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In vivo antibacterial activity against *Rhizobium vitis* and the induction of defense-related genes in grapevines (*Vitis* spp.) by hairy vetch and ryegrass extracts

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Abstract

Water based crude extracts from hairy vetch (*Vicia villosa*) and ryegrass (*Lolium perenne*) were investigated for their antimicrobial activity against *Rhizobium vitis*, the causal agent of crown gall of grapevine (*Vitis* spp.), in a greenhouse. A total of 100 ml of each extract (125, 250 and 500 $g \cdot L^{-1}$) prepared from fresh and pounded shoots and roots was applied to grapevine cuttings once every 10 days. The shoot and root extracts displayed remarkable *in vivo* antibacterial effects, as indicated by reductions in gall formation by *R. vitis* of 24.97% to 75.02% in 'Kyoho' (susceptible to crown gall) and 100% in 'Campbell Early'(moderately resistant to crown gall) grapevines compared with the untreated control. The expression of several defense-related genes was investigated by quantitative real-time and semi-quantitative RT-PCR in two grapevine cultivars. Treatment with the extracts of hairy vetch and ryegrass led to differential induction of the accumulation of defense-related genes including *β*-1,3-glucanase (PR-2), phenylalanine ammonia-lyase (PAL), thaumatin-like protein (TLP), leucine reach-repeat (LRR), polygalacturonase inhibiting protein (PGIP), stilbene synthase (STS) and catalase (CAT). Expression analysis of defense related genes revealed noticeable differences between 'Kyoho' and 'Campbell Early' grapevines. Based on these results, extracts from tested cover crops can act as efficient biological inducers and therefore serve as an alternative strategy for grapevine crown gall protection.

Keywords: Biological control, Biopesticides, Crown gall, Gene Expression, *Vitis* spp.
Abbreviations: PR-2_ *β*-1,3-glucanase; PAL _ Phenylalanine ammonia-lyase; TLP_ Thaumatin-like protein; LRR_Leucine reach-repeat; PGIP_Polygalacturonase inhibiting protein; STS_Stilbene synthase; CAT_Catalase.

Introduction

A number of diseases caused by bacterial, fungal, and viral pathogens have resulted in significant reductions in the yields of grape (*Vitis* spp.) (Montesinos, 2007; Wang et al., 2011). Crown gall disease of grapevines caused by *Rhizobium vitis* (formerly *Agrobacterium vitis*) causes severe economic loss via reduced fruit productivity and mainly occurs during early summer in Korea (Chung and Shim, 1996). Although there have been great increases in our understanding of the infection mechanism of *R. vitis* and ability to control crown gall in grapevines, few strategies are available for the efficient management of this disease in vineyards. *R. rhizogenes* strain K84 was commercially applied to control crown gall in peach trees as a biological control agent (Kerr, 1980); however, it is not effective at preventing grapevine infections caused by *R. vitis* (Chen et al., 2007). Moreover, application of synthetic pesticides exerted negative impacts such as toxicity toward other living organisms, and led to accumulation in the food chain. Consequently, there is an obvious requirement to identify effective alternatives to pesticides to control plant pathogens for agricultural applications that are nontoxic and nonpolluting (Costa et al., 2000). Naturally occurring and biologically active plant products such as plant extracts could be a source of alternatives to chemical pesticides for use as templates for new and more effective compounds for controlling plant pathogens (Gan-Mor and Matthews, 2003). Hairy vetch (*Vicia villosa*) and rye grass (*Lolium perenne*) are annual cover crops utilized to improve nitrogen status in soil in no-till cropping systems (Hoffman et al., 1993). Previous studies of the influence of cover cropping, no-till, and reduced tillage practices on soil organic matter were conducted in temperate regions (Grandy and Robertson, 2007; Hermle et al., 2008), but recent findings suggest that combining cover crops with no-till practices and shifting tillage intensity may similarly enhance ephemeral and longer-term pools of soil organic matter in Mediterranean and semiarid annual agroecosystems (A´ lvaro-Fuentes et al., 2008; Veenstra et al., 2007). However, there are limited reports suggesting the effectiveness of cover crop species for protection of plants against plant pathogens. It was previously reported that some cover crops such as *Brassica* species significantly inhibited the growth of various pathogens (Larkin and Griffin, 2007). In the last decade, a new technology for disease control involving the induction of host-defense mechanisms was developed as an alternative to chemical pesticides. This activation of plant defense systems, known as induced resistance (IR), can be achieved by the application of inducers that mimic pathogen invasion (Hamiduzzaman et al., 2005). The complex signaling network involved in plant defenses against bacterial, viral, and fungal pathogens has been extensively reviewed for dicotyledonous plants (Bari and Jones, 2009). Resistance in plants can be induced by exogenous application of a variety of molecules as elicitors, including oligo- and polysaccharides, peptides, proteins, and lipids in plants (Ahn et al., 2013; Boubakri et al., 2012 and 2013; Hammerschmidt, 2009; Rudrappa et al., 2010;

Trouvelot et al., 2008). Induction of defense-related genes leads to reinforcement of plant cell walls, accumulation of antimicrobial compounds such as phytoalexins, and synthesis of proteins with hydrolytic or inhibitory activity toward microbes by various signaling pathways, such as salicylic acid, jasmonic acid, and ethylene (Kunkel and Brooks, 2002). Phytoalexins are a heterogeneous group of compounds (Ahuja et al., 2012) that show biological activity toward a variety of pathogens and are considered molecular markers of disease resistance. We also recently reported biologically active water-based extracts of cover crop species against the grapevine crown gall pathogen (Islam et al., 2012) and isolated three different compounds that showed antimicrobial activity against *R. vitis* (Islam et al., 2013). In the present study, we investigated the *in-vivo* antibacterial activity of extracts from hairy vetch and rye grass and their ability to induce defense responses by monitoring the expression of selected defense-related genes against the grapevine crown gall pathogen *R. vitis*.

