Chapter 1

The Status and Prospects for Biotechnological Approaches for Attaining Sustainable Disease Resistance

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1.1 Introduction

Plant pathogens constitute major constraints on crop yield. In fact, a recent conservative estimate suggests that crop diseases are responsible for average annual yield losses of 10% (Chakraborty and Newton, 2011). For example, late blight of potatoes, caused by *Phytophthora infestans*, is estimated to cause annual losses of over €5 billion worldwide (Chapter 9). Another disease complex, Fusarium head blight, represents a more complex problem because the disease not only affects yield, but also contaminates food and fodder with mycotoxins which impact negatively on the health of both humans and livestock (Buerstmayr and Lemmens, 2015).

Several factors suggest that the negative impact of advancing plant diseases is increasing. For example, increasing areas of monoculture with reduced rotation to meet food productivity and profitability increases crop vulnerability to pathogenic microorganisms. This is matched by the erosion of crop management systems as witnessed by, for instance, the alarming increase in fungicide resistance within cereal pathogens (Cools and Fraaije, 2012). Furthermore, the passive spread of opportunistic pathogens has increased as a consequence of globalisation, which has promoted open markets across continents. A recent example in Europe is the East Asian fungus *Hymenoscyphus fraxineus,* a saprophyte of *Fraxinus mandshurica.* This was not known as a pathogen before colonisation and subsequent decimation of European ash (*Fraxinus excelsior*) populations was observed in Eastern Europe about 20 years ago (reviewed by McKinney et al., 2014). Climate changes are also assisting the spread of crop pathogens, as evidenced by the devastating migration of coffee rust (caused by *Hemileia*

Plant Pathogen Resistance Biotechnology, First Edition. Edited by David B. Collinge.

vastatrix) strains across the central and northern parts of South America into coffee plantations at higher altitudes, which were previously not attacked (Ghini et al., 2011).

But how can the alarming progression of crop diseases be halted? There are several methods which can contribute to the control of plant diseases. Good farm management is always a prerequisite, but other measures, especially disease resistance obtained by classical breeding and the use of pesticides, are highly important to secure food production worldwide. Furthermore, biological control and induced resistance are promising alternatives, especially in sustainable and integrated pest management strategies (Strange and Scott, 2005; Chapters 17 and 18). Yet, when taken individually, each of these methods has its limitations, and none can stand alone to solve all the problems in the effort to feed the increasing world population.

We believe that the employment of biotechnology-based approaches can contribute towards developing more effective and higher levels of disease control. The development of transgenic disease resistant plants is only one – albeit the most obvious – way of exploiting these biotechnological approaches (Campbell et al., 2002; Chen et al., 2012; Collinge et al., 2008; Collinge et al., 2010; Fuchs and Gonsalves, 2007; Gurr and Rushton, 2005a; Gurr and Rushton 2005b). Indirect biotechnological approaches, such as marker-assisted breeding, as well as the exploitation of association genetics and genomic selection, are closely-linked methods where the identification of genes responsible for specific traits can be used to develop gene-specific molecular makers to accelerate the process of conventional breeding and/or make it more efficient (Mammadov et al., 2007; Moose and Mumm 2008; Chapter 19, this volume). In addition, the development and understanding of alternative control measures, including induced disease resistance (Chapter 17) and biological control (Chapter 18), has benefited from the application of multiple biotechnological approaches coupled with molecular and cellular approaches.

Among the thousands of species of plant pathogenic microorganisms, only a small minority have the capacity to infect a broad range of plant species. Most pathogens instead exhibit a high degree of host specificity and only cause disease in one or a few hosts. On the other hand, most hosts are susceptible to a number of pathogenic species. Therefore, different host-pathogen interactions represent different challenges, agronomically, biologically and ecologically. This chapter provides an overview of the mechanisms of disease resistance, which show the greatest potential for being targeted by GM approaches, and discusses how our increased understanding of the processes of plant defence can lead to improved disease control. In addition, the technical and biological constraints which are likely to hamper the successful development of GM crops are exemplified and discussed.

1.2 Factors to consider when generating disease-resistant crops

Disease resistance or, at the cellular level, plant immunity, is complex and depends on a plethora of independent but interacting physiological mechanisms. This section introduces important pathogen and host factors involved in the interaction between pathogens and their hosts. This is the platform for successful manipulation of the plant to achieve resistance.

1.2.1 The diversity and life styles of microbial pathogens

Many types of organisms can cause diseases in plants. Prokaryotes and eukaryotes themselves are highly diverse, and the latter encompasses three important kingdoms: Fungi, Chromista (oomycetes) and Protozoa

(plasmodiophrids). In addition, viruses constitute a highly specialized type of pathogen. Collectively, this means that pathogens differ physiologically, and therefore different individual physiological mechanisms in the host plant (e.g., individual antimicrobial factors, such as chitinases or phytoalexins), will often contribute to arrest growth of specific pathogens.

Pathogenic microorganisms interacting with plants exhibit several lifestyles which are best characterized in terms of the trophic interactions at the different phases in their respective life cycles. The pathogenic lifestyles can be manifested as biotrophy (Fig. 1.1a), necrotrophy (Fig. 1.1b) or hemibiotrophy (Fig 1.1c), where the amount of direct visible damage to the host increases accordingly, dependent on the duration of infection. The biotrophic lifestyle is exhibited by pathogens that are dependent on acquiring nutrition from a living cell, so if a host cell dies, the pathogen also dies. Important examples are oomycetes causing downy mildews (e.g., *Peronspora* spp.), the ascomycetes causing powdery mildews (e.g., *Blumeria* spp.) and the basidiomycetes causing rust (e.g., *Puccinia* spp.). These biotrophic pathogens rely on specialized feeding structures termed haustoria to obtain nutrients from the host. In contrast, a pathogen with a necrotrophic lifestyle obtains its nutrition from dead cells. Necrotrophs typically use toxins and hydrolytic enzymes to kill the host and are often characterized by a broad host range. There appear to be relatively few true necrotrophic pathogens. Important examples are *Botrytis cinerea* and *Sclerotinia sclerotiorum*, although many organisms have been classified as such. Between the biotrophic and necrotrophic pathogens is a third group of pathogens classified as hemibiotrophic due to an apparent biotrophic, or perhaps endophytic, stage after initial infection, which is then followed by a visible necrotrophic phase leading to host death. Examples include *Phytophthora infestans* in potato and *Zymoseptoria tritici* in wheat, and many important bacteria especially *Pseudomonas syringae* and *Xanthomonas* spp pathovars. Hemibiotrophic pathogens are quite heterogeneous, ranging from organisms with a predominantly biotrophic lifestyle (*Z. tritici*) to organisms exhibiting predominantly necrotrophic features such as toxin production. Examples of the latter include victorin produced by *Bipolaris victoriae* which can introduce programmed cell death in its host, oat, and ToxA produced by *Drechslera tritici-repentis* in wheat (Curtis and Wolpert, 2002; Howlett, 2006; Pandelova et al., 2009).

