PROJECT REPORT No. 328

USE OF DNA FINGERPRINTING TO ASSESS GENETIC VARIABILITY AND PURIFICATION OF SPECIFIC PROTEINS FROM OILSEED RAPE MEAL (PART OF THE EU 'ENHANCE' PROJECT)

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USE OF DNA FINGERPRINTING TO ASSESS GENETIC VARIABILITY AND PURIFICATION OF SPECIFIC PROTEINS FROM OILSEED RAPE MEAL (PART OF THE EU 'ENHANCE' PROJECT)

by

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This is the final report of a thirty six-month project which started in July 2000. The work was carried out at the Central Science Laboratory and was funded by a grant of £55,032 from HGCA (project no. 2325). It formed part of the EU 'ENHANCE' project funded with a grant of £950,000 from the European Union and involving 14 research partners.

Contents

Abstract

The overall aim of this project was to explore the feasibility of obtaining added-value products from de-oiled oilseed rape (OSR) meal. The research and development work was carried out by 14 partners from different EU countries. The research carried out at CSL was jointly funded by the European Commission and HGCA.

As part of the project, two classes of bioactive proteins were purified and characterised at CSL. These included proteins with a potent antifungal activity (thionins), and proteins with a strong proteinase inhibitor action. A method for preparing semi-purified extracts with antifungal activity from larger quantities of OSR meal was also devised.

Research was also carried out at CSL into the application of DNA based methods for determining genetic variability of protein composition in OSR cultivars. The technique based on DNA fingerprinting could be useful in estimating the likely content and type of proteins in meals resulting from different breeding programmes. The technique could equally be useful in identifying a cultivar, and could be adapted to identify contamination of OSR seeds by other cultivars.

The R&D work carried out by other partners showed the potential for obtaining (or preparing) a number of value-added products from de-oiled OSR meal. These included protein-based polymers and films as alternative to synthetic plastics; extracts with pesticidal action for plant protection; glues, adhesives, products for cosmetics and paper industry; and natural dispersants for addition to detergents to aid shifting of grease and grime.

Summary

- The funding for this research project at Central Science Laboratory (CSL) was jointly provided by the European Commission and HGCA. The work carried out at CSL was aimed at estimating the genetic variability among different OSR varieties, and isolating and characterising certain bioactive protein components in de-oiled meal. This research formed part of a larger EC-funded project that involved 14 R&D and industrial partners from different EU countries. The overall aim of the ENHANCE project (QLKS CT 1999 01442: Green chemicals and biopolymers from rapeseed meal with enhanced end-uses performances) was to explore the feasibility of obtaining value-added products from de-oiled OSR meal for specific end uses.

- The studies carried out at CSL showed that DNA fingerprinting could be used to estimate variability in protein composition of the OSR meal. Research involved comparing the two main methods currently used for DNA fingerprinting, and results showed that microsatellite analysis was a more reliable method for estimating genetic variability among the OSR varieties tested than a method based on amplified fragment length polymorphism (AFLP). A further comparison of DNA data with protein data from INRA France showed that the breeding programmes devoted over the past twenty years to improve the oil fraction in OSR have led to a decrease in the seed protein content, with a concomitant enrichment in the level of cruciferins.

- The studies also showed that OSR meal could be a cheap source of useful bioactive proteins, such as thionins with potent antifungal properties, and proteinase inhibitors, that could also be used in plant protection applications.

- On the basis of these studies, it could be suggested that high-value components may be extracted from the meal prior to use in animal feed, and that protein quality of the seeds may be enhancied by breeding programmes for producing added-value meals.

- Research by other EU partners showed that a number of other useful products may be obtained (and/or prepared) from the de-oiled OSR meal. These include OSR protein based polymers and films as alternative to synthetic plastics;extracts with pesticidal and

fungicidal action for plant protection; glues, adhesives, products for cosmetics and paper industry; andnatural dispersants for addition to detergents to aid shifting of grease and grime. Further details of these developments can be found at the website <http://www.nf-2000.org/secure/FP5/F1186.htm>.

- The research work was written into two research papers, and presented at the 11th International Rapeseed Congress in Denmark in July 2003 (Atterby et al., 2003; Malabat et al., 2003).
- The outcome of research has also been presented at a number of scientific and public forums.

Introduction

Oilseed rape (OSR) (*Brassica napus L.*) is one of the major oilseed crops grown in Europe, with over 3.5 million hectares under cultivation in the EU in 1999. OSR is primarily grown for edible oil extraction. However, the process also generates a large quantity of de-fatted meal (around 1.5 million ton in the UK) which is mainly used as a low-value additive to animal feeds. The recent increasing production of biodiesel from OSR in Western Europe (around 2 million tons anticipated in 2003) is also expected to increase the meal production as a by-product. The opportunities for increasing use of OSR meal in animal feed are limited by the presence of glucosinolates, which impose limits on inclusion rates in diets for poultry and pork production. There are further difficulties in the utilisation of the meal from industrial oil crops such as high erucic acid rapeseed (HEAR) due to the presence of high levels of glucosinolates; that have been reduced by breeding in conventional '00' OSR crops.

The de-oiled OSR meal is a rich source of structural and bioactive proteins with a range of exploitable properties that are only just starting to be realised. A higher profitability of the rapeseed integrated chain would only be achieved by developing new higher-value applications for these protein fractions. Some recent results highlight the feasibility of protein extraction processes, and the economic potential of the resulting innovative protein products, especially in the non-feed area (Sanchez-Vioque et al., 2001, Gerbanowski et al. 2003).

During the last 20 years, the breeding objectives for the seed quality of OSR have been mainly concerned with the quantitative and qualitative improvement of the oil fraction and a decrease in the content of glucosinolates in meal. The studies on the alternative aqueous oil extraction processes have shown that there are some bottlenecks due to difficulties in separating the oil fraction from proteins because of the ability of the latter to stabilise emulsions. Consequently, the development of any innovative technologies and products from OSR needed a reliable account of the protein fraction from the seeds. Indeed, due to a number of emerging industrial applications for OSR, it is looks likely that OSR will be grown and used in the future for many non-food applications. The ENHANCE network funded by the European commission, therefore, undertook the task to study different aspects of OSR

processing and to develop value-added uses for the resulting by-products such as the deoiled meal. These included studying:

- Existing and new markets for OSR products
- Protein composition and genetic variability among different OSR cultivars
- Improved extraction process for proteins from OSR meal
- Identification and characterisation of value-added components from OSR meal
- Modification of OSR proteins to obtain desired functionality

In particular the project looked into the possibility of using components from OSR meal to develop products for specific industrial applications; such as

- Antimicrobial preparations
- Pesticides
- Glues and adhesives
- Paper coatings
- Polymer materials and films
- Surfactants
- Ingredients for cosmetics
- Additives to enhance performance of detergents

As the current methods for estimation of protein types and content in a de-oiled OSR meal involve growing, harvesting and processing a given cultivar, studies carried out at CSL were aimed at assessing DNA based methods for applicability for quickly determining the content and type of proteins in OSR cultivars. Studies were also aimed at isolating and characterising high-value bioactive proteins from deoiled OSR meal to add value to this important by product of the rapeseed industry.

