Role of ethylene in cotyledon development of microspore-derived embryos of Brassica napus

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Abstract

Ethylene production during seed development in Brassica napus occurs first at 20 d after pollination (DAP), while a second greater peak occurs at 35 DAP. Because of the inaccessible location of the embryo within the maternal tissue, microspore-derived embryos (MDEs) of B. napus were used as a model for studying the role of ethylene during embryogenesis. The MDEs also produced a peak in ethylene evolution at 20 DAC (i.e. the early cotyledonary stage), dropping to minimal levels by 25-30 DAC. At 20 DAC the excised cotyledon evolved 85% of the ethylene found in the whole MDE. To determine the role of ethylene, MDEs were treated with aminooxyvinylglycine (AVG, an inhibitor of ethylene biosynthesis), CoCl₂ (an inhibitor of 1-amino-cyclopropane-1-carboxylic acid (ACC) oxidase), and silver thiosulphate (STS, an inhibitor of ethylene action). An inhibition in ethylene production or action at 20 DAC resulted in diminished lateral cotyledon expansion, due to a reduction in the lateral expansion of cells within the cotyledon. Recovery to ‘control-type’ levels of cotyledon cell expansion was achieved by application of ACC (the metabolic precursor of ethylene) to AVG-treated MDEs. Thus, ethylene production at 20 DAP likely controls cotyledon expansion during embryogenesis.

Key words: Brassica napus, cotyledon expansion, ethylene production, microspore-derived embryos, seed development.

Introduction

Ethylene is an endogenous gaseous plant hormone which has been extensively studied in relation to a wide range of vegetative plant growth, seed dormancy and especially germination processes (Eisengen, 1983; Abeles et al., 1992). However, the role of ethylene during embryogenesis has not been extensively studied. Recently, ethylene evolution in desiccating silique and seeds of mustard and canola was measured, with the conclusion that ethylene within the silique may contribute to seed degreening (Johnson-Flanagan and Spencer, 1994). Ethylene production in seeds of both mustard and canola, i.e. Brassica napus (cv. Westar) peaked during the torpedo stage of embryogenesis, i.e. 20 d after pollination (DAP) (Johnson-Flanagan and Spencer, 1994). Similar results were obtained for B. napus cv. Global seeds (Jayasekera, 1993). These findings suggest that ethylene may be involved in zygotic embryogenesis. However, due to the location and relative inaccessibility of the embryo within maternal tissue, the ability to assess the role of ethylene during zygotic embryogenesis is difficult.

Somatic and anther embryo culture systems, which are not limited in tissue quantity and accessibility, may represent a useful model for investigating the role of ethylene during embryogenesis. Significant ethylene production has been demonstrated in microspore-derived embryo (MDE) cultures of B. oleracea (Biddington et al., 1993) and in somatic embryos of Medicago sativa (Meijer, 1989). In both in vitro embryo systems ethylene evolution occurred maximally during the middle stages of

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Abbreviations: ACC, 1-amino-cyclopropane-1-carboxylic acid; DAC, days after culture (for microspore-derived embryos); DAP, days after pollination; MDE(s), microspore-derived embryo(s); ZEs, zygotic embryo(s).
embryo development. In MDEs of *B. oleracea*, the earliest peak in ethylene evolution was correlated with an inhibition in embryogenesis (as demonstrated by separate trials where ethylene inhibitors were applied to embryo cultures) (Biddington et al., 1993). In contrast, in somatic embryos of *M. sativa*, inhibition of ethylene production using ethylene biosynthesis inhibitors did not inhibit embryo induction. Ethylene did, however, inhibit embryo growth after initiation of embryogenesis (Meijer, 1989). While these studies do suggest a possible role for ethylene during embryo development, no one study to date has been conclusive.

