

Identification of natural sources of resistance to bipartite begomovirus TYLCKaV in *Solanum melongena*

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Abstract

Yellow leaf curl disease caused by begomoviruses has emerged as a major limitation to the production of eggplant (*Solanum melongena*) in several regions of the world. Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) is a bipartite begomovirus isolated from diseased eggplant in the areas of the Indochinese peninsula, South China, and Maritime Southeast Asia. In this study, five begomovirus species isolated in Indonesia, including TYLCKaV, were inoculated to eggplant using infectious clones. Among the inoculated begomoviruses, TYLCKaV alone induced typical yellowing and curling symptoms in eggplant. Inoculation of TYLCKaV to a panel of crop species (eggplant, tomato, pepper, tobacco, cucumber, melon, and squash) commonly grown in Indonesia clarified that TYLCKaV infects and causes yellow leaf curl disease mainly in eggplant and tomato. Agroinfiltration of TYLCKaV to 736 accessions of eggplant enabled selection of 33 resistant candidates. Further evaluation by TYLCKaV graft-inoculation of the resistant candidates clarified that 10 accessions were classifiable as moderately resistant and 23 accessions as highly resistant. Among the highly resistant accessions, 22 accessions restricted viral DNA accumulation to a significantly lower amount than the susceptible control. These 22 highly resistant accessions represent valuable genetic resources for breeding begomovirus resistance in eggplant.

Introduction

The genus *Begomovirus* of the family *Geminiviridae* comprises 445 species (ICTV 2021), and thus is the largest plant-infecting virus genus. Begomoviruses have circular and single-stranded (ss) DNA genomes comprising either one or two genomic components (DNAs A and B) encapsidated in twinned icosahedral virions (Briddon et al. 2010). DNA A encodes a replication-associated protein (Rep), a coat protein, and proteins that participate in the control of replication and gene expression, whereas DNA B encodes proteins required for nuclear trafficking and cell-to-cell movement of the viral DNA. Begomoviruses are naturally transmitted by the insect vector whitefly (*Bemisia tabaci*) and severely threaten crop production worldwide.

Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) is a bipartite begomovirus first reported to infect tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*) in Kanchanaburi Province, Thailand, in 2001 (Green et al. 2003). Our previous field survey of begomoviruses infecting solanaceous and cucurbit crops cultivated in Aceh Province in Indonesia inferred that TYLCKaV is the predominant begomovirus that infects eggplant (Kesumawati et al. 2020). Given that TYLCKaV has been isolated from solanaceous crops, including eggplant in Vietnam (Ha et al. 2008), Laos (Tang et al. 2014), Cambodia (Bagewadi and Naidu 2016), Indonesia (Koeda et al. 2016), China (Zhang et al. 2018), and Myanmar (Kwak et al. 2022), this virus is indicated to pose a severe constraint to eggplant production in these areas.

In temperate regions, chemical control with insecticides and installation of fine-mesh screens across greenhouse openings to limit the vector population, in combination with the cultivation of virus-resistant cultivars, are widely implemented measures to control begomoviral-caused yellow leaf curl disease.

However, chemical control has been only partially effective because most crops in tropical and subtropical regions are grown in open fields. This has led to strong demand for genetic resistance to begomoviruses for improved control of diseases, and the cloning of resistance genes in tomato and pepper has enabled marker-assisted breeding of resistant cultivars (Verlaan et al. 2013; Lapidot et al. 2015; Yamaguchi et al. 2018; Koeda et al. 2021, 2022). However, breeding for begomovirus resistance is much less advanced in eggplant compared with that in tomato and pepper. To the best of our knowledge, no genetic source of resistance against begomoviruses in eggplant has been reported previously.

In this study, we used infectious clones of five begomoviruses isolated in Indonesia to confirm that TYLCKaV is the predominant species that causes yellow leaf curl disease in eggplant. In addition, natural genetic sources of resistance to TYLCKaV were screened from among 736 accessions of eggplant.

