, stable integration and expression in the cells of the target crop plant. For the most part, genes encoding for useful herbicide resistance in crops are isolated from herbicide degrading soil microorganisms.

Herbicide tolerance via genetic transformation can be conferred by one or a combination of these four mechanisms:

1. Introduction of a gene(s) encoding for a herbicide detoxifying enzyme(s);
2. Introduction of gene(s) encoding for a herbicide insensitive form of a normal functioning enzyme or over expression of the genes encoding for a herbicide target enzyme such that the normal metabolic functioning is still achieved in the plant even though some of the enzyme is inhibited;
3. Modification of the herbicide target enzyme in such a way that the herbicide molecule does not bind to it and;
4. More recently described engineering of active herbicide efflux from plant cells.

Glyphosate (Monsanto Technology LLC) is one of the most widely used herbicides in the world; it is relatively inexpensive and can be applied after the emergence of resistant crop seedlings. Nearly all broadleaf and grass weeds are eliminated, resulting in reduced competition, higher yields, and cleaner fields at harvest. Adoption of reduced and no-till practices, where dead vegetation is left in the field rather than plowed under, has been a significant unintended feature of herbicide-resistant crops, saving farmers money in fuel costs and reducing soil erosion.

Since 1996, glyphosate-tolerant or Roundup Ready crops have been developed and marketed for soybean and maize. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaf weeds and has superior environmental and toxicological characteristics, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence), as well as extremely low toxicity to mammals, birds and fish. Glyphosate resistance is achieved in Roundup Ready® brands by expression of a modified *Agrobacterium* gene encoding for the herbicide insensitive enzyme CP4 enolpyruvyl-shikimate-3-phosphate (Padgette et al. 1996). The GA21 trait for glyphosate-resistant maize relies on a modified maize *epsps* gene, but is largely being replaced by varieties with the NK603 trait which has two copies of CP4 *epsps* with different promoters for better expression in the meristems.

Traits for resistance to three other classes of herbicides have been developed, but have not reached the same level of diffusion as glyphosate resistance. Resistance to oxynil herbicides conferred by the BXN nitrilase from *Klebsiella pneumoniae* (subspecies *ozaenae*) (Stalker et al. 1988) was the first trait engineered in cotton (developed by Calgene, Davis, now Monsanto). Because glyphosate is less expensive and controls more weed species, interest in using the oxynil herbicides has waned and 2004 was the

final year of BXN® cotton sales. BXN canola was marketed by Rhone-Poulenc Canada (now Bayer CropScience, Monheim, Germany) and then discontinued.

Phosphinothricin acetyltransferase (PAT or BAR) detoxifies phosphinothricin- or bialaphos-based herbicides (glufosinate) by acetylation of the free NH_2 group of molecules. The *pat* gene is native to *Streptomyces viridichromogenes* and *bar* is from *S. hyhroscopicus* where they act in both the biosynthesis and detoxification of the tripeptide bialaphos (De Block et al. 1987). Like glyphosate, phosphinothricin herbicides control a broad spectrum of weed species and break down rapidly in the soil so that problems with residual activity and environmental impact are greatly reduced. Bayer CropScience markets this trait as Liberty Link® in several species. The *pat* and *bar* genes are also popular plant transformation markers in the research community.

As a technology, herbicide-resistant crops are a valuable tool for efficient weed control. However, doubts remain about the long-term viability of this strategy, particularly the emergence of herbicide-resistant weeds following widespread cultivation of herbicide-resistant crops (Sandermann 2006). Regardless, growers perceive that the benefits of the herbicide resistance characteristic outweigh the risks. It is clear that the widespread adoption of herbicide-resistant cultivars, particularly glyphosate-resistant crops, has dramatically impacted weed communities (Powles and Yu 2010). Weed population shifts to naturally resistant species, species with inherent biological characteristics that make the populations difficult to control, and the evolution of herbicide-resistant biotypes are real, as are the immediate economic issues attributable to the adoption of herbicide-resistant crops and the concomitant use of the herbicide. However, studies have shown the possibility of engineering multiple resistance in plants. In this respect strategies have been suggested to delay the development of herbicide-resistant weeds. These include combined or sequential use of herbicides with different modes of action, crop rotation, integrated weed management.