In order to examine the role of ethylene during embryo development the levels of ethylene produced in seeds of *B. napus* cv. Topaz have been established at different times after pollination. Topaz is a variety of canola exhibiting a high frequency of embryogenesis in isolated microspore cultures (Keller et al., 1987). As criteria for using MDEs as a model system, it has been established that MDEs produce a peak in ethylene evolution at a stage in development identical to zygotic embryos (ZEIs). Hence, MDEs have been used to show that ethylene biosynthesis, and ethylene action *in vivo*, are required for cell expansion in the cotyledon during MDE development.

**Materials and methods**

**Chemicals and growth regulators**

Sugar for MDE medium was obtained from BDH (Dorset, England). The following ethylene biosynthesis or action inhibitors were used in this study: CoCl₂, aminoethoxyvinylglycine (AVG) (Fluka, USA), 1-aminocyclopropane-1-carboxylic acid (ACC) (Calbiochem, USA). Silver thiosulphate (0.005 M) (STS: Ag₂S₂O₃) was freshly prepared using a 1:1 (v:v) ratio of stock solutions of 0.01 M AgNO₃ (Fisher Scientific Co., Fair Lawn, NJ, USA) and 0.04 M Na₂S₂O₃ (Chemical Scientific, USA). Both STS and other stock solutions were kept at 4 °C in light-tight vessels. All plant hormone precursors, and plant hormone inhibitors used for exogenous application to MDEs were made up in stock solutions with 50% ethanol as a solvent. For treatments, 10 µl of the stock solutions (at a final concentration of 8 mM ethanol) were applied to embryos in 10 ml of culture medium. Control embryos were treated with 10 µl of 50% ethanol.

**Plant materials**

*Brassica napus* cv. Topaz (Dr Keith Downey, Agriculture Canada, Saskatoon, Saskatchewan) plants used for zygotic embryo and seed dissection were grown at 25/16 °C day/night temperature with a 16 h photoperiod (400 mmol m⁻² s⁻¹). Plants for microspore culture were raised as above for 5 weeks and then transferred to 12/7 °C day/night temperatures until flower buds were harvested.

**Microspore-derived embryo (MDE) culture**

Methods for MDE production were as described previously (Hays et al., 1996, 1999). For ethylene inhibitor treatments 0.1–10 µM CoCl₂, STS, or AVG were added to MDE cultures at 12–14 d after culture (DAC) (or prior to the peak in endogenous ethylene that occurred at 20 DAC). For recovery of normal MDE development in inhibitor-treated MDEs, these MDEs were also treated with 0.1–100 µM of ACC at 20–22 DAC.

**Ethylene analysis**

Methods used for analysis of *in vivo* ethylene production have been previously described (Finlayson et al., 1991). Briefly, developing MDEs sampled at various times after culture, and dissected seeds harvested at various times after pollination, were placed in separate 10 ml syringes with the plunger adjusted to 2 ml. A 1 ml gas sample was then transferred to a second gas-tight syringe through a three-way valve after 20 min (it was found that the burst in wound-induced ethylene could not be detected for at least 30 min). This sample was then analysed for ethylene concentration using a Photovac 10S Plus GC with a 3.2 mm × 2.45 m 60/80 Carbopack B column (Photovac, Markham, Canada) fitted with a photoionization detector.

**Histological preparations**

Control and inhibitor-treated MDEs were collected at 25 DAC and 30 DAC. All tissues were fixed in 50 mM cacodylate buffer, containing 1.6% glutaraldehyde and 2.4% formaldehyde for 24 h at 4 ºC. They were then dehydrated in methyl cellulose for 24 h. Methyl cellulose was then replaced with absolute ethanol with two changes over 48 h. Infiltration of LKB Historesin was carried out at 4 ºC with daily changes at progressively higher concentrations of Historesin (1:1, 2:1, 1:1, 1:2, 1:3, pure Historesin). The pure Historesin solution was changed once more and left at 4 ºC for 3 d. This tissue was then embedded in 10 ml of Historesin solution, to which was added 0.6 ml of hardener and 0.4 ml PEG 200 (Yeung and Law, 1987). It was then allowed to polymerize for 5 h in plastic moulding trays with microtome mounting chucks. The polymerized tissue was sectioned into 3 mm thick sections with a LKB 2040 Histostage using a dry glass Ralph knife, collected in water on subbed slides and allowed to dry. Sections were stained with Periodic acid–Schiff’s (PAS) reagent for carbohydrates and counter-stained with toluidine blue O (TBO) or amido blue-black 10B for proteins (Yeung, 1984).

