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Early down-regulation of chorismate mutase 2 gene expression in a sugarcane cultivar tolerant to *Xanthomonas albilineans* infection

T.M. Rosa-Santos[1], L.F. Nogueira[2], T.A. Paz[3], R.G. da Silva[1], J.O.L. Silva[2], S. Creste[4,5] and S.M. Zingaretti[1,2]

[1] Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP, Brasil
[2] Universidade de Ribeirão Preto, Departamento de Biotecnologia, Ribeirão Preto, SP, Brasil
[3] Universidade Estadual Paulista, Instituto de Química, Araraquara, SP, Brasil
[4] Centro de Cana, Instituto Agronômico de Campinas, Campinas, SP, Brasil
[5] Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Corresponding author: S.M. Zingaretti
E-mail: szingaretti@unaerp.br

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ABSTRACT. *Xanthomonas albilineans* causes leaf scald disease, one of the most economically important diseases of sugarcane. Leaf scald is a systemic and vascular disease that can severely reduce productivity. The best and primary method of leaf scald control is the use of tolerant cultivars. Knowing that cell wall lignification is a plant defense mechanism and that chorismate mutase is relevant in this process, we analyzed the expression pattern of the chorismate mutase 2 (*SoCM2*) gene in two contrasting sugarcane cultivars by RT-qPCR. A similar pattern of downregulation for the *SoCM2* gene was observed in both cultivars; however, after 72h, the tolerant cultivar (RB867515) showed repression of the *SoCM2* gene, while for the susceptible cultivar (SP78-4467), *SoCM2* gene downregulation occurred only after 144h. Here we propose that early

repression of the *SoCM2* gene is responsible for tolerance to *X. albilineans* infection in the sugarcane cultivar RB867515.

Key words: Biotic stress; Chorismate mutase 2; Cytosolic; Sugarcane

INTRODUCTION

Sugarcane (*Saccharum* spp.) an important source of sugar and ethanol has become the third most commodity in the world (1.4G) and Brazil figures as a major producer (500M tons) (FAOSTAT, 2017). The increasing demand for food and reneweable energy has lead to the expansion of sugarcane cultivated areas, as well as productivity (Hoang et al., 2015). However, several factors such as drought, salinity, temperature and pathogenesis can affect productivity (Qin et al., 2011; Safarnejad et al., 2011).

Leaf scald, caused by *Xanthomonas albilineans*, is one of the most important and persistent sugarcane diseases (Birch, 2001), decreasing productivity and sugar quality (Rott et al., 2000). *Xanthomonas albilineans* is the only xanthomonad that produces albicidin, a phytotoxin responsible for a white leaf stripe with necrotic zones at leaf margin, rapid wilting and plant death. Albicidin is a DNA gyrase inhibitor that blocks chloroplast DNA replication and differentiation (Legaz et al., 2018). The disease can appear in three distinct forms: chronic, acute and latent. In the acute and chronic forms, the necrotic zones at leaf margins can easily be seen, but they are not seen in the latent form, and the bacteria can easily be spread by mechanical harvesting. The evolution of the latent to acute form of infection is not only based on the bacterial population, but also on abiotic stress and the cultivars in use (Rott et al., 2000; Birch, 2001).

Plants and microorganism interaction involves a complex and integrative system that includes the synthesis of different compounds, such as effector proteins (AvrB, HopPtoD2, AvrXv4 and XopD) (Beth Mudgett, 2005) and phytotoxins (Coronatine) (He et al., 2004) by the bacteria that will allow the establishment of infection. On the other hand, plants also produce compounds to prevent the establishment of bacteria, such as: phytohormones (ethylene, jasmonic acid, and salicylic acid) (Kazan and Lyons, 2014), transmembrane receptors (FLS2) (Gómez-Gómez and Boller, 2002), cell wall lignification (Vance et al., 1980), among others.

