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Microreview

Molecular mechanisms that limit the costs of NLR-mediated resistance in plants

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SUMMARY

Crop diseases cause significant yield losses, and the use of resistant cultivars can effectively mitigate these losses and control many plant diseases. Most plant resistance (*R*) genes encode immune receptors composed of nucleotide-binding and leucine-rich repeat (NLR) domains. These proteins mediate the specific recognition of pathogen avirulence effectors to induce defence responses. However, NLR-triggered immunity can be associated with a reduction in growth and yield, so-called 'fitness costs'. Recent data have shown that plants use an elaborate interplay of different mechanisms to control NLR gene transcript levels, as well as NLR protein abundance and activity, to avoid the associated cost of resistance in the absence of a pathogen. In this review, we discuss the different levels of NLR regulation (transcriptional, post-transcriptional and at the protein level). We address the apparent need for plants to maintain diverse modes of regulation. A recent model suggesting an equilibrium 'ON/OFF state' of NLR proteins, in the absence of a pathogen, provides the context for our discussion.

Keywords: cost, methylation, post-transcriptional regulation, plant disease resistance genes, small RNAs, transcriptional regulation.

INTRODUCTION

Plant innate immunity depends on the recognition of pathogen effectors by disease resistance (*R*) genes and the activation of host defences. The major class of *R* genes encodes nucleotide-binding and leucine-rich repeat immune receptors (NLRs) and corresponds to one of the largest and most diversified gene families in plant genomes (Michelmore *et al*., 2013). NLR genes are often localized in complex clusters, a structural organization that may favour the dynamic evolution and diversification of NLRs to cope with fast-evolving pathogens (Baggs *et al*., 2017). NLRs are activated by the recognition of specific pathogen effectors, leading to a strong immune response that is often associated with a localized programmed cell death, called the hypersensitive response (HR). If NLRs provide resistance to a variety of pathogens, their inherent cell death-inducing activity, combined with their abundance in plant genomes, requires strict regulation. Consequently, precise and timely control of NLR gene expression is needed to ensure appropriate immune responses in the case of pathogen attack, but also to avoid uncontrolled immune activation and massive fitness costs (Karasov *et al*., 2017). This so-called 'cost of resistance' is illustrated by the fitness compromise characterized for several *R* genes in the absence of disease, including *Rpm1* and *PigmR* (Deng *et al*., 2017; Tian *et al*., 2003). Recent data have shown that plants use an elaborate interplay of different mechanisms to control NLR gene expression and protein abundance to allow plants to maximize their defence capacity, whilst limiting the negative impact on their fitness. This fine-scale regulation is critical to alleviate the burden of NLR cost. In this review, we discuss the different levels of NLR regulation, including transcriptional, post-transcriptional and at the protein level, and attempt to address the apparent need to keep these various levels of regulation, in the light of a recent model suggesting an equilibrium 'ON/OFF state' of NLR proteins in the absence of a pathogen (Bernoux *et al*., 2016).

TRANSCRIPTIONAL REGULATION OF NLR GENES

Gene transcription is an early regulatory step in the modulation of NLR activities. Indeed, appropriate NLR gene transcription is required to achieve resistance, as excessive transcription can trigger programmed cell death, which is detrimental to plant development and growth. One of the most studied cases is the Toll-interleukin receptor (TIR)-NLR ('TNL') SNC1 gene located in the RPP5 cluster in Arabidopsis (Huang *et al*., 2013; Yi and

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Richards, 2007, 2009; Zhang and Li, 2005; Zhang *et al.*, 2003). Indeed, in the *bal* variant, corresponding to a duplication of the wild-type SNC1 locus, a subtle increase in SNC1 mRNA of less than four-fold is sufficient to induce an autoimmune phenotype (Yi and Richards, 2007, 2009). The *bal* plants are dwarf and display a strong and constitutive activation of the immune response (Stokes *et al.*, 2002). Consequently, even a slight increase in NLR gene transcription can have strong harmful effects (Lai and Eulgem, 2017).

