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Review

Transcript-level expression control of plant NLR genes

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SUMMARY

Plant NLR genes encode sensitive immune receptors that can mediate the specific recognition of pathogen avirulence effectors and activate a strong defence response, termed effector-triggered immunity. The expression of NLRs requires strict regulation, as their ability to trigger immunity is dependent on their dose, and overexpression of NLRs results in autoimmunity and massive fitness costs. An elaborate interplay of different mechanisms controlling NLR transcript levels allows plants to maximize their defence capacity, whilst limiting negative impact on their fitness. Global suppression of NLR transcripts may be a prerequisite for the fast evolution of new NLR variants and the expansion of this gene family. Here, we summarize recent progress made towards a comprehensive understanding of NLR transcript-level expression control. Multiple mechanistic steps, including transcription as well as co-/post-transcriptional processing and transcript turn-over, contribute to balanced base levels of NLR transcripts and allow for dynamic adjustments to defence situations.

Keywords: alternative polyadenylation, alternative splicing, nonsense-mediated decay, plant disease resistance genes, post-transcriptional regulation, small RNAs, transcriptional regulation.

INTRODUCTION

Like other multicellular organisms, plants have evolved immune receptors that allow them to recognize pathogenic microorganisms or pests (Jacob *et al.*, 2013). Particularly efficient are disease resistance (*R*) gene-encoded receptors, which typically mediate very strong immunity (Hammond-Kosack and Jones, 1996). On specific interactions with pathogen avirulence (avr) gene products, R proteins can induce the hypersensitive response (HR), a form of programmed cell death which, together with other defence reactions, provides efficient protection against biotrophic or hemibiotrophic pathogens (Coll *et al.*, 2010). Most known *R* genes encode immune receptors with a central ARC (APAF-1, disease resistance proteins, CED-4)-type nucleotide-binding site (NB) and a C-terminal leucine-rich repeat (LRR) domain (Jacob *et al.*, 2013; Jones *et al.*, 2016). In *Arabidopsis thaliana* (Arabidopsis), most of these NB-LRRs, or NLRs, contain at their N-terminus a coiled coil (CC) or Toll Interleukin-1 Receptor (TIR) domain (Meyers *et al.*, 2003). Examples of other domains present at the N-terminus of NLRs are Solanaceae domains (SDs) in some Solanaceae NLRs, BED-DNA-binding zinc-finger domains of *Populus trichocarpa* NLRs or the WRKY domains in the Arabidopsis proteins WRKY19/MEKK4 and RRS1. In some cases, additional domains occur in other parts of NLR proteins. Generally, CC-NB-LRR genes are present in monocot and dicot genomes, whereas TIR-NB-LRRs are entirely absent in monocots, but strongly represented in dicots (Jacob *et al.*, 2013). Although NLR repertoires are relatively compact in ancestral plant lineages (~25 NLRs in the bryophyte *Physcomitrella patens* and ~2 NLRs in the lycophyte *Selaginella mollendorffii*), the presence of these proteins massively expanded to one of the largest and most variable families in advanced plant clades, reaching more than 400 members in rice (*Oryza sativa*) or grape (*Vitis vinifera*) (Clark *et al.*, 2007; Jacob *et al.*, 2013; Ossowski *et al.*, 2008). The number of NLR genes seems generally to correlate with the total number of genes in a genome. However, there are some exceptions; with papaya, watermelon and cucumber featuring relatively low numbers of NLRs (Zhang *et al.*, 2016).

Pathogen *avr* genes are fast evolving and encode polymorphic effector proteins that are often transmitted into plant cells, where they interact with host proteins to either attenuate host immune responses or otherwise enhance pathogen fitness (virulence) in the host environment (Abramovitch *et al.*, 2006; Chisholm *et al.*, 2006; Dangl and McDowell, 2006). Effector recognition by NLR immune receptors triggers a set of defence reactions referred to as effector-triggered immunity (ETI). How NLRs induce ETI is poorly understood. At this point, no general pathway of NLR-mediated defence induction has been identified, and it seems that many different mechanisms have evolved that link NLR-facilitated effector recognition to downstream defence responses (Dodds and Rathjen, 2010; Li *et al.*, 2015). In addition to serving as specific receptors for pathogen effectors, several widely conserved members of this receptor family have been proposed to function

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as helper NLRs, that have a function in the transduction or amplification of defence signals (Bonardi *et al.*, 2011; Li *et al.*, 2015).

Appropriate homeostasis of NLR activity is critical for their function (Li *et al.*, 2015). NLR protein levels must be above a certain threshold to sufficiently activate defence signalling (Bieri *et al.*, 2004; Holt *et al.*, 2005). However, mutations that either constitutively activate NLR immune receptors or result in elevated NLR expression levels can lead to embryo lethality, spontaneous cell death and/or retarded plant growth (Mackey *et al.*, 2003; Palma *et al.*, 2010; Shirano *et al.*, 2002; Xiao *et al.*, 2003). NLR-mediated resistance can be associated with a substantial reduction in plant fitness (Tian *et al.*, 2003). The phenomenon of hybrid necrosis can be caused by aberrant genetic interactions involving NLR genes in hybrid genomes. Imbalanced activity of NLR genes in such hybrid contexts can result in autoimmunity-related phenomena, such as spontaneous HR (Bomblies and Weigel, 2007; Bomblies *et al.*, 2007). Similar effects are often observed in transgenic plant lines overexpressing NLRs (Li *et al.*, 2015; Stokes *et al.*, 2002).

Post-translational mechanisms involving the HSP90 chaperone, the co-chaperones RAR1 and SGT1, as well as other components of the SKP1-Cullin-F-box (SCF)-dependent protein degradation pathway, are known to control NLR protein levels and activity (Bieri *et al.*, 2004; Cheng *et al.*, 2011; Holt *et al.*, 2005; Hubert *et al.*, 2003; Schulze-Lefert, 2004). In addition, control of NLR transcript levels can be important for their function (Mohr *et al.*, 2010). For example, subtle increases in constitutive mRNA levels (of less than two-fold) of the Arabidopsis NLR gene *SNC1* convert normal-looking plants into dwarfed individuals with strong constitutively activated immune responses (Li *et al.*, 2007; Stokes *et al.*, 2002).

Transcripts of many NLR genes are known to accumulate in response to defence induction or related stimuli. Of 124 Arabidopsis NLR genes represented on the widely used ATH1 microarray, 75 were found to exhibit at least two-fold higher transcript levels in response to one or more of 15 examined defence-related treatments (Mohr *et al.*, 2010). A recent RNA-sequencing (RNA-seq) analysis uncovered 55 up-regulated NLR transcripts in response to treatment with the microbe-associated molecular pattern (MAMP) flg22 in Arabidopsis (Yu *et al.*, 2013). Massive up-regulation of NLR transcripts after defence induction has also been observed in other plant species, such as *Brassica rapa*, soybean and rice (Brechenmacher *et al.*, 2015; Chen *et al.*, 2015; Ribot *et al.*, 2008). Experimental detection of elevated transcript levels may reflect enhanced rates of transcription, but also changes in alternative transcript splicing or polyadenylation and/or altered transcript stability.

