ROLE OF CYTOKININ AND ETHYLENE DURING SENESCENCE IN BROCCOLI (*BRASSICA OLERACEA* VAR. *ITALICA*).

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

at

Massey University

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2003

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TO WHOM IT MAY CONCERN

This is to state that the research carried out for my PhD thesis entitled "Role of cytokinin and ethylene during senescence in broccoli (*Brassica oleracea* var *italica*)" in the Institute of Molecular BioSciences at Massey University, Palmerston North, New Zealand is all my own work. This is also to certify that thesis material has not been used for any other degree.

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TO WHOM IT MAY CONCERN

This is to state that the research carried out for the PhD thesis entitled "Role of cytokinin and ethylene during senescence in broccoli (*Brassica oleracea* var *italica*)" was done by Nigel Gapper in the Institute of Molecular BioSciences at Massey University, Palmerston North, New Zealand. This thesis material has not been used for any other degree.

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CERTIFICATE OF REGULATORY COMPLIANCE

This is to certify that the research carried out in the Doctoral Thesis entitled "Role of cytokinin and ethylene during senescence in broccoli (*Brassica oleracea* var *italica*)" in the Institute of Molecular BioSciences at Massey University, New Zealand:

(a) is the original work of the candidate, except as indicated by appropriate attribution in the text and/or in the acknowledgements;

(b) that the text, excluding appendices/annexes, does not exceed 100,000 words;

(c) all the ethical requirements applicable to this study have been complied with as required by Massey University, other organizations and/or committees which had a particular association with this study, and relevant legislation.

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Candidate's Name: Nigel Gapper

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Date: 14 April 2003

Supervisor's Name: Professor Paula Jameson

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Date: 14 April 2003

Dedication

This thesis is dedicated

to my parents

Brian and Linda

Abstract

Broccoli (*Brassica oleracea* var. *italica*) deteriorates rapidly following harvest. The two plant hormones ethylene and cytokinin are known to act antagonistically on harvest-induced senescence in broccoli: ethylene acts by accelerating the process, whereas additional cytokinin delays it. The overall aim of this thesis was to gain a better understanding of how these two hormones control postharvest senescence. The effects of exogenous cytokinin (6-benzyl aminopurine, 6-BAP), 1-aminocyclopropane-1-carboxylic acid (ACC) and sucrose on senescence-associated gene expression were measured in both wild-type plants and transgenic plants harbouring an antisense tomato ACC oxidase gene (*pTOM13*). Exogenous cytokinin caused both a reduction (*BoACO*) and an increase (*BoACS*) in ethylene biosynthetic gene expression as well as reduced expression of genes encoding sucrose transporters and carbohydrate metabolising enzymes, indicating a significant role for cytokinin in the delay of senescence.

Transgenic broccoli was produced using *Agrobacterium tumefaciens*-mediated transformation. Ethylene biosynthesis was targeted *via* an antisense *BoACO2* gene fused to the harvest-induced asparagine synthetase (AS) promoter from asparagus. In addition, broccoli was transformed with constructs harbouring the *Agrobacterium tumefaciens* isopentenyl transferase (*ipt*) gene using the senescence-associated SAG_{12} and floral-associated MYB_{305} gene promoters to enhance the levels of cytokinin either during senescence or in floral tissue, respectively.

The presence of the antisense AS-ACO construct was associated with an increased rate of transformation when compared to control constructs. Physiological analyses of mature plants showed that the antisense AS-ACO gene construct caused delayed senescence in both detached leaves and detached heads. Gene expression analyses of harvested floret tissue from AS-ACO lines showed decreases in transcript levels of senescence marker genes compared to wild-type and transgenic control lines, as well as a reduction in

expression of sucrose transporter and carbohydrate metabolising genes, confirming the key role of ethylene in the promotion of senescence.

In addition, genes involved with cytokinin biosynthesis and metabolism were isolated by PCR using primers based on *Arabidopsis* clones. The four broccoli *ipt* sequences aligned closely to four of the *Arabidopsis* sequences and were subsequently named *BoIPT4*, *BoIPT5*, *BoIPT6* and *BoIPT7*. A cytokinin oxidase clone (*BoCKX*) was also isolated from broccoli. The four *BoIPT* genes were expressed in a number of different tissues, suggesting that the different genes may be involved in different biological processes in the plant. *BoIPT4* was expressed early and *BoCKX* expressed late in florets during senescence.

A model depicting the regulation of senescence in broccoli through the expression of cytokinin biosynthesis and metabolism genes, and their interaction with ethylene and carbohydrate metabolism is presented and discussed.

