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ORIGINAL PAPER

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Genes involved in biosynthesis and signalisation of ethylene in *Brassica oleracea* and *Arabidopsis thaliana*: identification and genome comparative mapping of specific gene homologues

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Abstract The study reported was aimed at the identification and determination of the chromosomal organisation of genes involved in the ethylene biosynthesis and signalling pathways in *Brassica oleracea*, on the basis of the *Arabidopsis thaliana* DNA probes and in silico genome analysis. Because of its polyploidal origin, the *B. oleracea* genome is characterised by extensive gene redundancy. Therefore, an important aspect of gene expression in *B. oleracea* response to environmental stimuli is to identify the specific gene copy involved. This aspect should also be taken into consideration while studying the genetic basis of biosynthesis and signal transduction in relation to basic phytohormones. Our present work concerns the identification of homologue genes involved in ethylene biosynthesis such as *SAM*, *ACS* and *ACO*, as well as those involved in the ethylene signalling pathway, mainly *ETR1*, *CTR1*, *MKK4*, *MKK5*, *EIN2*, *EIN3, EREBP*, *ERF5* and *ERF7* on the basis of the restriction fragment length polymorphism (RFLP) and PCR mapping. In the case of ACC synthases, (ACSs) the in silico analysis of gene variants in the genome of *A. thaliana* was followed by the identification of homologues to *ACS2*, *ACS6* and *ACS7* in the *B. oleracea* database. In total, 22 loci with sequence homology to the genes under analysis were included in the existing *B. oleracea* RFLP chromosomal map. Based on the stress responsiveness of most of the *A. thaliana* genes analysed in this study, we performed initial functional analysis of some gene homologues mapped. With the use of the RT-PCR approach the conservation of differential transcriptional induction of *ACS* homologues in the *B. oleracea* and *A. thaliana* was demonstrated during ozone stress.

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Introduction

The information derived from the *Arabidopsis thaliana* genome, such as the primary gene structure, gene duplication level and proven or predicted gene function, may be helpful in studies of crop plants. It will provide insight into the organisation and functioning of larger and more complex genomes. The close relationship between *A. thaliana* and *Brassica* species and earlier comparative analyses suggests that at least some gene homologues are structurally and functionally conserved (Wiersma et al. 1989; Osborn et al. 1997; Quiros et al. 2001). It has also been reported that cloned genes from *A. thaliana* may have specific counterparts with similar functions in the *Brassica* species (Arondel et al. 1992; Fray et al. 1997; Vicente and King 2001). However, the other members of the gene families may differ even between closely related plants since frequent genome duplications and deletions are often found in cultivated species (Blanc and Wolfe 2004). The conserved gene order, together with sequence similarity, may be a valuable tool for defining functional relationships based on orthology among genes.

Following the split in the *Arabidopsis* and *Brassica* lineages, further polyploidisation cycles seem to be responsible for the formation of the present *Brassica* genomes and could be the main source of extensive gene redundancy. This gene multiplication may result at least partially from the increase in the number of chromosomal duplicated segments observed on the *B. oleracea* genetic (Kowalski et al. 1994) and physical (Ziolkowski and Sadowski 2002) maps versus the corresponding *A. thaliana* maps. This is in agreement with earlier reports that suggested two additional polyploidisation events that followed the speciation of the *Brassica* lineage from

a common ancestor of *Arabidopsis* and *Brassica* (the AGI 2000). Therefore, in further functional studies on gene expression and response to environmental stresses in *Brassica* it will be essential to identify and understand the function of individual gene homologues involved.

This report concerns the identification and chromosomal organisation of gene homologues involved in the ethylene biosynthesis and signalling pathways in the *Brassica oleracea* genome. These homologues have been recognised on the basis of the *A. thaliana* genome data and their differential ozone- and drought-responsiveness revealed by a DNA microarray experiment (Ludwików et al. 2004; Ludwików and Sadowski, unpublished). The genome collinearity was analysed in *A. thaliana* and *B. oleracea* on the basis of chromosomal regions carrying the gene homologues studied. Some homologues of selected genes have been identified and mapped with the use of *A. thaliana* gene-specific primers. Most importantly, we demonstrate by functional test (RT-PCR) homologue-specific responsiveness to ozone stress of three selected ACS genes in *B. oleracea* that is conserved with *A. thaliana* homologues. Finally, the identification of specific gene homologues in the *Arabidopsis*/*B. oleracea* model will facilitate their further functional analysis during ozone and drought stress response.

Materials and methods

Plant material

The *B. oleracea* segregating population derived from a cross between collard (*B. oleracea* var. *acephala*) and cauliflower (*B. oleracea* var. *botrytis*) was used for mapping as previously described (Babula et al. 2003). A RFLP map covering all the nine *B. oleracea* chromosomes was constructed on the basis of this segregating population (Hu et al. 1998; Babula et al. 2003).

To analyse ozone response at the transcription level of selected genes cauliflower (*B. oleracea* var. *botrytis* "Pionier") plants were used.

Gene probes

A set of probes used for Southern hybridisation consisted of three cDNA clones for ACC synthese 1 (*BoACS1*, X82273), ACC synthese 2 (*BoACS2*, AF338651) and ACC synthese 3 (*BoACS3*, AF338652) genes from *B. oleracea* var. *italica* (prefix Bo), genomic sequences of *B. oleracea* var. *alboglabra* for MAPKK4 kinase (*MKK4*) and MAPKK5 kinase (*MKK5*) genes and ten *Arabidopsis* ESTs corresponding to gene codings for S-adenosyl-L-methionine synthetase (*SAM1*), 1-aminocyclopropane-1-carboxylate oxidases (*EAT1* and *ACO2*), ethylene response protein (*ETR1*), serine/threonine protein kinase (*CTR1*), ethylene insensitive 2 (*EIN2*), ethylene-insensitive 3 (*EIN3*), putative ethylene response element binding protein (*EREBP*), ethylene response binding factor 5 (*ERF5*) and ethylene response binding factor 7 (*ERF7*). ESTs were received from the *Arabidopsis* Biological Resource Centre (Ohio State University, Columbus, OH); their accession numbers are given in Tables 1 and 2. The homology was searched for by comparison between the EST sequence and the *Arabidopsis* genomic sequence with the use of the BLASTN alignment with an expected value of $E < 10^{-10}$ for a significant homology (see the AGI 2000).

