Plant Physiology and Biochemistry 60 (2012) 119–128

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Effects of glucans and eicosapentaenoic acid on differential regulation of phenylpropanoid and mevalonic pathways during potato response to *Phytophthora infestans*

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ARTICLE INFO

Article history:
Received 6 June 2012
Accepted 30 July 2012
Available online 8 August 2012

Keywords:
Oomycetes
Effectors
Phenolics
Phenylpropanoids
Defense inducers
Defense suppressors
Late blight
Terpenoids

ABSTRACT

The effects of *Phytophthora infestans* glucans, eicosapentaenoic acid (EPA) and isolates of this pathogen, on the differential expression of eight genes from the phenylpropanoid and the mevalonate (Ac-MVA) pathways were analyzed in potato by semi-quantitative RT-PCR and qRT-PCR. The application of EPA had an elicitor effect in Russet Burbank (RB) and Defender (DF) in response to inoculation with a US8 isolate of *P. infestans*, thereby reducing symptoms of late blight. Such effect was associated with the expression of *PAL-1* and *PAL-2*, since the latter occurred only when EPA was followed by inoculation, whereas these genes were down-regulated in individual treatments RB + EPA, RB + US8, DF + EPA, and DF + US8. The glucan fraction did not by itself suppress phenylpropanoid genes, but its combination with the pathogen resulted in a down-regulation of *PAL-1*, *PAL-2* and *CHS*. The addition of the glucan fraction to the elicitor EPA, had a negative effect (RB + EPA + GL + US8) since plants showed higher disease symptoms than the ones pretreated with water then infected with US8, and in comparison with RB + EPA + US8 and RB + GL + US8. Exclusive up-regulation of *4CL* in DF + US11 and of *CHS* in DF + EPA + GL + US8, DF + EPA + US11, DF + GL + US11 and DF + EPA + GL + US11, where late blight lesions were not detected, could be associated with potato protection against late blight. Along with previous findings in this pathosystem, these data suggest that genetic resistance in potato against *P. infestans* is not the result of isolated reactions against the pathogen, but rather the combination of many factors in-line with a polygenic/horizontal resistance.

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1. Introduction

Plant pathogens have the ability to manipulate biochemical, physiological and morphological processes in their host plants through a diverse array of extracellular effector molecules that can either promote the infection or trigger defense responses [1]. Many of these effectors are soluble glucans, glycoproteins or proteinaceous compounds synthesized by the pathogen. However, many can be surface components released from the cell wall of other microbes or the host [2–4].

The oomycete *Phytophthora infestans*, which causes late blight disease in both potato and tomato, is heavily studied due to its great economic importance and the genotypic changes in its population structure in the last two decades [5–8]. The new populations of *P. infestans* are more damageable to potato and tomato crops, and such success has been ascribed at least partially to their differential ability to suppress plant defenses [9,10] probably using more efficient effectors.

Effectors that suppress host defense responses have been described in several pathosystems involving the genus *Phytophthora*. Sanchez et al. [11] showed that water-soluble glucans (WSG) from *Phytophthora capsici* suppress the elicitor-induced death of suspension culture cells of susceptible sweet pepper and tomato, but not that of resistant pepper and tobacco. Ozeretskovskaya et al. [12] also isolated suppressors identified as β-1,3-β-1,6-glucans from both mycelium and liquid culture of *P. infestans*. Glucans isolated

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http://dx.doi.org/10.1016/j.plaphy.2012.07.027

from *P. infestans*' cell walls suppress the accumulation of phytoalexins and reduce β-1,3-glucanase activity in potato tubers [13]. These glucans showed race specificity since they suppressed potato defenses only when they were obtained from the strain that was compatible with the tested potato cultivar [12–14]. Contrastingly, this pathogen also produces elicitors, i.e., arachidonic acid (AA) and eicosapentaenoic acid (EPA), which elicit phytoalexin accumulation [13,15].

It is increasingly evident that fungal pathogens and oomycetes tactically use their effectors to counteract plant defenses [16–18]. During plant–pathogen interactions, cell wall reinforcement through lignin synthesis, production and accumulation of secondary metabolites from the phenylpropanoid and mevalonate pathways, and the accumulation of phytoalexins are well-known plant defense responses [19–21]. Phenylpropanoids exhibit a broad-spectrum antimicrobial activity and are therefore believed to help the plant fight microbial disease [21]. On the other hand, terpenoids also recognized as isoprenoids and related plant secondary metabolites (sesquiterpenoids and sterols) have been shown to be important factors in resistance to several insect pest and pathogens [22].

The phenylpropanoid pathway occurs in the plastids, with the first step in the core pathway being the formation of trans-cinnamic acid by the elimination of an ammonia molecule from phenylalanine. This reaction is catalyzed by the enzyme phenylalanine ammonia lyase (PAL). Then, cinnamate 4-hyhrolase (C4H) introduces a hydroxyl group into the phenyl group of trans-cinnamic, forming *p*-coumaric acid. The final step in the core phenylpropanoid pathway catalized by the enzyme 4-coumarate: CoA ligase (4CL) to form *p*-coumaroyl-CoA from coumaric acid. This core phenylpropanoid pathway produces the ring B and C for the flavonoid synthesis and the malonic acid/acetate pathway provides the ring A with the participation of enzyme chalcone synthase (CHS) [21].

Terpenoids are synthesized from two common precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) by two separate routes, the cytoplasmic mevalonate (Ac-MVA) and the plastidial 2C-methyl-D-erythritol-4-phosphate (DOXP-MEP) pathways [23]. In the mevalonate (Ac-MVA) pathway that proceeds in the cytosol, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is formed from three acetyl-CoA. Then, mevalonic acid (MVA) is formed from HMG-CoA by a reaction catalyzed by the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). Downstream, squalene synthase and sesquiterpene cyclase catalyze the first steps in the branches leading to sterols and steroidal glycoalkaloids (SGA), and sesquiterpenoid phytoalexins, respectively [24].

