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Adaptation of *Cucumber mosaic virus* soybean strains (SSVs) to cultivated and wild soybeans

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Abstract *Cucumber mosaic virus* soybean strains formerly called soybean stunt virus (SSV) were inoculated onto 23 wild soybeans collected from four Asian countries to investigate their infectivity in order to improve understanding of the co-evolution of SSVs and soybean. SSV inoculation resulted in systemic infection in most of the wild soybeans used. However, an SSV strain (SSV-In), which was isolated in Indonesia, did not result in systemic infection of many of the wild soybeans distributed in southern Japan. This exceptional infectivity of SSV-In may be due to its specific adaptation to the local soybean population(s) of Indonesia, which has rarely been affected by gene flows from wild soybean. In the present study, the nucleotide sequences of the 3a and CP genes of SSV were determined, and the data were used to classify seven SSV isolates among known *Cucumber mosaic virus* (CMV) strains. The phylogenetic analysis showed that the seven SSVs formed a distinct cluster separated from the other CMV strains despite their different geographical origins; SSV-In was the most divergent of the seven isolates. Comparison of the rates of synonymous and nonsynonymous substitutions revealed that the SSV group had evolved faster than subgroup IA. The implications of the findings are discussed in relation to the so-called Red Queen hypothesis.

Keywords Soybean · Soybean stunt virus · Adaptation · Evolution · Host-virus interaction

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Introduction

The *Cucumber mosaic virus* (CMV) is a type member of the genus *Cucumovirus* and has a very broad host range (Roossinck et al. 2000). It contains a tripartite positive-sense RNA genome (RNAs1-3) and is distributed worldwide. RNAs1 and-2 encode the 1a and 2a proteins, respectively, which are necessary for viral replication. RNA3 contains the movement protein (3a) and the coat protein (CP) genes. Based on the sequence similarities, CMV strains are classified into two subgroups – I and II; the former is further divided into IA and IB (Roossinck et al. 1999; Roossinck 2002). CMV that infects soybean [*Glycine max* (L.) Merr. subsp. *max* Ohashi] used to be called soybean stunt virus (SSV) and is now called CMV soybean strain. For convenience, in this paper, SSV is used for the soybean strain. SSVs are vertically transmitted through seeds with a high efficiency and horizontally by aphids. SSV was first reported in Japan (Koshimizu and Iizuka 1958) and since then extensive characterization of SSV has been conducted by the same research group (Takahashi et al. 1980, 1987). From their results, it is known that the soybean response to SSV infection is determined by combination of soybean cultivars and SSV isolates, suggesting the existence of cultivar-specific resistance genes (Takahashi et al. 1980). Although the biological characteristics of SSV have been studied extensively, there is no available sequence information on SSV genomes.

The cultivated soybean is one of the most important grain legumes in the world and is considered to have been domesticated from its wild annual counterpart, *G. max* subsp. *soja* (Sib. et Zucc.) Ohashi in East Asia. The two taxa have few barriers to hybridization and produce a viable progeny. The wild soybean has thus been used in soybean breeding to furnish useful genes that are not in the cultivated gene pools. The wild soybean is native to China, the Korean peninsula, Japan, and far-eastern Russia. Weedy intermediate forms between wild and cultivated soybeans have also been found where the two taxa are growing sympatrically (Sekizuka and Yoshiyama

1960; Zheng and Chen 1980). The wild soybean does not possess any device for long-distance seed dispersal. Analyses of maternally inherited organelle genomes have revealed an extensive geographical differentiation in wild soybean (Tozuka et al. 1997; Shimamoto et al. 1998; Abe et al. 1999; Xu et al. 2002), although migration occurs in a local scale as a result of natural disturbance such as flooding and human interference (Abe 2000).

In this investigation reported here, we collected seven SSV isolates from four Asian countries and tested their infectivities to wild soybeans distributed in Japan, Korea, China and Russia to survey the variation of host-virus interactions between SSVs and wild soybean genotypes. We also conducted phylogenetic analysis using 3a and CP gene sequences to understand the evolutionary relationships among the collected SSVs.

