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Short Communication

Over expression of anti-apoptotic gene in banana cv Rasthali enhances resistance against *Fusarium oxysporum* f. sp. *cubense* Race 1

Umesha M.[1], Sunisha C.[2], Usharani T.R.[2*], Sowmya H.D.[2] and Sriram S.[3]

[1]Dept of Biotechnology and Biochemistry, Centre for Post-Graduate Studies
Jain University, Bengaluru - 560069, Karnataka, India
[2]Division of Basic Sciences, [3]Division of Crop Protection
ICAR-Indian Institute of Horticultural Research, Hesaraghatta, Bangalore - 560089, Karnataka, India
*Corresponding author Email : Usharani.TR@icar.gov.in

ABSTRACT

The most popular banana cv Rasthali was transformed with anti-apoptotic gene, *AtBAG4* regulated with two different promoters *viz*., ZmBgl and ubiquitin to enhance the tolerance levels to *Fusarium oxysporum* f. sp. *cubense* Race 1 (FOC1). The differences in gene expression driven by two promoters revealed that stronger expression of *AtBAG4* gene under the ubiquitin promoter suppressed the infection and spreading processes of FOC1 in transgenic banana under standard bioassay systems. Analysis using the real time PCR showed the varying levels of *AtBAG4* gene expression under two promoters. It was evident that ZmBgl driven *AtBAG4* lead to lower gene expression in leaves which correlated with lesser levels of resistance to FOC1. Constitutive expression of *AtBAG4* under the control of ubiquitin promoter showed increased transgene transcripts which directly correlated with the enhanced tolerance against FOC1 from seedlings stage to active vegetative phases. This study reveals the importance of constitutive expression of anti-apoptotic gene showing enhanced tolerance against the most dreaded FOC1 in highly susceptible variety Rasthali.

Keywords : Anti-apoptotic gene, banana, constitutive expression, fusarium wilt, rasthali

Fusarium wilt disease caused by fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (FOC) is one of the major threats to the banana cultivation (Ploetz and Pegg, 2000). The fungal mycelium clogs the xylem vessels and hinders the supply of water and nutrients, showing the symptoms of discoloration and drooping of leaves, splitting of stem, eventually leading to the collapse of the plant (Li *et al.,* 2011). Based on virulence and host specificity FOC has been differentiated to four physiological races *viz*., races 1-4 (Moore *et al.,* 1993). Banana cv. Rasthali is highly susceptible to FOC1, due to which the area of cultivation has declined from 146 to 20 hectares (Singhal, 1999), continued cultivation in same condition worsen the situation which threatens total extinction (Thangavelu *et al*., 2001). Currently available methods to control the disease are ineffective, hence deploying resistance in susceptible variety by genetic transformation served as an alternative strategy (Ploetz, 2015). *F. oxysporum* species known to exhibit short biotrophic phase followed by complete necrotrophy in host plant (Thaler *et al.,* 2004). In view of this, employing genes which negatively regulate the cell death pathway in host were used to enhance resistance against necrotrophic fungi (Dickman and de Figueiredo, 2013). BAG genes encoding multifunctional group of proteins function by interacting with the signaling molecules and molecular chaperones, such as heat shock proteins resulting in inhibition of PCD (Sondermann *et al.,* 2001; Doukhanina *et al.,* 2006; Jacobs and Marnett 2009; Ge *et al.,* 2016). Most of the time, exogenous genes have mostly been expressed by ubiquitin promoter that drives high-level expression of transgenes in monocot plants (Jiang *et al.,* 2018). Beta-glucosidase promoter isolated from *Zea mays* (ZmBgl) shows much stronger activity in root parts (vigorous cell division zones associated with vascular tissues) compared to mature seeds and edible part of the crop (Gu *et al.,* 2006). Over expression of programmed cell death (PCD) gene, *AtBAG4* under two different promoters namely ZmBgl promoter and ubiquitin promoter was studied to confer FOC1 resistance in banana cv. Rasthali.