Materials And Methods

Plant material

Plants of eggplant (*S. melongena*) 'Senryo nigo' (SN), tomato (*S. lycopersicum*) 'Momotaro', melon (*Cucumis melo*) 'Otome', cucumber (*Cucumis sativus*) 'Natsu suzumi', squash (*Cucurbita maxima*) 'Ebisu' (Takii Seed, Kyoto, Japan), pepper (*Capsicum annuum*) No. 218, and tobacco (*Nicotiana tabacum* 'Samsun-NN' and *N. benthamiana*) were used for evaluation of the host range of TYLCKaV. *N. benthamiana* used in this study is the internationally widespread laboratory line susceptible to many plant infecting viruses. Resistance sources to TYLCKaV were screened from among 736 accessions of eggplant (*S. melongena*). Most of these accessions are local traditional varieties or pre-breeding materials which were mainly collected from Asian countries and preserved at the National Agriculture and Food Research Organization (NARO) Genebank in Japan. Plants were grown in a growth room with the temperature ranging from 23°C to 30°C under a 13 h/11 h (light/dark) photoperiod.

Assessment of eggplant-infecting begomoviruses and host range of TYLCKaV

The bipartite begomoviruses TYLCKaV isolate BA_B6 (GenBank accession number for DNA A: LC051116, DNA B: LC177332), pepper yellow leaf curl Indonesia virus (PepYLCIV) isolate BA_D1-1 (DNA A: LC051114, DNA B: LC314794), pepper yellow leaf curl Aceh virus (PepYLCAV) isolate BAPep-V2 (DNA A: LC387327, DNA B: LC387329), tomato leaf curl New Delhi virus (ToLCNDV) isolate BACu-20 (DNA A: LC511775, DNA B: LC511780), and a monopartite begomovirus ageratum yellow vein virus (AYVV) isolate BA_C1-2 (DNA A-like: LC051118) originally isolated in Indonesia (Kesumawati et al. 2019, 2020; Koeda et al. 2016, 2017, 2018) were used in this study. Infectious clones of TYLCKaV, PepYLCIV, PepYLCAV, and ToLCNDV are described elsewhere (Koeda et al. 2017, 2018, 2021; Yamamoto et al. 2021). An infectious clone for AYVV accompanied by tomato leaf curl Malaysia betasatellite (ToLCMYB) isolate BA_C1-2 (LC511782) was obtained as reported by Koeda et al. (2017) using the primers listed in Supplementary Table S1. *Agrobacterium tumefaciens* strain EHA105 was used for inoculation of eggplant and tomato, and strain GV2260 was used for inoculation of pepper, cucumber, melon, squash, and tobacco. Agrobacterial suspensions with an optical density of 0.1 (eggplant, pepper, and tobacco) and 0.3

(cucumber and melon) were infiltrated through the abaxial surface of the cotyledons of seedlings before the development of true leaves. Agroinoculation of the hypocotyl of tomato and squash seedlings was conducted using the colony inoculation procedure described by Koeda et al. (2017). Seven to eight plants were inoculated for each treatment. Symptom surveys were conducted 28 days postinoculation (dpi), and young upper leaves were collected and stored at – 80°C until DNA extraction.

Screening of resistance to TYLCKaV among eggplant germplasm

In the initial screening of resistance to TYLCKaV, 736 eggplant accessions were agroinfiltrated with TYLCKaV. Two plants per accession were agroinfiltrated using *A. tumefaciens* strain EHA105 according to Koeda et al. (2018) and symptoms were evaluated at 28 dpi. For the second screening of TYLCKaV-resistant eggplant accessions, graft-inoculation was conducted. For graft-inoculation of TYLCKaV, agroinfiltrated susceptible SN plants were used as rootstocks and uninoculated resistant accessions were used as scions. The hypocotyls of rootstock and scion plants were cut when the diameter was approximately 1.7 mm and grafted using grafting clips (Nasunics, Tochigi, Japan). At 28, 42, and 56 days postgrafting, the symptoms of each plant were scored in accordance with the disease severity index (DSI) using an ordinal scale ranging from 0 to 4 as follows: 0, no symptoms; 1, very mild symptoms; 2, mild symptoms; 3, moderate symptoms; and 4, severe symptoms. Young upper leaves were collected at 28, 42, and 56 days postgrafting and stored at – 80°C until DNA extraction.

Diagnosis of begomovirus infection

DNA was extracted from the collected leaves using the Nucleon PhytoPure Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Specific primers for TYLCKaV, PepYLCIV, PepYLCAV, ToLCNDV, and AYVV were used (Kesumawati et al. 2019, 2020). Polymerase chain reaction (PCR) tests for viral DNA detection were performed using the EmeraldAmp PCR Master Mix (Takara Bio). The amplified PCR products were subjected to electrophoresis using 1.0% (w/v) agarose gel. Primer sequences and the PCR conditions used are listed in Supplementary Table S1.

