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Expressions of autophagy-associated *ATG* genes in response to fusarium wilt infection in banana

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Abstract

Host and pathogens have to cope with different types of environmental stresses during their developmental processes. Autophagy regulates programmed cell death as a response to pathogen infection and is emerging as key a defense module in host-pathogen interactions. The current study focuses on the differential pattern of expression of autophagy (ATG) genes in two contrasting banana genotypes, upon infection with fungal pathogen *Fusarium Oxysporum* f.sp. *cubense* (*Foc*1). The expression analyses of twelve ATG genes responding to biotic stress were investigated in the contrasting genotypes "Calcutta-4" (tolerant) and "Kadali" (susceptible). All the 12 ATG genes were upregulated in both contrasting genotypes, upon disease progression. After Foc1 infection, it was observed that the susceptible genotype "Kadali" showed an enhanced expression at 3dpi, in comparison to the tolerant genotype. An increased and sustained expression of ATG genes in tolerant genotype "Calcutta-4" at 10dpi was also observed suggesting accelerated defense response, under *Foc*1 infection, indicating an important role of autophagy in disease tolerance to regulate programmed cell death which is a key component of plant immunity response. The present survey suggests that both increased and sustained higher expression of ATG genes during disease progression, imparts tolerance to *Foc*1 in banana.

Keywords Defense response. Host-pathogen interaction · *Musa* · Necrotrophic pathogen · Pathogen triggered immunity

Plants, upon exposure to stress conditions, bring about degradation of cytoplasmic macromolecules inside the vacuole (Bassham 2007) and thus utilise the mechanism of recycling, when the nutrient supplies are limited by a process known as autophagy (Doelling et al. 2002).

Multiple roles have been identified and reported for autophagy-related proteins (*ATG*s) that participate in host defense responses (Klionsky et al. 2003; Zhou et al. 2014a). Various functions of macroautophagy in several plant species have been reported in response to stress conditions (Moriyasu and Ohsumi 1996; Doelling et al. 2002; Bassham 2007). Certain phenotypic analysis of *ATG* mutants indicates that autophagy plays a role in growth, development, and stress responses (Chetan et al. 2012). An overall schematic representation of autophagy pathway is shown in Fig. 1.

Although the role of *ATG*s and autophagy is well-known in model plants such as Arabidopsis (Su et al. 2006), Rice (Chung et al. 2009), Tobacco (Xia et al. 2011) and Maize (Li et al. 2015), the possible role of signalling pathways triggering the induction of plant autophagy upon fungal infection in banana, remains unknown (Wei et al. 2017). It is also important to understand the role of autophagy genes (*ATG*s) under biotic stress, as these genes are shown to regulate some Pathogen Triggered Immunity (PTI) responses, such as Reactive Oxygen Species (ROS) accumulation, programmed Cell Death (PCD), defense related gene expression and callose deposition.

The roles of autophagy genes in plants have been reported based on comparative phenotypic analysis of wild type and *ATG*-knockout or overexpressing plants, such as 25 At*ATG*s in *Arabidopsis* (Hanaoka et al. 2002), 33 Os*ATG*s in *Oryza sativa* (Xia et al. 2011), 24 Sl*ATG*s in *Solanum lycopersicum* (Wang et al. 2015). However, in banana recently, 32 Ma*ATG*s in response to fungal pathogen *Fusarium oxysporum* f. sp.

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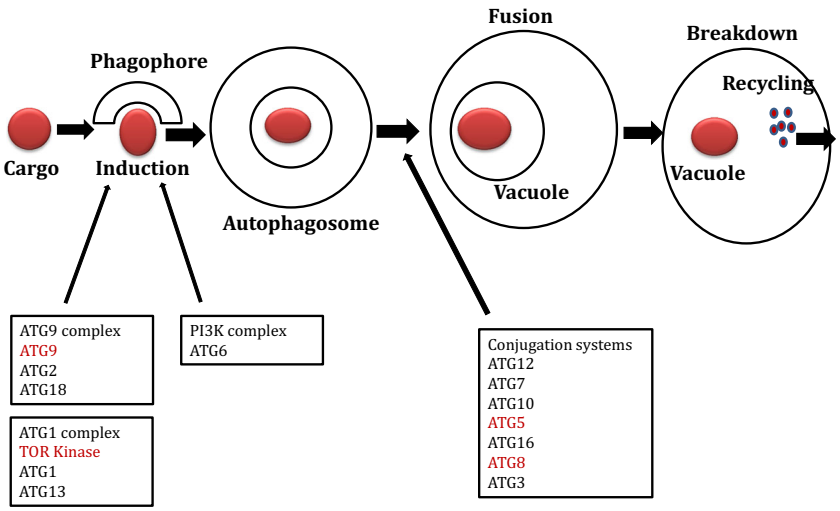

Fig. 1 Pathway of autophagy and role of different autophagy genes and their involvement during the process. In the figure, we can find the role of ATG9 during the formation of autophagosome formation and ATG5 and ATG8 involved during the core process of autophagy

cubense race 1 (*Foc*1) have been reported, of which, seven *ATG* genes (*ATG*1, *ATG*2, *ATG*5, *ATG*6, *ATG*7, *ATG*9 and *ATG*12) contained only one member, whereas, *ATG*8 had a maximum of 10 members (Wei et al. 2017).

