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RESEARCH PAPER

Application of Bruchin B to pea pods results in the up-regulation of *CYP93C18*, a putative isoflavone synthase gene, and an increase in the level of pisatin, an isoflavone phytoalexin*

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Abstract

Bruchins, mono and bis (3-hydroxypropanoate) esters of long chain α,ω-diols, are a recently discovered class of insect elicitors that stimulate cell division and neoplasm formation when applied to pods of peas and certain other legumes. Differential display analysis resulted in the identification of an mRNA whose level was increased by the application of Bruchin B to pea pods. The corresponding amplification product was cloned and sequenced and a full length cDNA sequence was obtained. This cDNA and the gene from which it was derived were assigned the name *CYP93C18* based upon sequence similarities to the cytochrome P450 monooxygenase CYP93C subfamily, which contains isoflavone synthase genes from legumes. RNA gel blots and quantitative RT-PCR demonstrated that expression of *CYP93C18* increased within 8 h of bruchin treatment to a maximum of 100–200-fold of the level in untreated pods, and then declined. The up-regulation of *CYP93C18* was followed by an increase in the level of the isoflavone phytoalexin, pisatin. Pisatin was detectable in the bruchin-treated pods after 16 h and reached a maximum between 32 h and 64 h. This, the first report of induction of phytoalexin biosynthesis by an insect elicitor, suggests that Bruchin B not only stimulates neoplasm formation, but also activates other plant defence responses.

Key words: Bruchin, *CYP93C18*, cytochrome P450, insect elicitor, *Neoplastic pod*, phytoalexin, pisatin, *Pisum sativum*.

Introduction

Bruchins, mono and bis (3-hydroxypropanoate) esters of long chain α,ω-diols, are a new class of insect-derived natural products that stimulate cell division and neoplasm formation when applied to pods of peas and certain other legumes (Doss *et al.*, 2000; Oliver *et al.*, 2000). The isolation and characterization of the bruchins followed the observation that oviposition by the pea weevil *Bruchus pisorum* L. (Coleoptera: Bruchidae), an important pest of pea, on pods of peas carrying the wild-type allele of the *Neoplastic pod* (*Np*) gene resulted in browning followed by cell division and callus formation at the site of oviposition (Berdnikov *et al.*, 1992; Hardie, 1993; Doss *et al.*, 1995). The calli formed in response to oviposition reduce weevil infestation of the pea seed via both indirect and direct mechanisms (Berdnikov *et al.*, 1992; Hardie, 1993; Doss *et al.*, 2000) and the bruchins have been referred to as 'herbivore-specific elicitors' (Kessler and Baldwin, 2002).

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Abbreviations: *N*, *Neoplastic pod*; IFS, isoflavone synthase; RACE, Rapid Amplification of cDNA Ends; QRT-PCR, quantitative reverse transcriptase-PCR; UV, ultraviolet.

Direct application of a bruchin to both *Np/Np* and *np/np* pods stimulates browning and swelling and ultimately results in callus formation. The calli formed on *np/np* pods are much smaller than those seen on *Np/Np* pods and much of their mass results from cell enlargement rather than cell division; however, the minimum dose required to elicit a response on pods of either genotype appears to be about the same (Doss *et al.*, 2000). Interestingly, the *Np* gene was described 30 years ago, long before its role in insect resistance was noted, because its presence causes pods grown under greenhouse conditions to develop patches of callus (neoplasms) on their surface (Dodds and Matthews, 1966; Snoad and Matthews, 1969). Such neoplasms do not form under natural sunlight, as their formation is stimulated by the attenuation of ultraviolet wavelengths (UV) by greenhouse coverings (Snoad and Matthews, 1969), nor do they form spontaneously in the greenhouse in peas homozygous for the recessive allele (*np*).

The work reported here was undertaken as part of a larger project to evaluate the changes in gene expression that are stimulated by bruchin application to pea pods. Among the genes up-regulated was *CYP93C18*, a gene in the CYP93C subfamily of cytochrome P450 mono-oxygenases. This subfamily comprises several (legume) isoflavone synthase (IFS) genes. Isoflavone synthases catalyse the first specific step in the biosynthesis of the isoflavone phytoalexins of legumes (Steele *et al.*, 1999). Phytoalexins are antimicrobial compounds produced by plants in response to infection by plant pathogens (Hammerschmidt and Nicholson, 1999). Bruchin B application also led to the biosynthesis of pisatin, an isoflavone phytoalexin of pea (Cruickshank and Perrin, 1962; Perrin and Bottomley, 1962). It has been hypothesized that phytoalexins play a role in disease resistance, but despite considerable study such a role has not been definitively demonstrated (Hammerschmidt, 1999, 2003). The up-regulation of *CYP93C18*, and the increase in pisatin content would appear to be, along with neoplasm formation, part of the defence response brought about by application of a bruchin to pods of pea.

