Recessive resistance governed by a major quantitative trait locus restricts clover yellow vein virus in mechanically but not graft-inoculated cultivated soybeans

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25 Clover yellow vein virus (ClYVV) infects and causes disease in legume plants.
26 However, here we found that ClYVV isolate No. 30 (ClYVV-No.30) inefficiently
27 multiplied or spread via cell-to-cell movement in mechanically inoculated leaves of a
28 dozen soybean (*Glycine max*) cultivars, and resulted in failure to spread systemically.
29 Soybean plants also had a similar resistance phenotype against additional ClYVV
30 isolates. In contrast, all but one of 24 tested accessions of wild soybeans (*G. soja*) were
31 susceptible to ClYVV-No.30. Graft inoculation of cultivated soybean TK780 with
32 ClYVV-No.30-infected wild soybean B01167 scion resulted in systemic infection of the
33 cultivated soybean rootstock. This suggests that upon mechanical inoculation the
34 cultivated soybean inhibits ClYVV-No.30 at infection steps prior to the systemic spread
35 of the virus via vascular systems. Systemic infection of all of F1 plants from crossing
36 between TK780 and B01167 and 68 out of 76 F2 plants with ClYVV-No.30 indicated
37 recessive inheritance of the resistance. Further genetic analysis using 64 recombinant
38 inbred lines between TK780 and B01167 detected one major quantitative trait locus,
39 designated *d-cv*, for the resistance that was positioned in the linkage group D1b
40 (chromosome 2). The mapped region on soybean genome suggests that *d-cv* is not an
41 allele of the known resistance genes against soybean mosaic virus.
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43
44 The cultivated soybean (*Glycine max*) is an important crop worldwide, supplying food
45 products such as boiled and fermented soybean (edamame and natto), soybean milk,
46 tofu and soy sauce, vegetable oil and proteins for human consumption and animal feed.
47 Its economic contribution worldwide is estimated conservatively at US$48.6
48 billion/year (Wilson, 2008). It is a diploidized tetraploid (2*n* = 40) species, classified in
49 the subgenus *Soja* in the legume family *Fabaceae*. The subgenus *Soja* includes

50 members of *G. soja*, which is thought to be an immediate wild ancestor of cultivated
51 soybean, and the domestication is supposed to have occurred 3000–5000 years ago
52 (Cregan, 2008). Wild soybean is distributed in China, Russian Far East region, Korea,
53 Taiwan and Japan (Lu, 2004). Since progeny derived from crosses between cultivated
54 and wild soybeans are fully fertile, wild soybean has been used as a genetic resource to
55 confer useful traits on soybean cultivars.
56 One hundred twelve viruses are known to infect, naturally or experimentally,
57 soybean plants. Among these, 46 have been isolated from field soybeans and are
58 currently a concern or could potentially be a problem in soybean production (Hill and
59 Whitham, 2014). *Soybean mosaic virus* belongs to the genus *Potyvirus* in the family
60 *Potyviridae*. Soybean mosaic virus (SMV) is the most prevalent virus that causes
61 significant crop losses in soybean production (Saghai Maroof et al., 2008; Hajimorad et
62 al., 2018). Members of the genus *Potyvirus*, including SMV, are single-stranded,
63 positive-sense RNA viruses, forming filamentous particles with 2,000 copies of coat
64 protein (CP). The genomic RNA is about 10 kb and encodes a large open reading frame
65 (ORF), from which a polyprotein is produced and processed into a dozen of mature
66 proteins by three viral-encoded proteases (Ivanov et al., 2014). A small ORF, *pipo*, is
67 embedded in the *P3* cistron at −1 frame (Chung et al., 2008). The *P3* cistron
68 additionally produces frameshift products, with/without the *pipo*-encoded peptide, P3N-
69 PIPO and P3N-ALT (Hagiwara-Komoda et al., 2016), which are involved in viral cell-
70 to-cell movement (Wei et al., 2010; Wen and Hajimorad, 2010), via transcriptional
71 slippage at the $G_{1\text{-}2}A_{6\text{-}7}$ motif upstream of *pipo* (Olspert et al., 2015; Rodamilans et al.,
72 2015; Hagiwara-Komoda et al., 2016). *Potyvirus* is the largest genus of plant RNA
73 viruses (Gibbs and Ohshima, 2010) and members of the genus cause diseases in diverse
74 crops. In addition to SMV, 18 members are able to infect soybean; however, only 9 have

been isolated from field-grown soybeans (Hill and Whitham, 2014). Peanut mottle virus (Bays et al., 1986), bean common mosaic virus (Zhou et al., 2014) and bean yellow mosaic virus (BYMV) (Campos et al., 2014) are at least associated with soybean diseases under field conditions. Clover yellow vein virus (ClYVV) is closely related to BYMV but had not been considered to be a causal agent of soybean disease until an isolate (ClYVV-Gm) was isolated recently from field-grown soybeans in South Korea (Shin et al., 2014).

ClYVV was initially isolated from white clover (*Trifolium repens*) and causes chlorotic and necrotic diseases in legume plants including broad bean (*Vicia fava*) and pea (*Pisum sativum*) (Bos et al., 1974; Tracy et al., 1992; Sasaya et al., 1997). ClYVV had not been clearly distinguished from BYMV by comparison of their host ranges and reactions as well as serological studies until their nucleotide sequences of *CP* were determined and compared (Uyeda et al., 1991; Tracy et al., 1992). Pea plants were shown to possess three resistance loci, *cyv1*, *cyv2* and *Cyn1*, against ClYVV (Andrade et al., 2007; Ravelo et al., 2007; Choi et al., 2012); *cyv1* and *cyv2* inhibit systemic infection of ClYVV and are recessively inherited, and *cyv2* encodes an eukaryotic initiation factor 4E (Andrade et al., 2009). The P1 protein is a virulence determinant of ClYVV in peas carrying *cyv2* (Nakahara et al., 2010). *Cyn1* is a semi-dominant gene that does not inhibit infection of ClYVV but induces a hypersensitive response (HR)-like systemic lethal necrosis via perception of ClYVV P3N-PIPO (Atsumi et al., 2009; Atsumi et al., 2016). We recently provided evidence that P3N-PIPO qualitatively and quantitatively determines virulence of ClYVV in peas carrying either *cyv1* or *Cyn1* and provides a consistent gradation of virulence for ClYVV isolates No.30, 90-1 and I89-1 in these resistant peas (Choi et al., 2013; Atsumi et al., 2016; Miyashita et al., 2016).

There is no published study regarding resistance against ClYVV in soybean cultivars. However, three inheritably dominant resistant loci, *Rsv1*, *Rsv3* and *Rsv4*, against SMV have been identified in cultivated soybeans (Whitham et al., 2016). To the best of our knowledge, no recessively inherited resistance in soybean cultivars against SMV has been reported to date though existence of recessive resistance against another potyvirus, peanut mottle virus, has been reported (Hill and Whitham, 2014; Hajimorad et al., 2018). *Rsv1* confers extreme resistance (ER) against majority of SMV strains (Hajimorad and Hill, 2001). The *Rsv1* locus located on chromosome 13 is multigenic with genes encoding nucleotide binding-leucine rich repeat proteins (NB-LRR) (Hayes et al., 2004; Wen et al., 2013). Two SMV-encoded proteins, helper-component proteinase (HC-Pro) and P3 are the target of recognition by the *Rsv1*-associated genes (Eggenberger et al., 2008; Hajimorad et al., 2008; Wen et al., 2013). The *Rsv3* belongs to coiled-coil class of NB-LRR resistance genes (Suh et al., 2011) that targets cylindrical inclusion (CI) for recognition (Seo et al., 2009; Zhang et al., 2009). This gene mediates local HR or ER against avirulent SMV strains (Seo et al., 2009; Zhang et al., 2009). The *Rsv4* belongs to an unidentified class of *R* genes that restricts SMV to inoculated leaves without apparent induction of HR (Saghai Maroof et al., 2010; Khatabi et al., 2012; Ilut et al., 2016). SMV determinant for virulence in soybean carrying *Rsv4* has been mapped to P3 (Chowda-Reddy et al., 2011; Khatabi et al., 2012; Wang et al., 2015).

In this study, we found that cultivated and wild soybeans are resistant and susceptible to ClYVV, respectively. The genetic analyses using F1 and F2 progenies and recombinant inbred lines (RILs) derived from a cross between a cultivated soybean cultivar and a wild soybean line revealed that resistance in cultivated soybean is

recessively inherited and governed by one major quantitative trait locus (QTL) on chromosome 2, apart from the three loci conferring resistance against SMV.

RESULTS

Cultivated soybean genotypes restrict ClYVV infection to limited foci in inoculated leaves

Based on the *uidA* gene expressing β-glucuronidase (GUS) assay, ClYVV-No.30-GUS was detected in inoculated leaves of a number of soybean genotypes regardless of carrying the anti SMV resistant genes (*Rsv1*, *Rsv4* and *3gG2*) or not (*rsv1*, *rsv4* and -*3gG2*), albeit in limited and restricted foci (Fig. 1). In none of the biolistically inoculated cultivated soybean (*Glycine max*) the virus was detected in the noninoculated leaves whether assayed for GUS expression or detection of the coat protein (CP) by ELISA (Table S1). We then mechanically inoculated cultivated soybean 'Williams' with sap extract derived from ClYVV-No.30-infected broad bean leaves in the absence of GUS. None of the 28 inoculated plants developed symptoms of infection on inoculated or noninoculated leaves, and ELISA failed to detect CP in the noninoculated leaves. Thus, failure of ClYVV-No.30-GUS to move systemically in cultivated soybean is not a consequence of GUS tagging of the genome as shown for another potyvirus (German-Retana et al., 2000). This result is further supported by the observations that ClYVV-No.30-GUS was able to move systemically in noninoculated leaves from all 5 biolistically inoculated broad bean cultivar "Windsor" and 5 out of 8 wild soybean accession B01167. We here note that the virus was detected in veinal tissues of inoculated leaves of wild soybean B01167 but not at all in cultivated soybean leaves (Fig. 1).

We then mechanically inoculated ClYVV-No.30 tagged with green fluorescent protein (ClYVV-No.30-GFP) onto cultivated and wild soybeans and compared its spread by continuously monitoring GFP fluorescence over time (Fig. 2A). ClYVV-No.30-GFP gradually spread in inoculated leaves of cultivated soybean TK780. However, cell-to-cell movement seemed repressed in soybean TK780 when compared with movement in inoculated wild soybean B01167 leaves. Consistently, viral CP accumulated to a relatively lower level in inoculated leaves of cultivated soybeans at 7 days post-inoculation (dpi) than in wild soybean leaves (Fig. 2B). We further monitored GFP fluorescence in systemically infected parts of cultivated and wild soybeans (Fig. 2C). No GFP signal derived from ClYVV-No.30-GFP infection was observed in noninoculated parts of cultivated soybean TK780 despite presence of GFP fluorescence in slices of stems in the upper part (cut 1), root (cut 3) and petiole of inoculated leaves (cut 2) of a wild soybean (B01167). Western blotting and RT-PCR assays to detect viral CP and genomic RNA in noninoculated upper leaves, respectively, confirmed that soybean cultivars Williams 82, Tanishidaizu and TK780 were not infected systemically with ClYVV-No.30-GFP (Fig. 3 and Table 1). Taken together, all the cultivated soybeans tested were able to reduce the multiplication and/or cell-to-cell movement of ClYVV-No.30 in inoculated leaves and to restrict it to inoculated leaves only.

