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ORIGINAL PAPER

Gene expression of serine and cysteine proteinase inhibitors during cereal leaf beetle larvae feeding on wheat: the role of insect-associated microorganisms

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Abstract
Plants and insects have been coexisting for more than 350 million years. During this time, both have evolved many strategies to successfully exploit or respond to reciprocal adaptation and defense reactions. Plants tend to minimize the damage caused by pest feeding, while pests tend to manipulate plant response by suppressing plant defense mechanisms or developing strategies to overcome plant defense systems. Plants recognize insect pests by the wounding that they cause and elicitors present in pest oral secretions (saliva and/or regurgitant). These elicitors or insect-associated microorganisms can modulate plant response to the benefit of their insect hosts. In this article, we have undertaken an analysis of gene expression in serine and cysteine proteinase inhibitors (*SerPI* and *CysPI*, respectively) in wheat (*Triticum aestivum*) plants exposed to cereal leaf beetle (CLB, *Oulema melanopus*, Coleoptera, Chrysomelidae) larvae feeding, and the impact of microbes associated with CLB on the expression levels of these genes. Using three wheat varieties and antibiotic-treated and untreated CLB larvae, we found that SerPI plays a more important role than CysPIs in plant defense against CLB larvae. Additionally, higher levels of *SerPI* gene expression were observed in systemic leaves in comparison to the wounded leaves (local response). Each of the tested wheat varieties reacted in a specific way to the particular treatment. Moreover, the presence of microbial components associated with insects influenced plant response to CLB larvae feeding.

Keywords Coleoptera · Pest feeding · Plant defense · Plant–insect–microbe interaction · Serine proteinase inhibitor · Gene expression · Plant response · Wheat

Introduction

Wheat (*Triticum aestivum*) is among the most common and widely consumed cereal in the world (Philips et al. 2011). However, it is also exposed to many insect pests, including the cereal leaf beetle (CLB, *Oulema melanopus*, Coleoptera, Chrysomelidae). Both the beetles and the larvae of this pest damage leaves of various cereals (oats, barley, rye, corn), but their favorite plant host is wheat (Bieńkowski 2010). Larvae are considered as the most damaging stage of CLB (Groll and Wetzel 1984), their feeding can lead to significant yield and quality reduction and thus to considerable economic losses (Dimitrijević et al. 2001).

Plants are continuously improving or developing new defense strategies against pests. Among the best-known of such defense responses is the production of proteins such as plant proteinase inhibitors (PIs) that have toxic, repellent or/and anti-nutritional effects on the herbivorous insects (Rani and Jyothsna 2010; War et al. 2011a, b). PIs belong to the sixth group of plant pathogenesis-related (PR) proteins and represent one of the most abundant defensive classes of proteins in plants. The amount of PR-6 significantly increases in response to wounding (Sharma 2015). Insect digestion of plant proteins may be disturbed by PIs, because PIs bind to insect digestive proteases in the insect gut (Oliveira et al. 2007; Gomes et al. 2005), resulting in a shortage of amino

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acids, slow development and/or starvation of insect pests (Azzouz et al. 2005).

On the other hand, insects are not passive, and tend to manipulate the host response by suppressing plant defense mechanisms or developing new strategies to overcome defense systems. For instance, insects responded to the activity of plant PIs by evolving adaptations to reduce their harmful effects, including increased activity of digestive enzymes, decreased production of inhibitor-sensitive enzymes (Brunelle et al. 2004), digestion of plant PIs (Girard et al. 1998b), decreased sensitivity of proteases to PIs (Brito et al. 2001) and synthesis of more PI-resistant enzymes (Paulillo et al. 2000). In the case of CLB larvae, such activity was found for four classes of proteases [cysteine, serine (trypsin-, chymotrypsin-like), aspartyl (cathepsin D) proteases and metalloproteases] (Wielkopolan et al. 2015). In response to synthetic serine PI [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)] feeding, two additional proteases were observed (Wielkopolan et al. 2015). Additionally, insects may use proteases of endosymbiotic bacteria inhabiting their gut (Shao et al. 2012; Chu et al. 2013; Wielkopolan and Obrępalska-Stęplowska 2016). Microbiota inhabiting the insect gut can regulate or contribute to digestive enzyme activity of their insect hosts. For instance, Chu et al. (2013) demonstrated that microbiota associated with a 'rotation–resistant' variant of western corn rootworm (RR-WCR, *Diabrotica virgifera virgifera*, Coleoptera, Chrysomelidae) contribute to the proteolysis and survival of this pest on soybean (digestive adaptation to soybean CysPIs). Thus, insect digestive enzyme profiles may undergo changes in response to plant PIs, along with the contribution of associated microorganisms.

It is believed that a specific plant response is activated by various elicitors present in insect oral secretion (saliva and/or regurgitant) that have direct contact with macerate of plant tissue and can interfere with plant cell response. The plant defense induced by various elicitors may differ. The effect is the result of the action of not only insect pests and plant species/variety, but also of microbes associated with them (commensal, symbiotic and pathogenic microorganisms) (Zhu et al. 2014; Wielkopolan and Obrępalska-Stęplowska 2016). For example, Chung et al. (2013) reported that some bacteria (microbial symbionts) from the secretion of larvae of the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae) and one of the bacterial effectors (flagellin from *Pseudomonas* sp.) interfered with plant (tomato, *Solanum lycopersicum*)–insect interaction. Bacteria and flagellin suppressed production of jasmonic acid (JA) and JA-responsive anti-herbivore defenses, and induced salicylic acid (SA)-regulated defenses. Plants recognized an insect attack as a microbial threat that resulted in changes in the expression of a specific set of defense-related genes. Our preliminary studies have shown that imago of CLB is associated with endosymbiotic bacteria *Wolbachia* and *Rickettsia* (data not shown). These bacteria occur widely among insect host populations (Toju and Fukatsu 2011). *Wolbachia* is known to be involved in modulation of plant defense response to insect feeding (Chung et al. 2013; Barr et al. 2010).

In this study, we have undertaken the study of wheat response induced by CLB larva feeding, with the aim of analyzing gene expression of serine and cysteine proteinase inhibitors (*SerPI* and *CysPIs*, respectively) in three wheat varieties in wounded and systemic leaves. We use a real-time PCR (RT-qPCR) approach to elucidate whether the elimination of microorganisms associated with CLB larvae through treatment of the insects with antibiotics will change the plant response to their feeding. We demonstrated that *SerPI* gene expression is considerably up-regulated in plants wounded by CLB larva, more so than gene expression levels of *CysPIs*, especially in systemic leaves. However, the plant response is dependent on the wheat variety. Importantly, a reduction in insect-associated microorganisms in CLB larvae frequently led to changes in the levels of the studied gene expression in challenged plants. This confirms the modifying role of microbial components of insects in shaping plant defense against insect feeding.

Materials and methods

Plant materials

Three varieties of wheat (seeds obtained from DANKO) were used: Arabella (spring wheat), Banderola and Arkadia (winter wheat). All plants were grown in a greenhouse (Research Centre of Quarantine, Invasive and Genetically Modified Organisms of Institute of Plant Protection – National Research Institute, Poznan) with a photoperiod of 16L:8D, 40% humidity, temperature 17 °C day/12 °C night. Forty-five-day-old plants were used for the experiments, which included the following: (a) control (untreated) plants, (b) plants wounded by larvae treated with antibiotic cocktail (AB + Triton X-100), (c) plants wounded by larvae not treated with AB (water + Triton X-100) and (d) mechanically wounded plants treated with water with the addition of Triton X-100. Foliar material (wounded and systemic leaves obtained from each plant) was collected at three time points (12, 24 and 48 h after treatment). Experiments were carried out in five biological replicates. All samples were frozen in liquid nitrogen and stored at − 80 °C until used.

