Characterization of Type Three Effector Genes of *A. citrulli*, the Causal Agent of Bacterial Fruit Blotch of Cucurbits

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Abstract

Bacterial fruit blotch (BFB) of cucurbits is caused by *Acidovorax citrulli,* a Gram-negative seedborne bacterium that can cause up to 100% fruit yield losses in the field. Currently, BFB is a major problem for the cucurbits industry worldwide. Thus far, attempts to identify resistance in cucurbit germplasm for controlling BFB have been unsuccessful. Despite the importance of the disease, little is known about the molecular mechanisms of *A. citrulli* pathogenicity, due to a lack of molecular tools for studying the *A. citrulli*/cucurbit interaction. The genomic sequence of *A. citrulli* strain AAC00-1 has been determined, and the components of type III secretion system have been identified. The goal of this research was to develop molecular tools for studying the BFB disease. Nineteen putative type III effector genes were cloned from two representative *A. citrulli* strains (AAC00-1 and M6). The distribution of 19 type III effectors among *A. citrulli* strains, collected worldwide, was studied. A novel Gateway-compatible binary vector was developed for transient expression of *A. citrulli* type III effectors genes *in planta*. A set of modified vectors for marker-exchange mutagenesis in *A. citrulli* were constructed. The model plant species *Nicotiana benthamiana* was found to be susceptible to *A. citrulli*, while *Nicotiana tabacum* was resistance to *A. citrulli*, so therefore could carry nonhost resistance genes. Two T3S effectors, Aave1548 and Aave2166, triggered water soaking-like cell death in *N. benthamiana,* but HR-like cell death in *N. tabacum*. Bacterial mutagenesis and *in planta* disease assay confirmed that both Aave1548 and Aave2166 have significant virulence

contributions to *A. citrulli* in *N. benthamiana* plant and melon seeds. *Aave2166* encodes a putative acetyltransferase that belongs to the YopJ super family, which is conserved in both animal and plant pathogenic bacteria. Wild type but not the putative catalytic mutant (C232A) of Aave2166 can trigger cell death phenotype in *N. benthamiana* and *N. tabacum*. *N. benthamiana* yeast two-hybrid cDNA library screening using Aave2166 identified six *N. benthamiana* proteins/peptides which specifically interacted with Aave2166. Further characterization of these Aave2166 interactors may allow us to understand the virulence mechanism provided by Aave2166. The identification of nonhost resistance genes that can recognize Aave2166 and other type III effectors may help to develop novel strategies to control BFB disease of cucurbit.

Keywords: *A. citrulli*, T3S effectors, marker-exchange mutagenesis, *Nicotiana benthamiana*, *Nicotiana tabacum*, plant immunity

Dedication

To My Mother, Awa Ouedraogo-Traore

I would like to dedicate this dissertation to my beloved late mother, Awa Ouedraogo-Traore, who unfortunately didn't get the chance to see this day. I remembered the last words I had with my mother, she said: "Sy, I know your passion for higher education, and I also know that you want to be somebody so you can take care of me. Please don't think because I'm not alive anymore, and give up." She continued, "even if I'm not in this world anymore, if you become successful in life people will still remember me through you." During my PhD journey, these words were my inspiration and will continue to be throughout my life.

Love and miss you dearly mom!

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Table of Contents

List of Tables

Chapter 3

Chapter 4

Chapter 5

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 1: Review Paper on *A. citrulli*/Cucurbit Pathosystem

Introduction

Cucurbitaceae is one of the largest crop families with more than 800 species in 130 genera, including the important vegetables watermelon, melon, pumpkin, cucumber; squash (Robinson and Decker-Walters, 1997). The cucurbitaceae family (cucurbits) members are classified mainly based on their vine-like structures and their special fruit called a pepo, derived from an inferior ovary. Cucurbits are grown worldwide and play an important role in human consumption. Cucurbits fruits have many uses and are made into salads, cakes, breads, fruit juices, and wines, some of the species also have medicinal value (Shrivastava & Roy, 2013). Like many other crop plants, the stable production of cucurbits is threatened by various diseases, caused by microorganisms such as bacteria, fungi, oomycetes, and viruses (Celetti & Roddy, 2010; Simmons, Holmes, & Stephenson, 2008). Bacterial fruit blotch (BFB) caused by *Acidovorax citrulli,* is one of the most devastating cucurbit diseases that is currently lacking an effective control strategy.

BFB was first identified in 1968 at the USDA station in Georgia, USA (N.W. Schaad, Sowell Jr, Goth, Colwell, & Webb, 1978; Wall & Santos, 1988). However, the first severe BFB outbreak did not occur until 1987 and 1989 in Georgia, Mariana Islands, South Carolina, North Carolina, Florida (Jacobs, Damicone, & McCraw, 1992; N. Schaad et al., 2003; Somodi et al., 1991). Recently, serious economic losses caused by BFB in watermelon and in other cultivated cucurbits, such as melon, cucumber, squash and pumpkin, have been reported elsewhere, including other regions of the US, Nicaragua, Costa Rica, Brazil, Australia, Japan, Korea, Thailand, Taiwan, China, Turkey, Hungary and Israel (S. Burdman, Kots, Kritzman, &

Kopelowitz, 2005; Walcott, Fessehaie, & Castro, 2004). During the first outbreak, hundreds of acres of watermelons were infected causing a tremendous loss to farmers as well seed companies. It is very hard to estimate the exact economic losses caused by BFB; however, it was estimated that BFB accounts for a 50-90% loss of the cucurbit fruit crop in the world market annually (Bahar O. & S., 2010). Today, BFB is still a big concern to farmers and seed companies because strategies for managing BFB are limited, and no commercial resistant cultivars have been developed (Bahar & Burdman, 2010). The development of a sustainable control strategy against BFB requires the deep understanding of the *A. citrulli* /cucurbit pathosystem. Thus far, the process of how *A. citrulli* colonizes a host and the pathogenesis mechanism of *A. citrulli* are poorly understood. In this chapter I will review, the *A. citrulli*/cucurbit pathosystem, the existing strategies to control BFB, and future perspectives on how to develop a sustainable control strategy against BFB.

Symptoms of BFB

The peculiarity of BFB disease is its resemblance to other necrotic or dried leaves symptoms making it hard to distinguish infected plants from normal plant stress (Latin & Hopkins, 1995). Nevertheless, unique symptoms occur at different developmental stages. Latin et al. (1995) described BFB symptoms in different parts of the watermelon plant (Fig. 1). The first symptoms in watermelon seedlings appear as dark, water-soaking areas on the lower surface of cotyledons and leaves followed by necrotic lesions, which may have chlorotic halos. In young seedlings, lesions develop on the hypocotyl, which collapses killing the plant (Fig. 1G, H). Foliar symptoms develop throughout the growing season. Leaf lesions are light to reddish-brown in color and often spread along the midrib of the leaf (Fig. 1 I and F). Under field conditions, the leave lesions usually do not cause defoliation, but are the main reservoirs of bacteria for fruit

infection (Fig. 1 F). Symptoms on the fruit surface begin as small, greasy appearing, water-soaked areas that are only a few millimeters in diameter (Fig. 1 A). The infected areas spread rapidly to form dark, green, water-soaked lesions several centimeters in diameter with irregular margins (Fig.1 B). Within a few days, the lesions could rapidly expand to cover the entire upper surface of the fruit, leaving only the ground spot symptomless. In the fruit of watermelon cultivars with dark green striped rinds, the water-soaking may be more restricted, resulting in smaller lesions on the lighter green stripes. Under certain environmental conditions and with infection of very young fruit, symptoms may not include the typical water-soaking symptoms. In young fruits, the lesions do not extend into the flesh of the melon (Fig. 1 D). With age, the center of the lesions may turn brown and crack (Fig. 1 C), and a total fruit soft rot may follow as white bacterial ooze or an effervescent exudate occurs during fruit decay (Fig. 1 E).

BFB Life Cycle

The developmental cycle of BFB was firstly described by Latin and Hopkins (1995) as illustrated in Figure 2. The nine key steps of the developmental cycle of BFB are: (1) contaminated seeds are usually the source of *A. citrulli*, (2) bacteria from the contaminated seeds will infest the developing seedlings as the cotyledons emerge from the seed coats in greenhouses or transplant facilities. The warm and humid greenhouse condition will facilitate the establishment of the *A. citrulli* on the transplants. (3) Irrigation systems used for transplant production disperse the pathogens onto the non-contaminated seedlings, (4) the contaminated seedlings are transplanted, (5) further development of infected plants occurs in the field where the pathogen can easily spread to newly emerging leaves. Lesions on infected leaves now serve as a source for further infection, (6) infected fruits develop symptoms before ripening and produce new contaminated seeds. (7) Escaped contaminated seeds from rotted fruits become

volunteer watermelon seedlings the following season (8) contaminated volunteer seedlings serve as the source of new inoculum. (9) Contaminated cucurbit weeds, volunteer plants, and new transplants serve as a source of contamination during the following season. However, the magnitude of this kind of infection is much lower than that derived from infested seeds.

Strategies to Control BFB Disease

Despite the importance of the BFB, little is known about the pathogenesis of *A. citrulli* and this lack of information limits the development of strategies for controlling BFB disease. The ideal control is to prevent the introduction of *A. citrulli* bacteria into a field, which is almost impossible (Hopkins & Thompson, 2002) since the fruit blotch bacterium may be introduced into a field by infested seeds, infected transplants, contaminated volunteer watermelons, or infected wild cucurbits (see above). Diseased transplants can cause severe problems because the bacterium may spread in the transplant house and result in high numbers of infected plants established in a field.

Chemical control. Several different methods have been tried to control BFB (Latin, 2000). Chemical control measures have been unsuccessful because *A. citrulli* is seed-transmitted (Hopkins, Thompson, Hilgren, & Lovic, 2003; Rane & Latin, 1992). Applications of copper-containing fungicides prior to fruit set reduce the incidence of fruit blotch symptoms. A minimum of two to three copper applications with thorough foliage coverage is essential for good control (Cheng, Hsu, Huang, & Wang, 2000). Applications should begin before flowering, or earlier, and continue until all fruits are mature. For control of other foliar diseases, copper-containing fungicides should be used in combination with a broad-spectrum fungicide, if labels permit such usage. However, early identification of BFB in the field is difficult because it resembles other diseases (Hopkins, 1991a).

Crop rotation. Crop rotation is an efficient method to control many diseases, but has been unsuccessful against BFB. For crop rotation to be effective against BFB, volunteer watermelon seedlings from previous crops must be destroyed as well as wild cucurbit plants both in production fields but also near transplant houses. Decontamination of irrigation or mechanical equipment before moving it from an infested field to a non-infested field must be practiced in conjunction with crop rotation (Hopkins et al., 1992).

Biological control. The use of biological agents to control BFB has been also explored even though this strategy is far away from field application. Potential biological control measures have been identified for use against BFB. Some strains of the bacterium, such as AAA-92 (*Acidovorax avenae avenae* a maize pathogen) (Fessehaie & Walcott, 2005) and the Aac/TTSS mutants, suppress BFB transmission in seed transmission assays. However, the mechanism by which these strains suppress *A. citrulli* growth is poorly understood. It is well established that biological control of plant diseases involves effective competition for nutrients and colonization sites, production of antimicrobial compounds, and induction of plant resistance (Bloemberg & Lugtenberg, 2001; Compant, Duffy, Nowak, Clément, & Barka, 2005). The Aac/TTSS mutant is an *A. citrulli* mutant without the needle-like structure that the pathogen uses to inject putative effector proteins into host cells. The effector proteins also interfere with PTI, the basal defense system used by plant cells, to suppress resistance (Alfano & Collmer, 2004). The *A. citrulli*/TTSS mutant (Fessehaie & Walcott, 2005) might compete with the wild type *Aac* for colonization sites or its presence could activate PTI; therefore, suppressing the establishment of the wild type *A. citrulli*. The TTSS mutant has been suggested as a biological control agent but is considered to be a genetically modified organism (GMO). The release of such organisms in the

ecosystem to compete against other pathogens may carry risks and needs to be further evaluated before it can be used to control the disease in the field.

Seed treatments. BFB is seed borne and transmitted so fermentation and chemical treatments have been tried. For fermentation, seeds are incubated in their fruit juice for 24-48h, after harvest then rinsed and dried. Hopkins *et al.* (2002) showed that fermentation could completely eliminated BFB seedling transmission without affecting germination (Hopkins & Thompson, 2002). However, Walcott, et al. (2003) demonstrated that fermentation could reduce germination performance (Walcott, Gitaitis, & Castro, 2003).

Many seed treatments have been reported to reduce BFB seedling transmission including treatments with 0.5-1% CaOCl, or NaOCl (Hopkins, 1991b; Hopkins et al., 2003), peroxyacetic acid (Hopkins et al., 2003) cupric sulfate, acidic electrolyzed water (AEW) (Feng, Li, Randhawa, Bonde, & Schaad, 2009). These chemical treatments can reduce the severity of BFB; however, failed to completely eliminate the pathogen from the seeds or if they did eradicate the pathogen, seed germination was negatively affected. Only AEW eradicated *A. citrulli* from infested seeds without decreasing seed germination or seedling establishment (Feng, Li, et al., 2009).

Exclusion. Seed health testing is the most widely used method for excluding *A. citrulli*. The most widely used seed health assay for BFB is the "seedling growing out" assay that includes growing out 10,000 – 50,000 seeds under greenhouse conditions and observing the seedlings for BFB symptoms followed by subsequent laboratory assays such as serological tests, semi-selective media, and polymerase chain reaction (PCR) assays to confirm the identity of bacteria recovered from seedlings. However, the method is time consuming, expensive and requires adequate facilities and qualified technicians. Moreover, Walcott *et al*., (2006) reported

that the "seedling grow out" assay could detect only 12.5% and 37.5% of seed lots infected when infestation is as low as 0.01% and 0.1%, respectively (Walcott, Castro, Fessehaie, & Ling, 2006).

Many studies have investigated the improvement of the efficiency of seed health testing. Immunomagnetic separation combined with polymerase chain reaction (IMS-PCR) was explored (Walcott & Gitaitis, 2000; Walcott et al., 2006). This approach was necessary because it increased the detection frequency of 25% and 87.5% for 0.01% and 0.1% infestation; therefore, IMS-PCR is an effective alternative for seed health testing.

The Causal Agent of BFB

The causal agent of BFB is a bacterium called *Acidovorax avenae citrulli* (*Aac*) formerly known as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (N.W. Schaad et al., 1978; Willems et al., 1992). *Aac* is a rod shaped Gram-negative bacterium with one flagellum; grows well at high temperature (42°C) and high humidity. *Aac* is a seed-born and seed-transmitted disease. Research studies about Aac can be divided in two main periods: the first period covers the discovery of the disease (1968) until the first outbreak. During that period not much molecular work was done. The studies that were done focused on the description of the pathogen, as well as the symptoms of BFB and the life cycle of the disease (Hopkins et al., 1992; Hopkins & Schenck, 1972; Latin & Hopkins, 1995; Willems et al., 1992).

The second period starts from the first outbreak until today. Until the first outbreak, Aac was known as *Pseudomonas pseudoalcaligenes* subsp. *citrulli.* Molecular analyses of the pathogen have allowed changing the taxonomy of the causal agent of BFB and the pathogen formerly known as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* was reclassified as *Acidovorax avenae citrulli* (*Aac*) (N.W. Schaad et al., 1978; Willems et al., 1992). Just after the first outbreak, research studies focused mainly on the development of techniques that could help

to detect easily and quickly *Aac* in infected cucurbit seeds, seedling and plants to avoid any further contamination and/or contaminated explants to cross the borders in countries where BFB outbreaks occurred. Then in countries such as Japan, Israel, US and Australia, different techniques such as semi-selective agar medium, serological methods, polymerase chain reaction and loop mediated isothermal amplification were developed to test seed health.

Other studies investigated the diversity of *Aac* as well as the hosts. In the study by Walcott et al. (2000), the assessment of the diversity among *Aac* strains revealed the existence of at least two well differentiated groups: group I strains (M6) mainly isolated from melon and pumpkin and group II (AAC001) mainly represented by the typical watermelon BFB causing strains (Walcott, Langston Jr, Sanders Jr, & Gitaitis, 2000). Another study lead by Walcott (2004) demonstrated that the group I strains appear to be moderately aggressive on a wide range of cucurbits, while the group II strains appear to be highly aggressive on watermelon but only mildly on melon (Walcott et al., 2004).

The Genetic Potential of *A. citrulli* AAC00-1

Despite the economic importance of BFB, little is known about the basic aspects of the biology and pathogenesis of *A. citrulli*. The genome sequence of a group II strain, AAC00-1, was released in 2007 by the Joint Genome Institute (JGI; GenBankNC_008752), providing a great contribution to the investigation of basic aspects of BFB. The AAC00-1 genome comprises a single circular chromosome of about 5.3 Mb and putatively encodes 4858 genes. Some of these genes may be involved in the virulence of *A. citrulli*, including the specialized secretion systems of pathogenic bacteria, toxins, plant hormones, bacterial surface attachment factors, flagella and etc. Below, we provide an overview of the current knowledge of *A. citrulli* pathogenesis-related

genes as well as the computational screening of AAC00-1 leading to the identification of useful genes that could be involved in the pathogenicity of *A. citrulli*.

Protein secretion systems. The genome of AAC00-1 contains genes that encode type I, II, III, IV, V and VI and twin-arginine transporter (Tat) secretion systems. Among these secretion systems only type II and III have been shown to be important in terms of pathogenicity.

The Tat system. The twin-arginine transporter system is well conserved among Gram-negative bacteria and plays the role of transporting folded proteins to the periplasmic space (Palmer & Berks, 2012). Defects in the Tat system in pathogenic bacteria such as *Pseudomonas syringae* pathovar *tomato (Pst)* DC300 was shown to be attenuated in fitness as well as virulence in *Arabidopsis thaliana* and tomato plants. Furthermore, this mutant has a reduced ability to elicit HR in tobacco plants. The Tat secretion system in *Pst* DC3000 is predicted to transport proteins, among which are at least 5 that have been confirmed to be virulence factors including phospholipase, amidase and amino-transpherase (Bronstein, Marrichi, Cartinhour, Schneider, & DeLisa, 2005; Caldelari, Mann, Crooks, & Palmer, 2006). The genome sequence of AAC00-1 has revealed the presence of the Tat secretion system. However, no functional studies have demonstrated the importance of the Tat secretion system in the pathogenicity of *A. citrulli*.

Type II secretion. In many Gram-negative bacteria, the type II secretion (T2S) system is responsible for the translocation of several pathogenesis-related proteins from the bacterial cytoplasm to the extracellular environment (Douzi, Filloux, & Voulhoux, 2012). T2S proteins include toxins and hydrolytic enzymes, such as phospholipases, cellulases, pectate lyases and proteases, and have been shown to be virulence factors in plant pathogenic bacteria, including *Pst* DC3000 (Bronstein et al., 2005; Joardar et al., 2005), Xanthomonas *oryzae* pv. *oryzae*, *X.*

campestris pv. *vesicatoria* and *R. solanacearum* (Hu, Qian, & He, 2007; H. Liu, Zhang, Schell, & Denny, 2005; Robert Szczesny et al., 2010).

The T2S system is conserved and generally comprises 12–16 proteins that form a complex (secreton) that spans the bacterial envelope. The subunits of the secreton are encoded by general secretion pathway (gsp) genes that are often organized into large operons. There are three major components of the secreton: an inner membrane platform, a piston-like pseudopilus and an outer membrane secretin. The inner membrane platform comprises the proteins Gsp C, F, L, M and E. The pseudopilus comprises five pseudopilin proteins, GspG, H, I, J and K, (Hansen & Forest, 2006; Nunn, 1999). The final component of the secreton, the outer membrane secretin, is encoded primarily by *gspD* and forms a pore through which proteins are translocated. A complete secreton operon includes *gspC, D, E, F, G, H, I, J, K, L, M, O* and, in some cases, *gspA/B* and *gspN/S* (Douzi et al., 2012). Previous studies have shown that bacterial species, such as *Pseudomonas aeruginosa*, *R. solanacearum* and *X. campestris*, have multiple operons encoding the secreton (Cianciotto, 2005). *A. citrulli* AAC00-1 possesses two sets of T2S gene clusters. One cluster, *gsp1*, lacks *gspA, S, B* and *N*, whereas the second cluster, *gsp2*, lacks *gsp A, B, S* and *F*. *Pst* DC3000 mutant in T2S components *gspD* and *gspE*, displayed reduced chlorotic cell death in *A. thaliana* plants. Deletion of both copies of *gspG* in *A. citrulli* AAC00-1 resulted in the loss of the ability to secrete endoglucanase (Johnson, 2010). Furthermore, the *gspG* mutant displayed a significant reduction in ability to colonize watermelon seedling cotyledons relative to the wild-type strain, suggesting that T2S enzymes may be virulence factors. Using a T3S system AAC00-1 mutant, Johnson, Minsavage, Jones, & Walcott (2011) demonstrated that in watermelon seed colonization and BFB seedling transmission assays, the *gspG1/G2* deletion (T2S) mutant of AAC00-1 displayed a significant reduction in seed colonization 96 h after

planting, and a significant reduction in seed-to-seedling BFB transmission (Johnson, 2010; Johnson et al., 2011).

If the contribution of the T2SS to virulence of AAC00-1 has been demonstrated; however, the functional analysis of the putative enzymes has not been investigated. Genomic analysis of AAC00-1 revealed the presence of putative cell wall-degrading enzymes, endoglucanase, xylanase, pectate lyase and phospholipases that could be secreted through the T2SS. Future studies need to focus on the identification of the T2S proteins involved in pathogenicity of *A. citrulli*.

Role of type 2 secretion in BFB seedling transmission

AAC00-1 T2SS genes		
Function	**Cluster 1**	**Cluster 2**
ATPase in the inner membrane	gspE	
Outer membrane pore	gspD	gspD
Interconnector between outer and inner membrane	gspN	Putative gspN or gspC
Platform on which pseudopilus is built	gspM	gspM
Platform on which pseudopilus is built	gspL	Putative gspL
Psuedopilus, inclusion may arrest elongation	gspK	gspK
Psuedopilus	gspJ	gspJ
Psuedopilus	gspI	gspI
Psuedopilus	gspH	gspH
Psuedopilus	gspG	gspG
Interconnector between outer and inner membrane	gspC	gspF
		gspE
absent	gspA,B,F,S	gspA,S,B, C?, N?

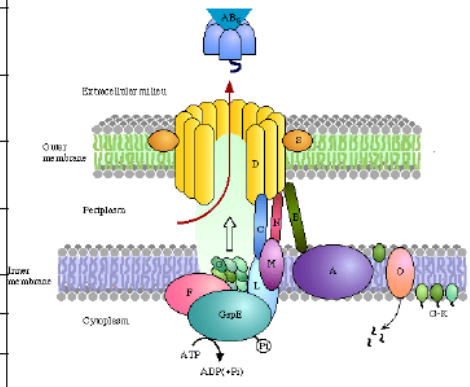

Figure 1: AAC00-1 T2SS genes; Burdman, S., & Walcott, R. (2012). *Acidovorax citrulli*: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. *Molecular plant pathology*, *13*(8), 805- 815 (used under fair use 2014).

Type IV secretion system. The type IV secretion system (T4SS) is one of several types of secretion systems, which microorganisms use for the transport of macromolecules such as proteins and DNA across the cell envelope (Rego, Chandran, & Waksman, 2010). It is the most versatile family of secretion systems, mediating transport of monomeric proteins as well as multi-subunit protein toxins and nucleoprotein complexes, and has been found in both Gram-positive and Gram-negative bacteria as well as in some archaea. There are broadly three functional types of T4SSs (Alvarez-Martinez & Christie, 2009). The first type, found in many Gram-positive and Gram-negative bacteria and some archaea, is used for transferring DNA molecules from one cell to the other in a cell-to-cell contact-dependent manner. This event is called conjugation, increases the genomic plasticity, helping microorganisms to adapt to changes in their environment and is the major mechanism behind the spread of antibiotic resistance genes among pathogenic bacteria. Conjugative T4SSs give pathogenic bacteria the advantage to transfer/receive transmissible plasmids with selective genes including antibiotic resistance, virulence and other genes that enhance survival. One example of T4SS transferring DNA is that of *Agrobacterium tumefaciens*, a well-characterized system specialized in delivering oncogenic nucleoprotein complexes into plant cells. The second type of T4SSs mediates DNA uptake (transformation) and release from the extracellular milieu. The two so far characterized systems are the *Helicobacter pylori* ComB system, which takes up DNA from the extracellular milieu, and the *Neisseria gonorrhoeae* gonococcal genetic island (GGI), which secretes DNA to the extracellular milieu. The third type of T4SSs is used to transfer proteins. Most T4SSs in that category are found in pathogenic bacteria where they play important roles in virulence such as establishing pathogen–host interaction and/or transferring toxic effector proteins or protein

complexes into the cytoplasm of the host cell. Important human pathogens like *H. pylori*, *Bordetella pertussis, Legionella pneumophila*, *Brucella* spp. and *Bartonella* spp. rely on T4SSs for their pathogenicity. The T4SS encode by the Ti plasmid of *A. tumefaciens* and the pKM101 of *E. coli* contain 12 proteins VirB1-VirB11 and VirD4 (Cascales & Christie, 2003; Wallden, Rivera-Calzada, & Waksman, 2010). The genomic analysis of AAC00-1 has shown the presence of the T4SS where the *vir* genes are clustered in an operon called tbl operon. This operon lacks *vir* genes such as *vir1, 3, 5* and *7*. If conjugation as well as transformation analyses have shown evidence of the functionality AAC00-1 T4SS; however, no studies have shown evidences of the involvement of the T4SS in the pathogenicity and virulence of AAC00-1.

Polar flagellum. Flagella are found on the surface of many bacteria, where they mediate motility and are involved in various processes, such as adhesion to and colonization of biotic and abiotic surfaces, and virulence on both animal and plant hosts (Macnab, 2003; Moens & Vanderleyden, 1996). Bahar et al. (2011) showed in a virulence screens with the *A. citrulli* M6 transposon library that a *fliR* mutant displayed reduced virulence in seed transmission assays. *fliR* encodes a flagellar biosynthetic protein involved in flagellin secretion. Further generation and characterization of an M6 mutant impaired in *fliC*, encoding flagellin, confirmed the polar flagellum as a virulence factor of *A. citrulli* (Bahar, Levi, & Burdman, 2011). However, the recognition of the flagellin of *A. citrulli* by the pathogen recognition receptors (PRR) (Delphine Chinchilla et al., 2007; Gómez-Gómez & Boller, 2000) has not been addressed with the *A. citrulli* flagellum. Future studies should investigate the role of the flagellum in triggering PTI, the first layer of defense against pathogens.

Type III secretion. Most Gram-negative bacteria infecting plants and animals use the TTSS as machinery to inject effector proteins into host cells (Alfano & Collmer, 2004;

Bogdanove et al., 1996). Studies of susceptible hosts have shown that some of the T3S effectors contribute to the virulence of the pathogen by reprogramming the host cell and suppressing host defense responses. While in resistant hosts, some of the T3S effectors are recognized by their cognate R (resistance) genes (Alfano & Collmer, 2004). This recognition event elicits effector-triggered immunity (ETI), resulting in a localized programmed cell death of infected tissue suppressing the proliferation of the pathogen. As in many Gram-negative plant-pathogenic bacteria, the T3S system is required for pathogenicity and HR induction in susceptible and resistant plants, respectively. On the basis of gene organization, sequence analysis and regulation, *hrp* clusters are divided into two classes: class I contains the clusters of *Pseudomonas syringae* and enteric plant-pathogenic bacteria, whereas class II contains the *hrp* clusters of Xanthomonas species and *Ralstonia solanacearum* (Bogdanove et al., 1996; Büttner & Bonas, 2002). The genome sequence of the *A. citrulli* group II strain AAC00-1 revealed the existence of a Hrp-T3S system. On the basis of sequence analysis and cluster organization, the *A. citrulli* hrp cluster belongs to class II. The generation and characterization of hrp mutants of both group I and II strains have revealed that, in *A. citrulli*, a functional Hrp-T3S system is required for both pathogenicity on cucurbit hosts and HR-inducing ability on non-host tobacco and tomato (Bahar et al., 2011; K. Johnson et al., 2011). Annotation of the AAC00-1 genome has also revealed the presence of at least 11 putative T3S effectors that are homologous to known effectors from Xanthomonas species, *P. syringae* and *R. solanacearum*. However, groups I and II differ significantly from each other in the sequence of most effectors. Moreover, some effectors that are present in strain AAC00-1 and all assessed group II strains seem to be absent or nonfunctional in group I strains (N. Levi, T. Zimmermann, A. Castro-Sparks, J. Sikorski, B. Zhao, G. Welbaum, R. Walcott and S. Burdman, unpublished data). We hypothesize that differences in the arsenal of

T3S effectors are responsible, at least in part, for the observed differences in host preference among isolates from these groups. Further functional studies involving the contribution of the T3S effectors as well as their function should be performed. Moreover, using the effectors as molecular probes to identify Resistance R genes in host and non-host plants could be a new and sustainable approach to control the BFB disease.

The first outbreak of BFB affected mainly watermelon. Since then, the disease has been reported to cause damage to others cucurbits fruit crops such as melon, pumpkin, squash *etc*. Research groups around the world have also focused on the identification of cucurbit lines, with resistance to BFB. However, until today, no cucurbit lines have been found to have a useful level of resistance. Nevertheless, some lines have been found to have partial level of resistance and could be used in breeding research against BFB (Wechter, Levi, Ling, Kousik, & Block, 2011). However, it should be noted that the screening of cucurbits has not covered the 130 genera of cucurbits yet.

Problem Statement

Research studies on BFB disease have mainly concentrated on the description of the disease, its life cycle, and the assessment of *A. citrulli* strains as well as the range of hosts in cucurbit crops. Recently, molecular studies have also investigated genes that are essential for the establishment and the pathogenicity of BFB disease (Liu et al., 2011). For example the TTSS is crucial for the virulence of *A. citrulli* (Johnson et al., 2011). Even though progress has been made in trying to understand the pathogenicity of BFB, the genetic and biochemical mechanism by which *A. citrulli* is so successful in cucurbits remain elusive. Therefore, research studies should now focus on the identification of a model plant that could be used to study the pathogenicity of *A. citrulli*, the identification of a model plant resistant to BFB disease that could be used to

isolate one or more resistance genes, and the characterization and determination of the biological function of putative type three effector genes present in the *A. citrulli* genome. These will be helpful for designing a sustainable approach to control BFB disease.

Since the discovery of BFB disease, researchers have focused on *A. citrulli* -watermelon and *A. citrulli* -melon interaction. However, studies have shown the existence of two main groups (I&II) of *A. citrulli* with a difference in virulence in watermelon and melon as well. Others studies have also investigated the use of Cucumber as a model plant because it presents a reduced gap of virulence between group I and group II strains of *A. citrulli* compared to watermelon and melon. The ideal model plant will be the one which presents a small difference of virulence between group I and group II strain. Studies should investigate non-host model plants such as *Nicotiana* and *A. thaliana* to identify line (s) that could be used in the lab to study the pathogenicity of *A. citrulli* and findings could then be transferred to the host plants.

After the first BFB outbreak, strains isolated from Florida have shown hypersensitive response on non-host plants such as tobacco and tomato (Johnson et al., 2011). However, studies have shown that the pathogenicity of *A. citrulli* is TTSS-dependent. Until now no putative T3S-effector genes has been characterized in *A. citrulli* genome. Gram-negative pathogenic bacteria possess TTSS a needle as structure that the bacteria uses to inject in the plant cell proteins known as effector proteins (Alfano & Collmer, 2004). The first group of effector proteins are known primarily to interfere with the receptor, inhibit the recognition of the elicitors, or block the transmission of the signal (Zipfel & Rathjen, 2008). These effectors are localized to the plasma membrane of the plant cell. For example, AvrPto as well as AvrPtoB are effector proteins from *Pst* DC300 (Chinchilla et al., 2007; Mersmann et al., 2008). These effectors are known to block PTI by interfering with the PRRs FLS2 and BAK1 (Zhang et al., 2007). The second group of

effectors is known to interfere with the signal transduction signal components in the cytosol to block the signal to reach the nucleus. This group of effectors is usually localized to the cytosol of the susceptible host plants. Finally, the third group of effector proteins is composed of transcriptional activation like effectors (TAL). Studies have shown that TAL effectors activate plant genes that could help the colonization of the plant cell (Boch et al., 2009; Moscou & Bogdanove, 2009).

Eckshtain-Levi and colleagues (unpublished results) have shown that the *A. citrulli* genome carries eleven putative T3-effector genes. Transient expression of these putative genes in *N. benthamiana* has shown that three of these putative genes trigger a cell death-like phenotype (Traore and al. unpublished results). Further characterization as well as the identification of the biological function of these putative effector genes could be the turning point to understand *A. citrulli* pathogenicity and, therefore, help to design a system to control BFB. For example, TAL effectors have been found to bind to the promoter of the genes that they reprogram. Identifying a TAL effector in the pool of *A. citrulli*, putative effector genes and their interactors will be useful because cucurbit crops could be engineered with a known R gene fused to the promoter to which the TAL effector binds to. After colonization, the TAL effector from *A. citrulli* would activate the expression of the R gene and trigger the plant defense mechanism.

