Molecular detection of luteovirus and polerovirus

In oat, broad bean, soybean and aphids

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Independent project in Biology, G2E • 15 hp
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Agricultural Programme – Food Science
Examensarbete / Institutionen för växtbiologi, SLU, 198
Uppsala 2023

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Molekylär detektion av luteovirus och polerovirus – i havre, åkerböna, sojaböna och bladlöss

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Credits:	15 hp
Level:	First cycle, G2E
Course title:	Independent project in Biology, G2E
Course code:	EX0894
Programme/education:	Agricultural Programme – Food Science
Course coordinating dept:	Department of Aquatic Sciences and Assessment
Place of publication:	Uppsala
Year of publication:	2023
Copyright:	All featured images are used with permission from the copyright owner.
Title of series:	Examensarbete / Institutionen för växtbiologi, SLU
Part number:	198

Keywords:	Barley yellow dwarf virus, bean leafroll virus, RT-PCR, food production, Sweden

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Sammanfattning

Havre, åkerböna och sojabönor är alla vanliga och viktiga livsmedelsgrödor. De spelar en viktig roll över hela världen när det kommer till både näring och ekonomi. Liksom för de flesta växter kan de drabbas av olika sjukdomar, vilket orsakar ansenliga skördeförluster. Ett stort bekymmer är virusinfektioner i växter. Rödsotvirus (engelska barley yellow dwarf virus, BYDV), bean leafroll virus (BLRV) och besläktade virus tillhörande släktena luteovirus och polerovirus infekterar ofta ovan nämnda grödor. Symptom på infektion inkluderar missfärgning och dvärgväxt, och spridning sker främst med bladlöss som vektorer.

Virusen som studeras i detta projekt har sitt genetiska material i form av RNA. Omvänd transkription och polymeraskedjereaktion (RT-PCR) utfördes för en nukleinsyrabaserad detektion av luteovirus och polerovirus i symptomatiska och asymptomatiska blad från ovan nämna växter, samt i bladlöss. Bladlössen samlades in på hösten år 2014 och 2020. Den stora mängden bladlöss dessa år orsakade omfattande virusspridning i stråsäd, och skador efterföljande år.

Sekvenser med härkomst från virus påvisades inte i något av proven, inte heller den positiva kontrollen innehållande bladlöss med känd förekomst av BYDV. För bladlusprovet som fungerade som positiv kontroll så skulle en hög bakgrundsamplifiering kunna vara anledningen till frånvaron av identifierade virus-RNA-sekvenser. För växtproverna tros de negativa resultaten bero på att de inte var infekterade med luteovirus eller polerovirus. Resultaten från analyser av äldre bladlusprover indikerade inte någon förekomst av virus, men på grund av osäkra resultat kan inga säkra slutsatser dras.

Fortsatt forskning på bladlöss kan leda till en ökad kännedom om korrelationen mellan den vektorburna virusöverföringen och virusepidemier i grödor. Denna kunskap kan i sin tur vara användbar i utformandet av riktlinjer av en precis användning av insekticider. Det skulle resultera i en säkrare skörd, reducerade ekonomiska förluster och en minskad miljöpåverkan.

Nyckelord: Rödsotvirus, bean leafroll virus, RT-PCR, livsmedelsproduktion, Sverige

Abstract

Oat, broad beans and soybeans are all common and important food crops. They play an important role worldwide in terms of both nutrition and economy. As for most plants, different diseases can emerge in these crops, causing considerable yield losses. One great concern is virus infections in plants. Barley yellow dwarf virus (BYDV), bean leafroll virus (BLRV), and other related viruses of the genera luteoviruses and poleroviruses are commonly infecting the aforementioned crops. Symptoms of infection include discolouration and dwarfism, and transmission is mainly vectored by aphids.

The viruses studied in this project have their genetic material in the form of RNA. Reverse transcription polymerase chain reaction (RT-PCR) was performed for a nucleic acid-based detection of luteovirus and polerovirus in symptomatic and non-symptomatic leaves from the plants mentioned above, and in aphids. The aphids were collected in 2014 and 2020. The great amount of aphids in these years caused extensive spread of virus in cereals and damage following years.

No sequence of viral origin was detected in any of the samples. This was also the case for the positive control sample, containing aphids known to carry BYDV. For the positive control, a high background amplification in the PCR might be the explanation for absence of identified viral RNA sequences. For the plant samples, the negative results are believed to be an indication that they were not infected with luteovirus or polerovirus. Results from analyses of older aphid samples did not indicate any presence of virus, although due to uncertain results, no definitive conclusions can be drawn.

More research on aphids could lead to an increased knowledge of the correlation between the vectored transmission and virus epidemics in crops. This knowledge could in turn be useful when creating guidelines of more accurate insecticide use, resulting in secured yields, reduced economical losses and less negative environmental impact.

Keywords: Barley yellow dwarf virus, bean leafroll virus, RT-PCR, food production, Sweden

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Abbreviations

BChV	Beet chlorosis virus (polerovirus)
BLAST	Basic Local Alignment Search Tool
BLRV	Bean leafroll virus (luteovirus)
BMYV	Beet mild yellowing virus (polerovirus)
bp	Base pairs
BYDV	Barley yellow dwarf virus (luteovirus)
cDNA	Complementary DNA
CP	Coat protein
CpCSV	Chickpea chlorotic stunt virus (polerovirus)
CYDV	Cereal yellow dwarf virus (polerovirus)
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
NGS	Next-generation sequencing
rRNA	Ribosomal RNA
(RT-)PCR	(Reverse transcription) polymerase chain reaction
SbDV	Soybean dwarf virus (luteovirus)
SJV	Swedish Board of Agriculture / Statens jordbruksverk
SLU	Swedish University of Agricultural Sciences
TuYV	Turnip yellows virus (polerovirus)

Introduction

1.1 Plant disease is a peril

Current global food production faces a number of challenges. Among the challenges are plant diseases (Ristaino et al. 2021), which cause more than 10 % of the world's food production to be lost (Strange & Scott 2005). This factor has a significant impact on the effectiveness of food production and must thus be studied.

The relevance of this topic is supported by the second of the 17 Sustainable Development Goals developed by the UN, "Zero Hunger" (United Nations n.d.). One of the objectives within this goal is to "ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production". A quarter of a billion people are already on the edge of starvation (United Nations n.d.).

This report focuses on a group of viruses that are important pathogens and infect a range of different food crops.

1.2 Barley yellow dwarf-associated viruses infect cererals

Cereals, such as oat (*Avena sativa*), are important staple crops (Strange & Scott 2005). For thousands of years, they have been a major component in the human diet, and still today cereals count as a crucial food commodity worldwide, which nourishes billions of people (Awika 2011; Garg et al. 2021). To a majority of people, especially in developing countries, cereal grains are the primary source of calories and contributing with essential nutrients (Awika 2011).

In 2022, cereals were cultivated on 962,454 hectares in Sweden, corresponding to 38.0 % of the total arable area. The cereal production includes oat, which was cultivated on 158,445 hectares (6.2 % of the total area) (Jordbruksverket 2022).

Barley yellow dwarf (BYD) associated viruses, such as BYDV-PAV, BYDV-PAS and BYDV-MAV, constitute a globally widespread virus complex, which endangers the production of cereal crops (Sõmera et al. 2021b; Yazdkhasti et al. 2021). BYD-associated viruses can also infect grasses and use these as virus

reservoirs (Yazdkhasti et al. 2021). They belong to either of the genera *Luteovirus* or *Polerovirus*.

Luteovirus has its name from the Latin word *luteus*, translating to 'yellowish' (Domier & D'Arcy 2008). One clear and visible symptom of infection by luteoviruses is yellowing of the crop leaves – hence the name. When infecting oat, there is instead a reddening of the leaves (Sigvald et al. 2019). Symptoms also include curled leaves and a reduced growth (Domier & D'Arcy 2008). A significant risk for decreased yield persists, depending on the severity of symptoms and the timing of infection (Domier & D'Arcy 2008; Sõmera et al. 2021b).

The genus *Polerovirus* contains more BYD-associated virus species, including cereal yellow dwarf viruses (CYDVs) (Sõmera et al. 2021b). Just like luteoviruses, poleroviruses cause symptoms like discolouration and stunting of crops (Domier & D'Arcy 2008).

Luteoviruses and poleroviruses are closely related, and are substantially alike at the 3' end of their genomes (Domier & D'Arcy 2008). This part encodes the important coat protein (CP) and shares a common evolutionary origin (Sõmera et al. 2021b). Luteoviruses belong to the family *Tombusviridae*, and poleroviruses to the family *Solemoviridae* (Sõmera et al. 2021a).

In Sweden, the occurrence of BYDV has been higher in certain years, with sporadical extensive outbreaks (Sigvald et al. 2019). Problems with BYDV infection are predicted to increase with a warmer climate.

1.3 Bean leafroll virus and soybean dwarf virus infect legumes

Broad bean (*Vicia faba*), also known as faba bean, is a legume widely consumed throughout the world (Muehlbauer & Tullu 1997). The protein content is high, varying between 20-41 % (Muehlbauer & Tullu 1997). Broad beans are both consumed by humans and used as animal feed.