Transcript up-regulation of NLRs may locally enhance their potency in pathogen recognition and defence induction (Mohr *et al.*, 2010). Transiently enhanced expression of NLRs in response to cues hinting at the presence of pathogens may increase sensitivity towards pathogen effectors, allowing for stronger and faster immune reactions. As the overexpression of NLR genes is known, in many cases, to lead to constitutive defence activation, broad-spectrum pathogen resistance as well as spontaneous HR cell death, the defence-associated up-regulation of these genes may also generally contribute to inducible immune responses, such as pattern-triggered immunity (PTI) or ETI.

The immunity of plants can be developmentally regulated (McDowell *et al.*, 2005) and NLR genes can be expressed in a tissue/organ-specific manner. A recent meta-analysis suggested that global NLR expression patterns of a plant species can reflect organ-specific effector challenges encountered by this organism (Much *et al.*, 2017). For example, Arabidopsis exhibits a high preference for NLR expression in shoots relative to roots, whereas the legume, Lotus, shows the opposite trend. Both species show low levels of NLR expression in reproductive tissues. Generally, NLRs seem to be preferentially expressed in tissues in which exposure to effectors is most anticipated. Circadian rhythms may play a similar role in preparing plants for possible pathogen attacks by transient up-regulation of NLR transcripts at certain times of the day (Bhardwaj *et al.*, 2011; Wang *et al.*, 2011)

During the past 10 years, a large body of literature has accumulated showing that regulatory mechanisms can target NLR transcripts at every step in their expression process (Fig. 1). Constitutive levels of NLR transcripts often seem to be set to a very narrowly defined ground state. In addition, many NLR genes exhibit differential expression of their full-length transcripts and/or specific transcript isoforms in response to defence induction. Here, we review transcript-level expression control processes of NLRs. Post-translational mechanisms of NLR regulation have been covered in several other reviews (Belkhadir *et al.*, 2004b; Li *et al.*, 2015; Schulze-Lefert, 2004; Shirasu, 2009).

DEFENCE SIGNALS REGULATING NLR TRANSCRIPT LEVELS

In addition to *R* gene-encoded NLRs as specific receptors of pathogen effectors, a second type of receptor, termed pattern recognition receptor (PRR), is involved in the recognition of pathogen-derived molecules. Molecular recognition of widely conserved MAMPs by PRRs triggers a broad set of defence reactions, jointly referred to as PTI (Couto and Zipfel, 2016). In contrast with NLR-triggered signalling processes, which are only peripherally understood, a variety of signalling mechanisms have been implicated in the regulation of PTI. These involve numerous regulatory proteins, such as protein kinases, ion channels, G-proteins, NADPH oxidases, lipase-like proteins and transcription factors (TFs). Details of such PTI-associated signalling processes have been summarized recently in several excellent reviews (Bigeard *et al.*, 2015; Couto and Zipfel, 2016; Tsuda and Katagiri, 2010).

Changes in NLR transcript levels can be controlled by unique stimuli, but may also respond to a wide variety of different defence signals. Some NLR genes, such as barley *Mla6* or *Mla13* (Halterman *et al.*, 2003), only seem to specifically respond to the recognition of their respective cognate avr effector, whereas others are less limited in the spectrum of stimuli to which they respond and exhibit transcript-level changes during a variety of different defence situations (Mohr *et al.*, 2010). In many cases,

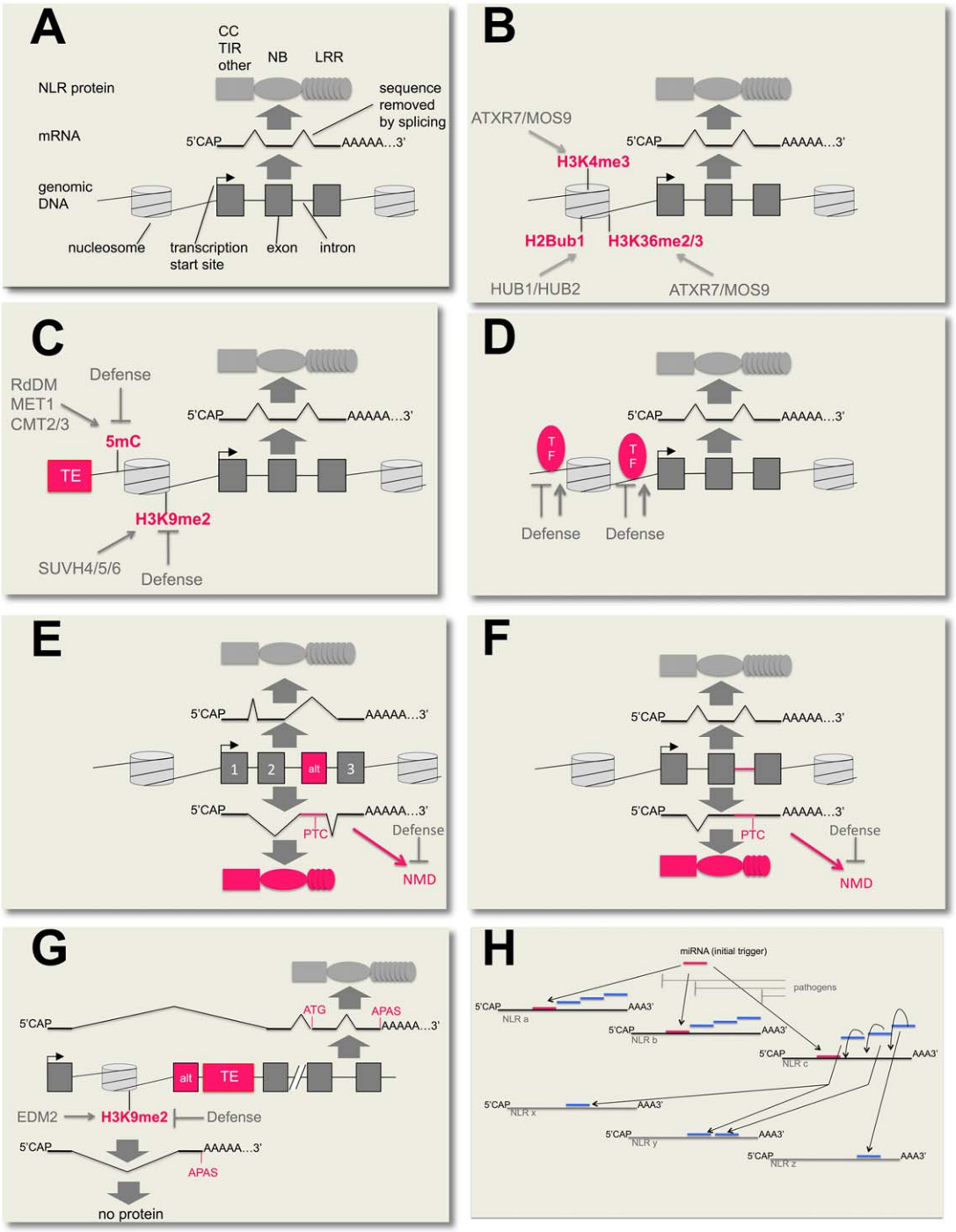

Fig. 1.

the universal defence hormone salicylic acid (SA) seems to be a critical regulator of NLR expression (Shirano *et al.*, 2002; Xiao *et al.*, 2003; Yang and Hua, 2004).

