Plant Cell Rep (2011) 30:2027–2036
DOI 10.1007/s00299-011-1110-2

ORIGINAL PAPER

Overexpression of a resveratrol synthase gene (*PcRS*) from *Polygonum cuspidatum* in transgenic *Arabidopsis* causes the accumulation of *trans*-piceid with antifungal activity

Zhongyu Liu · Chuxiong Zhuang · Shujing Sheng · Li Shao · Wei Zhao · Shujin Zhao

Received: 18 April 2011 / Revised: 12 June 2011 / Accepted: 15 June 2011 / Published online: 30 June 2011

Abstract Although resveratrol-forming stilbene synthase (STS) genes have been well characterized in many plant species, there are only a few descriptions about STS genes from *Polygonum cuspidatum* Sieb. et Zucc, an important medicinal crop in Asian countries. To evaluate the biological functions of a *Polygonum cuspidatum* resveratrol synthase gene (*PcRS*), the *PcRS* gene was expressed in *Arabidopsis* under the control of Cauliflower mosaic virus (CaMV) 35S promoter. Integration and expression of transgene in the plant genome of *Arabidopsis* was confirmed by Southern blot and Northern blot analyses. Transgenic plants accumulated a new compound in both the leaves and seeds, which was identified as *trans*-piceid by high-pressure liquid chromatography (HPLC) and electrospray mass spectrometry (HPLC–ESI–MS). Overexpression of *PcRS* in transgenic *Arabidopsis* caused restriction of *Colletotrichum higginsianum* colonization by inhibition of spore production, resulting in enhanced resistance against *C. higginsianum*. So, the *PcRS* gene could be deployed in other crop plants to significantly enhance resistance to fungal pathogens and improve the nutritional quality. In addition, altered seed coat pigmentation and significant reduction in anthocyanin levels were observed in transgenic *Arabidopsis*, while the expression of endogenous chalcone synthase (CHS) gene was not down-regulated. These results suggest that additional STS activities cause a lack of precursors for CHS which leads to the disturbance of the subsequent flavonoid biosynthesis steps in *Arabidopsis*.

Keywords *Polygonum cuspidatum* · Resveratrol · Stilbene synthase · *Arabidopsis*

Communicated by J. R. Liu.

Z. Liu · S. Sheng · L. Shao · W. Zhao
School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, Guangdong, China

C. Zhuang
College of Life Science, South China Agricultural University, Guangzhou 510642, Guangdong, China

S. Zhao (✉)
Department of Pharmacy, General Hospital of Guangzhou Military Command, Guangzhou 510010, Guangdong, China
e-mail: gzzsjzhs@163.com

Introduction

Resveratrol (3, 5, 4′-trihydroxystilbene) is known since the 1940s when it was first isolated from the roots of white hellebore and later from *Polygonum cuspidatum* Sieb. et Zucc (Aggarwal et al. 2004). *Polygonum cuspidatum* is a medicinal plant used historically in Asia, known for its medicinal properties and traditionally used in the treatment of neuropsychiatric disorders, such as Parkinson's disease (Chen et al. 2007; Wang et al. 2011). To date, it is known that resveratrol targets include a wide range of enzymes, such as cyclo- and lipooxygenases, protein and lipid kinases, ribonucleotide reductase and DNA polymerases (Pirola and Frojdo 2008). The biological activity of resveratrol has been well documented in recent years through a number of physiological and pharmacological studies which indicate that resveratrol plays an important role in prevention of cancers, heart diseases and neurodegenerative diseases (Anekonda 2006; Athar et al. 2007; Saiko et al. 2008). Moreover, resveratrol has been shown to increase longevity in eukaryotes through activation of sirtuins (Yang et al. 2009). Recently, Xu et al. (2010) reported

that the antidepressant-like effect of *trans*-resveratrol derived from *P. cuspidatum* might be related to serotonergic and noradrenergic activation.

Biosynthesis of resveratrol is catalyzed by resveratrol synthase (RS; EC 2.3.1.95), also known as stilbene synthase (STS), which utilizes the same substrates as chalcone synthase (CHS), but a different cyclization mechanism is involved. Resveratrol-forming stilbene synthase genes have been well characterized in many plant species, including peanut (Chung et al. 2001, 2003) and grapevine (Melchior and Kindl 1991; Wiese et al. 1994). Subsequently, a root-specific STS cDNA was identified in the medicinal plant *Rehmannia glutinosa* L., which accumulates resveratrol in roots (Samappito et al. 2003). The first example of a monocot STS gene, *SbSTS1*, was isolated from sorghum (Yu et al. 2005). More recent analyses of the draft assembly of the grapevine genome has revealed a large array of STS genes, with 43 genes identified and 20 of these being shown to be expressed (Jaillon et al. 2007).