Results

Efficacy of cover crops against R. vitis in grapevines

As shown in Table 2 and 3, the gall weight and gall incidence were reduced in grapevines by treatment with cover crop extracts when compared with the untreated controls. There were marked differences in gall incidence between 'Kyoho' (susceptible to crown gall) and 'Campbell Early' (moderately resistant to crown gall) grapevines. Water based shoot and root extracts from hairy vetch and ryegrass at the investigated concentrations showed remarkable antibacterial effects leading to reduction of gall formation by *R. vitis* of 24.97% to 75.02% in 'Kyoho,' and 0 to 100% for 'Campbell Early' grapevine when compared with the control (Table 2 and 3; Fig. 1). The crude extracts of shoots from hairy vetch and ryegrass at an initial concentration of 500 $g{\cdot}L^{-1}$ resulted in a significant decrease in disease severity (gall incidence) of over 75.02%, whereas extract of roots resulted in a decrease in disease severity of 50.05 to 62.54% of the control in 'Kyoho,' but 100% for 'Campbell Early' against *R. vitis*, the causal agent of crown gall of grapevine. Dilution of shoot extracts to 250 $g{\cdot}L^{-1}$ revealed potential antibacterial effects of 50.05 to 75.02%, whereas root extracts led to 37.57 to 50.05% decreases in gall incidence reduction in 'Kyoho,' but 100% decreases in 'Campbell Early' against tested bacterial strains (Table 2 and 3). However, treatment with 125 $g{\cdot}L^{-1}$ of shoot crude extracts exhibited moderate antibacterial efficacy against *R. vitis*, with gall incidence reductions of 24.97 to 37.45%, whereas for root extracts decreases of 25.08% were observed in 'Kyoho,' while no decrease was observed in gall incidence for 'Campbell Early'. Both gall weight and gall incidence were also significantly reduced in grapevines in response to tested cover crops after sowing seeds when compared with the control (Table 4). The recommended doses of sowed seeds of six cover crops showed moderate antibacterial effects leading to reduction of gall formation by *R. vitis* of up to 28.57 to 57.14% in 'Kyoho' grapevine when compared with the control (Table 4). The efficacy of controlling crown gall pathogen in tested cover crops also varied. Shoot extract treatments exerted greater antibacterial activity than root extracts and co-cultivation with tested cover crops after sowing seeds. Shoot and root extracts from hairy vetch and ryegrass applied at 250 $g{\cdot}L^{-1}$ promoted the shoot growth of grapevines relative to controls (data not shown), whereas co-cultivation by sowing seeds did not promote shoot growth.

Differential expression of defense-related genes in grapevine

In both 'Campbell Early' and 'Kyoho', the expression of the plant defense-related genes *PR-2, TLP, PAL, STS, LRR, PGIP* and *CAT* was strongly upregulated by treatment with hairy vetch (HV)/ryegrass (RG) extracts, *R. vitis* inoculation and salicylic acid (SA) at 0, 6, 24 and 48 h post treatment based on the ratio to the untreated control (RTC). Baseline expression of all selected defense-related genes was recorded in all analyzed samples at 0 h post treatment. Induction of defense-related genes was studied by quantitative real-time PCR and semi-quantitative RT-PCR approaches (Fig. 2 and 3, respectively). Grapevine actin was used as an internal control to normalize different samples to measure differences in the amount of cDNA. We considered both approaches to give similar results, even though some differences in the induction levels for various time points were observed. Nevertheless, there were marked differences between the two grapevine cultivars, and the results demonstrated that most genes were differentially activated by the tested treatments. The temporal expression of selected genes in 'Campbell Early' and 'Kyoho' grapevine leaves by *R. vitis*is is shown in Fig. 2A, 3A and 3B. Investigation of the expression profiles of all seven genes in 'Campbell Early' leaves inoculated with *R. vitis* revealed a general up-regulation pattern, including the notably higher up-regulation of *CAT* (RTC 10.97) by *R. vitis*, but down-regulation of the *PAL* gene at 24 h post inoculation. Conversely, *PAL* (RTC 5.37), *STS* (RTC 5.76) and *CAT* (RTC 6.42) genes were moderately up-regulated at 48 h post inoculation, while *PR-2* and *TLP* were down-regulated at 24 h post inoculation by *R. vitis* in the 'Kyoho' grapevine. As shown in Fig. 2B and 3A, much higher expression of the *PR-2* (RTC 15.14), *TLP* (RTC 41.98), *CAT* (RTC 23.15) and *PGIP* (RTC 34.18) transcripts was found in the 'Campbell Early' grapevine in response to hairy vetch (HV) shoot extract treatment at all-time points. Additionally, moderate expression of *PR-2* (RTC 12.75), *TLP* (RTC 14.44), *PAL* (RTC 15.19), and *CAT* (RTC 13.23) transcripts was observed in the 'Kyoho' cultivar (Fig. 2B and 3B). Accumulation of all transcripts in grapevine leaves by *R. vitis* inoculation following hairy vetch (HV) extract treatment is shown in Fig. 2C, 3A and 3B. In 'Campbell Early', accumulation of *PR-2* (RTC 41.37), *TLP* (RTC 45.64), *CAT* (RTC 21.06) and *PGIP* (RTC 20.5) genes expression was highly up-regulated (Fig. 2C and 3A), while in 'Kyoho' all transcripts were moderately expressed, with the exception of *CAT* **(**RTC 35.93), which was highly up-regulated (Fig. 2C and 3B). The expression of target genes in leaves at all time points in response to SA treatment is shown in Fig. 2D, 3A and 3B. Moderate expression of the *LRR* (RTC 5.94) and *CAT* (RTC 6.71) genes was observed in 'Campbell Early' grapevine leaves, whereas *PR-2, PAL, STS* and *LRR* were down-regulated at some time points. Conversely, the only noticeable up-regulation of *CAT* (RTC 14.23) was observed in response to SA treatment at 6 h post treatment in the 'Kyoho' grapevine.
The tested genes in grapevine leaves were induced at all time points by ryegrass (RG) shoot extract treatment (Fig. 2E, 3A and 3B). In Campbell Early, *TLP* (RTC 36.4), *PAL* (RTC 11.69), *LRR* (RTC 12.9), and *CAT* (RTC 26.83) genes showed higher expression in response to ryegrass extract. In 'Kyoho', *TLP* (RTC 13.98) and *CAT* (RTC 23.19) were most strongly up-regulated by ryegrass extract treatment. Interestingly, the *PR-2* gene was highly expressed at 6 h post treatment, whereas it was down-regulated at 24 or 48 h post treatment. As shown in Fig. 2F and 3A, the expression of all tested genes was up-regulated in 'Campbell Early' by *R. vitis*