Materials and methods

Virus and plant sources

Seven SSV isolates (B, C, D, AE, Ch, In, and WR) were used for inoculation experiments to wild soybean and sequencing analyses. Four SSV isolates (B, C, D, and AE) were obtained from the National Agricultural Research Center for the Hokkaido Region (Sapporo, Japan). These strains were isolated from soybean cultivars of northern Japan. SSV-Ch and SSV-In, which were isolated from the Jilin province of China and Indonesia, respectively, were obtained from the Japan International Research Center for Agricultural Sciences (Tsukuba, Japan). SSV-WR was isolated in our laboratory from an infected wild soybean, which was collected in the Khabarovsk region of Russia, following the screening of 120 wild soybeans collected in different sites of East Asia for CMV infection by ELISA. Wild soybeans collected in 23 different sites of China, far-eastern Russia, South Korea and Japan were used for the inoculation experiments. These were from the collection of the Laboratory of Plant Genetics and Evolution, Graduate School of Agriculture, Hokkaido University, Sapporo, Japan.

Inoculation experiments

Seeds of each wild plant were sown in pots, and the resultant seedlings were used for inoculation experiments with six SSV isolates except for SSV-AE. The first true leaves of wild soybean plants were dusted by carborundum, rub-inoculated with purified viruses at a concentration of 100 μg/ml and maintained in a greenhouse under conditions of natural light at 24 °C for 3 weeks. The inoculated leaves and uninoculated upper leaves were tested for viral infection by ELISA.

Sequence determination and phylogenetic analysis

Full-length cDNA cones of seven SSVs were constructed by reverse transcriptase-polymerase chain reaction (RT-PCR) using a TaKaRa RNA LA PCR kit (Takara Shuzo, Japan). The primer pair used for RNA3 was 3CL123 [5'-TGGTCTCCTTTGGA(AG)GCC-CCC-3'] and 5CL3T7G [5'-T7 promoter-GTAATCT(TA)AC-CACTGTGTGTG-3']. The 5' and 3' end sequences were confirmed by 5'/3' rapid amplification cDNA end (RACE). The sequences were determined using the Thermo Sequenase Cycle-Sequencing kit (Amersham, UK) for model 4000L LI-COR automated DNA sequencer (LI-COR, USA). A multiple alignment of the 3a and CP gene sequences for seven SSVs and 33 CMV strains was performed using the CLUSTAL W program (Thompson et al. 1994) with minor manual adjustments. Sequence data for the 3a and CP genes of 33 CMVs were referred to Roossink (2002) and Masuta et al. (2002). Parsimonious analysis was calculated by PAUP 4.0 beta version 10 (Hall 2001). Bootstrap values were generated with 100 replications of a heuristic search. Branches with low bootstrap support (less than 70%) were collapsed. Gaps were treated as a fifth-character state. The *Peanut stunt virus* (PSV) was included as an outgroup in the phylogenetic tree. Nucleotide substitution rates between the IA- and SSV-groups were calculated with MEGA2.1 using the Nei-Gojobori method (p-distance) (Kumar et al. 1993).

Results and discussion

Soybean responds to SSV infection in various manners; often the infected plants are severely stunted and eventually harbor few pods. Takahashi et al. (1987) surveyed resistances to five SSV strains isolated in northern Japan (A, AE, B, C and D) for 442 soybean accessions introduced from various countries and found that cultivated soybeans contained genetic variations with respect to resistance to SSVs. The accessions from China and Korea had a tendency to resist strains A and C, the most important SSVs in Japan. The other SSV isolates (SSV-In, Ch and WR) also showed various infection responses to soybean cultivars (our unpublished data). The accessions analyzed by Takahashi et al. (1987), however, contained many breeding lines and cultivars bred by outcrossing and, thus, it was impossible to precisely evaluate geographical effects on soybean susceptibility to SSVs. In the present research, to avoid influences on the soybean populations by crossbreeding, we inoculated the SSVs