Defence responses effective against biotrophic pathogens may not necessarily be effective against necrotrophic and hemibiotrophic pathogens, and in fact these pathogens may sometimes utilize the responses to facilitate their infection. This is outlined in Section 2.3.3 for the hypersensitive response and a further example is from barley, where *mlo*-based resistance is highly effective against *Blumeria graminis* f.sp. *hordei*, whereas cultivars with this recessive resistance are found to be highly susceptible to hemibiotrophic pathogens (e.g., *Pyricularia oryzae*, *Bipolaris sorokiniana*, *Ramularia collo-cygni*). It has indeed been suggested that one, *Ramularia collo-cygni*, has emerged as a pathogen due to the wide use of *mlo* resistance (Jarosch et al., 2003; McGrann et al., 2014).

1.2.2 Pathogenicity factors – the tools of pathogens

The term pathogenicity factor (Deslandes and Rivas, 2012; Tan et al., 2009) or effectors according to the broadest definition (Hogenhout et al., 2009; Vleeshouwers and Oliver, 2014) refers to the tools needed by a pathogen to colonize a plant. These include toxins (Fig. 1.1c), effectors (*sensu stricto* – Fig. 1.1a) degrading enzymes (Fig. 1.1d) and hormones (Fig. 1.1e) functioning by, e.g., killing, maiming, disarming, cheating

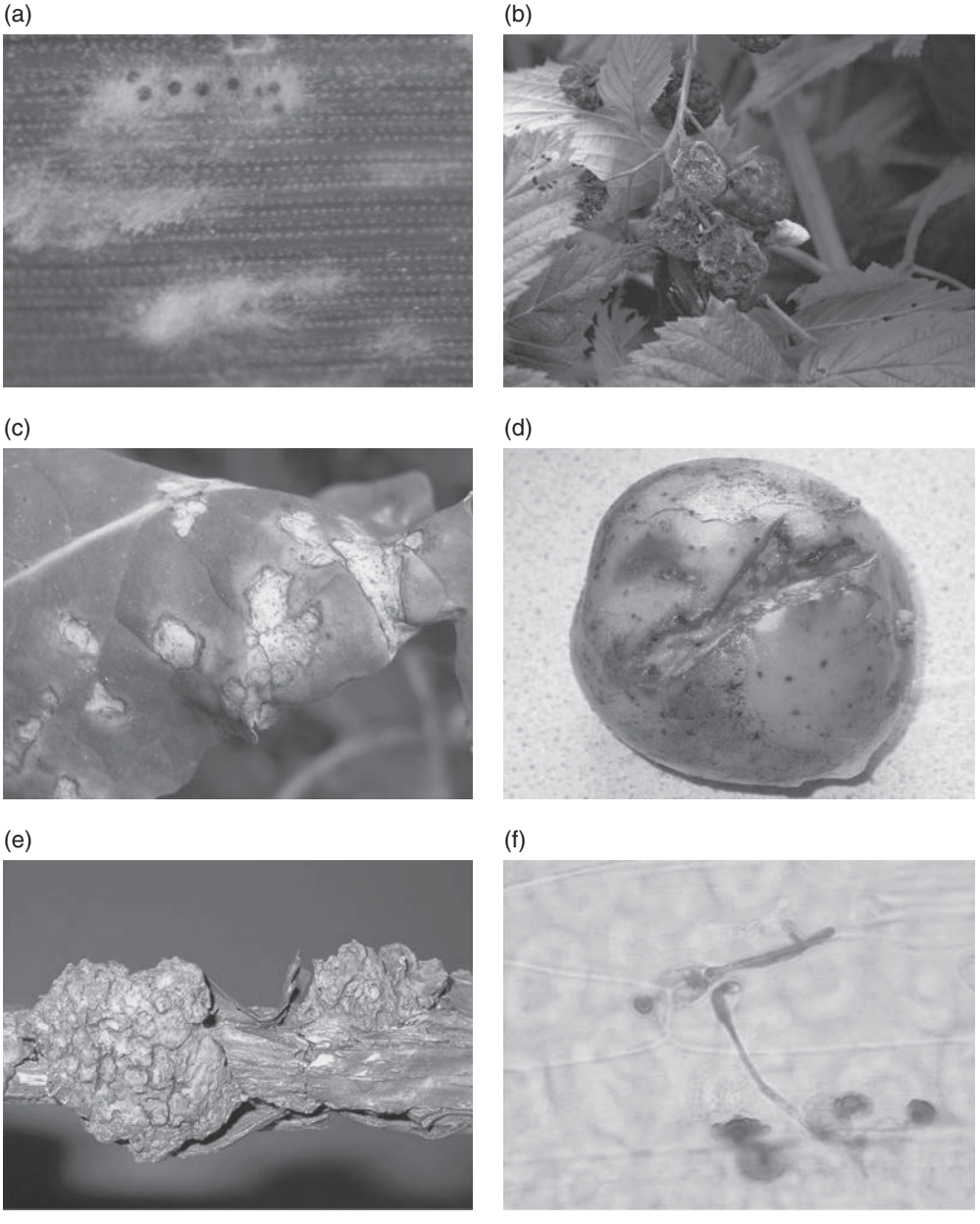

Fig. 1.1 Selected plant pathogen interactions illustrating lifestyle and the effects of specific types of pathogenicity factor. (a) The biotrophic pathogen *Blumeria graminis* f.sp. *tritici* (ascomycete) on wheat (*Triticum aestivum*). Note that the plant tissue is largely green and that there is profuse conidial sporulation as well as chasmothecia (cleistothecia). (b) The necrotrophic pathogen *Botrytis cinerea* on raspberry (*Rubus idaeus*). Note tissue collapse. (c) The hemibiotrophic fungal pathogen *Phoma lingam* on oilseed rape (*Brassica napus*). Note chlorosis in advance of necroses as an effect of the toxins. (d) Hydrolytic enzymes: rotting potato tuber tissue. (e) Hormones: *Agrobacterium tumefaciens* (bacteria) on rose (Rosa cultivar). Note tumours. (f) Effectors: *Blumeria graminis* f.sp. *hordei* on barley (*Hordeum vulgare*). Without effectors, the powdery mildew fungus would not be able to establish infection. Note lack of DAB staining (brown colour, Thordal-Christensen et al., 1997) where penetration has been successful and led to haustorial formation.