Part 1: Rapeseed Composition and Genetic Variability

Materials and Methods

DNA fingerprinting

DNA fingerprinting techniques were assessed for the estimation of genetic variability within an OSR cultivar, and between different cultivars. The methods tested were based on determination of microsatellite allele sizes, and amplified fragment length polymorphism (AFLP). The DNA fingerprinting data generated by microsatellite analysis were compared with that obtained by one of the research partners (INRA, France) on protein composition of different OSR cultivars to establish genetic variability among the cultivars tested.

For microsatellite analysis, DNA was isolated either from individual seeds of the variety 'Express', or from de-oiled OSR meals from 34 different cultivars. Each meal comprised of seeds collected from a number of plants of a given variety. For AFLP analysis, DNA was extracted from lyophilised leaf samples from individual plants of 14 different cultivars. A typical method involved grinding around 100 mg of OSR material to a powder in liquid nitrogen using a pestle and mortar. DNA was isolated using a NucleoSpin Plant Kit (Macherey-Nagel). All DNA extracts were run on a 1.5% agarose gel to check the quantity and quality of DNA.

The primers used for microsatellite analysis (Table 1) were derived from several members of *Brassiceae* family including *B. napus* L. (Uzunova and Ecke, 1999, Mitchell et al., 1997). These primers were optimised for routine use. PCR amplification of microsatellite alleles was carried out either by using DNA extracted from individual seeds or 100 mg of deoiled meal using 'NucleoSpin Plant Kit' (Macherey-Nagel). The PCR conditions were 94°C for 2 minutes, followed by 40 cycles at 94°C for 45 seconds, annealing at corresponding temperatures for 30 seconds, and extension at 72°C for 1 minute. This was followed by final extension at 72°C for 5 minutes and storage at 4°C. The PCR products obtained by microsatellite method were separated by electrophoresis and size of each product determined on an automated DNA analyser (ABI-Prism-377). A dendrogram was built based on allele sharing between the cultivars tested (Fig. 1).

For AFLP analysis, restriction digests, ligations and amplifications were performed as described in the AFLP Plant Mapping Protocol (ABI) using the primers supplied by All New England Biolabs, and products were run on a 1.5% agarose gel to check the success of pre-selective amplification. This was followed by selective amplification with the ABI kit using all the primer pair combinations (64 in total: 2 sets of eight primers). The resultant products were electrophoresed on a vertical 6% polyacrylamide gel on an ABI-377 prism automated DNA analyser at 2500V for 4 hours. The variation in size of each amplified fragment was analysed using Genescan 3.1 software, which scored each product according to known size standard fragments (Genescan Rox 500), run along with the samples in each lane. Polymorphism in different DNA templates was detected by the presence or absence of fluorescent bands. Bionumerics software was used to generate a dendrogram of the data based on the Pearson correlation (Fig. 2).

Table 1: Composition of PCR primers used in microsatellite analysis of OSR varieties

Primer	Forward primer sequence	Reverse primer sequence	Annealing temp (^{o}C)
BN01	H-AAAAATAGAAAGTTTGGAAGG	CCAACACACCTTTTACTCTT	47
BN02	T- TCGACATGGATTCTACCAAA	GAACTTGCAAGCTGCAATTA	47
BN03	F-TATGTACACATTCCTCATTTTC	CATTCGTCTCCACCTTCT	47
BN04	H-GATTGAACACCCCTAGTGAG	TATCAAAAACCCCAAGATTG	47
BN05	T-AGATTTGCATGTGGTTTGAC	ATTGCTTANTGATGTTGGGAA	47
BN06	F-GAGCCATCCCTAGCAAACAAG	CGTGGAAGCAAGTGAGAATGAT	54
BN6A2	T-CTTTGTGTGGACTTTTAGAACTTT	CGCAGCTTTTGGCCCACCTG	54
BN07	H-AAACCTCCTCAAAAACCCCTAAACG	TCCCCTCTTTCCTCTCTCTCTAGGC	47
BN08	T-GCCGTTCTAGGGTTTGTGGGA	GAGGAAGTGAGAGAGGGGGAAATC A	47
BN12A	H-GCCGTTCTAGGGTTTGTGGGA	GAGGAAGTGAGAGCGGGAAATCA	58
BN19A	F-CACAGCTCACACCAAACAAACCTA	CCCCGGGTTCGAAATCG	53
BN25C2	F-AAACCTCCTCAAAAACCCCTAAACG	TCCCCTCTTTCCTCTCTCTCTAAG	59
BN35D	F-GCAGAAGGAGGAGAAGAGTTGG	TTGAGCCGTAAAGTTGCTCACCT	57
BN38A	F-TGGTAACTGGTAACCGACGAAAATC	ACGCGTTCTTCAGGTCCCACTC	57
BN59AI	T-TGGCTCGAATCAACGGAC	TTGCACCAACAAGTCACTAAAGTT	54
BN72A	F-GCCCACCCACCTTCTTGTCCT	CCCTTCATCCAAACTCCTCCTCGT	58
BN83/1	F-GCCTTTCTTCACAACTGATAGCTAA	TCAGGTGCCTCGTTGAGTTC	57

H = ABI dye HEX
T = ABI dye TET
F = ABI dye FAM

Results

All preparations from seed, meal or lyophilised leaf samples showed successful extraction of DNA upon visualisation by agarose electrophoresis and ethidium bromide staining. PCR amplifications of the restriction/ligation reactions gave a characteristic smear of DNA (100-

500 bp), when run on 1.5% agarose gel. The separation and size determination of amplified products from 34 cultivars on an automated DNA sequencer (ABI-Prism-377) gave a table of allele sizes (Appendix A) that were used to build a dendrogram to establish the genetic proximity of these samples based on allele sharing. The results also indicated that there was no intra-varietal variability within the variety 'Express', whilst 34 other cultivars tested showed variability in microsatellite allele sizes. The method successfully grouped together cultivars that are known to be genetically-close, such as Darmor, Stellar and Yellowseed varieties. The genetic similarity between varieties obtained by microsatellite analysis also compared well with similar data obtained by protein profiling of the varieties. Out of a total of 34 varieties tested, 22 grouped in a similar manner by both protein profiling and DNA analysis, whilst 12 varieties (Arvor, Link, Zeruca, Bristol, Pacha, B001, ISLR3, Pollen, Matador, Gaspard, R33 and Hokkaido) were grouped differently by the two methods.