Quantification of TYLCKaV titer by real-time PCR

The TYLCKaV DNA was quantified using the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) as previously described (Koeda et al. 2020, 2021). The DNA A component of TYLCKaV was detected by quantitative PCR (qPCR) using the TYLCKaV A Real 2F and 2R primers, which amplified an 80-bp fragment. Data from the total DNA extracts were normalized relative to a 93-bp DNA fragment from the genomic 25S ribosomal-RNA multicopy gene amplified by qPCR using the 25S-rRNA 2F and 2R primers. The primers 25S-rRNA 2F and 2R were initially designed for the quantification of tomato genomic DNA in our previous study (Koeda et al. 2020). By BLASTn analysis, it was confirmed that this primer can also be used for the quantification of eggplant genomic DNA. The qPCR assays were performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) under the following conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 5 s, and 58°C for 10 s.

Statistical analysis was performed using Student's *t*-test and a *p*-value less than 0.05 was considered to be statistically significant. Primer sequences used for real-time PCR are listed in Supplementary Table S1.

Results

TYLCKaV is the predominant begomovirus infecting eggplant

Infectivity of eggplant SN and symptoms caused by isolates of TYLCKaV, PepYLCIV, PepYLCAV, ToLCNDV, and AYVV were assessed by agroinfiltration (Table 1). Because agroinfiltrated *N. benthamiana* plants (*n* = 8 for each begomovirus) showed disease symptoms, infectious clones used in this study were functional. At 28 dpi, TYLCKaV-infected SN showed typical begomoviral symptoms of yellowing, curling, and stunting. Although PepYLCAV infected SN, no disease symptoms were observed. Neither PepYLCIV nor ToLCNDV infected SN. Similar results were obtained in a repeated experiment. A single SN plant was infected by AYVV without symptoms in the first experiment, but no infection was detected in the second experiment. These results showed that TYLCKaV is the predominant begomovirus causing yellow leaf curl disease in eggplant.

To survey the host range of TYLCKaV, a panel of crop species (eggplant, tomato, pepper, *N. tabacum*, cucumber, melon, and squash) commonly grown in Indonesia were agroinfiltrated or agroinoculated. TYLCKaV was able to infect and caused disease symptoms in eggplant and tomato but not in tobacco, pepper, cucumber, and squash (Table 2). Interestingly, some of the TYLCKaV-inoculated melon plants were infected without showing disease symptoms. Symptomology observations were consistent across replications. From these results, we concluded that eggplant, tomato, and melon are the hosts of TYLCKaV from the list of the species tested in this study, and this virus causes yellow curl disease mainly in eggplant and tomato.

Screening of TYLCKaV-resistant accessions of eggplant

Following agroinfiltration, the eggplant accessions were screened for their reactions to TYLCKaV on the basis of development of disease symptoms. Of 736 accessions, 703 accessions were highly susceptible to TYLCKaV. In contrast, 33 accessions which were symptomless or only with slight symptoms were selected as TYLCKaV-resistant candidates. Five out of the 33 accessions showed slight TYLCKaV symptoms, and nine symptomless plants were positive for TYLCKaV based on conventional PCR detection. On the other hand, the remaining 19 symptomless accessions were negative for TYLCKaV detection by conventional PCR. Agroinfiltration of these 33 accessions was repeated and similar results were obtained. From these similar outcomes, we selected the 33 accessions as moderately and highly resistant candidates to TYLCKaV.

To further evaluate the resistance of the candidate accessions, TYLCKaV was graft-transmitted via a symptomatic SN rootstock to scions of the candidate accessions and symptoms were evaluated at 28, 42, and 56 days postgrafting (Table 3). Successfully grafted plants were all positive for TYLCKaV infection by conventional PCR detection and/or qPCR detection. The average disease severity index (DSI)

scores for the complete experimental period for the graft-inoculated susceptible controls SN and No. 47 were 3.6 and 2.8, respectively. In contrast, the average DSI scores of the candidate resistant accessions were less than 2. We classified the degree of resistance into three categories: susceptible (DSI score $\geq$ 2.5), moderately resistant (DSI score $\geq$ 1), and resistant (DSI score $<$ 1). Of the 33 accessions, 10 accessions were classified as moderately resistant and 23 accessions as highly resistant (Table 3; Fig. 1).

