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Polyamine contents, ethylene synthesis, and *Br*ACO2 expression during turnip germination

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Abstract

Contents of total free [PA(S)] and conjugated polyamines [PA(SH), PA(PH)] were higher in turnip (*Brassica rapa* L. cv. Rapa) seeds during imbibition (0 - 36 h) and radicle protrusion (36 - 48 h) than during the further growth (10 d). Ethylene production was activated with the protrusion, reaching a maximum at the second day of germination and dropping afterwards. The application of ethrel accelerated radicle emergence but the direct intervention of ethylene in the breaking of the seed coat was not clear from the use of ethylene-biosynthesis inhibitors ($CoCl_2$ and AVG). Finally, in this work the gene *Br*ACO2 was characterized. Although its expression was not detected in seeds through zygotic embryogenesis, it increased concomitantly with the germination process.

Additional key words: *Brassica rapa*, *Cruciferae*, conjugated polyamines, ethrel, free polyamines, seed.

Introduction

Seed germination involves a series of metabolic processes regulated by hormones (Koorneef *et al.* 2002). Many physiological and molecular studies have clearly demonstrated that the synthesis and perception of ethylene is required for several developmental stages (*e.g.* flowering, maturation, senescence) or response to pathogens (Imaseki 1999). Since ethylene regulates such diverse processes, its production in plants is assumed to be tightly regulated (Fluhr and Mattoo 1996). However, the role of ethylene in germination remains controversial, and, among seeds that require ethylene to germinate, some are extremely sensitive while others require relatively high content of the gas (Esashi 1991, Kepczynski and Kepczynska 1997, Matilla 2000). In contrast, some authors hold that ethylene production is a consequence of the germination process (Fu and Yang 1983). For the seeds that depend on ethylene to germinate, current evidence suggests that ethylene synthesis during imbibition interrupts dormancy maintained by ABA, thereby triggering germination (Beaudoin *et al.* 2000).

From the time of the discovery of methionine as the precursor of ethylene, major breakthroughs have been made in the knowledge and understanding of regulation of the methionine cycle and ethylene physiology. One was that the S-adenosyl-L-methionine (AdoMet) can be alternatively channelled towards the ethylene or towards the polyamine (PA) pathways, these polycations being related with various growth and development processes in higher plants (Walden *et al.* 1997, Kakkar *et al.* 2000, Pandey *et al.* 2000). On the other hand, it has also been demonstrated that an alteration in the synthesis of one or both metabolic pathways may alter the synthesis of the other (Matilla 1996). In addition, regulation of the

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Abbreviations: ACO - 1-aminocyclopropane-1-carboxylate oxidase; AdoMet - S-adenosyl-L-methionine; AVG - L-α-(2-aminoethoxyvinyl)glycine; Cad - cadaverine; Ethrel - 2-chloroethylphosphonic acid; PA - polyamine; PA(PH) - acid insoluble conjugated polyamine; Put - putrescine; PA(S) - free-polyamine; PA(SH) - acid soluble conjugated polyamine; Spd - spermidine; Spm - spermine.
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partitioning of AdoMet between the ethylene *vs*. PA biosynthetic pathways may be a way of controlling germination in some seeds (Gallardo *et al.* 1994, 1995, Matilla 1996, 2000, Matilla *et al.* 2005).

To advance our knowledge concerning the role of ethylene and PAs during seed germination and post-germinative growth of *Brassica rapa* L. cv. Rapa, an important crop in north-western Spain, in the present work, we *1*) evaluated the alterations in the levels of PAs in both phases of life cycle, *2*) studied the possible intervention of ethylene in the emergence of embryonic axis by using inhibitors of its synthesis, and *3*) quantified the expression of *Br*ACO2 which is the second member of 1-aminocyclopropane-1-carboxylate oxidase (ACO) family from turnip isolated by us, since the first one called *Br*ACO1 was recently reported (Rodríguez-Gacio *et al*. 2004).

Materials and methods

Chemicals: 1-aminocyclopropane-1-carboxylic-acid, L-α-(2-aminoethoxyvinyl)glycine, β-D-thiogalactopyranoside, 2-chloroethylphosphonic acid, cadaverine (dihydrochloride), putrescine (dihydrochloride), cobalt chloride, spermidine (trihydrochloride) and spermine (tetrahydrochloride) were obtained from *Sigma-Aldrich* (Madrid, Spain).