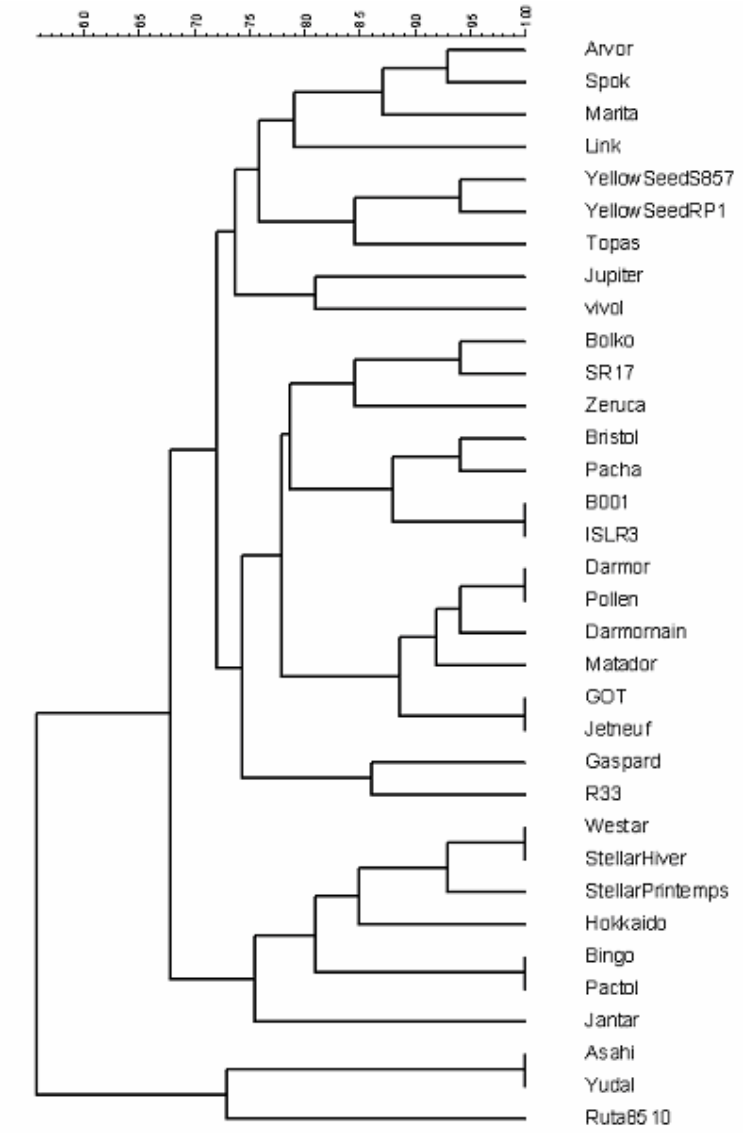

Fig. 1: A dendrogram based on microsatellite allele sharing between OSR genotypes

The data obtained by AFLP analysis was also used to build a dendrogram based on Pearson correlation (Fig. 2). However, a comparison of the results with those obtained by microsatellite analyses indicated that AFLP analysis produced entirely different results. For example, whilst microsatellite analysis grouped together a number of cultivars that are known to be genetically related (e.g. yellowseed varieties and Stellar varieties), they appeared genetically far away by AFLP analysis. This clearly showed that the latter method needed further refining and selection of better markers to indicate genetic variability.

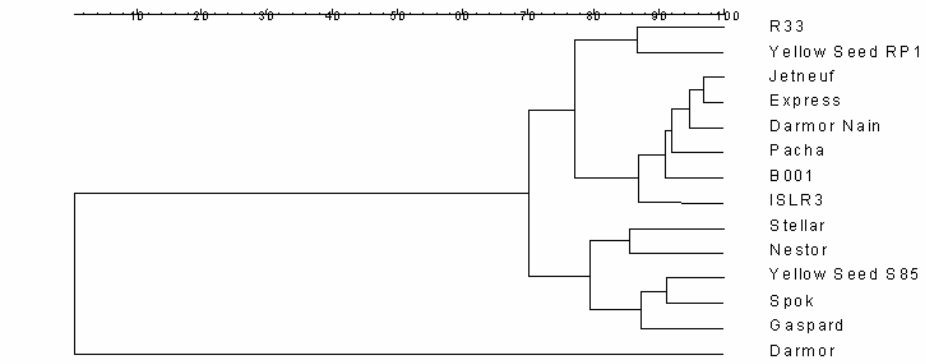

Fig. 2: A dendrogram based on Pearson correlation between AFLP data from OSR genotypes

Discussion

The DNA fingerprinting based on microsatellite analysis provides a useful tool for the identification of different genotypes and relative genetic linkages between them. The results obtained by AFLP analysis in this study, however, did not prove reliable. The initial results, nevertheless, indicated that further work was needed to improve the results obtained by AFLP analysis through selection of better markers to indicate genetic variability among OSR genotypes. Also, with further work, the microsatellite method could be refined to provide very useful tool for identifying contamination of OSR seeds by other varieties, possibly also those that have been genetically modified.

Combining the DNA fingerprinting data with that from INRA on major protein types, showed that the breeding programmes devoted over the past twenty years to improve the oil fraction in OSR have led to a decrease in the seed protein content, and a concomitant enrichment in the level of cruciferins (Malabat et al., in preparation). Cruciferin and napin are the two main classes of storage proteins in OSR seed, both with different nutritional and functional properties. The proportion of cruciferins and napins in seed proteins is a major determinant of the functional properties of the protein products that can be obtained from OSR meal. The similarities found by both DNA and protein methods further suggested that genetic factors are responsible for controlling the protein composition. Thus breeding efforts could be devoted to

improve the protein quality in seeds to adapt OSR meal to derive protein enriched products with desired functional properties for added-value uses.

