



Actinidia seed-borne latent virus (ASbLV, *Betaflexiviridae*) is transmitted paternally and maternally at high rates

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Abstract

Actinidia seed-borne latent virus (ASbLV, *Betaflexiviridae*, genus Prunevirus) was detected at high frequency in healthy seedlings grown from lines of imported seed in a New Zealand post-entry quarantine facility. To determine the route and efficiency of transmission of ASbLV in this dioecious crop species, we developed a rapid molecular protocol and identified a reliable progeny plant tissue to determine paternal and maternal transmission rates. The virus was detected at a high incidence (98%) in individual seeds, but cotyledon testing of seedlings from selected crosses confirmed staminate (male) transmission at high frequency (~60%), and pistillate (female) transmission at even higher frequency (~80%). The use of cotyledons allows non-destructive detection of ASbLV in very young seedlings that enables early screening of kiwifruit plants in nurseries to manage its spread to orchards. The high ASbLV transmission rates, whether from infected pollen or ovules, facilitate bulk testing of seed lots that could quickly detect infected parent plants (fruit bearing female or male pollinator) already in an orchard. The dioecious nature of *Actinidia* may provide a useful biological tool to further investigate ASbLV movement, transmission biology, and ultimately its impact on infected *Actinidia* plants.

Keywords Virus · Seed · Transmission · Cotyledons · Pollen · Kiwifruit

Introduction

In 2018, Veerakone et al. described the genome of actinidia seed-borne latent virus (ASbLV, a new member of the *Betaflexiviridae*, genus Prunevirus) in kiwifruit (*Actinidia* species) grown from seed in New Zealand but originating from China. Since *Actinidia* species are dioecious, viruses may be transmitted via pollen from the staminate (male) plants and/or from the pistillate (female) plants that bear the ovaries that form the fruit. Ovules in each fruit are

collectively fertilised by approximately a thousand pollen grains, with each fertilisation event resulting in a single seed. The majority of kiwifruit material transiting through quarantine into New Zealand has been seed, with some pollen or budwood also imported. The New Zealand kiwifruit industry mainly uses seed propagated rootstocks grafted to clonally propagated scions, though some clonally propagated rootstocks are also available.

Seed infection, including virus present in and/or on the seed, may result from either the maternal or paternal parent, or both (Albrechtsen 2006). Maternal transmission can result from indirect invasion of the embryo via infected meristematic tissue and derived megaspore mother cells; direct invasion of the embryo via the suspensor as a transient pathway; or by infection of maternal seed parts (e.g., integuments of the ovule) without embryo invasion. For instance, the potyvirus pea seed borne mosaic virus is able to infect the maternal tissue of the micropylar region, move via symplastic pore-like openings between the seed coat and endoplasm, to access the suspensor cells and directly invade the embryo in early development (Roberts et al. 2003). Paternal transmission can result from viruses being carried on or in

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pollen tubes to infect the embryo or endosperm at fertilisation or by viruses escaping from pollen tubes and thence onto or into the ovule (i.e., the prospective seed, Isogai et al. 2015). Furthermore, even if the embryo remains virus-free, the process of germination can result in mechanical infection of the emerging seedling via physical interaction with the seed coat carrying a virus. For example, ribgrass mosaic virus (Tobamovirus), that infects *Actinidia* (Chavan et al. 2012), can be transmitted from the surface of the seed coat.

There are several technical barriers to detecting the presence of seed transmitted viruses and understanding their biology, which has significance for detection in quarantine and subsequent management within germplasm collections and bred progeny. Firstly, direct screening of seed is destructive thereby requiring either inference of untested seed from the same lot or an alternative, non-destructive method. Secondly, seed transmission is typically at low rates therefore high-test numbers from each seed lot is required to gain confidence in a negative infection result. Thirdly, vertical transmission favours viruses that are not deleterious to their host and thus generally form asymptomatic infections (Villamor et al. 2019). Such asymptomatic infections may provide benefit to the host but undermine the importance of inspection methods in a quarantine context and visual assessment within orchards. The asymptomatic nature has led to under-representation within the literature and the development of few specific tests to detect asymptomatic viruses, although their identification is being remedied through high throughput sequencing (Villamor et al. 2019). The dioecious host kiwifruit offers a useful biological tool to understand the pollen and/or ovule transmission of ASbLV, to develop appropriate methods to retain ASbLV-free orchards if desired, and to generate biological resources for future host-virus interaction research. This study aimed to develop a non-destructive, reliable and rapid molecular method to detect ASbLV in F1 progeny and to determine the route and efficiency of ASbLV transmission.