The embryogenic cell suspension (ECS) initiated from male inflorescence of banana cv. Rasthali was maintained in controlled condition as previously

described (Sunisha *et al.,* 2020a). ECS was heat shocked at 45ºC for 5 min and transformed with *AtBag4* gene constructs obtained from Queensland University Technology, Australia using *Agrobacterium* strain AGL as described by (Khanna *et al.,* 2004). The putative transformants developed with *AtBAG4* genes driven by ZmBgl promoter as well as ubiquitin promoter were subjected to PCR analysis. Total RNA was extracted from leaf tissue of untransformed and transformed plants using plant RNA isolation kit (Sigma, Aldrich). Briefly, 4 µg of RNA was subjected for DNase treatment and first strand cDNA synthesized using 2µg of RNA, Oligo (dT) $_{12\text{-}18}$ primer (Sigma), RevertAid™ M-MuLV Reverse Transcriptase (Thermo Scientific). Five transgenic lines from each construct were individually subjected to qPCR in three replications with a total volume of 20 µL reaction mixture containing 10 µL of PCR mix from SYBR Green kit (Takara), 1 µL of gene primer set and 5 µL of cDNA template in a 7500 Real-Time PCR system (Applied Biosystems). Banana Actin gene was used as an endogenous gene control for qRT-PCR. Amplification conditions were, initially 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 60 °C for 45 sec, and 72 °C for 30 sec, each. Final PCR melt curve of 95°C for 1min and 60°C for 15 s was employed and $2^{-\Delta\Delta CT}$ method was used to analyze quantitative variation (Sunisha *et al.,* 2020b). Five transgenic lines from each construct along with the untransformed susceptible cv. Rasthali and resistant cv. Grand Naine controls were inoculated with FOC1 obtained from Plant pathology laboratory, ICAR-Indian Institute of Horticulture Research, Bangalore (India), as described previously by Smith *et al*. (2008) with slight modification in the potting mixture. External symptoms were recorded four weeks post inoculation by scoring each plant for yellowing and wilting symptoms (Paul *et al.,* 2011) using a 1–5 point scoring, where 1 represented -healthy, no symptoms; 2—slight symptoms (yellowing of the leaves); 3—advance symptoms (dropping of the leaves); 4—extensive symptoms (whole foliage got dried); 5—entire plant affected (complete dead plant). The data was analyzed by one-way analysis of variance (ANOVA) using GraphPad prism® software (USA).

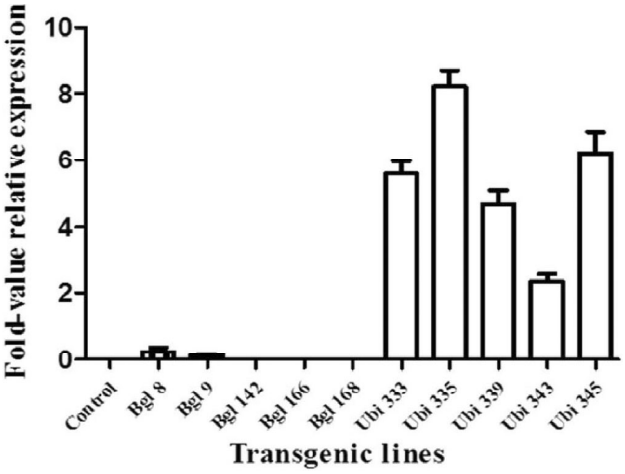

Fig. 1 Comparative *AtBAG4* gene expression analysis in leaves of transgenic plants

Lines 8, 9, 142, 166 and 168 represent transgenic banana cv Rasthali expressing AtBAG4 under ZmBgl promoter. Lines 333, 335, 339, 343 and 345 represent transgenic banana cv Rasthali expressing AtBAG4 under ubiquitin promoter

Table 1. Bioassay of *AtBAG4* transgenic lines of banana cv. Rasthali plants inoculated with FOC1 for symptom expression

