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Cellular and molecular aspects of ethylene on plant morphogenesis of recalcitrant *Brassica* species *in vitro*[1]

Eng-Chong Pua
Department of Botany, National University of Singapore,
Lower Kent Ridge Road, Singapore 0511, Republic of Singapore

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Introduction

Efficient systems for high frequency plant regeneration from cultured cells and tissues, via either *in vitro* organogenesis or somatic embryogenesis, are essential for genetic manipulation in plants, which is an important tool for the study of the organization and regulation of plant genes (Kuhlemeier *et al.*, 1987; Schell, 1987) and for the production of novel transgenic plants for crop improvement (Dennis and Llewellyn, 1991). Although to date plant regeneration from somatic and germinal cells has been reported for a wide range of species, our knowledge regarding *in vitro* plant morphogenesis has been mostly empirical, and many economically and agronomically important crop species

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and cultivars, particularly monocotyledonous and leguminous plants, remain recalcitrant in culture.

The genus *Brassica* comprises a wide range of crop plants with great economic value worldwide. Plants are utilized as a source of condiments, edible oils, vegetables, animal fodders and green manures. The genus is

color, with four petals forming a cross. In nature, there are six major *Brassica* species, among which the three diploid species, *B. nigra* (black mustard, n=8, BB), *B.*

studies also indicated that ethylene may be involved in regulation of plant morphogenesis of other plant species *in vitro*, including recalcitrant monocotyledons (Table 1) (Biddington, 1992).

Ethylene Biosynthesis and Action

Ethylene (C_2H_4) is a gaseous plant hormone, which is associated with various physiological responses, including seed germination, fruit ripening and senes-

Table 1. *The promotive effect of ethylene inhibitors on growth and morphogenesis of plant cells and tissues in vitro*

Plant species	Tissue/explant used	Ethylene inhibitor[a]	Enhanced morphogenesis/growth	Reference
Monocotyledons				
Triticum aestivum	Callus	$AgNO_3$	Shoots	Purnhauser *et al.*, 1987
Zea mays	Callus	$AgNO_3$, NBD	Plants	Songstad *et al.*, 1988
Zea mays	Immature embryos	$AgNO_3$, AVG	Type II callus	Vain *et al.*, 1989
Zea mays	Immature embryos	$AgNO_3$	Type II callus	Songstad *et al.*, 1991
Dicotyledons				
Arabidopsis thaliana	Roots	$AgNO_3$	Shoots	Marton and Browse, 1991
Brassica spp.	Cotyledons/hypocotyls	$AgNO_3$, AVG	Shoots	Chi *et al.*, 1990
Brassica campestris	Cotyledons	$AgNO_3$	Shoots	Palmer, 1992
Brassica campestris ssp. *chinensis*	Cotyledons	AVG, $AgNO_3$, Ag_2SO_4	Shoots	Chi and Pua, 1989
Brassica campestris ssp. *pekinensis*	Cotyledons	$AgNO_3$, AVG	Shoots	Chi *et al.*, 1991
Brassica juncea	Somatic embryos	$AgNO_3$	Plants	Pua, 1990
Brassica juncea	Leaf discs	$AgNO_3$, AVG	Shoots	Pua and Chi, 1993
Brassica oleracea var. *gemmifera*	Anthers	$AgNO_3$	Androgenesis	Biddingdon *et al.*, 1988
Brassica oleracea var. *italica*	Callus	$AgNO_3$, $CoCl_2$, AVG	Shoots	Sethi *et al.*, 1990
Brassica oleracea and *B. napus*	Hypocotyls	$AgNO_3$	Shoots	De Block *et al.*, 1989
Brassica rapa	Hypocotyls	$AgNO_3$	Shoots	Radke *et al.*, 1992
Cucumis melo	Cotyledons	$AgNO_3$	Shoots	Roustan *et al.*, 1992
Daucus carota	Cell suspension	$CoCl_2$, $NiCl_2$	Somatic embryos	Roustan *et al.*, 1989
Helianthus annuus	Hypocotyl callus	AVG	Plants	Robinson and Adams, 1987
Helianthus annuus	Cotyledons	$AgNO_3$, $CoCl_2$	Shoots	Chraibi *et al.*, 1991
Hevea brasiliensis	Callus	AOA, $AgNO_3$	Somatic embryos	Auboiron *et al.*, 1990
Hevea brasiliensis	Callus	AOA	Somatic embryos	Housti *et al.*, 1992
Lycopersicon esculentum	Cultured shoots	Ag thiosulfate	Protoplast yield and viability	Rethmeier *et al.*, 1991
Nicotiana tabacum	Callus	$AgNO_3$	Shoots	Purnhauser *et al.*, 1987
Solanum tubarosum	Leaves	Ag thiosulfate	Protoplast yield	Perl *et al.*, 1988
Solanum tuberosum	Anthers	$AgNO_3$, n-propylgallate	Androgenesis	Tiainen, 1992

[a]AOA, aminooxyacetic acid; AVG, aminoethoxyvinylglycine; NBD, 2,5-norbornadiene.