The effects of different glucan suppressors from *P. infestans* on potato defense responses have been previously reported [12–14]. However, most previous studies were carried out "*in vitro*" using either leaflets or tubers, and none have assessed these effects in whole plants. When used *in vitro*, plant tissues are usually in a surviving state, are stressed, and their metabolism is not necessarily matching their counterparts in whole plants. In addition, no studies have considered comparison of both susceptible and resistant varieties infected by either highly or weakly aggressive isolates of *P. infestans*. Therefore, the objective of this study was to integrate these levels of comparison, by using whole plants, from two species with different levels of susceptibility to late blight, inoculated with *P. infestans* isolates that have contrasting levels of aggressiveness, to investigate the expression of selected genes both in the phenylpropanoid and the mevalonate pathways after inoculation. In addition, a glucan fraction (suppressor), and eicosapentaenoic acid (EPA, elicitor) were used to investigate their effects on such expression and to better understand their role in this interaction.

2. Results

2.1. Disease development

The susceptible cultivar RB inoculated with US8 (RB + US8) showed typical late blight lesions, with a brown necrotic spot surrounded by a chlorotic ring (Fig. 1A). The leaves from plants pretreated with EPA and then inoculated with US8 (RB + EPA + US8) showed less and smaller lesions than those from plants only inoculated with US8 (RB + US8). However, when RB plants were treated with the glucan fraction and then infected with US8 (RB + GL + US8), the number and size of lesions were higher than in plants only infected with US8. In RB + EPA + GL + US8, plants had higher disease symptoms than the ones pretreated with water then infected with US8 (Fig. 1A).

The cultivar Defender inoculated with US8 (DF + US8) showed typical late blight lesions, but in smaller number and size than the susceptible cultivar RB (Fig. 1A). In addition, no symptoms were visible in Defender inoculated with US8 after EPA (DF + EPA + US8), or EPA with the glucan (DF + EPA + GL + US8) treatments. A hypersensitive-like reaction (HR) was detected in (DF + GL + US8) (Fig. 1A).

Russet Burbank inoculated with US11 after treatment with EPA, glucan, or EPA and glucan, showed typical late blight lesions. In RB + US11, the number and size of lesions were higher than DF + US8. However, in RB + EPA + US11, RB + GL + US11 and RB + EPA + GL + US11, lesions were reduced significantly (Fig. 1B). In addition, late blight lesions were not detected in DF + US11, DF + EPA + US11 or DF + GL + US11. A minor hypersensitive-like reaction (HR) was recorded in the DF + EPA + GL + US11 (Fig. 1B). Late blight symptoms were not detected in treatments without *P. infestans* inoculation, RB + H_2O, RB + EPA, RB + GL, RB + EPA + GL, DF + H_2O, DF + EPA, DF + GL, and DF + EPA + GL (data not shown).

2.2. Gene expression in the mevalonate pathway

Expression levels of the mevalonate pathway 3-hydroxy-3-methylglutaryl coenzyme A reductase *HMGR*, *HMGR3*, squalene synthase (*SQS*) and sesquiterpene cyclase (*SC*) genes were first assessed by semi-quantitative RT-PCR analysis. Statistical analysis showed no significant differences in RB or DF among treatments in expression levels of *HMGR*, *HMGR3*, *SQS* or *SC* among all treatments, except in a few instances, i.e., the *SC* gene was down-regulated in RB + US8 and RB + GL + US8 compared with RB + H_2O (Fig. 2). Therefore, qRT-PCR analysis was not pursued.

2.3. Gene expression analysis in the phenylpropanoid pathway

RT-PCR analysis of gene expression in the phenylpropanoid pathway showed several differences among the tested treatments. Therefore, we moved on to assess the expression levels of phenylalanine ammonia lyase (*PAL-1* and *PAL-2*), 4-coumarate:coenzyme A ligase (*4CL*) and chalcone synthase (*CHS*) genes using qRT-PCR. The differences were significant among all treatments in both RB and DF (Fig. 3). *PAL-1* and *PAL-2* were down-regulated in all treatments in Defender and RB, with the exception of RB + GL, RB + EPA + US8 and DF + EPA + US8 when compared with RB + H_2O and DF + H_2O, respectively. Similar to *PAL-1* and *PAL-2*, *4CL* was down-regulated in all treatments in RB, with the exception of RB + GL and RB + EPA + US8, but it was up-regulated in RB + GL + US8 when compared with RB + H_2O. On the other hand, *4CL* was only up-regulated in DF + US11 and down-regulated in the remaining treatments (Fig. 3). In addition, *CHS* was down-regulated in all treatments compared with RB + H_2O, with the