By modulation of the chromatin structure, epigenetic marks, such as post-translational modifications of histones and DNA methylation, can impact NLR gene expression; examples include *SNC1*, *RPP4*, *LAZ5* and *RPM1* (Palma *et al.*, 2010; Xia *et al.*, 2013). This is a potentially useful mechanism to minimize fitness costs. In plants, tri-methylation of lysine 4 of histone 3 (H3K4me3) and di- or tri-methylation of H3K36 are enriched at actively expressed genes. Screens for suppressors of autoimmune mutants identified histone lysine methyltransferases (HKMTs) in the regulation of NLR gene expression. This is exemplified by ATXR7, a nuclear Set1 class H3K4 methyltransferase, and SDG8 (/LAZ2), an H3K36 methyltransferase (Palma *et al.*, 2010; Xia *et al.*, 2013). Loss-of-function mutations in these two HKMTs were identified as suppressors of the autoimmune *snc1* and *acd11* mutants, respectively, and correspond to positive regulators for the transcription of the exemplified NLR genes. The activity of ATXR7 was shown to be required for the expression and resistance response triggered by both SNC1 and RPP4, whereas SDG8 is involved in the expression of *LAZ5*, encoding an RPS4-like TNL, as well as *RPM1* (Palma *et al.*, 2010; Xia *et al.*, 2013). Thus, several NLR genes have been demonstrated to be targets of various HKMTs that activate NLR gene transcription by the addition of di- or tri-methylated marks on defined residues of specific histones around these genes.

In order to avoid inappropriate NLR gene expression that could have detrimental effects in non-challenged plants, the activity of these HKMTs is tightly regulated; a subtle equilibrium between positive and negative histone marks on NLR genes fine tunes their transcription. Yet, no examples of histone modifications leading to a repression of NLR gene transcription have been published. This may be a result of an experimental bias related to the fact that autoimmune mutants with their obvious morphological phenotypes of dwarfism and spontaneous cell death are efficient tools to perform suppressor screens (to identify wild-type plants), leading to the identification of genes that activate, rather than suppress, the defence response.

Chromatin modifications, such as H3K9me2, and cytosine DNA methylation are hallmarks of transposable element (TE) silencing (transcriptional repression). The silencing of TE insertions can lead to local changes in chromatin structure, thereby impacting nearby genes. NLR gene clusters often contain repetitive sequences (David *et al.*, 2009). In one well-described example, maintenance of H3K9me2 on a Copia-type retrotransposon is important for correct transcription and splicing of an NLR gene. The TE is in the first intron of the coiled coil (CC)-NLR ('CNL') *RPP7*, conferring a resistance to isolate Hiks1 of *Hyaloperonospora arabidopsidis* (Tsuchiya and Eulgem, 2013). Low levels of H3K9me2 on the TE result in reduced full-length *RPP7* transcripts because of premature transcriptional termination.

DNA methylation in the promoter of NLR genes may influence their expression. The *Arabidopsis* TNL gene *RMG1* is methylated on two helitron repeats in its promoter (Yu *et al.*, 2013). After flg22 treatment, *RMG1* is transcriptionally induced partly as a result of active demethylation, suggesting dynamic DNA methylation in NLR promoters during biotic stress. The *Met-REP1* TNL in *Medicago truncatula* is similar: resistance in Jemalong A17 to powdery mildew (caused by the biotrophic fungus *Erysiphe pisi*) is a result of the demethylation of the promoter, leading to its constitutive expression and resistance, yet without any described fitness cost (Yang *et al.*, 2013). Promoter DNA methylation may also regulate organ-specific NLR gene expression. In rice, two MITEs in the promoter of the CNL *PigmS* determine its pollen-specific expression. During development, these MITEs are epigenetically regulated by DNA methylation dependent on RNA-directed DNA methylation (RdDM), repressing *PigmS*. In pollen, RdDM at these MITEs diminishes, derepressing *PigmS* (Deng *et al.*, 2017). These observations strongly suggest that DNA methylation in NLR gene promoters confers an important layer of transcriptional regulation. Yet, DNA methylation in promoters functions *in cis* to target individual NLR genes, and *in trans* regulation of larger numbers of genes requires other modes of regulation.

Genes can also be methylated in their transcribed regions, typically an enrichment of either methylated CG [gene body-methylated (gbM) genes] or methylated CHH [with possible enrichment in methylated CG and CHG, sometimes called TE-like-methylated (teM) genes (Kawakatsu *et al.*, 2016)]. The gbM genes often correspond to constitutively expressed housekeeping genes; teM genes are mostly silenced in *Arabidopsis*, perhaps resulting from RdDM. NLR genes methylated in their transcribed region are rarely described. In common bean, a genome-wide methylome analysis of the complete NLR gene family revealed that more than one-half of NLR genes are methylated in their transcribed region and resemble teM genes (Richard *et al.*, 2017). Among them, one-half are also targeted by 24-nucleotide small RNA (sRNA), suggesting that RdDM could direct DNA methylation to both promoters and transcribed regions of NLR genes. Whether NLR genes are widely methylated in other plant species is still an open question, but previous genome-wide analyses in *Arabidopsis* have shown an enrichment of defence genes among teM genes (Kawakatsu *et al.*, 2016). It is tempting to speculate that DNA methylation of NLR genes could ensure a low basal expression of NLR genes in the absence of pathogens. Increasing data suggest that DNA methylation dynamically