Table 1 Diagram of ethylene biosynthesis pathway showing involvement of *A. thaliana* gene homologues

	Gene in *A. thaliana*		Induction factor	Number of loci in the *Brassica oleracea* genome	
	Name (symbol)	Acc. number		Putative*	mapped
L-methionine					
SAM synthetase	*SAM1*	At1g02500 (T76645)**	–	3–5	1
S-adenosyl-L-methionine					
ACC Synthase	*ACS2*	At1g01480	Ethylene, cycloheximide, wouding, IAA	3	2 (*BoACS3*)
	ACS6	At4g11280	Ozone, ethylene, cyanide, touching, cycloheximide, IAA	3–4	2 (*BoACS1*)
	ACS7	At4g26200	Cycloheximide	3	1 (*BoACS2*)
1-aminocyclopropane-1-carboxylate					
ACC oxidase	*EAT1(ACO 1)*	At1g05010 (R90435)	Wounding, ethrel, $Fe2^{+}$	2–4	1
	ACO2	At1g62380 (N96535)	–	4	2
Ethylene					

The gene-specific ESTs used as probes for RFLP mapping in *B. oleracea* are shown. Information on the protein function was retrieved from the TAIR database. Numbers of loci mapped in *B. oleracea* genome are shown. * the copy number of a particular gene was estimated as described in Materials and Methods. ** Acc. number of EST used in this study

Table 2 Diagram of ethylene signal transduction pathway based on studied gene homologues in *A. thaliana*

	Gene	Gene product	Acc. number in *A. thaliana*	Induction factor	Number copy in *B. oleracea*	
					Putative*	mapped
Membrane-receptor	*ETR1*	Putative ethylene receptor	At1g66340 (AA067423)***	Jasmonic acid, ethylene	1	1
	CTR1	Homologues to the Raf family of Serine/threonine protein kinase	At5g03730 (W43603)	Ethylene	5	4
MAPK cascade	*MKK4* (*MAPKK4*)**	Mitogen-activated protein kinase kinase	At1g51660	Bacterial elicitor (flagellin)	1	0
	MKK5 (*MAPKK5*)**	Mitogen-activated protein kinase kinase	At3g21220	Bacterial elicitor (flagellin)	3	2
EIN2	*EIN2*	Ethylene insensitive 2	At5g03280 (N96212)	–	1	1
Transcription factor	*EIN3*	Ethylene insensitive 3	At3g20770 (R65273)	–	5	3
Transcription factor	*EREBP*	Ethylene response element binding protein	At2g44840 (N97133)	–	5	0
	ERF5	Ethylene response factor 5	At5g47230 (AA713176)	–	5	0
	ERF7	Ethylene response factor 7	At3g20310 (T04554)	–	4	2

ESTs from *A. thaliana* were used as probes for RFLP mapping in *B. oleracea*. Information on the protein function was retrieved from the TAIR database. * The gene copy number was estimated as described in Materials and Methods. ** *A. thaliana* genomic sequences amplified by PCR were used as probes in this case. *** Acc. number of EST used in this study

Restriction fragment length polymorphisms analysis

Procedures for DNA extraction, restriction enzyme digestion and Southern hybridisation were as described earlier (Babula et al. 2003) with a minor modification. The alkali transfer for Southern blotting was carried out according to the Roche manual (including denaturation and neutralisation steps). Hybridisation probes were prepared by PCR and labelled non-radioactively by the DIG system (Roche). After hybridisation, moderate stringency wash conditions were used. Segregation data for restriction fragment length polymorphism (RFLP) loci detected by the *A. thaliana* EST probes were analysed by using MapMaker ver. 3.0 (Lander et al. 1987). The loci detected with single EST probe were designated according to decreasing length of polymorphic fragments. The loci identified were assigned to the existing linkage groups (LGs) of *B. oleracea* (Kaczmarek et al., unpublished data). The names of the *B. oleracea* LGs (O1–O9) were assigned on the basis of the proposal presented in the Multinational *Brassica* Genome Project (http://www.brassica.bbsrc.ac.uk).

Estimation of the gene copy-number for the *A. thaliana* probes tested

The numbers of gene homologues related to the selected probes in the *A. thaliana* genome were estimated on the basis of a comparison between the EST sequence and the *Arabidopsis* genomic sequence with the use of the BLASTN alignment with an expected value of $E < 10^{-10}$ for a significant homology. The homologues identified were localised on the *A. thaliana* map, reconstructed in silico with the support of the data published by Blanc et al. (2003) (Fig. 1; the corresponding chart is available at http://www.wolfe.gen.tcd.ie/athal/dup). The physical and genetic positions of loci on the *A. thaliana* chromosomes were estimated on the basis of the TAIR Map Viewer (www.arabidopsis.org), at first attributing the adequate nucleic sequences to the BAC clone on the AGI map and then to genetic markers on the RI map (http://www.arabidopsis.org).

Identification of gene-specific homologues

Because both *Arabidopsis* and *Brassica* species experienced extensive gene duplication events, most genes belong to multigene families. Thus orthologs may not be distinguished easily from paralogs. For this reason the following assumptions have been made: first, *Arabidopsis* gene-specific probes or designed primers sets were selected, which were initially screened computationally against the whole genome sequence to avoid misidentification of gene families or paralogs; second, a locus was considered to be potentially orthologous if it was determined to be single and mapped to a collinear region in both species. Genes meeting these criteria are referred to here as putative orthologs, with the reservation that these data are considered insufficient to prove orthology in the strictest evolutionary context, but nevertheless their identification can be useful.