Seed germination: Seeds of turnip (*Brassica rapa* L. cv. Rapa), harvested in the experimental fields of the University of Santiago de Compostela, Spain, were stored in darkness at 4 °C until used. For germination experiments, seeds of uniform size were washed three times in sterile, double-distilled water, and then allowed to germinate in darkness at 30 °C (optimal conditions) and 70 % relative humidity on top of two layers of sterile filter paper (*Whatman No. 1*, 14 × 25 cm) in plastic trays (100 seeds and 40 cm^3 distilled water or ethrel and $CoCl_2$ at concentrations indicated in the text). Germination was scored as positive when the radicle tip had fully penetrated the seed coat (24 - 36 h) and the post-germinative growth was evaluated until 10 d.

Determination of free and conjugated polyamines: Plant material (300 mg) frozen at -80 ºC was powdered in a chilled mortar, and 0.2 M perchloric acid [4 cm^3 g^{-1}(f.m.)] containing 1,6-hexanediamine [1 μmol g^{-1}(f.m.)] as an internal standard was added. The homogenate was centrifuged at 27 000 *g* for 12 min at 4 ºC, and the supernatant was kept on ice for later use. The precipitate containing the PAs bound to macromolecules and an aliquot of the aforementioned supernatant were hydrolysed in the presence of 12 M HCl for 18 - 24 h at 110 ºC and then filtered through glass wool, using 5 - 10 drops of 10 mM HCl to clean out the vial. The supernatants and hydrolysed precipitate were dried at 70 ºC under a flow of N_2 before being redissolved with perchloric acid (0.2 M). An aliquot (0.2 cm^3) of supernatant, hydrolysed supernatant and hydrolysed pellet were mixed in a tapered vial with 0.2 cm^3 of saturated sodium carbonate and 0.4 cm^3 dansyl chloride in acetone (10 g dm^3). The mixture was incubated at 65 ºC for 60 min and 0.1 cm^3 of a solution of L-proline (100 g dm^3) was then added. After 30 min, dansylated PAs were extracted, first with 0.5 cm^3 toluene and then with 0.3 cm^3 toluene. The toluene extract was dried under a N_2 stream and the residue dissolved in acetonitrile (0.2 cm^3) and passed through *Millipore HV-4* filters for immediate analysis. The PAs present in the samples were analysed with a *Hewlett Packard* (Palo Alto, CA, USA) HPLC equipped with a fluorescence detector and a reverse-phase column (*Hypersyl ODS* 5 μm, 20 × 0.46 cm). As the elution solvent, a mixture of 60:40 acetonitrile: water was used at a flow rate of 1.5 cm^3 min^{-1}. The injection volume was 0.02 cm^3. The same procedure was applied to Put (dihydrochloride), Spd (trihydrochloride) and Spm (tetrahydrochloride), which were used as standards.

Ethylene production: Samples of germinated seeds (200 mg) were transferred to 12 cm^3 vials containing 0.25 cm^3 of sterile distilled water. The vials were hermetically sealed and incubated in darkness at 30 ºC. After 120 min, 1 cm^3 samples were withdrawn from the flasks and injected into a *Hewlett Packard HP 6890 Plus* gas chromatograph fitted with a flame-ionisation detector and a 10 m × 0.32 mm pseudocapillary *P-Pora Plot Q* column packed with polystyrene-divinylbenzene. Other characteristics were as previously described (Rodríguez-Gacio and Matilla 2001). Ethylene identification was based on the retention time compared with an ethylene standard (purity 99.9 %) supplied by Praxair España S.L., Pontevedra, Spain.

