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THE UNFOLDED PROTEIN RESPONSE (UPR) IN MICROSPORE-DERIVED EMBRYOS (MDEs) FROM *BRASSICA NAPUS*.

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Introduction

A major role of the endoplasmic reticulum (ER) is to maintain the quality of many proteins synthesized in the cell. Stressed ER can lead to misfolding of proteins, which in turn induces a response pathway aimed at mitigating the stress. This response is termed the unfolded protein response (UPR). The major characteristics of UPR are increased production of protein-folding chaperones and enhanced protein degradation. While the UPR has been studied in plant vegetative tissues, our knowledge of its effect on the developing plant seeds is limited. There is evidence that overexpression of a non-plant protein in the seed can induce UPR (*Oono et al 2010*). Thus, an understanding of the UPR in seeds is essential if one takes into account that the ability to use the seed as a vehicle for recombinant protein production resides in the ability of the ER to handle increased protein processing capacity. Furthermore, it is no known whether a persistent UPR in the developing seed could also be detrimental to seed maturation and stability.
Chemicals like Tunicamycin and DTT could be used to induce the UPR in plants (*Martinez and Chrispeels, 2003*). However, the chemical induction of UPR in developing zygotic seeds *in planta* is experimentally difficult. Cultured microspore derived embryos (MDEs) have been shown to reflect zygotic embryos in seed development in *Brassica*. Here we report the successful induction of UPR in *Brassica napus* microspore derived embryos.

Methods

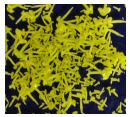

Figure 1

Microspore Culture: Donor plants, *B. napus topas* were grown for 16 hour photoperiod with full illumination and day/night temperatures of 10°C and 5°C respectively. Buds of 3-4 mm in length were selected, surface sterilized in bleach, and rinsed in sterile water. Sterile buds were crushed with glass rod in 5 mL ½ B5 media. Suspen-sion was filtered over 44um nylon filter by gravity. Eluate was spun down at 1000 rpm for 3 min to gather microspores. Resulting pellet was washed a further 2 times with ½ B5 media, then suspended at 10^5 microspores per mL in NLN media. The 10^5 suspension was dispensed 10mL volume per 100 x 15 mm Petri dish. Plates were wrapped and placed in darkness at 32°C for three days then at 25°C with gentle rotation (70 rpm). After one week, microspores were subcultured into larger 150 x 15 mm Petri dishes with a four fold dilution into fresh NLN. Figure 1 shows torpedo staged *B. napus* cv Topas MDEs used for induction of UPR with Tunicamycin.

Tumicamycin treatment of embryos: Induction conditions previously used in Arabidopsis culture (*Martinez and Chrispeels, 2003*) are used to induce UPR in *B. napus* microspore derived embryo (MDE) culture. On day 18 of culturing, (or) at or just before torpedo stage, microspores were treated with 5 ug/mL Tunicmaycin in DMSO or an equivalent amount of DMSO as negative control for 5 hours. Embryos were harvested over a 250um nylon mesh and flash frozen in liquid nitrogen.

Isolation of Total RNA: Total RNA was extracted from approximately 100 mg embryos using TRIzol reagent (with plant modification). DNaseI treatment was performed using QIAGEN Rnase-Free Dnase set and followed up with QIAGEN cleanup. RNA was evaluated by Bioanalyzer.

RT-PCR and Microarray Analysis: 2 ug total RNA was used in RT-PCR using Invitrogen SuperScript II and random hexamer for first strand synthesis and BiP specific primers in second strand. RNA samples were used on Agilent Brassica 2x105k arrays with one dye flip as technical replicate. Custom 2x105K Agilent 60-mer oligo Brassica arrays were scanned with a Genepix 4200A scanner (Molecular Devices) and the images analyzed with Genepix 6.0 (Molecular Devices). The data was imported in Acuity 4.0 (Molecular Devices) and Lowess normali-zation was applied to each array. Low intensity features with Lowess A median values of less than 8.5 in at least 50% of the arrays were removed from the dataset. Substances with a standard deviation ≥0.3 were also removed.