S. cerevisiae and 49% to *E. coli*. It has been shown to express differentially in plant organs, among which expression in stems and roots was 10- to 20-fold stronger than that in the leaves, inflorescences and seed pods (Peleman *et al.*, 1989). The expression pattern of the *sam-1* gene has been confirmed by histochemical analysis of transgenic *A. thaliana* plants carrying a chimeric gene consisting of a coding sequence of the *E. coli* β-glucuronidase (GUS) gene under the control of a 748-bp 5' regulatory element of *sam-1*. Furthermore, transgenic plants displayed tissue-specific expression of the GUS gene, which occurred mainly in the vascular tissues, sclerenchyma and root cortex (Peleman *et al.*, 1989). This strong cellular preference of *sam-1* gene expression may be related to lignin biosynthesis, in view of the SAM requirement for synthesis of monolignols which, after polymerization, form the lignin macromolecules (Grisebach, 1981).

ACC Synthase

The conversion of the methionyl side chain of SAM to the three-membered ring amino acid ACC is catalyzed by ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase, E.C.4.4.1.14), which requires pyridoxal phosphate for activity (Yang and Hoffman, 1984). This reaction is a rate limiting step of ethylene biosynthesis. The use of pyridoxal phosphate inhibitors, i.e. AVG or aminooxyacetic acid (AOA), results in a marked decrease in enzyme activity (McKeon and Yang, 1987). ACC synthase is believed to be encoded by a multigene family (Rottmann *et al.*, 1991; Yip *et al.*, 1992), and genes encoding ACC synthase have been cloned and sequenced. The enzyme was purified 5000-fold from *Lycopersicon esculentum* pericarp and its activity was associated with a 45 kD protein (van der Straeten *et al.*, 1989), which was similar to a 46 kD polypeptide for ACC synthase in *Cucurbita pepo* (Sato and Theologis, 1989). However, the same authors later isolated a cDNA clone, pcVV4A, encoding a larger protein (55 kD). They believed that the 45 kD polypeptide was probably derived from the 55 kD protein after C-terminal processing (van der Straeten *et al.*, 1990). A cDNA clone, pACC1, which encodes a protein of 493 amino acids with the molecular mass of 55 kD, has also been isolated from *C. pepo* fruits (Sato *et al.*, 1991). Using a partial sequence of pACC1 as a probe, two genomic clones (*CP-ACC1A* and *CP-ACC1B*) have been identified. *CP-ACC1A* and *CP-ACC1B* showed 97% DNA identity in the coding sequence, of which both were interupted by four highly conserved introns occurring at the identical positions (Huang *et al.*, 1991). Both genes expressed differentially in response to wounding and hormonal treatment. Several genes encoding ACC synthase, isolated from fruits and cell suspension culture (Yip *et al.*, 1992) or plants (Olson *et al.*, 1991) of *L. esculentum*, also showed differential expression in response to developmental, environmental and/or hormonal factors. In *L. esculentum*, at least six ACC synthase genes have been isolated so far (Theologis, 1992). Similar genes have also been cloned from *Vigna radiata* hypocotyls (Botella *et al.*, 1992), *Malus domestica* fruits (Dong *et al.*, 1991), *C. maxima* (Nakajima *et al.*, 1990), *Nicotiana tabacum* leaves (Bailey *et al.*, 1992) and *D. caryophyllus* flowers (Park *et al.*, 1992). A comparison of deduced amino acid sequence showed extensive homology among ACC synthase genes derived from different plant species (Theologis, 1992). We have recently cloned a ACC synthase genes, *MACC*, from *Brassica juncea* leaves (Wen *et al.*, 1993). *MACC* also contained all the conserved domains of ACC synthase genes, indicating the common evolutionary orgin of the genes.

ACC Oxidase

ACC oxidase, which is believed to be a hydroxylase (Fernandez-Maculet and Yang, 1992), catalyzes the last step of ethylene biosynthesis by converting ACC to ethylene (Yang and Hoffman, 1984). This oxidative reaction requires iron as a cofactor (Ververidis and John, 1991; Bouzayen *et al.*, 1991). Unlike SAM synthetase and ACC synthase, ACC oxidase characterization has mostly been conducted *in vivo*, because the enzyme had proved intractable to purification. Results of earlier work indicated that ACC oxidase was associated with vacuoles (Guy and Kende, 1984; Erdmann *et al.*, 1989) and that the enzyme required the membrane integrity for activity (McKeon and Yang, 1987; Kende, 1989). This was believed to explain the rapid loss of activity during enzyme isolation. However, the notion is not supported by the recent finding, in which the protein and activity of ACC oxidase were absent in vacuoles of transgenic *S. cerevisiae* carrying the ACC oxidase gene of *L. esculentum*, although it present in intact cells and protoplasts (Peck *et al.*, 1992). In addition, the intractibility of enzyme purification appears to be a technical rather than a conceptual one. This has been demonstrated by the full recovery of the ACC oxidase activity using Fe^{2+} and ascorbate (Ververidis and John, 1991), and enhanced enzyme activity in the presence of polyvinylpolypyrrolidone or Triton X-100 during enzyme purification (Fernandez-Maculet and Yang, 1992). The improved version of extraction procedure has enabled ACC oxidase to be isolated from *M. domestica* fruits (Dong *et al.*, 1992a). The ACC →ethylene reaction can be inhibited by exogenous application of cobaltous ions at 10-100 μM (Lurssen *et al.*, 1979; Yu *et al.*, 1979) or α-aminoisobutyric acid (AIB), which is a structural analog of ACC (Satoh and Esashi, 1980). Genes responsible for ACC oxidase have been cloned and characterized. A cDNA clone, pTOM13 encoding a protein of 35 kD, was first isolated from ripening fruits of *L. esculentum* (Holdsworth *et al.*, 1987). Accumulation of the corresponding mRNA of pTOM13 has been shown to be correlated with ethylene synthesis during

