

Full Length Research Paper

Differential protein expression in maize (*Zea mays*) in response to insect attack

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Maize (*Zea mays*) is a major food staple in sub-Saharan Africa. However, yields are constrained by insect pests. Insect feeding induces a number of changes in genes encoding different proteins and the plant's response can either be direct or indirect, or both. In this study, maize plants were infested with two insects with different feeding strategies (*Spodoptera littoralis*, chewing insect and *Busseola fusca*, stem borer) to investigate differential protein expression using the Proteomics technique. Infestation of *S. littoralis* (3rd instar larvae) resulted in 14 spots being up-regulated and 7 being down-regulated. Similarly, infestation of maize with *B. fusca* (3rd instar larvae) resulted in 12 spots being up-regulated and 9 spots being down-regulated. Interestingly, of those up-regulated only 9 were common to both insects, with only 4 common to both in terms of down regulation. Infestation of maize with *S. littoralis* resulted in a greater number of spots being up-regulated and less being down-regulated compared to maize infested with *B. fusca*. Unfortunately, we were unable to identify the proteins represented by these spots.

Key words: *Busseola fusca*, down-regulation, expression, proteomics, *Spodoptera littoralis*, up-regulation.

INTRODUCTION

Insect feeding induces a number of changes in plants, including those responsible for direct and indirect defence responses (Lawrence and Novak, 2004). Furthermore, defences may be classified as being either constitutive or inducible (Gatehouse, 2002). Direct defences are commonly metabolites that interfere with insect feeding and nutrition (Kessler and Baldwin, 2002); they also include proteinase inhibitors that inactivate digestive enzymes. On the other hand, indirect defences occur when products from infested plant attract natural enemies of the attacking insect. The induced defence responses are regulated by interconnecting signal transduction pathways in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play important roles (Thomma et al., 2001). JA, SA, and ET accumulate in response to insect feeding, resulting in the activation of distinct sets of defence-related genes (Schenk et al., 2000).

Insects with different feeding strategies affect the induction of plant genes in different ways; some induce the emission of volatiles in maize (*Zea mays*) and activate the wound signalling pathways, which are part of the induced defence reactions (Walling, 2000). In *Arabidopsis*, the quality and timing of the signal molecules SA, JA, and ET have been shown to be insect or pathogen – dependent (De Vos et al., 2005). The wounding of potato leaf tissue induces both local and systemic responses result in the accumulation of protease inhibitors (PI), pathogenesis-related (PR) proteins, RNAs or proteins. Proteinase inhibitors are normally produced in low levels in plants, their levels, however, increase when the plant is attacked by insects or suffer mechanical damage (Rakwal et al., 2001). These molecules can cause amino acid deficiency resulting in reduced insect growth and development, eventually leading to the death of the insect. This occurs either by inhibition of gut proteinases or due to an excessive overproduction of the digestive enzymes, reducing the availability of essential amino acids for the production of

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other proteins. Similarly, in maize, three major insect-induced sesquiterpenes have been reported; B-caryophyllene, (E), α -bergamotene and (E) - β -farnesene (Lawrence and Novak, 2004) while monoterpenes and diterpenes are produced in the plastid, the sesquiterpenes are produced in the cytosol.

Plant degradative enzymes are also induced by insect feeding. For example, arginine and threonine deaminase, which degrade arginine and threonine respectively are induced by insect feeding (Chen et al., 2004) while asparaginase has been shown to be up-regulated in resistant plants during insect feeding (Liu et al., 2007). Interestingly, most of these remain active and stable after they are ingested into the insect gut (Chen, 2008). While such details have been worked out in specific systems, very little is known regarding lepidopteran (both leaf feeders and stem borers) defence in maize.

Ecologists have long understood that plants exhibit multi-mechanistic resistance towards herbivores, but the molecular mechanisms underpinning these complicated responses have remained elusive (Baldwin et al., 2001). However, recent studies investigating the plant's herbivore-induced transcriptome, using microarrays and differential display technologies, have provided novel insights into plant-insect interactions. The jasmonic acid cascade plays a central role in transcript accumulation in plants exposed to herbivory (Hermsmeier et al., 2001). A single microarray based study revealed that the model plant *Arabidopsis* undergoes changes in levels of over 700 mRNAs during the defence response (Schenk et al., 2000). In contrast, only 100 mRNAs were up-regulated by spider mite (*Tetranychus urticae*) infestation in lima bean (*Phaseolus lunatus*); although, a further 200 mRNAs were up-regulated in an indirect response mediated by feeding-induced volatile signal molecules (Arimura et al., 2000). The effect of insect herbivore may be easily seen on the plant's response over that caused by mechanical tissue damage. Analysis of timing, dynamics and regulation of the expression of 150 genes in leaves of *Arabidopsis* showed that many genes strongly induced by mechanical damage were induced less, or not at all, when the plant was attacked by the lepidopteran pest *Pieris rapae*. The studies of Baldwin et al. (2001) on the interaction between insect herbivores and tobacco (*Nicotiana attenuata*) have provided new insights into the molecular basis of plant defense. They estimate that approximately 500 mRNAs constitute the insect-responsive transcriptome in tobacco (Hermsmeier et al., 2001). However, many of these genes are of unknown function, and many changes in gene expression do not represent induction of defence-related proteins. Photosynthetic genes, for example, are down-regulated in tobacco plants in response to insect attack. Further microarray analysis (Hui et al., 2003) has demonstrated putative up-regulation of defence-associated transcripts and down-regulation of growth associated transcripts. This analysis provided evidence for the simultaneous activation of