Table 1. Sequence of primers derived from *Vitisvinifera* sequences used for quantitative real-time and semi-quantitative RT-PCR analysis.

Gene	Accession No.	Forward and reverse primers (5'−3') for quantitative real-time PCR	Forward and reverse primers (5'−3') for semi-quantitative RT- PCR
Phenylalanine ammonia lyase (*PAL*)	X75967.1	5'-TGAACAATGGCGAAAGTGAGAA-3' 5'-TCTCTTGCGCTCTCAACCTCTT-3'	5'-AGCCACGTAGCTAAGAAAACTCTA-3' 5'-AGTAAACACCTTGTCAAAATCCTC-3'
β-1,3-glucanase (*PR-2*)	XM002274792.2	5'-GGGGTTATTTGGATCCCATCAT-3' 5'-CAGAAGCGGCGACTTATTGTCT-3'	5'-CATTCCGCCAAGAACTCATC-3' 5'-GCCCTCGACTGTAAATGGAA-3'
Leucine reach-repeat (*LRR*)	XM002285517.2	5'-GCCGATTTGGATCTCTCTCTGA-3' 5'-GTATGCTCACCGCCGAGTTAAT-3'	5'-TCGTGGAGTGGCTATGACTG-3' 5'-GTGTTGAGAGAACCGCCATT-3'
Thaumatin-like protein (*TLP*)	XM002282928.2	5'-TTCGCACTTAACCAATTCAGCA-3' 5'-TGCACCCATTGGAAGTAGGATT-3'	5'-CAATCCTGGAGCCTCAATGT-3' 5'-AACCCAAGGTGGAAACCATT-3'
Polygalacturonase inhibiting protein (*PGIP*)	AF305093.1	5'-GT TTACTCGCCACTCGTCCAT-3' 5'-GTTGGGATTCCACGAAGCTAGA-3'	5'-AAAAGTTCTCCTTCAAATCAAAAA-3' 5'-AATCCTCTGAAAGAATATGGGATT-3'
Stilbene synthase (*STS*)	X76892.1	5'-GGTGCCATTGCAGGAAACTTAC-3' 5'-CAAGTGGGTCAAAAGCCTGAGT-3'	5'-TTAACATACGCCAAGAGATTATCA-3' 5'-CCTGCAGAATTAGGAATAAATGTT-3'
Catalase (*CAT*)	XM003635412.1	5'-CAAGAGGCCCAGTTCTTCTTGA-3' 5'-CTAGCATGGACCACACGTTCTG-3'	5'-TCATGCTACTCAGGATCTCTATGA-3' 5'-CTTGAAATTGTTCTCCTTCTCAAT-3'
Actin (VACT)	AB372563	5'-ATGTGCCTGCCATGTATGTTGCC-3' 5'-AGCTGCTCTTTGCAGTTTCCAGC-3'	5'-ACTGTGCCAATTTATGAAGGTTAT-3' 5'-TCCAGACACTGTACTTTCTCTCAG-3'