Soybean (*Glycine max*) is a major protein source and is considered to be the most important food legume (Allen 2013). The protein content in the dry seed is typically 35 %. Just like broad beans, it is used both as human food and animal feed (Grassini et al. 2021).

The area used for legume cultivation in Sweden has increased during the last 22 years, from cultivation on 27,892 hectares in 2000, to 47,455 hectares in 2022, equivalent to 1.9 % of the arable land (Jordbruksverket 2022).

Bean leafroll virus (BLRV), just like BYDV, belongs to the genus *Luteovirus* (Ashby 1984), and is persistently transmitted by aphids. BLRV infects legumes (Ashby 1984; Domier et al. 2002) and as the name suggests, one symptom is rolled leaves of the plant. Further symptoms include yellowing of the leaves and fewer

pods. BLRV is distributed all over the world, and is common in Europe. It has for instance been detected in Germany (Ashby 1984; CABI 2022), however, BLRV has yet to be reported in Sweden.

Lastly, the luteovirus soybean dwarf virus (SbDV) is of interest for this study as well. It is suggested to be the closest relative to BLRV (Domier et al. 2002), also infecting legumes and causing symptoms such as yellowing and growth reduction (Damsteegt et al. 1995). SbDV has been reported worldwide, but never in Sweden. In Europe, it has been discovered only in Germany and Finland (Luoto et al. 2021).

1.4 Aphid vectored virus transmission

Transmission of BYD-associated viruses between plants is vectored by aphids (Blystad et al. 2020). The transmission is persistent, meaning that once the aphids carry the virus, they will be infectious throughout their whole life. Furthermore, aphids transmit BYD-associated viruses in a non-propagative manner (Ali et al. 2018; Garcia-Ruiz et al. 2021; ICTV 2012; Sõmera et al. 2021b), meaning that the viruses cannot propagate within the vector. Thereby, no virus transmission properties are inherited from parent to offspring.

Not every aphid species is able to carry any type of luteovirus. There is a high degree of specificity of the virus transfer (Blystad et al. 2020), and the properties of the transmitting vector is dependent on interaction with the viral coat protein (CP) (Sõmera et al. 2021b).

When it comes to BYD-associated viruses, there are at least 28 aphid species capable of transmission (Harrington 2002). An important and prevalent species involved in vectoring BYDV transmission is bird cherry-oat aphid (*Rhopalosiphum padi*) (Blystad et al. 2020; Sigvald et al. 2019).

1.5 Economical consequences of viral infections in plants

The viruses described belong to the most detrimental viruses out of those that infect cereals, and belong also to the most widespread globally (Sõmera et al. 2021b). Infected crops such as cereals are of substantial economical importance (Yazdkhasti et al. 2021).

The yield loss depends on several factors, such as the virus and vector species, and can vary broadly (Sõmera et al. 2021b). Losses have been estimated to be between 5 and 30 %, in some cases 50 % (Sigvald et al. 2019), and there have even been extreme examples reported, with up to 80 % loss of total yield (Sõmera et al. 2021b) due to YDVs. In 2015, yield losses were estimated to be around 30 million

SEK in the county of Skåne only, due to infections of BYDV (Holmblad et al. 2015).

As for broad beans, the number of pods is an important factor for the yield (Muehlbauer & Tullu 1997), thus also for the sales. Infection of BLRV has a close correlation with a decreased number of pods (Ashby 1984). In other words, infections of this virus causing fewer pods could have a negative impact for the farmer. Soybean is a central crop for the economy as well, since it belongs to the most cultivated legumes globally (Muehlbauer & Tullu 1997), and yield losses are by all means undesired.

1.6 Future challenges

When temperatures are high, aphids are more active and capable of transmitting viruses efficiently (Sigvald et al. 2019). Certain years, a high prevalence of virus infections in Sweden, predominantly in winter crops, has been observed to follow years of high temperature and an accordingly high aphid activity during autumn (af Geijersstam & Sohlman 2022; Holmblad et al. 2015).

The climate is changing and temperatures are increasing over time (IPCC 2018). A warmer climate in Sweden will imply a longer time throughout the year when aphids can be active and transmit viruses, since their reproduction is favoured by milder temperatures (Yazdkhasti et al. 2021). As a result, problems with BYDV in winter crops is expected to increase (Sigvald et al. 2019). This might cause a serious vulnerability for food production.

Especially winter crops are more prone to be susceptible for insect transmission, since these can be attacked by aphids as young plants, during autumn (Blystad et al. 2020; Roos et al. 2011).

When examining the possibility of developing an early-stage warning system for coming virus epidemics, a strong correlation between virus detection in aphids and spread in plants was discovered (Congdon et al. 2019). Considering this, it may be possible to predict viral diseases in plants more accurately, particularly for disease in winter crops in the following year. One aspect to consider when making predictions could be the number of virus-carrying aphids present in fields.

Such a method would enable the opportunity to develop guidelines for an accurate application of insecticides. A more precise use will result in increased economical efficiency in food production, with less negative impact on the environment. At the same time, yields will be better protected.

1.7 Methods of virus detection

There are different ways of detecting viruses. It can be done both directly and indirectly (Cassedy et al. 2021). Common methods include immunoassays (such as Enzyme-linked immunosorbent assay (ELISA), which is considered cheap and robust), serologically specific electron microscopy (sensitive but expensive and time consuming) (D'Arcy et al. 1999), or nucleic acid-based detection, which offers the highest sensitivity (Cassedy et al. 2021; D'Arcy et al. 1999).

Generally for luteoviruses, RT-PCR has been suggested to be the preferred method in cases of small sample quantities (D'Arcy et al. 1999). BYDV, BLRV and SbDV have all been detected by this method (Luoto et al. 2021; Ortiz et al. 2004; Robertson et al. 1991).

Another method that might complement the above mentioned ones and become more used in the future is next generation sequencing (NGS) (Cassedy et al. 2021). This time-efficient method is used to sequence genomes, and has the ability to differentiate between genes from hosts and viruses.

As for aphids specifically, all techniques described above have been used for detection of aphid-vectored plant viruses in aphids (Mehta et al. 1997; Plumb 1989).

In this project, RT-PCR was carried out on all samples in order to detect viral RNA.

1.8 Aim of this project

The goal with this project was to survey the presence of luteo- and polerovirus RNA in oat, broad beans and soybeans, as well as in the common virus vector aphid.

For the plant samples, the detection was conducted in order to determine if the observed symptoms emerged from infection of luteo- or polerovirus.

As for the aphids, the primary aim was to investigate the possibilities of detection of viral genes. If detection is achievable, the information will be used to examine potential correlations between virus outbreaks in plants in certain years, and aphids carrying viruses at the time.

More knowledge may facilitate future forecasting of virus epidemics. An accurate prediction of virus outbreaks can help to determine an appropriate dosage of insecticides. In the long run, the results of this study might be one factor that can help reducing economical losses and alleviating agricultural difficulties.

Materials and methods

RNA was isolated from plants and aphids, and reverse transcribed to complementary DNA (cDNA). Polymerase chain reaction (PCR) was carried out, using general primers for luteoviruses and poleroviruses, and products were run on gel electrophoresis to determine if fragments of the target size were present. When that was the case, DNA was purified from the gel. DNA fragments were cloned into plasmid vectors, which were grown in cells of *Escherichia coli* on selective medium. Out of growing colonies, overnight cultures were started, and plasmid DNA was isolated from these. Restriction enzyme digestion was done to see if the correct fragment had been cloned. Clones with insert of the expected size were sent for sequencing to see if they originated from luteo- or poleroviruses, and if so, of which virus genotype.

2.1 Material for analysis

2.1.1 Oat leaves

Two samples of dry leaves from oat (*Avena sativa*) grown in Falköping, Västra Götaland county, were received from the Swedish Board of Agriculture (Figure 1a; Table 1). The leaves were symptomatic as their leaves had turned reddish. For comparison, fresh leaves from white oat of the cultivar Belinda, growing in the demonstration field of the Swedish University of Agricultural Sciences (SLU) in Uppsala, were collected and tested (Figure 1b). Both symptomatic and asymptomatic leaves from SLU were tested.