CHROMATIN-ASSOCIATED MECHANISMS OF NLR TRANSCRIPT REGULATION

Studies on the *RPP5* NLR cluster in the Arabidopsis accession Col-0 have contributed substantially to our understanding of NLR expression control. Suppressor screens for mutations inhibiting autoimmunity in genetic backgrounds with enhanced activity of the *RPP5* cluster member *SNC1* have uncovered several *MOS* (*modifiers of snc1*) loci encoding regulators of NLR expression (Li *et al.*, 2015; van Wersch *et al.*, 2016). The Col-0 *RPP5* cluster is a complex 90-kb locus harbouring eight closely related NLR genes and two transposons, including the haplotype-specific *RPP4* and *SNC1*, two NLR genes which have been shown to be functional and mediate pathogen resistance (van der Biezen *et al.*, 2002; Noël *et al.*, 1999; Parker *et al.*, 1993, 1997; Stokes *et al.*, 2002). A first hint at the possible epigenetic regulation of *NLR* expression stems from the analysis of *SNC1*, as ectopic insertions of transgenes containing the promoter and transcribed region of this gene resulted in much higher transcript levels than the endogenous *SNC1* copy, which seems to be present in a repressive chromatin environment (Li *et al.*, 2007).

Trimethylation of lysine 4 of histone H3 (H3K4me3) has been identified as one possible epigenetic mark required for the appropriate expression of the adjacent *RPP5* cluster members *RPP4* and *SNC1* (Xia *et al.*, 2013). The SET (Su(var)3–9, Enhancer-of-zeste, Trithorax) type H3K4 methyltransferase ATXR7 is responsible for establishing this mark at the *RPP4* and *SNC1* loci. Transcript levels of both NLR genes are reduced in an *atxr7* mutant, as are the defence responses triggered by them.

Similar phenotypes are observed in the *mos9* mutant, which has been identified as a suppressor of the autoimmunity phenotype of *snc1-1* (Xia *et al.*, 2013). MOS9, a nuclear plant-specific protein, which bears no discernible motifs or domains, seems to be physically associated with ATXR7, assisting this histone methyltransferase in its catalytic function. H3K4me3 is a mark universally associated with actively transcribed genes (Kouzarides, 2007). A similar situation was observed for *LAZ5*, a TIR-NB-LRR-encoding gene unlinked to the *RPP5* cluster, which, for correct expression and function, requires the SET domain group 8 (SDG8)

Fig. 1 General mechanisms of NLR transcript regulation described in this review. (A) General flow of genetic information from chromatin-associated genomic DNA through transcription and transcript processing to mature mRNAs, and from mRNAs via translation to NLR proteins. CC, coiled coil; LRR, leucine-rich repeat; NB, nucleotide-binding site; TIR, Toll Interleukin-1 Receptor domain. (B) Post-translational histone modifications associated with active transcription. H3K4me3, H3K36me2, H3K36me3 and H2ub1 levels positively correlate with transcript levels of some NLRs. In Arabidopsis, the H3K4 and H3K36 methyltransferase ATXR7/MOS9 and the H2 ubiquitylases HUB1 and HUB2 are known to promote high levels of these epigenetic marks. Although not shown for NLR loci so far, some post-translational histone modifications, such as H3K4me3, can be inducible during pathogen defence. Post-translational modifications shown in this figure are not meant to be present at the exact location indicated, but rather in the genomic area of the NLR gene. (C) Transposon-associated silencing marks. Transposon insertions can recruit epigenetic marks silencing their expression into the context of NLR genes, where these marks can have suppressive effects on NLR transcription (or other expression-related processes; see G). At some NLR loci, the levels of these silencing marks, such as 5mC or H3K9me2, decrease after defence induction. In Arabidopsis, H3K9me2 is established by the SUVH (Suppressor of variegation homologue) 4, 5 and 6 H3 methyltransferases, whereas 5mC is established by the RNA-directed DNA methylation (RdDM) pathway and maintained by the MET1, CMT2 or CMT3 DNA methyltransferases. How these marks are removed during defence induction is not known. (D) Regulatory transcription factors (TFs), such as WRKYs, can affect the transcript levels of certain NLRs. A broad role of WRKYs in NLR transcription is likely, as their binding sites are enriched in the promoters of Arabidopsis NLRs. Many WRKY TFs are known to be controlled by defence signals and can play activating or suppressing roles in defence gene expression. (E, F) Expression of a large number of NLRs is known to be affected by alternative splicing. This process can result in the inclusion of alternative exons (E) or retained introns (F) in the respective mRNAs. Either one of these processes can lead to the inclusion of in-frame premature termination codons (PTCs), which may result in truncated NLR proteins and/or recruit the respective transcripts to the nonsense-mediated decay (NMD) RNA degradation pathway. In at least some cases, truncated NLRs generated by alternative splicing are probably functional by associating with full-length NLRs or acting as downstream signalling components. Defence-associated suppression of NMD can lead to increased abundance of some NLR isoforms, probably resulting in heightened states of immunity. (G) An increasing number of NLRs are found to be regulated by alternative polyadenylation. In the case of Arabidopsis *RPP7*, a transposon recruited the silencing mark H3K9me2 into the first intron of this NLR gene. High levels of H3K9me2 in this area suppress the use of a proximal polyadenylation site and promote the production of full-length RPP7 coding mRNAs. Reduced levels of H3K9me2 caused by defence induction or mutations in the *EDM2* gene lead to enhanced use of the proximal polyadenylation site, increased levels of a prematurely terminated non-coding transcript and, consequently, reduced levels of full-length RPP7 coding transcripts. Alternative polyadenylation can be combined with alternative splicing by the differential inclusion of alternative terminal exons, as in *RPP7*. (A–G) Basic graphical elements used in these panels are defined in (A). Features specifically important or unique for the respective mechanisms are shown in red. TE, transposable element; alt, alternative exon; Defence, defence-inducing signals; thin pointed arrows, activating interactions; thin blunt arrows, suppressing interactions. (H) MicroRNA (miRNA)-triggered cascades of secondary phased small interfering RNAs (phasiRNAs). In several cases, miRNAs targeting conserved regions in NLR transcripts were shown to serve as initial triggers (short red horizontal lines) of secondary phasiRNA (short blue horizontal lines) cascades, which comprehensively mediate post-transcriptional co-silencing of NLR gene families. On association with primary target NLR transcripts (e.g. NLRs a, b and c in the figure), these miRNAs trigger the production of phasiRNAs which amplify and diversify the initial trigger and reinforce the silencing of the primary NLR targets (e.g. NLRs a, b and c), but also secondary NLR targets (e.g. NLRs x, y and z). Consequently, the secondary phasiRNAs target a wider set of NLR transcripts than the initial miRNA trigger. Such NLR silencing cascades can be suppressed by microbial RNA silencing suppressors resulting in enhanced immunity.

histone methyltransferase (Palma *et al.*, 2010). SDG8 is responsible for di- or trimethylation of H3K36. Like methylation of H3K4, these marks are generally associated with active transcription. Whether the levels of any of these histone modifications change at their target NLR loci in response to defence induction, or whether they play any specific roles in defence-related regulation of other NLRs, has not been reported at this point. However, defence-inducing treatments have been found to increase H3K4me3 levels at other Arabidopsis loci (Jaskiewicz *et al.*, 2011).

