Development and utilization of plant vaccines in Japan

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ABSTRACT

Pepper mild mottle virus (PMMoV), which belongs to the genus *Tobamovirus*, causes serious damage on green pepper (*Capsicum annuum* L.) in Japan. Until 2012, disease caused by PMMoV in Japanese fields has been controlled by the soil fumigant methyl bromide. However, methyl bromide has not been used since, except in critical or quarantine cases in developed countries. Therefore, we developed an attenuated strain to be used as an alternative strategy to control PMMoV infection of cultivated green peppers. The vaccine, L3-163 was obtained by heat treatment of the plant infected with PMMoV-wild type. The vaccine was able to completely protect green peppers from infection by PMMoV-wild type. In addition, large-scale inoculation technique was developed to apply the vaccine. This report described the development of the vaccine and for its use on control of viral diseases, such as PMMoV.

Keywords: *Pepper mild mottle virus*, attenuated strain, plant vaccine, cross protection, bio-control

INTRODUCTION

Cross-protection was first demonstrated by McKinney (1929) [(14)], who observed that in tobacco plants systemically infected with *Tobacco mosaic virus* (TMV)-light green strain, the appearance of yellow symptoms caused by TMV-yellow mosaic strain was repressed. On the other hand, TMV-mild dark green strain did not repress the yellow symptoms. Ever since that observation, cross-protection have been demonstrated in several viruses such as *Potato virus* X, *Potato leafroll virus* and *Citrus tristeza virus* (Gal-On and Shiboleth 2006, Grant and Costa 1951, Salaman 1933, Webb

et al. 1952) [4, 6, 12, 22]. The phenomenon of cross protection is similar to that for vaccination: once a plant was inoculated and infected by an attenuated strain, that plant could not be re-infected by the same wild-type strain or a related viral species. Thus, the attenuated virus acts as a form of biological control. To date, many attenuated viruses have been developed for practical use worldwide (Fulton 1986, Lecoq, 1998, Ziegreen and Carr 2010) [3, 13, 24].

In Japan, many studies about cross-protection have been reported. For example, *Tomato mosaic virus* (ToMV)-L_{11}A isolate is one of such attenuated virus developed in Japan, which provides a high degree of protection against the wild-type ToMV in commercial tomato crops (*Solanum lycopersicum* L., Goto and Nemoto 1971) [5]. This isolate has been widely used throughout Japan. Around 20% of all tomato farmers in the Chiba Prefecture have introduced ToMV-L_{11}A before new tobamovirus-resistant tomato cultivars were developed (Nagai 1984) [15]. Recently, *Zucchini yellow mosaic virus*-2002 attenuated isolate was developed and registered as biotic pesticide. The isolate produced very mild or no symptoms on cucurbit plants. In addition, inoculated cucumber plants had very similar fruit productivity to healthy control plants under field conditions. In field experiments, when other viruses were also present, protected plants significantly suppressed infection with ZYMV, progression of disease severity, and reduction of fruit yield and quality (Kosaka et al. 2006) [11]. As described above, many researches about attenuated plant viruses have been conducted and the isolates have been used in Japan.

Green pepper is one of the important crops in Japan. The total amount of yield of green pepper is near 140,000 tons in a year, and the cultivated areas covered about 3000 hectares in Japan. The main production regions are Ibaraki and Miyazaki prefectures. There are many viral diseases, such as mosaic and necrosis diseases, have been reported on Green pepper in Japan (Phytopathological Society of Japan 2000) [19]. Of these, mosaic disease caused by *Pepper mild mottle virus* (PMMoV), which belongs to the genus *Tobamovirus*, lead to serious damage on green pepper (*Capsicum annuum* L.). PMMoV has a positive-strand RNA genome consisting of 6,357 nucleotides. The virus encodes at least four proteins: a 130 kDa replicase, a 180 kDa replicase, a movement protein, and a coat protein (Kirita et al. 1997) [10]. PMMoV infection reduces plant growth and induces leaf mosaic symptoms and fruit malformation (Wetter and Conti, 1988) [23]. This is of relevance as the shape of pepper fruits affects their salability in

Japan, where they are classified into the following three categories: class A (excellent), class B (good), and unsalable (not marketable).

