Optimization of a virus-induced gene silencing system and functional elucidation of *MaBAM9b* in postharvest banana fruits

juhua liu (✉ juhua69@126.com)

Institute of Tropical Bioscience and Biotechnology <https://orcid.org/0000-0002-3200-6672>

Meng Li

Nanjing Agricultural University

Jing Zhang

Institute of Tropical Bioscience and Biotechnology, CATAS

Hongxia Miao

Institute of Tropical Bioscience and Biotechnology, CATAS

Jingyi Wang

Institute of Tropical Bioscience and Biotechnology, CATAS

Caihong Jia

Institute of Tropical Bioscience and Biotechnology, CATAS

Jianbin Zhang

Institute of Tropical Bioscience and Biotechnology

Zhuo Wang

Institute of Tropical Bioscience and Biotechnology

Biyu Xu

Institute of Tropical Bioscience and Biotechnology

Zhiqiang Jin

Institute of Tropical Bioscience and Biotechnology

Research

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Abstract

Background: The genetic basis of metabolic pathways that operate during fruit ripening needs to be understood before the nutritional value of the banana can be improved. The banana is a typical starch conversion fruit, and β -amylase is a key enzyme that may play an important role in starch degradation during the ripening process. *Musa acuminata* β -amylase 9b (*MaBAM9b*) is closely related to starch degradation. However, its exact function in starch degradation has not been demonstrated in banana. Stable genetic transformation to identify gene function is time- and energy-consuming. Thus, an efficient and rapid method is needed for functional identification. Virus-induced gene silencing (VIGS) is a reverse-genetics method based on RNA-mediated antiviral plant defense that has been used to rapidly identify gene functions in plants. The aim of this study is to optimize a VIGS system and functional elucidation of *MaBAM9b* in postharvest banana fruits.

Results: Using 0.5% iodine-potassium-iodide (I_2 -KI) staining for 150 s, we found that 1:3 TRV1:TRV2-*MaBAM9b* cultivated at 30 mmHg for 30 s to an optical density (OD) of 0.8 at 600 nm, and kept on Murashige & Skoog (MS) media for 5 days produced the best silencing results. Under these conditions, the total starch content was greatly increased, whereas the β -amylase activity, soluble sugar content, and expression of endogenous *MaBAM9b* greatly decreased.

Conclusions: The system described here is particularly useful for studying genes and networks involved in starch conversion in fruit, which alone would not produce a visual phenotype. This system will provide a platform for functional genomics and fruit quality improvement in the banana.

Background

Banana is an important and pleasantly flavored tropical fruit that is popular with consumers worldwide [1]. As a typical starch conversion fruit, the starch content of banana fruit upon harvesting can reach 12–35% fresh weight (FW) [2]. Starch is degraded and converted to sucrose rapidly during the post-harvest banana fruit ripening process [3]. The soluble sugars may reach up to 20% FW of the pulp in the ripe fruit, with sucrose accounting for approximately 80%, whereas glucose and fructose make up almost all the remaining 20% of the soluble sugars in equal proportions [2]. During banana fruit ripening process, the sucrose content varied from 12.2 to 411.8 mg g⁻¹ FW, while the glucose and fructose contents varied from 2.1 to 100.5 mg g⁻¹ FW and 1.5 to 103.8 mg g⁻¹ FW, respectively (not published data). The conversion efficiency directly decides the fruit sweetness, firmness, flavor, nutritional quality, and shelf life. Therefore, banana is a model fruit for investigating fruit starch metabolism, β -amylase is a key enzyme that converts starch to glucose [4]. *Musa acuminata* β -amylase 9b (*MaBAM9b*, Ma05_t07800.1) is highly expressed during banana fruit ripening and is closely related to starch degradation [5-6]. However, its exact function in starch degradation has not been demonstrated in banana. Elucidating the function of *MaBAM9b* in banana using genetic transformation could take in excess of three years due to various banana-specific challenges such as slow growth process, low transgenic efficiency and high dependence

on genotype [7]. Therefore, developing an efficient way to rapidly and exactly identify gene function is important for banana functional genomics.