Materials and methods

To study transmission from the mega-gametophytes, the same ASbLV infected female was crossed with two unrelated males. For transmission from the micro-gametophyte, two unrelated, ASbLV infected males were crossed with two unrelated females; the ASbLV infected female and one of the ASbLV infected males were half-siblings, having originally been derived from seeds of the same fruit.

Crosses, seeds extraction and plant growth

Seed lots 'C15', 'C53', 'T66' and 'X84' were obtained as single-fruit extracted seed sublots derived from four separate controlled crosses performed between asymptomatic yet ASbLV-infected and ASbLV-uninfected *Actinidia chinensis* var. *deliciosa* parents (Fig. 1). Both the infected and non-infected asymptomatic parents were originally sourced as seed imported into New Zealand from open-pollinated fruit collected from wild vines in China. A range of crosses between ASbLV-infected and non-infected parents were performed in December 2015 using field-grown vines. The post entry quarantine (PEQ) facility housed uninfected *Actinidia* plants grown under a level one containment (established under the New Zealand's Biosecurity Act 1993) that were used as negative controls. These plants had previously been tested in the Palmerston North diagnostic laboratory by using reverse transcription-polymerase chain reaction (RT-PCR) as ASbLV negative. The ASbLV infection status of the parents was assessed by RT-PCR targeting a 278 bp sequence of the RNA-dependent RNA polymerase (RDRP) gene of the virus by testing leaf samples collected in March and December 2015 from field-grown vines and repeated in August 2016 by testing newly shoot buds just emerging from dormant budsticks.

On female vines, the terminal ends of shoots containing flower buds were covered with a brown paper bag prior to anthesis, and then sealed by folding and stapling so that the flowers were completely enclosed in the bag. Flower buds from male vines were collected just before the flower buds opened; anthers were extracted from the flower buds and air-dried to extract the pollen. Pollinations were then performed in the field by briefly opening the paper bags once the flowers inside had opened, and applying the isolated pollen using a small paintbrush. New brushes were used for each pollination. The flowers were then sealed in the paper bags again and left until fruitlets formed (December) when each paper bag was exchanged with an onion bag (5 mm mesh bag). Fruit from the crosses were harvested in May 2016 and seeds were extracted.

Whole seeds from lots 'X84', 'T66', 'C15' and 'C53' extracted from single fruit, respectively, were tested for the presence of ASbLV (Tables 1 and 2). Each seed lot consisted of over 1000 seeds extracted from a single kiwifruit berry. Seed from lots 'C15' and 'C53' are from the same ASbLV-infected *Actinidia* mother plant (DA51_03), which is a sibling to the infected male DA51_05 used to produce seed lot 'T66' (Fig. 1). Seed lot 'X84' was generated using an unrelated infected male DA102_03 and uninfected female DA64_02.

Prior to sowing, 100 *Actinidia* seeds from each test cross were surfaced sterilized in 1% bleach (sodium hypochlorite)

