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Responses of Wheat (*Triticum aestivum*) to Grain Aphid (*Sitobion avenae*) Infestation and Mechanical Wounding Using a cDNA Subtractitve Library Approach

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Abstract

Aphids are major insect pests of cereal crops, acting as virus vectors as well as causing direct damage. The responses of commercial wheat (cv. Claire) to grain aphid (*Sitobion avenae*) infestation and mechanical wounding were investigated in this study, with the aim to eventually identify a source of molecular markers to breed wheat for enhanced insect resistance, and in particular for enhanced resistance to phloem-feeding insects. Mechanical wounding was included in this study as a comparison with aphid feeding to distinguish between insect-specific responses in wheat plants to those involved in a general wounding response. Wheat (*Triticum spp.*) is known to have partial resistance toward aphids [1]. The plant response and defence against insect feeding are complicated, but always follow the same principle: insect detection, signal transmission to initiate defence, changes in plant gene expression and subsequent production of defensive compounds, which may be targeted to the wound site to deter or kill insects. Defensive gene products/proteins reach the target area and deter or kill insects. Whether the last step is successful or not depends on the resistance and susceptibility of the plant towards that particular pest. In the light of this principle, it is important to detect changes in gene expression, first at the transcriptional level, which is useful for detection of early-stage responses, and then once sufficient time is allowed for the plant to produce defensive gene products, responses at the proteome level can be identified. Work presented in this study focuses on the changes at the transcriptional level; differential responses at the proteome level were investigated and presented in Ferry *et al.* 2011 [2] and Guan *et al.* 2015 [3]. Two cDNA subtractive hybridization libraries were constructed, one to identify tran-

scripts involved in the responses to aphid infestation, and the second to identify transcripts involved in responses to mechanical wounding. Following subtractive hybridization, 520 and 800 clones were obtained from the subtractive hybridization between aphid-infested and un-infested wheat cDNAs and between mechanically wounded and un-wounded wheat cDNAs, respectively. Over 70% of the total clones were sequenced and 44% and 55% of sequenced clones were successfully identified by homology to known sequences held at NCBI with Blastx search engine in aphid-infested vs un-infested and mechanically wounded vs un-wounded cDNA subtractive libraries, respectively. These results reveal that the differences in the response of commercial wheat (cv. Claire) plants towards aphid infestation and mechanical wounding are subtle. Although the majority of differentially expressed putative genes after aphid infestation or mechanical wounding were involved in metabolic processes and photosynthesis, the majority of the genes expressed were different. Genes encoding glutathione transferase (GST), apoptosis and proteolysis were up-regulated after aphid feeding, suggesting their importance towards plant defence/tolerance against aphid attack. These results suggest that commercial wheat does have a certain degree of tolerance to aphids, but appears to lack a specific response to aphids; these findings are supported by those presented in Ferry *et al*. 2011 [2].

Keywords

Wheat, Grain Aphid, Response, Resistance, Tolerance,
cDNA Subtractive Library

1. Introduction

Wheat (*Triticum aestivum*) is one of the major global crops. The data from 2016 showed that the global planting area is over 2.2 million hectares, with an annual output of 7.3 billion tons, accounting for 1/3 of the world's total grain output (China Industry Information). Crop loss due to insect pests is one of the major problems challenging crop productivity, including wheat. Despite the current crop protection practices with the intensive use of pesticides, crop losses have not decreased significantly during the past 40 years. The crop loss maintains at a high level because of the increased susceptibility to the damaging effect of pests and an increase in attainable yields is often associated with an increased vulnerability to pest damage. Insect pests cause approximately 18% crop loss worldwide every year, ranking second after weeds (34%) and followed by pathogens (16%; [4]).

Aphids (Order Hemiptera) are major insect pests of world agriculture, damaging crops by removing photoassimilates and vectoring numerous plant viruses [5]. The grain aphid (*Sitobion avenae*) is considered a serious pest of commercial wheat. Many pest aphid species readily become resistant to insecti-

cides [6]. Bt is not effective against homopteran insects, such as aphids. Insect-related crop damage and insecticide resistance have made the development of aphid-resistant crop varieties a priority [7] [8]. Restriction on the availability of active ingredients for crop protection in Europe (European Directives 91/414/EEC) has made this even more desirable. Commercial wheat lines with partial resistance to aphids have been described by several sources [9] [10] [11] [12] [13].

Insect feeding usually causes major plant tissue damage, and induces a plant wounding response, mediated by jasmonic acid, which results in the synthesis of plant defensive compounds, such as proteinase inhibitors and polyphenol oxidases [14], [15]. Aphids and other hemipteran insects feed on plant phloem sap by inserting a stylet between the overlying cells, thus limiting cell damage and plant response [15] [16] [17]. Under these circumstances, plant responses have been reported as similar to responses to pathogen attack, with examples of gene-for-gene interactions, based on aphid-derived elicitors, and mediated by salicylic acid as a signalling molecule [5] [18] [19]. The detection of aphid stylet penetration by plant receptors are followed by the transmission of responsive signal cascades which involve various signalling molecules.

Plant signalling pathways mediated by jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), gibberellic acid (GA), reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and nitric oxide (NO) induce changes of defensive gene expression, which ends with changes at the metabolic level with the effect of improved plant defence. The recognition of aphid feeding by plants occurs through the use of transmembrane pattern recognition receptors (PRRS) or, acting largely inside the cell, polymorphic nucleotide-binding leucine-rich-repeat (NB-LRR) protein products, encoded by most R genes. Plant-induced defences are also regulated by a network of inter-connecting signalling pathways, in which JA, SA, and ET play dominant roles. Both synergistic and inhibitory aspects of the cross-talk among these pathways have been reported. In addition, the activation of transcription factors often enables a cross-talk between the pathways and determines the final gene expression profile of induced resistance. Furthermore, it has been demonstrated that activation of signalling pathways is integrated and temporally controlled. Much evidence suggests that ROS signalling pathways are closely linked with hormone-signalling pathways in plant-insect interactions [20]. Insect infestation can affect stress signalling network through effects on ROS and cellular redox metabolism [21]. Microarray and macroarray data have identified genes involved in oxidative stress, calcium-dependent signalling, pathogenesis-related responses, accumulation of camalexin and signalling as key components of the induced plant response to aphids [19] [22].