Cultivated soybeans show resistance to all tested ClYVV isolates

To examine whether the resistance of cultivated soybeans is specific to isolate No.30 of ClYVV or it is broad, we inoculated three soybean cultivars, Tanishidaizu, TK780 and Williams 82, with three ClYVV isolates, ClYVV-No.30-GFP, I89-1 (ClYVV-I89-1) and 90-1 (ClYVV-90-1) (Table 1 and Figs. 3, S1 and S2). No symptoms developed on the three soybean cultivars inoculated regardless of the ClYVV isolates, and GFP

fluorescence or viral CP were not detected in any of the noninoculated upper leaves, except for the TK780 plants inoculated with ClYVV-I89-1 (Fig. S1). RT-PCR assay to detect viral genomic RNA also confirmed that none of the three ClYVV isolates systemically infected the inoculated cultivars, except for the TK780 plants inoculated with ClYVV-I89-1 where that virus spread to noninoculated systemic leaves of all four inoculated plants (Fig. S1). However, we did not detect CP in two out of four of these plants by western immunoblotting, and in those where viral CP was detected, the level of viral accumulation was still lower compared to the level in the wild soybean tissues infected with ClYVV-I89-1 (Fig. S1). None of TK780 plants developed pronounced symptoms although one plant developed very mild symptoms at a later date (Table 1), indicating that TK780 may even be partially resistant to ClYVV-I89-1. Taken together, we conclude that cultivated soybeans commonly have resistance against ClYVV, or ClYVV is not adapted well to cultivated soybeans. Interestingly, phylogenetic analysis of *Clover yellow vein virus* with members belonging to nine other virus species within the *Potyvirus* genus, of which members infect soybeans naturally or experimentally (Hill and Whitham, 2014), showed that *Clover yellow vein virus* and its close relative, *Bean yellow mosaic virus*, forms a clade that is very distant from the well-adapted *Soybean mosaic virus* (Fig. S2).

Contrasting susceptibility of wild soybeans against ClYVV

In contrast to cultivated soybeans, most of wild soybeans inoculated were susceptible to ClYVV. When 24 accessions of wild soybeans were inoculated with ClYVV-No.30-GFP, all but one accession, B00197, developed various systemic symptoms including vein clearing, mosaic and necrotic spot on noninoculated upper leaves (Table 1). We confirmed systemic infection by observing GFP fluorescence and detecting viral CP in

198 noninoculated leaves of some of the accessions by western immunoblotting (Table 1 and
199 Figs. 2, 3 and 4). Wild-soybean accession B00092 plants, however, did not express GFP
200 fluorescence or symptoms on noninoculated leaves until 28 dpi (Table 1). B04009 and
201 B08034 plants developed relatively mild symptoms compared with those of B01167 and
202 B03015 (Fig. 4). These results indicate that wild soybeans are generally susceptible to
203 ClYVV though among those tested some were less susceptible than others and one was
204 resistant to systemic infection.
205
206 **Graft-inoculation of cultivated soybean with ClYVV-No.30 results in systemic**
207 **infection**
208 Grafting between wild and cultivated soybean is compatible. To examine where ClYVV
209 infection is blocked, we directly inoculated ClYVV-No.30-GFP into the vascular system
210 of cultivated soybean by grafting while using ClYVV-No.30-GFP infected wild soybean
211 (B01167) as a scion. One month post-grafting, the GFP signal was detected in leaves of
212 the cultivated soybean rootstock parts grown from axillary bud (Fig. 5A). Accumulation
213 of ClYVV-No.30-GFP genomic RNA was confirmed by RT-PCR in these leaves (Fig.
214 5A), indicating the systemic spread of ClYVV-No.30-GFP in the cultivated soybean
215 parts upon graft-inoculation. We were concerned that the systemic spread of ClYVV-
216 No.30-GFP in cultivated soybean was not due to the inoculation manner but rather to
217 the emergence of an adaptive ClYVV-No.30 mutant emerged during continuous forced
218 inoculation from ClYVV-No.30-GFP infected wild soybean scion serving as the
219 inoculum source. This possibility was examined by two experiments. First, we removed
220 the grafted wild soybean (scion) one week post graft-inoculation and investigated
221 ClYVV-No.30-GFP infection one and two months afterward. Both the GFP signal and
222 ClYVV-No.30 genomic RNA were still detected in the cultivated soybean parts (Fig.

5B). Second, ClYVV-No.30-GFP that systemically infected cultivated soybean leaves (tentatively designated ClYVV-TK) was back-inoculated onto cultivated and wild soybean and broad bean. ClYVV-TK failed to infect cultivated soybean though as expected infected wild soybean and broad bean systemically (Fig. 5C) displaying similar disease phenotypes comparable with the original ClYVV-No.30-GFP induced symptoms. The nucleotide sequence of the ClYVV-TK genomic RNA was determined and analyzed. No mutation was detected in the region encoding the polyprotein when compared with that of ClYVV-No.30-GFP that infected wild soybean. However, when compared with the sequences of the infectious cDNA clone (DDBJ/ENA/GenBank accession no. AB011819), both ClYVV-TK and ClYVV-No.30-GFP in wild soybean had the same non-synonymous single mutation (Fig. S3C). We concluded that the graft-inoculation of ClYVV-No.30 infects systemically cultivated soybean rootstocks. Thus, failure of ClYVV to move systemically in the mechanically-inoculated cultivated soybean is likely due to inhibition of ClYVV infection at step(s) before systemic spread via vascular systems.

Resistance against ClYVV in cultivated soybean is recessively inherited

Further experiments were done to genetically characterize the contrasting reactions of cultivated and wild soybeans against ClYVV. To determine whether the mode of inheritance for resistance in cultivated soybean is recessive or dominant, cultivated soybean TK780 was crossed with wild soybean B01167, and the F1 and F2 progeny plants were mechanically inoculated with ClYVV-No.30-GFP. As a result, ClYVV-No.30-GFP spread to noninoculated leaves and caused systemic symptoms in all the F1 plants at 14 dpi, comparable to the susceptibility of wild soybeans (Table 1). However, the inoculated F2 plants showed varied reactions; 40 out of the 76 inoculated plants

248 developed systemic symptoms within 14 dpi, and additional 14 showed systemic
249 symptoms at 21 dpi. Eight out of 76 inoculated F2 plants were not infected systemically
250 with ClYVV-No.30-GFP at least through 28 dpi. The above-results with F2 plants were
251 obtained by two independent inoculation tests where each test showed similar results.
252 These inoculation tests with F1 and F2 plants suggest that resistance against ClYVV in
253 cultivated soybean is recessively inherited. The ratio of the resistant and susceptible F2
254 plants to systemic infection was 8/68. The segregating ratio was not conformed to the
255 3:1 ratio (Chi square test: χ^2 =8.4912, p-value = 0.003569), indicating that two or
256 more host factors might be involved in the inhibition of systemic infection.
257
258 **One major QTL governs soybean resistance to ClYVV**
259 To elucidate how many loci are involved in cultivated soybean resistance to ClYVV and
260 to determine where they are located in the genome, 64 recombinant inbred lines (RILs)
261 derived from the cross between TK780 and B01167 soybeans (Liu et al., 2007) were
262 mechanically inoculated with ClYVV-No.30-GFP and evaluated for symptoms and GFP
263 fluorescence expression. Since various symptoms were observed on the inoculated
264 RILs, we classified them into four type groups (Fig. 6). Type 1 (score 1) had no
265 symptoms or GFP fluorescence expression on upper leaves similar to the parental
266 cultivated soybean TK780. Type 2 (score 2) rarely spread systemically prior to 28 dpi
267 and showed no or very weak symptoms or GFP expression. Type 3 (score 3) developed
268 visible but mild vein clearing symptoms between 14 and 21dpi with low, but detectable
269 level of GFP expression., Type 4 (score 4) developed severe mosaic or necrotic
270 symptoms similar to the parental wild soybean B01167 by 14 dpi combined with
271 pronounced level of GFP expression (Fig. 6A). The inoculated RILs were broadly
272 classified into the four types; 11, 15, 16 and 17 lines were grouped into types 1–4,

273 respectively, and the other 5 lines could not be classified into just one type group (Table
274 S2).
275 To quantify and map the genes that contribute to the soybean resistance against
276 ClYVV, multiple QTL Model (MQM) mapping was performed using 282 markers (Liu
277 et al., 2007) and the phenotypes of all 64 RILs. One major QTL significantly associated
278 with the resistance (logarithm of the likelihood ratio [LOD] > 2) was identified near the
279 top arm of linkage group D1b (chromosome 2) (Fig. 6B) and had an LOD score of 15.0,
280 accounting for 68.3% of the genetic variation. This major QTL was located between
281 simple sequence repeat (SSR) markers Satt095 and Satt558 (Liu et al., 2007) (Fig. 6B).
282 RILs that inherited the cultivated and wild soybean genotype between markers Satt095
283 and Satt558 were resistant and susceptible to ClYVV, respectively, except for RILs 022
284 and 030. Although inheriting the cultivated soybean genotype between markers Satt095
285 and Satt558, these RILs were moderately susceptible to ClYVV (assigned as Type 3 in
286 Table S2) implying that other loci confer the susceptibility. Our data indicate that one
287 major QTL, which is located on linkage group D1b, governs the resistance to ClYVV.
288 We here designate the resistant allele at this major QTL as domestication-related
289 resistance to ClYVV (*d-cv*).
290
291 **DISCUSSION**
292 This study revealed differences between cultivated soybean (*G. max*) and its ancestor
293 wild soybean (*G. soja*) against ClYVV. Among the *Glycine* spp. Lines tested, cultivated
294 and wild soybeans were resistant and susceptible, respectively. A dozen of the cultivated
295 soybeans tested expressed restricted local infection and were not infected systemically
296 with three ClYVV isolates used, except for cultivated soybean TK780 inoculated with
297 ClYVV-I89-1. Even though systemically infected, TK780 soybean was still partially

298 resistant to ClYVV-I89-1. Interestingly, the genomic sequence of ClYVV-Gm, which
299 was isolated from naturally infected field-grown soybean plants in South Korea (Shin et
300 al., 2014), has the highest genetic similarity to ClYVV-I89-1 among the three ClYVV
301 isolates used in this study (Fig. S2). However, in general ClYVV is not considered as a
302 major pathogen of soybean because ClYVV-Gm was isolated from only two field-
303 grown soybeans displaying mottle and mosaic among 151 plants exhibiting viral-like
304 leaf symptoms. Shin et al. (2014) study was the first report implying ClYVV causing
305 disease in South Korean soybean fields. To the best of our knowledge, there has been
306 only another earlier study reporting on a virus that was likely ClYVV isolated from a
307 field-grown soybean (Jones and Diachun, 1977). Contrasting susceptibility of almost all
308 wild soybeans used in this study to ClYVV suggests that ClYVV has intrinsically been
309 a pathogen of members of the subgenus *Soja*. Then, it is possible that resistance of
310 cultivated soybeans to viruses including ClYVV has been derived from the ancestral
311 wild soybeans by artificial selection during domestication thousands years ago.
312 Uncovering the molecular mechanism of nonhost resistance is important for
313 understanding how the host range of a pathogen is determined and also for obtaining
314 genetic resources to confer disease resistance in crops. However, in nonhost plants, the
315 factors that are involved in the resistance are not genetically tractable if all the members
316 of the species are resistant to a pathogen. That is probably why the molecular
317 mechanism(s) of most types of nonhost resistance remain poorly understood. One
318 exception is the study of the nonhost resistance in tomato (*Solanum lycopersicum*)
319 against tobacco mild green mosaic virus (TMGMV) and pepper mild mottle virus
320 (PoMMV) (Ishibashi et al., 2009). Ishibashi et al. (2009) initially identified the protein
321 encoded by tomato *Tm-1* that does not govern nonhost resistance but rather controls
322 cultivar-specific semidominant resistance to tomato mosaic virus. The *Tm-1*-encoded

protein inhibits replication of tomato mosaic virus by binding to viral replicase (Ishibashi et al., 2007). Ishibashi et al. (2009) later demonstrated that the recessive allele *tm-1* encodes the protein that governs tomato nonhost resistance against TMGMV and PMMoV via binding to and inhibiting viral replicases. Since the protein encoded by *tm-1* was easily identified using the information on its allelic *Tm-1*, they could create the *tm-1* protein-expressed transgenic tobacco, for which the wild-type host is susceptible to TMGMV and PoMMV. They showed that the *tm-1* confers resistance to these tobamoviruses in transgenic tobacco plants with the tomato *tm-1*.