Table 1 Infectivity of SSV isolates to wild soybeans

Collection site	Systemic infectivity of SSV isolates[a]					
	B	C	D	Ch	In	WR
Khabarovsk, Russia	+[b]	+	+	+	+	+
Primorsky, Russia	+	+	+	+	+	+
Niao Ning, China	+	+	+	+	+	+
Nan jing, China	+	–	–	+	+	+
Won ju, Korea	+	+	+	+	+	+
Kyung ju, Korea	+	+	+	+	+	+
Hidaka, Japan	+	+	+	+	+	+
Hiyama, Japan	+	+	+	+	+	+
Aomori, Japan	+	+	+	+	+	+
Iwate, Japan	+	+	+	+	+	+
Kanagawa, Japan	+	+	+	+	+	+
Yamanashi, Japan	+	+	+	+	–	+
Kaizu, Japan	+	+	+	+	+	+
Ueno, Japan	+	+	+	+	–	+
Hyougo, Japan	+	+	+	+	–	+
Hyougo, Japan	+	–	+	+	–	+
Tokushima, Japan	+	+	+	+	–	+
Ehime, Japan	+	+	+	+	–	+
Shimane, Japan	+	+	+	+	+	+
Hiroshima, Japan	+	+	+	+	–	+
Okayama, Japan	+	+	+	+	+	+
Nagasaki, Japan	+	+	+	+	–	+
Fukuoka, Japan	+	+	+	+	–	+

[a] Tested by ELISA
[b] +, Systemic infection; –, no infection or local infection

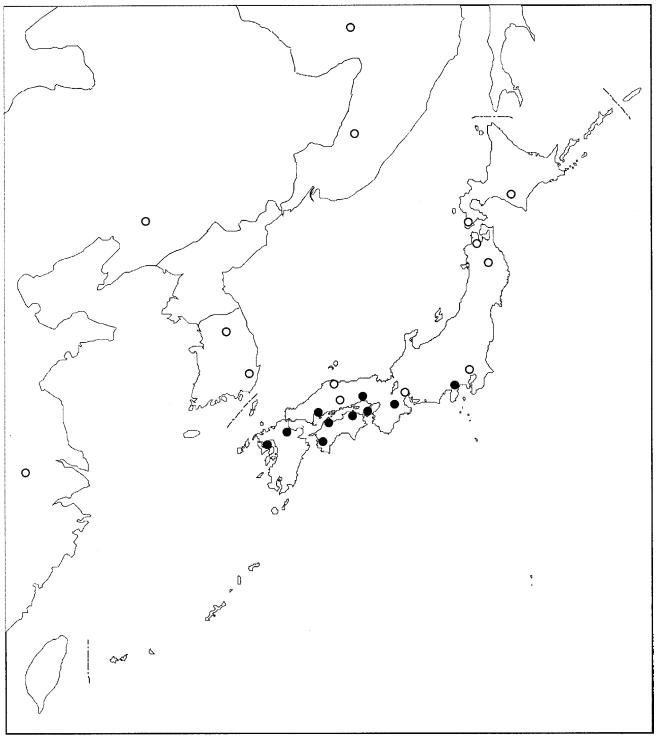

Fig. 1 Geographical distribution of the wild soybeans used in Table 1. *Open circles* ecotypes in which SSV-In systemically infected, *Solid circles* ecotypes in which SSV-In did not systemically infect