Most of the plant species transformed with various sources of STS genes showed increased tolerance against microbial pathogens (Hain et al. 1993; Coutos-Thévenot et al. 2001; Leckband and Lörz 1998; Stark-Lorenzen et al. 1997; Thomzik et al. 1997; Serazetdinova et al. 2005; Hipskind and Paiva 2000; Lim et al. 2005; Zhu et al. 2004). But no increased resistance against fungal pathogen was observed in transgenic poplar (Giorcelli et al. 2004) and kiwi (Kobayashi et al. 2000). On the other hand, engineering resveratrol might help increasing the nutritional quality of crops (Liu et al. 2006; Schwekendiek et al. 2007; D'Introno et al. 2009). Generally, stilbene accumulation does not trigger any detrimental effect on plant development, growth, fertility or morphology, and no influence of newly synthesized stilbenes has been reported on other secondary metabolite pools (Delaunois et al. 2009). Nevertheless, transformation events may have a negative impact on the general fitness of a plant. Fischer et al. (1997) reported altered flower morphology as well as male sterility in transgenic tobacco. The expression of the gene encoding the STS enzyme in tomato tissues resulted in an altered flavonoid profile (Nicoletti et al. 2007). The transgenic strawberry engineered with the *NS-vitis3* gene showed an altered phenylpropanoid metabolism, leading to increased sensitivity to grey mould infection (Hanhineva et al. 2009).

Despite the fact that STS has been well characterized in many plant species and widely introduced in transgenic plants, there are only a few descriptions about resveratrol biosynthesis-related genes in *P. cuspidatum*. *PcPKS2*, a three-intron type III polyketide synthase (PKS) gene was isolated from *P. cuspidatum* and functional analyses revealed recombinant PcPKS2 overexpressed in *Escherichia coli* to be a benzalacetone synthase (Ma et al. 2009a). Subsequently, another three-intron type III PKS gene (*PcPKS1*) was isolated from *P. cuspidatum*, and sequence analyses demonstrated that PcPKS1 is a chalcone synthase (Ma et al. 2009b). Recently, a *P. cuspidatum* resveratrol synthase gene (*PcRS*) was isolated (NCBI Accession No. DQ900615). However, the effectiveness of *PcRS* in improving stilbene accumulation and/or increasing tolerance to pathogenic microorganisms in foreign species has not been studied. In this paper, to assess the biological functions of *PcRS*, the *PcRS* gene was expressed in *Arabidopsis* under the control of CaMV 35S promoter. We showed that overexpression of *PcRS* in transgenic *Arabidopsis* resulted in accumulation of *trans*-piceid and enhanced resistance against *Colletotrichum higginsianum*. These observations indicate that the *PcRS* gene can be deployed in other crop plants to significantly enhance resistance to fungal pathogens and improve the nutritional quality.

Materials and methods

Plant material and growth conditions

Wild-type *Arabidopsis thaliana* (ecotype Columbia) was used for raising transgenics. *Arabidopsis* plants were grown in a growth chamber (22°C, 16-h light/8-h dark).

Overexpression construct and floral dip transformation of *Arabidopsis*

The full-length cDNA of *PcRS* (NCBI Accession No. DQ900615) was PCR amplified with gene-specific primers 5′-CCGGATCCATGGCAGCTTCAACTGAAG-3′ (forward, *Bam*HI site underlined) and 5′-GAAGATCTTTAAATGA TGGGCACACTTC-3′ (reverse, *Bgl* II site underlined). The PCR product was cloned into the *Bam*HI and *Bgl* II sites of pCAMBIA 1380 vector under the CaMV35S promoter. The *35S:PcRS* recombinant plasmid was introduced into *Agrobacterium tumefaciens* LBA 4404 by electroporation and transformed into *Arabidopsis* by the floral dip method (Clough and Bent 1998). The dipped plants were grown to maturity, and the seeds harvested. The seeds of transformed plants were selected after being sown on agar plates supplemented with hygromycin (25 μg/ml). Resistant seedlings were transferred to soil for further growth. Transformed plants were identified by PCR using gene-specific primers and their seeds were harvested separately. Independent transgenic lines were identified by Southern blot analysis, and *PcRS* expression was analyzed by Northern blot analysis. Homozygous T3 progenies were selected by hygromycin resistance and used for further experiments.

Southern blot analysis

About 10 μg of genomic DNA was isolated from leaves of wild-type and transgenic *Arabidopsis* and digested with *Eco*RI overnight. The completely digested DNA was size-fractioned in 0.8% agarose gel and transferred to a Hybond N+ membrane (Amersham). Southern blotting analysis was performed according to the DIG DNA Labeling and Detection Kit instruction (Roche). The full-length cDNA of *PcRS* labelled with the DIG-High Prime kit (Roche Diagnostics) was used as a probe.

Northern blot analysis

Total RNA was isolated from leaves of 4-week-old wild-type and transgenic *Arabidopsis*. About 20 μg of RNA was size-fractioned in 1% formaldehyde agarose gel and transferred to Hybond N+ membrane (Amersham). The hybridization conditions and labelling of probes were prepared as described above. For individual experiments, probes included the PcRS cDNA and cDNA of the *Arabidopsis* CHS gene (At5g13939).

