Attenuated Isolates of Soybean Mosaic Virus Derived at a Low Temperature

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ABSTRACT

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Attenuated isolates of soybean mosaic virus (SMV) were derived when black soybean (*Glycine max* cv. Shin Tambaguro) seedlings, inoculated with a virulent SMV isolate, were maintained at 15 C for 14 days, or alternatively, for 30 days. Two isolates obtained (designated Aa15-M1 and Aa15-M2), were serologically and biochemically similar to the initial isolate. However, they differed from the initial isolate in aphid and seed transmissibility, and in replication in soybean plants. In greenhouse and field experiments, Aa15-M1 and Aa15-M2 were effective in cross-protection against virulent SMV strains.

The potyvirus soybean mosaic virus (SMV) is transmitted through seed and by aphids in a stylet-borne manner (1). SMV is commonly found in soybean (*Glycine max* (L.) Merr.) in Japan (17) and in many other countries in the world (1). In Kyoto Prefecture, SMV causes chronic damage in the soybean cultivar Shin Tambaguro or Tambaguro, which is called a black soybean because of its black seed coat. Shin Tambaguro or Tambaguro is the most widely grown cultivar in all the prefectures around Kyoto. Therefore, the development of effective control of SMV is needed to stabilize the seed production of black soybean.

Attenuated strains have been exploited for the control of other viral diseases, including those caused by tobacco mosaic virus, papaya ringspot virus, and watermelon mosaic virus 2. In these examples, attenuated strains were produced by treatment with heat, nitrous acid, or nitrous acid and UV light (4–6, 14,15,19). In addition, a mild variant of zucchini yellow mosaic virus was selected from a severe strain (12). In this paper, we report some properties of attenuated SMV isolates obtained by cold treatment, which has not been previously reported, and their effectiveness against the virulent strains of SMV in greenhouse and field experiments. Partial reports of this work have been made (7,9,10).

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MATERIALS AND METHODS

Virus source and mechanical inoculations. The initial SMV isolate used in almost all of the experiments, designated Aa, was obtained from infected seed of black soybean and characterized after single-lesion isolation using soybean cv. Shiromame, a local lesion host. This isolate belongs to the A strain of SMV and induces mosaic and leaf roll symptoms on soybean plants. The six other isolates of SMV used in cross-protection tests are isolate R (8), obtained from the field-grown black soybean plant, and the five standard strains A, B, C, D, and E (16), supplied by I. Shigemori. All of these isolates were propagated in cv. Shin Tambaguro, and the infected leaves (the first to third trifoliolate leaves 15–20 days after inoculation) were stored at −70 C before being used as standard inoculum sources. Mechanical inoculation was made by rubbing Carborundum-dusted leaves of the test plants with cotton swabs dipped in

extract of infected leaves ground in approximately 20 volumes (ml/g) of 0.1 M phosphate buffer (pH 7.2). After inoculation the leaves were rinsed with tap water, and the inoculated plants were kept in the greenhouse.

Temperature treatments and single-lesion isolations. The seedlings of Shin Tambaguro were mechanically inoculated with the isolate Aa when the primary leaves were just fully expanded. Immediately after inoculation, the plants were cut off at the base of the hypocotyl; and the stems were immersed in distilled water in vials. The cuttings were then transferred into a growth cabinet, which was maintained at 25 C in a 24-hr photoperiod, at about 8,000 lx. After 8 days, temperature treatments were applied to the plants. Leaf extracts from the treated plants were used to inoculate the young primary leaves of cv. Shiromame or bean (*Phaseolus vulgaris* L.) cv. Top Crop (used for the treatments at 35 C for 8, 14, and 21 days). The inoculated plants were kept in growth cabinets at 28 C in a 14-hr photoperiod. Single local lesions, developed on the primary leaves, were transferred 5 or 6 days later to a seedling of Shin Tambaguro at the one trifoliolate leaf stage. In the greenhouse, all seedlings were grown in 0.8-L plastic pots containing a mixture of sand, vermiculite, and synthetic soil amended with fertilizers. Symptom development in all inoculated plants was monitored daily for 20–25 days. To check for SMV, all plants with no symptoms or mild symptoms were tested by double sandwich enzyme-linked immunosorbent assay (ELISA) (2) using an antiserum to SMV-C (18).

