Sukarta, Octavina C.A. and Townsend, Philip D and Llewelyn, Alexander and Dixon, Christopher H and Slootweg, Erik J and Pålsson, Lars-Olof and Takken, Frank L.W. and Goverse, Ask and Cann, Martin J (2020) 'A DNA-binding bromodomain-containing protein interacts with and reduces Rx1-mediated immune response to Potato Virus X.', Plant communications., 1 (4). p. 100086.

Further information on publisher’s website:
https://doi.org/10.1016/j.xplc.2020.100086

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A DNA-Binding Bromodomain-Containing Protein Interacts with and Reduces Rx1-Mediated Immune Response to Potato Virus X

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https://doi.org/10.1016/j.xplc.2020.100086

ABSTRACT

Plant NLR proteins enable the immune system to recognize and respond to pathogen attack. An early consequence of immune activation is transcriptional reprogramming. Some NLRs have been shown to act in the nucleus and interact with transcription factors. The Rx1 NLR protein of potato binds and distorts double-stranded DNA. However, the components of the chromatin-localized Rx1 complex are largely unknown. Here, we report a physical and functional interaction between Rx1 and NbDBCP, a bromodomain-containing chromatin-interacting protein. NbDBCP accumulates in the nucleoplasm and nucleolus, interacts with chromatin, and redistributes Rx1 to the nucleolus in a subpopulation of imaged cells. Rx1 overexpression reduces the interaction between NbDBCP and chromatin. NbDBCP is a negative regulator of Rx1-mediated immune responses to potato virus X (PVX), and this activity requires an intact bromodomain. Previously, Rx1 has been shown to regulate the DNA-binding activity of a Golden2-like transcription factor, NbGlk1. Rx1 and NbDBCP act synergistically to reduce NbGlk1 DNA binding, suggesting a mode of action for NbDBCP’s inhibitory effect on immunity. This study provides new mechanistic insight into the mechanism by which a chromatin-localized NLR complex co-ordinates immune signaling after pathogen perception.

Keywords: cellular immune response, DNA-binding protein, host–pathogen interaction, plant biochemistry, plant defense, plant virus

Sukarta O.C.A., Townsend P.D., Llewelyn A., Dixon C.H., Slootweg E.J., Pålsson L.-O., Takken F.L.W., Goverse A., and Cann M.J. (2020). A DNA-Binding Bromodomain-Containing Protein Interacts with and Reduces Rx1-Mediated Immune Response to Potato Virus X. Plant Comm. 1, 100086.

INTRODUCTION

The plant’s innate immune system enables cell-autonomous defense responses upon pathogen perception (Maekawa et al., 2011b; Jones et al., 2016). Plant nucleotide-binding leucine-rich repeat (NLR)-type immune receptors directly or indirectly detect pathogen-produced effector proteins and mediate immune responses to the invading pathogen (Staskawicz et al., 2001; Eitas and Dangl, 2010; Maekawa et al., 2011b). NLR proteins are signal-transduction ATPases with numerous domains and have a multidomain structure that allows them to function as a sensor, switch, and response factor (Leipe et al., 2004; Takken and Tameling, 2009).

Plant NLR immune receptors can be broadly divided into three distinct domains. The N terminus typically consists of either a coiled-coil (CC) or Toll–interleukin 1 receptor domain that is
involved in inter- and intramolecular interactions (Maekawa et al., 2011a). The nucleotide-binding (NB) domain, sometimes referred to as the NB, Apaf-1, R-proteins, or CED-4 (NB-ARC) domain, forms the central domain and is proposed to function as a nucleotide-dependent molecular switch in NLR activation (van der Biezen and Jones, 1998; Tranier et al., 2000; Takken et al., 2006; Takken and Tameling, 2009). Finally, the C-terminal leucine-rich repeat domain determines pathogen recognition specificity and maintains the NLR protein in an autoinhibited state in the absence of a pathogen-derived signal. The NB-ARC domains of NLRs are bound to ADP in the off state (Tameling et al., 2006; Maekawa et al., 2011a; Williams et al., 2011). Pathogen recognition by the NLR is hypothesized to activate the nucleotide exchange of ADP for ATP, allowing the NB-ARC domain to adopt an activated state and triggering immune signaling, whereas the hydrolysis of ATP to ADP is proposed to re-establish the inactivated state (Tameling et al., 2006). ATP binding in potato Rx1 has been linked to its in vitro activity (Fenyk et al., 2015). Recent elucidation of the cryoelectron microscopic structure of Arabidopsis ZAR1 in its autoinhibited and activated states provides structural support for this ADP/ATP exchange model (Wang et al., 2019a, 2019b). The NB-ARC domain, however, may not be a strict ATPase for all plant NLRs because, for example, the NB subdomains of rice Os2g_25 900, maize PSIP, and Arabidopsis Rpm1 can sequentially cleave phosphates from the nucleotide to the nucleoside in vitro, although the in vivo role of this activity has yet to be determined (Fenyk et al., 2012).

Proper NLR function often requires a nucleocytoplasmic distribution in the cell. A subset of NLR proteins, including Nb, MLA10, and Rx1, has a dynamic nuclear-cytoplasmic distribution, whereas RRS1-R becomes localized to the nucleus, dependent on the Pop2p effector (Deslandes et al., 2003; Burch-Smith et al., 2007; Shen et al., 2007; Caplan et al., 2008; Slootweg et al., 2010). Several other NLRs, including barley MLA1 and MLA10, Arabidopsis RPS4 and SNC1, and the tobacco N protein, also show nuclear localization (Shen et al., 2007; Wirthmueller et al., 2007; Zhu et al., 2010; Bai et al., 2012). Nuclear expulsion of Rx1, MLA10, N, RPS4, and SNC1 compromises immune activation, suggesting that NLR-dependent signaling components reside in the nucleus (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007; Cheng et al., 2009). The identity of these signaling components is therefore of considerable interest.