Larvae treatment with antibiotics

To test the effect of insect-associated microbes of CLB larvae, the presence of microbes in oral secretion was reduced

using an AB-cocktail, which consisted of 50 ml of cocktail streptomycin sulfate salt (0.00034 g, Sigma-Aldrich), chlortetracycline (0.05 g, Sigma-Aldrich), sorbic acid (0.03 g, Sigma-Aldrich) and methyl paraben (0.04 g, Sigma-Aldrich), with the addition of Triton X-100 (0.01%). The CLB larvae (collected from a wheat field in Słupia Wielka, Greater Poland district, Poland) were fed for 2 days on a sterilized piece of wheat leaf covered by the AB-cocktail. Control larvae received a piece of sterilized leaf covered with sterile water with the addition of Triton X-100. One larva was placed in a Petri dish containing one leaflet (AB- or water-treated) on top of a layer of 1% agar to maintain moisture. Leaves were freshly prepared daily.

Herbivore treatment

To investigate the effect of CLB-associated microbes, one AB-treated or untreated larva was placed on the second leaf (counting from the top). Larvae were allowed to feed for 12 h, after which the insects were collected from the plants. Undamaged plants were used as controls.

Total RNA extraction and cDNA synthesis

Each leaf was ground in a mortar using liquid nitrogen, then total RNA from samples was extracted using Tri Reagent solution (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. The RNA concentration of each sample was measured using Nano-Drop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed by 1% agarose gel electrophoresis through visualization of the ribosomal subunit. Genomic DNA was digested and removed from the extracted total RNA. Briefly, 3 µg of RNA were mixed with DNase buffer (3 µl), DNase (1 U/µl; DNase I RNase free + buffer DNase I + $MgCl_2$, Thermo Fisher Scientific) and appropriate volume of water to a final volume of 30 µl. Samples were incubated for 30 min at 37 °C, followed by the addition of lithium chloride (2.5 M) and overnight RNA precipitation. Samples were then centrifuged (12,000×*g*, 10 min, 4 °C), and the obtained pellet was washed with ethanol. After additional centrifugation (12,000×*g*, 10 min, 4 °C), the pellet was dried and suspended in 10 µl of RNase-free water, and 500 ng RNA was analyzed by 1% agarose gel electrophoresis to confirm sample integrity.

Purified RNA (500 ng) was mixed with 50 ng random hexamers (Thermo Fisher Scientific), heated at 65 °C for 5 min and rapidly cooled for 2 min on ice. Next, a reverse transcription mixture (1× buffer, 1 µl dNTPs, 200 U/µl of RevertAid Reverse Transcriptase; Thermo Fisher Scientific) was added to each sample. Samples were incubated at 25 °C for 10 min, and then at 42 °C for 60 min. The reaction was terminated at 75 °C for 5 min. The cDNA samples (10 µl) were diluted with 5 µl DNase-free water and stored at − 20 °C until use for RT-qPCR.

Primer design, PCR and RT-qPCR

Primers used in this study were designed on the basis of the wheat gene sequences available in the GenBank database (National Center for Biotechnology Information) for actin (*ACT*, accession no. AB181991.1), *CysPIs* (accession nos. AB038392.1, AB038394.1, FJ545271.1 belonging to cystatins), and *SerPI* [accession no. AY549888.2, from Bowman–Birk family (BBI)] (Table 1). The designed primers were checked using the OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc., Skokie, IL, USA). Protease inhibitors were chosen on the basis of previous mass spectrometry analysis of protease inhibitors accumulating in wheat upon wounding and larvae feeding and in untreated plants (data not shown; only *CysPI* [no. AB038392.1] due to the high identity of the core gene nt sequence to the other identified *CysPIs* was additionally chosen). In this study, four reference genes including *ACT*, 18S rRNA, *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) and *ARF* (ADP ribosylation factor) from *T. aestivum* were tested for their gene expression stability under CLB treatment as described previously (Wrzesińska et al. 2016). Based on the delta CT method, *ACT* was indicated as the gene with the most stable expression for the three tested varieties of wheat (data not shown) and used for normalization.

Table 1 Primer sequences used for RT-qPCR analysis

Gene	Accession number	Primer sequence (5′–3′)	Amplicon length [bp]	Annealing temperature [°C]
ACT	AB181991.1	F:CTCTATTTTGGCCTCTCTTAGCAC R:GACCAGACTCATCGTACTCCG	71	60
CysPI	AB038392.1 AB038394.1 FJ545271.1	F: CTGCTGGAGTTCGAGAATG R: CACACCTTAGCTTCATAGAG	119	60
SerPI	AY549888.2	F:CACTACCACAGAGCATTCTAC R:GTGCTCTTCATGCTTGCTGATG	91	60

The PCR reaction was performed in a 10 µl mixture consisting of 1 µl AllegroTaq reaction buffer with 2.5 mM Mg^{2+} (Novazym, Poznan, Poland), 0.5 µM forward and reverse primers (Table 1), 200 µM dNTPs, 1 U AllegroTag DNA polymerase (Novazym), and 1 µl cDNA obtained in the previous step, using the Biometra TProfessional Basic Thermocycler. The PCR conditions for all the primer pairs were as follows: 94 °C for 3 min, then 35 cycles of 25 s at 94 °C, 20 s at 55 °C, 72 °C for 25 s and a final elongation step at 72 °C for 5 min. The remainder of the procedure, i.e. product separation in gel, purification, cloning in vector, plasmid isolation and *Eco*RI digestion, was performed as previously described (Wrzesińska et al. 2016). DNA inserts were sequenced by Genomed S.A. (Warsaw, Poland). Sequencing data were analyzed using the BioEdit Sequence Alignment Editor 7.1.11 (Hall 1999).

The RT-qPCR was performed using the Mx3500P thermal cycler (Agilent Technologies, Santa Clara, CA, USA). The reaction was performed in a 10 µl solution containing 1x iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 0.5 µM forward and reverse primers (except for the reaction mix for *CysPIs*, where 0.125 µM was used; Table 1), and 1 µl of diluted cDNA. The thermal reaction profile for all the primer pairs consisted of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, and an annealing step at 60 °C for 30 s. Dissociation curves were generated from 65 to 95 °C to confirm the specificity of each primer pair. For each tested sample (five biological replicates), reactions were performed in triplicate (technical replicates). The amplification efficiency for each gene was validated by running RT-qPCR with serial dilutions of cDNA. Non-template controls were included to verify the absence of contamination. The expression levels of the studied genes were normalized to the level of the housekeeping gene *ACT*. The data obtained from the RT-qPCR were analyzed using the Relative Expression Software Tool (REST; 2009 V2.0.13).

Results

Contribution of CysPI and SerPI in plant response to mechanical wounding and CLB larvae feeding

Analysis of the wheat *SerPI* and *CysPIs* gene expression in wounded (local response) and systemic leaves revealed a stronger increase in *SerPI* gene expression upon mechanical wounding or CLB larvae feeding. Only in the case of the Arabella variety were changes in gene expression levels comparable between the tested PIs (plants wounded mechanically and by larval feeding). Also, for each tested wheat variety, higher up-regulation of *SerPI* than *CysPIs* gene expression was observed in systemic leaves (Fig. 1b, d, f). Additionally, higher increase in *SerPI* gene expression occurred locally in two wheat varieties, Arkadia and Banderola (Fig. 1c, e). Notably, increased gene expression in the systemic leaves of all tested plants was higher in response to mechanical wounding than to damage by CLB larvae feeding (Fig. 1b, d, f).