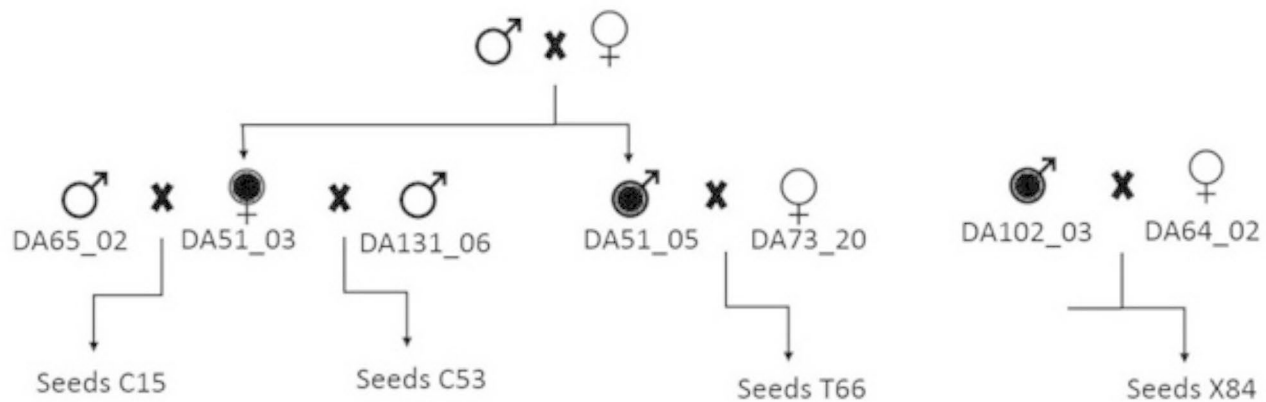


Fig. 1 Identification and relationship of parents used for crosses that generated seeds tested for actinidia latent seed-borne virus (ASbLV) transmission from maternal and paternal *Actinidia deliciosa* parents. Seeds of lot 'X84' were tested destructively as whole seeds, or grown before cotyledons, true leaves and roots were tested. Seeds of lot 'C15', 'C53' and 'T66' were tested by assay of cotyledons only. Open circles indicate uninfected parents and filled circles indicate ASbLV infected parents

and air dried under laminar flow prior to soaking in 1 mL of 3000 ppm gibberellic acid for 24 h, then spread over 100 mm diameter moist Whatman® filter paper (Buckinghamshire, United Kingdom). Each seeded filter paper was placed on top of a half filled pasteurised medium grade bark/

pumice potting media (Daltons Ltd Matamata) in 150 mm punnet container and covered with 4 mm particle size sterile sand. Seeds were germinated at 22.5°C for 1 week and 50 healthy looking seedlings were individually transplanted into 55 mm plastic pots filled with potting mix for growth.

Table 1 Transmission from infected staminate (male) parents used for crosses that generated seeds tested for actinidia latent seed-borne virus (ASbLV) infection. Seeds of lot 'X84' were tested destructively as whole seeds, or grown before cotyledons, true leaves and roots were tested. Seeds of lot 'T66' were tested by assay of cotyledons only

Cross ID	Pistillate vine ID ♀	Staminate vine ID ♂	Total samples (n)	Total infected	ASbLV detection rate (%)	Confidence intervals
'X84' Whole seed	DA64_02 (uninfected)	DA102_03 (infected)	50	49	98	89–100
'X84' cotyledons	DA64_02 (uninfected)	DA102_03 (infected)	50	32	64	47–75
'X84' Juvenile (leaf)	DA64_02 (uninfected)	DA102_03 (infected)	32	18	56	39–75
'X84' Juvenile (Roots)	DA64_02 (uninfected)	DA102_03 (infected)	20	12	60	36–81
'X84' Young leaves	DA64_02 (uninfected)	DA102_03 (infected)	13	8	61	32–86
'X84' Mature leaves	DA64_02 (uninfected)	DA102_03 (infected)	13	8	61	32–86
'T66' (Cotyledons)	DA73_20 (uninfected)	DA51_05 (infected)	49	29	59	44–73

Table 2 Transmission from infected pistillate (female) parents used for crosses that generated seeds tested for actinidia latent seed-borne virus (ASbLV) infection. Seeds of lot 'C15' and 'C53' were tested by assay of cotyledons only

Cross ID	Pistillate vine ID ♀	Staminate vine ID ♂	Total samples (n)	Total infected	ASbLV detection rate (%)	Confidence intervals
'C15' (Cotyledons)	DA51_03 (infected)	DA65_02 (uninfected)	53	43	81	68–91
'C53' (Cotyledons)	DA51_03 (infected)	DA131_06 (uninfected)	46	35	85	61–87

Tests for ASbLV transmission in cotyledons, leaves and roots were carried out when the plants were 1–4 weeks old.