In our case, all the cultivated soybeans are resistant to ClYVV-No.30 and thus a cross between a resistant and a susceptible cultivated soybean is not possible. Because wild soybeans are susceptible to ClYVV instead we were able to cross a resistant cultivated soybean with a ClYVV susceptible wild soybean to generate F1, F2 and RILs. All the F1 plants were systemically infected with ClYVV-No.30-GFP, suggesting the recessive inheritance of the resistance. Inoculation tests with F2 plants support the recessive inheritance of the resistance as well. The ratio of the resistant and susceptible plants to systemic infection by ClYVV among inoculated F2 plants implied that two or more host factors are involved in the resistance. Consistently, the inoculated RILs showed varied reactions to ClYVV and were divided into four categories according to their reactions to the virus in terms of symptoms and GFP fluorescence expression in the noninoculated leaves. The ratio (11/64 RILs) of the resistant plants that prevented the virus from infecting systemically was similar to that of the inoculated F2 plants. However, the QTL analysis using 64 RILs only detected one major peak for the logarithm of the likelihood ratio (LOD) in soybean linkage group D1b (chromosome 2) (Fig. 6B), at which we designated the resistant allele as *d-cv*. We are now continuing a finer genetic analysis to detect possible minor QTL(s) and identify the genes encoded

on *d-cv*. The involvement of one major QTL in the resistance to ClYVV and its recessive trait of inheritance supports its selection during domestication since most beneficial traits of cultivated plants are known to be acquired by loss of function mutations that are recessively inherited (Ladizinsky, 1985). It is known that most domestication-related traits are controlled by one or two major QTLs in soybean and other crops (Ross-Ibarra, 2005; Liu et al., 2007).

Soybean cultivars possess resistant genes, including *Rsv1, Rsv3* and *Rsv4*, against SMV that is a major pathogen of soybean and belongs to the genus *Potyvirus*, similar to ClYVV (Saghai Maroof et al., 2008). The *d-cv* locus seems not related to these resistant loci because these are all dominantly inherited. Furthermore, *Rsv1* and *Rsv3* are located on different chromosomes, 13 and 14, respectively, whereas that of the *Rsv4* position differs from the region where the *d-cv* is mapped by the QTL analysis (Saghai Maroof et al., 2008; Saghai Maroof et al., 2010); *d-cv* and *Rsv4* are located upstream and downstream of the marker Satt558 on chromosome 2, respectively (Fig. 6B). Considering the recessive traits of inheritance, the *d-cv* gene is supposed to encode a co-opted factor for ClYVV infection such as eIF4E (Andrade et al., 2009; Nakahara et al., 2010) and probably have mutations that disrupt the function required for ClYVV systemic infection, resulting in the resistance when plants are homozygous for the *d-cv* allele. Another possibility is that *d-cv* encodes a negative regulator for the soybean antiviral system against ClYVV.

Our study provides data for where ClYVV infection is blocked in cultivated soybean carrying *d-cv*. In general, in order for a mechanically inoculated virus to move systemically in an inoculated plant, it must infect and multiply in initially-invaded cells, moves cell-to-cell within the inoculated leaf followed by long-distance movement (i.e. entry into vascular system, systemic spread through phloem tissue, and exit into

mesophyll cells of noninoculated leaves). Systemic infection of ClYVV in soybean carrying *d-cv* upon direct delivery into phloem tissue by graft inoculation (Fig. 5) suggests that upon mechanical inoculation the cultivated soybean inhibits ClYVV at infection steps prior to the systemic spread of the virus via phloem tissue. Actually, ClYVV accumulated and spread to a lesser extent in mechanically inoculated leaves of cultivated soybean than in wild soybean (Figs. 1-3). Nevertheless, it is possible that movement of a susceptibility factor from ClYVV-infect wild soybean scion into cultivated soybean rootstock results in enhancement of ClYVV infection. It should be pointed out that there is no published evidence in support of this possibility.

To further investigate how cultivated soybean inhibits systemic infection of ClYVV, and whether the resistance of cultivated soybeans has been artificially selected during domestication, we need to identify not only the gene(s) at the *d-cv* locus but also viral factor(s) that interacts with *d-cv*. P1, P3, P3N-PIPO, CI and viral genome-linked protein of potyviruses have previously been reported as virulence determinants that interact with recessively inherited resistance factor(s) from plants (Nakahara et al., 2010; Tavert-Roudet et al., 2012; Wang and Krishnaswamy, 2012; Choi et al., 2013). HC-Pro, 6K2 and CP, which are involved in long-distance movement (Hipper et al., 2013), are also candidate viral factors that may interact with *d-cv*. Although ClYVV is not a major pathogen of cultivated soybean, if the gene(s) encoded by *d-cv* is a co-opted factor for ClYVV infection, it is highly likely that *d-cv*, its homologues and/or related genes are co-opted by other potyviruses, including SMV. Therefore, uncovering the mechanism of this *d-cv*-mediated recessive resistance might contribute to molecular breeding to confer resistance to SMV in soybean cultivars as well.

EXPERIMENTAL PROCEDURES

Construction of ClYVV-No.30 harboring the *uidA* gene expressing β-glucuronidase (GUS) (ClYVV-No.30-GUS)

To construct ClYVV-No.30-GUS, plasmid ClYVV-*Pst*I (a gift from Dr. I. Uyeda of Hokkaido University, Japan) was used as a backbone. To use the *Pst*I sites at positions 630 and 1098 (numbering form the first nucleotide of ClYVV genome) for cloning *uidA* gene, the *Pst*I site at position 9164 was destroyed by oligonucleotide-site directed mutagenesis (Masuta et al., 2000). The oligonucleotide primers used are listed in Table S2. PrimeSTAR HS DNA polymerase Premix (Takara Bio, Madison, WI, U.S.A.) was used. All plasmid constructs were amplified and maintained in *E. coli* DH5α. Initially, the 3' region of the *P1* cistron of ClYVV was amplified by PCR with primer C-GUS-1 and C-GUS-2 (Table S3) using ClYVV-*Pst*I as a template. The *uidA* gene was amplified with primers C-GUS-3 and C-GUS-4 while SMV-N-GUS (Wang et al., 2006) served as a template. The 3' region of the *P1* cistron and 5' termini of *uidA* gene were merged by fusion PCR (Charlier et al., 2003). The resultant two gel-purified fragments were mixed together at the same molar ratio to serve as a template in a 50-μL reaction mixture that consisted of 200 ng of mixed fragments, 25 μL of PrimeSTAR HS (Premix), and 0.5 μM each of primers C-GUS-1 and C-GUS-4. The PCR program for the amplification reaction was as follows: one cycle of 2 min at 95°C; 20 s at 95°C, 30 s at 58°C, and 2 min at 72°C for a total of 20 cycles; followed by a final extension cycle for 10 min at 72℃. The resultant amplified fragments were digested with *Pst*I and ligated into ClYVV-No.30 to generate ClYVV-No.30-GUS. The presence of P1 and NIa cleavage recognition sites between P1 and GUS as well as between GUS and HC-Pro, respectively, were verified by sequencing their encoded region. Furthermore, the junctions, 3' region of *P1* cistron and 5' termini of *uidA* gene as well as the junction

423 between 3' of *uidA* gene with the 5' terminus of *HC-Pro* cistron in the construct were
424 also confirmed by sequencing with primers C-531S, C-GUS-3 and C-1195a (Table S3).
425 Sequencing was done at The University of Tennessee DNA Sequencing Facility, and
426 sequences were edited using the DNA Star package and the Wisconsin package version
427 10.2 (Genetic Computer Group, Madison, WI, USA).
428
429 **Viruses, soybean genotypes and inoculation**
430 Wild soybean accessions, soybean cultivars, and RILs used in this study were obtained
431 from LegumeBase (https://www.legumebase.brc.miyazaki-u.ac.jp/), an integrated
432 resource database of *Lotus japonicus* and *G. max*, or from the Genebank of the National
433 Agriculture and Food Research Organization in Japan. Soybean genotypes (Williams
434 [*rsv1*, *rsv4*], Essex (*rsv1*, *rsv4*), Lee68 (*rsv1*, *rsv4*), York (*Rsv1*), PI96983 (*Rsv1*), L78-
435 379 (*Rsv1*), L800 (*3gG2*), L943 (-*3gG2*) and V94-5152 [*Rsv4*]) (Bernard et al., 1991),
436 which show variable reactions (from extreme resistance to susceptible) to SMV strains,
437 were used in this study. Plant materials used in this study are also listed in Table 1, S1
438 and S2. The RILs (F8) used in this study were developed previously by the single seed
439 descent method from an F2 population of a cross between TK780 (*G. max* parent) and
440 B01167 (alias: Hidaka 4, *G. soja* parent) at Hokkaido University (Liu et al., 2007).
441 To establish infection with plasmid DNAs, we biolistically inoculated fully
442 expanded primary leaves of soybean seedlings as described previously (Hajimorad et
443 al., 2003, 2008). For mechanical inoculations, sap extract from biolistically inoculated
444 and systemically infected leaf tissues from broad bean (*V. faba* L.) cultivar Windsor, in
445 10 mM phosphate buffer, pH7.0, was applied manually to fully developed primary
446 leaves. ClYVV-No.30-GFP, which was named pClYVV/C3-S65T previously (Sato et
447 al., 2003), was used to biolistically inoculate broad bean plants (*V. faba*, cv.