and/or modifying the host. Some pathogenicity factors are metabolites, others are proteins and it is now emerging that some pathogens (e.g., *Botrytis*) use microRNA molecules as effectors (Weiberg et al., 2013). In some cases, mutation in a single gene in a pathogen can mean that it is no longer capable of causing infection, or, on the contrary can 'break down' established host resistance delivered by an R gene. Not surprisingly, such pathogenicity genes are of significant interest since they can be targeted via the host. Thus, the plant may be rendered resistant by modifying the host target of the pathogenicity factor by using dsRNA molecules, or by interfering with the pathogen's miRNA signal to the plant (Weiberg et al., 2013; see Section 1.5).

For necrotrophic pathogens, a study of their pathogenicity mechanisms may give clues for the best approach. As discussed previously (Collinge et al., 2010), where phytotoxins of necrotrophic and hemibiotrophic pathogens are essential for achieving infection, a means of inhibiting the mode of action of the toxin may be effective in combatting the pathogen. Note that, for many systems, toxins are suspected to be important but the critical evidence is lacking (e.g., for *Mycosphaerella fijiensis*, causing Black Sigatoka disease in banana; Churchill, 2011). Resistance to phytotoxins could be achieved by transforming the plant with an alternative version of the target protein, which is not sensitive to the pathogen. An alternative approach is to use enzymes to detoxify the phytotoxin (Liu et al., 2015; Legrand et al., 2003; Pedras et al., 2001; Zhang et al., 1999). In the latter case, the potential of the product as a mycotoxin needs to be investigated. As mentioned in Section 2.3, the mode of action of some phytotoxins may act through their ability to induce a hypersensitive response (HR) in the host, which suggests as a third possible strategy the option to block the ability of plants to activate their HR response. This has indeed been achieved in tomato against *Alternaria alternata* f. sp. *lycopersici* (Lincoln et al., 2002). However, the trade-off of such a strategy needs to be carefully considered since it could potentially complicate the plant's ability to control biotrophic pathogens.

1.2.3 Plant defence mechanisms

The ability to withstand and repel a pathogen can be achieved by several independent means (illustrated in Fig. 1.2) and the successful arrest of a pathogen can be the result of a synergy between different mechanisms contributing individually and/or incrementally. This means that the modification of a single inhibitory mechanism alone may be insufficient to confer effective resistance. Nevertheless, strengthening of these apparently minor resistance mechanisms can make a positive contribution from an epidemiological perspective by slowing down the development of epidemics. The following sections describe individual physiological mechanisms for resistance of relevance to this issue.

1.2.3.1 Antimicrobial proteins and secondary metabolites

Antimicrobial proteins such as the pathogenesis (PR) proteins (Chapter 3) exhibit different levels of antimicrobial activities against different pathogen groups and types. For example, the synergistic action of PR2 and PR3 (β-1,3-glucanases and chitinases, respectively) is highly effective in inhibiting fungal growth through their activities on fungal cell walls whilst the PR1 and PR5 proteins act to inhibit oomycete growth. This has been observed in the efficacy of GM plants in combating diseases caused by diverse pathogens (Collinge et al., 2008; Kaur et al., 2011; van Loon et al., 2006; Chapter 3).

Furthermore, plants also possess antimicrobial metabolites, termed phytoalexins or