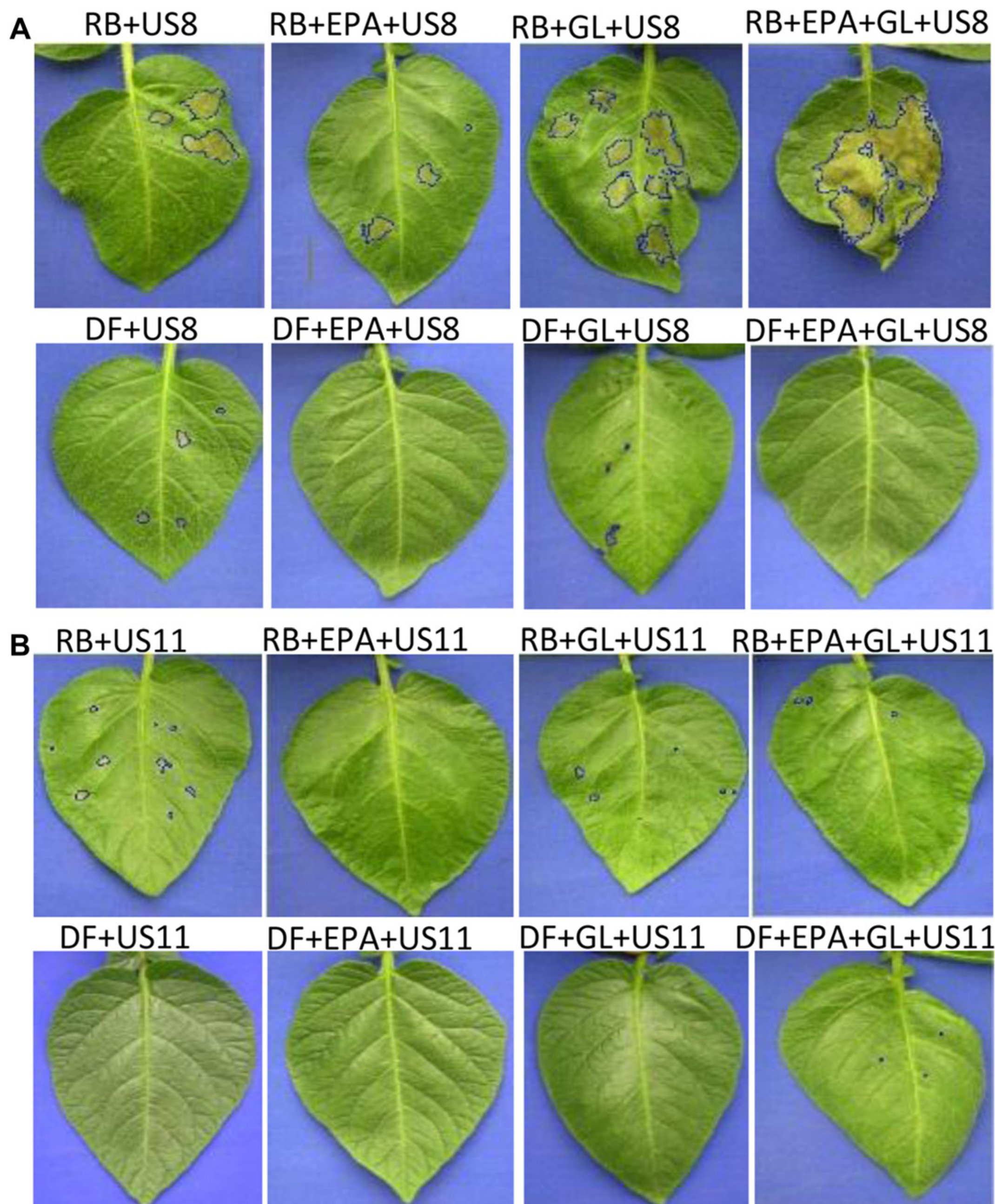

Fig. 1. Effect of EPA elicitor and Glucan suppressor on the disease development caused by *P. infestans* US8 (A) and US11 (B) in Russet Burbank and Defender cultivars. Potato plants from Russet Burbank (RB) and Defender (DF) were pretreated with eicosapentaenoic acid (EPA), Glucan race C (GL) or both as was described in Materials and methods. Then potato plants were infected with a spores suspension of *P. infestans* (4×10^4 sporangia/ml), US8 (highly aggressive) or US11 (weakly aggressive). Control plants were treated with water. The plants were placed in a growth chamber (20 °C, 16 h photoperiod, 100% relative humidity) during 48 h. The pictures are from one replicate representative out of three.

exception of RB + GL, whereas in Defender, it was up-regulated in DF + EPA + GL + US8, DF + EPA + US11, DF + GL + US11, and DF + EPA + GL + US11 (Fig. 3).

2.4. Analysis of variance

Data collected from the 2 × 3 × 4 factorial arrangement with two cultivars, three inoculums and four effectors from the expression levels were assessed by analysis of variance (ANOVA-PROC GLM) (Table 3). Comparisons between treatments (Figs. 2 and 3), for all primers was performed using PROC GLM, due to significant interactions ($p < 0.05$) between cultivar * inoculum, cultivar * effector or cultivar * inoculum * effector, with the exception of *HMGR3* and *SQS*.

3. Discussion

Several previous studies have demonstrated transcriptional activation of specific genes in potato in response to *P. infestans* or an elicitor [10,25–27], but most were conducted *in vitro*, and none have integrated more than one of the pathways potentially involved. In this study we combined both elicitors and suppressors originating from the pathogen to study their effects. In addition we did so using whole plants of both susceptible and moderately