Materials and methods

Plant material and bruchin treatment

The pea (*Pisum sativum* L.) lines used in these studies were derived from a cross between the lines C887–332 (*Np/Np*) and I_3 (*np/np*) (Doss *et al.*, 1995, 2000) and were homozygous for either the *Np* or *np* allele. Near-isogenic lines of the F_{12} generation were used throughout the study, with the exception of the differential display analysis (F_9 near-isogenic lines). Pea plants were either grown outdoors under natural light or in a greenhouse supplemented with UV light (UVA-340, O-Panel Co. USA) to prevent spontaneous callus formation (Doss *et al.*, 2000). Pods in the late flat pod stage (Meicenheimer and Muehlbauer, 1982), approximately 3.5–5 cm in length, were treated on one side with 6 ng bruchin B [(Z)-9-Docosen-1,22-diol *bis*-(3-hydroxypropanoate) ester, $C_{28}H_{52}O_6$] (Doss *et al.*, 2000; Oliver *et al.*, 2000) in 60 µl 50% ethanol, while on the opposite side, 50% ethanol was applied or it was left untreated. An untreated sample of pod tissue was collected at the time of treatment. After the appropriate treatment period, the pods were removed from the plant, placed onto ice, and split along the suture. The seeds and the untreated portions of the pod were removed. The pod samples were frozen immediately in liquid nitrogen and stored at −80 °C.

RNA extraction, poly(A)$^+$ RNA selection and cDNA synthesis

Total RNA was isolated from the pod tissue samples (10 g for the differential display (DD) study and 0.5 g for the time-course experiments) using a guanidine thiocyanate–phenol extraction solution based upon the method of Chomczynski (1993). Poly(A)$^+$ RNA was prepared from total RNA (in the DD study) using the PolyA-Tract® system (Promega Corp., Madison, WI), following the manufacturer's protocol. First-strand cDNA synthesis (primed with an oligo $(dT)_{12\text{-}18}$ primer) was performed using *Dnase* I-treated total RNA or poly(A)$^+$ RNA, and Superscript II reverse transcriptase (all from Life Technologies, now Invitrogen Corp., Carlsbad, CA), following the manufacturer's protocol. The cDNA samples were quantified using either ethidium bromide dot quantitation (Sambrook and Russell, 2001) or with a Dyna Quant 200 fluorometer (Amersham Biosciences, Piscataway, NJ), following the manufacturer's protocol.

Differential display analysis, cloning and sequencing

Np/Np and *np/np* pea plants (F_9 near-isogenic lines) were grown in a greenhouse, under supplemental ultraviolet light (to prevent spontaneous callus formation) with controlled temperatures. Pods were treated with Bruchin B or ethanol for 24 h, collected, and poly(A)$^+$ RNA was isolated (all steps as described above). A modified, non-radioactive version (Doss, 1999) of DD (Liang and Pardee, 1992) analysis was performed using cDNA synthesized from poly(A)$^+$ RNA using pairwise combinations of 12 anchored primers T_{11}MN (M=A, C or G and N=A, C, G, T) and a set of arbitrary ten base primers, OPA-01 through OPA-20 (all from Operon Technologies, now Qiagen Operon, Alameda, CA). The amplification products were fractionated using 6% non-denaturing PAGE and visualized by silver staining. The differentially displayed band (confirmed using an independent set of pod tissues), was excised from the polyacrylamide gel, reamplified and cloned using the pGem®-T Easy Vector System (Promega Corp, Madison, WI) and sequenced at the Center for Gene Research and Biotechnology (CGRB), Oregon State University. Sequences were analysed using BioEdit Sequence Alignment Editor (Hall, 1999).

5′ Rapid Amplification of cDNA Ends (RACE)

5′ RACE (GeneRacer®, Invitrogen Corp, Carlsbad, CA) was performed following the manufacturer's protocol using poly(A)$^+$ RNA isolated from bruchin-treated *Np/Np* pods (F_9 near-isogenic lines). Nested gene-specific primers were designed based upon the sequence of the fragment obtained from DD (GT13R1: 5′-TGG GTC TCT TCC CAC TGC CCA TAC A-3′ and GT13R2: 5′-CAC ATC CTC CTT CCT GAC CCA AAC G-3′). A single amplification product was obtained and was cloned using the TOPO TA® cloning vector pCR®4-TOPO® (Invitrogen Corp, Carlsbad, CA), sequenced and analysed as above.

RNA gel blot analysis of changes in CYP93C18 expression

Total RNA was isolated as above from bruchin- and ethanol-treated pod tissue (*Np/Np* and *np/np* F_{12} near-isogenic lines) over a 32 h time-course and was denatured with glyoxal (10 µg per treatment). After fractionation by electrophoresis (1.5% (w/v) agarose gel) in the presence of ethidium bromide, the RNA was transferred (downward capillary transfer with an alkaline buffer) and fixed to a positively charged nylon membrane (Zeta Probe®, Bio-Rad), following standard procedures for RNA gel blots (Sambrook and Russell, 2001). A

radiolabelled PCR product (amplified using the primers: GT13F3 (5′-CAT GCC CAC CAT TGT TGT ATC C-3′) and GT13R3 (5′-TGC TCA CTT TCT TCG ATT TCT CC-3′)) was prepared by random primer labelling (Feinberg and Vogelstein, 1983, 1984) (RadPrime® DNA Labeling System, Life Technologies, now Invitrogen) using 50 μCi [α-^{32}P]dCTP (New England Nuclear, now Perkin Elmer) in a 50 μl reaction. The probe was passed over a gel filtration column (Micro Bio-Spin® P-30, Bio-Rad) before addition to the prehybridization solution. Hybridization of the probe to the membrane, washing, and autoradiography were carried out using standard procedures (Sambrook and Russell, 2001). The images shown in Fig. 3 were prepared using an AlphaImager® System (Alpha Innotech) and Adobe Photoshop®.