In vitro biochemical experiments demonstrate that at least a subset of plant NLRs are directly active at DNA. Significant structural homology was proposed between the NLR NB-ARC domain and the DNA replication origin-binding Cdc6/Orc1 proteins (Tameling et al., 2006). In line with this observation, direct interaction with DNA has been observed in vitro for potato Rx1, tomato I-2, and the maize orphan NLR PSIP (Fenyk et al., 2015, 2016). The Rx1 gene, introgressed into potato from the wild species Solanum tuberosum ssp. andigena, confers resistance to potato virus X (PVX) upon recognition of its coat protein (Bendahmane et al., 1995, 1999). The Rx1 protein binds to genomic DNA in situ on immune activation (Fenyk et al., 2015). In addition, Rx1 induces the ATP-dependent bending and melting of DNA in vitro. Analysis of Rx1 binding to a variety of DNA structures demonstrated that it favors topologies that resembled transcription bubbles. Rx1 therefore binds, bends, and distorts DNA in a manner reminiscent of the formation of the transcription initiation complex (Finzi and Dunlap, 2010; Liu et al., 2010; Tang et al., 2011; Kim et al., 2012). A further interesting parallel between Rx1 and the eukaryotic Cdc6/Orc1 proteins is that eukaryotic ORCs lack DNA sequence specificity in vitro but show a higher affinity for specific DNA topologies (Remus et al., 2004; MacAlpine et al., 2010). Consistent with this, Rx1 showed no observed sequence specificity but did show an increased affinity for branched and melted DNA topologies over linear double-stranded DNA (dsDNA).

One of the most important and earliest consequences of immune activation is transcriptional reprogramming (Navarro et al., 2004; Tsuda et al., 2008; Garner et al., 2016). The associations of MLA10 with Myb and WRKY transcription factors (TFs) and N with the TF SPL6 suggest that plant NLRs themselves may directly regulate transcription during the immune response (Chang et al., 2013; Padmanabhan et al., 2013; Roberts et al., 2013). The specificity of an immune-dependent transcription response is difficult to reconcile with the observation that Rx1 DNA-binding specificity may be mediated by local DNA topology rather than sequence specificity, implying the involvement of other factors in conferring specificity. Indeed, the CC of Rx1 has been shown to interact with a Golden2-like (GLK) TF, NbGlk1 (Townsend et al., 2018). NbGlk1 binds distinct to consensus DNA sequences, and this binding affinity is reduced upon its interaction with Rx1 in vitro. Moreover, NbGlk1 overexpression activates immune responses to PVX. Such a direct involvement of GLK-like TFs in defense signaling has also been reported in Arabidopsis toward cucumber mosaic virus (Han et al., 2016) and the fungal pathogens Fusarium graminearum (Savitch et al., 2007) and Hyaloperonospora arabidopsidis (Murmu et al., 2014).

Transcriptional reprogramming initiated as part of an immune response must be under tight control and is likely to be exercised at multiple levels (Garner et al., 2016). Rx1 associates with NbGlk1 and prevents its assembly on DNA unless Rx1 is activated by PVX, representing one level of transcriptional control (Townsend et al., 2018). Among other potential mechanisms, histone modifications are of particular interest because they represent an important mechanism to control the transcription of defense-related genes (Espinas et al., 2016). For example, histone modification through the removal of acetyl groups from modified lysine residues by histone deacetylases can suppress immunity (Ding et al., 2012; Wang et al., 2017).

Here, we set out to identify nuclear regulators of Rx1 function and investigate their role in immunity. We report that Rx1 interacts directly with a DNA-binding bromodomain (BD)-containing protein (NbDBCP), thereby identifying a new member of the nuclear DNA bound NLR complexes that control plant immunity. The finding that NbDBCP acts as a negative regulator of Rx1-mediated immune responses to PVX provides a direct link between chromatin and immunity.

RESULTS

The CC Domain of Rx1 Interacts with a BD-Containing Protein

To provide further insight into Rx1’s mechanism of action and its control of the NbGlk1 TF, we screened for additional Rx1
Figure 1. Rx1 Binds NbDBCP In Vitro and In Planta.

(A) Top: diagram of the NbDBCP domain structure. Middle and bottom: NbDBCP-T and NbDBCP-BD represent proteins used in this study. Numbers represent amino acid residues. SANT, DNA-binding SANT-type helix-turn-helix domain. CC, coiled-coil domain; BD, bromodomain.

(B) Interaction of Rx1(GST-1–144) with NbDBCP-BD. On the left are representative gel filtration chromatograms of Rx1(GST-1–144), NbDBCP-BD, GST, and Rx1(GST-1–144) incubated with NbDBCP-BD, and GST incubated with NbDBCP-BD. Peak fractions were visualized by SDS–PAGE and are represented by capped bars.

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Plant Communications 1, 100086, July 13 2020 © 2020 The Author(s).
interactors. NbGk1 was previously identified in a yeast two-hybrid (Y2H) screen as an interactor of the CC domain of Rx1 (amino acids 1–144). Here, we used the same CC domain to perform an additional Y2H screen of a random-primed *Nicotiana benthamiana* mixed tissue cDNA library. Niben101Scf17137g00006.1 (https://solgenomics.net) was identified in seven clones corresponding to four individual cDNAs, two of which were isolated twice. Individual clones were presumably obtained multiple times due to the amplification of the random-primed cDNA library. The full-length cDNA for Niben101Scf17137g00006.1 encodes a protein of 664 amino acids with a predicted molecular weight of 74 814 Da. The protein carries a single Swi3, Ada2, N-Connector, and TFIIB (SANT)-type helix-turn-helix domain and a single BD of 111 amino acids (Figure 1A). We therefore named it *N. benthamiana* DNA-binding BD-containing protein (NbDBCP). We used the Simple Modular Architecture Research Tool (Letunic and Bork, 2018) to identify all proteins that had a similar domain structure consisting of a SANT-type domain and a BD. Proteins with domain structures similar to NbDBCP were identified as uncharacterized proteins in both dicots (e.g., *Arabidopsis thaliana*) and monocots (e.g., LOC4346003 of *Oriza sativa* Japonica Group). A maximum likelihood phylogenetic analysis demonstrated that the proteins formed two distinct clades, with branches for each clade receiving strong support with bootstrap values of 0.98 (Supplemental Figure 1). Of the 57 plant proteins that had domain structures similar to NbDBCP, 43 (including NbDBCP) also carried a CC domain between the SANT-type domain and the BD. NbDBCP is therefore representative of a larger protein family with a conserved domain structure.