salicylic acid, ethylene, cytokinin and jasmonic acid-regulated pathways during herbivore attack. Similar co-activation of numerous signaling cascades in response to various stresses has been found in *Arabidopsis* (Chen et al., 2002) and supports the idea of a network of interacting signal cascades. Microarray analysis also identified direct defensive responses in dramatic increases in PI transcripts, and increases in transcripts encoding putrescine N-methyl transferase, which catalyses the first committed step of nicotine biosynthesis, as well as metabolic commitment to terpenoid-based indirect defenses. Although, it may be argued that more information can be obtained by studying gene expression at the level of the proteome, the vast majority of studies that have been carried out to identify differentially expressed genes in plants in response to insect herbivory, have focused at the level of the transcriptome. However, one such study has investigated differential expression at the proteome level, comparing this with the appropriate transcript levels. In this study, the proteins present in phenolic extracts and in a nuclear fraction of *N. attenuata* leaves elicited by insect attack (*Manduca sexta*) were characterized using 2DE and MALDI (Giri et al., 2006). Phenolic extracts yielded approximately 600 protein spots, many of which were altered by elicitation, whereas nuclear protein fractions yielded approximately 100 spots, most of which were unchanged by elicitation. In general, proteins shown to be upregulated were involved in primary metabolism, defense, and transcriptional and translational regulation; those that were down regulated were involved in photosynthesis. Based on their results, the authors concluded that the response of the plant's proteome to herbivore elicitation is complex, and that integrated transcriptome-proteome-metabolome analysis is required to fully understand this ubiquitous ecological interaction.

Gene expression can be studied in several levels: DNA micro arrays, real-time polymerase chain reaction and northern blotting are the conventional methods to investigate changes at the transcript level (mRNA abundance). It is however, important to investigate gene expression of an organism or tissue at the level of the proteome for a number of reasons. Looking at the transcript level alone does not give the full picture as regulation of gene expression also occurs translationally and post-translationally (Saravanan and Rose, 2004). The correlation between mRNA and the corresponding proteins has been shown to be very poor (Ideter et al., 2001). Also, there are as many as 300 post-translational modifications that can occur physiologically (Witze et al., 2007). These modifications can result in a dramatic increase in protein complexity without any increase in gene expression (Saravanan and Rose, 2004). Proteomics allows the analysis of protein populations in a tissue, cell or subcellular compartment (Van Wijk, 2001). This method was applied in this study to gain a better understanding of maize endogenous responses to insect

pests for a directed strategy in crop improvement.

Maize is a major staple food in sub-Saharan Africa. However, its yield is constrained by pests and diseases (George, 2009; George et al., 2008). Little is known about the interaction between maize and either chewing and boring insects. This study was carried out to investigate differential protein expression in maize following infestation with both a chewing (*Spodoptera littoralis*) and a boring insect (*Busseola fusca*) attack, with a long term view for developing directed strategies for breeding maize for resistance to these devastating insect pests.

MATERIALS AND METHODS

Insect material

B. fusca larvae were obtained from the International Centre of Insect Physiology and Ecology, Nairobi, Kenya as first instar larvae; larvae were maintained on an artificial diet as previously described (Onyango and Ochieng-Odero, 1994). *S.littoralis* larvae were obtained from Science Laboratory, York, UK as first instar larvae and maintained on oil seed rape (*Brassica rapus*) plants. All the insects were kept in controlled environmental chambers under the following conditions: 80% RH, 25°C, L12:D12. Work was carried out under Defra Licence No: PHL 163/5509(11/2006).

Plant materials

Maize plants (supplied as seed by Monsanto Company, St Louis, USA) were grown in John Innes No 2 under controlled conditions at 25 ± 2°C, L16:D8. The plants were infested with third instar *B. fusca* and *S.littoralis* when they were 28 days old. For each insect species, five plants were infested, each with one third instar larva. Leaf tissues were harvested 24 h after infestation from plants showing signs of tissue damage and flash frozen in liquid nitrogen. Non-infested plants served as controls. Comparable tissues, in terms of age and position, were sampled from both experimental and control plants so as to minimise developmental effects.