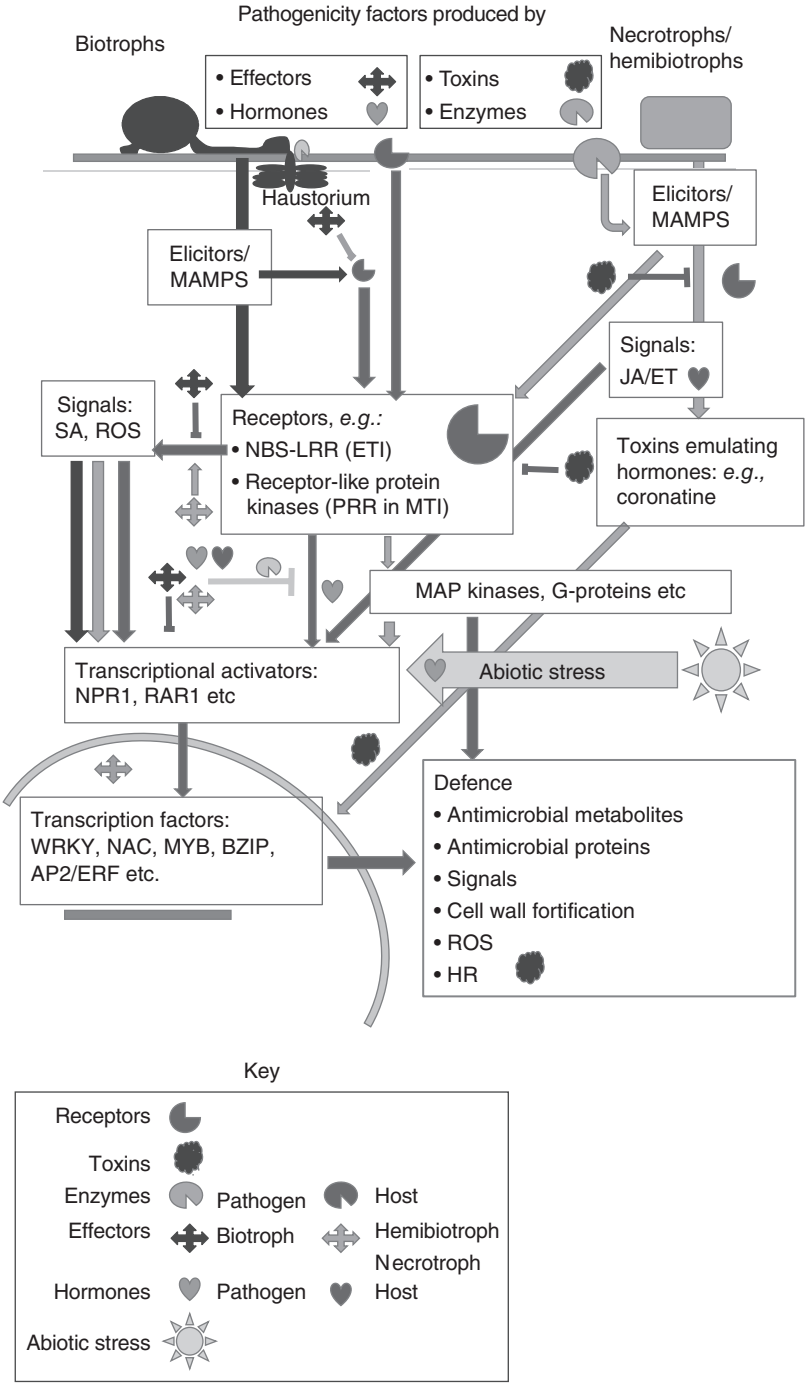

Fig. 1.2 Pathogenicity factors and regulation of defence. Biotrophic pathogens produce effectors and often hormones as their main pathogenicity factors. Small amounts of enzymes are also produced, but not toxins. The effectors interfere primarily with defence signalling. Stimulation is marked with a block arrow, inhibition by a "T". some fungal and oomycete biotrophic pathogens develop haustoria as feeding structures. Necrotrophic and

phytoanticipins, that can provide a level of protection especially against hemibiotrophic and necrotrophic pathogens. While a pathogen can often adapt to the phytoalexins produced by its host (Meyer et al., 2015; Osbourn, 1996), it will not be expected to have adapted to structurally unrelated phytoalexins produced by unrelated host species. Therefore the introduction of genes coding for novel phytoalexins is an obvious approach to investigate. However, a disadvantage of phytoalexins is that the production of secondary metabolites almost always requires the coordinated action of a series of genes to produce the enzymes required to produce the biosynthetic intermediates correctly. For pathogens that kill the tissue before invading in their necrotrophic phases means that, to be effective, these defences have to be activated before, or in response to, the activation of phytotoxins by these necrotrophs. The complexity of the issue of how to produce novel phytoalexins is discussed in depth in Chapter 4.

1.2.3.2 Physical barriers

Barriers constitute further mechanisms of defence and cover both passive barriers, such as a thick cuticle, as well as active barriers, such as papillae and tyloses. Constitutively, the structure of the pectin part of the cell wall is immensely complex (Scheller et al., 2007), requiring a plethora of enzymes to complete degradation (Zhang and van Kan, 2013). Surely, a proportion of this complexity lies in the need to stop those pathogens for which the cell wall is an important carbohydrate source. Tyloses and callose are fortifying carbohydrates found in the vascular tissue and the host cell wall, respectively (e.g., as cell wall appositions, which includes papillae). These structures can also be strengthened by the oxidative cross-linking of proteins and phenolic compounds via lignification (Collinge, 2009; Thordal-Christensen et al., 1997). It is important to note that a successful pathogen does not necessarily need to be able to degrade the cell wall in its entirety to successfully complete the

Fig. 1.2 (*Contiuned*)
hemibiotrophic pathogens generally produce tissue disrupting enzymes and/or toxins to damage host tissues, often remotely from their position. Effectors are also used. Elicitors are molecules of pathogen origin that the host can recognise via receptors. Some of these are specific to special groups or individual pathogen species, whereas others are widely produced. The latter are termed MAMPS (or PAMPS). Host receptors recognising PAMPS are termed pattern recognition receptors (PRRs). They stimulate signal transduction via other protein kinases such as MAP or calmodulin-dependent protein kinases. Likewise, G-proteins and transcription regulators activate defence using transcription factors. Effectors (*sensu stricto*) are proteins of pathogen origin, which are injected/taken up by the host cell where they interfere with the host transcriptional activation of defences or stimulate a biotrophic interaction to provide nutrients for the host, e.g., by establishing a haustorium. It has been proposed that the term "effector" should be considered synonymous with the term 'pathogenicity factor' (Hogenhout et al., 2009). For the purpose of assisting comprehension, we retain the original narrow sense meaning for the term effector, i.e., proteins introduced into the host cell to manipulate host defence or availability of nutrition (Chapter 2). Receptors for MAMP-triggered immunity (MTI - or PTI) can be receptor-like proteins, receptor-line protein kinases or nucleotide-binding site leucine-rich repeat proteins (NBS-LRR). Receptors for Effector-Triggered Immunity (ETI) are NBS-LRR proteins. These are the classic resistance genes. The subset of effectors that are documented to interact either directly or indirectly with described resistance genes are the avirulence gene (Avr) products. Hormones include the classic defence hormones salicylic acid (predominately biotrophic interactions) and jasmonic acid/ ethylene (predominately necrotrophic interactions), but it has been discovered that abscisic acid, cytokinins, brassinosteroids and strigolactones also play important roles in regulating defences and pathogenicity. Hormone levels can be modulated by abiotic stress or by pathogens. Pathogens can make or degrade hormones themselves, but also inhibit or stimulate production or degradation in the host. Hormones modulate host growth and defence mechanisms. Reactive oxygen species (ROS) and ions like Ca^{2+} play roles in the stimulation and regulation of defences. Enzymes can be used by pathogens to release nutrients and interfere with signal transduction. Their activity can inadvertently release elicitor active fragments from cell walls.

pathogenic life cycle. Furthermore, the attempts of pathogens to penetrate and degrade the cell wall in fact contribute significantly to defence signalling (Malinovsky et al., 2014). Thus, the degradation products released from partial degradation can be important in the outcome of the interaction as they act as stimulants to prime or induce the host defence mechanisms. An understanding of especially these signalling processes may lead to strategies for overcoming such enzyme-producing pathogens (see examples in Chapter 5).

