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Developing a molecular marker for the detection of asparagus virus II —final report for project 97/13

A report prepared for the
New Zealand Asparagus Council

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1 EXECUTIVE SUMMARY

Asparagus virus II (AVII) can cause yield losses of up to 50% in asparagus crops. However, infected plants bear no viral disease symptoms making their removal from a crop following a visual assessment impossible.

Currently available technologies for detecting virus in asparagus plants via ELISA or indicator plants are time consuming and involve repeated testing. The objective of this project was to develop a molecular marker for detecting AVII that would allow rapid screening of large numbers of samples, accurate detection, a small sample size and early virus detection.

An adapted Polymerase Chain Reaction process, Reverse-Transcriptase PCR, was used to detected the presence of the RNA AVII virus. A range of asparagus plant materials with known AVII infection status were tested.

Results confirmed that the RT-PCR test for AVII can accurately detect the presence of virus at very low concentrations. AVII can be detected in single seeds which may allow seed exporters to meet stringent import/export phytosanitary standards. The technique is also suited to testing the viral status of tissue culture plants and may be used by plant breeders to determine the level of resistance in breeding lines.

2 BACKGROUND

The objective of this project is to develop a molecular (PCR) marker for asparagus virus II, (AVII) enabling the rapid and accurate detection of AVII at an early stage.

Asparagus virus II has a detrimental effect on the production of asparagus, with yield losses of up to 50% reported (Marlene Jaspers, ARC meeting Palmerston North, May 1997). The most efficient way of avoiding such yield losses and/or the (mechanical) spread of the virus is to isolate and remove infected plants from the field. However, there are no visual symptoms on infected plants which makes their identification impractical. The preferred method would, therefore, be to test the planting material for the presence of the virus.

The current technique of ELISA and/or indicator plants is both time-consuming and involves repeated testing. Plants need to be grown for three to four months to enable the accumulation of large quantities of virus in order to obtain a virus concentration above the detection limit.

To overcome these difficulties, it is proposed to develop a molecular marker for the detection of AVII that would allow for:

- **rapid** screening of plant material,
- **accurate** detection of the presence of virus at low concentration,
- a **small sample size** to be used for detection,
- **early** detection of the virus, and
- **large numbers** of samples to be screened.

3 MATERIALS AND METHODS

The technique of **PCR** (Polymerase Chain Reaction) is based on the amplification of specific pieces of DNA, for which the nucleotide sequence is known, to such an extent that it is very easy to detect the product of the reaction. This technique is highly sensitive (several orders of magnitude more sensitive than ELISA).

Since AVII is an RNA virus, an additional step has to be built into the PCR protocol to first change the RNA virus molecule into a DNA molecule. This step is called 'Reverse Transcriptase' and hence the PCR reaction is called '**RT-PCR**'.

The amplification occurs with the help of '**primer**' sequences, short DNA sequences that are specific to the sequence of DNA/RNA to be detected. Primers are carefully selected on the basis of a few criteria that make them optimally suited for PCR. RNA sequences of AVII have been determined and are published on the internet. We used the putative coat protein gene as determined by Rafael-Martin & Rivera-Bustamente (1995, Nucleotide sequence of asparagus virus II (AVII) RNA3. GenBank accession X86352) and designed primers that amplify a 500 base pair fragment of the coat protein gene.

The **plant** materials used in this study consisted of infected samples, uninfected samples or 'unknown' samples. The different plant tissues used are listed in Table 1. Total RNA is extracted from the plant tissue samples and 1 μl of this extract is subjected to the RT-PCR.

The presence of the **AVII band is visualised** by performing gel-electrophoresis on agarose gels followed by staining with ethidium bromide.

4 RESULTS

The results are outlined in Table 1. Appendix I contains a poster outlining progress made in this research project.

Plant material with the status infected/uninfected originated from plants that had had their infection status determined by ELISA in fresh spears of full-grown plants (Marlene Jaspers, pers. comm.). Other plant material is listed as 'unknown'.

Infected seeds were from a cross between two infected plants. Siblings of these seeds were grown and proven to be infected (ELISA of spears). Uninfected seeds were from a cross between two uninfected plants. Siblings of these seeds were grown and proven to be uninfected (ELISA of spears).

It should be noted that at present it seems that dead fern is not a reliable source of material upon which to accurately detect virus. If desirable/required, further optimising of the protocol might allow the detection of virus in dead fern under all conditions.

Table 1: Type of asparagus plant material subjected to RT-PCR and its infection status.

Type of material	AVII detected (RT-PCR)
Spear, infected plant (frozen)	+[1]
Spear, uninfected plant (frozen)	-[2]
Spear, infected plant (dried)	+
Fern, infected plant	+
Fern, uninfected plant	-
Dead fern, unknown, plant #1	+
spear of the above plant (#1)	+
Dead fern, unknown, plant #2	-
spear of the above plant (#2)	+
Single seed, infected parent plants	+
Single seed, uninfected parent plants	-
Pollen, unknown (frozen)	+
Pollen, unknown (fresh)	-
Tissue culture plant, shoots #1	+
Tissue culture plant, shoots #2	-
Asparagus densiflorus Sprengeri	-
Spear of canned asparagus, can #1	+
Spear of canned asparagus, can #2	-

[1] + = AVII detected.

