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A Proteomic Analysis of Seed Proteins Expressed in a *Brassica* Somatic Hybrid and its two Parental Species

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Abstract

Cabbage (*Brassica oleracea*) is one of the world's major vegetable crops. However, the cabbage plant cannot be readily vernalized and produce seed in the tropics, whereas Chinese cabbage (*Brassica campestris*) can be conveniently vernalized at the seed stage. Proteins from seeds of an IPSA cabbage-Chinese cabbage somatic hybrid showing both hybrid vigour and the seed-stage vernalization characteristic were analyzed and compared with those of its two parents. 1D gel analysis indicated some differential expression of the seed proteins. Furthermore, in 2D, gel analysis, 13 differentially expressed protein spots were examined: of these ten were found to belong to the cruciferin seed storage protein complex, two were unidentifiable (no MASCOT match), and one was identified as late embryogenesis Abundant (LEA)-like protein, and was present only in the cabbage parent. This LEA-like protein in cabbage seed could therefore be implicated in interfering with the vernalization process in the tropics.

Introduction

Cabbage (*Brassica oleracea* L. var. *capitata*) and Chinese cabbage (*B. campestris* L. var. *pekinensis*) are important vegetables, cultivated worldwide. They are propagated entirely via seeds. However, the mild winters in the tropics and subtropical regions mean that seed production by cabbage (*Brassica oleracea* L. var. *capitata*) is very problematic: the cabbage needs vernalization at least 3 - 5ºC for four - six weeks, starting from the 7 - 8 leaf stage, to induce flowering. By contrast, Chinese cabbage can be vernalized at the seed stage by keeping the seeds at the germination stage at 4ºC for one - two weeks. To introduce the seed

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vernalization characteristic into the cabbage, a significant numbers of somatic hybrids have been produced between cabbage (cv. Yoshin) and Chinese cabbage (cv. Kenshin) through protoplast fusion using polyethylene glycol (PEG) (Hossain and Yazawa 1994, Yamagishi et al. 1992). Somatic hybridization is a developing field of great importance and has opened up the possibility of combining genetic factors between sexually incompatible species, thereby increasing the spectrum of genetic variation in higher plants (Yamagishi et al. 1992, Hossain and Asahira 1992, Choung et al. 1988). Interspecific crosses in the genus *Brassica* are very useful tools for the development of new vegetables, and oil seeds. Interspecific somatic hybridization in *Brassica* has various advantages over sexual hybridization. It dispenses with laborious procedures such as synchronization of flowering, embryo rescue, and chromosomal duplications of hybrids to obtain fertile amphidiploids (Yamagishi et al. 1992). However, success in interspecific sexual hybridization is limited and it is more difficult when cabbage is used as the female parent (Hossain et al. 1988, Inomata 1997).

Vernalization of seeds of somatic hybrids between cabbage and Chinese cabbage is similar to Chinese cabbage seed vernalization. Moreover, somatic hybrid cabbages can produce larger seeds (average weight of 3.4, 2 and 5 mg per seed of cabbage, Chinese cabbage and somatic hybrid respectively) and grow faster than their parents, starting at germination, which occurs earlier than either parent (Hossain and Asahira 1992).

These novel traits must reflect patterns of gene expression and biochemical activity unique to the hybrid(s), evidence of which would be provided by comparing the protein profiles of the hybrid(s) and the parents. While one-dimensional SDS-PAGE analysis can detect alterations in expression of a number of gene products, the resolution is limited. Two-dimensional gel electrophoresis (2-DGE) is increasingly used to gain new insights into plant functioning (Lery et al. 2008), as it allows the separation of several hundreds of gene products in a single gel and their subsequent identification and quantification. In recent years 2-DGE has been applied to the analysis of plant development (Hocholdinger et al. 2006, Mechin et al. 2004), deciphering of biotic or abiotic stress (Vincent et al. 2005), establishment of genetic distance and further phylogenetic studies (Marques et al. 2001) and characterization of plant mutants (Sorin et al. 2006). It has been used to establish proteomic maps derived from the identification by mass spectrometry of a great number of the protein spots present in the 2-D gel of a particular organ, tissue, or cell culture (Mathesius et al. 2001, Lei et al. 2005). These reference maps can provide clues about the nature of the proteins and their abundance in other studies, and may be used to deduce the main gene functions expressed and likely major metabolic pathways in operation (Schiltz

