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Full Length Article

Isolation and Characterization of an Endopolygalacturonase Produced by *Fusarium oxysporum* f. spp. *cubense* Race 4

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Abstract

A novel endopolygalacturonase, designated as PGC1, was purified from the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* race 4 (Foc4) infecting banana plant tissues. The molecular weight of PGC1 is 42.3 kDa; its $K_m$is 0.462 mg mL^{-1} and its $V_{max,}$ 256.41 units mg $protein^{-1}$ min^{-1}. The PGC1 gene was cloned into the eukaryotic expression vector pPICZaA and then expressed in strain SMD1168 of *Pichia pastoris*. Re-PGC1 was purified, and its properties were found to be comparable to those of PGC1 from Foc4. Optimum pH and temperature for activity of both PGC1 and re-PGC1 were 5.0 and 50°C, respectively. PGC1 and re-PGC1 caused tissue immersion necrosis in banana plants. These results indicate that PGC1 is an important pathogenic endoPG reported from pathogen Foc4. © 2020 Friends Science Publishers

Keywords: Banana; Endopolygalacturonase; *Fusarium oxysporum* f. sp. *cubense* race 4; *Fusarium* wilt; *Pichia pastoris*

Introduction

Among the many soil-borne species worldwide, *Fusarium* is considered a major group of plant pathogens (Dean *et al*. 2012). Specifically, *F. oxysporum*, which causes vascular wilt disease in many important cash crops including tomato, cucumber and banana, is one of the most common and highly pathogenic *Fusarium* species (Michielse and Rep 2009). Banana (*Musa* spp.) *Fusarium* wilt (Panama disease) caused by *F. oxysporum* f. spp. *cubense* (Foc) is a serious threat to banana production globally (Hwang and Ko 2004).

According to the sensitivity of different banana varieties in the field, banana Foc is divided into three races, Foc1, Foc2 and Foc4 (Ploetz 2015). Foc1 can infect banana cultivars "Gros Michel" (AAA) and "Silk" (AAB), while Foc2 infects only "Bluggoe" (ABB). In contrast, Foc4 readily attacks not only Cavendish (AAA) but also all other Foc1- and Foc2-sensitive cultivars (Ploetz 2015). Therefore, Foc4 is thought to be the most highly infectious race of the pathogen (Ploetz 2006). Indeed, Grimm (2008) pointed out that Latin America bananas might be destroyed if Foc4 invaded the region. To date, there are no 100% Foc4 resistant varieties or any suitable management strategy for *Fusarium* wilt in plants infected with Foc4 (Butler 2013; Ordonez *et al*. 2015).

Banana wilt disease has been reported in all the main banana producing areas in China and already the national banana industry, has been seriously affected, as many plantations have been completely devastated by this disease, causing great economic losses. Therefore, understanding the molecular basis of the pathogenicity of banana wilt might provide a method to counter the disease; however, its pathogenesis has not been fully elucidated, as to date, only some of the pathogenic genes associated with Foc4 have been reported (Ploetz and Randy 2015; Guo *et al*. 2016; Ding *et al*. 2020); thus pathogenesis of Foc4 warrants further study.

Plant cell walls, the first chemical and physical barriers against pathogen invasion, are mainly composed of three polysaccharides. Although the fungal cell wall *per se* cannot sense an external stimulus, a large number of glycoproteins surrounding the cell wall may participate in the interaction between pathogenic fungi and the host plant (Geoghegan *et al.* 2017). Some of the cell wall degrading enzymes (CWDEs) secreted by pathogenic fungi, including Foc4, have been proved to be important pathogenicity factors during infection (Dong and Wang 2011, 2015). Thus, for example, Guo *et al.* (2014) found that Foc1 and Foc4 genomes contained a large number of carbohydrate-active enzymes (CAZymes). Furthermore, there were more specific CAZymes expressed in Foc4 than Foc1 when grown in proximity to the host cell wall, implying that CAZymes may play an irreplaceable role in Foc pathogenicity, while there might still be some special

CAZymes in Foc4 contributing to make it more infective (Guo *et al.* 2014). In our previous studies, differences among polygalacturonases (PGs) produced by Foc1 and Foc4 and two other forma of *F. oxysporum* were compared (Dong *et al.* 2010). Now, in this study, a novel endoPG isozyme named as PGC1, was purified from *F. oxysporum* f. spp. *cubense* race 4 (Foc4) *in vitro* cultures. The cloned gene and heterologous expression of PGC1 were then tested for tissue maceration and necrosis. Our findings provide evidence that PGC1 may play a role in Foc4 pathogenicity to plants and that it can be produced as a fully functional PG in the *P. pastoris* protein expression system.