448 Komazakae). Approximately 10 dpi, sap derived from the first noninoculated
449 symptomatic leaves were used for mechanical inoculation. ClYVV-I89-1 and ClYVV-
450 90-1 were used to inoculate broad bean plants, and sap from the first noninoculated
451 symptomatic leaves were used as inocula. Inoculated plants were kept in a growth
452 chamber at 22°C with a photoperiod of 16 h. For segregation analysis, F1 plants were
453 obtained by crossing wild soybean B01167 and cultivated soybean TK780. F2 plants
454 were derived from self-pollination of F1 plants. These plants were inoculated with
455 ClYVV-No.30-GFP and incubated in a growth room as described above. For graft
456 inoculation, primary leaves of wild soybean seedlings B01167 were mechanically
457 inoculated with infectious sap containing ClYVV-No.30-GFP. One week post-
458 inoculation, upper part of wild soybean seedlings were grafted on seedlings of soybean
459 TK780; these served as scion and rootstock, respectively.
460
461 **Phylogenetic analysis**
462 Phylogenetic analysis done using full-length nucleotide sequences of ORFs encoding
463 the polyproteins or deduced amino acid sequences of polyproteins. Sequence alignment
464 was conducted by using MUSCLE (Edgar, 2004). Maximum likelihood tree was
465 inferred with substitution models and rates among sites which were determined using
466 MEGA6 package (Tamura et al., 2013). The significance of the nodes was estimated
467 with 1,000 bootstrap replicates.
468
469 **Detection of ClYVV**
470 Histochemical assay for GUS expression was done as described by Wen and Hajimorad
471 (2010). Infection of wild soybean plants and cultivated soybean plants with ClYVV-
472 No.30-GFP was monitored for GFP fluorescence expression at 3, 5, 7 dpi and on

systemic leaves at 21 dpi using a fluorescence microscope system (VB-7010; Keyence, Osaka, Japan) with a band-pass GFP filter (FF01-520/35-25; Semrock, Rochester, NY, USA). To detect ClYVV immunologically, mouse polyclonal antibodies against CP of ClYVV was used at a rate of 1:1000 in antigen-coated indirect ELISA and probed with goat anti-mouse IgG alkaline phosphatase conjugate (Bio-Rad, USA) at a rate of 1:5000. ClYVV CP was also detected by western blotting as previously described (Nakahara et al., 2012) using rabbit serum containing polyclonal antibodies raised against ClYVV CP at 7 dpi for inoculated leaves and at 21 dpi or 35 dpi for systemic leaves. For more sensitive detection of ClYVV, RT-PCR was performed at 28 dpi using primers for *NIb* cistron of ClYVV: 5′-CTTTAGACCTATGATGGGC-3′ (sense) and 5′-GTTCAAGCCCAATTCTTTG-3′ (antisense) as described previously (Choi et al., 2013).

Evaluation of resistance of RILs plants to ClYVV

The level of resistance of each RIL plant was determined by the severity of symptoms and the level of GFP expression monitored at 28 dpi and separated into four types (Type 1-4, as described in the Result section).

QTL mapping

The F8 generations of RILs derived from crosses between *G. max* and *G. soja* (parents) as described above were mechanically inoculated with ClYVV-No.30-GFP, and four phenotypes were used for scoring and QTL analysis. Each type or trait (Types 1-4) in response to ClYVV infection of 64 RILs was scored from 1 to 4, and the QTL mapping was performed as described next. Marker order and distance from expected QTL of *d-cv* were used to find the candidate QTL by composite interval mapping (CIM)

498 implemented by MapQTL 5 (van Ooijen, 2004). A total of 1000 permutations were
499 performed to establish the logarithm of the likelihood ratio (LOD) thresholds at 0.05
500 probability (Churchill and Doerge, 1994). QTLs were considered to exist only at
501 positions where the LOD score exceeded the corresponding significance threshold.
502
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509
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Table 1. Reactions of wild soybean (*Glycine soja*) and cultivated soybean (*G. max)* plants and F1 and F2 progeny derived from a cross between the two species to three strains of ClYVV. Plants were mechanically inoculated with sap derived from ClYVV-infected broad bean leaves. Viral infection was confirmed by visualization of GFP fluorescence expression (ClYVV-No.30-GFP), western blot analysis and RT-PCR.

		ClYVV-No.30-GFP								
		Number of plants which show symptoms				GFP	western blot	RT-PCR	symptom	total infected
	line	7 dpi.	14 dpi.	21 dpi.	28 dpi.					
G.soja	T106	0/9	9/9	9/9	9/9	9/9[a]	9/9	nt	++(M,VC)[b]	9/9
G.soja	B00046	0/3	3/3	3/3	3/3	3/3	3/3	nt	++(VC)	3/3
G.soja	B00090	3/3	3/3	3/3	3/3	3/3	3/3	nt	++(VC)	3/3
G.soja	B00092	0/5	0/5	0/5	4/5	4/5	4/5	nt	+(VC)	4/5
G.soja	B00197	0/3	0/3	0/3	0/3	0/3	0/3	nt	no symptom	0/3
G.soja	B00225	0/3	3/3	3/3	3/3	3/3	3/3	nt	+++(M,VC)	3/3
G.soja	B01160	0/2	2/2	2/2	2/2	2/2	2/2	nt	++(M,VC,NS)	2/2
G.soja	B02280	0/3	3/3	3/3	3/3	3/3	3/3	nt	++(VC)	3/3
G.soja	B03015	0/3	3/3	3/3	3/3	3/3	3/3	nt	++(M,VC)	3/3
G.soja	B03037	0/3	1/3	3/3	3/3	3/3	3/3	nt	+(VC)	3/3
G.soja	B04009	0/3	3/3	3/3	3/3	3/3	3/3	nt	+(VC)	3/3
G.soja	B04114	0/4	4/4	4/4	4/4	4/4	4/4	nt	+++(M.VC)	4/4
G.soja	B04158	0/4	4/4	4/4	4/4	4/4	4/4	nt	+++(M,VC)	4/4
G.soja	B05023	0/10	1/10	7/10	10/10	10/10	10/10	nt	+～++（VC）	10/10
G.soja	B06086	0/4	4/4	4/4	4/4	4/4	4/4	nt	++(M,VC)	4/4
G.soja	B06098	0/5	5/5	5/5	5/5	5/5	5/5	nt	++(M,VC)	5/5
G.soja	B07126	0/4	4/4	4/4	4/4	4/4	4/4	nt	+++(M,VC,NS)	4/4
G.soja	B07164	0/4	3/4	4/4	4/4	4/4	4/4	nt	++(VC)	4/4
G.soja	B08034	0/4	2/4	4/4	4/4	4/4	4/4	nt	+(VC)	4/4
G.soja	B08040	0/2	2/2	2/2	2/2	2/2	2/2	nt	++(M,VC)	2/2
G.soja	B08045	0/3	3/3	3/3	3/3	3/3	3/3	nt	++(M.VC)	3/3
G.soja	B09092	0/4	0/4	4/4	4/4	4/4	4/4	nt	++(VC)	4/4
G.soja	B09117	0/2	0/2	2/2	2/2	2/2	2/2	nt	+(VC)	2/2
G.soja	B01167	0/6	6/6	6/6	6/6	6/6	6/6	nt	++++(M,VC,NS)	6/6
G.max	Tanishidaizu	0/6	0/6	0/6	0/6	0/6	0/6	0/6	no symptom	0/6
G.max	Williams 82	0/3	0/3	0/3	0/3	0/3	0/3	0/3	no symptom	0/3
G.max	TK780	0/4	0/4	0/4	0/4	0/4	0/4	0/4	no symptom	0/4
G. soja × *G. max*[c]	F1	0/8	8/8	8/8	8/8	8/8	8/8	nt	+++	8/8
	F2	0/76	40/76	54/76	68/76	68/76	NT	nt	Variable	68/76
		ClYVV-I89-1								
G. soja	T106	3/3	3/3	3/3	3/3	–	3/3	nt	+++(M,VC,)	3/3
G. soja	B03015	4/4	4/4	4/4	4/4	–	4/4	nt	++++(M,VC,NS)	4/4
G. soja	B04158	3/4	4/4	4/4	4/4	–	4/4	nt	++++(M,VC,NS)	4/4
G. soja	B01167	2/4	4/4	4/4	4/4	–	4/4	nt	++++(M,VC,NS)	4/4
G. max	Tanishidaizu	0/3	0/3	0/3	0/3	–	0/3	0/3	no symptom	0/3
G. max	Williams 82	0/4	0/4	0/4	0/4	–	0/4	0/4	no symptom	0/4
G. max	TK780	0/4	0/4	0/4	1/4	–	3/4	4/4	+(M) 1/4	4/4
		ClYVV-90-1								
G. soja	T106	0/3	2/3	3/3	3/3	–	3/3	nt	+++(M,VC)	3/3
G. soja	B03015	0/2	2/2	2/2	2/2	–	2/2	nt	+++(M,VC)	2/2
G. soja	B04158	0/4	0/4	0/4	2/4	–	2/4	nt	+(VC)	2/4
G. soja	B01167	0/7	0/7	5/7	5/7	–	5/7	nt	+～+++(M,VC)	5/7
G. max	Tanishidaizu	0/3	0/3	0/3	0/3	–	0/3	0/3	no symptom	0/3
G. max	Williams 82	0/4	0/4	0/4	0/4	–	0/4	0/4	no symptom	0/4
G. max	TK780	0/4	0/4	0/4	0/4	–	0/4	0/4	no symptom	0/4

[a] Number of plants that showed the GFP fluorescence at 28 dpi

[b] +, ++, +++, ++++; severity of symptoms, such as vein clearing (VC), mosaic (M) and necrotic spots (NS) on noninoculated upper leaves

[c] F1 and F2 plants were derived from cross between cultivated soybean TK780 and wild-soybean B01167.

745 **Figure legend**

746

747 **Fig. 1.** ß-Glucuronidase (GUS)-histochemical analysis of clover yellow vein virus
748 (ClYVV) infection in biolistically inoculated soybean leaves. For inoculation, a full-
749 length infectious cDNA clone of ClYVV -No.30 harboring GUS was delivered
750 biolistically into fully developed attached primary leaves of approximately 2-week-old
751 soybean seedlings maintained at 22°C. Leaves were analyzed histochemically for GUS
752 expression at 21 days postinoculation. Note the presence of GUS in leaf veins of wild
753 soybean B01167 and its absence in veins of all leaves from cultivated soybean
754 genotypes. Scale bar = 2 cm.

755

756 **Fig. 2.** (A) ClYVV spread in ClYVV-No.30-GFP-inoculated cultivated soybean and
757 wild-soybean plants. (B) Accumulation of ClYVV CP in inoculated leaves at 7 dpi was
758 investigated by western blotting. Coomassie brilliant blue (CBB) gel shown in lower
759 panels is the loading control. (C) Systemic spread of ClYVV-No.30-GFP in cultivated
760 soybeans (cultivars Tanishidaizu and TK780) and wild-soybeans (lines T106 and
761 B01167) inoculated plants monitored for GFP fluorescence expression at 28 dpi. GFP
762 signal derived from ClYVV-No.30-GFP was observed in slices of stem at upper part
763 (cut 1), root (cut 3) and petiole of inoculated leaves (cut 2) of a wild-soybean (line
764 B01167) but not in those of cultivated soybean cultivar of TK780. Scale bars = 1 mm.