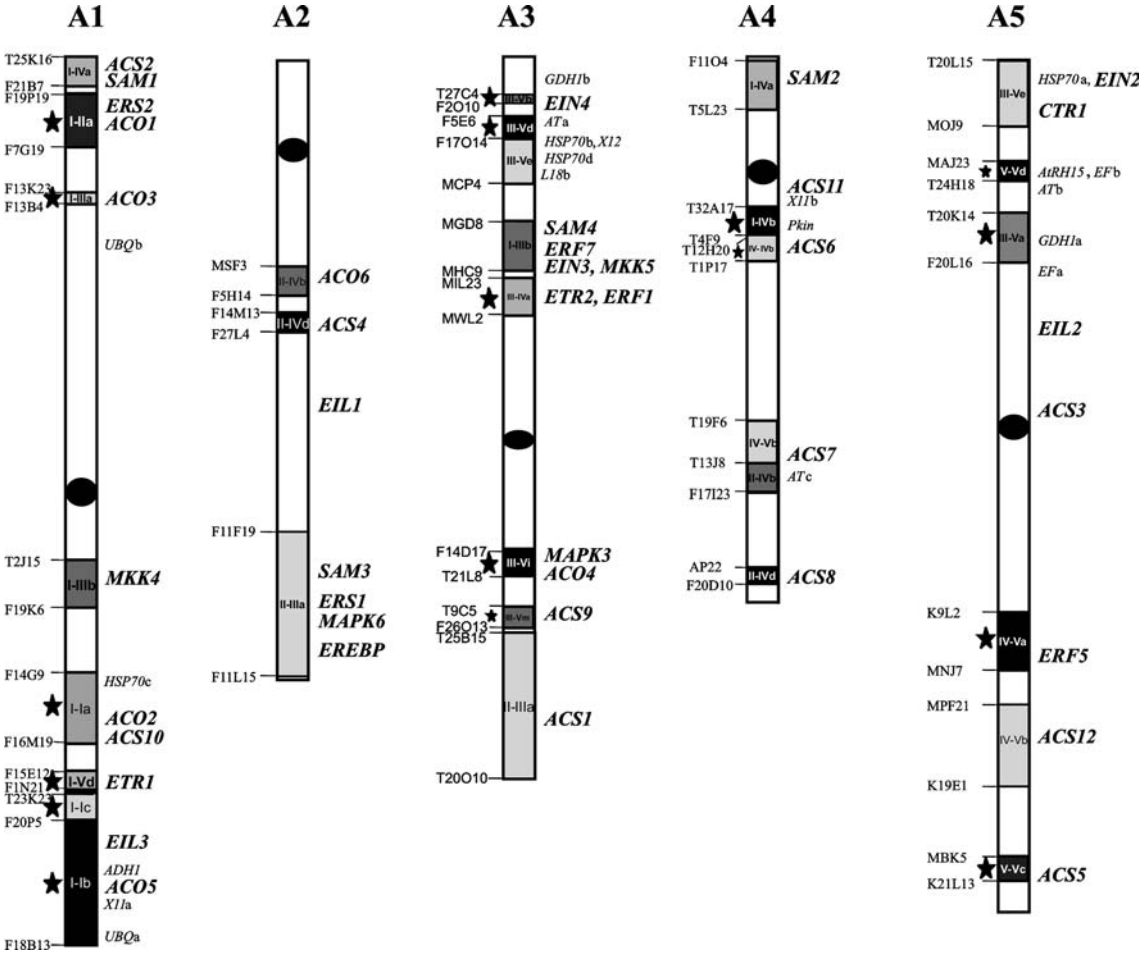

Fig. 1 Chromosomal distribution of loci corresponding to genes involved in ethylene biosynthesis and signalling transduction pathways in *A. thaliana*. The chromosomal map was reconstructed in silico with the support of data published by Blanc et al. 2003 and available at http://www.wolfe.gen.tcd.ie/athal/dup. *A. thaliana* chromosomes are designated A1–A5 (*above*). Duplicated regions are shown with loci analysed in this study (*in capitals*) and anchor loci for comparative analysis of *A. thaliana*/*B. oleracea*. Borders of duplicated segments are marked by BAC symbols on the left of each chromosome. Location of all loci along chromosomal segments approximates their actual physical position. Asterisk marks segment which counterpart was deleted from the map

Generally, gene-specific homologues in the *B. oleracea* genome have been identified using the *Arabidopsis* EST as probes on the basis of their sequence similarity. Identification of the loci corresponding to a particular probe was based on the analysis of restriction fragments disclosed by hybridisation. Additionally, true or spurious homologues of these genes were differentiated on the basis of the signal intensities of individual fragments under hybridisation.

Gene-specific primers based on the sequence of *B. oleracea* var. *italica* cDNA clones X82273 (*BoACS1*); AF338651 (*BoACS2*); AF338652 (*BoACS3*) (Pogson et al. 1995) and of *Brassica oleracea* Genome Database TIGR clones BOGJR53TF (*MKK4*) and BOGGG54TR (*MKK5*) were designed and used to amplify genomic DNA from *B. oleracea*. Three gene-specific primer pairs were designed to generate sequences: for *BoACS1* /F/ CGACGGTTTCTTGGTTCCGACT and /R/ TTATA TCTGTGCACG AACAAGCGGAG; for *BoACS2* /F/ GACGCGCTTCTCGTCCCCACG and /R/ CTCCGA GCAATGGCACGACGAT; for *BoACS3* /F/ GCCTC GCCGATCCAGGCGATG and /R/ TCCGTGCACT GGAAGGAAGAG; for *MKK4* /F/ GGTTCCCTTT CGCTGTGAGTAGA and /R/ GTTGGGAAGAAA GAAGAAGACGAA; for *MKK5* /F/ GGTTCCCTTT CGCTGTGAGTAGA and /R/ AGATTCGGACCAC CACTTGATTTA. The amplified sequences corresponded to the 3′ ends of putative genes, including coding as well as untranslated regions. The PCR products were verified by sequencing. Gene-specific mapping was further confirmed by detecting the presence of the genes within collinear regions in the comparative map for *A. thaliana* and *B. oleracea*.

Ozone treatment

To study induction of some genes during ozone stress cauliflower "Pionier" plants were grown in a growth chamber. Plants were cultivated with a 16-h photoperiod

and day/night temperatures of 21/17°C, respectively. Fifteen-week-old plants were treated for 40 and 80 min with ozone at 350 ppb. Leaves from control and ozone-treated plants were frozen in liquid nitrogen, and stored at −80°C.

