RNA silencing based resistance to Phytophthora cinnamomi.

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Abstract

English

Phytophthora root rot (PRR) caused by *Phytophthora cinnamomi* is an economically significant disease of avocados world wide. We aim to utilize RNA silencing technology to deliver PRR resistance to avocado trees by means of transgenic rootstocks.
RNA silencing is a conserved eukaryotic surveillance mechanism with similar pathways in plants, animals and fungi. A key conserved feature is that it is triggered by double-stranded RNA (dsRNA) that is processed into small interfering (si)RNAs which can direct degradation of RNA with complete sequence homology. RNA silencing can provide high-level pathogen resistance by specific targeting of essential pathogen genes via siRNAs taken up from transgenic plants.
We have engineered dsRNA constructs in plants to target essential genes in *P.cinnamomi* with the aim to deliver RNA silencing based resistance to this pathogen in avocado. Transgenic *Arabidopsis thaliana* plants have been generated with selected dsRNA constructs for proof of concept.

Spanish

Phytophthora pudricion de la raiz (PPR) en aguacates causada por *Phytophthora cinnamomi* es economicamente la enfermedad mas importante de este cultivo en el mundo. Nuestro objetivo es utilizar la tecnica de silenciamiento de genes por RNA de interferencia para obtener resistencia a PPR en selectos pies de injerto o patrones transgenicos de aguacate.
Silenciamiento del RNA es un mecanismo de defensa en eucariotes, es altamente conservado y muy similar en plantas, animales y hongos. El mecanismo es iniciado por la presencia de RNA de doble cadena (dsRNA) el cual es procesado por ribonucleasas en pequenas sequencias llamados RNAs de interferencia (siRNAs) que cuando hibridan con RNA homologos son degradados y proveen de alto nivel de resistencia a plantas transgenicas con sequencias especificas de patogenos.
Hemos disenado construcciones de dsRNA que son especificas para genes esenciales de *P. cinnamomi* con el objetivo de obtener resistencia en aguacate basada en silenciamiento de RNA del patogeno. Se han generado plantas transgenicas de Arabidopsis thaliana con construcciones de dsRNA seleccionadas para probar este concepto.

Key words: Avocado, Phytophthora, RNA silencing, Resistance, Zoospore, Oomycete.

Introduction

Avocado (*Persea americana* Miller) is a member of the Lauraceae family and is a tree native to Central Mexico (Chen et al, 2008). It is widely grown in tropical temperature climates throughout the world. In recent years, production of avocado in Australia has increased substantially with approximately 49500 tonnes being produced, worth around $120 million (FAO, 2008). The main growing areas in Australia for avocados are in Queensland, New South Wales and Western Australia. Many different varieties are grown, but most of the Australian industry is made up of Hass, Fuerte, Sharwil, Wurtz and Shepard. Orchards range from 50 to 35 000 trees and trees are grown from grafted nursery stock.

Production of avocado is limited by the serious disease Phytophthora root rot (PRR) and to date all cultivars obtained by conventional breeding are tolerant to PRR. The soil borne pathogen that causes this disease, *Phytophthora cinnamomi* Rands, is considered one of the world's worst invasive organisms and all areas in Australia producing avocados are affected (Gees and Coffey 1989). It causes substantial damage to trees in Queensland and New South Wales. The causal oomycete

fungus, *P. cinnamomi* Rands, was first isolated from cinnamon trees in Sumatra in 1922 and has since been reported from over 70 countries. It has an extremely wide host range including 1000 varieties and species of plants. Major hosts include avocado, pineapple, chestnut, eucalyptus, several species of pine, sycamore, peach, pear, many ornamentals (including azalea, camellia and rhododendron) and many indigenous Australian and South African plants.