Until 2012, disease caused by PMMoV in Japanese fields has been controlled by the soil fumigant methyl bromide (Ikegashira et al. 2004) [9]. However, in 1992 at the Fourth Meeting of the Parties to the Montreal Protocol on Substances that Deplete the Ozone Layer, methyl bromide was considered an ozone-depleting substance. In developed countries, therefore, methyl bromide has not been used since 2005, except in critical or quarantine cases. Although Japan has continued to use it for soil fumigation, the government has agreed to phase it out by the end of 2012 (Report of the 28th meeting of the Open-ended Working Group of the Parties to the Montreal Protocol 2008) [25]. Consequently, alternative strategies to control PMMoV infection of cultivated green peppers are urgently required. The use of an attenuated virus was investigated to provide cross protection from PMMoV-wild type.

Some wild species of *Capsicum* carry the hypersensitive response (HR)-associated tobamoviral resistance gene *L*, which exists as four different alleles, L^1, L^2, L^3, and L^4, inherited in a Mendelian fashion (Boukema 1984) [1]. The P_1, $P_{1,2}$, and $P_{1,2,3}$ pathotypes of PMMoV overcome L^1 alleles, L^1-L^2 alleles, and L^1-L^3 alleles, respectively (Rast 1988) [20]. In Japan, many pepper varieties now grown for market carry a resistance gene such as L^3 ,which also confers resistance to these attenuated isolates. In addition, some wild-type isolates of PMMoV ($P_{1,2,3}$) have been found in cultivated Japanese peppers that can overcome the L^3 resistance (Tsuda et al., 1998, Hamada et al., 2007) [7,21]. Therefore, the attenuated isolate was developed for use in green peppers carrying the L^3 resistance gene throughout Japan. This report would be used for the development of the integrated management strategy for control of viral disease.

Development and characterization of the vaccine

Traditionally, attenuated strains were isolated from naturally occurring variants or by mutagenesis of wild type strains using nitrous acid or ultraviolet (UV) irradiation. Nitrous acid is a well-known mutagen that deaminates cytosine and adenine to produce uracil and hypoxanthine, respectively. UV irradiation has been used for isolation of attenuated strain, which causes the formation of pyrimidine dimers in DNA as well as in RNA (Bre´geon and Sarasin 2005, Nishiguchi and Kobayashi 2011) [2, 16]. In addition, some attenuated strains have been isolated after cultivation at higher or lower

temperatures. In this study, the attenuated strains of PMMoV were isolated by cultivation at higher temperature. Firstly, the sap of the leaves infecting PMMoV-wild type was inoculated to the stem of green peppers. The inoculated plants were kept at 40 ℃ for three weeks in a growth chamber. The sap of the stems was inoculated to the leaves of *Nicotiana tabacum* cv. Xanthi-nc and from which single local lesion isolation was conducted. After several weeks, four plants, which did not show any symptoms, were selected as candidates for vaccine production.

Northern blot analysis and Double Antibody Sandwich-Enzymed liked immnosorbent assay (DAS-ELISA) (Fig. 1) were used to analyze the accumulation levels of the vaccine both in cells and the whole plant. Accumulation levels of a vaccine, L3-163 in cells was lower than that of other strains. Similar result was obtained from the analysis of accumulation levels in whole plant. Then the distribution of the vaccines in a plant was analyzed by hammer blot analysis. Distribution of the vaccines in a plant often correlated with cross protection effect against PMMoV-wild type. Therefore, a vaccine, L3-163, which showed a wide distribution in a plant was selected. The nucleotide sequence of the L3-163 was then determined and compared to that of PMMoV-wild type. Several mutations were observed in the genome of the L3-163. Of these, we confirmed that three mutations in 130kDa/180kDa protein genes play an important role for attenuation by the reverse genetic technique using infectious clone of PMMoV-wild type. Finally, an attenuated strain, L3-163, with pathotype $P_{1, 2, 3}$, was commercially registered as a biological control agent in 2012.

Cross protection effects of the vaccine

The effectiveness of the vaccine, L3-163, for the cross protection of peppers from the infection by wild-type PMMoV was evaluated in the greenhouse and in the field (Ogai *et al*. 2011, 2013) [17, 18].

In the first experiment, green peppers cv. Miogi L^3 inoculated with the attenuated isolate were completely protected from infection by wild-type PMMoV, whereas non-treated plants began to show symptoms of infection with the wild-type virus after starting agricultural work (Fig. 2). By the end of the cultivation period, 71.4% of the plants were infected. In the second experiment, diseased plants were identified after starting agricultural work and 76.2% of the plants were infected by the end of the cultivation period. In this experiment, some plants inoculated with the vaccine showed

mosaic symptoms on their upper leaves, but sequence analysis revealed that this was caused by PMMoV-wild type.