HPLC and HPLC–ESI–MS analysis of transgenic *Arabidopsis*

Transgenic lines with strong expression of *PcRS* were analysed for the presence of stilbene-related metabolites. T3 plants were grown to maturity, and the seeds harvested. Triplicate samples (0.5 g each) were collected from leaves of 4-week-old plants and ground to a fine powder in liquid nitrogen. Ground tissues were extracted with 5 ml of 80% methanol and supernatants were collected after centrifugation. The methanol fraction was evaporated to dryness under nitrogen. The residue was immediately redissolved in 0.5 ml 80% methanol. Extracts from seeds (20 mg) were prepared as described above. The sample was filtered through a 0.45 μl PVDF filter (Millipore) into HPLC vial. HPLC experiments were performed at room temperature on a HP 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) using a Nucleosil C18 column (5 mm, 4.6 × 250 mm) and H_2O-acetonitrile as eluent (acetonitrile: H_2O = 25:75, flow rate 0.2 ml/min), and 306 nm of detection wavelength was used. The LC effluent was then introduced into a turbo ion-spray source on a Q/STAR-XL quadrupole/time-of-flight (TOF) hybrid mass spectrometer (Applied Biosystems, Foster City, CA, USA). Negative ESI mass spectra were acquired over the range from m/z 100 to 400. The electrospray voltage was set at −4.5 kV and the source temperature was maintained at 350°C.

Disease resistance evaluation of wild-type and transgenic *Arabidopsis*

The *Colletotrichum higginsianum* strain IMI 349063 was kindly provided by Dr. Minhui Li (South China Agriculture University, China). Cultures of the isolate were maintained on potato dextrose agar (PDA) at 25°C in the dark. Four-week-old wild-type and transgenic *Arabidopsis* were used for inoculation test. For spot inoculations, 10 μl of spore suspension at concentrations of 10^4–10^6 spores/ml was used to inoculate detached leaves. Detached leaves were inoculated in petri dishes (with wet filter paper inside) and maintained with a 12-h photoperiod at a light intensity of 30 μE m^{-2} s^{-1}. The disease symptoms were evaluated by lesion size at 6 days after inoculation. Number of spore on inoculated leaves of *Arabidopsis* was evaluated by using a previous method (Kim et al. 1999). Each experiment was repeated at three times and each included 15 individual plants. Data are the mean ± standard errors from three independent experiments.

Quantitation of anthocyanin

To determine anthocyanin levels in transgenic *Arabidopsis*, seeds were sown on Murashige and Skoog (MS) basal medium without a nitrogen source and grown for 5 days under continuous white light (2 mW cm^{-2}). Accumulation of anthocyanin on cotyledons was observed in 4–5 days. The quantitation was done as described before (Shirley et al. 1995). Briefly, 50 seedlings were picked and soaked in 0.5 ml of the extraction solution (100% methanol + 0.5% HCl) overnight at 4°C. The next day, samples were centrifuged briefly and the supernatant was used for spectrophotometric assay. Anthocyanin was quantitated by absorbance at OD_{530}. Each experiment was run in triplicate.

Results

Molecular analysis of transgenic *Arabidopsis* overexpressing *PcRS*

The full-length cDNA of *PcRS* was expressed in *Arabidopsis* under the control of CaMV 35S promoter (Fig. 1a). The presence of *PcRS* in hygromycin-resistant transgenic *Arabidopsis* was confirmed by PCR using gene-specific primers (data not shown). Seven transgenic lines were further evaluated for the integration of the transgene by Southern blot analysis (Fig. 1b). Between one and seven insertions of *PcRS* were detected in transgenic lines. The hybridization pattern indicated that they carried independent integration events for the transgenes and some of them (L4, L6 and L15) are single-copy-carrying transgenic lines. The expression of the transgene in transgenic plants was analysed by Northern blot (Fig. 1c). No hybridization signal was observed in the wild-type plants. The single-copy-carrying transgenic lines (L4, L6 and L15) showed