responds to biotic stress (Deleris *et al.*, 2016). Consequently, NLR DNA methylation could be altered after pathogen infection, allowing a rapid induction of NLR gene expression only when needed. Similarly, epigenetic states of TEs are dynamically altered in response to biotic stress (Dowen *et al.*, 2012). Epigenetic control of TEs (in promoter and intronic regions) may thus act as a regulatory mechanism for NLR gene expression in plant–pathogen interactions.

Thus, NLR gene transcription can be tightly controlled through DNA methylation and/or histone modifications, linked or not with the presence of TEs. Further studies are still necessary to investigate the dynamic nature of these epigenetic marks during biotic stress and their consequences on NLR expression. This is challenging as NLR gene induction occurs in a precise time window after pathogen infection, and can be of low amplitude and highly localized. Future advances may be made by the development of sensitive, single-cell analyses that enable spatiotemporal maps to be obtained of transcription, chromatin and epigenetic marks at and around the site of infection.

POST-TRANSCRIPTIONAL REGULATION OF NLR GENE TRANSCRIPTS BY SMALL RNAS

The abundance of NLR gene transcripts is also regulated in a post-transcriptional manner *via* the action of sRNAs that function to direct the degradation of sequence-matched messenger RNAs (mRNAs) (Fei *et al.*, 2013). Indeed, seminal work in the Fabaceae and Solanaceae has demonstrated complex networks of sRNAs targeting NLR mRNAs, triggered by microRNAs (miRNAs) functioning as 'master regulators' (Li *et al.*, 2012; Shivaprasad *et al.*, 2012; Zhai *et al.*, 2011). A common feature of these miRNAs is that they target conserved, encoded motifs of NLRs, resulting in one miRNA that can potentially target tens to over 100 phylogenetically distant NLR genes. For example, members of the miR482/2118 superfamily target the highly conserved, P-loop-encoding region. This is an extremely powerful mechanism to modulate collectively the transcript abundance of the enormous and diversified NLR gene family. These NLR-modulating miRNAs are typically 22 nucleotides in length, a size that triggers the biogenesis of phased secondary small interfering RNAs (phasiRNAs) from their target genes (Fei *et al.*, 2013). Consequently, these resulting phasiRNAs amplify the regulatory network by also targeting *in trans* other NLR genes for post-transcriptional regulation. These networks of NLR-targeting miRNAs and phasiRNAs occur in a wide range of angiosperms (Zhang *et al.*, 2016), and are quite extensive in the gymnosperm Norway spruce (Xia *et al.*, 2015), suggesting that this control mechanism evolved in seed plants or earlier.

Although this mechanism shows wide prevalence in plant genomes, its functional relevance in NLR regulation remains a matter of conjecture. This is perhaps because of its relatively poor representation in the most tractable genetic system, Arabidopsis, which nonetheless shows enhanced resistance in the absence of phasiRNAs (Boccara *et al.*, 2014). An attractive hypothesis for the evolutionary benefits of the pathway relates to the fitness cost of NLRs. Indeed, widespread control of NLRs by sRNAs may collectively keep levels of NLR gene transcripts under a critical threshold, and thus minimize the fitness costs of overactive immunity pathways. Another possibility is that this regulatory network may help to maintain a stoichiometric balance across diverse NLR proteins by maintaining transcript levels, encoded genome wide, within the same range of abundance. The reason why this might be important is that some NLR proteins function in heteroduplexes (see later), often corresponding to genome-linked pairs of genes that have evolved in patterns to diversify and strengthen resistance (Wu *et al.*, 2017). miRNAs that target conserved motifs may target transcripts from tens to hundreds of diverse NLR-encoding genes (Arikit *et al.*, 2014; Zhai *et al.*, 2011). The pairing of NLRs may require balanced levels of these diverse gene products, i.e. a stoichiometric balance, to prevent unpaired proteins (resulting from unbalanced levels) from auto-activating and causing deleterious effects. In other words, we propose that retaining components of NLR complexes at balanced levels may be the basis for NLR control by sRNAs.