Protein preparation

Two grams of leaf tissue were ground in liquid nitrogen and total proteins were precipitated using the TCA/acetone precipitation. Briefly 10 ml of TCA/acetone (+0.07% 2-mercaptoethanol) was added to the plant material and incubated overnight at -20°C. This was centrifuged at 16000 g for 10 min at 4°C. The protein pellets were washed five times in ice cold acetone (+0.07% 2-mercaptoethanol) and then dried under vacuum. The pellets were resuspended in resolubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 0.5% ampholytes, and protease inhibitor cocktail, SIGMA). The proteins were solubilised by sonication for 5 min. Samples were then centrifuged at 16000 g for 30 min at 4°C to pellet insoluble proteins and cell debris. The supernatant was collected and the protein quantified using 2D Clean Up Kit according to the manufacturer's instructions (GE Bioscience).

The 2-DE PAGE

Each protein (400 µg) sample was applied to an IPG strip (18 cm, linear pH 3 to 10) (Immobilized dry strip, GE Biosciences) with rehydration buffer (Destreak rehydration solution, GE Bioscience).

After rehydration the strips were subjected to isoelectric focusing using the IPG-phor system (GE, Bioscience). The strips were then incubated in equilibration buffer (50mM Tris-HCL, pH 6.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulphate) containing 1% DTT for 15 min, and alkylated for 15 min in equilibration buffer containing 4.5% iodoacetamide. Proteins were then separated according to their molecular mass by sodium dodecyl polyacrylamide gel electrophoresis on a 12% gel using stained in colloidal Coomassie brilliant blue.

Image and data analysis

Wet, Coomassie stained gels were scanned using a LabScan 5.0 (GE Healthcare) at a resolution of 600 dots and 12-bits per inch. Image treatment, spot detection, and quantification were done using the Progenesis SameSpots software package (Non Linear Dynamics, Newcastle, U.K.). 2-D gels of the control and treatment groups were analysed. The gels were produced as a single batch using identical solutions, loaded equivalently and electrophoresed under the same conditions. Digitized images of stained gels were aligned using Progenesis SameSpots (Nonlinear Dynamics, Newcastle, UK), which addresses the problems of missing values and reduces the variance in spot volume across biological or technical replicates by applying the same spot outline to each gel in the experiment. Briefly, prominent spots were used to manually assign approximately 60 vectors to each gel image and the 'automatic vectors' feature of the software was used to add additional vectors, which were manually verified. The average total number of vectors per gel was 300 ± 26 and these were used to warp the images and align the spot positions to a common reference gel. Spot detection performed on this reference gel was edited and artefacts removed. The resulting spot outlines were applied consistently to each parent image and verified, producing a dataset with no missing values. Spot volume, expressed relative to total spot density, was used to identify spots that differed significantly in normalised volume between control and treated samples. The molecular masses of protein on gels were determined by co-electrophoresis of Mark 12 standard protein markers (Invitrogen) and pI of the protein spots on 18 cm gels were estimated by relating position on the second-dimension gel to its original position on the Immobiline Dry Strip as per the manufacturers recommendations (GE Healthcare), this was further calibrated by visual inspection of the migration of the protein subunits of RUBISCO to the expected pI. Spots were determined to be significantly up- or down regulated when P<0.05.

In gel digestion of protein spots

Spots selected by Progenesis were manually punched from the gel and then destained using dH₂O. For digestion protocol, each sample was completely destained using 50 mM ammonium hydrogen carbonate and acetonitrile at a 1:1 mix for 15 min before being dehydrated using acetonitrile. Samples were then rehydrated using 50 mM NH₄HCO₃ for 15 min before addition of an equal volume of acetonitrile to dehydrate before being air-dried. 10 mM DTT in 25 mM NH₄HCO₃ was added to the samples and then incubated for 20 min at 56°C. The liquid was then removed from the gel pieces and an equal amount of 55 mM IAA in 25 mM NH₄HCO₃ was added. All remaining liquid was removed and the samples were then dehydrated with acetonitrile and then air dried prior to addition of trypsin. Modified sequencing grade trypsin (Promega) was diluted to 20 ng/µl with 25 mM NH₄HCO₃ and 2 to 3 µl was added. Samples were incubated at 37°C overnight. Additional 25 mM NH₄HCO₃ was added for 30 min to rehydrate the samples, if required. Peptides were extracted from the gel pieces by sonication for 10min. An amount of 3 µl of the 50% acetonitrile, 1% trifluoroacetic

acid was added and followed by 10 min of sonication. Supernatants were collected and pooled.