et al. 2004). However, 2-DGE proteome maps are usually limited to a unique organ or tissue, such as the pollen of *Arabidopsis thaliana*, the endosperm of *Zea mays*, the root of barrel clover (*Medicago truncatula*) or cassava *Manihot esculenta*, and the phloem sap of *Brassica napus* (Mechin et al. 2004, Holmes-Davis et al. 2005, Sheffield et al. 2006).

Using 2-DGE, present authors have recently shown that photosynthesis-related proteins (e.g. Rubisco) are more highly expressed in the leaves of a cabbage (*Brassica oleracea*)-Chinese cabbage (*Brassica campestris*) somatic hybrid showing both growth vigour and seed-stage vernalization, than in the parents (Hossain et al. 2013). Here, a proteomic analysis of seeds from the same *Brassica oleracea-Brassica campestris* somatic hybrid, giving evidence of differential expression between the seed proteins of the hybrid and the parent species is presented. This is the first report of proteomic analysis of seeds from a *Brassica* somatic hybrid.

Materials and Methods

Seeds of cabbage (*Brassica oleracea* var. *capitata* L.), Chinese cabbage (*B. campestris* var. *pekinensis* L.), and a somatic hybrid (synthesized *B. napus* L.) variety IPSA (Institute of Postgraduate Studies in Agriculture, Bangladesh) cabbage were used as plant material. Seeds were collected from these plants and preserved in a desiccator until used.

Triplicate samples of seeds of the somatic hybrid and its parents were frozen in liquid nitrogen and ground into a fine powder using a sterile mortar and pestle with liquid nitrogen. For each sample 100 mg of seed powder were then measured into a sterile Eppendorf tube and 1 ml of ice-cold acetone containing 10% (w/v) trichloacetic acid (TCA), 0.07% (v/v) β-mercaptoethanol was added into the tube. The powdered samples were mixed by vortexing and then kept at –20ºC for 2 hrs. The extract was centrifuged at 16,000 g for 20 min at 4ºC and the supernatant discarded. Each pellet was resuspended and washed in 1 ml cold acetone containing 0.07% (v/v) β-mercaptoethanol. The samples were vortexed and centrifuged at 16,000 g for 20 min at 4ºC and the supernatant again discarded. Washing steps were repeated until all pellets were colourless. After discarding the final supernatants, the pellets were dried under vacuum for 2 - 5 min. Each vacuum-dried pellet was then resuspended in 950 µl freshly prepared lysis buffer (10% (w/v) TCA, 0.07% (v/v) β-mercaptoethanol in acetone) and kept at –20ºC for 2 h. The suspensions were then sonicated on ice for 3 - 6 sessions of 15 seconds each and further incubated on a rotary shaker for 10 - 15 min at room temperature. Then 5 µl of 99% N,N-dimethylacrylamide (DMA) were added to each lysate followed by incubation on a rotary shaker for 30 min at room

temperature, to effect alkylation. Excess DMA was quenched by adding 10 µl 2 M dithiothreitol (DTT) and insoluble material was removed by centrifugation at 16,000 g for 30 min at 4°C. Each supernatant was stored at –20°C until protein concentration determination by the Bradford method (Bradford 1976) and analysis by gel electrophoresis.

For both 1D and 2D gel analyses 100 µg protein were taken from each sample.

For 1D SDS PAGE analysis was carried out on NuPAGE gel (4 - 12%) using MES buffer and then stained by Simply Blue safety stain (Life Technologies) following the manufacturer's instructions. The gels were scanned using the Epichemi Gel Doc system (UVP Ltd., Cambridge, UK).

