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Enhancement of *PR1* and *PR5* gene expressions by chitosan treatment in kiwifruit plants inoculated with *Pseudomonas syringae* pv. *actinidiae*

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Abstract Kiwifruit, with a production of more than 1.5 million tons/year in the world, must be protected against attack by its most common pathogen. Following the European guidelines on the substitution of pesticides by safer alternatives, the aim of this work was to verify if kiwifruit plants are able to better resist pathogen infections through the use of chitosan, a biodegradable compound and a well-known elicitor of Systemic Acquired Resistance (SAR). To evaluate the chitosan's elicitation effect in plant during the treatment period, two genes involved in the metabolic pathway of SAR were chosen, Pathogenesis Related Protein 1 and 5 (PRs). Primers for both genes were designed and validated and chitosan's elicitation effect was tested in qRT-PCR. Elicitation of SAR was first evaluated in a model system with plants cultured in vitro and subsequently in 2 year old plants belonging to two different species (*Actinidia chinensis* Planch. and *A. deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson). To evaluate the effects of chitosan elicitation in the presence of the pathogen attack, the 2-year-old plants were inoculated with the bacterium *Pseudomonas syringae* pv. *actinidiae*. Micropropagated kiwifruit plants were a good model to test molecular markers for SAR onset. Moreover, *PR1* and *PR5* have also shown to be suitable candidates for the detection of the plant immune system activation. In this study, chitosan elicited a systemic response in kiwi plants with intensity comparable to other well-known signalling compounds (salicylic acid, methyl jasmonate or ethylene), as shown by the changes in *PR1*'s and *PR5*'s transcription profiles. The data obtained by chitosan treatments in in vitro cultures were confirmed in plants grown in greenhouse, in which, moreover, the combination of chitosan treatment and the bacterial inoculum had the greatest effect on PRs synthesis. This study also proved that chitosan, leading to an increased expression of both PRs, has a role in kiwifruit defense reactions.

Keywords Kiwifruit · Chitosan · Pathogenesis related proteins · Systemic acquired resistance

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Introduction

After record years in which the Italian production reached 402.900 tons (Fruit and Vegetables Service Centre, CSO, Italy 2013) there has been a sharp yield decline due to the spread of a booming epidemic. In recent years the most important pathogen that has endangered the kiwifruit cultivation is the bacterium *Pseudomonas syringae* pv. *actinidiae* (PSA). The economic impact for farmers is estimated to be € 20,000/ha/year in production losses, €

5000/ha for orchard investments and € 15,000/ha to destroy plants to stop the spread of infection (Cacioppo 2012).

This pathogen is notorious for causing serious epidemic outbreaks in all major production centres, showing its aggression against yellow (*Actinidia chinensis* Planch.) and green (*A. deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson) varieties of kiwifruit. It is the causal agent of the kiwifruit bacterial canker, which causes brown discolouration of the buds, dark brown spots surrounded by yellow haloes on leaves, cankers with reddish exudates on trunks and collapse of fruits (Balestra et al. 2009). Disease can also be very rapid and it leads to a sudden death of the plants attacked (Fratarcangeli et al. 2010), causing a total loss of production in a few years (Cacioppo 2012; Scortichini et al. 2014).

Until today one of the few effective strategies for the containment of such outbreaks is the use of pesticides, in particular all formulations based on copper (Cu). It has been estimated that less than 0.1 % of these compounds applied to crops actually reaches the target pest. The rest is dispersed in the environment, where it can adversely affect non-target organisms (Pimentel and Levitan 1986). In the ecosystem many pesticides can persist for long periods and several chemical classes of these molecules can potentially affect the health of humans and animals. The eventual presence of pesticide traces in treated products poses a real risk to the consumers. Therefore, enhanced efforts are necessary in order to control and possibly eliminate exposures wherever possible (Weisenburger 1993).

Recently, a new legislative framework (Council Regulation N° 889/2008, subsequently amended by Council Implementing Regulation (EU) N° 354/2014 on the organic agriculture) clearly stimulates a new agronomic course for the continued development of organic farming, aiming a sustainable cultivation systems and a variety of high-quality products. The use of synthetic chemical pesticides is strictly prohibited and for a small group of inorganic and naturally derived active agents it is precisely defined. Furthermore many plant protection products currently in use will be replaced by substances with less environmental impact by 2018 (Council Implementing Regulation (EU) N° 408/2015). This new legal framework is a marked path towards the abandoning of pesticides in agriculture and their substitution by safer alternatives.

In the last decade the effectiveness of substances that act as pest antagonist or stimulators of plant defences have been tested. In particular, *Bacillus amyloliquefaciens* subspecies *plantarum* (AMYLO-X, Intrachem Bio Italia Spa) has been used in Italy to control the bacterial canker of kiwifruit caused by PSA (Biondi et al. 2012; Reva et al. 2004) and the use of naturally occurred bacteriophages has also studied (Frampton et al. 2014; Di Lallo et al. 2014). Among potential elicitors of host resistance, one of the most effective on *Actinidia* was acibenzolar-S- methyl (ASM), a functional analogue of salicylic acid sold under the names of Bion® or Actigard® (Syngenta) (Reglinski et al. 2013).

The purpose of this work is in agreement with the European trend, starting with the prevention of disease outbreaks through plants with enhanced resistance to pathogen infection with the use of chitosan, an "environment friendly" compound (El Hadrami et al. 2010).

Chitosan is a linear amino-polysaccharide of glucosamine and *N*-acetylglucosamine units, obtained by alkaline deacetylation of chitin extracted from the exoskeleton of crustaceans, such as shrimps and crabs, as well as from the cell walls of some fungi (Badawi and Rabea 2011). Chitosan exhibits a variety of antimicrobial activities, which depend on the degree of polymerization, the chemical composition of the substrate and the environmental conditions. There is evidence of its mechanism of action through direct toxicity or chelation of nutrients and minerals, limiting their availability to pathogens (Kulikov et al. 2006). In some cases, especially with pathogens which enter into the plant through wounds, chitosan can form a physical barrier around the penetration site (Hirano et al. 1996).

Chitosan can also act as potent inducer, enhancing a battery of plant responses to alert healthy parts during a pathogen attack (Rabea et al. 2003; Kowalski et al. 2006; Orzali et al. 2014). It can stimulate the plant immune system, resulting in a longer lasting defense for the host plant and, in some cases, conferring broad-spectrum resistance to different pathogens (Falcón-Rodríguez et al. 2012). These mechanisms are known as Systemic Acquired Resistance (SAR), which include early signalling events, as well as the accumulation of defense-related metabolites and proteins, such as phytoalexins, β-1,3-glucanases and chitinases, which are members of the Pathogenesis Related Proteins (PRs) (van Loon et al. 1994).

PRs have been classified into 17 families. They possess antimicrobial properties in vitro, with hydrolytic activities on cell walls, and they are involved in defence signalling (van Loon et al. 2006). Their expression is

modulated by plant hormone networks, e.g. salicylic acid, jasmonic acid or ethylene (Sinha et al. 2014; Cellini et al. 2014). PR proteins have been studied in many model plant species. However, in *Actinidia* there is still little information related to induced genes during pathogen attack and preliminary studies were only published in 2013 (Petriccione et al. 2013; Petriccione et al. 2014).

Among the PR families, *PR1* genes have been frequently used as SAR molecular markers in many plant species (Mitsuhara et al. 2008). PR1 proteins have been discovered in *Arabidopsis, Hordeum vulgare* (barley), *Nicotiana tabacum* (tobacco), *Oryza sativa* (rice), *Piper longum* (pepper), *Solanum lycopersicum* (tomato), *Triticum* sp. (wheat) and *Zea mays* (maize). All characterized by a molecular weight ranging from 14 to 17 kDa and several isoforms localized in different cellular compartments. PR1 proteins have antifungal properties, at the micromolar level, against several plant pathogenic fungi, including *Uromyces fabae, Phytophthora infestans* and *Erysiphe graminis* (Linthorst et al. 1989). The exact modes of action of their antifungal activities are yet to be identified but a PR1-like protein, helothermine, from the Mexican banded lizard has been found to interact with the membrane-channel proteins of target cells, inhibiting the release of Ca^{2+} (Monzingo et al. 1996).