RT-PCR

Total RNA was isolated with RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed according to the basic protocols accompanying the reagents and enzymes supplied by Promega. Total RNA (2 µg) was reverse-transcribed in a reaction containing 2.5 mM of each dNTP, 0.5 µg oligo(dT), 20 Units of RNase inhibitor, and 2.5 Units reverse transcriptase in a final volume of 20 µl. The sample was incubated at 25°C for 10 min, followed by 90 min at 42°C, 15 min at 70°C and 5 min at 4°C in an Eppendorf Mastercycler. Reactions were diluted five times before PCR. Amplifications were performed in 10-µl reaction volumes containing 3 µl of the diluted reverse transcription mix, 0.5 Units of Taq DNA polymerase (Fermentas), and 2.5 pmol of each oligonucleotide primer. The following gene-specific forward (F) and reverse (R) primers were used: for the *A. thaliana actin 2* (acc. no. U41998) - /F/ GGTAACATTGTGCTCAGTGG TGG and /R/ CTCGGCCTTGGAGATCCACATC; for *BoACS1* - /F/ GCTGTGGCGTGTGATCGTCC and /R/ TTATATCTGTGCACGAACAAGCGGAG; for *BoACS2* - /F/ GGACAGCGAGAAGGATGTC GAG and /R/ CTCCGAGCAATGGCACGACAGT; for *BoACS3* - /F/ ACGACTCGGTTGTGTCATGCG and /R/ TCCGTGCACTGGAAGGAAGAG. PCR amplifications were carried out at 95°C for 90 s, followed by five cycles of 95°C for 10 s, 50°C for 10 s, 72°C for 1 min, then 25 cycles of 95°C for 10 s, 62°C for 10 s and 72°C for 1 min. A final extension was carried out at 72°C for 5 min, and the samples were incubated at 4°C until analysis. The *actin 2* gene expression level was used as a quantitative control. Amplification of *actin 2* was carried out with annealing at 55°C and only 25 cycles were performed, as *actin 2* has a high expression level and it was difficult to observe differences between control and other probes. Amplification of each RNA sample without prior reverse transcription confirmed the absence of contaminating genomic DNA.

Analysis of amplification products

Whole 10 µl of amplification reactions were analyzed in 1.5% agarose gels in TAE buffer following standard procedures with the Fermentas 100 bp ladder as a size standard. To quantify levels of transcripts corresponding to *BoACS1*, *BoACS2* and *BoACS3* genes the Multi Gauge v2.2 programme was used. In each case the product from the control reaction was taken as 100% and products from cDNA after 40 and 80 min ozone treatments were referred to the control. The control level of the *BoACS1* gene was compared to the control level of *actin 2*, the *BoACS1* level of a 40 min treated probe to the *actin 2* treated probe, etc.

Results

Identification and homology-based mapping of selected genes in the *A. thaliana* and *B. oleracea* genomes were performed taking the following steps: (1) Application of *A. thaliana* gene-specific ESTs as probes for RFLP mapping in *B. oleracea*, whenever it was possible. (2) PCR amplification of the *B. oleracea* genes based on *A. thaliana* gene-specific primers. (3) Gene mapping in the *B. oleracea* genome with the use of ESTs and the above-mentioned primers. (4) Construction of a comparative map for the *B. oleracea* and *A. thaliana* genomes. (5) Confirmation of gene homologues through the identification of their loci within unique/specific conserved regions of the *B. oleracea* and *A. thaliana* genomes. (6) Approximation of the loci number in the *B. oleracea* genome. The total number of homologous loci corresponding to each *A. thaliana* EST probe was estimated on the basis of Southern hybridisation banding patterns taking into account the number and length of the restriction fragments generated with different restriction endonucleases.

Identification and mapping of selected genes involved in ethylene biosynthesis

The in silico analysis of the *A. thaliana* genomic sequence indicated that crucial genes of the ethylene biosynthesis pathway such as s-adenosyl-L-methionine synthetase (*SAM*), 1-aminocyclopropane-1-carboxylate synthase (*ACS*) and 1-aminocyclopropane-1-carboxylate oxidase (*ACO*) belonged to multigene families with high conservation of nucleotide sequences (Johnson and Ecker 1998). Hence, in this study, specific members of *SAM*, *ACS* and *ACO* gene families were selected, with codes for the basic enzymes of the ethylene biosynthesis pathway whose expression can be induced by ozone and/or drought stress (Ludwików et al. 2004; A. Ludwików and J. Sadowski, unpublished data) (Table 1).

s-adenosyl-L-methionine synthetase (SAM)

Four members of the *SAM* gene family involved in ethylene biosynthesis (the database http://www.arabidopsis.org, Peleman et al. 1989) were identified in silico in the *Arabidopsis* genome. The EST corresponding to *SAM1* was used as the *A. thaliana* probe in cross-hybridisation to Southern blots carrying genomic DNA from the *B. oleracea* mapping population. The probe sequence similarity varies between 78 and 80% with corresponding regions in the other three *SAM* genes that