Plant response in wounded (local response) and systemic leaves

Plant response to insect feeding occurs not only at or near the site of damage, but also in other parts of plants. Overall, it was observed that the stronger plant response associated with PIs expression was located in systemic leaves, but this may be dependent on wheat variety.

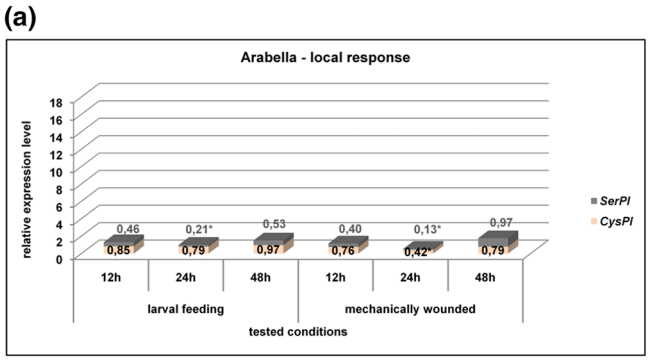
In the case of the *CysPI* genes, the change in expression level was generally comparable between wounded and systemic leaves. Moreover, in most cases, a decrease in *CysPI* gene expression was observed upon damage by both CLB larvae feeding and mechanical wounding in comparison to untreated control plants (Fig. 2a). In contrast, changes in expression levels of the *SerPI* gene were variety-specific. In two varieties (Arabella and Arkadia), stronger (or earlier) up-regulation of *SerPI* gene expression was observed systemically, whereas it was observed locally in one variety (Banderola; Fig. 2b).

Reduction of microorganisms associated with CLB larvae affects the gene expression levels of *CysPI* and *SerPI*

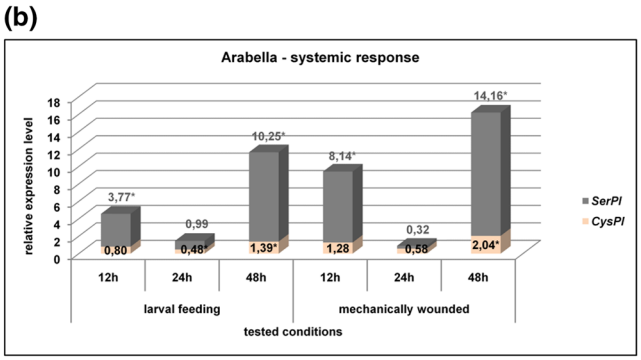
Treating the larvae with AB-cocktail (to reduce microbial components associated with CLB) and exposing plants to feeding by such larvae showed that there were differences in plant response to such insect attacks compared to CLB larvae not treated with AB-cocktail.

In the case of *CysPIs* in wheat, where the decrease in gene expression occurred mostly upon larvae feeding, the changes in *CysPI* genes expression levels in plants wounded by AB-treated or AB-untreated CLB larvae feeding were comparable (Fig. 3a). This indicates that the microbial component associated with CLB larvae has a minor effect on plant response associated with *CysPIs* expression.

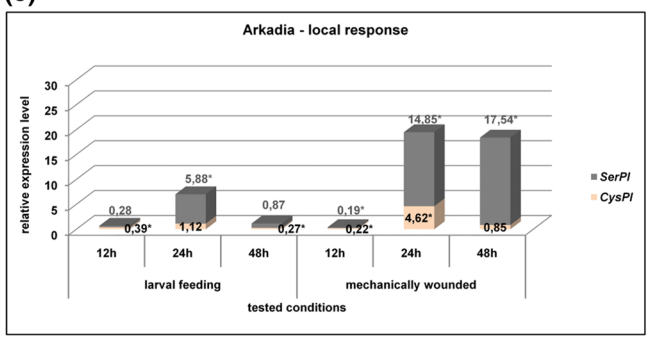
However, gene expression of *SerPI* in systemic leaves of all wheat varieties was clearly higher in plants exposed to CLB larvae in which insect-associated microbe content was reduced. This effect was also visible locally in Banderola and 48 h after treatment in the Arabella variety, but to a lesser extent than it was observed systemically (Fig. 3b). A comparison of gene expression levels of *SerPI* and *CysPIs* between plants damaged by AB-untreated and AB-treated CLB larvae shows statistically



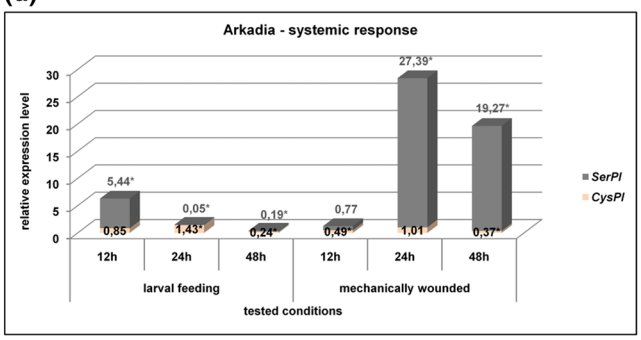
Arabella - local response		larval feeding			mechanically wounded		
time		12h	24h	48h	12h	24h	48h
SerPI	p	0,055	**0,003***	0,062	0,067	**0,004***	0,922
	SE	0,225-0,986	0,081-0,408	0,259-0,996	0,187-0,930	0,043-0,354	0,520-1,808
CysPI	p	0,335	0,279	0,918	0,291	**0,014***	0,294
	SE	0,508-1,074	0,564-1,240	0,672-1,507	0,481-1,157	0,288-0,664	0,546-1,206



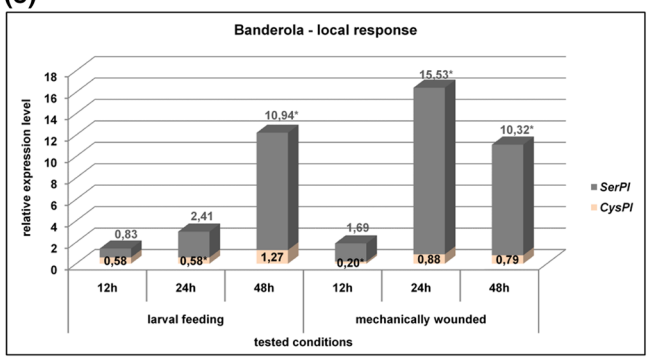
Arabella - systemic response		larval feeding			mechanically wounded		
time		12h	24h	48h	12h	24h	48h
SerPI	p	**0,033***	0,966	**0,001***	**0,04***	0,067	**0***
	SE	1,222-12,670	0,289-3,010	3,641-36,787	1,857-45,931	0,097-1,008	4,384-41,196
CysPI	p	0,309	**0,038***	**0***	0,477	0,098	**0,001***
	SE	0,522-1,140	0,260-0,885	1,105-1,695	0,680-3,279	0,299-1,233	1,680-2,501