In this study, in addition to *PR1* gene, we selected PR5 protein in kiwifruit plants. PR5 or Thaumatin-Like Protein family (TLPs) was isolated from many plant species (Zamani et al. 2004), including *Actinidia deliciosa* (kiwifruit; Crowhurst et al. 2008). TLP family comprises polypeptide classes that share homology with thaumatin, a sweet tasting protein from *Thaumatococcus danielli* Benth (Cornelissen et al. 1986). Most of the TLP/PR5s have a molecular weight in the range of 18 to 25 kDa and a pH in the range of 4.5 to 5.5. Constitutive levels of PR5s are typically absent in healthy plants and they are induced exclusively in response to wounding or pathogen attack (e.g. by *Uncinula necator* and *Phomopsis viticola;* Monteiro et al. 2003). Although the specific function of many PR5s in plants is unknown, these proteins can cause the inhibition of hyphal growth and reduction of spore germination, probably by a membrane permeabilization mechanism and/or by interaction with pathogen receptors (Thompson et al. 2006).

Chitosan treatments resulted quite promising for substituting chemicals employed in crop protection (Scortichini et al. 2014). In vitro trials confirmed an antimicrobial activity on PSA (Ferrante and Scortichini 2010). Furthermore, chitosan spray treatments showed an overall higher performance in field experiment than traditionally used copper-based compounds in reducing PSA disease symptoms, such as the presence of exudates, leaf spots, wiling twigs and necrotic flowers (Scortichini 2014).

However, there are many gaps to be filled before the mechanism of chitosan-treatment in reducing expression of the disease on kiwifruits is fully understood. The purpose of this work was to verify if chitosan was able to elicit the *Actinidia* defence response by developing a monitoring system for the onset of SAR along different nursery plant production steps, from in vitro cultures, through the breeding nursery, to the field plantation. As model system, in vitro cultures of kiwifruit plants were chosen to study the interaction between elicitor and host plant. To evaluate the plant's onset of defence response, the variation in the expression levels of *PR1* and *PR5* genes induced by chitosan was analysed in comparison to the action of the most common SAR elicitors, such as the salicylic acid (SA), methyl jasmonate (MEJA) and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). For this purpose new specific primers for qRT-PCR were designed. Furthermore, to confirm the effects of chitosan elicitation on pathogen resistance, adult plants were tested in greenhouse and in field conditions against PSA, one of the most harmful kiwifruit pathogens.

Materials and methods

In vitro cultures

In vitro cultures of A. deliciosa (A. Chev.) C. F. Liang & A. R. Ferguson cv. Hayward were obtained from Vitroplants Italia Srl Società Agricola (Cesena, Italy). Multiplication of the vegetative material was obtained on MS basal medium (Murashige and Skoog 1962), supplemented with 3 % (*w*/*v*) 6-benzyladenine (BAP), 1 % (*w*/*v*) 3-indoleacetic acid (IAA), 35 % (*w*/*v*) sucrose, 0.15 % (*w*/*v*) malt extract, 0.15 % (*w*/*v*) yeast extract and 7 % (*w*/*v*) agar, adjusted to pH 5.7. The medium was sterilized in an autoclave at 120 °C for 20 min. The medium was renewed every 21 days (subculture period). The plants were grown at 24 ± 2 °C, with relative humidity of 50 %, lighting set point of 60 % and with a

12 h light/12 h dark photoperiod. Light was provided by mercury fluorescent lamps (3000–4000 lx).

Two year old plants

In addition to in vitro cultures, 2 year old kiwifruit plants were used. Two year old plants belonging to *A. deliciosa* cv. Hayward and *A. chinensis* cv. Soreli were purchased from Co.n.vi Nursery (Ravenna, Itay). The plants were grown in 25-l pots, containing universal soil mixture (Zeoliter, Agricola2000) at 20 + 2 °C with 50 % relative humidity (RH) in a quarantine greenhouse. This was necessary because PSA is considered an A2 quarantine pest according to European and Mediterranean Plant Protection Organization (EPPO) standards (EPPO data sheet 2015).

Design and validation of the primers

PR5 and PR1 primers (Table 1) were designed on the sequences deposited at the National Centre for Biotechnology Information (NCBI). PR1 primers were based on a DNA sequence of a PR1-type (FG499230.1) protein from *Actinidia chinensis* with 70 % sequence homology with PR1 of *Vitis vinifera* (E2GEU6), identified by Petriccione et al. (2013). An EST (AJ871175.2) of the thaumatin-like protein from *Actinidia deliciosa* was used to design the PR5 primers (Crowhurst et al. 2008). Three couples of primers for each gene were chosen by the aid of Primer3web software (version 4.0) and synthesized.

The primers were evaluated against both plant genomic DNA and complementary DNA (cDNA) extracted from in vitro kiwi plants. Plant genomic DNA was obtained by a commercial kit for DNA extraction (Genomic DNA from plant, Machery-Nagel GmgH & Co, Germany), using 0.1 g of fully expanded leaves. RNeasy Plant Mini Kit manual (Qiagen) was used for RNA extraction from 200 mg of fresh tissue (from a pool of in vitro shoots). Quality and quantity RNA determination were performed using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham). The synthesis of the cDNA was carried out from from 1 μl of RNA derived from two independent extractions with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

Initially, the evaluation of the primers was done by end point PCR, subsequently the selected couple of primers was validated with a quantitative Real-Time PCR (qRT-PCR).

To set up the end point PCR protocol, a gradient PCR with an annealing temperature from 60 °C to 70 °C was performed, and the temperature at which the primers worked well was chosen as the proper annealing temperature. Hence, the regions of the PR1 and PR5 proteins were amplified using the following conditions: 50 μL reaction volume were prepared containing 10 mM dNTPs (Promega), 2.5 μL of each primer, 10 μL of 5× Phusion HF buffer and 0.5 μL of Phusion DNA Polymerase (Fynzime). The end point thermal cycler (MyCycler, Biorad) was programmed for an initial incubation of 98 °C (30 s), followed by 39 cycles at 98 °C (10 s), 68 °C (for PR1 primers)/65 °C (for PR5 primers) (15 s) and 72 °C (15 s) and a final extension at 72 °C for 7 min. Phusion DNA polymerase was chosen as an high fidelity enzyme. Amplification products (from genomic DNA and cDNA) of the expected sizes were purified from the gel using GeneJET Gel Extraction kit (Fermentas), ligated into plasmid pJET1.2 blunt (Thermo Scientific), cloned in *E. coli* XL-1 Blue and sequenced to confirm that the amplification product obtained was correct.

The qRT-PCR was carried out using 10 μL 2× GoTaq qPCR Master Mix (Promega), 5 μM of each primers and 5 μL of cDNA in a total volume of 20 μL. All samples were examined as three technical replicates. A non-template control with no genetic material was included to verify contaminations or nonspecific reactions. The optimal annealing temperature in the qRT-PCR cycles was 65 °C for PR1 and 62 °C for PR5. The thermal cycler (c1000 CFx96, BioRad) had been programmed for an initial incubation at 50 °C (2 min) and 95 °C (10 min), followed by 39 cycles at 95 °C (15 s), 65 °C (for PR1 primers)/62 °C (for PR5 primers) (30 s) and 72 °C (3 min). After each cycle the melting curve from 65 °C to 95 °C was determined, with readings every 0.5 °C. The constitutive expression of Actin gene was used as internal references for relative quantification

Table 1 List of primers used.