fruit ripening and wounding response of leaves (Slater *et al.*, 1985; Smith *et al.*, 1986; Holdsworth *et al.*, 1987). The function of the pTOM13 has been studied using the transgenic plant system. The transgenic plants expressing antisense pTOM13 RNA displayed a substantial reduction of ethylene synthesis, and endogenous ACC oxidase activity of heterozygous and homozygous [illegible]

tively (Hamilton *et al.*, 1990). This suggested that pTOM13 might encode at least part of ACC oxidase. More direct evidence showing pTOM13 responsible for ACC oxidase is derived from the study of gene expression in a heterologous system. pTOM13 fused with a 5′ fragment of a genomic clone, pETH1 (Kock *et al.*, 1991), was expressed in transformed *S. cerevisiae* (Hamilton *et al.*, 1991). The ACC oxidase activity in transformed *S. cerevisiae* exhibited similar properties to that found in plant tissues, i.e. conversion of the trans isomer of the ACC analog 1-amino-2-ethylcyclopropane-1-carboxylic acid to 1-butene in preference to

(Fig. 1). The reaction results in a decrease of the SAM pool, and thereby reducing ACC synthesis and ethylene production (Langhoff *et al.*, 1992). This has been demonstrated in transgenic plants of *N. tabacum* and *L. esculentum* carrying the SAM hydrolase gene originating from bacteriophage T3. The SAM hydrolase gene under the control of the cauliflower [illegible]

and constitutive expression and transgenic *N. tabacum* plant, but they were found to be dwarfed. However, transgenic *L. esculentum* plants carrying the chimeric gene under the control of E8 promoter, which only permits its expression in the ripening fruits of *L. esculentum* (Lincoln *et al.*, 1987), were phenotypically normal (Langhoff *et al.*, 1992). Although a decrease of the SAM pool results in reduced ethylene production, it may also affect polyamine metabolism, because SAM is a common precursor of both ethylene and polyamine biosynthesis (Evans and Malmberg, 1989).

ACC deaminase originating from *Pseudomonas*

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a MEKNIKFPVVDLSKL---IGEERDATMALINDACENWGFFEIVNHGLPHD  47
b ME-N--FPIINLEKL---NGDERANTMENIKDACENWGFFELVNHGIPHE  43
c M---ATFPVVDLSLV---NGEERAATLEKINDACENWGFFELVNHGMSTE  44
d M---DSFPVINMEKL---EGQERAATMKLINDACENWGFFELVNHSIPVE  44
e MANIVNFPIIDMEKLNNYNGVERSLVIDQIKDACHNWGFFQVVNHSLSHE  50

a LMDNVEKMTKEHYKISMEQKFNDMLKSKGLENLEREVEDVDWESTFYLRH  97
b VMDTVEKMTKGHYKKCMEQKFKELVASKGLEAVQAEVTDLDWESTFFLRH  93
c LLDTVEKMTKDHYKKTMEQRFKEMVAAKGLDDVQSEIHDLDWESTFFLRH  94
e LMDKVERMTKEHYKKFREQKFKDMVQTKGLVSAESQVNDIDWESTFYLRH 100

a LPQSNLYDTPDMSDEYRTAMKDFGKRLENLAEDLLDLLCENLGLEKGYLK 147
b LPTSNISQVPDMSDEYREVMRDFAKRLEKLAEELLDLLCENLGLEKGYLK 143
c LPSSNISEIPDLEEEYRKTMKEFAVELEKLAEKLLDLLCENLGLEKGYLK 144
d LPVSNLSEIPDLTDEHRKTMKEFAEKLEKLAEQVLDLLCENLGLEKGYLK 143
e RPDSNISEVPDLDDQYRKLMKEFAAQIERLSEQLLDLLCENLGLEKAYLK 150

a KVFHGTK-G-PTFGTKVSNYPACPKPEMIKGLRAHTDAGGIILLFQDDKV 195
b NAFYGSK-G-PNFGTKVSNYPPCPKPDLIKGLRAHTDAGGIILLFQDDKV 191
c KVFYGSK-G-PNFGTKVSNYPPCPKPDLIKGLRAHSDAGGIILLFQDDKV 192
d MAFAGTTTGLPTFGTKVSNYPPCPRPELFKGLRAHTDAGGLILLFQDDRV 193
e NAFYGAN-G-PTFGTKVSNYPPCPKPDLIKGLRAHTDAGGIILLFQDDKV 198
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of spermidine and spermine synthesis in both prokaryotes and eukaryotes (Fig. 1). Spermidine, spermine and other polyamines (PAs), e.g. putrescine, play an important role in cell growth and division (Evans and Malmberg, 1989; Heby and Persson, 1990), but the mechanism is not clear. It is believed that PA biosynthesis in plants may result in lowering the SAM pool leading to the inhibition of ethylene production.

