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Full Length Research Paper

Identification of a latent pathogen on mulberry tree with a disease of mosaic dwarf

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A disease on mulberries with the typical symptoms of mosaic and dwarf leaves was found in middle areas of China in 1980s. Presently, this disease became serious and spread out. Based on previous finding, we detected a viroid-like small molecular RNA in diseased mulberries tissues. In this paper, we further identified the pathogen of mulberry mosaic dwarf disease (MMDD) according to the Koch's postulates and reported the diagnostic method of the pathogen by using PCR with two sets of specific primers. The result might be helpful to control the disease extension.

Key words: Mulberry, mosaic dwarf disease, viroid-like RNA, identification.

INTRODUCTION

Mulberry dwarf disease (MDD) is also named as “Nong-sang”. There are many different symptoms of “Nong-sang” caused by different pathogens in China. One kind of “Nong-sang” is characterized by “mosaic” and “dwarf”, therefore, is named as MMDD (Figure 1a). In 1980s, The MMDD was noticed in the middle areas of China for its extension. Recently, the disease was found in the main silkworm-cultured areas, such as Zhejiang, Jiangsu, Sichuan and Anhui.

It has been assumed that MMDD was induced by some kinds of viruses for a long time before (Kuai, 1965; Zhang, 1988). However, this hypothesis had not been confirmed. Until 1990, a small circular viroid-like RNA pathogen was isolated by Kuai and his colleagues in diseased mulberry tissues using Return-directional-PAGE (one cycle of non-denaturing polyacrylamide gel electrophoresis followed by one cycle of denaturing gel electrophoresis at 70℃) and the partial sequence of this RNA was obtained (Kuai and Tian, 1990; Zhou and Kuai, 1993). However, at that time, it was not further confirmed that this newly found small RNA was the real pathogen of MMDD, according to the Koch's postulates. In this paper, we focus on the identification of the pathogen of MMDD according to Koch's postulates and the molecular diagnostic techniques of this disease.

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MATERIALS AND METHODS

Extraction of viroid-like RNA pathogen

Leaves collected from established standard diseased mulberries grown in the countryside of Huzhou city (Zhejiang Province) were frozen in -70℃ for further use. Total RNA was isolated from the mulberry leaves with characteristic symptoms of MMDD and was further detected by electrophoresis in the same direction consisted of consecutive electrophoresis on 5% polyacrylamide gels under non-denaturing and then denaturing conditions respectively, according to Fei et al. (2007).

Detection of the viroid-like RNA pathogen

RT-PCR was carried out to detect the viroid-like small molecule using the total RNA extractions. The primers were designed and synthesized according to the conserved sequences of PSTV as followers:

Forward: 5’-GTTTCCACCGGGTA-3’

Figure 1. The symptom observation of the diseased mulberry trees. **a**, mosaic and dwarf symptoms of spontaneous diseased mulberry leaves; **b**, inoculation by injection; **c**, inoculation by graft; and **d**, symptoms of diseased mulberry leaves inoculated.

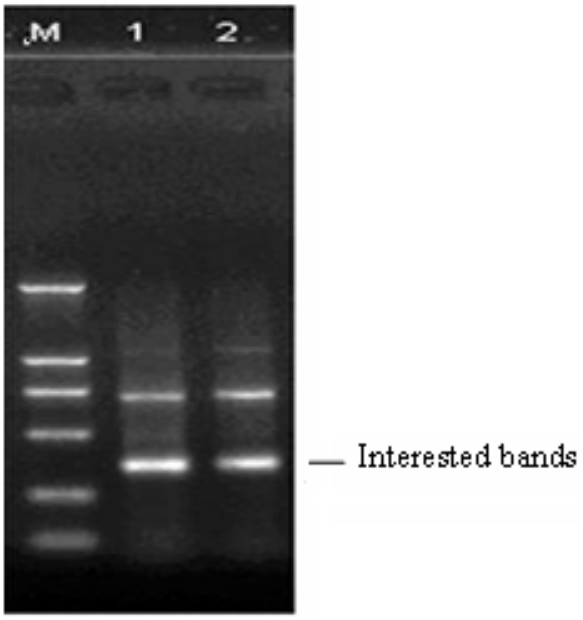

Figure 2. RT-PCR products form the leaves of diseased mulberries in field. Lane M, DNA markers; lane 1, samples obtained from Huzhou; lane 2, samples obtained from Zhenjiang (infected spontaneously).