MALDI-TOF-MS

Samples were mixed with matrix and spotted onto a steel target plate. The matrix was a saturated solution of 100 mg α -hydrocinnamic acid, 33% acetonitrile, 66% 0.1% TFA. The matrix solution was sonicated for 10 min after addition of the acetonitrile and then again for 10 min on the addition of the TFA. The solution was then centrifuged at 8000 g for 10 min at 4°C. Samples were mixed in a 1:1 ratio with the matrix solution. 1 μ l was spotted onto the target plate and allowed to air dry. The samples were then analysed using MALDI-TOF-MS (Bruker Daltonics). The equipment was calibrated against a set of peptide standards. A minimum of 1000 hits were used for the calibration. A size range of 400 to 5000 Da was the calibrated size range. Each spot was examined manually with the laser power between 30 and 50%. Each spot had a minimum of 1000 hits recorded. Maximum intensities were in the 1×10^5 ppm range. Peak masses were calibrated in the Flex – Analysis software (Bruker daltonics), using a supplied script. Peaks in the mass range 500-4000da only were identified.

MASCOT searches

The generated peak list for each sample was queried against the Swiss-Prot database using the MASCOT search engine. The *Arabidopsis thaliana* database was queried using 2 missed cleavages, carbamidomethylation (C) and oxidation (M) as modifications. Peptide tolerance was set to ± 1.2 Da. Protein scores, intensity coverage and sequence coverage were recorded in Biotools (Bruker).

RESULTS

Maize plants (28 days-old) were infested with either third instar *B. fusca* larvae or third instar *S. littoralis* larvae. Leaves were harvested after 24 h, ensuring that comparable tissues, in terms of age and position, were sampled from both experimental and control plants (non-infested) so as to minimise any developmental effects.

Protein samples were prepared as aforementioned and three gels were run for each of the four different groups (maize + *B. fusca*; maize - *B. fusca*; maize + *S. littoralis*; maize - *S. littoralis*). The protein spots were resolved on a 2-DE gel with colloidal Coomassie brilliant blue staining. In the first dimension an immobilised pH gradient strip (pH 3 to 10) was used and 12% SDS-gel electrophoresis used for the second dimension. Graphic analyses of the multiple gels for the different experimental groups were displayed as composite gel images (Figures 1 to 2). Under these conditions a total number of approximately four hundred spots were resolved, of which only approx 20 were reproducibly changed following insect feeding, although not all were common between the two species. When investigating differential expression, only 2-fold differences in protein spot volume/density were considered; differences in significance in expression levels were determined by ANOVA.

Differential protein expression in response to *Busseola fusca*

Infestation of 28-days old maize plants by third instar larvae of the stem borer *B. fusca*, resulted in the up-regulation of 12 protein spots, with 9 being down regulated; these are identified in Figure 1 as: 2, 3, 5, 6, 10, 12, 14, 15, 18, 19, 21, and: 1, 4, 7-9, 11, 13, 16, 17, 20, respectively. Although proteins that were differentially expressed in response to herbivory covered the full range of pls resolved, the majority exhibited pls in the range of 5 to 8; these proteins varied in molecular weight, from > 14 kDa to approximately 100 kDa, with the majority having higher M_r values.

Differential protein expressions in response to *Spodoptera littoralis*

Infestation of 28-days old maize plants by third instar larvae of the chewing insect *S. littoralis*, resulted in the up-regulation of 14 protein spots, with 7 being down regulated; these are identified in Figure 2 as: 2, 4-8, 10, 12-14, 16, 19-21 and: 1, 3, 9, 11, 15, 17, 18, respectively. As seen with *B. fusca*, while those protein spots that were differentially expressed in response to *S. littoralis* covered both the full range of pls and molecular weights resolved, the majority exhibited pls in the range of 5 to 8, with proteins resolved across the full mass range.

Interestingly, of those protein spots up-regulated in maize in response to infestation, only 9 were common to both insects, with only 4 common to both in terms of down regulation. These comparisons are clearly presented in the Venn diagram (Figure 3) where the numbers in the circle indicate the protein spots that are differentially accumulated (a, representing down-regulation; b, representing up-regulation) and the numbers in overlapping areas representing protein spots with similar patterns of accumulation.

Figure 4 shows magnified gel sections for both insects; as can be clearly seen; Spot number 5 is significantly up-regulated in both species, although no proteins in common appeared to be down-regulated. However protein Spot 16 is an example of a protein that is down-regulated in response to *B. fusca* feeding, but up-regulated in response to *S. littoralis* while Spot 21 shows the reverse effect.