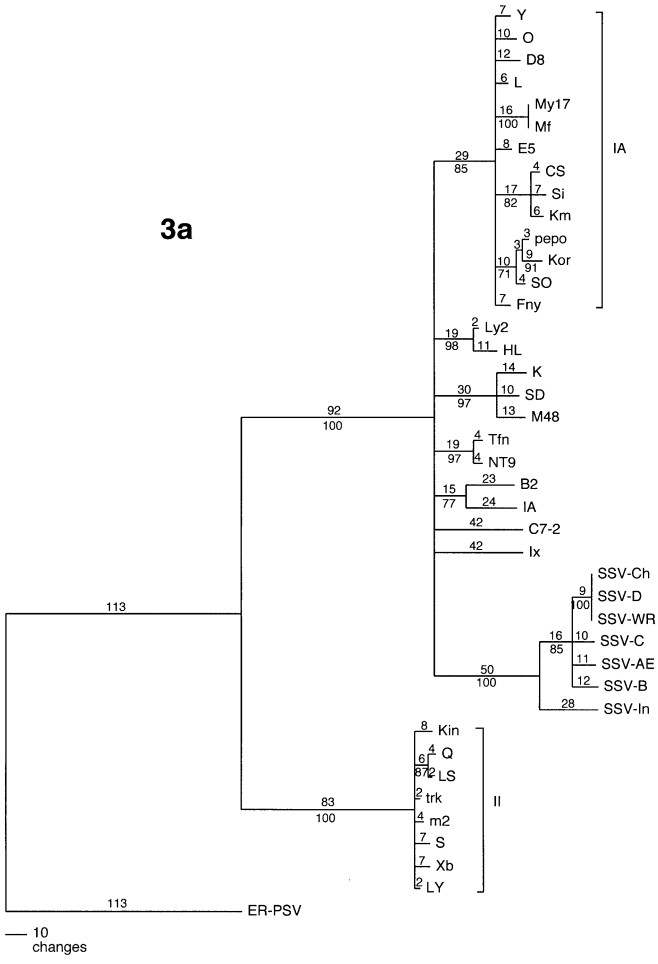

Fig. 2 Phylogenetic tree of 3a genes from 40 CMV isolates including seven SSVs constructed by the parsimony method based on the nucloeotide sequences. Bootstrap values are indicated at each branch point. Branch lengths are shown *above* the branch lines. The scale relates branch lengths, which are the nucleotide substitutions representing a distance. ER-PSV was used as the outgroup. *IA* CMV subgroup *IA*, *II* CMV subgroup II

onto wild soybeans collected from different geographical sites, and the viral adaptation to host plants was tested.

Six SSVs tested systemically infected most of the wild soybeans used, but some genotype-specific resistances were also observed in SSV-C, -D and -In (Table 1). Wild soybeans were therefore genetically variable in resistance to SSVs, as are cultivated soybeans. Our data further indicated a geographical differentiation of the resistance to SSVs. As shown in Table 1 and Fig. 1, SSV-In did not systemically infect the wild soybeans distributed in the southern areas of Japan. Considering its geographical separation from the other SSV strains, SSV-In may have independently evolved, adapting itself to the local soybean populations of Indonesia. Consequently, it perhaps lost its ability to infect the wild soybeans distributed in southern Japan.

Phylogenic analysis of CMV genes by Roossinck (2002) previously demonstrated that CMV 3a genes had evolved under more rigid host-interactive constraints than the other viral genes. In addition, when systemic infectivity of chimeric viruses was analyzed between SSV-C and SSV-D to a wild soybean plant collected in Hyougo, Japan, which is systemically infected by SSV-D but not by SSV-C (Table 1), the host specificity determinant was mapped to the 3a gene (our unpublished data). To evaluate the adaptation of SSV as a result of host-virus interactions, we sequenced the entire RNAs3 of seven SSVs and performed a phylogenetic analysis based on the nucleotide sequences of the 3a and CP genes from 40 CMVs including those seven SSVs. A phylogenetic tree for the 3a gene indicated that the seven SSV strains formed a distinct cluster (Fig. 2); a phylogenetic tree for the CP gene showed essentially a similar topology with a minor modification within the SSV group (data not shown). Based on the analysis of the 3a gene, SSV-Ch, SSV-WR, and SSV-D formed a single cluster within the SSV group, and SSV-In was found to be the most divergent among the SSVs analyzed. Synonymous substitutions (ds) and nonsynonymous substitutions (dn) per synonymous and nonsynonymous site, respectively, among strains were compared between the subgroup IA (14 isolates for 3a, 16 isolates for CP) and SSV (seven isolates) groups because the two groups formed discrete clusters and shared the same branching point. Average dn and ds were calculated with MEGA2.1 using the p-distance method (N-J method). dn (SSV)/dn (IA) (0.010/0.002) = 5 for the 3a gene indicates that the SSV group evolves five