Isolation of total RNA from seeds, cotyledons, leaves and roots

To test for the presence of ASbLV, total RNA was isolated from seeds, cotyledons, leaves (young and mature) and roots from parental and/or progeny *A. chinensis*. For seed, each total RNA isolation used one test seed combined with four *Actinidia* seeds from another source previously tested and known to be non-infected with ASbLV. This approach provided sufficient seeds for their effective grinding. For the extraction of total RNA from cotyledons, a single cotyledon (weighing ~1–8 mg depending on age) was harvested from each seedling 1–2 weeks after emergence. For total RNA extraction from leaf tissue, a fully formed and opened leaf (~10 mg) was harvested from field grown plant or from a seedling 3–4 weeks after emergence. For root tissue, a piece of root tip (~100 mg) was harvested from a seedling 3–4 weeks after emergence. Tissue isolated from plants that had previously been tested by RT-PCR as ASbLV negative that are grown in level 1 PEQ containment (established under Biosecurity Act 1993) were used as negative control. Plants (grown within The New Zealand Institute for Plant & Food Research Limited experimental orchard in Motueka, New Zealand) tested by RT-PCR and known to be infected with ASbLV were used as positive controls.

For all plant sample types, total RNA was isolated by the silica milk-based method developed by Menzel et al. (2002) initially using a grinding bag (10 cm × 8 cm, BIOREBA AG™, Switzerland) and a ball-bearing grinder. The RNA was stored in a freezer at -20°C prior to RT-PCR analysis.

Virus detection by RT-PCR

ASbLV was detected by one-step RT-PCR using ASbLV-specific primers that targeted the RDRP gene (BetaRDRP-F2: GAATCAGACTATGAAGCATTGATGC and BetaRDRP-R2: CACATATCGTCACCTGCAAATGCTATTG), in combination with a primer set that targeted the plant mitochondrial mRNA encoding subunit 5 of the NADH ubiquinone oxidoreductase complex (NAD5) (Menzel et al. 2002) to confirm RNA integrity. Three negative controls were an extract from non-ASbLV infected leaf, buffer and sterile water. Each 20 µL reaction contained PCR 1x PCR buffer (Invitrogen™, ThermoFisher Scientific, USA), DEPC-treated water, 2.5 mM MgCl₂, 0.4 mM dNTPs, each primer (0.4 µM for ASbLV primers, 0.1 µM for NAD5 primers), 5.0 mM DTT, 0.5U Platinum® Taq DNA polymerase and 10U SuperScript™ III reverse transcriptase (ThermoFisher Scientific, USA). Amplification using a thermal

cycler (Eppendorf MasterCycler Gradient, Netheler-Hinz GmbH, Hamburg) was achieved by the following temperature regime: Reverse transcription, 30 min for 48°C; Taq activation, 3 min at 96°C; followed by 35 cycles of amplification 30 s at 95°C, 30 s at 56°C and 45 s at 72°C, with a final extension of 2 min at 72°C. Amplicons were separated by electrophoresis in a 1.5% Ultrapure™ agarose gel in 0.5 × TBE buffer at 10 V/cm for 50 min. The gel was stained with 0.5 µg/ml ethidium bromide for 15 min, and visualised with ultraviolet light.

Statistical analysis

The ratios from male and female sources of ASbLV infection, and the ratios from the different tissue types of the 'X84' seed lot were compared using binomial generalized linear models. Confidence intervals for binomial proportions were calculated using Gentat (VSNi ltd, Hemel Hempstead, UK).

Results

ASbLV was originally identified in imported seed within a New Zealand PEQ facility, and subsequently identified in healthy-appearing *Actinidia* seedling collections. The ASbLV RDRP-specific primers amplified a 278 bp product from positive samples (Fig. 2). All *Actinidia* seeds from lot 'X84' (n = 50) tested positive for ASbLV amplification except one (lane 32) (Fig. 2). The ASbLV detection rate $n=49/50$ whole seeds provides a binomial confidence interval of 89–100% infection, $P=95%$ (Tables 1 and 3). Negative controls showed bands for NAD5 only, and blank extractions (buffer or water controls) did not amplify (Fig. 2). No viral-like symptoms were observed on any parents or progeny throughout the study.

