The Ups and Downs of Plant NLR Expression During Pathogen Infection

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Plant Nucleotide binding-Leucine rich repeat (NLR) proteins play a significant role in pathogen detection and the activation of effector-triggered immunity. NLR regulation has mainly been studied at a protein level, with large knowledge gaps remaining regarding the transcriptional control of NLR genes. The mis-regulation of NLR gene expression may lead to the inability of plants to recognize pathogen infection, lower levels of immune response activation, and ultimately plant susceptibility. This highlights the importance of understanding all aspects of NLR regulation. Three main mechanisms have been shown to control NLR expression: epigenetic modifications, cis elements which bind transcription factors, and post-transcriptional modifications. In this review, we aim to provide an overview of these mechanisms known to control NLR expression, and those which contribute toward successful immune responses. Furthermore, we discuss how pathogens can interfere with NLR expression to increase pathogen virulence. Understanding how these molecular mechanisms control NLR expression would contribute significantly toward building a complete picture of how plant immune responses are activated during pathogen infection—knowledge which can be applied during crop breeding programs aimed to increase resistance toward numerous plant pathogens.

Keywords: pathogen infection, NLR, epigenetics, transcriptional regulation, NB-LRR, cis elements, NLR expression

INTRODUCTION

Various pathogens, including bacteria, fungi, oomycetes, and viruses, constantly bombard plant species and may cause large crop losses in agricultural settings. Plants have in turn evolved a complex set of defense mechanisms to combat pathogen infection (Jones and Dangl, 2006). Understanding how these plant defense responses are regulated and activated during pathogen attack will accelerate crop breeding programs and may contribute to the development of transgenic crop species with the desired resistance characteristics (Wang et al., 2019). Research focused on unraveling plant immune responses has, unsurprisingly, been of particular interest for the past decade (Bezerra-Neto et al., 2020). All research studies have contributed to reveal an increasingly more complex system, with thousands of signaling molecules, receptors, and hormones, each playing a role in plant immune responses (Wan et al., 2012; Van den Berg et al., 2018; Adachi et al., 2019).
Jones and Dangl (2006) first explained plant immune responses with the well-known Zig-Zag model. This model explains that pathogens are first recognized when pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs). After PAMP recognition, PRRs activate a low amplitude immune response, known as PAMP-triggered immunity (PTI). This immune response is often able to overcome infection by suppressing pathogen growth. However, some pathogens can overcome PTI responses. Plant Resistance (R) proteins may then recognize Avirulence effector (Avr) proteins, secreted by pathogens, and trigger effector-triggered immunity (ETI; Davis and Hahlbrock, 1987). A successful ETI response leads to the reactive oxygen species (ROS) and activation of the hypersensitive response (HR)—leading to localized plant cell death and the suppression of pathogen growth. When a R protein is neither present, or unable to recognize a corresponding Avr protein, effector-triggered susceptibility (ETS) is triggered, often leading to plant death (Cui et al., 2015).

This model can, however, be deceivingly simple (Naveed et al., 2020). For example, the model explains that a successful ETI response (able to overcome host-adapted pathogen attack) can only be activated when R proteins recognize a respective Avr protein. However, an increasing amount of evidence has suggested that the expression levels of R genes also contribute toward a successful immune response (Bradeen et al., 2009; Andam et al., 2020). When R gene expression is mis-regulated, the amplitude of ETI activation decreases, and an ETI response strong enough to suppress pathogen growth cannot be triggered (Umadevi and Anandaraj, 2017; Xu et al., 2018). Thus, even when a R protein is able to recognize a corresponding Avr protein, insufficient levels of this protein would lead to susceptibility. Understanding how R gene expression is regulated is thus the first step in untangling the mechanisms behind successful immune responses. In this review, we will focus on the regulatory mechanisms controlling R gene expression during pathogen infection, and how pathogens interfere and hijack these mechanisms for their own advantage.

