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Involvement of *miR160*/*miR393* and their targets in cassava responses to anthracnose disease

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ABSTRACT

Cassava is a starchy root crop for food and industrial applications in many countries around the world. Among the factors that affect cassava production, diseases remain the major cause of yield loss. Cassava anthracnose disease is caused by the fungus *Colletotrichum gloeosporioides*. Severe anthracnose attacks can cause tip die-backs and stem cankers, which can affect the availability of planting materials especially in large-scale production systems. Recent studies indicate that plants over- or under-express certain microRNAs (miRNAs) to cope with various stresses. Understanding how a disease-resistant plant protects itself from pathogens should help to uncover the role of miRNAs in the plant immune system. In this study, the disease severity assay revealed different response to *C. gloeosporioides* infection in two cassava cultivars. Quantitative RT-PCR analysis uncovered the differential expression of the two miRNAs and their target genes in the two cassava cultivars that were subjected to fungal infection. The more resistant cultivar revealed the up-regulation of *miR160* and *miR393*, and consequently led to low transcript levels in their targets, *ARF10* and *TIR1*, respectively. The more susceptible cultivar exhibited the opposite pattern. The *cis*-regulatory elements relevant to defense and stress responsiveness, fungal elicitor responsiveness and hormonal responses were the most prevalent present in the miRNAs gene promoter regions. The possible dual role of these specific miRNAs and their target genes associated with cassava responses to *C. gloeosporioides* is discussed. This is the first study to address the molecular events by which miRNAs which might play a role in fungal-infected cassava. A better understanding of the functions of miRNAs target genes should greatly increase our knowledge of the mechanism underlying susceptibility and lead to new strategies to enhance disease tolerance in this economically important crop.

Introduction

Cassava (*Manihot esculenta* Crantz, Family *Euphorbiaceae*) is one of the most important staple crops that are commonly grown in tropical and subtropical areas. It is used as a major source of carbohydrates and stores important quantities of starch in its roots (El-Sharkawy, 2004; FAO, 2008). Cassava starch is used as raw material in many industries, including the paper, food and textile industries. In addition, the technology of producing ethanol from starch is internationally well developed (Kuiper et al, 2007). Cassava is facing a number of production challenges worldwide. Global climate change is one such challenge that has highlighted the importance of studies on plant responses to drought and disease stresses. There has been a recent increase in the movement of invasive exotic species from one region of the world to others. Cassava production in the field can be severely affected by cassava anthracnose disease (CAD), which is caused by *Colletotrichum gloeosporioides* f. sp. *manihotis* (Hahn et al., 1989a,b). This disease is present in all regions where cassava is grown and causes 12–30% of production losses (Hahn et al., 1989a,b). This major economically significant disease is characterized by cankers on stems, leaf spots and tip die-backs. The disease appearance depends on the cassava varieties and the infected plant parts. On susceptible varieties, the cankers spread towards the top of the plant and cause wilt, shoot death and easy breaking by wind action. Some resistant cassava plants can recover from *C. gloeosporioides* infection by sprouting new twigs from axillary buds below the necrotic area (Hahn et al., 1989a,b).

Abbreviations: ARFs, auxin-response factors; CAD, cassava anthracnose disease; IAA, indole-3-acetic acid; miR, microRNA; HB60, Huay Bong 60; HN, Hanatee; TIR1, Transport Inhibitor Response 1.

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MicroRNAs (miRNAs) are a class of small, single-stranded, non-coding RNAs of approximately 21 nt in length, and they regulate gene expression at the post-transcriptional level through base-pairing to target mRNAs (see Jones-Rhoades et al., 2006; Chen, 2009; Voinnet, 2009 for reviews on plant miRNAs). Mature miRNAs are derived from single-stranded primary miRNA transcripts (pri-miRNAs) that possess an imperfect stem–loop secondary structure. These hairpin RNAs, which are known as pre-miRNAs, are processed by DICER-LIKE 1 into the miRNA/miRNA* duplex in the nucleus and are transported to the cytoplasm. A mature miRNA is derived from one strand of the duplex and bound by an argonaute protein that is incorporated into the RNA-induced silencing complex, which in turn uses miRNAs as guides to recognize target complementary mRNAs and negatively regulate their expression through the degradation (Llave et al., 2002) or repression of productive translation (Gandikota et al., 2007). Plant miRNAs generally recognize their target site through perfect or near-perfect complementary direct cleavage of mRNAs. The targets of conserved miRNAs have a narrower range of functions than the targets of non-conserved miRNAs (Willmann and Poethig, 2007). Plant miRNAs play vital roles in multiple essential biological processes including plant development (Chen, 2009) and responses to environmental stimuli such as abiotic and biotic stresses (Sunkar, 2010).