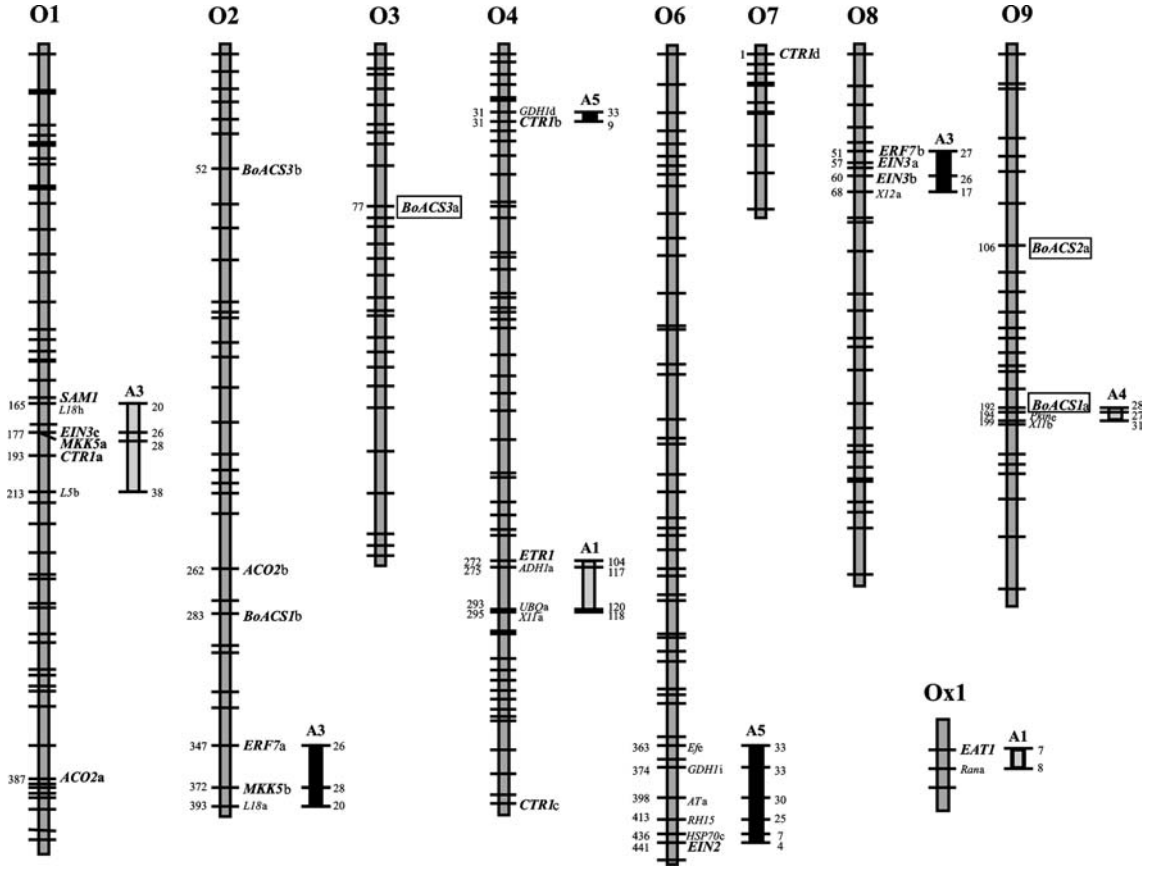

Fig. 2 Genetic map with conserved regions detected in the *A. thaliana* and *B. oleracea* genomes with homologous loci corresponding to genes involved in ethylene biosynthesis and signalisation pathways. Chromosomal segments of *B. oleracea* are marked by *vertical bars* with chromosome designation O1–O9 *above* them, except for O5. Additional LG is marked by Ox1. To the *right* of these groups there are chromosomal regions of *A. thaliana* designated A1–A5 (*black bars* inversion). Loci marked by *boxes* were mapped by PCR with *B. oleracea* primer sequences. Positions of loci on the *A. thaliana* and *B. oleracea* chromosomes are indicated in cM

preclude cross-hybridisation under experimental conditions of hybridisation and washings applied in the study. RFLP analysis of mapping blots made it possible to identify segregating restriction fragments corresponding to two genes (with sequence similarity to *SAM1*) in the *B. oleracea* genome. However, only one of them was localised on the existing genetic map of *B. oleracea*, on the LG O1 (Fig. 2). To verify if the *B. oleracea* locus identified is a true homologue of the *A. thaliana SAM1*, a local chromosome collinearity analysis for *A. thaliana* and *B. oleracea* was carried out. Comparative analysis for the two species did not make it possible to successfully identify a conserved region with common loci including *SAM1*. On the basis of the restriction fragment hybridising pattern it was estimated that this EST probe corresponding to a single *SAM1* gene of *A. thaliana* detected 3–5 gene homologues in the *B. oleracea* genome (Table 1). This estimation was made taking into account the presence of two additional sets of polymorphic fragments corresponding to two different loci. Additionally, three monomorphic fragments (> 10.0, 5.5 and 2.0 kb) were observed, suggesting the existence of at least one more locus.

ACC synthase

In *Arabidopsis*, the in silico analysis has shown that 12 members of the *ACS* gene family, previously identified (Ecker 1995), are dispersed along the whole genome (Fig. 1) (Yamagami et al. 2003).

Three cDNA clones were identified by searching the database from *B. oleracea* var. *italica* (broccoli) coding for ACC synthases (see Materials and Methods): BoACS1, BoACS2 and BoACS3. Sequences of these *B. oleracea* clones were compared with the *Arabidopsis* genomic sequence using the BLASTN alignment. The detected matches of BoACS1, BoACS2 and BoACS3 showed homology to three different members of the gene family coding for ACC synthases, i.e. *ACS6*, *ACS7* and *ACS2*, respectively (see Jakubowicz and Pacak 2004 for details). With the use of gene-specific primers, they were mapped on the *B. oleracea* chromosomes via the PCR-analysed segregating population. The *BoACS1*a (LG O9, see the O9/A4 conserved region), *BoACS2*a (LG O9) and *BoACS3*a (LG O3) loci identified via PCR correspond to homologues of *A. thaliana ACS6*, *ACS7* and *ACS2*, respectively

(Fig. 2). Additionally, these sequences were used as probes in Southern hybridisation. The nucleotide sequence similarity among the three members of the *BoACS* gene family essentially varies from 60 to 63%. Hence, it can be expected that cross-hybridisation will not yield false positive results. In fact, a comparison of hybridisation patterns of restriction fragments (the number and length of restriction fragments) revealed with these probes, indicated that they represented different sequences. The analysis of hybridisation patterns made it possible to estimate putative copy numbers of these genes. Thus, for *BoACS1* the hybridisation data indicated the presence of 3–4 genes (Table 1). However, mapping (based on PCR and Southern hybridisation) facilitated the localisation of only two of them on the map (Fig. 2; LGs O2 and O9 with *BoACS1*a). For *BoACS2*, the hybridisation pattern indicated the presence of three loci (Table 1); however, only one of the genes identified was localised on the *B. oleracea* genetic map (Fig. 2; LG O9 with the *BoACS2*a locus detected by PCR). Three loci were identified with the *BoACS3* probe (Table 1); however, only two were located on the *B. oleracea* map (Fig. 2; LGs O2 and O3 with the *BoACS3*a locus detected by PCR).

