Brief Communication

Host-induced gene silencing of Foc TR4 ERG6/11 genes exhibits superior resistance to Fusarium wilt of banana

Tongxin Dou1,†, Xiuhong Shao2,†, Chunhua Hu1,†, Siwen Liu3, Ou Sheng1, Fangcheng Bi1, Guiming Deng1, Lijie Ding2, Chunyu Li1, Tao Dong1, Huijun Gao1, Weidi He1, Xinxian Peng4, Sheng Zhang5, Heqiang Huo6, Qiaosong Yang1,† and Ganjun Yi1,*

1Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Key Laboratory of South Subtropical Fruit Biology and Genetic Resource Utilization (Ministry of Agriculture and Rural Affairs), Guangdong Province Key Laboratory of Tropical and Subtropical Fruit Tree Research, Guangzhou, China
2Horticulture and Landscape College, Hunan Agricultural University, Changsha, China
3College of Horticulture, Shenyang Agricultural University, Shenyang, China
4State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, South China Agricultural University, Guangzhou, China
5Institute of Biotechnology, Cornell University, Ithaca, NY, USA
6Department of Environmental Horticulture, University of Florida, Apopka, FL, USA

Received 22 April 2019; revised 14 June 2019; accepted 18 June 2019.
*Correspondence (Tel 86 020 87596278; fax 86 020 87565398; email yiganjun@vip.163.com (GY); Tel 86 020 38765390; fax 86 020 38765390; email soyang@hotmail.com (QY))
†These authors contributed equally to this work.

Keywords: banana, Fusarium wilt, HIGS, ihpRNA, siRNA, ergosterol biosynthesis.

Current banana cultivation is facing a serious threat known as Panama disease or Fusarium wilt, which is caused by the Fusarium oxysporum (Dale et al., 2017). To date, development of Fusarium wilt-resistant banana still faces challenges of various sexual reproduction barriers, such as high sterility, complex genetic background, polyopoid, and parthenogenesis (Ghag et al., 2014), making it extremely difficult to develop new disease-resistant bananas via cross-breeding (Czisloisvki et al., 2018). In recent years, host-induced gene silencing (HIGS) has increasingly become one of the most practical technologies for generating new disease-resistant crop varieties thanks to its high specificity and efficiency in silencing pathogen-derived genes (Qi et al., 2018; Zhang et al., 2016). Using this technology, Ghag et al. (2014) succeeded to silence two vital fungi genes, velvet, and fft1 (Fusarium transcription factor 1), from Foc Race 1 via dsRNA-mediated gene silencing and enabled the original susceptible banana cv. Rasthali to develop efficient resistance against Foc Race 1. Despite its resistance to Foc Race 1, the current Cavendish cultivars are highly susceptible to Foc TR4, and the global outbreak of this fungal disease could pose a serious threat to the present banana production, making the current world’s banana industry vulnerable (Dale et al., 2017). Therefore, development of Foc TR4-resistant banana cultivars will be a cost-efficient and effective solution to banana growers. However, selection of appropriate target genes is the most vital prerequisite for development of a HIGS technique against Fusarium wilt.