Identification of differentially expressed proteins

Selected differentially expressed protein spots, as identified by Progenesis software, were digested in gel, and subjected to MALDI-TOF mass spectrometry to generate peptide mass fingerprints (PMFs). However, no positive protein identifications were obtained when queried against the Swiss-Prot data base. Unfortunately

3pI

10pI

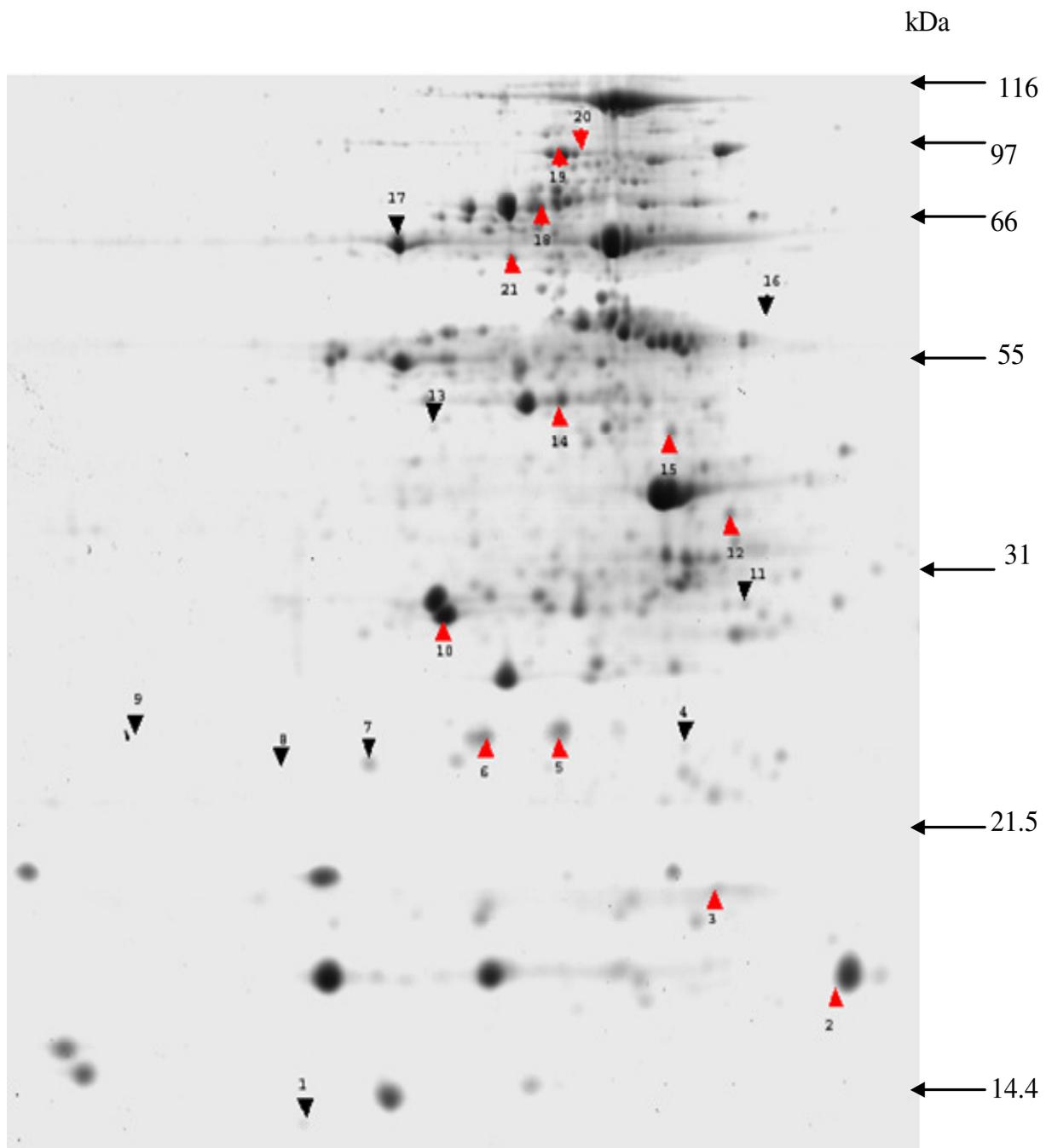


Figure 1. 2DE image of leaf proteins from 28 days old maize plants following infestation by 3rd instar *B. fusca* larvae. The numbers refer to proteins that are differentially expressed. 3gels representing composite image.

fund was not secured to re-run the samples.

DISCUSSION

Herbivory is known to elicit both local and systemic

responses in plants; furthermore, these can either be direct or indirect. Direct defences are compounds that exert repellent, anti-nutritive, or toxic effects on the herbivore (Howe and Jander, 2008), while indirect defences are mainly volatiles that attract natural enemies of the herbivore (Kessler and Baldwin, 2002) such as