In addition to methylation, ubiquitylation of histones is known to affect transcription. Two E3 ubiquitin ligases, HUB1 and HUB2, are known to mono-ubiquitylate histone H2B to H2Bub1 in Arabidopsis. Both of these enzymes control H2Bub1 levels at *SNC1* and *RPP4*, and positively contribute to the transcription of these NLR genes (Zou *et al.*, 2014). In response to defence induction, H2Bub1 levels at *SNC1* and *RPP4* exhibit a moderate increase, which is associated with elevated transcript levels of these genes.

Another example of a post-translational histone modification exhibiting differential levels at an NLR gene after defence induction is the canonical transposon silencing mark H3K9me2 (Tsuchiya and Eulgem, 2013). After induction of an ETI response, H3K9me2 levels transiently decrease within the first intron of *RPP7*, affecting alternative polyadenylation (APA) of this NLR gene (see below).

The histone mark H3K9me2 is functionally linked to cytosine methylation, another type of epigenetic signal found to have an impact on the expression of NLR genes. The complex interplay of *de novo* methylation, maintenance methylation and demethylation mechanisms controls the methylation of the nucleobase cytosine at position 5. Methylated cytosine (5mC) occurs in plants at the symmetrical GC and CHG motifs or asymmetrical CHH sites (H = any nucleobase, except G). *De novo* methylation seems to be globally controlled in Arabidopsis by the RNA-directed DNA methylation (RdDM) pathway (Law and Jacobsen, 2010). Well-characterized Arabidopsis maintenance methyltransferases include MET1, which methylates cytosines at hemimethylated GC, and CMT2 and CMT3, which catalyse cytosine methylation at CHH or CHG sites associated with H3K9me2. Cytosine demethylation requires active base excision followed by DNA repair. As in the case of H3K9me2, 5mC is primarily involved in the silencing of transposons. In the promoters of genes, 5mC typically has a repressive effect on transcription.

Transcript levels of several NLR genes have been found to be controlled by cytosine methylation. Generally, it seems that this mark is often recruited to NLR genes by transposon insertions. The Arabidopsis NLR gene *RMG1* (*Resistance Methylated Gene 1*), encoding a TIR-NB-LRR protein, is expressed at high levels in response to the MAMP flg22. RMG1 transcripts are suppressed by cytosine-methylated helitron transposon regions in this gene's promoter and flg22-induced processes seem to counteract this suppressive mechanism (Yu *et al.*, 2013). Although its details are unknown, this regulatory mechanism may be linked to the flg22-induced decrease of several transcripts encoding components of the RdDM and 5mC maintenance pathways (Yu *et al.*, 2013). Defence induction is known to globally affect 5mC levels in Arabidopsis (Deleris *et al.*, 2016; Dowen *et al.*, 2012; Pavet *et al.*, 2006; Yu *et al.*, 2013). For example, avr effectors of the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 induce hypomethylation and chromatin de-condensation at centromeric and pericentromeric repeats (Pavet *et al.*, 2006). SA, as well as various *P. syringae* strains, induce comprehensive changes in 5mC patterns in genic regions and transposons of the Arabidopsis genome (Dowen *et al.*, 2012). The MAMP flg22 has also been shown to induce globally differential cytosine methylation in transposons and transposon-associated defence genes (Yu *et al.*, 2013).

REGULATION OF NLR GENE EXPRESSION BY TFs

Major types of TF known to control transcript levels of defence-related genes are members of the WRKY, ERF and TGA-bZIP families (Eulgem, 2005). WRKY TFs typically bind to pathogen/elicitor-responsive W box *cis*-elements (hexameric consensus sequence: TTGACC/T) (Eulgem and Somssich, 2007; Eulgem *et al.*, 2000; Rushton *et al.*, 2010). Mohr *et al.* (2010) demonstrated a key role of W boxes in controlling constitutive levels and the defence-associated transient accumulation of RPP8 transcripts. *RPP8* encodes a CC-NB-LRR in the Arabidopsis accession Ler, which mediates strong race-specific resistance against isolates of the pathogenic oomycete *Hyaloperonospora arabidopsidis*. *RPP8* exhibits low basal transcript levels, which are transiently elevated in response to various defence-inducing stimuli. In contrast with a wild-type *RPP8* transgene, an *RPP8* construct containing mutated W boxes in its promoter failed to confer *RPP8*-mediated immunity against *H. arabidopsidis* in a susceptible Arabidopsis background. W boxes were further found to be generally enriched in promoter regions of Arabidopsis NLR genes (Mohr *et al.*, 2010), supporting a general role of these promoter elements and, possibly, WRKY TFs in the transcriptional regulation of NLR genes. Although, at this point, no other types of TF seem to be known to be involved in transcriptional regulation of NLR genes, it is unlikely that such roles are exclusively executed by WRKYs.

ALTERNATIVE SPLICING IN NLR EXPRESSION CONTROL

Alternative splicing (AS) has been recognized as a main contributor to transcriptome and proteome diversity. Dynamic AS processes, which are responsive to environmental or developmental stimuli, can regulate the abundance of defined functional mRNA

isoforms, and thereby serve as a mechanism of quantitative gene expression control.

In Arabidopsis, AS is known to affect approximately 60% of its multi-exon genes (Marquez *et al.*, 2012). Studies in other dicot or monocot angiosperm species have suggested that AS affects 30%–50% of their multi-exon genes (Mandadi *et al.*, 2014; Shen *et al.*, 2014; Thatcher *et al.*, 2014).

Multiple reports have linked dynamic AS events to the activation of plant immune responses (Dinesh-Kumar and Baker, 2000; Howard *et al.*, 2013; Mandadi *et al.*, 2014; Yang *et al.*, 2014; Zhang and Gassmann, 2007). Many examples of alternative NLR transcript splicing have been described, some of which seem to be constitutive and not affected by defence signalling, and some of which are dynamic and responsive to defence-related signals (Table 1) (Gassmann, 2008; Jordan *et al.*, 2002; Yang *et al.*, 2014). Although many cases of AS in plants result in a changed composition of exonic coding sequences and, consequently, in changed protein sequences, the retention of intronic sequences seems to be predominant in plants (Reddy *et al.*, 2013). This form of AS can introduce premature in-frame stop codons, which can destabilize transcripts by subjecting them to nonsense-mediated decay, a pathway of RNA degradation (see below). However, at least in some cases of NLR expression control, such alternative transcript isoforms seem to remain stable and to encode truncated protein versions that contribute to successful immunity (see below).

One of the first reports on AS in NLR regulation demonstrated the critical importance of two splice isoforms of the tobacco TIR-NB-LRR gene *N*, which mediates resistance against *Tobacco mosaic virus* (TMV) (Dinesh-Kumar and Baker, 2000; Whitham *et al.*, 1994). Both isoforms are required for full immunity: the full-length protein encoding the shorter isoform N_s and the longer N_l version, which encodes a truncated protein via a retained alternative exon leading to reading-frame shift and a premature translational termination codon. Interestingly, N-mediated TMV recognition in tobacco triggers a transient change in the N_s/N_l transcript ratio, which is essential for a successful defence against TMV. A 3′ untranslated region (3′ UTR) downstream of the *N* coding sequence seems to be critically important for the control of this ratio. The mechanistic details of this dynamic AS process are not known.