Materials and Methods

Fungal strain and culture conditions

The pathogenic strain Foc4 was preserved at South China Agriculture University, where it was cultured on SM medium with 1% [w/v] citrus pectin (Sigma) for pectinolytic enzyme production and RNA extraction (Pietro and Roncero 1996).

PG activity and protein assays

PG activity was assayed in a mixture (1 mL of total volume) containing 0.5% polygalacturonate (PGA) (w/v), 50 m*M* sodium acetate buffer (pH 4.5) and various amounts of enzyme solution at 50°C for 30 min. The number of reducing groups, expressed as galacturonic acid (GA) released by enzymatic action, was determined. One unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 μmol of GA per minute under the assay conditions. The molecular weight of the purified enzyme determined by SDS-PAGE was performed using 12% acrylamide.

Isolation and characterization of PGC1 from FOC4 culture supernatant

An Foc4 sample culture was centrifuged at 16,000 rpm for 20 min at 4°C. Then, the supernatant in the tube was transferred onto a new tube and concentrated 100-fold with an Amicon 8400 ultrafiltration system containing a 10 kDa MWCO membrane. This concentrated extract filtrate was added to a gel filtration column (Sephacryl S-100 16/60, Pharmacia) and eluted with 50 m*M* sodium acetate buffer (pH4.5) at a flow rate of 1 mL min^{-1}. The active eluate fraction containing PG was collected and added to a cation exchange column (Sepharose SP XL 16/10, Pharmacia) equilibrated with 20 m*M* sodium acetate buffer (pH 4.5). The column was eluted with an NaCl gradient (0–0.7 *M*) at a flow rate of 4 mL min^{-1}. The fractions were collected and added to another cation exchange column (Sepharose FF CM Hitrap 1 mL, Pharmacia) equilibrated with 20 m*M* sodium acetate buffer (pH 4.5). The column was then eluted with NaCl gradient (0–0.7 *M*) at a flow rate of 2 mL min^{-1}.

Purified PGC1 was run on a SDS-PAGE gel, then transferred onto a PVDF membrane and submitted for N-terminal analysis by automated Edman degradation (Pulsed liquid sequencer model 470A; Applied Biosystems).

Construction of plasmid vectors and transformation

Re-PGC1 expression vector pPICZαA-*pgc1*-Myc-His6 was constructed as follows: the *pgc1* cDNA was amplified using the forward primer (5′-GCGCTCGAGAAAAGAGATCCCTGCAGCGTCACTG ACT-3′) and the reverse primer (5′-CGCGCGGCCGCGTTAGGGCAAGTGTT-3′); *Xho*I and *Not*I restriction sites are underlined and the yeast consensus sequence is shown in bold. The primers are based on the sequence of *pgc1* (accession no. FJ593631). The PCR product was then cloned into the pPICZαA vector (Invitrogen) in fusion with a *C*-terminal Myc and His6 tag giving pPICZαA-*pgc1*-Myc-His6. The correct sequence was verified by sequencing. Yeast transformation was performed. *Sac*I was used to digest the recombinant plasmid pPICZαA-*pgc*1-Myc-His6. After that, the linearized part was transformed into *P. pastoris* strain SMD1168 by electroporation. Then, they were patterned at 28°C for 48 h in a yeast extract peptone dextrose (YPD) plate containing 1% yeast extract, 2% dextrose, 2% peptone, and 100 μg mL^{-1} of Zeocin.

Expression and purification of re-PGC1

Transformants containing pPICZαA-*pgc*1-Myc-His6 were inoculated in 10 mL of BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 m*M* potassium phosphate, 4×10^{-5}% biotin and 1% glycerol) at 28°C for 24 h and then inoculated in 200 mL BMMY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 m*M* potassium phosphate, 4×10^{-5}% biotin and 0.5% methanol). Samples were taken every 12 h. One milliliter of 100% methanol was added into it every 24 h to ensure a final methanol concentration of 0.5% assuming methanol was completely utilized in 24 h. The culture supernatant was analyzed by 10% (w/v) SDS-PAGE followed by silver staining.