Virus-induced gene silencing (VIGS) is a reverse-genetics tool that has powerful applications in functional genomics [8]. In VIGS, the recombinant virus carrying a plant cDNA fragment infects the plant tissue and spreads systemically throughout the tissue. Then, the endogenous gene transcripts, which are homologous to the insert fragment in the viral vector, are degraded by post-transcriptional gene silencing (PTGS). Finally, the gene function can be assessed based on the plant phenotype and [physiological index](#) alteration [9]. VIGS can be developed rapidly and has been applied to functional gene identification in fleshy fruits, such as tomato [8,10], strawberry [11-13], apple [14-15], grape [16], citrus [17], loquat [18], pear [19-20], peach [21-22], and sweet cherry [23-24]. However, there are no reports on VIGS in banana.

In this report, a VIGS-mediated banana fruit slice system was developed to rapidly elucidate the role of *MaBAM9b* in banana fruit starch metabolism. The effects of different ratios of pTRV2-*MaBAM9b* with pTRV1, the OD600 values of the bacterial fluid, and the co-cultivation times on the silencing results were carefully investigated. The results showed that the co-cultivation of 2–4-mm-thick fruit slices at a 1:3 ratio of pTRV1: pTRV2-*MaBAM9b* at OD600 0.8 at 30 mmHg for 30 sec and kept on Murashige & Skoog (MS) media for 5 d could obtain the most efficient silencing results based on an analysis of the contents of total starch, glucose, β -amylase activity, and *MaBAM9b* expression. These results will provide a platform for banana functional genomics on starch metabolism and should contribute towards developing a technique for controlling starch degradation.

Results

The effects of different I_2 -KI solutions and times on the staining results

As shown in Fig. 1, as the treatment duration and concentration increased, the blue stain gradually became darker. The 0.1% (a) and 0.25% (b) I_2 -KI solutions did not complete their staining in any of the tested treatment times (Fig. 1A–F). The 0.75% (d) and 1.0% (e) I_2 -KI solutions resulted in an uneven dark or light color under long or short treatment times, respectively. The banana fruit slices stained with 0.5% I_2 -KI solution for 150 s obtained a more uniform staining effect that allowed for easier identification.

The effects of different ratios of pTRV2-*MaBAM9b* with pTRV1 on the silencing results

As shown in Fig. 2A, when the ratio of pTRV2-*MaBAM9b* with pTRV1 reached 1:2, the blue stain of I_2 -KI became darker. The color became darkest at a ratio of 1:3 compared to the control (CK). This result indicated that ratios of 1:2 and 1:3 could efficiently suppress starch degradation. Further investigation found that the contents of soluble sugars, including glucose, fructose, and sucrose, were greatly decreased by 62.8%, 61.9%, and 62.6% compared to CK, respectively, at a ratio 1:3 (Fig. 2B). The β -amylase activities were also significantly decreased compared to CK, decreasing by 18.7%, 62.7%, and 76.7% at ratios of 1:1, 1:2, and 1:3, respectively (Fig. 2C). In contrast, the total starch contents at ratios of

1:1, 1:2, and 1:3 were 339.7, 398.8, and 436.2, indicating a 2.7%, 20.5%, and 31.9% increase compared to CK, respectively (Fig. 2D). The expression of endogenous *MaBAM9b* was greatly suppressed, decreasing by 28.0%, 42.0%, and 63.0% at ratios of 1:1, 1:2, and 1:3, respectively. These results indicated that the ratio of pTRV2-*MaBAM9b* together with pTRV1 at 1:3 obtained the best silencing efficiency.

The effects of different bacterial fluid OD values on the silencing results

As shown in Fig. 3A, with the increase in OD₆₀₀ value of the bacterial fluid from 0.4 to 0.6, the I₂-KI staining gradually became darker compared to CK. When the OD₆₀₀ value reached 0.8, the stain was darkest. However, when the OD₆₀₀ value increased to 1.0 or 1.2, the staining became weaker. This result demonstrated that an OD₆₀₀ of 0.8 obtained the best results for suppressing starch degradation. Further investigation indicated that the total starch contents at OD₆₀₀ values of 0.4, 0.6, 0.8, 1.0, and 1.2 were 235.7, 264.5, 326.0, 303.1, and 278.4, which were increased by 6.9%, 19.7%, 47.6%, 37.2%, and 26.0% relative to CK, respectively (Fig. 3B). Conversely, the β -amylase activities at OD₆₀₀ values of 0.4, 0.6, 0.8, 1.0, and 1.2 were significantly decreased compared to CK, decreasing by 9.2%, 15.3%, 27.6%, 22.4%, and 9.2%, respectively (Fig. 3C). The contents of soluble sugars, including glucose, fructose, and sucrose, were also greatly reduced by 67.2%, 67.8%, and 74.8% compared to CK, respectively, at OD₆₀₀ 0.8 (Fig. 3D). The expression of endogenous *MaBAM9b* was greatly suppressed, decreasing by 29.0%, 49.0%, 79.0%, 68.0%, and 52.0%, respectively. These results suggested that an OD₆₀₀ value of 0.8 obtained the best silencing efficiency.