Ethylene Action

The various genes encoding SAM synthetase, ACC synthase and ACC oxidase are characterized by the absence of a transit peptide at the N-terminal of the protein. It suggests that these enzymes may be located in the cytosol rather than organelles, i.e. chloroplasts and mitochondria. Evidence indicates that ethylene-in-

ene in the culture container. In rapid-cycling *B. campestris*, ethylene accumulated in the sealed culture container and caused hypocotyl swelling and inhibition of leaf expansion and flowering (Lentini *et al.*, 1988). The abnormal growth and development of the plant reverted to normal after an exogenous application of 250-500 ppm NBD. However, the role of ethylene in cultured plant cells and tissues remains unclear.

Earlier attempts in our laboratory to induce shoot morphogenesis from cultured explants of recalcitrant *Brassica*, via manipulation of the hormonal combinations in the culture medium and selection of the explants and genotypes, were not successful. We found that explants of *B. campestris* ssp. *pekinensis* and *B. juncea* usually gave rise to callus, which concomitantly produced high levels of ethylene during early stages of

to be effective in promoting shoot regeneration from cultured explants of at least 10 recalcitrant genotypes in the Cruciferae (Table 2) (Chi *et al.*, 1990; Pua *et al.*, 1990). $AgNO_3$ also enhanced plant regeneration from protoplast-derived somatic embryos of *B. juncea* (Pua, 1990) and androgenesis of *B. oleracea* var. *gemmifera* (Biddington *et al.*, 1988). In *B. rapa*, the use of 5-10 mg l^{-1} $AgNO_3$ enhanced formation of shoot primordia from hypocotyls by 4-fold (Radke *et al.*, 1992). In addition, the promotive effect of Ag^+ has been demonstrated in *A. thaliana*, which showed 10-fold increase in number of shoots regenerated from root explants in the presence of 25 mg l^{-1} $AgNO_3$, as a result, facilitated *A. thaliana* transformation (Marton and Browse, 1991). The similar results have also been reported by the use of 5 mg l^{-1} silver thiosulfate (Clarke *et al.*, 1992).

Ethylene production by cultured plant tissues is affected by the mode of action of the ethylene inhibitor. This was demonstrated by AVG and $AgNO_3$, which exerted two diverging effects on ethylene synthesis, although both were equally effective in promoting shoot regeneration from cultured explants of *B. campestris* ssp. *pekinensis* (Chi *et al.*, 1991) and *B. juncea* (Pua and Chi, 1993). AVG decreased ethylene pro-

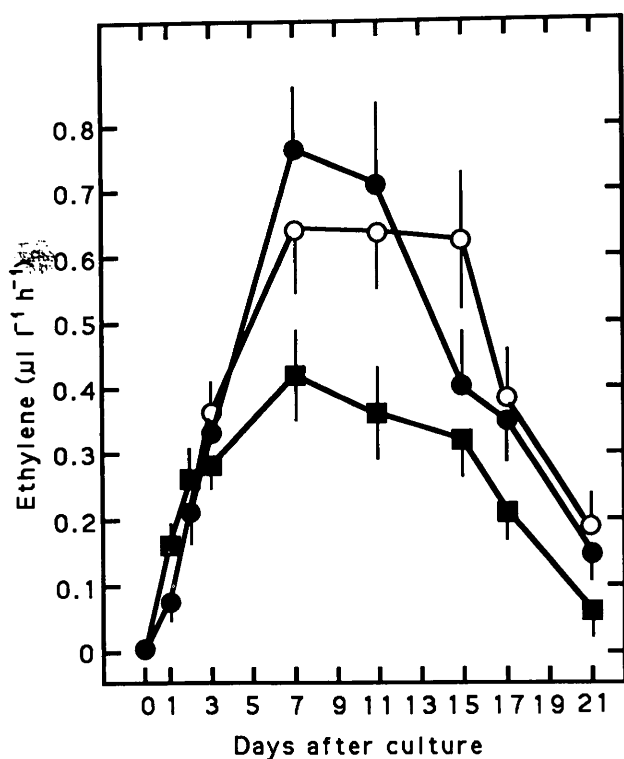

Fig. 3. Ethylene produced by cultured explants of *Brassica* during three weeks of culture. Cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Wong Bok (●) and cv. Shantung (■) and leaf discs of *B. juncea* var. India Mustard (○) were grown in a 50 ml flask containing 25 ml MS medium supplemented with 17.6 μM BA and 5.4 μM NAA. Each flask containing five explants was sealed with a serum rubber stopper. A 1 ml gas sample was withdrawn with a hypodermic syringe at different intervals from each flask for ethylene measurement using gas chromatography (Chi *et al.*, 1991). Vertical bars represent S.E.s of six measurements.

