SUPPLEMENTARY MATERIALS AND METHODS

Growth conditions of the donor plants

Plants of Brassica napus L. cv. Topas line DH 4079 were grown year-round in a greenhouse at a 20/18°C day/night temperature regime. Additional light (Philips SON-T lamps) was given from mid September to the end of April, which also served to obtain a photoperiod of 16 h. At the beginning of bolting, the plants were transferred to a growth chamber at continuous 10 °C with 16 h illumination provided by 150 $\mu E.m^{-2}.s^{-1}$ HPI (Philips) light, and watered twice a week with N:P:K=15:15:18 soluble fertilizer. Inflorescences were harvested from plants kept at least two weeks under these conditions, after the first flowers had opened.

Microspore isolation and culture

Media and other chemicals needed for microspore isolation, washing, and culture were taken from a 4°C refrigerator and were used immediately. The entire microspore isolation procedure was usually accomplished within one hour while the chemicals were still cool. Flower buds were sterilized for 10 min with 2% NaOCl + 0.05% (v/v) Tween-20, rinsed 3 times in sterile water for 1, 5 and 10 min, consecutively, and transferred to 50 ml disposable buckets containing 1.5 ml filter sterilized NLN-13 medium. This is a Nitsch and Nitsch medium as modified by Lichter (1982) with 13% (w/v) sucrose and without potato extract or growth regulators. The buds were gently squeezed for 5 to 10 sec with the back of the plunger of a disposable 50 ml syringe. The slurry was passed through two-layers of 44 μm nylon cloth, then bucket, plunger and cloth were rinsed with another 8.5 ml NLN-13 medium. The filtrate was centrifuged at 100g for 3 min in a refrigerated centrifuge at 4°C, and the pellet resuspended in fresh NLN-13 medium. After two additional washings, the microspores were resuspended at a density of 40,000 per ml in NLN-13 medium. Aliquots of 1 or 3 ml microspore suspension were plated in 3 or 6 cm Petri dishes, respectively, for culture. Developmental stages of the microspores at the start of culture was determined using 4',6-diamidino-2-phenylindole (DAPI) epifluorescence staining according to Custers et al. (1994). The cultures were incubated in the dark under different temperature regimes: (i) continuously 18.0 ±1.0°C to allow pollen maturation, (ii) 24 hrs 32.0 ±0.2°C followed by transfer to 25.0 ±1.0 °C for suspensor-bearing MDE development, and (iii) continuously at 32.0 ±0.2°C for production of conventional MDE development.

Microarray Slide Printing and Processing

Each cDNA probe was spotted in duplicate on the array. Three non-plant probes were spotted in four-fold on the array and used for the estimation of array-wide background signals: yeast aspartate kinase (Genbank accession number J03526), imidazoleglycerolphosphate dehydratase (Genbank accession number Z75110) and phosphoribo-sylaminoimidazole carboxylase (Genbank accession number Z75036). *Luciferase* cDNA probes were used as

spike-in controls for normalization of the expression data. The complete coding sequence of the firefly luciferase gene, and three partial luciferase clones encompassing the 5'-, middle- and 3'- part of the gene were spotted in 16- and four-fold, respectively. Spiking of the samples with luciferase mRNA prior to labeling allowed for correction of the expression ratios for channel-specific effects. The probes were resuspended in 10 μl 5 X SSC (~1 μg/μl) and then transferred to 384-well plates for spotting. The identity of a random selection of probes was verified by re-sequencing the DNA from the 384-well plates used for spotting of the array.
Probes were spotted on GAPS amino silane coated glass slides (Corning) using a PixSys 7500 arrayer (Cartesian Technologies) equipped with Chipmaker 3 quill pins (Telechem). Approximately 0.5 nl of probe was spotted, resulting in a spot diameter of 120 μm at a pitch of 160 μm. Each non-control probe was spotted in duplicate, 2.25 mm apart, resulting in a total spotted area of 9×9 mm. Slides were rehydrated by holding them over a hot water bath (~70°C) and then snap-dried on a 95-100°C hot plate (5-10 sec). The probe DNA was UV cross-linked (150 mJoules). The slides were processed by soaking them twice in 0.2% SDS for 2 min, twice in water for 2 min and once in 100 °C water for 2 min to allow DNA denaturation. The slides were dried (5 min) and then rinsed 3 times in 0.2% SDS for 1 min each, once in MQ water for 1 min, submerged in 100 °C MQ water (2 sec) and dried.

Microarray Target Amplification and Labeling

Material from two independent MDE cultures (culture 1 and culture 2), each harvested at seven time points (described above) was used to isolate total RNA (Plant RNAEasy, Qiagen). Total RNA was also isolated from the vegetative leaves of flowering plants and from flower buds at the same stage of development as those used for microspore isolation. Total RNA (1 μg) from each sample was converted into first strand cDNA using an oligo(dT) primer containing a T7 RNA polymerase promoter. Target amplification, cDNA synthesis and indirect labeling steps were performed using the MessageAmp aRNA Kit (Ambion). Luciferase mRNA (L4561 Promega) was added to each RNA sample (0.1 ng Luciferase/ 1 μg total RNA) prior to target amplification. The RNA quantity of the amplified RNA was measured by RiboGreen staining (Molecular Probes) using a molecular imager FX pro plus (Biorad). Amplified RNA (1μg for a Cy5 and 2,5 μg for a Cy3 labeling) was converted into first strand cDNA using random hexamer primers and 5-(3-aminoallyl)-dUTP. Cy3 or Cy5 monofunctional dyes were coupled to the amino-modified nucleotides as described in van Doorn et al. (2003).
The quality of the labeling reaction was determined by gel electrophoresis of 10 % of the labeled target and followed by measurement of the Cy5 and Cy3 fluorescence emissions on a Molecular Imager FX pro plus scanner. After amplification equal amounts of antisense RNA corresponding to each of the seven culture time points, from each of the two independent cultures was used to create a common reference sample. The labeled targets were resuspended in 8 μl MilliQ water.

