DETECTION OF CASSIICOLIN-ENCODING GENES IN *CORYNESPORA CASSIICOLA* ISOLATES FROM COTTON AND SOYBEAN

M.N. Rondon
N. Xiang
J. Koebernick
K.S. Lawrence
Auburn University
Auburn, AL

Abstract

Corynespora cassiicola is a fungal pathogen with increasing importance across cotton and soybean producing countries and is responsible for target spot disease in these crops. A small protein of 27 amino acids named cassiicolin produced by *C. cassiicola* isolates has been reported as an essential effector for the pathogenicity. Currently, information is lacking on the genetic interaction between the pathogen *C. cassiicola* and cotton and soybean. This lack of information makes development of resistant cultivars by breeding programs difficult. The goal of this project is to detect the cassiicolin-encoding genes (*Cas1* to *Cas6*) from *C. cassiicola* isolates from cotton and soybean in Alabama. Cotton and soybean leaf samples were collected from different locations of Alabama. Fungal isolates obtained from the samples were identified by morphological characters and subjected to DNA extraction. All 85 isolates obtained were submitted to polymerase chain reaction with specific primers covering *Cas* sequences for known gene detection. Among the 85 *C. cassiicola* isolates of our collection, we found four different profiles of clusters for cotton and soybean based on cassiicolin-encoding genes. We found isolates collected from cotton with the absence of cassiicolin-encoding genes (Cas0) and Cas2. For isolates from soybean, four different gene combinations, Cas0, Cas2, Cas6 and Cas2+6 were found. All fragments amplified were approximately 750 bp long. Cas2 was the dominant gene regardless of the crop. Higher diversity of Cas genotypes was found in isolates sampled from soybean. Results generated from this project will be useful for further studies of *C. cassiicola* as a pathogen in cotton and soybean. In addition, knowledge about pathogen diversity is fundamental to select isolates in a breeding program to screen for resistant cultivars.

Introduction

Corynespora cassiicola (Berk. & Curt.) C.T. Wei is a widespread plant pathogenic fungus that generates necrotic spots on plant leaves, stems, roots, flowers, and fruits. As a cosmopolitan pathogen, *C. cassiicola* has been reported from over 70 countries on more than 700 plant species including fruits, vegetables, grains, perennial crops, and various forest and ornamental plants (Farr & Rossman, 2017). Symptoms on the cotyledonary leaves appear as small circular spots. The foliar phase of the disease is characterized as small circular spots, varying between 2 mm and 10 mm. The well-developed lesions are necrotic and show typical "target spot" symptoms, with some depression at the center of the lesion. In severe cases of infection, the lesions coalesce and the leaves show severe necrosis followed by complete premature senescence and death of the leaf (Galbieri et al., 2014).

Onerirosan et al. (1975) first reported a toxin produced by highly pathogenic *C. cassiicola* isolates producing symptoms on susceptible cultivars of tomato; however, expression was not seen in resistant tomato cultivars. Déon et al. (2012) demonstrated that differences existed between aggressive and moderately aggressive isolates in their levels of a putative effector protein, cassiicolin. Further studies by Déon et al. (2014) found variation in the cassiicolin gene for several isolates of *C. cassiicola* possibly related to host range. The cassiicolin gene was expressed in the early phase of infection and six cassiicolin isoforms (Cas1, Cas2, Cas3, Cas4, Cas5 and Cas6) were identified by PCR. Some of the isolates from cotton had a combination of two Cas gene (Cas2+Cas6) and others with no detectable Cas gene.

As target spot becomes more relevant as a plant disease due to its increasing occurrence on several high economic value crops, it is important to improve understanding about its pathogen, *Corynespora cassiicola*, and its toxin, cassiicolin. With knowledge about genetic diversity, isolates from soybean and cotton could be separated and used in breeding programs for screening lineages. The main goal of this project is to detect the cassiicolin-encoding genes (*Cas1* to *Cas6*) from *C. cassiicola* isolates from cotton and soybean in Alabama.