**Rx1 Interacts Directly with NbDBCP In Vitro and In Vivo**

A BD typically recognizes acetylated lysine residues. Since all NbDBCP clones identified from the Y2H screen encompassed the BD, Rx1 probably interacts with this domain. To test this hypothesis, we assessed the binding of Rx1 to the BD domain of NbDBCP (referred to as NbDBCP-BD hereafter) in vitro. NbDBCP-BD is encoded by amino acids 293–414 of the NbDBCP ORF (Figure 1A). We therefore expressed amino acids 293–414 of NbDBCP (NbDBCP-BD) as a recombinant protein and examined its interaction with a glutathione-S-transferase (GST)-tagged Rx1-CC domain (Rx1-GST (1–144)) by size-exclusion chromatography. The extreme C terminus of Rx1-GST (1–144) was susceptible to some proteolytic cleavage during purification, causing the purified protein to run as a doublet in SDS–PAGE. We noted shifts in the peak bands that corresponded to Rx1(GST–1–144) (Figure 1B, SDS–PAGE panel 1, capped green bar) and NbDBCP-BD (Figure 1B, SDS–PAGE panel 2, capped red bar) when they were co-incubated (Figure 1B, panel 3). By contrast, GST alone (Figure 1B, SDS–PAGE panel 4) showed no peak shift when co-incubated with NbDBCP-BD (Figure 1B, SDS–PAGE panel 5). These results suggest that NbDBCP-BD and Rx1-CC interact in vitro.

We then examined whether full-length NbDBCP interacts with full-length Rx1 in * planta*. Co-immunoprecipitation experiments were performed using full-length NbDBCP fused to an N- or C-terminal 4xHA epitope tag (Figure 1C; 4xHA-NbDBCP and NbDBCP-4xHA) with full-length Rx1 fused to a 4xMyc epitope tag (Figure 1C; Rx1–4xMyc). Experiments were also performed with the Rx1 CC domain fused to a 4xMyc epitope tag (Figure 1C; Rx1-CC-4xMyc). GFP fused to a 4xMyc epitope tag (Figure 1C; 4xMyc-GFP) served a control for nonspecific interactions with NbDBCP. Two different elution protocols were used to optimize the recovery of potential complexes formed between NbDBCP and Rx1-4xMyc (Figure 1C; elution 1) or Rx1-CC-4xMyc (Figure 1C; elution 2). Both the N- and C-terminal 4xHA NbDBCP fusions could be detected with an anti-HA tag antibody after immunoprecipitation with an anti-myc antibody when they were co-expressed with Rx1-CC-4xMyc (Figure 1C; elution 2) or Rx1-4xMyc (Figure 1C; elution 1). The amount of N- and C-terminally tagged NbDBCP that co-immunoprecipitated with the full-length Rx1-4xMyc was less than that with Rx1-CC-4xMyc. Nevertheless, the amount of N- and C-terminally tagged NbDBCP that co-immunoprecipitated with the full-length Rx1 construct was consistently and repeatedly above background levels. Elution 1 did not liberate Rx1-CC-4xMyc (Figure 1C; elution 1; 4xMyc panel), whereas elution 2 did release Rx1-4xMyc (Figure 1C; elution 2; 4xMyc panel). However, the 4xHA western blot was optimized to observe interactions between NbDBCP and Rx1-CC-4xMyc and was not exposed for a sufficient length of time to observe interactions between NbDBCP and Rx1-4xMyc. Why less NbDBCP was immunoprecipitated with the full-length Rx1 molecule compared with the CC domain is unknown. Nonetheless, the interaction between the full-length NbDBCP and Rx1 in * planta* was observed across independent experiments and thus represents a genuine interaction.

**Rx1 and NbDBCP Localize to the Nucleus and Nucleolus In Situ**

Having established that Rx1 interacts with NbDBCP both in vitro and in * planta*, we set out to identify the intracellular localization of this event. We used confocal laser scanning microscopy to examine the cellular localization of a C-terminal fusion of NbDBCP with GFP (NbDBCP-GFP) in *N. benthamiana* epidermal cells in the absence and presence of Rx1 (Rx1-mCherry). The subcellular distribution of Rx1-mCherry in the cytoplasm and nucleus was within the range reported in existing studies (Supplemental Figure 2) (Slootweg et al., 2010; Tameling et al., 2010). NbDBCP-GFP in combination with free mCherry (Figure 2A; mCh) is localized in the nucleoplasm and nucleolus (Figure 2A; left and middle panels; Supplemental Figure 3). These localization patterns do not vary substantially between 2 and 3 dpi or in the presence or absence of the P19 silencing suppressor, minimizing the likelihood that the nucleolar distribution of NbDBCP-GFP is an artifact of overexpression (Supplemental Figure 3). The amount of nucleoplasmic signal

(C) Co-immunoprecipitation of 4xMyc-tagged full-length Rx1 (Rx1–4xMyc) or Rx1-CC (Rx1-CC-4xMyc) when co-expressed in * planta* with N- and C-terminally 4xHA-tagged NbDBCP (4xHA-NbDBCP and NbDBCP-4xHA). The labels on the figure are as follows: input denotes the constructs agroinfiltrated into *N. benthamiana* leaves; 4xMyc, an immunoblot performed using an anti-myc epitope tag antibody; 4xHA, an immunoblot performed using an anti-HA epitope tag antibody; 4xMyc IP, immunoprecipitation of the denoted input samples with an anti-myc epitope tag antibody; elution indicates the method used to release immunoprecipitated protein (see Supplemental Information). Immunoblot bands corresponding to Rx1–4xMyc, Rx1-CC-4xMyc, 4xMyc-GFP, and NbDBCP from the 4xMyc and 4xHA immunoblots are indicated. See also Supplemental Figure 1.
varied between cells (compare Figure 2A with the GFP channel of Figure 2B, panels 4–9), and in a few cells, NbDBCP-GFP aggregated in subnuclear bodies (Figure 2A; image at lower right panel taken at 3 dpi). No signal was observed in uninfiltrated cells. The distribution of NbDBCP-GFP differed from that of free GFP, confirming that the localization of NbDBCP-GFP is genuine (Figure 2A; upper and lower right panels).