1.2.3.3 Programmed cell death

An important defence mechanism against biotrophic and, to some extent, hemibiotrophic pathogens is a form of programmed cell death, termed the hypersensitive response (HR). This phenomenon probably covers different cell death pathways related to apoptosis and autophagy as described from animal systems (Hofius et al., 2007; Hayward and Dinesh-Kumar, 2011). HR stops biotrophic pathogens efficiently whereas it has been observed that some necrotrophic pathogens exploit induction of the HR as a way to make the plant commit suicide and provide nutrition from the dead cells, although the general validity of this has been questioned (Shetty et al., 2008). The environment in a cell undergoing HR is very harsh and at least hemibiotrophic pathogens are often inhibited to some extent here even though, at least in some cases, they can tolerate and eventually overcome the initial inhibition. Under all circumstances, the tightly regulated process of the HR is a complex, energy-requiring process with the potential to affect (benefit) certain types of pathogens whilst arresting infection by others.

1.2.4 Plant immunity and the regulation of defence

Disease-resistance genes – or at the cellular level ETI receptors (Section 1.2.4.1) – have received an excessive focus from plant breeders and molecular plant pathologists. For breeders, they are generally simple dominant genes conferring absolute resistance. For the molecular plant pathologists, the biological question of mode of action is intriguing and the biotechnological potential clear. Basal resistance – or, at the cellular level, MAMP-triggered immunity (MTI) – has been less popular since the rewards (partial or quantitative resistance) and mode of action are less clear and the molecular and genetic tools underlying their study and utilisation have been developed more slowly.

1.2.4.1 MAMP-triggered immunity (MTI) and effector-triggered immunity (ETI)

Many defences are constitutively present, including the antimicrobial phytoanticipins and the chemically complex cell wall. Others, such as certain antimicrobial proteins, are produced in specific phases of the life cycle where the plant is particularly vulnerable, such as in young roots or in flowers (van Loon et al., 2006). However, many host defences are only activated once the plant perceives that it is being attacked by microorganisms. According to the 'zig-zag' model, host perception and subsequent reactions occur in two phases and pathogens can counter-attack them in both phases (Jones and Dangl, 2006), though this is a literal interpretation and describes the evolutionary timescale (or arms race) rather than physiological timescale of activation.

Microbes possess a range of molecules and structures which are associated with broad taxonomic groups. For example, bacteria possess the protein flagellin and EF-Tu (elongation factor thermo unstable) and fungi have specific glucans and chitin as important structural components of their cell walls. The presence of these compounds, collectively termed MAMPs (originally PAMPS) for microbe (pathogen)-associated molecular

patterns, in the host cell environment is a strong indicator of pathogen attack. Hence, the host reacts accordingly following perception via the process of M(P)AMP-triggered immunity (MTI or PTI), which uses receptor-like protein kinases (RLK) as Pattern Recognition Receptors (PRRs) (Antolín-Llovera et al., 2014; Beck et al., 2012; Boller and Felix, 2009; Jones and Dangl, 2006; Zipfel et al., 2006).

The natural variants at the resistance gene loci have evolved under balancing selection with their corresponding cognate effectors in the pathogen (i.e., avirulence genes) and these gene families are among the most rapidly evolving in plants (McDowell and Simon, 2006). This can result in a strong selection pressure to favour pathogen effectors which are not recognized by the host. New variants arise by mutation but, in practice, their appearance more usually reflects migration from the centre of origin of the crop plant (and coevolution with the pathogen), which is particularly likely for long-distance migrants such as rusts and powdery mildews and less so for soil-borne pathogens (McDonald and Linde, 2003). Host plant species have evolved to counter this pathogenic strategy by having an *in situ* surveillance system looking for perturbations in the plant's MTI (and ETI). Thus some disease-resistance genes (ETI receptors) operate through direct recognition of pathogen factors, namely effector proteins, others via guardees, for example PTO and RIN4 (Belkhadir et al., 2004; Oh and Martin, 2011), which are decoy molecules with the purpose of warning of pathogen attack. In response, the best adapted pathogens react by using tools – effector proteins – to inhibit the host signalling pathways, which induce the basal defence of the plant. These effector recognition proteins in the host include the classic disease-resistance genes (R-genes), which confer effective disease resistance against the pathogen but, at the same time, are vulnerable to 'break down'. These processes are presented in more detail in Chapter 2 on effectors, Chapter 9 on potato late blight and in Chapter 20. Ultimately, deciding on whether to engineer disease resistance focusing on MTI or ETI depends on the potential of the target pathogen to evolve in response to the resistance strategy adopted, which will affect the durability of the phenotypic resistance.

1.2.4.2 Receptor-like protein kinases

Not surprisingly, receptor-like protein kinases (RLKs) and other protein kinases are large groups of gene families in plants, with more than 600 and 1100 members alone in the *Arabidopsis* and rice genomes, respectively (Shiu et al., 2004). Several of these RLK families have members exhibiting roles in plant defence. For example, Wrzaczek et al. (2010) found that in one *Arabidopsis* RLK family, comprising 44 CRK (cysteine-rich kinase), several family members had roles in defence against *Pseudomonas syringe* and the powdery mildew fungus *Golovinomyces orontii.* Other CRK family members play roles in relation to cell death in response to ozone, excess light or UV-B stress, stomatal regulation, plant development, hormone signalling, seed germination, as well as photosynthetic processes (Bourdais et al., 2015). Interestingly, suppression of a barley CRK protein kinase by RNAi in transient assays increased resistance to penetration by the powdery mildew fungus *Blumeria graminis* (Rayapuram et al., 2012). The latter suggests that the effects of manipulation (over-expression or suppression) of specific receptor-like protein kinases may represent tools for manipulating plant disease resistance but, by doing so, there may be interactions with other adaptive physiological processes such as abiotic stress tolerance and, indeed, opposite effects may be seen for pathogens exhibiting different lifestyles.

1.2.4.3 *The regulation of defences by phytohormones*

Phytohormones regulate all aspects of plant growth and development as well as responses to biotic and abiotic stress. In particular, the hormones salicylic acid (SA), ethylene (ET), jasmonic acid (JA), abscisic acid (ABA) and cytokinins play roles in defence. The hormonal regulation of defence is highly complex and sometimes precisely the same proteins and molecules are involved in more than one process, with the same hormone having a contradictory effect on different interactions, i.e., promoting resistance or susceptibility to different pathogens. Many receptors and components of signal transduction, such as protein kinases, as well as transcription factors and their regulators, are used in radically different processes in the plant. Further details are given in Chapter 6 (transcription factors), in Chapter 9 (resistance genes) and in Chapter 7 (hormonal regulation of defence).