To assess the association between symptom expression and accumulation of viral DNA, quantification of TYLCKaV viral DNA was conducted by qPCR using DNA extracted from systemic young upper leaves collected at 28, 42, and 56 days postgrafting (Fig. 2). The highly resistant accessions, with the exception of No. 754, restricted viral DNA accumulation (mean accumulating viral DNA included in Table 3) to a significantly lower amount than that of the susceptible control SN throughout the experimental period (Log_{10} less than -0.7 with minimum value of -5.2). In contrast, of the 10 moderately resistant accessions, only No. 763 and No. 766 restricted viral DNA accumulation to an amount significantly lower than that of the susceptible control SN throughout the experimental period. From these results, we concluded that there was a good correspondence in general between the symptomology observed and the amount of TYLCKaV viral DNA accumulated.

Discussion

Over the past three decades, epidemics of diseases caused by begomoviruses have emerged to be an important constraint to the production of solanaceous crops in many tropical and subtropical regions of the world (Kenyon et al. 2014). Genetic resistance to begomoviruses is an ideal means of controlling the diseases, and the isolation of several resistance genes from tomato and pepper has accelerated marker-assisted breeding; the genes *Ty-1/Ty-3*, *Ty-2*, and *ty-5* provide resistance against tomato yellow leaf curl virus, and *pepy-1* and *PepY-2* provide resistance against PepYLCIV and PepYLCAV (Verlaan et al. 2013; Lapidot et al. 2015; Yamaguchi et al. 2018; Koeda et al. 2021, 2022). However, breeding for resistance to begomoviruses is much less advanced in eggplant compared with that in tomato and pepper. In the present study, we identified multiple eggplant accessions resistant to TYLCKaV, which is widespread in Southeast Asia, through large-scale germplasm screening.

In the present study, five begomovirus species present in Indonesia were inoculated to eggplant using infectious clones. Among the inoculated plants, only those inoculated with TYLCKaV showed typical begomoviral yellowing and curling symptoms (Table 1). This finding is consistent with our previous field survey conducted in Indonesia in which only TYLCKaV was detected from diseased eggplant plants (Kesumawati et al., 2020). Moreover, Charoenvilaisir et al. (2020) surveyed the infection of 15 begomovirus species in Thailand and only TYLCKaV was detected from symptomatic eggplant. It should also be noted that agroinfiltrated PepYLCAV consistently infected eggplant without causing any symptoms (Table 1). PepYLCAV is a recombinant begomovirus of PepYLCIV isolated from pepper, tomato, and tobacco in Indonesia (Kesumawati et al. 2019). Given that PepYLCIV was not able to infect eggplant in our present repeated experiments, PepYLCAV might have attained a broader host range through recombination. Parrella et al. (2020) isolated the Es strain of ToLCNDV from diseased eggplant in

Italy. In the present study, the Southeast Asian isolate of ToLCNDV was not able to infect eggplant (Table 1), and an identical result was obtained for ToLCNDV-[ES-Alm-Cuc-16] (DNA A: LC596380, DNA B: LC596383), an Es strain of ToLCNDV (data not shown). From these results, we concluded that TYLCKaV is the predominant begomovirus species infecting and causing yellow leaf curl disease in eggplant.

Inoculation of TYLCKaV to solanaceous and cucurbit crops resulted in infection of eggplant and tomato accompanied with diseased symptoms (Table 2). This result is consistent with previous findings that most isolates of TYLCKaV have been identified from eggplant or tomato (Green et al. 2003; Ha et al. 2008; Tang et al. 2014; Bagewadi and Naidu 2016). Therefore, TYLCKaV should be the target of resistance breeding of eggplant in Southeast Asian countries. Moreover, because resistance conferred by *Ty-2* is ineffective against TYLCKaV, and the resistance conferred by *Ty-3a* is only partially effective (Koeda et al., 2020), TYLCKaV is also an important target of resistance breeding in tomato. In the repeated experiments, melon was infected by TYLCKaV without any disease symptoms (Table 2). It should be noted that melon plants may serve as reservoir host for disease spread.