Microarray Hybridization, Scanning and Data Acquisition

Two-color microarray experiments were performed in which targets from each of the 16 samples was hybridized with the common reference. In one set of experiments the 14 targets from cultures 1 and 2, as well as the leaf and flower bud targets were each labeled with Cy5 and hybridized with a Cy3-labeled common reference target. A swapped-dye experiment was conducted for the series of seven targets from one of the independent cultures and for the single leaf and flower bud targets. In total, 25 hybridizations were performed. One μg of Cy5-labeled and 2.5 μg of Cy3-labeled target were used per hybridization. The labeled targets were added to 64 μl hybridization mix (50% formamide, 5 X Denhardt's reagent, 5 X SSC, 0.2% SDS, 0.1 mg/ml sonicated/fragmented salmon sperm DNA) and denatured by 1 min 94°C. Slide hybridization, washing and scanning was performed as in Soeda et al. (2005)

Real-time RT-PCR

Expression profiles for selected differentially expressed probes were validated using quantitative real time RT-PCR. Total RNA from the seven individual time points from culture 2, and from the common reference were used for the analysis. The cDNA synthesis and real-time PCRs were performed using the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster City) and the SYBR-Green PCR Master kit containing a Hot Start Taq polymerase (AmpliTaq Gold) on a ABI Prism 7700 (Applied Biosystems), respectively. PCR amplification was followed by a DNA melting curve analysis to verify primer specificity. DNA amplification efficiencies were assessed for each primer pair using a cDNA dilution series under Real-Time PCR conditions. The comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to compare the relative level of gene expression for a given primer set in different tissues. 18S rRNA genes were used as the reference and the common reference sample from the microarray experiments as the calibrator. The primer sequences and Ct values are available as supplementary data (Table S4, Figure S1)

Protein Isolation and Sample Preparation

For 2-D-GE analysis of proteins cells were collected by centrifugation at 1000g (5 min, 4 ^{o}C) and immediately frozen in liquid nitrogen. The frozen pellet was resuspended in 1 ml of ice-cold 10% TCA, 0.5% DTT by vortex-mixing. The mixture was sonicated for 5 min in ice-water and then allowed to stand for 30 min, after which the proteins were pelleted by centrifugation at 20,000g for 15 min. The supernatant was removed and the pellet was washed with 1 ml of 100% acetone, 0.1% DTT by incubation for at least 1 h at -20°C. The tube was vortexed every 15 min to disperse the pellet. After centrifugation at 20,000g for 10 min. the pellet was washed once with 1 ml of ethanol:ether (1:1, -20°C) and air dried briefly. The proteins were resuspended in 0.2 ml of isoelectric focusing (IEF) sample buffer (8 M urea, 2 M thiourea, 2% CHAPS, 1% DTT) by stirring for several hours on a shaker at room temperature. After centrifugation at 20,000g for 10 min. the soluble proteins were transferred to a new tube and re-precipitated by using the 2-D Clean-Up kit from Amersham Biosciences following the

instructions of the supplier. Finally, the pellet was resuspended in an appropriate volume of IEF sample buffer for protein determination and first dimension IEF. Protein concentrations were determined with the RC DC Protein Assay kit (BioRad) using protein aliquots in IEF sample buffer.

2-D Gel Electrophoresis

IEF was carried out with 400 µg of protein for each of the culture extracts using precast Immobiline DryStrip gels (pH 4 to 7, 18 cm; Amersham Biosciences). IEF was performed at 20°C on the IPGphor system (Amersham Biosciences) for 25 h (160,000 Vh) after overnight (≈18 h) rehydration of the strips with protein sample, Strips were stored at – 70 °C until the second-dimension electrophoresis was performed. For the second-dimension electrophoresis, the strips were first incubated for 15 min. in reducing equilibration buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 1% DTT) and then for 15 min in alkylation buffer that contained 2.5% iodoacetamide instead of 1% DTT. Equilibrated strips were placed on top of vertical 11% polyacrylamide gels (23 cm x 19 cm x 1 mm) and overlaid with hot (70°C) 0.5% agarose in SDS running buffer with a trace of bromophenol blue. After agarose solidification, electrophoresis was performed at 10 °C for 1 h at 30 V, followed by 100 V until the dye front reached the bottom of the gel (overnight). The 2-D gel-separated protein spots were visualized with the fluorescent stain SYPRO Ruby (BioRad). Fluorescent scanning was performed using the Molecular Imager FX and the digitized gel images were analyzed using the software packages PDQuest version 7.1.0 (BioRad) and Progenesis Discovery version *2003.03* (Nonlinear Dynamics*)*.

REFERENCES

Custers JBM, Cordewener JHG, Nöllen Y, Dons JJM, Van Lookeren Campagne MM (1994) Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep **13:** 267-271

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus.* Z. Pflanzenphysiol **105:** 427-434

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25:** 402-408

Soeda Y, Konings MC, Vorst O, van Houwelingen AM, Stoopen GM, Maliepaard CA, Kodde J, Bino RJ, Groot SPC, van der Geest AHM (2005) Gene expression programs during *Brassica oleracea* seed maturation, osmopriming, and germination are indicators of progression of the germination process and the stress tolerance level. Plant Physiol **137:** 354-368

van Doorn WG, Balk PA, van Houwelingen AM, Hoeberichts FA, Hall RD, Vorst O, van der Schoot C, van Wordragen MF (2003) Gene expression during anthesis and senescence in Iris flowers. Plant Mol Biol **53:** 845-63