765

766 **Fig. 3.** Reactions of cultivated soybean Williams 82 and wild soybean line T106
767 mechanically inoculated with three isolates of ClYVV (No.30-GFP, I89-1 and 90-1).
768 (A) Noninoculated upper leaves were photographed at 21 dpi. Scale bars = 10 mm. (B)
769 Western blotting analysis to detect coat protein (CP) of ClYVV in samples from

770 noninoculated leaves at 21 dpi using anti-CP rabbit polyclonal antiserum. It should be
771 noted that ClYVV CP was not detected in any samples from cultivated soybean
772 Williams 82. In contrast, CP was detected from all noninoculated leaves of wild-
773 soybean T106 inoculated on primary leaves with any of the three ClYVV isolates.
774 Coomassie brilliant blue (CBB) stained gel is shown as a loading control (lower panel).
775 (C) RT-PCR analysis of RNA extractions from cultivated soybean Williams 82 at 28 dpi
776 amplifying part of the nuclear inclusion protein b (NIb) region of ClYVV genome.
777 Control is RT-PCR with an RNA extract from wild soybean line T106 at 28 dpi. DW is a
778 product of RT-PCR without RNA extract from a sample.
779
780 **Fig. 4.** Various symptoms and GFP signals on noninoculated leaves from wild-soybean
781 lines (B01167, B03015, B01160, B04158, B08034, B04009, B00197) or that of
782 cultivated soybean TK780 inoculated mechanically with sap containing ClYVV-No.30-
783 GFP are shown. Leaves were visualized at 21 dpi for GFP expression indicating viral
784 distribution in tissues. Inoculated leaves of B01167 and B03015 developed severe
785 mosaic symptoms while those of B01160 and B04158 developed vein clearing, mottle
786 and mosaic. Leaves of B08034 and B04009 showed very weak vein clearing; those of
787 B00197 and *G. max* TK780 did not have any symptoms or GFP signals. Scale bars = 10
788 mm.
789
790 **Fig. 5.** Systemic infection of ClYVV-No.30-GFP in leaves of cultivated soybean TK780
791 rootstock onto which ClYVV-No.30-GFP-infected wild soybean (B01167) (scion) was
792 grafted. (A) One week after inoculation of seedling, ClYVV-No.30-GFP-infected wild
793 soybean B01167 scion was grafted onto cultivated soybean TK780. GFP signal derived
794 from ClYVV infection was detected one month post grafting. ClYVV-No.30-GFP

infection in the TK 780 rootstock (cultivated soybean) tissues was investigated by RT-PCR one month post grafting. (B) ClYVV-infected wild soybean scion was temporarily (for one week) grafted onto TK 780 cultivated soybean serving as a rootstock. GFP signal was also detected one month post grafting. ClYVV infection in the grafted cultivated soybean rootstock was investigated by RT-PCR one and two months post grafting. Cultivated soybean leaves at the positions (a-d) were harvested for the RT-PCR assay. (C) Cultivated and wild soybean plants were inoculated with sap containing ClYVV that infected cultivated soybean (tentatively designated ClYVV-TK) (back-inoculation). One month post back-inoculation, non-inoculated upper leaves of the plants were harvested and their ClYVV-TK infection was investigated by RT-PCR.

Fig. 6. Genetic elucidation of the soybean anti-ClYVV resistance genes using recombinant inbred lines (RILs). (A) Phenotypes of RILs noninoculated leaves from plants mechanically inoculated with ClYVV-No.30-GFP on primary leaves. Type 1 (Score 1) did not show any symptoms or GFP signals on upper noninoculated leaves similar to the parental soybean TK780. Type 2 (Score 2) showed no or very weak symptoms or GFP signals until 28 dpi. Type 3 (Score 3) developed visible but mild vein clearing symptoms with low, but detectable GFP expression at 21 dpi, and Type 4 (Score 4) developed severe mosaic or necrotic symptoms similar to the parental wild soybean B01167 at 14 dpi combined with pronounced level of GFP expression. Scale bar = 1 mm. (B) To quantify and map gene(s) contributing to cultivated soybean resistance to ClYVV, multiple QTL Model (MQM) mapping was performed using 282 markers (Liu et al. 2007) and the phenotypes of all 64 RILs (A). One major QTL (*d-cv*) significantly associated with the resistance was located at 33.6 cM (logarithm of the

likelihood ratio [LOD] > 2) was identified near the top arm of soybean linkage group D1b (chromosome 2); LOD score of 14.95 accounted for 68.3% of the genetic variation.

Fig. S1. Reactions of TK780 cultivated soybean plants to ClYVV-I89-1 inoculation. (A) TK780 soybean plants that were mechanically inoculated on primary leaves with ClYVV-I89-1 developed no symptoms or displayed weak mottling and mosaic at 35 days postinoculation (dpi) on noninoculated leaves. (B) Level of accumulated viral CP of ClYVV-I89-1 in noninoculated leaves of TK780 plants was lower than the level detected by western blotting in those of susceptible wild soybeans (T106) inoculated with ClYVV-I89-1 or ClYVV-No.30. Coomassie brilliant blue (CBB) stained gel is shown as a loading control (lower panel). (C) RT-PCR detected viral genomic RNA in noninoculated, systemically infected leaves of all four TK780 plants at 28 dpi following mechanical inoculation of primary leaves with ClYVV-I89-1. DW is a product of RT-PCR without RNA extract from a sample.

Fig. S2. Phylogenetic analysis of full-length nucleotide sequences of open reading frame (ORF) encoding the polyprotein (A) or deduced amino acid sequence of the encoded polyprotein (B). The sequences were aligned by using MUSCLE, and maximum likelihood tree was inferred. The significance of the nodes was estimated with 1,000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Turnip mosaic virus (TuMV-Tu-2R1) was set as an outgroup. Each accession number is indicated after the underscore of each taxon name. Abbreviations are BCMV = bean common mosaic virus; BYMV = Bean yellow mosaic virus; BtV = beet mosaic virus; BlCMV = blackeye cowpea mosaic virus; ClYVV = clover yellow vein virus; CABMV = cowpea aphid-borne mosaic virus;

844 PWV = passion fruit woodiness virus; PeMoV = peanut mottle virus; PStV = peanut
845 stripe virus; SMV = soybean mosaic virus; TuMV = turnip mosaic virus; WMV =
846 watermelon mosaic virus; WVMV = wisteria vein mosaic virus.
847
848 **Fig. S3.** Sequences of primers used for determining genome sequences of ClYVV (A)
849 and their positions on ClYVV genome (B) are shown. (C) This panel shows a portion of
850 alignment of nucleotide sequences of the main ORF (upper panel) and a portion of
851 alignment of the corresponding amino acids from the encoded polyproteins (lower
852 panel) among the original ClYVV-No.30 clone (DDBJ/ENA/GenBank accession no.
853 AB011819), progeny viruses derived from wild-soybean B01167 scion (ClYVV-No.30-
854 GFP) and progeny viruses derived from systemically infected cultivated soybean TK780
855 rootstock (tentatively designated ClYVV-TK), which was graft-inoculated with the
856 wild-soybean B01167 scion infected with ClYVV-No.30-GFP. RT-PCR amplicons
857 consisted of the entire ORFs were generated while using total RNAs isolated
858 from systemically infected leaves of the scion and the rootstock as templates and
859 directly sequenced. It should be noted that sequences of both progeny viruses derived
860 from scion and rootstock differed only by a single common nucleotide and amino acid
861 compared with the corresponding sequences of the original parental clone. Hence,
862 systemic infection of ClYVV-No.30 in cultivated soybean TK780 was not associated
863 with emergence of a new adaptive mutation.

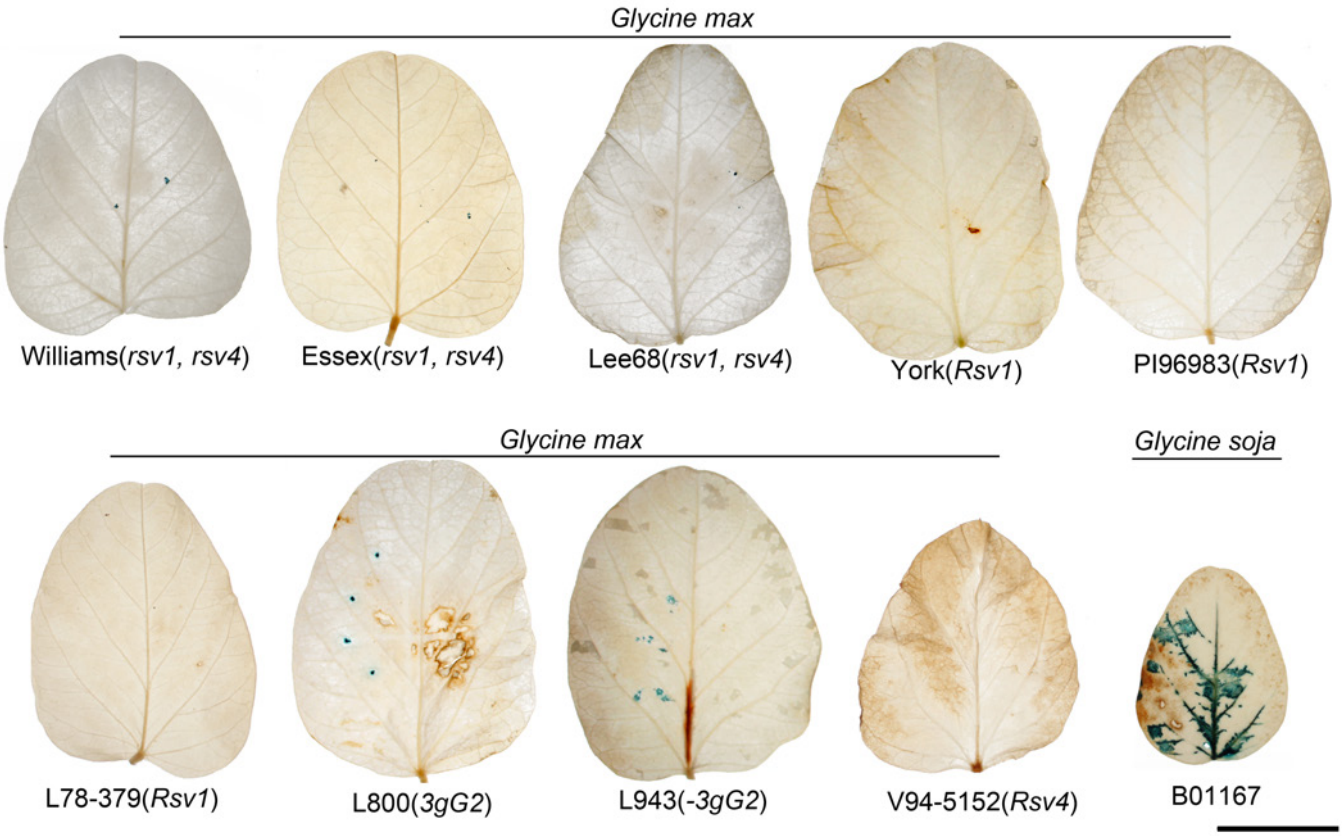

Glycine max
Williams(rsv1, rsv4)
Essex(rsv1, rsv4)
Lee68(rsv1, rsv4)
York(Rsv1)
PI96983(Rsv1)
Glycine max
Glycine soja
L78-379(Rsv1)
L800(3gG2)
L943(-3gG2)
V94-5152(Rsv4)
B01167