**THE MAIN CHARACTER: NLR PROTEINS**

NLR proteins, also known as NB-LRRs, constitute the largest subclass of R proteins and are characterized based on containing a Nucleotide binding (NB/NB-ARC) domain and a Leucine rich repeat (LRR) domain. NLRs can further be classified based on whether or not they contain a C-terminal domain. The LRR domain is often present in these proteins (Bentham et al., 2017). In NLR dimer pairs, one NLR often acts as a “sensor” NLR, able to recognize pathogen Avr proteins (Bonardi et al., 2011). The second NLR acts as a “helper” NLR, triggering the ETI response following activation by the sensor NLR. NB domains remain largely conserved between species and are often used during phylogenetic studies (Maity et al., 2014). NB domains function as molecular switches for NLR proteins, determining whether the protein is in an active or inactive state. This molecular switch is controlled by ADP and ATP binding to the NB domain P-loop, with ATP binding to activate NLRs following Avr recognition (Steele et al., 2019). The LRR domain, however, is variable in length and shows large sequence variations since this domain is responsible for Avr recognition (Maule et al., 2007). The LRR domain also exerts a negative regulatory effect on the NLR, with loss of this domain leading to increased cell necrosis (Bentham et al., 2017).

The recognition of Avr proteins can either occur through direct binding to LRR domains, with the use of a guard protein, or through sensor NLR proteins (Chiang and Coaker, 2015). The use of a guard, or sensor NLRs, to recognize Avr proteins have been shown to significantly increase the variety of Avr proteins recognized by a particular NLR protein. Furthermore, many guard proteins including RIN4, are under the surveillance of multiple NLR proteins (Su et al., 2018). This increases the chances of Avr recognition and successful ETI activation. Arabidopsis RPP1 proteins serve as an example for NLR proteins which directly recognize Avr proteins (Botella et al., 1998). Some NLR proteins also contain integrated domains (ID), which may resemble Avr targets. For example, a WRKY domain was identified in the Arabidopsis RRS1-R protein which recognizes *Ralstonia solanacearum* effectors (Deslandes et al., 2002; Grund et al., 2019). *Ralstonia solanacearum* PopP2 and AvrRps4 effectors normally target WRKY transcription factors (TFs), which abolishes transcriptional activation of defense-related genes. However, when these effectors bind to RRS1-R proteins, the RRS1-R/RPP4 complex is activated and triggers defense responses (Ma et al., 2018). Thus, the WRKY ID acts as a decoy for *R. solanacearum* effectors.

**MORE IS BETTER—SOMETIMES: NLR EXPRESSION**

NLR regulation has mainly been studied on protein level, and very little is known regarding NLR transcriptional regulation (Yu et al., 2022). Studies focusing on the expression of NLR genes have highlighted the importance of proper timing and level of NLR expression to activate successful immune responses during pathogen attack (Liu et al., 2020a). The overexpression of NLR genes leads to stunted growth and Avr-independent cell death (Palma et al., 2010; Li et al., 2015). However, rice mutants in which the APIP4 NLR was knocked down showed increased susceptibility when infected with *Magnaporthe oryzae* (Zhang et al., 2020). Thus, enough NLR proteins need to be activated to trigger successful immune responses during pathogen infection. This indicates that NLR expression needs to be above a certain threshold (Bieri et al., 2004). These factors make it unsurprising to observe differences in NLR expression levels between resistant and susceptible plant genotypes when infected with a pathogen. For example, 22 NLRs were upregulated in *Raphanus sativus* resistant to *Plasmodiophora brassicae*, but not in a susceptible genotype during *P. brassicae* infection.
Furthermore, significant differences in \textit{NLR} expression were observed between a partially resistant and susceptible \textit{Persea americana} rootstocks infected with \textit{Phytophthora cinnamomi}, especially after 6h post-inoculation (Fick et al., 2022). The expression of \textit{NLR} genes is regulated by three main mechanisms: (1) epigenetic mechanisms, (2) \textit{cis} elements and TFs, and (3) post-transcriptional modifications (Figure 2; Bezerra-Neto et al., 2020). Epigenetic mechanisms include histone modifications and DNA methylation, which influence chromatin density, and subsequently the ability of TFs and transcription machinery to bind to gene promoter sequences. TFs, which bind to \textit{cis} elements in gene promoter sequences either exert

![Picture](image-url)
FIGURE 2 | Schematic illustration of the main regulatory mechanisms of plant NLR expression. (A) Before pathogen infection, a heterochromatin structure is
a positive or negative regulatory effect on gene transcription. Furthermore, post-transcriptional modifications include alternative splicing patterns and small RNA, which can introduce stop codons, change protein structure or cause NLR mRNA degradation.