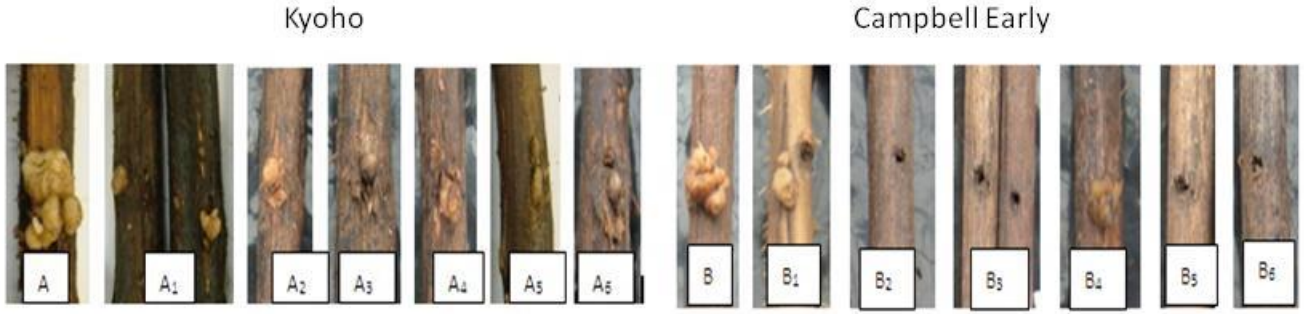

Fig 1. *In vivo* antibacterial efficacy of the crude shoot extracts from hairy vetch (*Vicia villosa*) and ryegrass (*Lolium perenne*) against *Rhizobium vitis* on greenhouse-grown 'Kyoho' (A_1 to A_6) and 'Campbell Early' (B_1 to B_6) grapevines. (A and B) Control (treated with pathogen only); (A_1 to A_6) and (B_1 to B_6) treated with pathogen and different concentrations of crude extracts (125, 250 and 500 $g \cdot L^{-1}$) of hairy vetch and ryegrass, respectively, in water.

Table 2. Efficacy of fresh shoots extracts from hairy vetch and ryegrass against crown gall in 'Kyoho' and 'Campbell Early' grapevines under greenhouse conditions.

Cultivar	Treatment	Dosage ($g \cdot L^{-1}$)	Gall wt (mg)	% GW[z] reduction over control	Rate of gall incidence (%)	% GI[y] reduction over control
Kyoho	Hairy vetch	125	46.0±8.2 c	65.57	55.5±0.1 c	37.45
		250	13.7±1.2 d	89.74	22.2±0.1 e	75.02
		500	10.3±0.6 d	92.29	22.2±0.1 e	75.02
	Ryegrass	125	81.7±12.5b	38.84	66.7±0.1 b	24.97
		250	47.7±7.2 c	64.29	44.4±0.1 d	50.05
		500	13.0±1.7 d	90.26	22.2±0.1 e	75.02
	Control (PI[x])	0	133.6±3.4 a	-	88.9±0.1 a	-
Campbell Early	Hairy vetch	125	10.7±0.6 b	73.44	33.3 a	0
		250	0.0±0.0 c	100	0	100
		500	0.0±0.0 c	100	0	100
	Ryegrass	125	12.3±0.6 b	69.47	33.3 a	0
		250	0.0±0.0 c	100	0	100
		500	0.0±0.0 c	100	0	100
	Control (PI[x])	0	40.3±4.2 a	-	33.3 a	-

GW[z], gall weight; GI[y], gall incidence; PI[x], pathogen inoculated. Means within a column followed by different letter(s) are significantly different (DMRT, $p < 0.05$).