Name	Sequence 5′ → 3′	PCR's product size
PR1fKw	CACGGTTCACTCCTCCTTAG	500 bp
PR1rKw	CCCACTTACTAGCACACACAG	
PR5fKw	GGATCATCAATCCTGGTGCAGGC	378 bp
PR5rKw	CCGATCAGTACTGTTGCAACTCCG	

analyses (Genbank: FG440519.1, Walton et al. 2009). A dissociation curve was included at the end of qRT-PCR program to evaluate potential primer-dimers and nonspecific amplification products. The results were analysed by the CFX Manager Software version 2.1 (Biorad).

SAR's onset monitoring system

The ability of chitosan to stimulate natural plant defense barriers has been verified by developing a monitoring system for SAR's onset using PR1 and PR5 as molecular markers. The strength of this monitoring system was first tested on in vitro cultures, subsequently, on 2 year old plants.

Fifty micropropagated plants grown for 35 days in a multiplication medium were transferred to another one enriched with chitosan (0.05 g/L). It was purchased from ChitoPlant® (ChiPro GmBH, Bremen), composed by 99 % (*w*/w) low-molecular-weight Chitosan (70–90 % deacetylation) plus boron (0.05 % *w*/w) and zinc (0.05 % *w*/w). Three general elicitors, salicylic acid, methyl jasmonate and a precursor of ethylene, the 1-aminocyclopropane-1-carboxylic acid, were used to compare their profiles of elicitation with that of chitosan. Fifty plants for each treatment, grown as described before, were transferred to a multiplication medium supplemented with salicylic acid (SA, 1 mM, Sigma-Aldrich), 1-aminocyclopropane-1-carboxylic acid (ACC, 100 μM, Sigma-Aldrich) and methyl jasmonate (MeJA, 50 μM, Sigma-Aldrich). Only MeJA was added after autoclaving to avoid its degradation. A stock of fifty plants grown only on multiplication medium was used as control. The plantlets were sampled as following: (time point 1), immediately before the elicitation; (time point 2), six hours after the beginning of the treatment; (from 3 to 5 time points), 1, 2, and 3 days after the elicitor's application. All shoots were sampled and stored at −80 °C until the quantification by RT-PCR. The experiments were repeated twice. The results were analysed by the CFX Manager Software version 2.1 (Biorad). and the cycle at which the increase of fluorescence exceeded the threshold setting (Cq) was used to calculate the fold changes (defined as relative normalized expression) in each infected sample compared to the expression level detected in the corresponding sample under control conditions, plants not treated and not inoculated (baseline).

Fifty 2 year old kiwifruit plants (25 for each cultivar) were treated with chitosan soil amendments 2 days before the inoculum: 0.25 L each plant with a chitosan solution at 0.05 g/L. Fifty not treated plants were used as controls. Twenty-four hours before inoculation, all plants were placed in humidity chambers. These were created by closing each plant in a plastic bag. Humidity chambers affect the duration of the congestion water stomata and stomatal opening to increase the pathogen inoculum effectiveness. The following day, a PSA solution of 108 CFU was sprayed on the abaxial surface of the leaves of 25 treated and 25 not treated plants, up to drip. Twenty-four hours after infection the plastic bags were opened.

For SAR induction evaluation, all plants were sampled one week after inoculation, collecting 10/15 leaves each plant. The samples were stored at −80 °C until analyses. Two independently experiment were performed on adult kiwifruit plants and each considered as a dataset. For each individual dataset, the control values (plants not treated and not inoculated, negative controls) has been set to 100 % and all the individual valued has been reported in percentage respect to the controls. As percentage both datasets were combined and a unique average ± standard error (SE) were reported in the graphics.

For the disease assessment, leaf symptoms were evaluated 21 days after inoculation according to the McKinney Index (McKinney 1923). A scale was created based on the lesions covering leaf surface from 0 score (no symptoms) to 4 (necrotic lesions spread over the entire leaf surface) (Table 2).

Table 2 Disease symptoms evaluation on the basis of a 5 level scale.

Value	Percentage	Symptoms
0	0	No symptoms
1	25	Necrotic spots spread over a quarter of the leaf surface
2	50	Necrotic lesions spread over half of the leaf surface
3	75	Necrotic lesions spread of ¾ of the leaf surface
4	100	Necrotic lesions spread over the entire leaf surface

Statistical analysis

Two way analysis of variance (ANOVA) on qRT-PCR data was performed using the CoStat-200 Statistics Software version 6.4. The data were subsequently evaluated with a post hoc Duncan's new multiple range test (significance level $*P = 0.05$) to compare changes within a group over the study period and between groups at the same time. Data reported were the means of three repetitions + standard error (SE).

Results

PR1 and PR5 specific primer

Among the primers designed and evaluated, the two couples of specific primers, PR1fKw - PR1rKw, and PR5fKw - PR5rKw, were validated (Table 1). The optimal amplification conditions obtained were 68 °C for PR1 primers and 65 °C for PR5 primers in PCR cycles; 65 °C for PR1 and 62 °C for PR5 in qRT-PCR cycles.

Amplification products, from both plant genomic DNA and cDNA obtained from in vitro plants, checked by agarose gel electrophoresis, showed bands of the expected sizes from both couple of primers. Amplification products of PR1 gene obtained from *A. chinensis* (cv. Soreli) differed in only 1 of 473 nucleotides from the deposited EST sequence obtained from *A. chinensis*. For *A. deliciosa* (cv. Hayward) the results of the sequencing showed that the amplified PR1 shared 94 % nucleotide sequence identity with the deposited PR1 sequence (Fig. 1). To test whether the difference at the nucleotide level would led to the synthesis of a different protein, the cloned sequence was in silico translated into the amino acids string using the ExPASy program (http://web.expasy.org/translate). Alignment of the cloned PR1 amino acid sequence with the deposited PR1 protein showed that they had 92 % identity. Considering amino acids with similar physical-chemical characteristics, the amplification product of PR1 gene had 95 % sequence homology compared to the reference one (Fig. 2). A comparison between the two predicted secondary structures was also made using the "Prediction of protein conformation" software (Chou and Fasman 1974). The analysis revealed that the two PR1 proteins have identical structures (Fig. 3). Primers PR5fKw and PR5rKw, corresponding to the PR5 gene, were designed based on the sequence of an EST of A. deliciosa (Genebank #AJ871175.2, Petriccione et al. 2013). The resulting PCR product was identical to the reference sequence on both Actinidia species (Fig. 4).

qRT-PCR of micropropagated plants

To monitor the elicitation, the expression of the PR1 and PR5 genes was quantified through qRT-PCR. Constitutive expression of the Actin gene (Walton et al. 2009) was used as internal reference for relative quantification analyses. Basal level of expression of an untreated sample was also identical to that of a sample obtained before the treatment. During the whole time course, the controls also did not show significant changes in expression ($P > 0.05$) for all tested genes.

Chitosan as well as all the other elicitors significantly influenced the PR1 transcription profile during the experiment. Chitosan resulted in a 3.5 fold increase after 3 days (72 h) compared to untreated plants ($*P < 0.05$). In the following sampling (96 h) the amount of mRNA synthesis decline, although it remained higher than the basal level at time 0 (Fig. 5).

SA increased the expression of PR1 gene after six hours from the treatment ($*P < 0.05$). The amount of transcripts continued to increase up to 3 days (72 h), after which a modest decline was observed. MeJA treatment resulted in a 9-fold increase after 24 h compared to untreated plants ($*P < 0.05$). At 48 h the amount of mRNA synthesis decline to the baseline expression level at time 0. ACC induced a 3-fold increase in PR1 expression after 24 h ($*P < 0.05$) compared to untreated plants. The amount of expression remained high in the next three days (Fig. 5).

Generally, all elicitor treatments have led to an increased expression of the PR1 gene. Compared to the other inducers, chitosan seemed to have a delayed action in inducing the plant immune system, but it was not a less efficient inducer respect to the others.

All the elicitors also produced a change in expression of the PR5 gene. They showed, with few exceptions, the same trend of elicitation observed for PR1. The controls did not have significant changes in expression ($P > 0.05$) during the experiment.

The treatment with chitosan increased significantly ($*P < 0.05$) the PR5 expression level within 24 h (2.5 fold increase compared to untreated plants). The transcription rate of mRNA declined to the basal level at 48 h (Fig. 6).