### Epigenetic Control of NLR Genes

Epigenetic modifications regulate whether the chromatin is in a euchromatin (open) or heterochromatin (condensed) structure, thus controlling whether NLR transcription can be activated (Figures 2A, B). Histone modifications and DNA methylation patterns are dynamic molecular mechanisms able to change chromatin structure following pathogen infection (Zhang et al., 2018). Histone modifications have mostly been studied for Arabidopsis NLR genes. One histone modification often associated with transcriptional activation is the trimethylation of lysine 4 of histone H3 (H3K4me3) and is observed to regulate the expression of the Arabidopsis RPP4 and SNC1 NLR genes (Kouzarides, 2007; Xia et al., 2013). This histone mark is established by the histone methyltransferase ATXR7, with the expression of both NLRs being reduced in atrx7 mutants (Xia et al., 2013). Expression of LAZ5, another Arabidopsis NLR gene, is also controlled by histone methylation. Histone methyltransferase SDG8 is responsible for di- and trimethylated H3K36, activating LAZ5 transcription (Palma et al., 2010). The di- and trimethylated H3K36 mark, is interestingly associated with alternative splicing patterns (discussed below) of NLR genes. H3K36me2/me3 levels were significantly higher at the 5’ UTR (untranslated region) of the ARG1 NLR gene in resistant Sorghum bicolor genotypes when infected with Colletotrichum sublineola (Lee et al., 2022). H3K36me2/me3 was shown to increase the expression of ARG1 and regulate alternative splicing patterns to produce a full-length ARG1 mRNA transcript. In the susceptible S. bicolor genotype, lower H3K36me2/me3 marks and expression of ARG1 was observed, together with truncated ARG1 mRNA. This indicates that histone modifications also control NLR expression in an indirect manner at post-transcriptional levels.

Histone acetylation is also associated with active transcription of NLR genes (Luna et al., 2012). Histone acetylation is established by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs; Bannister and Kouzarides, 2011). Acetyl groups are negatively charged, and histone acetylation would thus result in the chromatin adopting a euchromatin structure (Luna et al., 2012). One HDAC, HD9, in association with Aragonate 4 (AGO4), domains rearranged methyltransferase 2 (DRM2), and RNA-dependent DNA methyltransferase 1 (FD1M1) proteins. DNA methylation is further maintained by DNA methyltransferase 1 (MET1), CMT3 (Chromomethylase 3), and CMT2/DRM2. Following pathogen infection, a euchromatin structure is adopted which allows for the activation of NLR expression. Histone marks H3K36me3 and H3K36me2/me3 are established by Histone methyl transferases (HMT), H3K9ac by Histone acetyltransferases (HAT), and H2Bub1 by Histone monoUbiquitination 1 and 2 (HUB1/2). Repressor of silencing 1 (ROS1) and DEMETER enzymes (DME) antagonize the RNA-directed DNA methylation pathway, and lower levels of DNA methylation is observed. A euchromatin structure allows for transcription factors (TFs) to bind to cis elements within NLR promoter sequences located upstream from the transcription start site (TSS). Most TFs are activated following stress hormone (H) detection, but may also be activated by pathogen effectors (E). The four most common cis elements identified within NLR promoter sequences include W-boxes, ABRE, MYB, and MYC elements. Following NLR expression, alternative splicing (AS) patterns may contribute to different NLR mRNA isoforms, and thus, different levels of NLR proteins. AS may produce mRNAs containing (1) different exons, (2) different Untranslated regions (UTRs), (3) or a retained intron which may code for a stop codon, producing a truncated NLR protein following translation. MicroRNA (miRNA) molecules in association with AGO1 can downregulate NLR expression by binding to NLR mRNAs to either block mRNA translation or cause mRNA degradation. Phased secondary RNA (phasiRNA) molecules can also be produced when diced NLR mRNAs are reverse transcribed by RNA-directed RNA polymerase 6 (RDR6) and diced by Dicer-like 4 (DCL4). These phasiRNA molecules may then target more NLR mRNA molecules to further contribute to NLR suppression (DCL3—Dicer-like 3; Pol IV—RNA polymerase IV).
then incorporated into Argonaute 4 (AGO4) as single-stranded siRNA. In association with the AGO4-siRNA complex, Domains rearranged methyltransferase 2 (DRM2) and RNA-dependent DNA methylation 1 (RDML1) establishes de novo DNA methylation (Wendte and Pikaard, 2017). Thereafter, DNA methylation is maintained by MET 1 (DNA methyltransferase 1) at CG sites, CMT3 (Chromomethylase 3) at CHG sites, and CMT2/DRM2 at CHH sites. DNA methylation defective Arabidopsis plants showed increased resistance levels toward P. syringae pv. tomato DC3000 and H. arabidopsidis, indicating that decreased levels of DNA methylation may lead to increased NLR expression and immune activation (Dowen Robert et al., 2012; López Sánchez et al., 2016).