Transmission from infected staminate (male) parents

ASbLV was detected in approximately three-fifths of cotyledons of germinated seedlings from the same 'X84' seed lot ($n=31/50$ detected; confidence interval 47–75% infected, $P=95%$, Tables 1 and 3). When testing juvenile leaves produced from the 32 surviving seedlings (previously tested by cotyledon) approximately half were positive for the ASbLV ($n=18/32$; confidence interval 39–75% infected, $P=95%$, Tables 1 and 3). Of the 32 surviving plants, only 13 had multiple fully formed leaves after 2 weeks' growth. When two leaves from the same plant (one younger and one older) were tested from each of these 13 seedlings, about half of the plants (for both leaf ages) tested positive for ASbLV

Table 3 Infection status of *Actinidia* seedlings from 'X84' cross, tested by end-point reverse transcription-polymerase chain reaction (RT-PCR) for RNA of actinidia seed borne latent virus and using RNA for the plant NAD5 gene as an internal reference. Grey backgrounds indicate dead plants. (PCR target band strength: +++ = much stronger than NAD5 reference; ++ = similar to NAD5 reference, + = weaker than NAD5 reference, - = target not detected.)

Plant ID	Germinating	Juvenile seedling		Young plant	
	Cotyledon	Leaf	Root	Mature	Immature
'X84'-01	+++				
'X84'-02	+++				
'X84'-03	+++				
'X84'-04	+++				
'X84'-05	-	-	-	-	-
'X84'-06	+++	+++	+++	+	+
'X84'-07	-				
'X84'-08	-	-	-	-	-
'X84'-09	+++	+++	+++	+++	+++
'X84'-10	+++	+++	+++	+++	+++
'X84'-11	+++	+++	+++	+	+++
'X84'-12	+++				
'X84'-13	-	-	-	-	-
'X84'-14	+++	+++	+++	++	+
'X84'-15	+++	+++	+++	+++	+++
'X84'-16	-	-	-	-	-
'X84'-17	-	-	-	-	-
'X84'-18	+++	+++	+++	+	+++
'X84'-19	+++	+++	+++	++	+++
'X84'-20	+++	+++	+++		
'X84'-21	-	-	-		
'X84'-22	-				
'X84'-23	+++	+++	+++		
'X84'-24	+++				
'X84'-25	+++				
'X84'-26	+++	++	++		
'X84'-27	+++				
'X84'-28	-				
'X84'-29	+++				
'X84'-30	-	-	-		
'X84'-31	-	failed	-		
'X84'-32	+++	++	++		
'X84'-33	+++				
'X84'-34	+++	+++			
'X84'-35	+++				
'X84'-36	-	-			
'X84'-37	+++	+++			
'X84'-38	+++	+++			
'X84'-39	+++	+++			
'X84'-40	-	-			
'X84'-41	-				
'X84'-42	+++	+++			
'X84'-43	-	-			
'X84'-44	+++	+++			
'X84'-45	-				
'X84'-46	+++				
'X84'-47	-	-			
'X84'-48	-	-			
'X84'-49	+++				
'X84'-50	-	-			
Detection	31/50	18/32	12/20	8/13	8/13