The molecular bases of plant-aphid interactions have begun to be understood [5], as a result of intensive studies in both model plants [19] [23] [24] [25] [26] and crop plants in the past two decades [22] [27] [28] [29] [30]. Extensive gene

reprogramming in the plant can occur in response to aphid herbivory [24] [31] [32] [33] [34].

Analysis of global gene expression profiles in Arabidopsis in response to aphid and pathogen attacks revealed consistent changes induced by both pathogens and insects, with attacker-specific responses but considerable overlap in the sets of genes up- or down-regulated by different treatments [31]. Similar Studies have been extended to crop plants. Changes in wheat gene expression have been shown to occur after aphid infestation; these comprise two distinct phrases, an immediate response from 0 - 24 h after infestation, and a second prolonged response that prevails in the tissue for an extended period of time, up to 8 days. Previous studies on wheat (*Triticum aestivum* L. cv. "Tugela DN") resistant to the Russian wheat aphid (RWA, *Diuraphis noxia*), showed that changes in ethylene production were observed within the first 24 h after infestation by the RWA in resistant cultivar "Tugela DN", but not in the susceptible near-isogenic line (NIL) "Tugela" [35].

Plant tolerance to an insect pest is considered as an excellent defensive strategy. Transcript profiling of tolerant and susceptible barley genotypes after three hours, three days and six days of *Diuraphis noxia* feeding identified a total of 909 genes significantly up-regulated in the tolerant barley as compared to susceptible plants. Of these genes, several associated with plant defence and scavenging of reactive oxygen species (ROS), two peroxidase genes, designated HvPRXAI and HvPRXA2, were up-regulated, indicating that specific peroxidases could be important for the tolerance process. These findings show that the ability to raise and maintain levels of ROS-scavenging enzymes is important to the tolerance of aphids [30].

Myzus persicae (green peach aphid) feeding on *Arabidopsis thaliana* induces a defence response, which leads to reduced aphid progeny production, in infested leaves but not in other parts of the plant, suggesting the localisation of the response. This response is also independent of the known defence signalling pathways involving salicylic acid, jasmonate and ethylene. The expression of a set of O-methyltransferases, which may be involved in the synthesis of aphid-repellent glucosinolates, was significantly up-regulated in infested leaves by both *M. persicae* feeding and treatment with aphid saliva [36].

Trehalose phosphate synthase 11 (TPS11) gene-dependent trehalose metabolism promotes *Arabidopsis thaliana* defence against *Myzus persicae* (Sulzer), commonly known as the green peach aphid (GPA). TPS11 gene encodes a trehalose-6-phosphate synthase/phosphatase. Evidence indicates that TPS11-dependent trehalose regulates expression of the phytoalexin deficient4 gene, which is a key modulator of defences against GPA. TPS11 also promotes the re-allocation of carbon into starch at the expense of sucrose, the primary plant-derived carbon and energy source for the insect, which results in a decrease of the severity of aphid infestation [37].

A study on the importance of jasmonate signalling in the induction of plant

defences upon cabbage aphid (*Brevicoryne brassicae*) attack revealed that the aphid-induced response of more than 800 transcripts was regulated by jasmonate signalling. Thus, in plants lacking jasmonates many of the defence-related responses induced by infestation in wild-type plants were impaired [38].

Transcriptional and metabolic study of maize response to aphid feeding revealed a previously uncharacterized terpene synthase activity, which strongly influences aphid reproduction on maize.

A study on zucchini plants (*Cucurbita pepo* L., Cucurbitaceae) by Aphids gossypii Glover (Hemiptera: Aphididae, Aphidini) showed an elicitor from aphid saliva triggering a plant response affecting the strategy of host - plant colonization by *A. gossypii*. Aphid infestation showed a transcriptional up - regulation of genes underlying the biosynthesis of SA and of genes modulating the SA-mediated defence response.

Proteomic Analysis of aphid-resistant (Stella and Alibaba) and -sensitive rose (*Rosa Hybrida*, (Sun star and Haetsal)) cultivars showed that proteins related to ubiquitin metabolism and the stress response, such as stress-responsive proteins superoxide dismutase (SOD) and ascorbate peroxidase (APX) were differentially expressed due to aphid infestation [39]. This study also identified proteins related to defence response like pathogenesis related proteins both in aphid-resistant and -sensitive cultivars. Ubiquitin conjugation is a major regulator to stress response by modulating the activity of stress-responsive proteins required for adaptation to stress, for example, E3 ubiquitin ligase is involved in regulating drought and salinity stress through abscisic acid signalling [40] [41].

Research with other innovative methods was also carried out in the past two decades, not only to understand the plant-aphid interaction but also to improve plant defence against aphid attacks.

Some plant defensive compounds work in a more direct way, such as plant lectins. Genetically modified crops with inserted lectin genes may have improved defensive advantage over aphids [42].

Some research has indicated the resistance genes to hessian fly also work for aphids Insecticidal activity of wheat Hessian fly (*Mayetiola destructor*) responsive proteins HFR-1 and HFR-3 towards cereal aphid (*Sitobion avenae* F.) were reported by Pyati *et al*. in 2012 [43]. HFR-1 and HFR-3 were found to be insecticidal towards *S. avenae* when fed in an artificial diet, whilst HFR-3 was almost non-toxic to *S. avenae*.

Pitino *et al.* [44] demonstrated the feasibility of a plant-mediated RNAi approach for aphid control. They achieved up to 60% knock down in target gene expression and silenced aphids produced less progeny due to the importance of the chosen target genes to those aphids.

Recombinant fusion proteins containing arthropod toxins have been developed as a new class of biopesticides. Fusion protein Hv1a (a spider venom toxin)/GNA was shown to work against peach-potato aphid (*Myzus persicae*) through an artificial diet or transgenic Arabidopsis. Grain aphids (*Sitobion ave-*

nae) were shown to be even more susceptible than *M. persicae* to the Hv1a/GNA fusion protein in artificial diet bioassays, as they were not able to hydrolyse the fusion protein as readily as *M. persicae* [45].