ACC oxidase

In the *Arabidopsis* genome, six genes coding for ACC oxidases were identified in silico: four copies were located on chromosome A1 and single loci on chromosomes A2 and A3 (Fig. 1). Until now, only *EAT1* and *ACO2* gene expression patterns and protein activities have been investigated (Gomez-Lim et al. 1993). Therefore, two ESTs corresponding to *EAT1* and *ACO2* genes were selected for analysis in this study. The BLAST alignment (by threshold expected value of $E < 10^{-10}$) of EST sequences against the *A. thaliana* genomic sequence indicated that *EAT1* had only one match and thus was treated as a single-copy gene in *A. thaliana*. However, the EST sequence for *ACO2* showed two matches at a high similarity level: one corresponding to the *ACO2* gene and the other to the other *ACO* gene, which indicates a possibility of detecting by hybridisation the gene family members in *Brassica*. A comparison of the obtained hybridisation patterns for selected ESTs made it possible to exclude the possibility of cross-hybridisation between *EAT1* and *ACO2*. In the mapping process, the EST probes corresponding to *EAT1* and *ACO2* facilitated the mapping of single and two genes, respectively (Table 1, Fig. 2; LGs O1, O2 and Ox1). However, the complex hybridisation pattern indicated the presence of a greater number of copies in the *B. oleracea* genome (Table 1). For the *EAT1* probe a single homologue in the *B. oleracea* genome was identified in the Ox1/A1 conserved region (Fig. 2). The strongly hybridising probe suggests that this locus is a homologue of the *A. thaliana EAT1*.

Analysis of selected genes involved in the ethylene signal transduction pathway

The first stage of the ethylene signalling pathway involves the perception of the ethylene signal through the five ethylene-binding receptors: ETR1, ERS1, ETR2, EIN4 and ERS2 (Hua and Meyerowitz 1998; Sakai et al. 1998). Their genes compose a common multigene family with a high nucleotide sequence similarity. A *Raf*—like kinase—*CTR1* negatively regulating the downstream ethylene response pathway through the MAP kinase cascade, is activated in the absence of the ethylene signal. The cascade system is based on three multigene families, namely MAPKKKs, MAPKKs and MAPKs. From the last cascade step (MAPK) the ethylene signal is carried to *EIN2* gene, which acts as a positive regulator of the ethylene pathway. *EIN2* positively regulates transcription factors belonging to the EIN3 gene family. This family includes six members; among them EIN3, EIN1 and EIL2 are best characterised at the gene level. These factors bind to primary ethylene response elements (PERE) in the promoters of *EREBP* genes, whose products interact with the GCC box in the promoters of target genes (Wang et al. 2002). In silico chromosomal analysis of these genes in the *A. thaliana* genome indicates that they are dispersed over all chromosomes. Some of them, functionally characterised in previous reports, were chosen for genetic mapping in *B. oleracea*.

Membrane-localised receptors

In this study, an EST corresponding to the *A. thaliana ETR1* was selected as a probe (Table 2). A comparison of its sequence with the genomic sequence of *Arabidopsis* (BLASTN with a cutoff of $E < 10^{-10}$) excludes the possibility of cross-hybridisation with the other related sequences from the *A. thaliana* genome. In the mapping process, a single gene coding for ETR1 was detected and located on the *B. oleracea* map (Fig. 2; O4). The analysis of the hybridisation pattern after *Eco*RI digestion indicated that *ETR1* is a single-copy gene in the *B. oleracea* genome (Table 2), similar to the *A. thaliana* genome and it could be considered as unique. The collinear segments carrying the *ETR1* locus were detected in the two genomes (Fig. 2; O4/A1). This region contains four loci and its length is 16 and 23 cM in *A. thaliana* and *B. oleracea*, respectively. A significant sequence conservation between the *A. thaliana ETR1* gene and its *Brassica* homologue, their location within the collinear regions, in view of the fact that these genes are single in both species, raises the possibility of identifying a homologue with the conserved functional role (Fulton et al. 2002).

MAP kinase kinases

Three gene homologues in the *B. oleracea* genome were identified with the *MKK5* probe. Two homologues were

included in the *B. oleracea* LGs O1 and O2 (Fig. 2). An analysis of the hybridisation patterns indicated that *MKK5* had three homologues in the *B. oleracea* genome (Table 2). A comparative analysis of the *B. oleracea* and *A. thaliana* genomes revealed that both homologues identified were located in the collinear regions. The first was found in the LG O1 in the conserved region including four loci, together with the ethylene-specific gene *EIN3*. Its length was 18 and 48 cM in *A. thaliana* and *B. oleracea*, respectively. The other region included three loci (Fig. 2; LG O2) together with the other ethylene-specific gene *ERF7*; however, the gene order was disrupted. In fact, it hindered the identification of a *MKK5* homologue. For *MKK4*, only monomorphic restriction fragments were identified and no corresponding loci were included in the *B. oleracea* map. No common cross-hybridising restriction fragments were identified when hybridising patterns for *MKK4* and *MKK5* were compared.

Ethylene insensitive

Restriction fragment length polymorphisms mapping proved the presence of a single locus for *EIN2* detected in the *B. oleracea* genome similar to *A. thaliana*. The locus was included in the *B. oleracea* LG O6 and placed in the conserved region (Fig. 2; O6/A5). This chromosomal segment contains six loci and its length is 29 and 78 cM in *A. thaliana* and *B. oleracea*, respectively. It suggests that the locus detected in *B. oleracea* is a homologue of the *EIN2* gene in *A. thaliana*, likewise the *ETR1* gene.

