Plant Science 239 (2015) 26–35

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Mitochondrial alternative oxidase is involved in both compatible and incompatible host-virus combinations in *Nicotiana benthamiana*

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ARTICLE INFO

Article history:
Received 2 April 2015
Received in revised form 8 July 2015
Accepted 10 July 2015
Available online 19 July 2015

Keywords:
Alternative oxidase (AOX)
Systemic resistance
Tobacco mosaic virus (TMV)
Turnip crinkle virus (TCV)
Virus-induced gene-silencing (VIGS)

ABSTRACT

The alternative oxidase (AOX) functions in the resistance to biotic stress. However, the mechanisms of AOX in the systemic antiviral defense response and *N* (a typical resistance gene)-mediated resistance to *Tobacco mosaic virus* (TMV) are elusive. A chemical approach was undertaken to investigate the role of NbAOX in the systemic resistance to RNA viruses. Furthermore, we used a virus-induced gene-silencing (VIGS)-based genetics approach to investigate the function of AOX in the *N*-mediated resistance to TMV. The inoculation of virus significantly increased the NbAOX transcript and protein levels and the cyanide-resistant respiration in the upper un-inoculated leaves. Pretreatment with potassium cyanide greatly increased the plant's systemic resistance, whereas the application of salicylhydroxamic acid significantly compromised the plant's systemic resistance. Additionally, in *NbAOX1a*-silenced *N*-transgenic *Nicotiana benthamiana* plants, the inoculated leaf collapsed and the movement of TMV into the systemic tissue eventually led to the spreading of HR-PCD and the death of the whole plant. The hypersensitive response marker gene *HIN1* was significantly increased in the *NbAOX1a*-silenced plants. Significant amounts of *TMV-CP* mRNA and protein were detected in the *NbAOX1a*-silenced plants but not in the control plants. Overall, evidence is provided that AOX plays important roles in both compatible and incompatible plant-virus combinations.

1. Introduction

The mitochondrion is the main site responsible for energy production in both animals and plants. In addition, mitochondria have other important functions, such as their active role in the programmed cell death (PCD) pathways of animals and plants [1–5]. The plant mitochondrial electron transport chain supporting oxidative phosphorylation branches at ubiquinone [6]. Electrons can flow from ubiquinone (UQ) through the usual cytochrome (Cyt) pathway or through an alternative oxidase (AOX) pathway. Under stress, electrons are frequently rerouted through the AOX pathway, which branches from the Cyt pathway at the level of the UQ pool [7]. The alternative pathway bypasses two important proton translocation sites (complex III and IV) and then transfers electrons from the reduced forms of UQ to O_2 directly via AOX, thereby avoiding oxidative phosphorylation and ATP production [8,9]. Alternative respiration is widespread in plants, fungi, and some prokaryotes [7], but its physiological functional characteristics are not well understood.

AOX was initially investigated in the thermogenic flowers of Araceae [10]. The functions of AOX have been extensively investigated. Several studies have indicated that the AOX plays an important role in fruit ripening [11,12]. Moreover, AOX has been proved as the important defense components in the plant response to abiotic stresses, including drought, high salt, chilling, and wounding [13–16]. In addition, abundant evidence suggests that AOX also functions in the resistance to biotic stresses, such as pathogen attack. For example, the AOX protein levels increased in *Nicotiana attenuata* leaves infected with *Manduca sexta*, and the silencing of AOX in *N. attenuata* makes the plants more susceptible to *Empoasca* [4]. In addition, the role of AOX signaling during the defense against plant viruses was also investigated [17–19]. Overexpression of AOX in the TMV-infected tobacco leaves resulted in reducing hypersensitive response (HR) lesions, suggesting a link

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http://dx.doi.org/10.1016/j.plantsci.2015.07.009
0168-9452/

between AOX and PCD [20]. However, overexpression of AOX in tobacco cultivar Samsun NN plants allowed an increase in TMV spread and the development of severe symptoms [21]. Therefore, the role of AOX in virus resistance has been reported, however, the mechanisms of this antiviral action are complex and varied [9,22,23].

The reactive oxygen species (ROS) equilibrium is maintained between production and scavenging under normal metabolic conditions [24]. For a long time, ROS was considered to be a harmful byproduct. Extensive studies suggest that high levels of ROS cause cell death inside the cell. However, low levels of ROS possess regulatory effect in plant stress responses. The ROS production is involved in plant responses to different types of stresses, which suggests that ROS may act as a defense signal in the plant stress responses [25]. Several lines of evidence have suggested that AOX may have a general function by limiting the production of ROS [26]. This evidence indicates that the levels of ROS production affected by AOX proteins can influence the plant defense against viruses [27]. However, Murphy et al. (2004) [21] suggested that a high level of the AOX protein accelerates the systemic movement of TMV and the accumulation of ROS in *N. benthamiana* plants. Hence, the roles of ROS and AOX in plant defense pathway may be elusive.