The aim of the present study was to use cDNA subtractive library analysis to identify putative defence responses in a commercial wheat variety when subjected to grain aphid (*S. avenae*) feeding. The winter wheat (cv. Claire) was selected for study as a high-yielding variety widely grown in the UK, and recommended by the HGCA (https://www.gov.uk/government/organisations/home-grown-cereals-authority). This study provides a baseline for future attempts to improve the resistance of wheat to grain aphids. A 24 h time point (early phase, as opposed to a late phase, which is 24 h - 8 days) was selected for studies to investigate transcriptional responses to aphid infestation [46]. However, studies to investigate proteome responses [2] [3] were carried out at both 24 h and 8 days post infestation. Furthermore, since the plant response to insect damage differs to some extent from that caused by mechanical wounding [15] [47], mechanical wounding of plants was included to distinguish insect specific responses in wheat as opposed to general stress responses. Insect-derived damage can cause a greater accumulation of proteinase inhibitors than mechanical damage [48]. Insect damage of plants often causes different physiological and biochemical responses than mechanical damage alone, which is based on the measurement of a number of parameters such as levels of induced phenolics [49], enzymatic activities [50], amino acid profiles [51], plant regrowth [52] [53], and release of volatile compounds [54].

Subtractive hybridization is a powerful technique that enables comparison between two populations of mRNA and allows clones of genes differentially expressed to be obtained. The advantages of the subtractive hybridization technique include: first, subtractive hybridization is a simplified, fast and reliable method for generating subtracted probes or subtracted cDNA libraries; second, only a small amount of sample/mRNA is required; third, magnetic handling minimizes loss at each step; fourth, specific mRNAs are highly enriched during the process; fifth, magnetic handling enables simple and rapid buffer changes to optimize conditions for hybridization and specific enzymatic reactions; last, the subtractor Dynabeads can easily be regenerated, stored and reused. The basic theory behind subtraction is simple. First, both mRNA populations are converted into cDNA. Second, two cDNA populations are hybridized and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are differentially expressed. In this study, mRNA from 24 h aphid-infested wheat leaf tissue and non-infested leaf tissue were used to obtain differentially expressed genes in wheat after 24 h aphid feeding.

2. Materials and Methods

2.1. Selection of Wheat Line

Wheat cv. Claire was chosen for this study because it is s a commonly used

commercial wheat line and its average performance in the bioassay study (**Figure 1**). Wheat (cv. Claire, seeds obtained from British Wheat Breeders) seedlings were grown to the four-leaf stage in soil (John Innes, No. 2) under controlled environmental conditions in custom built growth rooms (HA Davie Ltd, U.K.) under the following conditions: light intensity; photosynthetically active photon flux: 600 μmol/m^2/s, 16 h light/8h dark, with a temperature regime of 18˚C (day): 16˚C (night) and 70% relative humidity [55].

2.2. Aphid Infestation

Clonal grain aphids, *Sitobion avenae*, were initially reared on oats (*Avena sativa*, cv. Black Coast) maintained at 20˚C, 55% R.H. under a 16:8 h L: D light regime [1]. Prior to infestation studies, aphids were established on wheat (*T. aestivum*, cv. Claire) for 4 weeks under the same conditions. New plant material was supplied weekly. For transcription studies, plants at the 4-leaf stage were used; 2 leaves per plant were infested with aphids, with 20 aphids per leaf. Aphids were confined to specific leaves using clip cages, which are made from a 7 cm Petri dish, attached to a wooden stick with a twisted paper clip, as seen in the image (**Figure 2** and **Figure 3**). Non-infested plants at the same developmental stage were used as control plants (48 plants with the treatment, 48 plants for control).

2.3. Mechanical Wounding, Tissue Collection and Storage

Wheat plants, at the 4-leaf stage, were mechanically wounded with a sharp wheel roller (24 plants with the treatment). The wheel roller has sharp points around the wheel, and when rolling along the leaf it leaves tracks of small holes, mimicking chewing insect feeding wounds. Within 24 hours of initial wounding (9 am), the plants were wounded another 5 times during the day at 2 hr intervals

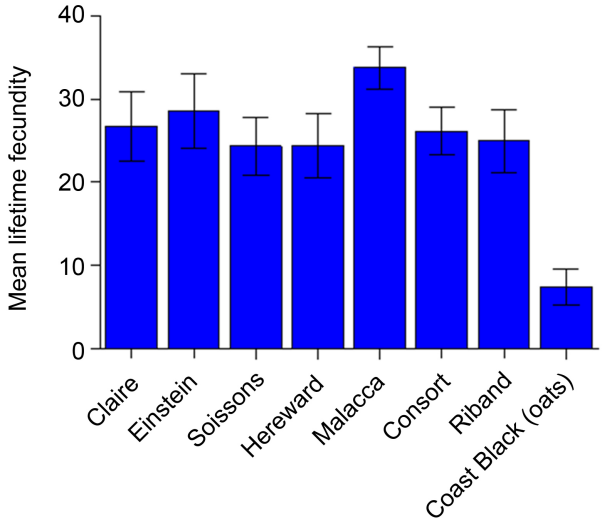

Figure 1. Bioassay mean fecundity (n = 16 - 19) of *S. avenae* when developing on different winter wheat varieties and oats (n = 16 - 19).

Figure 2. Clip cages used to confine aphids.

Figure 3. Aphids within clip cages.

(11 am, 1 pm, 3 pm, 5 pm, 7 pm), and were finally wounded the next day 9 am just before tissue collection (24 h post initial wounding). The rationale for repeated wounding was to ensure that the plants were severely wounded and that it occurred over the same time period as for aphid probing. An image of plants 24 hours post initial wounding is shown in **Figure 4**. Wheel rollers used to cause mechanical wounding are shown in **Figure 5**.

Tissue Collection and Storage

After 24 hours infestation, aphids were removed from the leaves with a fine camel hair brush, and all leaves of the infested plants were collected, wrapped with tin foil and flash frozen in liquid nitrogen (−70˚C) to prevent any reactions that

Figure 4. Plants at 24 h post initial wounding.

Figure 5. Wheel rollers.

may occur post harvesting. The collected leaf tissues were then transferred to −80˚C freezers for long-term storage. Leaf tissue 24 h post wounding was collected and stored in the same way. Control leaf tissues were also collected and stored.