Part 2: Isolation and Characterisation of Bioactive Proteins

Materials and Methods

Antifungal proteins

A number of plant seeds, including *Brassica* spp., are known to contain the antifungal proteins called thionins (Vernon et al., 1985). Attempts were made to isolate similar proteins from cold-pressed de-oiled OSR meal (variety Express) using a method described by Vernon et al. (1985). The method involved homogenising the meal in 0.1M phosphate buffer, fractionating solubilised proteins by salt precipitation, purifying by ion-exchange chromatography, and desalting by gel filtration.

For this purpose, 300 g of rapeseed were initially ground using a ceramic mortar and pestle on ice, and further homogenised in 30 g batches in 100 ml 0.1M phosphate buffer (pH 7.0) using an Ultra Turrax tissue disperser. The homogenate was frozen overnight and re-extracted in 50 ml 0.1M phosphate buffer. The resulting extracts were then pooled, centrifuged at 1000*g* for 10 minutes and the supernatant removed. Ammonium sulphate was added at a rate of 20g per 100ml of the supernatant, left to dissolve for 10 minutes with occasional stirring and centrifuged at 20,000*g* to sediment proteins (Fraction 1). The process was repeated twice with additions of 10g of ammonium sulphate per 100ml of the supernatant (Fractions 2 and 3). The latter fractions were each suspended in 300ml 0.05M phosphate buffer (pH 7.2) with stirring, and were dialysed overnight in 5mM phosphate buffer (pH 7.2). A gel filtration column was packed with 50g of pre-swollen carboxymethyl cellulose (CM52), suspended in 96 ml 0.5M phosphate buffer, using a flow rate of at least 3.7 ml/ minute. The column was equilibrated with 0.05M phosphate buffer, and 300 ml of fraction-3 were loaded onto the column. A linear gradient of 0.1 to 1M NaCl was used to elute proteins from the column, which were collected in 15 ml fractions. The column was re-equilibrated with 0.05M phosphate buffer, and the process repeated for fraction-2.

The fungal species *Fusarium culmorum* was used to determine anti-fungal activity in the 15 ml fractions as thionins have been shown to specifically inhibit their activity (Terras *et al*., 1996). Four plates of sucrose nutrient agar were inoculated with *F. culmorum* and grown for 7 days at 25°C. Spores were harvested from the plates in 2ml sterile distilled water. The spore suspension was filtered through a double layer of lens tissue to remove any agar fragments. 0.1 ml of the *F. culmorum* spore suspension was spread onto plates of potato dextrose agar (PDA) amended with streptomycin (3.165 ml of a 1% streptomycin solution in 250 ml PDA). Three sterile filter paper circles (6 mm in diameter) were dipped into one of the extract or control solutions and the excess wiped off. The two replicate filter paper circles were placed onto one of the seeded PDA plates. After four days incubation at 25°C, the inhibition zone around the filter paper circles was measured. A zero score was assigned to fractions where *F. culmorum* grew over the pre-soaked filter paper.

Protein concentrations in fractions eluted from carboxylmethylcellulose column were estimated by measuring absorbance at 290nm against BSA standards. Aliquots of each fraction were run on a10-20% gradient SDS-PAGE gel using kaleidoscope polypeptide standards (MW range 3,500-39,000) at 150 volts for 1 hour, and gels were stained using Coomassie Brilliant Blue R-250. The observation of two main protein bands (around 6 and 12 KDa) on SDS-PAGE gels (Fig. 4) led to further investigations to determine whether these were mono- and dimers of the same proteins linked through disulphide bonds. Dithiothreitol was added to the samples prior to electrophoresis at a final concentration of 1%. The results showed that addition of dithiothreitol made no difference to the size of protein bands. It was, therefore, concluded that in order to carry out further analysis by HPLC the two bands would have to be separated. The use of a meltable synthetic acrylamide system 'Protoprep II' (National Diagnostics) was investigated for this purpose. However, although the medium allowed protein bands to be excised and recovered by melting; a number of problems were encountered, mainly due to limitations on the resolving power of the gel medium, which could only separate proteins with a molecular weight of 10 KDa or higher. The separation of individual proteins was, therefore, attempted by size exclusion chromatography. The results again showed that, because of the close range of sizes (6-12 KDa), it was not possible to obtain any significant resolution of individual components by this technique. Further separation was, therefore, carried out on an HPLC system, which comprised of two Waters-510 pumps, an autosampler, an interface Module, Lambda–Max 480 spectrophotometer and a

Millennium Chromatography Manager software. The HPLC column used was a Vydac C_{18}, 300Å pore size (5 micron particle size), 4.6mm x 250mm (218TP54) analytical column maintained at 35°C throughout the experiment. The mobile phase used was a gradient of acetonitrile and Milli-Q grade water both containing 0.1%TFA at 1ml / min. The lyophilised thionin-rich proteins (approximately 60µg) were dissolved in water and injected on to the column in 20µl volumes. The eluted proteins were detected by measurement of UV absorbance at 280nm, and fractions corresponding to each eluted peak were collected manually. The fractions were concentrated by centrifuging under vacuum (Speed-Vac), and subjected to MALDI-TOF analysis for molecular weight determination.

A simple method for processing relatively larger amounts of OSR meal for the isolation of thionin-rich fraction was also devised. For this purpose, one Kg meal (variety 'Express') was processed in batches by blending with approximately 8 litres of 0.1M phosphate buffer using a Tercator homogeniser. The resultant suspension was then spun at 1000g for 10 minutes and the supernatant retained. Ammonium sulphate was added at a rate of 60g per 200ml to precipitate out the fractions that were not required. The solution was left to stand for 10 minutes and centrifuged at 27,504 RCF to sediment the precipitate. 40g of ammonium sulphate were then added to precipitate the fraction that was previously found to have high antifungal activity. This was left to stand for 10 minutes and then centrifuged to sediment the proteins. The fraction was then suspended in approximately 1.2 litres of 0.05M phosphate buffer (pH 7.2) by stirring overnight. The suspended extract was dialysed using 3,500 MW cut off tubing against 0.005M phosphate buffer (pH 7.2) for approximately 48 hours to remove ammonium sulphate. The fraction was purified on an ion exchange column (carboxymethyl-cellulose), and dialysed again to remove sodium chloride. The anti-fungal activity in the isolated fraction was confirmed, and the preparation was freeze dried to lyophilise the proteins (around 5 grams final weight was obtained). The freeze-dried preparations were again tested to ensure that they retained antifungal activity.