The widespread loss of DNA methylation (hypomethylation) at TEs has been observed to occur during the activation of immune responses following pathogen infection (Anaccondia et al., 2021). Demethylation of promoters leads to cis elements being more accessible to TFs, ultimately leading to increased NLR gene expression and disease resistance. In poplar trees infected with Lonsdaila populi, hypomethylation occurred at CH sites within promoter regions of defense-related genes (Xiao et al., 2021). Higher levels of hypomethylation were particularly noted in poplar trees with increased resistance toward L. populi when compared to susceptible trees. This observation suggests that hypomethylation results in increased defense-related gene expression which may ultimately lead to increased resistance levels. This could be explained by the observation that AGO4 is repressed after Arabidopsis treatment with PAMP flagellin-22 (flg22), and Aegilops tauschii infection with Blumeria graminis f. sp. tritici (Yu et al., 2013; Geng et al., 2019). The repression of AGO4 leads to lower levels of DNA methylation, which decreases transcriptional repression. Furthermore, ROS1 (Repressor of silencing 1) antagonizes RdDM mediated DNA methylation and promotes resistance toward P. syringae pv. tomato DC3000 (Halter et al., 2021). ROS1 has specifically been implicated in the regulation of some Arabidopsis NLR genes. In ros1 mutants, four NLR genes showed decreased expression levels, due to active demethylation being repressed (Kong et al., 2020). ROS1 was also shown to demethylate promoter regions in which WRKY TFs bind (Halter et al., 2021). In particular, ROS1 demethylated promoter regions of RGM1 TNL after PTI activation by P. syringae pv. tomato DC3000 flagellin proteins. Lastly, DNA demethylation by DEMETER (DME) enzymes also contributes to enhanced resistance toward Verticillium dahlia and P. syringae pv. tomato infection in Arabidopsis (Zeng et al., 2021). In dme mutants, a hypermethylated region was associated with the AtPRX34 TNL gene. This gene showed lower expression levels following P. syringae pv. tomato infection in dme mutants when compared to wild-type plants. These results indicate that DME demethylates NLR sequences in response to bacterial and fungal infection.

**Cis Elements of NLR Genes**

Cis elements of NLR genes remain largely unknown due to these genes having unusually large promoter sequences (Yu et al., 2022). An NLR promoter sequence has often been defined as the 2kb region upstream from the NLR transcription start site (Figure 2C).

Multiple cis elements are frequently identified within NLR promoter sequences, many being pathogen-inducible cis elements (Table 1). These cis elements may be found in different arrangements within promoter sequences, resulting in complex gene regulatory mechanisms (Wang et al., 2021). Regulatory mechanisms are further complicated by the fact that the TFs which bind to these cis elements either exert a positive or negative regulatory effect on gene expression. Both a positive and negative cis-acting element were identified within the SNC1 NLR gene promoter using CRISPR/Cas9 directed mutations in Arabidopsis (Yu et al., 2022). This study further identified that two other NLR genes, RPP4 and SIKIC2, are also affected by these mutations. This may indicate that these genes share the same cis elements. This hypothesis is supported by the fact that plant NLRs are often found within gene clusters and arranged in a head-to-head configuration (Narusaka et al., 2009; Van Wersch and Li, 2019). Many of these NLRs are often co-expressed following infection, further suggesting that these genes might be under the control of the same promoter, or promoters with the same cis elements (Liang et al., 2019; Yang et al., 2021).