AS has also been described for the barley CC-NB-LRR encoding *Mla6* and *Mla13* genes (Halterman and Wise, 2004; Halterman *et al.*, 2001, 2003; Shen *et al.*, 2003). *Mla13* is a particularly interesting example as the ratio of its five different splice isoforms changes after defence induction by its cognate powdery mildew strain (Halterman *et al.*, 2003). Even more complex is the situation for the rice CC-NB-LRR gene *Pi-ta*, which expresses 12 different transcript splice forms, 11 of which are predicted to encode proteins and some of which are differentially expressed after defence induction by rice blast (Costanzo and Jia, 2009).

Additional examples of alternative NLR transcript splicing include wheat *LR10* and *Sr35* (Saintenac *et al.*, 2013; Sela *et al.*, 2012), rice *RGA5* (Cesari *et al.*, 2013), flax *L6* and *M* (Anderson *et al.*, 1997; Lawrence *et al.*, 1995), tomato *Bs4* (Schornack *et al.*, 2004), potato *Y-1* (Vidal *et al.*, 2002), *Medicago truncatula RCT1* (Tang *et al.*, 2013), common bean *JA1tr* (Ferrier-Cana *et al.*, 2005), soybean *Rj2* (Tang *et al.*, 2016) and the Arabidopsis genes *RPS4* (Gassmann *et al.*, 1999) and *RAC1* (Borhan *et al.*, 2004). As in the case of *N*, *MLA13* and *Pi-ta*, the relative abundance of its six splice forms changes for *RPS4* (Zhang and Gassmann, 2007) and, similar to *N*, AS of *RPS4* was found to be critical for its function (Zhang and Gassmann, 2003). This also applies to *RCT1*, for which the presence or absence of alternative transcripts affects the outcome of pathogenic interactions of *M. truncatula* and races of the fungus *Colletotrichum trifolii* (Tang *et al.*, 2013). Strikingly, the AS pattern of *RCT1* orthologues is conserved between *M. truncatula* and its close relative *M. sativa*. A recent RNA-seq study found nine NLR genes to exhibit differential accumulation of alternative splice isoforms after viral infections in the monocot model system *Brachypodium distachyon* (Mandadi *et al.*, 2014).

Interestingly, most TIR-NB-LRR genes share a common gene structure, with the first exon encoding the TIR domain, the second exon encoding the NB domain and the remaining exons encoding the LRR region as well as additional C-terminal parts (Yang *et al.*, 2014). Common to several NLR genes encoding TIR-NB-LRR proteins, such as *N* or *RPS4*, AS leads to the expression of truncated proteins partially or fully lacking their LRR, NB-LRR or other C-terminal portions (Gassmann, 2008; Yang *et al.*, 2014). Typically, this is a consequence of the retention of intronic sequences introducing premature in-frame stop codons into the mature mRNAs. The resulting truncated protein variants may serve critical functions during defence activation, as the transcript isoforms encoding them seem to be needed for full immunity in many cases. Multiple studies have shown intramolecular or intermolecular interactions between individual NLR protein domains to have important roles in the regulation of their activity (Belkhadir *et al.*, 2004a; Bendahmane *et al.*, 2002; Hwang *et al.*, 2000; Moffett *et al.*, 2002; Rairdan and Moffett, 2006; Takken and Goverse, 2012). Thus, by engaging in protein–protein interactions with defined NLR partners, accumulating TIR-NB-LRR protein fragments resulting from induced splicing processes may serve to regulate NLR activity (Gassmann, 2008). Systematic protein–protein interaction studies support such a role of truncated NLRs (Nandety *et al.*, 2013). Alternatively or additionally, truncated NLRs may also play a role in downstream defence signalling processes, as has been demonstrated for the mammalian TIR domain protein MyD88 (Kenny and O'Neill, 2008). This possibility is supported by the fact that the overexpression of isolated TIR or CC domains of some plant NLRs can constitutively induce defence activation

Table 1 Examples of alternative splicing in NLR gene expression.

Species/NLR	Known variants	Comments	Reference
Tobacco *N*	2	Alternative exon in intron 3; both isoforms are required for full immunity	Whitham *et al.* (1994); Dinesh-Kumar and Baker (2000)
Tomato *Bs4*	2	Alternative splice donor sites	Schornack *et al.* (2013)
Potato *Y-1*	4	Differential use of cryptic intron combined with alternative polyadenylation	Vidal *et al.* (2002)
Medicago truncatula RCT1	>4	Combined alternative splicing/polyadenylation leading to numerous different transcript isoforms representing at least four distinct coding sequence variants	Tang *et al.* (2013)
Common bean *JA1tr*	7	Result suggest the existence of seven transcripts generated by alternative use splice donor–acceptor sites and polyadenylation	Ferrier-Cana *et al.* (2005)
Soybean *Rj2*	2	Intron 4 retention; regular transcript is sufficient for function	Tang *et al.* (2016)
Flax *L6*	4	Intron 3 retention; alternative splice donor or acceptor sites	Lawrence *et al.* (1995)
Flax *M*	12	Intron retention, exon skipping and use of multiple splice acceptor sites	Schmidt *et al.* (2007)
Arabidopsis *RPS4*	3	Intron 2 and/or intron 3 retention; all isoforms are required for full immunity	Gassmann *et al.* (1999); Zhang and Gassmann (2003)
Arabidopsis *RAC1*	2	Intron 1 retention	Borhan *et al.* (2004)
Barley *Mla6-2*	2	Intron retention	Halterman *et al.* (2001)
Barley *Mla13*	5	Alternative spliced introns in 5′ untranslated region (5′ UTR)	Halterman *et al.* (2003)
Rice *Pi-ta*	12	12 different transcript isoforms generated by combined alternative splicing and polyadenylation with some forms also having different 5′ ends	Costanzo and Jia (2009)
Rice *RGA5*	2	Intron 3 retention; isoform with retained intron does not confer resistance to *Magnaporthe oryzae*	Cesari *et al.* (2013)
Wheat *LR10*	2	Intron retention	Sela *et al.* (2012)
Wheat *Sr35*	2	Intron 3 retention (second intron in 3′ UTR)	Saintenac *et al.* (2013)

(Bernoux *et al.*, 2011; Collier *et al.*, 2011; Maekawa *et al.*, 2011; Swiderski *et al.*, 2009; Zhang *et al.*, 2004).