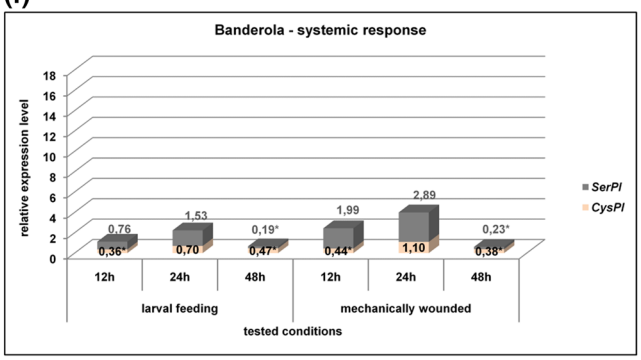
Arkadia - local response		larval feeding			mechanically wounded		
time		12h	24h	48h	12h	24h	48h
SerPI	p	0,105	**0,014***	0,822	**0,028***	**0,008***	**0,009***
	SE	0,065-1,146	2,116-15,238	0,211-3,943	0,059-0,626	7,982-27,024	3,364-60,807
CysPI	p	**0,009***	0,749	**0,005***	**0,005***	**0,005***	0,761
	SE	0,202-0,714	0,617-2,088	0,057-0,661	0,159-0,315	2,136-10,657	0,294-1,944



Arkadia - systemic response		larval feeding			mechanically wounded		
time		12h	24h	48h	12h	24h	48h
SerPI	p	**0,021***	**0,001***	**0,008***	0,555	**0,001***	**0,009***
	SE	2,288-17,574	0,021-0,177	0,080-0,571	0,255-1,945	9,761-89,237	9,490-61,534
CysPI	p	0,584	**0,037***	**0***	**0,006***	0,971	**0***
	SE	0,472-1,658	0,997-2,284	0,080-0,635	0,350-0,688	0,719-1,486	0,232-0,567



Banderola - local response		larval feeding			mechanically wounded		
time		12h	24h	48h	12h	24h	48h
SerPI	p	0,437	0,173	**0,007***	0,155	**0***	**0,005***
	SE	0,509-1,431	0,494-6,663	3,851-25,565	0,946-3,335	9,005-26,340	3,738-29,850
CysPI	p	0,095	**0,001***	0,083	**0,036***	0,374	0,147
	SE	0,359-1,209	0,471-0,716	0,974-1,713	0,039-0,744	0,678-1,119	0,609-0,969



Banderola - systemic response		larval feeding			mechanically wounded		
time		12h	24h	48h	12h	24h	48h
SerPI	p	0,7	0,611	**0,003***	0,208	0,099	**0,038***
	SE	0,206-2,655	0,322-7,655	0,089-0,403	0,840-5,249	0,935-7,954	0,062-0,975
CysPI	p	**0,003***	0,14	**0,009***	**0,007***	0,688	**0,01***
	SE	0,232-0,508	0,464-1,137	0,323-0,700	0,315-0,645	0,738-1,895	0,233-0,691

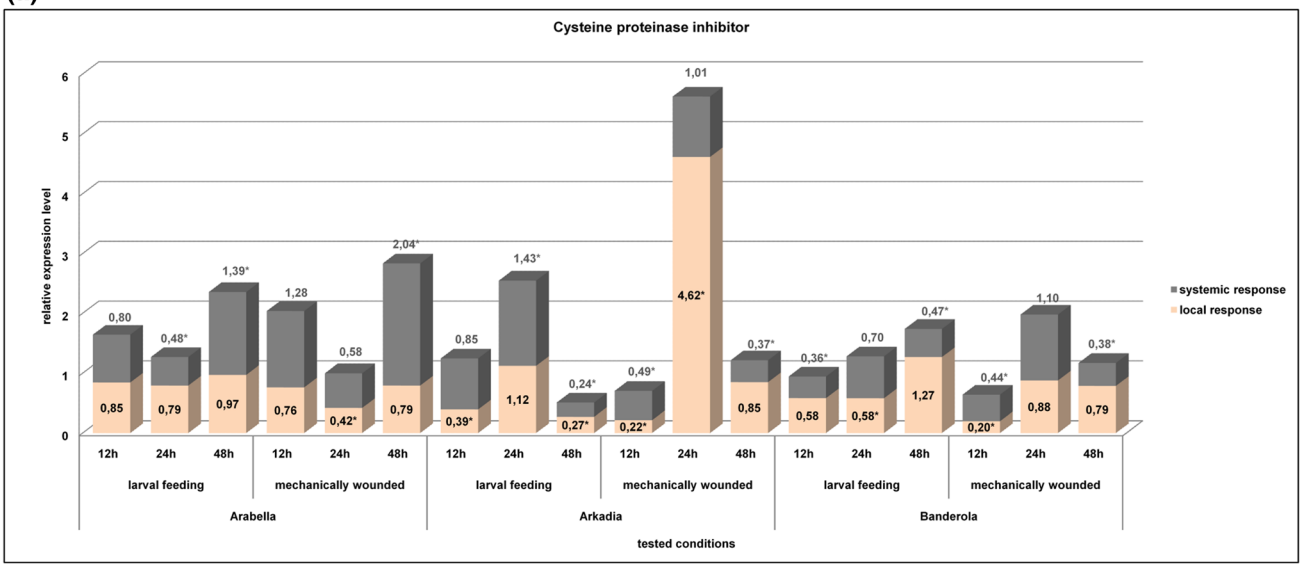
Fig. 1 Analysis of relative expression levels of cysteine proteinase inhibitors (*CysPIs*, **a, c, e**) and serine proteinase inhibitor (*SerPI*, **b, d, f**) genes in plants challenged with cereal leaf beetle larvae or mechanically wounded for three wheat varieties [Arabella (**a, b**), Arkadia (**c, d**), Banderola (**e, f**)]. The tables contain the data on the *p*-value and standard error (SE) for each treatment. Asterisks indicate statistically significant results in comparison to the control (undamaged plants, value 1; $p < 0.05$)

significant up-regulation of expression of these genes in plants wounded by CLB with a reduced microbial component (Fig. 4).

Discussion

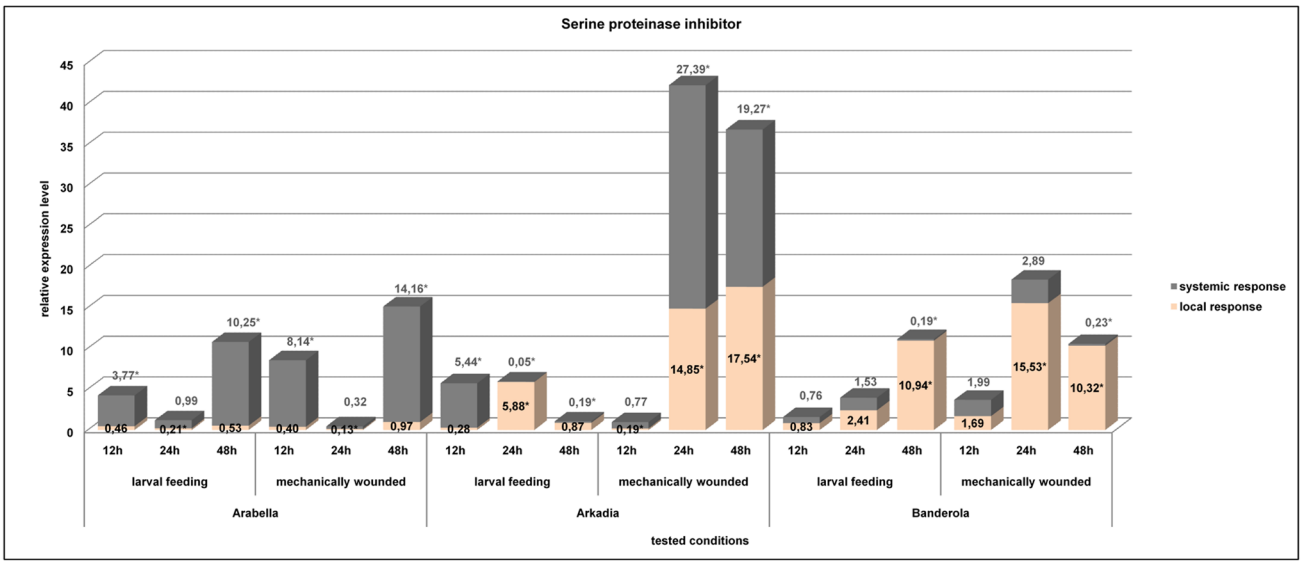
Plants develop a wide range of strategies for defense against insect attacks and their effects, including specialized morphological structures (e.g. hairs, trichomes, thorns, spines),