The recombinant protein was extracted from the culture supernatant and purified using a Ni-NTA His Bind resin column as per manufacturer instructions (Novagen). All steps were carried out at 4°C. Protein concentrations were determined by the Bradford assay and analyzed by 10% (w/v) SDS-PAGE. Proteins were transferred onto a PVDF membrane for Western blot analysis. Re-PGC1 was detected using an Anti-Myc-HRP Antibody (Invitrogen). The membrane was developed using the chemiluminescent substrate HRP-DAB Kit (TIANTEN), according to manufacturer instructions.

Biochemical characterization of PGC1 and re-PGC1

To analyze the hydrolysis products, the samples (0.02 U enzyme in 0.5 mL water) were added to 1 mL of 0.5% (w/v)

PGA in 50 m*M* sodium acetate buffer (pH 4.5) and incubated at 50°C, for 10, 20, 30, 40, 50, 60 min and then used for PG activity assay. The 3% of the substrate being hydrolyzed by endo-PGs can lead to 50% reduction of viscosity, while exo-PGs need 20% of the substrate being hydrolyzed.

The Michaelis constant (*K*m) and *V*max values were determined from Lineweaver–Burk plots of enzyme activity measured with the PGA as substrates, at concentrations between 0.25 and 1.25% at optimum pH and temperature, and then plotted the results.

To determine the optimal pH, the PG activity was assayed using 100 m*M* potassium phosphate buffer for pH values between 3 and 10 at 50°C and 0.5% (*w*/*v*) PGA as substrates. The consequence of temperature on PG activity was determined in 100 m*M* potassium phosphate buffer at pH 4.5, between 10 and 90°C. Three replicates were tested per treatment as well as the negative control.

Tissue maceration and necrosis assayed with PGC1 and re-PGC1

To evaluate banana tissue maceration, the cultivars tested comprised *Musa* AAA 'Cavendish' cv. 'Baxi', resistant to FOC1 and susceptible to FOC4; *Musa* AAB cv. 'Guangfen-1', susceptible to FOC1 and FOC4. 1 cm lengths of tissue (0.5 g) were taken from the healthy stems of the four-leaf stage banana and placed in test tubes. A mixture of 1 unit of purified enzyme with 1 ml of 50 m*M* sodium acetate buffer, pH 5.0, was inoculated with the sterilized banana tissue and maceration was evaluated after 48 h at 45°C. Control tubes contained the same buffer without enzyme. Released reducing sugar was calculated from standards of GalA after incubation 48 h at 45°C.

For the tissue necrosis assay, 1 unit of enzyme was applied to the stems of healthy banana plant by injection. For each treatment, stems were cut (vertical-sectioned) 5 day later to observe vascular necrosis, ten replicates. Sterile double distilled water and 50 m*M* sodium acetate buffer pH 5.0 were used as controls.

Results

Purification of PGC1

Polygalacturonase PGC1 was finally purified from Foc4 through several steps including three steps of ultra-filtration, gel filtration chromatography, and cation exchange chromatography. The enzyme activity increased from 3.57 to 21.46 units mg protein^{-1} min^{-1} after the purification process (Table 1). Concentrated crude PG from the shaking culture showed one unclear single PG-activity peak when applied onto a Sephacryl S-100 16/60 gel filtration column. Subsequently, the fraction with PG activity was collected and applied onto a cation exchange (Sepharose SP XL 16/10) chromatography column that yielded a significantly enriched single PG peak. This concentrated PG sample was loaded onto another cation exchange column (Sepharose FF CM Hitrap 1 mL) that yielded a single PG and protein peak. SDS-PAGE showed a single protein band, thus proving that PGC1 was totally purified to homogeneity (Fig. 1) with molecular weight of a 42.3 kDa.

Table 1: Purification of the PGC1 from FOC4 cultured with synthetic medium (SM) medium supplemented with 1% citrus pectin

	Protein (mg)	Activity (Unit)	Yield (%)	Specific activity (Unit/mg)
Crude	36.6	131.2476	100	3.586
Ultrafiltration	8.21	70.2604	53.53	8.5579
Sephacryl S-100 16/60	1.85	20.35	15.51	11
Sepharose SP XL 16/10	0.66	12.50	9.52	18.9394
Sepharose FF CM Hitrap	0.15	3.22	2.45	21.4667