In sugarcane, several genes have been identified as involved in defense mechanisms to biotic stress; e.g. β-1,3-glucanase was identified in *Sporisorium scitamineum* sugarcane interaction (Su et al., 2013), NBS-LRR genes acting in systemic acquired resistance (SAR) against *Colletotrichum falcatum* fungal infection (Selvaraj et al., 2014) and glycoproteins that are produced to inhibit the effect of enzymes secreted by *X. albilineans* during invasion (Legaz et al., 2011). Dabbas et al., (2006) selected differently expressed genes, with different biological functions, when comparing two contrasting sugarcane cultivars (33 induced and 67 repressed) and (53 induced and 47 repressed) for the susceptible and tolerant cultivars respectively, evidencing the complexity of the sugarcane response to *X. albilinea*ns. Among those, an Expressed Sequence Tag (EST) similar to chorismate mutase was induced.

It is known that chorismate mutase (CM) catalyzes the conversion of chorismate to prephenate and this, in turn, will participate in the biosynthesis of the aromatic amino acids phenylalanine and tyrosine, precursors of secondary metabolites such as lignin, alkaloids

and cinnamic acid (Mobley et al., 1999; Anterola and Lewis, 2002; Tzin and Galili, 2010; Westfall et al., 2014). In Arabidopsis, three chorismate mutase isoforms (CM1, CM2 and CM3) were identified (Woodin et al., 1978); CM1 and CM3 are located in the plastids, while CM2 is found in the cytosol (Eberhard et al., 1996; Mobley et al., 1999).

Knowing that the membrane lignification is one of the main plant defense mechanisms and that chorismate mutase is relevant in this process, we analyzed the expression pattern of the chorismate mutase 2 (*SoCM2*) gene in two contrasting sugarcane cultivars by RT-qPCR.

MATERIAL AND METHODS

Biological material

Inoculation experiments were conducted at IAC (Sugarcane Center of the Agronomic Institute of Campinas, at Ribeirão Preto, Brazil) in a greenhouse using healthy plants derived from disease-free tissue-cultured plantlets of sugarcane cultivars: SP78-4467 (sensitive to *X. albilineans* Cultivar - SXaC) and RB867515 (tolerant to *X. albilineans* Cultivar - TXaC). Plants were grown in individual pots containing a mixture of soil, sand, pine, and coconut bark substrate at 3:1:1:1 proportion. Twenty-five sugarcane stalks per cultivar were inoculated by the decapitation method using *X. albilineans* strain IACXa11, considered one of the most aggressive *X. albilineans* isolates found in Brazil (Tardiani et al., 2014), as described by Rott et al., (2011) with modifications. Stalks were cut off using sterile scissors just above leaf+2, and inoculated using 100 µL of bacterial suspension calibrated to $1x10^8$ CFU mL^{-1} in sterile PBS buffer. Control plants were inoculated using 100 µL of sterile PBS buffer. Leaf samples from inoculated and control plants were collected at 24, 72, 144, 360, and 720 h after inoculation, immediately frozen in liquid nitrogen, and stored at -80°C for further processing.

RNA extraction

Total RNA was extracted from 200 mg of leaf tissue using the Trizol reagent (Invitrogen, USA), following the manufacturer's recommendations. After extraction, total RNA was quantified in NanoPhotometer P360 (Implen, Germany) and its quality was evaluated by denaturing agarose gel electrophoresis.

RT-qPCR

For the *chorismate mutase 2* (*SoCM2*) gene expression analyses, a quantitative real time polymerase chain reaction (RT-qPCR) was performed. First strand cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer´s recommendations, and the RT-qPCR was performed using 20 ng of cDNA, 200 nM of each primer (Table 1), KAPA SYBR Fast qPCR Master Mix Kit (2X) Universal (Kapa Biosystems, USA), and water with final volume of 20 µL. In RT-qPCR experiments, cDNA concentration was standardized for each sample, dissociation curve analysis was performed to check primer specificity, and teal time PCR was carried out in a Stratagene MX3005P thermocycler, (California, USA).

Thermal cycling conditions were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The dissociation curve was constructed based on: 95°C - 1 m; 55°C - 30 s, and 95°C - 30 s.

Table 1. Nucleotide sequence for primers used in RT-qPCR analyses of the sugarcane *chorismate mutase 2* (*SoCM2*) gene from leaves infected and not infected by *Xanthomonas albilineans*.