Plants limit the spread of pathogen that attack them by inducing defense responses. One of these is the HR cell death, which is a form of PCD that limits the spread of the pathogen. When a plant resistance (*R*) gene product recognizes specific molecules or proteins produced by pathogen avirulence genes, then the HR is activated [28,29]. Numerous individual *R* genes have been identified from various plant species that confer specific resistance to insects, bacteria, oomycetes, viruses, fungi, and nematodes [30]. The tobacco plant resistance gene *N* is a classic *R* gene, and the *N* gene encodes a protein which belongs to the TIR-NBS-LRR class [31]. The *N* protein specifically can recognize the helicase domain (50 kDa) of the TMV replicase and then triggers a signal transduction cascade that leads to the induction of HR in the form of local necrotic lesions, the restriction of virus spread, and the onset systemic acquired resistance (SAR) [32]. Salicylic acid (SA) and jasmonic acid (JA) signaling play an important role in the *N* gene-mediated resistance to TMV. However, the biological role of AOX in *N* gene-mediated resistance to TMV is elusive. Tobacco plants transformed with the salicylic acid hydroxylase gene could not restrict TMV to the inoculation site in TMV-infected NN-type tobacco leaves. However, treatment with KCN restored TMV localization [33]. These results suggested that SA play a role as potential integrator of mitochondrial function and AOX expression [33]. In addition, the overexpression of AOX in tobacco resulted in slightly smaller TMV-induced HR lesions [20]. However, high levels of AOX in NN-type tobacco allowed an increase in TMV spread [21].Therefore, it is necessary to further investigate the biological role of AOX in *N* gene-mediated resistance to TMV.

In this study, a chemical approach was undertaken to investigate the role of AOX in the viral resistance of *N. benthamiana* plants. Furthermore, a virus induced gene silencing-based (VIGS) approach [34] was used to investigate the function of AOX in *N* gene-mediated resistance to TMV. Our results indicate that NbAOX plays an important role in the systemic resistance to virus infection and that NbAOX is required for *N* gene-mediated resistance to TMV.

2. Materials and methods

2.1. Multiple alignments of AOX

We cloned the complete open reading frame (ORF) of *NbAOX1a* and *NbAOX1b* from *Nicotiana benthamiana* plants using the specific primers AOX1a-ORF-F (5′-ATGATGACACGTGGAGCGA-3′), AOX1a-ORF-R (5′-TTAGTGATACCCAATTGGTGCT-3′), AOX1b-ORF-F (5′-ATGTGGGTTAGG CATTTTCCAG-3′), and AOX1b-ORF-R (5′-TTAGTGATACCCAATTGGTGCT-3′). The sequences of *NbAOX1a* and *NbAOX1b* were uploaded to the National Center for Biotechnology Information (NCBI). A multiple alignment of the amino acid sequences of *NbAOX1a* (GenBank accession KF367455), AtAOX1a (GenBank accession NM_113135), NbAOX1b (GenBank accession KF367456), and AtAOX1b (GenBank accession NM_113134) was performed with CLUSTAL 2.1.

2.2. Plant materials and growth conditions

The *N*-transgenic *N. benthamiana* and wild-type *N. benthamiana* plants were grown in a greenhouse at 25 °C with a 16-h-light/8-h-dark cycle (100 μmol m^{-2} s^{-1}). Six- to seven-week-old seedlings were used in the experiments.

2.3. Chemical treatments and pathogen inoculations

At least six wild-type *N. benthamiana* seedlings were subjected to pharmacological pretreatment and subsequent virus inoculation. In brief, 3–4 upper, fully developed wild-type *N. benthamiana* leaves were treated at one site on the primary leaf with a final concentration of 1 mM KCN and a final concentration of 3.5 mM SHAM three days before inoculation with the virus, and then inoculated with TMV-GFP or TCV on the secondary (systemic) leaves. Seedlings sprayed with distilled water three days before virus inoculation were used as controls. The common strain of *Turnip crinkle virus* (TCV) was maintained in an aqueous suspension of 0.02 M sodium phosphate buffer (PBS) at 4 °C. The inocula preparation was performed as described previously [35]. PBS buffer (pH 7.0) only inoculation was carried out as control. Stock inocula of TCV were performed mechanically by dusting two leaves with sterile Carborundum. For *Tobacco mosaic virus* (TMV) inoculation, *Agrobacterium* cultures at OD_{600} = 0.5 containing the TMV-GFP construct were infiltrated into *N. benthamiana* leaves [36]. Empty *Agrobacterium* culture only infiltration was carried out as control in TMV-GFP experiments. In the *NbAOX* transcript levels in response to virus infection experiments, the inoculated leaves and systemic leaves were collected to detect the expression of *NbAOX1a* and *NbAOX1b*. In other experiments, the systemic leaves were collected. All experiments were repeated with similar results.

2.4. Construction of VIGS vectors and TRV-mediated VIGS assay

The pTRV vector for VIGS were carried out as described by Liu et al. (2002) [37]. Partial cDNA of the *NbAOX1a* was amplified by PCR from a cDNA library of *N*-transgenic *N. benthamiana* leaf tissues using gene-specific primers. The gene-specific primers are shown in Table S1. The RT-PCR products were cloned into the pCR®8/GW/TOPO® vector using a TOPO TA cloning kit (Invitrogen, USA) according to the instructions of the manufacturer. Partial fragments of *NbAOX1a* were then inserted into the *Tobacco rattle virus* (TRV) vector (pTRV-RNA2).

The VIGS assay was performed as described previously [37]. Simply, pTRV1 or pTRV2 and pTRV-*NbAOX1a* were respectively introduced into *Agrobacterium* strain GV2260 via electroporation method (BIO-RAD, USA). *Agrobacterium* cultures containing pTRV1 and pTRV2 or pTRV-*NbAOX1a* were mixed at a 1:1 ratio and infiltrated into the lower leaf of 4-leaf stage plants using a 1-ml needleless syringe. In addition, we used 12 days post-silenced plants for virus infections because the RT-PCR analysis of the silenced plants showed a significant reduction in the RNA levels at this time point compared with non-silenced plants.