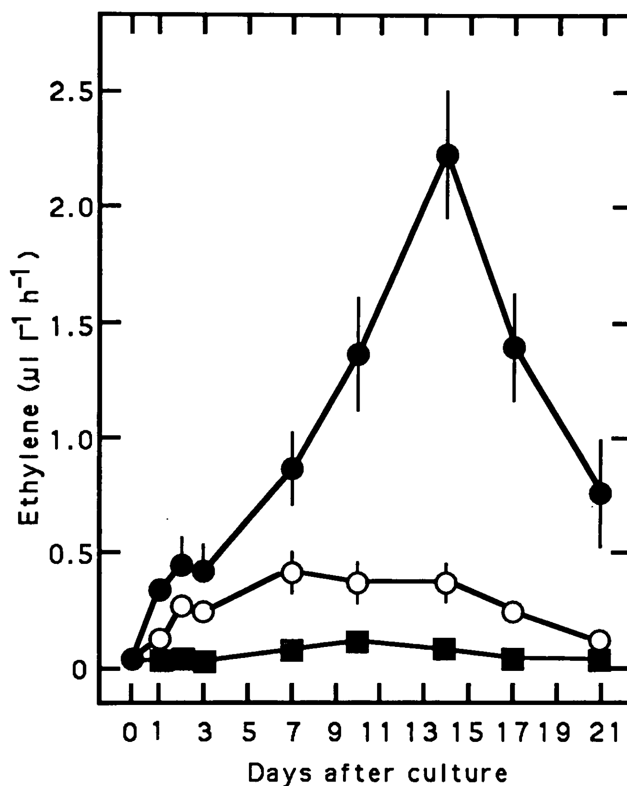

Fig. 4. Effect of ethylene inhibitor on ethylene production by cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung after three weeks in culture. Explants were grown on MS medium supplemented with 8.8 μM BA and 5.4 μM NAA in the absence (○) or presence of 30 μM $AgNO_3$ (●) or 5 μM AVG (■). Ethylene measurement was conducted as described in Fig. 3. Vertical bars represent S.E.s of 5-6 measurements. Redrawn from Chi *et al.*, 1991.

duction (Fig. 4), endogenous ACC synthase activity and ACC level of the explants (Table 3). The results are in agreement with previous findings (see McKeon and Yang, 1987). To the contrary, all parameters of ethylene synthesis in cultured explants were enhanced substantially in the presence of $AgNO_3$ (Chi *et al.*, 1991; Pua and Chi, 1993). $AgNO_3$-stimulated ethylene production has also been reported in *L. esculentum* fruits (Penarrubia *et al.*, 1992) and in ethylene-overproducing *A. thaliana* mutants (Guzman and Ecker, 1990). The mechanism of ethylene overproduction in response to $AgNO_3$ is not clear. It is speculated that $AgNO_3$-stimulated ethylene production may be a consequence of stress-induced response, because Ag^+ is a heavy

Table 2. *Effect of ethylene inhibitors on shoot regeneration from various explants of members in Cruciferae*

Plant species and cultivar	Explant[a]	Medium[b]		
		No inhibitor	$AgNO_3$	AVG
		Shoot regeneration (%)		
B. campestris ssp. *chinensis*				
cv. Gracious	Cotyledon	26	53	–
cv. Ching Chang	Cotyledon	20	61	–
B. campestris ssp. *pekinensis*				
cv. Chefoo cabbage	Cotyledon	25	80	94
cv. Shantung	Cotyledon	50	91	85
	Hypocotyl	12	70	85
cv. White Sun	Cotyledon	17	60	60
cv. Wong Bok	Cotyledon	17	73	87
	Hypocotyl	20	78	78
B. campestris ssp. *parachinensis*	Cotyledon	13	68	–
B. juncea				
var. Leaf Heading	Hypocotyl	5	70	55
var. India Mustard	Hypocotyl	21	100	–
	Leaf disc	10	75	85
Raphanus sativus var. *longipinnatus*	Hypocotyl	0	40	40

[a]0.8% Difco-Bacto agar was used as a gelling agent in the medium for cotyledonary explants and 0.4% agarose was used for hypocotyl explants.
[b]Explants were grown on MS medium containing 8.8 μM BA and 5.4 μM NAA in the absence (no inhibitor) or presence of 10-30 μM $AgNO_3$ or 5-20 μM AVG. Explants were evaluated for shoot regeneration after four weeks in culture.