3pI

10pI

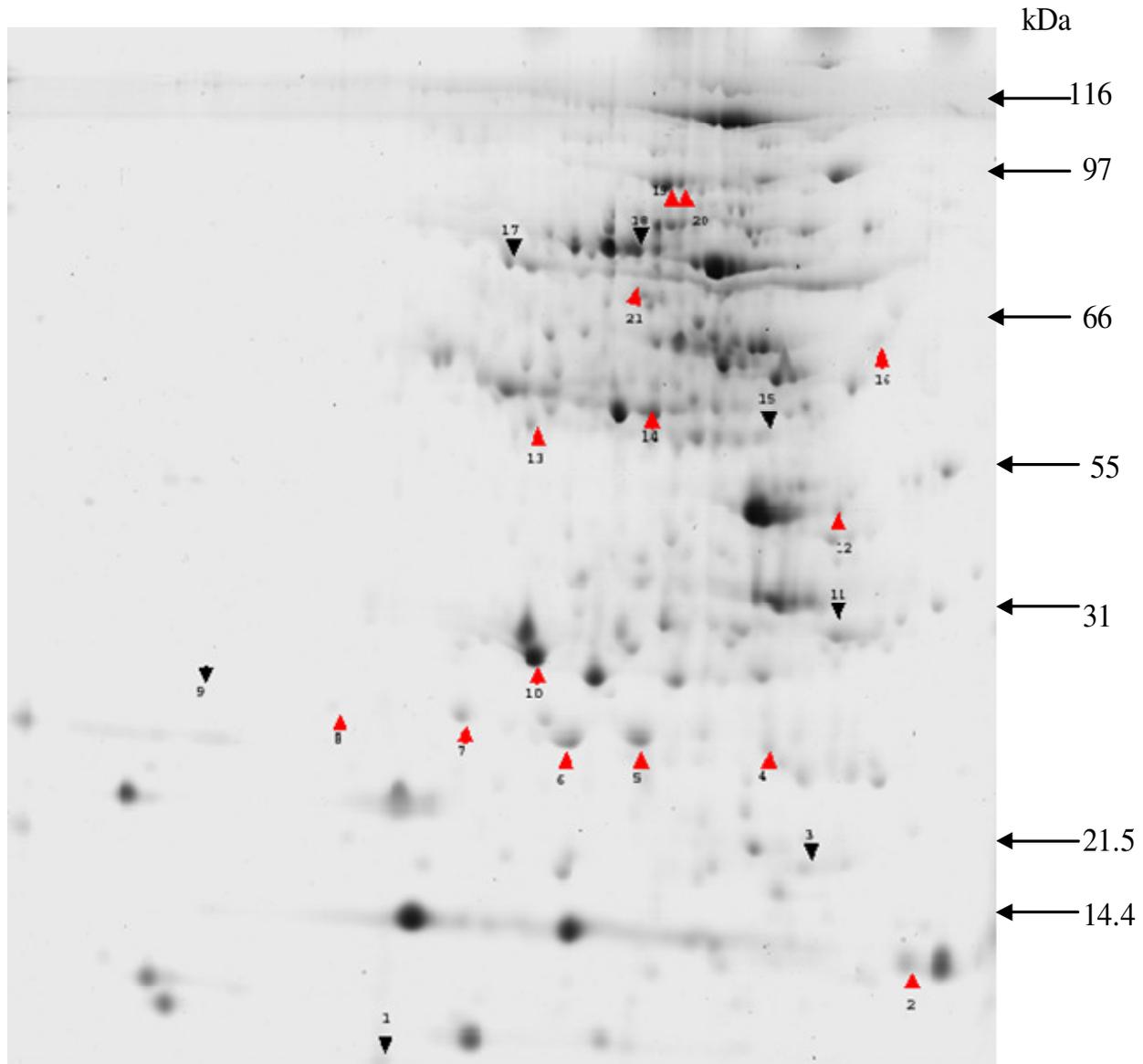


Figure 2. 2DE image of leaf proteins from 28 day old maize plants following infestation by 3rd instar *S. littoralis* larvae. The numbers refer to proteins that are differentially expressed. Composite image representing 3 gels

terpenes, green leaf volatiles, and ethylene (Howe and Jander, 2008). However, volatiles can also repel potential herbivores due to the toxic compounds released in the air (Mello and Silva-Filho, 2002). β -Galactosidase from *Pieris brassicae* caterpillars and a low Mr fatty acid derivative, N-17-hydroxylinoyl-L-glutamine (volicitin) from *Spodoptera exigua* (beet army worm) oral secretions can trigger the synthesis and emission of volatile chemical signals in plants. Several genes have been reported to be

activated by volicitin, systemin or volatiles released from attacked plants. The genes encoding indole -3 glycerol phosphate lysate (IGL), that catalyses the formation of free indole, (Frey et al., 2000), and allene synthase (AOS), that catalyse the first step in JA biosynthesis (Reymond et al., 2000), are induced by herbivory.