Molecular processes controlling alternative NLR transcript splicing do not seem to be understood in great detail at this point. Some splicing factors, such as serine and arginine-rich (SR) proteins, are known to be stress regulated (Ali and Reddy, 2008; Palusa *et al.*, 2007). In *B. distachyon*, multiple genes encoding putative splicing regulators have been found to exhibit differential splicing after viral infections (Mandadi and Scholthof, 2015). Thus, in some cases, differential activity of regulatory proteins may contribute to dynamic NLR transcript splicing after defence induction. A partial loss-of-function mutation of *MOS12* encoding an arginine-rich protein related to the human SR-type splicing regulator cyclin 1 has been reported to affect the splicing patterns of the Arabidopsis NLR genes *SNC1* and *RPS4* (Xu *et al.*, 2012). Components of the evolutionarily conserved nuclear spliceosome-associated MOS4-associated complex (MAC) (Monaghan *et al.*, 2009, 2010; Palma *et al.*, 2007) have been found to physically interact with the MOS12 protein (Xu *et al.*, 2012). Mutations in genes encoding other MAC components have also been shown to affect splicing-related processes controlled by *MOS12* (Xu *et al.*, 2012). Roles of SR proteins, like MOS12, in *SNC1* and *RPS4* transcript splicing are further supported by splicing defects of these NLRs in a mutant of the SR importin-encoding *MOS4* gene (Xu *et al.*, 2012). SR importins are known to function as nuclear import receptors for SR proteins (Ström and Weis, 2001). Although these results strongly link MAC components to alternative *SNC1* splicing, direct physical interactions of these proteins with SNC1 transcripts have not been reported so far.

In addition to direct involvement of splicing regulators, AS can be affected by the rate of Pol II elongation during transcription (de la Mata *et al.*, 2010). Low rates of Pol II elongation can favour the use of upstream splice sites over their downstream alternatives. Such kinetic coupling between transcription and splicing can theoretically be controlled by defence-associated changes in chromatin states. Epigenetic marks can also serve the recruitment of splicing factors to sites engaged in co-transcriptional transcript processing. It will be interesting to see whether future studies can establish functional links between defence-associated changes in cytosine methylation or histone modifications and dynamic alternative splicing processes affecting the expression of defence genes in general or, specifically, NLR genes.

ALTERNATIVE POLYADENYLATION AS A MODE OF NLR REGULATION

In contrast with AS, the significance of APA for global gene expression has been realized only very recently (Proudfoot, 2011). At least 70% of all Arabidopsis genes are known to be subject to APA (Sherstnev *et al.*, 2012; Wu *et al.*, 2011). APA can result in distinct transcript isoforms that differ in their coding sequences, but also their 3′ UTRs, which are typically unique for each transcript and important for their stability (Proudfoot, 2011).

Mechanistically, the processes of AS and APA are distinct, although they can also act together (see Fig. 1G). Cleavage of native mRNAs at their 3′ end and the addition of a poly A tail can be controlled by a set of *cis*-elements at the 3′ end of the pre-mRNA, referred to as polyadenylation signals (Proudfoot, 2011; Tian and Graber, 2012). A highly conserved protein complex of more than 50 subunits is typically recruited to polyadenylation sites (Hunt *et al.*, 2008; Lutz, 2008).

Several recently published studies have indicated that APA is also involved in the regulation of plant immune responses (Lyons *et al.*, 2013; Shen *et al.*, 2011; Thomas *et al.*, 2012, Tsuchiya and Eulgem, 2013). For example, the Arabidopsis RNA-binding protein FPA affects APA of the ERF4 TF gene in response to the MAMP flg22 (Lyons *et al.*, 2013). This mechanism seems to limit the extent of MAMP-induced defence responses. Furthermore, the Arabidopsis polyadenylation complex component CPSF30 controls APA site use in introns and 3′ UTRs of a large number of defence-associated genes, and positively contributes to SA-dependent defence responses and HR-like cell death (Bruggeman *et al.*, 2014; Thomas *et al.*, 2012).

Early evidence for an important role of APA in NLR regulation stems from a massive parallel signature sequencing analysis, which showed that transcripts of 72 Arabidopsis NLR genes are possibly alternatively polyadenylated (Tan *et al.*, 2007). The only in-depth analysis of APA in NLR regulation has been reported for the Arabidopsis NLR gene *RPP7*. This CC-NB-LRR gene mediates strong ETI of the Arabidopsis accession Col against the Hiks1 isolate of *H. arabidopsidis* (McDowell *et al.*, 2000). The gene structure of *RPP7* is unusually complex and features three non-coding upstream exons, three central coding exons and three non-coding downstream exons. The *COPIA-R7* retrotransposon, which is inserted in the first *RPP7* intron, recruits high levels of the transposon silencing mark H3K9me2 into this region of *RPP7*, together with a polyadenylation signal in its 5′ long terminal repeat (Tsuchiya and Eulgem, 2013). Loss of *RPP7*-mediated resistance in *edm2* (*enhanced downy mildew 2*) (Eulgem *et al.*, 2007) mutants is associated with a strong shift in *RPP7* polyadenylation site use, leading to strong accumulation of proximally polyadenylated non-coding transcripts and, consequently, reduced production of full-length RPP7 coding transcripts (Lei *et al.*, 2013; Tsuchiya and Eulgem, 2013). Similar effects were observed in mutants of *ASI1/IBM2* (Saze *et al.*, 2013; Wang *et al.*, 2013). *EDM2* encodes a histone H3-binding PHD-finger protein (Eulgem *et al.*, 2007; Lei *et al.*, 2013; Tsuchiya and Eulgem, 2014), whereas *IBM2* encodes an RNA-binding protein (Saze *et al.*, 2013; Wang *et al.*, 2013). Thus, an unconventional mechanism may link histone marks to the RNA processing machinery.

EDM2 promotes high levels of H3K9me2 in the first *RPP7* intron and suppresses the use of the proximal polyadenylation site in this area. H3K9me2 is critical for this process, as the *suvh4/5/6* triple mutant, which is deficient in three Arabidopsis H3K9 methyltransferases (Ebbs and Bender, 2006), phenocopies the effects of EDM2 on *RPP7* expression and function. The EDM2- and H3K9me2-dependent polyadenylation mechanism is responsive to pathogen recognition and dynamically adjusts *RPP7* expression levels during the induction of immune responses (Tsuchiya and Eulgem, 2013).

POST-TRANSCRIPTIONAL REGULATION OF NLR TRANSCRIPTS BY SMALL RNAs

In addition to silencing at the transcriptional level by the mediation of cytosine methylation, plant small RNAs can also suppress the expression of target genes at the post-transcriptional level. MicroRNAs (miRNAs) and some types of small interfering RNAs (siRNAs), such as secondary *cis*- or *trans*-acting siRNAs (casiRNAs; tasiRNAs), can bind to complementary sequences in target mRNAs and suppress their expression, either by endonucleolytic cleavage followed by degradation or translational suppression (Axtell, 2013).

The first evidence for a role of small RNAs in the suppression of NLR genes was provided by Yi and Richards (2007), who found small RNAs to co-suppress several members of the Arabidopsis Col-0 *RPP5* cluster, including *SNC1* and *RPP4*. Although the mechanistic details of this process are not known, this process appears to involve 21-nucleotide siRNAs generated from overlapping sense and antisense transcripts within the *RPP5* cluster, as well as the Dicer-like protein DCL4 and the Argonaute member AGO1. Additional studies have suggested roles of miRNAs in the cleavage of NLR transcripts in *Brassica rapa*, *Vitis vinifera*, *Pinus taeda* and *Citrus trifoliata* (Carra *et al.*, 2009; He *et al.*, 2008; Lu *et al.*, 2007; Park and Shin, 2015; Song *et al.*, 2010).