How are NLR gene transcript levels modulated after pathogen infection? One answer comes from the pathogen side, as pathogens (viruses, bacteria and oomycetes) have evolved suppressors of RNA silencing that hijack plant sRNA pathways at diverse steps, and suppress plant immunity (Fei *et al.*, 2016). These suppressors presumably evolved to mitigate the impact of the host plant sRNA machinery on pathogen success—a particularly clear example of this would be viruses that are directly silenced by the host sRNA machinery. However, plants may have sneakily co-opted this pathogen interference; in infected tissues, suppressors would diminish silencing at all levels, including the miRNA/phasiRNA silencing cascade. As a result, NLR gene transcript levels would increase, theoretically resulting in enhanced basal resistance (Boccara *et al.*, 2014). Under the perspective of cost incurred by resistance, this would then represent a subtle host exploitation of the pathogen RNA suppressor activity (Fei *et al.*, 2016; Li *et al.*, 2012), which would allow plants to transiently alter a broad set of NLR levels only in infected cells, thus limiting the fitness costs associated with longer term disregulation. This is largely speculative at this point, as the global increase in NLR levels and a resulting change in resistance have yet to be demonstrated definitively.

The NLR gene family is large in most land plants, a size paralleled in many genomes by the number of miRNAs targeting NLRs. However, the representation of NLR-encoding genes is variable when comparing plants with relatively similar genome sizes, e.g. when comparing peach (408 NLR-encoding genes in 265 Mb) and melon (104 NLR-encoding genes in 450 Mb). miRNAs may emerge in parallel to the expansion of NLRs in a given plant lineage, or at

least in parallel to their sequence diversity, reducing fitness costs of individual genes to enable both the amplification and diversification of NLR gene families (Zhang *et al*., 2016). In this context, Gonzalez *et al.* (2015) proposed that the cost of multiple NLR genes may be compensated by the diversity of the miR482 superfamily. In agreement with this hypothesis, they found a positive correlation across species between the diversity of the miR482 superfamily and the number of NLR genes, suggesting that, in plants with fewer NLR genes, the intrinsic cost of NLRs would be relatively low and the advantage of a diverse miR482 superfamily would be reduced. This correlation is especially pronounced in cucurbits (watermelon; 55 NLRs) and papaya (34 NLRs) with few NLR genes and lacking the miR482 family, and in the closely related genomes of peach (408 NLRs) and apple (737 NLRs) with numerous miR482 family members and many NLR genes. Recently, from a larger analysis including 70 land plants, a tight association was described between the diversity of NLRs and the emergence of miRNAs targeting them (Zhang *et al*., 2016); one interpretation is that this supports a conclusion of evolutionary benefits for the miRNA–NLR regulatory system. A co-evolutionary model was proposed, according to which miRNAs targeting the same conserved motifs of NLRs are frequently generated *de novo* from highly duplicated NLR genes, suggesting that they have arisen through convergent evolution (Zhang *et al*., 2016).

It may take relatively few evolutionary events to generate a miRNA that coordinately regulates numerous NLR genes—an observation supported by the frequency with which new such miRNAs emerge (Zhang *et al*., 2016). It is therefore a parsimonious system to modulate at a genome-scale level the large NLR gene family, relative to the individual optimization of regulatory sequences of many genes (i.e. promoters), or to the accumulation of point mutations in many NLR genes (i.e. pseudogenization). In addition, regulation by miRNAs can maintain a repertoire of active NLR genes which, relative to evolutionary controls dependent on pseudogenization, can be more easily cycled between active and inactive. However, these rules may not be universal: in the Brassicaceae and Poaceae genomes, NLRs are rarely targeted by miRNAs, suggesting that these plant families have evolved alternative strategies to dampen NLR costs.