Table 2 Comparison of synonymous and nonsynonymous substitutions in the viral genes within the SSV and IA groups

Virus gene	ds[a]			dn[a]		
	SSV	IA	SSV/IA	SSV	IA	SSV/IA
CP	0.123±0.016[b]	0.089±0.012	1.38	0.021±0.004	0.012±0.002	1.75
3a	0.086±0.000	0.091±0.000	0.95	0.010±0.003	0.002±0.001	5.00

[a] ds, Synonymous substitutions per synonymous site; dn, nonsynonymous substitutions per nonsynonymous site; SE, standard error
[b] Standard error

times as fast as the IA group (Table 2). On the other hand, the values of ds for the IA and SSV groups are barely different (0.091 and 0.086, respectively), suggesting that the different evolutionary rates between the two groups are due to changes at the amino acid level. No significant difference was found between the two groups from the analysis of average dn and ds in the CP gene (Table 2). These results indicate that the degree of the functional constraint in the 3a gene is larger than that in the CP gene; the 3a proteins of SSVs were modified under selection pressure, perhaps host interactive constraints.

The present study has shown that the adaptation of SSVs to cultivated and wild soybeans is oriented to one phylogenetic group despite their different geographical origins. The relatively fast evolutionary rates observed in the 3a gene of the SSV group may result from the genetic diversity in the resistance of soybeans to SSVs, which probably have been provided by genetic recombination as a result of outcrossing. It is obvious that geographical separation has somehow contributed to the relative divergence of SSV-In from the other SSV strains. Wild soybeans, especially, are not distributed in Indonesia and, thus, SSV-In had been maintained in cultivated soybeans. The genetic variation in the host soybean population, in which SSV-In has evolved, may have been much simpler than those of other Asian countries, which appeared to have been repeatedly influenced by gene flows from wild soybean (Abe et al. 1999; Xu et al. 2002).

Finally, an interesting possibility is that our observations may somehow meet the criteria (Clay and Kover 1996) for the so-called Red Queen hypothesis (RQH) (Hamilton 1990). The criteria are: (1) genetic variation for resistance and virulence, (2) selective impact of pathogen infection and (3) frequency-dependent selection (host genotype frequency responds to change in pathogen genotype frequency, and vice versa). Our observations satisfy the criteria as follows: (1) cultivated and wild soybeans exhibit various host-specific resistance against SSV infection; (2) SSV infection causes severe damage to cultivated soybeans, and SSV-infected wild soybeans are often severely stunted, resulting in few flowers; (3) as demonstrated by SSV-In, an SSV isolate can genetically adapt to a soybean population; but, at present, there is no evidence showing how soybeans change in response to SSV infection.

Both cultivated and wild soybeans are basically self-pollinating. An average outcrossing rate was estimated to be between 2.4% and 3.0% in the cultivated soybean (Ahrent and Caviness 1994; Chiang and Kiang 1987), while wild soybeans showed a relatively high outcrossing rate (9 to 19%) in natural habitats (Fujita et al. 1997). Genetic recombination by outcrossing, which mimics sexual reproduction as a force of genetic shuffle favored by the RQH,would have generated new genetic variations in SSV resistance. In turn, in response to a change in host, SSV strains then change, adapting to the region-specific soybean cultivars (populations) by increasing amino acid replacement, as observed in the 3a proteins. Further analysis of the co-evolutionary interactions between soybean and SSV is necessary to examine whether the RQH is applicable for the SSV-soybean system.