P. cinnamomi forms several different spore stages that are involved in infection, disease development and survival; these include zoospores, chlamydospores and oospores. Zoospores are motile spores which are disseminated by flowing water and on reaching the roots; they penetrate, germinate and infect tender root tissue. Root lesions can be found throughout the small absorbing roots within 72 hrs. Under dry soil conditions the fungus may produce chlamydospores which are survival structures that can survive for several years. These spores are formed within the roots and are released into the soil when the roots decay. Where *P. cinnamomi* is not native to an area, the primary method of introduction of the disease into orchards is by infected nursery trees.

RNA silencing has proven to be an emerging strategy to control plant viruses and nematodes in agricultural crops. It is a conserved eukaryotic surveillance mechanism thought to play a role in protection against invading nucleic acids such as viruses, transposons and transgenes. The RNA silencing pathways that exist in plants (post transcriptional gene silencing), animals (RNA interference) and fungi (quelling) have similar genetic requirements and biochemical features. A key conserved feature is that it is triggered by double-stranded RNA (dsRNA) that is processed into 21-25 nt short interfering (si)RNAs by the activity of an RNase III like enzyme called Dicer. The siRNAs are then incorporated into an RNA-induced silencing complex (RISC), so ensuring that it specifically degrades any RNA sharing close sequence similarity with the inducing dsRNA (Baulcombe 2005). Transgene-mediated virus resistance is a classical example of RNA silencing and its role in antiviral defence in plants (Dietzgen and Mitter 2006).

In the current study *Arabidopsis thaliana* plants were transformed with constructs targeting essential genes in *P. cinnamomi* with the aim to impart RNA silencing based resistance to this pathogen.

Materials and Methods

Phytophthora cinnamomi Zoospore Production

From the margin of an actively growing *P. cinnamomi* colony (ca 5-7days old) 5mm cubes of agar were cut out and 5 pieces placed onto a clean empty petri dish. The agar cubes were bathed in 10mL clarified 20% V8 liquid (V8 clarified by addition of 10g/L $CaCO_3$, centrifuge 7000rpm 10 mins and collect supernatant) and incubated for 5 days at 24ºC+/- 1ºC in darkness. After 5 days the V8 liquid was removed and the petri dish rinsed 3 times with sterile deionised water. The agar cubes were bathed in 10mL soil filtrate (100g/L garden soil in sterile deionised water was agitated and left to stand 5 hrs before carefully pipetting off liquid and filtering through Whatman's® No 1 filter paper) and incubated under continuous light overnight and up to three days to produce sporangia. After sporangia were produced the soil filtrate was pipetted off and the culture bathed in 10mL sterile chilled 4ºC deionised water. The petri dish was placed at 4ºC for 30 mins followed by incubation at room temperature 30 mins up to 1 hour to release zoospores.

Construct Design

Hairpin RNA constructs targeting *P. cinnamomi* essential genes were designed and cloned into a binary vector carrying Basta® resistance (provided by Prof Bernie Carroll, University of Queensland). *A. tumefaciens* LBA4404 transformed with the constructs was used for stable transformation of *Arabidopsis thaliana* ecotype Landsberg.

Arabidopsis transformation via floral dip

A. thaliana ecotype Landsberg plants were grown until floral buds formed. A 250mL *Agrobacterium tumefacians* (LBA4404 pUQC477) (Brosnan et al.,2007) culture containing the construct of interest was grown overnight with antibiotic selection at 28°C in the dark before being centrifuged

3000rpm/4°C/10 min. The supernatant was removed and the pellet resuspended in 500mL of a 5% sucrose solution. The solution was spun vigorously on a magnetic stirrer and 187.5µL of Silwet L-77® added. The floral buds were submerged 5 times in the stirring solution until a film covered all the buds. The plants were sealed in a foil wrapped tray to create a moist dark environment for 24 hrs. The floral dip process was repeated one week later with the same plants. T_1 generation seeds collected from transformed T_0 generation plants were bulked and sown in a seedling tray. The bar gene (D'Halluin et al., 1992) from the binary vector serves as a negative selectable marker and allows the selection of putative transformants with the herbicide Basta® (Bayer®, Australia). Two Basta treatments three days apart were administered as soon as the cotyledons appeared.