2.4. Total RNA Extraction and mRNA Isolation

Total RNA was extracted from 24 h aphid-infested, 24 h post wounding and control leaf tissue using Tri-Reagent according to the manufacturer's protocol (Applied Biosystems/Ambion, TRI Reagent→). The extracted RNA was re-suspended in DEPC-treated water (DEPC: diethylpyrocarbonate, RNase inhibitor, protects RNA against RNase digestion). Total RNA was quantified using a Thermo Scientific NanoDrop 1000 Spectrophotometer. After quantification, appropriate amounts of total RNA was then used for messenger RNA (mRNA)

isolation using a Promega mRNA Isolation Kit. The isolated mRNA was then stored at −80˚C.

2.5. cDNA Synthesis and Subtraction

cDNAs from 24 h aphid-infested, 24 h post wounding and control wheat plants were synthesized from their mRNAs and then subtracted using a Clontech PCR-Select cDNA Subtraction Kit. During the subtraction, cDNAs common to both aphid-infested and control tissues, both mechanically wounded and control tissues were discarded and only cDNAs that were differentially expressed in either aphid-infested, mechanically wounded or control tissue were amplified by PCR (Polymerase Chain Reaction). Following subtraction and PCR, four groups of PCR products were generated. These were: 1) Two forward subtractions, contain differentially expressed cDNA, which are over-expressed or switched on for aphid-infested or mechanically wounded wheat in comparison with the control group, *i.e*. these genes are up-regulated in wheat after aphid infestation or mechanical wounding; 2) the other two are reverse subtracted, containing differentially expressed cDNA, which are expressed less or switched off after aphid feeding or mechanical wounding, *i.e*. these genes are down-regulated in wheat after aphid infestation or mechanical wounding. The whole profile of each cDNA subtraction group constitutes one cDNA subtractive library.

2.6. Cloning, Sequencing and Database Search

The PCR amplified differentially expressed cDNAs were then cloned using a Strataclone PCR Cloning Kit. First, the cDNAs were inserted into StrataClone PCR cloning vector pSCA by ligation reaction. These vectors were then transformed into StrataClone competent cells to be cloned. The competent cells were grown on LB-ampicillin-X-gal plates overnight at 37˚C. After overnight growth, cell colonies with vectors containing a cDNA insert were identified using blue/white selection; white or light blue colonies were subsequently used for plasmid DNA extraction. Plasmid DNAs, which contain the vector with differentially expressed cDNAs, were sequenced by Macrogen Sequencing Services (Korea). Annotation of the *Triticum aestivum* transcriptome sequences was based on sequence similarity, namely sequence-based alignments. The sequence-based alignments were performed against the non-redundant protein database (NR) at NCBI (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) using BLASTx algorithm with a significant E-value threshold of 1e-5 [56].

2.7. Validation of Differentially Expressed Genes by Real-Time PCR

Full-length sequence of GST and Tritican gamma (a cystain protease) were obtained from NCBI Gene Bank and approximately 20 bp primers were used in real-time PCR to amplify GST, Tritican gamma, and housekeeping gene GAPDH

from control tissue (wheat cv. Claire, 4 leaf stage, cDNA) and 24 h grain aphid-infested leaf tissues (cDNA), in order to compare the expression level of the 3 genes in control and 24 h infested tissue.

3. Results

3.1. Resistance of Commercial Wheat to Grain Aphid

It is important to know whether the widely used commercial wheat line (*Triticum aestivum* cv. Claire) has partial resistance against phloem-feeding insects, and if the resistance does occur, to understand the basis of this resistance/tolerance. Bioassay carried out previously and published in Ferry *et al*. 2011 [2] established that of the 8 cultivars of wheat screened, representative of current commercial practice, there were no significant differences in terms of aphid fecundity (see **Figure 1**). Based on this screening, the cultivar Claire, which gave intermediate values in terms of aphid tolerance/susceptibility compared to the other cultivars tested, was used as the "representative" commercial cultivar for investigation of the endogenous response in wheat to aphid infestation. This cultivar was therefore used to generate the cDNA subtractive libraries used in this study.

3.2. Gene ID and Annotation of cDNA Subtractive Library

3.2.1. Aphid-Infested vs Control

Following subtractive hybridization of the two cDNA pools (aphid-infested vs control), 166 clones appeared to be up-regulated in response to aphid feeding (*i.e*. forward subtraction). Of these, 91 (55% of all obtained clones) were able to grow in liquid culture and were sequenced, out of which 49 (54% of all sequenced clones) were putatively identified.

Similarly there were 354 clones from the control tissue, representing those that were down-regulated after aphid feeding (*i.e*. reverse subtraction). Of these 88 (25% of all obtained clones) were sequenced, out of which, 16 (18% of all sequenced clones) were putatively identified. Sequence analysis showed that there were many clones that had identical or very similar sequence, and will be treated as repeats of the same gene.

Analysis of sequence data and database annotation show that there were 22 genes putatively up-regulated after aphid feeding, *i.e*. these genes were over-expressed or switched on after aphid feeding (**Table 1**). Five putative genes were identified as being down-regulated after aphid feeding, *i.e*. these genes were expressed less or switched off after aphid feeding (**Table 2**). Predicted gene product IDs and the corresponding gene annotation were obtained from NCBI Blastx search and Uniprot search. For clarity, putative gene products that were differentially expressed were grouped by function (**Table 1** & **Table 2**).

1) Genes up-regulated after aphid feeding

As can be seen in **Table 1**, there were 22 putative gene products up-regulated after 24 h aphid feeding. The number of sequences identified is not necessarily

Table 1. Protein name/organism.