The effects of different co-cultivation times on the silencing results

As shown in Fig. 4A, as the co-cultivation time progressed, the blue stain of I₂-KI became gradually darker. When the co-cultivation time reached 5 d, the color was the darkest. However, when the co-cultivation time increased to 7 d, the staining became weaker. This result demonstrated that co-cultivation for 5 d was best for suppressing starch degradation. Further investigation found that the total starch contents at 3 d, 5 d, and 7 d were 198.4, 315.7, and 301.4, increasing by 12.5%, 79.1%, and 71.0% compared to 1 d, respectively (Fig. 4B). Conversely, the β -amylase activities at co-cultivation times of 3 d, 5 d, and 7 d were significantly reduced compared to 1 d, decreasing by 10.7%, 29.8%, and 27.4%, respectively (Fig. 4C). The contents of glucose, fructose, and sucrose also decreased greatly by 60.5%, 53.8%, and 64.1% at an co-cultivation time of 5 d, respectively (Fig. 4D). The expression of endogenous *MaBAM9b* was greatly suppressed by 26.0%, 58.0%, and 42.0%, respectively. These results suggested that 5 d of co-cultivation obtained the best silencing efficiency.

Discussion

Banana is an important global tropical climacteric fruit with high nutritional value. Fruit ripening involves a quality formation process, during which many irreversible physiological and biochemical changes occur. Starch is the main component and the building block of banana fruit. Starch conversion to sugar is a primary metabolic process that directly influences the fruit quality, such as the texture, flavor, and shelf

life [3]. Understanding the molecular mechanism of fruit starch degradation metabolism is necessary for improving fruit quality. *MaBAM9b* is a key enzyme gene that might play an important role in starch degradation during the banana fruit ripening process [5]. However, its function has not been explicitly identified in banana. Identifying gene functions using stable genetic transformation is time- and energy-consuming. It is thus necessary that an efficient means for rapid functional identification is developed. VIGS is a reverse-genetics method based on RNA-mediated antiviral plant defense that has been used to rapidly identify gene function in plants [25]. The efficiency of VIGS in inducing endogenous gene silencing is related to multiple factors, such as the correct viral vectors, the characteristics of the target gene, the infection buffer, the activity of *Agrobacterium* in the bacterial suspension, and the plant growth environment suitable for silencing [26]. Using *MaBAM9b* as an example, we optimized the VIGS system to facilitate gene function analysis.

The selection of experimental material is the first important step for VIGS. In sweet cherry, Li et al. (2015) [23] selected 28 days after full bloom (DAFB) fruits as material for TRV vector injection. In Hanfu' apple, pear, and loquat, 15 DAFB, 30 DAFB, and breaker stage fruits were used as materials for TRV vector injection, respectively [14,18-19]. We selected 8 days postharvest (DPH) fruits to investigate the function of *MaBAM9b* by VIGS, as 8 DPH is the key stage of rapid starch degradation, as has been demonstrated by Wang et al. (2019) [6].

I₂-KI staining is a visual technique for quickly identifying starch contents in plant organs. This technique has been widely applied in rice [27], *Zea mays* [28], and *Arabidopsis* [29]. The result of I₂-KI staining is influenced by factors such as staining solution and time. However, these factors have not been optimized in banana. Here, we investigated the effects of the concentration of the staining solution and the staining duration on the staining efficiency. The results indicated that 2-4 mm-thick banana fruit slices stained with 0.5% I₂-KI solution for 150 s obtained the best staining effect. This result provides a reference for the rapid identification of starch contents, especially for high-starch fruits.