Sections were viewed and photographed with a Leitz microscope with Kodak Technical Pan or Kodak SuperTM 200 ASA print film. Determination of lateral cell expansion in the MDE cotyledon was made on sections that were cut longitudinal median with respect to the embryo axis using a Zeiss light microscope with a built-in eyepiece micrometer. Lateral cell expansion measurements were made at three points in the cotyledon; the tip (or most distal with respect to the shoot apical meristem), the mid-point between tip and axis, and adjacent to the shoot apical meristem.

**Conversion frequency measurements**

The phenotype of MDEs treated with and without inhibitors of ethylene biosynthesis and action was assessed. An assessment of conversion, defined as the survival and development of MDEs in an *ex situ* environment (Stuart and Strickland, 1984), was performed based on the methods and definitions defined earlier (Yeung et al., 1996). Embryos were carefully removed from the liquid microspore media 25–30 DAC and placed on 0.7% agar media with half-strength Murashige and Skoog medium supplemented with 1% sucrose, with plastic Petri dishes being used.
for growth containers (Murashige and Skoog, 1962). Embryos were maintained at 25 °C under a 16 h photoperiod (Sylvania Gro-lux F40T132 Gro-WS lamps at 80 μmol m−2 s−1). Germination and conversion were scored after 2 weeks in the germination medium. Conversion frequency was defined as the number of embryos that developed primary leaves, divided by the total number of embryos plated (Merkle et al., 1990).

Statistical analysis

Values are the mean ± standard error of three replicates. An analysis of variance with multiple comparisons was conducted for all experiments with the SPSS general linear models procedure (SPSS Institute, 1994). When significant differences between unequal variables occurred, means were separated using the Student–Newman–Keuls test (P < 0.05; SPSS software package, SPSS Inc., Chicago, Illinois, USA).

Results

Ethylene production in embryos of Brassica napus

As a prerequisite for using MDEs of B. napus cv. Topaz as a model for examining the role of ethylene during embryo development, the pattern of ethylene production was determined in developing seeds of donor plants (i.e. B. napus cv. Topaz). Developing and maturing seeds were harvested for determining ethylene levels at 5 d intervals beginning at 15 DAP, i.e. the torpedo stage in embryo development (Fig. 1). Ethylene evolution from developing seeds and collected MDEs was linear for at least 30 min, indicating developmental ethylene synthesis. Thereafter, the 1 ml headspace gas sample was transferred to the gas-tight syringe for ethylene measurement after 20 min (see Materials and methods). The overall pattern of ethylene evolution in developing seeds shows a peak occurring at the early cotyledonary stage (20 DAP), while a second peak (approximately 2-fold greater) occurs at 35 DAP. Ethylene evolution decreases during the later stages of seed drying (Fig. 1).

Prior to using MDEs as a model for investigating the role of ethylene in embryo development, it was felt important to demonstrate that MDEs mimic the zygotic embryos in terms of ethylene evolution during corresponding stages of embryo development. Therefore, ethylene evolution was analysed during MDE development (Fig. 2). In order to optimize the comparison, both the zygotic system and MDE systems were maintained under the same temperature regime and ethylene measurements were made at the same time of day. As was the case for zygotic embryos (ZEs), ethylene evolution peaked in MDEs at 20 DAC (i.e. the early cotyledonary stage), dropping to minimal levels by 25–30 DAC. However, unlike ZEs, a peak in ethylene evolution did not occur at 35 DAC for MDEs (Fig. 2). The production of ethylene was then localized to the cotyledon and axis of 20 DAC MDEs. Interestingly, the cotyledon produced significantly (P < 0.05) more of the total ethylene, i.e.
CoCl₂ and AVG inhibited ethylene biosynthesis, ethylene evolution being reduced to one-third to one-sixth of that measured from control MDEs of the same age in culture (Fig. 3).