Recent studies have provided substantial evidence for the use of phased siRNAs (phasiRNAs) in the broad silencing of NLR family members in legumes and Solanaceae (Park and Shin, 2015). This type of secondary siRNA can be triggered in Arabidopsis by 22-nucleotide miRNAs (Allen and Howell, 2010). By targeting conserved regions in 74 NLR transcripts of the legume *M. truncatula*, the miR1507, miR2109 and miR2118 families of highly abundant 22-nucleotide miRNAs trigger a cascade of phasiRNAs that probably serve as casiRNAs or tasiRNAs, suppressing the expression of at least 60% of the 540 NLR genes of this organism (Zhai *et al.*, 2011). One of these miRNA families, miR2118, is closely related to the miR482 family of Solanaceae, which can also serve as a miRNA trigger of a regulatory cascade suppressing NLR expression (Shivaprasad *et al.*, 2012). Although variable in sequence, members of the widely conserved miR482/miR2118 superfamily target transcript sequences encoding the P-loop motif of NLR nucleotide-binding sites, as well as LRR motifs of these immune receptors. Transient expression in *Nicotiana benthamiana* leaves supported a role of miR482 in NLR transcript decay associated with RDR6-dependent production of secondary phasiRNAs (Shivaprasad

et al., 2012). Intriguingly, this silencing cascade can be suppressed in plants infected with various viral or bacterial pathogens, resulting in enhanced levels of miR482-targeted NLR transcripts and, as a likely consequence, enhanced basal defence states, as NLR overexpression is known to trigger defence reactions even in the absence of the respective cognate avr ligands.

In addition to miR482-type miRNAs, six additional miRNA families from the three Solanaceae species tobacco, tomato and potato were also found to cleave NLR transcripts and to trigger secondary phasiRNAs (Li *et al.*, 2012). These include two miRNAs (nta-miR6019 and nta-miR6020) that guide the cleavage of the tobacco TMV resistance gene *N*. Transient overexpression of these two miRNAs in *N. benthamiana* leaves attenuated *N*-mediated TMV resistance, demonstrating the significance of these miRNAs for plant immunity (Li *et al.*, 2012). Evidence for similar phasiRNA networks suppressing NLR expression was also found in many other plant species, including gymnosperms (Howell *et al.*, 2007; Johnson *et al.*, 2009; Klevebring *et al.*, 2009; Zhai *et al.*, 2011), suggesting that this type of control mechanism evolved early in land plants. The ancient gymnosperm Norway spruce features 19 miRNA families targeting over 750 NLR genes to generate phasiRNAs. This also includes members of the miR482/miR2118 superfamily (Xia *et al.*, 2015). Based on an extensive analysis of the NLR contingents of 70 land plants, coupled with extensive small RNA data, Zhang *et al.* (2016) observed a tight association between the diversity of NLRs and the emergence of miRNAs targeting them. They proposed a co-evolutionary model according to which miRNAs targeting NLRs are frequently generated *de novo* from highly duplicated NLR genes.

Consistent with the involvement of RDR6 in the generation of secondary siRNAs and the roles of these small RNAs in NLR suppression, an Arabidopsis *rdr6* mutant exhibits enhanced resistance against virulent and avirulent strains of *P. syringae* (Boccara *et al.*, 2014). Enhanced resistance against *P. syringae* was also observed by knock down of the miR482-related Arabidopsis miR472, whereas overexpression of this NLR-targeting miRNA reduced pathogen resistance. In RDR6- and miR472-deficient lines, numerous NLR transcripts exhibit altered constitutive levels and a stronger defence-associated induction compared with wild-type Arabidopsis.

Taken together, all these observations indicate that miRNAs can serve as master regulators triggering cascades of secondary phasiRNAs, which collectively post-transcriptionally suppress the expression of a wide set of NLRs sharing conserved target motifs. This type of mechanism may serve one or several important purposes (see below).

1. Enhanced NLR evolvability by global NLR transcript dosage control

The necessity for fast-paced NLR evolution inevitably must lead to the occasional emergence of highly expressed variants causing a substantial fitness penalty for the plant. Even if expression levels of individual NLRs are limited, expansion of this gene family may result in intolerably high levels of NLR transcripts and, consequently, NLR proteins. Thus, miRNA-controlled global NLR suppression may keep collective levels of NLR transcripts under a critical threshold and provide a background allowing for safe diversification and expansion within NLR gene families (Shivaprasad *et al.*, 2012). An miRNA-triggered phasiRNA cascade globally suppressing transcripts of the pentatricopeptide repeat gene family in Arabidopsis has been proposed to serve a similar purpose and to quench rapid expansion of this fast-evolving and diversifying gene family (Allen and Howell, 2010; Lurin *et al.*, 2004; Saha *et al.*, 2007).

2. Counter-counter defence by pathogen-induced transient overexpression of NLR genes

As described above, the miR482-triggered silencing cascade can be suppressed by viral or bacterial pathogens (Shivaprasad *et al.*, 2012). This effect may be caused by microbial RNA silencing suppressors, which attenuate miR482 levels. Various types of pathogen seem to be able to suppress the host RNA silencing machinery, which originally evolved as a mechanism enhancing pathogen virulence (Li and Ding, 2006; Navarro *et al.*, 2008; Pumplin and Voinnet, 2013; Qiao *et al.*, 2013). By non-specifically counteracting miRNA-dependent defence responses, pathogens may also counteract the miR482/miR2118-mediated NLR suppression mechanism and transiently elicit high NLR transcript levels and, consequently, a broad activation of defence reactions, as observed in NLR-overexpressing plant lines (Park and Shin, 2015; Shivaprasad *et al.*, 2012).

3. Immune suppression to enable symbioses with microbes

In legumes, highly abundant miRNAs and associated phasiRNAs regulate NLR expression *en masse* (Zhai *et al.*, 2011). The extent of this NLR suppression system may imply a function in symbiosis with nitrogen-fixing rhizobia bacteria, a process specific to legumes. One of the NLR-targeting miRNAs, miR482, triggers hypernodulation in soybean and is transiently upregulated in this host in response to inoculation with rhizobia (Li *et al.*, 2010). Another NLR-targeting miRNA, miR1507, is upregulated in a hypernodulating soybean mutant (Li *et al.*, 2010). Thus, global suppression of NLR transcripts associated with rhizobial colonization may enable interactions with these beneficial symbionts and support the nodulation process. As some NLR-associated miRNAs are also conserved in Solanaceae and other plant clades, they may have a wider role in supporting plant–microbial symbioses, such as the mycorrhizal colonization of roots (Zhai *et al.*, 2011).