Materials and Methods

Isolates

Isolates of *C. cassiicola* were recovered from cotton and soybean leaves showing typical target leaf spot symptoms collected at seven different locations in Alabama. Direct isolation was used and small amounts of mycelia and conidia on the surface of the lesions were transferred onto potato dextrose agar (PDA; DIFCO Laboratories) enriched with 0.1% lactic acid (Fisher Scientific, Pittsburgh, PA). Plastic bags were used to induce fungal sporulation when it was not already present. Plates were incubated at room temperature and pure cultures were transferred to PDA plates. Morphological characters were used to identify the fungi (Ellis, 1971) and pure cultures were stored at 4˚C on PDA slants.

DNA extraction

Mycelial plugs of each isolate were placed over a cellophane membrane onto a fresh APDA plate. After 10 days of growth, DNA was extracted from each isolate using a ZR Fungal/Bacterial MiniPrepTM kit from Zymo Research (California, USA). Extracted DNA concentration was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and stored at -20˚C prior to use.

Detection of the cassiicolin-encoding genes

Primers encompassing available Cas sequences (Table 1) were used for polymerase chain reaction (PCR) amplification (Déon et al., 2014). PCR was performed on 20 ng/µl of *C. cassiicola* genomic DNA. A 50-µl reaction mix was prepared for each isolate containing 2 µl of each purified DNA sample, 1 µl of forward primers, 1 µl of reverse primers, 21 µl of deionized water, and 25 µl of JumpStart *Taq* ReadyMix (Sigma-Aldrich, St. Louis, MO, USA). Reactions (50 µl) were pipetted into 8-tube strips, and PCR was conducted. DNA amplification was conducted in a MultiGene DNA thermal cycler (Labnet International; Edison, NJ) with a program consisting of initial denaturation for 5 min at 94˚C; followed by 35 cycles of 1 min at 94˚C, 1 min at 55˚C, and 2 min at 72˚C; and a final cycle of 5 min at 72˚C. Amplification products were visualized under UV light on 1.5% agarose gels stained with GelRed 10,000X in DMSO (Biotium, Inc., Hayward, CA) to detect the presence of the cassiicolin-encoding genes. A 1 kb DNA ladder (Biolabs, California, USA) was used as the marker and water, without DNA extract, was used as the negative control.

Table 1. Description of the primers used to identify cassiicolin-encoding genes.

Target gene	Sequences (5'…3')	Primer name
Cas1	CCCAAGATACATGTTTTGAATGT	CasF18
	CCACACAAAGCAAGATACAGAATGAGC	CasR27
Cas2	GGATTTGCCTGAGATCCTA	CasF17
	CAAACAATGCTAACCAAACAAAC	CasR24
Cas3, Cas4	GTCGGCTAACTTGGGAAAAACTCT	CasF20
	GCAGGAAGCAAAACACAGAACAAG	CasR28
Cas5	CGGGGAGGTATCAGGTGTGAGATA	CasF19
	CAGAACAAGCCAAAAGAGAACTAC	CasR26
Cas6	GCTTGATTTGCCTGTGAGATACT	CasF16
	AAAACGATGCTAAACAAAAGGA	CasR25

Results and Discussion

Eighty-five cotton and soybean *C. cassiicola* isolates fell into different profile clusters for cassiicolin-encoding genes. Only two clusters of isolates from cotton were found, while four clusters of isolates from soybean were identified. There were seven cotton isolates from which no cassiicolin-encoding genes were detected (Cas0) and 39 isolates with Cas2. We obtained *C. cassiicola* isolates from soybean with the absence of cassiicolin-encoding genes (Cas0), Cas2, Cas6, and Cas2+6 (Table 2). The two cassiicolin genes (Cas2+6) were found together for 12 isolates sampled from soybean only and not for isolates sampled from cotton as previously reported by Déon et al. (2014).