For 2D gels separation in the first-dimension, by isoelectric focusing (IEF), was performed using 13 cm linear IPG strips (pH 3 - 10) in the IPGphor system. All IPG strips were rehydrated with 250 µl of dehydration buffer (8 M urea, 2% CHAPS, 0.5% ampholytes, 0.002% bromophenol blue) containing 100 µg protein. The voltage settings for IEF were 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and 2000 V for 30 min. Separation in the second dimension followed immediately. The IPG gel strips were incubated in equilibration buffer 1 for 10 min (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT), followed by equilibration buffer 2 (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% v/v glycerol, 2% SDS, 0.002% bromophenol blue, 2.5% iodoacetamide), for 15 min. The strips were subsequently placed onto a 4 - 12% IPG-well NuPAGE Tris-Bis gel (18 × 16 cm) with a Tris- glycine buffer system, as described by Natarajan et al. 2005. Strips were overlaid with 0.5% agarose. The electrophoresis was performed using the XCell SureLock MinI-Cell electrophoresis system from Life Technologies according to the manufacturer's recommendations (Invitrogen). The 2D-PAGE gels were then stained with colloidal Coomassie Blue for 3 h, de-stained with distilled water, and finally stored in 2% NaCl solution at room temperature. The gels were scanned using the Epichemi Gel Doc system.

Protein spot detection was performed by comparing and analysing the scanned images of 2-DGE proteome using a commercial software package (Melanie). Triplicate 2-DGE analysis was performed on the protein samples from each of the three plant types. Normalization of the intensity of the spots was calculated by quantifying the intensity of each spot in a gel as a proportion of the total protein intensity detected for the entire gel. After normalization spot intensities were compared between the replicate groups and group differences were subjected to statistical analysis. Statistical data showed high level of reproducibility for replicate groups. The spots showing significant differences

between replicate groups - either increases or decreases in protein quantity - were selected for protein identification. Protein spots were accordingly excised from the stained gel as "gel plugs" and washed with ultrapure water and then sent to the Department of Biology of University of York, UK for trypsin digestion and protein identification by MS. The protocols followed are described below.

In-gel tryptic digestion was performed after reduction with DTE and S-carbamidomethylation with iodoacetamide. Gel pieces were washed two times with 50% (v/v) aqueous acetonitrile containing 25 mM ammonium bicarbonate, then once with acetonitrile and dried in a vacuum concentrator for 20 min. Sequencing-grade, modified porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold by adding 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02 μg/μl. Gel pieces were rehydrated by adding 10 μl of trypsin solution, and after 30 min enough 25 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37ºC.

A 1 μl aliquot of each peptide mixture was applied directly to the ground steel MALDI target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/ml solution of 4-hydroxy-∞-cyano-cinnamic acid (Sigma) in 50% aqueous (v /v) acetonitrile containing 0.1% , trifluoroacetic acid (v/ v).

Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800 - 4000. Final mass spectra were externally calibrated against an adjacent spot containing 6 peptides (des-Arg^1-Bradykinin, 904.681; Angiotensin I, 1296.685; Glu^1-Fibrinopeptide B, 1750.677; ACTH (1-17 clip), 2093.086; ACTH (18-39 clip), 2465.198; ACTH (7-38 clip), 3657.929). Monoisotopic masses were obtained using a SNAP averaging algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a S/N threshold of 2.

For each spot ten strongest peaks of interest, with an S/N greater than 30, were selected for MS/MS fragmentation. Fragmentation was performed in LIFT mode without the introduction of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection used a SNAP averaging algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 6. Bruker flexAnalysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.1), through the Bruker ProteinScape interface (version 2.1). Search criteria included:

Enzyme, Trypsin; Fixed modifications, carbamidomethyl (C); Variable modifications, oxidation (M); peptide tolerance, 250 ppm; MS/MS tolerance, 0.5 Da.