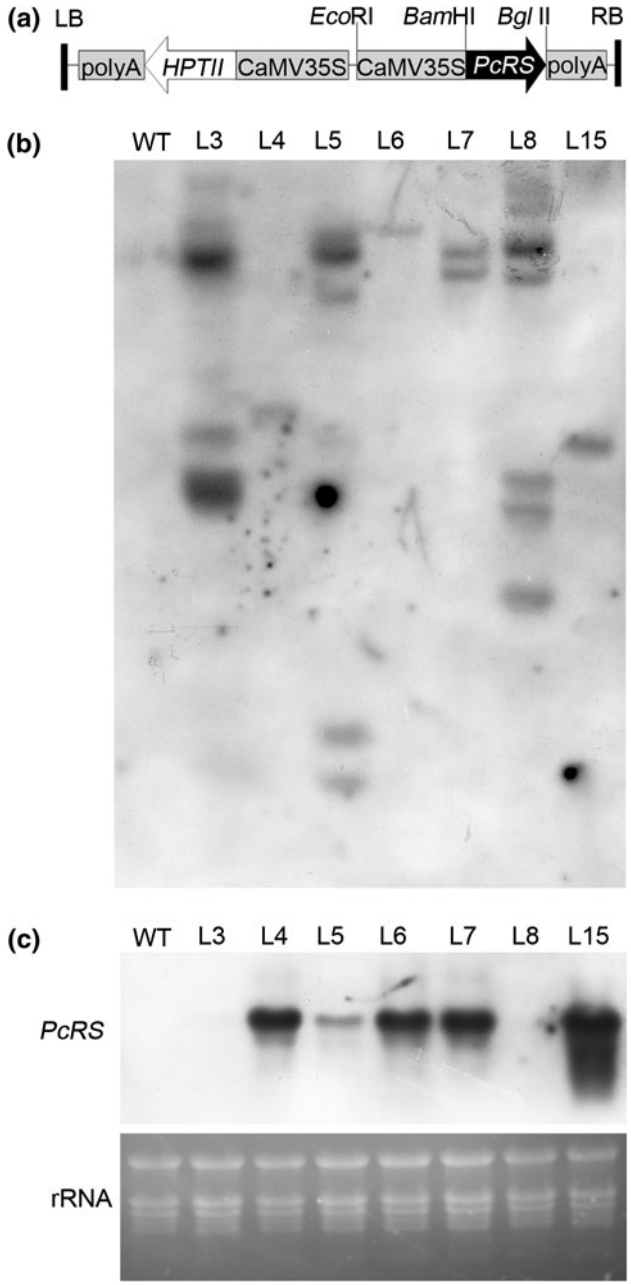

Fig. 1 Identification of transgenic *Arabidopsis* overexpressing *PcRS*. **a** Schematic representation of the construct used to overexpress *PcRS* in *Arabidopsis*. **b** Southern blot analysis to confirm the presence and copy number of transgene. DNA samples were digested by *Eco*RI and blots were probed by a PCR amplified fragment of *PcRS*. Lines L4, L6 and L15 represent single copy integration. **c** Northern analysis of transgenic *Arabidopsis* using the PCR amplified *PcRS* as a probe. Lines L4, L6, L7 and L15 represent relatively high expression of *PcRS*. rRNA was used as a loading control

relatively high-level expression of *PcRS*. The expression level of the transgene was very low or absent in the high-copy intergrated transgenic lines (L3,L5 and L8). The homozygous T3 progenies of three transgenic lines (L4, L6 and L15) were selected for further studies.

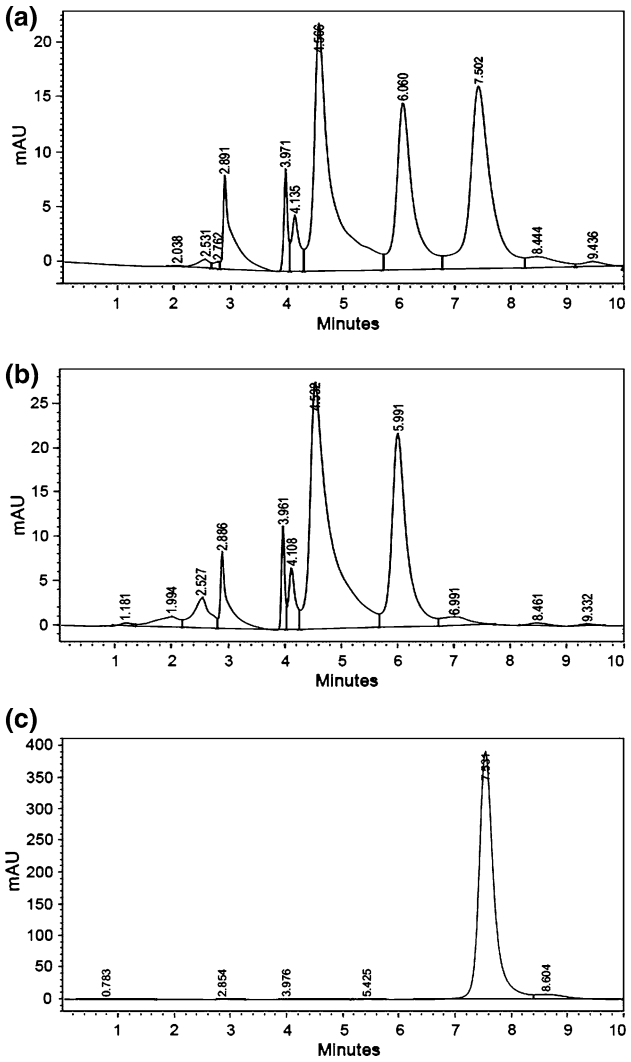

Fig. 2 HPLC analysis of extracts from leaves of 4-week-old *Arabidopsis*. **a** Transgenic line L4. **b** Wild-type plant. **c** Standard *trans*-piceid. Chromatographic profiles reveal a peak at 7.5 min in the transgenic line L4 and standard *trans*-piceid, which is not present in the wild-type plant

High levels of piceid were detected in transgenic *Arabidopsis* overexpressing *PcRS*

For analysis of plant metabolites, extracts were prepared from leaves of 4-week-old plants. HPLC chromatograms of the extracts from transgenic plants had an obvious peak (Fig. 2a) which was not detected in the extracts of the wild-type plants (Fig. 2b). The retention time (7.5 min) of the peak was the same as the *trans*-piceid standard (Fig. 2c), indicating the production of *trans*-piceid in transgenic plants. HPLC–ESI–MS was performed to further confirm the new compound. We confirmed its identity as *trans*-piceid from the spectrum (Fig. 3) of the $[M–H]^-$ ion (m/z 389) with a prominent $[M–H–C_6H_{10}O_5]^-$ product ion at m/z 227 (deprotonated resveratrol). As shown in Table 1,