(a)



CysPI		Arabella						Arkadia						Banderola					
treatment		larval feeding			mechanically wounded			larval feeding			mechanically wounded			larval feeding			mechanically wounded		
time		12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h
systemic response	P	0,309	**0,038***	**0***	0,477	0,098	**0,001***	0,584	**0,037***	**0***	**0,006***	0,971	**0***	**0,003***	0,14	**0,009***	**0,007***	0,688	**0,01***
	SE	0,522-1,140	0,260-0,885	1,105-1,695	0,680-3,279	0,299-1,233	1,680-2,501	0,472-1,658	0,997-2,284	0,080-0,635	0,350-0,688	0,719-1,486	0,232-0,567	0,232-0,508	0,464-1,137	0,323-0,700	0,315-0,645	0,738-1,895	0,233-0,691
local response	p	0,335	0,279	0,918	0,291	**0,014***	0,294	**0,009***	0,749	**0,005***	**0,005***	**0,005***	0,761	0,095	**0,001***	0,083	**0,036***	0,374	0,147
	SE	0,508-1,074	0,564-1,240	0,672-1,507	0,481-1,157	0,288-0,664	0,546-1,206	0,202-0,714	0,617-2,088	0,057-0,661	0,159-0,315	2,136-10,657	0,294-1,944	0,359-1,209	0,471-0,716	0,974-1,713	0,039-0,744	0,678-1,119	0,609-0,969

(b)



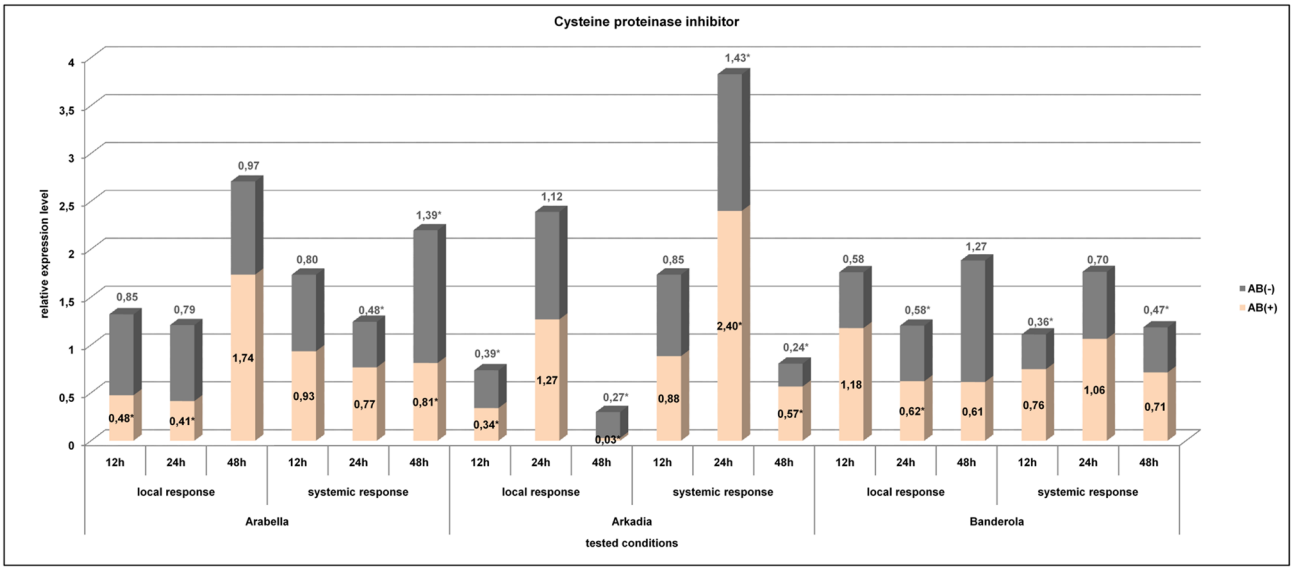
SerPI		Arabella						Arkadia						Banderola					
treatment		larval feeding			mechanically wounded			larval feeding			mechanically wounded			larval feeding			mechanically wounded		
time		12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h
systemic response	p	**0,033***	0,966	**0,001***	**0,04***	0,067	**0***	**0,021***	**0,001***	**0,008***	0,555	**0,001***	**0,009***	0,7	0,611	**0,003***	0,208	0,099	**0,038***
	SE	1,222-12,670	0,289-3,010	3,641-36,787	1,857-45,931	0,097-1,008	4,384-41,196	2,288-17,574	0,021-0,177	0,080-0,571	0,255-1,945	9,761-89,237	9,490-61,534	0,206 -2,655	0,322-7,655	0,089-0,403	0,840-5,249	0,935-7,954	0,036-0,975
local response	p	0,055	**0,003***	0,062	0,067	**0,004***	0,922	0,105	**0,014***	0,822	**0,028***	**0,008***	**0,009***	0,437	0,173	**0,007***	0,155	**0***	**0,005***
	SE	0,225-0,986	0,081-0,408	0,259-0,996	0,187-0,930	0,043-0,354	0,520-1,808	0,065-1,146	2,116-15,238	0,211-3,943	0,059-0,626	7,982-27,024	3,364-60,807	0,509-1,431	0,494-6,663	3,851-25,565	0,946-3,335	9,005-26,340	3,738-29,850

Fig. 2 Analysis of relative gene expression levels of cysteine proteinase inhibitors (**a**) and serine proteinase inhibitor (**b**) genes in wheat plants wounded mechanically or by cereal leaf beetle larvae feeding in wounded (local response) and systemic leaves. Three wheat varieties (Arabella, Arkadia, Banderola) were tested. The tables contain the data on the *p* value and the standard error (SE) for each treatment. Asterisks indicate statistically significant results in comparison to the control (undamaged plants, value 1; $p < 0.05$)

production of secondary metabolites (e.g. alkaloids, terpenoids, phenols) (Hanley et al. 2007), the emission of volatiles (attraction of insect natural enemies, induction of defense in neighboring plants), or synthesis of compounds considered as natural insecticides (e.g. PIs) (Wielkopolan and Obrępalska-Stęplowska 2016).