Protein identification was performed by searching the National Center for Biotechnology Information (NCBI) non-redundant database using the Mascot search engine, which uses a probability-based scoring system. The following parameters were used for database searches with MALDI-TOF peptide mass fingerprinting data: monoisotopic mass, 25 ppm mass accuracy, trypsin as digesting enzyme with 1 missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. For database searches with MS/MS spectra, the following parameters were used: average mass; 1.5 Da peptide and MS/MS mass tolerance ; peptide charge of +1, +2, or +3; trypsin as digesting enzyme with 1 missed cleavage allowed; carbamidomethylation of cysteine as a fixed modification; oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. Taxonomy was limited to green plants for both MALDI and MS/MS ion searches. For MALDI-TOF-MS data to qualify as a positive identification, a protein's score had to equal or exceed the minimum significant score. Positive identification of proteins by MS/MS analysis required a minimum of two unique peptides, with at least one having a significant ion score.

Results and Discussion

The proteins extracted from seeds of cabbage, Chinese cabbage, and their somatic hybrid, were subjected to 1D gel analysis. Fig. 1 shows the results, and it is clear that while some bands are very similar in all three Brassicas, there are also significant differences. Some of the differences are in abundance levels, for example the c. 60 kDa species (A) is abundant in cabbage, but much less so in Chinese cabbage and the hybrid, and again, the c. 35 kDa species (B) appears more abundant in the hybrid than Chinese cabbage but absent in cabbage. Similarly, a c. 34 kDa species (C) appears more abundant in Chinese cabbage than in the hybrid, but is absent in cabbage. There is a heavy band at around 31 kDa in cabbage, which appears to be shared by Chinese cabbage and the hybrid at significantly reduced level (D). The band at 28 kDa (E) is shown by all three Brassicas at similar level. There appear to be two doublets present in cabbage, two extremely close bands averaging at c. 24 kDa band (F, G) and bands at c. 22 kDa (H) and c. 21kD (I). The upper element of the upper doublet (F) and the lower element of the lower doublet (I) are present in all three Brassicas at similar

levels. However, the lower element of the upper doublet (G) and the upper element of the lower doublet (H) are only present in cabbage, but apparently absent in the other two. 1D analysis of the seed proteins showed at least 15 protein bands, with significant variation between the three Brassicas in terms of protein band intensities (Fig. 1: cabbage (track 1), Chinese cabbage (track 2) and the somatic hybrid (track 3). The most prominent bands (c.14KDa) are probably due to napin and cruciferin, because these two classes of protein are the most abundant in *Brassica* seeds, comprising more than 60% of the seed proteins (Miernyk and Hajduch 2011).

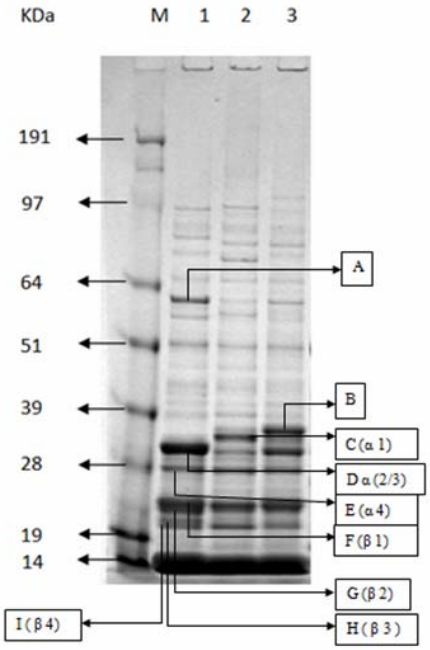

Fig. 1. 1D gel analysis of seed proteins. M = Marker, Lane 1 = Cabbage, 2 = Chinese cabbage and Lane 3 = somatic hybrid. Marker used: See Blue Plus2 Pre-stained standard (Invitrogen). C - I are cruciferin subunits. C = α1 c.34kDa, D = α2/3 c. 31kDa, E = α4 28kDa, F = β1 c. 23kDa, G = β2 c. 23kDa, H = β3 c. 20kDa, I = β4 c. 20kDa.