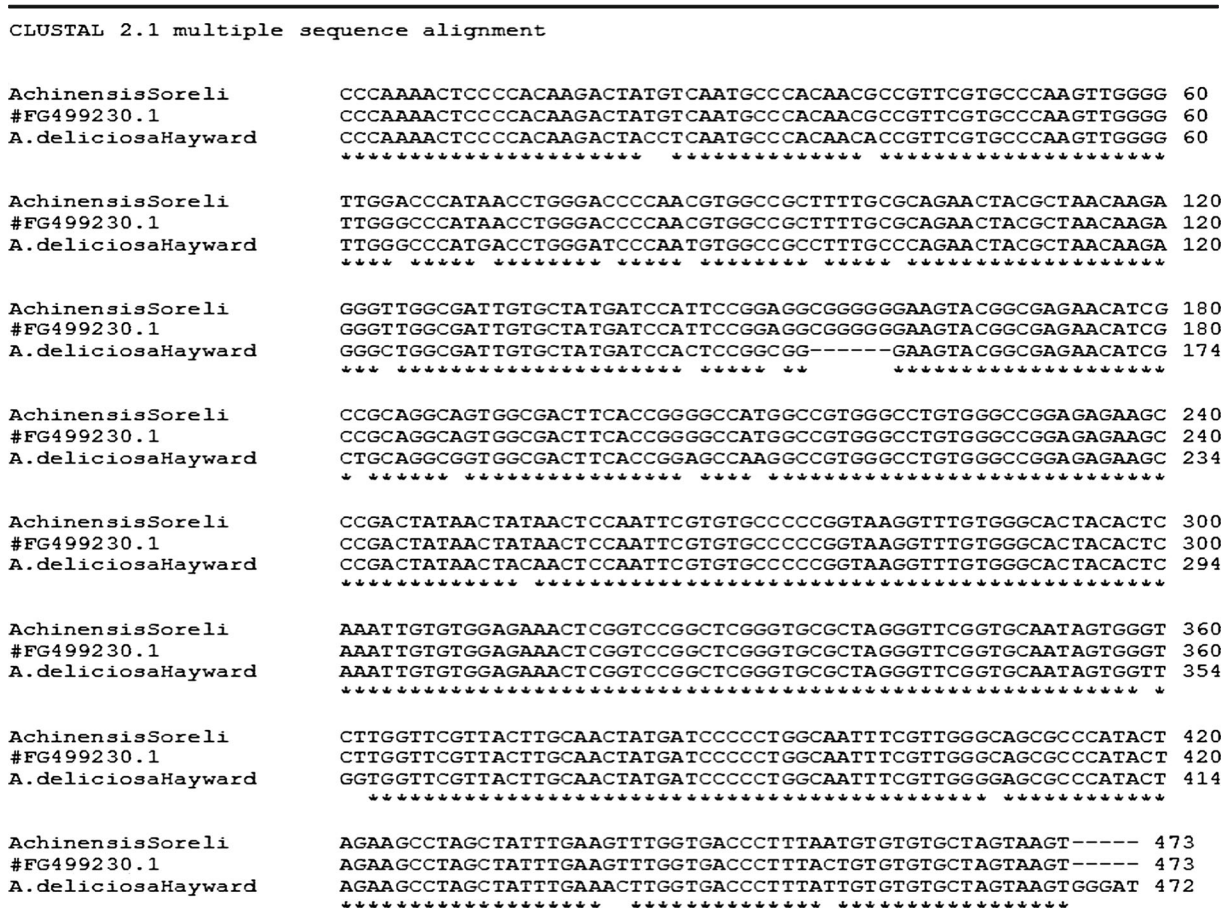

Fig. 1 Alignment of PR-1 EST deposited in Genbank (#FG499230.1) with sequences of *A. chinensis* cv. Soreli and *A. deliciosa* cv. Hayward

SA has led to a 4-fold increase in PR5 expression after 72 h (*$P < 0.05$). In the following sampling (96 h), there was a decline in mRNA synthesis. MeJA induced a significant increase in the transcription profile of PR5 after 24 h (*$P < 0.05$), after which (48 h) the transcription rate of mRNA decreased. ACC treatment resulted in a 4-fold increase in transcription level at 24 h (*$P < 0.05$). The mRNA synthesis was maintained up to 48 h, after that a decline was detected (Fig. 6).

All the elicitors produce PR transcription profiles different in timing and efficiency. In particular, chitosan was able to activate PR5 expression, resulting in mRNA accumulation comparable to that of already known SAR elicitors, like SA or MeJA.

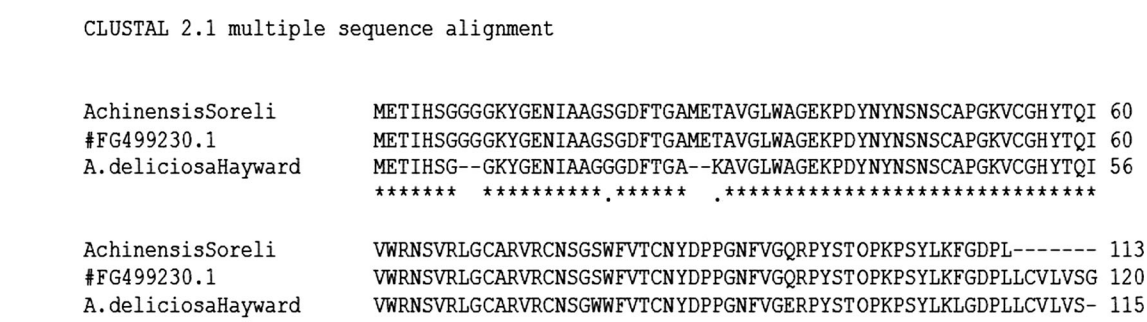

Fig. 2 Alignment of PR-1 EST deposited in Genbank (#FG499230.1) with sequences of *A. chinensis* cv. Soreli and *A. deliciosa* cv. Hayward at amino acid level

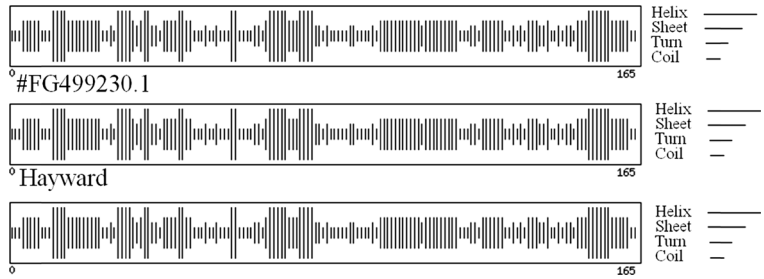

Fig. 3 Prediction of secondary structure, obtained using "Prediction of protein conformation" Software

qRT-PCR of two year old plants

In both cultivars, chitosan increased the expression levels of PR1 gene. The transcription profiles confirmed the data obtained with the in vitro cultures. In the cv. Hayward, chitosan treatment slightly increased the expression of PR1 gene and the bacterial inoculum resulted in a 2-fold increase in expression ($*P < 0.05$) compared to untreated plant. Furthermore, the mRNA synthesis continued to increase in treated and infected plants: 2-fold increase compared to treated plants, 1.5-fold increase compared to infect ones (Fig. 7). In Soreli plants, chitosan treatment moderately increased the PR1 expression and PSA inoculation has led to a significant increase in the transcription level compared to controls ($*P < 0.05$). The two treatments together further enhanced the amount of PR1 transcripts (Fig. 7).

In Hayward plants, the treatment increased the amount of expressed PR5 mRNA compared to untreated plants. The transcription level increased ($*P < 0.05$) in infected plants and it was even more enhanced ($*P < 0.05$) in treated and inoculated plants (Fig. 8). In Soreli plants, chitosan treatment increased the PR5 expression. However, the change in the transcription profile became statistically significant ($*P < 0.05$) only after pathogen infection. The combination of chitosan treatment and the bacterial inoculum had the greatest effect on mRNA synthesis ($*P < 0.05$) compared to control (Fig. 8).