Quantitative reverse transcriptase-PCR (QRT-PCR)

Total RNA was isolated from bruchin- and ethanol-treated pod tissue over a 32 h time-course, *DNase* I-treated and reverse transcribed as above. Quantitative reverse transcriptase-PCR (QRT-PCR) was performed using the SYBR® green master mix (Applied Biosystems Inc, Foster City, CA), as recommended by the manufacturer, except that the reactions were done in 25 μl volumes and the final concentration of the primers used was 500 nM. Primers for QRT-PCR (GT13 315F 5′-GGA GAA CGT TAT GGC CCT TTG-3′ and GT13 388R 5′-ATG AGT TTG AAG GAA GAG GTT GAA GA-3′) were designed using Primer Express software (Applied Biosystems, Inc) and synthesized by Qiagen Operon. Primers (*Ps*ActF2 5′-CAC AAT TGG CGC TGA AAG AAT-3′ and *Ps*ActR2 5′-TAA TTG AGT TAA ATG TCG TCT CAT GGA T-3′), based upon the sequence of a highly conserved region of pea β-actin (PEAc9, GenBank Accession no. PSU81047), were used to prepare an amplification product that served as an endogenous control for the QRT-PCR assays.

Quantitative RT-PCR was performed on a PE Biosystems ABI Prism® 7700 Sequence Detection System (Applied Biosystems Inc) using the default program. After amplification, the PCR products were subjected to a heat dissociation program consisting of a 35 °C temperature gradient from 60 °C to 95 °C over approximately 20 min and analysed using the ABI Prism® Dissociation Analysis software (Applied Biosystems, Inc) to distinguish the target amplicon from non-specific PCR products. For the initial runs, the real-time RT-PCR products were also analysed by agarose gel electrophoresis to verify that a single amplicon of the appropriate size had been produced.

Estimations of relative changes in expression of *CYP93C18* in bruchin- and ethanol-treated pods was carried out using the Comparative C_T method (Applied Biosystems Inc, 2001). Each data point is an average of the $2^{-\Delta\Delta CT}$ values calculated for three independently performed QRT-PCR measurements (i.e. three individual plates), while on each plate, each reaction was replicated three times. Relative quantitation of gene expression depends upon the assumption that the efficiencies of amplification of the target and reference sequences are approximately equal. The absolute value of the slope of log input versus ΔCT should be less than ±0.1 (Applied Biosystems Inc, 2001). That this was the case was verified by amplification using each of the primer sets; *CYP93C18*, the target gene, and pea β-actin, the endogenous control gene, using standard curves of pod cDNA from 1 pg to 10 ng and plotting the ΔCt ($Ct_{CYP93C18}$–CT_{PsAct}) versus the log quantity of cDNA (data not shown).

Pisatin determinations

Pisatin was extracted from Bruchin B-treated pod tissue (*Np/Np* and *np/np* F_{12} near-isogenic lines) over an 80 h time-course, based on a procedure described by Schwochau and Hadwiger (1969). A 0.5 g FW portion of frozen pod tissue was ground to a fine powder in a mortar and pestle cooled with liquid nitrogen. After the powder was extracted three times with 4 ml portions of 95% ethanol, the ethanolic extracts were filtered, combined and taken to dryness *in vacuo*. The residue was taken up in 5 ml water and partitioned five times against equal volumes of hexanes. The combined hexanes fractions were taken to dryness *in vacuo*, and the residue was dissolved in 4 ml absolute ethanol. The ethanolic solution was taken to dryness under nitrogen, and the residue was redissolved in 50–200 μl of absolute ethanol. Three independent extractions were performed for each time point, the pisatin values (μg pisatin g^{-1} FW of pod tissue) are presented with the standard error of the mean of the three determinations.

Pisatin concentration was determined using HPLC with a 250×4.6 mm column packed with Adsorbosil® C-18 (5 μm) (Alltech). A flow rate of 1 ml min^{-1} with a linear gradient of 70–100% methanol was used. The gradient was preceded by 5 min at 70% methanol, and followed by 15 min at 100% methanol. Under these conditions pisatin had a retention time of approximately 8 min. Spectrophotometric (309 nm) detection was used, and estimates of pisatin concentration were based on peak area. The identity of the pisatin peak was confirmed using liquid chromatography-mass spectrometry (LC-MS), and a set of spiked samples allowed an estimate of recovery (87.5±4.9%, mean ±standard error of the mean for n=5), which was used in the estimates of pisatin level. A sample of authentic pisatin (recrystallized from heptane) was prepared from naturally infected pea seedlings using solvent partitioning, column, and thin-layer chromatography (Van Etten *et al.*, 1975). The identity of this sample, which was used for development of HPLC methods, and as a pisatin standard for HPLC determinations, was confirmed using MS (Kobayashi *et al.*, 1993).