Napin is a 2S albumin class of protein with a size range of 12.5 - 14.5 kDa (Berot et al. 2004). The prominent 14 kDa band present in all three lanes (Fig. 1) can be attributed to napin. In all three samples the napin band is very dense, and no expression differences between the three Brassicas are evident.

Cruciferin is a 12S globulin class of protein consisting of six subunits with a molecular weight of c. 300 kDa. Each subunit is composed of an alpha chain, approximately 29 - 34 kDa, and a beta chain in the range 20 - 23 kDa, derived from at least three major precursor forms (Ochuodho et al. 2006), and *Brassica* has

four α chains (α 1 - 4) and four β chains (β1 - 4). The alpha and beta chains form four cruciferin subunits (cru1 - cru4), and complexes with different subunit compositions make cruciferin a complex, heterogeneous protein (Rodin et al. 1990). In a 1D gel analysis of rapeseed proteins, more than ten cruciferin polypeptides were found (Wu and Muir 2008).

In the gel in Fig. 1, all eight cruciferin subunit chains (α 1 - 4 and β 1 - 4) can be identified, but relative abundances differ significantly between cabbage, Chinese cabbage and the somatic hybrid. The heaviest chain, at c. 34 kDa, α1 (labeled as C), appears absent from cabbage, is well represented in Chinese cabbage, and at a much lower level in the hybrid. The 31 kDa band (D), apparently α2 and α3, is up-regulated in cabbage, much less prominent in the Chinese cabbage, and intermediate in the hybrid. The 28 kDa band, α4, is at a very similar, fairly low level in all three Brassicas. Bands F and G form a close doublet at c. 23 kDa, corresponding to β1 and β2: β1 appears most highly expressed in cabbage, followed by the hybrid, then Chinese cabbage, but β2 appears to be absent from Chinese cabbage and the hybrid. Another doublet (H and I, c. 20 kDa) corresponding to β3 and β4, is evident in cabbage, with β4 expressed similarly by the three Brassicas, but β3 is at a much lower level in both Chinese cabbage and the hybrid.

Other much less prominent bands, and the evident band A (cabbage) and even stronger band B (hybrid) may reflect different conformation states of cruciferin (Berot et al. 2004) or incomplete reduction of cruciferin hexamers by the reducing agents β-mercaptoethanol and DTT, or limited proteolytic activity occurring in dry seed (Ochuodho et al. 2006). Similar findings were observed in *Cleome gynandra*, where the dimeric cruciferin polypeptides of ~50KDa were reduced to M_rs of 30, 28, 18 and 13 - 15 kDa (Ochuodho et al. 2006), and in rape seed (Rodin et al. 1990). In essence, the 1D gel analysis for the three Brassica seed samples indicates that while overall napin contents appear similar, cruciferin profiles are distinctive for each plant type, and overall cruciferin content appears highest in the cabbage, and lowest in the Chinese cabbage.

The 2D analyses presented in Fig. 2 show broadly similar spot patterns for all three Brassicas (cabbage - gel S1, Chinese cabbage - gel S2, somatic hybrid - gel S3), but a number of differences of abundance or presence *versus* absence are discernible. Most of these differentially expressed proteins lie in the 19 - 39 kDa zone. Spot S1-1 is intense in gel S1, but the equivalent spots are less so in the other two gels. Spots S1-2 and S3-2 were at same positions, but no such spot was present in S2. Spot S1-3, in gel S1, was absent in gels S2 and S3. Spots S2-2 and S3-3 were similar in abundance, and at the same positions on their respective gels, but lower in level in gel S3, there being no equivalent spot in gel S1. Spots

S1-4 and S3-4 were at the same position on gels S1 and S3 respectively, but no equivalent spot occurred in gel S2. These differentially expressed spots (except S1-4 but equivalent spot S3-4 in S3 was analysed) were excised and analysed by MS for protein identification, as were spots S1-6, S2-1 and S3-1, which, being similar in abundance and identically positioned on their respective gels, were used as controls.