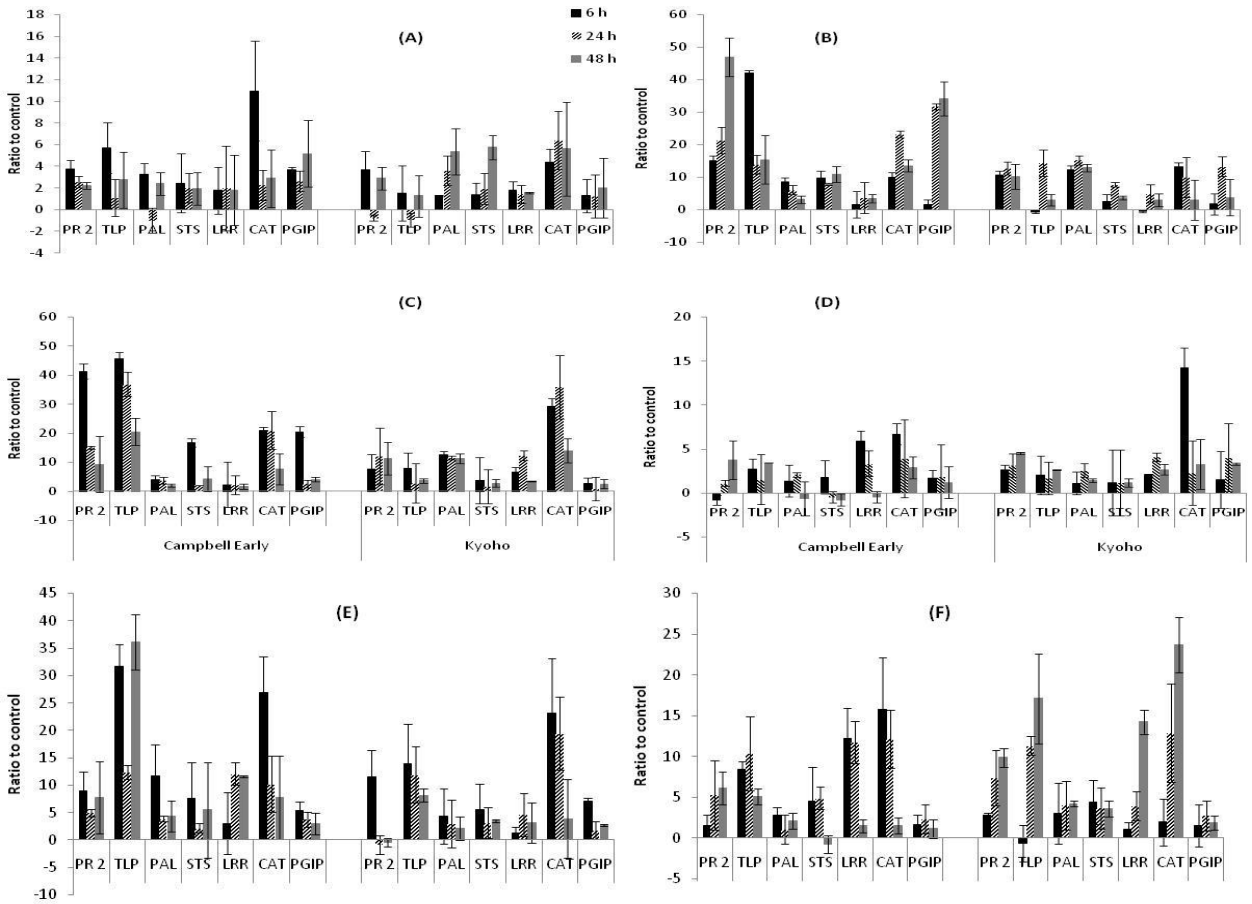

Fig 2. Quantitative real-time PCR analysis of the expression of seven defense-related genes induced by hairy vetch (HV) and ryegrass (RG) extract treatment and *R. vitis* inoculation. *R. vitis* inoculation and HV/RG extract treatment were performed as described for the *in-vivo* experiment. Expression of defense-related genes in the leaves of 'Campbell Early' and 'Kyoho' grapevines in *R. vitis* inoculated leaves (A), plants treated with hairy vetch (HV) extract (B), HV extract followed by *R. vitis* inoculation (C), salicylic acid (SA) (D), ryegrass (RG) (E), and RG extract followed by *R. vitis* inoculation (F) at 0, 6, 24 and 48 h post treatment based on the ratio to the untreated control. Genes of interest encoded *beta*-1,3-glucanase (*PR-2)*, thaumatin-like protein (*TLP*), phenylalanine ammonia lyase (*PAL*), stilbene synthase (*StSy*), leucine reach-repeat (*LRR*), polygalacturonase inhibiting protein (*PGIP*) and catalase (*CAT*). Transcripts levels were calculated using the standard curve method from triplicate data, with the grapevine actin gene as an internal control and nontreated leaves (at time zero) as reference samples. Vertical bars represent the SEs (n=3).

inoculation following ryegrass (RG) treatment at all time points. Among these, *TLP* (RTC 10.29), *LRR* (RTC 12.19) and *CAT* (15.81) showed higher expression in 'Campbell Early', whereas *TLP* (RTC 17.12), *LRR* (RTC 14.23) and *CAT* (RTC 23.65) were highly up-regulated by ryegrass treatment followed by *R. vitis* inoculation in 'Kyoho' grapevine (Fig. 2F and 3B).

Discussion

In the present study, the *in vivo* antibacterial activities of water based crude extracts from hairy vetch and rye grass were effective against *R. vitis* to prevent crown gall disease in greenhouse-grown grapevines. Our data showed that the gall incidence and gall weight were significantly reduced by treatment with cover crop extracts and co-cultivation by sowing seeds treatment when compared with the untreated control in 'Kyoho' (susceptible to crown gall) and 'Campbell Early' (moderately resistant to crown gall) grapevines (Table 2,3,4, and Fig. 1). Previous *in vivo* studies of the antibacterial effects of various organic extracts showed that they had varying degrees of antibacterial activity against different pathogenic bacteria (Hevesi et al., 2006; Bajpai et al., 2007a). Our study revealed similar results of *in vivo* antibacterial effects of hairy vetch and rye grass extracts against *R. vitis* under greenhouse conditions. The results of this study are consistent with those of *in vitro* screening (Islam et al., 2012). The present study also investigated 'Kyoho' and 'Campbell Early' grapevine leaves, which are susceptible and resistant to crown gall, respectively, in response to hairy vetch (HV) and ryegrass (RG) extracts applied as potential biological inducers of the grapevine's defenses against *R. vitis* (Fig. 2 and 3). The transcripts of all selected defense-related genes accumulated within 48 h of hairy vetch/ryegrass extract treatment and/or pathogen inoculation, but the high transcript levels did not persist. The constitutive expression of tested genes was higher in the resistant 'Campbell Early' than in the susceptible 'Kyoho' in response to the tested treatments. Interestingly, expression was constitutively higher in grapevine leaves in response to hairy vetch/ryegrass extract treatment or pathogen inoculation following plant extract treatment. Similar findings were reported in previous studies (Ahn et al., 2005a and 2005b). Plants show defense responses against biotic attacks by employing a complex array of physical and chemical defense mechanisms, including production of various signal compounds, production of PR proteins, and the buildup of histological barriers (Ortíz-Castro et al., 2009). Important changes in the plant transcriptome pattern have been reported in elicitor or pathogen-treated plants (Dangl and Jones, 2001). In the present study, PR genes such as *β*-1,3-glucanase (*PR-2*) and thaumatin-like proteins (*PR-5*) were confirmed to be expressed by exogenous application of HV/RG extract and pathogen inoculation following plant extract treatment (Fig 2 and 3). These findings are accordance with previous reports that PR genes were induced by some plant extracts (Erika et al., 2010; Medeiros et al., 2009). *PAL* is involved in the phenylpropanoid pathway, which leads to synthesis of defense-related phenolic compounds such as lignin and phytoalexins (Daayf et al., 1997). Induction of the *PAL* gene is consistent with the production of phytoalexin, which is associated with wounding and abiotic or biotic stresses (Coutos-Thevenot et al., 2001). This is consistent with the results of the present study, in which *PAL* and *STS* were strongly stimulated by hairy vetch or ryegrass extract treatment and extract application combined with pathogen inoculation. Grapevine phytoalexins are formed via the phenylpropanoid pathway, and synthesis of stilbenes (*STS)* only occurs if *PAL* and subsequent genes are induced (Jeandet et al., 2002). The product of *PAL* is *trans*-cinnamic acid, which is an immediate precursor for the biosynthesis of salicylic acid, a signal molecule in systemic acquired resistance (SAR) (Klessig and Malamy, 1994). The results of the present study support the view that development of acquired resistance mediated by hairy vetch or ryegrass extract such as SA may be attributed, at least in part, to the tested plant extracts induced *PAL* gene expression and activation. *STS* is a further key branch point enzyme in the phenylpropanoid pathway, and its enzymatic activities and gene expression are closely related to phytoalexins biosynthesis in grapevine (Jeandet et al., 2002). Signal transduction related genes and *LRR* proteins were up-regulated after all treatments. The results showed that the expressed genes encode *LRR* resistance protein-like proteins, which constitute the largest and most diverse family of resistance genes in plants (Wroblewski et al., 2007). In 'Campbell Early' and 'Kyoho' grapevine leaves, genes related to cell wall modification, such as *PGIP*, accumulated in all tested treatments, which is consistent with reports that *WRKY* transcription factor 10 (*WRKY*) and *PGIP* were regulated by pathogen attack fungal elicitors and salicylic acid (Eulgem et al., 2000). Therefore, the reduction of crown gall development by *R. vitis* was shown to be caused by higher expression of defense-related genes induced by plant extract treatment in tested grapevines. In the present study, the leaves of SA and hairy vetch or ryegrass extract treated plants showed strong catalase gene up-regulation. Chen et al. (1993) were first to identify catalase as an SA-binding receptor protein that becomes inactive after SA binding, leading to H_2O_2 accumulation, which acts as a secondary messenger inducing the expression of PR genes. Catalase plays an important role in the plant signal transduction pathway that leads to the development of SAR (Bagnoli et al., 2004).