Molecular pathology has identified useful avirulent (*avr*) and resistance (*R*) genes that have been used to control many different diseases caused by pathogenic bacteria. These R genes have been transferred to cultivars through traditional breeding or gene transfer using molecular biology tools (genetic engineering) (Rommens, Salmeron, Oldroyd, & Staskawicz, 1995). The identification and characterization of the pool of the pool of putative type three effector genes

could help to identified *R* genes in the host and non-host plant; therefore, use their interaction to control BFB.

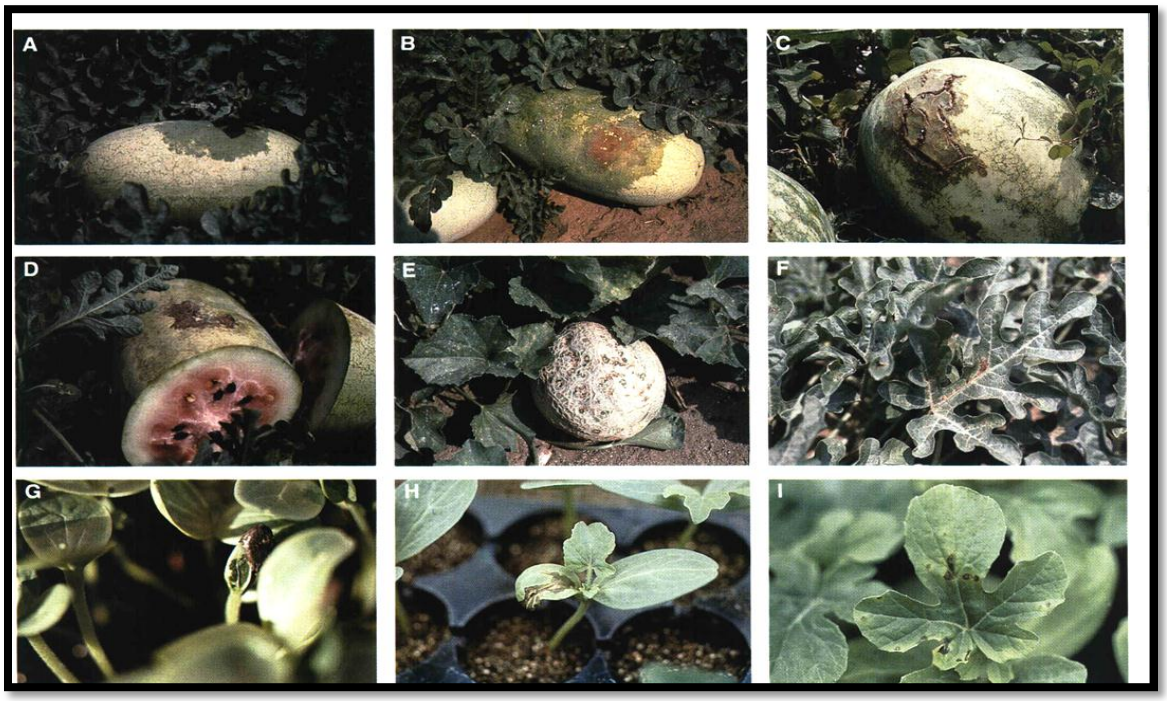

Figure 2: Symptoms of BFB on different organs of host plants; Latin, R., & Hopkins, D. (1995). Bacterial fruit blotch of watermelon. *Plant Disease, 79*(8), 761-765.(Used under fair use, 2014)

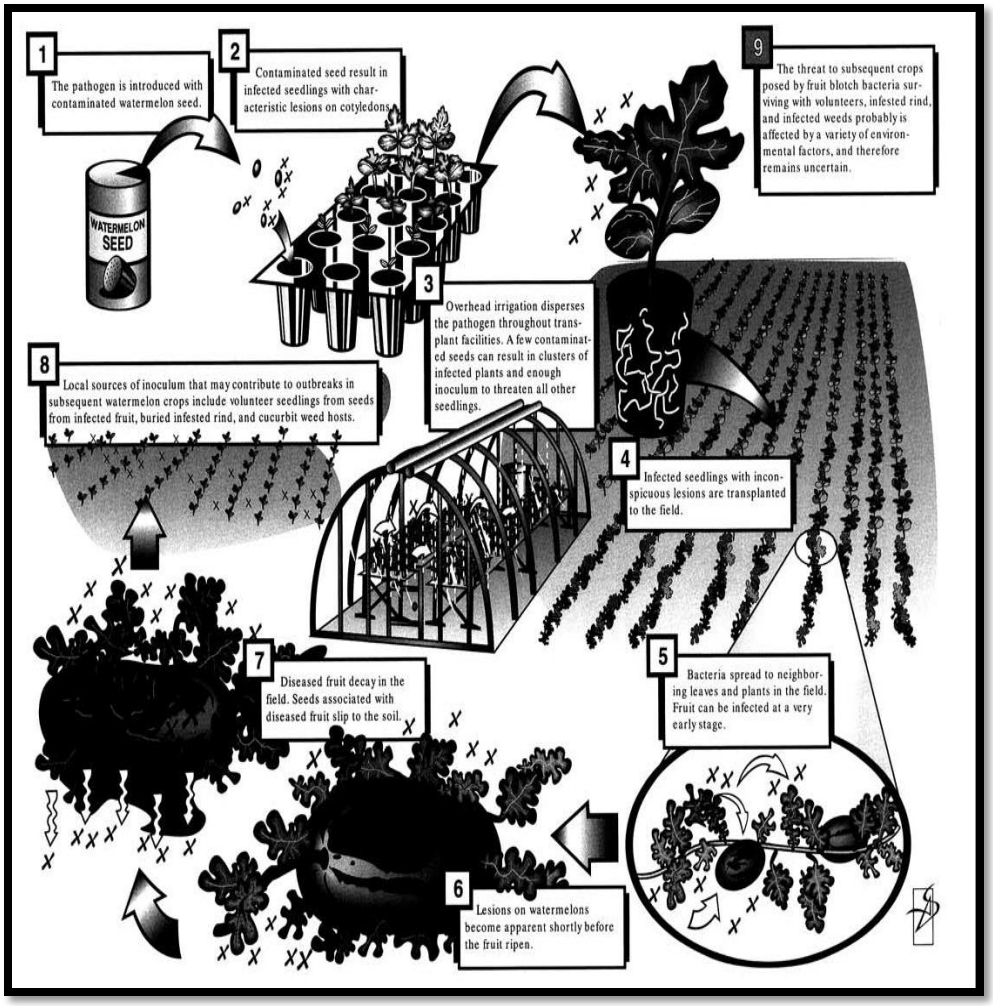

Figure 3: Cycle of BFB as described by Latin, R., & Hopkins, D. (1995). Bacterial fruit blotch of watermelon. *Plant Disease, 79*(8), 761-765.(Used under fair use, 2014)

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Chapter 2: A Novel Gateway®-Compatible Binary Vector Allows Direct Selection of Recombinant Clones in *Agrobacterium tumefaciens*

Traore, S. M., & Zhao, B. (2011). A novel Gateway®-compatible binary vector allows direct selection of recombinant clones in Agrobacterium tumefaciens. *Plant methods*, *7*(1), 42.
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Abstract

Background. Cloning genes into plasmid vectors is one of the key first steps for studying gene function. Recently, Invitrogen™ developed a convenient Gateway® cloning system based on the site-specific DNA recombination properties of bacteriophage lambda and the cytotoxic protein ccdB, which is lethal to most *E. coli* strains. The ccdB protein, however, is not toxic to *Agrobacterium tumefaciens*, an important player often used for studying gene function *in planta*. This limits the direct application of the Gateway® cloning system in plant transformation-mediated research.

Results. In this study, we constructed a novel Gateway®-compatible destination vector, pEG101-SacB/R/R, by replacing the *ccdB* gene with a *SacB/R-SacR* gene cassette as the negative selectable marker.

Conclusion. Our results demonstrate that the new pEG101-SacB/R/R destination vector can be used for Gateway® cloning in *Agrobacterium tumefaciens*. pEG101-SacB/R/R will be a valuable tool for high-throughput functional analysis of genes *in planta*.

Introduction

The wide-spread availability of genomic sequences from many organisms has raised interest in characterizing the biological functions of newly discovered genes through various high-throughput methodologies ("Summaries of National Science Foundation-Sponsored Arabidopsis 2010 Projects and National Science Foundation-Sponsored Plant Genome Projects That Are Generating Arabidopsis Resources for the Community," 2002). In order to study gene function, a gene-of-interest needs to be cloned into different plasmid vectors. In plant biology research, the gene-of-interest is frequently cloned into binary vectors that can be used for *Agrobacterium*-mediated transformation (Chakrabarty et al., 2007).

Gateway® technology was developed as a convenient and fast gene cloning system (Invitrogen, Carlsbad, CA). This method involves three key steps: (1) amplifying the targeted gene by PCR; (2) directly cloning the PCR product into a TopoEntr/D® vector without digestion/ligation; and (3) subcloning the targeted gene from the entry vector into any destination vector using the Gateway LR® cloning technique. The Gateway® LR cloning reaction is based on the site-specific homologous DNA recombination properties of bacteriophage lambda (Papagiannis et al., 2007). This method is more convenient than other methods because it does not involve either DNA digestion or ligation, two processes that can hinder the cloning process (Co, 2004). The Gateway®-compatible destination vector harbors a negative selectable marker, the *ccdB* gene, which produces a toxic protein that is lethal to most *Escherichia coli* strains, including *DH5α* (Bernard & Couturier, 1992; Bernard, Gabant, Bahassi, & Couturier, 1994). During the Gateway® LR reaction, the *ccdB* gene in the destination vector is replaced by the targeted gene from an entry vector through site-specific homologous DNA recombination. The LR reaction mixture containing both recombinant and non-recombinant plasmids are subsequently transferred into an *E. coli* strain, such as *DH5α*, that is sensitive to the toxic effect

of ccdB. Only recombinant destination plasmids that have lost the *ccdB* gene are able to survive in the transformed *E. coli* cells.

Recently, a collection of Gateway®-compatible binary destination vectors, that can be used for *Agrobacterium*-mediated transformation, have been constructed and are available to the plant research community (Chen, Songkumarn, Liu, & Wang, 2009; Curtis & Grossniklaus, 2003; Earley et al., 2006b; Karimi, Inze, & Depicker, 2002). Targeted genes can be easily cloned into these binary vectors through Gateway® LR cloning. However, the Gateway® LR cloning mixture has to first be transformed and screened in *E. coli* strains before the recombinant plasmid can be mobilized to *Agrobacterium tumefaciens* (*A. tumefaciens*) by either conjugation or electroporation. This process could be simplified if the recombinant binary plasmids from the Gateway® LR reaction could be directly transformed and selected in *A. tumefaciens*. Unfortunately, *A. tumefaciens* is insensitive to the toxic effect of ccdB (Bernard & Couturier, 1992). Thus, the binary vectors that contain the *ccdB* gene cannot be negatively selected against in *A. tumefaciens*. To overcome this problem, a new negative selectable marker that is functional in *A. tumefaciens* must be tested.

The *SacB/R-SacR* genes were originally isolated from *Bacillus subtilis* and encode levansucrase, an enzyme involved in both the hydrolysis of sucrose and the biosynthesis of levan (R. Chambert & Petitglatron, 1989; Quandt & Hynes, 1993). Levan cannot be metabolized by most gram-negative bacteria, including *E. coli* and *A. tumefaciens*, and is therefore toxic to this group of organisms (Gay, Lecoq, Steinmetz, Berkelman, & Kado, 1985; Schweizer, 1992). The *SacB/R-SacR* gene cassette, driven by its native promoter, has been used as a negative selectable marker for many Gram-negative bacteria and works by preventing the transformed bacterial strains from growing on culture medium supplemented with 5% sucrose (J. L. Ried & A.

Collmer, 1987). Therefore, we hypothesized that the *ccdB* gene in any Gateway®-compatible binary vector could be replaced with the *SacB/R-SacR* gene cassette as the negative selectable marker. This would allow for direct transformation and screening of the Gateway® LR cloning product in *A. tumefaciens*.

In this study, one popular Gateway® compatible destination binary vector, pEarleyGate101 (Earley et al., 2006b), was modified by replacing the *ccdB* gene with a *SacB/R-SacR* gene cassette. The so contructed novel destination binary vector, pEG101-SacB/R/R, should allow for the direct selection of Gateway® recombinant clones in *A. tumefaciens*. To test the efficiency and functionality of pEG101-SacB/R/R, a *luciferase* gene (*LUC*) and a bacterial effector gene *Aae2166* were used as reporters and cloned into the pEG101-SacB/R/R destination vector. The *LUC* gene encodes the fire fly luciferase protein, an enzyme that catalyzes luciferin and produces a luminescence signal when expressed in plant cells. This makes the *LUC* gene a convenient reporter gene for this study (Gould & Subramani, 1988). The putative bacterial type III effector gene *Aae2166*, isolated from the bacterial pathogen *Acidovorax avenae* pv. *citrulli*, encodes a homolog of *AvrBsT* (Kim, Choi, & Hwang, 2010b). Transient expression of *Aae2166* in the leaves of *Nicotiana benthamiana* triggers a cell death phenotype (Traore et al, unpublished results). Therefore, *Aae2166* is also a convenient marker for testing the functionality of the newly developed pEG101-SacB/R/R destination vector. The results of this study demonstrate that both genes were successfully cloned, selected for in *A. tumefaciens*, and expressed in *N. benthamiana* leaves. Therefore, the new destination vector is a convenient tool for cloning and expressing genes in *A. tumefaciens*. The new vector also has potential to be used in high-throughput cloning applications *in planta*.

Materials and Methods

Bacterial strains. *E. coli* DH5α [F^- endA glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15Δ(lacZYA-argF)U169, hsdR17($r_K^- m_K^+$), λ–] and *A. tumefaciens* (GV2260) [C58 background, rifampicin resistant with the Ti plasmid (pTiB6s3)]

Preparation of electroporation competent cells.

1. *E. coli DH5α* and *A. tumefaciens GV2260* strains were streaked on Luria Broth (LB) agar media supplemented with appropriate antibiotics and incubated at 37°C and 28°C, respectively.
2. Single colonies of *DH5α* and *A. tumefaciens* (*GV2260*) were inoculated in 50 ml of LB liquid media, incubated at 37° and 28 °C, respectively, and shaken at 200 rpm for 24 hours.
3. A new 500 ml LB liquid culture was re-inoculated from the initial saturated bacterial culture to produce a final concentration of OD_{600nm}= 0.1. The liquid cultures were incubated at 18°C and 28°C for *DH5α* and *A. tumefaciens (GV2260),* respectively, until the OD at 600 nm reached 0.6.
4. The bacterial liquid culture was subsequently chilled on ice for 20 minutes.
5. Bacterial cells were harvested by centrifugation at 6000 x g for 10 minutes at 4°C.
6. The bacterial cells were washed twice with 250 ml of ice-cold water, and then finally washed with 25 ml of 10% ice-cold glycerol.
7. The bacterial cells were pelleted by centrifugation and re-suspended in 5 ml of 10% ice-cold glycerol.
8. A 100 µl aliquot of bacterial cells was transferred to 1.5 ml microtubes and immediately frozen in liquid nitrogen.

9. The cells were stored at –80°C for at least six months without loss of competency.

Plant materials. *N. benthamiana* (PI 555478) plants were propagated in a growth chamber programmed for 16 hours light (140 µmol/m^2/s cool white fluorescent irradiance) at 28°C and 8 hours dark at 24°C. *Agrobacterium*-mediated transient assays were conducted on three to four week-old plants.

Construction of the destination binary pEG101-SacB/R/R vector. The *SacB/R-SacR* genes were amplified by PCR from plasmid pMSacB/R (M. Metz et al., 2005) using primers SacB/R/R Forward: 5'-AATCAGGAAGGGATG**GCTGAGG**GATATCGGATCGATCCTTTTTAACCCATCAC-3'and SacB/R/R Reverse: 5'-AGACCGGCACACTGGCCATATCGGTGGGATATCTTATTTGTTAACTGTTAATTGTCCT-3'. The *Bbv*CI and *Bst*XI restriction sites appear in the primers as bolded and underlined text, respectively. The nucleotide sequences of the *SacB/R-SacR* genes, including their native promoter, can be found in GenBank (#AB183144).

The iProof ™ high fidelity Taq DNA polymerase (Bio-Rad, Hercules, CA) PCR program consisted of 1 cycle at 98°C (2 min), followed by 30 cycles at 98°C (30s), 55°C (45s), and 72 °C (1 min), and finished with a 1 cycle extension at 72°C (7 min). The PCR products were digested with *Bbv*CI and *Bst*XI (New England BioLabs Inc, Ipswich, MA), and gel purified using an AccuPrep™ Gel Purification Kit (Bioneer, Alameda, CA).

The destination binary plasmid pEarleyGate101 (Earley et al., 2006b) was obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH) and digested with *Bbv*CI and *Bst*XI to remove the *ccdB* gene. The gel-purified PCR product of the *SacB/R-SacR* gene cassette was then cloned into the *Bbv*CI and *Bst*XI restriction sites of

pEarleyGate101. The ligation products were transformed into *DH5α* using a Bio-Rad gene pulse electroporation apparatus (2.5 Kv/uM/mSec). Successfully transformed bacteria were selected for on LB medium supplemented with kanamycin (50 mg/ml) and chloramphenicol (25mg/ml). The derived new destination vector was re-named pEG101-SacB/R/R.

Plasmid DNA isolation. All plasmid DNA was isolated using an AccuPrep Plasmid Extraction Kit (Bioneer, Alameda, CA).

Negative selection for the SacB/R-SacR gene. Bacterial cells that were successfully transformed with the pEG101-SacB/R/R plasmid were negatively selected for on LB medium supplemented with 5% sucrose as described previously (Gay et al., 1985; Schweizer, 1992).

Cloning of the LUC gene and Aae2166 into the TopoEntry/D vector. The open reading frame (ORF) of the *LUC* gene was cloned from plasmid pDesA-luc (Ow et al., 1986) with primers: Sal_LucFor:5'-caccgtcgacGGCGGTGGCTCATCTGGCGGAGGT**atg**gaagacgccaaaaac-3' and Pst_LucRev: 5'-ctgcag**TTA**CAATTTGGACTTTCCGCCCTTCTTGG-3'. The start and stop codons of the *LUC* gene are bolded and underlined in the forward and reverse primers, respectively. The ORF of the *Aae2166* gene was amplified from genomic DNA of *Acidovorax avenae* subs *citrulli* strain AAC-001 using primers: Aae2166 For: 5'-caccAGATCT**ATG**AAGAATTTCATGCGATCGAT -3' and Rev: 5'-GTCGACTTCGATAGCTTTTCTGATTTTTCTCA-3'. The start codon of Aae2166 is bolded and underlined. The PCR products of the *LUC* and *Aae2166* genes were gel purified and cloned into the TopoEntr/D® vector (Invitrogen, Carlsbad, CA) following the instructions of the user's manual. Plasmid DNA was sequenced at the Core facility of the Virginia Bioinformatics Institute (Blacksburg, VA).

LR reaction and transformation of Agrobacterium tumefaciens by electroporation. The Gateway® LR clonase™ enzyme mix kit (Invitrogen, Carlsbad, CA) was used for the LR recombination reaction. In brief, the LR reaction mixture contained: 1-5 µl (100 ng) of entry clone plasmid DNA, 1 µl (100ng) of destination vector pEG101-SacB/R/R plasmid DNA, 2 µl of 5xLR clonase reaction buffer, and 2 µl of LR clonase™ enzyme. The reaction mixture was brought to a final volume of 10 µl with TE buffer (pH 8.0) and incubated at room temperature for 1 hour. Following the incubation, a 3-5 µl aliquot was mixed with 100 µl of competent *A. tumefaciens* (GV2260) cells. A gene pulse apparatus (Bio-Rad, Hercules, CA), programmed for 2.5 Kv/uM/mSec, was used to transform the cells by electroporation. Following electroporation, the mixture was resuspended in 1 ml of liquid LB and incubated shaking at 28°C/200 rpm for 3 hours. Successfully transformed cells were then selected for on LB agar supplemented with kanamycin (50mg/ml), rifampicin (100mg/ml), and 5% sucrose. The LB plates were incubated at 28°C for 2-3 days. After incubation, ten colonies were randomly selected for further analysis. Recombinant plasmids were confirmed by PCR with primers: 35S forward 5'-AAGAAGACGTTCCAACCACGTC-3' and NLuc reverse: 5'-AACTGCAGTCATCCATCCTTGTCAATCAAG-3' to detect the firefly *luciferase* gene (1.4 Kb) (Ow et al., 1986). Recombinant plasmids were also confirmed by PCR using primers 35S forward plus Aae2166 reverse: 5'- GTCGACTTCGATAGCTTTTCTGATTTTTCTCA-3' to detect the *Aae2166* gene (1.1 kb). The PCR fragments were separated on a 0.8 % agarose gel, stained with 0.01% ethidium bromide solution, and visualized using the Gel-Document Image System™ under UV light (Bio-Rad, Hercules, CA).

Agrobacterium-mediated transient assays in N. benthamiana plants. Agrobacterium-mediated transient assays in *N. benthamiana* plants were performed as described previously

(Wydro, Kozubek, & Lehmann, 2006). In brief, the *Agrobacterium* strain was streaked on LB medium supplemented with appropriate antibiotics and incubated at 28°C for 2 days. Bacterial cells were harvested and re-suspended in induction buffer composed of 10 mM $MgCl_2$, 10 mM MES (pH 5.6), and 100 µM acetosyringone and incubated for 3 hours at room temperature. The bacterial inoculums were adjusted to OD_{600nm} = 0.6 and infiltrated into the stomata of fully expanded *N. benthamiana* leaves using a 1 ml blunt-end syringe without a needle. The inoculated plants were incubated at room temperature under continuous light for 20-48 hours before the detection of expressed proteins. Transient expression of the *luciferase* gene was detected by applying 1 µM luciferin to the inoculation site (H. M. Chen et al., 2008). The chemical fluorescent signals were captured with a CCD camera and visualized using the Gel-Document Image System (Bio-Rad, Hercules, CA). The fluorescent signal of the Aae2166-GFP fusion protein was monitored 20 hours after inoculation by fluorescent microscopy (Zeiss Axio Observer.A1, Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Results

Constructing a novel Gateway®-compatible binary vector pEG101-SacB/R/R. In order to construct a new Gateway®-compatible destination binary vector that can be used for selection of recombinant plasmids in *A. tumefaciens*, the *SacB/R-SacR* gene cassette was amplified and cloned into the *Bbv*CI and *Bst*XI restriction sites of pEarleyGate 101 (Earley et al., 2006b). The *SacB/R-SacR* genes replaced *ccdB* to generate a novel destination vector, pEG101-SacB/R/R. The key components of pEG101-SacB/R/R are illustrated in Figure 1A. To test the functionality of the *SacB/R-SacR* gene cassette as an effective negative selectable marker, *A. tumefaciens* strain GV2260, *A. tumefaciens* strain GV2260 carrying pEarleyGate101, and *A. tumefaciens* strain GV2260 carrying pEG101-SacB/R/R were grown on LB agar medium

supplemented with or without 5% sucrose (Figure 2). As shown in Figure 2A and 2B, *A. tumefaciens* grew equally well on LB medium with or without 5% sucrose, which suggested that sucrose in LB agar medium has no inhibitory effect to *A. tumefaciens*. The *A. tumefaciens* strain GV2260 carrying pEarleyGate101 also grew well on LB agar medium with or without 5% sucrose (Figure 2C and 2D). This result demonstrates that the *ccdb* gene cassette in pEarleyGate101 cannot be used as a negative selectable marker in *A. tumefaciens*. To test the functionality of the *SacB/R-SacR* gene cassette as an effective negative selectable marker in *A. tumefaciens*, the pEG101-SacB/R/R plasmid DNA was transformed into *A. tumefaciens* strain GV2260. After transformation, ten randomly selected clones were raised on LB medium supplemented with or without 5% sucrose. *A. tumefaciens* carrying pEG101-SacB/R/R grew well on LB agar medium without sucrose (Figure 2E); however, their growth was completely inhibited when the LB agar medium was supplemented with 5% sucrose (Figure 2F). These results show that the new destination vector, pEG101-SacB/R/R, can efficiently select against *A. tumefaciens* when grown on LB agar medium supplemented with 5% sucrose.

Testing the stability of binary vector pEG101-SacB/R/R. To test the stability of destination vector pEG101-SacB/R/R in *E. coli*, the restriction patterns of pEG101-SacB/R/R were compared before and after repeated sub-culturing. A single *E. coli* clone carrying pEG101-SacB/R/R was grown overnight in LB liquid medium supplemented with both kanamycin and chloramphenicol. The overnight bacterial culture was diluted (5000x) with LB medium and sub-cultured for an additional 24 hours. This process was repeated five times as described previously (Shizuya et al., 1992). The plasmid DNA of pEG101-SacB/R/R was isolated after each generation and digested with *Bst*XI and *Bbv*CI to remove a 2 kb fragment corresponding to the inserted *SacB/R-SacR* gene cassette. As shown in Figure 3, the restriction digestion patterns of

pEG101-SacB/R/R from each generation were identical. All plasmid DNAs were further transformed into both *E. coli* and *Agrobacterium tumefaciens* and the derived clones were re-plated on LB agar medium supplemented with or without 5% sucrose. All transformed bacteria colonies failed to grow on LB agar medium supplemented with 5% sucrose, confirming the functionality of the *SacB/R-SacR* genes. These results demonstrate that pEG101-SacB/R/R can be stably propagated in *E. coli* grown on LB agar medium without sucrose.

Generating recombinant clones in Agrobacterium tumefaciens through Gateway LR cloning. The functionality of pEG101-SacB/R/R for Gateway® LR cloning was tested in *A. tumefaciens* using two entry clones carrying the *LUC* and *Aae2166* genes, respectively. *A. tumefaciens* strain GV2260 was directly transformed with the LR reaction mixture for each gene by electroporation. Putative recombinant clones were selected on LB agar medium supplemented with kanamycin (50mg/ml) and 5% sucrose. Ten randomly selected *A. tumefaciens* clones were analyzed by PCR using either *LUC* or *Aae2166* gene specific primers, or a combination of gene specific primers with a primer designed using the 35S promoter in the vector pEG101-SacB/R/R as a template (Figure 1A). As shown in Figure 4, all colonies tested were positive, suggesting a high cloning efficiency of targeted genes into pEG101-SacB/R/R in *A. tumefaciens*. We also used the vector to clone twenty two bacterial effector genes of *Acidovorax avenae pv. citrulli*, which gave a similar cloning efficiency (data not shown).

As shown in Figure 1A, the cloned *LUC* and *Aae2166* genes are driven by the 35S promoter. The ORF of *Aae2166* was cloned, without a stop codon, into the pEntry/D vector. After LR cloning, the lack of a stop codon allowed the *Aae2166* ORF to be fused in-frame with the N-terminus of the GFP gene in the recombinant pEG101-*Aae2166* plasmid. Conversely, a

stop codon was added to the C-terminus of the *LUC* gene; therefore, GFP fusion would not have occurred.

Validating the function of recombinant binary plasmids by Agrobacterium-mediated transient assays. To examine the expression of the *LUC* and *Aae2166* genes cloned in pEG101-SacB/R/R, *A. tumefaciens* colonies carrying the recombinant plasmids pEG101-*LUC* or pEG101-*Aae2166* were inoculated into the leaves of *N. benthamiana*. As shown in Figure 5B, *A. tumefaciens* colonies carrying pEG101-*LUC* (Figure 5 B-2) but not pEarleyGate 101 (Figure 5 B-1) triggered strong luminescence after treatment with luciferin, thereby confirming the expression of *luciferase*. As a negative control, the same *A. tumefaciens* strain, carrying pEG101-*LUC*, was spotted onto either the leaf surface of *N. benthamiana* or onto a LB agar plate. After treatment with luciferin, the samples were examined under a CCD camera and no luminescence signal was detected (data not shown). This result confirmed that the luminescence signal in Figure 5B-2 was from the transiently transformed *N. benthamiana* plant cells. When *N. benthamiana* plants were inoculated with *A. tumefaciens* colonies carrying pEG101-*Aae2166*, cell death was observed at 48 hours after inoculation (Figure 5C-2). As a negative control, an *A. tumefaciens* strain carrying an un-modified pEarleyGate101 was inoculated into the leaves of *N. benthamiana*. The results of this assay did not trigger cell death at 48 hours after inoculation (Figure 5C-1). Since we modified Aae2166 to contain a C-terminal GFP tag (Figure 1A), pEG101-*Aae2166* expressed an Aae2166-GFP fusion protein. As shown in Figure 5E, the fluorescent signal of the Aae2166-GFP fusion protein was visible by fluorescent microscopy. The picture in Figure 5E was taken at 20 hours after inoculation, at which point cell death had not been induced. This eliminated the possibility of autofluorescent signals from dead cells.

Aae2166-GFP proteins were predominantly localized in the cytosol of transformed cells, although this observation requires further confirmation by an independent method. As a negative control, *A. tumefaciens* carrying an un-modified pEarleyGate101 did not incite a fluorescent signal in *N. benthamiana* (Figure 5D). These results demonstrate that two different genes can be efficiently cloned into pEG101-SacB/R/R using the Gateway LR reaction with direct selection in *A. tumefaciens*. The two genes were functionally expressed when assayed by *Agrobacterium*-mediated transient assays.

Conclusion

In this report, a novel Gateway®-compatible binary vector pEG101-SacB/R/R is described that allows direct transformation and selection of recombinant LR-generated clones in *A. tumefaciens.* The *LUC* gene, along with a putative bacterial effector gene, *Aae2166*, were used to demonstrate the efficiency and functionality of this newly developed Gateway®-compatible binary vector. The ability to directly clone and select recombinant genes in *A. tumefaciens* would save time, labor, and would also minimize potential contamination problems associated with conjugation or transformation manipulation. The new vector pEG101-SacB/R/R described here has great potential for simplifying and improving the efficiency of cloning genes in *A. tumefaciens* for plant transformation research. The pEG101-SacB/R/R vector can also be adapted for high- throughput applications. For example, a gene-of-interest mutant library could be generated by Error-Prone PCR from a TopoEntr/D™ (Invitrogen, Carlsbad, CA) plasmid template. The loss-of-function mutants could be identified through *Agrobacterium*-mediated transient assays in *N. benthamiana* leaves. Therefore, the new destination binary vector pEG101-*SacB/R/R* may be a valuable tool for high-throughput functional analysis of genes *in planta*.

Competing Interests

No competing interests were claimed by the authors. The vector pEG101-*SacB/R/R* is available upon request from the authors.

Authors' Contributions

ST designed and performed the experiments. BZ is the the principal investigator who conceived the project and the cloning strategy. ST and BZ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Figure Legends

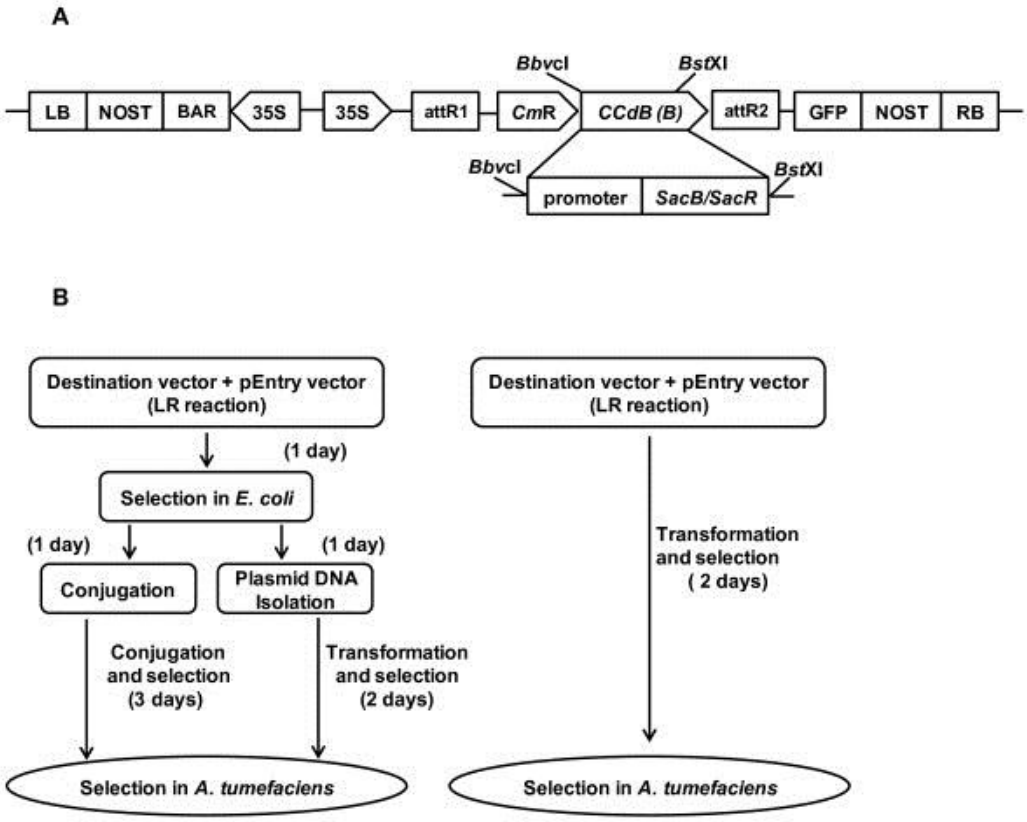

***Figure* 1. Schematic cloning of a gene into a binary expression vector and selection in *Agrobacterium tumefaciens*.** (A) Key features of pEG101-SacB/R/R. LB: T-DNA left border; RB: T-DNA right border; Cm^R: chloramphenicol resistance gene; attL1 and attL2: recognition sites of the LR clonase; *ccdB*: the Invitrogen *ccdB* gene cassette frame B; *SacB/R/SacR*: Levansucrase genes along with their native promoter; *Bbv*CI and *Bst*XI: restriction enzyme sites for replacing the *ccdB* gene fragment with the *SacB/R/SacR* gene cassette. (B). Compared to the regular LR cloning procedure, the new protocol is more convenient and time-saving.