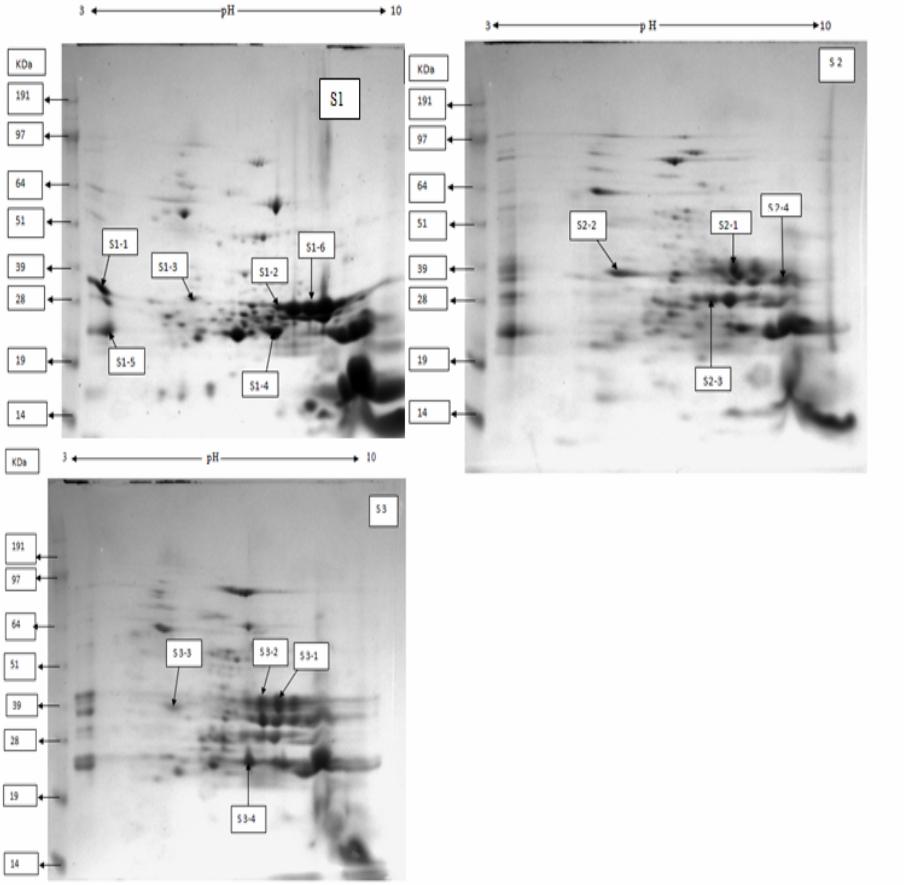

Fig. 2. 2-DGE protein profiles of seed proteins of cabbage (S1), Chinese cabbage (S2) and the somatic hybrid (S3). The spot numbers are differentially expressed proteins selected for protein identification (except S1-4). The relative expression levels are indicated in the text and in Table 1. Markers used: SeeBlue Plus2 Pre-stained standards (Invitrogen).

A total of 13 spots were analysed, including the three controls (Table 1), and all MASCOT scores were substantially higher than 70 - the usual criterion of reliability in protein identification (Perkins et al. 1999). Two proteins, S2-2 and S3-3, could not be identified, ten were the well-known cruciferin storage proteins, and cruciferin precursors, and the remaining protein (S1-3) was

Table 1. Seed proteins from selected 2 D gel spots putatively identified via mass spectrometric analysis of tryptic digests. S1, S2 and S3 refer to proteins from cabbage, Chinese cabbage and somatic hybrid samples, respectively.