Of 46 cotton isolates and 39 soybean isolates assayed, 39 (84.8%) cotton and 19 (48.7%) soybean isolates were positive for the Cas2 by PCR detection, therefore Cas2 was the dominant gene in our samples. Three *C. cassiicola* isolates from soybean were positive for Cas6 gene alone, while Déon et al. (2014) did not find any isolate with Cas6

alone even assessing 70 isolates from most of the *C. cassiicola* host plants. Previous work found no genetic diversity in *C. cassiicola* isolates from cotton sampled in the southeastern U.S. and low diversity for isolates from soybean (Sumabat et al., 2016).

Table 2. *Corynespora cassiicola* isolates from cotton and soybean with the corresponded target gene.

Target gene	*Corynespora cassiicola* isolates		Total
	Cotton	Soybean	
Cas0	7	5	**12**
Cas2	39	19	**58**
Cas6	-	3	**3**
Cas2+6	-	12	**12**
Total	**46**	**39**	**85**

The PCR products obtained from genomic DNA of the *C. cassiicola* isolates from cotton (HSV 01, FHP 22, BRW 03, EVS 01, HSV 12 and FHP 01) and soybean (LIM 02, PBU 06, LIM 14, PBU 07, LIM 13 and PBU 04) used for illustration here represent 6 isolates from each host (Figure 1). The primers covering the Cas sequences were able to amplify fragments around 750 bases of pairs on agarose gels. No amplification product was obtained from the negative controls, where water was used instead of DNA. Between the five gene variants encoding cassiicolin isoforms, just two genes (Cas2 and Cas6) were amplified in our *C. cassiicola* isolates.

The diversity of the cited genes with different forms and combinations of the cassiicolin toxin found for cotton and soybean isolates could explain some symptom differentiation. Further work assessing the pathogenicity, the cultivar preferences, and a comparison of disease caused by isolates with different cassiicolin toxin genes on a range of cultivars is required.