Reverse: 5'-GTGGTTCCTGTG-3'

The PCR cycling parameters of the amplification of the viroid-like molecule were thirty cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec. The PCR products were visualized by electrophoresis in 2% agarose gels with stained with ethidium bromide. The PCR product was further cloned into pGEMT vector and sequenced. In order to obtain the complete sequence of the viroid-like RNA, another-paired primers were designed according to the obtained sequenced as follows:

Forward: 5'- GTGGTTCCTGTG- 3'
Reverse: 5'- CACACCAAGGAC- 3'.

The later obtained PCR product was sequenced as well. The integrated sequence of the viroid-like RNA was detected.

Pathogen inoculation and comparison

The healthy mulberry trees grown in Wuxing Station of Silkworm and Mulberry Technical Guidance, Huzhou (the area without diseased mulberries) and Institute of Sericultural Reseach, Chinese Academy of Agricultura Sciences (Zhenjiang City, Jiangsu Province) were inoculated with the RNA extract containing viroid-like molecule prepared from the trees with typical MMDD symptoms (Figure 1a). The pathogen mixture was inoculated into the leaves or branches of the mulberries by injection or graft (drop into the section of graft), respectively (Figures 1b and 1c). The total RNA was isolated from the leaves of the inoculated mulberries, which were induced MMDD symptoms. RT-PCR was further performed to detect the viroid –like molecules.

RESULTS

Comparative analyses of the inoculums

The mulberry trees inoculated with crude nucleic acid extracts at 16th, Aug., 2008, were investigated at 16th, Nov., 2008. Three months after inoculation, some of the inoculums were induced symptoms of MMDD (Figure 1d).

Among these inoculums, viroid-like pathogen was transmitted to three mulberries in Zhenjiang by needle injection. Two of the three mulberries were infected, but the rest one did not induced typical symptoms of MMDD. Nine mulberries were inoculated by grafting test. Three mulberries were infected, while the three control mulberries, which were grafted by healthy branches, remained healthy. The healthy mulberries grown in Wuxing area were inoculated by injecting with the pathogen into the bark at June, 2007. When checked at November, 2008, the leaves and branches of these trees displayed typical symptoms (Figure 1d). However, in rubbing test, all mulberries inoculated did not fall to infection and are still kept for future investigation.

Detection of the viroid-like RNA

The leaves obtained from healthy or infected mulberries in field were detected by RT-PCR using the above primers. As shown in Figure 2, the viroid-like molecule could be detected in the infected mulberries.

The pathogen bands were detected in the leaves samples obtained from the mulberries inoculated by pathogens using the same primers as before (Figure 3). The isolation of the pathogen from the inoculated mulberries

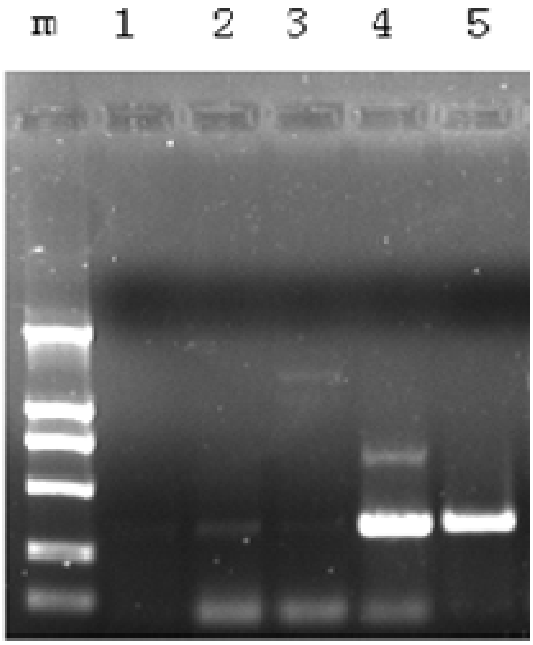

Figure 3. RT-PCR products of the leaves of diseased mulberries inoculated with pathogen. The result indicated that the specific band can be amplified only from the diseased mulberry trees. Lane M, DNA markers; lane 1, healthy leaves; lanes 2, 4, and 5, infected leaves of mulberries inoculated with pathogen; lane 3, leaves of anti-disease mulberry tree inoculated with pathogen.

suggests that the identification of the viroid-like pathogen was successful.