The nuclear localization pattern of NbDBCP-GFP was unaltered when it was co-expressed with Rx1-mCherry (Figure 2B; GFP channel; compare panel 1 with panels 4–9). In addition, the cellular distribution of Rx1-mCherry in the nucleus relative to the cytoplasm was mostly unaffected and similar to that observed upon co-expression with GFP-GUS (Figure 2B; mCherry channel; compare panel 3 with panels 4–6). We confirmed this result by determining the intensity ratios of the mCherry signal in the nucleus relative to the cytoplasm ($I_N/I_C$), as described previously (Slootweg et al., 2010). The $I_N/I_C$ ratios of Rx1-mCherry did not differ significantly in the presence or absence of NbDBCP-GFP (Supplemental Figure 2). However, in some cells, we observed that Rx1-mCherry relocalized to the nucleolus and to subnuclear bodies when it was co-expressed with NbDBCP-GFP (23% ± 7% of imaged cells (SD; 51 cells imaged in four biologically independent experiments); Figure 2B; mCherry channel; compare panels 4–6 with panels 7–9). Rx1-mCherry was present in the cytoplasm and nuclear envelope when it was not relocalized to the nucleolus (Figure 2B, panels 4–6). The weaker signal of mCherry was due to a lower quantum yield compared with GFP, and the apparent brighter signal of Rx1 in the nucleolus (Figure 2B, panels 7–9) likely reflected a high local concentration. In addition, the Rx1-mCherry construct used in this study is intact (as demonstrated by immunoblotting in Supplemental Figure 4) but expressed at significantly lower levels than free mCherry.

Given that only a low proportion of cells showed the redistribution of Rx1-mCherry into the nucleolus, we quantified this shift in all
A DNA-Binding Protein Reduces Rx1 Function

We studied Rx1-mCherry in the nucleolus relative to the nucleoplasm (\(\text{Hnucleolus}/\text{Hnucleoplasm}\)) were determined. Data from independent experiments indicated that there was a statistically significant increase in nucleolar signal (Supplemental Figure 5). Notably, the possibility of an overexpression artifact still remains. To explore this, we demonstrated that NbDBCP-GFP exhibited very low levels of bleed-through in the mCherry channel when expressed alone (Figure 2B, panel 1; note the lack of signal in the mCherry channel compared with the GFP channel). The observed redistribution of Rx1-mCherry upon co-expression with NbDBCP-GFP far exceeds this background level (Figure 2B; mCherry channel; panels 2–9). In addition, this redistribution was not observed when Rx1-mCherry was expressed with GFP-GUS (Figure 2B; mCherry channel; panel 3; Supplemental Figure 5). Together, these observations minimize the likelihood that the accumulation of Rx1 into subnuclear bodies is due to experimental artifacts. In summary, the observed shared subcellular distribution of Rx1 and NbDBCP in the nucleus (and the nucleolus in a subset of cells) is consistent with their ability to interact.

Rx1 and NbDBCP Interact with DNA In Situ

The co-localization of Rx1 and NbDBCP to the nucleus prompted us to investigate whether Rx1 and NbDBCP interact at DNA in planta.

We studied Rx1-NbDBCP-DNA interactions using a Förster resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM). GFP (negative control), histone H2B fused to GFP (GFP-H2B; positive control), full-length Rx1 with or without an N-terminal GFP tag, or full-length NbDBCP with or without an N-terminal GFP tag were transiently expressed in N. benthamiana. The constituent fluorescence lifetimes for the GFP tag were examined in leaves counterstained with the nucleic acid stain LDS 751 (Figure 3). GFP showed two distinct fluorescence lifetimes at \(\sim 0.5\) and \(\sim 1.5\) ns. The fluorescence lifetime at \(\sim 0.5\) ns can be explained by energy transfer from GFP to an acceptor fluorophore (Fenyk et al., 2015). Increased energy transfer from GFP to acceptor LDS 751 (indicative of physical proximity to nucleic acids) increases the relative contribution of the \(\sim 0.5\)-ns fluorescent lifetime. A shift in the ratio of the \(\sim 1.5\) (long)- to \(\sim 0.5\) (short)-ns GFP lifetimes is indicative of interactions with DNA (Fenyk et al., 2015) at distances within the Förster radius (likely <50 Å). We monitored the interaction of an NbDBCP-GFP fusion with DNA with or without Rx1 (untagged) in the presence or absence of the Rx1-activating PVX coat protein CP106. This experiment would reveal whether NbDBCP interacts with chromatin in situ and whether this interaction is altered by the co-expression of Rx1 and CP106. Consistent with the localization of NbDBCP to the nucleus (Figure 2), NbDBCP-GFP bound DNA when expressed alone (Figure 3A). However, NbDBCP did not bind DNA when it was co-expressed with either Rx1 or CP106, despite an equivalent fluorescent signal indicating that NbDBCP was still expressed. The ability of CP106 alone to displace NbDBCP from DNA prompted us to ask whether CP106 and NbDBCP can physically interact. CP106 did not interact with NbDBCP from DNA and whether this interaction in planta. While we cannot formally exclude such an interaction, there are currently no data to support it. Future experiments using a time course of co-immunoprecipitation and bifluorescence complementation experiments on the co-expression of CP106 and NbDBCP in planta will help to resolve whether this is a true negative result.

Next, we monitored the interaction of an Rx1-GFP fusion with chromatin with or without NbDBCP (untagged) and in the presence or absence of the Rx1-activating (avirulent) PVX coat protein (CP106) (Figure 3B). In line with our previous studies, Rx1-GFP expressed without NbDBCP only bound DNA in the presence of CP106 (Fenyk et al., 2012). The Rx1-GFP fusion did not interact with chromatin when it was co-expressed with NbDBCP (untagged) (Figure 3B). This result is consistent with the observation that NbDBCP-GFP did not interact with chromatin when it was co-expressed with Rx1 (untagged) (Figure 3A). Interestingly, when Rx1-GFP was co-expressed with NbDBCP and CP106, it was observed to interact with chromatin (Figure 3B).

NbDBCP Reduces Rx1-Mediated Immune Responses

The observed interaction between NbDBCP and Rx1 suggests a functional relationship between these proteins. Rx1 can trigger two distinct types of immune outputs: (1) a cell death response that can be induced by overexpression of the PVX coat protein,
and (2) a symptomless extreme resistance response that results in the inhibition of viral replication (Bendahmane et al., 1999).

Virus-induced gene silencing (VIGS) uses a recombinant TRV virus that carries part of a designated plant gene sequence that is targeted for silencing (Lange et al., 2013). We used VIGS to silence the endogenous NbDBCP-encoding gene of N. benthamiana to investigate whether it is required for Rx1-mediated immune responses. Two independent VIGS constructs were designed to target different regions of the NbDBCP transcript to ensure that the results were not due to off-target effects.