Spot	Putative identity of protein	Mascot score	MWt (Da)	pI	Accession number	Reference organism	Relative abundance*		
							S1	S2	S3
S1-1	*Cruciferin precursor*	171	56429	6.84	gi|167136	*Brassica napus*	+++	++	+
S1-2	*Cruciferin precursor*	296	56429	6.84	gi|167136	*B. napus*	++	-	+
S1-3	LEA-like protein	134	31408	5.21	gi|4140257	*Arabidopsis thaliana*	+	-	-
S1-5	Cruciferin CRU1, CRU4 and single peptide match to CRU2/3	390	56867	7.64	gi|461840	*Brassica napus*	++	++	+
S1-6	*Cruciferin precursor*	169	56429	6.84	gi|167136	*B. napus*	++	++	++
S2-1	*Cruciferin precursor*	169	56429	6.84	gi|167136	*B. napus*	++	++	++
S2-2	No match						-	++	+
S2-3	Cruciferin CRU4	219	51630	7.70	gi|461841	*B. napus*	+	++	+
S2-4	Cruciferin BNC2, BNC1	458	54542	8.72	gi|1345841	*B. napus*	++	++	++
S3-1	Cruciferin precursor	237	56429	6.84	gi|167136	*B. napus*	++	++	++
S3-2	Cruciferin precursor	258	56429	6.84	gi|167136	*B. napus*	++	-	+
S3-3	No match						-	++	+
S3-4	Cruciferin CRU2/3 subunit and CRU1, plus single peptide matches to cupin family protein, seed storage β-China 6 and ribosomal protein L2	234	12000	9.30	gi|17803	*B. napus*	++	-	++

*This column indicates apparent similarities and differences in expression levels for the relevant protein in different proteomes examined. +++ = high, ++ = medium, + = low, - = absent.

identified as an LEA-like protein. The gels also show that the detectable proteins present in these seeds are mostly neutral to basic.; very few protein spots observed are acidic, such as S1-1 (Mr 39 - 51 KDa).

2-DGE analyses of seed proteins from the three Brassicas (cabbage - S1, Chinese cabbage - S2, somatic hybrid - S3 - see Fig. 2) showed most detected polypeptides are observed between 10 and 39 kDa, and with pIs above neutrality.

Spots in the 10 - 14 kDa molecular weight range and isoelectric points of ≥ 10 are most probably napins: these are strongly present in all gels, consistent with the 1D gel result (Fig. 1), but the superior resolution of the 2D gels indicates a higher napin content in cabbage than Chinese cabbage and the hybrid, which are fairly similar.

Thirteen differentially expressed protein spots were identified and selected for protein identification analysis, and were mostly in the 14 to 39 kDa molecular weight range, with pIs from 4 - 10; an earlier report detected distributions of *Brassica* cruciferin proteins at pIs 4.75 - 9.15 (Mackenzie 1975). Of the 13 spots, ten are indeed cruciferin precursors and cruciferin subunits, two could not be matched via Mascot, and one spot is an LEA-like protein, showing that most of the samples of differentially expressed proteins examined here are derived from seed storage proteins (SSP) (Table 1). Given the abundance of SSP in seeds, this is not surprising, and SSP-derived spot patterns and comparisons are made more complex by the extended SSP gene families and heterogeneity in both proteolytic and glycolytic processing (Miernyk and Hajduch 2011). Nevertheless, comparing relative abundance levels of the ten cruciferin-related proteins in the three Brassicas indicates overall cruciferin content is highest in the cabbage and lowest in the Chinese cabbage (Table 1), consistent with the 1D result. Unexpectedly, for dry seeds, cruciferin precursors were found in seeds of all types (Table 1). However, it is possible that the α and β chain maturation processes were incomplete when the developing seeds entered into quiescence (Gallardo et al. 2001).

Spots S2-2 (Chinese cabbage) and S3-3 (somatic hybrid) were in the same position (~39 kDa) in their respective gels but protein analysis scored no match, presumably because of database limitations (Song et al. 2007).