Isolation of cDNA clone (*Br*ACO2): The total RNA from embryonic axes of seeds germinated for 2 d was extracted using the *Qiagen pack-500* cartridge (Valencia, CA, USA) following the manufacturer's instructions. The cDNA was synthesised from 5 μg of total RNA prepared from embryonic axes using the 1st Strand cDNA Synthesis kit for RT-PCR AMV (*Roche Diagnostic*, Mannheim, Germany) with specific primers and following the manufacturer's instructions. The cDNA was used as a template for a PCR reaction using degenerated oligonucleotides corresponding to conserved regions among ACOs. The forward primer of *Br*ACO2

consisted of a 26-mer of the sequence 5´-ATGGAG AAGAAGAACATTAAGTT(C,T)CC(G,A,C)GT-3´ encoding the MEKNIKFPV amino-acid sequence and the reverse primer was a 24-mer of the sequence 5´-TTA GAAAGTCTCTACGGCTGC(G,A,C)GT-3´ corresponding to the TAAVETFZ amino-acid sequence. The PCR conditions were as follows: 1 min 94 ºC, 2 min 55 ºC, 2 min 72 ºC for 30 cycles and 10 min 72 ºC. The PCR product was cloned into the *pGEM T* easy vector (*Promega*, Madison, WI, USA) and sequenced. As the predicted gene product encoded by this clone revealed similarity to different ACOs, it was called *Br*ACO2 (AJ309322). As in the case of *Br*ACO1 (Rodríguez-Gacio *et al.* 2004), the Southern-blot analysis revealed that *Br*ACO2 belongs to a multigene family, and the recombinant protein expressed in *E. coli* (BL21DE3) also displayed ACO activity (data not shown).

Results and discussion

Viable dry seeds of *B. rapa* cv. Rapa have similar contents of free PA(S) and acid-soluble conjugated PA(SH), these PAs being quantitatively six-fold more abundant than acid insoluble conjugated PA(PH). Total amounts of free and conjugated PAs increased during imbibition (0 - 36 h) and decreased from radicle protrusion (36 - 48 h) onwards (Table 1). Similar variation patterns for PA(S) were previously reported during germination of chick-pea seeds (Gallardo *et al.* 1992) and tobacco-pollen grains (Chibi *et al.* 1994). During the entire study period the turnip seeds contained the most important PAs (Put, Spd and Spm) (Fig. 1), in addition to Cad, the distribution of which in higher plants is much more limited than the former. All these PAs appeared both in their free and conjugated forms. During both imbibition and radicle-emergence processes, the most abundant PA(S) was Spd, whereas over the post-germination growth period it was Put. Also, Spd was the most abundant PA(S) in seeds of *Picea abies* (Huang and Villanueva 1992), *Triticum durum* (Anguillesi *et al.* 1990), *Zea mays* (Lozano *et al.* 1989), *Hordeum vulgare* (Nielsen 1990) and *Cicer arietinum* (Gallardo *et al.* 1992). The endosperm of *Zea mays* contained no Spd, and the most abundant PA(S) was Put, followed by Spm (Torrigiani *et al.* 1988). However, this fact cannot be generalized for higher plants and varies among species (Matilla 1996). In turnip, Spd(S) peaked at 48 h, coinciding with radicle emergence and with the lowest levels of Put(S) (Fig. 1), suggesting high Spd-synthase activity. At this point, the Put/Spd relationship was very low (<<1), as is typical in meristematic tissues with mitotic activity (Walden *et al.* 1997, Applewhite *et al.* 2000). Nevertheless, the Put/Spd, Spm relationship was > 1 from 48 h on, indicating a predominance of cell elongation (Schwartz *et al.* 1986, Ruiz *et al.* 2000). These di/tri, tetra-amine relationships were also measured in chick-pea seeds in which a detailed study was made of the distribution of PA(S) in the meristematic, elongation, and differentiation zones of the embryonic axis (Gallardo *et al.* 1994).