[2] - = AVII not detected.

Two additional experiments were performed in which an attempt was made to determine the detection limit of the method.

1. A dilution series was made of a known positive sample.
   In the 1000x dilution the AVII band was still detectable. In the 10 000x dilution the band was not present in one RT-PCR experiment, but was present in the parallel duplo RT-PCR experiment. In the 100 000x dilution the AVII band was not detected.

Note: the RNA concentration of the original sample was not determined, as it will not be possible to accurately estimate the AVII particles. The RNA sample will contain plant RNA as well as virus RNA and it is not possible to determine the proportion of the total RNA that is virus RNA.

2. A 'spiking' experiment was performed.
In this experiment RNA from a virus-free sample was mixed 1:1 with the dilution series of the virus-infected sample used for the dilution series described above. The detection limit in this experiment was virtually the same as in the dilution series experiment; the 1:1 mix of virus-free and 10 000x virus infected showed the AVII specific band, while the 1:1 mix of virus-free and 100 000x virus infected did not show the AVII specific band.

These results confirm that the RT-PCR test for AVII can accurately detect the presence of virus at very low concentrations.

5 CONCLUSIONS

A molecular (PCR) marker for the detection of AVII has been developed. This marker allows for:

- a **rapid** screening of plant material
  In the protocol that was developed samples can be dealt with in a single day (i.e. from extraction, through PCR and detection of a potential AVII presence),

- an **accurate** detection of the presence of virus at low concentration
  We have been able to dilute an infected sample up to 1000 times and still detect the presence of the virus,

- a **small sample size** to be used for detection
  Virus could be detected in single seeds and pollen samples,

- **early** detection of the virus
  Plant tissues from pollen and seeds to spears and mature fern caused no serious problem for virus detection,

- **large numbers** of samples to be screened
  We have not concentrated on determining the number of samples that can be handled in a single day, but expect that it would be feasible to handle up to 96 samples per day (i.e. one standard 8 x 12 assay plate for PCR).

6 FUTURE PROSPECTS

The protocol developed allows for a very rapid, efficient and accurate screening of asparagus tissues for the presence of AVII. The test can be performed at any time, on many types of tissue, e.g. fern, tissue culture plants, seedlings, single seeds, and harvested spears (fresh, dried or frozen). Detecting the presence of AVII on single seeds is especially important for import/export purposes. The protocol will allow for earlier testing of tissue culture plants following viral elimination via meristem culture. Furthermore, it will allow for virus testing of seed blocks to ensure the production of virus-free seed. The method is a potentially critical component of breeding programmes allowing germplasm to be efficiently screening for resistance to AVII (i.e. absence of virus = resistance).

7 APPENDIX

Appendix I Poster—Developing a molecular marker for the detection of Asparagus Virus II

Developing a Molecular Marker for the Detection of Asparagus Virus II

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Introduction

Asparagus virus II (AVII) has a detrimental effect on the production of asparagus, with yield losses of up to 50% reported . The most efficient way of avoiding such yield losses and/or the (mechanical) spread of the virus, is to isolate and remove infected plants from the field. However, there are no visual symptoms on infected plants which makes their identification impractical. The preferred method would therefore be to test the planting material for presence of the virus.

The current technique of ELISA and/or indicator plants is both time-consuming and involves repeated testing. Plants need to be grown for a period of 3-4 months to enable the accumulation of large quantities of virus in order to obtain a virus concentration above the detection limit.

The objective of this project is to develop a molecular marker for AV II, enabling rapid and accurate detection at an early stage, on large numbers of small samples.

The jargon.

PCR (=Polymerase Chain Reaction): the amplification of specific pieces of DNA, for which the sequence is known, to such an extent that it is very easy to visualise the product of the reaction. This technique is highly sensitive (several orders of magnitude more sensitive than ELISA).
RT-PCR: AVII is a RNA virus: an additional step has to performed to first change the RNA molecule into a DNA molecule. This step is called 'Reverse Transcriptase' and hence the PCR reaction is called RT-PCR.
Primers: short DNA sequences that are specific for the DNA/RNA to be detected, necessary for amplification of the target sequence (here: AVII coat protein). Primers are carefully selected on basis of a few criteria that make them optimally suited for PCR
Plants: infected samples, uninfected samples or 'unknown' samples were used. The different plant tissues used are listed in the table. Total RNA is extracted from the individual plant tissue samples and 1 µl of this extract is subjected to the RT-PCR.
Visualise AVII: gel-electrophoresis on agarose gels followed by staining with ethidium bromide (see photo).