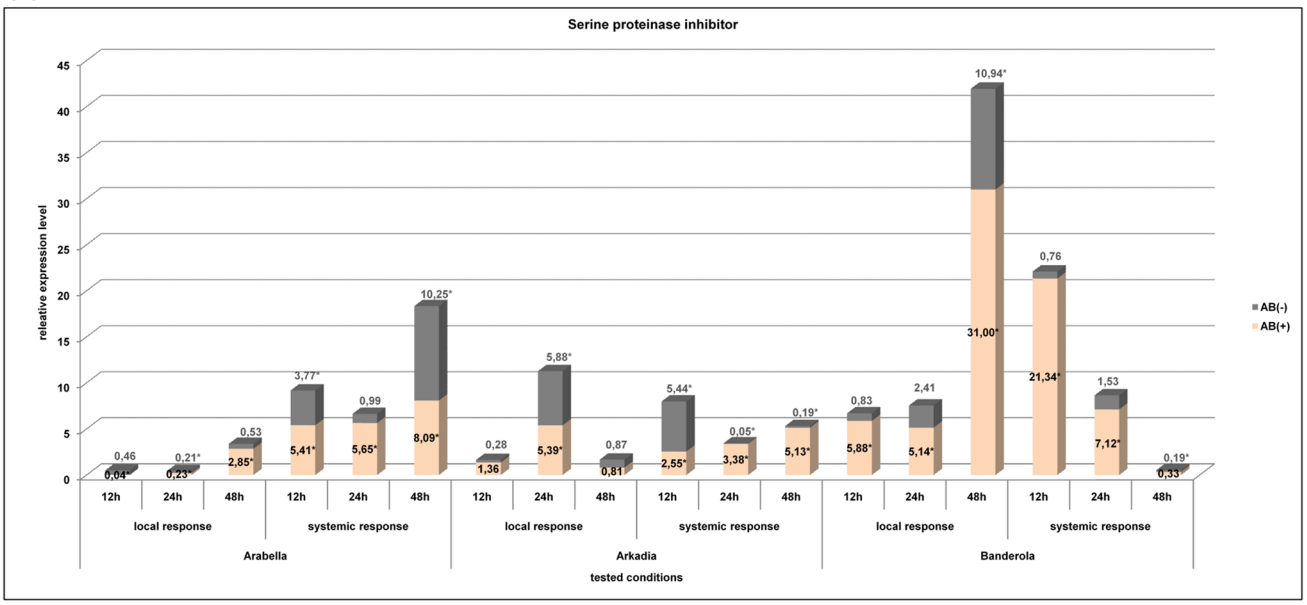
Plant PIs often occur in plant tissue in quite high concentrations (Murdock and Shade 2002; Menon and Rao 2012)

(a)



CysPI		Arabella						Arkadia						Banderola					
treatment		local response			systemic response			local response			systemic response			local response			systemic response		
time		12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h
AB(-)	p	0,335	0,279	0,918	0,309	**0,038***	**0***	**0,009***	0,749	**0,005***	0,584	**0,037***	**0***	0,095	**0,001***	0,083	**0,003***	0,14	**0,009***
	SE	0,508 - 1,074	0,564-1,240	0,672-1,507	0,522-1,140	0,260-0,885	1,105 -1,695	0,202-0,714	0,617 -2,088	0,057 -0,661	0,472-1,658	0,997-2,284	0,080 -0,635	0,359-1,209	0,471-0,716	0,974-1,713	0,232-0,508	0,464-1,137	0,323-0,700
AB(+)	p	**0,019***	**0,008***	0,086	0,809	0,34	**0,05***	**0,01***	0,498	**0,041***	0,511	**0,027***	**0,027***	0,716	**0,012***	0,487	0,331	0,833	0,23
	SE	0,273 - 0,865	0,262-0,603	0,982-3,337	0,613-1,521	0,403-1,610	0,681-0,982	0,091-0,676	0,670-2,515	0,002-0,887	0,551-1,451	1,041-4,185	0,367-0,844	0,713-2,388	0,406-0,862	0,142-2,203	0,437-1,167	0,527-1,885	0,430-1,236

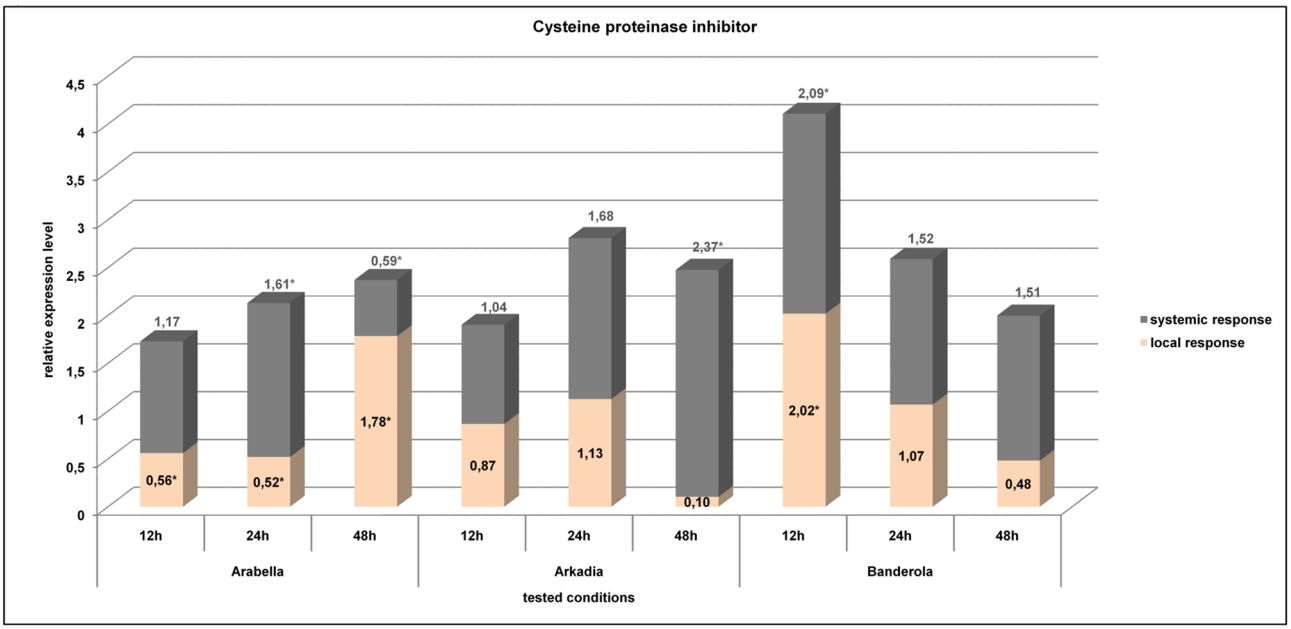
(b)



SerPI		Arabella						Arkadia						Banderola					
treatment		local response			systemic response			local response			systemic response			local response			systemic response		
time		12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h
AB(-)	p	0,055	**0,003***	0,062	**0,033***	0,966	**0,001***	0,105	**0,014***	0,822	**0,021***	**0,001***	**0,008***	0,437	0,173	**0,007***	0,7	0,611	**0,003***
	SE	0,225-0,986	0,081-0,408	0,259-0,996	1,222-12,670	0,289-3,010	3,641-36,787	0,065-1,146	2,116-15,238	0,211-3,943	2,288-17,574	0,021-0,177	0,080-0,571	0,509-1,431	0,494-6,663	3,851-25,565	0,206-2,655	0,322-7,655	0,089-0,403
AB(+)	p	**0,009***	**0,034***	**0,038***	**0,018***	**0,016***	**0***	0,64	**0,008***	0,726	**0,048***	**0,001***	**0,017***	**0***	**0***	**0,004***	**0,005***	**0,008***	0,112
	SE	0,011-0,115	0,091-0,538	1,197-7,084	1,950-20,225	1,615-15,077	2,444-22,861	0,601-6,047	2,411-10,821	0,243-2,132	1,038-5,308	1,496-7,765	1,482-17,082	3,257-10,784	2,774-8,309	11,925-81,405	6,967- 55,227	2,043-24,726	0,054-1,240