Table 3. *Effect of ethylene inhibitor on endogenous levels of ACC synthase activity and ACC in cotyledonary explants of* ***B. campestris*** *ssp.* ***pekinensis*** *cv. Shantung in vitro (Chi et al., 1991)*

Medium[a]	Days after culture				
	0	3	7	10	14
	ACC synthase, units (mg protein)$^{-1}$				
No inhibitor	1.7±0.2	9.8±0.8	3.9±0.6	1.5±0.1	1.5±0.3
$AgNO_3$		223±16	226±16	191±10	23±5
AVG		2.1±0.7	1.3±0.3	1.2±0.4	1.2±0.3
	ACC, nmol (g tissue)$^{-1}$				
No inhibitor	0.5±0.1	7.5±0.8	2.3±0.5	1.1±0.3	0.4±0.1
$AgNO_3$		115±5	181±13	111±10	25±9
AVG		0.3±0	0.9±0.1	0.2±0	0.2±0

[a]Explants were grown on MS medium, solidified with 0.8% Difco-Bacto agar, containing 8.8 μM BA and 5.4 μM NAA in the absence (no inhibitor) or presence of 30 μM $AgNO_3$ or 5 μM AVG.

metal and its presence may be stressful to plant cells and tissues *in vitro* (Chi *et al.*, 1991). It has also been explained, according to Theologis (1992), by receptor interference by Ag^+, which resulted in low ethylene reception, thereby triggering cells to overproduce ethylene. $AgNO_3$ also enhanced ethylene production in *B. juncea* plants grown *in vitro*, but at a slower rate and lower level, as compared with cultured explants (Pua and Chi, 1993). However, $AgNO_3$ was inhibitory to growth of the plants which were severaly stunted. These results suggest a differential Ag^+ tolerance between cultured tissues and whole plants, which may be attributed to the difference in metabolic activity between the processes of cell differentiation and growth and development. Interestingly, tissues derived from the stunted plants were capable of shoot regeneration at high frequencies on medium without any ethylene inhibitor. This enhanced regeneration response is believed to be conferred by a carried [illegible]

[illegible]

Ag^+ in the tissues (Pua and Chi, 1993). In addition to *Brassica* species, the promotive effect of ethylene inhibitors has been demonstrated in a number of dicotyledonous and monocotyledonous species, with respect to protoplast yield, embryogenesis from somatic and germinal cells and shoot organogenesis from cultured cells and tissues (Table 1) [illegible]

tant for embryogenesis from anther cultures of *Hordeum vulgare* (Cho and Kasha, 1989) and flower bud formation from thin cell layer explants of *N. tabacum* (Smulders *et al.*, 1990). In *Medicago sativa*, AVG, AOA, dinitrophenol and salicylic acid have been shown to be inhibitory to somatic embryo formation from petiole explants (Meijer and Brown, 1988). This discrepancy may be due in part to the variation of the genotype, physiological stage of donor tissues, culture type, ethylene inhibitor concentration, and/or culture condition employed.

The evidence of the regulatory role of ethylene on *in vitro* shoot regeneration is partly derived from exogenous application of ACC or 2-chloroethylphosphonic acid (CEPA), which is an ethylene producing compound. We found that the promotive effect of AVG could be overcome by the use of 10-100 μM ACC or 50 μM CEPA, whereas regeneration in the presence of [illegible]

[illegible]

(Chi *et al.*, 1991; Pua and Chi, 1993). The differential effect of AVG and $AgNO_3$ on regeneration in response to ACC and CEPA may be explained by the different modes of action of ethylene inhibitors. As discussed earlier, AVG inhibits only endogenous ethylene production by inhibiting ACC synthase and therefore tissues [illegible]