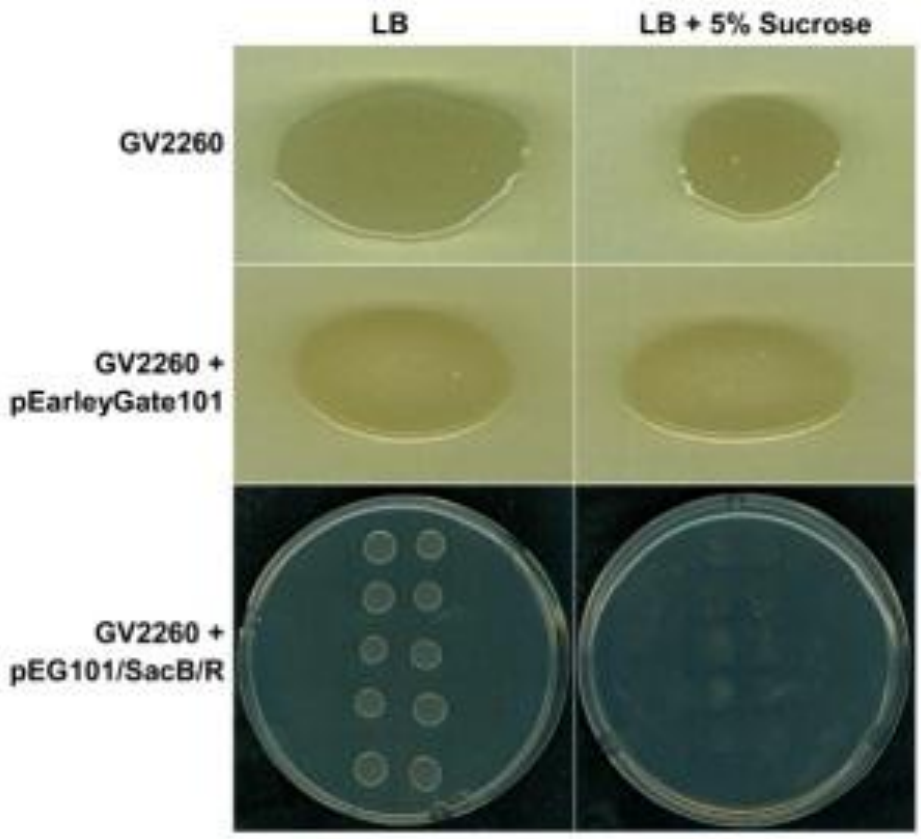

***Figure 2*. The growth of *Agrobacterium tumefaciens* strain on LB agar plates with different selections.** A. tumefaciens strain GV2260 grew equally well on LB medium with (A) or without 5% sucrose (B). The ccdB gene cassette in the pEarleyGate 101 vector could not inhibit the growth of A. tumefaciens on LB medium with (C) or without 5% sucrose (D). A. tumefaciens strain GV2260 carrying the newly generated pEG101-SacB/R/R destination vector grew well on LB medium without sucrose (E), but growth was inhibited on LB medium supplemented with 5% sucrose (F).

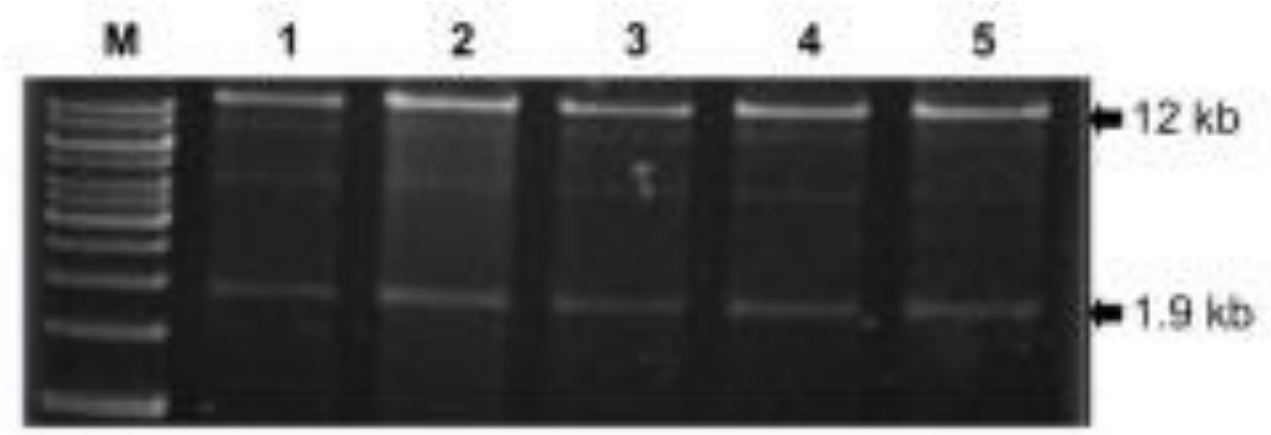

***Figure* 3. The destination vector pEG101-SacB/R/R can be stably propagated in the *E. coli* strain *DH5α*.** Restriction digestion (*Bst*XI and *Bbv*CI) of plasmid DNAs, isolated from five repeated overnight cultures of *E. coli* carrying the pEG101-SacB/R/R destination vector, showed identical restriction patterns. M: 1Kb marker. Lanes 1 through 5 were digested plasmid DNAs of five repeated subcultures.

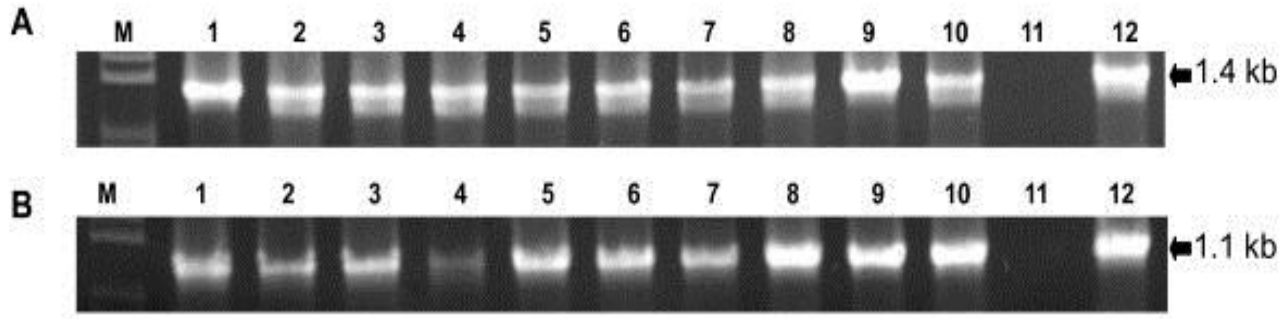

***Figure* 4. PCR analysis of recombinant clones of *Agrobacterium tumefaciens*.** (A). Clones of *A. tumefaciens* transformed with pEG101-*LUC*. M: 1Kb marker; lanes 1 to 10 were from bacteria colonies carrying the recombinant plasmid pEG101-*LUC*, where a 1.4 kb band was amplified corresponding with the expected 35S-*LUC* gene; lane 11 was a negative PCR control using *A. tumefaciens* only as a template; lane 12 was a positive PCR control using plasmid DNA of pEG101-*LUC* isolated from *E. coli* as a template. (B). Clones of *A. tumefaciens* transformed with pEG101-*Aae2166*. M: 1Kb marker; lanes 1 to 10 were clones carrying the recombinant plasmid pEG101-*Aae2166*. Amplified fragments corresponded to 35S-*Aae2166* (1.1 Kb). Lane 11 was a negative PCR control using *A. tumefaciens* only as a template. Lane 12 was a positive control using plasmid DNA of pEG101-*Aae2166* isolated from *E. coli* as a template.

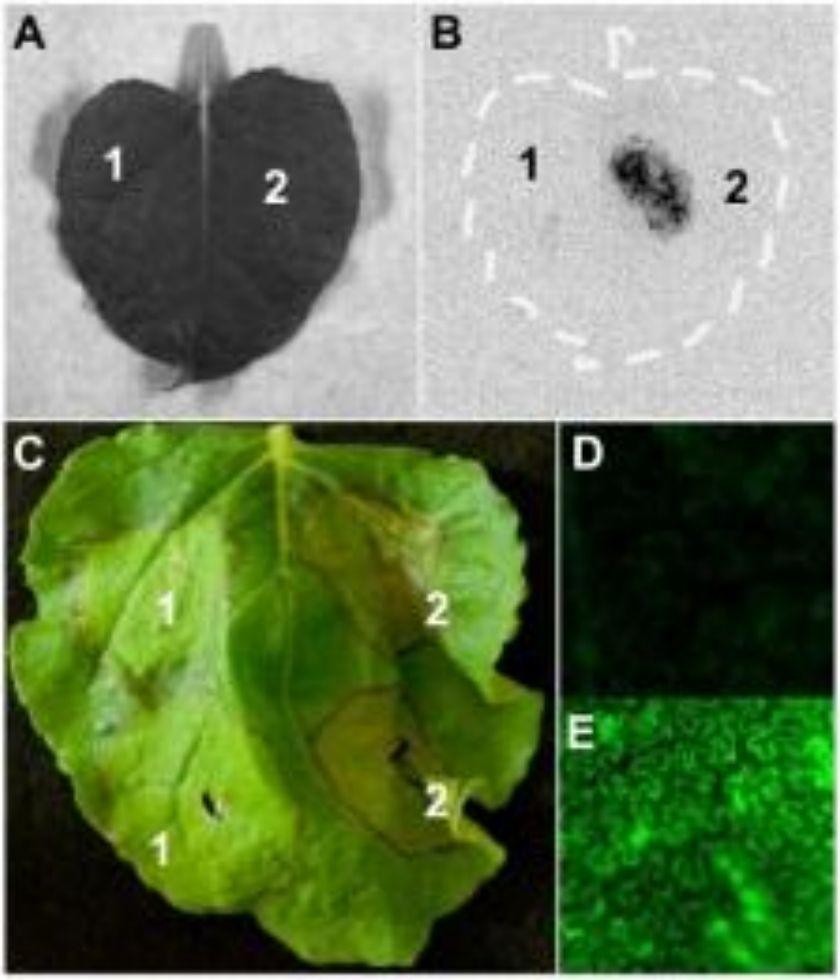

***Figure* 5. Functionality test of two genes, cloned into the pEG101-SacB/R/R destination vector, by means of *Agrobacterium*-mediated transient assays in the leaves of *Nicotiana benthamiana*.** *A. tumefaciens* GV2260 strains, carrying either pEarleyGate 101 empty vector (A-1) or pEG101-*LUC* (A-2), were inoculated in *N. benthamiana* leaves. The *A.tumefaciens* GV2260 strain carrying pEG101-*LUC* (B-2) produced a strong luminescent signal under dark conditions after luciferin treatment, while the strain carrying the empty vector pEarleyGate 101 (B-1) did not show any signal. The luminescent signal was detected using the Gel Document Image System equipment with a CCD camera. *A. tumefaciens* strain GV2260 carrying pEG101-*Aae2166* (C-2), but not the empty vector pEarleyGate 101 (C-1), was able to trigger programmed cell death in the leaf of *N. benthamiana*. The fluorescent signal of the Aae2166-GFP fusion protein was detected by fluorescent microscopy (200 x magnifications) (E). As the negative control, GFP signal was not detected in the leaf of *N. benthamiana* that was inoculated with *A. tumefaciens* carrying pEarleyGate 101 (D).

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Chapter 3: Sequential Knockout of Type III Effectors in *Acidovorax avenae subs. citrulli* Using a Modified SacB/R Gene Cassette as the Counter Selectable Markers

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Greg Welbaum, Department of Horticulture, Virginia Polytechnique and State University, Blacksburg, VA, 24061 helped with the writing and revision of the manuscript

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Abstract

A. citrulli is the causal agent of bacterial fruit blight (BFB) disease of cucurbit. Despite the importance of the disease in cucurbits, little is known about the pathogenicity of *A. citrulli*. The release of the genomic sequence of AAC00-1 was the turning point in regard to understanding the molecular mechanisms by which *A. citrulli* infects its host plants. Annotation of the genomic sequence of *A. citrulli* has revealed the presence of type 3 secretion systems (T3SS) along with at least 11 type 3 effector (T3E) genes. Thus far, none of these T3Es have been functionally characterized by comparing the pathogenicity of wild type and effector-deleted mutant *A. citrulli* strains. Marker-exchange mutagenesis is routinely used for generating effector-deletion bacterial mutant strains. However, T3Es usually have redundant virulence functions, and deletion of individual effectors frequently shows no obvious effect on the virulence of the mutant

bacterial strains. Furthermore, *A. citrulli* is a less-studied pathogen lacking tools that could be used to manipulate its genome. In this study, we tried to develop a method allowing sequential deletion of T3E genes to generate poly-mutant strains. Two modified marker-exchange mutagenesis vectors were developed by incorporating the counter selectable marker gene cassette *SacB/R*, the FRT-flanked antibiotic resistance marker gene, and the Flipase gene, which are all driven by the *Npt*II-promoter. We also optimized a protocol for selection of marker-exchanged mutants and generated mutant *A. citrulli* strains with deletions in two T3Es. The virulence of the mutant *A. citrulli* strains were evaluated on melon by using the seed transmission efficiency assay. We expect the new bacterial marker-exchange mutagenesis vectors and mutant selection method should be suitable for bacterial mutagenesis in other phytopathogenic bacterial species too.

Keywords: *A. citrulli*, Marker-exchange mutagenesis, *SacB/R*, Flp-mediated recombination, *Npt*II-promoter, polymutant

Introduction

Bacterial fruit blotch (BFB) is an economically important disease of cucurbits, causes by the gram negative bacterium *A. citrulli* (Bahar & Burdman, 2010). The release of the genomic sequence of the strain AAC00-1 was an important step for our understanding of the pathogenicity of *A. citrulli*. One of current research topics is to identify virulence genes that are important for the pathogenicity of *A. citrulli* (Johnson et al., 2011; Liu et al., 2012; ZhengGuang, Lei, ZhiGuo, & LiQun, 2009). The annotation of the AAC00-1 genome has revealed the presence of eleven T3E genes (Eckshtain-Levi et al., 2014). Understanding the contribution of each or multiple effector genes to the pathogenicity of *A. citrulli* requires the

development of mutant strains with deletions of single or multiple T3E genes. Unfortunately, an efficient gene knockout protocol for *A. citrulli* is currently not available.

There are at least two standard methods that have been frequently used for knocking out bacterial genes. The first method consists of inserting DNA fragments in the middle of targeted genes (Bahar, Goffer, & Burdman, 2009a; Penfold & Pemberton, 1992). An internal DNA fragment of the gene of interest (which does not span the 3′ and 5′ ends of this gene) can be sub-cloned into a suicide vector for bacterial transformation. The whole plasmid will integrate into the bacterial genome through homologous DNA recombination and disrupt the function of the targeted gene. This method has been successfully applied to the genetic analysis of *A. citrulli* (O. Bahar et al., 2009a; Johnson et al., 2011; Penfold & Pemberton, 1992). However, this method could encounter several problems. First of all, this method depends on the deletion of both 5' and 3' ends of the targeted genes, where the small deletions may not completely block the gene function. The second issue with this method is that it only allows the generation of a single deletion mutant, where the backbone of the suicide vector will be integrated into the bacterial genome, in some cases the plasmid could be lost resulting in the loss of the mutant phenotype. The insertion of large DNA fragments could also affect the expression of adjacent bacterial genes in the genome. The second method is based on gene replacement, which is also based on the homologous recombination and involves a double crossover event. In this method, an antibiotic marker gene is flanked by DNA fragments upstream and downstream of the open reading frame of the targeted gene. The DNA fragment is then cloned into a suicide vector and transformed into the bacterial cells. Homologous recombination with a double crossover event will lead to the replacement of the gene of interest (Baba et al., 2006). The double crossover event will lead to the deletion of the plasmid backbone along with the gene of interest and

generate “cleaner” polar mutant strains. A second bacterial gene can be further deleted by using different antibiotic marker genes. However, the choice of antibiotic marker genes may be limited for a given bacterial species.

More recently, an improved gene replacement method was developed by introducing a counter selectable marker, the *SacB/R* expression cassette, in the suicide vectors, where the bacterial cells carrying *SacB/R* genes are not able to grow on the medium supplemented with 5 % sucrose (Régis Chambert & Petit-Glatron, 1989; Quandt & Hynes, 1993; Reyrat, Pelicic, Gicquel, & Rappuoli, 1998). Therefore, the removal of the plasmid backbone can be selected for on medium supplemented with sucrose. However, it was reported that the original *SacB/R* genes may not function in some bacterial species probably due to the inefficient promoter activity (Jeffrey L Ried & Alan Collmer, 1987). Another significant improvement is the introduction of the flipase recombination enzyme that can recognize the FTR sequence flanking the antibiotic marker genes in the marker-exchanged mutant strains (Jittawuttipoka et al., 2009a). The Flp-mediated recombination can remove the antibiotic marker gene after gene replacement. The removal of the marker gene allows the use of the same antibiotic marker gene for the deletion of another bacterial gene therefore, generating a polymutant strain.

The plasmid carrying the flipase enzyme needs to be removed for sequential gene knockout experiments. Several methods have been reported for curing plasmids from bacterial cells including the growth of bacteria at higher temperature, the exposure to UV and the use of certain chemicals etc. (Sharma & Laxminarayana, 1989; Spengler et al., 2006). More recently, the chemical acridine-orange was reported to be an effective reagent for curing plasmids from *E. coli* (Zaman, Pasha, & Akhter, 2010).

In this report, an improved Gateway-compatible suicide vector, pVLC18L/*Npt*II-*SacB/R*-Des, was constructed with a strong *SacB/R* expression cassette allowing gene replacement in *A. citrulli*. Another new vector expressing the Flipase enzyme in *A. citrulli* was also developed. Furthermore, the chemical acridine-orange was adapted to efficiently cure plasmids from *A. citrulli*. We demonstrated that this optimized protocol is suitable for deletion of multiple T3S effector genes from the *A. citrulli* genome. The new vectors and the optimized marker-exchange protocols should also be valuable rescues for genetic studies of other bacterial species.

Material and Methods

Bacterial strains. *Escherichia coli* (*E. coli*) *DH5α* [F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–]; helper strain p600 (Figurski & Helinski, 1979), *A. citrulli* strains M6 (Rif^r) (O. Bahar et al., 2009) and AAC00-1 (Rif^r) (Johnson et al., 2011).

Construction of the marker-exchange mutagenesis vector for clean genes knockout suicide vector pLVC18L/NptII-SacB/R/R construction. The *Npt*II promoter was amplified from pDSK519-GFP (Matthysse, Stretton, Dandie, McClure, & Goodman, 1996) using primes: 1776*Npt*IIPro_EcoRv For1, 5'-caccGATATCACATGGCGATAGCTAGACTG-3', and 1777*Npt*IIPro_*SacB/R*Rev1, 5'-GTGATGGGTTAAAAAGGATCGATCCGCGCCATCAGATCCTTG-3'. The *SacB/R* gene cassette was amplified from vector pMac/SacB (M. Metz et al., 2005) using primers 1778 *Npt*IIPro_*SacB/R* For2, 5'-CAAGGATCTGATGGCGCGGATCGATCCTTTTTAACCCATCAC-3' and 2221Infus *SacB/R* Rev, CCAGTCTAGCTATCGCCATGTGCCgtCTTATTTGTTAACTGTTAATTGT. Using the overlapping PCR method the *Npt*II promoter was fused in front of the *SacB/R* gene cassette.

The PCR product was amplified and cloned into the pCR8/GW (Invitrogen, Carlsbad, CA) to generate PCR8/GW-*Npt*II-*SacB/R*. The *Npt*II-*SacB/R* fragment was re-amplified from PCR8/GW-*Npt*II-*SacB/R* using primers P1846, 5'-TGCCATTGCTGCAGGTCGAC*TCTAGA*GATATCACATGGCGATAGCTAGACTG-3' P1847, 5'-CTCGGTACCCGGGGATCC*TCTAGA*GATATCTTATTTGTTAACTGTTAATTGTCCT-3'. The PCR fragment was then cloned into the *Xba*I site of the suicide vector pVLC18L (Staskawicz, Dahlbeck, Keen, & Napoli, 1987; Zhao, Dahlbeck, Krasileva, Fong, & Staskawicz, 2011) by using the Gibson cloning kit (New England Biolabs Inc., Ipswich, MA) generating pVLC18L-*Npt*II-*SacB/R* suicide vector. The *ccdB* gene expression cassette flanked by the *EcoRV* sites (Invitrogen) was further cloned into the *Sma*I site of pVLC18L-*Npt*II-*SacB/R*. The derived vector was designated as pVLC18L-*Npt*II-*SacB/R*-Des.

Construction of the broad host range vector expressing the Flp recombinase gene. The pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flipase vector carries the counter selectable selection *SacB/R* gene cassette and the Flipase gene, all driven by the strong and constitutive *NptII* promoter. The pEDV6-Des is a broad host range expression vector (Cabral et al., 2011; Sohn, Lei, Nemri, & Jones, 2007) that can survive in most phytopathogenic bacterial species including *A. citrulli*. The vector construction was performed in two steps. First, the *NptII* promoter amplified from pDSK519-GFP (Matthysse et al., 1996) and the flipase gene from pBBR1_FLP2 (Jittawuttipoka et al., 2009a) using set of primers NaeI *Npt*IIPro For, 5'-caccGCCGGCACATGGCGATAGCTAGAC-3' and NaeI NptIIPro Rev, 5'-cataatataccaaattgtggCATATGTATATCTCCTTTCT-3', The flipase gene open reading frame was amplified from pBBR1_FLP2 using primers *Npt*IIPro-Flpase For, 5'-

AGAAAGGAGATATACATATGccacaatttggtatattatg-3’ and Flpase-6His Rev, 5’-ttaGTGATGATGATGATGATGtatgcgtctatttatgtaggatg-3’.

By using the overlapping PCR method the *Npt*II promoter was fused to the flipase gene. The resulting PCR product was cloned into Topo vector pEntr/D (Invitrogen) to generate TopoEntry/D-NaeI-*Npt*IIPromoter-Flpase-6xHis tag-Stop. Second, the *Npt*II-*SacB/R* cassette was amplified from the plasmid PCR8/GW/*Npt*II-*SacB/R* using primer Infus_*SacB/R*_For, 5’-GGCTCCGCGGCCGCCCCCTTCACCGCCGGCACATGGCGATAGCTAGACT-3’, and Infus SacBR Rev, 5’-CCAGTCTAGCTATCGCCATGTGCCgtCTTATTTGTTAACTGTTAATTGT-3’ and cloned into the *Nae*I site of the vector TopoEntry/D-NaeI-*Npt*II Promoter-Flpase-6xHis tag-Stop to generate the construct TopoEnd-*Npt*II-Flipase-NaeI-*Npt*II-*SacB/R*. Finally, the *Npt*II-Flipase-NaeI-*Npt*II-*SacB/R* was further subcloned into a broad host spectrum destination vector pEDV6-Des (Cabral et al., 2011; Sohn et al., 2007) through LR Gateway cloning to generate the plasmid vector pEDV6-*Npt*II-*SacB/R-Npt*II-flipase.

Marker-exchange mutagenesis fragment construction. DNA fragments about 1.3 kb upstream of the start codon of the effector genes Aave1548 and Aave2166 were amplified from the genomic DNA of AAC00-1 using primer sets Aave1548_f1, 5’-cccggatcgaaaggcgagcgcacg-3’ and Aave1548_R2, 5’-CCTACACAATCGCTCAAGACGTGTGCTGCAGCGTGCCATGGACAA-3’; and Aave2166_forward, 5’-gaagcccacgtgcgcgtgctggcc-3’, and Aave2166_Reverse, CCTACACAATCGCTCAAGACGTGCCATTCTCAATTGCAAATATTCAG; respectively. Additional 1.4 kb fragments downstream to the stop codon of effector genes *Aave1548* and *Aave2166* were amplified from the genomic DNA of AAC00-1 using primers set Aave1548_forward, 5’-ggaccatggctaattcccatgtcccttggggacgggggagtctggga-3’,

Aave1548_Reverse, 5'-GGTGAGCCAGTGGAAGCGCGCCCGG-3'; Aave2166_forward, 5'-ggaccatggctaattcccatgtcgcaccgggaatgataatccccgg-3' and Aave2166_Reverse, CGCTGTCGTAGCCCACGGTGATGTTCG; respectively. A kanamycin resistance gene (about 1.5 kb) was amplified from the pKD4 vector (Jittawuttipoka et al., 2009b) using primers forward: 5'-cacgtcttgagcgattgtgtagg-3' and reverse 5'-GACATGGGAATTAGCCATGGTCC-3'. By using overlapping-PCR, the upstream fragment was fused to the N-terminal region of the kanamycin resistance gene while the downstream fragment was fused to the C-terminal region. The 4.5 kb fragments were cloned into the pCR8/GW vector (Invitrogen) and then sub-cloned into the suicide vector pVLC18L-*Npt*II-*SacB/R*-Des by LR Gateway cloning (Invitrogen). The derived vector was named pVLC18L-*Npt*II-*SacB/R*-ΔAave1548 or pVLC18L-*Npt*II-*SacB/R*-ΔAave2166, which was transferred by conjugation into *A. citrulli* strains AAC00-1 and M6 for gene knock out by marker-exchange mutagenesis as described previously (Zhao et al., 2011).

Bacterial conjugation and marker-exchange mutagenesis The helper strain p600 (Zhao et al., 2011), the donor *E. coli* strain carrying either pVLC18L-*Npt*II-*SacB/R*-ΔAave1548 or pVLC18L-*Npt*II-*SacB/R*-ΔAave2166 and the recipient strain *A. citrulli* (AAC00-1 and M6) were grown separately on Nutrient Broth Agar (NA) plate medium supplemented with appropriated antibiotics. Overnight cultures of the helper, donor, and recipient bacteria strains were mixed in 1:1:1 ratio, and plated on NA medium without antibiotics. The mixed bacteria strains were grown overnight at 28 °C. The conjugated bacterial mixture was streaked on NA medium supplemented with Kanamycin 25 µg/ml, Tetracycline 10 25 µg/ml, and Rifampin 100 µg/ml, and incubated at 28 °C for 48 h. Single colonies grown on selection medium were picked up and tested on NA medium supplemented with Kanamycin 25 µg/ml, Tetracycline 10 µg/ml, and Rifampin 100 µg/ml, plus 5% sucrose. The colonies that are sensitive to 5% sucrose were

chosen for inoculation of 5 ml liquid NB medium supplemented with Rifampin 100 µg/ml and Kanamycin 25 µg/ml and grown at 28 °C till the concentration reached an A600 = 0.8-1. The bacterial cultures were diluted (1:1,000) into new liquid NB medium supplemented with Rifampin 100 µg/ml and Kanamycin 25 µg/ml for 12 h. The derived culture were then span down at low speed (3,000 x g) for 10 minutes at 4 °C and the bacterial pellet was plated on NA medium supplemented with Rifampin 100 µg/ml, Kanamycin 25 µg/ml and 5 % sucrose, and grown at 28 °C to select colonies that are resistance to Kanamycin and Rifampin but insensitive to 5 % sucrose. Twenty colonies were selected and re-streaked on NA plates (supplemented with Rifampin 100 µg/ml, Kanamycin 25 µg/ml and 5 % sucrose) with or without Tetracycline (10 µg/ml). Colonies that are sensitive to Tetracycline were selected for PCR analyses to detect the *SacB/R*, Tetracycline, Kanamycin genes, and the Aave2166 and Aave1548 genes using primers listed in Table 2.

Mellon seed transmission efficiency assay. Melon hybrid cantaloupe/muskmelon seeds were incubated for 2 h with gentle agitation in 50-ml tubes containing 10-ml suspensions of *A. citrulli* (~$0.1x10^6$). As a control, melon seeds were incubated in sterile distilled water for 2 h. Following incubation, melon seeds were collected and air-dried in a laminar flow hood for 4 h. Seeds were sown in 600-ml pots filled with peat (one seed per pot). The pots were kept in a greenhouse at 26-28°C for 10-14 days after which, the above ground parts of the seedlings were collected and their fresh weight was determined. We have previously shown that seedling fresh weight directly correlates with disease severity (Bahar, Kritzman et al. 2009). Disease severity was determined using a 0 to 7 scale, based on the wet weight of inoculated plants relative to the average weight of non-inoculated control plants (Ofir Bahar, Kritzman, & Burdman, 2009): 0, weight higher than 90% of average control weight; 1 to 5, weight equal to 76-90%, 61-75%, 46-

60%, 31-45% and 16-30% of average control weight, respectively; 6, weight equal to or lower than 15% of average control weight; 7, dead seedling.

Statistical analysis. In order to detect significant differences between bacterial strains in *in planta* virulence assays, the comparison of treatment means was analyzed by Ture-Kramer HSD multiple comparison procedure using the JMP software (version 7, SAS Inst., Cary NC).

Results

Testing the functionality of SacB/R as an effective counter selectable marker in *Aac*.

The *SacB/R* gene expression cassette has been used as a counter selectable marker in gene deletion strategy. In presence of high concentration of sucrose, *SacB/R* is toxic to many gram negative bacteria including *E. coli, Xanthomonas, Erwinia* and *Agrobacterium tumefaciens etc.* (M. Metz et al., 2005; Ried & Collmer, 1987; Traore & Zhao, 2011). However, it is not clear if the *SacB/R* gene expression cassette is also functional in *A. citrulli*. As preliminary test, the original *SacB/R* expression cassette from the pMSacB vector (Matthew Metz et al., 2005) was cloned into a broad host spectrum vector pVSP61 (Century et al., 1995). The derived vectors pVSP61-*SacB/R* vector was conjugated into *A. citrulli* strain M6 and AAC00-1. The derived *A. citrulli* strains were plated on NA medium supplemented with different concentration of sucrose. The original *SacB/R* expression cassette can inhibit bacterial growth but with high background colonies that are still tolerant to 5 % sucrose (Figure 1A). Increased concentration of sucrose (up to 30%) could not reduce the background colony growth. We hypothesized that the high background colony growth could be due to the inefficient expression of the *SacB/R* genes. To test this hypothesis the *SacB/R* gene was fused to the universal and strong *NptII* promoter (Han, Park, Lee, & Ronald, 2008). The new *Npt*II-*SacB/R* gene cassette was cloned into the Topo PCR8 G/W vector and then subcloned into pVSP61-Destination vector. The derived vector pVSP61-*Npt*II-*SacB/R* was transformed into *A. citrulli* strain AAC00-1 and M6. Both *A. citrulli* strains carrying pVSP61-*Npt*II- *SacB/R* plasmid were not able to grow on NB medium supplemented with 5% sucrose without any background colonies (Figure 1B).

Modification of the suicide vector pLVC18L by incorporating the new *Npt*II-pro-*SacB/R* gene expression cassette. The new *Npt*II-*SacB/R* gene expression cassette was subcloned into vector the pLVC18L (Staskawicz et al., 1987) (Figure 2). The derived clone was further modified by insertion of the Gateway cloning cassette frame A. The novel vector was designated as pLVC18L-*Npt*II-*SacB/R*-DesA. *E. coli* strain carrying pLVC18L-*Npt*II-*SacB/R*-DesA could grow on LB medium supplemented with Tetracycline but could not grow on medium supplemented with Tetracycline and 5 % sucrose (Figure 1C).