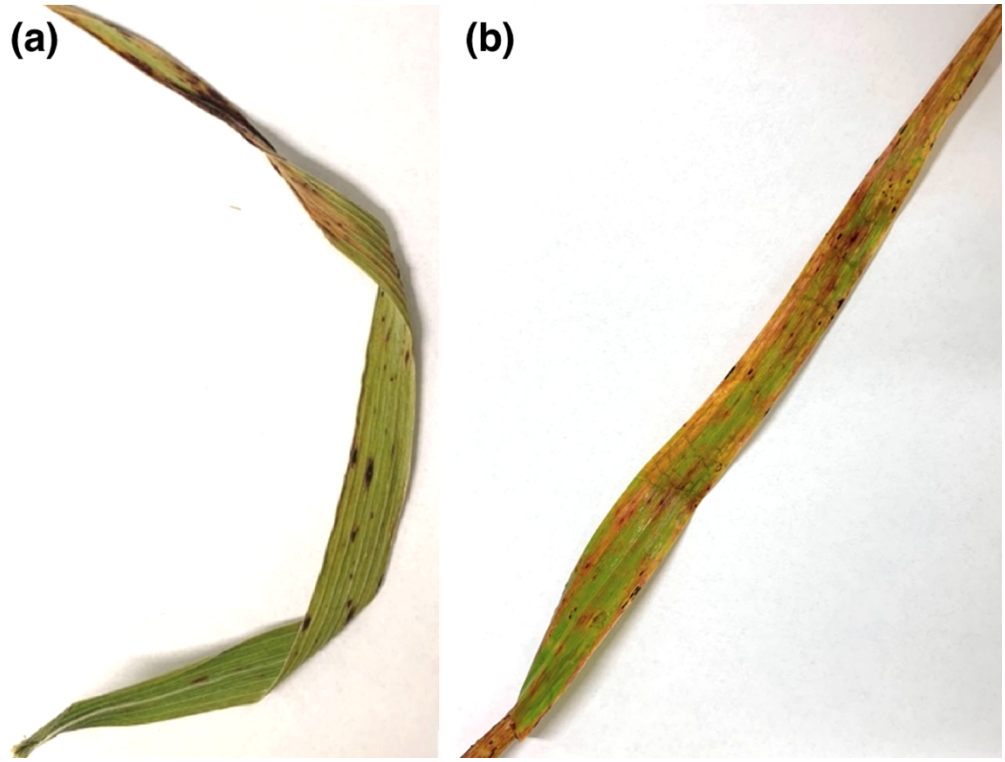

Figure 1. Symptomatic oat leaves (a) from Falköping, Västra Götaland, dry, received from the Swedish Board of Agriculture and (b) collected fresh in the demonstration field of SLU, Uppsala. This leaf has symptoms of reddening.

2.1.2 Broad bean and soybean leaves

The broad beans tested in this study were leaves from symptomatic plants received from the Swedish Board of Agriculture, and grown in Mellerud, county of Västra Götaland (Figure 2a; Table 1). Their leaves were curled and the plants displayed an inhibited growth, symptoms that could potentially be caused by a virus infection.

For comparison, broad beans collected from the demonstration field of SLU in Uppsala were tested (Figure 2b). These showed mild symptoms of rolled leaves, that could have been due to virus infection. The mild symptoms were more clearly visible on plants of cultivar Birgit than of cultivar Taifun.

Two samples of soybean were collected from the SLU field and tested as well. They did not display symptoms of virus infection.

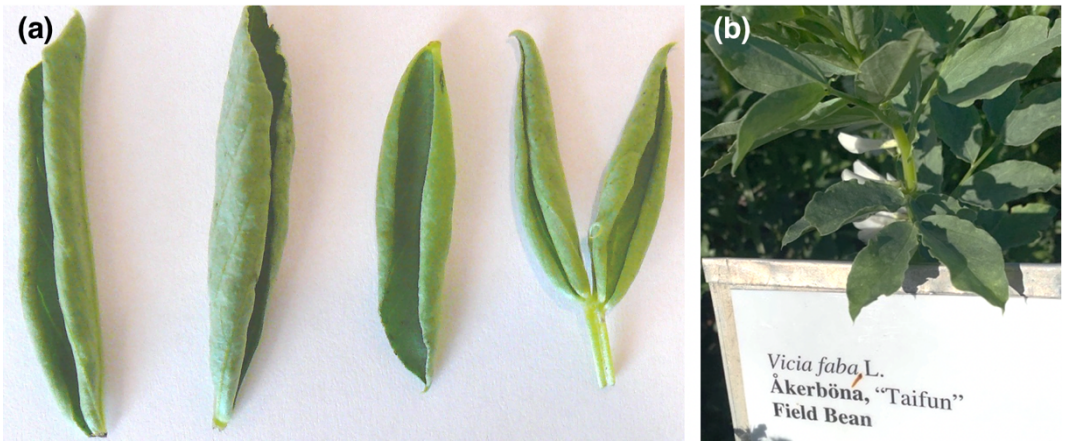

Figure 2. Broad bean leaves; (a) Samples collected in Mellerud, Västra Götaland and received from the Swedish Board of Agriculture. Symptoms including rolled leaves are displayed. (b) Broad bean leaves growing in the demonstration field of SLU, Uppsala.

2.1.3 Aphids

Bird cherry-oat aphids (*Rhopalosiphum padi)* were kindly provided by Velemir Ninkovic and Maria Kedmark from the Department of Ecology, SLU, Uppsala.

Three groups of aphids served as positive and negative controls, with approximately 40 aphids in each: two groups carrying BYDV-PAV, and one group without virus (Figure 3a; Table 1). They were collected living in September, 2022, put into 70 % ethanol and kept at room temperature until usage. One of the positive control aphid samples was tested together with the plant samples, this sample was named A-pos-test.

Aphids for testing had been captured through suction traps (Figure 3b) located in Ingelstorp, county of Kalmar, and Alnarp, county of Skåne, Sweden. There were approximately 25 aphids in each of these samples. The aphids were collected in autumns of 2014 and 2020, years that preceeded severe virus infection outbreaks in winter cereals. These aphids had been stored cool in 70 % ethanol.

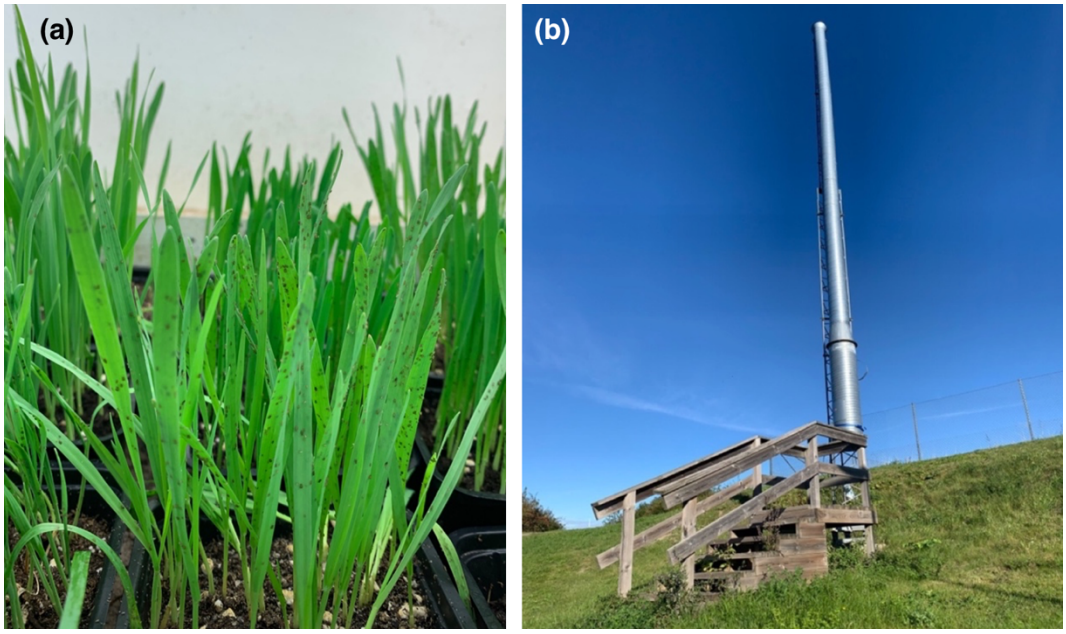

Figure 3. (a) Culture of virus-carrying aphids at the Department of Ecology, SLU, and (b) suction trap, the same type of device that was used to collect aphids in Alnarp and Ingelstorp.

2.1.4 Additional cDNA for evaluation of primer pairs

To select primers for RT-PCR detection of poleroviruses and luteoviruses, cDNA from three samples (named sugar beet 19, sugar beet 22 and sugar beet 24; Table 1) were kindly provided by Vinitha Puthanveed. These were derived from sugar beet plants from Alnarp and Kongsmarken, county of Skåne, and had previously tested positive for beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV) (Pettersson, 2020). Sample sugar beet 24 had also tested positive for turnip yellows virus (TuYV). BMYV, BChV and TuYV belong to the genus *Polerovirus*.