Fig. 1

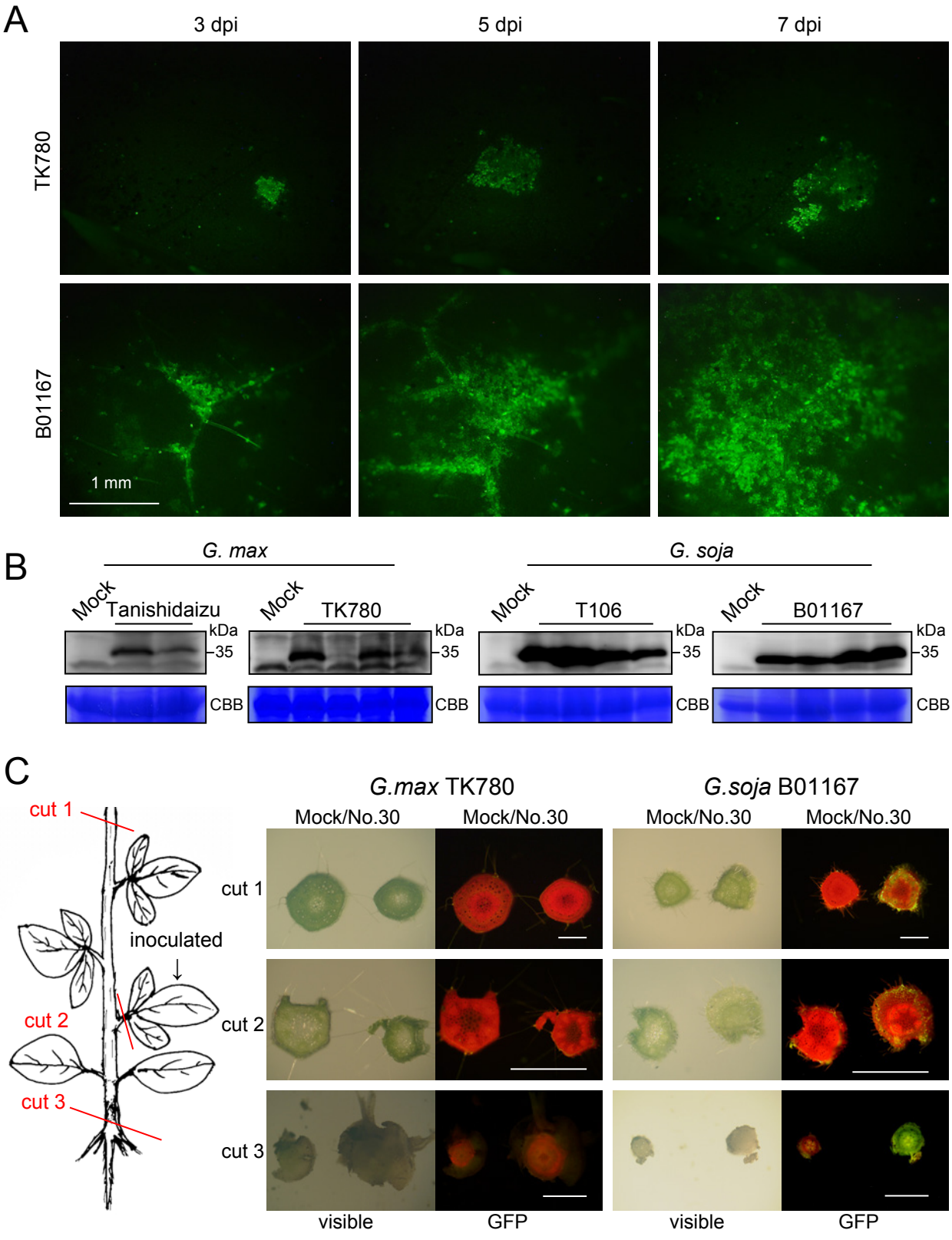

A
3 dpi
5 dpi
7 dpi
TK780
B01167
1 mm
B
G. max
G. soja
Mock
Tanishidaizu
TK780
T106
B01167
kDa
35
CBB
C
G.max TK780
G.soja B01167
Mock/No.30
cut 1
cut 2
cut 3
inoculated
visible
GFP

Fig. 2

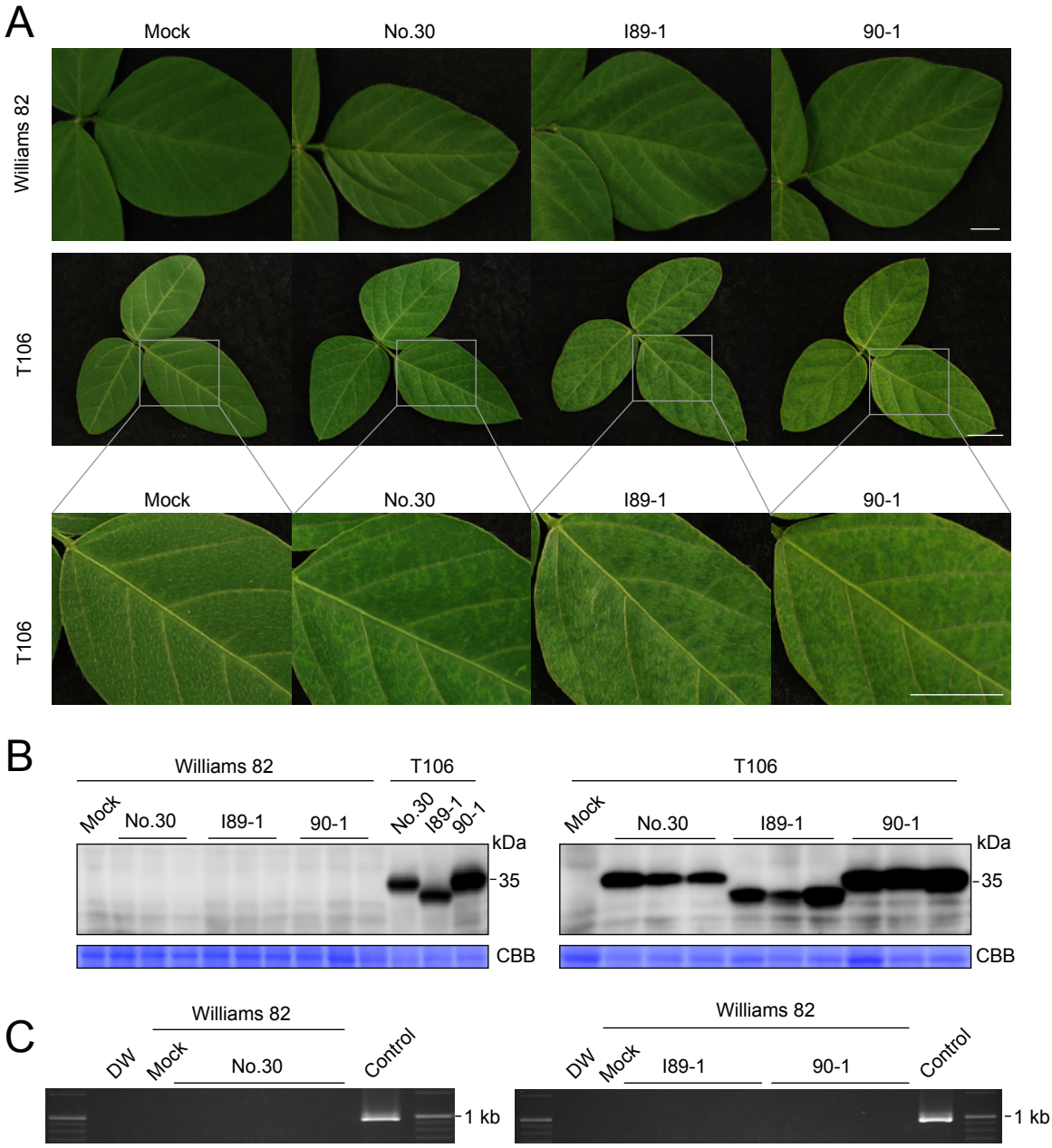

A
Mock
No.30
I89-1
90-1
Williams 82
T106
T106
Mock
No.30
I89-1
90-1
B
Williams 82
T106
T106
Mock
No.30
I89-1
90-1
No.30
I89-1
90-1
Mock
No.30
I89-1
90-1
kDa
35
CBB
kDa
35
CBB
C
Williams 82
Williams 82
DW
Mock
No.30
Control
DW
Mock
I89-1
90-1
Control
1 kb
1 kb