The downregulation of NbDBCP mRNA by VIGS was examined by real-time qPCR (Figure 4A). Rx1-mediated immunity was assessed by measuring PVX coat protein accumulation following agroinfiltration using an infectious PVX clone. A VIGS construct targeting GFP served as a negative control for Rx1-mediated immune responses. No change in PVX coat protein accumulation (in the absence of Rx1) was observed in NbDBCP-silenced plants (pTRV2-NbDBCP-1 and -2) compared with GFP-silenced plants (pTRV2-GFP; Figure 4B). Therefore, NbDBCP alone does not influence PVX accumulation.

As expected, PVX coat protein accumulation was reduced when Rx1 was co-expressed with PVX in either non-silenced or GFP-silenced plants (pTRV2-GFP; Figure 4B). A TRV::SGT1 silencing construct was used as a positive control for compromised Rx1 function. SGT1 is a homolog of a yeast ubiquitin ligase-associated protein; it has a general role in NLR-mediated immunity and is required for Rx1 function (Pearl et al., 2002; Lu et al., 2003; Slootweg et al., 2010; Hoser et al., 2014). PVX coat protein accumulation was unaffected in SGT1-silenced plants (pTRV2-SGT1) in the absence of Rx1. However, in the presence of Rx1, SGT1 silencing eliminated immunity, resulting in increased accumulation of PVX coat protein (Figure 4B). In addition, PVX coat protein accumulation was significantly reduced when Rx1 and PVX were co-expressed in NbDBCP-silenced plants (pTRV2-NbDBCP-1 and -2) compared with GFP-silenced plants (pTRV2-GFP; Figure 4B). These findings suggest that NbDBCP suppresses Rx1-mediated resistance response. Our qRT–PCR analysis demonstrated a trend of decreased relative NbDBCP expression in pTRV2-NbDBCP-1 and -2 compared with pTRV2-GFP plants. Despite the minor effect and variability in gene silencing (see Figure 4A, pTRV2-NbDBCP-1), the findings of our disease assays were robust and replicable across experiments (Figure 4). This indicate that Rx1-mediated immunity is sensitive to a relatively small decrease in NbDBCP expression. Overall, these data suggest that NbDBCP is a negative regulator of Rx1-mediated immunity.

**NbDBCP-BD Affects Immune Responses to PVX**

NbDBCP-BD is sufficient for the interaction between Rx1 and NbDBCP (Figure 1B). We therefore investigated whether the BD domain was crucial to NbDBCP-mediated immunity to PVX.

NbDBCP-BD was analyzed using the Phyre² protein fold recognition engine and modeled using the BPTF (Bromodomain and PHD finger-containing transcription factor) BD in complex with histone H4 acetylated at Lys16 (PDB: 3QZT). Y336 and E386 in NbDBCP were identified as candidate residues that interact with acetyl-lysine and therefore required for BD-dependent function (Figure 5A). To study their effect on immune responses to PVX, NbDBCP-Y336F and NbDBCP-E386L mutants were generated, conserving amino acid side-chain bulk while ablating intermolecular interactions at the site. Unfortunately, the NbDBCP-Y336F variant could not be expressed in planta and was excluded from further studies.

The cellular localization of a C-terminal fusion of NbDBCP-E386L with GFP (NbDBCP-E386L-GFP) in N. benthamiana epidermal cells was determined using confocal laser scanning microscopy. NbDBCP-E386L-GFP was co-expressed with the P19 silencing suppressor to enhance expression, and the fusion protein was found to localize to the nucleoplasm and nucleolus (Figure 5B; GFP channel), similar to that observed for the NbDBCP-WT-GFP protein.

NbDBCP-E386L expression in planta was compared with that of the wild-type NbDBCP by western blot. The NbDBCP-E386L protein was detectable but its expression level was consistently
lower than that of NbDBCP-WT (Figure 5C). This prevented us from further assessing whether and how E386L affected the suppressive effect of NbDBCP on Rx1-mediated immune responses by studying the loss-of-function phenotype, as it can be explained by reduced NbDBCP-E386L protein levels. We therefore investigated whether NbDBCP-E386L confers a possible gain-of-function phenotype in planta, as such a phenotype is less likely a result of decreased protein expression. We investigated the effect of overexpression wild-type and mutant NbDBCP (NbDBCP-GFP-E386L) on phenotypes induced by PVX. NbDBCP and PVX were transiently expressed in N. benthamiana upon A. tumefaciens infiltration. As expected, no immune response was observed when PVX was co-expressed with GFP (Figure 5D and Supplemental Figure 7; A1), NbDBCP-GFP-WT (Figure 5D and Supplemental Figure 7; A2), or NbDBCP-GFP-E386L (Figure 5D and Supplemental Figure 7; A3) in the absence of Rx1. However, a hypersensitive response was observed when PVX and GFP were co-expressed with Rx1 (Figure 5D and Supplemental Figure 7; B1) and when PVX and NbDBCP-GFP-WT were co-expressed with Rx1 (Figure 5D and Supplemental Figure 7; B2). Interestingly, the levels of cell death were qualitatively reduced when PVX and NbDBCP-GFP-E386L were co-expressed with Rx1 (Figure 5D and Supplemental Figure 7; B3). The gain-of-function phenotype for NbDBCP-E386L cannot be explained by a failure to interact with Rx1, as both the NbDBCP-WT and NbDBCP-E386L interact with the CC domain of Rx1 in a Y2H assay (Supplemental Figure 8).

To further validate these data, we directly measured virus coat protein accumulation in infiltrated N. benthamiana leaves to provide quantitative support for the qualitative analysis of Figure 5D. Virus coat protein accumulation in leaf infiltrates was measured by double antibody sandwich ELISA (DAS–ELISA) using an antibody that recognizes the PVX coat protein. In the absence of Rx1, both the NbDBCP-WT and NbDBCP-E386L proteins increased the basal immune response of N. benthamiana cells (Figure 5E; compare the first and second datasets with the first), which is evident through a decrease in the accumulation of PVX coat protein. Consistent with our hypothesis, Rx1 co-expression indeed significantly reduced PVX virus expression (Figure 5E; compare the first and fourth datasets). Notably, more viruses accumulated when Rx1 was co-expressed with NbDBCP-WT-GFP than with GFP (Figure 5E; compare the fourth and sixth datasets). This supports the observation from VIGS that NbDBCP-WT suppresses Rx1 activity. Significantly, less viral accumulation was observed when Rx1 was co-expressed with NbDBCP-E386L-GFP (Figure 5E; compare fourth and fifth datasets). The significant increase in Rx1-mediated immunity can therefore be attributed to a gain-of-
function phenotype conferred by the NbDBCP-E386L-GFP variant, demonstrating that NbDBCP is a negative regulator of Rx1 function.