Table 1. Samples used for RT-PCR tests of poleroviruses and luteoviruses

Sample name	Species	Collection location	Collection date	Cultivar
Oat-SLU-1 Oat-SLU-2 Oat-SLU-3	*Avena sativa* (oat)	Uppsala	September 8, 2022	"Belinda"
Oat-SJV-1 Oat-SJV-2	*Avena sativa*	Björkhagen, Falköping	July 2022	Unknown
SJV-BBG-1 SJV-BBG-2	*Vicia faba* (broad bean)	Tängelsbol, Mellerud	July 4, 2022	"Gloria"
SLU-BBB-1 SLU-BBB-2	*Vicia faba*	Uppsala	September 8, 2022	"Birgit"
SLU-BBT-1 SLU-BBT-2	*Vicia faba*	Uppsala	September 8, 2022	"Taifun"
SLU-SB-1 SLU-SB-2	*Glycine max* (soybean)	Uppsala	September 8, 2022	"Obelix"
A-pos-test, A-pos (controls with virus), A-neg (control without virus)	*Rhopalosiphum padi* (Bird cherry-oat aphid)	Uppsala	September 16 (pos) and 28 (neg), 2022	
I-14-1, I-14-2	*Rhopalosiphum padi*	Ingelstorp, Kalmar	October 13, 2014	
I-20-1, I-20-2	*Rhopalosiphum padi*	Ingelstorp, Kalmar	September 25, 2020	
Aln-14-1, Aln-14-2	*Rhopalosiphum padi*	Alnarp	October 13, 2014	
Aln-20-1, Aln-20-2	*Rhopalosiphum padi*	Alnarp	September 28, 2020	
Sugar beet 19, sugar beet 22	*Beta vulgaris* (sugar beet)	Alnarp	October 2019	Unknown
Sugar beet 24	*Beta vulgaris*	Kongsmarken	October 2019	Unknown

2.2 RNA extraction

Extraction of RNA from oat, broad bean and soybean leaves was carried out using Sigma Spectrum Plant Total RNA Kit (Sigma-Aldrich), according to the protocol from the manufacturer. All plant tissues were ground using Tissuelyser II (QIAGEN) for 40 seconds at an oscillation frequency of 30 Hz. In the tubes, two stainless steal beads with size 5 mm (QIAGEN) were placed together with the samples. A few modifications to the protocol were made: 1) Centrifugtion was carried out at 13,300 × g. 2) For step 4 in the procedure, the instructions for 4a were used. 3) No DNase was used. 4) Elution buffer was pre-heated to 65°C before usage. No second elution was done.

For RNA extraction from aphids, a protocol published by Oñate-Sánchez & Vicente-Carbajosa (2008) was used. Aphids were homogenized using the Tissuelyser as well, with an oscillation frequency of 30 Hz. The DNase treatment (step 4 and 5 in protocol) was not carried out.

Total RNA concentrations were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The ratio of absorbance between 260 / 280 nm shows the purity of the RNA. A result above 2.0 is an indication of pure RNA, and a verification that the isolation was successful.

2.3 Gel electrophoresis

The negative charge of the nucleic acid forces its movement towards the positive electrode of the gel when an electric currency is applied. Fragments of different sizes then move at a different pace in the gel. This brings the opportunity to separate RNA or DNA fragments according to their size. Using a size reference, it is possible to compare with the present fragments, and thus enable to determine whether or not a fragment has the expected size. In this project, size validation was achieved using Gene Ruler 1 kb DNA Ladder (Thermo Fisher Scientific). The ladder was mixed with dye and water in accordance with the manufacturer's protocol.

Throughout the project, 1 % agarose gel with 1 x Tris-acetate-EDTA (TAE) buffer was used together with 4 µL Midori Green (Nippon Genetics) per 100 mL gel for staining of nucleic acids. The loading dye used was TriTrack DNA Loading Dye (6x) (Thermo Fisher Scientific).

2.3.1 Gel electrophoresis of RNA

To check if the RNA seemed intact, a gel was run on selected samples after the RNA extraction. For this, 2 µL of RNA was used for each sample.

In order to avoid any RNase contamination, all equipment used for the gel electrophoresis of RNA was carefully washed beforehand.

2.4 cDNA synthesis

RNA from all samples was used to synthesise complementary DNA (cDNA). This was done using RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific) according to the manufacturer's protocol, with random hexamer primers from the kit. For each sample, 1 µg of RNA was used for the reverse transcription.

2.5 PCR

To amplify the targeted viral DNA, polymerase chain reaction (PCR) was run using Bio-Rad T100 Thermal cycler. The use of specific primers allows this method to amplify sequences to which these primers bind – in this case, a gene specific to luteoviruses and poleroviruses.

Multiple runs of PCR were carried out in order to find the best possible combination of primer pair and polymerase enzyme. For all PCR tests, sterile water was used as a negative control. After every PCR run, the amplification products were stored in a freezer or immediately run on a gel to check for amplicons of the size of the CP gene. For the verification, 5 µL of PCR product was used for each sample.

2.5.1 PCR primers

Two primer pairs were evaluated and used for the PCR, both pairs targeting a part of the CP gene of luteo- and poleroviruses. This is a conserved part of the genome, and it is in many cases highly similar between species of the two genera (Domier & D'Arcy, 2008). The primers are called Lu1 and Lu4 (Robertson et al. 1991), and Luteo F and Luteo R (Abraham et al. 2006). Both primer pairs were ordered from TAG Copenhagen.

Lu1 and Lu4 had previously been used successfully when amplifying the CP gene from instance-wise BYDV, and a few poleroviruses (Bisnieks et al. 2004; Robertson et al. 1991). The amplicons should be around 530 base pairs (bp).

Luteo F and Luteo R had successfully been used to amplify the CP gene of the polerovirus BMYV and several other poleroviruses in PCR assays (Abraham et al. 2006; Pettersson 2020). The amplicons from these primers should be around 600 bp.

2.5.2 PCR on cDNA from plant and aphid samples

Two initial PCR tests were carried out in order to evaluate primers. To evaluate the primer pair Lu1 / Lu4, 10 µL DreamTaq Green PCR Master Mix (2x) (Thermo Fisher Scientific), 0.25 µM each of Lu1 and Lu4, and 1 µL cDNA were mixed with nuclease-free water in a total volume of 20 µL. The program for this combination

was 95 °C for 2 min and 30 s, then 40 cycles of 95 °C for 30 s, 42 °C for 1 min, 72°C for 2 min, and thereafter 72 °C for 10 min before cooled down.

To evaluate the primer pair Luteo F / Luteo R, 10 µL Phusion High-fidelity PCR Master Mix with HF buffer (2x) (Thermo Fisher Scientific), 0.25 µM each of Luteo F and Luteo R, and 1 µL of cDNA template were mixed with nuclease-free water, in a total volume of 20 µL. The program for this run was 98 °C for 30 s, then 35 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 20 s, and thereafter 72 °C for 5 min before cooled down.

After the initial trials with plant and aphid samples, Lu1 and Lu4 proved to be the better primer pair to use. The Phusion High-fidelity PCR Master Mix with HF buffer was chosen to be the master mix to use, because of its high fidelity and accuracy, which is described in the protocol from the manufacturer. Therefore, Lu1 and Lu4 were used together with the Phusion Master Mix in a subsequent test of samples of plants and aphids. For this PCR, the same volumes were used as previously, and the PCR program with this combination was 98 °C for 30 s, then 40 cycles of 98 °C for 10 s, 55 °C for 20 s and 72 °C for 20 s, followed by 72 °C for 5 min before cooled down.

Three PCR runs were carried out to analyse older aphid samples. For the first and second run, the reaction mix contained the Phusion High-fidelity PCR Master Mix with HF buffer (2x) (Thermo Fisher Scientific), and the primer pair Lu1 / Lu4 with the same volumes and program as mentioned above for the combination of the Lu primer pair with Phusion.

Because of possible contamination, a third set of PCR tests for aphids were run, where all solutions were replaced to the greatest extent possible. New dilutions of the Lu1 and Lu4 primers were made, new nuclease-free water was used, and the master mix was changed to DreamTaq Green PCR Master Mix (2x) (Thermo Fisher Scientific). The same volumes as previously were used. The program was the same as used for the combination of DreamTaq Master Mix and Lu1 and Lu4 primers for the plant and test aphid samples.

2.6 Cloning of fragments

2.6.1 Purification of DNA fragments from gel

PCR products for samples Oat-SLU-3, Oat-SJV-1, SJV-BBG-2, SLU-SB-2, and A-pos-test were selected for cloning. For each sample, 12 µL of PCR product was used for gel purification.

For the purification of the DNA fragments of correct size, GeneJET Gel Extraction Kit (Thermo Scientific) was used according to the manufacturer's protocol. During the purification, no sodium acetate was used, and step 4 was not carried out.

The decision to purify the PCR products from the gel, instead of directly from the PCR solution, was based on the fact that the gel electrophoresis had shown the presence of multiple amplification products. Even if the non-target bands were faint, there would be a higher risk of cloning the wrong DNA fragments if using the PCR product without gel purification.

2.6.2 Ligation and transformation

Cloning enables the possibility to ligate a DNA fragment into a plasmid vector, which can be transformed into bacteria. The vector has several clever built-in features, one of them being a positive selection system, which only enables bacteria with an insert to grow on a selective medium.

CloneJET PCR Cloning Kit (Thermo Fisher Scientific) was used for the process, and the protocol from the manufacturer was followed accordingly. During the ligation step, instructions for blunt-end Cloning Protocol were followed.

The amount of DNA included depended on the yield from the DNA purification from the gels, and varied between the samples. For each ligation, 8 µL of purified PCR fragment was used. The ligation products were stored in freezer until transformation.

2.6.3 Transformation and growth of *E. coli*

Subcloning Efficiency DH5α Competent Cells of *Escherichia coli* (Invitrogen) were used for the transformation, which was done following the manufacturer's protocol. For each transformation, 5 µL of the ligation mix was used. In order to calculate the transformation efficiency, a transformation with 0.25 ng of pUC19 control DNA was carried out.