Proteinase inhibitors

A protein fraction with strong proteinase-inhibitor activity was isolated from defatted OSR meal using a method adapted from Genov *et al.*, (1997). For this purpose, 100g of deoiled meal was extracted with 1 litre of water using an Ultra-turrax homogeniser. The resultant suspension was centrifuged at 3,000g for 25 minutes and the supernatant collected. The pellet

was re-extracted with 1 litre of water and centrifuged as above. The two supernatants were combined and adjusted to pH 5.0 with 4N HCl. The precipitated proteins were centrifuged as described before and the pellet discarded. The resultant supernatant was heated at 70°C for 5 minutes and cooled to room temperature. The precipitated (heat denatured) proteins were again removed by centrifugation. The supernatant was filtered through Whatman No1 filter paper and lyophilised by freeze-drying.

For further purification, the lyophilised powder was rehydrated in 200ml of 0.05M ammonium acetate buffer (pH 5.0) containing 5% glycerol. Approximately 3ml of this extract were filtered through 0.45μm filter (Gelman Sciences) and 2ml injected onto Sephadex G-50 column (1.5cm x 80cm) at a flow rate of 1 ml/minute, using a UK6 injector. The equilibration and elution buffer was 0.05M ammonium acetate buffer (pH 5.0). The UV cord was set to 280 nm and 1.9 ml fractions were collected continuously until no further proteins eluted from the column. To reduce the number of samples tested, each even fraction showing significant absorption at 280nm was desalted using a PD10 column (Amersham Biosciences). Each column was washed with 25ml of water and the volume of each fraction was adjusted to 2.5ml with water. This was added to the column, eluate discarded, and desalted protein eluted with 3.5ml water.

The proteinase inhibitor activity was determined by using a method adapted from Smith et al. (1980). For this purpose, 40 mg of the substrate BAPNA (N-α Benzoyl-DL-arginine-p-nitroanilide hydrochloride) were dissolved in 1ml of Dimethylsulphoxide. The mixture was heated to 40°C, and added with 99ml of water already heated to 40°C and the mixture was kept at this temperature during use. 40 mg of bovine pancreatic trypsin were dissolved 1ml of 1mM HCl and made up to 2 litres with 1mM HCl. Tubes containing aliquots of the desalted fractions and control blanks were vortexed and incubated for 10 minutes at 40°C. BAPNA substrate (2.5ml) was added to each tube, vortexed and incubated for exactly 10 minutes at this temperature. To stop the reaction, 0.5ml of 30% acetic acid was added to each tube. Trypsin (1ml) was added to the reagent blank and the sample blank. Each tube was centrifuged for 3 minutes at 8000 g. UV absorption was measured at 410nm to detect the presence of p-nitroaniline. The fractions that produced significant inhibition of trypsin were pooled and subjected to further analysis by MALDI-TOF.

Results

Antifungal proteins

The fractions isolated from OSR meal in this study by salt precipitation, ion-exchange chromatography, and gel filtration were tested for antifungal activity against *Fusarium culmorum* at each stage of purification. The results (Fig. 3) showed increasing antifungal activity with increasing stage of purification. The highest activity detected in subfractions 7 to 12 from a carboxymethylcellulose column was also consistent with the appearance of a major protein band in the same fractions, when resolved by SDS-PAGE (Fig. 4), and which corresponded to approximate size of 6KDa reported for thionins. These fractions were pooled and further purified to obtain a thionin rich extract.

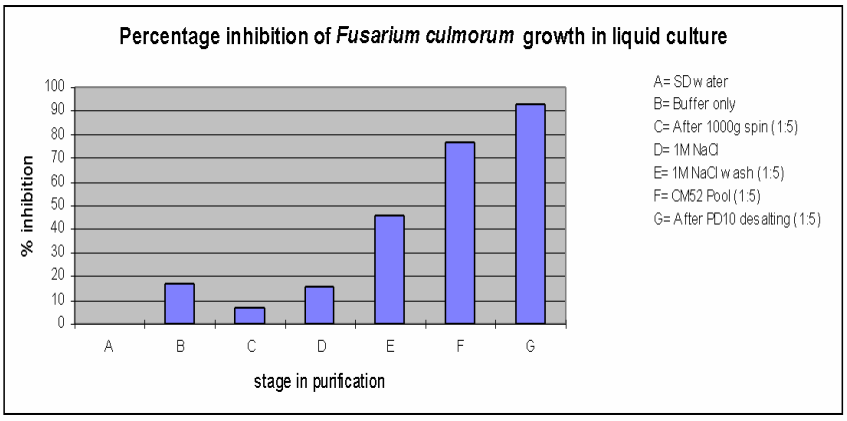

Fig. 3: Antifungal activity of the OSR fractions at different stages of purification

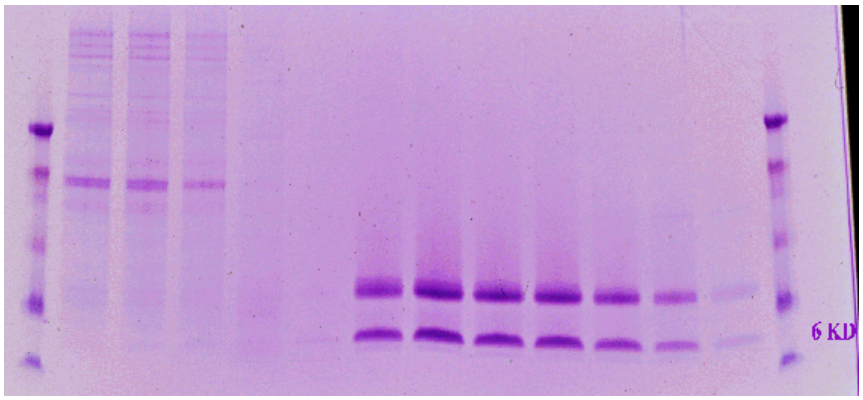

Fig. 4: SDS-PAGE separation of the protein fractions 2-14 (L to R) purified by HPLC. Lanes 1 and 14 show size markers.