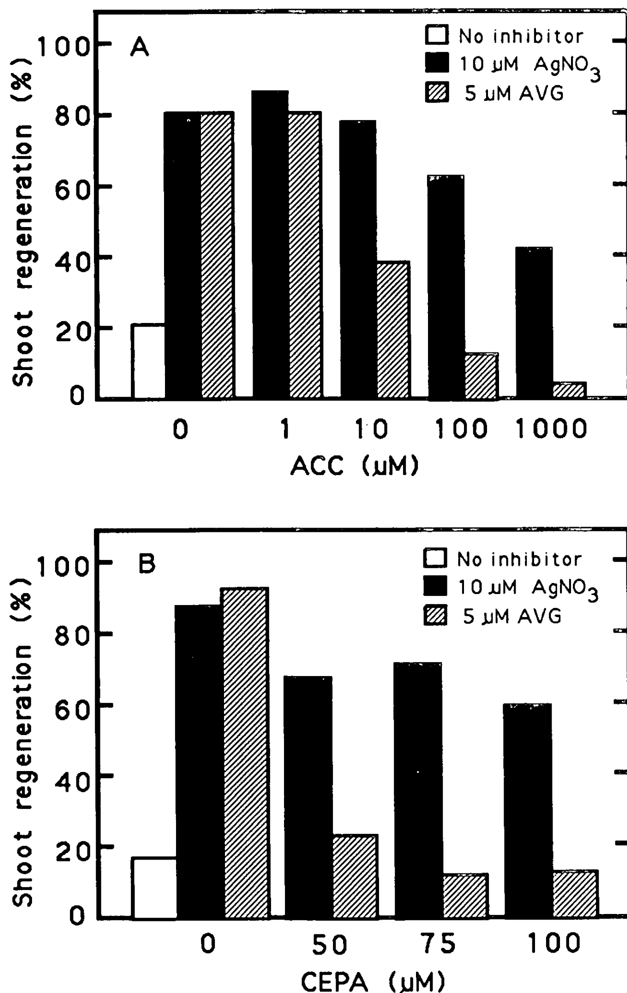

Fig. 5. Effect of exogenous supplied ACC (A) and CEPA (B) on shoot regeneration from cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Wong Bok after four weeks in culture. Explants were grown on MS medium supplemented with 8.8 μM BA and 5.4 μM NAA in the absence or presence of ethylene inhibitor. (B) was redrawn from Chi *et al.*, 1991.

AVG or $AgNO_3$ (Pua and Chi, 1993). It indicates that factors other than ethylene may be involved in cell recalcitrancy of *R. sativus* var. *longipinnatus*.

Culture Medium

The event of organogenesis, e.g. shoot and root formation, in plant cells and tissues *in vitro* is markedly affected by the presence of hormones in the medium. This was clearly demonstrated by Skoog and Miller in the 1950s. While the requirements for the type and concentration of hormones for shoot regeneration vary with plant species, we found that 9-20 μM cytokinin, e. g. benzyladenine (BA), kinentin, 6-γ-γ-dimethylallylamino purine or zeatin, in combinaton with 2.7-5.4 μM naphthaleneacetic acid (NAA) in the presence of ethylene inhibitor were effective for shoot regeneration from cotyledons of *B. campestris*, but hypocotyls were not responsive (Chi *et al.*, 1990). The recalcitrancy of hypocotyls was later discovered to be due to the use of Difco-Bacto agar, a gelling agent, in the medium. The substitution of Difco-Bacto agar with 0.4% agarose (Ultrapure, electrophoresis grade, BRL) greatly enhanced hypocotyl regenerabilty (Barfield and Pua, 1991). We speculated that the promotive effect of agarose might be attributed to the lack of growth inhibitors, because agarose is a purified form of agar. Agar is a rich source of impurities, especially Na^+ and Cu^{2+} (Debergh, 1983), which may be inhibitory to regeneration.

PAs

Ethylene and PA biosynthesis are closely related, because both utilized the same precursor SAM (Fig. 1). It is therefore believed that ethylene and PAs may regulate each other's synthesis (Evans and Malmberg, 1989). Although several studies, using PAs or its synthesis inhibitors, supported the above hypothesis (Apelbaum *et al.*, 1981; Suttle, 1981; Roberts *et al.*, 1983), results of other studies did not show the correlation between ethylene and PA synthesis (Downs and Lovell, 1986; Wang and Kramer, 1990; Chi *et al.*, 1993). The discrepancy indicates that the relationship between ethylene and PAs may be more complex than the currently prevailing precursor competition hypothesis. Nevertheless, PAs have been shown to be involved in somatic embryogenesis of *Daucus carota* (Montague *et al.*, 1979; Feirer *et al.*, 1984; Fienberg *et al.*, 1984) and *M. sativa* (Meijer and Simmonds, 1988) and formation of floral buds from thin-layer explants of *N. tabacum* (Torrigiani *et al.*, 1987). We have also shown that exogenous application of 1-20 mM putrescine, 0.1-2.5 mM spermidine or 0.1-0.5 mM spermine markedly enhanced *de novo* shoot regeneration of *B. campestris* ssp. *pekinensis in vitro*, but it did not affect ethylene production (Chi *et al.*, 1993). It is not clear how differentiation of cultured plant cells and tissues is triggered by PAs. Further studies are required to elucidate the regulatory mechanism of PAs and its relationship with ethylene to gain a better understanding of plant mor-

phogenesis *in vitro*.

Evidence from the study of ethylene inhibitors clearly indicates that the capacity of plant morphogenesis *in vitro* is closely associated with ethylene produced by cultured cells and tissues. However, the definitive role of ethylene on plant morphogenesis is not conclusive, since ethylene inhibitors possess some inherent problems, e.g. slow mobility, non-specificity and carcinogenesis (Roberts *et al.*, 1987). The use of mutant plants will be a useful tool in providing more direct evidence for the impact of impaired ethylene biosynthesis or sensitivity on *in vitro* morphogenesis and the subsequent plant growth and development, and also facilitate the study of ethylene action and signal transduction at the molecular level, e.g. *etr* and *etr1* mutants of *A. thaliana* (Bleecker *et al.*, 1988; Kieber *et al.*, 1993).