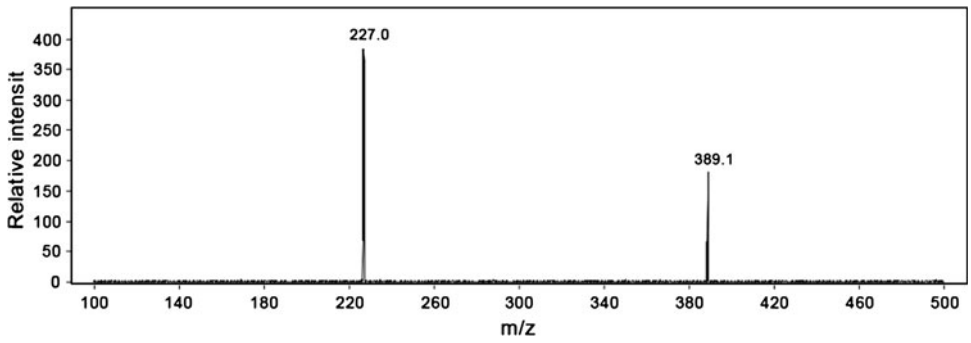

Fig. 3 HPLC–ESI–MS spectra of *trans*-piceid extracted from transgenic *Arabidopsis*

trans-piceid accumulated in the leaves of T3 transgenic lines ranged from 93.31 to 183.73 μg/g fresh weight (FW). In addition, *trans*-piceid was also detected in the extracts of transgenic seeds, although to a much lesser extent. The amount of *trans*-piceid reached an average of 26.77 μg/g FW in seeds (mean value of lines L4, L6 and L15).

Table 1 *trans*-Piceid content (μg/g fresh weight) estimated by HPLC analysis from leaves of T3 plants and seeds

Line	Leaves	Seeds
Wild-type	0.00	0.00
L4	183.73 ± 8.50	36.30 ± 4.71
L6	93.31 ± 4.41	15.24 ± 2.42
L15	104.52 ± 5.27	28.79 ± 5.26

The values represent the mean of different samples ($n = 15$) ±SD

Enhanced resistance of transgenic *Arabidopsis* overexpressing *PcRS* against *Colletotrichum higginsianum*

We investigated whether the accumulation of *trans*-piceid in transgenic *Arabidopsis* would improve fungal resistance. Three independent homozygous T3 lines (L4, L6 and L15) that consistently accumulated the high levels of *trans*-piceid and the wild-type plants were tested for resistance to *C. higginsianum*. The detached leaves of 4-week-old *Arabidopsis* plants were used for spot inoculations. Fungal colonization was arrested around the inoculation sites in the transgenic plants; by contrast, wild-type plants showed the typical susceptible symptoms in which spreading lesions were formed (Fig. 4a). At 6 days after inoculation, the lesion diameter of the transgenic plants was 2.5- to threefold smaller than that of the wild-type plants (Fig. 4b). To verify if the restricted symptom of transgenic plants is caused by inhibited fungal colonization, spore production on infected plants was measured. At 6 days after inoculation, spore production of the transgenic plants was over fivefold less than the wild-type plants (Fig. 4c). This observation was consistent with the smaller lesion size of the resistance response.

Reduced pigmentation in transgenic *Arabidopsis* overexpressing *PcRS*

To study the effect of overexpression of *PcRS* on growth and development of transgenic *Arabidopsis*, the homozygous T3 progenies of three transgenic lines (L4, L6 and L15) were selected for phenotypic studies. The mature seed coats of wild-type *Arabidopsis* were brown, resulting from the presence of tannins in the endothelium, whereas the seeds of transgenic *Arabidopsis* appeared pale fawn (Fig. 5a). To test if the overexpression of *PcRS* influences accumulation of anthocyanin, both wild-type and transgenic *Arabidopsis* plants were grown on medium devoid of nitrogen sources and anthocyanin content was quantitated using a spectrophotometer. Transgenic seedlings showed reduced anthocyanin pigmentation in contrast to the purple cotyledons of the wild-type plants (Fig. 5a). The transgenic lines had a remarkable decrease in anthocyanin accumulation, resulting in OD_{530} values approximately 50% of those in the wild-type plants (Fig. 5b). Northern analysis showed that expression of endogenous *Arabidopsis* chalcone synthase gene (*AtCHS)* in the transgenic lines was not decreased (Fig. 5c). No significant phenotypic difference was observed in adult plants overexpressing *PcRS.*

Discussion

Our results demonstrated that *PcRS* was integrated into the *Arabidopsis* genome in varying copy numbers and expressed to varying degrees. There are conflicting reports about the relationship between transgene copy number and expression. They have been shown to be negatively correlated (Tang et al. 2007), not correlated (Kobayashi et al. 2000; Zhu et al. 2004) or positively correlated (Hobbs et al. 1993). In white poplar, a high transgene copy number correlating with low levels of transcripts suggest that homology-dependent gene silencing should have occurred in these transgenic lines (Giorcelli et al. 2004). This is in agreement with the findings of the present study that the