Gene	Sequence	Amplicon (bp)
SoCM2	F: 5' - GCTCCCGTTATTCACTGTTG - 3'	134
	R: 5'- CGTCTTTGAACTTCACCTCAG - 3'	
α-Tub	F: 5' - CCATTGGCAAGGAGATTGTT - 3'	104
	R: 5' - TCCACCAACTGCATTGAAGA - 3'	
GAPDH	F: 5' - CACGGCCACTGGAAGCA - 3'	100
	R: 5' - TCCTCAGGGTTCCTGATGCC - 3'	

The mRNAs expression level was quantified after normalization with constitutive genes *glyceraldehyde-phosphate dehydrogenase (GAPDH)* and *alpha-tubulin (α-TUB)* (Thiebaut et al., 2012; Silva et al., 2014) used as internal controls. In the RT-qPCR procedure, all time points were analyzed and three biological replicates were examined to ensure reproducibility.

Statistical analysis

Expression levels were analyzed using the MxPro QPCR Software version 4.10 (Stratagene, California, USA) and the relative expression values were established by the ratio between the expression of the target gene and the control that was considered 1.

RESULTS AND DISCUSSION

The RT-qPCR analysis revealed a similar pattern of downregulation for the *SoCM2* gene in the two cultivars. Although gene repression was evident for the two cultivars, down regulation was different at two points, 72 and 144 h after infection. At 24, 360 and 720 h, the *SoCM2* gene exhibited no significant differences in expression levels (Figure 1).

After 72h, the tolerant cultivar (RB867515) showed the first signaling in response to infection with repression of the *SoCM2* gene expression from 0.926 to 0.565. In contrast in the susceptible cultivar (SP78-4467), *SoCM2* gene down regulation occurred only after 144h (0.809 to 0.278).

As it is already known, the main difference between tolerance and susceptibility of plants may be a result of the timing of the response; the most tolerant plant perceives the pathogens and immediately activates the necessary mechanism to prevent pathogen invasion (Yang et al., 1997). The mechanisms utilized by sugarcane plants, especially cultivar RB867515 (tolerant) were activated faster, controlling the pathogenic effect caused by the bacterium *X. albilineans.* This is reinforced by the result of the *SoCM2* gene expression at 144h for the susceptible cultivar, where it reached its maximum repression (Figure 1). Plants of cultivar SP78-4467 remained susceptible to the free action of *X. albilineans* for six days before their defense mechanisms signaled by *SoCM2* were actually activated. In this

cultivar, the decrease in gene expression is probably related to late molecular recognition of the pathogen by the sugarcane plant.

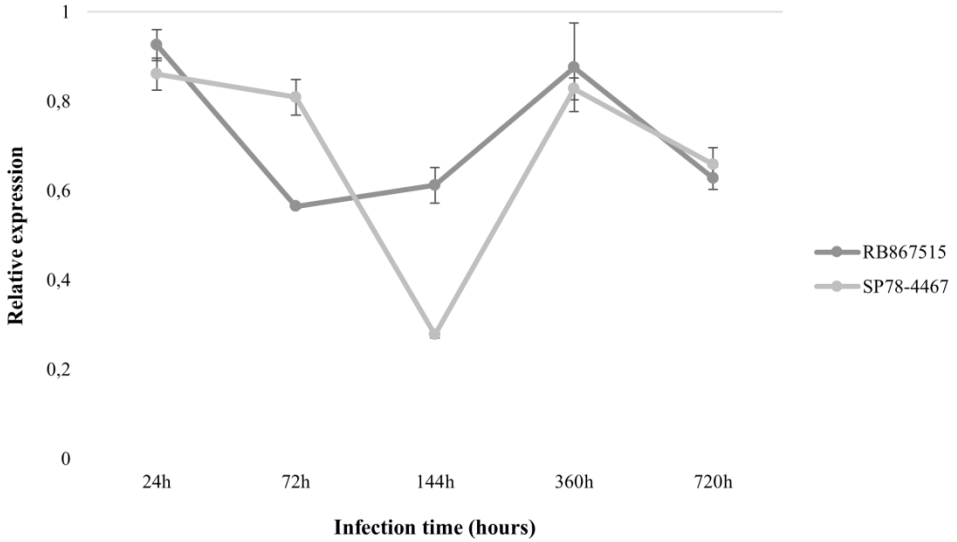

Figure 1. Relative expression profile of the *SoCM2* gene in the leaves of cultivars SP78-4467 (susceptible) and RB867515 (tolerant) submitted to infection by *Xanthomonas albilineans* during the periods of 24, 72, 144, 360 and 720 h, compared to control plants. The relative expression values were established by the ratio between the expression of the target and the control gene, which was considered 1.