NONSENSE-MEDIATED DECAY OF NLR TRANSCRIPTS

Originally, nonsense-mediated mRNA decay (NMD) was identified as a cytoplasmic surveillance mechanism degrading aberrant mRNAs (Maquat, 2004; Schweingruber *et al.*, 2013). In many cases, the NMD machinery recognizes premature translational termination codons (PTCs) in conjunction with downstream exon junction complexes. NMD-targeted transcripts enter general mRNA degradation processes (Nicholson and Mühlemann, 2010). An increasing body of literature supports a wide role of NMD in eukaryotic gene expression control, and NMD seems to regulate the transcript levels of specific genes in response to a wide variety of biological signals (Bruno *et al.*, 2011; Drechsel *et al.*, 2013; Gloggnitzer *et al.*, 2014). Interestingly, impairment of NMD by mutations in Arabidopsis leads to constitutive immunity (Jeong *et al.*, 2011; Rayson *et al.*, 2012; Riehs-Kearnan *et al.*, 2012). This effect can be rescued by mutations in defence signalling components (Riehs-Kearnan *et al.*, 2012). A recent RNA-seq analysis found that transcripts of 50 Arabidopsis NLR genes were up-regulated in an NMD-deficient background, and were therefore probably suppressed by the NMD machinery (Gloggnitzer *et al.*, 2014). Splice variants of a subset of these, all of the TIR-NB-LRR-type, have typical features of NMD targets, such as PTCs, and were found to exhibit increased half-lives in the NMD- and defence-deficient *smg7*/*pad4* background. The Ler allele of one of these NLRs, $RPS6^{Ler}$, was found to be causal for the strong constitutive immunity phenotype observed in an NMD-deficient mixed Col-0/Ler ecotype background. As proposed for NLR-targeting miRNA/phasiRNA cascades, NMD may also enhance plant fitness and NLR evolvability by controlling global NLR transcript dosage and preventing inappropriately high NLR activity levels. Intriguingly, NMD appears to play a role in defence induction similar to the miRNA/phasiRNA cascades described above. The perception of MAMPs can trigger transient suppression of NMD in Arabidopsis and, consequently, a temporary increase in NMD target transcripts, including those of NLRs, which is associated with enhanced disease resistance against the virulent DC3000 strain of *P. syringae* (Gloggnitzer *et al.*, 2014).

AS can generate NLR transcripts with NMD-eliciting features. At least in some cases, excision of cryptic introns results in NMD-susceptible NLR transcripts, whereas transcripts containing retained introns, the most prevalent form of alternative splice products in plants, are often not targeted by NMD, even if featuring PTCs (Gloggnitzer *et al.*, 2014; Gohring *et al.*, 2014). Instead, such transcripts can be retained in the nucleus, where they are inaccessible to the NMD machinery (Gohring *et al.*, 2014).

ROLES OF TRANSPOSONS IN THE EVOLUTION OF NLR REGULATORY MECHANISMS

NLR genes are one of the fastest evolving gene families in plants. Their often-found organization in complex gene clusters is believed to be critical for the fast pace of their structural and functional diversification (Meyers *et al.*, 2003; Michelmore and Meyers, 1998). Frequent recombination events at such loci, followed by diversifying selection, may generate the structural diversity needed to match high avr effector evolution rates in the microbial world (Jacob *et al.*, 2013; McDowell and Simon, 2006). The need for well-balanced expression probably forces on new NLR varieties a maturation process to evolve suitable regulatory mechanisms. High enrichment of transposons in NLR clusters is often observed (Miyao *et al.*, 2003; Wei *et al.*, 2002). In the recent past, transposons have emerged as a major source of the 'raw material' needed for the fast evolution of nuanced NLR expression control. For example, in Solanaceae, the insertion of miniature inverted-repeat transposable elements (MITEs) within NLR regions appears to be conserved, as numerous families of these transposons were found to reside within six syntenic NLR regions in tomato, potato and tobacco (Kuang *et al.*, 2009). These insertions are suspected to affect gene function and regulation in many cases. One of them, *MiS1-1*, is critical for the function and regulation of the tobacco TIR-NB-LRR gene *N*, by providing the alternative exon, which is retained in the alternatively spliced N_I transcript (see above) (Dinesh-Kumar and Baker, 2000; Kuang *et al.*, 2009).

The insertion of the LTR retrotransposon Renovator upstream of the rice NLR gene *Pit* provided promoter sequences directing sufficiently high transcript levels of this gene for effective disease resistance against the fungal pathogen *Magnaporthe oryzae* (Hayashi and Yoshida, 2009). Likewise, insertion of the *COPIA-R7* LTR retrotransposon into the first intron of the 5′ UTR of the Arabidopsis NLR gene *RPP7* recruited an H3K9me2-dependent APA mechanism critical for the function of this gene (Tsuchiya and Eulgem, 2013). In addition to *COPIA-R7*, additional transposon sequences affect the complex regulation of *RPP7*. Several transposon and other repeat sequences in its 5′ UTR seem to attract massive cytosine methylation to a region directly upstream of the transcription start-site (Le *et al.*, 2014). However, cytosine methylation in this region is suppressed by demethylases to a level that allows for sufficient levels of transcription of this gene. Furthermore, electrophoretic mobility shift experiments with Arabidopsis nuclear protein extracts have shown that the Simplehat transposon in the distal promoter of *RPP7* provides a docking site for an unknown DNA-binding activity and serves as a *cis*-element mediating elevated *GUS* reporter gene expression in response to the recognition of *H. arabidopsidis* (Y.-H. Wei and T. Eulgem, unpublished).

CONCLUSIONS

The need for tightly regulated and balanced expression of NLR genes has led to the evolution of multiple mechanisms of NLR transcript-level expression control (Fig. 1). Several themes in this context reoccur.

1. The involvement of AS in NLR expression control is widespread (Table 1). Defence-associated changes in the ratios between different splice forms often seem to be critical for immunity. In some cases, AS seems to result in splice forms encoding truncated NLRs, which can be functionally important for defence induction.
2. Numerous examples of NLR suppression by miRNA-induced phasiRNA cascades as well as NMD are emerging. A probably common role of these mechanisms is global NLR transcript dosage control, which may be a critical prerequisite for the evolution of new NLRs and the expansion of this gene family. Pathogen-triggered down-regulation of these NLR suppressive mechanisms allows for a transient defence induction.
3. Transposons are likely to play an important role in the fast evolution of NLR genes by providing the 'raw material' for regulatory sequences and chromatin states serving their expression control.
4. Defence induction can trigger a transient global reduction of transposon silencing marks, such as 5mC and H3K9me2. At least in some cases, these epigenetic signals affect NLR expression and function.

In addition to NLR genes, the expression of other components of the plant immune system must be tightly controlled. The overexpression and misregulation of numerous defence regulators are known to result in autoimmunity and growth retardation, similar to the effects observed in plant lines with enhanced NLR activity. Thus, the regulatory mechanisms discussed here are unlikely to be limited to NLR genes. For example, the involvement of WRKY TFs and W box promoter elements is clearly widespread amongst many genes encoding components of the plant immune system. However, the tolerance of plants to a relaxation of NLR control mechanisms is low, and mutations affecting these are associated with reduced plant fitness.

A comprehensive understanding of NLR expression control is not only of academic interest. Efforts to increase pathogen and pest resistance of crops are strongly dependent on breeding-based transfer of NLR genes into different genetic backgrounds. The design of genetically engineered crops expressing new NLR derivatives is a promising approach for the near future. An accurate knowledge of the gene regulatory requirements for optimal expression of the respective NLR genes is likely to increase the success of breeding- and gene transfer-based approaches targeting enhanced pathogen resistance of crop cultivars.

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