Results and discussion

CYP93C18*, a cytochrome P450, was isolated by differential display*

Differential display, performed with cDNA populations from pea pods treated either with Bruchin B or ethanol, yielded 19 amplification products whose appearance was either bruchin treatment-specific or genotype-specific (data not shown). One of these, designated GT13, obtained with the primer combination T_{11}GT/OPA-13, appeared to have been derived from a gene that was up-regulated by bruchin treatment, with stronger up-regulation in *Np/Np* pods than in *np/np* pods. After cloning and sequencing, the length of the GT13 clone was 710 bp, which corresponded to the size of the band observed on the DD gels.

The sequence of the GT13 cDNA clone (boxed region in Fig. 1) extended from the open reading frame of the gene into the 3′ untranslated region (UTR). The full-length cDNA sequence was compiled from the sequence of the GT13 clone and that of the 5′ RACE product. The full-length cDNA, and the gene from which it was derived, were assigned the name *CYP93C18* by Dr David Nelson (personal communication) based upon motifs indicative of a cytochrome P450 mono-oxygenase and sequence similarity to isoflavone synthase genes from other members of the legume family.

CYP93C18 contains the characteristic haem-binding domain, highlighted in Fig. 1, FgsGrRmCpG, where the capital letters represent the conserved amino acids common to all classic cytochrome P450 mono-oxygenases (Schuler, 1996). Cytochrome P450 mono-oxygenases, which often

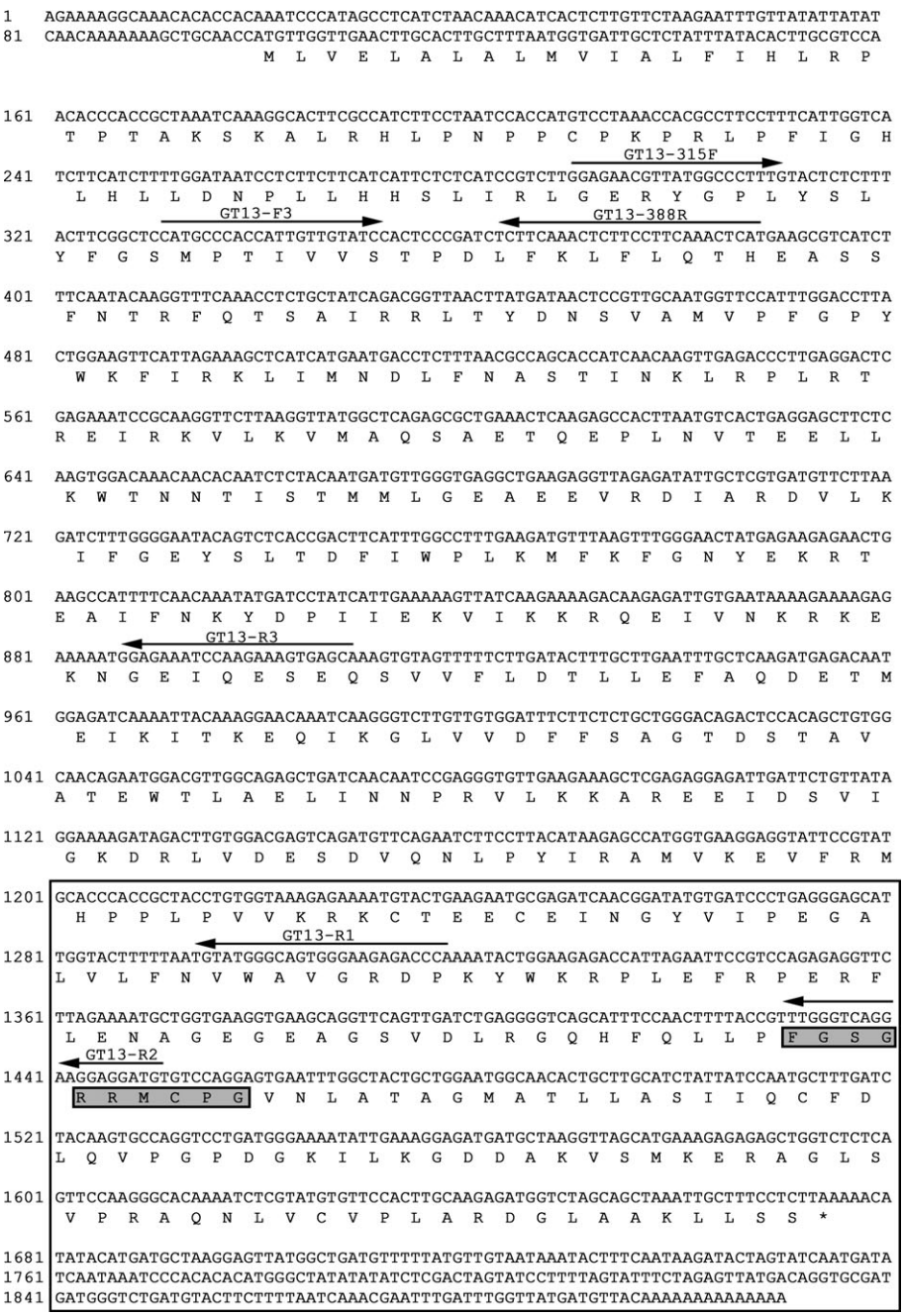

Fig. 1. The nucleotide sequence and deduced amino acid translation of *CYP93C18*. The full-length cDNA sequence was compiled from the sequence of the GT13 clone from differential display and from the sequence of the 5′ RACE product. The deduced amino acid sequence is shown in a single letter code below the nucleotide sequence. The boxed region illustrates the sequence of the GT13 cDNA clone, isolated from Bruchin B-treated pod tissues by differential display. Primers for 5′ RACE (GT13-R1 and GT13-R2), QRT-PCR (GT13-315F and GT13-388R) and to generate the probe for the RNA gel blot (GT13-F3 and GT13-R3) are indicated above the nucleotide sequence. The cytochrome P450 mono-oxygenase haem-binding motif is shaded. The asterisk denotes the termination codon. The full-length cDNA sequence was assigned GenBank Accession no. AF532999.