Knockout of the T3S effector Aave1548 by marker-exchange mutagenesis. To test the function of pLVC18L-*Npt*II-*SacB/R*-DesA, *A. citrulli* T3S effector gene *Aave1548* was selected for marker-exchange mutagenesis. As outlined in Figure 2, the fragment A (about 1. 3Kb) upstream and part of the open reading frame (ORF) of Aave1548 and fragment B (about 1.4Kb) downstream of Aave1548 from the AAC00-1 genomic DNA were amplified. A kanamycin resistant gene expression cassette was inserted between fragment A and B. The Kanamycin gene cassette was flanking by the FTR sites that can be recognized by the flipase (Cherepanov & Wackernagel, 1995) for later removal of the antibiotic marker genes. The vector pLVC18L-*Npt*II-*SacB/R*-ΔAave1548 was conjugated into *A. citrulli* strain AAC00-1. The AAC00-1 strain with integration of the suicide plasmid pLVC18L-*Npt*II-*SacB/R*-ΔAave1548 can grow on NA medium supplemented with kanamycin, Tetracycline, but not on NA medium supplemented with Tetracycline and 5 % sucrose (Figure 3A). This result confirmed the insertion of the plasmid into the genome of AAC00-1. Therefore, the new *Npt*II-*SacB/R* expression cassette is functional in *A. citrulli*. To induce the double crossover event, the AAC00-1 derived strains from the single crossover event, were sub-cultured twice on liquid medium supplemented with kanamycin. Mutant strains which lost the plasmid backbone pLVC18L-*Npt*II-*SacB/R* were

sensitive to Tetracycline but resistant to Kanamycin and 5 % sucrose (Figure 3B). These marker-exchanged AAC00-1 clones were designated AAC00-1-ΔAave1548.

To further confirm the marker-exchanged mutant clones, PCR analyses with primers specific to the Kanamycin, Tetracycline, and Aave1548 genes were performed. As shown in Figure 4, the selected mutant strains carry the Kanamycin gene, but not the Tetracycline and the *Aave1548* genes. Therefore, we confirmed the partial deletion of the *Aave1548* open reading frame.

Construction of the pEDV6/NptII-SacB/R/Flipase vector. The Kanamycin resistance gene used for marker-exchange mutagenesis was flanked by the FTR sites, a repeat sequence that can be recognized by the Flipase enzyme, allowing the removal of the targeting marker gene for further gene knock out analysis (Figure 2). We tested if a modified pBBR1_FLP2 (Gentamycin resistance) (Jittawuttipoka et al., 2009b) can be used to express the Flipase enzyme in mutant *A. citrulli* strain AAC00-1-ΔAave1548. Unfortunately, transformed AAC00-1-ΔAave1548 strains carrying pBBR1_FLP2 (Gentamycin resistance) were always Kanamycin resistant even after many cycle of subculture (Data not shown). Therefore, we conclude that the flipase on pBBR1_FLP2 (Gentamycin resistance) might not be express in *A. citrulli* because of the promoter. To solve this problem, a plasmid pEDV6-*Npt*II-SacB/R-*Npt*II-Flipase was constructed where the Flipase gene is driven by the *Npt*II promoter. With the aim to facilitate the removal of the Flipase construct after the removal of the antibiotic marker gene, we also cloned the counter selectable marker *Npt*II-*SacB/R* gene expression cassette along with the *Npt*II-Flipase as described in the material and methods section. The derived vector pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flp was illustrated in Figure 2.

The new vector pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flipase was conjugated into mutant strain AAC00-1-ΔAave1548 and further selected on NA medium supplemented with Gentamycin (a Gentamycin resistance gene was used as the selection marker on vector pEDV6). As shown in Figure 5 B&C, the derived AAC00-1colonies were also sensitive to Kanamycin, suggesting the removal of Kanamycin resistance gene cassette. The absence of Kanamycin gene was confirmed by PCR analysis using Kanamycin specific primers (Figure 5 A, D&E).

Removal of the pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flpase plasmid from the transformed *A. citrulli* strains. To further delete other T3S effector genes from the mutant strain AAC00-1-ΔAave1548; the plasmid pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flpase needed to be cured from the transformed AAC00-1-ΔAave1548 strain. To this end, AAC00-1-ΔAave1548 (pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flpase) was selected on NA medium supplemented with 5% sucrose only. Unexpectedly, pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flpase could not be recovered in any viable clones even after long incubation periods, or with sequential subcultures before the selection on sucrose medium (data not shown). The use of other methods including high temperature and subcultures didn't enhance the removal of plasmid pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flpase.

Previous reports suggested that the use of acridine orange hermizine in liquid culture medium could enhance the elimination of plasmid from *E. coli* strains (Hara, Aumayr, Fujio, & Ueda, 1982; Zaman et al., 2010). To test if the supplemental of acridine orange in NB liquid culture could facilitate the removal of pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flpase from *A. citrulli* strain AAC00-1-ΔAave1548, the clones were grown on liquid culture supplanted with acridine orange (75g/ml). The overnight culture of AAC00-1-ΔAave1548 strains was plated on NA medium supplemented with 5% sucrose. A lot of single clones were grown on NA medium supplemented with 5% sucrose suggesting the successful removal of the pEDV6-*Npt*II-*SacB/R*-*Npt*II-Flpase

plasmid. About 1 out of 1,000 AAC00-1 lost the plasmid after an overnight liquid culture in medium supplemented with acridine orange (75g/ml). Selected single clones were further confirmed to be sensitive to Gentamycin (50µg/ml) (data not shown).

Knockout of the T3S effector Aave2166 gene in AAC00-1 and AAC00-1-ΔAave1548 mutant strain. To generate a mutant *A. citrulli* strain with deletions of two effector genes, Aave2166 and Aave1548, the pLVC18L-*Npt*II-*SacB/R*-ΔAave2166 was conjugated into mutant strain AAC00-1-ΔAave1548. Marker-exchange mutagenesis was performed as described previously to delete the *Aave2166* mutant in the AAC00-1 background. The deletion of Aave2166 was confirmed by PCR amplification of Aave2166 and Kanamycin resistance genes (Figure 6 A, B &C).

Virulence assay of the single and double effector knock-out *A. citrulli* strains using a melon seed transmission assay. The wild type AAC00-1, AAC00-1-ΔAave1548, AAC00-1-ΔAave2166, and AAC00-1-ΔAave1548-ΔAave2166 strains were inoculated on melon seeds and their seed transmission efficiency was evaluated. As summarized in Figure 7 and Table 2, the deletion of *Aave1548* significantly reduced disease severity, while deletion of *Aave2166* alone has no significant effect on the virulence of *A. citrulli.*

Summary

The Gram-negative pathogenic bacterium *A. citrulli* causes bacterial fruit blotch disease of different cucurbit species including watermelon, melon and cucumber etc. As a relative new disease identified in the field, current research has focused on the identification and characterization of virulence related genes in *A. citrulli*. However these studies have been hampered by the lack of efficient tools and methods for gene replacement in *A. citrulli*. Redundancy of function of different bacterial genes also makes it difficult to identify virulence related genes in *A. citrulli*. Here, we described an optimized pipeline that allowed us to

sequentially delete two bacterial T3S effector genes in *A. citrulli*. The disease assay using the mutant *A. citrulli* strains allowed us to identify one T3E effector, Aae1548, as important for seed transmission efficiency. The new vectors and the optimized marker-exchange mutagenesis protocol may be valuable for studying other phytopathogenic bacterial species.

Table Legends

Table 1.

List of primers used for detection of the AAC00-1 mutant strains

Primer Name	Primer Sequence	Note
Tetra For	ATGAAATCTAACAATGCGCTCAT	For detect the tetracycline resistance gene
Tetra Rev	TACGAGTTGCATGATAAAGAAGACA	
Kan For	5′-**cacgtcttgagcgattgtgtagg**-3′	
Kan Rev	5′-GACATGGGAATTAGCCATGGTCC-3′	
Aave1548 flanking For	5'-caccTCTAGAATGCCTCTACAGTCCATTTCCAT-3'	
Aave1548 flanking rev	5'-GTCGACTGGTTGATCCCCCGTCCGAGCAT-3'	
P1	5′-cccggatcgaaaggcgagcgcacg-3′	
P2	5'-caccTCTAGAATGCCTCTACAGTCCATTTCCAT-3'	
P3	5'-GTCGACTGGTTGATCCCCCGTCCGAGCAT-3'	
P4	5′-GGTGAGCCAGTGGAAGCGCGCCCGG-3′	
Aave2166 For	5'-cacc**AGATCT**ATGAAGAATTTCATGCGATCGAT-3′	
Aave2166 Rev	5′-**GTCGAC**TTCGATAGCTTTTCTGATTTTTCTCA-3′	

Table 2
Seed Transmission Assay of Wild Type Strains AAC00-1 and Mutant Strains Impaired on Certain Effectors on Melon

Bacterial Strains	Plant Weight[a] (mg)	Disease Severity[b]
AAC00-1	186±42 [A]	3.9±0.22
AAC00-1/*Δ Aave1548/ ΔAave2166*	439±82[B]	1.25±0.7
AAC00-1/*ΔAave2166*	218223±43 [A]	3.3±0.61
AAC00-1/*Δ Aave1548*	448±72[B]	0.71±0.49
Control (H_2O)	471±61[B]	0

[a] Hybrid cantaloupe/muskmelon seeds were used for AAC00-1 inoculation. All inoculated and control seeds were incubated in the bacterial inoculum ($1x10^6$ CFU/ml for the hybrid seeds) for two hours. Infected seed were blot dried and planted in four per pots two pots each. The weights of the plantlets were measured at 12 days post inoculation and compared to the control (H_2O).
[b]Disease severity is scored on a scale of 0 to 7, based on plant weight values of inoculated plants relative to the average shoot weight of non-inoculated controls (Bahar et al. 2008): 0, weight higher than 90% of average control weight; 1 to 5, weight equal to 76-90%, 61-75%, 46-60%, 31-45% and 16-30% of average control weight, respectively; 6, weight equal to or lower than 15% of average control weight; 7, dead seedling. Data was presented as Mean ± Standard Error, n=16.

[c]Different letters indicate significant difference between treatments ($P \leq 0.05$) by Turkey-Kramer HSD test.

Figure Legends

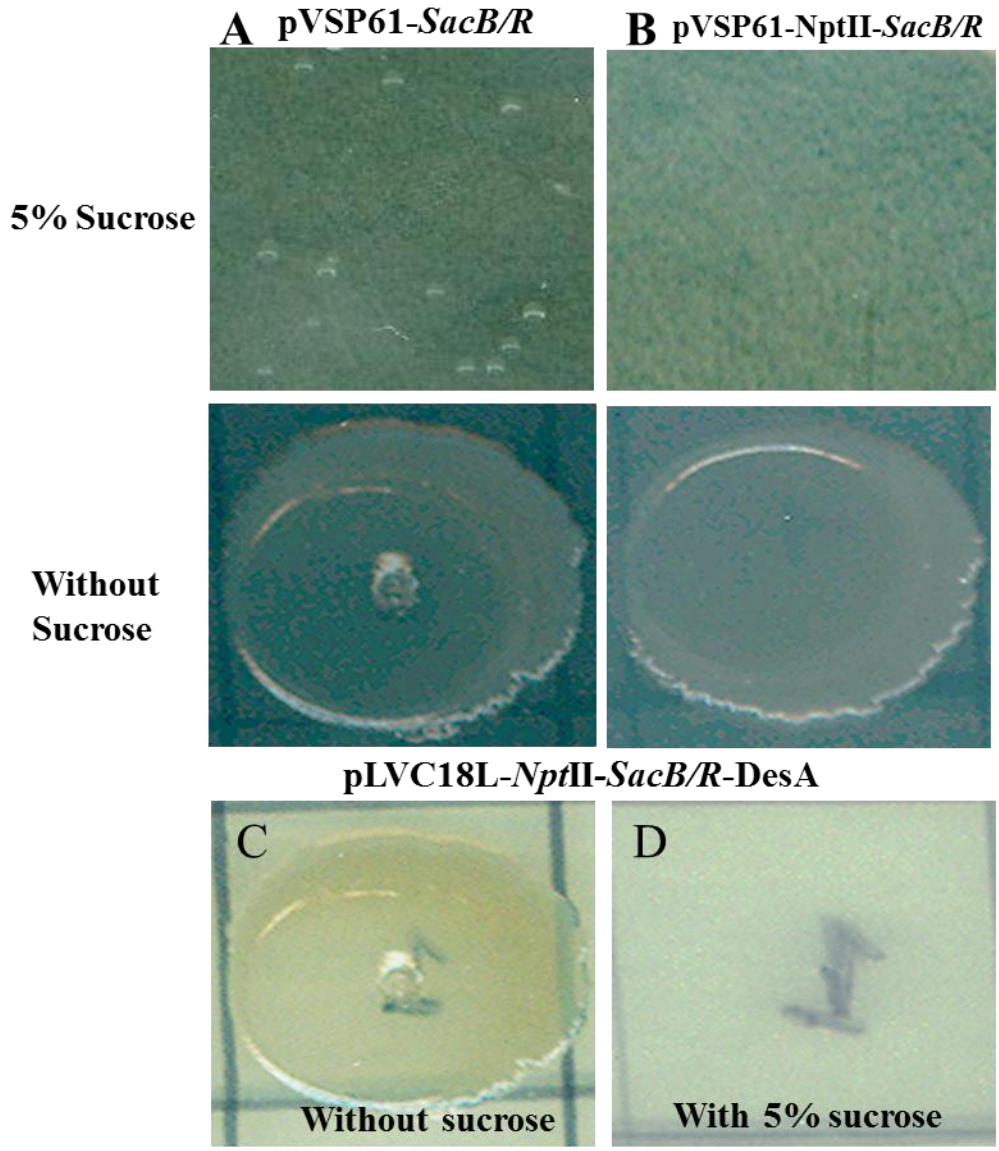

***Figure* 1. The *Npt*II-SacB/R cassette has improved counter selective activity. (A)** Some *A. citrulli* (AAC00-1) cells carrying the original SacB/R cassette can grow on NA medium supplemented with 5 % sucrose. (**B)** *A. citrulli* (AAC00-1) cells expressing the *Npt*II-*SacB/R* cassette are completely inhibited by 5% sucrose. Pictures were taken at 3 days after incubation at 28C. **(C) &(D)** The growth of *E. coli* strain *ccdB* survivor (Invitrogen) carrying pLVC18L-NptII-Pro-SACb/R-DesA can grow on LB supplemented with Tetracycline (**C**) but cannot grow on LB supplemented with Tetracycline and 5% sucrose.

All bacterial strains were adjusted to $1x10^8$ CFU/ml and 50 ul bacterial culture was spotted on NA or LB plate supplemented with appropriate antibiotics with or without sucrose.

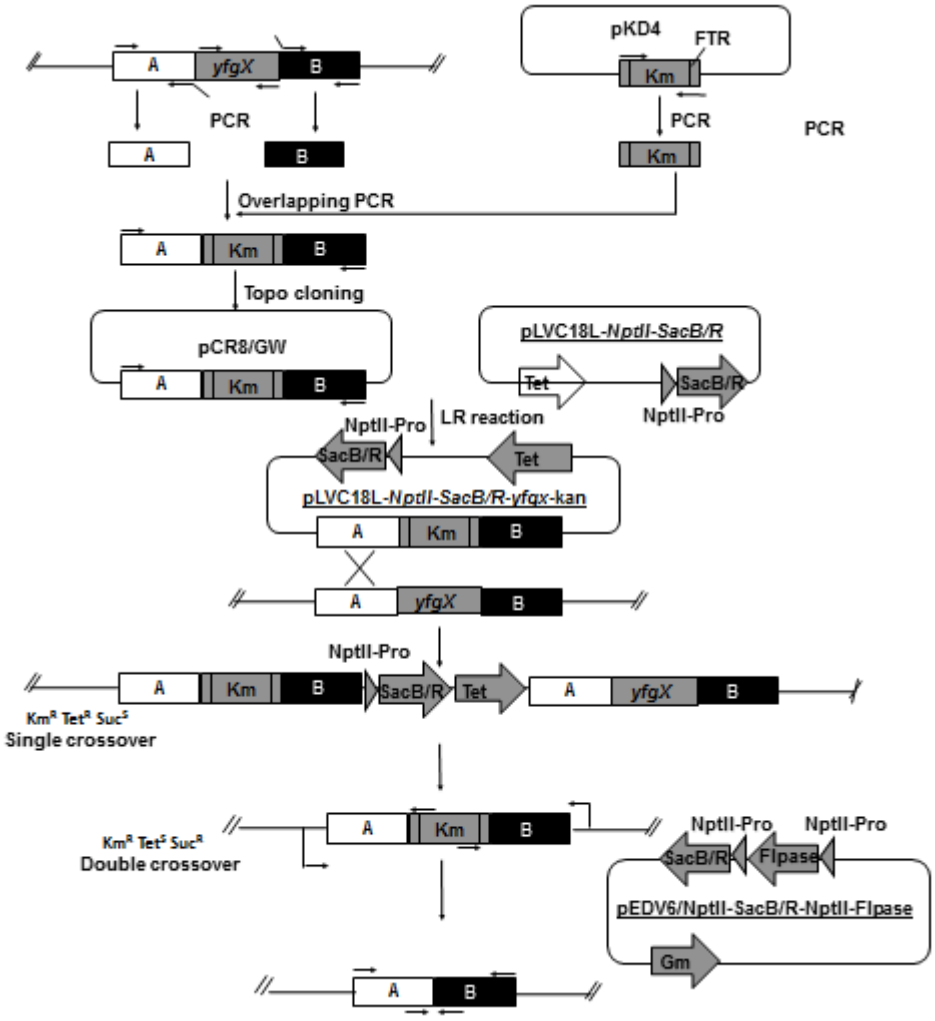

***Figure 2*: Diagram explaining the steps of single and multiple gene knockout. (**A) Fragment upstream of gene of interest. (B) Fragment downstream of gene of interest. (YFP) Your favorite gene X. (Km) kanamycin resistant gene flanked by the FTR sites. (FLP) Flipase gene.

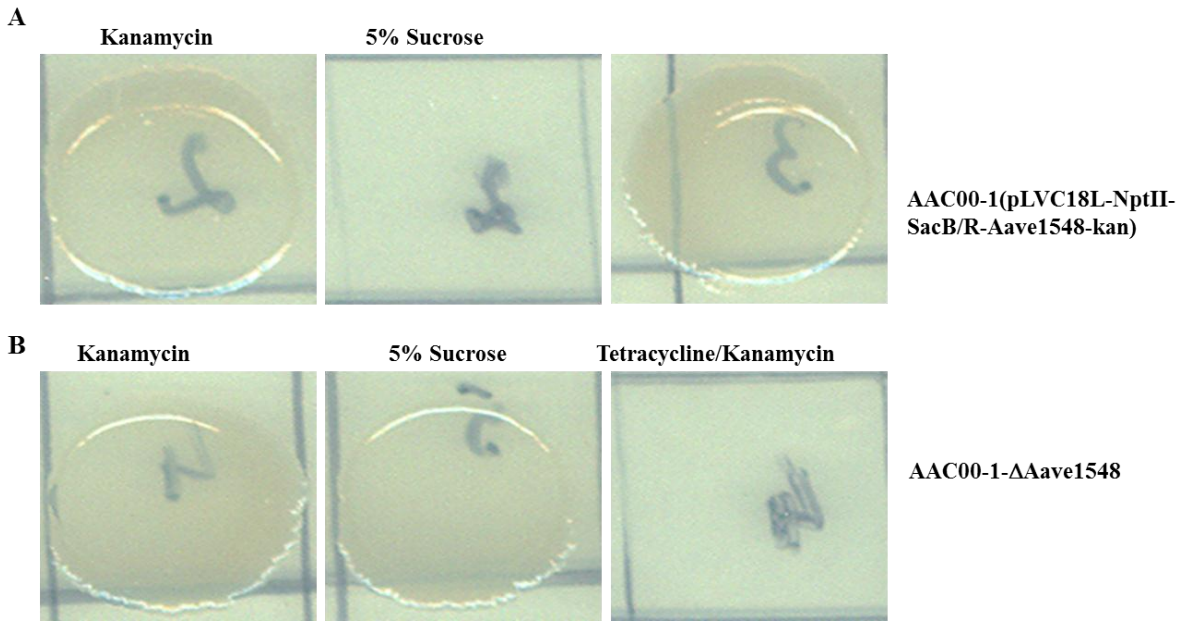

***Figure* 3**. **AAC00-1(pLVC18L-NptII-SacB/R-Aave1548-kan) and AAC00-1-ΔAave1548 grown on medium supplemented with different selection reagents.** (**A**) Bacterial cells of AAC00-1(pLVC18L-NptII-SacB/R-Aave1548-kan) are resistant to Kanamycin and tetracycline, but sensitive to 5 % sucrose. (**B**) Bacterial cells of AAC00-1-ΔAave1548 are resistant to Kanamycin and 5 % sucrose, but sensitive to tetracycline.

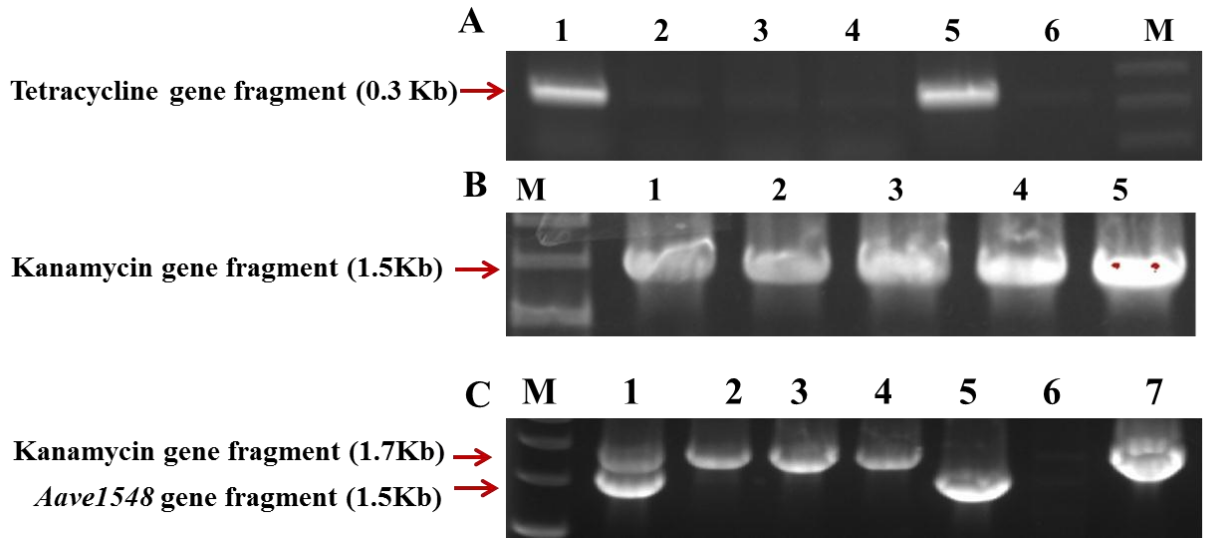

***Figure* 4. *Aave1548* was replaced with a kanamycin resistance gene in mutant strain AAC00-1-ΔAave1548-kan.** (**A**) A Tetracycline resistance gene fragment was detected from the genomic DNAs of AAC00-1 derived mutants. **M**: 1Kb marker; **1**: single crossing over mutant; **2-4**: double crossing over mutants**; 5.** pLVC18L-NptII-SacB/R-Aave1548-kan plasmid DNA control **6**: AAC00-1 gDNA control. (**B**) Detection of the Kanamycin gene from AAC00-1 derived mutants. : **M**: 1Kb marker; **1**: Single crossing over mutant; **2-4**: double crossing over mutants**; 5.** pLVC18L-NptII-SacB/R-Aave1548-kan plasmid DNA control. (**C**) Replacement of the Aave1548 with a Kanamycin resistance gene was detected using primers that are flanking Aave1548 open reading frame. **M**: 1Kb marker; **1**: single crossing over mutant; **2-4**: double crossing over mutants**; 5:** AAC00-1 gDNA control **6**: H20 **7:** pLVC18L-NptII-SacB/R-Aave1548-kan plasmid DNA control.

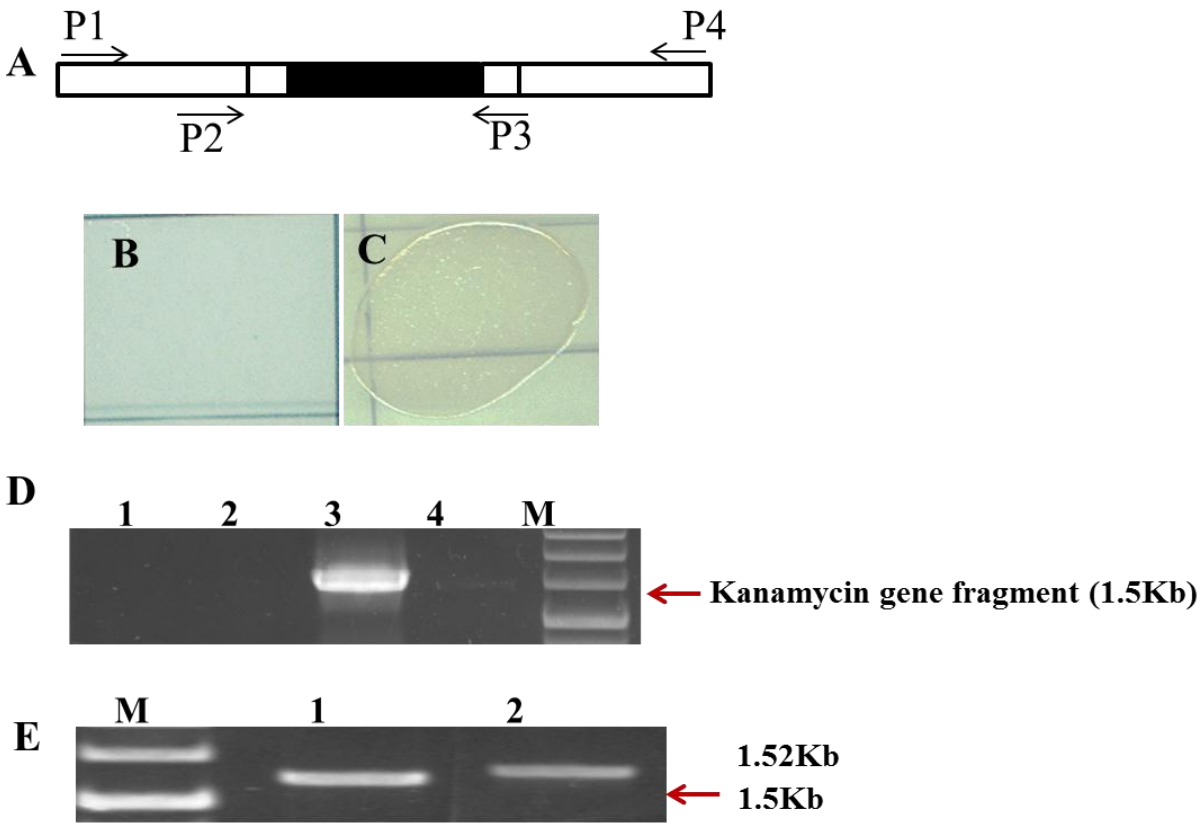

***Figure* 5. FLP-mediated excision of antibiotic resistance gene.** **(A)** Diagram showing the location of primers used to confirm the excision of the kanamycin gene.**(B)** AAC00-1-ΔAave1548 (pEDV6-NptII-SacB/R-NptII-Flp) is kanamycin sensitive. **(C)** AAC00-1-ΔAave1548-kan is kanamycin resistant**. (D)** Amplification of the kanamycin resistance gene from AAC00-1 derived mutants. The DNA templates used for PCR: lane 1-2 are two clones of AAC00-1-ΔAave1548 (pEDV6-NptII-SacB/R-NptII-Flp); lane 3 is AAC00-1-ΔAave1548-kan; lane 4 is the wild type AAC00-1; M: 1kb marker. **(E)** The up- and downstream DNA fragments flanking Aave1548 in mutant strain AAC00-1-ΔAave1548 were detected using specific PCR primers. Lane 1 is showing the upstream DNA fragment of Aave1548 that was amplified with primers P1 and P3; lane 2 is showing the downstream DNA of Aave1548 that was amplified with primers P2 (**A**). and P4 M: 1Kb marker.

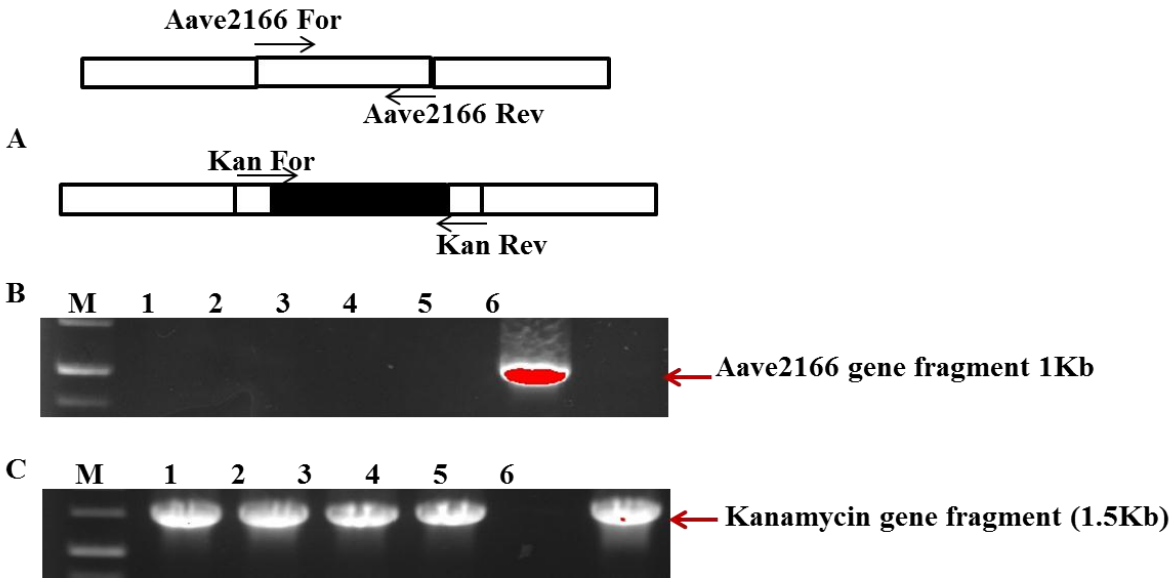

***Figure* 6. The Aave2166 gene in mutant strain AAC00-1-ΔAave1548-ΔAave2166 was replaced with a kanamycin resistance gene.** **(A)** Diagram showing the location of primers used to detect Aave2166 and the Kanamycin resistance gene. **(B)** Detection of the Aave2166 gene using specific primers (Aave2166For and Aave2166Rev) in four double-deletion mutant AAC00-1-ΔAave1548-ΔAave2166 and wild type AAC00-1 strains. **(C)** Amplification of the kanamycin resistance gene (Kan For and Kan Rev) from four double-deletion mutant AAC00-1-ΔAave1548-ΔAave2166 and wild type AAC00-1 strains. The DNA template used for PCR analysis: lanes 1-4 are four AAC00-1-ΔAave1548-ΔAave2166 mutant clones; lane **5** is the wild type AAC00-1 control; lane 6 control plasmid pLVC18L-NptII-Pro-SacB/R-ΔAave2166. M: 1Kb marker.

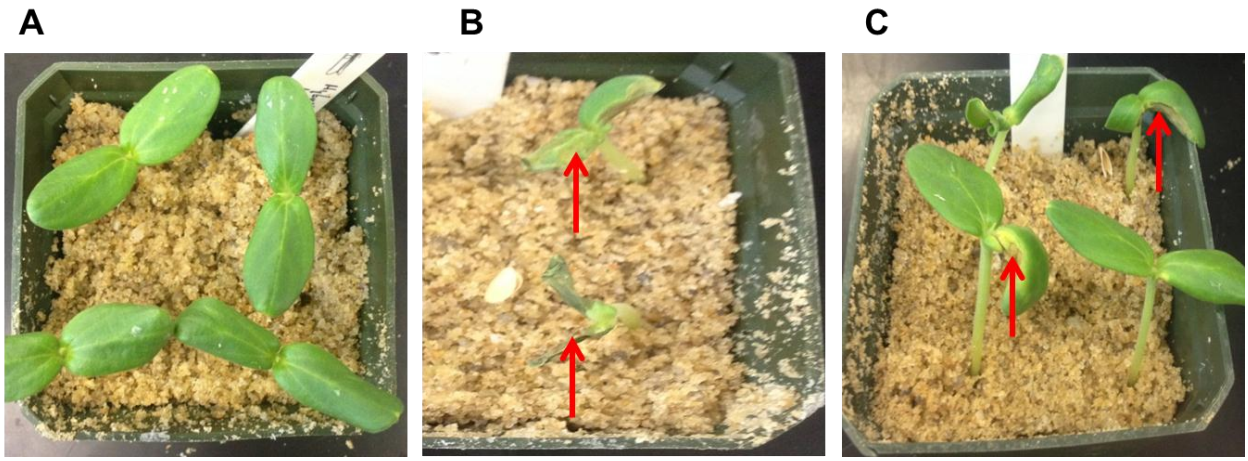

***Figure* 7. Evaluate the virulence of *A. citrulli* mutant strains by seed transmission efficiency assay.** (**A**) hybrid cantaloupe/muskmelon seeds were incubated with water for two hours before planting. (**B**) hybrid cantaloupe/muskmelon seeds were incubated with wild type AAC00-1 strain for two hours before planting. (**C**) hybrid cantaloupe/muskmelon seeds were incubated with the AAC00-1-ΔAave1548-ΔAave2166 double mutant strains for two hours before planting. Red arrows are showing the symptoms of BFB on cotyledons. Pictures were taken at 12 days post inoculation.