Biological process	Protein name/organism	Ac. No.	E-value
Detoxification, antioxidant	Glutathione transferase (GST)/ *Tritium aestivum*	P30111	7.00E−55
DNA regulation	Serine hydroxymethyltransferase/ *Triticum monococcum*	A6XMY5	9.00E−17
Photosynthesis	Chloroplast 29 kDa ribonucleoprotein/Oryza sativa subsp. indica	A6N1F5	7.00E−42
	Putative rubisco small subunit/ *Triticum turgidum* subsp. Durum	Q575T3	2.00E−15
	Ribulosebisphosphate carboxylase small chain/ *Triticum aestivum*	Q41582	6.00E−14
	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit/*Triticum urartu*	Q9ZWG3	2.00E−16
	Ribulose 1,5-bisphosphate Carboxylase activase isoform 1/*Hordeum Vulgare* subsp. Vulgare	Q40073	2.00E−99
	Ribulose bisphosphate carboxylase small chain clone 512/Triticum aestivum	P07398	1.00E−15
	Putative oxygen-evolving complex precursor/*Triticum aestivum*	A4UQP4	2.00E−27
	LHCI-680, photosystem I antenna protein/*Hordeum vulgare* subsp.vulgare	Q43485	9.00E−13
	Chloroplast chlorophyll a/b-binding protein/*Agave tequilana*	A4ZGB5	4.00E−08
	Ribulose-1,5-bisphosphate carboxylase activase/ *Triticum aestivum*	Q6XW16	4.00E−88
Propanoate metabolism	Tyrosine/nicotianamine aminotransferases family protein, expressed/Oryza sativa subsp. japonica	Q2R0I0	2.00E−68
	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit/ *Triticum aestivum*	Q9FRZ4	2.00E−50
Proteolysis	Triticain gamma (cysteine protease)/ *Triticum aestivum*	Q0WXG6	2.00E−33
	Cysteine protease/*Lolium multiflorum*	Q9M4E5	1.00E−30
	Ubiquitin-conjugating enzyme spm2, putative, expressed/Oryza sativa subsp. japonica	Q2QMG7	8.00E−09
Stress response	Heat shock protein 70/*Zea mays*	Q5EBY7	2.00E−40
	Heat shock cognate 70 kDa protein, putative, expressed/*Oryza sativa*	Q2QZ41	3.00E−51
Unknown	Light-induced protein 1-like/*Lolium perenne*	Q3HNF6	7.00E−08
	Putative uncharacterized protein/*Zea mays*	B4FN76	2.00E−11
	Hypothetical protein OsJ_009954/Oryza sativa subsp. japonica	EAZ26471	2.00E−34

Notes: Up-regulated genes were identified from the forward subtraction of the cDNA subtractive library (aphid-infested vs control). Keys: gene products up-regulated in both aphid-infested and mechanical wounding (common to both treatment). gene products of most interest.

Table 2. Biological process.

Biological process	Protein name/organism	Accession No.	E-value
Cytoskeleton components	Actin/*Oryza sativa* (indica cultivar)	A2XLF2	1.00E−84
DNA repair	Putative reverse transcriptase/*Zingiber officinale*	A0ST23	1.00E−08
GAPDH/glycolysis/initiation of apoptosis	Glyceraldehyde-3-phosphate dehydrogenase subunit A/*Zea mays*	Q8LPT4	4.00E−07
Resistance to beta-lactam antibiotics	Beta-lactamase/*Zea mays*	Q285M4	3.00E−32
	Putative uncharacterized protein (Fragment)/*Hordeum vulgare*	Q6KB67	3.00E−32

Notes: Down-regulated gene products were identified from reverse subtraction of cDNA subtractive library (aphid-infested vs control). Keys: genes products down-regulated in response to both aphid infestation and mechancial wounding. The gene highlighted in blue is used as a housekeeping gene in the qRT-PCR. The gene highlighted in pink is of most interest.

representative of the corresponding gene copy number. The number of copies of each gene must be taken into account when the distribution of gene products in each category (biological process) is considered. Therefore, in Table 1, there are various number of genes listed in each category and the number of copies for each gene is presented followed by protein names (IDs) and the names of the organism in which the protein is originally discovered from. The Accession Number (Ac. No.) from Uniprot is also listed in order to link the data from this chapter to the corresponding chapter where gene expression studies were investigated at the proteome level [2]. The E-value is also listed to show the chance of the identification being generated randomly. Since all the E-value figures were very small (≤1.00E−5), all sequences presented were considered to significantly align with the data in NCBI database and were considered true IDs of the differentially expressed genes/transcripts/gene products.

The majority of genes up-regulated in response to aphid feeding are known to be involved in photosynthesis. However, of particular interest was the up-regulation of a putative glutathione transferase (GST); this enzyme is known to be an antioxidant and plays a role in detoxification. Two further putative genes associated with the stress response were also identified as being up-regulated in response to aphid infestation (Table 1). These two genes are both putative heat shock proteins.

2) Genes down-regulated after aphid feeding

As can be seen in Table 2, there were 5 gene products down-regulated after 24 h aphid feeding. Again, the number of copies of each gene was taken into account and in Table 2, the number of copies for each gene is presented followed by protein names (IDs) and the organism name. Accession No. and E-value is presented as well. Same threshold for E-value is used. None of these genes have been reported to be directly involved in either defence or the stress response.

3.2.2. Mechanically Wounded vs Control

Analysis of sequence data and database annotation show that there were 11 genes up-regulated after mechanical wounding, *i.e.* these genes were over-expressed or switched on after mechanical wounding. However, approximately twice the number of genes [23] was down-regulated after mechanical wounding, *i.e.* these genes were expressed less or switched off after mechanical wounding. The predicted gene product IDs and the corresponding gene annotation were obtained from NCBI Blast search and Uniprot search, and are presented in Table 3 & Table 4.

3) Genes up-regulated after mechanical wounding

Genes which encode products of similar function were grouped together. Of the 11 genes up-regulated in response to mechanical wounding, the majority were involved in either metabolism or photosynthesis (Table 3). Of potential interest was the up-regulation of a putative RING-type zinc finger gene since it has proposed function in stress response in plants.

4) Genes down-regulated after mechanical wounding

The majority of genes differentially regulated in response to mechanical wounding were down-regulated. Of these 23 putative genes, the majority are involved in metabolism and photosynthesis (Table 4). Of particular interest was the decrease in transcript level of glutaredoxin, which is involved in maintaining

Table 3. Putative gene products in wheat up-regulated in response to mechanical wounding (24 h post wounding).