REGULATION AT THE PROTEIN LEVEL

The activity of NLRs is also regulated at the protein level. In the absence of pathogen infection, NLR proteins exist in an auto-inhibited conformation in the cell (the OFF state) because of intramolecular interactions. After pathogen recognition, NLR proteins switch to an activated conformation (the ON state), which can trigger resistance responses often associated with cell death. The central NB-ARC domain plays a key role in this activation, allowing NLR switching between OFF/ON states (Takken *et al*., 2006). In the OFF state, the nucleotide-binding pocket formed by the NB-ARC domain has a closed conformation centred on bound adenosine diphosphate (ADP). This inactive conformation is tightly maintained by intramolecular interactions of the three different subdomains of the central NB-ARC domain (NB, ARC1 and ARC2) with each other and with other NLR domains (Sukarta *et al*., 2016). For instance, cooperation between the C-terminus of ARC2 and the N-terminal LRRs of the CNLs Rx1 and Gpa2 from potato, or Rp1-D and Rp1-dp2 from maize, has been shown to repress NLR signalling by maintaining the protein in an auto-inhibited conformation (Rairdan and Moffett, 2006; Slootweg *et al*., 2013; Wang *et al*., 2015). After pathogen recognition, NLR activation is associated with partial opening of the binding pocket, allowing the exchange of ADP for adenosine triphosphate (ATP). ATP hydrolysis into ADP enables the NLR to return to its inactive conformation. These auto-inhibitory intramolecular interactions constitute a powerful lock to NLR activation, and therefore reduce costly defence induction. Mutations that disrupt this repressive mechanism result in inappropriate NLR activation. For example, in both TNLs and CNLs, mutation of the histidine or the aspartate of the MHD motif in the ARC2 domain constrains NLRs to adopt a permanent open structure, mimicking the active conformation and resulting in the auto-activity of the mutated NLR. This has been observed in various NLRs, including the CNLs RPM1 in Arabidopsis (Gao *et al*., 2011), Rx in potato (Bendahmane *et al*., 2002), and Mi-1 and I-2 in tomato (Van Ooijen *et al*., 2008), and the TNLs L6, L7 and M in flax (Bernoux *et al*., 2016; Williams *et al*., 2011). Similarly, mutations in the Kinase 2 motif lead to auto-activity by affecting the NLR ATPase activity and therefore locking the NLR in the ATP-bound ON state (for I-2 and Mi-1.2) (Tameling *et al*., 2006). In addition, domain-swapping experiments between closely related NLRs, such as Rx1 and Gpa2 from potato, and RPS5 and RPS2 from Arabidopsis, can result in incompatibility between subdomains, leading to either constitutively activated or non-functional proteins (Qi *et al*., 2012; Rairdan and Moffett, 2006; Slootweg *et al*., 2013). Taken together, these data indicate that NLRs are self-optimized entities, in which even very subtle sequence change can result in incompatibility between subdomains. This sophisticated structure that keeps them safely inactive in the absence of a pathogen, but allows them to switch efficiently to an active signalling conformation after pathogen perception, is probably the result of complex intramolecular co-evolution. As erroneous activation of defence, in the absence of a pathogen, compromises plant growth and often triggers spontaneous cell death, auto-activating mutations are probably highly counter-selected in nature.

Recent work has characterized numerous examples in which resistance is mediated by a pair of NLRs. For example, the CNL pair RGA4 and RGA5 is required to confer resistance against a strain of *Magnaporthe oryzae* in rice, and the TNL pair RPS4 and RRS1 is able to confer resistance against *Colletotrichum higginsianum*, *Ralstonia solanacearum* and *Pseudomonas syringae* in Arabidopsis (Cesari *et al*., 2014; Narusaka *et al*., 2009). In these two cases, one of the NLRs, RGA4 or RPS4, triggers cell death

in the absence of pathogen elicitation and when expressed alone without its cognate NLR partner. These two 'naturally auto-active' NLRs are repressed by the second NLR of the pair, RGA5 or RRS1, respectively. In both cases, effector perception by RGA5 or RRS1 relieves the repression on RGA4 and RPS4, respectively, unleashing their signalling potential. Consequently, intermolecular interactions and, in particular, cooperative interactions between paired NLR proteins represent another way to regulate NLR activity. However, NLR–NLR cooperation needs to be carefully regulated to avoid inappropriate immunity activation. Such incompatibilities between independently evolved NLRs have been described in Arabidopsis; DM1 and DM2d are two TNLs from two different Arabidopsis ecotypes, Uk-3 and Uk-1, and underlie hybrid necrosis observed in F1 progeny. The necrosis results from incompatibility between these two TNLs that triggers the auto-activation of immune responses (Chae *et al.*, 2014; Tran *et al.*, 2017). Similarly, earlier work in Arabidopsis has also established NLR incompatibility as the basis for hybrid necrosis (Bomblies *et al.*, 2007).

NLR interactions with other proteins, such as chaperones, the proteasome machinery and effector targets (in the case of indirect recognition of pathogen effector by the NLR, guard or decoy model), are also required for appropriate regulation of NLR activity (He *et al.*, 2017; Kadota and Shirasu, 2012; Li *et al.*, 2015). For example, the over-accumulation of NLRs as a result of a deficiency in the regulation of their turnover *via* proteasomal degradation can lead to autoimmune responses (Cheng *et al.*, 2011).