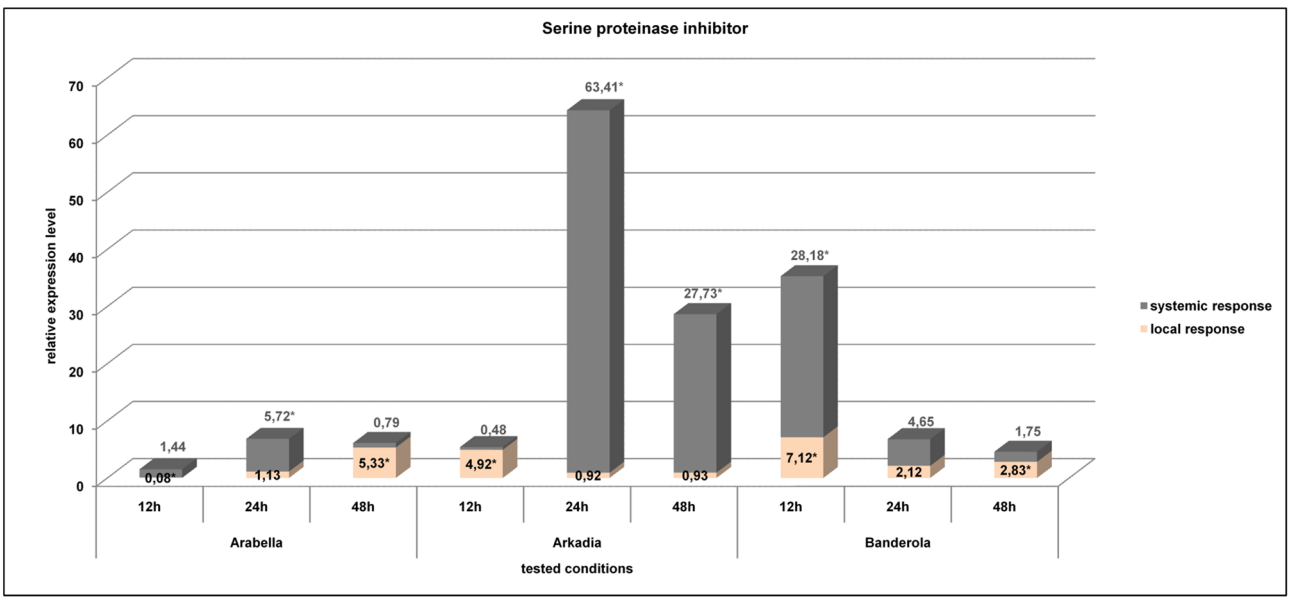
Fig. 3 Analysis of the gene expression levels of cysteine proteinase inhibitors (**a**) and serine proteinase inhibitor (**b**) genes in plants wounded by antibiotic-treated [AB(+)] or antibiotic-untreated [AB(−)] cereal leaf beetle larvae feeding. Expression levels of inhibitors genes were assessed in wounded (local response) and systemic leaves. Three varieties of wheat (Arabella, Arkadia, Banderola) were tested. The tables contain the data on the *p* value and the standard error (SE) for each treatment. Asterisks indicate statistically significant differences in expression for inhibitors in comparison to the control (undamaged plants, value 1; $p < 0.05$)

(a)



CysPI		Arabella			Arkadia			Banderola		
time		12h	24h	48h	12h	24h	48h	12h	24h	48h
systemic response	p	0,453	**0,012***	**0***	0,909	0,152	**0,049***	**0,026***	0,184	0,111
	SE	0,771-1,737	1,260-2,128	0,462-0,701	0,472-2,009	0,898-3,093	1,022-6,917	1,494-3,445	0,943-2,646	0,921-2,277
local response	p	**0,043***	**0,018***	**0,02***	0,761	0,562	0,148	**0,003***	0,731	0,319
	SE	0,334-1,013	0,377-0,770	1,157-4,087	0,400-2,393	0,760-1,553	0,006-2,238	1,554-2,724	0,684-1,494	0,089-1,602

(b)



SerPI		Arabella			Arkadia			Banderola		
time		12h	24h	48h	12h	24h	48h	12h	24h	48h
systemic response	p	0,331	**0,008***	0,63	0,19	**0,001***	**0,001***	**0,008***	0,066	0,418
	SE	0,689-3,054	3,125-10,749	0,318-1,483	0,163-1,731	10,723-134,309	11,351-62,503	5,852-111,910	1,054-19,944	0,517-6,155
local response	p	**0,014***	0,843	**0,004***	**0,045***	0,855	0,958	**0,002***	0,287	**0,041***
	SE	0,021-0,302	0,333-2,373	2,650-11,604	1,604-16,207	0,296-3,049	0,235-4,860	3,991-12,936	0,626-8,993	1,416-6,525

Fig. 4 Analysis of the gene expression levels of cysteine proteinase inhibitors (**a**) and serine proteinase inhibitor (**b**) in plants wounded by antibiotic-treated larvae of cereal leaf beetle feeding. Expression levels of tested genes was assessed in wounded (local response) and systemic leaves. Three varieties of wheat (Arabella, Arkadia, Banderola) were tested. The tables contain the data on the p value and the standard error (SE) for each treatment. Asterisks indicate statistically significant results in comparison to the control (plants wounded by antibiotic-untreated cereal leaf beetle larvae feeding, value 1; $p < 0.05$)

and are considered anti-metabolic proteins which interfere with the digestive process (suppression of proteolytic activity) of insect pests (Sharma 2015) or microorganisms. As a result, the availability of amino acids necessary for insect growth and development is reduced (Sharma 2015). For example, trypsin inhibitors present in soybean were shown to be toxic to the larvae of the flour beetle (*Tribolium castaneum*, Coleoptera, Tenebrionidae) (Lawrence and Koundal 2002). Cingel et al. (2016) showed a reduction in development time (thus decreasing plant damage) of CPB larvae which were reared on potato leaves (*Solanum tuberosum* L.) with co-expression of genes encoding oryzacystatin I and II (*OCI*/*OCII*). Koiwa et al. (1998) reported that soybean cystatin (scN) delayed the growth and development of the cowpea weevil (*Callosobruchus maculatus*, Coleoptera, Chrysomelidae). On the other hand, Girard et al. (1998a) indicated differential susceptibility of two strains of the cabbage seed weevil (*Ceutorhynchus assimilis*, Coleoptera, Curculionidae) to oilseed rape plants over-expressing *OCI* cystatin. Zhu-Salzman et al. (2003) demonstrated that cowpea weevil was susceptible (feeding inhibition, developmental delay) for scN only during the early developmental stages.

Plant defense response occurs not only at or near the site of damage by insect feeding, but also in other parts of plants, thanks to signaling molecule-based communication between different plant parts. In wounded and systemic leaves, the same plant defense proteins can be synthesized but the kinetics of their production may differ. Green and Ryan (1972) demonstrated that wounding of tomato and potato leaves by CPB induced a rapid accumulation of SerPI I (PINI) not only in damaged leaves, but also in distal, undamaged leaves. On the other hand, Mishra et al. (2012) showed significantly higher levels of PI activity (trypsin inhibitor) in the pepper (*Capsicum annuum*) in systemic leaves of plants wounded and treated with water. In our study, plant defense in wheat leaves in response to CLB larvae feeding occurred both locally and systemically. However, in systemic leaves, the expression of inhibitors, and especially the *SerPI* gene, was generally higher than that in damaged leaves (local response). It is likely that it was more profitable for plants to accumulate the stronger defense response in their distal part to effectively reduce further damage.