The results shown in Fig. 5 indicated that it was possible to further separate individual components of the protein fractions by reverse-phase HPLC. Five (5) sub-fractions were collected from the HPLC column eluents (referred to as fractions 1-5). Analysis of these fractions by MALDI-TOF indicated that fraction 1 contained a mixture of two main proteins with masses of 4714 and 6365 Dalton, and also that their respective, apparent, dimeric forms were present in the solution. Similarly, fraction 2 contained the protein around 4710 Dalton as

well as its dimeric form (Fig. 6). When these fractions were separated by SDS-PAGE, two main monomeric and respective dimeric bands were observed.

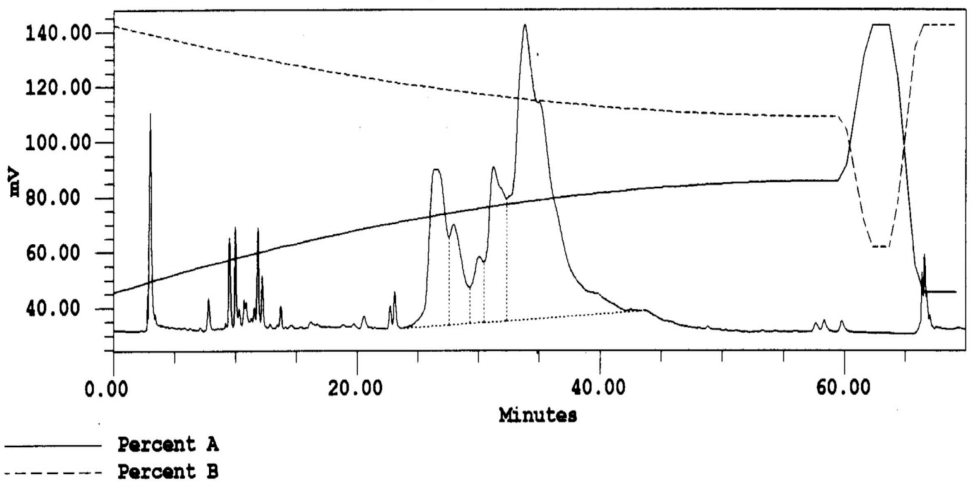

Figure. 5: showing separation of individual protein components by HPLC

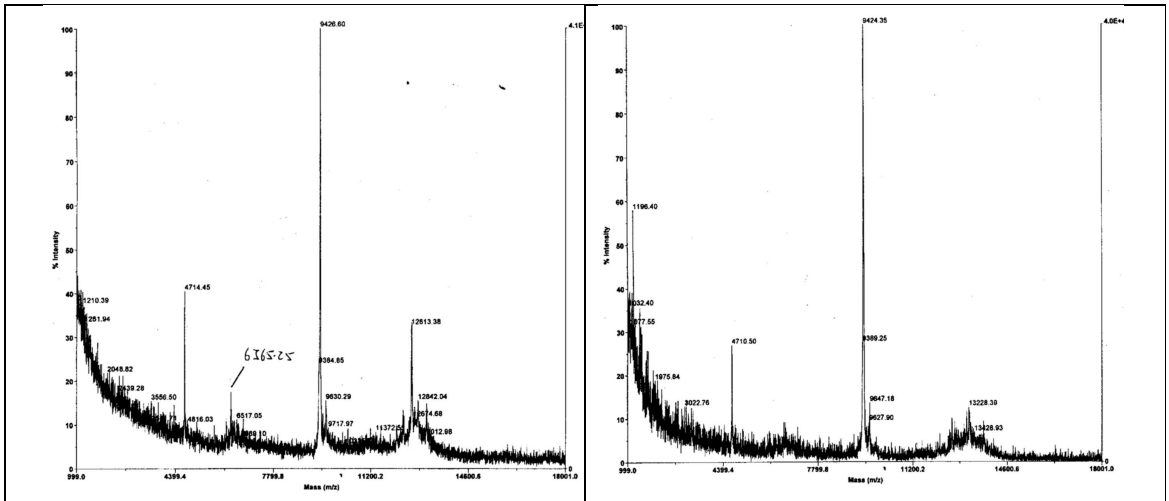

Fig. 6: showing MALDI-TOF analysis of HPLC purified antifungal fractions 1 (A) and 2 (B) from OSR meal

Proteinase inhibitors

The heat-stable protein fractions isolated from OSR meal were tested for proteinase inhibitor activity, and individual components were further purified by HPLC.

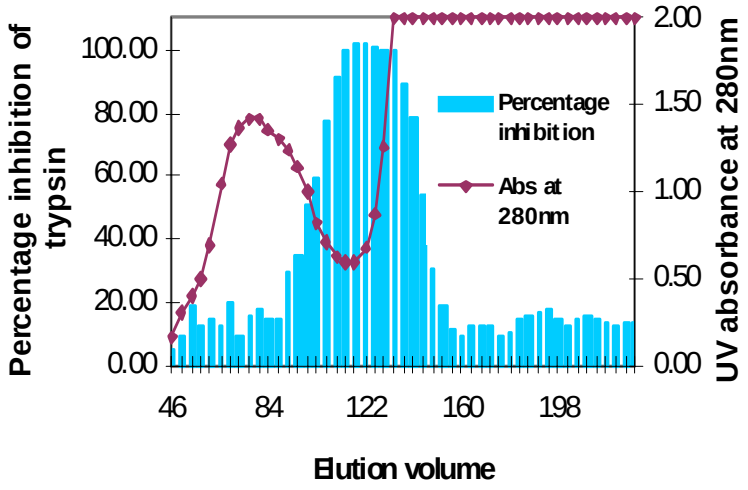

Fig. 7: Proteinase inhibitor activity in different protein fractions

The results (Fig. 7) showed a substantial inhibition of trypsin activity by fractions in the elution range of 106 to 140 ml from the Sephadex G-50 column. Further analysis by MALDI-TOF of the fractions showing inhibitory activity indicated 2 main proteins of MW 3643 and 5677 Da (Fig. 9). The results of HPLC showed that although the best separation was achieved by using a gradient of 10-32% acetonitrile over 60 min. The peak corresponding to the highest activity (F4, see Fig. 8) was broad, indicating that either there were several forms of the protein with different polarities, or different proteins of a very similar polarity.