Banana is severely affected by *Foc* (Ploetz et al. 2015). To analyse the role of autophagy genes in banana upon fungal infection, we chose to focus on banana *ATG*5, *ATG*8 and *ATG*9 as potential targets for the study, as their products are required for the core process of autophagy and also for the regulation of autophagosomes (Yoshimoto et al. 2009, Lai et al. 2011). Aiming to understand the pattern of expression of *ATG* genes upon infection with *Foc*1 in contrasting genotypes, the present study was performed. This would help in understanding their role in *Foc*1 tolerance in banana.

The fungus pathogen *Foc*1 (*Foc*1- VCG group 0125; deposited in the Queensland Department of Primary Industries South Johnstone Australia; Pegg et al. 1993) used in this study, was isolated from infected banana corm and were characterised using translation elongation factor primers, and subsequently sequenced (GenBank MH746761, Divakara et al. 2014). Two consequently contrasting diploid *Musa* genotypes "Calcutta-4" (C4-tolerant; AA; IC-395097) and "Kadali" (K-susceptible; AA; IC- 250622) were used (Ravishankar et al. 2011). For inoculations, wounds were made in the newly emerged roots of the plantlets with sterilised needles (2 months old plantlets in pots containing autoclaved sterilised cocopeat) and infected with 50 ml conidial suspension poured over the injured roots (*Foc*1; 1×10^4 spores ml^{-1}). Three plantlets for each treatment were inoculated, while three uninfected plantlets served as controls. Root samples were harvested before inoculation (0) and at 3 and 10 days post inoculation (dpi). These samples were immediately frozen using liquid nitrogen and kept at −80 °C until further use (Swarupa et al. 2013).

In the present study, we selected 12 ATGs (*ATG*5a, *ATG*5b, *ATG*9a, *ATG*9b, *ATG*8ca, *ATG*8cb, *ATG*8fa, *ATG*8fb, *ATG*8ga, *ATG*8gab, *ATG*8ia, *ATG*8ib; (Wei et al. 2017) to explore their role and expression pattern during disease development in contrasting genotypes. We analysed their expression at two different time points of disease progression using qPCR.

Total RNA was extracted by the modified pine tree method (Chang et al. 1993). Extracted RNA was later subjected to RNase-free DNase treatment using TURBO DNA-free kit (Ambion, Cat#AM1907) followed by cDNA synthesis using RevertAid First-Strand cDNA Synthesis kit (Thermo Scientific; Cat no. K1622). Quantitative RT-PCR was performed in 20 μL reaction volume with DyNAmo Flash SYBR green qPCR kit (Thermo Scientific, Catalog No.F-416 L). PCR reactions were performed on a fast Real-Time PCR System Quant Studio 7 Flex (No. 278871032 Applied Biosystems). Primers used for qPCR analysis are listed in Table 1. To compare untreated and treated expression levels, the function $^{\Delta\Delta}$CT was determined and the induction ratio of treatment/control was calculated using the eq. $2 - {}^{\Delta\Delta}$ CT.

The gene expression data for *ATG* genes was represented as mean values with standard error (mean ± SE) and the significant differences between treatments were compared statistically by one way ANOVA using MS-Excel software as described by Yamada et al. (2007).

All of the *ATG* genes showed differential expression profiles in response to the *Foc*1 treatment at some time intervals