catalyse NADPH and oxygen-dependent hydroxylations (Schuler, 1996; Chapple, 1998) are represented by more than 1000 enzymes in 62 families in plants (cytochrome P450 website http://drnelson.utmem.edu/CytochromeP450.html). A recent study of the available rice and *Arabidopsis* genome sequences revealed more than 600 P450 genes in those two species alone (Nelson *et al.*, 2004). The predicted amino acid start codon (ATG) is at nucleotide 101 and the open reading frame runs to a stop codon (TAA) at position 1673, encoding a predicted protein of 524 amino acids. The full-length cDNA sequence has been deposited in the GenBank database under the Accession no. AF532999.

CYP93C18 is a putative isoflavone synthase (IFS)

The isoflavone synthases (also referred to as 2-hydroxyisoflavanone synthase) of legumes are members of a small subfamily within the cytochrome P450s. Isoflavone synthases catalyse the first committed step in the biosynthesis of the isoflavone phytoalexins, hydroxylation at carbon-2 and the 2,3-aryl migration, giving rise to the isoflavonoid skeleton (Dixon and Paiva, 1995; Akashi *et al.*, 1999; Overkamp *et al.*, 2000).

A PSI-BLAST (Altschul *et al.*, 1997) search of the GenBank non-redundant database carried out with the deduced amino acid sequence of *CYP93C18* revealed sequence similarity with other isoflavone synthase genes from legumes. The highest similarities at the amino acid level were to isoflavone synthases from barrel medic (*Medicago truncatula*) 88%, chickpea (*Cicer arietinum*) *CYP93C3* (Overkamp *et al.*, 2000) 86%, licorice (*Glycyrrhiza echinata*) *CYP93C2* (Akashi *et al.*, 1999) 83%, and soybean (*Glycine max*) *GmIFS1* (Jung *et al.*, 2000) 82%. Another pea isoflavone synthase, *PsIFS1* (Jung *et al.*, 2000) is only 80% similar at the amino acid level to the deduced amino acid sequence of *CYP93C18*. The alignment of the amino acid sequences of *CYP93C18* with isoflavone synthases from other Fabaceae (Fig. 2) shows where the similarities and differences lie.

The function of *CYP93C18* was inferred based upon a strong similarity to the sequences of legume isoflavone synthase genes already characterized. *CYP93C3* was identified after suspension-cultured cells of chickpea were treated with fungal elicitors (Overkamp *et al.*, 2000). *CYP93C2* from licorice was expressed in recombinant yeast microsomes and shown to encode 2-hydroxyisoflavanone synthase (Akashi *et al.*, 1999). Similarly, in assays of the proteins encoded by *PsIFS1* and *GmIFS1* and *GmIFS2* in engineered yeast strains, the flavanone naringenin was converted to the isoflavone genistein (Jung *et al.*, 2000).

CYP93C18 *expression increases within 4 h of Bruchin B application*

RNA gel blots and QRT-PCR were used to assess the changes in the level of CYP93C18 mRNA over a 32 h time-course of bruchin treatment. With RNA gel blots the amount of *CYP93C18* mRNA (Fig. 3A, C) increased from an undetectable level at the time of treatment (time <1.5 h) to a maximum at 8 h, then declined again to an undetectable level in the bruchin-treated pods of both lines. The treatment effect appeared to be more pronounced in the pods of the *np/np* peas. No effect of ethanol treatment could be detected in either line. The observed size of the transcript on the RNA gel blot (~1.6 kb) matched the length of the open reading frame, 1572 bp.

This pattern of expression was confirmed using quantitative RT-PCR (Fig. 3B, D). There was a 30-fold increase in the amount of *CYP93C18* mRNA in the *Np/Np* pods within 4 h after bruchin treatment with a further increase to almost a 100-fold by 8 h, relative to the untreated control pod tissue. In the *np/np* pods there was an even greater up-regulation of *CYP93C18*. Four h after the application of bruchin to the pods, the level of *CYP93C18* mRNA was more than 50 times greater than that in the untreated control pods, and by 8 h it had increased to a level about 230-fold higher.

This pattern is similar to what was seen in the RNA gel blot analysis of *CYP93C2* from licorice, which revealed that the IFS mRNA accumulated transiently upon elicitation of the cells. Intense signals were observed in preparations from the cells 3–6 h post-elicitation, prior to the accumulation of the end-product, medicarpin, after 24 h (Akashi *et al.*, 1999). In chickpea suspension cultures *CYPC93C3* transcript levels reached a maximum 4–6 h after elicitor treatment, but the transcript was also expressed constitutively, and only a slight increase was noted upon elicitation (Overkamp *et al.*, 2000).