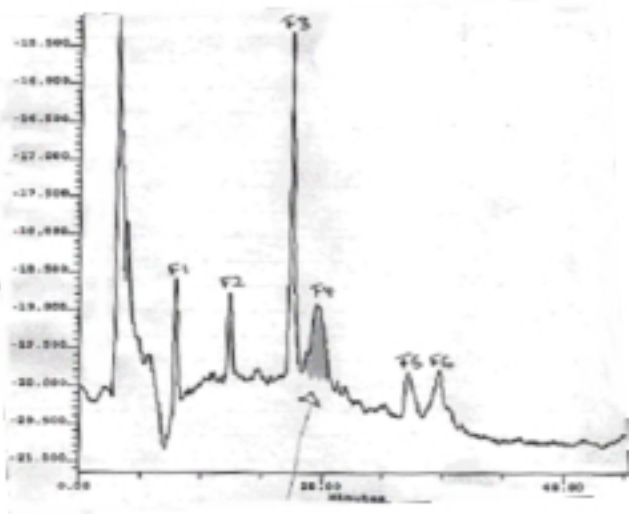

Fig. 8: showing HPLC separation of different OSR fractions with trypsin inhibition activity

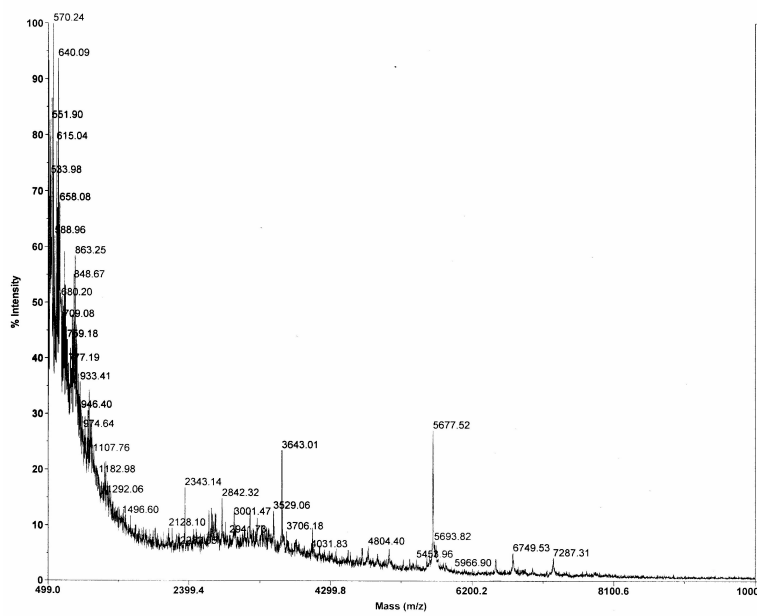

Fig. 9: MALDI-TOF analysis of pooled fractions of OSR meal extract that gave substantial inhibition of trypsin following separation on a G-50 sephadex column

Discussion

A number of plants, including *Brassica* spp., are known to contain small basic proteins called thionins that have potent antifungal activity. Although the exact mode of their antifungal action is not known, it appears to be at the cell membrane level. Thionins are also shown to form disulphide linkages with other proteins. Most mature plant thionins are 45-47 amino acids in length (Approx. Mol.Wt. 5 KDa), whilst their precursors usually contain 70-80 amino acids. Thionins are mostly found in seeds but may also be found in the cell wall of leaves (Vernon et al., 1985; Bohlmann, 1994; Neumann, et al., 1996). Similarly, the presence of proteinase inhibitors has been reported in a number of plant species (Genov et al., 1997). Present studies were successful in isolating both of the high-value bioactive proteins from de-oiled OSR meal. Purification of some of the active components was also achieved, although a semi-purified extract may suffice for practical use in plant protection applications. The development of such a plant derived antifungal extract would be of special interest to the

rapidly growing organic food sector in the UK. Further work is, however, needed into purification and amino acid sequence determination of individual components with antifungal, proteinase inhibitor, and other biological activities for their potential utilisation in developing varieties of other plants with desirable traits such as enhanced pest resistance. Efforts are also being made to amplify and sequence thionin genes from OSR to obtain the information on the composition of individual thionin proteins at a molecular level (Appendix B).

The de-oiled OSR meal is currently a very cheap agricultural by product (around £90-£100/ ton), whilst at the same time a rich source of proteins and other useful chemicals. The multi-dimensional research that has been carried out under the EU-ENHANCE project has indicated that breeding programmes aimed at improving the oil fraction in OSR in the past decades appeared to have led to a decrease in the overall seed protein content, and also an increase in the level of cruciferins. Any major alteration in the ratio of the two major storage proteins, cruciferins and napins, could severely jeoperdise quality and functional properties of proteins products that can be obtained from the de-oiled meal. As genetic factors mainly govern the protein composition, it should be possible to aim breeding efforts to re-adjust protein ratios in the seeds to derive products enriched in proteins with desired functional properties from the meal for specific added-value uses.

Research by other EU partners showed that a number of other useful products may be obtained (or prepared) from the deoiled OSR meal. For example, around 40% crude proteins may be extracted from OSR meal for high value uses such as synthesis of polymer materials as alternative to synthetic plastics; extracts with pesticidal and fungicidal action for plant protection; glues, adhesives, products for cosmetics and paper industry; and natural dispersants for addition to detergents to aid shifting of grease and grime. Further details of these developments can be found at the website <http://www.nf-2000.org/secure/FP5/F1186.htm>.

The research has also highlighted the need for further research to fully exploit the bioactive components identified in OSR at a commercial scale. Further research is also needed to identify other yet-unexplored high-value components in OSR meal.

Publications and dissemination of the outcome of research

Based on the research reported here, the following two research articles were prepared and presentated at the 11th International Rapeseed Congress in Denmark in July 2003:

1. Atterby, H., Larré, C., Chaudhry, M.Q., Malabat, C., Bérot, S., Compoint, J.P., Douliez, J.P., MacNicoll, A.D., Gueguen, J. and Price N.R. (2003) Isolation and characterisation of certain bioactive proteins from deoiled rapemeal, in Proceedings of the 11th International Rapeseed Congress "Towards Enhanced Value of Cruciferous Oilseed Crops by Optimal Production and use of the High Quality Seed Components", Sorensen, H. (Editor), 6-10 July 2003, The Royal Veterinary and Agricultural University, Copenhagen, Denmark, pp. 651-653.