Similar to PA(S), the seeds of *B. rapa* cv. Rapa contained the highest levels of PA(SH) and PA(PH) at the end of germination (Fig. 1). We have noted this fact in the dicotyledonous chick-pea (Muñoz De Rueda *et al.*

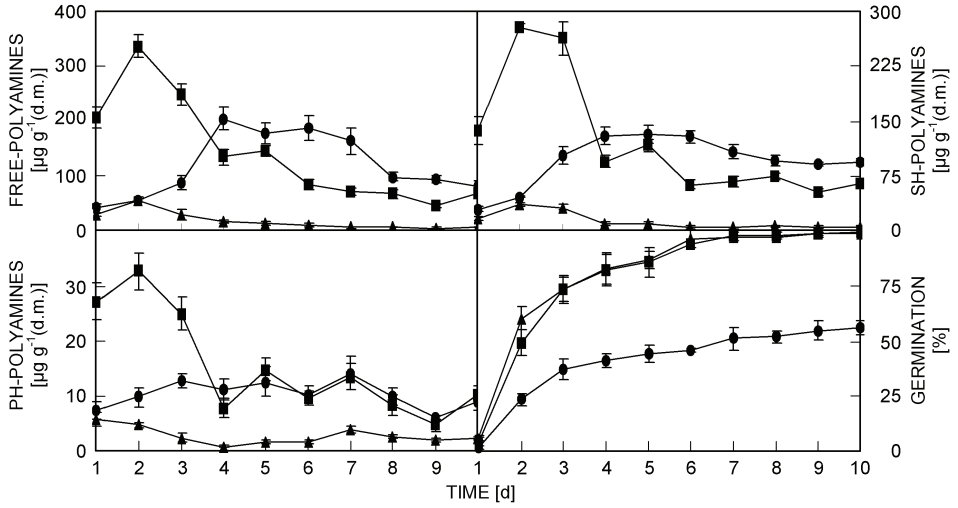

Fig. 1. Variation in polyamines (free, SH and PH) during germination of turnip seeds (*circles* - Put, *squares* - Spd, and *triangles* - Spm) and germination percentage in the presence of water (*circles*), 7 µM (*squares*) and 70 µM ethrel (*triangles*). Data represent the mean of 3 - 4 replicate samples ± SD.

1993). Kaur-Sawhney and Applewhite (1993) and Bouchereau *et al*. (1999) have also proposed that the free and conjugated PAs reach high levels when the mitotic activity is also high. The necessity of tight control of the intracellular level of free PAs is the same as in the case of plant phytohormones. Apart from their biosynthesis rate, the intracellular concentrations of free PAs could be maintained by conjugation, PA degradation through oxidative deamination and transport. From the data collected during germination of *Glycine max* and *Phaseolus vulgaris*, it has been hypothesised that diamine-oxidase (DAO) is involved in the regulation of the intracellular level of PAs (Scoccianti *et al*. 1990). In *P. vulgaris*, DAO was founded in young, actively growing parts (*e.g.* shoot apex), while in *G. max* it occurred mainly in elongating tissues (*e.g.* hypocotyls), which contain high Cad contents (Scoccianti *et al.* 1990). In *Cicer arietinum* the highest DAO activity was registered during the post-germination period and fundamentally in the differentiation and elongation zones of the embryonic axis (Matilla *et al.* 2002). Bonneau *et al.* (1994) related conjugated PAs to seed viability. In the case of dry *B. rapa* seeds, the Spd(SH) levels were also higher with greater viability (Puga-Hermida 2003).

Given that PAs and ethylene share AdoMet as a common precursor (Kushad and Dumbroff 1991, Pandey *et al.* 2000, Matilla 1996, 2000), and that during the germination period of turnip seeds high contents of free and conjugated PAs were found (Fig. 1), in the present work we quantify the ethylene production in the embryonic axis and also discuss its possible participation in radicle emergence. The embryonic axis of *B. rapa* seeds began their protrusion after 24 - 36 h of imbibition at 30 ºC (optimal temperature), their growth progressing afterwards (Fig. 1). Temperatures slightly higher and lower than 30 ºC (30 ± 5 ºC) strongly inhibited protrusion. The presence of ethrel (7 µM) in the germination medium accelerated radicle emergence, reaching 100 % germination in 6 - 7 d, while control reached only 50 % (Fig. 1). Ethylene production in the embryonic axis was activated at the onset of protrusion, reaching a high at 48 h and dropping afterwards concomitantly with radicle growth (Table 2). This ethylene synthesis was inhibited by $CoCl_2$ (inhibitor of ACC-oxidase, ACO), while no gas production was detected from 1 mM on (Table 2), although protrusion and growth of the embryonic axis did take place (Table 2). The addition of ethrel (7 µM),

Table 1. Variation in total free, PA(S), and conjugated-polyamines, PA(SH) and PA(PH), during germination and post-germinative growth of embryonic axis of turnip seeds. Data represent the mean of 3 - 4 replicate samples ± SD.