Disease symptoms appeared after two weeks from the inoculum in greenhouse experiments: dark brown spots surrounded by a yellow chlorotic halo on the leaves. Disease severity evaluated after 21 days from the inoculum is reported in Table 2. Soreli cultivar

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CLUSTAL 2.1 multiple sequence alignment

#AJ871175.2             GGATCATCAATCCTGGTGCAGGCACCAAAGGCGCTAGAGTATGGCCCCGTACCGGCTGCA 60
A.deliciosaHayward      GGATCATCAATCCTGGTGCAGGCACCAAAGGCGCTAGAGTATGGCCCCGTACCGGCTGCA 60
                        ************************************************************

#AJ871175.2             ATTTTGATGGGGCAGGGCGGGGCAAGTGCCAAACCGGTGACTGCAACGGCCTCCTCCAAT 120
A.deliciosaHayward      ATTTTGATGGGGCAGGGCGGGGCAAGTGCCAAACCGGTGACTGCAACGGCCTCCTCCAAT 120
                        ************************************************************

#AJ871175.2             GCCAAGCCTTTGGTCAACCCCCTAACACACTAGCTGAATATGCCCTAAACCAATTCAACA 180
A.deliciosaHayward      GCCAAGCCTTTGGTCAACCCCCTAACACACTAGCTGAATATGCCCTAAACCAATTCAACA 180
                        ************************************************************

#AJ871175.2             ACTTGGACTTCTTTGACATATCCCTCGTTGATGGGTTCAATGTGGCGATGGAATTTAGCC 240
A.deliciosaHayward      ACTTGGACTTCTTTGACATATCCCTCGTTGATGGGTTCAATGTGGCGATGGAATTTAGCC 240
                        ************************************************************

#AJ871175.2             CTACGTCTGGTGGGTGCACCCGTGGCATCAAATGTACTGCAGATATAAACGGGCAGTGCC 300
A.deliciosaHayward      CTACGTCTGGTGGGTGCACCCGTGGCATCAAATGTACTGCAGATATAAACGGGCAGTGCC 300
                        ************************************************************

#AJ871175.2             CAAATGAGTTACGTGCCCCTGGTGGGTGTAATAACCCATGTACCGTATTCAAGACCGATC 360
A.deliciosaHayward      CAAATGAGTTACGTGCCCCTGGTGGGTGTAATAACCCATGTACCGTATTCAAGACCGATC 360
                        ************************************************************

#AJ871175.2             AGTACTGTTGCAACTCCGG 379
A.deliciosaHayward      AGTACTGTTGCAACTCCGG 379
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Fig. 4 Alignment of PR-5 EST deposited in Genbank (#AJ871175.2) with sequences of *A. deliciosa* cv. Hayward

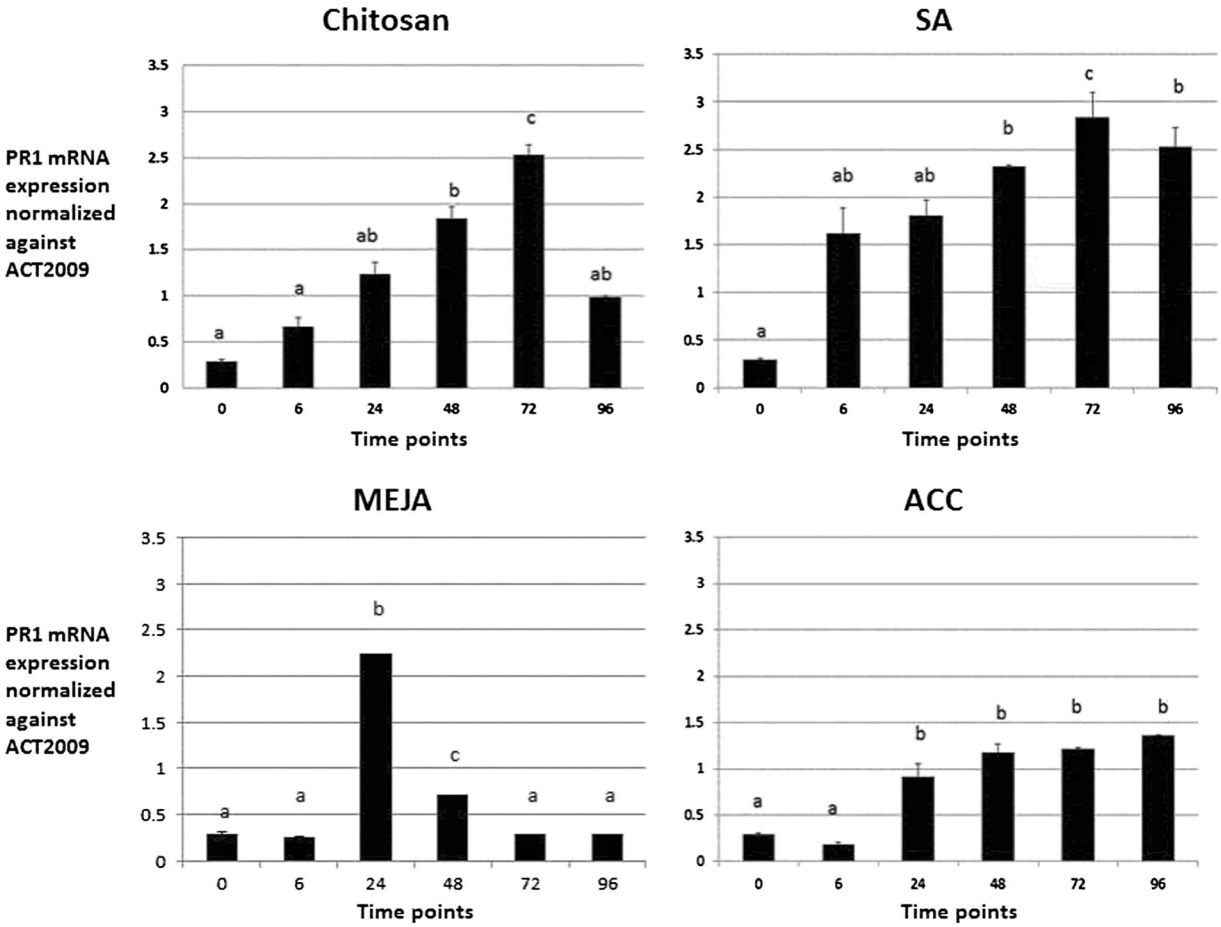

Fig. 5 PR1 mRNA fold increase expression normalized against ACT2009 after elicitation with chitosan, salicylic acid, MEJA and ACC. For each transcription profile, significance differences (*$P < 0.05$) between different sampling times within the same treatment found using the SNK test are indicated as letters a-c: values followed by the same letter within the same treatment do not differ significantly. The statistical analysis was separately performed for PRs and the housekeeping gene (Actin) transcription profiles

appeared less sensitive (26 % severity disease) than Hayward plants (38 % severity disease; $p < 0.05$). In both cultivar the chitosan treated plants showed a decreased severity index, even if statistically significant only for Hayward plants ($p < 0.05$) (Fig. 9).

Discussion

One of the goals of this study was to identify candidate genes to use as SAR molecular markers in kiwifruit. Members of the PR family were chosen because their constitutive expression is generally associated with SAR's onset and they are commonly conserved among all higher plant species (Borad and Sriram 2008). PRs sequences are available for many model plants like *Arabidopsis* (Hamamouch et al. 2011) or tobacco (Lotan et al. 1989).

Although a draft genome of kiwifruit (*A. chinensis*) was published in 2013 (Huang et al. 2013), *Actinidia* PR proteins have not been fully characterized. Only the EST sequences were available for *PR1* and *PR5* genes (Crowhurst et al. 2008; Petriccione et al. 2013). Two couples of primers based on these ESTs were designed, validated and used in PCR on kiwifruit cDNA to obtain fragments that were cloned and sequenced. Subsequently, amplified *PR* fragments were analysed to detect sequence variability in comparison with the reference ESTs and its significance at structural level.