Infestation of maize with beet army worm (BAW; *S. exigua*) has been reported to cause the production of volatile compounds that attract the generalist parasitoid

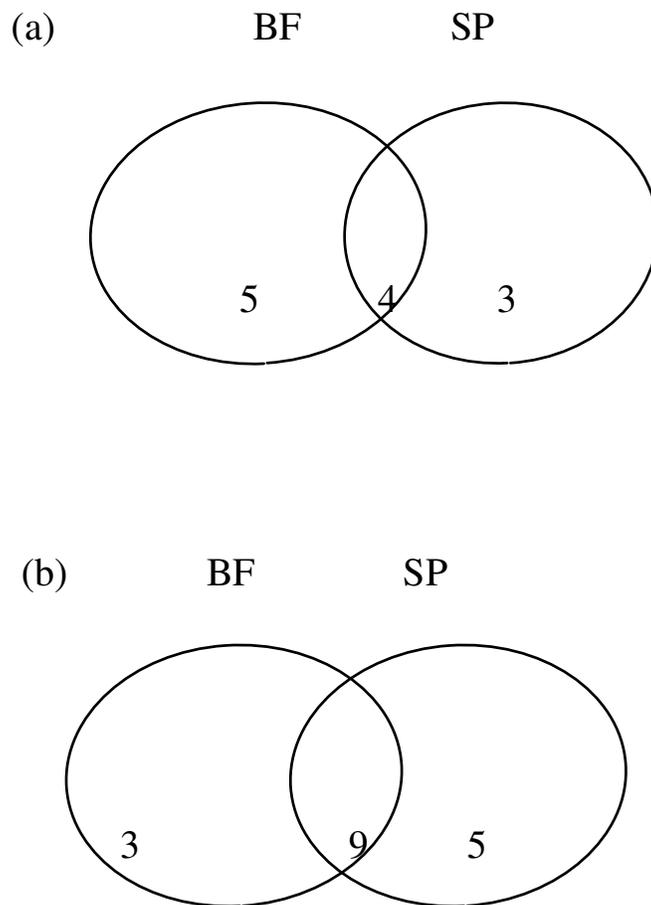


Figure 3. Comparison of differentially expressed proteins in maize leaves as a result of feeding by two different insect species. The Venn diagram presents the number of protein spots that exhibit differential accumulation patterns among different insects compared to the control leaves. Shown are the comparisons between *B.fusca* (BF) infested maize leaves and *S.littoralis* (SP) infested maize leaves; (a) represents down-regulation; (b) represents up-regulation. The numbers in the circle indicate the protein spots that are differentially accumulated and the numbers in overlapping areas represent protein spots with similar patterns of accumulation.

Cotesia marginiventris to the BAW larval host (Turlings et al., 1990). It has also been reported that volatiles released by tobacco plants attract predatory bugs to tobacco hornworm eggs (Kessler and Baldwin, 2001). Each plant is said to produce an herbivore-specific blend of volatiles in response to a specific elicitor from a particular herbivore species. Artificial leaf damage of growing maize plants can increase the concentration of DIMBOA (hydroxamic acid), relative to the control plants. Such damage leaves negatively affect the growth and survival of *Rhopalosiphum padi*.

Proteinase inhibitors are able to inhibit insect digestive proteases (Chen, 2008). The inhibition of gut proteases results in amino acid deficiencies and this negatively

affects the growth and development of the herbivore (Lison et al., 2006). For example, maize plants have been shown to overproduce a 33 kDa cysteine protease inhibitor at the site of insect attack (Pechan et al., 2000), retarding insect growth by up to 80% (Pechan et al., 2002). It has been demonstrated that the leaves of *Populus tremuloides* accumulate trypsin inhibitors within two days following insect damage and that this helps reduce further insect attack (Haruta et al., 2001). Herbivory has also been reported to suppress some plant genes. Photosynthesis, latex and cytoskeleton related genes have been shown to be suppressed in *N. attenuate* as a result of *Manduca sexta* attack (Hermsmeier et al., 2001).