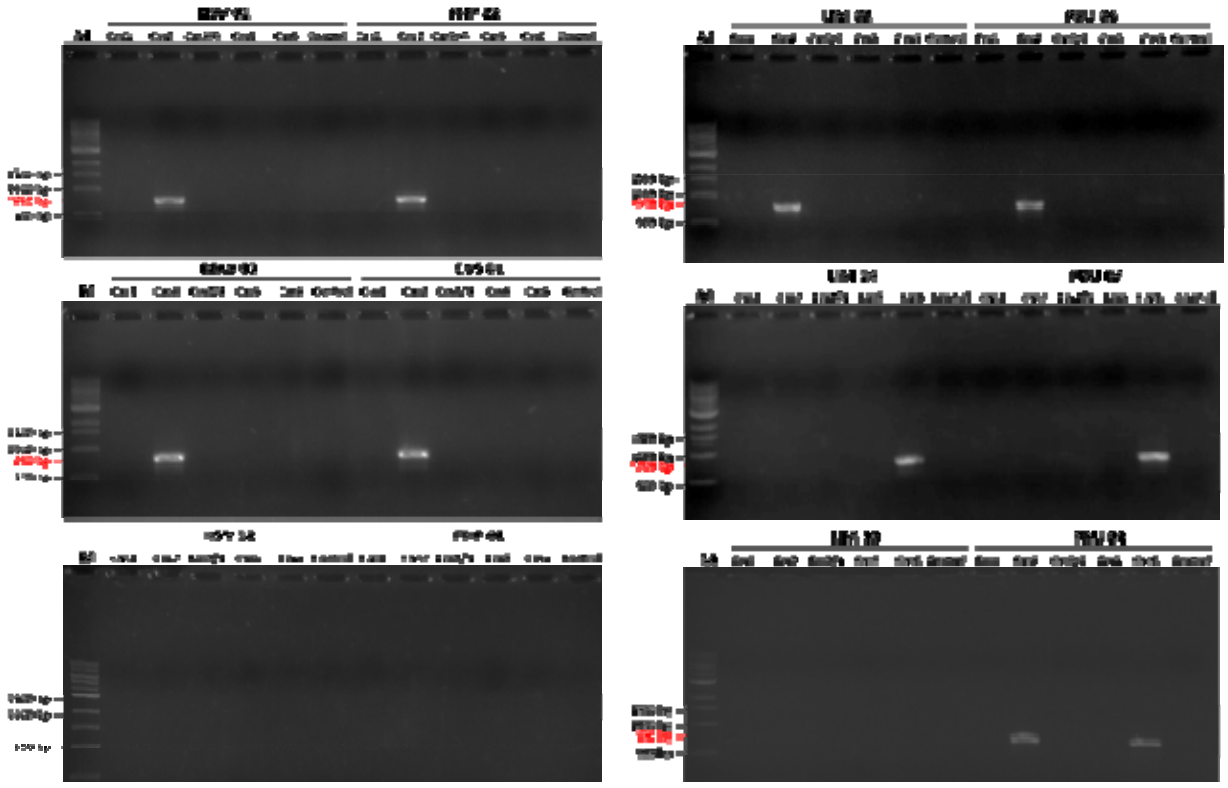

Figure 1. Detection of cassiicolin-encoding genes in *Corynespora cassiicola* isolates from cotton, at the left; and soybean at the right. M, molecular weight marker; Control, water (without DNA extract).

Summary

We characterized cassiicolin-encoding gene diversity of *C. cassiicola* isolates collected in Alabama U.S. Cas gene diversity was higher for isolates from soybean compared to those from cotton. Additional sampling of isolates from cotton may reveal higher diversity. Twelve *C. cassiicola* isolates from soybean have co-existence of different Cas genes (Cas2+6). Our findings may help to develop ways to assess susceptibile and resistant cultivars to *C. cassiicola* in a breeding program by testing genetically distinct isolates.

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References

Déon, M., Bourre, Y., Gimenez, S., Berger, A., Bieysse, D., De Lamotte, F., Poncet, J., Roussel, V., Bonnot, F., Olivier, G., Franchel, J., Seguin, M., Leroy, T., Roeckel-Drevet, P., Pujade-Renaud, V. 2012. Characterization of a cassiicolin-encoding gene from *Corynespora cassiicola*, pathogen of rubber tree (*Hevea brasiliensis*). Plant Science, 185, 227-237. https://doi.org/10.1016/j.plantsci.2011.10.017

Déon, M., Fumanal, B., Gimenez, S., Bieysse, D., Oliveira, R.R., Shuib, S.S., Breton, F., Elumalai, S., Vida, J.B., Seguin, M., Leroy, T., Roeckel-Drevet, P., Pujade-Renaud, V. 2014. Diversity of the cassiicolin gene in *Corynespora cassiicola* and relation with the pathogenicity in *Hevea brasiliensis*. Fungal Biology, 118 (1), 32-47. https://doi.org/10.1016/j.funbio.2013.10.011

Ellis, M.B. 1971. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute: Kew, Surrey, UK.

Farr, D.F., & Rossman, A.Y. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Retrieved October 1, 2017, from https://nt.ars-grin.gov/fungaldatabases/

Galbieri, R., Araújo, D.C.E.B., Kobayasti, L., Girotto, L., Matos, J.N., Marangoni, M., Almeida, W.P., Mehta, Y.R. 2014. Corynespora leaf blight of cotton in Brazil and its management. American Journal of Plant Sciences, 5, 3805-3811. http://dx.doi.org/10.4236/ajps.2014.526398

Onesirosan, P.T., Mabuni, C.T., Durbin, R.D., Morin, R.B., Righ, D.H., and Arny, D.C. 1975. Toxin production of *Corynespora cassiicola*. Physiological Plant Pathology, 5, 289-295.

Sumabat, L.G., Kemerait R.C., Brewer, M.T. 2017. Host-specialized populations of *Corynespora cassiicola* causing emerging target spot epidemics in the southeastern U.S. (Abstr.). Phytopathology 107:S31. http://dxdoiorg/101094/PHYTO-107-4-S31.