NB: Grey or blank area means seedlings did not survived

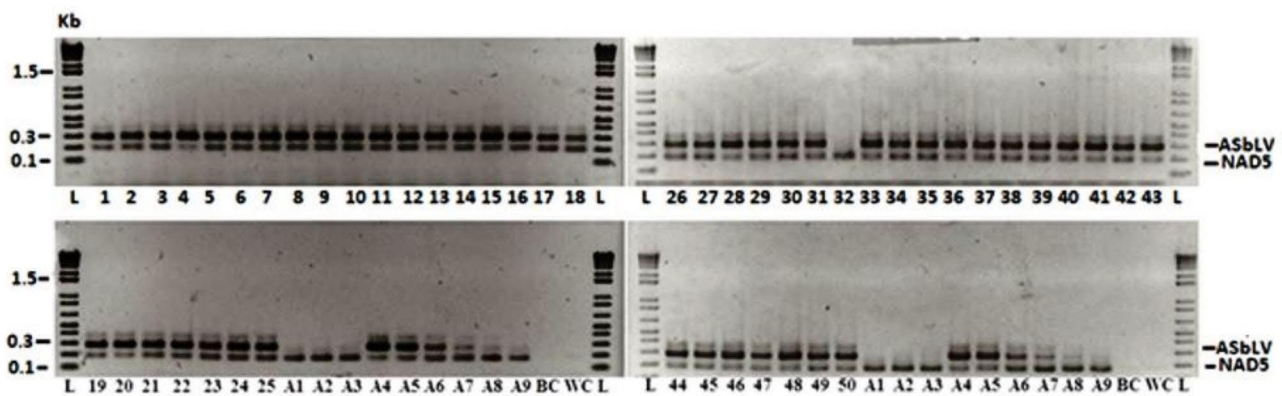


Fig. 2 Actinidia latent seed-borne virus (ASbLV) reverse transcription-polymerase chain reaction (RT-PCR) product from positive and negative samples. Lanes L is 1 kb + ladder (Invitrogen™); the numbered lanes 1–50 represents the test samples; Lanes A1–A3 are *Actinidia* seed negative controls; Lanes A4–A9 are positive control dilution series (10^0 to 10^{-5}); BC buffer control; WC water control. ASbLV is amplicon size is 278 bp and NAD5 internal control is 181 bp

($n=8/13$; confidence interval 32–86% infected, $P=95\%$, Tables 1 and 3). Approximately half of the roots sampled from 20 surviving ASbLV-positive seedlings (previously tested by cotyledon) tested positive for the virus ($n=12/20$; confidence interval 36–81% infected, $P=95\%$, Tables 1 and 2).

The detection rates in ‘T66’ and ‘X84’ seedlings indicates similar transmission of ASbLV from both infected staminate parents (Table 1). ASbLV was detected in 29 seedlings from the ‘T66’ seed family when cotyledons were individually tested ($n=29/49$; confidence interval 44–73% infected, $P=95\%$) (Table 1).

Transmission from infected pistillate (female) parents

ASbLV was detected in a high proportion of cotyledons from *Actinidia* seedlings grown from two seed families with an infected pistillate (female) parent (Table 2). ASbLV was detected in 81% seedling cotyledons grown from seed lot ‘C15’ ($n=43/53$; confidence interval 68–91% infected, $P=95\%$, Table 2). ASbLV was detected in 85% cotyledons sampled from ‘C53’ seedlings ($n=35/46$; confidence interval 61–87% infected, $P=95\%$, Table 2).

Comparison of ASbLV detection in cotyledons from crosses involving maternal- and paternal- infection showed a significantly higher proportion of infected seedlings arising from maternal infection (deviance = 7.8, 1 df, $P=0.005$). There was no significant difference in transmission frequency of ASbLV to different seedling tissues in the case of the one infected paternal parent crossing (‘X84’, Table 1) where this was investigated (deviance = 0.1, 2 df, $P=0.967$).

Discussion

In this study, ASbLV was found to be high in paternal (~60%) transmission and even higher maternal transmission efficiency (~80%) from asymptomatic infected parents. About 25% plant viruses are vertically transmitted in at least one host, however, the infection traits associated with the efficiency of virus seed transmission are largely unknown (Simmons and Munkvold 2014; Cobos et al. 2019). Generally, viruses do not enter the meristem or reproductive cells and most are not vertically transmitted (Bradamante et al. 2021). Seed-borne transmission of viruses is generally at low rates, e.g. ~1%, with exceptions including some nepo-viruses, and other specific viruses including pea seed borne mosaic virus, cucumber mosaic virus and red clover vein mosaic virus and bean common mosaic virus which can reach 100% transmission (Sastry 2013). The transmission frequency observed for ASbLV is very high and similar to pea seed borne mosaic virus into susceptible *Pisum sativum* genotypes (Maule and Wang 1996). Such high and directionally-balanced transmission of ASbLV (seed infection equally, or in this case at high rates, from both the maternal and paternal sporophyte parents), along with absence of symptoms is consistent with the virus being well-habilitated as a parasitic ‘guest’ within *Actinidia*. Other viruses, such as turnip yellow mosaic virus (TYMV) infection of *Arabidopsis thaliana*, are transmitted by both parents but not in, or close to, a balanced manner (de Assis Filho and Sherwood 2000). The frequency of ASbLV infection was highest in seeds rather than seedling tissues (cotyledons, roots, and juvenile or matured leaves), suggesting some degree of virus presence in or on the seed coat.