Biological process	Protein name/organism	Accession No.	E-value
Membrane component	Putative uncharacterized protein/*Zea mays*	B4FM33	6.00E−23
Membrane protein	putative membrane protein/Triticum aestivum	A0MAU8	3.00E−61
Metabolism	LAs17 Binding protein-like (Phosphatidylinositol 4-kinase)/*Oryza sativa* (japonica cultivar)	Q5Z9E4	5.00E−62
Photosynthesis	Phosphoglycerate kinase, chloroplast precursor/*Triticum aestivum*	P12782	2.00E−86
	putative oxygen-evolving complex/*Triticum aestivum*	A4UQP4	3.00E−16
	Ribulose bisphosphate carboxylase small chain clone 512/*Triticum aestivum*	P07398	3.00E−12
	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic/*Triticum aestivum*	P00871	6.00E−15
	Os01g0720500/*Oryza sativa* (japonica cultivar)	Q8W0E6	1.00E−124
Unknown	hypothetical protein OsI_019373/*Oryza sativa* (indica cultivar)	EAY98140.1	7.00E−63
	Os03g0857400/*Oryza sativa* (japonica cultivar)	Q84M73	6.00E−25
Zinc ion binding/redox	Yrg1 (Ring-type zinc finger)/*Hordeum vulgare* var.distichum (two-rowed barley)	B0FLE0	3.00E−100

Notes: Above gene products were identified from forward subtraction of cDNA subtractive libarary (wounded vs control). Gene products up-regulated in response to both aphid infestation and mechancial wounding.

Table 4. Chlorophyll a-b binding protein of LHCII type III, chloroplastic/Hordeum vulgare.

Biological process	Gene product name/organism	Accession No.	E-value
Antioxidant defence	Glutaredoxin/ *Triticum aestivum*	Q7XY25	2.00E-37
DNA synthesis	putative reverse transcriptase/ *Zingiber officinale*—Ginger	A0ST23	1.00E-16
Endoplasmic regiculum component/protein folding/calcium ion binding	Calnexin/ *Zea mays*	B6TNF1	9.00E-18
	reticulon/ *Hordeum vulgare*	Q306I3	1.00E-15
	Putative uncharacterized protein/ *Zea mays*	B4FAK8	2.00E-18
Membrane component	Transmembrane bax inhibitor motif-containing protein4/ *Oryza sativa*	A6N0U8	1.00E-05
Metabolism	xyloglucan xyloglucosyl transferase/ *Hordeum vulgare*—Barley	B1P1S7	4.00E-13
	cell wall invertase/ *Zingiber officinale*—Ginger	Q2QI10	5.00E-16
	IMP dehydrogenase/ *Zea mays*	B6T3S5	2.00E-27
	cystathionin beta synthase protein/ *Zea mays*	B6SRQ8	9.00E-63
Metabolism/apoptosis	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)/ *Triticum aestivum*	A5YVV3	3.00E-07
photosynthesis	Chlorophyll a-b binding protein of LHCII type III, chloroplastic/ *Hordeum vulgare*	P27523	4.00E-36
	Os01g0720500 protein/Oryza sativa (japonica cultivar)	Q8W0E6	1.00E-124
	Chlorophyll a/b-binding protein WCAB/ *Triticum aestivum*	O24401	1.00E-32
	Ribulose bisphosphate carboxylase small chain/ *Triticum aestivum*	Q41582	1.00E-15
	Ribulose bisphosphate carboxylase small chain/ *Triticum Turgidum subsp. Durum*	Q575T3	3.00E-15
Photosynthesis/magnesium ion binding/protein-chromophore linkage	Chlorophyll a-b binding protein 1B-21, chloroplastic/ *Hordeum vulgare*	Q9SDM1	2.00E-21
Plant defence/disease response	jasmonate-induced protein/ *Triticum aestivum*	Q564C9	2.00E-22
Ribosomal protein	60S ribosomal protein L10-2/ *Oryza sativa* (indica cultivar)	A2Y0T4	4.00E-63
Unknown	hypothetical protein OsI_033526/Oryza sativa (indica cultivar)	EAY79567.1	2.00E-44
	hypothetical protein OsI_013926/Oryza sativa (indica cultivar)	EAY92693.1	9.00E-62
	hypothetical protein OsJ_025758/ *Oryza sativa* (japonica cultivar)	EAZ42275.1	6.00E-67
	Os08g0313200 protein/Oryza sativa (japonica cultivar)	Q69LL7	3.00E-62

Notes: Down-regulated gene products were identified from reverse subtraction of cDNA subtractive library (wounded vs control). Keys: The two gene product names highlighted in orange colour are two putative IDs of the same sequence. Gene products down-regualted in response to both aphid infestation and mechanical wounding. The gene highlighted in pink is of most interest. The gene highlighted in blue is used as a housekeeping gene in the qRT-PCR verification.

the redox status of proteins during oxidative stress.

5) Verification of selected genes

All the above putative genes of interest require verification. In the present study Real-time PCR was used in an attempt to verify the up-regulation of specific transcripts (*i.e.* GST and tritican gamma) in response to aphid feeding. The housekeeping gene GAPDH was used as an internal marker gene. Unfortunately the two target genes investigated showed no significant difference in transcript levels between the aphid-infested and control wheat (data not presented).

4. Discussion and Conclusions

The results of gene IDs and gene annotation suggest that the wheat plant (cv. Claire) undergoes metabolic reprogramming in response to aphid infestation, most probably in an attempt to compensate for nutritional loss caused by these phloem feeders. In the present study, transcripts/gene products involved in metabolism and photosynthesis represent a large percentage of all unique genes identified in response to both aphid feeding and mechanical wounding, being 40% and 39% respectively. Furthermore, there are more genes involved in photosynthesis than in any other plant metabolic processes, for example, gene encoding ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo) small subunit were routinely up-regulated in wheat leaves after both aphid feeding and mechanical wounding. Ribulose-1,5-bisphosphate carboxylase activase and putative oxygen-evolving complex precursor were also up-regulated in response to aphid feeding. Up-regulation in expression of photosynthetic or photorespiration genes has also been observed in leaves of celery in response to feeding by the aphid *Myzus persicae*, in wheat leaves in response to the Russian wheat aphid *Diuraphis noxia* feeding and in coyote tobacco *Nicotiana attenuata* leaves in response to *Myzus nicotianae* feeding. However, different genes involved in photosynthesis have also been shown to be down-regulated after feeding by *M. nicotianae* or *Schizaphis graminum* [57].