Results

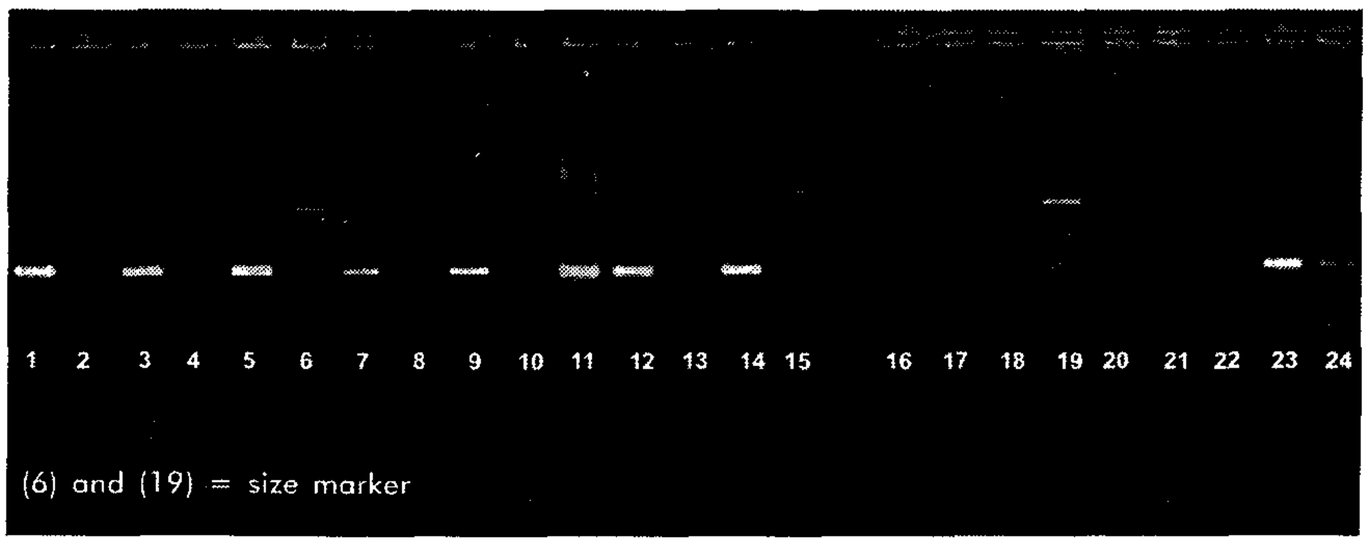
Type of material	AVII detected (RT-PCR)	Photo lane
Spear, infected plant (frozen)	+	12
Spear, uninfected plant (frozen)	–	13
Spear, infected plant (dried)	+	11
Spear, unknown (frozen)	+	5
Spear, unknown (fresh)	–	22
Fern, infected plant	+	14
Fern, uninfected plant	–	15
Fern, unknown - meristem-cultured plants	+	1
Fern, unknown - meristem-cultured plants	–	2
Dead fern, unknown, plant #1	+	24
spear of the above plant (#1)	+	23
Dead fern, unknown, plant #2	–	not shown
spear of the above plant (#2)	+	not shown
Single seed, infected parent plants	+	9
Single seed, uninfected parent plants	–	10
Pollen, unknown (frozen)	+	3
Pollen, unknown (fresh)	–	4
Tissue culture plant, unknown, shoots #1	+	21
Tissue culture plant, unknown, shoots #2	–	20
Spear, unknown - uninfected female x infected male, plant #1	+	7
Spear, unknown - uninfected female x infected male, plant #2	–	8
Spear of canned asparagus, can #1	+	16
Spear of canned asparagus, can #2	–	17
Asparagus densiflorus 'Sprengeri'	–	18

Plant material for which the status infected/uninfected is listed came from plants for which the infection was determined by ELISA in fresh spears of full-grown plants (Marlene Jaspers, pers. comm.). Other plant material is listed 'unknown'.

Infected seeds were from a cross between 2 infected plants. Siblings of these seeds were grown and proven to be infected (ELISA of spears).

Uninfected seed were from a cross between 2 uninfected plants. Siblings of these seeds were grown and proven to be uninfected (ELISA of spears).

At present it seems that dead fern is not reliable enough for accurate detection of virus. Further optimising of the protocol might allow the detection of virus in dead fern under all conditions.



Conclusion.

A molecular (PCR) marker for the detection of AV II has been developed. This marker allows for:

- a **rapid** screening of plant material- samples can be dealt with in one single day *i.e.* from extraction, through PCR and detection of a potential AVII presence.
- an **accurate** detection of the presence of virus at low concentration- a dilution of up to 1,000 times still detects the presence of the virus.
- a **small sample size** to be used - detection in single seeds and pollen samples.
- **early** detection of the virus- virus was detected in many different plant tissues
- **large numbers** of samples to be screened- it is easily feasible for one person to handle up to 96 samples per day (*i.e.* one standard 8x12 assay plate for PCR).

Future applications.

- Rapid, efficient and accurate screening of asparagus tissues for the presence of AVII on a routine basis
- Possibility to detect AVII in single seeds- especially important for import/export
- Earlier testing of tissue culture plants following virus-elimination via meristem culture
- Virus testing of seed blocks to ensure the production of virus-free seed
- Efficiently screen germplasm for resistance to AVII in breeding programmes

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