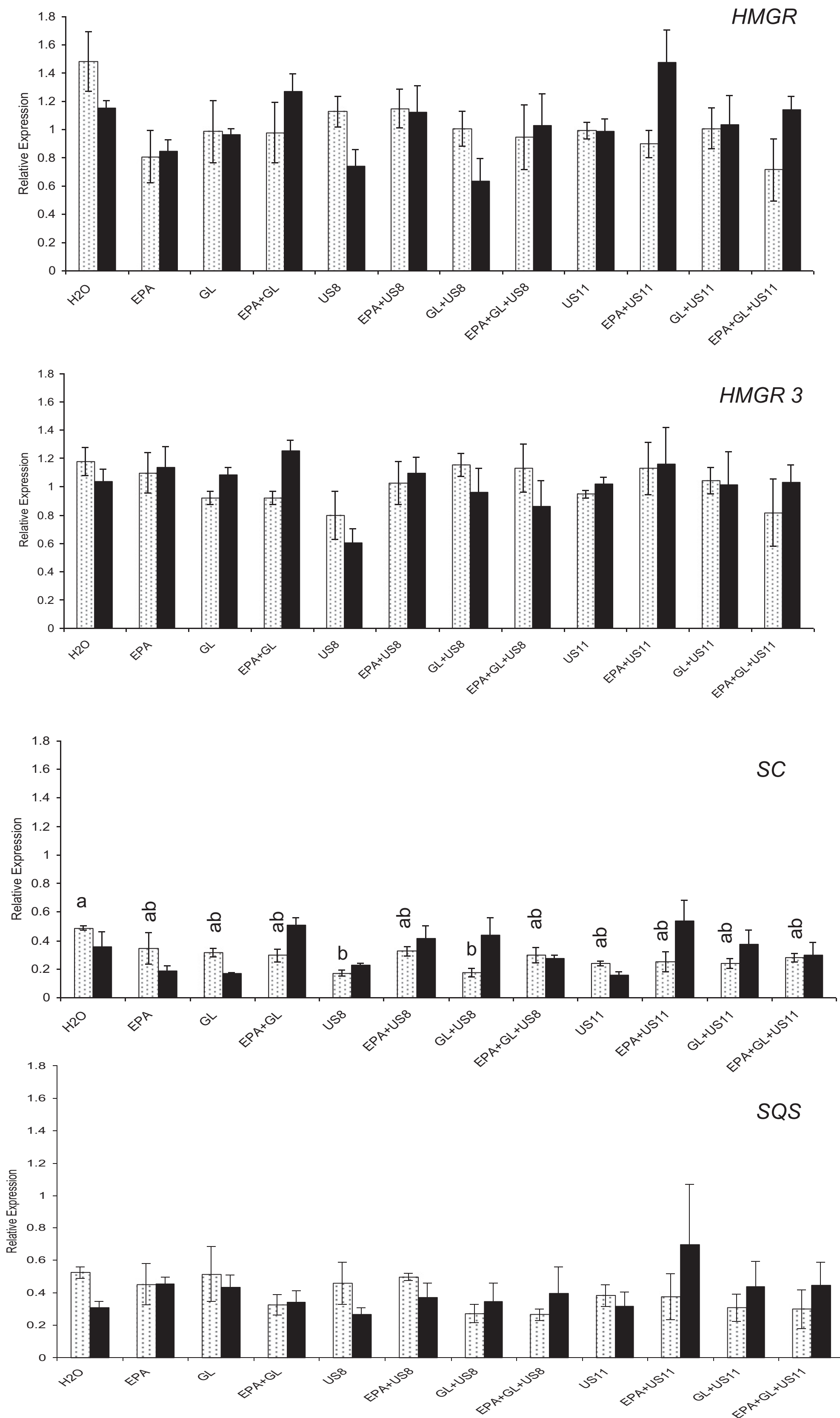

Fig. 2. RT-PCR analysis of the relative expression of *HMGR*, *HMGR3*, *SC* and *SQS*. RB: Russet Burbank (white bar), DF: Defender (black bar), H20: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentaenoic acid (EPA), GL: Glucans race C. Means with the same letter are not significantly different according to Tukey's Studentized Range test (Lowercase: Russet Burbank; Uppercase:Defender). A $p < 0.05$ was considered to indicate significant differences.

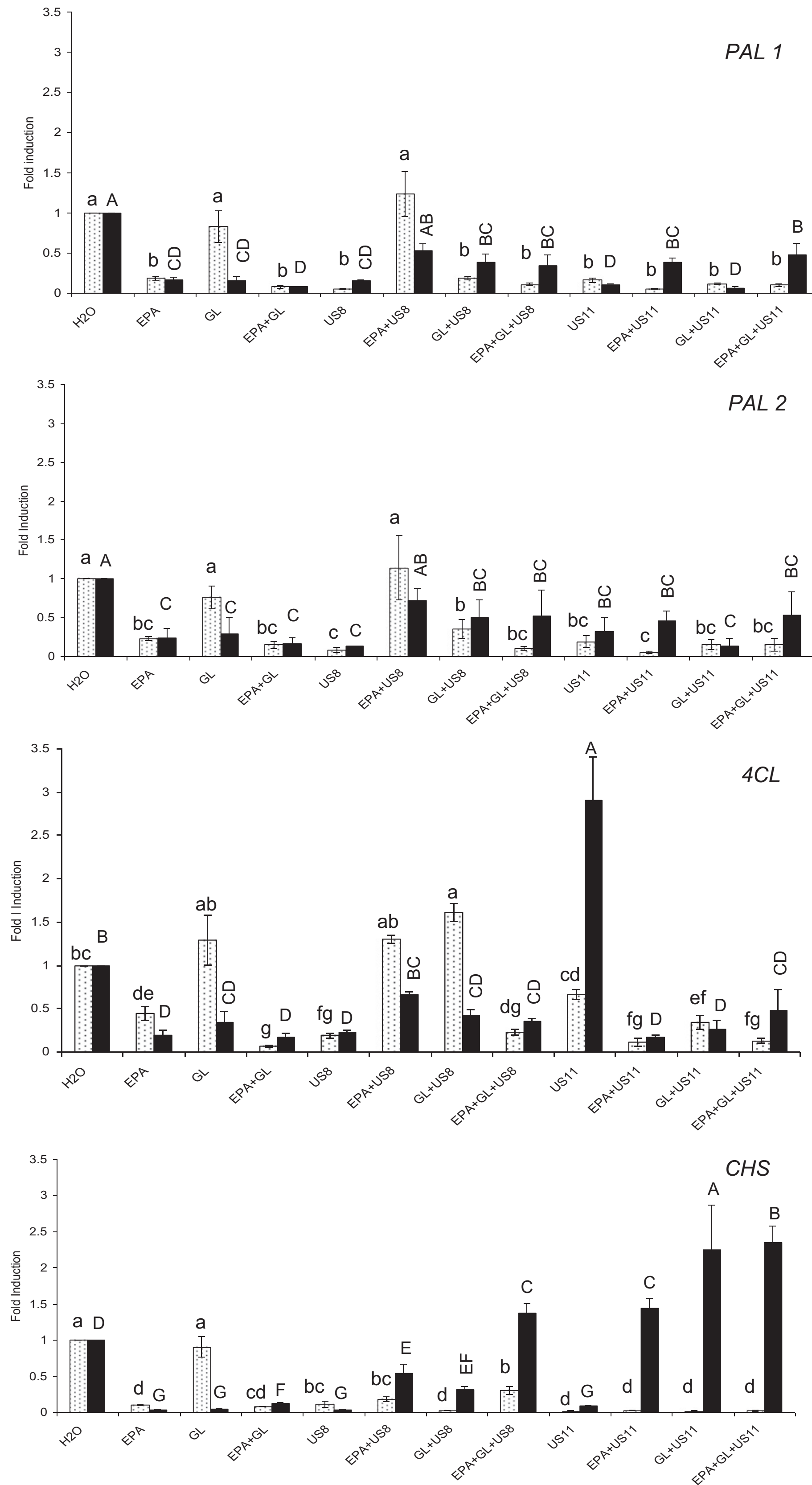

Fig. 3. qRT-PCR analysis of the relative expression of *PAL-1*, *PAL-2*, *4CL* and *CHS*. RB: Russet Burbank (white bar), DF: Defender (black bar), H_20: sterile water, US8: *P. infestans* strain D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strain D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentaenoic acid (EPA), GL: Glucans race C. Means with the same letter are not significantly different, according to Tukey's Studentized Range test (Lowercase: Russet Burbank; Uppercase:Defender). A $p < 0.05$ was considered to indicate significant differences.

Table 1
Treatments from the 2 × 3 × 4 factorial design.