In the present study, there were some similarities between the insect response and the wounding response. Of those gene products identified, there were 3 genes in common between the two treatments, including putative reverse transcriptase, ribulose bisphosphate carboxylase small chain clone 512 and putative oxygen-evolving complex precursor (these 3 genes are highlighted in green in **Tables 1-4**). There were 22 putative genes and 29 putative genes up-regulated after aphid feeding or mechanical wounding, respectively. However, the differences between treatments were greater, indicating the ability of the plant to distinguish between insect attack (in this case aphid feeding) and mechanical wounding, probably due to its recognition of insect salivary compounds [36] [47] [58] [59] [60].

In aphid-infested wheat leaves, transcripts for glutathione transferase (GST) were up-regulated after aphid feeding, but not up-regulated in response to mechanical wounding. The finding that this gene is over-expressed or switched on

within the first 24 hours of aphid infestation suggests its importance towards plant defence/tolerance against aphid attack. Glutathione transferase (GST) is a major antioxidant enzyme of ROS (reactive oxygen species) scavenging [61] and has also been shown to play a major role in detoxification of both internal and external compounds. This further supports the role of this gene in plant defense/tolerance, as there are a number of studies that describe the importance of tolerance to oxidative stress towards the survival of a plant under insect attack [18] [31] [62]. In addition to its role as an antioxidant, the ability of GST to detoxify endogenous and xenogenous compounds may also be important in plant defence/tolerance against aphid attack because phloem-feeding insects are known to inject their salivary compounds to sabotage plant defence [36] [47] [58] [59] [60].

There were two putative genes encoding heat shock proteins that were up-regulated in response to aphid feeding, but not up-regulated in response to mechanical wounding. The two putative gene products are named heat shock protein 70 (*Zea mays*) and heat shock cognate 70 kDa protein (*Oryza sativa*). Heat shock proteins are a class of functionally related proteins involved in the folding/unfolding of other proteins. It is known that their expression is increased when cells are exposed to stress. They are involved in plant abiotic stress and biotic stress [63] [64], including cold, osmotic, salt, drought stress, ultraviolet light, oxidative stress, wounding, and pathogen infection. Therefore, the up-regulation of these two heat shock proteins suggested that the grain aphid triggered a stress response in wheat.

The putative gene encoding Yrg1 (RING-type zinc finger, *Hordeum vulgare*) was up-regulated in wheat 24 h post mechanical wounding. Ascorbic acid is known to be involved in diverse physiological processes in plants and its ability to scavenge reactive oxygen species (ROS) and possible influence on cellular redox status [65] is of particular interest to this study. At1g22400, a C3HC4-type RING finger gene found in Arabidopsis was suggested to be crucial for the functionality of ascorbic acid [66] and therefore may impact ROS scavenging and regulation of cellular redox status through ascorbic acid. Therefore, the up-regulation of Yrg1 in the present study may indicate the activation of the ROS scavenging and the regulation of cellular redox status in wheat 24 h post mechanical wounding.

The putative gene encoding glutaredoxin (*Triticum aestivum*) was down-regulated 24 h post mechanical wounding. Glutaredoxin 1, a cytosolic thiol-disulfide oxido-reductase, was reported to be involved in the maintenance of redox status of proteins during oxidative stress in the pathogenesis of neurodegenerative diseases [67]. This suggests that the ability to maintain the redox status may be disrupted following the down-regulation of glutaredoxin in wheat in response to mechanical wounding. This suggests a tightly regulated antioxidant response to ROS, *i.e.* the plant controls antioxidant levels to prevent cellular damage. It is assumed that the glutathione-ascorbate cycle plays a key role in H_2O_2 detoxification. It is also known that H_2O_2 plays a key role in the synthesis of defence genes

in response to insect attacks via the octadecanoid pathway [15].

Genes involved in apoptosis were also up-regulated after aphid feeding, but were not up-regulated by mechanical wounding. Again this suggests their involvement in plant defence/tolerance towards insect attack, and towards aphids in particular. The presence of proteins involved in apoptosis such as glyceraldehyde-3-phosphate dehydrogenase subunit A suggests programmed cell death (PCD), a defence mechanism that has many features in common with the plant hypersensitive response (HR, [68]). Apoptosis is known to play an important role in the plant defence response [69] [70] as it isolates the infested cells from the rest of the plant.

The involvement of plant proteases in signalling during HR has been predicted [70] and further evidence that plant proteases are involved in defence emerged with the identification of RCR3 (Required for Cladosporium Resistance-3), a secreted cysteine protease that is required for the function of the resistance gene Cf-2 (Cladosporium fulvum resistance-2, [71]), and CDR1 (Constitutive Disease Resistance-1), a secreted aspartic protease that regulates defence responses [72].

The evidence of an increased expression of Mir1 (Maize inbred resistant-1) at the site of larval feeding and wounding [73] combined with the 80% decrease in larval growth after the overexpression of Mir1 in maize callus used for feeding showed that Mir1 is another papain-like cysteine protease (C1) that plays a role in defence against herbivorous insects. Mir1 may be directly toxic to larvae, but its proteolytic activity may also result in the release of other toxic compounds or essential signalling inter-mediates [68]. The example of Mir1 indicates that proteases may have many different roles in defence. They can act at the level of perception, signalling and execution, each according to different models as summarised in **Figure 6** [68]. Proteases are implicated in perception, signalling and execution leading to plant defence. Proteases can help perceive the invader in many different ways. They may release elicitors from the invader which can be recognised elsewhere in the plant (**Figure 6(a)**). They can be activated by specific binding elicitors, leading to the activation of downstream signalling components by proteolytic cleavage (**Figure 6(b)**). This mechanism was found to regulate a serine protease of a horseshoe crab that becomes activated upon binding of pathogen-derived lipopolysaccharide (LPS). This activation leads to a proteolytic cascade that results in a defence response that includes blood clotting. The binding of the elicitor to the protease may inhibit its activity, and the elicitor-protease complex or altered proteolytic activity might induce signalling (**Figure 6(c)**). This model may well apply to RCR3 (Required for Cladosporium Resistance-3). Signalling proteases may act by releasing positive regulators (**Figure 6(d)**) or by degrading negative regulators (**Figure 6(e)**). Proteases may also execute the defence response. They can directly degrade proteins from the invader (**Figure 6(f)**), release peptide-based toxins (**Figure 6(g)**), or activate enzymes from their precursor proteins (**Figure 6(h)**).