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Chapter 4: Employing *Nicotiana benthamiana* as a Surrogate Host for Studying the Pathogenicity of *Acidovorax citrulli*, the Causal Agent of Bacterial Fruit Blotch of Cucurbits

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Noam Eckshtain-Levi[2], The Hebrew University of Jerusalem, Rehovot, Israel; contributed in this research by performing the deletion of effector genes in M6 genome.

Anita Castro Sparks[3], [3]Department of Plant Pathology, University of Georgia, Athens, GA, USA, investigated the presence of the T3S genes in different strains of *A. citrulli*

Saul Burdman[2], The Hebrew University of Jerusalem, Rehovot, Israel, is co-PI and contribute in the writing and revision of the manuscript.

Ron Walcott[3], [3]Department of Plant Pathology, University of Georgia, Athens, GA, USA, is co-PI and contribute in the writing and revision of the manuscript.

Gregory E. Welbaum[1], [1]Department of Horticulture, Virginia Polytechnique and State University, Blacksburg, USA, , is co-PI and contribute in the writing and revision of the manuscript.

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Abstract

Acidovorax citrulli is the causal agent of bacterial fruit blotch (BFB) of cucurbits. To date chemical and other cultural practices have been ineffective in managing BFB. Despite its significant agricultural importance, the mechanisms associated with *A. citrulli* pathogenicity

have not been extensively investigated. Additionally, complete genetic resistance resources for BFB have not yet been identified from cucurbit germplasm. In this study, we explored the use of the model plant species *Nicotiana benthamiana* and *Nicotiana tabacum* as surrogate hosts for studying *A. citrulli* virulence and non-host resistance triggered by bacterial type III-secreted (T3S) effectors. Here we show that two *A. citrulli* strains, M6 and AAC00-1, that represent the two major groups among *A. citrulli* populations, can induce disease symptoms on *N. benthamiana*, but trigger hypersensitive response (HR) on *N. tabacum* plants. Moreover, we show that these reactions were associated with certain *A. citrulli* T3S effectors. We isolated 19 genes encoding putative T3S effectors from the genomes of strains AAC00-1 and M6. Transient expression in *N. benthamiana* and *N. tabacum* leaves revealed that three effectors, Aave1548, Aave2708, and Aave2166, triggered water-soaking-like cell death in *N. benthamiana* and HR in *N. tabacum*. Knockout mutations of effector genes, *Aave1548* and *Aave2166* in strains M6 and AAC00-1 reduced their virulence on *N. benthamiana* and melon. This suggested that *N. benthamiana* can be used to study a range of *A. citrulli*-host interactions that contribute to virulence. Both Aave1548 and Aave2166 effectors triggered non-host HR in *N. tabacum*, and this reaction depended on the functionality of the immune signaling component, *NtSGT1*. Based n these observations, identification and transfer of *N. tabacum* disease resistance gene(s) to cucurbits may offer a new strategy for controlling BFB in the future.

Keywords: *Acidovorax citrulli*, non-host resistance, type III effectors, effector triggered immunity, seed transmission

Introduction

Bacterial fruit blotch (BFB) of cucurbit is caused by the seed-borne Gram- negative phytobacterium *Acidovorax citrulli* (formerly *A. avenae* subsp. *citrulli*) (Schaad et al., 2008). *A. citrulli* gained importance after severe outbreaks in watermelon fields in several US states in the late 1980s'. Subsequently, the pathogen has spread to many parts of the world, mainly by seed transmission, and has become a serious threat to the cucurbit industry worldwide, (Burdman & Walcott, 2012). Several research groups have screened plant germplasm for resistance to BFB. Although several cucurbit germplasm lines have been reported to be partially tolerant to *A. citrulli* (Bahar, Kritzman, & Burdman, 2009; Wechter et al., 2011), to date, no cucurbit lines have been identified with complete resistance. Hence all commercial varieties of watermelon and melon are susceptible to *A. citrulli*. Despite the economic importance of BFB, little is known about the molecular basis of *A. citrulli*-cucurbit interactions that contribute to pathogenicity and virulence (Bahar O. & S., 2010; Burdman & Walcott, 2012).

Two evolutionary lineages of *A. citrulli* have been identified through DNA fingerprinting and Multi Locus Sequence Typing analyses (Feng, Schuenzel, Li, & Schaad, 2009; Walcott et al., 2000). Group I *A. citrulli* strains have a broad host range and have been isolated from various cucurbit species, but mainly from melon (S. Burdman et al., 2005; Walcott et al., 2004). M6, an *A. citrulli* strain isolated from melon plants in Israel, is representative of group I. In contrast, group II strains are highly virulent on watermelon but less virulent on melon and other cucurbit species (Eckshtain-Levi et al., 2014; Walcott et al., 2004). The group II representative strain AAC00-1 was isolated from watermelon in the United States and its genome has been completely sequenced (GenBank accession NC_008752).

Annotation of the AAC00-1 genome revealed the presence of genes encoding components of a type III secretion system (T3SS), which, in many Gram-negative plant pathogenic bacteria, is responsible for secretion of effector proteins into the cytosol of their hosts (Hueck, 1998). Most T3S effectors characterized so far contribute to suppression of host immunity in susceptible plants (Alfano & Collmer, 2004), while others can be recognized by specific plant disease resistance (*R*) genes to elicit effector triggered immunity (ETI) in resistant plants. Plant *R* genes mostly encode NB-LRR type proteins (Staal, Kaliff, Bohman, & Dixelius, 2006; Tameling & Baulcombe, 2007; Van Der Biezen & Jones, 1998) and their functionality frequently requires the conserved immune signaling component, SGT1 (Peart et al., 2002). Impairment of the *A. citrulli* T3SS abolished its ability to trigger HR in non-host plants (i.e., tobacco and tomato) as well as pathogenicity in watermelon and melon plants (Bahar & Burdman, 2010; K. Johnson et al., 2011; ZhengGuang et al., 2009).

Annotation of the *A. citrulli* AAC00-1 genome also revealed at least eleven putative T3S effector genes, based on their homology to known effectors in other bacterial species (Eckshtain-Levi et al., 2014). Comparative analyses of the eleven effector genes from 22 *A. citrulli* strains indicated that group I and II strains of *A. citrulli* have evolved different T3S effector repertoires (Eckshtain-Levi et al., 2014). It was proposed that differences in the repertoire of T3S effectors between the groups may contribute to observed differences in preferential host associations between these two lineages. However, thus far, none of these putative effectors have been functionally characterized in regard to their contribution to virulence on susceptible host plants or ability to trigger ETI on resistant plants.

Various model plant species have been used for studying important plant diseases (Glazebrook, Rogers, & Ausubel, 1997; Goodin, Zaitlin, Naidu, & Lommel, 2008). For instance,

the tomato pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000, has been found to be virulent on certain genotypes of *Arabidopsis thaliana* (Dangl et al., 1992; Dong, Mindrinos, Davis, & Ausubel, 1991; M. C. Whalen, Innes, Bent, & Staskawicz, 1991). Subsequently, the molecular interactions between *A. thaliana* and *Pst* DC3000 have been extensively studied, leading to a detailed understanding of the molecular mechanisms of bacterial virulence and plant immunity. Tobacco species, including *Nicotiana benthamiana* and *Nicotiana tabacum*, have been used to study virus-host interactions for several decades, largely because of their susceptibility to various virus species (Goodin et al., 2008). *N. benthamiana* has also been used to study other plant pathogens including bacteria (M. Metz et al., 2005; Wei et al., 2007), fungi (Dean, Goodwin, & Hsiang, 2005; Rivas-San Vicente, Larios-Zarate, & Plasencia, 2013) and oomycetes (Chaparro-Garcia et al., 2011). In addition, both *N. benthamiana* and *N. tabacum* can be easily transformed by *Agrobacterium tumefaciens*, allowing efficient protein expression through *Agrobacterium*-mediated transient assays. Tobacco rattle virus (TRV)-based virus-induced gene silencing has been developed to efficiently silence *N. benthamiana* genes that may be involved in immunity (Zhu & Dinesh-Kumar, 2009). Recently, the draft genomes of *N. benthamiana* and *N. tabacum* have been determined (Bombarely et al., 2012). All these proprieties make *N. benthamiana* and *N. tabacum* attractive model plants for studying host-pathogen interactions.

In this study the goals were (1) to determine if tobacco could serve as a surrogate host for studying pathogenicity mechanisms of *A. citrulli*, and (2) to characterize the virulence and avirulence functions of selected T3S effector genes of *A. citrulli* on *N. benthamiana* and *N. tabacum*. Here we demonstrate that both *A. citrulli* strains M6 and AAC00-1 can induce disease

symptoms on *N. benthamiana* and that the susceptibility of *N. benthamiana* to *A. citrulli* is T3S effector-dependent.

Cloning and transient expression of 19 putative T3S effector genes from AAC00-1 and M6 showed that three effectors, Aave1548, Aave2708 and Aave2166, could trigger water-soaking-like cell death in *N. benthamiana*. Deletion of *Aave1548* in M6 and AAC00-1, and *Aave2166* in AAC00-1 reduced virulence of these strains in *N. benthamiana*. Furthermore, strains M6 and AAC00-1 lacking a functional *Aave1548* displayed a significant reduction of virulence on cucurbit plants. Altogether, our data indicate that *N. benthamiana* can be used as a surrogate host for studying *A. citrulli* pathogenicity. In addition, transient expression of effectors Aave1548 and Aave2166 triggered HR-like cell death in *N. tabacum*. Moreover, transient expression of Aave1548 and Aave2166 as well as M6 and AAC00-1 inoculation onto *NtSGT1* silenced lines of *N. tabacum* inhibited the HR phenotype triggered by M6 and ACC00-1 strains or effectors Aave1548 and Aave2166 in wild type *N. tabacum*. This is the first report to elucidate the biological functions of *A. citrulli* T3S effectors. Employing *Nicotiana* species as surrogate hosts for studying the pathogenicity of *A. citrulli* may help to elucidate the function of *A. citrulli* T3S effectors and facilitate the development of new strategies for BFB management.

Materials and Methods

Bacterial strains, plasmids and plant material. Bacterial strains and plasmids used in this study are listed in Table 1. *N. benthamiana* PI 555478 and *N. tabacum* cv. Samsun-NN plants were propagated from seed in a growth chamber programmed for 16 h light (140 µmol/m^2/s cool white fluorescent irradiance) at 28°C and 8 h dark at 25°C.

***Agrobacterium*-mediated transient assays in tobacco plants.** The open reading frames (ORFs) of putative T3S effector genes were PCR-amplified from AAC00-1 and M6 genomic

DNAs using the primers listed in Table S1. The amplified DNA fragments were cloned into the Topo ENTR/D vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The sequences of all cloned genes, including the construct described below, were verified by DNA sequencing at the core facility of the Virginia Bioinformatics Institute, Blacksburg, VA. The effector genes were subcloned into the plant expression vector pEG101-SacB/R/R (Traore & Zhao, 2011) by LR reaction (Invitrogen). The pEG101-SacB/R/R vector has a C-terminal YFP fusion. After subcloning, the effector genes were fused with the N-terminal of YFP (Traore & Zhao, 2011). Recombinant plasmids were electroporated into *Agrobacterium tumefaciens* strain GV2260 as described previously (Traore & Zhao, 2011).

Transient expression assays in *N. benthamiana* or *N. tabacum* plants were performed as described previously (Traore & Zhao, 2011). Briefly, the *Agrobacterium* strains were streaked onto LB agar medium supplemented with appropriate antibiotics and incubated at 28°C for 2 days. Bacterial cells were harvested and resuspended in induction buffer [10 mM $MgCl_2$, 10 mM MES (pH 5.6) and 100 µM acetosyringone] and incubated for 3 h at room temperature. Bacterial cell suspensions were then adjusted to OD_{600}=0.6 and infiltrated into substomatal spaces of fully expanded *N. benthamiana* leaves using a 1-mL blunt-end syringe. The inoculated plants were incubated at 25°C under continuous light for 20-48 h before observation of expressed proteins or cell death phenotypes. The fluorescent signal of the effector-YFP fusion protein was observed 20 h after inoculation by fluorescence microscopy (Zeiss Axio Observer.A1, Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Knockout of T3S effectors by marker-exchange mutagenesis and mutant strain complementation. To knockout *A. citrulli* T3S effectors, 1.4 kb and 1.5 kb DNA fragments from regions flanking the ORFs of effector genes, *Aave2166* and *Aave1548*

were amplified using the following primers: Aave2166_f1 and Aave2166_R2 (upstream region); Aave2166_f5 and Aave2166_R6 (downstream region) for gene *Aave2166*; and Aave1548_f1 and Aave1548_R2 (upstream region); Aave1548_f5 and Aave1548_R6 (downstream region) for gene *Aave1548* (Table S2). A kanamycin resistance gene (*nptII*) was amplified from pDSK519-GFP (Matthysse, Stretton, Dandie, McClure, & Goodman, 1996) with primers kan_for and kan_rev (Table S2). The two flanking fragments from both genes were then fused to the *nptII* gene by overlap PCR (Higuchi, 1990). The derived cassettes were cloned into the PCR8/GW-Topo vector (Invitrogen), and cloned into the suicide vector pLVC18L-Des (Staskawicz et al., 1987; Zhao, Dahlbeck, Krasileva, Fong, & Staskawicz, 2011) by using the LR clonase (Invitrogen). The derived construct was then mobilized into *A. citrulli* strains by tri-parental mating as previously described (Ditta, Stanfield, Corbin, & Helinski, 1980). Double crossover mutants were selected using marker-exchange mutagenesis as previously reported (Zhao et al., 2011). *A. citrulli* strains that contained mutant genes were selected on Nutrien agar (NA) medium supplemented with rifampicin (100µg/ml) and kanamycin (50µg/ml). To complement the knockout mutants, the *Aave1548* effector gene, including its native promoter and ORFs, was amplified by PCR using the following primers: Aave_1548comp for and Aave_1548comp rev (Table S2). The PCR products were cloned into the PCR8/GW-Topo vector (Invitrogen), and then sub-cloned into the broad host range vector pVSP61-Des-GM (Century et al., 1995). Successful transformants were selected on NA supplemented with gentamycin at 50 µg/ml. The derived constructs were then mobilized into *A. citrulli* by conjugation.

An M6 mutant impaired in *Aave1548* was generated as previously described (Bahar et al., 2009a). An internal fragment of Aave1548 (which does not span the 3′ and 5′ ends of this gene) was PCR amplified with suitable primers (Table S2). The PCR product was cloned into pTZ57R/T (Fermentas), verified by sequencing, excised with appropriate restriction enzymes, and then cloned into pJP5603 (Penfold & Pemberton, 1992) conferring kanamycin (Km) resistance. Transformation into strain M6 was carried out as described previously (O. Bahar et al., 2009a). Putative mutants were selected on NA with Km and verified by Southern blot.

Virulence assays on *N. benthamiana* and *N. tabacum* plants. *A. citrulli* strains AAC00-1, M6, the derived mutants and complemented strains, as well as *Pseudomonas syringae* pv. *tabaci* (*Pta*11528) were grown on NA supplemented with rifampicin (100 µg/ml) at 28ºC for 48 h before inoculation. On the day of inoculation, bacterial cells were resuspended in 10 mM $MgCl_2$. The inoculum concentration was adjusted to OD_{600}=0.3 (~ 0.3 x 10^8 CFU/ml) for the HR assay and ~ 0.2 x 10^{-5} CFU/ml) for the infiltration-based virulence assay. For spray inoculation, the bacterial concentration was adjusted to OD_{600} =0.2 (~ 2 x 10^7 CFU/ml) and 0.01% of Silwet L-77 was added to the cell suspensions Before inoculation, four-week-old *N. benthamiana* and *N. tabacum* plants were covered with plastic bags for 12 h to maintain high relative humidity. The plants were then spray-inoculated using a mini spray bottle and covered with a plastic bag for 24 h. Inoculated plants were maintained in a growth chamber as described above. Two hours after inoculation, spray-inoculated leaves were harvested, soaked into 5% hydrogen peroxide solution for 2 minutes, and then washed in distilled water at least three times before collecting the leaf disks (Ishiga, Ishiga, Uppalapati, & Mysore, 2011). Leaf disks were used for bacterial extraction and quantification of the bacterial population by ten-fold serial dilution plating. The

same procedure was used to determine the bacterial population at three and six days post inoculation.

Developing an RNAi construct for silencing the *NtSGT1* gene in *N. tabacum*. A partial *NtSGT1* gene fragment was amplified by PCR from the cDNA of *N. tabacum* (cv. Samsun-NN) using primers NtSgt1-H3-RI, and NtSgt1-Xba-Sal (Table S2). The PCR product was digested with either *Xba*I and *Hind*III or *Eco*RI and *Sal*I and sub-cloned into the Topo-Cannibal vector as described previously (Xu et al., 2011). The derived Topo-Entry construct consisted of a DNA fragment carrying an antisense-*NtSGT1* DNA fragment, a castor bean intron spacer sequence, and a sense-*NtSGT1* DNA fragment. The DNA fragment was released from the TOPO-Cannibal vector using *Not*I, and sub-cloned into plasmid pORE-E2 (Coutu et al., 2007) that was digested with *Sal*I and filled in with the Klenow enzyme. The derived construct was named pORE-E2-SGT1-RNAi.

Generating *N. tabacum*-RNAi-NtSGT1 transgenic plants. The pORE-E2-SGT1-RNAi construct was transferred into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Traore & Zhao, 2011). *Agrobacterium*-mediated leaf disk transformation was performed as described previously (Horsch et al., 1989). Briefly, fully expanded leaves of four-week-old *N. tabacum* plants (cv. Samsum-NN) were collected and surface sterilized with 10% bleach. Small leaf disks (about 1 cm^2) were cut from the disinfected leaves and inoculated with an *A. tumefaciens* cell suspension (OD_{600}=0.1). Inoculated leaf disks were incubated on MS medium (PhytoTechnology Laboratories, Shawnee Mission, KS) in the dark at 25°C for 2-3 days. After co-cultivation, the transformed leaf disks were washed with liquid MS medium and cultured on MS medium supplemented with kanamycin (300 µg/ml), carbenecillin (500 µg/ml), NAA (100 mM), and BAP (50 mM) for 4 weeks. Regenerated transgenic shoots were further selected on

MS medium supplemented with kanamycin (100µg/ml) and carbenecillin (300µg/ml). Fully rooted transgenic plants were transplanted into soil and maintained in a growth chamber under conditions of 16 h of light daily at 28°C, and 8 h dark at 25°C.

Monitoring the silencing of *NtSGT1* by RT-PCR. Total mRNA of pORE-E2-SGT1-RNAi transgenic and non-transgenic control plants were isolated using the TRIzol reagent (Invitrogen) according to manufacturer's instructions. First-strand cDNA synthesis was performed using the DyNAmo cDNA Synthesis Kit (Thermo Scientific Inc. Pittsburgh, PA). *NtSGT1* transcripts were detected by qPCR with primers NtSGT1-Cter For and NtSGT1-Cter Rev (Table S2), which amplify an *NtSGT1* fragment outside of the region used for developing the RNAi construct. The *N. tabacum Actin* gene was used as the reference gene and was amplified using primers Nt_actin For and Nt_actin Rev (Table S2).

Melon seed transmission assays. Melon (Cucumis melo cv. Ofir (Zeraim Gedera, Israel) for inoculation of M6 related strains, and hybrid cantaloupe/muskmelon used for AAC00-1 related strains] seeds were incubated for 2 h with gentle agitation in 50-ml tubes containing 10-ml suspensions of *A. citrulli* (~$0.1x10^6$ and $0.1x10^7$ CFU/ml for AAC00-1-derived and M6-derived strains, respectively). As a control, melon seeds were incubated in sterile distilled water for 2 h. Following incubation, melon seeds were collected and air-dried in a laminar flow for 4 h. Seeds were sown in 600-ml pots filled with peat (one seed per pot). The pots were kept in a greenhouse at 26-28°C for 10-14 days after which, the above ground parts of the seedlings were collected and their fresh weight was determined. We have previously shown that seedling fresh weight directly correlates with disease severity (Bahar, et al. 2009). Disease severity was determined using a 0 to 7 scale, based on the wet weight of inoculated plants relative to the average weight of non-inoculated control plants (Bahar, et al., 2009): 0, weight higher than 90%

of average control weight; 1 to 5, weight equal to 76-90%, 61-75%, 46-60%, 31-45% and 16-30% of average control weight, respectively; 6, weight equal to or lower than 15% of average control weight; 7, dead seedling.

Statistical analysis. In order to detect significant differences between bacterial strains in *in planta* virulence assays, the comparison of treatment means was analyzed by Tukey-Kramer HSD multiple comparison procedure using the JMP software (version 7, SAS Inst., Cary NC).

Results

***N. benthamiana* is susceptible to *A. citrulli* infection while *N. tabacum* is resistant.** To determine if *A. citrulli* strains could infect *N. benthamiana,* four-week-old plants were inoculated by tissue infiltration and spray inoculation methods. Infiltration of high concentrations of bacterial cell suspensions (~0.3 x 10^8 CFU/ml) into *N. benthamiana* leaves triggered a strong water-soaking-like cell death at the inoculation site by two days post inoculation (dpi) (Figure 1A). When infiltrated with lower concentrations (~ 0.3 x 10^5 CFU/ml), the inoculated leaves of *N. benthamiana* turned chlorotic and black necrotic symptoms appeared along the veins at 6 dpi (data not shown). At 9 dpi, brown necrotic symptoms were also observed on the stem near the petioles of inoculated leaves. Plating of petiole and stem extracts confirmed the presence of *A. citrulli* (data not shown). As a control, *N. benthamiana* plants were inoculated with *Pseudomonas syringae* pv. *tabaci* (*Pta*) (Oh & Collmer, 2005) using a high bacterial concentration (~0.3 x 10^8 CFU/ml). This inoculation triggered a similar water-soaking-like cell death (Figure 1A). The *hrcV* and *hrcC* deletion mutants of M6 and AAC00-1, respectively (Bahar O. & S., 2010; K. Johnson et al., 2011) did not trigger-water soaking-like cell death (Figure 1A).

To determine if *A. citrulli* could infect other tobacco species, we infiltrated high concentrations (~0.3 x 10^8 CFU/ml) of M6 and AAC00-1 cell suspensions into *N. tabacum* (cv. Samson) leaves. Both M6 and AAC00-1 triggered a strong HR-like cell death 24 h after infiltration. However M6Δ*Hrc*V and AAC00-1Δ*hrcC* failed to trigger HR (Figure 1B). These results suggest that secretion of specific T3S effectors from *A. citrulli* trigger non-host HR in *N. tabacum*.

We further characterized the susceptibility of *N. benthamiana* and the resistance of *N. tabacum* to *A. citrulli* by using the spray inoculation method. We sprayed four-week-old plants of both species with bacterial cell suspensions containing ~0.2 x 10^8 CFU/ml. As shown in Figure 1C, spray inoculation of *A. citrulli* strains M6 and AAC00-1 onto *N. benthamiana* led to the development of necrotic lesions, which progressed along the veins of *N. benthamiana* leaves. Interestingly, the group I strain, M6 consistently resulted in greater disease severity than the group II strain, AAC00-1. This observation is important considering that group II strains are highly virulent on watermelon but weakly to moderately virulent on other cucurbits (and likely *N. benthamiana*), while group I strains are moderately to highly virulent in diverse cucurbits (and likely *N. benthamiana*). The difference in disease severity between these two strains also correlated with bacterial populations at 6 dpi (Fig. 1E). AAC00-1 Δ*hrc*C was unable to induce disease symptoms on *N. benthamiana* plants (Fig. 1C).

In contrast to *N. benthamiana*, symptoms were not observed on *N. tabacum* plants spray-inoculated with different *A. citrulli* strains (Figure 1D). Bacterial population growth assays confirmed that *N. benthamiana*, but not *N. tabacum*, is susceptible to *A. citrulli* infection (Figure 1E & F). In agreement with the observed differences in symptom severity, at 6 dpi, strain M6 reached significantly ($P \leq 0.05$) higher populations than strain AAC00-1, and AAC00-1 Δ*hrc*C

was significantly (P≤0.05) impaired in growth in *N. benthamiana* leaves relative to the wild type strains (Figure 1E).

Four T3S effectors triggered water-soaking-like cell death on *N. benthamiana* and HR-like cell death in *N. tabacum*. Nineteen putative T3S effector genes were cloned, eleven of which were from AAC00-1 and eight were from M6. Only eight effector genes were cloned from M6 because three, *Aave2166, Aave2708,* and *Aave3602* were either absent or truncated (Eckshtain-Levi et al., 2014).

To assess if any of the 19 T3S effector genes contributed to *A. citrulli's* ability to trigger water soaking-like cell death in *N. benthamiana* and HR in *N. tabacum*, all 19 T3S effector genes were sub-cloned into the binary vector pEG101-SacB/R/R for *Agrobacterium*-mediated transient assays (Traore & Zhao, 2011). The pEG101-SacB/R/R vector has an YFP tag fused at the C-terminal of the expression cassette and this allowed us to monitor *in planta* effector expression using fluorescence microscopy. Transient expression of three T3S effector genes from AAC00-1 (*Aave1548, Aave2166* and *Aave2708*), and one T3S effector gene from M6 (*Aave1548*), triggered water-soaking-like cell death on *N. benthamiana* (Figure 2A) and HR-like cell death on *N. tabacum* (Figure 2B). The water-soaking cell death phenotype on *N. benthamiana* was observed at 48 h after inoculation, while the HR-like cell death on *N. tabacum* plants was observed within 24 h after inoculation. Of the three genes cloned from AAC00-1, *Aave2166* triggered the strongest cell death on *N. tabacum*. Interestingly, the *Aave1548* effector gene from M6 triggerd a stronger cell death than *Aave1548* from AAC00-1 (Figure 2B). Interestingly, the Aave1548 effector is highly variable in the nucleotide sequence of its central region between group I and II strains (Eckshtain-Levi et al., 2014) (shown for AAC00-1 and M6 in Figure S1). As shown in Figure 2C, the *Aave2166*-YFP fusion protein from AAC00-1 was localized to both

the cytosol and nucleus (as indicated by the arrow), whereas the other three effector proteins were predominately localized in the cytosol.

***Aave1548* and *Aave2166* are important for *A. citrulli* virulence on *N. benthamiana* plants.** To determine if any of the above effector genes contributed significantly to *A. citrulli* virulence on *N. benthamiana*, *Aave1548* and *Aave2166* were deleted in AAC00-1 and M6 by marker exchange mutagenesis. The mutants and the strains complemented for *Aave1548* were inoculated onto *N. benthamiana* plants using syringe infiltration for phenotype observation and spray inoculation for growth curve analysis. When inoculated at a high concentration (~0.3 x 10^8 CFU/ml), the *Aave1548* mutant strains triggered weaker cell death on *N. benthamiana* compared to wild type and complemented strains (Figure 3A). Similarly, at 6 dpi, the *Aave1548* mutant of AAC00-1 grew to a significantly lower population level than both the wild type and the *Aave1548*-complemented strains on *N. benthamiana (*Figure 3B). Similar observations were made with the *Aave1548* deletion mutant in the M6 background (Figure 3A and3C). Therefore, the T3S effector *Aave1548* may contribute substantially to the virulence of both *A. citrulli* strains M6 and AAC00-1 on *N. benthamiana* (Figure 3A, 3B and 3C).

Interestingly, higher concentrations of the *Aave2166* mutant of AAC00-1 also triggered a weaker cell death than the wild type control strain (Figure 3A). Unfortunately, attempts to generate a complemented strain expressing *Aave2166* were unsuccessful, so it was unavailable as a control. At 3 dpi, the *Aave2166* mutant grew to a significantly lower population than the wild type strain AAC00-1 on *N. benthamiana* (Figure 3B). However, at 6 dpi, AAC00-1Δ*Aave2166* grew to a significantly higher population than the wild type strain on *N. benthamiana* plants (Figure 3B).

When inoculated at high concentrations (~0.3 x 10^8 CFU/ml) into *N. tabacum*, the *Aave1548* and *Aave2166* deletion mutants still triggered HR-like cell death (Figure 3D), which suggests that *A. citrulli* has additional T3S effectors or other components that trigger HR on *N. tabacum*. These results suggest that two T3S effector genes, *Aave1548* and *Aave2166*, contribute substantially to *A. citrulli* virulence and its ability to infect *N. benthamiana* plants.

Aave1548-AAC00-1 and Aave1548-M6 contribute to *A. citrulli* virulence on melon plants. To further characterize the roles of Aave1548 and Aave2166 in *A. citrulli* virulence, we compared the wild type and mutant strains on melon plants by seedling transmission assays. M6*ΔAave1548* and AAC00-1*ΔAave1548* showed significant reductions in seed transmission efficiency compared to the wild type control strains. Melon seeds inoculated with AAC00-1*ΔAae2166* developed seedlings that weighed slightly less than the wild type AAC00-1, but this difference was not significant l (Table 2). Therefore, it was confirmed that *Aave1548*, which is present in all strains of *A. citrulli* tested so far (Eckshtain-Levi et al., 2014), is important for virulence on the surrogate and natural host plants.

Silencing the NtSGT1 gene in *N. tabacum* compromised its resistance to *A. citrulli.* As at least three *A. citrulli* T3S effectors elicited HR on *N. tabacum*, and we hypothesized that this HR was the result of specific recognition of T3S effector(s) by unknown cognate plant *R* gene(s). Since the function of many *R* genes depends on the presence of the conserved immune signaling component, *SGT1* (Peart et al., 2002), we tested whether *NtSGT1* is required for elicitation of HR in *N. tabacum* in response to *A. citrulli*. To this end, we silenced the *NtSGT1* gene in this plant using an RNAi approach. Two independent *NtSGT1*-RNAi transgenic lines were used for this study, and silencing of *NtSGT1* was confirmed using RT-PCR (Figure 4A). The transgenic plants along with non-transgenic *N. tabacum* plants were used for inoculation and

in *planta* bacterial growth assays with *A. citrulli* strains. Inoculation with AAC00-1 and M6 failed to trigger HR cell death in the RNAi-*NtSGT1* transgenic *N. tabacum* plants (Fig. 4B). Transient expression of *Aave2166*-AAC00-1, *Aave1548*-AAC00-1, and *Aave1548*-M6 also failed to trigger the cell death phenotype on the RNAi-*NtSGT1* plants (Figure 4C), even though the fluorescent signal of the effector-YFP fusion proteins was detected (data not shown). To further test if silencing of *NtSGT1* in *N. tabacum* could compromise its resistance to *A. citrulli*, we monitored the bacterial growth of AAC00-1 and M6 on the RNAi-*NtSGT1* plants. As shown in Figure 4D and 4E, AAC00-1 induced weak lesions on the inoculated leaves at 9 dpi, while strain M6 failed to incite disease lesions at 9 dpi. However, the RNAi-*NtSGT1* plants supported higher bacterial growth of both AAC00-1 and M6 compared to the wild type *N. tabacum* plants (Figure 4F and 4G). These results suggest that the HR response observed in *N. tabacum* could be caused by the recognition of one or more of the effector genes present in *A. citrulli* by unknown *R* gene(s) that require the function of *NtSGT1*.

Discussion

Despite frequent BFB outbreaks and the significant threat posed to the cucurbit industry, limited information is available about the biology and pathogenicity mechanisms of the causal agent, *Acidovorax citrulli* (Bahar & Burdman, 2010; Burdman & Walcott, 2012). In this report, we demonstrated that *N. benthamiana* can be used as a surrogate host for studying *A. citrulli* pathogenicity and virulence, while *N. tabacum* may contain non-host *R* genes that can recognize *A. citrulli* T3S effectors and trigger disease resistance. By employing an *Agrobacterium*-mediated transient assay, bacterial mutagenesis, and growth curve assays on *N. benthamiana* plants, we identified the first *A. citrulli* T3S effector, Aave1548 that contributes significantly to

the virulence of *A. citrulli*. Furthermore we validated *N. benthamiana* as a system to study *A. citrulli* virulence.

N. benthamiana is an excellent model plant species for studying molecular plant- microbe interactions. In this study, we demonstrated that the symptoms caused by *A. citrulli* on *N. benthamiana* leaves are similar to those observed on its natural hosts. Spray inoculation with cell suspensions containing ~10^8 CFU/ml of *A. citrulli* onto *N. benthamiana* leaves resulted in necrotic lesions that appeared on the inoculated leaves and progressed along the secondary veins. Similar symptoms were observed in cucurbit plants (Hopkins & Thompson, 2002). By 9 dpi, brown lesions were observed on petioles and stems of inoculated tobacco leaves, as a possible result of systemic infection of *A. citrulli* (data not shown). The ability of *A. citrulli* to infect the vascular tissue of cucurbit plants was previously demonstrated in melon seedlings (Bahar, Goffer, & Burdman, 2009b) and squash leaves (O. Bahar et al., 2009a; Makizumi et al., 2011).