CONCLUSION

Altogether, increasing evidence shows that plants possess a multi-layered system regulating NLR protein activity *via* intra- and intermolecular interactions to avoid NLR auto-activity and to ensure a turnover of these useful, but potentially dangerous, immune receptors. In unchallenged cells, recent data have shown that NLRs are in an equilibrium between the OFF and ON states. Pathogen perception shifts the balance towards the ON state, resulting in an accumulation of active NLRs, probably exceeding a critical 'threshold' required for the induction of immunity (Bernoux *et al.*, 2016; Zhang *et al.*, 2017). Considering this equilibrium without pathogen infection, it makes sense that NLR protein production needs to be tightly regulated (*via* transcriptional, post-transcriptional and post-translational turnover/degradation regulation) in order to prevent widespread NLR over-accumulation or imbalances that could lead to the induction of immunity in the absence of a pathogen. However, plants still need to continuously produce NLRs in order to have them 'ready to detect' any potential pathogen that overcomes the first barrier of defence.

Plant NLRs are sensors of the non-self that are tightly regulated in a complex manner to avoid fitness costs. To that end, plants implement several mechanisms to control the levels and activities of NLR resistance proteins, such as transcriptional and post-transcriptional control of mRNA, and regulation of protein activity. These mechanisms vary in their magnitude of action, from single genes or proteins to broad and genome-wide activities. These mechanisms are known from studies in a wide variety of plant species, with certain types of NLR regulation present in some, but not all, species. For example, the miRNA/phasiRNA system is minimally represented in studied members of the Brassicaceae and Poaceae. However, the overall outcome of this regulation is that basal levels of cellular NLRs are sufficient to balance the monitoring of non-self-mediated changes, whilst minimizing the costs of this monitoring.

Theoretically, if the three regulation levels were completely efficient, resistance costs in the absence of a pathogen would not be observed. However, field trials using transgenic lines have revealed a high fitness cost in the absence of the cognate pathogen for two isolated *R* genes in Arabidopsis (*RPS5* and *RPM1*), for which a yield penalty of 5%–10% was observed in the transgenic lines containing the *R* gene compared with the lines without the *R* gene (Karasov *et al.*, 2014; Tian *et al.*, 2003). The biological basis of this cost is not completely understood. One hypothesis is that these NLRs induce the over-stimulation or misregulation of the immune system in the absence of the pathogen, presumably as a result of incomplete efficiency of at least one level of the NLR regulatory system. One clear example of this was published recently: the rice *PigmR R* gene located in a cluster was shown to exhibit a cost in the absence of the pathogen, demonstrating that some NLR genes can exhibit costs whatever their genomic organization (cluster or isolated). Conversely, such a high yield penalty was not observed for another isolated Arabidopsis NLR gene, *RPS2*, suggesting that cost is not ubiquitous to all NLR genes (MacQueen *et al.*, 2016). Despite the agronomic importance of yield losses, there are limited numbers of transgenic studies of NLR costs in the absence of the pathogen. In the future, one challenge to the minimization of yield penalties is to predict costly *R* genes and to better understand the regulatory mechanisms that dampen the plant immune system.

The various levels of regulation of NLRs suggest that the evolution of the plant immune system is not only driven by pathogen selective pressures, but also by internal constraints of the plant genome and genetic background. These various levels of regulation have important consequences in breeding for disease resistance. A classical strategy to achieve durable resistance is to pyramid resistance genes in an elite cultivar. However, back-crossing for introgression of one specific NLR gene in an elite cultivar background might be associated with a fitness cost if an associated miRNA is not present in the selected elite genomic background. The importance of the genomic context is underlined by the observation that resistance might be lost because of protein interaction between antagonistic NLR proteins. This was observed in rice for PigmR and PigmS (Deng *et al.*, 2017), but also in wheat

for the combination of *Pm3* alleles with different recognition specificities (Stirnweis *et al*., 2014), and for *Pm3* with *Pm8* from rye (Hurni *et al*., 2014). Alternatively, when NLRs are functioning as a pair, introgression of only the 'executor' NLR might lead to autoimmunity. In the case of *R* genes presenting gene-specific regulation features (such as methylated promoters), it might be important to include these components in a transgenic strategy. Consequently, one important message is that NLR genes cannot be considered as individual entities, but rather as components co-evolving within a complex genomic network.

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