An important aspect of plant defence regulation is the mobility of host signals around the plant. This is especially the case with respect to herbivory, and perhaps pathogens, where volatile signalling molecules can 'warn' neighbouring plants (Baldwin et al., 2002; Holopainen and Blande, 2012; Shulaev et al., 1997). This form of defence signalling was alluded to above in the need to discriminate between biotrophic and necrotrophic pathogens. The best characterized forms of induced resistance are termed SAR (systemic induced resistance) and ISR (induced systemic resistance), which are regulated by salicylic acid (SA) and ethylene-jasmonic acid (ET/JA) signalling pathways, respectively (Pieterse et al., 2012). The role of these signalling pathways is presented in detail in Chapter 17.

Studies from the model host plant *Arabidopsis thaliana* indicate that the ability of a plant to distinguish between biotrophic and necrotrophic pathogens with this differential response is important in the activation of host resistance (Chen et al., 2012; Glazebrook, 2005). This makes biological sense, since certain necrotrophic pathogens exploit ETI to induce programmed cell death in the host. The key regulator in these processes in Arabidopsis is NPR1 (reviewed by Chen et al., 2012). In contrast, work from rice and a series of transgenic studies where the gene *NPR1* and its orthologues have been over-expressed, suggest that this situation might not be so simple (de Vleesschauwer et al., 2013). Thus, over-expression of the key regulator NPR1 or its orthologues can give enhanced broad spectrum resistance active against biotrophic and *bone fide* necrotrophic, as well as hemibiotrophic pathogens (Chen et al., 2012; De Vleesschauwer et al., 2013; Pieterse et al., 2012). This supports the hope that it might be feasible to develop transgenic cultivars with disease resistance against both biotrophic and necrotrophic pathogens, at least for some crops.

1.3 Opportunities to engineer novel cultivars for disease resistance

Breeding for disease resistance presents substantial challenges in several important crops exemplified in this section. Here, genetic engineering could be considered an effective option, especially for species with a long generation time, such as fruit trees and species, where only clonally propagated plants are of interest (e.g., grapevine and banana). In addition, conventional breeding can be challenged with respect to keeping up with the adaptation of the pathogen population to the resistance sources used. Hence, employing technologies that, in effect, accelerate the breeding process to deploy new resistances would be highly advantageous.

1.3.1 Grape vine (Vitis vinifera)

The grape vine is important as a fresh fruit and as the main raw material for wine production. In the latter case, specific cultivars play a major role in the production of many fine wines which are based on specific named cultivars, e.g., Chardonnay, Merlot, Shiraz/Syrah. Conventional plant breeding cannot be used to improve disease resistance in these because then a new grape cultivar would be made and, even if it is basically similar to the original, it could not be used for the vintage wine market (although specific clonal variants are chosen at present which are best adapted to specific regions and climates). Grape vines are the subject of Chapter 10.

1.3.2 *Potato* (Solanum tuberosum)

Potatoes are used for two main purposes: industrial and culinary. Often specific cultivars have a major market share. The potato cultivar Russet Burbank, developed in the 1870s, is to this day the preferred potato chip, (N.Am. French Fry) cultivar across North America. In addition, consumers in many cultures are often conservative regarding their choice of potato cultivars, preferring well-known cultivars for their taste, look, etc. However, old cultivars are generally considerably more susceptible to diseases than new and their continued cultivation results in an increased need for disease control, especially of late blight. Furthermore, the remarkable ability of the causal agent *Phytophthora infestans* to adapt to resistance in the potato plant and to develop fungicide resistance, increases the need for pesticide use and therefore fast development of new resistant cultivars are needed. Indeed, since potatoes are so badly affected by late blight, it is often considered that the development of transgenic (or cisgenic) cultivars may offer the only real hope for controlling this incredibly adaptive pathogen in a sustainable manner (Chapter 9).

There are good reasons why genetic engineering of disease resistance in potato is particularly attractive. Thus, conventional breeding of potato is slow, taking 13–15 years to develop a new cultivar. If genes need to be introgressed from wild relatives, the time required can be significantly longer. In addition, it should be possible to incorporate resistance against the multitude of viruses (e.g., PVX, PVY, PSV, PLRV. PVS and PMTV) that cause significant problems in potato production, especially since very little natural resistance is available (Park et al., 2009).

1.3.3 Banana (Musa *spp*.)

Banana suffers from several major diseases and pests of both international and regional concern, each of which can cause major losses (Shotkoski et al., 2010). The two major fungal diseases are Panama disease (Fusarium wilt of banana), caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Swarupa et al., 2014) and Black Sigatoka caused by *Mycosphaerella fijiensis* (Churchill, 2011). The former is characterized by race-specific resistance and resistance genes are available, whereas no good sources of resistance are available for the latter, which is therefore controlled primarily by chemicals. Two bacterial diseases cause severe problems. The Moko wilt disease of banana is caused by *Ralstonia solanacearum* (race 2) and is a problem across the tropics. Another wilt disease caused by *Xanthomonas campestris* pv. *musacearum* is lethal and is a serious threat to banana production, particularly in East Africa (Tripathi et al., 2009; Tushemereirwe et al., 2004).