Sl. No.	Transgenic Lines		External symtoms			
	ZmBgl: AtBAG4	Uboquitin: AtBAG4	ZmBgl:AtBAG4		Ubiquitin:AtBAG4	
			Yellowing	Wilting	Yellowing	Wilting
1	168	333	3.3	1.3	2.3	1.6
2	166	335	3.0	2.0	2.6	1.3
3	142	339	3.3	2.3	2.6	1.6
4	8	343	2.6	1.6	2.3	2.0
5	9	345	2.3	2.6	1.6	2.0
6	RS CONTOL		4.3	4.6	5.0	4.3
	C.D.		0.9	1.2	0.8	1.1

Transgenic lines expressing AtBAG4 gene; RS—control are untransformed cv Rasthali. Results are presented as score—yellowing and wilting: 1–5 scale, stem splitting: 1–3 scale. Five to seven leaf stage plants were subjected for the FOC root-challenge bioassay. The treatments were significantly dissimilar from susceptible RS control lines as $P < 0.05$ based on LSD post hoc test

Agrobacterium mediated transformation of ECS with binary vector containing *AtBAG4* gene, driven by ZmBgl and ubiquitin promoter resulted in total of 23 PCR confirmed putative transformants free from somaclonal variation. The well rooted plantlets were acclimatized in net house for further development. The efficient overexpression and transcript levels of the *AtBAG4* gene were examined using cDNAs derived from five selected transgenic lines of each construct and also in un-transformed control. The qRT-PCR data showed that the expression of *AtBAG4* gene driven by ubiquitin promoter ranged from 2 to 8.5-fold whereas the transgenic lines expressing *AtBAG4* gene under ZmBgl promoter showed less than one-fold expression in leaves compared to untransformed plants (Fig 1). The differential expression in the transgenic and non-transformed plants were statistically significant ($F = 131.7$; $P = < 0.0001$). Transformants expressing *AtBAG4* driven by ubiquitin and ZmBgl promoter showed reduced external symptoms compared to untransformed control plants four weeks post inoculation.

The ubiquitin promoter resulting transgenic events exhibited higher gene expression level in leaves compared to transgenic events developed with ZmBgl promoter. Also, transgenic lines over expressing anti-apoptotic *AtBAG4* gene driven by ubiquitin promoter exhibited reduction in external symptoms (< 3 rating for yellowing and wilting) and enhanced tolerance to Fusarium wilt caused by FOC 1 under pot condition. Similarly, the ubiquitin promoter used in transgenic plants for over expressing MusaBAG1 showed enhanced resistance to FOC infection (Ghag *et al.,* 2014). It is evident from the present study that anti-apoptotic *AtBAG4* gene driven by root specific ZmBgl promoter was having lower levels of expression in leaves. Similarly, the root specific ZmRCP-1 promoter isolated from maize failed to deliver gusA expression in leaves of transgenic plantains (Onyango *et al.,* 2016). However, the gene expression in roots and vascular discoloration index post FOC inoculation are to be further studied to know the efficacy of root specific ZmBgl promoter over constitutive ubiquitin promoter. Further, the ZmBgl promoter expressing in vascular tissues is developmentally and spatially regulated promoter and such promoters are under the influence of several endogenous elements (Schmitz *et al.,* 2022) which could be the reason for lower levels of transgene expression in leaves. In contrast the constitutively over expressing *AtBAG4* gene are expressed in all the tissues leading to higher oxygen scavenging activity and preventing tissue damage restricting the faster multiplication of FOC1 (Paul *et al.,* 2011).

Owing to the multiple reports confirming host PCD manipulation induced resistance to various FOC races (Magambo *et al.,* 2012; Dale *et al.,* 2017), the modification of genes regulating PCD pathways in plants is emerging as a promising strategy to provide broad-spectrum resistance to both biotic and abiotic stresses (Lincoln *et al.,* 2002; Li and Dickman, 2004;). Hence, our research mainly focused on improving resistance to Fusarium wilt by manipulating PCD pathways.

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