Acknowledgements

There are many people that have been involved with my PhD project, both directly and indirectly, I wish to thank you all for your help and support over the past four years of my life. First and foremost I would like to express my many thanks to my supervisors, Professor Paula Jameson, Dr Ross Lill, Dr Marian McKenzie and Dr Mary Christey. Paula, thank you for believing in my ability over the past seven years. You took me on when others might have thought twice, these hands that are large, are very capable after all. You nurtured, moulded and made me the scientist I am today, I cannot thank you enough. Ross, my gratitude for giving me the opportunity to work within the infrastructure at Crop & Food Research. You have taught me to think outside of the square, I have very much enjoyed discussions we have had. Marian, your scientific input into the project has been very prominent from the outset. Thank you for your role as a mentor on a day-to-day basis, your door was always open and I appreciated this very much. Mary, thank you for sharing your tissue culture expertise. Without your skills, the project would never have taken shape the way it has.

I must pay a very special acknowledgement to Dr Simon Coupe. Your scientific input throughout the duration of my PhD was nothing less than outstanding. You taught me many of the skills that I have used throughout my PhD for which I am very grateful. You call me the 'PCR God', well in turn I must call you the 'Molecular Biology God'. Thank you Simon, working with you was always a pleasure, and I wish you all the best with your career.

My gratitude for the help I received from Drs Michael McManus and Richard Scott for the ACC oxidase biochemistry. I must also thank the people who have provided me with technical assistance. Robert Braun, Gill Reader and Astrid Erasmuson from the Lincoln C&FR. Ian King, Steve Arathoon, Andrew Mullan, Beverley Hoffmann, Jan Manson and Ranjith Pathirana from Palmerston North C&FR. Robert Lamberts for photography and

Anthony Corbett for photography and excellent poster preparation. For advice on statistical analyses, I must pay a special thank you to Dr John Koolard.

Drs Erin O'Donohue, Jocelyn Eason, Chris Winefield, Donald Hunter, Kevin Davies, Karen Neilsen, Huaibi Zhang, Julian Heyes, Kathy Schwinn, Simon Deroles, Mr Brent Page, Mr Murray Boase and Mr Ed Morgan thank you for useful discussions we have had either in the lab, at the card table or while walking the corridors. Theresa Lill, Tatyana Pinkney, Lyn Watson, Sheryl Somerfield, Dacey Ryan, Yongjin Shang, Michael Bennett, David Horgan, John Javellana, Glen Shingleton, Jason Johnston, Gayle Marshall, Toni Waugh, Lisa Harvey, Lei Wang and Vern Collette, thank you all for making the Lab a very happy place to work in. Thank you also to Dr John Clemens and members of Paula and John's Research group at Massey, for friendship and scientific discussion.

I would like to make a special thank you to Malcolm McKinnon, Managing Director of Audio Progress Ltd, Craig Black and Hayden Sinclair from Creative Sounds, Jordan Reyne, Rob McDermott, Anna McKenzie (Fran), Regan Hawea and the many bands and artists I have worked with over the past four years. Also, the C&FR indoor cricket team, "The Chosen Ones", and the C&FR "Hackers and Hunters Elite Golf Group". Thank you all for your friendship and the chance to do something other than science. You have all given me opportunities I have relished that have stopped me going insane. Big ups to you all!!!!!!

No acknowledgement would be complete without thanking the funding bodies. Thank you to the Foundation for Research Science and Technology for supplying the funding for this project through C&FR. In addition, The Royal Society of New Zealand, The New Zealand Society for Horticultural Science, and The Institute of Molecular BioSciences, Massey University, provided funds for international conference travel.

Finally I would like to thank my family, my mum and dad, Linda and Brian, my nana Winefred, and my brother Leyton. Your support and encouragement during my PhD has been invaluable.

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Abbreviations

A_{260}	absorbance at 260 nm
A	adenine
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
ADP	adenosine diphosphate
AHK	*Arabidopsis* histidine kinase
AHP	*Arabidopsis* histidine phosphotransfer
AMP	adenosine monophosphate
ARR	*Arabidopsis* response regulator
AS	Asparagine synthetase
AS-ACO	AS-promoter, antisense BoACO2 gene construct
AtCKX	*Arabidopsis thaliana* cytokinin oxidase
AtIPT	*Arabidopsis thaliana* isopentenyl transferase
ATP	adenosine triphosphate
6-BAP	6-benzylamino purine
BCIP	5-bromo-4-chloro-3-indoyl phosphate
Bo18S	*Brassica oleracea* 18S ribosomal subunit
BoACO	*Brassica oleracea* 1-aminocyclopropane-1-carboxylic acid oxidase
BoACS	*Brassica oleracea* 1-aminocyclopropane-1-carboxylic acid synthase
BoCAB	*Brassica oleracea* chlorophyll a/b binding protein
BoCKX	*Brassica oleracea* cytokinin oxidase
BoCP	*Brassica oleracea* cysteine protease
BoHK	*Brassica oleracea* hexokinase
BoIPT	*Brassica oleracea* isopentenyl transferase
BoINV	*Brassica oleracea* acid invertase
BoMLP	*Brassica oleracea* metallothionein-like protein
BoPI	*Brassica oleracea* protease inhibitor