Fig. 3

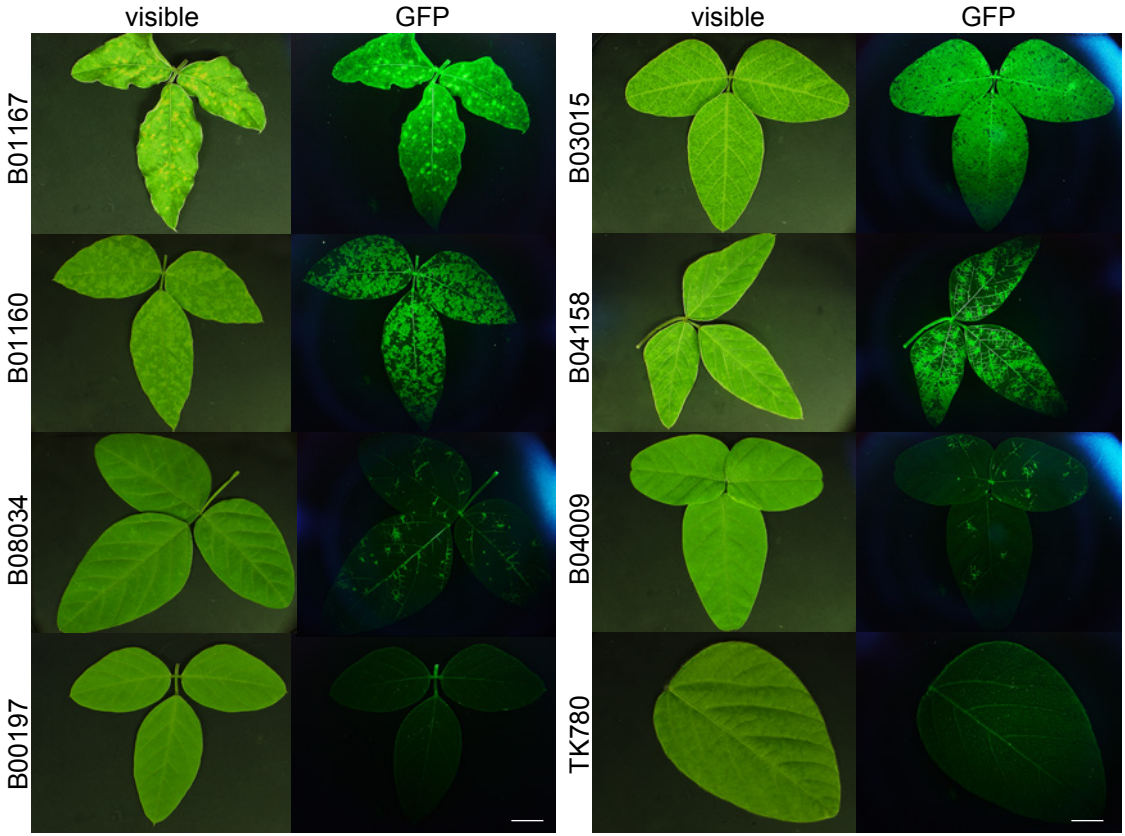

visible
GFP
visible
GFP
B01167
B01160
B08034
B00197
B03015
B04158
B04009
TK780

Fig. 4

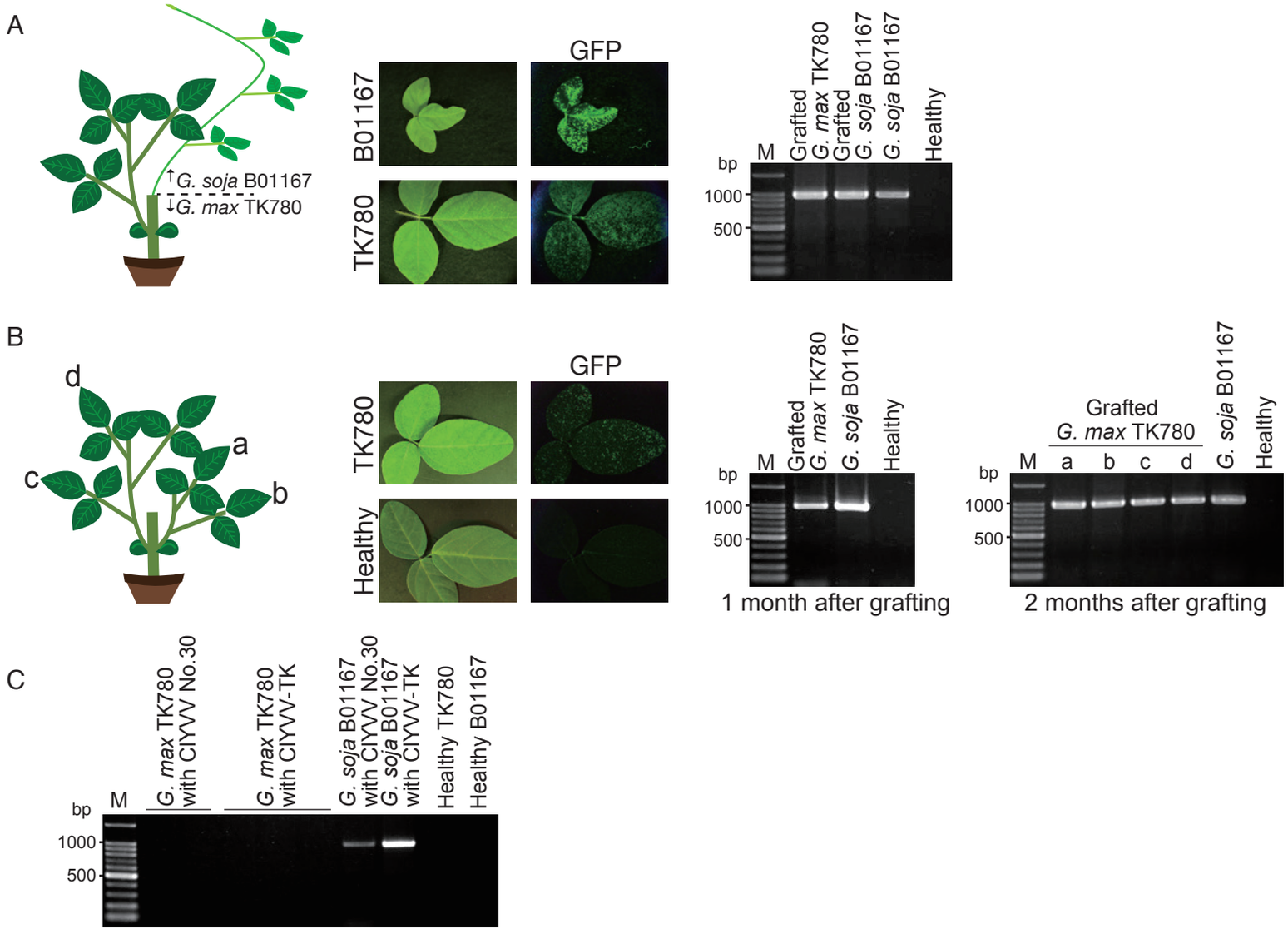

A
↑G. soja B01167
↓G. max TK780
GFP
B01167
TK780
M
Grafted G. max TK780
Grafted G. soja B01167
G. soja B01167
Healthy
bp
1000
500
B
d
a
c
b
GFP
TK780
Healthy
M
Grafted G. max TK780
G. soja B01167
Healthy
bp
1000
500
1 month after grafting
Grafted G. max TK780
M
a
b
c
d
G. soja B01167
Healthy
bp
1000
500
2 months after grafting
C
M
G. max TK780 with ClYVV No.30
G. max TK780 with ClYVV-TK
G. soja B01167 with ClYVV No.30
G. soja B01167 with ClYVV-TK
Healthy TK780
Healthy B01167
bp
1000
500

Fig. 5

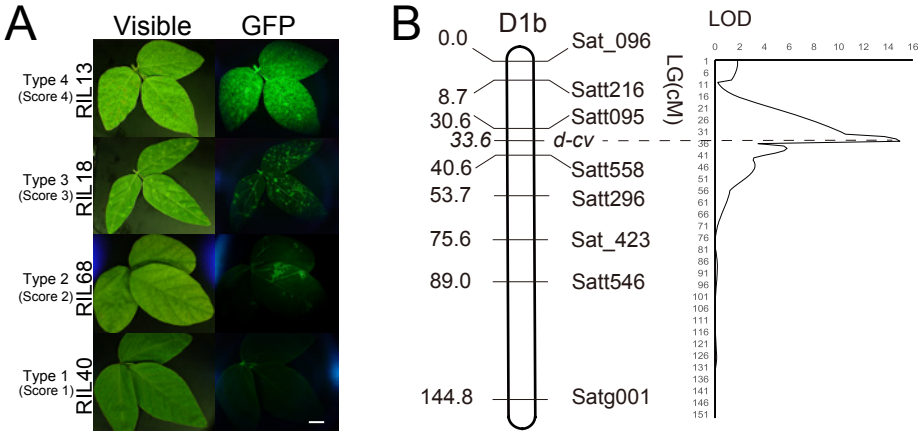

A
Visible
GFP
Type 4
(Score 4)
RIL13
Type 3
(Score 3)
RIL18
Type 2
(Score 2)
RIL68
Type 1
(Score 1)
RIL40
B
D1b
LOD
LG(cM)
0.0
Sat_096
8.7
Satt216
30.6
Satt095
33.6
d-cv
40.6
Satt558
53.7
Satt296
75.6
Sat_423
89.0
Satt546
144.8
Satg001

Fig. 6

Table S1. Evaluation of cultivated and wild-type soybean genotypes inoculated biolistically with ClYVV-GUS for expression of β-glucuronidase (GUS) via histochemical assay and detection of coat protein (CP) by ELISA in the inoculated and non-inoculated leaves, respectively.

Soybean genotype	No. of plants Inoculated	Inoculated leaves (GUS Expression)	Non-inoculated leaves (CP detection*)
Williams (*rsv1*, *rsv4*)	4	1#/4	0/4
Essex (*rsv1*, *rsv4*)	2	2/2	0/2
Lee 68 (*rsv1*, *rsv4*)	7	2/7	0/7
York (*Rsv1*)	3	0/3	0/3
PI96983 (*Rsv1*)	3	0/3	0/3
L78-379 (*Rsv1*)	3	0/3	0/3
L800 (*3gG2*)	3	2/3	0/3
L943 (-*3gG2*)	3	1/3	0/3
V94-5152 (*Rsv4*)	2	1/2	0/2
B01167&	8	5/8	5/8

*Indicates coat protein (CP) detection was done by ELISA.

#Indicates number of leaves expressing GUS/total number of leaves assayed.

&Indicates B01167 is a wild-type soybean genotype (*Glycine soja*) whereas all the other genotypes represent cultivated soybeans (*G. max*).

Table S2. TK780 (*Glycine max*) × B01167 (*G. soja*) recombinant inbred line (RIL) inoculated with ClYVV-No.30-GFP and evaluated at various days post-inoculation (dpi) for the expression of GFP, symptoms, presence of virus or virulence.

RIL	7 dpi	14 dpi	21 dpi	28 dpi	PCR	virulence	RIL	7 dpi	14 dpi	21dpi	28 dpi	PCR	virulence
001	0/3[*1]	0/3	0/3	0/3	-[*2]	1[*3]	046	0/3	0/3	0/3	2/3	nt	2
002	0/4	0/3	0/3	0/3	-	1	047	0/3	0/3	0/3	3/3	nt	2
004	0/4	0/4	0/4	1/4	+	2	048	0/3	3/3	3/3	3/3	+	4
005	0/3	3/3	3/3	3/3	+	4	051	0/3	3/3	3/3	3/3	nt	4
009	0/3	0/3	0/3	0/3	-	1	052	0/2	0/2	0/2	1/2	nt	2
012	0/4	0/4	0/4	3/4	nt	2	054	0/4	0/4	4/4	4/4	nt	3
013	0/2	2/2	2/2	2/2	+	4	055	0/3	3/3	3/3	3/3	+	4
014	0/3	0/3	0/3	3/3	nt	2	056	0/3	0/3	2/3	3/3	nt	3
015	0/3	0/3	1/3	3/3	nt	2-3	057	0/3	0/3	3/3	3/3	nt	2
016	0/3	0/3	3/3	3/3	nt	3	058	0/3	0/3	3/3	3/3	nt	3
018	0/3	0/3	3/3	3/3	nt	3	059	0/3	3/3	3/3	3/3	+	4
020	0/3	0/3	1/3	1/3	nt	2	060	0/3	0/3	0/3	0/3	-	1
021	0/3	0/3	0/3	3/3	nt	2	061	0/3	0/3	0/3	3/3	nt	2
022	0/3	0/3	3/3	3/3	nt	3	062	0/3	0/3	2/3	3/3	nt	3
023	0/2	0/2	2/2	2/2	nt	3	063	0/3	0/3	0/3	3/3	nt	2
025	0/2	2/2	2/2	2/2	+	4	064	0/2	0/2?	0/2	2/2	nt	4
026	0/3	0/3	0/3	0/3	-	1	065	0/3	3/3	3/3	3/3	nt	4
028	0/3	0/3	2/3	3/3	nt	2-3	066	0/2	2/2	2/2	2/2	+	4
029	0/1	1/1	1/1	1/1	nt	4	067	0/3	0/3	0/3	3/3	nt	2
030	0/3	0/3	0/3	3/3	nt	3	068	0/3	0/3	0/3	3/3	nt	2
031	0/4	0/4	0/4	0/4	nt	1	069	0/3	0/3	0/3	0/3	-	1
032	0/2	2/2	2/2	2/2	nt	4	070	0/3	1/3	2/3	3/3	nt	2-4
033	0/3	3/3	3/3	3/3	nt	4	071	0/3	1/3	2/3	3/3	nt	3-4
034	0/2	0/2	2/2	2/2	nt	3	072	0/3	0/3	3/3	3/3	nt	2
037	0/3	3/3	3/3	3/3	nt	4	073	0/1	0/1	1/1	1/1	nt	3
038	0/2	0/2	1/2	2/2	nt	2-3	074	0/2	0/2	2/2	2/2	nt	3
039	0/3	0/3?	3/3	3/3	nt	3	075	0/3	0/3	0/3	0/3	-	1
040	0/3	0/3	0/3	0/3	-	1	076	0/3	0/3	0/3	3/3	nt	2
041	0/3	0/3	0/3	0/3	+[*4]	2	077	0/3	0/3	3/3	3/3	nt	3
042	0/2	0/2	2/2	2/2	nt	3	078	0/2	2/2	2/2	2/2	+	4
043	0/2	1/2	1/2	2/2	nt	3	083	0/3	1/3	3/3	3/3	nt	4
045	0/3	0/3	0/3	3/3	nt	2	084	0/4	4/4	4/4	4/4	+	4

*1 Number of plants showing symptoms or GFP signal/total number of plants tested

*2 + and - indicate that ClYVV genomic RNA was detected (+) or not (-) in noninoculated upper leaves by RT-PCR. nt indicates not tested.