These studies have opened the avenue for the targeted development of crops that would reduce the environmental chemical load due to the use of different herbicides in crop rotation programs. A greater number of, and more various, modes of resistance have evolved in weeds than in other organisms because herbicides are used far more extensively than other pesticides, and because weed seed output is so prolific. Weeds have evolved unknown mechanisms, even antibiotic, as well as other drug and pesticide resistance. It is also possible that cases of epigenetic resistance may have appeared (Gressel 2009).

6 Conclusions and Future Prospects

Plant pests and diseases have major effects on agricultural production and the food supply. Although application of fungicides and pesticides has helped control plant diseases, chemical control is economically costly as well as environmentally undesirable. The development of new strategies based on a plant's own defense mechanisms for disease control is therefore critical for sustaining agricultural production and improving

our environment and health. Basic research on the genetic bases of pest and disease resistance in plants and of host-pathogen interactions has greatly improved the efficiency of manipulating disease resistance genes in practical breeding programs and resulted in the deployment of high-yielding genetically resistant crop cultivars that in some cases have been grown over vast areas, but much remains to be learnt at the interface of the genetics of resistance and crop physiology. The cloning of resistance genes and corresponding avirulence genes has indicated considerable complexity not only in structure but also in the way in which gene products interact and trigger resistance. Hence, our overall understanding of the process is still fragmentary. Furthermore, many gaps remain in our models of the defense signal transduction network and these must be bridged before we can design truly rational strategies to activate the network. Similarly, genetic mapping of plant mutations that alter herbivore resistance, or perhaps responses to purified insect elicitors, will almost certainly lead to the identification of previously unknown defense pathways.

A further point worth noting is that although major genes and QTLs for resistance to numerous pathogens and insect pests have been mapped, the usefulness of this information for MAS breeding programs has not yet been demonstrated. In this respect, the development of new technologies, such as high-throughput DNA sequencing and microarray analysis to facilitate the mapping and cloning of major genes and QTLs for routine use will provide an assemblage of new tools to facilitate the development of crops resistant to pests and pathogens, while analysis of signaling and metabolic pathways will be harnessed to increase the power of MAS and genetic engineering for crop improvement. Furthermore, the complexity of plant-insect interactions makes it difficult to determine which anatomical features, metabolites, and signaling pathways effectively limit pathogen and pest infestations. Genomic information from both host plants and pests and pathogens should accelerate the rate of discovery in this field. The field of genomics will provide powerful tools to investigate these critical factors. Transcript profiling techniques allow the simultaneous examination of thousands of genes, and can be utilized to study changes in gene expression that are transcriptionally regulated. Beyond transcript profiling, genomics also facilitates the functional analysis of genes implicated in resistance and susceptibility. As signaling cascades and metabolic pathways are elucidated in model systems and crop plants, key regulatory genes can be targeted for silencing or overexpression to study the role of these pathways in plant-insect and -pathogen interactions. To achieve a detailed understanding of plant interactions with pathogens and pests, it will ultimately be necessary to combine transcriptomic approaches with proteomic, metabolomic, and mutational analyses. While plant responses have been the focus of most transcriptomic studies, additional levels of complexity can also be analyzed with genomic tools. Investigating changes that occur concurrently within the pathogens and insects is essential to understand the basis of an effective plant defense. Therefore, knowledge accumulated in these studies will help us to establish economical and sustainable strategies to fight insects and diseases of many important crops. Beyond genome sequencing, additional effort should be targeted at identifying pathogens and insect genetic markers, studying natural variation in host plant utilization, and

developing methods such as RNA interference for manipulating insect gene expression. The development of such research tools will facilitate studies on both sides of the plant-insect and -pathogen interactions and thereby achieve a more complete understanding of plant response to pathogens and insects.

Since the first transgenic plants appeared almost two decades ago, this technology has contributed to develop new methods of crop protection aiming to increase world food production. It is certain that the methodology developed for creating *Bt* plants will ultimately make the objective of having highly productive, pest- and pathogen-resistant, and environmentally friendly crops, become a reality. The promising alternative of genetic engineering of insect- and pathogen-resistant plants, relying exclusively on the repertoire of plant defense genes, should be thoroughly investigated as it may provide solutions to the problem of increasing plant productivity for future needs. Success in developing transgenic organisms will also benefit from knowledge of the signal transduction pathways that regulate pathogenesis, particularly host range and the availability of a wide range of suitable genes that can be used to increase virulence. Genetic engineering strategies require information on the roles and consequences of these genes, leading to enhanced exploitation of the genetic resources present in plants.

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