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Nitric oxide associated protein 1 is associated with chloroplast perturbation and disease symptoms in *Nicotiana benthamiana* infected with *South African cassava mosaic virus*

Imanu Mwaba, Marie Emma Christine Rey*

School of Molecular and Cell Biology, University of the Witwatersrand, 1, Jan Smuts Avenue, Braamfontein, Johannesburg 2000, South Africa

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ABSTRACT

Nitric oxide associated 1 (NOA1) in plants is a cyclic GTPase involved in protein translation in the chloroplast and has been indirectly linked to nitric oxide (NO) accumulation and response to biotic stress. The association between NOA1 and NO accumulation in Arabidopsis *noa1* mutants has been linked to the inability of *noa1* mutants to accumulate carbon reserves such as fumarate, leading to chloroplast dysfunction and a pale green leaf phenotype. To understand the role played by NOA1 in response to *South African cassava mosaic virus* infection in *Nicotiana benthamiana*, the expression of *NbNOA1* and the accumulation of NO in leaf samples was compared between south african cassava mosaic (SACMV)-infected and mock-infected plants at 14 and 28 dpi. Real-time qPCR was used to measure SACMV viral load which increased significantly by 20% from 14 to 28 dpi as chlorosis and symptom severity progressed. At 14 and 28 dpi, *NbNOA1* expression was significantly lower than mock inoculated plants (2-fold lower at 14 dpi, p-value = 0.01 and 5-fold lower at 28, p-value = 0.00). At 14 dpi, NO accumulation remained unchanged in infected leaf tissue compared to mock inoculated, while at 28 dpi, NO accumulation was 40% lower (p-value = 0.01). At 28 dpi, the decrease in *NbNOA1* expression and NO accumulation was accompanied by chloroplast dysfunction, evident from the significant reduction in chlorophylls *a* and *b* and carotenoids in SACMV-infected leaves. Furthermore, the expression of chloroplast translation factors (chloroplast RNA binding, chloroplast elongation factor G, translation elongation factor Tu, translation initiation factor 3-2, plastid-specific ribosomal protein 6 and plastid ribosome recycling factor) were found to be repressed in infected *N. benthamiana*. GC–MS analysis showed a decrease in fumarate and an increase in glucose in SACMV-infected *N. benthamiana* in comparison to mock samples suggesting a decrease in carbon stores. Collectively, these results provide evidence that in response to SACMV infection, a decrease in photopigments and carbon stores, accompanied by an increase in glucose and decrease in fumarate, leads to a decline in *NbNOA1*expression and NO levels. This is manifested by suppressed translation factors and disruption of chloroplast function, thereby contributing to chlorotic disease symptoms.

1. Introduction

Understanding resistance or susceptibility to plant viruses is a major step toward potentially engineering virus-resistant plants. A model for defence response to viruses suggests that the host recognises viral proteins as microbe associated molecular patterns (MAMPs) triggering pathogen triggered immunity (PTI), or virus-encoded proteins act as effectors that are recognized by resistance (R) proteins, triggering antiviral effector triggered immunity (ETI) (Jones and Dangl, 2006; Mandadi and Scholthof, 2013; Nicaise, 2014). In addition to the plant's innate defence responses, plants are known to respond to virus infection by triggering RNA silencing, known as adaptive immunity, where viral dsRNA is targeted by the host post transcriptional gene silencing (PTGS) machinery (Aregger et al., 2012; Zvereva and Pooggin, 2012). In return, plant viruses encode silencing suppressors to circumvent this antiviral response, for example the geminivirus *trans*-activating proteins AC2, AC4, AC5, AV2 and ß satellite (ßC1) (Trinks et al., 2005; Fondong et al., 2007; Zrachya et al., 2007; Chowda-Reddy et al., 2008; Amin et al., 2011; Li et al., 2015b). Downstream of the onset of these antiviral responses lies signalling cascades which trigger activation of defence-responsive genes. At the heart of this cascade is nitric oxide (NO), a ubiquitous signalling molecule involved in various processes in plants including responses to biotic and abiotic stress (León et al., 2013; Domingos et al., 2015). The link between NO and disease resistance was

* Corresponding author.
E-mail address: chrissie.rey@wits.ac.za (M.E.C. Rey).