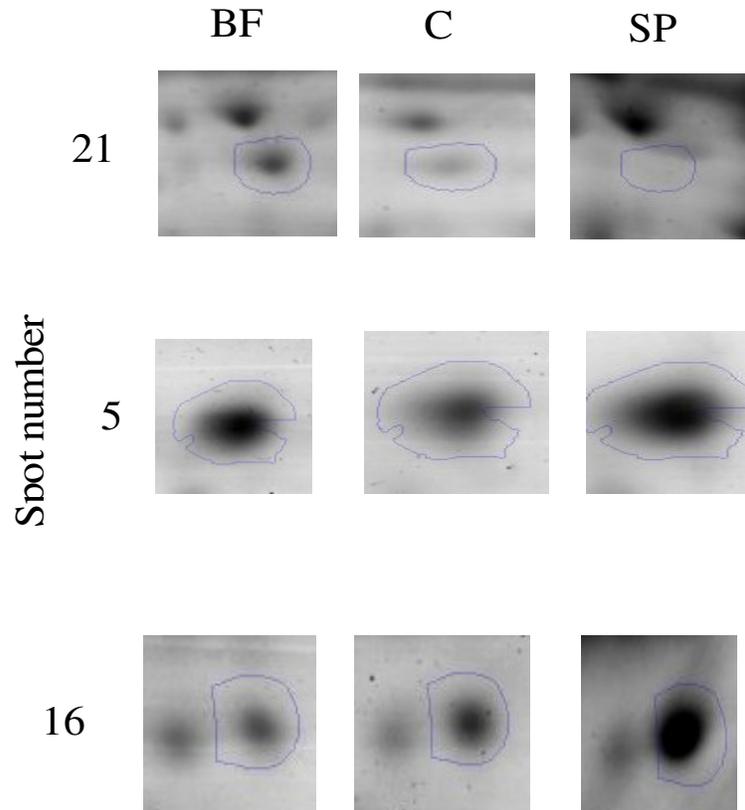


Figure 4. Magnified areas of the two dimensional gels. The three sections show magnified areas of *B. fusca* (BF), control (C), and *S. littoralis* (SP) fed maize plants.

Thus, while numerous studies have been carried out to investigate the products and intermediaries of the various wound response pathways known to be present in plants as part of the general defence mechanism (Ferry et al., 2004; Gatehouse, 2002), and more recently gene expression at the transcriptional level, studies carried out on maize, as opposed to model systems, have been somewhat limited. Furthermore, only one such study appears to have investigated gene expression at the level of the proteome, albeit in the plant model *A. thaliana*. This study thus attempted to address this gap by studying changes in the proteome profile of an economically important crop, maize (*Z. mays*), in response to two devastating insect pests which possess different feeding behaviours. Although, all phytophagous insects inflict mechanical damage on plant tissues, the nature and level of injury varies greatly depending on the feeding behaviour (Howe and Jander, 2008) and this may result in differential gene expression. Caterpillars that damage leaf tissues are thought to induce a stronger reaction in plants than stem borers (Turlings et al., 1990). In this study, maize plants attacked by *B. fusca* differentially expressed 21 spots. Of these protein spots, 12 were up-regulated and 9 were down-regulated. Similarly, maize infested with *S. littoralis* also caused differential

expression of protein spots, where 14 spots were up-regulated and 9 were down-regulated. Interestingly, 9 spots in common were up-regulated in both insects, with only 4 common to both in terms of down-regulation. These results would suggest that the two different feeding strategies did not result in marked differences in the induction of maize genes. However, infestation of maize with *S. littoralis* resulted in a greater number of spots being up-regulated and less spots being down-regulated. Other studies have reported that chewing insects induce a stronger reaction in plants than stem borers (Turlings et al., 1998).

There are, however, limitations to the use of 2DE as a method of protein separation, most notably its dynamic range. Proteomes can contain both high and low abundant proteins and if they are analysed on 2-DE only highly abundant proteins will be detected. Thus, low abundant proteins are normally under-represented although, most of these proteins are regulatory and signal transduction proteins (Rakwal et al., 2001). Prefractionation techniques have been used to enrich low abundant proteins (Kim et al., 2001). The technique, however, is time consuming and costly since different fractions have to be run on the gel. The other major limitation is that protein content and concentrations in

plant cell are low compared to those obtained from microorganisms and animals (Jacobs et al., 2000). Plant cells also contain a number of interfering compounds (salts, proteases, phenolics, terpenes etc) which exert a negative effect on protein extraction and IEF. Furthermore, phenolics and proteases have been reported to modify proteins by causing changes to their molecular weights and isoelectric points (pI) (Jacobs et al., 2000).

Following protein separation by pI, subsequent protein identification in this study was carried out using MALDI-MS. However, peptide mass fingerprinting (PMF) does not provide amino acid sequence information and hence cannot be used for *de novo* sequencing. This means that for proteins to be identified they must be in the database. Secondly, MALDI peptide fingerprinting information cannot be used to analyse post-translational modifications (Powell and Timperman, 2004). Most proteins undergo post-translational modifications in order for them to be functionally active. Lastly, information provided by MALDI peptide fingerprinting does not work well with protein mixtures (Powell and Timperman, 2004). However, despite these limitations, MALDI-MS remains a powerful analytical tool for studying the proteome and while the genome data base may not be available for this crop; there are extensive EST databases available as well as a fully annotated rice genome database. In this study, although, PMFs were obtained, none had high scores with any of these available databases. Unfortunately, due to constraints on time, it was thus not possible to identify the proteins represented by the different spots.

For future studies, more sensitive staining techniques such as silver staining could be employed for the construction of high resolution 2-DE maps. In this study, the leaves were harvested 24 h after infestation. It will be of interest to have more time points to capture those proteins that may have been expressed earlier but were later degraded. Strips with narrow pH ranges could also be used to capture as many proteins as possible in the different pH ranges.

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