Materials and Methods

Plant materials

Two cover crops, ryegrass (*Lolium perenne*) and hairy vetch (*Vicia villosa*), were grown in a greenhouse at Yeungnam University (Republic of Korea). Seeds of the cover crops were provided by a local seed supplier (Haewae Traders, Anyang, Republic of Korea). Dormant cuttings 30 cm long and 8-10 mm in diameter of two grapevine cultivars, 'Kyoho' (susceptible to crown gall) and 'Campbell Early' (moderately resistant to crown gall) that originated from 6- to 7-year-old vines without any visible symptoms of crown gall were collected in late autumn, stored at 4-5°C, and used for experiments the next spring.

Pathogen inoculation and crown gall disease evaluation in greenhouse

Based on previous *in vitro* screening conducted by Islam et al. (2012), extracts of hairy vetch (*Vicia villosa*) and ryegrass (*Lolium perenne*) were applied to evaluate the *in vivo* antibacterial activity against crown gall caused by *R. vitis* in grapevines under greenhouse conditions. *R. vitis* was stored at -20°C in Luria Bertani (LB) broth medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) containing 20% (v/v) glycerol. Cell suspensions were prepared from early

Table 3. Efficacy of fresh root extracts from hairy vetch and ryegrass against crown gall in 'Kyoho' and 'Campbell Early' grapevines under greenhouse conditions.

Cultivar	Treatment	Dosage ($g \cdot L^{-1}$)	Gall wt (mg)	% GW^z reduction over control	Rate of gall incidence (%)	% GI^y reduction over control
Kyoho	Hairy vetch	125	75.3±9.6 c	43.63	66.6±0.1 b	25.08
		250	32.3±4.2 e	75.6	44.4±0.1 d	50.05
		500	18.3±0.6 e	86.3	33.3±0.1 e	62.54
	Ryegrass	125	104.7±24.8 b	21.4	66.6±0.1 b	25.08
		250	55.7±11.5 d	58.3	55.5±0.1 c	37.57
		500	19.5±2.3 e	85.4	44.4±0.1 d	50.05
	Control (PI^x)	0	133.6±3.4 a	-	88.9±0.1 a	-
Campbell Early	Hairy vetch	125	15.0±1.0 b	62.77	33.3	0
		250	0.0±0.0 c	100	0	100
		500	0.0±0.0 c	100	0	100
	Ryegrass	125	15.7±1.5 b	61.04	33.3	0
		250	0.0±0.0 c	100	0	100
		500	0.0±0.0 c	100	0	100
	Control (PI^x)	0	40.3±4.1 a	-	33.3	-

GW^z, gall weight; GI^y, gall incidence; PI^x, pathogen inoculated. Means within a column followed by different letter(s) are significantly different (DMRT, $p < 0.05$).