DISCUSSION

The identification of current plant viruses and other pathogens was usually according to Koch's postulates (Prescott et al., 1996). The usage of this principle in identification of plant virus was summarized as follows:

1. The virus must be detected in large numbers of infected plants.
2. The pathogen must be isolated from the plants infected by the same pathogen and the characteristic should be described.
3. When the pathogen was inoculated into the plant, the induced symptoms should be in accordance with that of the plant in field.
4. The pathogen should be isolated from the inoculated plants again and the characteristics should be the same as before.

The identification of the pathogen in this paper was fitted with the Koch's postulates. The isolated small molecule, viroid-like RNA, was confirmed to be pathogen of MMDD. Therefore, we suggested that the viroid-like RNA should be named as MMDVd.

As far as 300 years ago, the pathogen of MMDD was recorded in ancient agriculture book (Sheng agriculture book, the end of Ming dynasty, 40s of 17th century) as " if the diseased mulberry tree was found, it must be got rid of and can not be kept. The disease might be spread by scissors when the mulberry branches were cut down." The author of the agricultural book was a native of Liang Shi, Huzhou municipality. At that time, Huzhou municipality was prosperous in silk breeding and mulberry planting and MMDD was the most serious disease in mulberry trees. Therefore, they accumulated abundant experiences to control MMDD. In our opinions:

1. MMDD was one kind of infectious diseases.
2. When the mulberries were cut or pruned, the pathogen might be spread in the sap.

Currently, the possibility of this kind of sap infection was confirmed by our injection experiment which induced the symptoms of MMDD in healthy mulberries. Furthermore, the distribution of mulberries with MMDD in field also supported the possibility of tree sap infection indirectly (Bai et al., 2005).

The identification of plant viroid using RT-PCR analysis has been reported (Arezou et al., 2008; Hosokawa et al., 2006; Schnell et al., 2001). In this study, we could detect the infected saplings in latent stage using this approach. The MMDD has been listed as one of the plant medical inspection diseases in Zhejiang province. By now, in order to control this disease, the mulberries were inspected in growing period to discard the infected saplings according to the symptoms on the leaves. However, it was difficult to detect the infected trees in latent stage. The technology of RT-PCR provides a molecular biological method in detection of the RNA pathogen.

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REFERENCES

Arezou Y, Jafarpour B, Rastegar MF, Javadmanesh A (2008). Molecular detection of potato spindle tuber viroid in Razavi and Northern Khorasan province. Pak. J. Biol. Sci. 11(12): 1642-1645

Bai XC, Fei JM, Yang HJ, Wang WB, Kuai YZ (2005). Analysis of mulberry mosaic dwarf disease in Huzhou area. Chinese Sericologica. 26(4):84-86

Fei JM, Bai XC, Yu F, Zhao H, Wang WB, Kuai YZ (2007). Detection of pathogen of mulberry mosaic dwarf disease by molecular biology techniques. Acta Agric. Zhejiang, 19(2): 115-118.

Hosokawa M, Matsushita Y, Uchida H, Yazawa S (2006). Direct RT-PCR method for detecting two Chrysanthemum viroids using minial amounts of Plant tissue. J. Virol. Methods, 131(1): 28-33.

Kuai YZ (1965). Styudies on three type of mulberry mosaic dwarf disease. Acta sericologica sinica, 3(4): 207-217.

Kuai YZ, Tian B (1990). Discovered on new pathogen of mulberry

mosaic dwarf disease. Collectanea of secondary a national science symposium of mulberry protection, pp. 40-41
Prescott LM, Harley JP, Klein DA (1996). Microbiology 3rd, Wm. C. Brown Publishers, USA, pp 8-9.
Schnell RJ, Olano CT, Kuhn DN (2001). Detection of avocado sunblotch viroid variants using fluorescent single-strand conformation polymorphism analysis. Electrophoresis, 22(3): 427-432.
Zhang YJ (1988) Study on mulberry mosaic dwarf disease. Acta sericologica sinica, 9(2): 74-79.
Zhou H, Kuai YZ (1993) Primarily nucleotide sequence of pathogen of mulberry mosaic dwarf disease. Bull. phytopathol. association Jiangsu. 2: 61-69.