**NbDBCP Reduces NbGlk1-Mediated DNA Binding**

We hypothesized that NbDBCP might inhibit the pro-immune activity of Rx1 as part of a larger complex. NbGlk1 binds to consensus GLK DNA binding sites, and its binding affinity for these sites is reduced by Rx1 (Townsend et al., 2018). Because Rx1 is a negative regulator of NbGlk1 DNA binding, we investigated whether NbDBCP reduces Rx1-mediated immunity via affecting NbGlk1 DNA binding.

We were unable to express the full-length NbDBCP molecule as a recombinant protein in vitro. However, we were able to express a C-terminally truncated variant of NbDBCP that consisted of amino acids 1–414 (NbDBCP-T). We measured the Kd value of NbGlk1 for a dsDNA substrate that contained a concatenated GGATATCC NbGlk1 binding site (Townsend et al., 2018) in the presence or absence of Rx1(GST-1–144) and/or NbDBCP-T by fluorescence anisotropy (Figure 6A). Rx1(GST-1–144) reduced the binding affinity of NbGlk1 for its dsDNA substrate, as expected, from 0.11 ± 0.00 μM (SD) to 0.15 ± 0.01 μM (SD). Interestingly, NbDBCP-T also reduced the binding affinity of NbGlk1 for its dsDNA substrate to 0.16 ± 0.01 μM (SD). In addition, Rx1(GST-1–144) and NbDBCP-T exhibited a synergistic effect, leading to a much greater reduction in the binding affinity of NbGlk1 for its dsDNA substrate to 0.26 ± 0.03 μM (SD).

As the interaction between Rx1 NbDBCP is BD-dependent, we investigated whether NbDBCP-BD alone was sufficient to reduce the binding affinity of NbGlk1 for its dsDNA substrate. NbDBCP-BD did not alter NbGlk1 binding to dsDNA in either the presence or absence of Rx1, indicating that the site of the Rx1-NbDBCP interaction is not required for the influence of NbDBCP on NbGlk1 DNA binding (Figure 6B). The experiment of Figure 6B was performed using NbDBCP-T instead of NbDBCP-BD (Figure 6C). NbDBCP-T reduced NbGlk1 binding to dsDNA in the presence of Rx1, supporting the findings of Figures 6A and 6B.

**Figure 6. Interactions between NbDBCP, Rx1, and NbGlk1 In Vitro.**

(A) The influence of Rx1 and NbDBCP on NbGlk1 DNA binding. Fluorescence anisotropy values plotted against log protein concentration for NbGlk1(83–402) in the presence or absence of Rx1(1–144) or NbDBCP-T (n = 3).

(B) DNA binding of NbGlk1(83–402) was measured by fluorescence anisotropy in the presence or absence of GST, Rx1(GST-1–144), and NbDBCP-BD (means ± SEM; n = 3; *p < 0.05, #p > 0.05; one-way ANOVA with post hoc Tukey multiple comparison test).

(C) DNA binding of NbGlk1(83–402) was measured by fluorescence anisotropy in the presence or absence of GST, Rx1(GST-1–144), and NbDBCP-T (means ± SEM; n = 3; *p < 0.05, #p > 0.05; one-way ANOVA with post hoc Tukey multiple comparison test).

(D) Interaction of NbGlk1(83–402) with NbDBCP-T. On the left are representative gel filtration chromatograms of NbGlk1(83–402), NbDBCP-T, and NbGlk1(83–402) incubated with NbDBCP-T. Peak fractions were visualized by SDS–PAGE and are represented by capped bars.

See also Supplemental Figure 9.
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confirming that the failure of NbDBCP-BD to reduce NbGlk1 DNA binding was not specific to the assay conditions.

The ability of Rx1 and NbDBCP to synergistically occlude NbGlk1 DNA binding suggests that they are able to form a larger protein complex. We therefore investigated whether NbGlk1 and NbDBCP form a complex in vitro. We expressed amino acids 1–414 of NbDBCP (NbDBCP-T) as a recombinant protein and examined its interaction with amino acids 83–402 of NbGlk1 (NbGlk1(83–402)) by size-exclusion chromatography. Both NbDBCP-T and NbGlk1(83–402) represent the largest recombinant protein variants we have been able to produce as soluble proteins in E. coli. We noted a shift in the peak band corresponding to NbGlk1(83–402) (Figure 6D, SDS–PAGE panel 1, capped green bar) when it was co-incubated with NbDBCP-T (Figure 6D, panel 3, capped green bar), which was indicative of complex formation. No shift in the peak band corresponding to NbDBCP-T (Figure 6D, SDS–PAGE panel 2, capped red bar) was obvious when it was incubated with NbGlk1(83–402) (Figure 6D, panel 3, capped red bar). However, a more sensitive quantitative examination of the distribution of NbDBCP protein to the left- (higher molecular weight) and right-hand sides of the protein peak at an elution volume of 12 mL by densitometry revealed an enhanced distribution of NbDBCP-T toward a higher molecular weight. The left:right ratio of NbDBCP within the 12-mL elution volume was 1.37 for NbDBCP-T alone but 1.60 when NbDBCP-T was incubated with NbGlk1(83–402), indicative of a shift due to complex formation.

The interaction between NbGlk1 and NbDBCP observed through fluorescence anisotropy was therefore demonstrated through gel filtration analysis. However, gel filtration analysis indicated that the interaction is relatively transient and only weakly detectable on the transit of the complex through a gel filtration column. Accordingly, we were unable to observe a three-way interaction between Rx1, NbDBCP, and NbGlk1 by gel filtration chromatography.