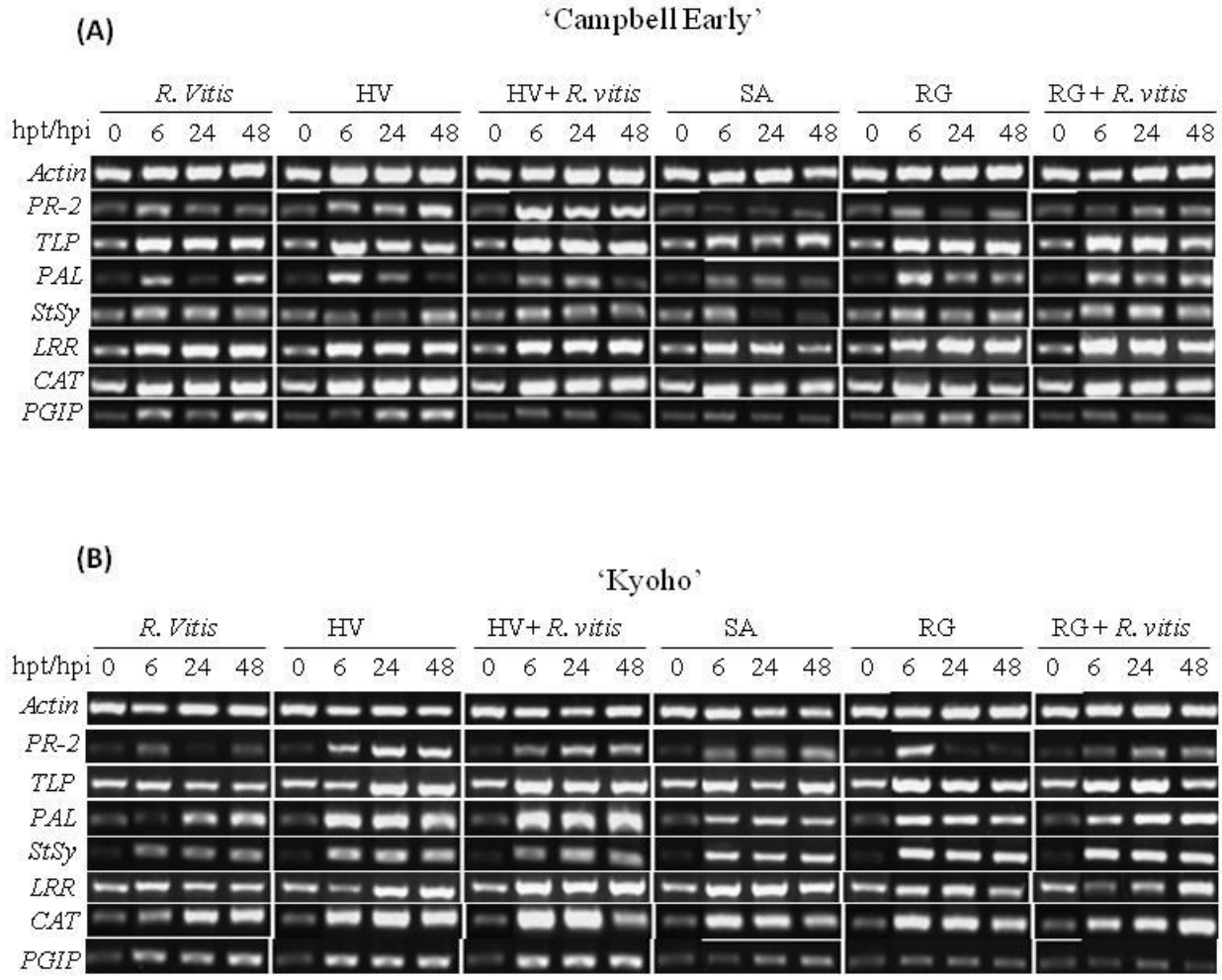

Fig 3. Semi-quantitative RT-PCR analysis to screen genes induced in leaves of (A) 'Campbell Early' and (B) 'Kyoho' in response to *R. vitis* inoculation, hairy vetch (HV) extract application, HV extract challenged with *R. vitis*, salicylic acid (SA) treatment, ryegrass (RG) extract and RG extract followed by *R. vitis* inoculation under greenhouse conditions. Samples were collected at different time points as indicated. hpt/hpi: hours post inoculation/treatment; 0, untreated leaves at the beginning of the experiment. The actin gene was used as an internal control. Results shown are one of three independent replicates.

Table 4. Efficacy of co-cultivation after sowing seeds of six cover crops against crown gall in 'Kyoho' and 'Campbell Early' grapevines under greenhouse conditions.

Cultivar	Treatment	Seed rate ($kg \cdot 10a^{-1}$)	Gall wt (mg)	% GW^z reduction over control	Rate of gall incidence (%)	% GI^y reduction over control
Kyoho	Chinese milk vetch	3.5	15.3±2.1 b	53.21	44.4±0.1 c	42.86
	Forage rape	0.7	15.3±0.6 b	53.21	55.5±0.1 b	28.57
	Orchard grass	1.2	31.0±10.0a	5.11	55.5±0.1 b	28.57
	Ryegrass	3.0	15.7±2.9 b	51.98	44.4±0.1 c	42.86
	Italian ryegrass	1.0	31.7±3.2 a	3.05	55.5±0.1 b	28.57
	Hairy vetch	7.5	13.7±0.6 b	58.1	33.3±0.1 c	57.14
	Control (PI^x)	0	32.7±0.6 a	-	77.7±0.1 a	-
Campbell Early	Chinese milk vetch	3.5	0.0±0.0 b	100	0±0.0 b	100
	Forage rape	0.7	0.0±0.0 b	100	0.0±0.0 b	100
	Orchard grass	1.2	16.8±13.9 a	30.86	33.3±0.1 a	0
	Ryegrass	3.0	0.0±0.0 b	100	0.0±0.0 b	100
	Italian ryegrass	1.0	0.0±0.0 b	100	0.0±0.0 b	100
	Hairy vetch	7.5	0.0±0.0 b	100	0.0±0.0 b	100
	Control (PI^x)	0	24.3±2.5 a	-	33.3±0.1 a	-