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References

Abe J (2000) The genetic structure of natural populations of wild soybean revealed by isozymes and RFLPs of mitochondrial DNAs: possible influence of seed dispersal, cross-pollination and demography. In: Oono K (ed) 7th MAFF Int Workshop Genet Resources. AFFRC and NIAR, Tsukuba, Japan, pp 143–158

Abe J, Hasegawa A, Fukushi H, Mikami T, Ohara M, Shimamoto Y (1999) Introgression between wild and cultivated soybeans of Japan revealed by RFLP analysis of chloroplast DNAs. Econ Bot 53:285–291

Ahrent DK, Caviness CE (1994) Natural cross pollination of twelve soybean cultivars in Arkansas. Crop Sci 34:376–378

Chiang YC, Kiang YT (1987) Geometric position of genotypes, honeybee foraging patterns and out-crossing in soybean. Bot Bull Acad Sin 28:1–11

Clay K, Kover PX (1996) The red queen hypothesis and plant/pathogen interactions. Annu Rev Phytopathol 34:29–50

Fujita R, Ohara M, Okazaki K, Shimamoto Y (1997) The extent of natural cross-pollination in wild soybean (*Glycine soja*). J Hered 88:124–128

Hall BG (2001) Phylogenetic trees made easy. Sinauer, Sunderland

Hamilton WD, Axelrod R, Tanase R (1990) Sexual reproduction as an adaptation to resistant parasites (a review). Proc Natl Acad Sci USA 87:3566–3573

Koshimizu Y, Iizuka N (1958) Soybean stunt disease (in Japanese). Ann Phytopathol Soc Japan 23:27

Kumar S, Tamura K, Nei M (1993) MEGA: molecular evolutionary genetic analysis. version 1.01. Pennsylvania State University, University Park

Masuta C, Seshimo Y, Mukohara M, Jung HJ, Ueda S, Ryu KH, Choi JK (2002) Evolutionary characterization of two lily isolates of *Cucumber mosaic virus* isolated in Japan and Korea. J Gen Plant Pathol 68:163–168

Roossinck MJ (2002) Evolutionary history of *Cucumber mosaic virus* deduced by phylogenetic analyses. J Virol 76:3382–3387

Roossinck MJ, Zhang L, Hellwald KH (1999) Rearrangements in the 5'nontranslated region and phylogenetic analyses of

Cucumber mosaic virus RNA3 indicate radial evolution of three subgroups. J Virol 73:6752–6758

Roossinck MJ, Bujarski J, Ding SW, Hajimorad R, Hanada K, Scott S, Tousignant M (2000) Cucumovirus. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (eds) Virus taxonomy, 7th Rep Int Committee Taxon Viruses. Academic Press, San Diego pp 929–930

Sekizuka S, Yoshiyama T (1960) Studies on the native wild grasses for fodder. (IV) Crop-scientific studies on wild species of *Glycine soja* in Japan, (in Japanese with English abstract) Kantou-Tosan Agric Exp Stn 15:57–73

Shimamoto Y, Fukushi H, Abe J, Kanazawa A, Gai J, Gao Z, Xu D (1998) RFLPs of chloroplast and mitochondrial DNA in wild soybean, *Glycine soja*, growing in China. Genet Resour Crop Evol 45:433–439

Takahashi K, Tanaka T, Iida W, Tsuda Y (1980) Studies on virus diseases and causal viruses of soybean in Japan (in Japanese). Bull Tohoku Natl Agric Exp Stn 62:1–130

Takahashi K, Nagasawa J, Tanaka T, Iizuka N (1987) Reaction of soybean varieties to the strains of soybean mosaic virus and soybean stunt virus in Japan (in Japanese). Misc Publ Tohoku Natl Agric Exp Stn 7:1–35

Tozuka A, Fukushi H, Hirata T, Ohara M,Kanazawa A, Mikami T, Abe J, Shimamoto Y (1997) Composite and clinal distribution of *Glycine soja* in Japan revealed by RFLP analysis of mitochondrial DNA. Theor Appl Genet 96:170–176

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Xu DH, Abe J, Gai JY, Shimamoto Y (2002) Diversity of chloroplast DNA SSRs in wild and cultivated soybean: evidence for multiple origins of cultivated soybean. Theor Appl Genet 105:645–653

Zheng HY, Chen HD (1980) A preliminary study of the resources of wild soybean in Jilin province (in Chinese with English abstract). Sci Agric Sin 13:26–32