Recently, the expression patterns of the genes encoding the two IFS isoforms in soybean were characterized. Isoflavone synthase transcripts were found primarily in the roots and seeds and the expression pattern of *GmIFS1* was found to be consistent with the physiological roles of isoflavonoids as defence compounds against pathogens and as signal molecules to symbiotic bacteria in soybean (Subramanian *et al*., 2004).

The pattern of expression inferred from examination of band intensity with differential display suggested that *CYP93C18* expression 24 h after application of a bruchin was greater in *NP/NP* pods than *np/np* pods. By contrast, in the time-course experiment (Fig. 3), greater up-regulation was seen in the *np/np* pods than in the *NP/NP* pods at all the time points where significant amounts of transcript could be detected. This discrepancy may be partially explained by the differences in the growing conditions used for the two experiments. The pea plants used for the differential display study were grown inside a greenhouse, under supplemental ultraviolet light (to prevent spontaneous callus formation) and contolled temperatures, while the samples for the time-course study were taken from pea plants grown outside under ambient temperatures and natural sunlight. It may also be significant that differential display was carried out using poly(A)$^+$ RNA isolated from F_9 near-isogenic lines; whereas the time-course experiment was carried out using F_{12} near-isogenic lines.

Changes in level of the phytoalexin pisatin result from Bruchin B application and follow up-regulation of CYP93C18

The biosynthesis of pisatin, the well-studied phytoalexin of pea, is induced by infection with fungal pathogens as well as by exposure to a number of abiotic stimuli (Cruickshank and Perrin, 1962). Pisatin is an isoflavone, as are the phytoalexins of a number of other legumes (Jung *et al*.,