It is important to remember that the abundance of TFs and certain arrangements of cis elements also influence gene expression levels, and the simple binding of a specific TF does not necessarily activate gene expression (Hoang et al., 2017). Thus, the identification of NLR cis elements alone cannot be used to predict the level of NLR expression, or when transcription will be activated. In tomato plants, a single nucleotide difference was identified in the promoter region of the Sl3SR-1 NLR gene when compared between a Tomato spotted wilt virus (TSWV) resistant and susceptible plant (Qi et al., 2022). This single nucleotide deletion in resistant tomato plants resulted in a new TF binding site to be formed, which increases Sl3SR-1 expression and subsequent resistance. Importantly, cis elements are not the only regulatory sequences to control NLR expression—the tobacco N TNL contains two introns which contribute to increased expression levels of this gene during Tobacco mosaic virus (TMV) infection (Ikeda et al., 2021). Transient expression of the N gene without these introns showed lower levels of expression.

Cis elements identified most often in NLR promoter sequences include W-boxes, ABRE, MYB, and MYC elements (Mohr et al., 2010; Ding et al., 2020). W-boxes bind WRKY TFs, which is a large, diverse group of zinc finger TFs (Babu et al., 2006). These TFs are mostly activated by pathogen infection, effectors, and stress hormones, such as salicylic acid (SA) and JA. Following activation, a subset of WRKYs trigger the expression of PTI and ETI-related proteins, and the synthesis of stress hormones (Chen et al., 2019). Interestingly, an apple (Malus domestica) NLR gene, MdNLR16, is under the control of the MdWRKY79 TF which is responsive to sorbitol levels (Meng et al., 2018). Higher sorbitol levels lead to increased MdNLR16 expression and subsequently enhanced resistance levels toward Alternaria alternata. ABRE elements are abscisic acid responsive elements which are recognized by bZIP proteins (Hobo et al., 1999). A single ABRE element, however, is not able to activate transcription, instead multiple elements are needed for transcriptional activation (Shen et al., 1996). Lastly, MYB and MYC elements have been shown to activate gene transcription in response to both abiotic...
| Cis element                  | Species                        | Putative function                     | Reference                                |
|-----------------------------|--------------------------------|---------------------------------------|------------------------------------------|
| Common CAAT                 | Pinus monticola                | Common element                        | Liu and Xiang, 2019                     |
|                             | Lagenaria siceraria            |                                       | Wang et al., 2022b                      |
|                             | Triticum durum                 |                                       | Rampino et al., 2017                    |
| TATA-box                    | P. monticola                   | Core element                          | Liu and Xiang, 2019                     |
|                             | L. siceraria                   |                                       | Wang et al., 2022b                      |
|                             | Tomato                         |                                       | Qi et al., 2022                         |
| Pathogen-inducible/stress   |                                |                                       | Rampino et al., 2017                    |
| ABRE                        | Rice                           | Abscisic acid responsive element      | Ding et al., 2020                       |
|                             | L. siceraria                   |                                       | Wang et al., 2022b                      |
|                             | T. durum                       |                                       | Rampino et al., 2017                    |
|                             | Vitis vinifera                 | Salicylic acid responsive element     | Goyal et al., 2021                      |
| AS-1 (TGACG)                |                                |                                       | Rao et al., 2018                        |
|                             | P. monticola                   | Regulation of defense-related genes   | Liu and Xiang, 2019                     |
|                             | T. durum                       | Stress tolerance genes                | Liu and Xiang, 2019                     |
| BIHD-binding site (TGTCGA)  |                                | Methyl jasmonate responsive element   | Wang et al., 2022b                      |