Transcription factors

These genes were mapped on the basis of RFLP analysis. Transcription factor gene EIN3 was found to be coded by a multigene family in *A. thaliana*, containing six gene members dispersed in the genome (Wang et al. 2002). In this study, *A. thaliana* ESTs representing four genes of the *EIN3* family were selected (Table 2). Unfortunately, only non-segregating restriction fragments were scored for the *EREBP* probe. For *ERF5*, the homologous genes identified on the basis of segregating restriction fragments remained unlinked with any of the *B. oleracea* LG.

Three genes for the *EIN3* probe were located on the existing *B. oleracea* map (Fig. 2; LGs O1 and O8). An analysis of the hybridisation pattern indicated the presence of five *EIN3* loci in the *B. oleracea* genome (Table 2). A comparative analysis of the *B. oleracea* and *A. thaliana* genomes facilitated the identification of collinear segments, which included the *EIN3* locus (Fig. 2; LGs O1 and O8). Two of the three *EIN3* loci identified in the *B. oleracea* genome occurred in the collinear segments (Fig. 2; O1/A3 and O8/A3). The first segment is located on the *B. oleracea* LG O1/A3 and covers four loci including *MKK5* known to be involved in the ethylene signal transduction pathway (Asai et al. 2000). Its length comprises 18 and 48 cM in *A. thaliana* and *B. oleracea*, respectively. The second segment is located on the *B. oleracea* LG O8 and includes three loci only (Fig. 2; LG O8/A3).

For *ERF7*, two loci were identified and localised on the existing *B. oleracea* map (Fig. 2; LGs O2 and O8). An analysis of the hybridisation pattern indicated the presence of four *ERF7* loci. Two *ERF7* loci mapped were present in the collinear segments (Fig. 2; LGs O2/A3 and O8/A3). The LG O2/A3 segment includes three loci, among them *MKK5*, involved in the signal transduction pathway. The LG O8/A3 segment includes three loci, among them *EIN3*, involved in the signal transduction pathway too.

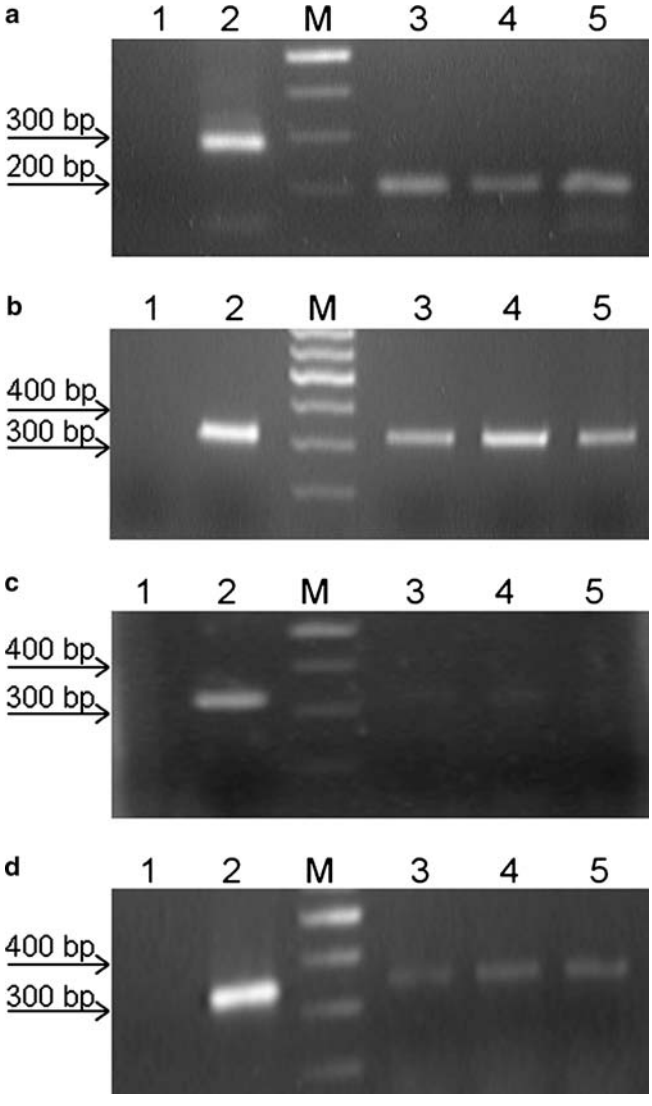

Fig. 3 RT-PCR and analysis of the *actin 2* gene expression (**a**), *BoACS1* gene expression (**b**), *BoACS2* gene expression (**c**), *BoACS3* gene expression (**d**); amplification products using cDNA as templates from control plants (*lane 3*), from plants ozone-treated by 40 min (*lane 4*) and 80 min (*lane 5*). Negative and positive PCR controls were carried out using the same respective primers without templates (*lane 1*) and with genomic templates (*lane 2*)

Functional analysis of selected gene homologues mapped in the *B. oleracea* genome

RT-PCR assays were carried out using RNA isolated from ozone-treated and untreated plants of *B. oleracea* var. *botrytis*.

Semi-quantitative RT-PCR of RNA from ozone-treated cauliflower

After reverse transcriptions, PCR reactions using gene-specific primers to 3′ ends of *BoACS1*, *BoACS2*, *BoACS3* and *actin 2* genes were performed. The changes of transcript level for appropriate synthases in stressed and unstressed plants were determined by quantitative comparison of PCR products with the *actin 2* product obtained from respective plant samples (Fig. 3). We found that *BoACS1* and *BoACS3* genes are threefold and 1.5-fold transcriptionally induced after 40 min of ozone treatment, respectively. After 80 min of ozone treatment the level of *BoACS1* and *BoACS3* transcripts were similar as in unstressed plants (Fig. 3b, d, respectively). Transcriptional activity of the *BoACS2* gene was not affected throughout ozone stress. In fact, very low level of the *BoACS2* gene transcript was hardly detected under such conditions (Fig. 3c).