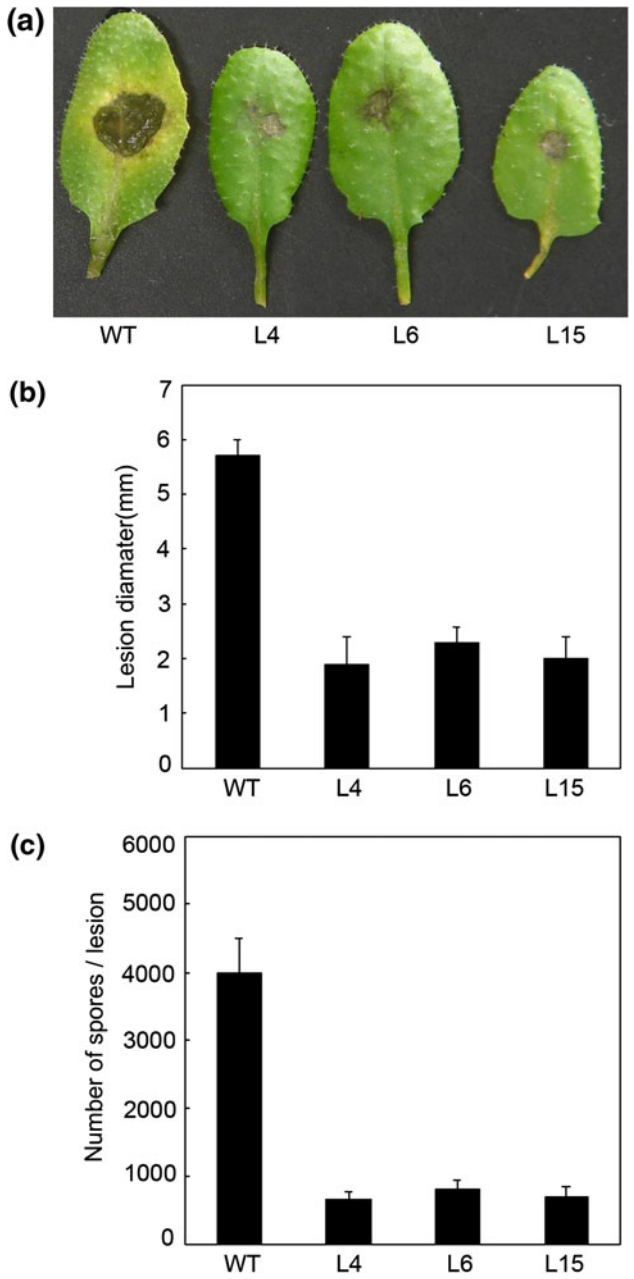

Fig. 4 Disease evaluation of the *PcRS* transgenic T3 *Arabidopsis* plants. **a** Comparison of lesion formation. Four-week-old wild-type plants (WT) and *PcRS* transgenic T3 plants were inoculated with *Colletotrichum higginsianum*. Pictures were made 6 days post infection. **b** Comparison of lesion diameter. Data were recorded 6 days post infection and are the mean ± SE of three replications, with each replication comprising three subreplications. **c** Comparison of spore production. Data were taken 6 days post infection and are the mean ± SE of three replications, with each replication comprising three subreplications

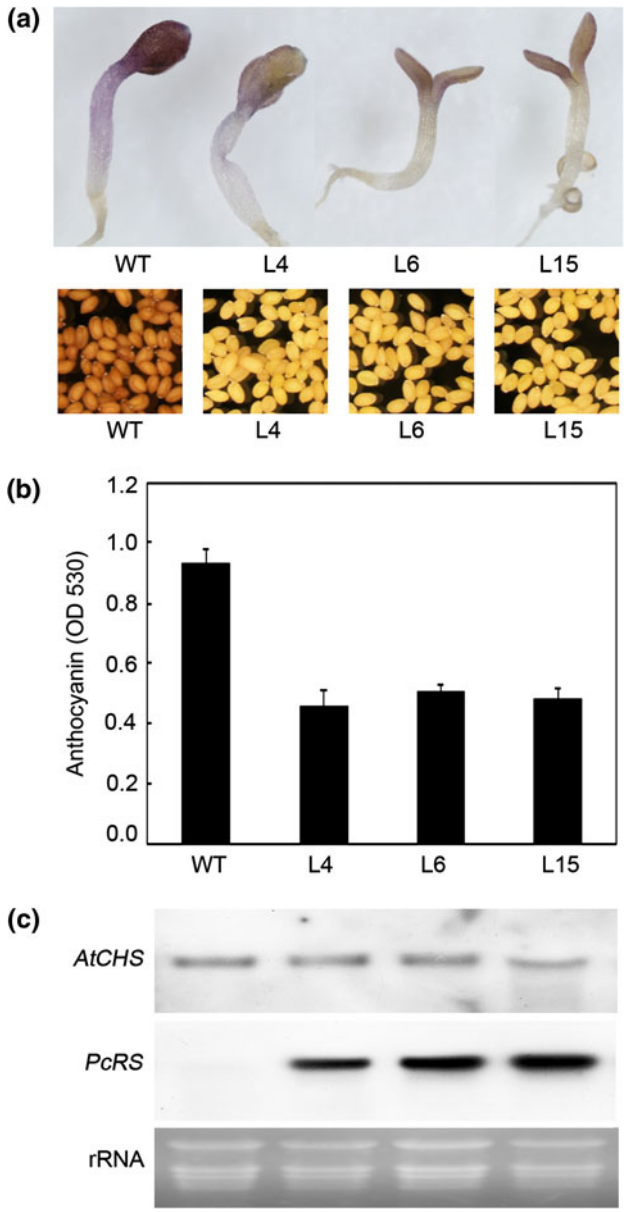

Fig. 5 Reduced pigmentation in transgenic *Arabidopsis*. **a** Pigment accumulation of 5-day-old *Arabidopsis* seedlings grown on MS plates without nitrogen sources and of seed coats. **b** Anthocyanin content in 5-day-old seedlings was quantitated by a spectrophotometer. Data are the mean ± SE of three replications, with each replication comprising three subreplications. **c** Endogenous *AtCHS* and ectopic *PcRS* expression in wild-type and transgenic plants. Blots were probed by the PCR amplified fragment of *PcRS* and *AtCHS*, respectively