The high frequency of ASbLV vertical transmission requires active intervention to manage its dispersal

nationally or internationally. Even where infection is asymptomatic, virus infection of new cultivars is generally managed until the biology is well understood. This approach eliminates perceived risks that may limit opportunities for their commercial exploitation and potential ramifications from future growth abnormalities under specific conditions including co-infections. For instance, *Actinidia* hosts a growing number of identified viruses that may lead to symptomatic co-infections (Clover et al. 2003; Chavan et al. 2012, 2013; Chavan and Pearson 2016; Zhao et al. 2019, 2020). And the list of viruses identified in *Actinidia* continues to grow (Blouin et al. 2013; Zheng et al. 2014; Biccheri 2015; James and Phelan 2016; Lebas et al. 2016; Zheng et al. 2017; Blouin et al. 2018; Veerakone et al. 2018; Wang et al. 2018; Wen et al. 2018; Zhao et al. 2019; Rasool et al. 2020; Wang et al. 2020; Zhao et al. 2020, Zhang et al., 2021). In plant breeding, virus-free progeny are generally preferred in case viruses are detrimental in new host genotypes. Should management of ASbLV be desired, the detection method reported here allows non-destructive detection of ASbLV in very young seedlings that enables early screening of kiwifruit plants in nurseries. One of the major factors contributing to plant virus long-distance dispersal is the global trade of seeds. The high ASbLV transmission rates, whether from infected pollen or ovules, facilitate bulk testing of seed lots that could quickly detect infected seeds or their parent plants (fruit bearing female or male pollinator) at the border or in an orchard.

ASbLV may have co-evolved with the wild *Actinidia* species and possibly provide a benefit to the host, supporting its persistence in the host plant. The high seed transmission efficiency of ASbLV from asymptomatic infected parents has evolved arguably the most important determinant of parasitic fitness (Alizon et al. 2009). Reduced virulence (or latency at an extreme) can be manipulated to evolve through serial vertical passaging (Pagán et al. 2014). ASbLV likely relies on cell division to move from seed to all successive plant organs as it cannot be transmitted mechanically and may not encode an effective movement protein, or effective suppressor of RNA silencing that are generally also associated with symptom expression (Chapman et al. 2004; Veerakone et al. 2018). Passive movement is a hallmark of persistent viruses that are present in all host cells and, like ASbLV, do not display disease symptoms (Roossinck 2010). ASbLV presence in multiple wild-sourced accessions selected by humans for their potential horticultural traits correlates with ASbLV providing benefit to the plant host. Even viruses traditionally known as phytopathogenic such as brome mosaic virus (*Bromoviridae*), cucumber mosaic virus (*Bromoviridae*), tobacco rattle virus (*Virgaviridae*), TMV *Virgaviridae* and tomato yellow leaf curl virus (*Begamoviridae*) can be beneficial to crops as

they can confer tolerance to drought or freezing temperatures in several different crops (Roossinck 2011; Mishra et al. 2022). Specific research is required to understand the ASbLV-*Actinidia* interaction including ASbLV movement and whether it confers benefit under a range of biotic and abiotic conditions. For instance, it would be intriguing to determine whether ASbLV provides any benefit to kiwifruit challenged with *Pseudomonas syringae* pv. *actinidiae* (Psa), a bacterium that has co-evolved with *Actinidia* species. The dioecious nature of *Actinidia* may provide a biological lever to prise apart cell division and ASbLV movement to understand its transmission biology. As identified here, up to ~40% seeds from a cross involving only a single ASbLV-infected parent can yield seedlings without detectable virus thereby providing sufficient numbers of sibling progeny kiwifruit with and without the virus for future research on impacts of the ASbLV on *Actinidia* species.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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