Agroinfiltration of TYLCKaV to 736 eggplant accessions revealed that 703 accessions were susceptible and enabled the selection of 33 accessions as resistant candidates from repeated experiments. To analyze the resistance of these candidate accessions in further detail, we conducted graft-inoculation of TYLCKaV. Graft-inoculation of a begomovirus is a reliable method to deliver a begomovirus to plants without inoculation escapes (Anaya-López et al. 2003; Vanderschuren et al. 2012; Koeda et al. 2018, 2021, 2022; Mori et al. 2022). From the graft-inoculation experiment, 23 accessions were classified as highly resistant and 10 accessions as moderately resistant to TYLCKaV (Table 3). Agroinfiltration and graft-inoculation consistently demonstrated that these 33 accessions are resistant to TYLCKaV. Moreover, 22 highly resistant accessions restricted viral DNA accumulation to a significantly lower amount than that of the susceptible control SN throughout the experimental period at 28 to 56 days postgrafting (Fig. 2). Because restriction of systemic virus accumulation occurred in highly resistant plants, it is suggested that viral replication or viral movement (cell-to-cell or long distance) may have been impaired.

TYLCKaV is reported to infect eggplant in the areas of the Indochinese peninsula, South China, and Maritime Southeast Asia as the predominant begomovirus causing yellow leaf curl disease. In this study, we selected TYLCKaV-resistant eggplant accessions based on experimental inoculations using an infectious clone. The 22 highly resistant accessions represent a valuable genetic resource for resistance breeding. Further genetic studies are needed to accelerate the marker-assisted breeding for begomovirus resistance in eggplant.

Declarations

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Author contributions

KK and SK designed experiment, interpreted the results, and wrote the manuscript. KK analyzed the host range of TYLCKaV and evaluated the begomovirus resistance of eggplants. MT prepared the infectious clone of AYVV. EK provided the material. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Tables 1-3 are available in the Supplementary Files section.

Figures

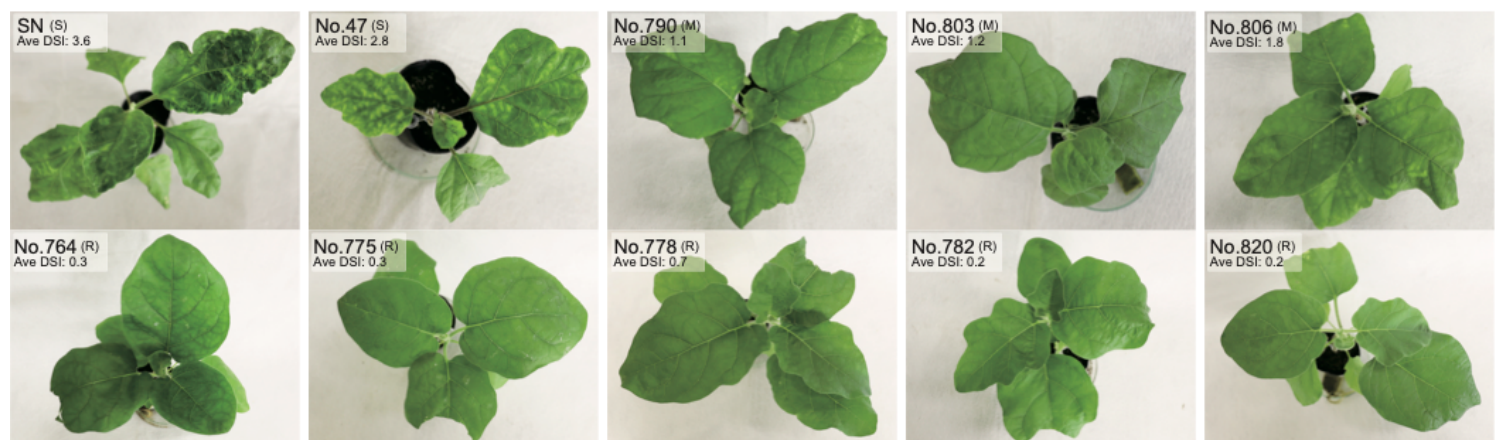


Figure 1

Disease symptoms of susceptible, moderately resistant, and highly resistant eggplants infected with tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) by grafting. The disease severity index (DSI) score was assessed using an ordinal scale ranging from 0 to 4 as follows: 0, no symptoms; 1 very mild symptoms; 2, mild symptoms; 3, moderate symptoms; 4, severe symptoms. SN = ‘Senryo nigo’ and No.47 are the susceptible controls. S, M, and R indicate susceptible, moderately resistant, and highly resistant, respectively. Pictures were taken at 56 days from grafting.