Previous studies in our group have indicated that ergosterol biosynthesis, especially C24 sterol methyltransferase (ERG6), cytochrome P450 lanosterol C-14-demethylase (ERG11), hydroxymethylglutaryl-CoA synthase (ERG13) and C-4 sterol methyl oxidase (ERG25), play crucial roles in Foc TR4 conidial germination (Deng et al., 2015). In this study, a newly stacked ERG6 (ERG6-2RNA) from two fragments of Foc TR4 ERG6A and ERG6B, a newly stacked ERG11 (ERG11-3RNA) from three fragments of Foc TR4 ERG11B, ERG11A, and ERG11C, a new ERG13 fragment and a new ERG25 fragment were synthesized at Gene Create Biotechnology Co., Ltd., respectively (Figure 1a). Through the transient silencing test, it was found that ERG11-3RNA showed the highest fungistatic effects compared to the other three ihpRNAs—ERG6-2RNA, ERG13-3RNA, and ERG25-3RNA (Figure 1b). Similarly, previous reports have demonstrated that silencing of ERG11 homologous genes in other crop species could improve resistance to fungus disease (Koch et al., 2013). Furthermore, the Foc TR4 ERG6-2RNA knockout mutant with significantly compromised virulence (Figure 1b), inspiring us to develop Foc TR4-resistant banana through HIGS technology against ERG6 or ERG11. Transgenic Cavendish banana ERG6-RNAi and ERG11-RNAi plants were obtained as described in our previous report (Dou et al., 2016). After PCR genotyping, a total of 48 ERG6-RNAi and 51 ERG11-RNAi transgenic banana lines were obtained, and the Southern blotting results further confirmed the stable integration of transgenes into Cavendish banana genomes (Figure 1c). Under a healthy growth condition, no phenotypic differences between ERGs-RNAi silencing lines and the wild-type (WT) Cavendish banana were observed. In order to verify disease resistance of ERG6-RNAi and ERG11-RNAi in transgenic banana plants, a preliminary screening experiment involving healthy and growth-consistent transgenic (10 ERG6-RNAi lines and 10 ERG11-RNAi lines) and WT banana plants were carried. At 14 days after inoculation, 87.65% of WT control plants died, while ERG6-RNAi’s two transgenic lines (ERG6-RNAi line5 and ERG6-RNAi line36) and ERG11-RNAi’s two transgenic lines (ERG11-RNAi line34 and ERG11-RNAi line49) exhibited optimum performance with no or slight disease symptoms (i.e., leaf wilting) (Figure 1c). The corm necrotic spot area reached 82.35% of the total corm area in WT control plants, while only 8.31%–22.35% of the total corm area in 4 ERGs-RNAi silencing lines exhibited necrosis. Next, it was examined whether this resistance of ERGs-RNAi silencing lines to the Foc TR4 was derived
from the decline in transcript level of two ERGs. At 14 days after Foc TR4 inoculation, the relative mRNA levels of the two ERG6 homologous genes (ERG6A and ERG6B) decreased by 6.57–8.11 and 4.06–4.23 folds in the two ERG6-RNAi silencing lines (line5 and line36), respectively, when compared to those in the WT control plants (Figure 1d); similar mRNA degradation were also triggered by RNAi silencing in two ERG11-RNAi transgenic bananas, with a 3.32–5.40, 4.49–6.89 and 6.19–7.66 folds decline in ERG11A, ERG11B, and ERG11C, respectively (Figure 1d). These results suggest that silencing of ergosterol biosynthetic genes ERG6s and ERG11s may effectively improve resistance of Cavendish bananas to Foc TR4.

Next, whether resistance of Cavendish bananas to Foc TR4 due to ERGs silencing is environmentally dependent in the open field was investigated, which is a crucial factor for application of HIGS technology in combating fungal diseases. Two ERG6-RNAi lines (line5 and line36), two ERG11-RNAi lines (line34 and line49) along with WT control banana plants were grown for two growth periods/two years in a field plot that was heavily infected by Fusarium wilt. All tested banana plants were grown in early spring, since the warm and humid climate at this planting season could promote the growth of Foc TR4, and disease symptoms like leaf wilting/yellowing of all tested plants were closely monitored. Results showed that all four tested ERG6- or ERG11-RNAi lines