Successful gene silencing depends upon a dynamic interplay between virus spread and plant growth, both of which can be influenced by various conditions [26]. Different fruits have different optimized conditions for VIGS. For *Lycium* plants, the optimized conditions included an OD600 of 1.0, with the cells incubated at room temperature for 3–4 h. The pTRV1 and pTRV2 solutions were mixed at a ratio of 1:1 for injection [30]. In tomato, the OD600 was adjusted to 0.5 or 1.0 for all of the vectors, and the resuspended cells with pTRV1 and pTRV2 were then combined at a ratio of 1:1 and kept for more than 10 d in a plastic tray [8, 31]. In loquat, the OD600 was adjusted to 1.2, and the resuspended cells with pTRV1 and pTRV2 were combined at a ratio of 1:1 and co-cultivated for a week [18]. In the present study, the effects of different ratios of pTRV2-*MaBAM9b* and pTRV1, OD600 values, and co-cultivation durations on the silencing results were systematically investigated. The results showed that a 1:3 ratio of pTRV1: pTRV2-*MaBAM9b*, an OD600 of 0.8, and 5 d of cultivation obtained the most efficient silencing results. This system was something different from other fruits and reflected the [distinguishing qualities](#) of banana fruit.

Conclusion

A TRV-mediated VIGS system was successfully constructed and used to efficiently silence *MaBAM9b*, which resulted in increased total starch contents and decreased β -amylase activity, soluble sugar content, and endogenous *MaBAM9b* expression. These findings support that *MaBAM9b* plays a vital role in banana fruit starch degradation metabolism, thus providing a platform for banana functional genomics and for the breeding of new banana varieties.

Methods

Plant materials

Banana (*Musa* AAA group cv Brazilian) fruits were harvested from the banana plantation of the Institute of Tropical Bioscience and Biotechnology (Chengmai, Hainan, 20N, 110E) and transported back to the laboratory. The healthy middle fruit comb was selected and separated into a single fruit finger. After surface-sterilization for 10 min with 0.1% sodium hypochlorite, the fruit fingers were air-dried and stored for 8 d at 22°C and 80% relative humidity.

A sharp blade that has been surface-sterilized with 75% ethanol was used to uniformly section the fruit fingers into 2–4-mm-thick slices, with the fruit ends discarded.

Iodine-potassium-iodide (I₂-KI) staining and treatments

The banana fruit slices were immersed in 0.1%, 0.25%, 0.5%, 0.75%, and 1.0% I₂-KI solutions for 30 s, 60 s, 90 s, 120 s, 150 s, and 180 s. The effect of dyeing was assessed based on the intensity of the stain.

Plasmid construction

The tobacco rattle virus (TRV)-based VIGS vectors pTRV1 and pTRV2 (described in Liu et al., 2002) [32] were kindly offered by Dr H. Shi from Hainan University. A 586-bp gene fragment corresponding to bases 912–1498 of *MaBAM9b* (Ma05_t07800.1) was PCR-amplified from banana cDNA templates using primers (forward: 5'-GGTCTAGATCCGGACTTCGGCAACTTT-3' with an *Xba* I restriction site and reverse: 5'-CGGGATCCCGCCATGCTCCTGATGAAC-3' with an *Bam*H I restriction site). The resulting product was cloned into pTRV2 to form pTRV2- *MaBAM9b*.

VIGS in the banana fruit slices

The *Agrobacterium tumefaciens* GV3101 cell cultures harboring pTRV2 and pTRV2- *MaBAM9b* with pTRV1 were mixed at ratios of 1:1, 1:2, and 1:3, and the resuspended *A. tumefaciens* GV3101 cells were cultivated at OD600 values of 0.4, 0.6, 0.8, 1.0, and 1.2. The banana fruit slices were vacuum-infiltrated with the *Agrobacterium* solution at 30 mmHg for 30 s. After the release of the vacuum, the fruit slices were maintained for 1 d, 3 d, 5 d, and 7 d on Murashige & Skoog (MS) medium at 23°C. The banana fruit slices at each point were sampled for I₂-KI staining, gene expression, and physiological index analysis.