Expression and purification of re-PGC1 in *P. pastoris*

The *pgc1* gene (GenBank accession no. FJ593631) fragment encoding the mature PGC1 was amplified by RT-PCR and cloned into pPICZαA to construct the recombinant pPICZαA-*pgc1* plasmid, which was then transformed into the sensitive cells of strain SMD1168 by electroporation. A mut$^+$ SMD1168 recombinant strain was inoculated in 200 mL BMMY and post induction samples from the cultures at different sampling time points were run on SDS-PAGE and analyzed by silver staining. These showed protein expression as early as 24 h after induction, which continued to increase from 24 to 72 h. Re-PGC1 was purified from 100 mL of crude extract by using a Ni-NTA His Bind resin column. SDS-PAGE of the purified re-PGC1 showed only one band on a 10% polyacrylamide gel. Together with our Western blot results, this indicated that the protein was probably a native protein of the SMD1168 transformant induced by methanol (Fig. 2).

Biochemical characterization of PGC1 and re-PGC1

The K_ms of purified PGC1 was 0.462 mg mL^{-1}, while the V_{max} was 256.41 units mg protein^{-1} min^{-1} (Fig. 3). The final products of enzymatic hydrolysis of PGA were analyzed using paper chromatography. The intermediate products that appeared during hydrolysis suggest an endoPG activity for PGC1. The optimum pH and temperature for PGC1 and re-PGC1 activity were 5.0 and 50°C, respectively (Fig. 4). Both natural PGC1 and re-PGC1 showed the same variation trend as a function of temperature and pH.

Active PGC1 and re-PGC1 cause tissue maceration and necrosis

Either one unit of PGC1 or of r-PGC1, mixed with one mL of 50 m*M* sodium acetate buffer (pH 5.0), were inoculated to the sterilized banana tissues to check whether they can macerate the tissue; observations were made after 48 h.

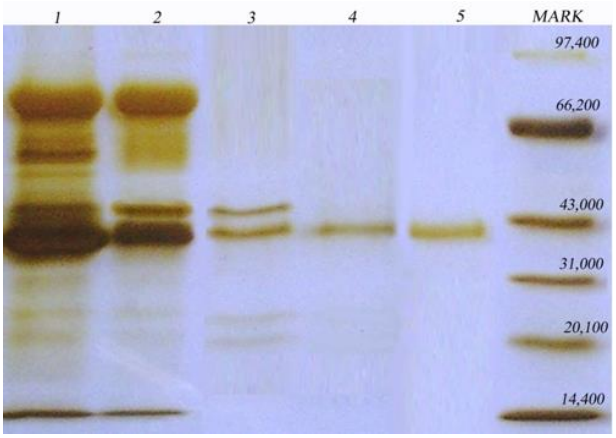

Fig. 1: Purification of PGC1 from FOC4 cultured with synthetic medium supplemented with 1% citrus pectin

Lane M: protein marker. Line 1: Concentrated culture. Line 2: PG after gel filtration. Line 3: PGC1 after sepharose SP cation exchange. Line 4-5: PGC1 after sepharose FF CM

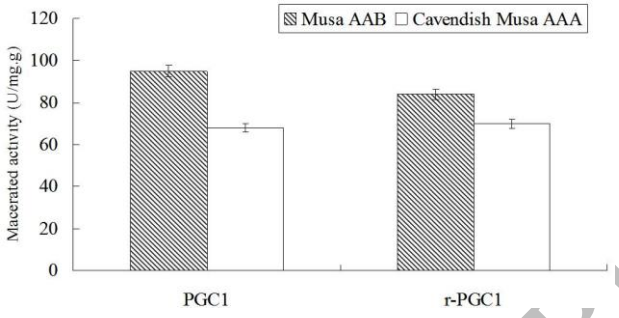

Fig. 2: The maceration activity of PGC1 to banana tissue

Musa AAB: *Musa* AAB cv. Guangfen-1; Cavendish Musa AAA: *Musa* AAA Cavendish cv. Baxi)

The results showed that the maceration activity of PGC1 on tissues of banana cultivar 'Guangfen-1' was much more extensive than that of re-PGC1, while the maceration activity of PGC1 on 'Baxi', a 'Cavendish' banana, was slightly lower than that of re-PGC1. The maceration activities of PGC1 and re-PGC1 on 'Guangfen-1' were higher than those on 'Baxi' (Fig. 5); PG showed differences in maceration ability on 'Guangfen-1' and 'Baxi' banana cultivars.