Table 1 ATG specific primer sequences used for qPCR analysis

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
*ATG*5a	CGGACTAAGGAGTAGCGAAATC	CACCACAAGTCGAGTAGCATAG
*ATG*5b	GTCGGTCGCCTTCCTATTATT	TGAGAGACAGTGAGAGAGAG AG
*ATG*8ca	CGCCACAGAGAAACGTAAGA	CACCTAACGCAACGAGGATAG
*ATG*8cb	CACATTAGGACGAAGGGTAGAG	GAACCCTCAACCTCAGAATAG
*ATG*8fa	CGGCCTTTCCTTGTTTGATTC	TGCTCTGACTCCTCGACATA
*ATG*8fb	GTGTGTTCTTG*ATG*CGTG*ATG*	GGAAACCTGCCAGGGTATTTA
*ATG*8ga	GCCTTAGTGGTTCTGTCACTT	TGGCCTTTCATCTCCTTCATC
*ATG*8gb	GAAAG*ATG*AAG*ATG*GGTT CCTTTAC	GAACTGTGTTGGTGGCATAATC
*ATG*8ia	GACGAATCTCGCCCTTCTTATTA	TCCAACCGCGG*ATG*AATAAA
*ATG*8ib	TGGCAAGAAGTCGCGTATAAT	CGGAAGATCACTCCTTGAGAAC
*ATG*9a	AGGACATC*ATG*GCAGTTTCTT	GCTACAGCAGCTCGACTTATAG
*ATG*9b	TGTTGATTGGCCTGCTCTC	AAGGTACAAGTGGGTGCTTATT
Rbp12	AGGGTTCATAGCCACACCAC	CCGAACTGAGAAGCCCCTAC

of infection. It was observed that the pattern of expression of most of the *ATG* homologues was altered during the early stages of infection (Fig. 2). In particular, we observed that expression patterns of *ATG* genes had enhanced in both tolerant and susceptible genotypes at 3dpi in comparison to the control (untreated) samples (Fig. 2). Further, we also observed a sustained and increased expression in tolerant genotype "Calcutta-4", but in the case of susceptible genotype "Kadali" there was a decrease with disease progression (10dpi; Fig. 2). We also observed that the multiple members of the same subfamilies, for example *ATG*8ga (Fig. 2g) and *ATG*8gb (Fig. 2h), had similar patterns of expression, confirming shared similar induction kinetics with an increase in expression levels during disease progression (Fig. 2).

Necrotrophic pathogens cause death of host cells at the early stage of infection and absorb the released nutrients for their survival and multiplication (Glazebrook 2005; van Kan 2006). They make use of virulent factors (toxins, ROS and hydrolytic enzymes) to cause death of the host cells (Glazebrook 2005; van Kan 2006). In turn, plants have evolved inbuilt strategies to respond to particular stress conditions, to enable growth and survival (Ahuja et al. 2010). A study reported by Xiong et al. (2007), describes the role of *ATG* proteins in plant responses to various environmental stresses. The finding presented here supports the previous studies in confirming the induction of ATG genes, when subjected to fusarium wilt infection in banana plants.

The majority of plant autophagy associated *ATG* genes are, for instance, expressed preferentially upon challenge with the *Foc*1 (Fig. 2). In this study, it has been observed that the *ATG* genes studied here are induced in both tolerant and susceptible genotype at 3dpi (Fig. 2). However, at 10 dpi, tolerant genotype sustained higher expression of *ATG* genes (Fig. 2). In the case of "Kadali", there is a drastic reduction in *ATG* gene expression from 3 dpi to 10dpi (Fig. 2). This suggests that sustained higher expression plays an important role in imparting tolerance to *Foc*1. The results of our study supports the well-substantiated pathway of plant-defense mechanism that involves the recognition of pathogen associated molecular patterns (PAMPs) and thus brings about plant triggered immunity (PTI) to prevent further infection, spread of disease, hypersensitive response (HR) and pathogen-induced hypersensitive cell death (PCD) (Apel and Hirt 2004; Jones and Dangl 2006; Kwon et al. 2013).

In recent years, there is much evidence that shows the involvement of autophagy in protection of plants against pathogenic infections by three ways, namely, PCD, Salicylic acid (SA) and Jasmonic acid (JA) regulated defense pathways (Zhou et al. 2014b). In our study, the patterns of expression of ATG genes (ATG5, ATG8 and ATG9) along with their subfamilies were up-regulated throughout the disease progression (3dpi and 10dpi) in tolerant genotype, indicating the positive role of *ATG*s in response to *Foc*1 infection.

A total of 12 putative *ATG* genes identified in banana were selected to analyse the pattern of expression in two contrasting genotypes, upon *Foc*1 infection at two time points of disease progression (3 and 10 dpi). The *ATG*s were induced in both the genotypes at 3 dpi. However, sustained and higher expression was observed in the tolerant genotype. This might be one of the mechanisms involved in imparting tolerance to "Calcutta-4" genotypes. The involvement of autophagy in plant defense against *Foc*1 is consistent with the induction