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highlighted when it was found that application of NO scavengers and inhibitors of NO synthesis in *Arabidopsis thaliana* rendered it susceptible to *Pseudomonas syringae* (Delledonne et al., 1998; Zeier et al., 2004). In susceptible tomato, tobacco mosaic virus (TMV) infection results in NO production which leads to the induction of mitochondrial alternative electron transport resulting in the induction of basal defence (Fu et al., 2010). Infection of resistant, but not susceptible, tobacco with TMV results in enhanced NO production (Durner et al., 1998). Treatment with NO donors in tobacco triggers expression of defence genes (Durner et al., 1998; Song and Goodman, 2001) and has been shown to prevent the spread of TMV (Durner et al., 1998) and potato virus X (Li et al., 2014). In *Hibiscus cannabinus*, infection with the geminivirus mesta yellow vein mosaic virus (MYVMV) resulted in an increase in NO production, and an increase in tyrosine-nitrated proteins (Sarkar et al., 2010). Nitric oxide has also been shown to be involved in modulating the activity of many defence-related proteins through *S*-nitrosylation (Tada et al., 2008; Kovacs et al., 2015).

In animals, nitric oxide synthases (NOS) are the main NO producing enzymes, and they catalyse the conversion of L-arginine to NO and L-citrulline, requiring (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphyrin IX (haem) as co-factors (Moncada et al., 1989; Mayer and Hemmens, 1997; Alderton et al., 2001). Despite being ubiquitously produced in plants, there is no consensus on the central source of NO (Domingos et al., 2015). A NOS has recently been identified from the algae *Ostreococcus tauri* and it bears sequence and structural similarities to mammalian NOS albeit with different co-factor requirements (Foresi et al., 2010; Correa-aragunde et al., 2013). *Arabidopsis* nitric oxide associated protein 1 (AtNOA1), formerly referred to as Arabidopsis nitric oxide synthase 1 (AtNOS1), was previously thought to be a plant NOS (Guo et al., 2003), and AtNOA1 homologues have been identified in different plants (Kato et al., 2007; Qiao et al., 2009; Yang et al., 2011; Kwan et al., 2015). It is now known that AtNOA1 is not a plant NOS but a member of the conserved circularly permutated GTPase (cGTPase) family YlqF/YawG with nucleic acids and protein binding abilities, but no binding sites for L-arginine and any NOS associated co-factors (Moreau et al., 2008; Sudhamsu et al., 2008; Moreau et al., 2010). AtNOA1 is a nuclear encoded, chloroplast translation factor (Flores et al., 2008; Moreau et al., 2008). Phenotype of NOA1 knockdown is lethal in maize (Li et al., 2015a) and in Arabidopsis, *noa1* mutants have a decreased chlorophyll biosynthesis, resulting in pale cotyledons, delayed development and greening of true leaves and smaller plants with pale young leaves compared to wild-type (Flores-Pérez et al., 2008; Van Ree et al., 2011; Kim et al., 2013).

Exactly how NOA1/cGTPase homologues participate in disease or defence responses is speculative. Expression of AtNOA1 and its plant homologues is differentially regulated in response to disease (Kato et al., 2007 Wünsche et al., 2011; Mandal et al., 2012; Kwan et al., 2015) and downregulation of NOA1 activity renders the plant more susceptible to invading pathogens (Zeidler et al., 2004; Zeier et al., 2004; Kato et al., 2007; Qiao et al., 2009). It is possible that NOA1 participation in disease response is through its association with the chloroplast (Reinero and Beachy, 1989; Bhat et al., 2012; Liu et al., 2014). In addition to its role as the cellular energy generator, chloroplasts are main sites of defence molecule production such as ROS, salicylic acid (SA) and jasmonic acid (Rodio et al., 2007; Padmanabhan and Dinesh-Kumar, 2010; Palukaitis et al., 2013; Caplan et al., 2015; Serrano et al., 2016). Chloroplasts can be directly targeted during plant virus infection (de Torres Zabala et al., 2015) as potyviruses, turnip yellow mosaic virus (TYMV) and turnip mosaic virus (TuMV), and the geminivirus abutilon mosaic virus (AbMV), have been shown to replicate within the chloroplast (Gröning et al., 1987; Bhattacharyya and Chakraborty, 2017). Different chloroplast proteins are targeted by pathogen effectors during infection (reviewed in Bobik and Burch-Smith, 2015), and the geminivirus-associated betasatellite βC1 and movement protein (MP) of AbMV were shown to target the chloroplast (Krenz et al., 2010; Bhattacharyya et al., 2015).