The transformed cells were grown on plates with LB agar high salt medium (Duchefa Biochemie) containing 100 ng / µL ampicillin (Duchefa Biochemie). For each sample, 100 µL of the culture of transformed bacteria was first spread onto a plate. Next, the culture was centrifuged for two minutes at 3,000 × g, the supernatant was decanted, and the remaining pellet was resuspended and spread on a plate. The bacteria were grown at 37 °C overnight.

2.6.4 Plasmid isolation and analysis

For each sample, 3-4 single colonies were transferred to glass tubes containing 3 mL LB Broth high salt (Duchefa Biochemie) with 50 ng / µL ampicillin (Duchefa Biochemie). The tubes were incubated at 37 °C overnight while shaking at 196 rpm.

To extract plasmid DNA from the overnight cultures, GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific) was used and the purification was done according to the instructions of protocol A. In the purification, step 7 was not

carried out, and no second elution was done. DNA concentrations were measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

To verify that the correct insert had been cloned into the vector, the plasmids were digested with restriction enzyme FastDigest *Bgl*II (Thermo Scientific). Volumes used were 13 µL of autoclaved water, 2 µL 10X FastDigest, 4 µL of plasmid DNA, and 1 µL of FastDigest enzyme.

2.7 Sequencing and sequence analyses

Plasmid DNA for a total of 13 clones were Sanger sequenced by Macrogen Europe: all four clones for Oat-SJV-1, all three clones of the positive aphid control, Oat-SLU-3_3, SJV-BBG-2_3, SJV-BBG-2_4, SLU-SB-2_2, SLU-SB-2_3 and SLU-SB-2_4. Sequencing was carried out using forward and reverse primers.

Presence of primers were identified in the insert sequences, and they were removed before further analyses.

Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) was used to search GenBank for highest nucleotide identities (BLASTn). When no significant identities were obtained with BLASTn, searches of the NCBI protein database were carried out using BLASTx.

Results

3.1 RNA extraction from plant leaf samples and aphids

Table 2. RNA concentration and A260 / 280 ratio measured after RNA extraction

Sample	RNA conc. (ng / µL)	A260 / 280
Oat-SLU-1	454.4	2.10
Oat-SLU-2	354.6	2.06
Oat-SLU-3	375.5	2.09
Oat-SJV-1	448.7	2.11
Oat-SJV-2	298.2	2.12
SJV-BBG-1	581.1	2.14
SJV-BBG-2	589.0	2.13
SLU-BBB-1	721.5	2.12
SLU-BBB-2	876.5	2.13
SLU-BBT-1	1322.4	2.11
SLU-BBT-2	1973.9	2.12
SLU-SB-1	706.1	2.13
SLU-SB-2	812.5	2.12
A-pos-test	521.3	1.74
A-neg	1577.6	1.75
I-14-1	654.7	1.60
I-14-2	205.3	1.47
I-20-1	674.4	1.60
I-20-2	696.8	1.49
Aln 14-1	549.8	1.66
Aln-14-2	631.1	1.68
Aln-20-1	1091.8	1.62
Aln-20-2	720.2	1.62
A-pos	459.4	1.65

RNA from ten of the oat and bean samples was analysed after RNA extraction. Absorbance measurements confirmed presence of RNA for all samples. For the bean and oat samples, RNA concentrations were high and the RNA extracts had an absorbance ratio at 260 / 280 nm above 2 (Table 2). These results indicated good possibilities to proceed successfully with the following steps. The extracted RNA from aphids had high concentrations as well, but not as high ratio of absorbance at 260 / 280 nm.

3.1.1 Gel electrophoresis after RNA extraction from plant samples

To see if the RNA seemed to be intact, RNA from ten of the oat and bean samples were run on a gel after extraction (Figure 4).

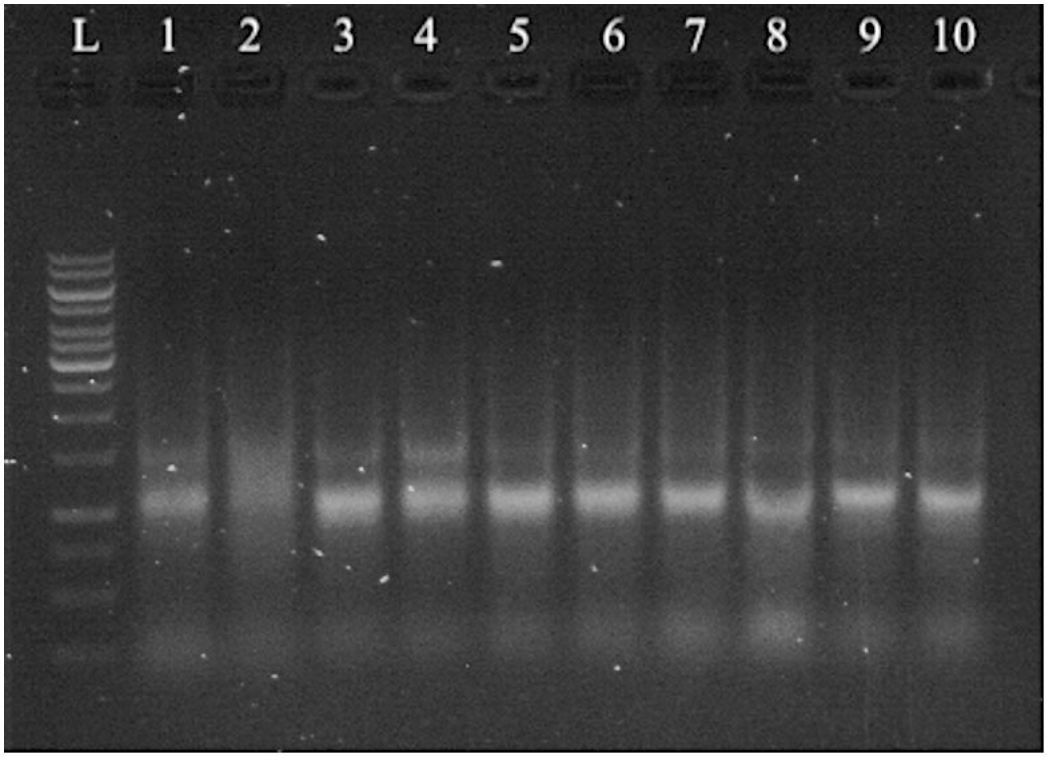

Figure 4. Gel results from the RNA extraction of oat and beans. Ladder used: GeneRuler 1 kb DNA Ladder. Samples: 1. Oat-SLU-1, 2. Oat-SJV-1, 3. SJV-BBG-1, 4. SJV-BBG-2, 5. SLU-BBB-1, 6. SLU-BBB-2, 7. SLU-BBT-1, 8. SLU-BBT-2, 9. SLU-SB-1, 10. SLU-SB-2.

The RNA extracts showed two bands, one bright and one faint (Figure 4). For Oat-SJV-1, the bands were not distinct. Two bands are expected, representing units of the ribosomal RNA (rRNA).

3.1.2 Gel electrophoresis after RNA extraction from aphids

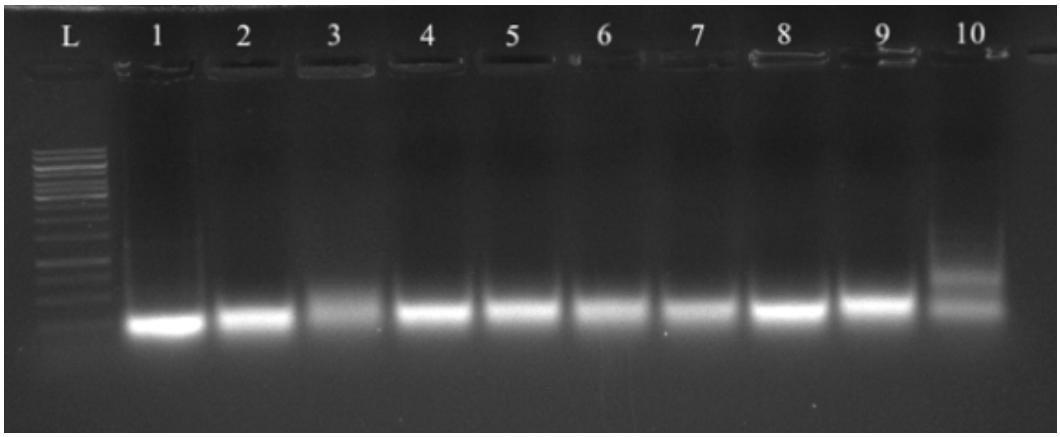

Figure 5. Results from gel after RNA extraction from aphids. Ladder used: GeneRuler 1 kb DNA Ladder. Samples: 1. A-neg, 2. I-14-1, 3. I-14-2, 4. I-20-1, 5. I-20-2, 6. Aln-14-1, 7. Aln-14-2, 8. Aln-20-1, 9. Aln-20-2, 10. A-pos.

RNA extracted from all aphids showed bands in the gel, however, the fragments were very small (Figure 5). Only RNA extracted from the positive control aphids showed two bands, however, these were smaller than the bands in RNA extracted from plants (Figure 4).