Figure 1 HIGS of Foc TR4 ERG6/11 results in strong resistance to Fusarium wilt in banana. (a) New synthesized ERG6/11/13/25 fragments (different colours represent different homologous gene fragments). (b) Up: growth inhibition of GFP-tagged Foc TR4 spore grown in axenic culture following treatment with total RNAs from E. coli HT115 (Control), E. coli HT115-contained empty vector (Empty vector), E. coli HT115-contained pYL-Di-New ERG6 vector (ERG6 ihpRNA), E. coli HT115-contained pYL-Di-New ERG11 vector (ERG11 ihpRNA), E. coli HT115-contained pYL-Di-New ERG13 vector (ERG13 ihpRNA) or E. coli HT115-contained pYL-Di-New ERG25 vector (ERG25 ihpRNA), respectively. 1 x 10^5 Foc TR4 filtrated spore/mL were suspended in 250 μL of PDB medium, treated with 2 μg/μL final concentrations of total RNAs, and microscopically evaluated at 0 hpt, 12 hpt, 24 hpt, and 48 hpt. Scale bar = 50 μm. Down: characterization and vegetable growth of ERG6 gene knockout Foc TR4 mutants. Representative figure of at least 3 independent experiments. (c) Molecular analysis and preliminary screening of the elite transgenic lines. 50 plants per line for preliminary screening inoculation experiments. (d) Abundance of ERG6s (left) and ERG11s (right) genes transcripts in Foc TR4-infected banana roots at 14-day post-inoculation. (e) Phenotypes of the WT (50 plants) and transgenic banana plants (50 plants per line) in serious-disease nursery after one growth period/one year and two growth periods/two years. (f) Unique read length count of small RNA of ERG6-RNAi line36 (Left) and ERG11-RNAi line49 (Right) of the second year.
have much lower infection rate compared to the WT control plants both in the first year and second year (Figure 1e). For example, more than 85% of the WT control plants showed apparent Fusarium wilt symptoms, whereas only 30% of ERG6-RNAi or 15% of ERG11-RNAi plants were sensitive to Foc TR4 in the second year. Interestingly, it was also found that disease resistance of ERG11-RNAi lines was superior to that of ERG6-RNAi lines, while disease resistance of ERG11-RNAi line34 with multicopy was superior to that of ERG11-RNAi line49 with a single copy. Data on complete banana life histories in a serious disease nursery for 2 years, indicated rate of transgenic banana plants was significantly lower than the WT control plants, consistent with the results of preliminary screening experiment. This could be explained by the more effective silencing of ERG11 due to multiple copies of T-DNA insertion in the genome of this transgenic line.

Mobility of small RNAs within organisms is a well-known phenomenon, facilitating gene silencing in adjacent cells and surrounding or even distant tissues (Jahan et al., 2015). Over recent years, several examples of exchange of small RNAs between host plants and invading pathogens have been described, although the mechanistic details of the actual exchange remain to be elucidated. For example, endogenous small RNAs from the fungus Botrytis cinerea have been proposed to transfer into host plants to target defence-related plant transcripts to promote disease development (Weiberg et al., 2015). In this paper, to confirm whether the disease resistance of transgenic banana plants is related to Foc TR4 ERG6 and ERG11-derived specific siRNAs, a siRNA in two ERG6-RNAi lines (line5 and line36), two ERG11-RNAi lines (line34 and line49) and WT plants of the second growth period/second year was performed by small RNA sequencing. The sequencing results showed that ERG6 and ERG11-derived specific siRNAs were significantly enriched in the root systems of the four transgenic lines and their lengths were mainly distributed between 18–25 bp, wherein 21, 22, and 24 bp small RNAs were the most abundant (Figure 1f). For instance, ERG6-derived specific small RNAs in the root systems of ERG6-RNAi line5 and line36 reached 2.02% and 2.38% of total small RNAs, respectively; similarly ERG11 target-specific small RNAs in the root systems of ERG11-RNAi line34 and line49 accounted for 5.22% and 3.35% of total small RNAs, respectively; small RNA abundance of line34 with multi-copies of T-DNA insertions was significantly higher than line49 with a single copy of transgene, which is consistent with the lower transcript level of ERG11 in line34 (Figure 1d). These findings provide further evidence that siRNA expression levels of the target genes in specific transgenic lines is an important factor to determine its disease resistance.

Taken together, HIGS has been employed to target two ergosterol biosynthetic genes—ERG6/ERG11 that were identified and confirmed as effective target genes by transient silencing and knockout mutants test, and ERG6-RNAi and ERG11-RNAi transgenic bananas were generated using HIGS technology; their disease resistance traits were dramatically enhanced by inhibition of growth and development of the infected Foc TR4. The data not only provide reference for disease-resistance breeding in bananas using HIGS technology, but also pave a theoretical foundation for developing double stranded RNA fungicide to control crop fungal diseases.