Aphid and seed transmission. Transmission by *Myzus persicae* (Sulzer) and *Aphis glycines* Matsumura were tested three and five times, respectively. *M. persicae* and *A. glycines* were reared on eggplant (*Solanum melongena* L.) and soybean, respectively. They were starved for 1.5–2 hr, then placed on SMV-infected soybean leaves. After a 5–10 min acquisition feeding period, 10–15 aphids were transferred to each seedling (four to six plants for each treatment) of Shin Tambaguro, allowed to feed for 3 hr, then killed with dichlorvos (DDVP). Twenty days after inoculation, the plants which showed no symptoms were tested for SMV by ELISA. To determine the rate of seed transmission of SMV in soybean, seedlings of cvs. Shin Tambaguro and Tokachinagaba were mechanically inoculated with each isolate at the one trifoliolate leaf stage. Tokachinagaba was grown in 3.5-L pots in the greenhouse (two or three plants for each treatment in four experiments). Shin Tambaguro (two plants for each treatment) was grown in 14-L pots in the greenhouse and in 1.2-m-wide rows in a screenhouse. Pods were harvested when mature and dried in the greenhouse. Seeds from these pods were sown in flats containing a mixture of sand and vermiculite in a temperature-controlled (23–30 C) greenhouse. The plants were grown to the two trifoliolate leaf stage to show the appearance of the symptoms. All plants were checked for the presence of SMV by ELISA.

Cross-protection tests. The primary leaves of Shin Tambaguro seedlings were inoculated with a mild isolate at the fully expanded primary leaf stage. Control plants were inoculated with buffer only. Four plants were used for each treatment. In the first experiment, the plants were challenge-inoculated with the isolate Aa either 4, 6, 8, or 12 days after the initial inoculation. This challenge was performed by mechanical inoculation on the upper fully expanded trifoliolate leaves (one to three leaves) or with viruliferous aphids (*M. persicae,* 10 aphids per plant). In the second experiment, the plants were mechanically inoculated on their second trifoliolate leaves, 12 days after the initial inoculation, with the R isolate or with each of the standard strains (A–E) of SMV which induce severe symptoms. The tests were done in triplicate. Mechanical challenge inoculation was made with crude leaf extracts (1 g/20 ml of buffer). Observations for severe symptoms were made 1 mo after the challenge inoculation in the greenhouse, from June to September.

Field experiments. Two experiments were performed in a grower's field from June to November 1990 in Obata, Wachi, Kyoto. In a greenhouse, mild isolates were mechanically inoculated on the fully expanded primary leaves of Shin Tambaguro seedlings (11 days old) grown in 105-cell paper pots containing 8 L of a mixture of sand and vermiculite. This inoculation was made with crude leaf extracts (1 g/10 ml of buffer). The inoculated seedlings were kept in a greenhouse until they were transplanted to the field. In the first experiment, the seedlings were transplanted 3 days after inoculation (4 June). In the second experiment, the seedlings were transplanted 8 days after inoculation (13 June) to the same field used in the first experiment. These seedlings were transplanted after the observation of transient veinclearing caused by mild isolates on the young trifoliolate leaves. In the fields around the experimental plots, seedlings grown from commercial seeds (the seed transmission rate of SMV, 2.6%) were transplanted by local farmers (9 June). In both experiments, the noninoculated seedlings in the same growth stage as the inoculated ones were transplanted as controls. The seedlings were grown in 2 × 10 m plots (two 1-m-wide rows, 45 plants) arranged in a completely randomized design with three replications. The percentage of diseased plants was calculated from the number of plants showing leaf roll and rugose symptoms characteristic of the virulent SMV infection. Plants with severe symptoms were randomly selected from the noninoculated plots and nearby fields, and tested for SMV by ELISA. The number of diseased plants was recorded four times from July to August, because yield loss of black soybean was found to be caused by the SMV infection before the flowering stage (8). Throughout this period, treatments with organophosphates to control aphids were done twice. To determine the effect on seed production, mature pods obtained from 50 randomly selected plants for each treatment (harvested 20 November) were air-dried and cleaned. Normal seeds (excluding the small and malformed, and those damaged by fungi and insect pests) were collected and weighed.