3.2 RT-PCR tests of luteovirus and polerovirus in plants and aphids

From the RNA, cDNA was synthesised and this was used for the PCR. The goal was to amplify a gene encoding the CP for luteo- and poleroviruses. The expected sizes of the PCR products were around 600 bp and 530 bp, respectively, for primer pairs Luteo F / Luteo R and Lu1 / Lu4.

3.2.1 Gel electrophoresis after RT-PCR on oat, bean and test aphid samples

Initial RT-PCR tests using RNA from the plant and aphid samples enabled selection of the best primers. The primer pair Lu1 / Lu4 together with the DreamTaq master mix yielded bands of the expected size for PCR products of oat, bean and aphid samples, while the use of the primer pair Luteo F / Luteo R did not result in any amplification (results not shown).

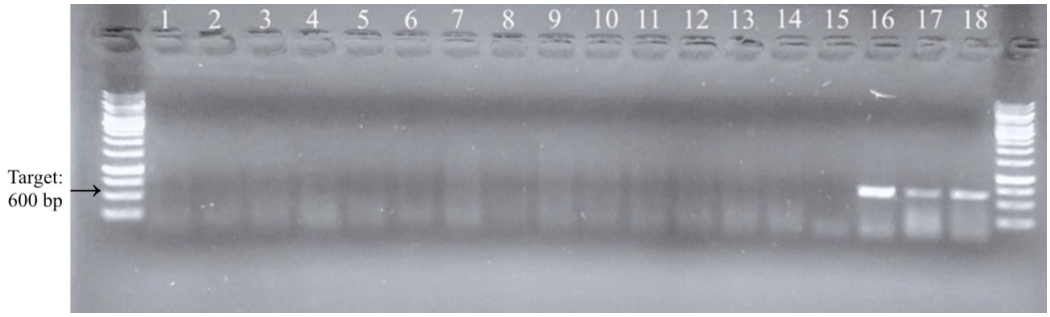

Figure 6. Results from gel after RT-PCR using the Luteo primer pair and Phusion master mix. Ladder used: GeneRuler 1 kb DNA Ladder. Samples: 1. Oat-SLU-1, 2. Oat-SLU-2, 3. Oat-SLU-3, 4. Oat-SJV-1, 5. Oat-SJV-2, 6. SJV-BBG-1, 7. SJV-BBG-2, 8. SLU-BBB-1, 9. SLU-BBB-2, 10. SLU-BBT-1, 11. SLU-BBT-2, 12. SLU-SB-1, 13. SLU-SB-2, 14. A-pos-test, 15. H_2O (negative control), 16. Sugar beet 19, 17. Sugar beet 22, 18. Sugar beet 24.

In a second test with the Luteo primer pair, cDNA was included of three sugar beet samples previously testing positive for polerovirus (Pettersson 2020). Amplicons of the expected size were only obtained for the three additional samples named sugar beet 19, sugar beet 22 and sugar beet 24 (Figure 6). No PCR bands were present for the samples from oat, beans or the test aphids.

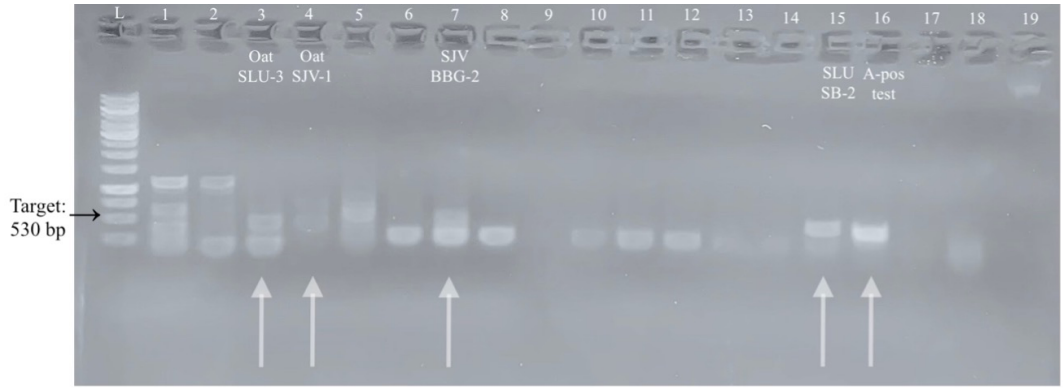

Figure 7. Results from gel after RT-PCR, using the Lu1 and Lu4 primers and the Phusion master mix. Indicated with arrows are the bands of expected size, that potentially could be the target. These indicated bands were purified from the gel. Ladder used: GeneRuler 1 kb DNA Ladder. Samples: 1. Oat-SLU-1, 2. Oat-SLU-2, 3. Oat-SLU-3, 4. Oat-SJV-1, 5. Oat-SJV-2, 6. SJV-BBG-1, 7. SJV-BBG-2, 8. SLU-BBB-1, 9. Empty lane, 10. SLU-BBB-2, 11. SLU-BBT-1, 12. SLU-BBT-2, 13. Empty lane, 14. SLU-SB-1, 15. SLU-SB-2, 16. A-pos-test, 17. Sugar beet 19, 18. Sugar beet 22, 19. H_2O (negative control).

Amplification using the primer pair Lu1 and Lu4 together with Phusion yielded bands of the expected size for PCR products of oat, bean and aphid samples (Figure 7).

3.2.2 Gel electrophoresis after RT-PCR on aphid samples

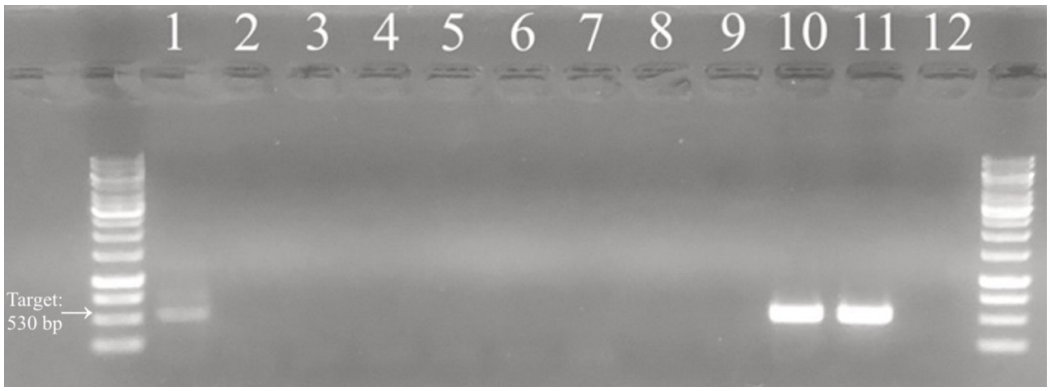

Figure 8. Results from gel after RT-PCR, using the Lu1 and Lu4 primers and DreamTaq Master Mix. Ladder used: GeneRuler 1 kb DNA Ladder. Samples: 1. A-neg, 2. I-14-1, 3. I-14-2, 4. I-20-1, 5. I-20-2, 6. Aln-14-1, 7. Aln-14-2, 8. Aln-20-1, 9. Aln-20-2, 10. A-pos, 11. A-pos-test, 12. H_2O (negative control).

After gel electrophoresis, the PCR products for the positive control aphids were visible as clear and bright bands of the size of the target (Figure 8). For the negative control, only multiple faint bands were visible that could be background amplification and / or traces of virus contamination. One of these bands had the same size as the target.

PCR tests of the older aphid samples from Ingelstorp and Alnarp did not result in any bands except for primer dimers. Neither amplicons of the size of the CP gene nor any of the background amplification found for the negative aphid control was present.

3.3 Gel electrophoresis after DNA purification from gel

The PCR products of expected size from Oat-SLU-3, Oat-SJV-1, SJV-BBG-2, SLU-SB-2 and A-pos-test (Figure 7) were purified. To verify a successful purification, a gel with the purified DNA was run.

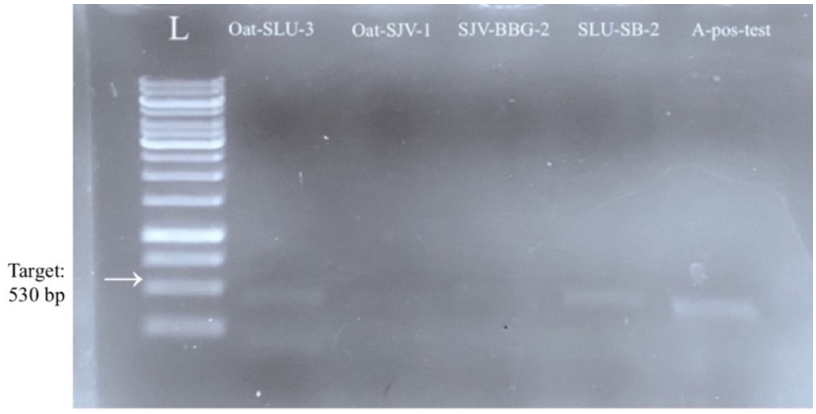

Figure 9. Results from gel after DNA purification from gel. Ladder used: GeneRuler 1 kb DNA Ladder. Samples: Oat-SLU-3, Oat-SJV-1, SJV-BBG2, SLU-SB-2 and A-pos-test.