The most commonly cultivated form of banana that is traded especially for western consumers is the Cavendish type, which is a sterile triploid hybrid between diploid and

tetraploid species. Thus, crossing the ancestral types is the only way to produce new banana cultivars of this type (Ortiz and Swennen, 2014). Given the difficulty in making crosses and the lack of sources of natural resistance to these diseases, genetic engineering is attractive as an option. At present, various approaches are being tried to provide disease resistance against the major diseases. These are usually approached on a case-by-case basis, and usually the transgenics developed have only been tested against a single pathogen. For example, mechanisms of defence and disease resistance in banana against Panama disease were reviewed recently (Swarupa et al., 2014). Other studies have approached the control of Black Sigatoka with promising results. Thus, improved disease tolerance was obtained by insertion of a cassette comprising three defence genes of unrelated function, namely the endochitinase gene ThEn-42 from the fungus *Trichoderma harzianum*, the grape stilbene synthase (StSy) and superoxide dismutase Cu,Zn-SOD from tomato (Vishnevetsky et al., 2011). The use of the hypersensitivity response-assisting protein (*Hrap*) gene from sweet pepper (*Capsicum annuum*) (Nordling, 2010; Tripathi et al., 2010) and a plant ferredoxin-like protein (*Pflp*) gene (Namukwaya et al., 2012) are also being assessed for the control of *Xanthomonas campestris* pv. *musacearum.* Another promising strategy is to alter the expression of the banana NPR1 gene since this can often impart broad spectrum resistance (Chen et al., 2012; Zhao et al., 2009). One of the most exciting recent developments in engineering disease resistance is the use of RNA interference (RNAi) technology to control disease (Niu et al., 2010). In several recent studies, (Ghag et al., 2014; Hu et al., 2015; Mumbanza et al., 2013,), transgenic banana plants were made, with small interfering RNAs (siRNAs) targeted against specific fungal genes and with the transformants exhibiting effective resistance against Panama disease in growth chamber experiments. This approach has wide implications for the future engineering of pathogen resistance in other crops (see Section 1.6.5).

1.3.4 *Fusarium* mycotoxins in cereals

A range of *Fusarium* species are important pathogens of cereals. For instance in Denmark, eight different species of *Fusarium*, each producing several (often chemically diverse) mycotoxins, are present in the five most commonly grown small grain cereals (Nielsen et al., 2011). As is typical for hemibiotrophic and necrotrophic pathogens, the only sources of resistance are in the form of quantitative trait loci (QTLs) (Buerstmayr et al., 2009; Buerstmayr and Lemmens, 2015; Walter et al., 2009). This combination of prevalence, the production of mycotoxins and lack of effective natural resistance makes *Fusarium* spp. a particularly attractive target for developing transgenic or cisgenic (Holme et al., 2013) disease-resistant plants. The cereal-*Fusarium* system, with *F. graminearum* as the model pathogen (Kazan et al., 2011; Walter et al., 2009), represents one of the most researched host-pathogen interactions and a number of different approaches has been tried to develop transgenic disease resistance (reviewed by Collinge et al., 2010). These include the use of PR-protein genes, NPR1 and others. Note that, as these pathogens do not require the mycotoxins for their growth as a pathogen, and although deoxynivalenol can contribute to virulence, detoxification approaches will not be useful for preventing infection (Lysøe et al., 2006; Maier et al., 2006). Many of the same *Fusarium* species infect maize, and it is of more than of anecdotal interest to note that BT-maize, designed to confer resistance against insects such as the European corn borer (*Ostrinia nubilalis*), confers significant

resistance to *Fusarium* species and, most importantly, reduces the mycotoxin levels significantly. This is, however, a side effect: the fungus has evolved to use the bore holes in the cob made by insects to gain entry (e.g., Bakan et al., 2002; Collinge et al., 2008) (see also Chapters 15 and 16).

1.3.5 Biotic and abiotic stresses

A pressing biological challenge includes the need to understand the interplay between biotic and abiotic stresses. For example, it is becoming increasingly clear that a range of hormones plays an important role in both stress types. Manipulation of hormone levels via their regulators thus requires large-scale phenotypic testing as it is clear that the results of manipulation experiments may have unpredictable consequences for the phenotype. Different pathogens can react in different ways to the same alteration as, e.g., evidenced by effects observed by alteration in the ATAF1/NAC6 transcription family members in *Arabidopsis*, barley or rice (reviewed by Chen et al., 2012). Field testing is the ultimate way to elucidate what and whether a particular gene works under a series of different environmental conditions, but may not provide as clear results as can be obtained through the use of controlled growth conditions. Key recent developments include the development of robotic phenotyping facilities, allowing multispectral analysis of plants, deep sequencing for transcriptome analyses and metabolomic analyses for measuring the levels of hormones and other metabolites.

1.4 Technical barriers to engineering novel cultivars for disease resistance

In addition to biological challenges related to plant species and pathogen types, there are specific technical challenges related to production of GM crops.

1.4.1 Regeneration and transformation efficiency

One of the biggest challenges in the quest to engineer resistance lies in the very process of engineering novel germplasm itself, including the ability to regenerate plants from tissue culture, etc. Some species are notoriously difficult to transform (e.g., coffee), others are easy (such as rice). These issues are addressed specifically in Chapters 10, 11 and 12. Furthermore, there can be huge variation between cultivars as to the efficiency of transformation, irrespective of which transformation method is used. Thus, for barley, the majority of successful experiments are performed using the cultivar "Golden Promise" by *Agrobacterium*-mediated transformation (reviewed by Harwood, 2012) while other cultivars have proven difficult to transform (reviewed by Harwood, 2012). "Cadenza" and "Bobwhite" are the primary wheat cultivars amenable to *Agrobacterium*-mediated transformation whereas many other cultivars are more efficiently transformed by particle bombardment (Sparks and Jones, 2009).

1.4.2 Availability of appropriate promoters

A second constraint is the availability of species-specific promoters. The 35S promoter of tobacco mosaic virus was the first, and is still the most widely used, especially in dicots (e.g., Broglie et al., 1991). However, there remains a need to develop a toolbox of organ-specific and response-specific promoters, in particular pathogen-inducible promoters, which work in different crop plants to provide expression of the target genes in the tissues where they are needed, thus, in principle, saving energy for the plant. Several pathogen-inducible promotors have been characterized (Himmelbach et al., 2010; van de Rhee et al., 1993). An additional advantage of using organ- and response-specific

promoters is to avoid expression in the tissues where they are consumed, thus eliminating any perceived allergenicity risk associated with the generated protein, e.g., (http://www.who.int/foodsafety/areas_work/food-technology/faq-genetically-modified-food/en/).

1.5 Approaches for identification and selection of genes important for disease resistance

A major effort in plant breeding research is the search for new sources of resistance and these have often been found in, and introduced from, the same or closely-related plant species. Understanding the genetic makeup of the plant and its interaction with its pathogens drives the discovery of new resistance genes. With the advent of genetic engineering technologies, it has become far easier to incorporate potential resistance genes even from more distantly-related species.