RNA isolation and quantitative real-time (qRT) PCR

The total RNA in the banana fruit slices was extracted using an RNAPrep Pure Plant Kit (Tiangen, DP441, Beijing, China) together with RNase free DNase (NEB, M0303S, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit and 20 µL was further diluted in 1:50 and quantified using a Nanodrop 2000 (both Thermo Scientific, Waltham, MA, USA). The changes in the transcriptome of *MaBAM9b* were evaluated by qRT-PCR analysis on a Stratagene Mx3000P Real-Time PCR system with SYBR® Premix Ex Taq™ (TaKaRa, Japan) using the 2- $\Delta\Delta$ Ct method [33]. *MaRPS2* (HQ853246) and *MaUBQ2* (HQ853254) constituted the internal controls that were used to normalize the target gene expression [34]. The primers for *MaBAM9b* expression are as follows: F: 5-GCCCGCAGTCACTACTATCT-3, R: 5-GAAGAA CTCAGCA CCCATCC-3.

Quantification of physiological index

The total starch content was detected following the method of Miao et al. (2014) [35]. The β -amylase activity was measured according to the method of Hou et al. (2017) [36]. The soluble sugar (fructose, glucose and sucrose) contents were analyzed using High-Performance Liquid Chromatography (HPLC; Waters, Milford, CT, USA). The HPLC method was developed and validated using an AMINEX 87H column, 10 mmol L⁻¹ sulfuric acid as eluent and refraction index detection[37].

Statistical analysis

At least three biological replicates were assessed, and all of the data were analyzed using One-way analysis of variance (ANOVA) and Student's *t*-tests for determination of significant differences.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LJH, XBY and JZQ conceived and designed the experiments. LJH and LM performed the experiments and analyzed the data, wrote the paper, prepared the figures, and reviewed drafts of the paper. ZJ, MHX, WJY, JCH, ZJB and WZ executed the experimental materials and laboratory experiments, acquired image data. All authors read and approved the final manuscript.

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Abbreviations

ANOVA, analysis of variance; DAFB, days after full bloom; DPH, days postharvest; FW, fresh weight; I₂-KI, iodine-potassium-iodide; *MaBAM9b*, *Musa acuminata* β -amylase 9b; MS, Murashige & Skoog; OD, optical density; PTGS, post-transcriptional gene silencing; qRT, quantitative real-time; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing.

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Figures

Figure 1

The effects of different I2-KI solution concentrations and treatment times on the staining results. A-F, staining for 30 s, 60 s, 90 s, 120 s, 150 s, and 180 s, respectively; a-e, I2-KI solutions of 0.1%, 0.25%, 0.5%, 0.75%, and 1.0%, respectively

Figure 2

The effects of different ratios of pTRV1: pTRV2-MaBAM9b on the silencing results. A, the effects of I2-KI staining; B, soluble sugar contents; C, β-amylase activity; D, total contents of starch; E, expression of MaBAM9b. a–c, the ratios of 1:1, 1:2, and 1:3, respectively. d, empty TRV2 plasmid as a negative control. The a1-a3, b1-b3, c2-c3, and d1-d3 represent three repeats for a, b, c, and d, respectively. The statistical significance of the differences was assessed by ANOVA (*, $p < 0.05$; **, $p < 0.01$).

Figure 3

The effects of different OD values of the bacterial fluid on the silencing results. A, the effects of I2-KI staining; B, total contents of starch; C, β-amylase activity; D, soluble sugar contents; E, expression of MaBAM9b. a–e, the OD value of 0.4, 0.6, 0.8, 1.0, and 1.2, respectively. f, empty TRV2 plasmid as a negative control. The a1-a3, b1-b3, c1-c3, d1-d3, e1-e3, and f1-f3 represent three repeats for a, b, c, d, e,

and f, respectively. The statistical significance of the differences was assessed by ANOVA (*, $p < 0.05$; **, $p < 0.01$).

Figure 4

The effects of co-cultivation time on the silencing results. A, the effects of I2-KI staining; B, total contents of starch; C, β -amylase activity; D, soluble sugar contents; E, expression of MaBAM9b. a–d, the co-cultivation time for 1 d, 3 d, 5 d, and 7 d, respectively. The a1–a3, b1–b3, c2–c3, and d1–d3 represent three

repeats for a, b, c, and d, respectively. The statistical significance of the differences was assessed by ANOVA (*, $p < 0.05$; **, $p < 0.01$).