2. Malabat, C., Atterby, H., Chaudhry, Q., Renard, M. and Guéguen, J. (2003) Genetic variability of rapeseed protein composition, in Proceedings of the 11th International Rapeseed Congress "Towards Enhanced Value of Cruciferous Oilseed Crops by Optimal Production and use of the High Quality Seed Components", Sorensen, H. (Editor), 6-10 July 2003, The Royal Veterinary and Agricultural University, Copenhagen, Denmark, pp. 205-208.

- The outcome of research has also been presented at the EU-ENHANCE and HGCA annual meetings, HGCA-Plant Breeders Conference, and the Cereals-2003 event.

- A news item on the successful isolation of useful proteins from rapemeal on CSL website (www.csl.gov.uk) on 09/10/02

3. A news item in *Farmers Guardian* 25 October 2002, page-18

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Appendix-A

Table. 2: showing typical microsatellite allele sizes

Primers ➔ OSR varieties🡻	1	2	4	6A2	7	8	11	12	19	5C2	83/1
Asahi	185	135	130,134	93	140	254,280	224,228	439	195	141	195
Hokkaido	***	135,151	134	97	140	254,318	224,226	439	195	141	195
Matador	187	135,151	134	97	140	280,288	224,226	447	195	141	217
Yudal	***	135	130,134	97	140	254,280	224,228	439	195	141	195
Gaspard	187	135	134	97	140	280,288	224,226	447	217	141	195
R33	***	135,151	132,134	97	140	280,288	224,226	447	217	141	195
Pacha	183	135,151	134,136	97	140	280,288	224,226	447	195	141	195
Jupiter	181	135,151	130	97	140	280,288	224,226	447	195	141	195
Jetneuf	187	135	132,134	97	140	***	224,226	447	195	141	195
Arvor	183	135,151	132	97	140	288,318	224,226	447	195	141	195
Ruta8510	185,189	135	***	98?	132,140	280,288	224,226	439	195	141	195
Darmor nain	187	135	132,134	97	140	280,288	224,226	447	195	141	195
Stellar Hiver	181,185	135,151	134	97	140	254,318	224,226	447	195	141	195
GOT	187	135	***	97	140	254,318	224,226	447	195	141	195
B001	183	135	134	*	140	280,288	224,226	447	195	141	195
Bolko	185	135	134	97	140	280,288	224,226	447	195	141	195
Darmor	187	135	134	97	140	280,288	224,226	447	195	141	195
Vivol	181	135,151	132,134	97	140	280,288	224,226	447	195	141	195
Zeruca	185	135	132	97	140	280	224,226	447	195	141	195
Jantar	185	135	***	97	136,140	254,318	224,228	447	195	137,141	195
Link	191	135	***	97	140	288,318	224,226	447	195	141	195
Marita	187	135,151	132	97	140	288,318	224,226	447	195	141	195
Bristol	183	135,151	132,134	97	140	280,288	224,226	447	195	141	195
Pollen	187	135	134	97	140	280,288	224,226	447	195	141	195
ISLR3	183	135	134	97	140	280,288	224,226	447	195	141	195
SR17	185	135,151	134	97	140	280,288	224,226	447	195	141	195
Yellow Seed S857	185,193	135,151	166,168	97	140	280,288	224,226	447	195	141	195
Yellow Seed RP1	185,193	135,151	166,168	97	140	288,318	224,226	447	195	141	195
Stellar Printemps	185	135,135	***	97	140	254,318	224,226	447	195	137	195
Pactol	193	135,151	170,172	97	140	254,318	224,226	447	195	141	195
Topas	185,193	135,151	172,174	97	140	280,288	224,226	447	195	137	195
Westar	181,185	135,151	134	97	140	254,318	224,226	447	195	137	195
Bingo	193	135,151	***	97	140	254,318	224,226	447	195	141	195
Spok	183,185	135,151	***	97	140	288,318	224,226	447	195	141	195

Appendix-B

AMINO ACID SEQUENCE ALIGNMENT OF VARIOUS THIONINS, AND DESIGNING OF PRIMERS FOR AMPLIFICATION OF THE GENES FROM OSR

1. Chinese cabbage (*Brassica rapa* subsp. pekinensis)
2. Abyssinian Cabbage (*Crambe abyssinica*)
3. Wheat (*Triticum Aestivum*)
4. European Mistletoe (*Viscum album*)
5. European mistletoe (*Viscum album*)
6. Barley (*Hordeum vulgare*)
7. Oil nut (*Pyrularia pubera*)
8. Argentine mistletoe (*Phoradendron liga*)
9. California mistletoe (*Phoradendron tomentosum*)
10. Columbian mistletoe (*Dendrophthora clavate*)

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1.          25 KICCPRTIDRNIYNACRLTGAS-MTNCANLSGCKIVSGTTCPPGYTH 70
2.           1 TTCCPSIVARSNFNVCRLPGTP-EALCATYTGCIIIPGATCPGDYAN 46
3.           1 KSCCRSTLGRNCYNLCRARGAQ--KLCAGVCRCKISSGLSCPKGFPK 45
4.           1 KSCCPNTTGRNIYNACRLTGAP-RPTCAKLSGCKIISGSTCPSDYPK 46
5.           1 KSCCPBTTGRBIYBTCRFGGGS-RZVCARISGCKIISASTCPSYPBK 46
6.          29 KSCCKDTLARNCYNTCHFAGGS-RPVCAGACRCKIISGPKCPSDYPK 74
7.           1 KSCCRNTWARNCYNVCRLPGTIsREICAKKCDCKIISGTTCPSDYPK 47
8.           1 KSCCPSTTARNIYNTCRLTGTS-RPTCASLSGCKIISGSTCBSGWBH 46
9.           1 KSCCPTTTARNIYNTCRFGGGS-RPVCAKLSGCKIISGTKCDSGWNH 46
10.          1 KSCCPTTAARNQYNICRLPGTP-RPVCAALSGCKIISGTGCPPGYRH 46
Consensus    1 KSCCPSTTARNIYNTCRLPGTS-RPVCAKLSGCKIISGSTCPSDYPK 46
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Amino acid sequence	Primer code	Oligonucleotide sequence
For all thionin genes:		
KSCCPST	Thio-1F	AAR WSI TGY TGY CCI WSI AC
CPSDYPK	Thio-1R	TT IGG RTA RTC ISW IGG RCA
For *Brassica* species only:		
MEGKTVIL	Thio-2F	ATG GAR GGI AAR ACI GTI ATI CT
GSAKVVETA	Thio-2R	YTC IAC IAC YTT IGC ISW ICC