The only identified non-SSP protein found to be differentially expressed (S1-3) is an LEA-like protein, found in cabbage seed at c. 31 kDa, but not observed in gels containing protein from Chinese cabbage (S2) or the somatic hybrid (S3) seeds. At 31 kDa, the molecular weight of this LEA-like protein is within the range (6.98 - 36.76 kDa) found in other species (Su et al. 2011). LEA protein accumulates in large quantities at the late seed maturation stage absent and

degradation during the germination stage (Tai et al. 2005). There are six forms of LEA-protein, according to their expression and sequence motifs, the major groups being forms 1 - 3 (Veerranagamallaiah et al. 2011). The amount of LEA present in dry seeds varies from 1.5 - 3% (Delseny et al. 2001), which is very low compared to SSP (> 60%). Although LEA-protein is regarded as a "multi-talented protein", its exact functions are still unclear (Tunnacliffe and Wise 2007). However, putative functions include desiccation tolerance, redox balance through anti-oxidant activity, chaperone roles, and the provision of increased resistance to osmotic and freezing stress (Ginger et al. 1999, Zhang et al. 2000).

Extensive literature searching has revealed no report of the presence of LEA-protein in Chinese cabbage seed. However, several papers have reported production of transgenic Chinese cabbage using a heterologous LEA gene to confer salt and drought resistance (Park et al. 2005). Since cabbage and Chinese cabbage require cold stress at different life stages for vernalization leading to early flowering, we postulate that the LEA-protein may play an important role in this regard. Vernalization, the cold-induced acceleration of flowering, involves epigenetic silencing of the floral repressor gene FLOWERING LOCUS C (FLC) (Shindo et al. 2006), and this epigenetic silencing, via histone methylation, is maintained throughout subsequent mitotic cell divisions in shoot apical meristem during spring growth (Sung et al. 2006). FLC also has a role in mediating temperature-dependent germination via regulation of two hormones, abscissic acid, (which induces and maintains dormancy), and gibberellins, (which stimulate germination) and the effect is maternal, with overexpression of the FLC gene occurring during the late seed maturation stage (Chiang et al. 2009). In Chinese cabbage vernalization is performed on the seeds, incubating at 4 - 6°C in the fridge. On the other hand, cabbage seeds cannot be vernalized in the same way, and vernalization must take place at the seedling stage.

Present authors postulate that the presence of LEA-like protein in cabbage seed protects them from cold stress, preventing the switching-on of the flowering developmental programme - if seed vernalization process is attempted at 4°C in the fridge. By contrast, seeds of Chinese cabbage and the somatic hybrid, lacking adequate levels of LEA-protein, are not protected from the cold stress which causes epigenetic repression of FLC gene, and as a result the flowering programme can be triggered. As LEA-proteins present in seed degrade during germination (Tai et al. 2005), seedlings exposed to cold stress would initiate the epigenetic repression of the FLC gene, which may be the process in cabbage vernalization. The somatic hybrid contains the genomes of both cabbage and Chinese cabbage, so present authors assume that there is some genetic

interaction repressing the cabbage LEA gene during seed development, or the amount of LEA-protein per hybrid seed may be just too low for cold induction.

Earlier study has revealed that the seed of the somatic hybrid germinate earlier than those of the parents, indicating lack of LEA could be a factor. LEA can interact with oligosaccharides to form a bioglass to relieve from different types of stress, including osmotic pressure (Zhang et al. 2000). The latter may also interfere with the imbibition process during germination. It has been suggested that bioglass in dry seed limits the mobility of molecules, slowing down different reactions (Kalemba and Pukacka 2008).

A final point is that the presence of LEA, and a greater abundance of storage proteins, is expected to give cabbage seed better storage ability than that of Chinese cabbage or the hybrid, and indeed the cabbage seed has shown superior storage ability compared to other Brassica seeds (personal communication). In a recent report (Rajjou and Debeaujon 2008), abundance of LEA protein, seed storage and heat shock proteins (HSP) have been linked to the storage capacity of the seed.

Further study needs to be carried out to confirm the type of LEA-like protein found in cabbage and as reported here, to assess its possible biotechnological applications.

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