Confirmation of transgenic status via PCR

The plants that survived this primary selection were further confirmed for transgenesis by PCR to account for any escapes. Genomic DNA was extracted from transformants via hexadecyltrimethylammonium bromide (CTAB) method as described by Springer (2010) except that leaves were ground in liquid nitrogen. Plants were confirmed by PCR for the presence of the *P. cinnamomi* specific gene.

siRNA

Total RNA from transgenic plants was isolated with TRIzol® reagent (Invitrogen, USA). Small RNA was separated on 17% acrylamide- 7M urea gels and transferred to nylon membrane by semi-dry blotting (BioRad) (Mitter et al 2003). Specific siRNAs were detected by radioactively labelled probes by the random-priming method using the Megaprime kit (GE Healthcare-Amersham). Hybridization was done at 40ºC in Ultrahyb buffer (Ambion) and after washing, bands were detected on Agfa-Curix X-ray film.

Results

P. cinnamomi zoospore production was optimised and the presence of sporangia confirmed by observation under a microscope. After the sporangia were cold shocked at 4ºC for 30 mins they were incubated for 30 mins at room temperature to allow the release of zoospores. The presence of motile zoospores was confirmed by observation under the microscope.

Agrobacterium carrying the construct targeting essential genes in *P. cinnamomi* was used to transform wild type *Arabidopsis thaliana* ecotype Landsberg via the floral dip method. Seeds harvested from T_0 generation plants were bulked and sown. The seedlings were subjected to primary screening with the herbicide Basta®.

Putative transformants that survived this primary screening were further confirmed for transgenesis via the extraction of genomic DNA and PCR analysis to confirm the presence of the *P. cinnamomi* specific gene. PCR-positive transgenic plants were used to investigate the expression of the siRNA. The presence of *P. cinnamomi* specific siRNAs in transgenic *Arabidopsis* Landsberg plants has been confirmed by RNA blot analysis.

The zoospore production method has been optimised to facilitate resistance screening experiments, and resistant plants have been identified. Resistance screening and further molecular analysis of transgenic plants is in progress. Transformation of avocado somatic embryos of selected rootstocks with these dsRNA constructs is also underway.

Conclusions

World-wide Phytophthora root rot is the most significant problem facing the production of avocado and currently methods for controlling the oomycete have had limited success. This is due to the pathogen being present in all Australian orchards and the lack of genuine resistance within the subgenus *Persea*. If left untreated the fungus has the potential of making orchards unprofitable. Even when avocado trees are grown in suitable soils, trees need a continuous and regular program of cultural and chemical control measures.

The best method for controlling PRR disease is to use resistant root stocks. However, resistance in avocado rootstocks is difficult to find because of the wide host range of *P. cinnamomi* (over 10000 different host species), and lack of *P. cinnamomi* resistant avocado cultivars. Raharjo et al., (2008) has reported resistance to PRR in the subgenus *Eriodaphne*. This subgenus is however sexually incompatible and graft incompatible with avocado (subgenus *Persea*) (Litz et al., 2005). The only other method to produce avocado plants with disease resistance to PRR is through genetic transformation.

RNA silencing has proven to be an emerging strategy to control plant viruses and nematodes in agricultural crops. A key conserved feature is that it can degrade RNA sharing a complete homology and provide high-level pathogen resistance by specific targeting of essential pathogen genes via siRNAs taken up from transgenic plants (Baulcombe, 2005).

We have chosen *A. thaliana* as our model system and shown proof of concept for plant-delivered RNA silencing based resistance to *P. cinnamomi*. The final aim is to deliver RNA silencing based resistance to *P. cinnamomi* in avocado through the transformation of avocado somatic embryos of selected rootstocks with these constructs. The genetic modification for imparting resistance will be limited to the rootstock; therefore the grafted scion will remain non-transgenic and produce GM-free fruit.

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