While the *PR5* sequence corresponded to an identical protein reported in the literature (Crowhurst et al. 2008), for the *PR1* several differences were detected. In

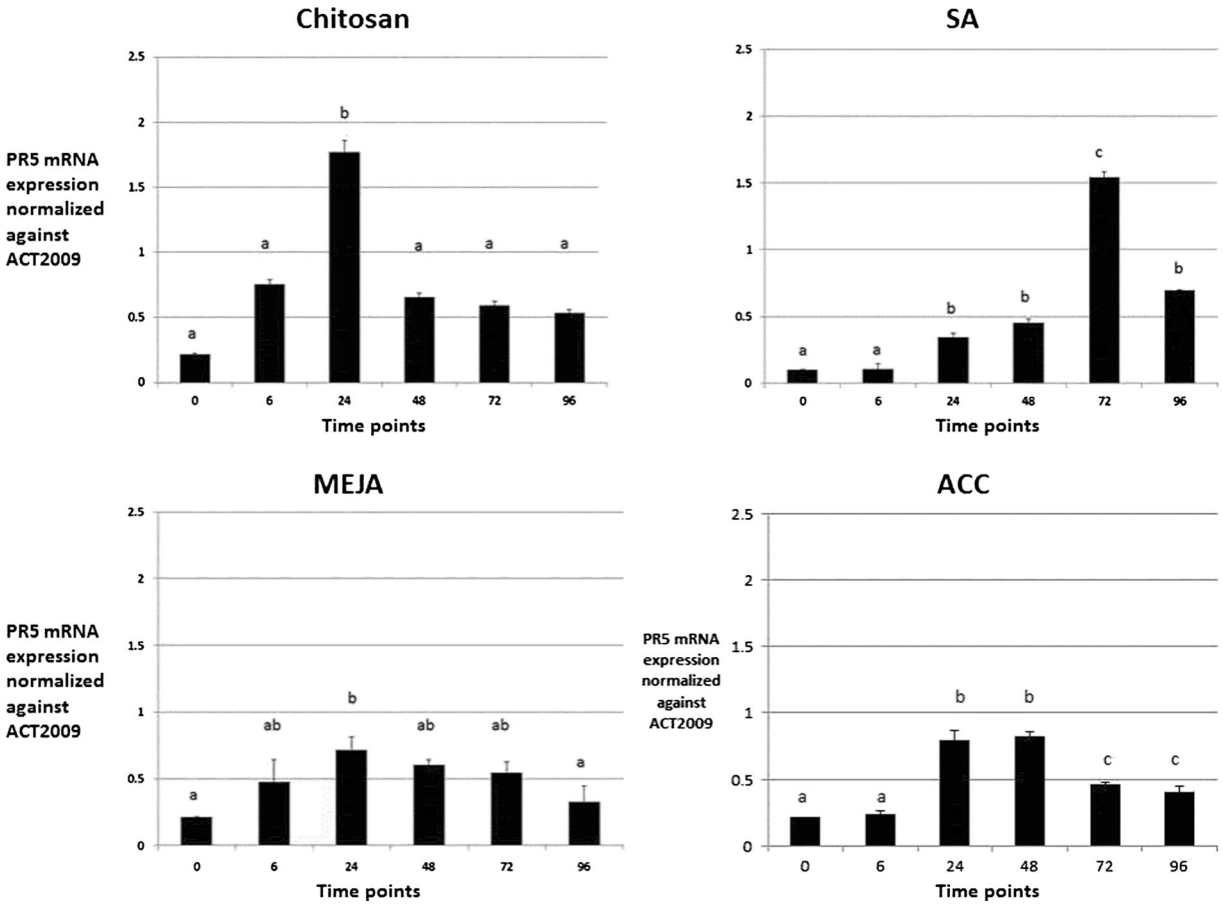

Fig. 6 PR5 mRNA fold increase expression normalized against ACT2009 after elicitation with chitosan, salicylic acid, MEJA and ACC. For each transcription profile, significance differences (*$P < 0.05$) between different sampling times within the same treatment found using the SNK test are indicated as letters a-c: values followed by the same letter within the same treatment do not differ significantly. The statistical analysis was separately performed for PRs and the housekeeping gene (Actin) transcription profiles

A. chinensis cv. Soreli, the amplified *PR1* fragment was identical to the deposited EST (FG499230.1), as expected. In *A. deliciosa* cv. Hayward the cloned sequence showed 94 % homology at DNA level with the reference sequence. The differences detected correspond to the change of single nucleotides (29 out of 469 nucleotides, 6 %). Few differences were also present at the amino acid level, resulting in 92 % sequence identity between the two proteins (12 amino acids different of 166 total amino acids). The sequence variability did not affect the functionality of the proteins; in fact, the two proteins had the same highly conserved secondary structure. The differences are probably related to an interspecies variability between *A. chinensis* and *A. deliciosa*. PR1 proteins, albeit displaying some interspecies variability, are highly conserved. Due to a common compact structure, stabilized by disulphide bridges, PR1 proteins have evolved to be inherently stable under varying conditions existing in the vacuole, apoplast or intercellular space where they are usually localized (Gorjanović 2009). Though their roles in establishing SAR are still unclear, *PR* genes are useful molecular markers for the onset of plant defence response (Taheri and Taghiri 2012). In this study, they were previously tested on micropropagated plants for some of their valuable features as model system, e.g. the large amount of clones produced and the easy handling.

Three endogenously produced signal molecules have been found to be important for induced defence response in *Arabidopsis:* SA, jasmonic acid (JA) and ethylene. SA is involved in a signalling cascade that results in induced resistance to bio-trophic pathogenic microorganisms (Vlot et al. 2009). JA mediates defense responses against necrotrophic pathogens and