Time [d]	PA(S)	PA(SH)	PA(PH)
dry seed	169.32 ± 14.7	159.83 ± 2.30	27.04 ± 1.8
1	277.50 ± 18.3	183.78 ± 19.1	40.22 ± 6.3
2	447.59 ± 24.7	362.41 ± 14.3	46.90 ± 7.2
3	364.14 ± 22.2	399.39 ± 25.2	39.98 ± 4.5
4	353.85 ± 23.5	234.09 ± 18.3	19.49 ± 2.4
5	336.06 ± 19.3	258.89 ± 16.5	28.80 ± 2.0
6	280.94 ± 21.4	197.07 ± 10.1	21.18 ± 2.1
7	239.81 ± 17.4	179.59 ± 12.4	31.39 ± 4.6
8	170.72 ± 16.3	176.94 ± 17.1	20.59 ± 2.7
9	143.04 ± 11.2	150.17 ± 12.6	12.48 ± 1.3
10	156.55 ± 12.2	163.56 ± 15.3	21.31 ± 2.2

Table 2. Ethylene production, EP [pmol g^{-1}(f.m.) h^{-1}] and germination percentage, G [%] during turnip seed germination in the presence of ethrel and cobalt chloride. Data represent the mean of 4 - 5 replicate samples ± SD. nd - not detected.

Treatment	[mM]		1 d	2 d	3 d	4 d	5 d
Control		EP	nd	0.80 ± 0.1	0.58 ± 0.1	0.44 ± 0.2	0.29 ± 0.1
		G	nd	24.00 ± 0.3	36.00 ± 0.2	42.70 ± 0.5	45.30 ± 0.1
$CoCl_2$	0.01	EP	nd	0.40 ± 0.1	0.45 ± 0.2	0.41 ± 0.1	0.36 ± 0.2
		G	2.00 ± 0.5	20.70 ± 3.0	33.10 ± 4.1	42.50 ± 3.2	38.50 ± 3.8
	0.10	EP	nd	0.24 ± 0.1	0.18 ± 0.1	0.38 ± 0.2	0.37 ± 0.1
		G	1.00 ± 0.2	19.90 ± 2.7	31.50 ± 3.5	36.30 ± 4.5	35.40 ± 4.6
	1.00	EP	nd	nd	nd	nd	nd
		G	1.00 ± 0.2	14.70 ± 3.4	20.00 ± 4.7	29.10 ± 3.6	29.10 ± 3.6
	10.00	EP	nd	nd	nd	nd	nd
		G	nd	0.35 ± 0.1	0.95 ± 0.2	1.27 ± 0.7	1.60 ± 0.6
Ethrel	0.007	EP	0.62 ± 0.2	3.17 ± 0.5	1.37 ± 0.4	1.51 ± 0.5	1.42 ± 0.3
		G	50.00 ± 0.2	72.20 ± 0.1	82.03 ± 0.2	85.02 ± 0.3	94.01 ± 0.5
Ethrel + $CoCl_2$	1.00	EP	0.60 ± 0.2	0.83 ± 0.3	0.92 ± 0.3	1.08 ± 0.4	0.89 ± 0.2
		G	6.00 ± 1.5	44.80 ± 1.7	57.50 ± 5.0	62.80 ± 4.4	56.10 ± 5.8
	10.00	EP	0.30 ± 0.1	0.25 ± 0.1	0.37 ± 0.2	0.32 ± 0.1	0.42 ± 0.2
		G	1.30 ± 0.3	25.70 ± 3.6	33.40 ± 4.6	42.10 ± 4.9	42.70 ± 3.6