T3S effectors usually have additive contributions to bacterial virulence on host plants. Bacterial strains with deletions of individual T3S effectors may not result in detectable phenotype changes on highly susceptible host plants due to functional redundancy of effectors, or lack of sensitivity of assays to subtle virulence phenotypes (Alfano & Collmer, 2004). Utilization of a weakly virulent pathogen or a less susceptible plant species may increase the likelihood of detecting the effects of individual T3S effectors. In this study, we found that at least one *A. citrulli* T3S effector, Aave1548, contributed significantly to *A. citrulli* virulence on *N. benthamiana* leaves. Importantly, inoculation of wild type and mutant *A. citrulli* strains onto watermelon leaves did not reveal significant contributions of *Aave1548* or *Aave2166* (data not shown) to virulence. Therefore, our results suggest that *N. benthamiana* could be a valuable tool for studying the roles of T3S effectors in *A. citrulli* virulence.

In this study the group I strain, M6, was consistently more aggressive than AAC00-1 on *N. benthamiana* plants. Group I strains cause moderate to high disease severity on a wide range of cucurbits, while group II strains are more aggressive on watermelon (Burdman & Walcott, 2012). It will be interesting to determine if the difference in aggressiveness between M6 and AAC00-1 on *N. benthamiana* reflect the general differences between the two groups, by screening more representative strains on this plant species.

Eckshtain-Levi et al. (in press) recently showed that all tested group I strains, including M6, lack functional effector genes, *Aave2166* and *Aave2708.* Group I strains were also shown to possess a copy of *Aave3602* that is truncated in the C-terminal domain. Deletion of *Aave2166* in AAC00-1 enhanced bacterial growth *in N. benthamiana* plants at 6 dpi, but reduced bacterial growth at 3 dpi. Previous studies have shown that some T3S effectors can suppress the activity of other T3S effector proteins when co-expressed in plant cells (Guo, Tian, Wamboldt, & Alfano, 2009). It is possible that Aave2166 and Aave3062 have an inhibitory effect on other T3S effectors, like Aave1548, which could mask their phenotype in group II strains. However, the transient co-expression of Aave2166-AAC00-1, Aave1548-AAC00-1 and Aave1548-M6 in pairs did not trigger different phenotypes compared to the expression T3S effectors individually (data not shown). Therefore, we were not able to conclude if AAC00-1 Aave2166 contributed to the reduced virulence relative to M6 on *N. benthamiana*. This question requires further investigation.

R-gene-mediated resistance can select for the loss of avirulent T3S effector genes (Kearney, Ronald, Dahlbeck, & Staskawicz, 1988; Vera Cruz et al., 2000; Yang, Sugio, & White, 2005). It is also possible that *N. benthamiana* and some cucurbit species have evolved weak *R* genes that can partially recognize Aave2166, Aave2708 and Aave3602 effectors

produced by group II strains. The fact that group I strains do not express these effectors in a functional state could partially explain the broad cucurbit range of these strains relative to group II strains. In future studies it would be interesting to express these T3S effector genes in M6 to test if they reduce virulence on *N. benthamiana* and other cucurbits.

Aave2166 is a homolog of *avrBsT*, a gene that encodes a protein with acetyltransferases enzyme activity, that was originally reported from *Xanthomonas campestris* pv. *vesicatoria* (Cheong et al., 2014; Thieme et al., 2005). AvrBsT belongs to a large family of effectors called YopJ and is conserved in a wide range of plant and animal pathogens (Ciesiolka et al., 1999; Hardt & Galán, 1997; M. Whalen et al., 1992). Previous studies demonstrated that the homolog of YopJ from *Yersinia pestis* suppressed the host immune response and induced apoptosis by blocking multiple signaling pathways, including the MAPK and NFκB pathways in the infected cell (Orth, 2002a). AvrBsT cloned from *X. campestris* pv. *vesicatoria* is also a critical virulence effector that may target important plant immune signaling components (Cheong et al., 2014; Kim, Choi, & Hwang, 2010a; R. Szczesny et al., 2010). Functional characterization of Aave2166 from AAC00-1 could be beneficial for understanding the virulence functions of YopJ homologs. In this study, we demonstrated that Aave2166 triggered non-host HR in *N. tabacum*; however, deletion of *Aave2166* did not significantly compromise the virulence of *A. citrulli* in melon seedling transmission assays (Table 3). Interestingly, an additional T3S effector gene from *A. citrulli*, *Aave2708*, also encoded a homolog of AvrBsT (Table 1). This raises the possibility that Aave2166 and Aave2708 may have redundant functions when translocated into plant cells. It will be interesting to test if double knockout mutants of both *Aave2166* and *Aave2708* significantly increase AAC00-1 virulence on melon hosts.

Aave1548 is a homolog of HopW1 isolated from *Pst DC3000*, an effector that has the ability to suppress the salicylic acid (SA)-mediated immune signaling pathway (Lee, Jelenska, & Greenberg, 2008). In this study, we demonstrated *Aave1548* contributes significantly to *A. citrulli* virulence (Figures 3 A, B and C). It will be interesting to test whether Aave1548 also has a similar role in suppressing SA production in both *N. benthamiana* and cucurbit hosts. Aave1548 also triggered a HR-like cell death in *N. tabacum* (Figure 2B) that was stronger for Aave1548-M6 than for Aave1548-AAC00-1. Interestingly, amino acid sequence alignment between the Aave1548 sequences of strains M6 and AAC00-1 revealed a small central domain (46 amino acids) with high polymorphism (Figure S1). Eckshtain-Levi et al. (in press) showed that this polymorphism exists among all group I and II strains assessed so far. It will be interesting to perform site direct mutagenesis to narrow down the critical amino acid residues that are responsible for the difference in HR observed between the two *Aave1548* homologues.

N. tabacum is naturally resistant to *A. citrulli* (N.W. Schaad et al., 1978). There are several explanations for the non-host resistance of *N. tabacum* to *A. citrulli*: (1) the structure of *N. tabacum* leaves could render a physical barrier for the establishment of *A. citrulli* populations; (2) recognition of conserved pathogen associated molecular patterns (PAMPs) by tobacco pattern recognition receptors (PRRs) and subsequent initiation of PAMP-triggered immunity (PTI); and/or (3) the presence of putative *R* genes that may recognize one or more effectors and initiate effector-triggered immunity (ETI). Our data showed that at least three *A. citrulli* T3S effectors triggered the HR in *N. tabacum*. Furthermore, silencing of *NtSGT1*, a putative R-protein chaperone, compromised the resistance of *N. tabacum* to *A. citrulli* (Figure 4C). Therefore, we conclude that ETI contributes, at least partially, to the non-host resistance of *N. tabacum* to *A. citrulli*. In future studies, it will be interesting to silence *NbSGT1* in *N. benthamiana* through

VIGS and test the virulence/avirulence functions of *A. citrulli* T3S effectors. In this study, *Aave2166* and *Aave1548* deletion mutants of *A. citrulli* did not completely abolish the non-host resistance of *N. tabacum* to *A. citrulli*. This result could be explained by the fact that *A. citrulli* has more than 11 T3S effectors, and multiple T3S effectors could be recognized by unknown *R* gene(s). Importantly, isolation of the non-host resistance genes from *N. tabacum* and transferring them to cucurbits may offer a new strategy of controlling BFB. In the future we will generate *A. citrulli* mutants with multiple deletions of T3S effector genes, to assess whether some combinations of impaired effector genes lead to abolishment of non-host resistance in *N. tabacum*. This information could help to focus screens for non-host resistance genes against *A. citrulli*.

Recently, several cucurbit genomes (watermelon, melon, and cucumber) have been sequenced (Garcia-Mas et al., 2012; S. Guo et al., 2013; Huang et al., 2009). Interestingly, genome annotation of these species revealed that some of these domesticated cucurbit species have significantly lower numbers of NB-LRR type *R* genes in their genomes (S. Guo et al., 2013). In comparison to the commercial lines, the wild-type watermelons contain more NB-LRR genes (S. Guo et al., 2013). It is possible that during domestication of cucurbits, through breeding and selection for fruit quality, many *R* genes were lost. This may partially explain the lack of resistance to BFB in commercial cucurbit cultivars. One strategy to improve the resistance of cucurbits to BFB could be the screening of wild cucurbit lines for disease resistance genes. Screening of cucurbit germplasm only identified partial resistant lines against BFB, which suggests that there are no race-specific *R* genes in cucurbits (Wechter, Levi, Ling, Kousik, & Block, 2011). However, it is still possible that those partial resistant lines may carry weak *R* genes that do not trigger strong and rapid ETI when screened with *A. citrulli* strains. By

employing T3S effectors as molecular probes, we may be able to identify those weak individual *R* genes from partially resistant germplasm. Pyramiding those partial resistance genes into elite cucurbit cultivars may allow us to achieve complete disease resistance to *A. citrulli* in the future.

Acknowledgments

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Table Legends

Table 1
List of Bacterial Strains and Plasmids

Strain/Plasmid	Characteristics	Reference
***Acidovorax citrulli* strains[a]**		
AAC00-1	Amp, Rif, wild type group II strain	Johnson, Minsavage et al. 2011
M6	Amp, Rif, wild type group I strain	Bahar O. and S. 2010
AAC00-1/ΔhrcC	Amp, Rif, Km, AAC00-1 mutant defective in *hrcC*	Johnson, Minsavage et al. 2011
M6/ΔhrcV	Amp, Rif, Km, M6 mutant defective in *hrcV*	Bahar O. and S. 2010
AAC001/ΔAave2166	Amp, Rif, Km, AAC00-1 mutant defective in *Aave2166*	This work
AAC00-1/ΔAave1548	Amp, Rif, Km, AAC00-1 mutant defective in *Aave1548*	This work
AAC00-1/ ΔAave1548(pAave1548)	Amp, Rif, Km, AAC00-1 mutant defective in *Aave1548* that was complemented with pVSP61-*Aave1548*	This work
M6/ΔAave1548	Amp, Rif, Km, M6 mutant defective in *Aave1548*	This work
***Escherichia coli* strains[b]**		
DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Invitrogen Inc.
DH5α(RK600)	Cm^r, helper plasmid	Figurski et al, 1979
***Agrobacterium tumefaciens* strains[c]**		
GV2260	C58 background, Rif^r	This work
LBA4404	TiAch5	Clontech
***Pseudomonas syringae tabaci* strains[d]**		
P. syringe* pv. *tabaci* (*Pta 11528)	Rif^r, wild type	Wei et al, 2007
P. syringe* pv.*tabaci 11528*/ Δ*hrcV	Rif^r, Km^r, *P. tabaci* mutant defective in *hrcV*	Wei et al, 2007
Plasmids		
pEG101/SacB/R/R	Km^r, toxic on high concentration sucrose, expression vector	Traore and Zhao, 2011
pLVC18L-Des	Suicide vector, Tet^r, low copy	Zhao, Dahlbeck et al.

Strain/Plasmid	Characteristics	Reference
		2011
pVSP61-Des-GM	Km^r, Broad range expression vector	(Century et al., 1995)
pORE E3	Km^r, plant expression vector,	Coutu, Brandle et al.
		2007
pORE-E2-SGT1-RNAi	Km^r, RNAi construct for silencing *NtSGT1*	This work
pLVC18L-Aave1548-Kan	Km^r,	This work
pLVC18L-Aave2166-Kan	Km^r,	This work
pVSP61-GM-Aave1548	Km^r,, Gm^r	This work

[a]All *A. citrulli* strains were grown on nutrient agar and incubated at 28°C

[b]*E. coli* strains were grown on Luria Broth plate and incubated at 37°C

[c]*A. tumefaciens* strains were grown on Luria-Bertani agar and incubated at 28°C

[d]*P. syringae* pv. *tabaci* strains were grown on NYGA medium and incubated at 28°C

Km^r: Kanamycin resistant, Gm^r: Gentamycin resistant, Rif^r: Rifampicin resistant, Tet^r: Tetracycline resistant, Cm^r: Chloramphenicol resistant.

Table 2
Seed Transmission Assay of Wild Type Strains AAC00-1 and M6 and Mutant Strains Impaired on Certain Effectors on Melon

Bacterial Strains	Plant weight[a] (mg)	Disease severity[b]
AAC00-1	170±33 [A]	3.8±0.52
AAC00-1/*ΔAave2166*	218±58 [A]	3.1±0.4
AAC00-1/*Δ Aave1548*	441±35[B]	0.62±0.2
Control (H_2O)	460±61[B]	0
M6	690.20±177[A]	4.97±0.34
M6/ *Δ Aave1548*	1060.45±181[B]	4.1±0.4
Control (H_2O)	2549.74±103[C]	0

[a] Hybrid cantaloupe/muskmelon seeds were used for AAC00-1 inoculation, while melon cv. Ofir seeds were used for M6 inoculation. All inoculated and control seeds were incubated in bacterial cell suspensions (~1 x 10^6 and 1 x 10^7 CFU/ml for the hybrid and Ofir seeds, respectively) for 2 h. Infected seed were blot dried and planted four seeds/per pot with two pots for each treatment. The weights of the seedlings were measured at 12 days post inoculation and compared to the control (H_2O).

[b]Disease severity is scored on a scale of 0 to 7, based on plant weight values of inoculated plants relative to the average shoot weight of non-inoculated controls (Bahar et al. 2008): 0, weight higher than 90% of average control weight; 1 to 5, weight equal to 76-90%, 61-75%, 46-60%, 31-45% and 16-30% of average control weight, respectively; 6, weight equal to or lower than 15% of average control weight; 7, dead seedling. Data was presented as Mean ± Standard Error, n=8.

[c]Different letters indicate significant difference between treatments ($P \leq 0.05$) by Tukey-Kramer HSD test.

Table Supplemental 1
List of the Putative AAC00-1 T3S Effector Genes, their Annotation, and Primers used to Amplify the Open Reading Frames from AAC00-1 and M6

Locus Tag	Gene and Description	Primer Sequences
Aave0277	Type III effector HopG1 (*Pseudomonas syringae* pv. *tomato*)	5'-cacc**GGATCC**ATGAGAAAAGAACTAGCCAGCTGT-3' 5'-**GTCGAC**GGCAGGCCTGCCCGGCAGCCCGGCCT-3'
Aave1548	Type III secreted effector HopPmaA (*Pseudomonas syringae* pv. *maculicola)*	5'-cacc**TCTAGA**ATGCCTCTACAGTCCATTTCCAT-3' 5'-**GTCGAC**TGGTTGATCCCCCGTCCGAGCAT-3'
Aave2166	Avirulence protein AvrBsT (*Xanthomonas campestris* pv. *vesicatoria*)	5'-cacc**AGATCT**ATGAAGAATTTCATGCGATCGAT-3'5'- **GTCGAC**TTCGATAGCTTTTCTGATTTTTCTCA-3'
Aave2173	Avirulence protein AvrBs1 (*Xanthomonas campestris* pv. *vesicatoria*)	5'-cacc**GGATCC**ATGCTGGAGAAAAAGAGGCAGGT-3' 5'-**GTCGAC**GGCAACATGCTGCACCTTGGAGGA-3'
Aave2708	Avirulence protein AvrBsT (*Xanthomonas campestris* pv. *vesicatoria*)	5'-cacc**GGATCC**ATGGGTCTATGCGTTTCAAA-3' 5'-**GTCGAC**TGACTGGCGATCAGAGATAGCT-3'
Aave2876	Type III effector HopH1 (*Pseudomonas syringae* pv. *tomato*)	5'-cacc**GGATCC**GTGAACGTCCATCTGCATGCCA-3' 5'-**GTCGAC**GTCGCTTCGGTAACCTGAATAAT-3'
Aave3062	Putative avirulence protein AvrRxo1 (*Xanthomonas oryzae* pv. *oryzicola*)	5'-cacc**GGATCC**GCCCGCCTCCTCGACCAGGAT-3' 5'- **GTCGAC**AGTCAGTGAACTATGGGCGGCCA-3'
Aave3237	Type III effector HopAJ2 (*Pseudomonas syringae* pv. *phaseolicola*)	5'-cacc**GGATCC**ATGCAACGAAGAAACACGACGA-3' 5'-**GTCGAC**GGGCGCCTGCAGTCGCTGCAGCA-3'
Aave3452	Avirulence protein AvrPphe (*Xanthomonas axonopodis* pv. *citri*)	5'-cacc**GGATCC**ATGGCAACTTTCATCATCTCGTCCAT-3' 5'-**GTCGAC**GGACGTTCTCCGGCGGAGCCGGCA-3'
Aave3462	Type III effector PopP3 protein (*Ralstonia solanacearum*)	5'-cacc**GGATCC**ATGCCCCGTTCCGTCACATCCT-3' 5'-**GTCGAC**GTGCCGATACCAGTCGCGCCA-3'
Aave4728	Type III effector HopAE1 (*Pseudomonas syringae* pv. *phaseolicola*)	5'-cacc**AGATCT**ATGTCTGCGATCAACAGTTCCT-3' 5'-**CTCGAG**GGGGGCCACGGGCGCGTGGCGCGGAA-3'

Table Supplemental 2
List of Additional Primers Used in This Study

Primer names	Primer sequences
Aave2166_f1	5'-GAAGCCCACGTGCGCGTGCTGGCC-3'
Aave2166_R2	5'CCTACACAATCGCTCAAGACGTGCCATTCTCAATTGCAAATATTCAG-3'
Aave2166_f5	5'GGACCATGGCTAATTCCCATGTCGCACCGGGAATGATAATCCCCGG-3'
Aave2166_R6:	5'- CGCTGTCGTAGCCCACGGTGATGTTCG-3'
Aave1548_f1	5'-CCCGGATCGAAAGGCGAGCGCACG-3'
Aave1548_R2	5'-CCTACACAATCGCTCAAGACGTGTGCTGCAGCGTGCCATGGACAA-3'
Aave1548_f5	5'-GGACCATGGCTAATTCCCATGTCCCTTGGGGACGGGGGAGTCTGGGA-3'
Aave1548_R6	5'-GGTGAGCCAGTGGAAGCGCGCCCGG-3'
Aave_1548comp for	5'-CACCTCTAGACACCAGCGCGTCGAACAGA-3'
Aave_1548comp rev	5'-GTCGACTGGTTGATCCCCCGTCCGAGCAT-3'
kan_for	5'-CACGTCTTGAGCGATTGTGTAGG-3'
kan_rev	5'-GACATGGGAATTAGCCATGGTCC-3'
Inter1548F	5'-GCGTCGACGAAGATTTCTTCAACGGCTA-3'
Inter1548R	5'-CCGGTACCCATCAACTCGTCCTGGTAGA-3'
NtSgt1-H3-RI (restriction enzyme sequence in bold)	5'-**AGCTACCaagcttGAATTC**GCTGGAGAACTACCTAATCAGTC-3'
NtSgt1-Xba-Sal (restriction enzyme site in bold)	5'**GATGCTCTAGAGTCGAC**TTCGAGAGATGTCCAGTGTAAGG-3'
NtSGT1-Cter For	5'-AGAGAGTCTGCTGTAGTGCAGAG-3'
NtSGT1-Cter Rev	5'-TTCTTCAGCTCCATGCCATCTGGA-3'
Nt_actin For	5'-TTGACGGAAAGAGGTTAT-3'
Nt_actin Rev	5'-GTTGGAAGGTGCTGAGAG-3'

Figure Legends

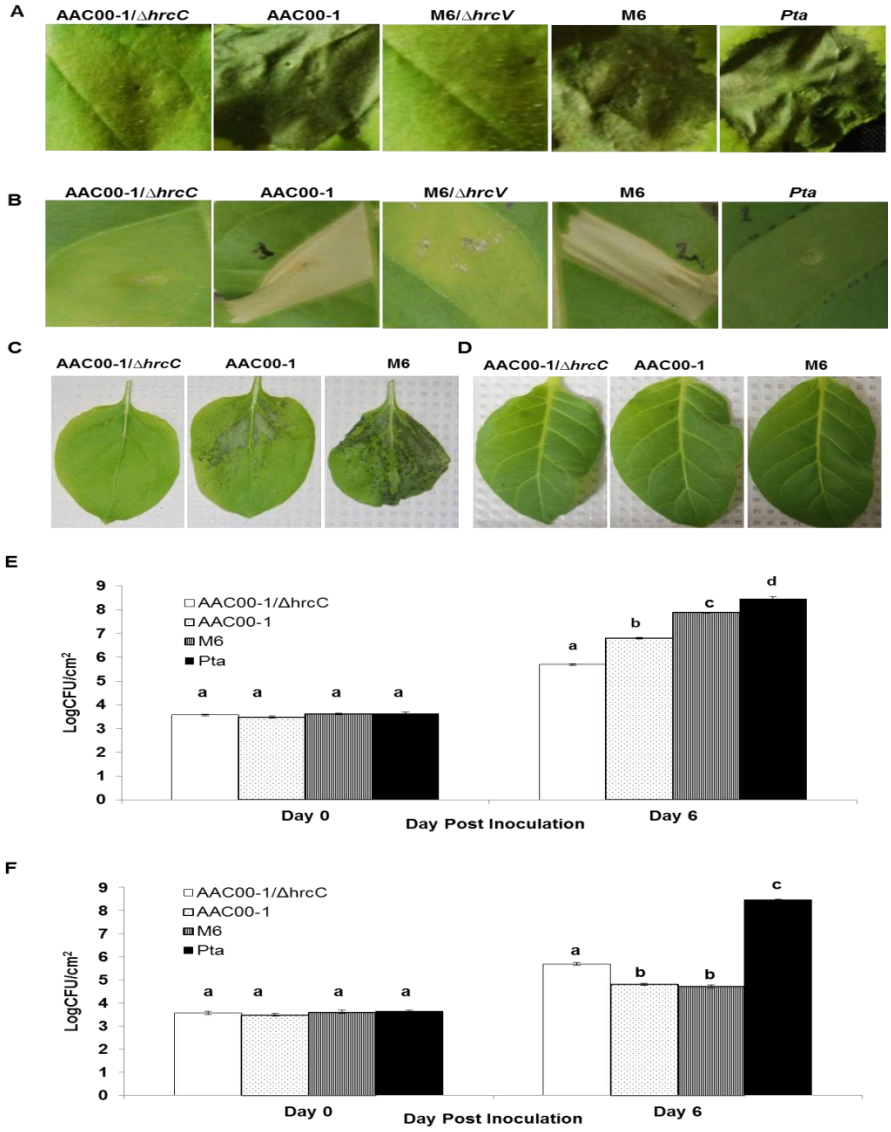

***Figure* 1. Inoculation of *Acidovorax citrulli* on *N. benthamiana and N. tabacum* plants.**
A & B. *A. citrulli* strains trigger a water-soaking-like cell death response in *N. benthamiana* (A) and HR-like cell death response in *N. tabacum* (B) plants. Four-week-old plants were infiltrated with AAC00-1Δ*hrcC*, AAC00-1 M6*ΔhrcV*, M6 or *Pseudomonas syringae* pv. *tabaci* at ~0.3 x 10^8 CFU/ml. The plants were incubated at 25°C for three days before photographs were taken. Pta: *P. syringae* pv. *tabaci.*
C & D. Symptoms of *N. benthamiana* (C) and *N. tabacum* (D) plants after spray-inoculation with *A. citrulli* strains. Four-week-old plants were spray-inoculated with AAC00-1Δ*hrcC*, AAC00-1 or M6 at 0.~2 x 10^8 CFU/ml. The inoculated plants were incubated at 30°C for two days before photographs were taken.
E & F. Bacterial growth of *A. citrulli* strains on *N. benthamiana* (E) and *N. tabacum* (F) plants**.** Four-week-old plants were infiltrated with AACΔhrcC, AAC00-1, M6 and *Pseudomonas syringae* pv. *tabaci* at ~ 0.3 x 10^5 CFU/ml. Bacterial populations were monitored at 0 and 6 days post inoculation (dpi). Data represent mean and standard errors from one representative experiment of three, with 3 replicates per treatment. Different letters indicate significant differences (P≤0.05) among treatments within each time point by Tukey-Kramer HSD test.

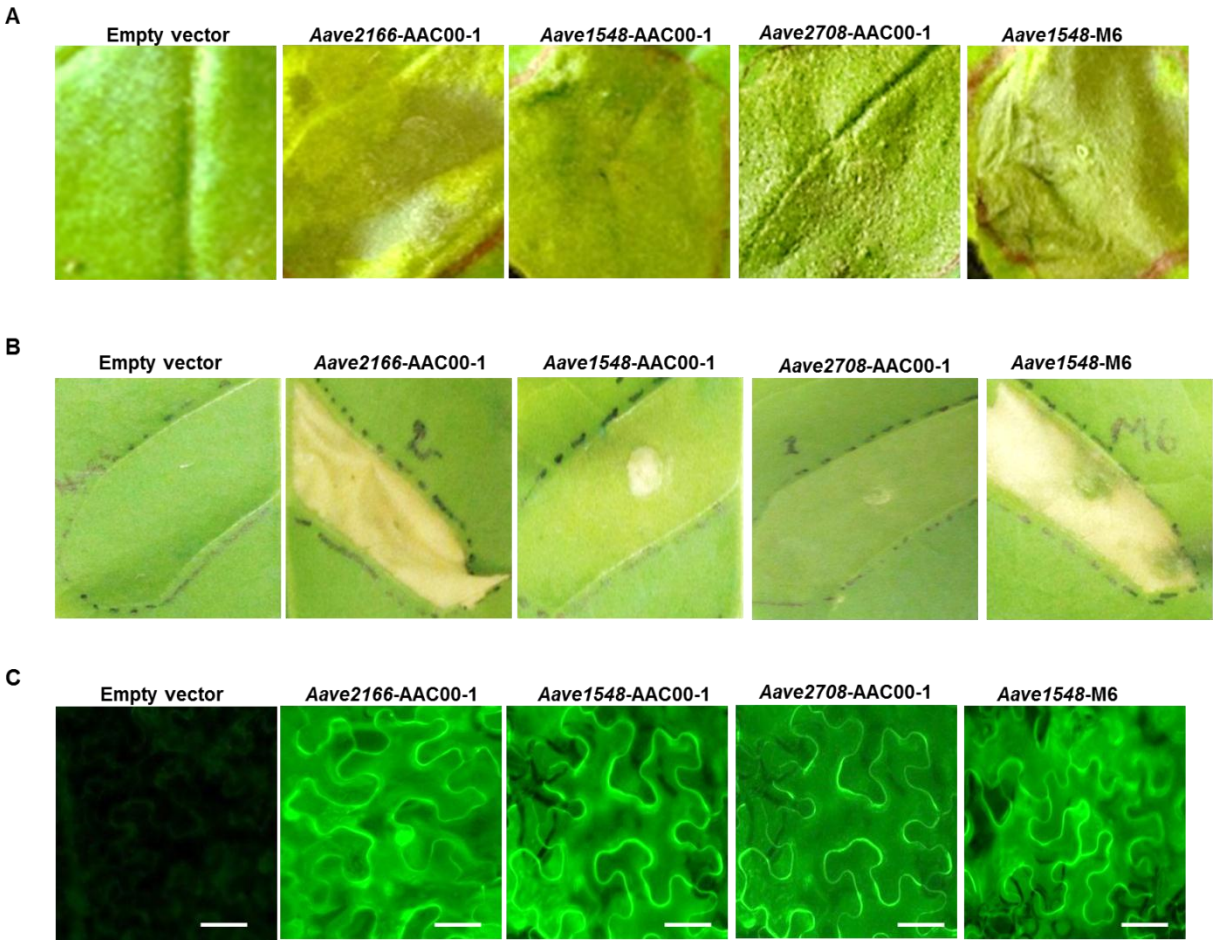

***Figure* 2**. **Transient expression of three *A. citrulli* T3S effectors trigger water-soaking-like cell death on *N. benthamiana* and HR-like cell death on *N. tabacum* plants**.

A. *Agrobacterium*-mediated transient expression of T3S effectors in *N. benthamiana*. Effectors Aave1548 from strains M6 and AAC00-1, and Aave2166 and Aave2708 from strain AAC00-1 triggered water-soaking like cell death on *N. benthamiana* at the concentration of ~0.3 x 10^8 CFU/ml. Pictures were taken at 2 dpi.

B. Transient expression of *Aave2166, Aave1548* on *N. tabacum* triggered HR-like cell death at 1 dpi.

C. YFP fluorescence showing the expression of the effector genes in *N. benthamiana* cells**.** The fluorescent signal of the effector-YFP fusion protein expressed in *N. benthamiana* was detected by fluorescence microscopy. Aave2166 was localized in the nucleus (arrow), cytosol and plasma membrane, while Aave1548 and Aave2708 were localized in the cytosol and plasma membrane. Bars represent 20 µm.

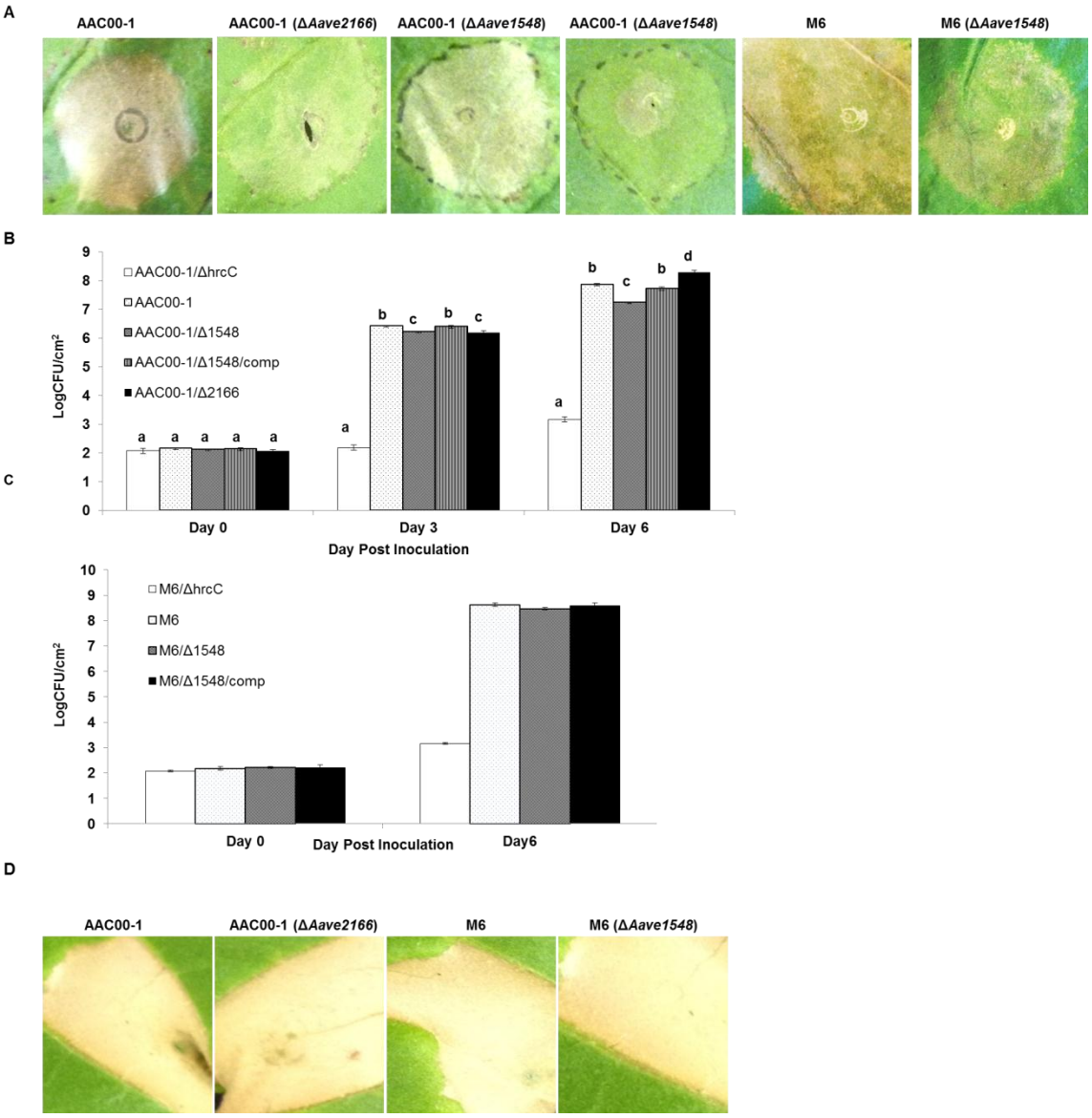

***Figure* 3. Effects of T3S effector gene mutation on the *Acidovorax citrulli* growth on *N. benthamiana* and *N. tabacum* plants.**

A. Inoculation *N. benthamiana* plants with *A. citrulli* strains : Four-week-old *N. benthamiana* leaves were infiltrated with AAC00-1, M6, AAC00-1Δ*2166*, and M6Δ*1548* at a concentration of ~0.3 x 10^8 CFU/ml. Mutant strains AAC00-1Δ*2166*, and M6Δ*1548*, triggered weaker cell death phenotypes compared to the wild type strains on *N. benthamiana*. Pictures were taken at 2 dpi.