We performed additional confocal imaging to investigate the influence of NbDBCP overexpression on the cellular localization of NbGlk1-GFP. Experiments were performed using an HA-tagged version of NbDBCP because an NbDBCP-mCherry construct was not available. Immunoblotting demonstrated that all proteins used for imaging were expressed and intact (Supplemental Figure 9A). The data demonstrated that NbDBCP-HA overexpression does not alter the subcellular distribution of NbGlk1-GFP (Supplemental Figure 9B). This does not preclude the possibility of a transient interaction between the two proteins.

In conclusion, NbDBCP is an immune regulator that acts on an Rx1 and NbGlk1 complex at chromatin, and its ability to modulate Rx1 activity is dependent on intact BD.

DISCUSSION

Here, we identify the BD-containing protein NbDBCP as an Rx1-interacting protein (Figure 1). At least one NbDBCP-like protein is predicted within the genome sequences of a range of dicot and monocot species (Supplemental Figure 1). NbDBCP-like proteins are therefore widespread in higher plants, but their functions are unknown. The localization of NbDBCP to the nucleolus suggests a broadly conserved function in plant ribosome biogenesis that awaits further investigation. The identification of NbDBCP provides a potential direct link between histone modification and NLR activity. Although antibodies that detect endogenous NbDBCP were not available, (Figure 4A), we can infer its relative expression levels by comparison with actin in TRV:GFP plants based on qPCR analysis. Data from independent repeats demonstrate that the average Ct values between actin and NbDBCP are within 1 Ct difference. This indicates that the expression levels of endogenous NbDBCP are comparable to those of actin.

NbDBCP was shown to localize to the nucleolus (Figure 2), and approximately a quarter of the cells showed the redistribution of Rx1 to the nucleolus upon NbDBCP and Rx1 co-expression (Figure 2). However, the reasons for this localization is unknown. We note that the nucleolus is a key target for plant viruses (Ding and Lozano-Duran, 2020; Kalinina et al., 2018), and nucleolar chromat is subject to histone modification (Saez-Vasquez and Delseny, 2019). Therefore, it is possible that the localization of Rx1 and NbDBCP to the nucleolus functions as part of sub-organellar specific defense response, but this awaits future investigation. Whether Rx1 and NbDBCP are further redistributed upon PVX infection is an open question. When expressed alone, NbDBCP-GFP interacts with plant chromatin in situ (Figure 3A), and its DNA binding in situ appears to be inhibited by either Rx1 or CP106 overexpression. It is interesting to note that Rx1-GFP does interact with chromatin when co-expressed with NbDBCP and CP106 (Figure 3B). This suggests that CP106, or the encoded mRNA, rearranges a complex at chromatin that results in the loss of an NbDBCP–chromatin interaction but permits the interaction of Rx1 with chromatin. The FRET-FLIM experiment measures a ratio of long to short GFP lifetimes, and so this observation is not an artifact of protein expression. This surprising finding suggests that CP106, or its mRNA, can affect NbDBCP activity in an Rx1-independent manner. The PVX coat protein has been proposed to interact with multiple host proteins in N. benthamiana (Park et al., 2009), and their identities remain a question for future research. Our interpretation of the confocal microscopy and FRET-FLIM data is that NbDBCP localizes in the nucleolus where it interacts—or is closely associated—with chromatin. Such an interaction is consistent with the known role of BDs to bind acetyl-lysine that is typically found in histones (Dhalluin et al., 1999; Marmorstein and Berger, 2001). These latter interactions can be disturbed upon the overexpression of Rx1 or the CP, possibly via a third protein that interacts with all partners. However, a caveat for the interpretation of the FRET-FLIM data is the possibility of a false-negative result. If the expressed NbDBCP-GFP fusion protein has saturated all available DNA-binding sites, the accumulation of an increased pool of non-DNA-bound protein will shift the ratio of the long to short lifetimes to the GFP negative control. In the absence of an available alternative method that is not susceptible to the same issue of false negatives, however, the interpretation of a negative result should be viewed with some caution. A conservative interpretation of the data is therefore that NbDBCP is able to interact with DNA in situ, with some evidence that either Rx1 or CP106 redistributes NbDBCP from this site. It is interesting to note that a similar observation was made previously for the interaction of NbGlk1

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with DNA in situ (Townsend et al., 2018). Rx1 redistributed NbGlk1 from DNA in situ and reduced its binding affinity for DNA in vitro.

We were unable to demonstrate an interaction between NbDBCP-BD and small molecules N-acetyl-lysine and 6-acetyl-histamine (which mimics the N-acetyl-lysine side chain) by isothermal calorimetry. However, the substrate specificity of BD for acetylated proteins is also dependent on binding interactions with the amino acids that surround the acetylated lysine (Mujtaba et al., 2002). The binding affinity for the acetylated lysine is therefore possibly too low to observe in the absence of an appropriate peptide sequence. An alternative possibility is that the BD shows specificity for a different chromatin modification. For example, the typical YN motif of the BD L-BD loop is replaced by YF in NbDBCP. The bulky phenylalanine may therefore clash with N-acetyl-lysine, as observed previously for BDs with alternative residues at this site (Wen et al., 2014).

We used an indirect approach to determine whether histone modification binding was required for NbDBCP function. Using the structural modeling of NbDBCP-BD, we identified two residues (Y336 and E386) that were possibly involved in NbDBCP-BD interactions with a modified target protein (Figure 5A). Only the NbDBCP-E386L variant could be expressed in planta, but it accumulated at levels below that of the wild-type protein. However, upon its accumulation, the NbDBCP-E386L variant showed a gain-of-function phenotype that resulted in a potentiated Rx1-mediated immune response to PVX compared with the wild-type protein. This finding is consistent with the VIGS data of Figure 4B, demonstrating that NbDBCP is a negative regulator of Rx1-mediated immunity. Extreme resistance and cell death are thought to constitute distinct pathways of Rx1 immunity (Bendahmane et al., 1999). The common view is that cell death occurs as a secondary resistance response when extreme resistance proves insufficient. As cell death was qualitatively more prominent, our data suggest that NbDBCP wild-type expression reduces extreme resistance (consistent with the gene-silencing data of Figure 4B), whereas NbDBCP-E386L expression enhances extreme resistance (due to less cell death). The precise mechanism of the NbDBCP-E386L gain-of-function phenotype is not known. NbDBCP-E386L may, for example, form non-functional complexes with the wild-type protein, causing a net reduction in the negative effect on Rx1 responses. This mechanism awaits the future investigation of whether wild-type NbDBCP co-expression can rescue the phenotype associated with NbDBCP overexpression.