1.5.1 The wealth of plant genes

There are roughly 30,000 genes in a typical plant genome and many genes are present in large families. For example, the regulatory genes, like receptor-like protein kinases (RLKs) and transcription factors, can be present in families of 40 to 100 members (Shiu et al., 2004, and Chapter 6, respectively) and the individual members can exhibit redundancy of function or even counteract each other. Great advances have been made in understanding the function of many individual genes in these large families but, even within *Arabidopsis*, there are still huge gaps in our knowledge. In short, we need to be able to translate the results and knowledge gained to economically important crops. Much of the knowledge about the function of specific genes in the model plant *Arabidopsis* has been validated in rice, the best-researched crop plant species (Chapter 14), and other crops and this alone justifies the effort put into the Arabidopsis. However, there are also many differences between *Arabidopsis* and crop plants. For instance, several of the regulatory gene families (e.g., resistance genes and RLKs) are evolving rapidly (e.g., McDowell and Simon, 2006). This means that a well-characterized gene from *Arabidopsis* might not have a clear homologue in another plants species or vice versa.

1.5.2 Identification of target genes

At the molecular level, there are different approaches which can be utilized to find and select genes of interest. Molecular genetic approaches have often provided an indication that a particular gene might be useful for preparing a transgenic/cisgenic plant. These include the identification of mutants exhibiting altered phenotypic response to the pathogen to disease, e.g., susceptibility, altered penetration resistace (Glazebrook, 2001; Lenk and Thordal-Christensen, 2009), as well as proteomics and transcriptome studies, which show expression of specific genes at specific time points (e.g., Delaunois et al., 2014; Eulgem, 2005; Weng et al., 2014). However, there are several important limitations of the mutational approach for dissecting signalling pathways. Especially, lethality, pleiotropy (gene redundancy, where several gene products have similar and overlapping functions) and epistasis (where the effects of one gene are modified by one or several other – modifier – genes) need to be considered. Several approaches supplement the traditional genetic approach. Firstly, where there is sufficient overlap in the gene sequences, RNAi/gene silencing technologies can be used to 'knock down' several genes at once, effectively reducing the efficiency of specific gene functions and, at least to an extent, overcoming one form of pleiotropy (e.g., Miki et al., 2005). Secondly, protein-protein interaction studies may

provide evidence that two proteins indeed contribute to the same regulatory pathway (e.g., Guo et al., 2013; Tang et al., 2015). The yeast two-hybrid approach often provides the screening tool and various other techniques, especially immunoprecipitation ('pull down assays'), can provide the clinching evidence that two proteins indeed interact *in planta* (e.g., Axtell and Staskawicz, 2003; Mackey et al., 2003). Indeed, the tools for the simultaneous large-scale phenotypic analyses of many mutants, natural variants (breeding material) or transgenes are only now really being developed (e.g., Schweizer and Stein, 2011). However, a major challenge remains with respect to identification of these genes in the large gene families in non-hosts to allow exploitation of non-host pathogen recognition. This suggests that an opportunity for more rapid identification of non-host resistance genes can lie in the development of means for high-throughput screening resistance genes in related plant species. These can be introduced subsequently into the main crop either by conventional breeding or by transgenic approaches. In the latter case, genetic drag (the unwanted introduction of undesirable genes conferring agronomically negative traits) can be avoided, although state-of-the-art molecular breeding techniques are making this issue less significant (Chapter 19).

1.6 Promising strategies for engineering disease-resistant crops

New strategies are constantly being developed and several new approaches have emerged within the past few years. Undoubtedly, new and currently unimagined approaches will continue to emerge in the future. A very promising approach is the use of RNA interference (RNAi) technologies, where RNA molecules inhibit gene expression, often by destroying specific mRNA molecules. A special type is host-induced gene silencing (HIGS) where small interfering RNAs (siRNAs) are produced by the host plant and can silence genes in an attacking pathogen (see examples in Chapter 2). Transgenic banana plants have been made using this approach (Section 1.3.3). The promising perspective of this method is to engineer the plant to make miRNA that can be taken up by pathogens to target either effectors or essential basal physiological processes in the pathogen (Niu et al., 2010). A caution with this strategy is that each transgenic event will, as a starting point, target a single pathogen genotype and clearly the development of this kind of approach requires many resources to ensure that allelic variation within a particular pathogen species is covered; however, it should have the advantage of being able to create resistance that does not burden the plant negatively in terms of energy costs and therefore yield. Interestingly, some pathogens, e.g., the fungus *Botrytis* and the bacterium *Xanthomonas*, inject miRNAs or effector proteins which apparently act by manipulating miRNA regulatory pathways in the host (Kurubanjerdjit et al., 2014; Weiberg et al., 2013). This discovery has led to the development of genome editing technologies and two techniques have emerged: TALEN (transcription activator-like effector nucleases) and CRISPR (clustered, regularly interspaced short palindromic repeats), which use endonucleases to cleave both strands in genomic DNA. TALEN/CRISPR has been suggested as a possible way to target specific host genes to block development of biotrophic pathogens (Belhaj et al., 2013).

1.7 Future directions and issues

We still have much to learn about the biology of plants, pathogens and their interactions. We also need to understand the interactions

between the physical and biological environments, i.e., abiotic stress. We have a good, but not complete, understanding of the means by which we can generate resistance against virus (see Chapter 8) and some very good leads for biotrophic pathogens, but the potential repertoire for necrotrophic and many hemibiotrophic pathogens remains small. We suggest a need to focus future research and development on several issues:

- Make more targeted use of basal resistance (MTI) to boost its effects against specific pathogens. For example, would it be possible to improve the perception and regulation of induced resistance whilst avoiding metabolic costs?
- Find and understand the function of many individual genes in large families.
- Identify resistance genes in the large gene families in non-hosts to allow exploitation of non-host pathogen recognition. Here, transfer of resistance genes between unrelated species and precision insertion needs to be addressed.
- Be aware that insertion of new resistance against one pathogen in a plant may compromise the ability of defence against other types of pathogens.
- Deploy new technologies TALEN/CRISPR, HIGS, i.e., siRNAs, miRNA in an effective manner that addresses regulatory constraints.
- Employ high-throughput screening in phenomics, genomics, metabolomics, transcriptomics and other omics coupled with association genetics to deliver cultivars of interest.

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