RESULTS

Attenuated isolates derived by low temperature treatments. One of 17 single-lesion isolates obtained from Aa-infected soybean plants maintained at 15 C for 14 days caused only very mild mosaic symptoms on Shin Tambaguro (Aa15-M1). The others caused mosaic and leaf roll symptoms characteristic of the original isolate Aa. None of the total of 164 single-lesion isolates obtained from other treatments (45 C for 2 days; 42 C for 9 days; 35 C for 8, 14, 21, 32, and 39 days; and 12 C for 20 days followed by 10 C for 20, 30, and 55 days) caused mild symptoms. Likewise, none of the 26 single-lesion isolates obtained from control Aa-inoculated plants produced mild symptoms. In order to demonstrate

Fig. 1. Symptoms on soybean cv. Hyuga caused by two mild isolates of soybean mosaic virus and the original isolate Aa (left to right): plants infected with Aa15-M1, Aa15-M2, and Aa, and noninoculated healthy plant.

the effect of the low-temperature treatment on the derivation of mild isolates, Aa-infected soybean plants were maintained at 10, 13, 15, and 25 C for 30 days. None of the 141 single-lesion isolates from the plants at 10, 13, and 25 C produced mild symptoms. However, three out of the 96 single-lesion isolates obtained from the plants treated at 15 C induced very mild mosaic symptoms. These were designated Aa15-M2, -M3, and -M4.

The host ranges of each of the four mild isolates and the original isolate Aa were very similar and were restricted to leguminous plants. Of the leguminous crops tested other than soybean, only one bean cultivar, Kintoki, was systemically infected with mild isolates and Aa. It showed very mild mosaic symptoms when infected with the four mild isolates.

To further evaluate the effects of the mild isolates on soybean, nine soybean cultivars were inoculated. Out of the nine cultivars tested, Shin Tambaguro, Tokachinagaba, Karibatakiya No. 28, and Hyuga were systemically infected with all four mild isolates and produced very mild mosaic symptoms. All four cultivars produced mosaic and leaf roll symptoms when infected with Aa (Fig. 1). The symptomatological comparison among the four mild isolates was conducted in a greenhouse from June to September. All isolates caused very mild mosaic symptoms on cvs. Shin Tambaguro and Tokachinagaba about 1 mo after the inoculation. From 10 to 20 days later, Aa15-M1 induced relatively prominent mosaic symptoms on Shin Tambaguro, although it did not cause such symptoms on Tokachinagaba. On the other hand, Aa15-M2, -M3, and -M4 produced almost no symptoms. Of the three isolates, Aa15-M2 was used in almost all the following experiments. There seemed to be no differences in seed yield (an average number and weight of normal seeds from 10 plants) between Aa15-M2-infected and noninoculated healthy plants of Shin Tambaguro grown in a screenhouse. Replication of Aa15-M1 and -M2 in soybean plants, examined by ELISA and local lesion assay with Top Crop bean (1), was apparently less than that of Aa (*data not shown*).

The mild isolates Aa15-M1 and -M2 did not differ from isolate Aa in serological properties in agar gel diffusion tests using SMV-C antiserum (*data not shown*). Electrophoretic patterns of viral RNA and coat protein of the three isolates were analyzed by the methods described by Fukumoto et al (3) and were found to be indistinguishable from each other (*data not shown*).

In aphid transmission tests, Aa15-M2 and Aa were efficiently transmitted by two aphid species. However, Aa15-M1 was rarely transmitted (No. of infected plants/No. of plants tested: *M. persicae,* M1 = 3/12, M2 = 9/12, Aa = 12/12; and *A. glycines,* M1 = 1/23, M2 = 11/23, Aa = 17/23).

The isolate Aa was transmitted through seed in Tokachinagaba and Shin Tambaguro at average rates of 18.4 and 7.6%, respectively. All seedlings infected with Aa through seed showed mosaic and leaf roll symptoms. On the other hand, Aa15-M1 and -M2 were seed transmitted at significantly lower rates (at $P = 0.05$ based on the least significant difference [LSD] test) than Aa (Tokachinagaba, M1 = 0%, M2 = 1.0%; Shin Tambaguro, M1 = 1.2%, M2 = 0.2%). All seedlings grown from seeds infected with Aa15-M2 showed very mild mosaic symptoms, while all seedlings from seeds of Shin Tambaguro infected with Aa15-M1 produced prominent mosaic symptoms. Aa15-M1 and -M2 produced mild mottle symptoms on seeds of Tokachinagaba at average rates of 1.9 and 6.6%, respectively, while Aa produced prominent mottle symptoms at 71.9% (LSD = 11.3%). Shin Tambaguro showed no SMV symptoms because of its black seed coat (Fig. 2).