Prior to 20 DAC the embryos appeared morphologically unaffected by treatment with any of the inhibitors. However, by 25 DAC the expanded cotyledons of MDEs treated with the three inhibitors were significantly reduced in size (P < 0.05) to half that of control MDEs (Figs 4, 5). Trends of reduced length and width of the axis for treated MDEs, relative to controls were, however, non-significant (Fig. 5). When control and inhibitor-treated MDEs were scored for expanded cotyledons (according to the morphological size of the control embryo at 25 DAC), the number of inhibitor-treated MDEs with fully expanded cotyledons ranged from 10–40%, depending upon inhibitor treatment. In fact, treatment with 10 μM STS resulted in embryos with no expanded cotyledons, a likely toxic effect of STS.

Conversion frequency of MDEs treated with inhibitors

In order to assess the viability of embryos treated with ethylene inhibitors, conversion tests were performed. All MDEs developed normal root growth when plated on germination medium at 25–30 DAC, and the conversion frequency of inhibitor-treated embryos was 1.2–2.5-fold greater than for control, untreated embryos (Table 1).

Recovery of lateral cotyledon expansion

In order to establish that the inhibition in lateral cotyledon expansion was caused by an inhibition in ethylene biosynthesis and was not a secondary effect, it was necessary to establish that normal cotyledon development could be re-established in inhibitor-treated MDEs. Thus
### Table 1. Percentage conversion of germinated MDEs treated with two concentrations of three ethylene inhibitors during embryo culture

Conversion frequency is defined by the embryo’s ability to germinate and produce a shoot via the apical meristems. Numbers represent the results of three independent experiments where 10 embryos were scored per trial. Errors represent ±SE. Embryos were treated at 12 DAC and then transferred to hormone-free MS media at 25 DAC containing 1% sucrose. Embryos were scored for conversion at 15 d post germination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage conversion</th>
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<tbody>
<tr>
<td>Control</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>1 μM AVG</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>10 μM AVG</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>1 μM STS</td>
<td>56 ± 15</td>
</tr>
<tr>
<td>1 μM CoCl₂</td>
<td>83 ± 4</td>
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<tr>
<td>10 μM CoCl₂</td>
<td>46 ± 18</td>
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two approaches were used. In one ethylene was applied (in the form of ACC) to MDEs that had been treated with AVG. As an alternative, the inhibitor was simply washed out of the MDEs (i.e. by three washes in fresh culture media). Both approaches were successful and normal cotyledon development can indeed be recovered in inhibitor-treated MDEs. For example, MDEs treated with 1 μM AVG at 12 DAC were cultured in the presence of AVG for 8 d, at which time 10 μM ACC was added to the MDE medium. Ninety per cent of MDEs treated in this manner showed subsequent lateral cotyledon expansion in a range consistent with control. Additionally, 85% of MDEs treated with either AVG (0.5 μM or 10 μM) or with 1 μM STS at 12 DAC, and then washed at 19 DAC with three changes of fresh NLN culture medium exhibited renewed lateral cotyledon expansion that was comparable to controls. Washing control embryos at 19 DAC with NLN medium had no effect. Results of the morphological recovery of the inhibitor-treated MDEs are best illustrated in the following paragraph where a corresponding recovery at the cellular level (see below) is shown.