| CCGC-box (ACCGGT)           |                                |                                      | Rampino et al., 2017                    |
| CGTCA-motif and TACG-motif  |                                |                                      | Cui et al., 2017                        |
| E-box (CANNTG)              | Rice                           | Jasmonic acid responsive element       | Miyamoto et al., 2012                   |
| ERE-box (ATTTCAAA)          | P. monticola                   | Ethylene responsive element           | Liu and Xiang, 2019                     |
| G-box                       | Rice                           | Regulation of defense-related genes   | Wang et al., 2020                       |
|                             | T. durum                       |                                       | Rampino et al., 2017                    |
| GARE-motif, P-box, and TATC-box | T. siceraria                  | Gibberellic responsive element         | Wang et al., 2020                       |
| GCC-box (AGCCGCCC)          | Rice                           | Ethylene and pathogen responsive gene | Kong et al., 2018                       |
| GT1-box (GAAAAAA)           | Saccharum spontaneum           | Pathogen and salt-induced gene         | Liu and Xiang, 2019                     |
| GTTGA                       | P. monticola                   | Rhizoctonia solani inducible          | Li et al., 2017                         |
| H-box (CCTACCTCT)           | Zea mays                       | Regulation of defense-related genes   | Kong et al., 2018                       |
| MYB recognition elements    | Rice                           | Stress responsive elements            | Kong et al., 2018                       |
| Myb1-box (GTAGTT)           | P. monticola                   | Regulation of defense and drought-     | Liu and Xiang, 2019                     |
|                             | L. siceraria                   | related genes                         | Wang et al., 2022b                      |
| MYC elements                | Rice                           | Stress responsive elements            | Wang et al., 2020                       |
| STRE                        | Rice                           |                                       | Ding et al., 2020                       |
| TRATTT                      | Z. mays                        | Rhizoctonia solani inducible          | Li et al., 2017                         |
| TC-rich repeats             | V. vinifera                    | Stress responsive element             | Goyal et al., 2021                      |
|                             | L. siceraria                   |                                       | Wang et al., 2020                       |
|                             | G. max                         |                                       | Cui et al., 2017                        |
| TCA element                 | V. vinifera                    | Salicylic acid responsive element     | Goyal et al., 2021                      |
|                             | G. max                         |                                       | Cui et al., 2017                        |
| TGA element                 | V. vinifera                    | Auxin responsive element              | Goyal et al., 2021                      |
| W-box (TTGACY)              | P. monticola                   | Regulation of defense-related genes   | Liu and Xiang, 2019                     |
|                             | V. vinifera                    |                                       | Wang et al., 2020                       |
|                             | Arabidopsis                    |                                       | Wang et al., 2020                       |
|                             | S. spontaneum                  |                                       |                                         |
|                             | A. chinensis                   |                                       |                                         |
and biotic stressors (Feng et al., 2013; Fang et al., 2018; Wu et al., 2019). NLR cis element identification studies have thus shown that the TFs controlling NLR expression is mostly activated by abiotic and biotic stress. Cis element studies may then also be used to identify putative NLR function. For example, in *Pinus monticola* Douglas ex D. (Western white pine trees), cis element identification of the *PmtNL2* gene suggested that this NLR might be important for both plant immune responses, as well as growth and development (Liu and Xiang, 2019).