expression level of *PcRS* was very low or absent in the high-copy intergrated transgenic lines (L3, L5 and L8).

In a number of cases, transformation of plants with STS has led to the production of resveratrol or piceid. But the anticipated resveratrol or its derivatives were not detected in transgenic strawberry (Hanhineva et al. 2009). The products of PcRS found in the transgenic *Arabidopsis* were identified piceid instead of resveratrol. This is consistent with the reports from poplar (Giorcelli et al. 2004), kiwi (Kobayashi et al. 2000), and *Brassica napus* seeds (Hüsken et al. 2005). Furthermore, we found that only *trans*-piceid was detected in *35S:PcRS* transgenic *Arabidopsis*. By contrast, Yu et al. (2006) reported that *cis*-piceid was

accumulated as the major stilbene in the *35S:SbSTS1 Arabidopsis* plants and inferred that the *cis*-isoform were likely to result from endogenous isomerase activities in *Arabidopsis*. The quantitative HPLC analysis of leaves from selected transgenic lines allowed us to demonstrate a high *PcRS* expression, with levels of *trans*-piceid in the range 93.31–183.73 μg/g FW. The stilbene contents were higher than those in transgenic alfalfa (Hipskind and Paiva 2000), pea (Richter et al. 2006) and lettuce (Liu et al. 2006), but lower than those in transgenic poplar (Giorcelli et al. 2004) and hop (Schwekendiek et al. 2007). In addition, *trans*-piceid was also detected in the seeds of transgenic *Arabidopsis*. The amount of *trans*-piceid produced in transgenic *Arabidopsis* seeds, 36.30 μg/g FW, was much lower than that produced in transgenic *Brassica napus* (Hüsken et al. 2005), the maximum value of piceid being around 616 μg/g FW in seeds of a homozygous T3-plant. Though piceid contents in our STS-overexpressing *Arabidopsis* seeds were much lower than those in STS-overexpressing *Brassica napus* seeds, altered seed coats pigmentation was observed in our report, which is not present in the transgenic *Brassica napus* (Hüsken et al. 2005).

The accumulation of stilbene derivatives has been reported to enhance protection in plants against various fungal pathogens; however, no significant increase in resistance was reported to *Colletotrichum* fungi. The hemibiotrophic fungus *Colletotrichum higginsianum* is pathogenic to *Arabidopsis* (Narusaka et al. 2004; O'Connell et al. 2004). *Colletotrichum higginsianum* was reported to cause typical anthracnose lesions on the leaves, petioles and stems. The anthracnose disease caused on the model plant *Arabidopsis* by *C. higginsianum* provides a valuable new system for analysis of host responses in a hemibiotrophic disease interaction. Using the new system, Narusaka et al. (2004) provided evidence that Glycerol-3-phosphate (G3P) levels in plants were associated with defence to *C. higginsianum*. In our experiments, *35S:PcRS* transgenic plants accumulating piceid showed a significant reduction in the size of lesions following infection with the pathogen *C. higginsianum*. Transgenic plants over-expressing *PcRS* had restriction of *C. higginsianum* colonization by inhibition of spore production. Taken together, these results suggest that *PcRS* products in the transgenic plants may inhibit fungal mycelia formation of *C. higginsianum*, resulting in enhanced resistance against *C. higginsianum*.

A clear relationship between stilbenes and disease resistance has been demonstrated in most of transgenic plants except kiwifruit (Kobayashi et al. 2000) and poplar (Giorcelli et al. 2004). Based on the fact that piceid was not synthesized in wild-type *Arabidopsis,* we refer the reduction of susceptibility in transgenic *Arabidopsis* lines to the presence of stilbenes. Disease symptoms in transgenic *Arabidopsis* were reduced but not completely inhibited as described for a horizontal type of resistance. This type of disease resistance was characteristic for other transgenic plant species expressing stilbene synthase genes (Hain et al. 1993; Thomzik et al. 1997; Hipskind and Paiva 2000; Zhu et al. 2004; Serazetdinova et al. 2005). Transgenic wheat plants showed a different level of resistance against two different pathogens (Serazetdinova et al. 2005). A pathogen-dependent resistance response was also reported for transgenic tomato (Thomzik et al. 1997). Therefore, further experiments in planta, and especially with other fungal and bacterial pathogens, are necessary in larger-scale experiments to determine whether the PcRS-overexpressing lines have broad-spectrum resistance to pathogen.