Rx1 and NbDBCP acted synergistically to reduce the affinity of NbGlk1 for dsDNA (Figure 6). The DNA-binding assay used had no histones present, and so the observed effect on NbGlk1 unlikely involved binding to chromatin modification. This is further demonstrated by the observation that isolated NbDBCP-BD does not influence NbGlk1 DNA binding (Figure 6). It is most likely, therefore, that Rx1 and NbDBCP act in a complex to physically occlude NbGlk1 from DNA. We have several lines of evidence that NbDBCP and NbGlk1 are able to form a complex (Figure 6). It is interesting to note that the NbDBCP-T protein had an approximate molecular weight consistent with the formation of a dimer, as assessed by gel filtration chromatography (Figure 6D). The functional relevance of a higher-order structure for NbDBCP-T is unknown. Unfortunately, NbDBCP-T-E386L could not be produced as a

Figure 7. Model for Protein Interactions with Rx1.
(A) NbDBCP interacts with DNA.
(B) In the absence of PVX, Rx1 interacts with NbDBCP and separately with NbGlk1 at DNA and reduces the affinity of NbGlk1 for DNA (Townsend et al., 2018). NbDBCP suppresses immunity downstream of the Rx1/NbGlk1-DNA interaction.
(C) PVX acts via Rx1 and/or NbDBCP, resulting in a complex where Rx1 but not NbDBCP interacts with chromatin. Instead, NbGlk1 interacts with chromatin (Townsend et al., 2018).
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recombinant protein in E. coli to investigate whether the altered formation of such a higher-order structure could explain the mutant phenotype and to investigate the relevance of such a structure. A crucial direction for future work, therefore, will be to fully define this larger Rx1-NbGlk1-NBDBCP complex (and potential homo-oligomerization) in planta and determine the requirements for its formation and complete role in immunity.

A possible interpretation of the data presented here and elsewhere (Townsend et al., 2018) centers on DNA. NbDBCP interacts with DNA (Figures 3A and 7A), presumably at the nucleolus (Figure 2), and suppresses immune responses associated with Rx1 through an unknown mechanism that likely depends on a functional BD (Figures 4 and 5). Co-expressed Rx1 and NbGlk1 form an inactive complex at DNA (Townsend et al., 2018). Co-expressed Rx1 and NbDBCP also form a complex in planta (Figure 1C). When Rx1 and NbDBCP are co-expressed, neither interact with DNA (Figures 3 and 7B). However, the nature of the DNA contacts formed by Rx1, NbGlk1, and NbDBCP at endogenous levels in the absence of PVX is not fully resolved. The model shown in Figure 7B should therefore be viewed with some caution, as the multi-protein complex and its interactions with DNA may well be dynamic and/or transient. Size-exclusion chromatography (Figure 6D) suggests that the multi-protein complex formed between Rx1, NbGlk1, and NbDBCP (Figure 7B) is possible, albeit transient, in the absence of PVX, although proof of its existence in planta awaits further experiment. The immune activation of Rx1 and/or NbDBCP via PVX CP, whether direct or indirect, permits an uncharacterized change in the relative orientation of Rx1, NbGlk1, and NbDBCP with respect to DNA (Figure 7C). Rx1 and NbGlk1 interact with DNA in the presence of PVX when co-expressed (Townsend et al., 2018). NbDBCP does not interact with DNA in the presence of PVX when co-expressed, whereas Rx1 does (Figures 3 and 7C). These data are consistent with the orientation of molecules with respect to DNA that is shown in Figure 7C. However, the exact nature of the multi-protein complex and its composition when Rx1, NbGlk1, NBDBCP, and PVX CP are all present may be different from that shown and awaits further investigation. Immune signaling is likely to be activated when NbGlk1 is stably bound to its consensus sequences (Townsend et al., 2018) and NbDBCP is removed from its inhibitory site (Figure 3A).

In conclusion, we identify NbDBCP as an immune-suppressing protein that acts at chromatin and is regulated by Rx1. Rx1 provides a direct link between PVX perception and transcriptional processes at DNA. Furthermore, the CC domain appears to function as a complex scaffold for nuclear proteins involved in transcriptional reprogramming. This immune-regulated complex at chromatin is regulated by nuclear-localized Rx1 to suppress immune activation until the perception of an appropriate pathogen signal.

METHODS SUMMARY

All plasmids were produced using standard molecular biology techniques. Proteins for in vitro analysis were produced as recombinants in E. coli and purified by affinity chromatography. Y2H analysis was performed by Biogenics Services SAS (Paris, France). Protein interaction studies were performed by gel filtration chromatography or immunoprecipitation after

expression in planta. The protein-DNA binding was analyzed by fluorescence anisotropy. Protein subcellular distribution studies were performed by laser scanning confocal microscopy. Gene knockdown in planta was performed by VIGS. Protein expression in plants was performed by Agrobacterium tumefaciens-mediated agroinfiltration. Several differences with the computed open reading frame for Niben101Scf17137g00006.1 were noted. The cloned NbDBCP open reading frame is deposited at NCBI under accession number MN594539. Full methods details are provided in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Plant Communications Online.

FUNDING

This work was supported by Biotechnology and Biological Sciences Research Council grant BB/M007405/1 (to M.J.C. and L.-O.P.), the Dutch Technology Foundation STW and Earth and Life Sciences ALW (to E.J.S., O.C.A.S., and A.G.), and VIVO project no. 865.14.003 (to F.L.W.T.) (Netherlands Organization for Scientific Research).

AUTHOR CONTRIBUTIONS

Conceptualization, M.J.C. and A.G.; Methodology, M.J.C., A.G., P.D.T., O.C.A.S., C.H.D., E.J.S., and L.-O.P.; Investigation, P.D.T., O.C.A.S., C.H.D., and E.J.S.; Writing – Original Draft, M.J.C.; Writing – Review & Editing, M.J.C., A.G., F.L.W.T., and O.C.A.S.; Funding Acquisition, M.J.C., L.-O.P., A.G., E.J.S., and F.L.W.T.; Resources, A.L. and F.L.W.T.; Supervision, M.J.C., A.G., and L.-O.P.

ACKNOWLEDGMENTS

The authors declare that they have no conflicts of interest with the contents of this article.

Received: November 18, 2019
Revised: June 9, 2020
Accepted: June 11, 2020
Published: June 16, 2020

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