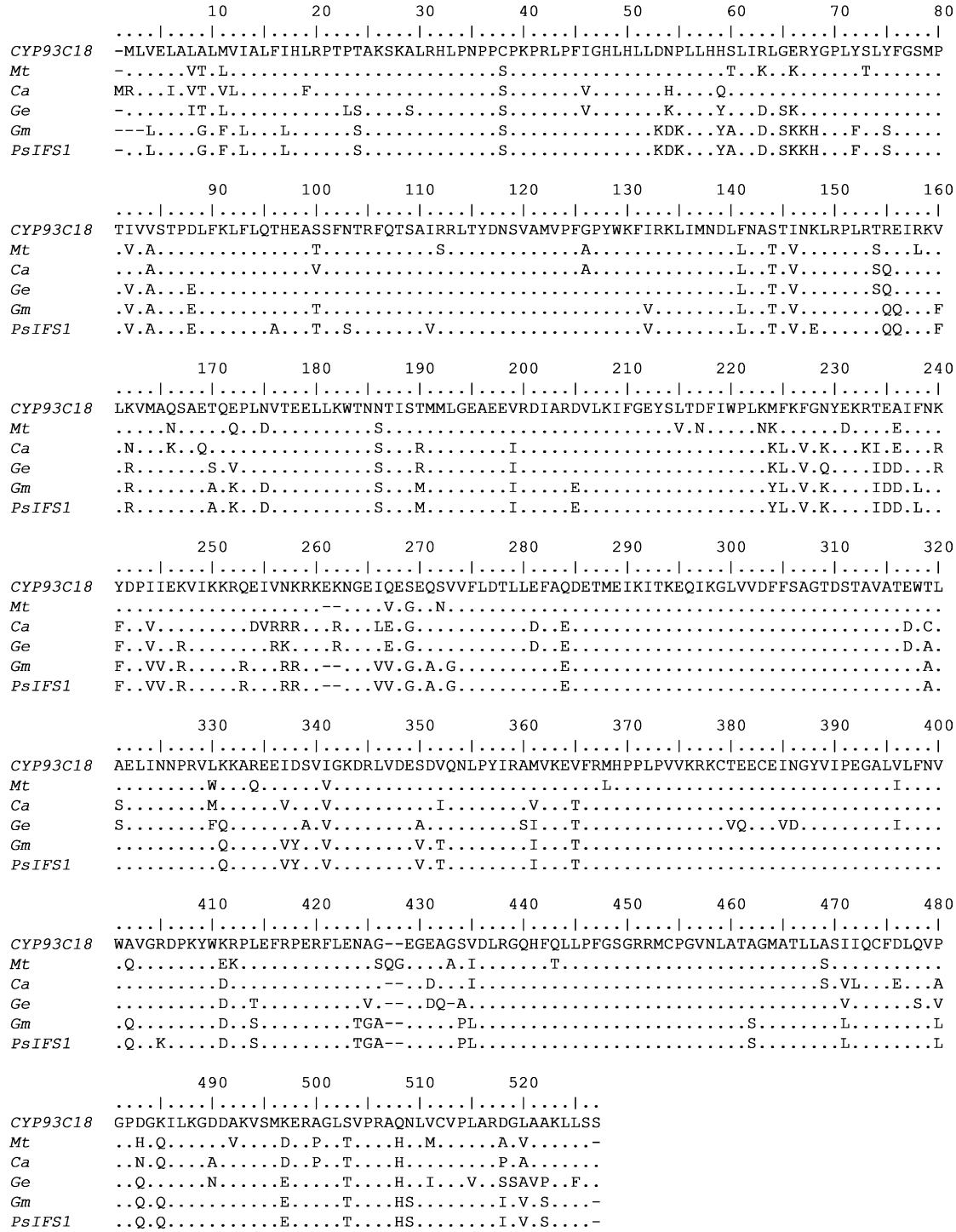

Fig. 2. *CYP93C18* deduced amino acid translation in alignment with other isoflavone synthases from the legume family. The deduced amino acid sequence of *CYP93C18* was aligned with the amino acid sequences of the closely related isoflavone synthases using ClustalW. Amino acid identities with CYP93C18 are represented as dots in the alignments. *Mt*, *Medicago truncatula* (AY167424); *Ca*, *Cicer arietinum* (*CYP93C3*, AJ243804); *Ge*, *Glycyrrhiza echinata* (*CYP93C2*, ABO23636); *Gm*, *Glycine max IFS1* (AF195798), and *PsIFS1*, *Pisum sativum IFS1* (AF195812).

2000), and isoflavone synthases catalyse the first committed step in their biosynthesis.

Given the up-regulation of an isoflavone synthase by treatment of pea pods with a bruchin, it was reasonable to investigate the effect of bruchin treatment on pisatin biosynthesis. Pisatin was barely detectible in untreated pods, but increased about 11-fold in both *np/np* and *Np/Np* pods after 16 h of treatment (Fig. 4). *Np/Np* pods accumulated more pisatin than did *np/np* pods. This pattern contrasted with that seen with CYP93C18 expression suggesting that

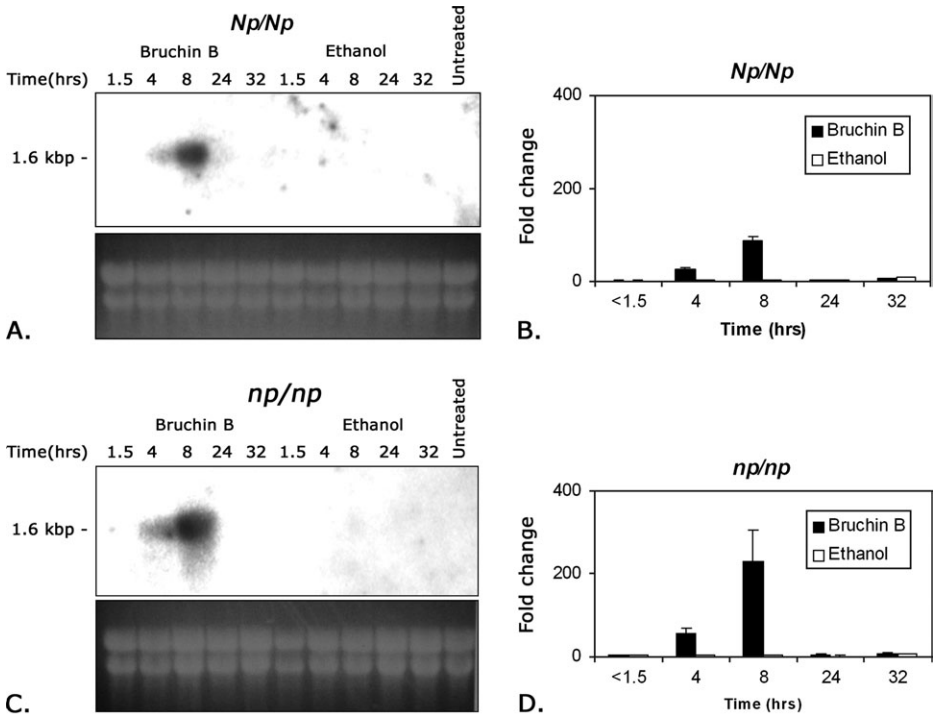

Fig. 3. Changes in expression of *CYP93C18* in bruchin- and ethanol-treated pods over a 32 h time-course. Either Bruchin B (6 ng in 50% ethanol) or 50% ethanol alone was applied to pea pods for the indicated periods of time. A sample of treated pod tissue was collected immediately after the Bruchin B solution or ethanol had evaporated (up to 1.5 h). Total RNA (10 μg per sample) from bruchin- or ethanol-treated pods of *Np/Np* (A) or *np/np* (C) peas was fractionated on a 1.5% agarose gel, transferred to nylon membrane, and probed with a random-primed [α-^{32}P]dCTP radiolabelled PCR product produced using the primers GT13F3/R3. A photograph of the ethidium bromide-stained ribosomal RNA bands on each gel is shown to verify equal loading. QRT-PCR was performed using cDNA prepared from *DNase I*-treated total RNA of either Bruchin B- or 50% ethanol-treated *Np/Np* (B) or *np/np* (D) pods. A sample of untreated pod tissue from each line was collected at the time of treatment and used as the calibrator for the QRT-PCR analysis. Estimations of the fold changes in gene expression were determined using the Comparative C_T method. Each data point represents the average of three replicate QRT-PCR analyses. The bars show the standard deviation of the means (n=3).

factors other than CYP93C18 transcription levels are rate-limiting for pisatin production.