Results

Tunicamycin induces the UPR in Brassica MDEs

Microarray analyses indicated that the highest levels of transcripts accumulated in the MDEs in response to Tunicamycin encoded ER-resident UPR housekeeping proteins (Table 1A). As well, these transcripts dominated the top 100 genes observed to be overexpressed 2+ fold (data not shown).

The UPR in the MDEs results in down regulation of genes critical to seed development

Interestingly, induction of UPR in *Brassica* MDEs resulted in the downregulation of LEA or LEA-like (late embryogenesis abundant) proteins (Table 1B). LEA-like proteins are also encoded by EM, low temperature induced 65kD, and BN28b transcripts (Table 1B).

The down regulation of transcripts encoding LEA-like proteins is likely to be the direct effect of the UPR induced by Tunicamycin. Overexpression of a mammalian beta-amyloid protein in rice seeds induced a UPR also with a concomitant downregulation of LEA-like genes (Oono *et al* , 2010). These observations underscore the importance of the UPR in seed development and in the use of the seed in protein hyperexpression.

Table 1A

Probe id	Fold change	Gene description	A.th. Ortholog
B_1061386	5.50	Luminal binding protein (BiP-1)	AtSG28540
X_1004736	5.20	Luminal binding protein (BiP-1)	AtSG28540
B_1089330	5.20	SHD (SHEPHERD); GRP94, ER-resident HSP90-like	AT4G24190
B_1041549	5.06	SHD (SHEPHERD); GRP94, ER-resident HSP90-like	AT4G24190
X_1009352	5.02	SHD (SHEPHERD); GRP94, an ER-resident HSP90-like protein	AT4G24190
B_1046951	4.86	ATPDIL2-3 (PDI-LIKE 2-3); protein disulfide isomerase	AT2G32920
B_1064156	4.79	HSP70; putative	AT4G16660
X_1022119	4.74	DER2.1 (DERLIN-2.1) like	AT4G21810
X_1004619	4.65	HSP70; putative	AT4G16660
X_1004278	4.58	HSP70; putative	AT4G16660
X_1029901	4.45	Fructose-bisphosphate aldolase, putative	AT4G38970
X_1016467	4.38	CALRETICULIN 1 (CRT1)	AT1G56340
X_1007113	4.37	HSP70; putative	AT4G16660
X_1062061	4.33	CALRETICULIN 1 (CRT1)	AT1G56340
X_1016728	4.27	UDP-GALACTOSE TRANSPORTER 3 (UTR3)	AT1G14360
B_1013287	4.25	ATERDJ3A; DNAJ/HSP40	AT3G08970
B_1055116	4.18	SHD (SHEPHERD); GRP94, ER-resident HSP90-like	AT4G24190
B_1055946	4.18	Unknown endoplasmic reticulum protein	AT4G29520
B_1052585	4.15	Conserved ER armadillo/beta-catenin repeat binding protein	AT3G51980
X_1083381	4.12	Calnexin 1 (CNX1)	AT5G61790
B_1060906	4.11	Calreticulin 2 (CRT2)	AT1G09210
B_1060940	4.10	Unknown endoplasmic reticulum protein	AT4G29520
B_1031934	4.08	SHD (SHEPHERD); GRP94, ER-resident HSP90-like	AT3G62600
X_1039818	4.04	Calnexin 1 (CNX1)	AT5G61790
B_1069079	4.03	UDP-GALACTOSE TRANSPORTER 3 (UTR3)	AT1G14360
X_1055886	4.01	Protein disulfide isomerase-like (PDIL)	AT2G32920
B_1057029	4.00	HSP40-like J domain ER lumen protein	AT3G08970
B_1066950	3.99	SHD (SHEPHERD); GRP94, ER-resident HSP90-like	AT4G24190
B_1072592	3.97	P-P-bond-hydrolysis-driven protein transmembrane transporter	AT1G29310
B_1077094	3.96	Zinc finger (C3HC4-type RING finger) family protein	AT1G22510
B_1051272	3.93	Unknown ER located protein	AT5G64510
B_1057509	3.85	BiP2	AtBG42020