Stem vascular tissues of 'Cavendish' cultivar 'Baxi' showed partial necrosis after inoculation with PGC1 or re-PGC1.In contrast, 'Cavendish' cultivar 'Baxi' inoculated with non-active PGC1 orsterile double-distilled water developed no necrotic spots (Fig. 6).

Discussion

Purification of the PG protein does not seem to be highly effective due to low activity. In order to collect PG more effectively, it should be increased and concentrated. The Sephacryl S-100 column for coarse separation can help to remove many contaminating proteins. For most PG isozymes usually produced under acidic conditions, weak

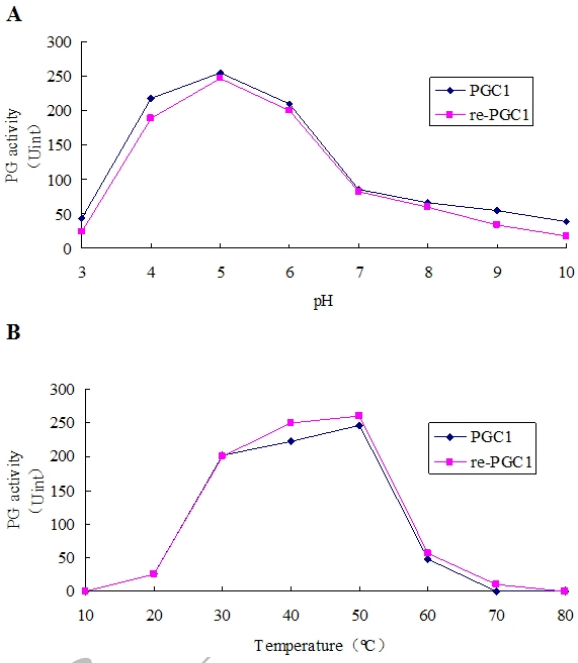

Fig. 3: Enzymatic activity of PGC1

A: Optimal pH. **B**: Optimal temperature

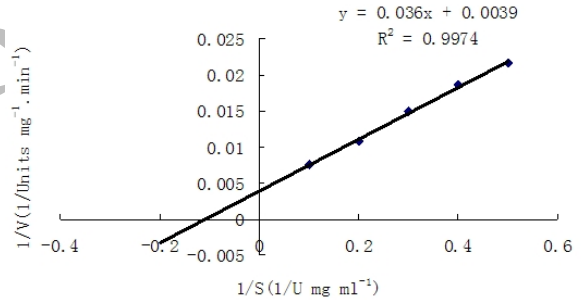

Fig. 4: The Michaelis-Menten curve of PGC1

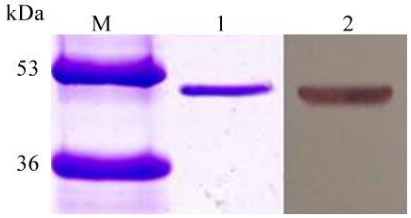

Fig. 5: SDS-PAGE and Western blot analysis of the purified re-PGC1

Lane 1: Markers, Lane 2: Purified re-PGC1 with Coomassie blue R-250, Lane 3: Western blot analysis of the purified re-PGC1 after SDS-PAGE with the Anti-Myc-HRP antibody

cation columns are used to separate them from middle and advanced ones. Obtaining protein purified to a greater degree can be achieved by regulating the collected volume from collection pipes. Similar methods were successfully used to purify other PGs from Foc4 (Dong and Wang 2011, 2015).

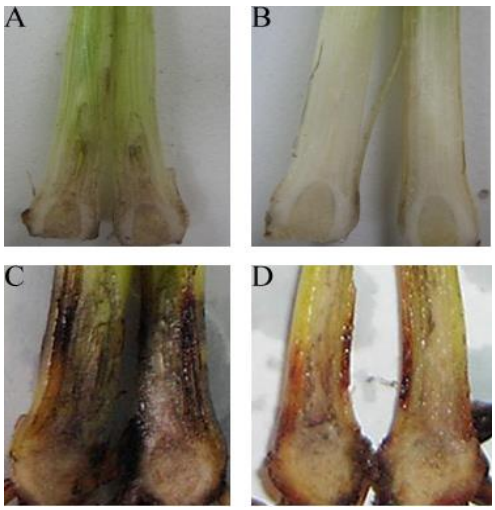

Fig. 6: Tissue necrosis analysis of Baxi banana tissue
A: Banana plant was injected with PGC1 of non-activity. **B**: Banana plant was injected with sterile double distilled water. **C**: Banana plant was injected with purified PGC1. **D**: Banana plant was injected with re-PGC1