Bruchins and plant defence

It is reported here that the application of Bruchin B to pea pods results in an increased expression of a putative isoflavone synthase gene *CYP93C18*, which encodes an enzyme involved in phytoalexin synthesis in the Fabaceae. Moreover, it is demonstrated that pisatin, the well-studied isoflavone phytoalexin from pea, increases in concentration in pea pods that have been treated with a bruchin. An obvious question is, why does an insect elicitor such as bruchin induce the biosynthesis of a compound usually thought to be involved in induced resistance to fungal attack (Cruickshank and Perrin, 1962; George and Van Etten, 2001). While the answer to this question must await additional study, there are several possible explanations. First, it is possible that bruchins, or compounds that induce the same reaction by the plant as the bruchins, are not restricted to insects. Indeed, bruchin-like activity in a pea pod bioassay (Doss *et al*., 2000) has been shown in chromatographic fractions prepared from a number of organisms (RP Doss, JE Oliver, and WM Proebsting, unpublished results), including *Botrytis cinerea* Pers:Fr., a plant pathogenic fungus.

However, a more probable explanation for the ability of Bruchin B to induce phytoalexin production follows from a consideration of recent reports that show that insect attack can result in resistance against various plant pathogens (Hatcher, 1995). This probably occurs because of the lack of specificity in the stimuli that induce resistance (Karban and Kuc, 1999). This is advantageous to the plant because many of the defensive substances produced are active against a range of organisms. In particular, Berenbaum (1988) has pointed out that phytoalexins '... are non-specific in terms of their mode of action and ... are broadly biocidal.' Within the legumes, Sutherland (1980) demonstrated that a number of isoflavonoid phytoalexins, including pisatin, can deter feeding of *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae) larvae, subterranean pests of pasture plants in New Zealand. Similarly, Hart (1983) found that the addition of glyceolin, the isoflavone phytoalexin of soybean, to artificial diet, could reduce the efficiency of food utilization by the soybean looper, *Pseudoplusia includens* (Walker), (Lepidoptera: Noctuidae), and that feeding by the Mexican bean beetle *Epilachna varivestis* Mulsant, (Coleoptera: Coccinellidae) was

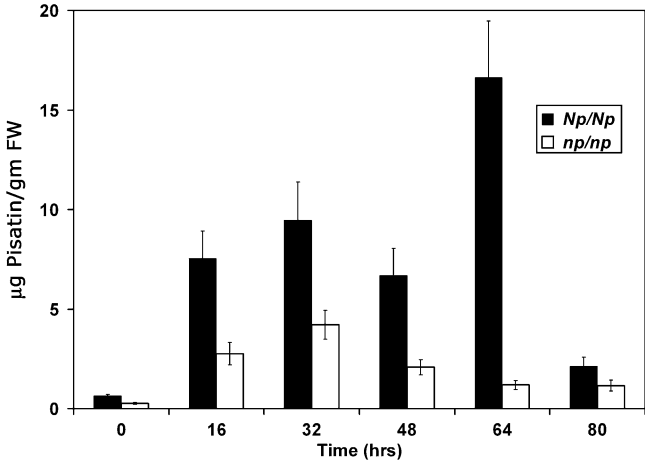

Fig. 4. Changes in pisatin levels in bruchin-treated pods over an 80 h time-course. Pisatin was extracted from Bruchin B-treated pod tissue (*Np/Np* and *np/np* F_{12} near-isogenic lines) over an 80 h time-course. Pisatin concentration was determined using HPLC with a 250×4.6 mm column packed with Adsorbosil® C-18 (5 µm) (Alltech). The identity of the pisatin peak was confirmed using liquid chromatography mass spectrometry (LCMS), and a set of spiked samples allowed an estimate of recovery (87.5±4.9%, mean ±standard error of the mean for n=5), which was used in the estimates of pisatin level. Three independent extractions were performed for each time point, the pisatin values (µg pisatin g^{-1} FW of pod tissue) are presented with the standard error of the mean of the three determinations.

strongly deterred on soybean cotyledons in which high glyceolin levels were present.

Pea pods are susceptible to infection by a number of fungal diseases in nature, among which are downy mildew, powdery mildew, and *Botrytis* mould (Hagedorn, 1991). The prior induction of pisatin accumulation by bruchin application or, indirectly by pea weevil oviposition might have an impact on the development of subsequent disease symptoms.

Conversely, Hammerschmidt (1999), noted that preformed antimicrobial compounds played a minor role in resistance compared with defences activated after infection. In addition, many fungi have the ability to demethylate and thus detoxify the pea phytoalexin pisatin (George and Van Etten, 2001). Further experimentation will be necessary to determine if bruchin-induced pisatin would play a role in protecting peas against fungal infection.

Bruchin B, an insect-derived compound, stimulates cell division and neoplasm formation when applied to pods of peas homozygous for *Np* and *np* (Doss *et al.*, 2000; Oliver *et al.*, 2000). The application of Bruchin B also resulted in the up-regulation of *CYP93C18*, a putative isoflavone synthase gene, and a subsequent increase in the level of the phytoalexin, pisatin. The responses observed in the near-isogenic *Np/Np* and *np/np* lines varied, indicating that there are, most likely, other influencing factors. These factors may be abiotic, such as the exposure of the pea plants to natural sunlight or supplemental UV light or there may be uncharacterized genetic differences that account for the variation in responses. Nevertheless, it appears that the induction of *CYP93C18* and the increase in pisatin content, along with neoplasm formation, form part of the defence response brought about by application of Bruchin B to pods of pea.

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