Virus-induced disease symptoms such as chlorosis, bleaching and mosaic suggest a decrease in chlorophyll synthesis and disruption of chloroplasts (Liu et al., 2014; Li et al., 2016). The expression of many genes associated with chloroplast function and photosynthesis were shown to be differentially expressed in african cassava mosaic virus (ACMV) infected cassava (Liu et al., 2014). Perturbation of chloroplast functionality arising during infection could explain the involvement of translation factors such as AtNOA1 in defence responses, and although AtNOA1 cannot directly produce NO, a decrease in NOA1 expression can negatively affect NO accumulation, as it has been shown that *atnoa1* mutants accumulate lower NO (Guo et al., 2003; Zeidler et al., 2004; Guo and Crawford, 2005; Bright et al., 2006; Zhao et al., 2007; Li et al., 2009; Chen et al., 2010). The decrease in accumulation of NO in *atnoa1* mutants is believed to be linked to the inability of *atnoa1* to fix carbon, resulting in a decrease in fumarate/fumarate stores inherently leading to a decrease in L-arginine accumulation in *Arabidopsis* (Van Ree et al., 2011). Although no plants enzyme catalysing L-arginine conversion to L-citrulline and NO has been found, the conversion has been measured in plants (del Río et al., 2004; Corpas et al., 2009) and therefore a decrease in L-arginine negatively contributes to the overall NO accumulation, linking chloroplast translation to carbon storage and NO.

To date, the relationship between AtNOA1, the indirect accumulation of NO and the chloroplast during geminivirus infection and symptom development is not documented. Cassava mosaic geminiviruses (CMGs) belong to the circular single-stranded DNA (ssDNA) *Begomovirus* genus of the family *Geminiviridae*, transmitted by the whitefly species *Bemisia tabaci* Gennadius. South african cassava mosaic virus (SACMV) (Berrie et al., 2001) is one of nine species causing cassava mosaic disease (CMD) which is a major threat to cassava (*Manihot esculenta* Crantz) production (Graziosi et al., 2016). In this study the aim was to evaluate if NOA1 plays a role in SACMV pathogenicity in the susceptible experimental host *N. benthamiana*. From the results, we demonstrate that the NbNOA1 status is important in terms of chloroplast 'health' and plant growth, and that infection with SACMV influences this status, leading to physiological perturbations and disease symptoms.

2. Material and methods

2.1. Plant growth and virus inoculation

Unless indicated otherwise, reagents used for this section were purchased from Sigma Aldrich. *Nicotiana benthamiana* plants were grown from seed at 25 °C, with a 16 h light and 8 h dark photoperiod under 4000 lx. For infection, *N. benthamiana* plants were grown to the 4–6 leaf stage and the source leaves were infiltrated using a needless syringe.

Agrobacterium tumefaciens C58C1 containing head-to-tail infectious clones of SACMV DNA-A or DNA-B (Berrie et al., 2001) were inoculated into YEP medium containing 100 mG/L kanamycin and 50 mG/L rifampicin and grown overnight at 28 °C. Fifty mL of fresh YEP media supplemented with 10 mM morpholino-ethane sulfonic acid (MES), 20 μM acetosyringone, 100 mG/L kanamycin and 50 mG/L rifampicin was inoculated with the overnight culture and allowed to grow overnight. The cells were collected by centrifugation and the pellet resuspended in infiltration media (10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone) to an OD_{600} of 0.4–0.6. The cultures were incubated for 3 h at room temperature before proceeding with agroinfiltration. Three independent biological experiments were carried out and each experimental replicate consisted of three plants. Plants were inoculated with the infectious clones and 9 plants were mock inoculated with C58C1 harbouring no clones (Supplementary Fig. S1). Symptoms on infected leaves were scored at 14 and 28 dpi, using a scale of 0–5, minimum score of 0 to asymptomatic plants and the maximum