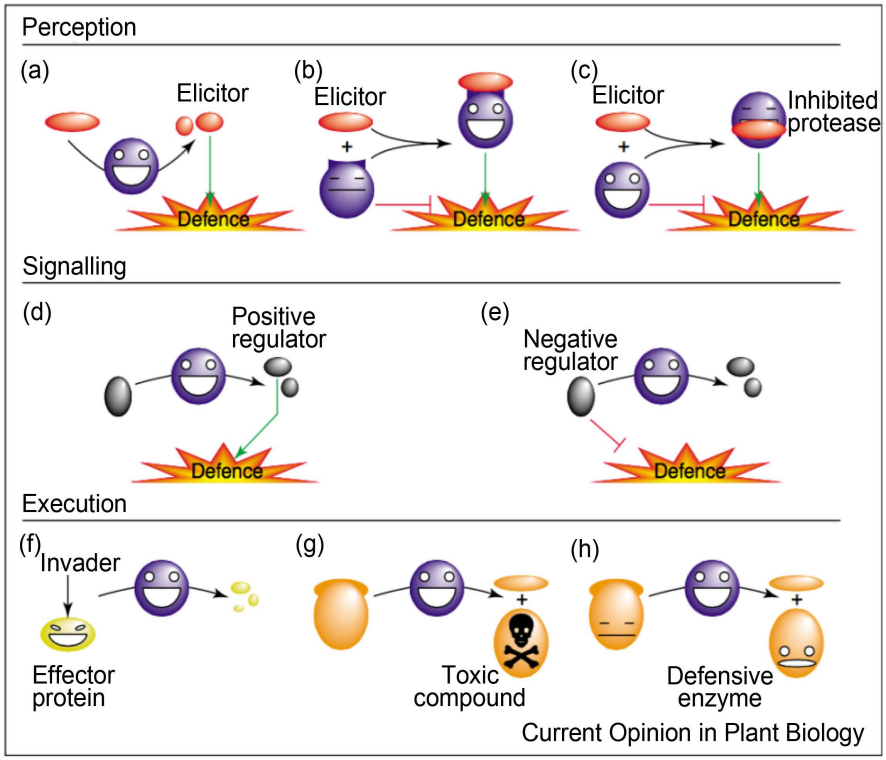

Figure 6. Models for the various roles of proteases in plant defence. (a)-(c) Proteases (blue balls) may act in perception, (d), (e) signalling or (f)-(h) execution. Green arrows and red t-bars indicate positive and negative signalling to defence responses, respectively.

Two cysteine proteases were found to be up-regulated in the subtractive library of aphid-infested vs control. As a proteolysis enzyme, cysteine protease is known to be involved in plant defence [12] [68] [74] [75].

The up-regulation of the gene encoding ubiquitin-conjugating enzyme spm2 was also of much interest because ubiquitin conjugation is a major regulator to stress response by modulating the activity of stress-responsive proteins required for adaptation to stress, for example, E3 ubiquitin ligase is involved in regulating drought and salinity stress through abscisic acid signalling [40] [76].

In the present study, real-time PCR was used in an attempt to verify the up-regulation of specific transcripts in response to aphid feeding. The housekeeping gene GAPDH was used as internal marker gene. Unfortunately, the two target genes investigated, namely GST and tritican gamma showed no significant difference in transcript levels between the aphid-infested and control wheat (data not presented). There are two possibilities that may account for this: 1) The qRT-PCR failed to work. This can be caused by primer design errors, amplification of non-target DNA and/or poor choice of the housekeeping gene (GAPDH). RNA samples used to verify the two target genes were quantified using a Nanodrop quantification device. This device cannot detect possible DNA contamination in RNA samples and the amplification of these DNAs can interfere with the qRT-PCR results. Furthermore, GAPDH is a housekeeping gene commonly used

for qRT-PCR and was used in this verification. In order to verify the up-regulation of GST and tritican gamma after 24 h aphid infestation, a housekeeping gene with no expression changes under the treatment should be used. However, it was noticed after the verification that GAPDH was one of many genes detected to be down-regulated after the treatment. This cannot explain why the up-regulation of target genes was not detected under the background of a down-regulated housekeeping gene. However, an alternative housekeeping gene with no expression changes has to be used in future attempts of this verification. 2) The original subtractive hybridisation was not entirely successful. This can be caused by possible inequality of the amount of mRNA used in samples that were being compared, due to possible contamination of other RNA residue or DNA residue. The contamination cannot be detected by the Nanodrop quantification device, so this contamination is a possible factor to consider in the explanation of this failed attempt.

Commercial wheat Claire does exhibit some degree of tolerance towards aphid attack, but this was not statistically significant as demonstrated in bioassay data [2]. The present study suggests that: 1) wheat (cv. Claire) over-express or switch on photosynthesis genes in order to boost its photosynthetic capacity to copy with the nutrition loss from aphid attack; 2) at least one antioxidant enzyme (GST) is up-regulated in response to aphid attack, which suggests that the plant is able to respond to oxidative stress caused by insect attack, although the level of antioxidant expression might not be enough to cope with the oxidative burst caused by insect feeding; 3) Two cysteine proteases were up-regulated in the subtractive library of aphid-infested vs control and they are known to be involved in plant defence [12] [68] [74] [75]; 4) The up-regulation of the gene encoding ubiquitin-conjugating enzyme spm2 in the subtractive library of aphid-infested vs control showed an emphasis on the regulation and adaptation of stress; 5) the commercial wheat cultivar Claire appears to lack a specific response to insect pests, or at least to aphids. Another study was carried out with Russian wheat aphid (RWA, *Diuraphis noxia*, Mordvilko) infested wheat plants, in which an RWA induced β-1,3-glucanase activity in resistant wheat cultivars closely resembled defence responses during pathogenesis and seemed to be part of a general defence response like the hypersensitive reaction (HR, [27] [77]). However, commercial wheat does exhibit a general stress response and some level of defence response. These responsive genes are not insect specific and cannot be used for the improvement of crop protection. However, because of the findings of potentially useful aphid responsive genes in diploid wheat (*Triticum monococcum*, 3) and durum wheat (*Triticum durum*, [77]), further study is required to investigate the potential of aphid-responsive genes in diploid wheat in the breeding (traditional or molecular) of commercial wheat in order to improve the resistance/tolerance of commercial wheat cultivars to grain aphids.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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