We examined effectiveness of the attenuated isolate for other cultivars of green pepper grown in a commercial field situation: TM suzunami, Kyosuzu, and Mihata 2-go. The wild-type PMMoV failed to infect all three vaccine-inoculated cultivars at the end of the cultivation period (Table 1). When growth yields were compared between vaccine-treated and non-treated plants, the yields of both class A and B vaccine-treated plants were significantly higher than those in non-treated plants in all four cultivars. Some plants of Mihata 2-go and Miogi L^3 showed mosaic symptoms that led to loss of fruits. The viral sequence isolated from these plants was shown to be identical to that of PMMoV-wild type.

Accordingly, we examined the levels of protection on the commercial production of pepper fruits provided by the attenuated isolate. Four plants each with the following four levels of infection with the attenuated isolates, e.g., 0, 50%, 75% and 100% were grown in the greenhouse. Mosaic symptoms initially appeared one month after starting agricultural work in all plots, except the 100% plot. The plants in 0% and 50% plots showed a significant yield loss in fruit weights and numbers due to severe mosaic symptoms and fruit malformation. There was no significant difference in numbers, yields, and salable and unsalable peppers among plants grown in 75% and 100% plots. Therefore, these findings suggest that the yields are statistically equal between pepper seedlings vaccinated with attenuated isolate and the healthy seedlings when greater than 75% of the seedlings were successfully vaccinated.

Lecoq (1998) [13] reported the following characteristics of attenuated isolate for cross protection in the field. 1) It can cause milder symptoms on crops in the field, but the crops still produce sufficiently marketable yield and quality. 2) It possesses high histocompatibility to inoculated plants. 3) It is genetically stable. 4) It should retain limited non-intentional spread. 5) It should ideally possess the ability to protect plants from wide range of severe challenging isolates. 6) It should be well control and easy to apply. In Japan, an attenuated isolate for cross protection in commercial fields must also be officially registered as a biological control agent (biotic pesticide). Currently, an attenuated isolate of *Zucchini yellow mosaic virus* (ZYMV), designated ZYMV-2002, is widely used in *Cucumis sativus* L.) cultivation for cross protection against ZYMV wild type (Kosaka et al., 2006) [11], which is the first attenuated virus registered

in Japan as a trade name “Cubio ZY-02” (MAFF Reg. No. 22152; Kyoto Biken Laboratories, Inc., Kyoto, Japan). Based on our results, the vaccine of PMMoV will be registered as a biological control agent to protect varieties of pepper plants grown in Japan. In order to help our farmers familiarize with the vaccine, we also made a video for them.

Inoculation technique of the vaccine

For inoculation of the vaccine, sap of the inoculum was prepared from leaves of *Nicotiana benthamiana* inoculated with the L3-163. The sap was mechanically inoculated with carborundum onto the cotyledons of 10-day-old green pepper seedlings. Mechanically inoculation is useful for the small number of the seedlings. However, it is not convenient for farmers, because farmers generally cultivate more than thousands of seedlings in Japan. To date, large-scale inoculation, which utilizes a high-pressure spray gun, was developed for inoculating tobacco plants with the *Tomato spotted wilt virus* (Hull, 2014) [8]. Therefore, the large-scale inoculation technique was improved for practical applications wherein thousands of green pepper seedlings need to be simultaneously inoculated.

The seedling fixator stand (commercially available from Kyoto Biken Laboratories, Inc., Kyoto, Japan) was developed for large-scale inoculation of vaccine. Place the plug tray planting of pepper seedlings on the seedling fixator and then sticks of comb-shaped structure are placed underneath the cotyledon leaves for support during inoculation. Place netting over the seedlings. Spray the inoculum to the seedlings using the spray gun and air compressor. In this technique, at least 0.3 ml of inoculum solution is required for inoculation of one seedling. More than 98% of seedlings were successfully infected with the vaccine using this method. This inoculation method and the apparatus have been patented to Kyoto Biken Laboratories, Inc. (Japanese Patent No. 5938798).

Future prospects

Plant viruses are intracellular parasites that cannot replicate themselves without a host plant. To date, no antiviral compounds are available for cure of plants with viral diseases. Instead, cross protection has [been used as one of the effective tools for control of viral diseases worldwide. Although it is relatively classical method, it is drawing

great attention again in Japan because it is considered as an environmentally friendly technique.

This study could be useful for effective control of plant viral diseases because the L3-163 strain was shown to protect pepper plants with a high degree of cross protection efficacy against the PMMoV-wild type infection, which resulted in a better yield in greenhouse settings. In the future, new strategies such as developing multiple vaccines that combine several attenuated viruses or exploiting a single attenuated virus that can effectively protect against multiple viruses warrant further investigation.