Mutant Production Using Antisense RNA

Gene isolation and cloning allow construction of plant mutants using reversed genetics. In view of the lack of gene replacement technology, the antisense RNA technology has been proven, for the last few years, to be an important tool for production of novel plant mutants, which have been used for the study of expression and function of the target genes in plants (Bird *et al.*, 1991; Schuch, 1991; Gray *et al.*, 1992). Expression of antisense RNA usually leads to the downregulation of gene expression. This has been demonstrated in the study of the molecular biology of fruit ripening in *L. esculentum*. Transgenic plants expressing antisense RNA of the genes encoding ACC synthase (Oeller *et al.*, 1991) and ACC oxidase (Hamilton *et al.*, 1990) showed inhibition of the respective mRNA and a great reduction of ethylene production. Fruits originating from these antisense plants displayed a delay of the fruit ripening process and an increase of shelf life.

Gene Cloning

Cloning of the target genes and development of an efficient plant transformation system are important for production of plant mutants using antisense RNA. To construct the antisense gene that inhibits ethylene production, we have cloned a ACC oxidase gene, pMEFE5, from *B. juncea* (Pua *et al.*, 1992b). The gene was inserted in an antisense orientation between CaMV 35S promoter and the coding sequence of the GUS gene in a binary vector pBI121 (Jefferson, 1987). The fusion of the antisense ACC oxidase gene and GUS facilitates early and rapid detection of transformants using a histochemical assay for the GUS activity. The chimeric gene, using the three-way mating (Rogers *et al.*, 1986), was transferred to *Agrobacterium tumefaciens* LBA4404, which was subsequently used for plant transformation.

Plant Transformation

The use of an appropriate gene transfer technique is crucial for the success of plant transformation. To date, *A. tumefaciens*-mediated transformation (Pua *et al.*, 1987) has been most widely used, because the transformation procedure is straightforward and transgenic plants can be obtained in 3-4 months. However, successful plant transformation depends largely on the compatible relationship between the host plants and *A. tumefaciens*, and an efficient tissue culture system in which shoots or plants can be regenerated from cultured cells and tissues at high freguencies. In *Brassica*, various species have been known to be highly susceptible to *A. tumefaciens* (Charest *et al.*, 1989) and transgenic plants have also been obtained in *B. napus* (Pua *et al.*, 1987; Fry *et al.*, 1987), *B. oleracea* (David and Tempe, 1988; Srivastava *et al.*, 1988; Eimert and Siegemund, 1992), *B. rapa* (Radke *et al.*, 1992), *B. carinata* (Narasimhulu *et al.*, 1992) and *B. juncea* (Mathews *et al.*, 1990). In our laboratory, an efficient transformation system for *B. juncea*, in which transgenic plants can be routinely produced at a 9-10% frequency, was developed via optimization of the tissue culture system, pre-culture treatment of explants and the use of nopaline strain *A. tumefaciens* carrying the disarmed Ti plasmid (Barfield and Pua, 1991). Transgenic shoots were regenerated from inoculated hypocotyl explants grown on Murashige and Skoog's (MS) medium (1962), solidified with 0.4% agarose, containing 10-30 μM $AgNO_3$ and 2-8 μM BA in combination with 0.05-0.25 μM 2,4-D or 0.5-5 μM NAA. Using this transformation procedure, we have introduced the antisense ACC oxidase gene into *B. juncea* plants (Pua *et al.*, 1992a).

Characterization of Transgenic Plants

The presence of antisense transcript in transgenic plants is usually verified by northern analysis using a strand-specific RNA probe (riboprobe). The transgenic plants expressing the antisense transcript are further

analysed for the function of the endogenous gene at transcriptional, translational and/or post-translational levels. Several lines of study have shown that antisense transcript in transgenic plants is not always detectable, for instance, *L. esculentum* plants carrying antisense ACC oxidase gene (Hamilton *et al.*, 1990) or pTOM5

teristics of enhanced regeneration and impaired ethylene biosynthesis in transgenic plants were also transmitted to the R1 progeny (unpublished results). This clearly shows that ethylene produced by cultured explants is responsible for cell recalcitrancy *in vitro*, and

vitro organ formation can be controlled by the ratio of exogenous application of auxin and cytokinin (Skoog and Miller, 1957) or by mutation of T-DNA genes of *A. tumefaciens* (Inze *et al.*, 1984; Schroder *et al.*, 1984; Akiyoshi *et al.*, 1984). In addition, there is a close relationship between ethylene and polyamine biosynthesis in that both compete for the same precursor, SAM (Evans and Malmberg, 1989). This, together with the promotive effect of polyamines on somatic embryogenesis of *D. carota* (Feirer *et al.*, 1984), indicates that *in vitro* plant morphogenesis may also be related to polyamine metabolism.

The capacity for plant morphogenesis *in vitro* may be genetically controlled (Halperin, 1986; Narasimhulu and Chopra, 1988). This notion is supported by our study using antisense RNA technology and a recent report, using morphological and restriction fragment length polymorphism markers, that a gene controlling shoot regeneration from cultured root explants of *L. esculentum* has been mapped and characterized (Koornneef *et al.*, 1993). Although the DNA sequence of the gene responsible for shoot morphogenesis has not been isolated and the gene product is also not known, the finding of our study suggests that the genetic control of *in vitro* plant morphogenesis may be related to genes responsible for regulation of ethylene biosynthesis.

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