Acknowledgements

This work was supported by the National Natural Science Foundation of China, No. 31801843 and 31872939; Natural Science Foundation of Guangdong Province, No. 2017A030310351 and No. 2016A030310326; Guangdong Academy of Agricultural Sciences Foundation, No. 201815; Projects from Ministry of Science and Technology of China, No. 2016B020233002, 2018YFD1000302, and 2014B050502007; and Modern Agricultural Innovation Team Project of Guangdong Province No. 2018LM2150; and Science and Technology Plan Project of Guangdong Province No. 2015B070701011.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Q.-S.Y., J.-Y.J., and T.-X.D conceived the study and analysed the data. T.-X.D., X.-H.S., C.-H.Hu., S.-W.L., O.S., F.-C.B., G.-M.D., L.-J.D., C.-Y.L., T.D., H.-J.G., and W.-D.H performed the experiments. T.-X.D., Q.-Y.S., and H.-Q.H drafted the manuscript. G.-J.Y., H.-Q.H., S.Z., and X.-X.P. revised the manuscript.

References

Czisloowski, E., Fraser-Smith, S., Zander, M., O’Neill, W.T., Meldrum, R.A., Tran-Nguyen, L.T.T., Batley, J. et al. (2018) Investigation of the diversity of effector genes in the banana pathogen, Fusarium oxysporum f. sp. cubense, reveals evidence of horizontal gene transfer. Mol. Plant Pathol. 19, 1155–1171.

Dale, J., James, A., Paul, J., Khanna, H., Smith, M., Peraza-Echeverría, S., García-Bastidas, F. et al. (2017) Transgenic Cavendish bananas with resistance to Fusarium wilt tropical race 4. Nat. Commun. 8, 1–8.

Deng, G., Yang, Q., He, W., Li, C., Yang, J., Zuo, C., Gao, J. et al. (2015) Proteomic analysis of conidia germination in Fusarium oxysporum f. sp. cubense tropical race 4 reveals new targets in ergosterol biosynthesis pathway for controlling Fusarium wilt of banana. Appl. Microbiol. Biot. 99, 7189–7207.

Dou, T., Hu, C., Sun, X., Shao, X., Wu, J., Ding, L., Gao, J. et al. (2016) MpMYB53 as a crucial transcription factor of cold signaling confers the cold tolerance of banana. Plant Cell. Tiss. Org. 125, 93–106.

Ghag, S.B., Shekhawat, U.K. and Ganapathi, T.R. (2014) Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against Fusarium wilt in banana. Plant Biotechnol. J. 12, 541–553.

Jahan, S.N., Asman, A.K., Corcoran, P., Fogelqvist, J., Vetukuri, R.R. and Dixelius, C. (2015) Plant-mediated gene silencing restricts growth of the potato late blight pathogen Phytophthora infestans. J. Exp. Bot. 66, 2785–2794.

Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J. and Kogel, K.H. (2013) Host-induced gene silencing of cytochrome P450 lanosterol C14-demethylase-encoding genes confers strong resistance to Fusarium species. Plant Physiol. 161, 932–942.

Qi, T., Zhu, X., Tan, C., Liu, P., Guo, J., Kang, Z. and Guo, J. (2018) Host-induced gene silencing of an important pathogenicity factor PsCPK1 in Puccinia striiformis f. sp. tritici enhances resistance of wheat to stripe rust. Plant Biotechnol. J. 16, 797–807.

Weiberg, A., Bellinger, M. and Jin, H.L. (2015) Conversations between kingdoms: small RNAs. Curr. Opin. Biotechnol. 32, 207–215.

Zhang, T., Jin, Y., Zhao, J., Gao, F., Zhou, B., Fang, Y. and Guo, H. (2016) Host-induced gene silencing of the target gene in fungal cells confers effective resistance to the cotton wilt disease pathogen Verticillium dahliae. Mol. Plant. 9, 939–942.