GW^z, gall weight; GI^y, gall incidence; PI^x, pathogen inoculated. Means within a column followed by different letter(s) are significantly different (DMRT, $p < 0.05$).

logarithmic phase cells obtained by culturing the bacterium in LB in a sterile flask at 28°C on a shaker at 140 rpm for 18 h. The bacterial cells were then collected by centrifugation at 3200 rpm for 15 min, re-suspended in sterile distilled water, and adjusted to 1×10^8cfu/ml (OD_{600}=1.0). Three grapevine cuttings were used per treatment, and each treatment was performed in triplicate. Cuttings were subsequently wounded by drilling holes in internodes to the depth of the pith, after which 30 µl bacterial cell suspensions were injected into the holes. Inoculated sites were subsequently wrapped with paraffin tape and cuttings were planted in separate pots and maintained in a greenhouse (26°C). Crude extracts from each cover crop were prepared by mixing 1 kg of pounded fresh shoots and roots in 1 liter of distilled water. Each cover crop extract was then filtered using sieves made of fine cloth mesh to yield a concentrated spray formulation. The concentrated extracts were diluted in distilled water prior to application (Obongoya et al., 2010) to give initial concentrations of 125, 250 and 500 $g \cdot L^{-1}$, after which 100 ml of the diluted extracts were applied to grapevine cuttings once every 10 days for two months. Water without any plant extract (0 $g \cdot L^{-1}$) was applied as a control. Gall formation was assessed two months after inoculation of the pathogen.

Treatments for defense-related gene expression

Grapevines of 'Kyoho' and 'Campbell Early' were grown in a greenhouse at 25-30°C under natural light, and vine leaves were sprayed with 50 $mg \cdot mL^{-1}$ of hairy vetch (HV)/ryegrass (RG) shoot extracts (Islam et al., 2012) or 1 mM salicylic acid (SA) as a positive control to induce defense responses in plants. Vines were inoculated with *R. vitis* following HV/RG extracts application. Leaves were then harvested at 0, 6, 24 and 48 h after treatment, immediately frozen in nitrogen, and stored at −80°C until RNA extraction.

RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from grapevine leaves inoculated with/without cover crops extract and control plant leaves using the modified pine tree method for removing polysaccharide and phenolic compounds (Chang et al., 1993). Total RNA from all time courses was collected in tubes for each series (control, cover crops extract treated, SA treated, extract treated and/or *R. vitis* inoculated). The quality of the RNA was then checked on an agarose gel and the concentration and purity were determined using a spectrophotometer (model S-3130, Sinco Co. Ltd.) based on the absorbance at 260 nm and the A_{260}/A_{280} ratio. Next, first-strand cDNA synthesis was conducted using a PrimeScript^{TM}1st strand cDNA synthesis kit (Takara Bio Inc., Japan) according to the manufacturer's recommendations. The synthesized complementary DNA was subsequently used for assessment of the defense-related genes *β*-1,3-glucanase (*PR-2*), phenylalanine ammonia-lyase (*PAL*), thaumatin-like protein (*TLP*), leucine reach-repeat (*LRR*), polygalacturonase inhibiting protein (*PGIP*), stilbene synthase (*STS*) and catalase (*CAT*). The constitutively expressed actin gene was used as an internal control.

Real-time and semi-quantitative RT-PCR

Primer pairs for each targeted defense-related gene were designed using the Primer 3 software (Rozen and Skaletsky, 2000) as indicated in Table 1. Real-time PCR was performed on a C1000^{TM} Thermal Cycler (BioRad, USA) using SYBR Premix Ex (Takara Bio Inc., Japan) as the florescent dye. Amplification was conducted by subjecting the samples to one cycle at 95°C for 5 min and then 40 cycles of 95°C for 5 s and 60°C for 30 s. All reactions were performed in triplicate to ensure consistency of results. Transcript levels were calculated using the standard-curve method and normalized using the grapevine actin gene (AB 372563) as an internal control, after which melting curves of the amplified products were recorded. Semi-quantitative RT-PCR was then performed by subjecting the samples to the following conditions: initial denaturation at 94°C for 5 min followed by 30 reaction cycles consisting of 45s denaturation at 94°C, 45s annealing at 55°C and 1 min elongation at 72°C, and then final extension for 7 min at 72°C. The PCR products were subsequently analyzed by electrophoresis using a 1.5% (w/v) agarose gel containing 0.01% ethidium bromide at 105 V for 30 min with 0.5X TBE running buffer. After taking the gel picture using a transilluminator (UVP, CA, USA), the expression levels were measured by analysis of the image using a public domain image analysis system (NIH ImageJ, NIH Image, Bethesda, USA).

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (IBM SPSS, Inc., Chicago, USA). The collected data were analyzed by ANOVA, with significant differences ($p < 0.05$) between the control and treated groups identified by Duncan's multiple mean comparison test (DMRT).

Conclusions

The results of this study clearly demonstrate that exogenous application of extracts from hairy vetch and ryegrass could inhibit development of crown gall caused by *R. vitis* in grapevines under greenhouse conditions through both direct antibacterial activity and induction of host-defense mechanisms. To the best of our knowledge, this is the first report describing the efficacy of tested cover crops to manage crown gall disease with *in vivo* antibacterial activity and to induce expression of genes related to defense in grapevines. In vineyard management programs, cultural management by co-cultivation of hairy vetch and ryegrass would be very valuable in sustainably reducing the loss of yield caused by crown gall disease in grapevines.

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