Table 1B

Probe id	Fold Change	Gene Description	A.th. Ortholog
B_1064732	-5.418	Em protein	AT2G40170.1
B_1047320	-5.183	AtHSP22.0 precursor	AT4G10250.1
B_1051522	-5.088	late embryogenesis abundant domain-containing protein	AT3G17520.1
B_1064074	-4.932	Low-temperature-induced 65 kDa protein	AT5G52300.1
B_1056758	-4.852	Gibberellin 2-beta-dioxygenase 8	AT4G21200.1
B_1062677	-4.552	Putative glutathione transferase	AT1G17170.1
B_1055747	-4.388	Lipase SIL1	AT5G40990.1
X_1055673	-4.333	Cytochrome P450	AT3G14650.1
B_1056739	-4.286	Cytochrome P450 71A1	AT3G26180.2
X_1058622	-4.231	Ethylene-responsive transcription factor 1A	AT4G17500.1
B_1075287	-4.16	Cytochrome P450-like protein	AT4G37370.1
B_1060265	-4.137	PJDC23.16 protein	AT1G78410.1
X_1063509	-4.1	Late embryogenesis abundant protein-like	AT5G44310.2
B_1001981	-4.067	22.0 kDa class IV heat shock protein precursor	AT4G10250.1
X_1081627	-3.997	Flavonol-3-O-glycoside-7-O-glucosyltransferase 1	AT2G36750.1
X_1031159	-3.971	Cytochrome P450	AT3G14650.1
B_1064864	-3.964	Putative disease resistance protein	AT1G33590.1
B_1060201	-3.964	Chloroplast serine acetyltransferase	AT1G55920.1
B_1035745	-3.945	Putative alanine acetyl transferase	AT2G32020.1
B_1056995	-3.894	Glutathione S-transferase ERD13	AT2G30870.1
B_1055780	-3.862	Type I inositol-1,4,5-trisphosphate 5-phosphatase 11	AT1G47510.1
B_1053494	-3.841	Group I late embryogenesis abundant protein	AT5G06760.1
B_1076615	-3.84	Ethylene-responsive transcription factor 1B	AT3G23240.1
B_1068146	-3.821	Thi2-like protein	AT3G14990.1
B_1049073	-3.79	Putative seed maturation protein	AT2G42560.1
B_1051422	-3.774	Cytochrome P450	AT3G14690.1
B_1055776	-3.72	Probable WRKY transcription factor 33	AT2G38470.1
B_1067336	-3.695	BN28b	AT5G15970.1
B_1062568	-3.672	17.6 kDa class II heat shock protein	AT5G12020.1
B_1057413	-3.666	Embryonic abundant protein-like	AT3G54150.1
B_1063172	-3.664	Putative myb-related protein, 33.3K	AT5G67300.1

Figure 2 shows RT-PCR validation of the microarray data with selected typical UPR induced as well as LEA genes observed to be up- and down-regulated respectively by microarray analyses. The designation next to the gene description is the Probe ID for the element on the array.

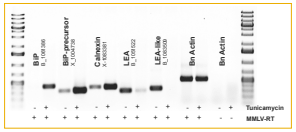

Figure 2

Summary

1) We show that *Brassica* microspore derived embryos (MDEs) can be chemically induced to produce the unfolded protein response (UPR). This is indicated by the upregulation of transcripts encoding ER resident proteins involved in protein folding and ER protein trafficking. These results suggest that MDEs constitute a valid model system for studying ER stress and the UPR in seeds and in relation to recombinant protein production and seed development.
2) Unlike the UPR induced in vegetative tissues in Arabidopsis (Martinez and Chrispeels, 2003), the UPR in MDEs resulted in the downregulation of genes critical for the devel-opment of desiccation and abiotic/biotic stress tolerances in the seed. We are in the process of determining the mechanisms of the downregulation of stress-related genes by the UPR and its effect on the ability of the MDEs to develop desiccation tolerance.

References

Martínez I, Chrispeels M (2003) Genomic Analysis of the Unfolded Protein Response in Arabidopsis Shows Its Connection to Important Cellular Processes. The Plant Cell 15, 561–576
Oono Y, Wakasa Y, Hirose S, Yang L, Sakuta C, Takaiwa F (2010) Analysis of ER stress in developing rice endosperm accu-mulating b-amyloid peptide. Plant Biotech J 8, 1–28

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