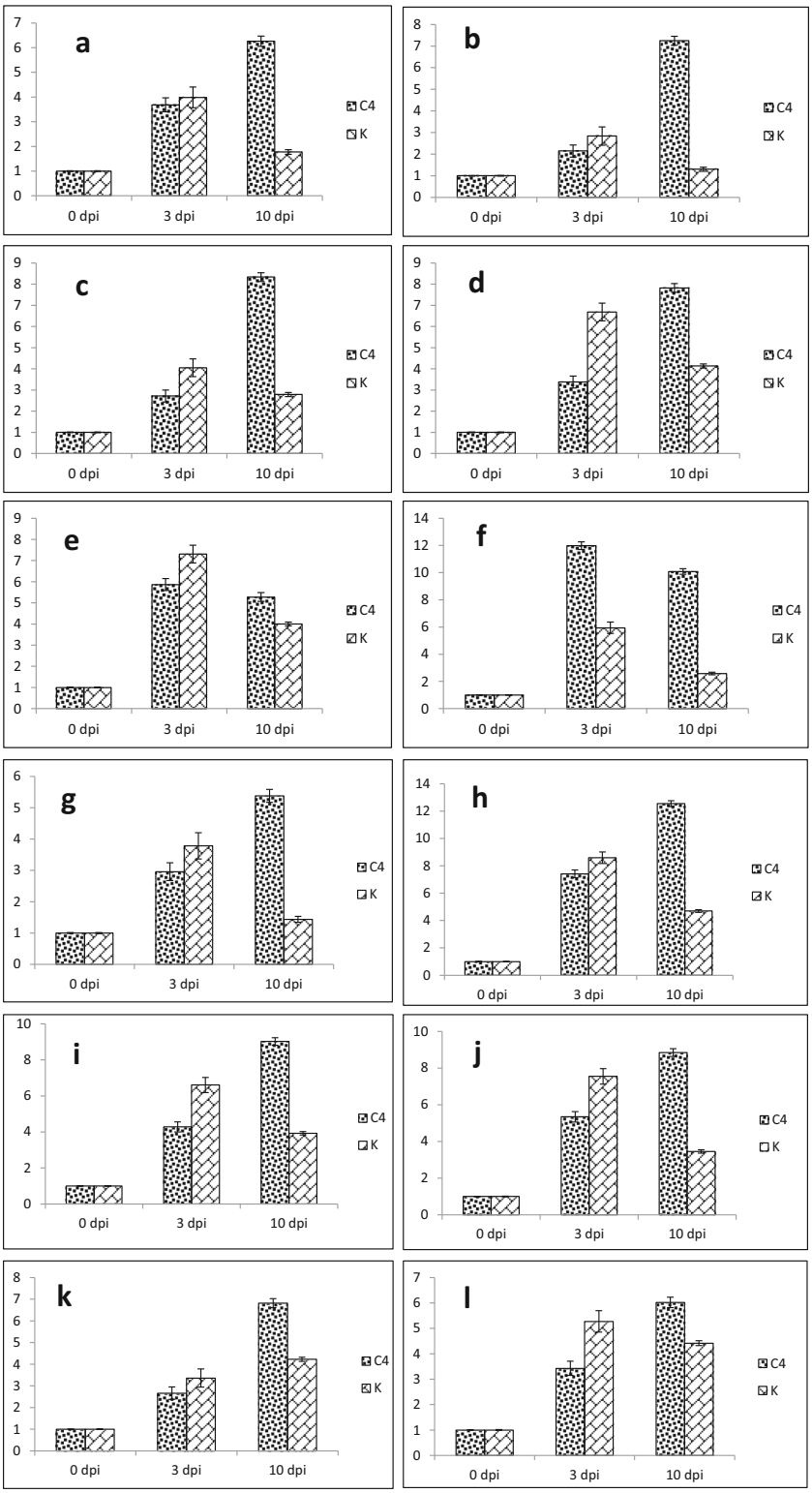

Fig. 2 Expression profile of 12 *ATG* genes ATG5a(2a), ATG5b(2b), ATG8ca(2c), ATG8cb(2d), ATG8fa(2e), ATG8fb(2f), ATG8ga(2 g), ATG8gb(2 h), ATG9b(2i), ATG8ia(2j), ATG8ib(2 k) and ATG9a(2 l) in response to *Fusarium oxysporum* f.sp.*cubense* (*Foc*1) treatment. Relative expression levels of each *ATG* gene in banana upon Foc1 were normalised to *Rbp12* endogenous reference gene. The expression level of each *ATG* gene was calculated and compared with uninfected plantlets. *C4- "Calcutta4" (tolerant genotype). *K- "Kadali" (susceptible genotype)

of autophagy genes in treated samples imparting tolerance to *Foc*1 infection. This is the first study in banana showing the pattern of autophagy gene expression among contrasting genotypes upon *Foc*1 infection.

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