**Post-transcriptional Modifications of NLRs**

Alternative splicing (AS) contributes significantly toward the diversity of the NLR transcriptome and NLR proteome—altering levels of different mRNA isoforms in response to developmental and environmental conditions (Kellemen et al., 2013). AS can result in NLR mRNAs to contain different exons, 5'- and 3' untranslated regions, and introns which may introduce stop codons resulting in truncated NLR proteins (Figure 2D). The example used most often for AS of *NLR* genes is the N TNL protein associated with resistance toward TMV. The *N* gene produces either a short *N* mRNA (*N*) or a long *N* mRNA (*N*; Erickson et al., 1999). *N* contains an exon which encodes a stop codon, resulting in a truncated protein. *N* is however, is translated into a complete protein. Both these proteins are expressed during TMV infection and needed for full TMV resistance. One rice *CNL*, *Pi-ta*, produces up to 11 different protein isoforms as a result of AS (Costanzo and Jia, 2009). In response to *M. oryzae* infection, a resistant rice genotype showed increased expression levels of a *Pi-ta* protein with a C-terminus thioredoxin domain, when compared to a susceptible genotype.

In both barley and wheat, AS was seen to regulate whether and which IDAs were present in NLR proteins in response to different experimental conditions (Halterman et al., 2003; Andersen et al., 2020). Different IDs may influence where NLR proteins localize to in the plant cell or may even cause the NLR protein to act as a decoy target for Avr proteins (Yang et al., 2014). It is worth noting that AS may also have an impact on proteins guarded by NLRs, suggesting that AS may regulate NLR activity in an indirect manner. For example, a NLR Rpi-vnt1.1 guards the GLYK (Glycerate 3-kinase) protein in potato (Gao et al., 2020). A truncated isoform of GLYK, which does not contain a chloroplast transit peptide-encoding sequence, is expressed in dark conditions, and cannot be recognized by a *Phytophthora infestans* Avr protein AVRvnt1. Thus, Rpi-vnt1.1 cannot activate immune responses. In light conditions, the full-length GLYK mRNA is expressed, and this protein isoform binds to the Avr during infection, leading to Rpi-vnt1.1 being activated to trigger ETI.

**NLR repression can be regulated at a post-transcriptional level using siRNAs and micro RNAs (miRNAs; Figure 2E).** miRNAs are non-coding RNAs between 20 and 24 nucleotides in length. They are encoded by *miRNA* genes, which are transcribed by RNA polymerase II to produce a long, primary *miRNA* (pre-miRNA) is produced which forms a hairpin structure with a self-complementary stem loop. This pre-miRNA molecule is then converted to a double-stranded *miRNA*, which is exported to the cytoplasm (Sun et al., 2019). An RNA-induced silencing complex (RISC) is then formed when the mature *miRNA* binds to an AGO1 protein. *miRNAs* guide AGO1 proteins to target mRNAs, either resulting in endonucleolytic cleavage and degradation or inhibition of translation. The P-loop domain, important for ATP binding and NLR protein activation, is a common target for *miRNAs* (Zhai et al., 2011; Fei et al., 2015). NLR *miRNA* cleavage by *miRNAs* may also produce *phasiRNAs* (phased secondary small interfering RNAs), which then target other mRNAs with the same sequence (Liu et al., 2020b). Three *Medicago truncatula* *miRNA* families target *miRNA* transcripts of 74 NLRs, leading to the production of *phasiRNAs* which suppress the expression of 324 NLR genes (Zhai et al., 2011). Liu et al. (2014) showed that the barley *miR9863* family targets *MAL1 CNL* transcripts, with the resulting *phasiRNAs* also leading to *MAL1* mRNAs being degraded. The authors suspect that this pathway prevents immune responses from being overloaded, and thus, NLR downregulation may have a positive effect on plant resistance levels. In Arabidopsis *miR472* knock-down mutants, increased resistance levels were observed toward *P. syringae*, and reduced resistance levels were observed when this *miRNA* was overexpressed (Boccara et al., 2014). This presents an interesting method of pathogen control—transient expression of *miRNA* targets in host plants may increase resistance levels toward various pathogens. In tomato, transient expression of short tandem target mimic *miRNAs* increased resistance levels toward *P. infestans* and *P. syringae* (Canto-Pastor et al., 2019). These mimic *miRNAs* acted
as targets for miR482/211b, which resulted in increased NLR expression and enhanced disease resistance.