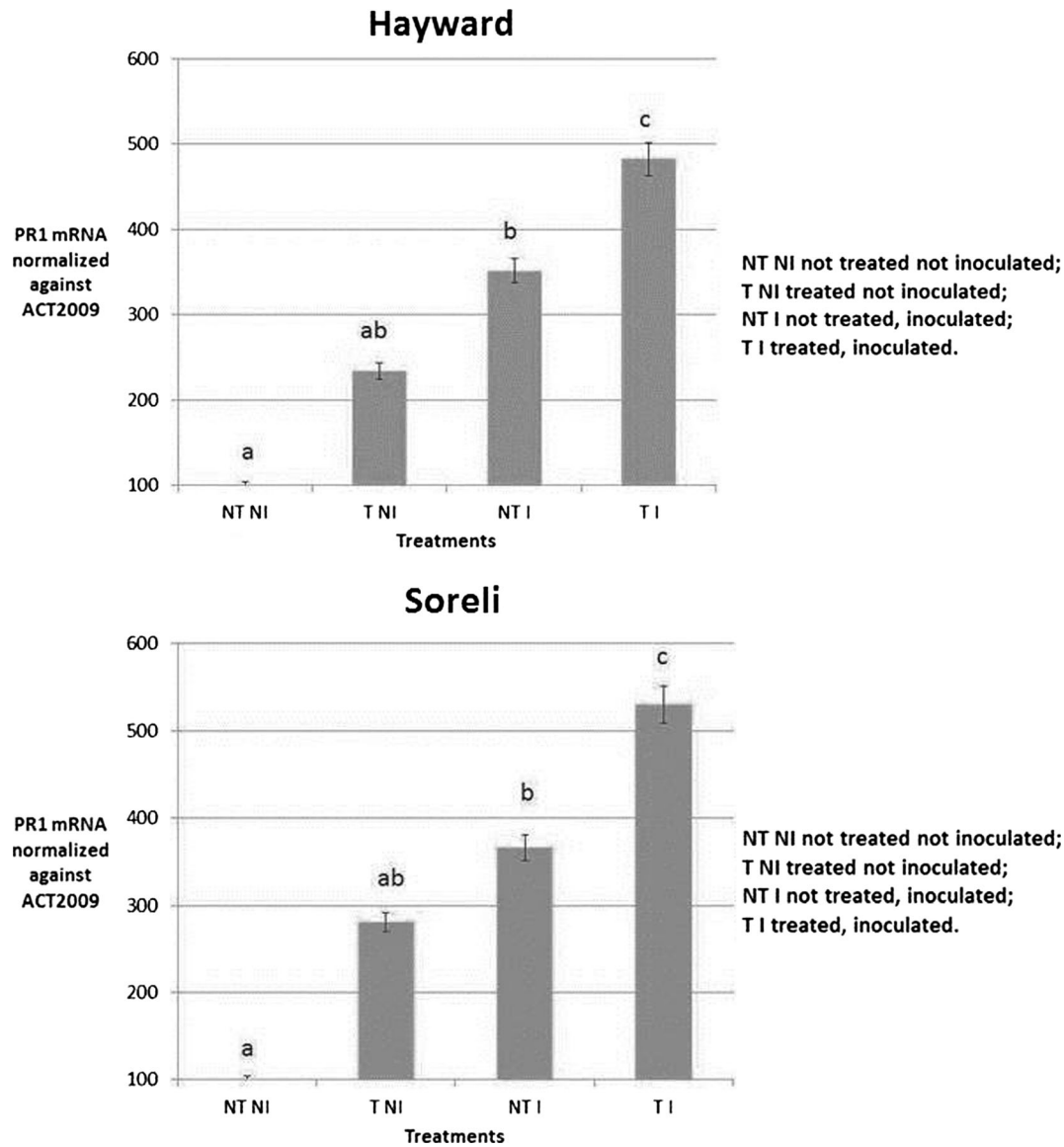

Fig. 7 PR1 expression normalized against ACT2009 in Hayward and Soreli cultivars. Control sample has been reported to 100. For each transcription profile, significance differences (*$P < 0.05$) between different sampling times within the same treatment found using the SNK test are indicated as letters a-c: values followed by the same letter within the same treatment do not differ significantly

wounding. Ethylene is involved in induction of several *PR* genes (Lawton et al. 1994). Their mechanism of action is based on the controlled activation of the expression of defense-related genes encoding for PR proteins (Taheri and Taghiri 2012). Since the nature of the plant immune defense signals in *Actinidia* remains unknown, these three plants potential SAR signals were tested in this study. Being a hydrocarbon gas, ethylene was substituted with one of its biochemical precursor, the 1-aminocyclopropane-1-carboxylic acid (ACC). The variation in the expression levels of *PR1* and *PR5* genes induced by SA, MeJA and ACC was used to compare the transcriptional profile produced by chitosan treatment.

Quantification by RT-PCR has shown that all the SAR elicitors where shown to act positively in PR expression. *PR* expression produced a transcription profile with different timing and efficiency for each. Those results are consistent even considering that the chosen PRs, belonging to different families, respond to several stimuli modulated by crosstalk between signal-transduction pathways, thus allowing the onset of a

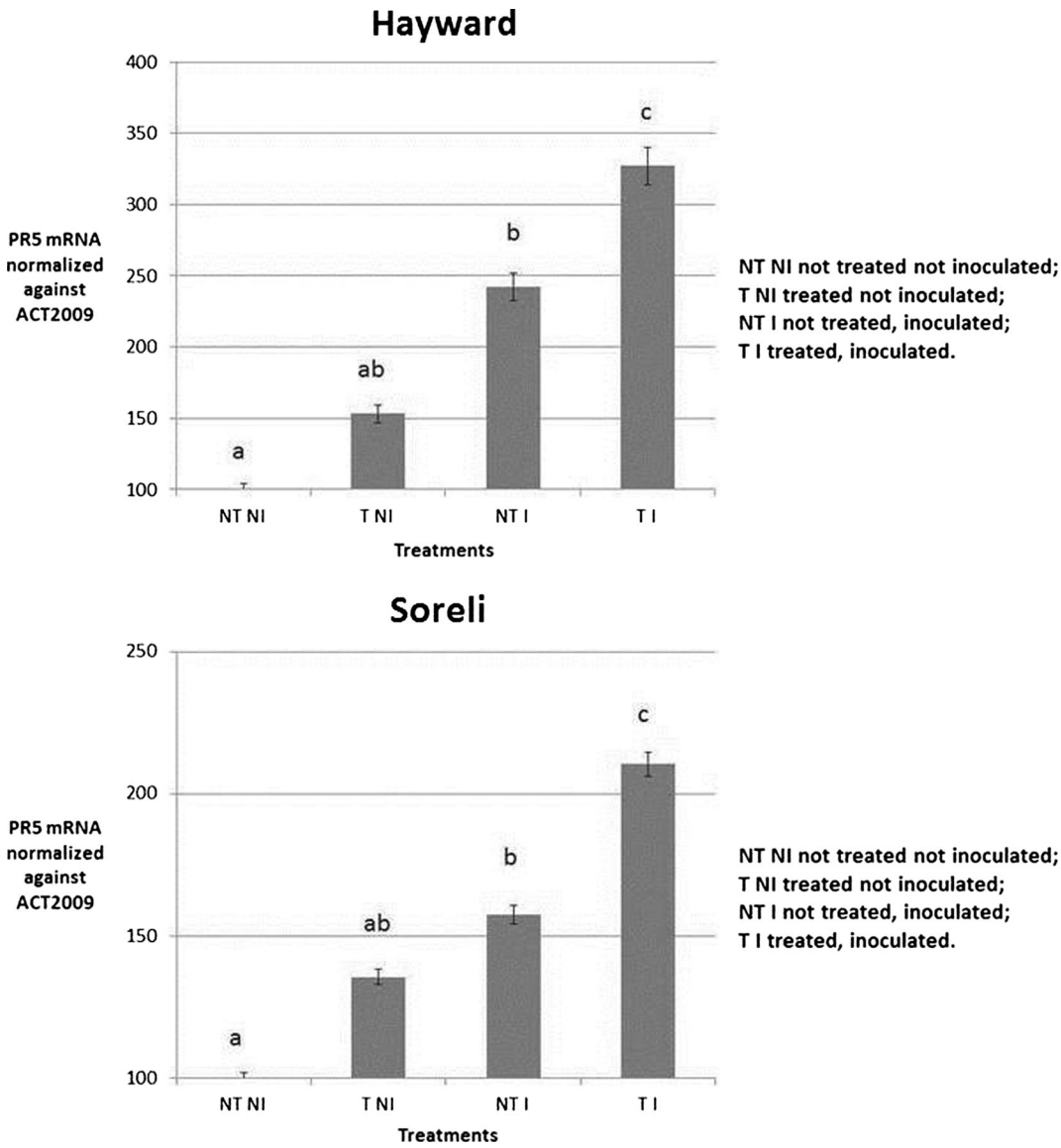

Fig. 8 PR5 expression normalized against ACT2009 in Hayward and Soreli cultivars. Control sample has been reported to 100. For each transcription profile, significance differences (*$P < 0.05$) between different sampling times within the same treatment found using the SNK test are indicated as letters a-c: values followed by the same letter within the same treatment do not differ significantly

complex signalling network that mediates the fine-tuning of plant defenses (Pieterse et al. 2012).

Confirming data present in literature, SA treatment has stimulated *PR1* gene expression within 24 h. The ability of salicylic acid to induce PR proteins was already known, also in the case of exogenous application of SA and its functional analogues (Maier et al. 2011; Cellini et al. 2014). This phenolic acid is necessary and sufficient for SAR onset and its key role in inducing *PR1* gene expression is generally recognized (Moreau et al. 2012).