Discussion

The completed genome sequencing of model plants, namely *A. thaliana* and rice, and the established extensive set of resources (DNA markers, EST collections, genetic and physical maps and genomic libraries) have changed the strategy of searching for genes, both those controlling basic processes and agronomically important traits in crops. Thus, RFLP marker loci originating from one species can be used for the construction of linkage maps in related species. The establishment of structural similarity between different plant genomes provides us with some insight into plant genome evolution and can facilitate linking positional information on genes and their functions available in one species to another, where the same information is not available or is more difficult to obtain (Lagercrantz et al. 1996). Furthermore, segmental duplications in the *Brassica* genome, as revealed by comparative mapping with *A. thaliana*, may correspond to functional duplications. An analysis of the sequence and function of genes from one genome segment can provide guidelines for the functional analysis of genes in duplicated regions (Gebhardt et al. 2003).

The experimentally recognised ozone- and/or drought-based induction of most of the 15 *A. thaliana* genes reported here makes it possible to draw conclusions on putative induction of the *B. oleracea* homologues. The first stage of this study was aimed at identifying the total number of homologues of the genes tested and estimating their chromosomal localisation in the *B. oleracea* genome. Our results indicate that the *B. oleracea* genome is characterised by a higher number of copies for the genes under study; among the fraction of 15 single-copy genes in the *A. thaliana* genome, only for three genes (20%) unique homologues were found in the *B. oleracea* genome. Equally, importantly, 11 (nearly 75%) out of the 15 genes are represented by 3–4 homologues in the *Brassica* genome. Therefore, the 13 *A. thaliana* probes selected specifically for the genes involved in the ethylene biosynthesis and signalling pathways correspond to at least 53 loci in the *B. oleracea* genome (average 3.5 loci/probe). It reflects the evolutionary history of the *Brassica* genus, characterised by two additional polyploidisation events following the speciation of *Brassica* from a common ancestor of Brassiceae and Arabidae.

The *B. oleracea* genome sequence is not yet available, thus access to most gene sequences and their chromosomal localisation for functional studies is limited. It can be assumed that a particular known homologue of *A. thaliana* having a specific partner in the *Brassica* genome can provide predictable data in terms of their specific function, as has been shown in several reports (e.g. Wiersma et al. 1989; Arondel et al. 1992; Osborn et al. 1997). Additional support for such a prediction would be the information on the collinearity of the corresponding chromosomal regions in both species (Fulton et al. 2002; Gebhardt et al. 2003). Thus, an important key element of comparative mapping is the identification of conserved segments with a similar conserved gene content and order in the two species. Earlier studies based on genetic mapping with anonymous genomic DNA fragments as probes (e.g. Kowalski et al. 1994) and ESTs (Lan et al. 2000; Babula et al. 2003) indicated the presence of collinearity across the short chromosomal regions of *A. thaliana* and *B. oleracea*. These observations were confirmed at the micro-collinearity level (gene-by-gene analysis) in selected chromosomal regions (Sadowski et al. 1996; O'Neill and Bancroft 2000; Quiros et al. 2001). However, within the conserved blocks detected, a divergence in gene content and order was observed. In the present study, a comparison of the loci order within the *A. thaliana* and *B. oleracea* chromosomes indicates that many of these loci are localised in the conserved chromosomal regions (Fig. 2). The sequence homology between *A. thaliana* and *B. oleracea* and the identification of the conserved chromosomal regions allowed us to initially identify specific homologous gene copies involved in the ethylene signalling pathway and deduce functions of the *B. oleracea* genes mapped. We confirmed the prediction in terms of the function conservation for some gene homologues by a transcription analysis (Fig. 3). We demonstrated with the use of the RT-PCR approach the conservation of differential transcriptional induction of the *ACS* homologues for *B. oleracea* and *A. thaliana* during ozone stress. It was already reported that within a large family of ACC synthases in *A. thaliana*, the *ACS6* is characterised by a

distinct ozone stress-based induction (Arteca and Arteca 1999). In addition, our recent microarray experiment designed to elaborate the ozone-inducible transcription profile in *A. thaliana* (Ludwików et al. 2004) showed, apart from the *ACS6* gene, another, although weaker, induction of the *ACS2* gene (A. Ludwików and P. Sadowski, unpublished data). The transcriptional analysis conducted here proved that *B. oleracea* homologues: *BoACS1* (threefold induction), *BoACS2* (no induction) and *BoACS3* (1.5-fold induction) are conserved with *AtACS6*, *AtACS7* (no induction) and *AtACS2* not only at the sequence and conserved chromosomal segment levels, but also at the ozone stress-response level, respectively. Whether the 3–4 duplicated homologues identified in the *B. oleracea* genome by Southern hybridisation to each of the three *A. thaliana* ACS genes are conserved in relation to ozone responsiveness remains to be answered. Here, with the use of the *Brassica* homologue-specific primers we were able to map on chromosomes (by PCR) and estimate the ozone responsiveness (by RT-PCR) of the individual gene copy of each of the three ACSs.

It should be emphasised that the presence of duplicated gene blocks in both the *Arabidopsis* and *Brassica* genomes and the gene loss phenomenon makes a comparative analysis difficult. The main problem is to distinguish which gene copy comprises the true ortholog of a particular *Arabidopsis* gene. In some homologous segments the lack of loci in the counterparts can be caused by gene loss and transposition, which frequently follow genomic duplication events (the AGI 2000). In this study, two aspects of comparative mapping should be emphasised. First, the localisation of such homologous genes in structurally more conserved regions of the genome points to their common origin, which can indicate that the encoded functions are still conserved in *Arabidopsis* and *Brassica*. Some genes after sequence divergence and/or deletion may exist in the *Brassica* genomes as unique copies, as it was observed in this study for *ETR1* and *EIN2*. One could expect that single copies of a given gene in related species can be more conserved at the functional level, assuming they play specific roles. It is true not only for the unique homologues of one copy of the *Arabidopsis* genes, such as *ETR1* and *EIN2*, but also for those of the *Brassica* homologues present in more copies, with one of them located in the conserved region only, such as *CTR1* and *ERF7*. However, some segmental duplications in the *Brassica* genome, as revealed by comparative mapping with *Arabidopsis*, may correspond to functional duplications. In conclusion, this study confirmed that conservation at the gene sequence and collinearity levels can provide guidelines for the functional analysis of gene homologues from closely related species.

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