**OH NO YOU DO NOT: HOW PATHOGENS INTERFERE WITH NLR EXPRESSION**

With multiple proteins contributing to the regulation of NLR expression, comes multiple opportunities for pathogen interference. Despite this, very few cases are documented in which pathogen Avr proteins influence NLR expression. However, Avr targets remain largely unknown, and it is yet to be discovered how NLR regulation is hijacked by pathogens (Wu et al., 2022). An average of 32% of Avr proteins from bacteria, fungi, and oomycetes localize in the plant cell nucleus, indicating that these Avr proteins may interfere with NLR transcription (Khan et al., 2018). Two cytoplasmic effectors from *M. oryzae*, MoHTR1, and MoHTR2, bind to effector binding elements (EBE) in rice gene promoters and function as transcription repressors (Kim et al., 2020). These EBEs were present in many defense-related gene promoters, and the binding of these effectors led to significant transcription repression. Transient expression of MoHTR1 and MoHTR2 in rice not only led to increased susceptibility toward *M. oryzae*, but also to *Xanthomonas oryzae* pv. *oryzae* and *Cochliobolus miyabeanus*. It remains unclear whether these effectors bind host repressor proteins, or whether they interfere with the binding of transcription activators. A *Melampsora larici-populina* effector, Mlp124478, also interferes with the transcription of WRKY TFs which indirectly inhibits the activation of defense-related gene expression, including the RPP8 NLR (Ahmed et al., 2018). Some pathogen Avr proteins also interfere with the synthesis of stress hormones. SA metabolism is inhibited by *Phytophthora sojae* PsIsc1 and *Verticillium dahliae* VdIsc1 enzymatic effectors, which redirects the precursor molecule of SA from the plastid into the cytosol (Liu et al., 2014). The metabolism of SA decreases and thus SA-mediated immune responses cannot be activated. Since some TFs which bind to NLR cis elements are activated by SA, lower SA levels may disrupt the activation of NLR expression (Goyal et al., 2021).

From another perspective, the suppression of NLR expression may not be the ultimate goal of the pathogen. *Phytophthora* species are hemibiotrophic oomycetes, which switch from a biotrophic to necrotrophic phase during infection (Zuluaga et al., 2016). During the necrotrophic phase, increased NLR expression may be beneficial to the pathogen since NLRs activate the HR and thus, plant cell death. *P. sojae* RxLR effectors PSR1 and PSR2 suppress plant RNA silencing by interfering with the miRNA synthesis pathway, which increased susceptibility in *Nicotiana benthamiana* (Qiao et al., 2013). This may lead to higher NLR levels and activation of HR, resulting in a more favorable environment for necrotrophic pathogens. This hypothesis is further supported by the fact that PSR2 is only expressed during the later stages of infection, when *P. sojae* switches to a necrotrophic stage (Qiao et al., 2013). Furthermore, the *V. dahliae* VdSSR1 protein was shown to interfere with the nuclear exportation of AGO1-miRNA complexes in *N. benthamiana* (Zhu et al., 2022). Decreased AGO1-miRNA exportation would subsequently lead to decreased suppression of NLR expression, which may contribute to the observed increased susceptibility in transgenic plants expressing VdSSR1 at higher levels. However, VdSSR1 expression data is needed to indicate whether this protein is only expressed during the necrotrophic stage of this hemibiotrophic fungus. Lastly, a necrotrophic fungus, *Botrytis cinerea*, is able to translocate siRNAs into plant cells and may redirect host siRNA machinery (Weiberg et al., 2013). *B. cinerea* siRNAs were associated with AGO1 proteins during infection of Arabidopsis, indicating that *B. cinerea* may hijack RISC to increase virulence (Ellendorff et al., 2009). It would be interesting to investigate whether these siRNA molecules cause siRNA-directed cleavage and degradation of NLR mRNA transcripts (Qiao et al., 2021). It may also be of interest to investigate whether pathogen-derived siRNAs influence DNA methylation patterns during infection.

**CONCLUSION**

NLR proteins play a significant role in activating plant immune responses during pathogen attack. The mis-regulation of NLR-encoding genes considerably impairs the plant’s ability to detect pathogen Avr proteins, which ultimately leads to susceptibility. Thus, a comprehensive understanding of NLR gene regulation is of particular interest. Unfortunately, NLR protein regulation has mainly been studied on a post-translational level, with a large knowledge gap remaining regarding the transcriptional- and post-transcriptional regulation of these proteins. Identifying epigenetic marks, and *cis* elements which control NLR expression in response to pathogen attack provides the first step in unraveling these complex regulatory mechanisms. These mechanisms can further be compared between susceptible and resistant plant genotypes to understand the factors which contribute to a successful immune response. Furthermore, investigating how pathogens interfere with these mechanisms would provide much needed insight into plant–pathogen interactions. Ultimately, knowledge in these areas may be used during plant breeding programs which aim to produce genotypes with increased resistance toward a variety of pathogens. These mechanisms can also be used to drive the expression of trans-NLR genes in genetically modified crops, with the goal of increasing resistance toward both biotic and abiotic stresses.

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AF conceptualized, drafted, and reviewed the manuscript. VS and NB reviewed and assisted in the drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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