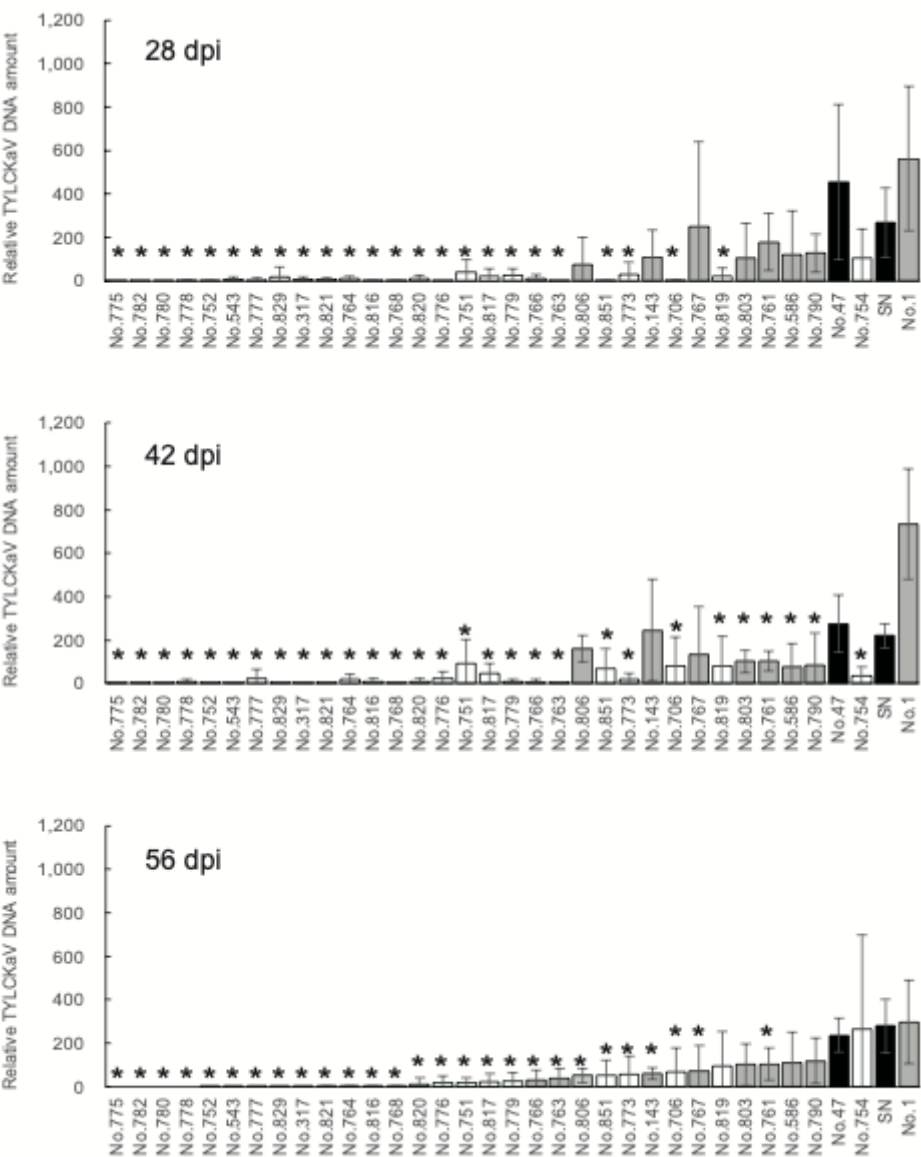


Figure 2

Relative quantification of graft-infected tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) DNA in eggplant accessions. Young upper leaves in the 33 eggplant accessions selected as resistant candidates and the two susceptible controls were collected at 28, 42, and 56 days after grafting for DNA extraction. SN = ‘Senryo nigo’ (a susceptible cultivar). Black, gray, and white bars indicate susceptible, moderately resistant, and highly resistant, respectively. Viral DNA values were normalized to genomic DNA from the

25S rRNA multicopy gene. An asterisk indicates a significant difference from the mean for SN (Student's *t*-test, $P < 0.05$).

Supplementary Files

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