#	Treatments	#	Treatments	#	Treatments
1	RB + H_2O	9	DF + H_2O	17	RB − US11
2	RB + US8	10	DF + US8	18	RB + EPA + US11
3	RB + EPA	11	DF + EPA	19	RB + GL + US11
4	RB + GL	12	DF + GL	20	RB + EPA + GL + US11
5	RB + EPA + GL	13	DF + EPA + GL	21	DF − US11
6	RB + EPA + US8	14	DF + EPA + US8	22	DF + EPA + US11
7	RB + GL + US8	15	DF + GL + US8	23	DF + GL + US11
8	RB + EPA + GL + US8	16	DF + EPA + GL + US8	24	DF + EPA + GL + US11

RB: Russet Burbank, DF: Defender, H_2O: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentaenoic acid (EPA), Gl: Glucan race C.

resistant potato cultivars, and assessed gene expression both in the phenylpropanoid and the mevalonic pathways.

3.1. Disease symptoms and potato defense responses

Glucan oligosaccharides, which are released from pathogen cell walls, are effectors that either induce a wide range of plant defense responses [28] or suppress them [12,13]. In our experiments, the suppression of defense responses in potato by the glucan fraction was specific to the treated potato cultivar and the pathogen genotype. Whereas RB treated with the glucan fraction and then infected with US8 (RB + GL + US8) showed higher number and size of lesions than in plants only infected with US8 (suppression effect), in RB + GL + US11 lesions were reduced significantly (elicitor effect). In Defender (DF + GL + US8) there was a hypersensitive-like reaction (HR), while in DF + GL + US11 late blight lesions were not detected. However, the reason why the glucan fraction used in this study either suppressed or elicited defense responses remains to be investigated.

EPA elicited potato defense responses in both Russet Burbank and Defender plants inoculated with either US8 or US11, thereby reducing symptoms of late blight. Cohen et al. [29] had reported that EPA induces systemic resistance in potato against *P. infestans.* Therefore, disease symptoms can be associated either with the suppression or elicitation of potato defense responses.

Table 2
Primers used for the qRT-PCR and RT-PCR analysis.

Primer name	Gene	Primer sequence 5′−3′	GenBank Accession
QRT-chalc-synth-F	*CHS*	TGGTGGTTGAAGTACCAAAACTTG	X14599
QRT-chalc-synth-R3		AGTACAGTGCCACCAGCAAA	
QRT-4CL-F	*4CL*	TCGTAGCGCTGCCGTATTC	AF150686
QRT-4CL-R2		AAATTGCTGCTCCGACTCTC	
PAL1-F	*PAL-1*	TGCACAAGTTGCATCCATT	X63103
PAL1-R		AAGAGCACCACCATTTTTGG	
PAL2-F	*PAL-2*	GCACCATCAATTGCACAAAA	X63104
PAL2-R		TGCAACTTGTGCAACAGTCA	
QRT-hmg-F	*HMGR*	CGTTCTGGATTACCTTCAGAGTGA	L01400
QRT-hmg-R2		CACAAGAGCAGCAACCTCAG	
QRT-hmg3-F	*HMGR3*	TCATCGGCATATCTGGGAACT	U51986
QRT-hmg3-R2		GAGACAATATTGCTGGCATGG	
SqualeneSyn-F	*SQS*	ATGGCACTTTGCATGTGGTA	AB022599
SqualeneSyn-R		CCAGAGGCATGGAACAGTTT	
SesqCyclas-F	*SC*	TCCAATTCCGATTGCTTAGG	gi|3108342
SesqCyclas-R		AGTGGAGAAAGCGAGTGCAT	
QRT-EF-1-alpha-F	*Efactor*	GATGGTCAGACCCGTGAACAT	AB061263
QRT-EF-1-alpha-R2		GGGGATTTTGTCAGGGTTGT	

Gene: Phenylalanine ammonia lyase (*PAL-1* and *PAL-2*), 4-coumarate:coenzyme A ligase (*4CL*) and chalcone synthase (*CHS*), 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*, *HMGR3*), squalene synthase (*SQS*), sesquiterpen cyclase (*SC*), Elongation factor (*Efactor*).

Interestingly, the addition of the glucan fraction to the elicitor EPA, induced a synergistic effect in RB + EPA + GL + US8, since plants showed higher disease symptoms than the ones pretreated with water then infected with US8 (RB + EPA + US8 and RB + GL + US8). This suggests that the potency to suppress defense responses by the glucan fraction in potato can considerably increase by pretreatment with the elicitor EPA before inoculation with the highly aggressive US8 isolate. EPA does not have suppression properties, but it is possible that EPA has an effect on potato defenses such that it allows the tested glucans to have a more negative effect when combined with the US8 isolate.

3.2. Defender's responses to P. infestans

Defender is originated from a cross between KSA195-90 and Ranger Russet, where KSA195-90 is the source of resistance to late blight. Corsini et al. [30] suggested that R-genes from *Solanum demissum* are likely present in KSA195-90, thereby conferring some resistance to *P. infestans*. Elicitors from *P. infestans* could be recognized by Defender receptors or R proteins before initiating signaling pathways, followed by defense reactions such as synthesis of pathogenesis-related proteins, or defense secondary metabolites as suggested by our results with tested genes from the phenylpropanoid and mevalonate (Ac-MVA) pathways. In Defender, inoculation with US8 or US11 affects the expression of *PAL-1*, *PAL-2*, *4CL* and *CHS*. Therefore, preformed secondary metabolites, phytoalexins, signaling molecules, and lignin synthesis [31] may have been strongly affected by inoculation of this cultivar. A down-regulation of *PAL-1* and *PAL-2* in all treatments in Defender, with the exception of DF + EPA + US8 when compared with DF + H_2O, indicated that the elicitor effect of EPA in Defender can be associated with the expression of these two genes. Expression of *PAL-1* and *PAL-2* in DF + EPA + US8 could translate into an increase in abundance of phenolic compounds, including salicylic acid, because it has been shown that induction of *PAL-2* may result into an increase in abundance of phenolic compounds, including salicylic acid [32]. On the other hand, the expression of *4CL* plays an important role in potato defense responses, because it was only up-regulation of DF + US11, where late blight lesions were not detected and down-regulated in the remaining treatments. Similar importance in defense responses should be attributed to *CHS*, due to its exclusive up-regulation in DF + EPA + GL + US8, DF + EPA + US11, DF + GL + US11 and DF + EPA + GL + US11, where late blight lesions were not detected. These results indicate that the phenylpropanoid pathway is playing an important role in potato defense responses.