It was hypothesized that the reduced cotyledon expansion was due to an inhibition in lateral cell expansion. To verify this a histological analysis of cotyledons was performed to compare treated MDEs with controls. Based on median longitudinal sections of the cotyledon, it is apparent that the cells of the inhibitor-treated embryos (Fig. 6B) were small (12–18 μm), i.e. had less lateral cell expansion relative to control MDEs (Fig. 6A). The intracellular spaces are also reduced in cotyledons of inhibitor-treated embryos relative to control embryos (Fig. 6A, B). The inhibition in lateral cell expansion of the cotyledons were recoverable, either by addition of ACC (Fig. 6C), or by washing treated embryos in fresh NLN. Intracellular spaces also return to near control levels for inhibitor-treated MDEs that were also treated with 10 μM ACC at 20 DAC (Fig. 6C). A quantitative analysis of lateral cell expansion is shown in Fig. 7. Here,

**Fig. 6.** The inhibition of cotyledon expansion by an ethylene biosynthesis inhibitor (AVG) and an ethylene action inhibitor (STS) is reversible at the cellular level. Histological comparisons of control MDEs versus MDEs that had been treated with an ethylene biosynthesis inhibitor (AVG) and an ethylene action inhibitor (STS). Embryos were treated at 12 DAC and harvested for serial sectioning at 30 DAC. Cotyledon sections shown are longitudinal median with respect to the embryo axis. The middle portion of the cotyledon is thus shown with respect to its tip and shoot apical meristem. Sections were stained with PAS for carbohydrates and amido black for proteins (Yeung and Law, 1987; Yeung, 1984). Sections are (A) control, (B) 1.0 μM AVG and (C) MDEs treated with 1 μM AVG at 12 DAC followed by addition of 10 μM ACC at 19 DAC. Scale bar in (A) equals 25 μm in length.

effects on MDEs treated with the inhibitors varied, depending on the segment of the cotyledon assessed. In fact, a gradient in inhibition occurred, with the most
Fig. 7. Measurements of lateral cell expansion in the cotyledons of MDEs treated with or without ethylene biosynthesis or action inhibitors showing ‘recovery’ after inhibitor treatment. Measurements were made using a light microscope equipped with an eyepiece micrometer. Embryos were treated at 12 DAC, washed with fresh medium at 19 DAC, or treated with 10 μM ACC at 20–21 DAC. Embryos were harvested for sectioning at 30 DAC. Cell size measurements were made at three regions of the cotyledon in a vertical plane as follows; (A) the end of the expanding cotyledon (i.e. most distal to the shoot apical meristem), (B) mid-way between the tip and apical meristem and (C) adjacent to the shoot apical meristem. Measurements were made on MDE cotyledons that had been sectioned longitudinal median with respect to the embryo axis. Cell measurements were taken from 18 embryos and three separate MDE culture treatment trials. 25 cells were counted for each embryo in each of the three regions of the cotyledon. n=450; values are the means±SE. Letters above the bars represent significant difference between treatments based on (P<0.05) via the SNK test.

severely inhibited cells (50% inhibition, significant at P<0.05) being located at the tip of the expanding cotyledon, i.e. most distal from the shoot apical meristem (Fig. 7A). Cells closer to the shoot apical meristem were also significantly inhibited (P<0.05) but to a lesser degree (by c. 25% (Fig. 7C)). When embryos treated with 1 μM AVG were treated with 10 μM ACC or were washed (as described earlier) cell expansion was equal to that for control embryos (Fig. 7).