A recent comprehensive study on miRNAs involvement in plant immunity showed that the miRNA expression is altered in response to adverse environmental conditions, including biotic stresses. With respect to cassava, Patanun and coworkers identified the 169 potential cassava miRNAs belonging to 34 miRNA families, and the *cis*-regulatory elements relevant to abiotic and biotic stresses were analyzed in the promoter regions of those miRNA genes (Patanun et al., 2013). MiRNAs have broad functions in regulating plant responses to various microbes. Some miRNAs reportedly respond to bacterial infection as in the cassava-*Xanthomonas axonopodis* pv. *manihotis* interaction. miRNA induction was found to be involved in regulating auxin signaling *via miR160*, *miR167* and *miR393* thus, auxin signaling seems to be an important strategy for impairing bacterial growth in plants (Pérez-Quintero et al., 2012). In addition, *Arabidopsis-miR393* mediates antibacterial resistance by repressing auxin signaling (Navarro et al., 2006). There are reports indicating cross-talk among jasmonate and salicylate signaling in pathogen attack (Kazan and Lyons, 2014). Moreover, the other repressed families were *miR397*, *miR398* and *miR408*, which are known to be involved in copper regulation, and they were differentially expressed in response to biotic stress, suggesting that the miRNA-mediated regulation of copper homeostasis could also be a crucial defense mechanism against bacteria (Pérez-Quintero et al., 2012). miRNAs were also shown to play a role in fungal infection. Various miRNA families were differentially expressed in response to infection by *Cronartium quercuum* f. sp. *fusiforme*, which caused fusiform rust disease in pines for example, *miR156* and *miR160* were repressed in pine stems infected with the rust fungus (Lu et al., 2007). By contrast, *miR156a-e* were induced in *Populus* infected by *Dothiorella gregaria*, suggesting that members of the same miRNA family might perform different functions in different plants (Chen et al., 2012). Recent data have indicated that some of the conserved miRNAs may affect the response of wheat (*Triticum aestivum* L.) to powdery mildew infection. Several miRNAs, such as *miR156*, *miR159*, *miR164*, *miR171* and *miR396*, were down-regulated, and *miR393*, *miR444* and *miR827* were up-regulated (Xin 2010). *miR408* was up-regulated during *Puccinia graminis* f.sp. *tritici* infection in wheat. The target of *miR408* is plantacyanin, which is known to be involved in copper regulation and is differentially expressed in response to biotic stress. Lignin biosynthesis and the regulation of protein synthesis machinery were reported to be down-regulated to maintain cellular homeostasis when *miR408* was up-regulated (Gupta et al., 2012).

Plants respond to stress by using multiple gene regulatory mechanisms including the post-transcriptional regulation of gene expression (Kawaguchi et al., 2004). Based on the fact that the same miRNA family might perform substantially different functions in different plants, the regulatory roles of miRNAs in their target genes in a specific pathogen response in a specific plant should be studied. A disease-resistant plant protects itself from pathogens by performing mechanisms and/or by infection-induced responses of the immune system. Understanding the regulation of miRNA genes in response to pathogens should uncover the role of those miRNAs. To test whether cassava miRNAs are involved in the stress responses associated with CAD, we employed the two cassava cultivars, which exhibited different reactions in response to *C. gloeosporioides*, Hanatee (HN) and Huay Bong 60 (HB60). The expression patterns of miRNAs-related to auxin signaling, namely *miR160* and *miR393* with their target genes-mediated response to *C. gloeosporioides*-infected cassava were investigated. The potential roles of these specific miRNAs and their target genes were associated with cassava susceptibility to *C. gloeosporioides* as discussed.

Materials and methods

Microbial strain

Colletotrichum gloeosporioides f. sp. *manihotis* strain CAD-A was kindly provided by Dr. Kanokporn Triwitayakorn of the Institute of Molecular Biosciences at Mahidol University, Thailand. The fungal type was identified by PCR using primers specific to *Colletrotrichum* spp. (Col_F and Col_R) (Kunkeaw et al., 2010) (Supplementary Table S1). A CAD-A colony was cultured on potato dextrose agar (PDA) at 30 °C for 10 days before use.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2014.09.006.

The detached leaf method for the infection assay

A detached leaf assay was performed by using a method adapted from Kunkeaw et al. (2010). The detached leaf method can be used as a rapid screening tool that can be undertaken within a short time and without contaminating the environment with the pathogen. The two Thai cassava varieties, namely Hanatee (HN) and Huay Bong 60 (HB60), were infected with CAD-A. The healthy mature leaves of each variety were collected from 2-month-old cassava plants grown in a pot. The third and fourth leaves from the top of each plant were collected and surface-sterilized with 70% ethanol and sterilized distilled water. The middle lobe of a cassava leaf was wounded by cutting the middle vein with a cork-borer. An agar plug cut with a 0.8-cm-diameter cork-borer from the edge of a CAD-A colony was placed face down in the center of the freshly wounded leaf. The inoculated leaves were placed in wet cotton wool to maintain sample moisture and then incubated at 30 °C. Each inoculation experiment was undertaken independently in triplicate and a physical control (PC) was set up by using a cassava leaf without either PDA agar or fungal plugs to observe any changes in the leaf samples. A cassava leaf inoculated with a PDA agar plug was used as a negative control (NC) in order to observe the effects of the agar on the inoculated area.

The inoculated and control leaf samples were observed daily until the seventh day after inoculation for lesion development. The lesion lengths were measured and recorded. The lesion size was determined by measuring the longest length (A) and width (B) of the lesion and size calculated by using the following formula: $(A+B)/2$. One-way ANOVA was used to analyze the significant differences