AKNOWLEDGMENTS

Part of this study was supported by a Grant-in-Aid for "The research project for utilizing advanced technologies in agriculture, forestry and fisheries" administered by the Ministry of Agriculture, Forestry, and Fisheries of Japan.

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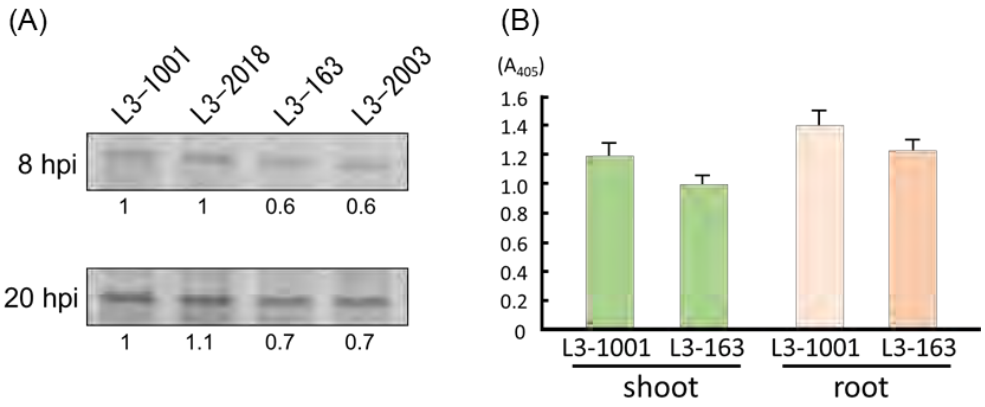


Fig. 1. Accumulation levels of the candidate vaccines, L3-1001, L3-2018, L3-163, L3-2003 in cells (A) and in whole plant (B). Accumulation levels in cells and whole plant were analyzed by northern blot analysis and double antibody sandwich enzyme-linked immunosorbent assay, respectively.

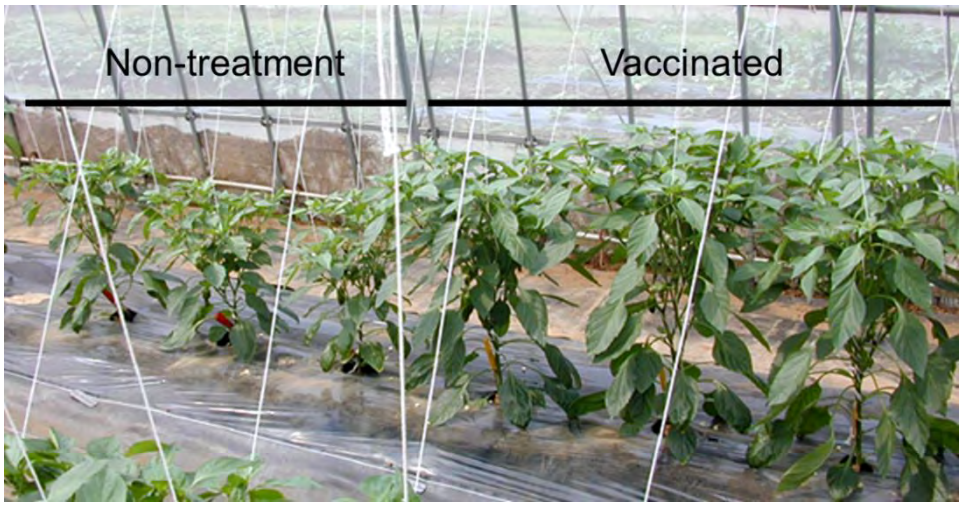


Fig. 2. Symptom comparison between the non-vaccinated and vaccinated plants in field experiments. Vaccinated plants remained asymptomatic throughout the study.

Table 1 Percentages of diseased plants of four green peppers cultivars inoculated with L3-163 and non-inoculated in the field.

Cultivar	Treatment	Diseased plant (%)
TM suzunami	Vaccinated	0.0±0.0
	Non-treatment	100.0±0.0
Kyosuzu	Vaccinated	0.0±0.0
	Non-treatment	100.0±0.0
Mihara 2-go	Vaccinated	3.7±3.0
	Non-treatment	96.3±3.0
Miogi *L*[3]	Vaccinated	3.7±3.0
	Non-treatment	100.0±0.0

The data was modified from Ogai et al. (2013).