The final products of enzymatic hydrolysis of PGA were analyzed; the results suggested that PGC1 showed endoPG activity. Endopolygalacturonases (endoPGs; poly-a-1, 4-galacturonide glycanohydrolase, EC 3.2.1.15) have long been proposed to play an important role in fungal pathogenicity to plants by depolymerizing homogalacturonan, a major component of the plant cell wall (Liu *et al*. 2017). In some cases, inactivation of individual PG genes by targeted disruption had no apparent effect on virulence, probably due to the presence of multiple or alternative PG genes (Gao *et al*. 1996). However, in other cases, inactivating PG did reduce pathogenicity. Thus, using UV irradiation to produce *F. oxysporum* f. spp. *lycopersici* mutants lacking PG activity, Mann (1962) found that the mutants were still pathogenic to tomato plants, but noted their virulence was reduced. Therefore, whether PGC1 is related to pathogenicity warrants further study.

We found that PGC1 and re-PGC1 caused tissue maceration and necrosis on banana plants. Many pectinases have been purified and found to macerate vascular plant tissues. For example, *Phytophthora capsici* Pcipg5 was found to increase leaves symptom development on pepper (Li *et al*. 2012). Purified recombinant RsPG2 from *Rhizoctonia solani* degraded rice tissue 48 h after inoculation (Chen *et al*. 2017). However, PG1 was purified and cloned from *F. oxysporum* f. spp. *lycopersici* and then introduced into *F. oxysporum* f. spp. *melonis* without altering the virulence pattern toward muskmelon, suggesting that PG1 could not macerate the vascular tissues of muskmelon (Pietro and Roncero 1998). Here, we found that banana inoculated with PG showed the same symptoms upon infection by *Fusarium* wilt disease. Although the maceration activity of re-PGC1 was lower than that of PGC1, its maceration ability was high enough to allow detection of the activity.

It seems that future PGC1 mass production in *Pichia pastoris* is possible. The pectin of 'Baxi' banana varieties was a poor substrate for PGC1, compared with that from 'Guangfen-1', suggesting that the structure of pectin polymers in 'Guangfen-1' and 'Baxi' might differ from one another. Nonetheless, how PGs function and whether a single gene can cause the disease or whether they work together, needs to be further researched.

Optimum pH for PGC1 and re-PGC1 activity was 5.0, while optimum temperature was 50°C. Further, both natural PGC1 and re-PGC1 showed the same variation trend in when considered as a function of temperature and pH. In areas where bananas are grown, ambient temperature is generally close to 30 degrees, indicating that the enzyme is more active at a relatively high ambient temperature, which can play a role in promoting pathogen infection. It also shows that the enzyme has the ability to be industrialized, because it is optimally active at 50°C and can be used for industrial purposes, such as plant stem-pectin digestion.

In vitro expression and preparation of active proteins is important to better research the function of the gene involved. Although the prokaryotic protein-expression system is widely used in various fields, the eukaryotic expression system displays more advantages. The eukaryotic protein expression system in yeast has been successfully used in different species (Chen *et al.* 2015; Meng *et al*. 2017). Here, PGC1 can be expressed in the yeast eukaryotic protein expression system quite satisfactorily. Purified PGC1 and re-PGC1 have a similar function; this implies that the activity of re-PGC1 is very similar to that of purified PGC1 from Foc4 directly. Additionally, further successful purification of the protein made functionality research more convenient. So considering maceration activity level, high expression level, optimal temperature and pH, we propose that re-PGC1 is a candidate endoPG for use in further researching fungal pathogenicity and in developing the de-pectinization industry.

Conclusion

The present study provides the basic characterization of a newly purified PGC1 protein. PGC1 can be produced as a fully functional PG by using the *P. pastoris* protein expression system. As purified PGC1 and re-PGC1 proteins both caused maceration of banana tissue, PGC1 seemingly plays an important role in Foc4 pathogenicity. However, further studies are needed to elucidate the role of PGC1 in pathogenicity role and its interactive target in host banana cultivars. Functional analysis of pathogenic factors will provide new ideas for the prevention and control of banana wilt.

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Author Contributions

ZY Dong planned the basic research and conducted the experiments. ZY Dong and M Luo analyzed the data. ZY Dong, M Luo prepared the manuscript. ZZ Wang revised the manuscript.

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