Induced defence responses are regulated through a network of signal transduction pathways in which the small molecules, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), act as secondary messengers. Although signalling pathways regulating stilbene biosynthesis in plant cells are not fully understood. The results from previous study clearly demonstrated that the stress hormones (such as ET, SA and JA) were involved in the resveratrol synthase gene expression in grapevine (Melchior and Kindl 1991; Wiese et al. 1994) and peanut (Chung et al. 2001, 2003). In *Arabidopsis*, SA controls the expression of *PR1*, *PR2* (*β*-1.3-glucanase) and *PR5* (thaumatin-like) genes and is required for the resistance to certain biotrophic pathogens as well as for mediating systemic acquired resistance (Thomma et al. 1998). In contrast, induction of distinct defense genes, including the defensin *PDF1.2*, the *PR3* (basic chitinase) and *PR4* (chitinase type I and II) genes together with the resistance to necrotrophic pathogens are abolished in mutant plants compromised in JA- or ET-dependent pathways (Glazebrook 2005). It is possible that enhanced resistance to pathogen attack is a result of the constitutive expression of *PcRS* in transgenic *Arabidopsis* plants. But the possibility cannot be completely excluded that overexpression of *PcRS* in *Arabidopsis* may result in activation of a signal transduction pathway that may be directly or indirectly related to pathogen disease resistance. To ascertain whether *PcRS* overexpression affects the expression levels of a series of defense genes (such as *PR1, PR2* and *PDF1.2*), expression analysis of these defense genes by RT–PCR or Northern blot are planned.

Generally, stilbene accumulation does not affect plant development, growth, fertility, morphology and other secondary metabolite pools (Giovinazzo et al. 2005; Schwekendiek et al. 2007; Delaunois et al. 2009). However, very high constitutive STS expression also has a dramatic influence on flower colour and pollen development. These undesirable side effects may be due to a competition between CHS and STS for their common substrates,

leading to reduced levels of flavonoids, which may give rise to male sterility in some species (Van der Meer et al. 1992; Ylstra et al. 1992). Other reports suggest that the reason for diminished flower pigmentation and male sterility is competition for the substrates rather than suppression of endogenous CHS expression by STS sequences (Fettig and Hess 1999; Fischer et al. 1997; Jeandet et al. 2002). More evidence for the hypothesis that the observed pleiotropic effects in STS-overexpressing plants are due to reduced flavonoid levels comes from partial chemical complementation of the male sterility phenotype by exogenous addition of flavonols as well as flavonoid precursors from the phenylpropanoid pathway (Fischer et al. 1997). In this paper, we observed that transgenic plants had seeds with a pale fawn colour and accumulated less anthocyanin in cotyledons in contrast to the wild-type plants. These results indicate that both tannin and anthocyanin accumulation are decreased due to ectopic expression of *PcRS*, though this reduction was not as dramatic as in the *Arabidopsis tt4* mutants which has yellow seeds and cotyledons. Moreover, we found that the expression of endogenous *AtCHS* in PcRS-overexpressing *Arabidopsis* was not down-regulated. These results suggest that the significant reduction in flavonoid levels in transgenic plants may be due to competition for the substrates, instead of suppression of endogenous *AtCHS* expression. None of the previous studies on transgenic plants modified by introducing STS demonstrates down-regulation of endogenous genes. Recently, one paper described a significant decrease in flavonols in transgenic strawberry modified with STS gene (*NS-Vitis3*) from *Vitis riparia* (Hanhineva et al. 2009). But the endogenous CHS mRNA level was dramatically diminished in the fully expanded leaves of the *35S:NS-Vitis3* line. One explanation for the changes in the *CHS* expression levels may lay in the regulatory control of the phenylpropanoid pathway; the introduction of the transgene may have resulted in changes in the control of gene expression, shown as diminished CHS mRNA levels (Hanhineva et al. 2009).

Remarkably, significant reduction in anthocyanin levels observed in PcRS-overexpressing *Arabidopsis* was similar to that previously observed in transgenic *Arabidopsis* expressing the mutated CHS genes (Hanumappa et al. 2007). Likewise, altered flower colour observed in STS-overexpressing tobacco (Fischer et al. 1997) is in line with the observation of (Nakatsuka et al. 2007) that the pigmentation of CHS RNAi tobacco flowers was suppressed in comparison with that of the wild type. Similarly, the occurrence of parthenocarpic fruits observed in STS-overexpressing tomato (Giovinazzo et al. 2005; Schijlen et al. 2006) has also been reported in CHS RNAi tomato (Schijlen et al. 2007), indicating that overexpressing of STS and silencing of CHS may cause a similar phenotype.

The present investigation shows that the expression of *PcRS* in transgenic *Arabidopsis* leads to accumulation of *trans*-piceid and also confers enhanced resistance against *C. higginsianum*. Moreover, additional PcRS activities cause a lack of precursors for CHS which leads to the disturbance of the subsequent flavonoid biosynthesis steps in *Arabidopsis*.

Acknowledgments We are grateful to Dr. Minhui Li (South China Agriculture University, China) for providing *Colletotrichum higginsianum* strain. We thank Dr. Yuchun Luo (University of Colorado, USA) for critical reading of the manuscript. This work was funded by grants from Guangdong Provincial Natural Science Foundation (10151001002000012) and Technologies Program of Guangdong Province Foundation (2010B060200009).

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