Bands of varying brightness were present. Purified PCR products for Oat-SLU-3, SLU-SB-2 and A-pos-test showed brighter bands, whereas the products for Oat-SJV-1 and SJV-BBG-2 showed very faint bands.

For Oat-SLU-3, an additional, small second fragment was seemingly present as well. This smaller fragment was probably purified together with the larger target fragment.

3.4 Colonies after transformation

Ligation for five samples were used to transform competent cells of *E. coli*, which were grown on selective plates. For each sample, bacteria were grown on two plates (Table 3).

Table 3. Number of bacterial colonies on selective plates after transformation

DNA from sample	100 µL culture	Remaining pellet
Oat-SLU-3	19	328
Oat-SJV-1	2	39
SJV-BBG-2	3	36
SJV-SB-2	1	36
A-pos-test	1	48
pUC19	220	overgrown

Single colonies were found on all plates, and used to start overnight cultures. Using the results for the pUC19 transformation control, the transformation efficiency was calculated to be 8.8 x 10^6 transformants / µg DNA. This counts as a good result, as a number above 10^6 suggests a successful transformation for this type of competent cells.

3.5 Analysis of plasmid DNA by restriction enzyme digestion

The purified plasmids were digested with restriction enzyme *Bgl*II, making it possible to determine the size of the insert.

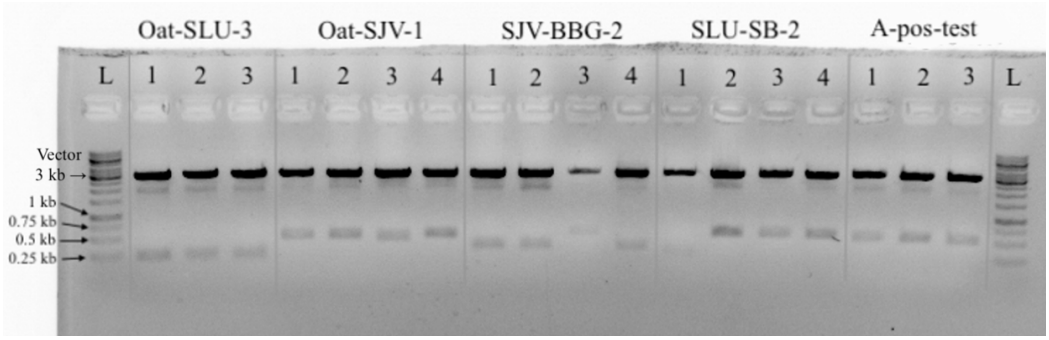

Figure 10. Fragments after restriction enzyme digestion of isolated plasmids. Ladder used: GeneRuler 1 kb DNA Ladder. Clones: Oat-SLU-3_1, Oat-SLU-3_2, Oat-SLU-3_3, Oat-SJV-1_1 Oat-SJV-1_2, Oat-SJV-1_3, Oat-SJV-1_4, SJV-BBG-2_1, SJV-BBG-2_2, SJV-BBG-2_3, SJV-BBG-2_4, SLU-SB-2_1, SLU-SB-2_2, SLU-SB-2_3, SLU-SB-2_4, A-pos-test_1, A-pos-test_2, A-pos-test_3.

For the majority of the samples, DNA fragments with the correct size of 530 bp were successfully cloned into a plasmid (Figure 10). All clones for Oat-SJV-1 and A-pos-test, as well as the clones SJV-BBG-2_3, SLU-SB-2_2, SLU-SB-2_3 and SLU-SB-2_4, contained inserts of the expected size. For the clones of sample Oat-SLU-3, this was not the case. These clones had small inserts, whose size did not match the target. The same was true for clones SJV-BBG-2_1, SJV-BBG-2_2, SJV-BBG-2_4, and SLU-SB-2_1, all having a small insert not matching the targeted size.

3.6 Sequencing results

All clones for Oat-SJV-1 and A-pos-test, as well as clones Oat-SLU-3_3, SJV-BBG-2_3, SJV-BBG-2_4, SLU-SB-2_2, SLU-SB-2_3, SLU-SB-2_4 (Figure 10) were sequenced.

Three clones, all from beans, had chromatograms with very low signal or unclear peaks, and could not be analysed further. These were (all forward reactions): SJV-BBG-2_3, SJV-BBG-2_4 and SLU-SB-2_3. As for the rest of the clones, most had an insert of a length between 408 and 461 bp, which is slightly shorter than the targeted length of 530 bp. The clone from Oat-SLU-3 had a shorter length of 152 bp (Table 4). Searches in nucleotide and protein databases did not for any insert sequence result in a significant identity with virus sequences.

Table 4. Sequence identities for cloned PCR products

Clone	Insert length (bp)	Highest sequence identity		% id.	Found in BLAST n / x
		Species	Accession number		
Oat-SLU-3_3 (F)*	152	*Hordeum vulgare*	XM_045089868.1	95.36	n
Oat-SJV-1_1 (F, R)	408	*Triticum dicoccoides*	XP_037438825.1	48.10	x
Oat-SJV-1_2 (F)	408	*Triticum dicoccoides*	XP_037438825.1	48.10	x
Oat-SJV-1_3 (F)	427	*Avena sativa*	OV702300.1	89.66	n
Oat-SJV-1_4 (F)	408	*Triticum dicoccoides*	XP_037438825.1	48.10	x
SJV-BBG-2_3 (R)	455	*Vicia faba*	KF658615.1	96.41	n
SLU-SB-2_2 (F, R)	461	*Glycine soja*	XM_028384848.1	95.37	n
SLU-SB-2_4 (F)	461	*Glycine soja*	XM_028384848.1	95.37	n
A-pos-test_1 (F, R)	416	*Rhopalosiphum maidis*	XM_026967293.1	96.03	n
A-pos-test_2 (F)	416	*Rhopalosiphum maidis*	XM_026967293.1	96.03	n
A-pos-test_3 (F)	416	*Rhopalosiphum maidis*	XM_026967293.1	96.03	n

*Forward (F) sequencing was carried out for all inserts, and reverse (R) sequencing for some of the inserts.

All clones for the oat samples had inserts with highest sequence identity to genes from oat, wheat (*Triticum dicoccoides)* and barley (*Hordeum vulgare)*. For the clones possible to analyse for bean samples, inserts with highest sequence identities only to genes from soybean and broad beans were found. Thus, the conclusion can be drawn that the sequences originated from the plant genomes. The inserts of the three clones for the aphid sample showed highest sequence identity with a gene originating from corn aphids (*Rhopalosiphum maidis*). Thus, the insert sequence from the aphids originated surely from their own genomes as well.

All clones that were possible to analyse had the Lu1 primer at both ends of the insert. Primer Lu4 was not present in any of the inserts.

Discussion

The goal of this project was to survey the presence of luteo- and polerovirus by detection of a specific gene. Throughout the process, indications of viral sequences were observed, however, in the end no viral sequences were found. Does this mean that all samples were virus free? In the following sections, reasons for the absence of detected virus genes are discussed.

In addition to the concrete factors listed below, there are as in all experiments a risk for human errors, for instance when pipetting or measuring. None of the end results in this study are, however, noticeably affected in such a way that the human factor can be assumed to have had a decisive impact.

4.1 Possible degradation of RNA in old aphid samples

When analyzing the RNA extracted from aphids, it was found that the RNA fragments from the older samples were very small. RNA from the aphids also had a lower purity than the plant samples, most probably because the method used for the extraction was different and did not include any column purification.

As for the small fragment sizes, this might be due to degradation. It may have been a limitation for the amplification, and the reason for the absence of bands in the gel after PCR. If this was the case, the possibility of determining whether the aphids were carrying virus or not was reduced. A potential degradation could be due to a too harsh RNA extraction method, or possibly, the RNA was degraded before the aphids were put in 70 % ethanol. However, short fragments are not necessarily a proof of RNA degradation (Winnebeck et al. 2010). In fact, this phenomenon is commonly observed also for non-degraded insect RNA, although there is no proposed explanation for it.

The age of the samples is another interesting point to discuss. There has been successful BYDV detection through RNA extraction and RT-PCR for very old samples earlier. Researchers have succeeded in detecting viral sequences of BYDV in material dating from 1917 (Malmstrom et al. 2007). Thus, the age of the samples should not be an issue. However, this detection was from dried grasses and not from aphids, and it is a difficult comparison to make.

The risk of RNase contamination during gel electrophoresis was minimised, since the gel equipment was carefully washed before use.

The uncertain results for the aphids hindered reliable results of whether or not the samples contained BYDV. They might have been virus free, but it is not necessarily the case.

4.2 Primer functioning

Amplification using the Luteo primer pair resulted in bands only for the additional sugar beet samples infected by the poleroviruses BMYV, BChV and TuYV. One reason for this could be that the samples were free from infection by luteoviruses or poleroviruses. Another reason might be that BLRV and BYDV are, despite their relatedness, too distinct from the virus species, which earlier have been detected using these primers. In the original publication, the Luteo primers were used to amplify the CP gene of mainly the polerovirus chickpea chlorotic stunt virus (CpCSV). The amino acid identity of the CP of CpCSV is 59 % to that of BLRV, and only 44 % to BYDV-PAV (Abraham et al. 2006). If there would be BYD-associated viruses or BLRV present in these samples, the conclusion could be drawn that the Luteo primers most probably do not work for amplification of them.