BoSUC	*Brassica oleracea* sucrose transporter
bp	base-pairs
BSA	bovine serum albumin
°C	degrees Celsius
C	cytosine
CA	controlled atmosphere
CaMV 35S	cauliflower mosaic virus 35S promoter
cDNA	cDNA clone
CKI	cytokinin insensitive
cm	centimetre
CPPU	*N*-2-2chloro-4 pyridyl-*N'*-phenylurea
CRE	cytokinin receptor
Da	Daltons
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMAPP	dimethylallylpyrophophate
DMP	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DPU	diphenyl urea
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetracetic acid
ETR	ethylene receptor
EREBP	ethylene response element biding protein
ERF	ethylene response factor
FPLC	fast performance liquid chromatography

g	gram
G	guanine
GC	gas chromatography
GCR	G-protein coupled receptor
GFP	green fluorescent protein
GMO	genetically modified organism
GTP	guanosine triphosphate
GUS	ß-glucuronidase
h	hour
Hyg	hygromycin
hpt	hygomycin phosphotransferase gene
IAA	indole acetic acid
iP	isopentenyladenine
iPA	isopentenyladenosine
iPDP	isopentenyladenosine-5-diphosphate
iPMP	isopentenyladenosine-5-monophosphate
ipt	isopentenyl transferase gene
IPT	isopentenyl transferase protein
IPTG	isopropyl-β-*D*-thiogalactopyranoside
IPTP	isopentenyladenosine-5-triphosphate
k	kilo
Kan	kanamycin
kb	kilo base-pairs
kDa	kilo Daltons
KV	kilo volts
L	litre
LB	Luria-Bertani (media or broth)
LSC	leaf senescence clone
LSD	least significant difference
M	molar, moles per litre

MAPK	mitogen activating protein kinase
min	minute
µg	micro gram
mg	milligram
µL	microlitre
mL	millilitre
µM	micro molar, micro moles per litre
mM	milli-molar, milli moles per litre
MOPS	3-[*N*-morpholino] propanesulphonic acid
mRNA	messenger ribonucleic acid
MYB_{305}	floral specific MYB_{305} promoter
NaHAc	sodium acetate
NBT	*p*-nitroblue tetrazolium chloride
ng	nanogram
nM	nano-molar, nano moles per litre
NOS	nopaline synthase
nptII	neomycin phosphotransferase gene
OCS	octopine synthetase
OD_{600}	optical density at 600 nm
OD_{660}	optical density at 660 nm
ORF	open reading frame
PCR	polymerase chain reaction
PIPES	piperazine-*N,N'-bis*-2-ethanesulphonic acid
PFU	plaque forming unit
pg	picogram
pmol	pico-molar, pico moles per litre
pTOM13	tomato 1-aminocyclopropane-1-carboxylic acid oxidase
rATP	ribooxyyadenosine triphosphate
rCTP	ribooxyycytidine triphosphate
rGTP	ribooxyyguanosine triphosphate

RH	relative humidity
Ri	root-inducing (plasmid)
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
rUTP	ribooxyyuradine triphosphate
SAG	senescence-associated gene
SAG_{12}	senescence-associated gene promoter
SAM	*S*-adenosyl-*L*-methionine
SAP	shrimp alkaline phosphatase
SDG	senescence-down-regulated
SDS	sodium dodecyl suphate
SSC	saline sodium citrate buffer
T	thymine
TBS	tris-buffered saline solution
T-DNA	transfer DNA
TE	tris-EDTA buffer
TEMED	*N*,*N*,*N'*,*N'*-tetramethylethylenediamine
Ti	tumour-inducing (plasmid)
Tris	tris(hydroxymethyl)aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
t-RNA	transfer-RNA
U	uracil
UV	ultra violet light
V	volts
vir	virulence gene
v/v	volume per volume
v/v/v	volume per volume per volume

w/v	weight per volume
Z	zeatin
ZR	zeatin riboside