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  1 atg gag aag aac att aag ttt ccg gtt gta gac ttg tcc aag ctc att ggt gaa gag aga
     M   E   K   N   I   K   F   P   V   V   D   L   S   K   L   I   G   E   E   R
 61 gac caa acc atg gct ttg atc aac gat gct tgt gag aat tgg ggc ttc ttt gag ata gtg
     D   Q   T   M   A   L   I   N   D   A   C   E   N   W   G   F   F   E   I   V
121 aac cat ggt tta cca cat gat ttg atg gac aac gtc gag aag atg aca aag gaa cat tac
     N   H   G   L   P   H   D   L   M   D   N   V   E   K   M   T   K   E   H   Y
181 aag ata tca atg gaa caa aag ttc aac gac atg ctc aaa tcc aaa ggt ttg gaa aat ctt
     K   I   S   M   E   Q   K   F   N   D   M   L   K   S   K   G   L   E   N   L
241 gag cga gaa gtt gag gat gtt gat tgg gaa agc act ttc tac ctt cgt cat ctc cct cag
     E   R   E   V   E   D   V   D   W   E   S   T   F   Y   L   R   H   L   P   Q
301 tcc aat ctc tac gac att cct gat atg tct gat gaa tac cgg acg gcc atg aaa gat ttt
     S   N   L   Y   D   I   P   D   M   S   D   E   Y   R   T   A   M   K   D   F
361 ggt aag aga ttg gag aat ctt gct gag gat ttg ttg gat cta ttg tgt gag aat tta ggg
     G   K   R   L   E   N   L   A   E   D   L   L   D   L   L   C   E   N   L   G
421 tta gag aaa ggg tac ttg aag aaa gtg ttt cat gga aca aaa ggt cca acc ttt ggg act
     L   E   K   G   Y   L   K   K   V   F   H   G   T   K   G   P   T   F   G   T
481 aag gtg agc aac tat cca gct tgt cct aag cca gag atg ata aaa ggt ctt agg gcc cac
     K   V   S   N   Y   P   A   C   P   K   P   E   M   I   K   G   L   R   A   H
541 act gat gca gga ggc atc atc ttg ttg ttt caa gat gac aag gtc agt ggt ctc cag ctt
     T   D   A   G   G   I   I   L   L   F   Q   D   D   K   V   S   G   L   Q   L
601 ctt aaa gat ggt gac tgg att gat gtt cct cca ctc aac cac tct att gtc atc aat ctt
     L   K   D   G   D   W   I   D   V   P   P   L   N   H   S   I   V   I   N   L
661 ggt gac caa ctt gag gtg ata act aac ggc agg tac aag agt gtg atg cac cgt gtg gtg
     G   D   Q   L   E   V   I   T   N   G   R   Y   K   S   V   M   H   R   V   V
721 act cag aaa gaa gga aac aga atg tca att gca tct ttc tac aac ccg gga agc gat gct
     T   Q   K   E   G   N   R   M   S   I   A   S   F   Y   N   P   G   S   D   A
781 gag atc tct cca gct tca tcg ctt gcc tgt aaa gaa acc gag tac ccg agt ttt gtt ttt
     E   I   S   P   A   S   S   L   A   C   K   E   T   E   Y   P   S   F   V   F
841 gat gac tac atg aag ctc tat gct ggg gtc aag ttt cag cct aag gag cca cgc ttc gag
     D   D   Y   M   K   L   Y   A   G   V   K   F   Q   P   K   E   P   R   F   E
901 gca atg aag aat gct aat gca gtt aca gaa ttg aac cca acc gca gcc gta gag act ttc
     A   M   K   N   A   N   A   V   T   E   L   N   P   T   A   A   V   E   T   F
961 taa
    OCH
```

Fig. 2. Nucleotide sequence of *Brassica rapa* L. cv. Rapa *Br*ACO2 and deduced amino acid sequence.

together with 1 - 50 mM $CoCl_2$ which were inhibitory for production of the gas, induced germination (Table 2), but the resulting seedlings were not viable. This suggests that the mutual effects of ethylene and $CoCl_2$ were in this case artefacts. These results cast doubt on the role of ethylene in the breaking of the seed coat in turnip, because the presence of AVG (10 µM), an inhibitor of ACC-synthase activity in the germination medium, strongly depressed ethylene production as well as ACC and MACC synthesis while hardly affecting radicle emergence (data not shown). Nevertheless, if radicle emergence in *B. rapa* is related to ethylene production, then sensitivity to the gas must be very high and therefore the ethylene produced under the detection threshold of GC for triggering germination would be sufficient. On the other hand, the pronounced alterations in the synthesis of ethylene and free and conjugated PAs at the onset of germination in turnip seeds, as demonstrated in the present work, and in other biological systems (Mehta *et al*. 