Discussion

There are two major peaks in ethylene evolution during B. napus seed development (Fig. 1). The first occurs at a developmental stage characterized by lateral cell expansion in the cotyledon (Tykarska, 1979; Yeung et al., 1996). The second occurs during the beginning of the programmed dehydration of the zygotic embryo and seed coat. Ethylene evolution in MDEs was similar, peaking during the early cotyledonary stage (20 DAC). The several-fold higher levels of ethylene seen in MDEs at 20 DAC, compared to ZEs, was not, however, seen in control MDEs of the same stage (Fig. 3). This may be due to a slight difference in time of day that the ethylene was measured, since circadian and diurnal changes in ethylene production have been reported for Stellaria longipes (Katherison et al., 1996), Chenopodium rubrum (Macháčková et al., 1997) and sorghum (Finlayson et al., 1998). Based on histological comparisons it is known that the early cotyledonary stage (20 DAC) in MDEs is characterized by the same lateral cell expansion found for ZEs at 20 DAP (Yeung et al., 1996). It seems likely then that the peak in ethylene production seen at 20 DAC in MDEs corresponds to 20 DAP peak seen in ZEs. Additionally, the MDEs have an early peak in ethylene evolution of 4050 pmol g⁻¹ DW h⁻¹ at 10 DAC (i.e. the transition period from globular to heart stage) (not included on graph). A corresponding peak in ethylene at 10 DAP could not be localized for seeds due to the difficulty of harvesting the seeds at that stage. However, it is assumed that the same peak in ethylene occurs in ZEs. A peak in ethylene at 10 DAP was localized to developing pods of tagged flowers, while the pod tissue alone produced little to none of the ethylene being evolved at this time (Jayasekera, 1993).

Interestingly, approximately 85% of the ethylene evolved by MDEs at this stage originates in the cotyledon. However, the peak in ethylene evolution that occurs when the initiation of programmed dehydration occurs in seeds, does not occur for MDEs. This is not surprising, given that MDEs were maintained in a liquid medium throughout development, and no attempts were made to modify the medium or mimic embryo/seed desiccation (i.e. MDEs were not treated as per Polsoni et al., 1988 or Brown et al., 1993).

In order to assess the role of ethylene on embryo development further, three classes of ethylene inhibitors were used. Each acts at a unique site in the ethylene biosynthesis/perception pathways. Application of the ethylene biosynthesis inhibitors AVG and CoCl₂ at 1 and 10 μM to MDEs was quite effective in inhibiting ethylene evolution (Fig. 3). These inhibitors have been
widely used, and their mode of action and saturable nature is well documented (Yang and Hoffman, 1984; Lee and Reid, 1997; Abeles et al., 1992). Application of 10 μM STS, an ethylene action inhibitor, resulted in a 2-fold increase in ethylene production. However, this is likely due to a toxic effect of free Ag⁺ ions, that are known to increase ethylene evolution (Aharoni and Lieberman, 1979; Attia-Aly et al., 1987). Thus, for the remainder of the study only 1 μM STS was used. Inhibition of in vivo ethylene production by AVG and CoCl₂ and the inhibition of ethylene action by STS, resulted in MDEs at the mid-cotyledonary stage that exhibited a morphological appearance equivalent to early-cotyledonary stage embryos (i.e. cotyledons unexpanded). Quantification of this observation demonstrated a predictable population effect, with some embryos being unaffected by the treatment. As well, the treatments affected only the transition from the early cotyledonary to the expanded cotyledonary stage, even though inhibitor treatments were begun 8 d prior to the expected peak in ethylene (Fig. 4). The ability to apply the inhibitors prior to embryo axis elongation (at 12 DAC) and influence cotyledon expansion while leaving axis elongation unaffected, demonstrates several key points. First, the inhibitors are acting specifically on ethylene biosynthesis/ action, thereby interfering with ethylene’s ‘normal’ regulation of lateral cell expansion during the transition from the early- to mid-cotyledonary stage. Second, the inhibitors are not causing a general growth inhibition (Fig. 5).

Based on histological comparisons, lateral cell expansion in the cotyledons was significantly diminished by use of the inhibitors (Figs 6, 7). The inhibition in lateral cell expansion occurred as a gradient, with cells in the cotyledon most distal to the shoot apical meristem being most severely affected (Fig. 7A). This inhibition in lateral cotyledon expansion is likely a direct effect of an inhibition in ethylene biosynthesis, as demonstrated by the normal development obtained with 1.0 μM AVG-treated MDEs which were later treated with ACC. These embryos were, in essence, rescued by addition of 10 μM ACC to their medium during the expected peak in ethylene production, and they were statistically indistinguishable from untreated MDEs at the cellular level (Fig. 7A, C). Similarly, MDEs that were treated with 1 μM STS or 10 μM AVG for 7 d followed by washing with fresh culture medium, were statistically indistinguishable from untreated embryos. This ability to reverse the effects of the inhibitors was seen both at the morphological and histological levels (Figs 6, 7).