Cross-protection. In the mechanical challenge inoculation with the isolate Aa, the seedlings inoculated with Aa15-M2 prior to inoculation with Aa did not produce leaf roll symptoms characteristic of Aa, except when plants were challenged 4 days after the initial inoculation with Aa15-M2. All the seedlings challenge-inoculated with Aa at 4 or 6 days after initial inoculation with Aa15-M1 showed leaf roll symptoms, while none of the seedlings challenged after 8 or 12 days produced such prominent symptoms. In the challenge inoculation with Aa by aphids, none of the seedlings inoculated with Aa15-M2 showed leaf roll symptoms. Seedlings that were inoculated with Aa15-M1 did not produce prominent symptoms except when the challenge treatment was made 4 days after inoculation. Mock-inoculated seedlings were infected when challenged with Aa by the mechanical inoculation and aphid transmission throughout the experiments (Fig. 3). To check for the absence of challenge Aa in test plants with no prominent symptoms, leaf samples were collected from plants challenged with aphids 6 days after initial inoculation. None of the many single-lesion isolates obtained from the samples produced the severe symptoms characteristic of Aa. These results showed that mild isolates completely protected soybean plants against infection with the Aa isolate when these plants were challenged 6–8 days after inoculation.

Aa15-M1 and -M2 also protected against the isolate R and the five standard strains A, B, C, D, and E of SMV. These results were reproducible in three experiments. Mild isolates did not protect against alfalfa mosaic virus (11), soybean stunt virus (17), or peanut stunt virus (PSV) isolated from black soybean. Synergistic symptoms due to mixed infections were not observed (*data not shown*).

Field experiments. Two experiments in a grower's field were conducted to demonstrate the protective effect of inoculation with a mild isolate, Aa15-M1 or -M2, on black soybean seedlings. In the control plots in both experiments, the number of plants with leaf roll and rugose symptoms reached approximately 50% at the flowering stage. On the other hand, the incidence of disease where plants received the protective inoculation

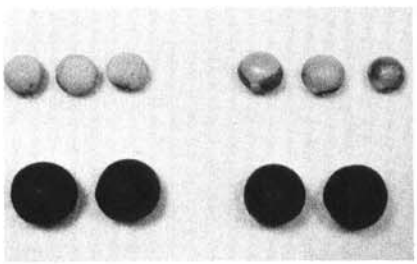

Fig. 2. Symptoms on soybean seeds caused by mild and original isolates of soybean mosaic virus. Seeds obtained from cvs. Tokachinagaba (upper) and Shin Tambaguro (lower) infected with Aa15-M2 (left) and Aa (right).

Fig. 3. Cross-protection effects of preinoculation with two mild isolates of soybean mosaic virus against the original isolate Aa on soybean cv. Shin Tambaguro (left to right): plants preinoculated with Aa15-M2, Aa15-M1, and buffer only. Each plant was challenge-inoculated with Aa by aphid transmission 6 days after the initial inoculation.

remained very low. The isolate Aa15-M2 was more effective than Aa15-M1 (data points for 17 August in two experiments are significantly different at $P < 0.05$ based on the chi-square test of independence). In addition, the Aa15-M2-protected plants showed few or no symptoms as late as 50–60 days after inoculation (preflowering–flowering stage). The incidence of leaf roll and rugose symptoms caused by SMV in a nearby field was about 80% at the flowering stage (Fig. 4). Other viruses, soybean dwarf virus and PSV, occurred at very low rates. The yields of seed from the plots with Aa15-M1 and -M2 inoculated plants were approximately 3–8% and 10–15%, respectively, greater than those of the control plots in two experiments. Since the incidence of severe symptoms in the control plots was apparently lower than that of a nearby field, leaf samples were collected at random from the noninoculated plants with no severe symptoms at the end of the survey (17 August). All field samples that were tested by ELISA (24 for each experiment) showed positive reactions to SMV. To determine whether the samples of SMV detected were mild isolates, the randomly selected samples (15 for each) were inoculated into soybean seedlings. Almost all samples produced very mild mosaic symptoms except one which produced mosaic and rugose symptoms. This indicates that mild isolates were naturally spread out by aphids within the experimental plots, suggesting that they would provide protective effects to the noninoculated plants.