B. Inoculation of *A. citrulli* strains onto *N. tabacum* plants. Four-week-old *N. tabacum* leaves were infiltrated with AAC00-1, M6, AAC00-1Δ*2116*, and M6Δ*1548* at a concentrations of ~0.3 x 10^8 CFU/ml. Both wild type and mutant *A. citrulli* strains triggered strong HR on *N. tabacum*. Pictures were taken at 2dpi.

C&D. *In planta* bacterial growth of *A. citrulli* strains on *N. benthamiana* plants. Four-week-old *N. benthamiana* leaves were spray-inoculated with *A. citrulli* strains at concentrations of 0.~2 x 10^8 CFU/ml. Bacteria growth was monitored at 0, 3 and 6 dpi for AAC00-1 derived strains and 0 and 6 dpi for M6 derived strains. N.B. AAC00-1Δ*1548comp* and M6Δ*1548comp* are the plasmid-borne complement strains of the *Aave1548* deletion mutants. Data represent mean and standard errors of one representative experiment of three, with 3 replicates per treatment. Different letters indicate significant differences ($P \leq 0.05$) among treatments within each time point by Tukey-Kramer HSD test.

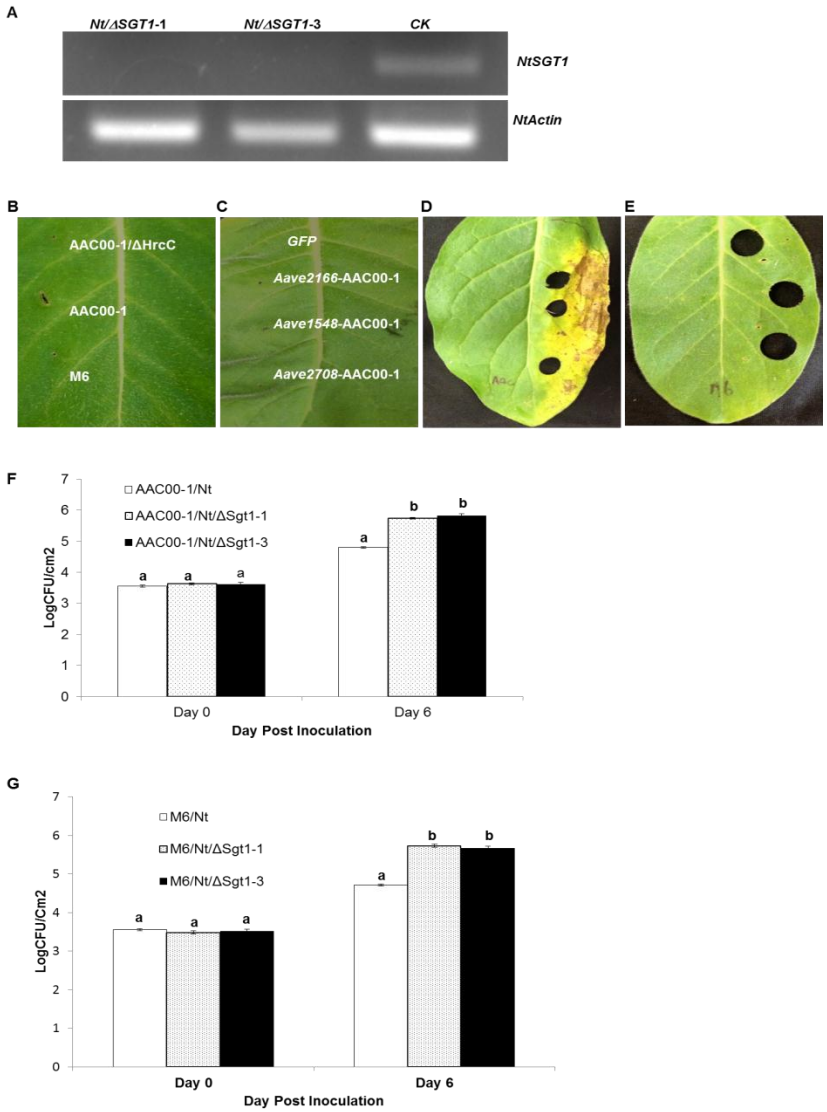

Figure 4. Silencing of *NtSGT1* in *N. tabacum* suppressed the HR phenotype triggered by *Acidovorax citrulli* strains and individual T3S effectors.

A. Validation of *NtSGT1* gene silencing by RT-PCR analysis. *NtSGT1* gene specific primers flanking the region outside of the RNAi construct was used for RT-PCR analysis. *NtActin* primers were used as internal control. AAC: AAC00-1, NtΔSgt1: *NtSGT1*-RNAi line.

B. HR was not triggered in *NtSGT1*-RNAi lines inoculated with *A. citrulli* strains. Two independent *NtSGT1*-RNAi lines were inoculated with AAC00-1, M6, and AAC00-1Δ*Hrc*C strains (shown for one of the lines) at the concentration of ~0.3 x 10^8 CFU/ml**.**

C. *Agrobacterium*-mediated transient expression of T3S effectors did not trigger HR in *NtSGT1*-RNAi plants. *Agrobacterium tumefaciens* strains carrying the effector genes *Aave2166*-AAC00-1, *Aave1548*-AAC00-1 and *Aave2708*-AAC00-1 were inoculated on *NtSGT1*-RNAi plants at the concentration of ~0.3 x 10^8 CFU/ml. Pictures were taken at 2dpi.

D&E. *A. citrulli* strain AAC00-1 triggered disease lesions on *NtSGT1*-RNAi plants (D) while M6 did not (E). The *NtSGT1*-RNAi transgenic plants were infiltrated with *A. citrulli* strains at a concentration of ~0.3 x 10^5 CFU/ml. Pictures were taken at 9 dpi.

F&G. In *planta* growth of *A. citrulli* populations on *NtSGT1*-RNAi and wild type *N. tabacum* plants. AAC00-1(F) and M6 (G)were inoculated onto *NtSGT1*-RNAi plants at the concentration of ~0.3 x 10^5 CFU/ml. Data represent mean and standard errors of one representative experiment of three, with 3 replicates per treatment. Different letters indicate significant difference between treatments ($P \leq 0.05$) by Tukey-Kramer HSD test.

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Aae1548-M6          1  MPLQSISIHRLDAVRAAFPFGNTPTGSSSRPRAGESPRLPRPQGLSPRSTGARQLPCPVA
Aae1548-AAC00-1     1  MPLQSISIHRLDAVRAAFPFGNTPTGSSSRPRAGESPRLPRPQGLSPRSTGARQLPCPVA

Aae1548-M6         61 REAAAQPAVALERSRLGWTQAGTIADRCIHKALSLLGLRTARDQAADFIRFMADADGQPG
Aae1548-AAC00-1    61 REAAAQPAVALERSRLGWTQAGTIADRCIHKALSLLGLRTARDQAADFIRFMADADGQPG

Aae1548-M6        121 TSSLPLGDGWVRNTRRVKDQVATLDLSMDGHGSVIDVRRPPRGSRIPEGQEREAFATVLE
Aae1548-AAC00-1   121 TSSLPLGDGWVRNTRRVKDQVATLDLRMDTNGAIIDVRRPEQPSRIAEGQERETFAMVLE
Sh951_

M6-Aae1548-1      181 EMRNRGKDTLRTVPVHYVNRNTHGYFLPTHGYVVAGDPGKGRKSGAVLYGVGGDPVRGPV
Aae1548-AAC00-1   181 EMRSRGKDMLRPVPVHYVNRNTHGYFLPTHGYVVAGDPGKGRKSGAVLYGVGGDPVRGPV

M6-Aae1548-1      241 LLDRPLLKHLLSSMDKAGPARFPAAVRATIAQLAGQRFASREDFFNGYRSVRGDAVDPAA
Aae1548-AAC00-1   241 LLDRPLLKHLLSSMDKAGPARFPAAVRATIAQLAGQRFASREDFFNGYRSVRGDAVDPAA

M6-Aae1548-1      301 IRKEVASIYRLLPMNTMELWPKTTGDYRAPRPPAPERDIRAFESPPQGLARKVYMRRIEG
Aae1548-AAC00-1   301 IRKEVASIYRLLPMNTMESWPKTTGDYRAPRPPAPERDIRAFESPPQGLARKVYMRRIEG

M6-Aae1548-1      361 ARQTDLQDARRQFLLHRLYQDELMGRDGSGFPPESVQPRADSPRRHRLVENTPRFQRLPP
Aae1548-AAC00-1   361 ARQTDLQDARRQFLLHRLYQDELMGRDGSGFPPESVQPRADSPRRHRLVENTPRFQRLPP

M6-Aae1548-1      421 HASGKVGNCNSGAASLLQRAMDNHARSTPGARAGKAAAASVFGLGSGHRIDLWDPLQDAR
Aae1548-AAC00-1   421 HASGKVGNCNSGAASLLQRAMDNHARSTPGARAGKAAAASVFGLGSGHRIDLWDPLQDAR

M6-Aae1548-1      481 TGDQP
Aae1548-AAC00-1   481 TGDQP
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Figure **Supplemental 1. Partial Amino Acid Sequence Alignment of the Aave1548 Effectors Identified from AAC00-1 and M6.** A small domain that is polymorphic between the two Aave1548 homologs is highlighted in yellow

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Chapter 5: Functional Characterization of a T3S effector Aave2166 Cloned from *Acidovorax citrulli* that Causes Bacterial Fruit Blotch Disease

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Abstract

A. citrulli is a Gram-negative bacterium that causes the bacterial fruit blotch disease of cucurbits. *A. citrulli* strains collected worldwide can be classified into at least two major groups. Previous work demonstrated that the Type Three Secretion System (T3SS) is critical for the virulence of both group I and II strains of *A. citrulli* on their natural host cucurbits, and the surrogate host *Nicotiana benthamiana* plants. This study demonstrates that the full length open reading frame of one T3S effector, Aave2166, is only present in the genome of group II strains of *A. citrulli*, which may be used as a marker for distinguishing the two groups of *A. citrulli*. Ectopic expression of Aave2166 in transgenic Arabidopsis plants could suppress the plant's basal defense response, which suggests it may have important virulence function. Aave2166 belongs to the widely distributed YopJ/HopZ effector family that may have cysteine proteinase or acetyltransferases enzyme activity. Transient expression of the wild type Aave2166, but not

the putative catalytic mutant Aave2166-C232A could trigger water-soaking like cell death in *N. benthamiana* and *N. tabacum*. Aave2166 was used as bait to screen a yeast-two hybrid cDNA library of *N. benthamiana*, which lead to the identification of six plant proteins that may specifically interact with Aave2166. Further characterization of these putative interactors may help to identify the true virulence targets of Aave2166.

Keywords: *A. citrulli*, TTSS, T3S effector, immunity, yeast two-hybrid, subcellular localization

Introduction

Almost all Gram-negative phytopathogenic bacteria possess the Type Three Secretion System (T3SS), which they use to inject a collection of T3S effectors into host cells. Some of these T3S effectors are known to inhibit plant immunity in susceptible host, which can enhance proliferation in the infected plant tissues (Alfano & Collmer, 2004). In certain plant genotypes, these T3S effectors can be recognized by cognate disease resistance gene (*R*) gene(s) (Alfano & Collmer, 2004). This R-effector interaction typically triggers hypersensitive response (HR), resulting in a rapid programmed cell death at the infection site. This HR may prevent the further proliferation of the pathogen and confer resistance to the plant (Song et al., 1995).

T3S effectors from some important phytopathogenic bacteria species including, *Pseudomonas syringae*, *Xanthomonas campestris pv. vesicatoria*, *Ralstonia solanacearum and Erwinia amylovora* have been intensively characterized for their virulence and biochemical functions (Cunnac, Occhialini, Barberis, Boucher, & Genin, 2004; Guttman et al., 2002; Nissinen, Ytterberg, Bogdanove, Van Wijk, & Beer, 2007; White, Potnis, Jones, & Koebnik, 2009). However, effectors from some other plant pathogens, such as *A. citrulli* that cause bacterial fruit blotch (BFB) disease of cucurbits, have never been studied at all (Burdman & Walcott, 2012).

Once inside of plant cells, T3S effectors are localized into different subcellular compartments where they target diverse immune signaling components. Based on their subcellular localization, T3S effectors can be classified into at least three groups. The first group of T3S effectors is localized to the plasma membrane of the host plant cells. They usually possess a myristiolation signal, which drives the effector into the plasma membrane. These effectors may be associated with the pathogen recognition receptors (PRR) and inhibit Pathogen Associated Molecular Patterns (PAMP) trigger immunity (PTI) that constitutes the first layer of defense against pathogens. One example is the *Pseudomonas* T3S effector AvrPtoB that can interact with FLS2 at the plasma membrane. The second group of effectors is localized to the nucleus (Gohre et al., 2008). Some of these effectors are characterized as transcriptional activator like (TAL) effectors. One example is the TAL effector family cloned from *Xanthomonas* and *Ralstonia* species (Doyle, Stoddard, Voytas, & Bogdanove; Li et al., 2013). Research has shown that these TAL effectors carry a nuclear localization signal (NLS). Once in the nucleus, the TAL effector can bind to the promoters of host genes and regulate their expression. Those targeted genes are host susceptibility genes, where their induction can suppress host immunity and promote the proliferation of the pathogens. The third group of T3S effectors is localized to the plastid or cytosol. One example is the *Pseudomonas* T3S effector AvrRps4 that is localized in the chloroplast, and may affect the salicylic acid biosynthesis (Li et al., 2014). Some effectors can also localize to different organelles, which suggests that these effectors could target multiple proteins in multiple complex pools. Nevertheless, the characterization of the subcellular localizations of T3S effectors could help us gain insight into the mechanism of T3S effector-mediated host susceptibility.

Most of the T3S effectors have no protein sequence similarity to each other, which makes it difficult to predict their biochemical functions based on sequence homology. One of the few exceptions is the YopJ/HopZ effector family that is highly conserved and widely distributed in diverse bacterial species infecting both animals and plants (Lewis et al., 2011). The archetypal member YopJ was found in the human pathogen *Yersinia pestis*, its homologue AvrA VopJ and AopP was also identified from animal pathogens: *Salmonella, Vibrio, and Aeromonas* respectively (Collier-Hyams et al., 2002; Fehr et al., 2006; Mukherjee et al., 2006; Noriea Iii, Johnson, Griffitt, & Grimes, 2010). YopJ homologues are also found in diverse plant pathogens that include Pseudomonas (the HopZ family), Xanthomonas (AvrRxv, AvrXv4, AvrBsT and XopJ), Erwinia (ORFB), and Ralstonia (PopP1 and PopP2), and the plant symbiont Rhizobium (Y4LO) (Ciesiolka et al., 1999; Deslandes et al., 2003; Lewis, Abada, Ma, Guttman, & Desveaux, 2008; Lewis et al., 2011; Noël, Thieme, Gäbler, Büttner, & Bonas, 2003). Previous studies have characterized the YopJ effector proteins as a cysteine protease. However, recent studies have shown that the YopJ protein isolated from *Yersinia pestis* possessed an acetyltransferase activity (Bliska, 2006; Lewis et al., 2011). These studies also demonstrated that YopJ suppresses the immune response in animals by acetylating serine and threonine residues in the activation loop of members of the mitogen-activated protein kinase-kinase superfamily, including MAPKK6, MEK2, and IκB kinase, which prevents their activation by phosphorylation and consequently inhibits their downstream signaling (Mittal, Peak-Chew, & McMahon, 2006).

The Gram-negative pathogenic bacterial species *Acidovorax citrulli* (formerly *A. avenae* subsp. *citrulli*) is the causal agent of BFB disease, which is one of most devastating diseases threatening the cucurbits industry world widely (Schaad et al., 2008). Despite the economic importance of the disease, little is known about the molecular basis of *A. citrulli*-cucurbit

interactions (Bahar & Burdman, 2010; Burdman & Walcott, 2012). Two evolutionary lineages of *A. citrulli* have been identified through DNA fingerprinting analyses and Multi Locus Sequence Typing (Feng, Schuenzel, et al., 2009; Walcott et al., 2000). The group I *A. citrulli* strains have a broad host range and have been isolated from various cucurbit family members, but mainly from melon (S. Burdman et al., 2005; Walcott et al., 2004). M6, an *A. citrulli* strain isolated from melon plants in Israel, has been considered as the Group I representative strain used for pathogenicity studies. In contrast, group II strains are highly virulent on watermelon, but less virulent on melon and other cucurbit species. The group II representative strain AAC00-1 was isolated from watermelon in the United States and was used for *A. citrulli* genome sequencing (GenBank accession NC_008752).

The annotation of the AAC00-1 genome revealed the presence of at least eleven T3S effectors based on their sequence similarly to T3S effectors identified in other bacterial species. Two T3S effectors, Aave2166 and Aave2708, present in the genome of AAC00-1 are homologous to AvrBsT, which belong to the YopJ effector family. Interestingly, this study revealed that both, Aave2166 and Aave2708, are present in all group II strains, but either absent or truncated in all group I strains that have been tested, which suggest those effectors may be responsible, at least in part, for the host preference among groups I and II isolates. Our previous studies demonstrated that transient expression of Aave2166 and Aave2708 in *Nicotiana benthamiana* (*N. benthamiana*) could trigger a water-soaking like cell death phenotype in *N. benthamiana.* They can also trigger an HR in *Nicotiana tabacum* (*N. tabacum*), which depends on the functionality of the immune signaling component NtSGT1. Furthermore, gene deletion analyses have revealed that the Aave2166 could contribute to the virulence of AAC00-1 in *N. benthamiana* at the early stage of infection (Traore et al, unpublished). Therefore, the YopJ-like effector, Aave2166 may target *N. benthamiana* proteins to suppress immunity and interact with

N. tabacum proteins to trigger nonhost resistance to *A. citrulli*. Identifying host proteins that interact with Aave2166 may help characterize its avirulence and virulence functions, and determine if it really contributes to host preference of the two lineage of *A. citrulli*.

This study characterizes the subcellular localization of Aave2166. The transient assay result suggests that Aave2166-YFP fusion proteins are localized into both cytosol and the nucleus of transformed *N. benthamiana* plant cells. Transient expression of Aave2166 could trigger cell death in *N. benthamiana*, which depends on the putative acetyltransferase catalytic site. We also generated transgenic Arabidopsis plants expressing Aave2166, where the expression of Aave2166 suppressed the host basal defense response. To identify *N. benthamiana* proteins that may interact with Aave2166, we screened a *N. benthamiana* yeast two-hybrid (Y2H) cDNA library using mutant Aave2166-C23A as bait, which lead to the identification of six tobacco proteins that specifically interact with Aave2166-C232A. Further characterization of these interactors may help identify the true virulence targets of Aave2166 in the future.

Materials and Methods

Bacterial strains. *Escherichia coli* (*E. coli*) *DH5α* [*F⁻ endA glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15Δ(lacZYA-argF)U169, hsdR17*($r_K^- m_K^+$)*, λ–*] and *A. tumefaciens* (GV2260) [C58 background, rifampicin resistant with the Ti plasmid (pTiB6s3)]

Plant materials. *N. benthamiana* (PI 555478) plants were propagated in a growth chamber programmed for 16 hours light (140 µmol/m^2/s cool white fluorescent irradiance) at 28°C and 8 hours dark at 24°C. *Agrobacterium*-mediated transient assays were conducted on three to four week old plants.

Arabidopsis thaliana ecotype Col-0 were grown in a growth chamber with setting of 12 hours light (140 µmol/m2/s cool white fluorescent irradiance) at 22°C and 12 hours dark at 20°C. Transgenic Arabidopsis plants were generated by using the flowering dipping method (ref). Transgenic seeds were selected on half MS medium supplemented with Hygromycin (50mg/L).

Cloning the wild type and mutant Aave2166 genes into various vectors. The open reading frame (ORF) of Aave2166 was PCR-cloned into TopoEntrD (Invitrogen, Carlsbad, CA) from the genomic DNA of AAC00-1 using primers Aave2166-for 5'-caccAGATCTATGAAGAATTTCATGCGATCGAT-3' Aave2166-rev 5'-GTCGACTTCGATAGCTTTTCTGATTTTTCTCA-3'. A putative catalytic mutant Aave2166-C232A was generated by employing the site-directed mutagenesis with primers Aae2116-C232A for: 5'-AGAAGTCGCATTACGACgcCATTATTTTTTCTTTGAA-3', and Aae2116-C232A rev, 5'-TTCAAAGAAAAAATAATGgcGTCGTAATGCGACTTCT-3'. The derived clone was designated TopoEntrD-Aave2166-C232A. Wild type and mutant Aave2166 was subcloned into plant expression vector pEG101-SacB/R/R to generate pEG101-Aave2166, and pEG101-Aave2166-C232A through Gateway LR cloning (Invitrogen, Carlsbad, CA) as described previously (Traore S. and B. Zhao, 2010).

To construct pEarleygate101-NES and pEarleygate101-mNES, the HIV Rev NES sequence (LQLPPLERLTL) and NES mutant sequence (LQAPPAERATL) (Burch-Smith et al., 2007) were fused to YFP by PCR using primers NES_Rev or mNES_Rev and YFP_For, and replaced the original YFP gene in pEarleygate101. To construct the pEarleygate101-NLS, the *Agrobacterium tumefaciens* VirD2 NLS sequence (KRPREDDDGEPSERKRERDER) (Shurvinton, Hodges, & Ream, 1992) was fused to YFP by PCR amplification with primers NLS_Rev and YFP_For, and replaced the original YFP gene in pEarleygate101. To construct

pEarleygate203-Myr-YFP, a lyn kinase palmityolation-myristyolation signal membrane anchor (MGSSKSKPKDPSQRRGSAASENLYFQG) (Hadjieconomou et al., 2011) was fused to YFP by overlap PCR using primers: Myr_For1, 2, 3 and YFP reverse. The Myr-YFP was cloned into the *Xho*I site of pEarleygate203. Aave2166 and Ave2166-C232A was clone into the subcellular localization vectors by LR Gateway cloning (Invitrogen).

To express Aave2166 in *A. thaliana*, the wild type and mutant Aave2166 was also subcloned into an inducible expression vector pTA7001(Aoyama T & NH, 1997) for generating transgenic Arabidopsis plants. The original pTA7001 vector was modified to be Gateway-compatible with YFP fusions at the C-terminal. In brief, the ccdb expression cassette fused to the N-terminal of YFP was dropped from pEarleyGate101 (Earley et al., 2006a) using *XhoI*, and subcloned into the *Xho*I site of pTA7001. The derived vector, pTA7001 was designated pTA7001-Des-YFP. The Wild type and mutant Aave2166 was LR (Invitrogen) cloned pTA7001-Des-YFP to generate pTA7001-Aave2166 and pTA7001-Aave2166-C232A.

***Agrobacterium*-mediated transient assays in *N. benthamiana* plants.** *Agrobacterium*-mediated transient assays in *N. benthamiana* plants were performed as described previously (Wydro et al., 2006). The *Agrobacterium* strain was streaked on LB medium supplemented with appropriate antibiotics and incubated at 28°C for 2 days. Bacterial cells were harvested and re-suspended in induction buffer composed of 10 mM $MgCl_2$, 10 mM MES (pH 5.6), and 100 µM acetosyringone and incubated for 3 hours at room temperature. The bacterial inocula were adjusted to OD_{600nm} = 0.6 and stomatal-infiltrated into fully expanded leaves of *N. benthamiana* plants using a 1 ml blunt-end syringe without a needle. The inoculated plants were incubated at room temperature under continuous light for 20-48 hours before the detection of expressed proteins. Transient expression of the luciferase gene was detected by applying 1 µM luciferin to

the inoculation site (Chen et al., 2008). The chemical fluorescent signals were captured with a CCD camera and visualized using the Gel-Document Image System (Bio-Rad, Hercules, CA). The fluorescent signal of the Aae2166-GFP fusion protein was monitored 20 hours after inoculation by fluorescent microscopy (Zeiss Axio Observer.A1, Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Electrolyte leakage. Leaves were pre-selected and marked based on similarity in size before plants were inoculated at 3 x 10^7 cfu mL-1. After inoculation, plants were covered with a clear plastic dome for 30 minutes before the 0 hour time point leaves were removed. The remaining pots were left covered for another 24 hours. A single leaf was removed from a plant and a disc (0.7 cm diameter) was harvested using a number 3 size cork borer. Excised leaf discs were floated in a bath of sterile dH2O and quickly swirled before being placed in a tube containing 3 mL of sterile dH2O. Four plants were used for each replicate. Tubes containing leaf disks were shaken on an orbital rocker at 35 rpm for 3 hours. After 3 hours, leaf disks were removed and the solution was assayed for conductance using a conductance meter (Traceable 23226-505; VWR Scientific). Leaf disks were frozen at -80°C for 1 hour. After the freeze cycle, the leaf punch was returned to the original sample tube and rocked for an additional 3 hours at room temperature. After 3 hours, the leaf punch was removed and the conductance was measured, recorded as total leakage. Electrolyte leakage was recorded and calculated as percent leakage of total (i.e., first reading/second reading) adjusted to percent maximal.

Construct a *N. benthamiana* Y2H library. A GAL4-based *N. benthamiana* Y2H cDNA library was obtained from Dr. Staskawicz, University of California at Berkeley (unpublished data). In brief, fully expended leaves of four-week old *N. benthamiana* plants were used to

isolate total RNAs. The derived cDNAs were cloned into the yeast two hybrid prey vector pGADT7 (Clontech, Palo Alto, CA) following the instructions of the user manual.

Yeast two-hybrid screening. The yeast two-hybrid assay was performed according to the user's manual of the Matchmaker Gold yeast 2-hybrid system (Clontech). The bait vectors pGBKT7 were modified as Gateway compatible vectors (pGBKT7-Des) by inserting the Gateway recombination cassette (invitrogen) at the *Nde*I site. The Aave2166 and Aave2166/C232A genes were then subcloned from TopoEntrD vectors into pGBKT7-Des vectors resulting in pGBKT7-Aave2166 and pGBKT7-Aave2166/C232A.

The yeast strain Y2HGold (Clontech) transformed with wild type pGBKT7-Aave2166 did grow well; therefore, were used for the Y2H assay. The indicated prey vector harboring the *N. benthamiana* library (pGADT7-Nb-Lib) and bait pGBKT7-Aave2166 construct were co-transformed into the Y2HGold Yeast Strain (Clontech), and selected on yeast SD medium that is lacking leucin and tryptophan (-LT). The transformed yeast clone was regrown on yeast SD medium lacking leucin, tryptophan, and histidine (-LTH) supplemented with 3mM 3-Amino-1,2,4-triazole (3-AT). The alpha-galactosidase activity was measured according to the user's manual of the Matchmaker Gold yeast 2-hybrid system (Clontech).

Results

Full length Aave2166 genes are presented in group II but truncated in group I *A. citrulli* strains. To determine if *Aave2166* genes are conserved in *A. citrulli* strains, a set of conserved primers flanking the ORF of Aave2166 were used to amplify Aave2166 from the genomic DNAs of 7 Group I and 10 Group II strains. The larger DNA fragment (~1.5Kb) was amplified from all group II strains, but a smaller DNA fragment (~1.2Kb) was amplified from all group I strains (Figure 1). Sequencing analysis of the PCR product revealed that the larger DNA

fragment contains the full length of ORF of Aave2166, while the smaller DNA fragment contains a truncated ORF of Aave2166 (data not shown). Therefore, we conclude that full length Aave2166 is only conserved in group II *A. citrulli* strains.

Aave2166 encodes T3S effector protein with high homology to AvrBsT and other YopJ proteins. The alignment of the homologs of AvrBsT in AAC00-1 (Aave2166 and Aave2708) showed that Aave2166 and Aave2708 possessed the conserved catalytic triad of the YopJ family of effectors as shown in Figure 2.

The putative catalytic mutant of Aave2166 could not trigger cell death in *N. benthamiana* and *N. tabacum*. The multiple sequence alignment of Aave2166 along with other YopJ effector family members has shown a conserved cysteine residue. To test whether the conserved cysteine residue or the catalytic activity of the cysteine protease was responsible for the cell death phenotype observed when Aave2166 (Figure 3A) was transiently expressed in *N. benthamiana*, we generated a Aave2166 mutant where the amino acid cysteine at position 232 was mutated to the amino acid alanine using site-specific mutagenesis resulting in the mutant Aave2166/C232A. Transient expression of the Aave2166/C232A mutant in the expression vector pEG101 showed that the cell death phenotype observed with the wild type was abolished (Figure3B). Aave2166 but not Aave2166-C232A triggered cell death in *N. benthamiana*. Similarly to other YopJ family effectors (Navarro, Alto, & Dixon, 2005), the catalytic activity of the effector Aave2166 could be critical for its virulence activity in *N. benthamiana*. However, the mutation of the cysteine residue to alanine (Aave2166/C232A) did not impair the localization of the protein in different compartments of the plant cell including nucleus, cytosol and plasma membrane and was the same as the one observed with the wild type (Figure 3C and D).

Subcellular localization of Aave2166. The YFP fusion to Aave2166 has shown nuclear as well as cytosol localization of the Aave2166 effector protein. However, the sequence of Aave 2166 does not show the presence of any localization signal. To further investigate the subcellular fraction which is important for Aave2166 ability to trigger cell death in the plant cell, we generated constructs harboring different localization signals including nls (nuclear localization signal), nes (nuclear exclusion signal) as well its mutant mnes and myr (the myristiolation signal) as described in the methodology section. As shown in Figure 4A and C, the expression of Aave2166 in the plasma membrane did not trigger cell death. However, when expressed in the cytosol (nes) and nucleus (nls) Aave2166 did trigger strong cell death (Figure 4A and C). To quantify the cell death between the subcellular compartments, we performed an ion leakage analysis. Ion leakage results showed that the cell death phenotype observed when Aave2166 was expressed in the cytosol was significantly stronger than when expressed in the nucleus. Only a weak cell death was observed when Aave2166 was expressed in the plasma membrane (Figure 4B). These results suggest that either the cytosol or the nucleus could be the subcellular fraction which is very important for the virulence of Aave2166 in *N. benthamiana*.

Aave2166 doesn't suppress the HR triggered by the interaction of AvrBs1 and Bs1 on pepper. Previous study revealed that AvrBsT could suppress the HR triggered by interaction between the resistance gene Bs1 and the effector AvrBs1 (Robert Szczesny et al., 2010). Because Aave2166 is a homologue of AvrBsT, we tested whether Aave2166 could also suppress the HR triggered by Bs1 andAvrBs1. Transient co-expression of Aave2166 and AvrBs1 in pepper leaves (R10) harboring the *Bs1* gene still triggered HR-like cell death (Figure 5). Therefore, unlike AvrBsT, Aave2166 cannot suppress the Bs1/AvrBs1-mediated HR.

Aave2166 contributes to virulence in the early stage of infection of *A. citrulli* on *N. benthamiana*. The Aave2166 effector gene has been identified along with its homologue Aave2708 in AAC00-1 genomic sequence. Screening of other strains of *A. citrulli* revealed the presence of both homologues in group II strains while in the group I strains, these effector genes were missing. Diseases assay results had shown that group I strains are more virulent compared to the group II strains. More importantly, group I strains have a broad range host compared to group II strains. To test whether this difference in virulence was due to the absence of effector genes in the group I strains, we performed a gene deletion analysis in group II strain AAC00-1. Deletion of Aave2166 in AAC00-1 showed a reduction of virulence at early stage; however, at late stage, the virulence of AAC00-1/Aave2166 significantly increased compared to wild type AAC00-1 in *N. benthamiana* plant leaves (Figure 6). These results suggest that Aave2166 may either be partially recognized by a weak R gene in *N. benthamiana* or inhibit the function of other effectors during their translocation through the TTSS to the plant cell.

Expression of Aave2166 in transgenic Arabidopsis plants suppressed the basal defense. To further investigate the virulence function of Aave2166 *in planta*, we subcloned the effector gene into the expression vector pTA7001-DesA-GFP along with the dexamethasone inducible promoter. *A. thaliana* plants were transformed using the floral dipping methodology. Putative transgenic *A. thaliana* were painted with 30mM of Dexamethasone to induce the expression of the Aave2166 in Arabidopsis. The induction of the expression of Aave2166 showed cell death phenotype at the area of inoculation on the leave surfaces. Moreover, induction followed by the inoculation of *Pst* DC3000/Δ*hrcC* at a concentration of $5x10^6$ showed that the *hrcC* mutant grew well on the transgenic *A. thaliana* expressing Aave2166 compared to the empty vector (Figure 7).