There is little evidence in the literature that directly implicates a role for ethylene in seed or embryo development. Supporting evidence for the role of ethylene in embryo development may, however, be drawn from other processes in plants where ethylene is known to play an important developmental role. In many species, increases in ethylene evolution are correlated with rapid leaf expansion (Goodwin and Erwee, 1983, and references therein; Lee and Reid, 1997), tracheid differentiation (Zobel and Roberts, 1978), and adventitious root formation (Liu et al., 1990). These reports lend support to conclusions that have been drawn in this study. Collectively, they indicate that ethylene, at low levels, is involved in responses where growth is characterized, at least in part, by cell expansion in a fashion that is lateral with respect to the plant axis. However, there is also evidence from ethylene-deficient mutants that ethylene can inhibit leaf expansion (Blecker et al., 1988; Guzman and Ecker, 1990).

Another example is the AINTEGUMETA (ANT) gene of Arabidopsis which regulates normal ovule and female gametophyte development (Klucher et al., 1996). ANT is similar to a family of genes encoding AP2 domain proteins. The AP2 domain is related to the DNA binding region of ethylene response element binding proteins, proteins that are involved in ethylene signal transduction (Ecker, 1995; Ohme-Takagi and Shinshi, 1995; Weigel, 1995). ANT-1 and ANT-3 mutants of Arabidopsis have ovules that fail to form integuments or normal female gametophytes (Klucher et al., 1996). Interestingly, cells within the outer and inner integuments, and cells within the adjoining funiculus of these mutants display a lack of lateral cell expansion (Klucher et al., 1996) similar to what was observed for cotyledons of the inhibitor-treated MDEs. However, it is important to note that the effect seen in ethylene-deficient mutants and mutants apparently influenced by ethylene, may also be caused by alterations in other phytohormones as a downstream result of the reduced ethylene production. For example, the ABA-deficient mutants of tomato have alterations in other hormone levels (Tal et al., 1979). Thus, it is necessary to be aware of the possible effects that reduced ethylene levels in MDEs may have on the biosynthesis and action of other hormones such as auxins, gibberellins (Pearce et al., 1991), and abscisic acid (Kong and Yeung, 1994). However, the ability to add the inhibitors prior to axis elongation, and only affect cotyledon expansion, argues strongly in favour of a role for ethylene in this cell expansion process (Fig. 6).

A reduction in plantlet conversion from MDEs of B. napus occurred at 15–20 DAC (Yeung et al., 1996), a time that corresponds with the major peak in ethylene (shown in Fig. 2). This is consistent with the promotive effect on embryo conversion that can be obtained by reducing ethylene production (Table 1). In the anatomical sections used in this study intracellular spaces are prominent in control embryos, in embryos treated with AVG plus ACC (Fig. 6A, C) and in embryos treated with ACC alone (data not shown). Intracellular spaces are, however, not common in inhibitor-treated embryos (Fig. 6B), nor are they common in ZEs of B. napus at an equivalent stage (Yeung et al., 1996). However, this ‘intercellular
space’ phenomenon is common in somatic embryos, where ethylene precursors are trafficked in and out of the liquid medium, and where ABA levels are low (Hays et al., 2000).

These results clearly demonstrate that ethylene is involved in normal MDE development. It is likely that ethylene plays a similar role in zygotic embryo development. The use of MDEs, which undergo lateral cell expansion in the cotyledon in response to ethylene, may thus represent a useful model system for studying the mechanisms of ethylene-regulated changes in the cytoskeleton.

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