Chitosan also induced *PR1* gene expression. Chitosan and SA had the same trend of elicitation, which started within the first 24 h, increased in the following two sampling (48 and 72 h) and then, gradually decreased. Other studies have demonstrated that chitosan induces the expression of defense genes in several species, e.g. rice (Rakwal et al. 2002), strawberry (Landi et al. 2014) and potato (Wang et al. 2008). Different hypothesis have been formulated to explain chitosan's mechanism of action (Weake and Workman 2008; Iriti et al. 2010;

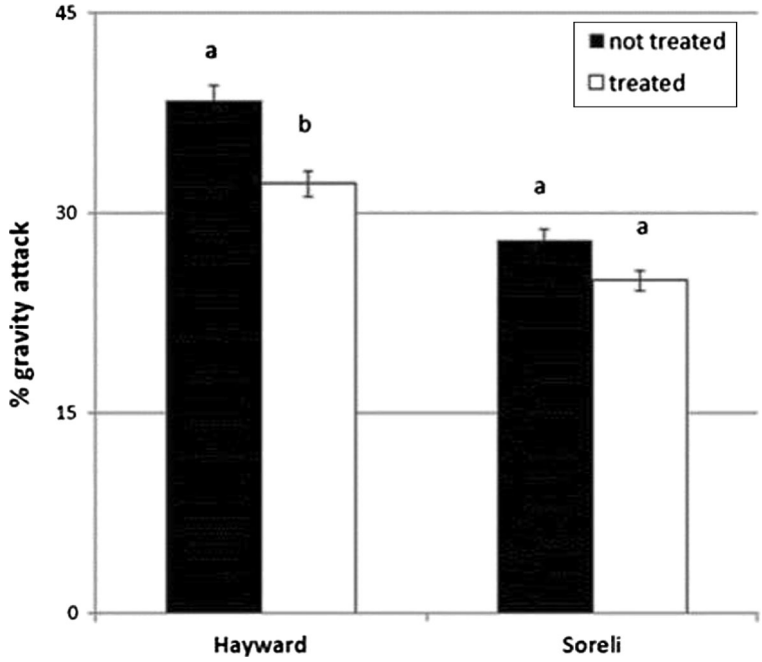

Fig. 9 McKinney Index for Hayward and Soreli cultivars at 14 days after inoculation Significant differences ($p \leq 0.05$) among the treatments found using the Duncan's new multiple range test are indicated as letters a-c: values indicated by the same letter do not differ significantly

Hadwiger and Polashock 2013). In kiwifruit plants, chitosan may stimulate a defense mechanism which modulates a cascade of related pathways similar to that induced by the SA. However, still little is known about *PRs* gene expression induced by chitosan and even less is known about interaction of the elicitor and kiwifruit plants. Thus, these results are important to confirm the ability of this compound to stimulate *PR1* in *Actinidia*.

In this study, MeJA showed a completely different elicitation trend compared to chitosan. It rapidly increased the *PR1* transcripts amount within 24 h and then returned to the baseline expression level. In the past few years, it has become evident that JA plays an important role in regulation of pathogen defenses. JA signalling has systemic effects, suggesting that JA-dependent responses are also important in resistance to pathogen (Holopainen et al. 2009). For example, plants in which only a few leaves were infected with *Alternaria brassicicola* expressed defensin gene *PDF1.2* throughout the plant (Penninckx et al. 1996).

In kiwifruit, the ethylene precursor, ACC increased *PR1* expression within 24 h and it remained relatively high expressed in the following sampling times (48, 72 and 96 h). Components of the ethylene-signalling pathway are already known for their ability in inducing *PR* gene expression in several species (e.g. tobacco, parsley, kiwifruit and brassicae; Zuo and Chua 2000; Wurms et al. 2011) and in response to plant pathogenic bacteria (e.g. *Pseudomonas*, *Xanthomonas* and *Erwinia*; Sanchez and Singh 2002). This study confirms that this elicitor is able to also activate the kiwifruit immune system.

Other studies already demonstrated that *PR5* overexpression corresponds to an increase of the disease resistance in several plant species. For example, potato osmotin enhanced resistance to potato late blight pathogen *Phytophthora infestans* (Liu et al. 1994); in rice TLP-D34 increased plant defence to the sheath blight pathogen *Rhizoctonia solani* (Datta et al. 1999) and in wheat TLP induced the plant's immune system to the head blight pathogen *Fusarium graminearum* (Mackintosh et al. 2007). Grapevines engineered to express VVTL-1, a *Vitis vinifera* thaumatin-like protein, exhibit a sustained resistance to several fungal pathogens such as *Uncinula necator* and *Botrytis cinerea* (Dhekney et al. 2011).

Even in the case of *PR5*, all the elicitors induced a greater amount of its transcripts, activating kiwifruit self-defense. Chitosan increased the amount of transcripts in 24 h, decreasing in the following sampling (48 h). MeJA showed a trend of elicitation similar to chitosan's one, which started within the first 24 h and then, gradually decreased. ACC also induced a significantly increase in transcription rate one day after treatment, but it maintained an high amount transcripts for 24 h before returning to basal level of transcription.

Conversely, SA had a completely different *PR5* transcriptional profile compared to that of chitosan. In fact, SA showed the maximum transcription rate of mRNA at 72 h after treatment.

In this study, the molecular markers were also tested in 2 year old kiwifruits to detect chitosan elicitation in adult plants. Usually at this stage of development, coming out from nurseries, they are planted in the field and most likely subject to pathogens. The results with the two year old plants confirmed those obtained with the in vitro cultures. Chitosan treatment induced a similar elicitation trend for the two molecular markers, suggesting a common mechanism of action within *Actinidia* species. In fact, it is already known in literature to be a non-species-specific elicitor of the plant defense response, like other compounds, e.g. oligogalacturonic acid (Lee et al. 1999).

SAR occurs when the plant infected with a virulent pathogen is able to generate a resistance reaction, making it less sensitive to a second, related or unrelated, pathogen to which it is normally susceptible (Dodds 1999). Hence, to verify chitosan's action concurrently with pathogen presence, 2-year-old plants were inoculated with PSA, the most virulent pathogen present in the Italian territory, which in recent years has led to the loss of entire harvests. Basing on transcripts amount, the combined action of the two elicitors (pathogen and chitosan) appeared to have an almost synergistic action, in comparison to the transcriptional level detected in plants that were treated only with the chitosan or inoculated with PSA alone. Thus, there has been a considerable activation of SAR in response to the presence of both elicitors together. Frequently the plant responses to multiple stresses led to the identification of overlapping sets of genes which are simultaneously regulated by stresses (Atkinson and Urwin 2012).

This study also proved that chitosan, leading to an increased expression of both PRs, has a role in plant defense reaction. In agreement with this hypothesis, the McKinney index revealed a statistically significant decrease of PSA symptoms in treated 2 year old Hayward plants compared to the control plants (Fig. 9), result not confirmed for Soreli cultivar in experimental conditions, although in field condition Soreli cultivar is generally less susceptible to PSA. The positive interaction between chitosan treatment and PSA infection observed in this work confirms the results obtained in other interactions (Grover et al. 2011). The changes in PR transcription profiles may also explain the chitosan efficacy in reducing PSA symptoms, as observed in field trials when the chitosan was applied as a spray (Scortichini 2014). The results of monitoring the induced plant immune system will be useful to plan chitosan treatments in relation to the life cycle of pathogenic bacteria.

Conclusion

In brief, in vitro cultures have enabled us to test molecular markers for the onset of SAR in kiwifruit. Moreover, *PR1* and *PR5* have shown to be suitable candidates for the detection of plant immune system activation in *Actinidia* and the selected couple of primers developed in this study could be a valuable tool to study the interaction with other elicitors.

A controlled induction of SAR can be considered an important and eco-friendly strategic tool to control plant pathogens in a modern management and protection of crops based on integrated control programs, including the use of environmentally safe products. Moreover, chitosan could be a useful product to alternate, even substitute, chemicals for disease management in field for the following valuable characteristics: simple and inexpensive synthesis, stability in long term usage and storage, solubility in water, absence of toxic products of decomposition, safety in handling (Badawi and Rabea 2011).

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Compliance with ethical standards

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