The Lu primers, on the other hand, are short (14 and 15 bp) (Robertson et al. 1991), and not very specific. They anneal easily. This enables an easy and straightforward detection, but can also cause complications, since also non-targeted fragments may be amplified to a higher extent. The Lu primers did amplify what seemed to be the target sequence for several of the samples. However, since the sequence results only matched with genes from host genomes of each sample, they might have been too general and allowing too much background amplification.

In addition, Lu4 was not found in any of the cloned PCR products. This could possibly mean that the primer was not working, e.g., because it was too old, or for other reasons degraded. However, it is also possible that Lu4 simply did not have any matching sequence to anneal with, whereas Lu1 matched with the sample DNA at two non-targeted positions. Both Lu1 and Lu4 would most probably have annealed to the viral sequence if there was any present. Thereby, the probability for presence of luteovirus or polerovirus RNA in these samples is very low.

4.3 Optimisation of PCR

One key factor with high impact on the end result is the PCR. Optimisation of this step is advantageous for the further procedures.

A few things that could be examined in order to improve the PCR results would be an increased annealing temperature and / or to run fewer cycles – or to use other primers, which are more specific for BYD-associated viruses and BLRV. By these means the high background amplification would presumably decrease, meaning

there would be less risk of amplifying and purifying the wrong fragments. A higher specificity could, however, also have a negative impact on the detection of targeted fragments.

4.4 Unintended purification of small, non-targeted DNA fragments

One crucial step when extracting the sought DNA, is the purification from the gel. One risk is remains of smaller fragments lagging behind and "hiding" in a band of bigger size. Therefore, the more non-specific the PCR is, the higher the risk will be to purify a fragment from background amplification instead of the intended one. This is likely what happened for the Oat-SLU-3_3 sample.

4.5 Ligation not a limiting factor

The ligation plays a critical role, too. In this case, the molar ratio between vector and insert did not reach the optimal. However, this should not be a reason of wrong fragments being cloned. Rather, if the non-optimal ratio was limiting in any way, it is more probable that no bacteria would have grown at all, since the lethal gene of the plasmid vector would then have been uninterrupted. The conclusion is therefore that ligation was not a limiting factor.

Nevertheless, something that was a disadvantage during the ligation process, was that small fragments were cloned into the vector instead of the correct larger fragment for the samples Oat-SLU-3, SJV-BBG-2 and SLU-SB-2. Again, the best way to avoid this from occurring would be to minimise the risk of amplification of the wrong fragments.

4.6 Cloning of potentially positive fragment

Although no clones with the sought sequence were found, it does not mean that they were completely absent. The end result might still show negative for virus, when in fact the samples had been infected. Here is one explanation.

4.6.1 Purification of DNA fragments

When purifying DNA from gel, it is not always possible to leave out non-targeted fragments, particularly in this case when background amplification was apparent. Amplification of the targeted CP gene might have worked, so correct fragments were present and perhaps even purified from the gel. However, also non-targeted

fragments of roughly the same size were cut out. This was especially the case for the aphid sample acting as positive control.

4.6.2 Ligation and transformation

The purification of non-targeted fragments together with the targeted, led to them being ligated into the plasmid vector, and they were consequently used for transformation. Colonies of transformed bacteria were later selected and used to generate overnight cultures, from which the plasmids subsequently were isolated.

Consequent to this, purified non-targeted fragments follow the different steps until restriction enzyme digestion analysis and finally sequencing. The non-target fragments still "pass the test" in all analyses, since they are of expected size.

The targeted fragments might have been present, and also been cloned, but not detected after the transformation. Picking and growing more colonies from the plates with transformed bacteria would have increased the likelihood of finding clones with the correct insert.

4.7 How expected are these results, given the current situation for luteo- / polerovirus in Sweden?

A Swedish study surveyed the presence of BYD-associated viruses in ryegrass samples collected in 2012 and 2013 (Yazdkhasti et al. 2021). In this study, infections with BYDV and CYDV were detected in Västra Götaland, which is the same county as for the oat samples in this study. Out of 423 samples, two samples tested positive for BYDV-PAV, 17 for BYDV-MAV, and 16 for CYDV. No infection was found in any of the 400 samples from Uppsala or the 20 samples from Stockholm.

In 2021, BYDV was detected in oat plants in the county of Västra Götaland (Johansson et al. 2021). In other words, there is a recurrent presence of BYD-associated viruses in Västra Götaland. This fact, together with the observed symptoms in this study's oat samples from the Swedish Board of Agriculture, suggested infection with BYDV or CYDV. However, the samples analysed in this project did not test positive for the virus.

As for the oat samples from Uppsala, the negative results in this study are in line with the previous study (Yazdkhasti et al. 2021). However, BYDV has been detected in the area previously (Bisnieks et al. 2004). Additionally, as recently as 2021, bird cherry-oat aphids were abundantly found in the area and up to three oat plants / m^2 in the fields displayed symptoms of BYDV infection (Norrlund et al. 2021).

BLRV and SbDV have been detected in Europe earlier. There are no reports of BLRV or SbDV in Sweden specifically, however, in countries such as Germany

and Finland (CABI 2022; Luoto et al. 2021). These countries are not geographically far from Sweden. Thus, findings of these viruses might be expected also in Sweden.

4.8 Suggestions for further research

Some factors might have changed the results if they were done differently. In this section a few suggestions are listed for what could be changed in order to identify the cause of the observed symptoms.

A first suggestion is to sequence non-purified products directly after RT-PCR, without cloning and transformation. This alternative might simplify findings of other sequences than the ones that were ultimately sequenced in this case. However, it would also require more difficult interpretation work of the sequence analyses, since there could be mixed sequences that needed to be distinguished. In this case, however, Lu1 and Lu4 primers were shorter than the sequencing company's minimum requirement of primer length for non-purified PCR products.

Secondly, if the symptoms on the plants were due to infections of other viruses, the same method, but with other primers for the PCR could have been useful. This could reveal infections of virus species that are not recognised by the Lu or Luteo primers. There are also alternative methods available to detect viruses, such as ELISA or NGS (Cassedy et al. 2021). Especially application of NGS offers a high specificity. Using these methods might have changed the results.

Lastly, even if the observed symptoms in the plants were in fact not caused by viruses, they could still lead to yield losses and have a negative economical impact. The symptoms might have been due to other pathogens, such as fungi or bacteria, or due to stress caused by insects or environmental factors. Further investigation would be needed to find an explanation for the symptoms in these plants.

This is true also for the aphids. Further investigations would be needed in order to learn more about their role of transmission and susequent virus outbreaks in plants.

4.9 Conclusion

BYDV has previously been detected in Sweden repeatedly. BLRV and SbDV have been detected in countries in close proximity. Findings of any of these viruses would thereby not have been surprising. However, no viral genes were detected, neither in the field samples from 2022, nor in the aphids. In the cases of the plants, it is most likely that these samples were not infected by any luteovirus or polerovirus. The symptoms that were displayed in some of the plants were most probably effects of other biotic or abiotic factors. The likelihood of virus detection might have increased by testing a larger quantity of samples.

In the case of the positive control aphids, the targeted viral gene was seemingly amplified, but so was an other amplification product of the same size, originating from the host genome. This was the case for all clones of the positive aphid control that were sequenced.

DNA from the negative control aphids showed only faint bands after the PCR, these could be assumed to be aphid genes (same amplicon as was sequenced from the positive control).

In the case of the older aphid samples, no certain conclusions can be drawn about the presence of virus. They could be virus free, or the viral RNA could have been degraded. Nothing can be concluded about correlations between the occurrence of virus-carrying aphids and the virus outbreaks in plants in 2014-2015 and 2020-2021. Other results might have been obtained if the methods used were optimised or changed.

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Acknowledgements

I would first of all like to direct a huge thank you to my supervisor Anders Kvarnheden. You have been the most important person during this project, and I could not have asked for a better guidance. You have been far more generous with your time, tips, patience and shared knowledge than could ever be expected, and I am so grateful to have had you as my supervisor.

A big thanks also to my lab supervisor Vinitha Puthanveed. I am happy for all you managed to teach me in this short time, and that you provided me with everything I needed.

Thank you to Velemir Ninkovic and Maria Kedmark for providing me with (double sets!) of aphids.

The rest of the department of Plant Biology deserves a thank you for including me in such a nice way during my short time of doing this. There was always someone around to patiently help me with every question that popped up.

Till sist vill jag gå utanför skolan och skicka uppskattning till min familj och mina vänner. Ni gör allt lite bättre, och det gör mig trygg och lycklig att veta att jag har så många fantastiska människor i mitt liv! Ni fortsätter se igenom alla mina tvivel och påminner mig om att jag klarar mer än vad jag själv inser. Det betyder mest. Jag älskar er.

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