Identification of putative Aave2166 interactors. To identify the putative interactors of Aave2166 in *N. benthamiana*, we subcloned the mutant Aave2166 into the pGDKT7 yeast vector and used it as a bait to screen the *N. benthamiana* yeast two-hybrid cDNA library. Co-transformation of the bait vector harboring the Aave2166 and *N. benthamiana* yeast two hybrid library in yeast cells, revealed about 9-10 thousands primary yeast transformants on SD medium lacking LT. Moreover screening of the primary yeast transformants on SD medium lacking amino-acids LTH revealed about two hundred putative interactors. Further screening on SD medium lacking LTH and supplemented with X-gal showed 88 interactor clones. Sequence analysis of these putative Aave2166 interactor clones indicated that 24 clones were in frame with the AD domain of the prey vector used in the construction of *N. benthamiana* library. Sequence annotation of the 24 clones using BLAST searches against the Arabidopsis protein database (TAIR) demonstrated that the homologues of these putative interactors of Aave2166 in Arabidopsis could be involved in the plant defense system as shown in Table 1. Therefore, these putative candidates were selected for further confirmation. Co-transformation of the selected putative candidates (prey vector) and the bait vector harboring Aave2166 confirmed interaction of 6 candidates with Aave2166 (Figure 8 and Table 1).

Discussion

The YopJ family of T3S effectors is one of the most conserved families of effectors identified in pathogenic gram negative bacteria affecting plants and animals (Orth, 2002b). Two homologues of the YopJ family T3S effectors were identified in the genome of the group II strains of *A. citrulli* while they were found to be absent in the group I strains (Eckshtain-Levi et al., 2014). In this study, we demonstrated that Aave2166, the homologue of AvrBsT, triggered

cell death in *N. benthamiana*, while the mutant (Aave2166/C232A) could not trigger the cell death phenotype; therefore, the cell death phenotype of Aave2166 is dependent on the catalytic activity of the cysteine protease. Further characterization of the virulence of Aave2166 showed that two subcellular localizations (nucleus and cytosol) were important for its ability to elicit the cell death activity. More importantly, the expression of Aave2166 in Arabidopsis indicated that Aave2166 suppressed plant immunity by targeting different interactors including WRKY, ERF and Ubi10 that are involved in plant defense system.

YopJ-like T3S effectors are one of most conserved T3S effector families identified in many different animal and plant pathogenic bacterial species including Ralstonia, Erwinia, Pseudomonas, Yersinia, Xanthomonas and Acidovorax. Studies have shown that YopJ effectors suppress the plant defense system and contribute to the proliferation of the pathogens. YopJ of Yersinia suppresses the animal cell defense system by inhibiting signal transduction components including MAPKK (Orth et al., 1999). Due to the importance of YopJ in the suppression of defense, it is possible that pathogenic bacteria have acquired YopJ by horizontal transfer. That could explain the reason why YopJ is so conserved in so many bacterial species. Our screening results showed that the YopJ homologs were present in all the group II strains of *A. citrulli*. Deletion of Aave2166 showed a reduced virulence of AAC00-1/Aave2166 mutant at early stages of infection. In contrast, the screening of the group I strains of *A. citrulli* indicated the absence of the YopJ family of effectors. Interestingly, the group I strains have lost the two homologs of AvrBsT (Aave2166 and Aave2708) and are in general more virulent on the host plant. AvrBsT is the only family member of YopJ present in *Xcv*. However, if the presence of AvrBsT extended its host range to tomato; all *Xcv* strains carrying AvrBsT are avirulent on pepper. Interestingly, another study previously showed that some Arabidopsis genotypes recognize the YopJ homolog

HopZ from *Pseudomonas syringae* (Cunnac et al., 2007). A study by (Michelmore & Meyers, 1998) also showed that plants evolved genotype-specific R genes to recognize conserved T3S effectors. It is possible that host plants possessing a weak R gene can partially select against these YopJ homologs present in the group I strains of *A. citrulli*; therefore, thereby explaining why the group I strains are more virulent compared to the group II strains. It will be interesting to express the Aave2166 gene in the group I background and test whether it will reduce its virulence as observed with the group II strains. Another interesting experiment would be to generate a double knockout mutant of Aave2166 and Aave2708 in the AAC00-1 background and test whether it enhances the virulence of the bacteria.

The comparison of the sequence of the two homologs of YopJ (Aave2166 and Aave2708) with other homologs have shown a conserved cysteine protease domain. YopJ contains a conserved catalytic triad [histidine (His), glutamate (Glu) and cysteine (Cys)], and mutations in these catalytic residues disrupt the ability of YopJ to inhibit the MAPK and NFκB pathways (Orth et al., 2000). However, the catalytic domain of YopJ was predicted to be a cysteine protease (Orth et al., 2000). Enzymatic assays have shown that AvrBsT, HopZ and HopZ2 have a weak cysteine protease activity. The YopJ homolog from Yersinia has been shown to be an acetyltransferases. Nevertheless, the catalytic activity of the YopJ homolog is dependent on the cysteine residue. As observed for other homologs of YopJ, Aave2166 triggers cell death on *N. benthamiana* leaves (Figure 3A); however, the cell death was dependent on the cysteine residue at position 232, since the substitution from cysteine to alanine abolished its ability to trigger cell death in *N. benthamiana* leaves.

Among the YopJ family members AvrBsT is the only homolog that has been shown to inhibit the HR response triggered by the interaction of AvrBs1 and its cognate R gene Bs1 (R.

Szczesny et al., 2010). XopJ or avrRxv from Xcv were not found to suppress the HR elicited by AvrB1/Bs1. The same result was observed when Aave2166 and AvrBs1 were co-expressed in pepper (ECW-R10) leaves. The inhibition of the HR triggered by avrBs1/Bs1 was SnRK1 dependent. However, screening the *N. benthamiana* library to identify putative interactors of Aave2166 did not reveal SnRK1 as a putative interactor of Aave2166. The interactors of Aave2166 including the 23 proteasome subunit, WRKY, and Ethylene Responsive Factors (ERF) have been all shown to be involved in the plant defense system. However, it is interesting to note that the 23 proteasome subunit has been found to be a putative interactor of AvrBsT. Studies (Spoel et al., 2009) have shown that T3S effectors use the host proteasome to control protein turnover, playing the role of an ubiquitin ligase. Another putative interactor of Aave2166 is the WRKY transcription factor. The WRKY family is a family of transcription factors which is involved in various plant processes but more importantly coping with biotic and abiotic stresses (Pandey & Somssich, 2009). Systemic acquired resistance (SAR) modulated by SA (biotrophic pathogens) and JA (necrotrophic pathogens) is regulated by the WRKY transcription factors (Eulgem, 2005). Studies have shown that the AtWRKY52 transcription factor interacts with the effector PopP2 in the nucleus (Yu, Chen, & Chen, 2001) and other studies have shown that WRKY regulated the expression of the NPR gene following biotrophic infection (Yu et al., 2001). Furthermore, the yeast two hybrid screening revealed that ERF, a transcription factor that regulates the expression of defense related genes to prevent pathogen proliferation, is a putative interactor of Aave2166. A study has demonstrated that ERF1 acts downstream of the intersection between the ethylene and jasmonate pathways and suggested that this transcription factor is a key element in the integration of both signals for the regulation of defense response genes (Lorenzo, Piqueras, Sánchez-Serrano, & Solano, 2003).

Our finding suggests that the homolog of YopJ from the group II strains of *A. citrulli*, suppresses the plant immune system. This suppression is dependent on the catalytic activity of the cysteine residue at position 232. We also demonstrated that Aave2166 interacts with the transcription factors WRKY, the 23 proteasome and finally the ERF. Further studies should investigate the mechanism by which Aave2166 inhibits or modifies its interactors.

Table Legends

Table 1

Putative *N. benthamiana* proteins/peptides that are specifically interacting with Aave2166-C232A in Y2H assay

Test ID	AD clone	Co-transformed with BD-empty vector (growth on LTH-)	Co-transformed with BD-Aave2166 (growth on LTH-)	Arabidopsis homologue locus	Annotation
9-1	10-4-1	-	+	AT3G56400.1	WRKY70, ATWRKY70 | WRKY DNA-binding protein
9-2	1-1	-	+	AT3G02750.2	Protein phosphatase 2C family
9-3	8-5	-	+	AT5G43410.1	Integrase-type ATERF14, ERF14
9-4	5-8-1	-	+	AT5G66140.1	PAD2,proteasome alpha subunit D2
9-5	26-1	-	+	AT4G05320.4	UBQ10 | polyubiquitin 10
9-6	10-1	-	+	AT3G55470.2	Calcium-dependent lipid-binding protein

Figure Legends

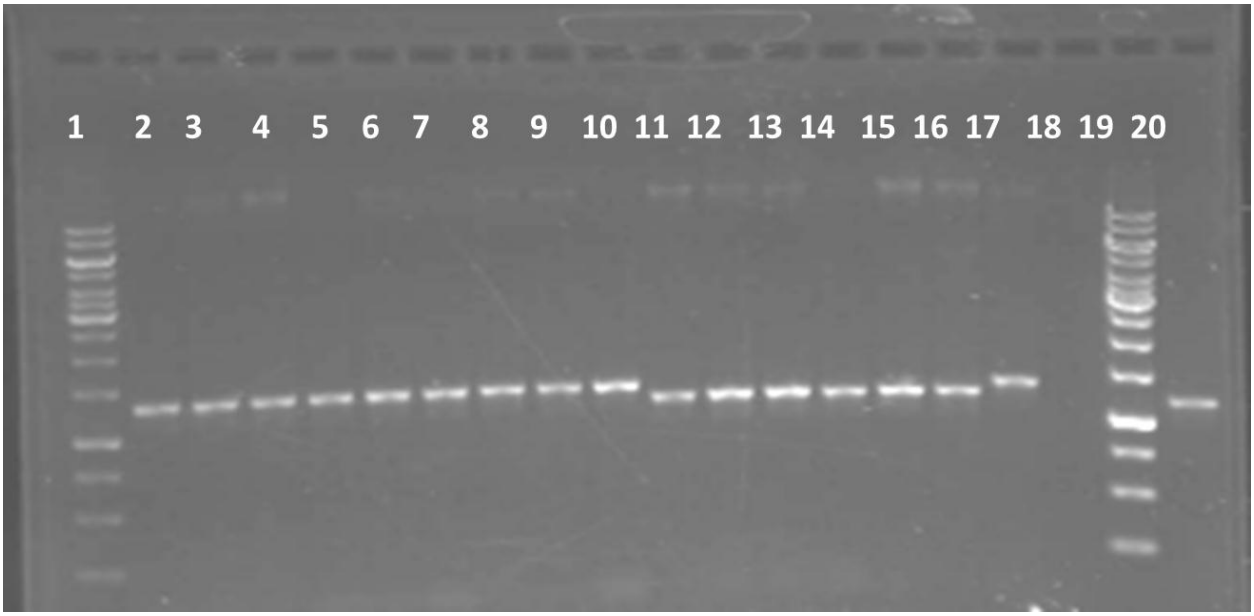

***Figure* 1. PCR analysis of the Aave2166 gene homologues in group I and II strains of *A. citrulli*.** Lane 1, 1kb marker; 2, 206-102(II); 3, 208-10 (II); 4, 208-27(II); 5, 94-48 (II); 6, 94-21 (II); 7, 201-12 (II); 8, 92-17 (II); 9, 94-12 (II); 10,94-39 (II); 11, 92-300 (I); 12, Au-9 (I); 13, 203-57 (I); 14, 99-5 (I); 15, 98-17 (I); 16, 92-305 (I); 17, ACC00-1 (II); 18, H2O CK; 19,1kb marker; 20, M6 (I). The group name (I) or (II) was labeled behind the name of each strain.

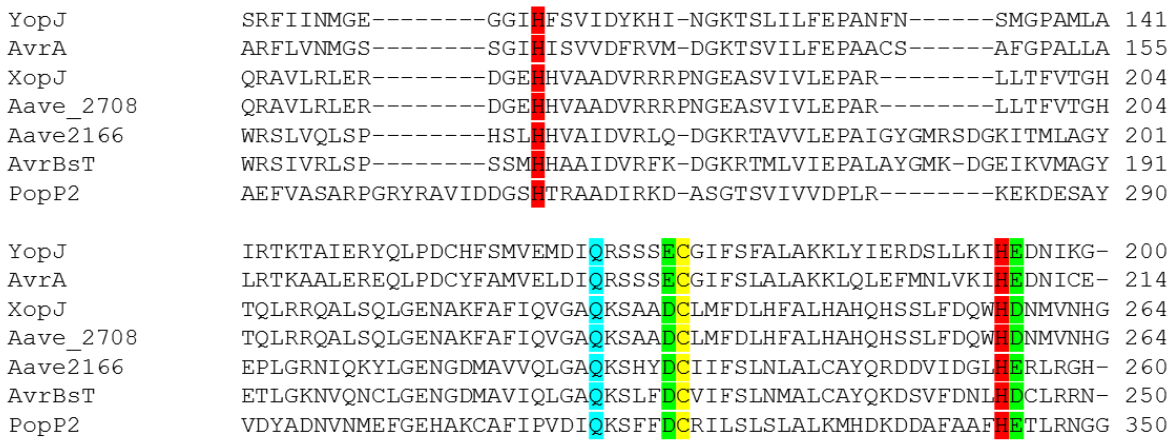

Figure 2. Amino-acid sequence alignment of some YopJ homologues including Aave2166 and Aave2708. Colored amino-acids showed the catalytic triad of YopJ protein histidine cysteine and the glutamate/Aspartate.

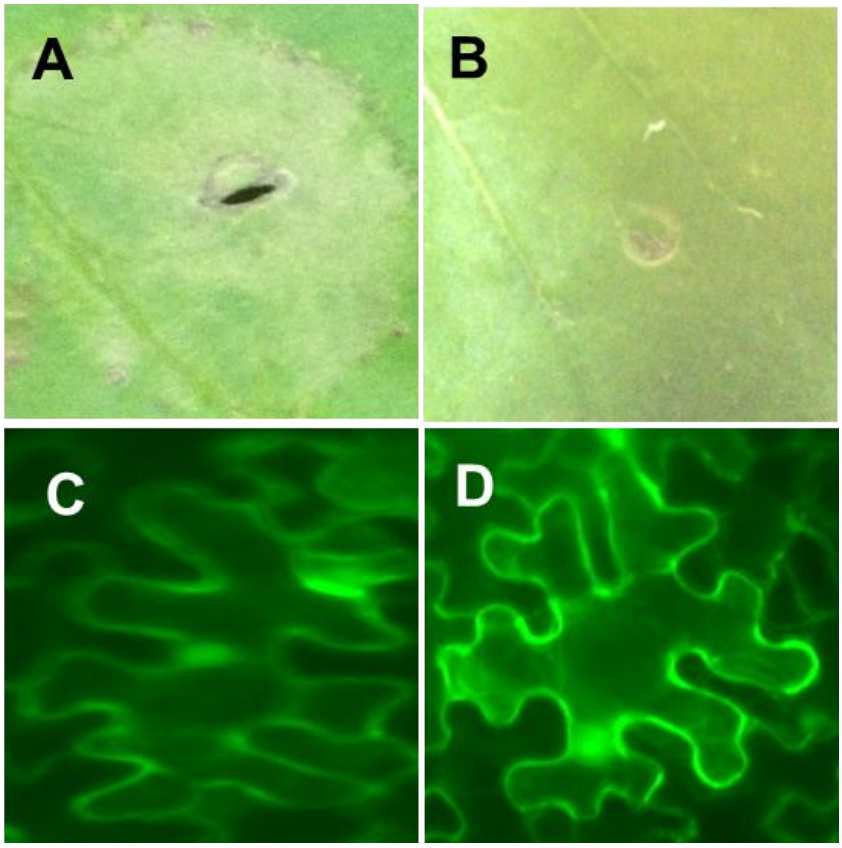

***Figure* 3. Transient expression of Aave2166 and Aave2166-C232A in *N. benthamiana*.**
(**A**) Transient expression of Aave2166 triggered cell death in *N. benthamiana*. (**B**) Transient expression of Aave2166-C232A did not trigger cell death in *N. benthamiana*. (**C**) YFP fluorescence signal showing the expression and the localization of Aave2166-YFP. (**D**) YFP fluorescence signal showing the expression and the localization of Aave2166-C232A-YFP. The inoculated leaves were photographed at 2 days post inoculation. The fluorescent signal was detected by a fluorescent microscopy (20x objective).

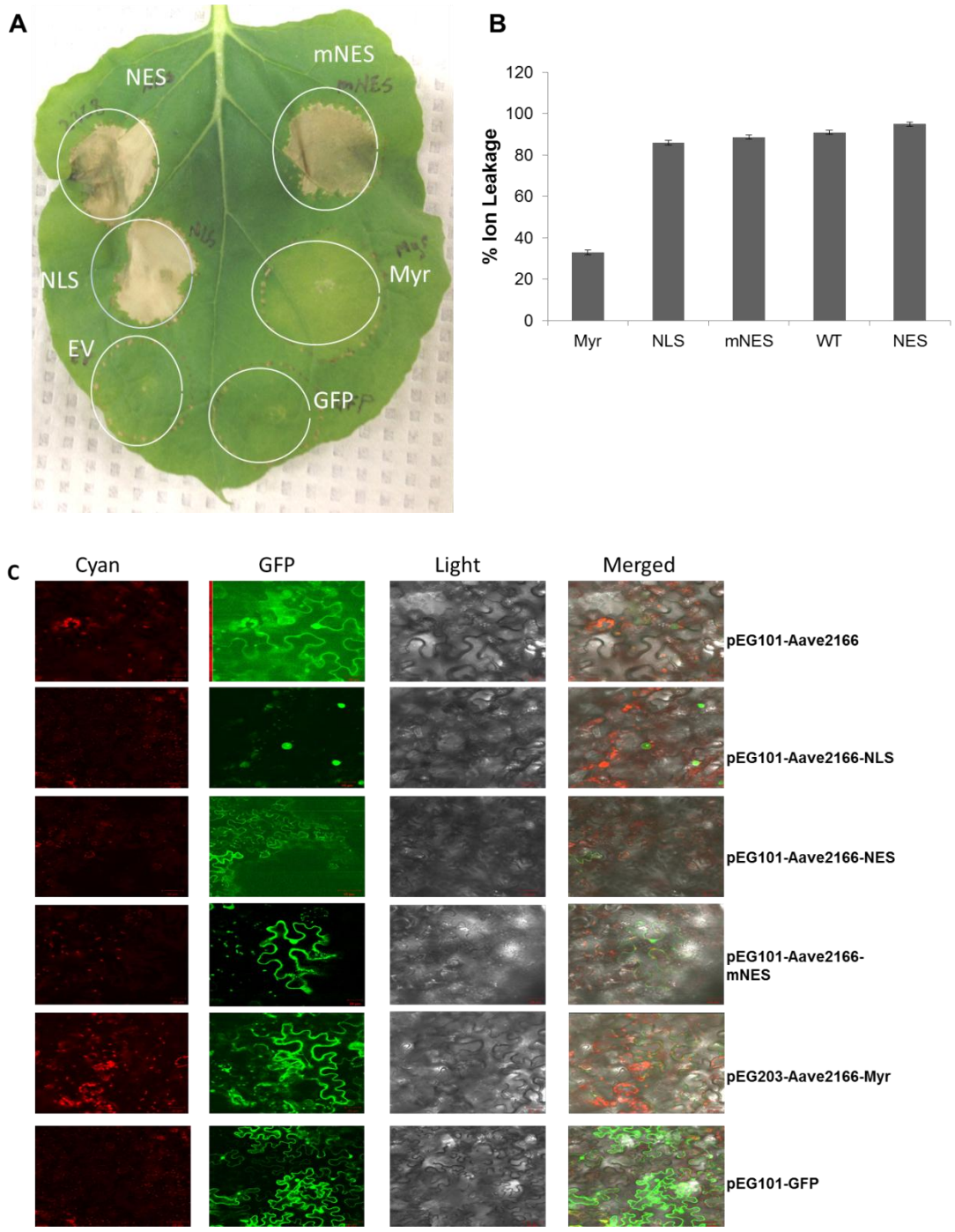

***Figure* 4: Transient expression Aave2166 fused with different subcellular localization signals triggers different levels of cell death in *N. benthamiana* leaves.** (**A**) Transient expression of Aave2166 fused to different localization signal. NES: nuclear exclusion signal, mNES: mutant NES, NLS: nuclear localization signal, Myr, myristoylation signal, EV: empty vector (**B**) Quantification of the cell death phenotype triggered by Aave2166 fused with different localization signals. (**C**) YFP fluorescence showing the expression of Aave2166 fused with different localization signals. Data represent mean and standard errors from one representative experiment of three, with 3 replicates per treatment. Different letters indicate significant differences ($P \leq 0.05$) among treatments within each time point by Tukey-Kramer HSD test.

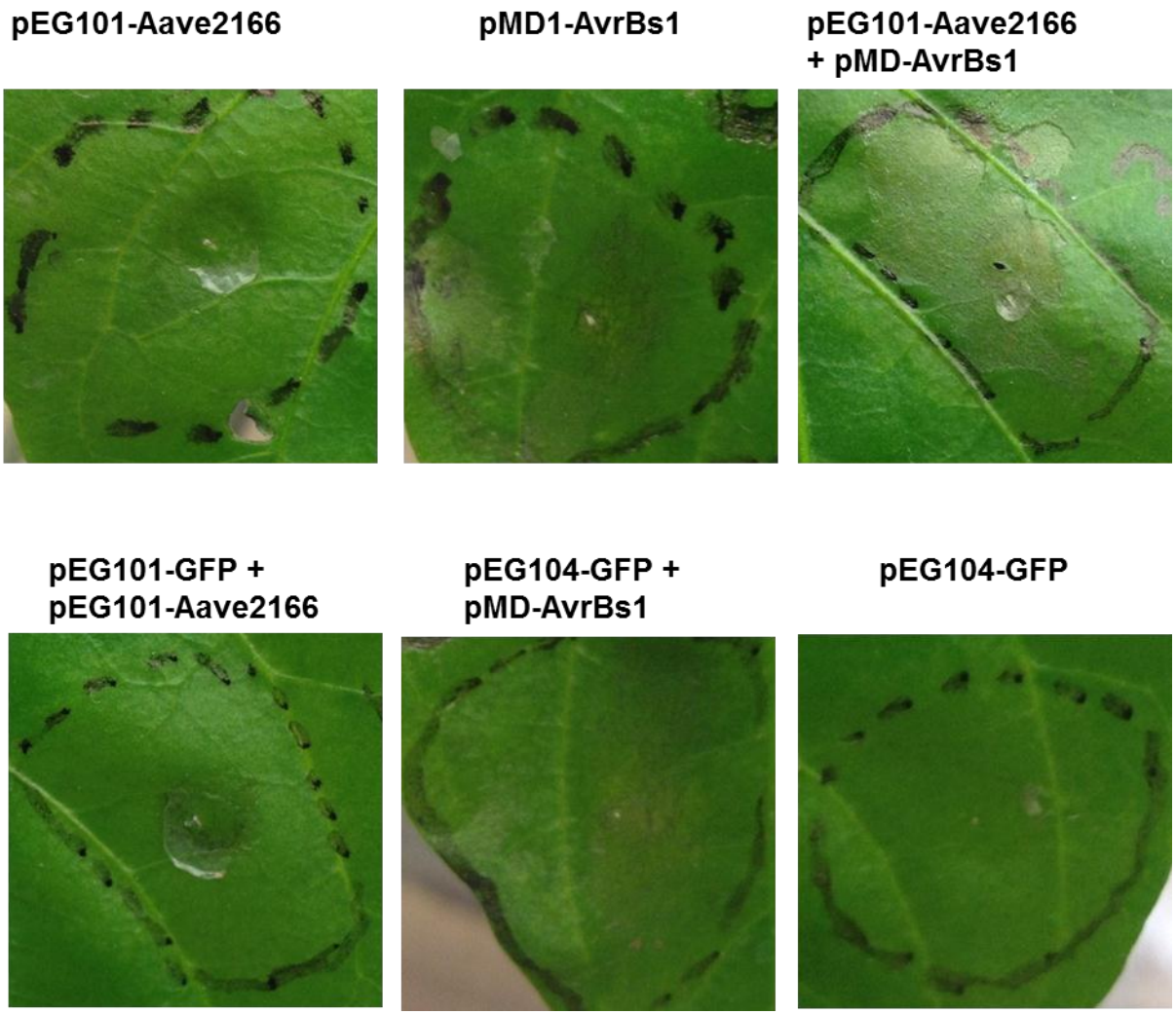

***Figure* 5. Aave2166 did not inhibit the HR trigger by AvrB1/Bs1 interaction.** Transient expression of Aave2166 did not trigger HR while AvrBs1 triggered cell death in pepper (ECW/R10). Transient co-expression of Aave2166 and AvrBs1 triggered a HR response. Expression of GFP and co-expression of GFP/Aave2166 did not trigger HR while co-expression of GFP/AvrBs1 triggered HR.

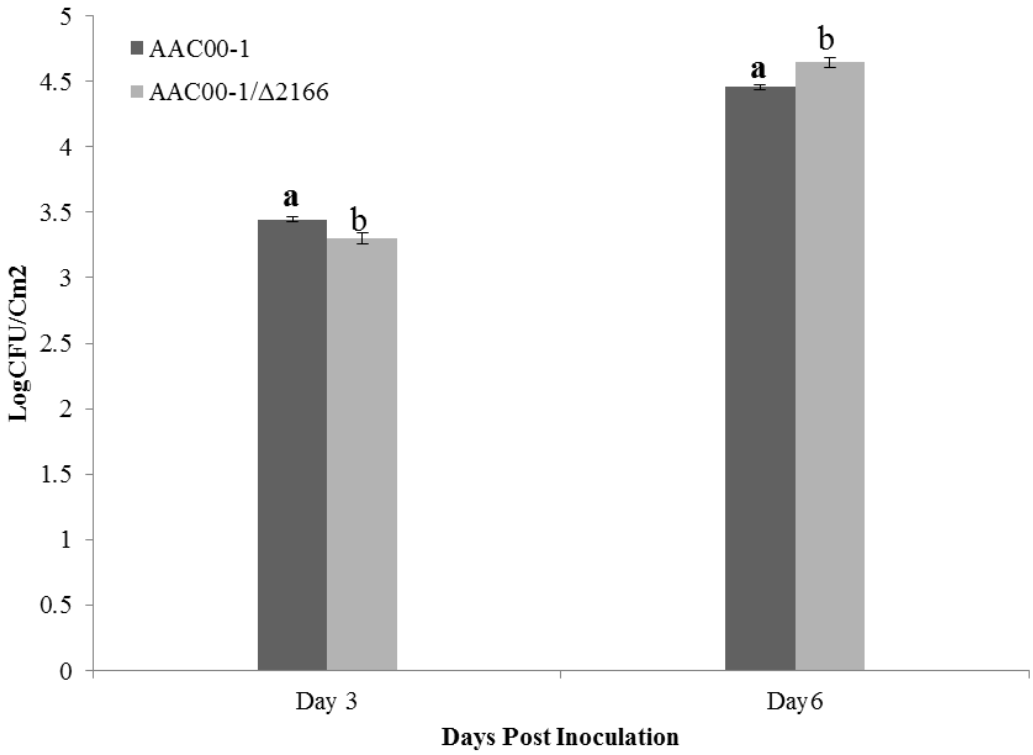

***Figure* 6. In *Planta* growth curve assay of AAC00-1 and AAC00-1-ΔAave2166 strains.** Four week-old *N. benthamiana* leaves were spray inoculated with $2x10^6$ CFU/ml of AAC00-1 and AAC00-1-ΔAave2166 strains. Bacteria growth was monitored at 3 and 6 dpi. Data represent mean and standard errors from one representative experiment of three, with 3 replicates per treatment. Different letters indicate significant differences ($P \leq 0.05$) among treatments within each time point by Tukey-Kramer HSD test.

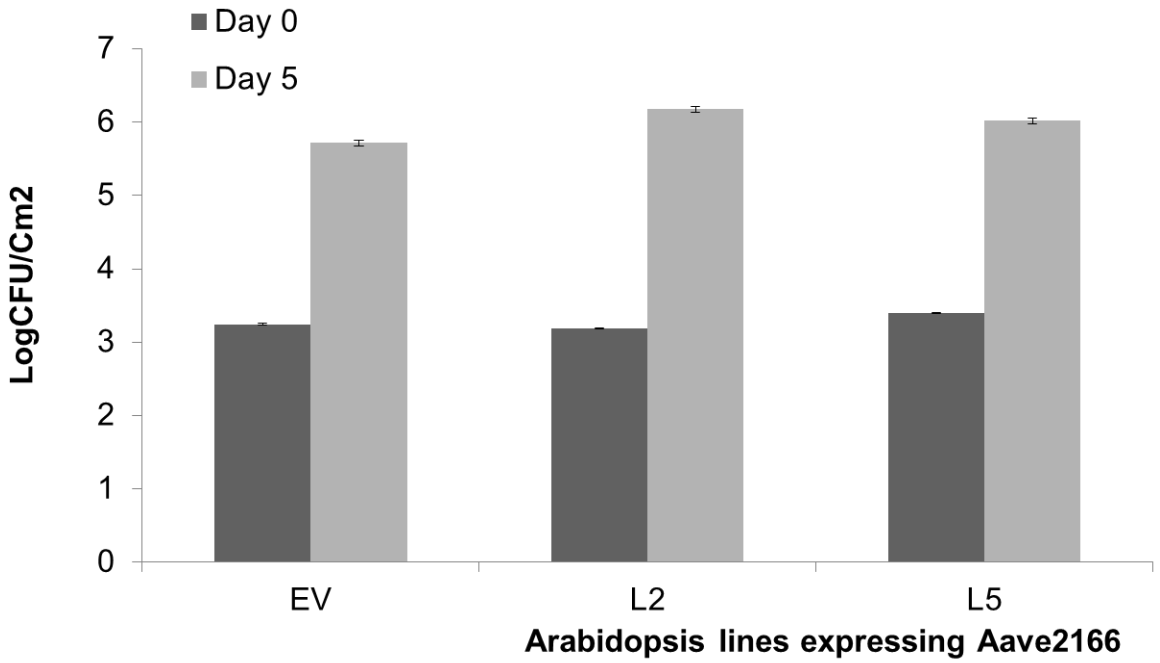

***Figure* 7. In *Planta* growth curve analysis of *Pst* DC3000-Δ*Hrc*C in *Arabidopsis thaliana* lines expressing *Aave2166*.** All lines were sprayed with Dexterian to activate the *Aave2166* expression. The following day, all lines were inoculated with *Pst* DC3000-Δ*Hrc*C at OD_{600}=0.05. Leaves samples were collected 5 days after inoculation. Two independent transgenic lines, L2 and L5, showed an increase in bacterial growth compared to the empty vector (EV) line. The expression of Aave2166 could suppress the basal defense in Arabidopsis. Data represent mean and standard errors from one representative experiment of three, with 3 replicates per treatment. Different letters indicate significant differences ($P \leq 0.05$) among treatments within each time point by Tukey-Kramer HSD test.

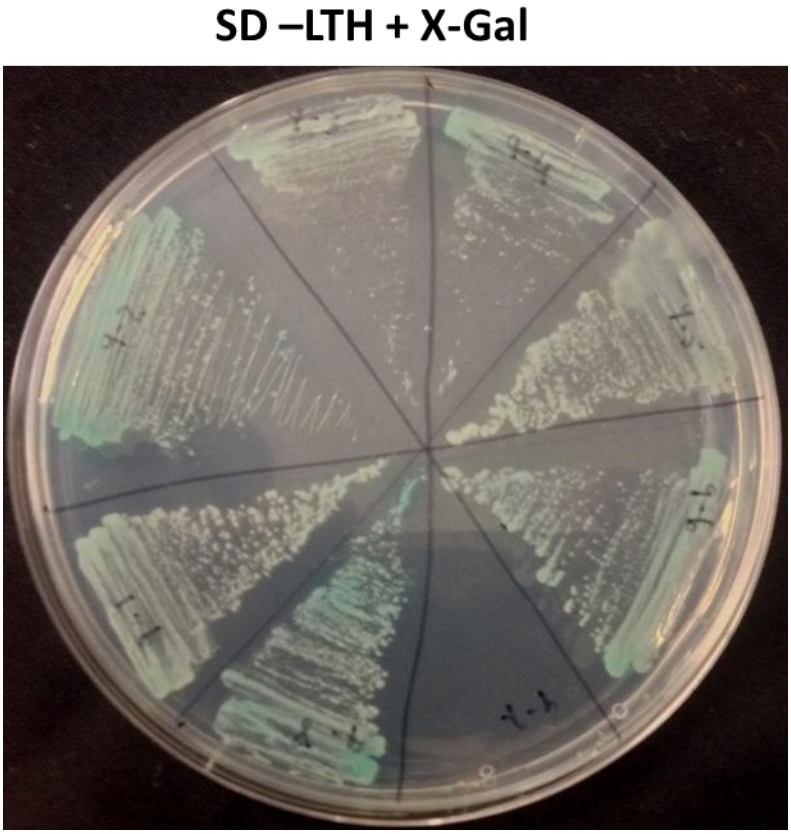

***Figure* 8. Yeast two-hybrid analysis showing the putative interactors of Aave2166.** Petri plate represents some of the putative interactors clones on SD medium lacking LTH and supplemented with X-Gal.

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