In Defender, the expression of *HMGR*, *HMGR3*, *SQS* and *SC* did not show variations in response to US8 or US11. Terpenoids also participate in essential plant processes such as respiration, growth, and development [33,34] more that phenolics do, which may explain a diluted variation in the expression of the mevalonate pathway genes. However, we did recently identify a preformed terpenoid compound in potato tissues which may contribute to Defender's moderate resistance to *P. infestans* [35].

3.3. Russet Burbank defense responses to P. infestans

In Russet Burbank, inoculation with US8 or US11 affects the expression of *PAL-1*, *PAL-2*, *4CL* and *CHS*. Similar to Defender, there was a down-regulation of *PAL-1* and *PAL-2* in all treatments in Russet Burbank, with the exception of RB + GL and RB + EPA + US8 when compared with RB + H_2O, indicating that the elicitor effect of EPA in RB is associated with the expression of these two genes. In fact, expression of these two genes in Russet Burbank is only achieved by the combination of EPA with the

Table 3
Significance (*p*-values) of interactions with respect to genes.

	PAL-1	*PAL-2*	*4CL*	*CHS*	*HMGR1*	*HMGR3*	*SQS*	*SC*
Cultivar	0.9263	0.5744	0.4856	0.422	0.7139	0.9274	0.9039	0.1991
Fungus	0.2313	**0.0003**	0.9631	**<0.0001**	0.4395	0.143	0.4554	0.4353
Elicitor	0.1227	**<0.0001**	0.5458	0.4813	0.3845	0.1688	0.2153	0.3661
Cultivar * Inoculum	**0.0117**	**<0.0001**	0.6227	0.0874	**0.0187**	0.1412	0.1812	0.0826
Cultivar * Effector	**0.0777**	**<0.0001**	**0.0468**	**0.0044**	**0.0131**	0.6554	0.1398	0.4544
Fungi * Effector	0.3153	**<0.0001**	0.148	**<0.0001**	**0.0299**	0.1861	0.8984	**0.0373**
Cultivar * Effector * Inoculum	0.2798	**0.0004**	0.4921	**0.0251**	0.9612	0.6002	0.8913	**0.0172**

Significant interactions are shown in bold ($p < 0.05$). Cultivar: Russet Burbank, Defender; Inoculum: H_2O (control), *P. infestans* strain D1901 (lineage US8, A2 mating type, highly aggressive), *P. infestans* strain D-03 (lineage US11, A1 mating type, weakly aggressive); Effector: eicosapentaenoic acid (EPA), Glucan race C (GL), EPA + GL and H_2O.

pathogen, since the individual treatments RB + EPA and RB + US8, showed down-regulation of *PAL-1* and *PAL-2.* Similar results, were observed in Defender. The suppression effect of glucans described above can be explained with the down-regulation of all phenylpropanoid genes in Russet Burbank when inoculated with US8. The glucan fraction did not by itself suppress phenylpropanoid genes, but its combination with the pathogen resulted in a down-regulation of *PAL-1*, *PAL-2* and *CHS*. *4CL* was up-regulated in RB + GL + US8 when compared with RB + H_2O, even though this treatment showed a number and size of lesions higher than in plants only infected with US8. In addition, similar to Defender, where *4CL* may play an important role in potato defense in DF + US11 and RB + US11, the expression of this gene was not affected when compared with RB + H_2O, leading to a reduction in the number and size of late blight lesions compared to RB + US8. Transcriptional activation of *4CL* has been reported in potato infected with *P. infestans* and other pathosystems [36,37]. Despite an up-regulation of *4CL* in RB + GL + US8, the suppression effect of the glucan fraction in Russet Burbank resulted in the down-regulation of *CHS*, which may be affecting flavonoid synthesis. Despite up-regulation of *4CL* in RB + GL + US8, there were considerable disease symptoms in this treatment, which could be explained by down-regulation of *CHS* as well as *CHS* gene homologs or downstream genes for flavonoid synthesis. Even though a down-regulation of *CHS* in Defender inoculated with US11, late blight lesions were not detected, suggesting that *CHS* gene homologs or downstream genes for flavonoid synthesis may have been unaffected by inoculation of this cultivar by this isolate. The amount of *CHS* mRNA was previously reported to increase significantly in roots inoculated with zoospores of either an avirulent or virulent race of *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*) [38]. However, inoculation of bean with *Pseudomonas syringae* pv. *phaseolicola* (*Pph*) inhibited the activity of PAL, CHS and Chalcone isomerase (CHI) [39].

Expression levels of *HMGR*, *HMGR3*, *SQS* and *SC* were not affected in any of the tested treatments, except for *SC* transcripts which where down-regulated in RB + US8 and RB + GL + US8. This specific down-regulation of sesquiterpene cyclase in the treatments showing most symptoms would be clearly associated with a significant role of sesquiterpenoid compounds in potato defense to *P. infestans.* Sesquiterpene cyclase (*SC*) catalyzes the first steps in the branch leading to sesquiterpenoid phytoalexins, such as rishitin, lubimin and phytuberin, which have been previously linked to potato resistance against *P. infestans* [24,40,41]. A reduction in the accumulation of these phytoalexins in RB + US8 and RB + GL + US8 would be directly associated with their late blight disease. In fact, Wang et al. [10] showed that the highly aggressive *P. infestans* genotypes (US8) led to a reduced accumulation of rishitin at the inoculation site in potato leaves. The fact that sesquiterpenes are affected by highly aggressive isolates is not surprising or novel. However, knowing that sesquiterpene cyclase is directly affected is an important piece of information to initiate more specific investigation to study the mechanisms of such suppression, especially that we have observed differential activity between the two isolates and between the two cultivars.