*3 For scoring for virulence of ClYVV in each of RIL see Fig. 6.

*4 RIL (no. 41) where all the inoculated plants expressed no symptoms or GFP signal even at 28 dpi, was scored as Type 2 virulence mainly because RT-PCR detected ClYVV-no. 30 in systemically infected leaves.

Table S3. Oligonucleotide primers used to construct ClYVV-No.30-GUS

Name	Sequences[a] (5'-3')	Position[b]
C-GUS-1	*ACTCCAAAAA**CTGCAG**AAAAACTGAAAGTG*	620-649
C-GUS-2	ACAGGACGGACCAT*AGAGAATTCTCTTATCCTAC*	1099-1080
C-GUS-3	*AAGAGAATTCTCT*ATGGTCCGTCCTGTAGAA	1087-1099
C-GUS-4	CTCTGCGC***CTGCAG***ATTGGAAAACAAATTTCATTTGTTTGCC TCCCTGC	8576-8595
C-531S	*AGAGATCGATCCTGATGCTG*	531-550
C-1195a	*CCATCACAGAACCACATTG*	1213-1195

[a] ClYVV sequences are italicized, *uidA* sequences are underlined, NIa Cleavage recognition site is bordered and *Pst*I sequences are bold.

[b] The position of oligonucleotides on the ClYVV genome are based on the full-length sequences of ClYVV (GenBank accession no. NC_003536).

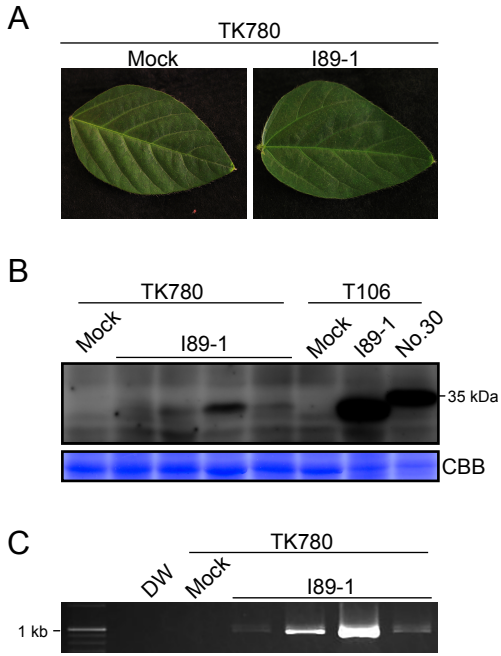


Fig. S1. Reactions of TK780 cultivated soybean plants to ClYVV-I89-1 inoculation. (A) TK780 soybean plants that were mechanically inoculated on primary leaves with ClYVV-I89-1 developed no symptoms or displayed weak mottling and mosaic at 35 days postinoculation (dpi) on noninoculated leaves. (B) Level of accumulated viral CP of ClYVV-I89-1 in noninoculated leaves of TK780 plants was lower than the level detected by western blotting in those of susceptible wild soybeans (T106) inoculated with ClYVV-I89-1 or ClYVV-No.30. Coomassie brilliant blue (CBB) stained gel is shown as a loading control (lower panel). (C) RT-PCR detected viral genomic RNA in noninoculated, systemically infected leaves of all four TK780 plants at 28 dpi following mechanical inoculation of primary leaves with ClYVV-I89-1. DW is a product of RT-PCR without RNA extract from a sample.

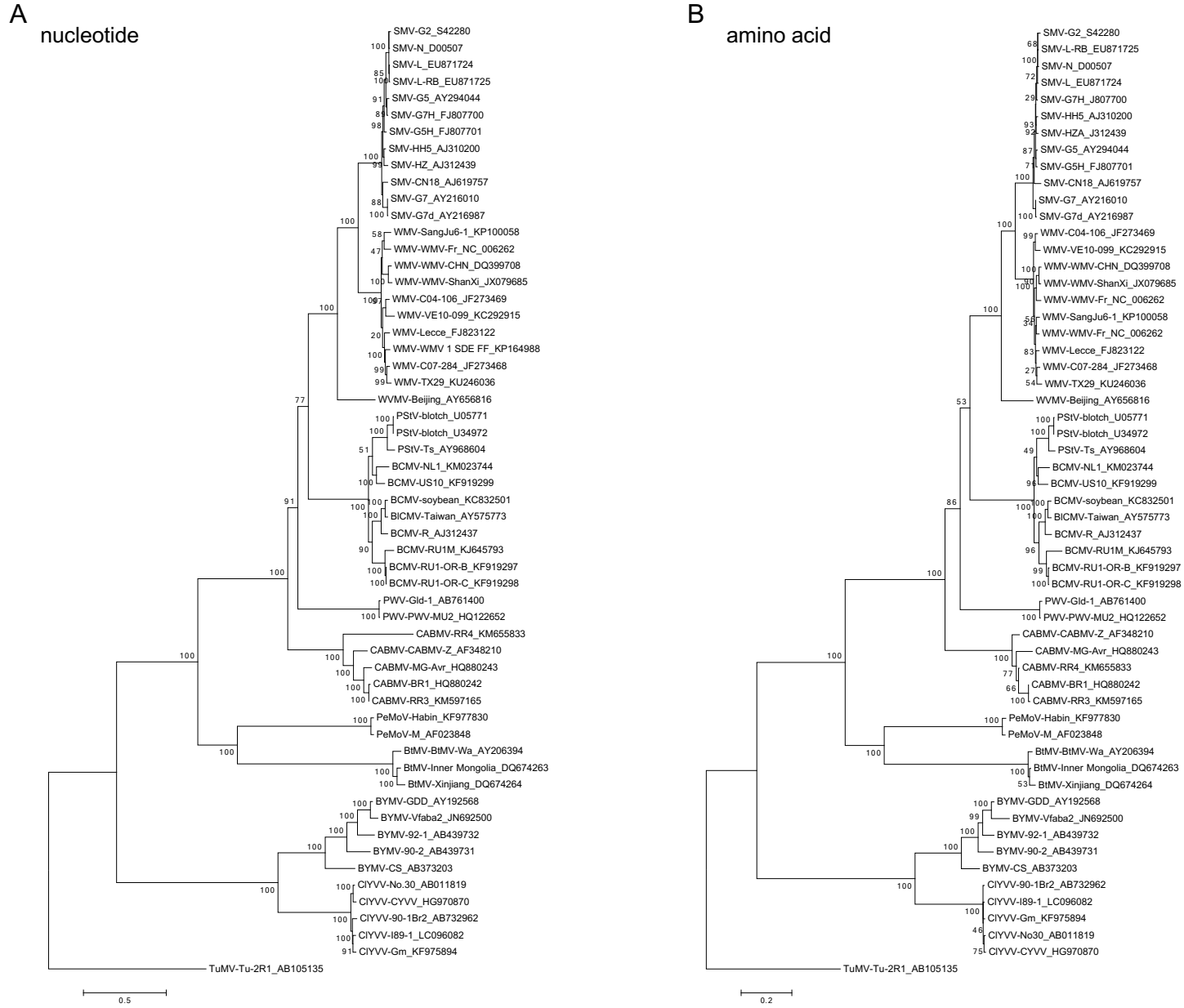
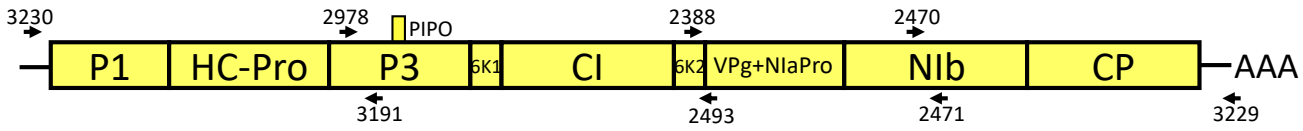


Fig. S2. Phylogenetic analysis of full-length nucleotide sequences of open reading frame (ORF) encoding the polyprotein (A) or deduced amino acid sequence of the encoded polyprotein (B). The sequences were aligned by using MUSCLE, and maximum likelihood tree was inferred. The significance of the nodes was estimated with 1,000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Turnip mosaic virus (TuMV-Tu-2R1) was set as an outgroup. Each accession number is indicated after the underscore of each taxon name. Abbreviations are BCMV = bean common mosaic virus; BYMV = Bean yellow mosaic virus; BtV = beet mosaic virus; BlCMV = blackeye cowpea mosaic virus; ClYVV = clover yellow vein virus; CABMV = cowpea aphid-borne mosaic virus; PWV = passion fruit woodiness virus; PeMoV = peanut mottle virus; PStV = peanut stripe virus; SMV = soybean mosaic virus; TuMV = turnip mosaic virus; WMV = watermelon mosaic virus; WVMV = wisteria vein mosaic virus.

A

Primers	Nucleotide sequences (5'-3')
3230	CACCAAAATATAAAATCAATACAAGACAAATACAGACAAAGC
2978	GAGTCAGATTTGAAGTTTTACAGAGTTGG
2493	GTTCTCTTACTTTTCCCTTGG
2388	CAGTCAGAGAGCTCAAATATG
2471	CTTGATTATTGTACCATCAGG
2470	CTTTAGACCTATGATGGGC
3229	TTTTTTTTTTTTTTTTTTTTCTCGCTCTATAAAGATCAGATTCACAACGAGGTATAACC
2552	TGATGTGCGGCTTAATGGTC
3435	GTGTGCTGACATTCAGCTTTTC
3130	GAATGACCTAAGTAAGTTCATAAACAAGATTTCCTC
2481	GCCAGCGACAAGTTCAGCG
157	GATGTCAGATCTCACTTGAC
2621	AAGCAACTCAAAAGTGATCC
2559	TCATGTACACAGTTAACTTGG
2464	TGCTTGCTAACAAATGAGTC
3436	GATGTTGATTTGTAATGATCACCG
2470	CTTTAGACCTATGATGGGC
2451	CGTGCTGTTCTTGATGGATC
2491	CTAAAATCGTGCTCCAGCAATG
2372	GTACTGGTTTTTGTTTTAGG
4235	GAAAGAGTAGTCTCAATCCT

B



C

Fig. S3. Sequences of primers used for determining genome sequences of ClYVV (A) and their positions on ClYVV genome (B) are shown. (C) This panel shows a portion of alignment of nucleotide sequences of the main ORF (upper panel) and a portion of alignment of the corresponding amino acids from the encoded polyproteins (lower panel) among the original ClYVV-No.30 clone (DDBJ/ENA/GenBank accession no. AB011819), progeny viruses derived from wild-soybean B01167 scion (ClYVV-No.30-GFP) and progeny viruses derived from systemically infected cultivated soybean TK780 rootstock (tentatively designated ClYVV-TK), which was graft-inoculated with the wild-soybean B01167 scion infected with ClYVV-No.30-GFP. RT-PCR amplicons consisted of the entire ORFs were generated while using total RNAs isolated from systemically infected leaves of the scion and the rootstock as templates and directly sequenced. It should be noted that sequences of both progeny viruses derived from scion and rootstock differed only by a single common nucleotide and amino acid compared with the corresponding sequences of the original parental clone. Hence, systemic infection of ClYVV-No.30 in cultivated soybean TK780 was not associated with emergence of a new adaptive mutation.