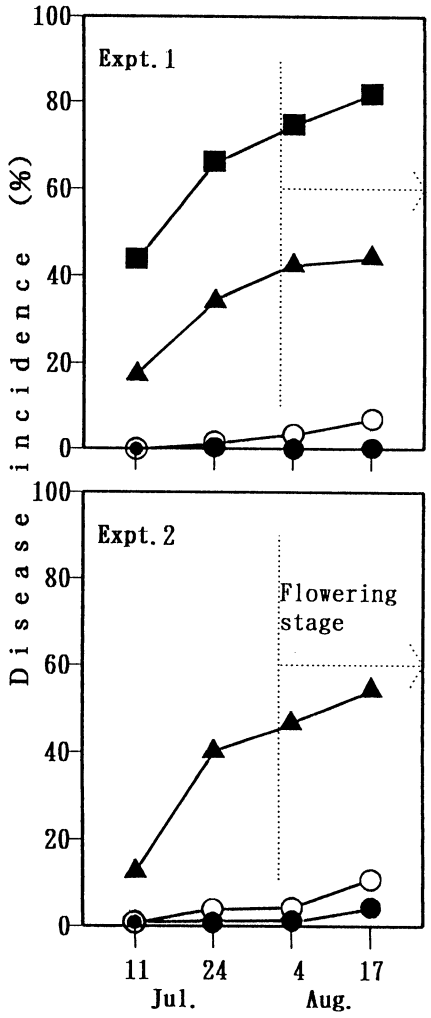

Fig. 4. The occurrence of leaf roll and rugose symptoms in the experimental plots in 1990. The plots were at Obata in Wachi, Kyoto. At 3 days (Experiment 1) and 8 days (Experiment 2) after inoculation (4 and 13 June, respectively), the seedlings were transplanted. The transplanting date in nearby fields was 9 June. ○ = Aa15-M1-inoculated plot; ● = Aa15-M2-inoculated plot; ▲ = noninoculated plot; and ■ = a nearby field. Data points for noninoculated plots in the two experiments are significantly different at $P < 0.001$ from those for Aa15-M1- and Aa15-M2-inoculated plots and for the nearby field, based on the chi-square test of independence.

DISCUSSION

The attenuated isolates of SMV obtained by the low-temperature treatments protected soybean plants from virulent isolates of SMV. The isolation of attenuated viruses by temperature treatments of infected plants has been conducted only by heat treatment at 35 C (4,5,14,15). The cold treatment described here appears to be the first report of obtaining attenuated viruses by relatively low temperature. The attenuated isolates of SMV obtained by this cold treatment were similar in many ways to the original Aa isolate; but they differed from Aa in symptoms produced, aphid and seed transmissibility, and accumulation in soybean. No experiments were conducted to determine whether the attenuated isolates were derived from selection of preexisting mild contaminants or from mutation by low-temperature treatments. McGovern and Kuhn (13) reported that a new strain of southern bean mosaic virus (SBMV) was derived at a relatively low temperature, and they suggested that the derivation of the SBMV mutant was caused by a highly specific selection pressure in the host at a specific temperature. In the combination of SMV and soybean, a relatively low temperature of 15 C was indispensable for obtaining mild isolates. Cold treatment might also be useful for obtaining mild isolates of other potyviruses. Further works on the biochemical and molecular analyses of mild isolates and the original isolate of SMV may give information concerning the symptom attenuation of potyviruses.

SMVs isolated in Japan can be divided into five strains, A, B, C, D, and E, based on the symptoms produced in several soybean cultivars (17). Although black soybean is sensitive to all these strains, the A strain predominates in Kyoto Prefecture (8). The attenuated isolates from Aa classified in the A strain protected soybean plants from virulent strains when challenge treatments were made 6–8 days after the protective inoculation. The inoculated seedlings must be maintained under the required conditions to avoid infection with virulent strains by aphids during this initial period. However, the results of the field experiments indicated that we could grow the seedlings only a few days after inoculation in the field under certain conditions, for instance, if protected plants were transplanted about 1 wk before the earliest planting date in the commercial fields, including infected seedlings as the inoculum source of the secondary spread.

Although the natural spread of attenuated isolates of SMV was observed in the field experiments, their dissemination by aphids would hardly cause a hazard to other leguminous crops because of their extremely restricted host ranges. The attenuated isolates produced mild mottle symptoms on seeds of soybean with a normal-colored seed coat at a low rate. However, almost no soybean cultivars grown in all prefectures around Kyoto were systemically infected with these isolates except black soybean. Currently, the incidence of viruses other than SMV is very low in black soybean-growing areas; so risks due to mixed infections with other viruses in fields of cross-protected plants are minimal. This cross-protection strategy to control the mosaic disease caused by SMV seems feasible for black soybean production, because the cultivation of black soybean is usually conducted by transplanting.

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