Fig. 4 summarizes the effects of *P. infestans* US8 and US11 isolates on the expression of selected phenylpropanoid pathway genes from this study and downstream accumulation of flavonoids in Russet Burbank and Defender at 120 hpi from a previous time course study [35]. A down-regulation of chalcone synthase (*CHS*) in Russet Burbank inoculated with US8 and US11 compared with the control may be associated with the decrease in the concentration of rutin and other flavonoids. However, a flavanone's concentration increased in response to inoculation, and more in response to the highly than the weakly aggressive isolate. In addition, it was only detected in the susceptible potato cultivar Russet Burbank and not in the moderately potato cultivar Defender [35]. Therefore, Henriquez et al. [35] suggested its possible implication in the susceptibility of Russet Burbank to *P. infestans*. On the other hand, a flavonol-glycoside induced only in cultivar Defender was suggested to be associated with potato resistance to *P. infestans*. In turn, down-regulation of chalcone synthase (*CHS*) is probably involved in its reduced accumulation in Defender inoculated with US8 (DF + US8) where typical late blight lesions were present. An extensive list of potential genes suppressed or induced in potato by *P. infestans* was previously reported [42], and much still needs to be done to better understand the complex functions of these compounds in this pathosystem and their feed-back effects of defense pathways.

To our knowledge, no other reports have used whole plant systems (*in planta*) to integrate analyses of the effects of suppressors (glucans from *P. infestans*), elicitors (eicosapentaenoic acid, EPA) and of *P. infestans* on the differential expression of genes encoding key checkpoint enzymes from the phenylpropanoid and mevalonate (Ac-MVA) pathways in potato. Here we provide experimental evidence for one particular case, which presents more insight into potato mechanisms of disease resistance and complements a previous study on the accumulation of secondary metabolites [35]. The current findings infer that sustainable genetic resistance in potato against *P. infestans* will not be the result of isolated reactions against the pathogen, but rather a combination of factors recalling a polygenic, horizontal resistance. They also highlight the need to investigate molecular responses of potato to *P. infestans* in an integrative manner, not only of individual genes from the phenylpropanoid or mevalonate (Ac-MVA) pathways, but also other genes and their homologs. Such analysis should also assess the concurrent alteration of secondary metabolites' profiles in potato tissues after inoculation with *P. infestans*, and how they respond to regulation at the gene level, as previously reported for interaction with *Verticillium dahliae* [43].

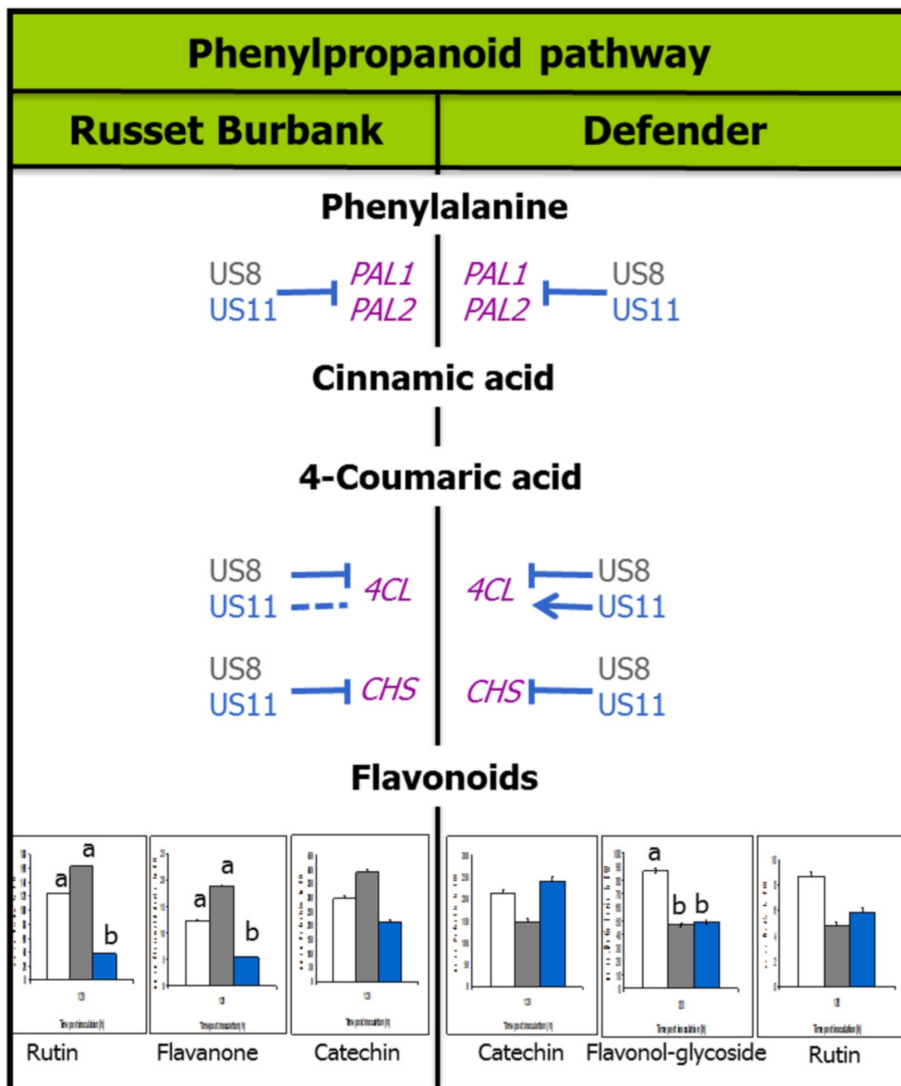

Fig. 4. Diagram representing the general effects of US8 and US11 isolates on the expression of selected phenylpropanoid pathway genes and on the accumulation of subsequent phenolics in potato cultivars Russet Burbank and Defender. ▭: H_2O control; ▬: potato inoculated with *P. infestans* US8; ▬: potato inoculated with *P. infestans* US11; ⊣: gene down-regulation; ←: gene up-regulation; - - -: no significant difference. Means with different letters are significantly different ($p < 0.05$).

4. Methods

4.1. Plant, fungal races and growth conditions

The highly susceptible cultivar "Russet Burbank" (RB) and the moderately resistant cultivar "Defender"(DF) were used. Plants were produced from high quality tubers and planted in clay pots containing soil–sand–peat–perlite mixture (4:4:4:1) and kept in a growth chamber at 20 ± 2 °C and 16 h photoperiod [44]. The *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive) and D1901 (lineage US8, A2 mating type, highly aggressive) were grown on rye agar medium supplemented with 2% sucrose at 18 °C [9].