1997), suggest that AdoMet-decarboxylase must be highly regulated and PAs must have some function during the process studied. However, the fact that no PA synthesis was stimulated by ethrel during the imbibition phase (Puga-Hermida *et al.* 2003), and that radicle emergence was strongly stimulated, suggest that it is ethylene itself and the sensitivity to this phytohormone, and not the PAs, that accelerate the breaking of the seed coat.

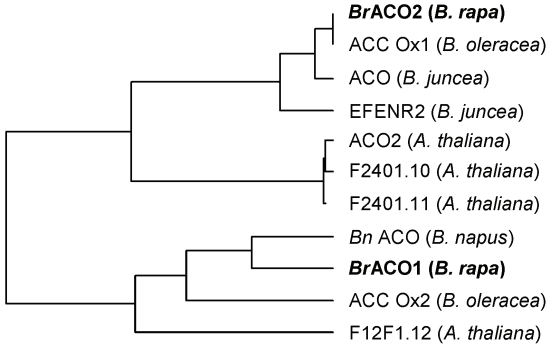

Fig. 3. Phylogenetic tree of several ACO described in higher plants including *Br*ACO1 and *Br*ACO2 from *Brassica rapa* L. cv. Rapa.

With the use of degenerated oligonucleotides designed from highly conserved regions of other ACO described in plants, cDNA synthesised from the total RNA of *B. rapa* cv. Rapa was amplified. PCR provided one fragment having an open reading frame of 636 pb (Fig. 2), termed *Br*ACO2 (AJ309322), which showed some similarity with ACO of flower tissues of *B. oleraceae* (100 %) (Pogson *et al*. 1995) and developing leaves of *B. juncea* (99 %) (Pua *et al*. 1992) (Fig. 3). To ascertain whether the DNA clone encodes

functional enzyme, we expressed the *Br*ACO2 sequence obtained (restriction sites *NdeI* and *XhoI*) in the *E. coli* heterologous system using the pET28a(+) as the expression vector. After induction of the bacterial *E. coli* strain (BL21DE3) with IPTG, a soluble protein with an electrophoretic mobility in SDS/PAGE of 36 kDa and *in vitro* ACO activity was detected (data not shown). The different phylogenetic position of *Br*ACO2 with respect to *Br*ACO1 (Fig. 3), the use of different primers for their cloning (*see* Materials and methods; Rodríguez-Gacio *et al*. 2004), and, mainly, the differential expression found in the two genes during zygotic embryogenesis of *B. rapa* cv. Rapa (Rodríguez-Gacio 2002) indicate that *Br*ACO1 and *Br*ACO2 clones codify two different ACO. The expression of the gene *Br*ACO2 was not detected in seeds throughout zygotic embryogenesis nor in the dry viable seeds (data not shown). However, although it was weakly expressed during the first instants of imbibition, it increased progressively in the radicle with the germination time (Fig. 4). In addition, the last step of the ethylene synthesis (ACO activity) did not appear to be very controlled in the germination or post-germination period of *B. rapa* seeds, since both the gene *Br*ACO1 (Rodríguez-Gacio *et al*. 2004) as well as *Br*ACO2 (characterized in this work) did not show different expression patterns during the two periods. However, *Br*ACO1 was in fact expressed exclusively at the onset of seed development. Its function must be directly involved in the ethylene synthesis observed at these early developmental stages (Rodríguez-Gacio and Matilla 2001, Rodríguez-Gacio *et al*. 2004).

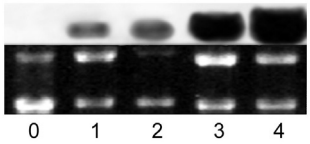

Fig. 4. Northern-blot analysis of *Br*ACO2 gene expression during the first four days of growing embryonic axis of *Brassica rapa* L. cv. Rapa (*upper)*. The 25S rRNA probe was used as a charge control (*lower*).

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