Chorismate mutase 2 is a cytosolic enzyme, and the reason for its presence in plants is still unknown (Westfall et al., 2014); however, repression of the CM2 gene has been associated with plant defense mechanisms in *Arabidopsis thaliana* leaves infected with *Fusarium oxysporum* (Eberhard et al., 1996) and wounding stress (Mobley et al., 1999).

During *X. albilineans* infection, the cell wall begins to be degraded, and it is worth mentioning that *X. albilineans* has 19 enzymes that degrade the cell wall of the host (Pieretti et al., 2009). As the need for protein in the lignification process increases, *SoCM2* is repressed and releases chorismate to be used by CM1 and CM3 (primary metabolism) for the production of aromatic amino acids, e.g. phenylalanine and tyrosine required for the biosynthesis of lignin precursors (Lewis and Yamamoto, 1990). CM2 is modulated by lignin precursors (Woodin et al., 1978); when the lignification process occurs by the induction of CM1 and CM3, the CM2 gene is repressed, as had been reported by Eberhard et al., (1996) CM1 increases in *A. thaliana* during fungal infection. CM2 has higher catalytic efficiency than the other two isoforms (CM1 and CM3), probably because it is in an environment (cytosol) with low supply of Chorismate, since all mechanisms for amino acid biosynthesis are found in plastids (Westfall et al., 2014).

Our results show that in the RB867515 cultivar, the repression of *SoCM2* after 72 h of infection releasing Chorismate for CM1 and CM3 to produce aromatic amino acids to be used in the lignification process can be responsible for its pathogen tolerance capacity (Figure 2).

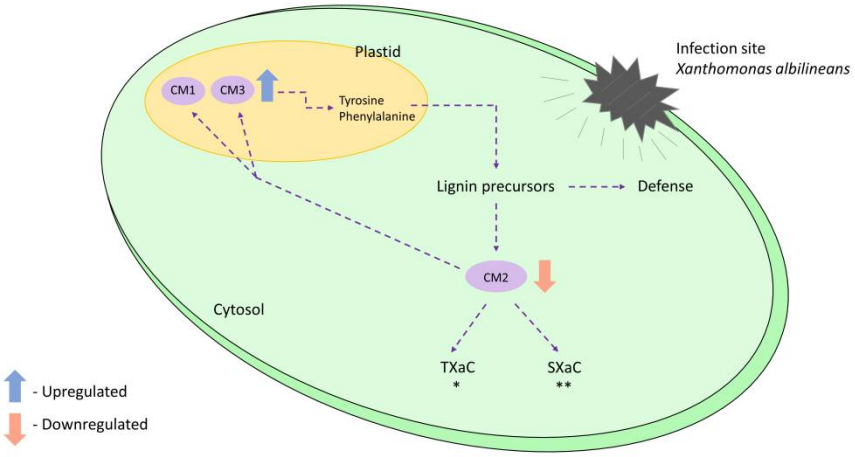

Figure 2. Role of the CM2 sugarcane gene in response to *Xanthomonas albilineans* infection. During the infection process, the bacterium causes physical damage to the plant, triggering the defense process through lignification. CM1 and CM3 are upregulated (blue arrow), and later the lignification precursors signal to *SoCM2* to be downregulated (red arrow), making all the chorismate available in the plastid only for CM1 and CM3. * Early repression in response - ** Later repression in response.

Here we describe how SoCM2 can influence the lignification process in sugarcane. The CM2 decrease will lead to the increase of CM1 and CM3 expression, increasing the production of aromatic amino acids essential in the lignification process.

CONCLUSIONS

The early repression of *SoCM2* gene appears to be responsible for the RB867515 sugarcane cultivar tolerance capacity to *Xanthomonas albilineans* infection.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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