A decrease in *CysPI* gene expression (cystatins) was observed for almost all treatments with CLB larvae, and especially in the case of local response (Fig. 2a). The mechanism for suppressing the expression of genes encoding CysPI is unknown. It is possible, however, that genes encoding other plant defense proteins, including other CysPI family members, are up-regulated upon CLB larvae feeding. For instance, Pechan et al. (2000) demonstrated that a unique 33 kDa cysteine proteinase, without involvement of a CysPI, was induced in maize in response to feeding by the fall armyworm (*Spodoptera frugiperda*, Lepidoptera, Noctuidae) and southwestern corn borer (*Diatraea grandiosella*, Lepidoptera, Crambidae). Cysteine proteinase was also accumulated (to a lesser extent) in leaves distal to the feeding site and expressed in response to wounding. In addition, this protease has been found to inhibit larval growth (Pechan et al. 2000); thus, down-regulation of *CysPI* expression might be desirable for this process.

As previously noted, gene expression in response to mechanical wounding differs from that in response to insect feeding. For example, Korth and Dixon (1997) demonstrated that in potato plants damaged by larvae of the tobacco hornworm (*Manduca sexta*, Lepidoptera, Sphingidae), production of PIs occurred more quickly than in plants damaged mechanically. This rapid expression of PIs was induced by insect-associated factor, which was recognized by plant. The Differences in the expression of PIs in response to mechanical damage and CLB larvae feeding were also observed in this study (Fig. 2). It suggests the presence of additional factors associated with insects, including these occurring in their oral secretions that may affect plant response to pest feeding. It can be associated with herbivorous pest elicitors and/or organisms [herbivory-associated organisms and elicitors (HAOEs)] (Wielkopolan and Obrępalska-Stęplowska 2016). During insect feeding, macerate of plant tissue has direct contact with insect oral secretions (saliva and/or regurgitates), which contain factors affecting plant response such as enzymes (Mattiacci et al. 1995; Eichenseer et al. 1999), modified forms of lipids (Alborn et al. 2007; Hilker and Meiners 2010), cell wall fragments (Bergey et al. 1999), proteins from digested plant proteins (Schmelz et al. 2006), and organisms or proteins derived from them (Chung et al. 2013).

Plants are able to recognize herbivore pests and induce responses to deter them by identifying compounds in insect oral secretion. For example, plants were found to recognize caeliferins secreted by the American bird grasshopper (*Schistocerca americana*, Orthoptera, Acrididae) (Alborn et al. 2007) and bruchins from the pea weevil (*Bruchus pisorum*, Coleoptera, Chrysomelidae) and cowpea weevil (Doss et al. 2000). In our research, differences were also observed between PI gene expression in response to mechanical wounding and to CLB larvae feeding. However, higher (or faster) PI gene expression occurred in plants that were mechanically wounded, especially in the case of the *SerPI* gene (Fig. 1). Only in the case of the Arabella variety were expression levels of the two PI genes comparable (in both wounded and systemic leaves) in response to CLB larvae feeding and mechanical damage (Fig. 1a, b). In the Arkadia and Banderola varieties, the differences between these two treatments were more pronounced, especially in the *SerPI* case. Much higher/faster *SerPI* gene expression occurred in mechanically damaged plants in both wounded (Fig. 1c, e) and systemic leaves (Fig. 1d, f). In addition, suppression of

SerPI gene expression was observed in systemic leaves in response to larvae feeding (Fig. 1d).

These results suggest that there are some insect salivary components that suppress plant response to wounding. For example, glucose oxidase secreted by the corn earworm (*Helicoverpa zea*, Lepidoptera, Noctuidae) not only protects the herbivore against pathogens, but also suppresses the synthesis of nicotine, normally induced in wounded tobacco plants (*Nicotiana tabacum*) attacked by these insects (Alba et al. 2011). Insects are also associated with microorganisms that play a crucial role in plant–insect interaction. Microorganisms have a huge impact on insect life. For example, bacteria inhabiting the insect gut in most cases are nonpathogenic, and positively affect the insect life, including their nutrition (Koga et al. 2003), digestion, reproduction, protection against pathogens (Dillon and Dillon 2004) and natural enemies (predator, parasites) (Oliver et al. 2010), genetic differentiation (Charlat et al. 2009) or insect sensitivity to environmental factors (Montllor et al. 2002). Bacteria (such as symbionts) may also act as elicitors or effectors in manipulating plant–insect interaction to the benefit of their insect hosts (Chung et al. 2013; Wielkopolan and Obrępalska-Stęplowska 2016). For example, Chung et al. (2013) reported that larvae of CPB secreted symbiotic bacteria at the wound site to suppress anti-herbivore plant response. On the other hand, Barr et al. (2010) found that the AB-untreated WCR induced down-regulation of plant defense genes (e.g. genes encoding cinnamoyl-CoA reductase, cinnamyl alcohol dehydrogenase, shikimate kinase, polyphenol oxidase) in comparison to the AB-treated WCR (up-regulation of maize defenses) and the control treatment.

In our research, in all varieties of wheat, we observed much higher or faster *SerPI* gene expression in plants wounded by AB-treated CLB larva (with a reduced microbial component associated with CLB) feeding compared to the control (undamaged) plants and plants wounded by AB-untreated larva (Fig. 3b). This indicates that microbial factors associated with insects may have been responsible for suppressing expression of the gene encoding this proteinase inhibitor. On the basis of our preliminary results showing the presence of *Wolbachia* and *Rickettsia* endosymbionts in CLB larvae, it can be concluded that these bacteria may be at least partly responsible for this effect. It was previously documented that *Wolbachia*-infected leaf miners (*Phyllonorycter blancardella*, Lepidoptera, Gracillariidae) modulate plant physiology to the benefit of their insect hosts (increased leaf miner performance, manipulation of plant cytokinin levels) (Kaiser et al. 2010). This should be confirmed by further research on CLB-associated bacteria and their role in plant–insect interaction.

It is also notable that in plant defense against CLB, SerPI may play a more important role than CysPIs. PIs are grouped into four families, according to the four types of proteinases that they inhibit (cysteine, serine, aspartyl proteases and metalloproteases) (Lawrence and Koundal 2002). These families are further subdivided into families based on amino acid sequences (e.g. eight families of serine proteinase inhibitors) (Mosolov and Valueva 2005). In this study, the expression of genes encoding inhibitors belonging to the cysteine (cystatins) and serine proteinase (Bowman–Birk) families were analyzed. Depending on various factors such as the developmental stage of insects as well as plant species/variety, environmental conditions, and length of time after wounding or larval feeding, the expression levels of PIs can vary and can be more complex.

In conclusion, the results provided in this article indicate the contribution of PIs, especially up-regulated gene expression of *SerPI* from the Bowman–Birk family, in wheat response against CLB larvae feeding. Secondly, the plant response was typically much stronger in systemic leaves (distal to damage site). Third, microorganisms associated with CLB larvae were found to modify gene expression of PIs—especially *SerPI*—which are known as the most common inhibitors in the plant world (Jamal et al. 2013) and are involved in plant defense response against insect pests. Lastly, each of the tested wheat varieties reacted in a specific way to the particular treatment. Our results demonstrate that gene expression of *CysPIs* and *SerPI* is regulated spatially and temporally.

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