4.2. Extraction and purification of glucans from P. infestans cell walls

Mycelium from *P. infestans* (4–5 g) was homogenized in 500 mM potassium phosphate buffer pH 7.2 and filtered through glass fiber filters (Millipore type 1, Sigma). The solid residue was washed with 1:1 $CHCl_3$–MeOH and dehydrated with acetone. The dried cell walls (2–3 g) were incubated with protease from *Streptomyces griseus* (Sigma) 50 μg/ml in Tris–HCl, 10 mM, pH 7.2 at 35 °C for 45 min. The incubation mixture was centrifuged and the solid residue remaining after protease treatment was resuspended in distilled water (100 ml/g) and autoclaved at 120 °C for 3 h. The autoclaved suspension was centrifuged, the supernatant filtered and dialysed against water. Purification of the glucan was carried out as described by Andreu et al. [13].

4.3. Experimental design

A full 2 × 3 × 4 factorial design, completely randomized and replicated, was used. This 2 × 3 × 4 factorial arrangement consists of the following treatments: two cultivars: Russet Burbank and Defender; three inoculums: H_2O (control), *P. infestans* strains D1901 and D-03; and four effectors: eicosapentaenoic acid (EPA), Glucans (GL), EPA + GL, and H_2O. Twenty-four treatment combinations were used in total (Table 1). The primary leaflet of the fourth fully-grown potato leaf in each stem of 8-wk-old Russet Burbank and Defender plants, were treated as follows: 1) 100 μl of 0.2 μM EPA

applied on the leaflet (as multiple tiny droplets using a micropipette to prevent run-off), 2) 100 μl of glucans from race C (race 1,4,10,11; Mating type A2) (200 μg in 100 μl of water as emulsion), 6 h after EPA treatment, and 3) Ten 10-μl-droplets of *P. infestans* spore suspension (4×10^4 sporangia/ml) 12 h after EPA application. Control plants were treated with water. The plants were placed in a moist chamber (20 ± 2 °C, 16 photoperiod, 100% relative humidity) for 48 h, and then in a growth chamber at 20°C ± 2 and 16 h photoperiod. We used three replicates per treatment.

4.4. RT-PCR analysis and sequence analysis

Five micrograms of total RNA extracted with Trizol (Invitrogen) from three replicates of each treatment (Table 1) were used to obtain the first-strand synthesis following the M-MLV (RT) enzyme (Invitrogen) manufacturer's recommendations. The RT-PCR was performed in 25 μl reaction volumes containing 1 μl of the first-strand synthesis pool from each treatment, 1X Buffer (500 mM KCl, 100 mM Tris–HCl, 1% Triton), 0.1 mM of dNTP mix, 2 mM of $MgCl_2$, 0.25 μM of each primer and 1 unit of Taq DNA polymerase (Invitrogen) in a thermocycler programmed for 35 cycles of 30 s at 94 °C, 30 s at 55 °C for all the primers, with the exception of *SQS* which has annealing temperature of 45 °C, and 60 s at 72 °C, followed by a final extension for 10 min at 72 °C. The sequences of primers used are listed in Table 2. The potato elongation factor gene was included in the RT-PCR assay as a constitutively expressed internal control. In addition, to verify the correct amplification of each gene, the PCR product from each one was isolated with the Qiaex II gel-extraction kit (Qiagen Inc., Alameda, CA, USA) following the manufacturer's instructions. The isolated fragments were cloned into the bacterial plasmid pGEM-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's instructions. The plasmids were then transformed into *Escherichia coli* DH5α, sequenced and analyzed with Seqman within the DNAStar program (DNAStar, Madison, WI, USA). Sequences were examined with Seqman within the DNAStar program (DNAStar, Madison, WI, USA) and analyzed using BLASTn algorithm in GenBank.

The PCR products were resolved by electrophoresis on ethidium bromide stained gels, photographed utilizing an Alpha Imager (Alpha Innotech Corporation, San Leandro, Calif.), and the intensity of the bands was evaluated using the densitometry function and numerically expressed as the relative density in comparison to the optical density of the background. Furthermore, all results were normalized to the expression of the elongation factor housekeeping gene. Each sample was assayed in triplicate.

4.5. qRT-PCR and data analyses

Five micrograms of total RNA from three replicates of each treatment (Table 1) were used to obtain the first-strand synthesis following the M-MLV (RT) enzyme (Invitrogen) manufacturer's recommendations. Quantitative Real time PCR (qRT-PCR) was performed using the IQ SYB Green Supermix (Biorad), in 20 μl containing 2 μl diluted template, 6.5 μL of IQ SYB Green Supermix, and 0.375 μM of each primer. Phenylpropanoid primers used for qRT-PCR are shown in Table 2. Amplicons from each primer pair were cloned and sequenced to confirm primer specificity. Gene expression was quantified using a Stratagene Mx3005p cycler, and the following qPCR cycling program was used for all sets of primers: The thermocycle program included 95 °C (2 min), followed by 40 cycles of 95 °C (15 s), 50 °C (45 s) and 72 °C (45 s). Melt-curve analysis was performed to observe primer–dimer formation and to check amplification of gene-specific products. All PCR reactions were performed from triplicate biological samples. The 2-ΔΔC(T) method [45] was used to calculate the fold expression relative to the control inoculated with water. The reference gene elongation factor was used to normalize small differences in template amounts.

4.6. Statistical analysis

All statistical analyses were performed with the Statistical Analysis Software (SAS) (SAS Institute, Cary, NC; release 9.1 for Windows). Prior to analysis, expression levels of phenylalanine ammonia lyase *PAL-1* and *PAL-2*, 4-coumarate: coenzyme A ligase (*4CL*) and chalcone synthase (*CHS*) gene data sets were checked for normality (PROC Univariate). Thus, data were normalized by log +0.5 transformations for analysis. Data collected from the $2 \times 3 \times 4$ factorial arrangement with two cultivars, three levels of inoculums and four levels of effectors were analyzed by analysis of variance (ANOVA-PROC GLM). Comparisons between grouped treatments within cultivars were made when a significant *F*-test ($p < 0.05$) for the cultivars * inoculum, cultivars * effector or cultivars * inoculum * effector interactions were found (Table 3). One-way ANOVA analysis for each individual group was performed using PROC GLM. Treatment means were separated using the Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

Acknowledgments

This work was financially supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Manitoba Agri-Food Research Development Initiative program to F. Daayf.

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