GOBIERNO DE CHILE
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INNOVACIÓN AGRARIA

INFORME TÉCNICO Y DE DIFUSIÓN

ACTIVIDAD DE FORMACIÓN

"Comparación de la expresión de genes durante la senescencia de hojas y pétalos de *Erysimum linifolium*"

BID-FP-V-2005-1-A-097

Ing Agr. DANILO FERNANDO AROS ORELLANA

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CONTENIDO DEL INFORME TÉCNICO Y DE DIFUSIÓN

Fecha de entrega del Informe
07 de Julio de 2006
Nombre del coordinador de la ejecución
Danilo Fernando Aros Orellana
Firma del Coordinador de la Ejecución


1. ANTECEDENTES GENERALES DE LA PROPUESTA

Nombre de la propuesta
"Comparación de la expresión de genes durante la senescencia de hojas y pétalos de *Erysimum linifolium*"
Código
BID-FP-V-2005-1-A-097
Postulante o Postulantes
Danilo Fernando Aros Orellana
Entidad Patrocinante o Responsable
Facultad de Ciencias Agronómicas – Universidad de Chile
Lugar de Formación (País, Región, Ciudad, Localidad)
Reino Unido – Gales, Cardiff (School of Biosciences – Cardiff University)
Tipo o Modalidad de Formación (curso, pasantía, otros)
Pasantía
Fecha de realización (Inicio y término)
26 de enero al 03 de abril de 2006

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2. ALCANCES Y LOGROS DE LA PROPUESTA

Justificación y objetivos planteados inicialmente en la propuesta

General:
Identificar genes relacionados con la senescencia de hojas y pétalos de *Erysimum linifolium*.

Específicos:

- Extraer RNA y luego obtener cDNA desde distintos estados de desarrollo de hojas y pétalos de *Erysimum linifolium*.
- Optimizar condiciones de PCR en función de partidores que amplifiquen para genes específicos descritos previamente en *Arabidopsis thaliana*.
- Identificar la presencia en el genoma Wallflower de algunos genes descritos previamente en Arabidopsis.
- Conocer en detalle los patrones de expresión de tres genes seleccionados, durante la senescencia de Wallflower.

Objetivos alcanzados tras la realización de la propuesta

ARN desde 7 estados de desarrollo en hojas y 8 estados de desarrollo en pétalos extraído exitosamente.

Condiciones de PCR optimizados en función del número de ciclos, temperatura de *annealing* y cantidad de Cdna.

Cuatro genes relacionados con el proceso de senescencia en *Erysimum linifolium*: "P1F4", "SAG 12", "WGST" y "LPH9" fueron identificados y caracterizados.

Fueron secuenciados 10 clones de cDNA en Cardiff y 96 clones de cDNA en Warwick.

Resultados e impactos esperados inicialmente en la propuesta

El principal resultado de esta actividad de formación será la capacitación de un miembro del grupo de Floricultura de la Facultad de Cs. Agronómicas, U. de Chile, en el uso de herramientas biotecnológicas que permitan conocer los procesos fisiológicos y la expresión de genes involucrados en la senescencia de pétalos y hojas de plantas ornamentales. Se buscará aplicar estas metodologías para reproducir o continuar este tipo de investigaciones en el país y de este modo acercarse a la solución de problemas más concretos que afectan a la floricultura nacional.

La experiencia adquirida por el beneficiario de esta actividad, le permitirá difundir sus conocimientos en el medio nacional, captando el interés de investigadores y académicos del

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sector floricultor. Estos conocimientos fortalecerán también la consolidación del grupo de Floricultura de la Facultad de Cs. Agronómicas, U. de Chile y permitirán desarrollar nuevas líneas de trabajo, orientadas hacia la biotecnología aplicada en la floricultura.

Además, esta experiencia permitirá establecer y luego consolidar redes de contacto entre la Universidad de Chile y otros centros de investigación reconocidos a nivel mundial, para desarrollar actividades de investigación en conjunto o establecer acuerdos de colaboración. Este tipo de contactos fomentará y enriquecerá el estudio de líneas de investigación asociadas a la biotecnología.

Resultados alcanzados

Durante esta pasantía de investigación se han cumplido con todos lo objetivos planteados en el proyecto (Anexo 1). Esto ha permitido al participante de la actividad, adquirir sólidos conocimientos en el tema desarrollado. En este sentido, surgen interesantes expectativas para la aplicación de estas nuevas tecnologías en el medio nacional.

Por otra parte, se realizaron interesantes contactos con importantes centros de investigación en Europa. Cardiff University, Warwick University, London University (Reino Unido) y ENEA C.R. Trisaia (Italia) participaron durante esta actividad y existen las intenciones de desarrollar en conjunto nuevos proyectos a futuro. Actualmente se está realizando un trabajo en conjunto para postular a un fondo denominado "Funding Opp: The British Council – Italy, British – Italian Partnership Programme" (Anexo 2). Este programa especial permitiría al participante de la presente actividad, desarrollar una investigación en una universidad italiana, siempre en el tema de genómica aplicada en la Floricultura.

Resultados adicionales

A través de esta actividad fue posible conseguir una carta de aceptación para seguir estudios de postgrado en la Universidad de Cardiff (Anexo 3). En este sentido, esta actividad fue fundamental para establecer un contacto más directo con profesores de la Universidad y además permitió trabajar de manera preliminar en el proyecto de investigación que debería desarrollar durante el postgrado.

Con el financiamiento del Ministerio de planificación a través de su programa "Beca Presidente de la República" comenzaré mis estudios de postgrado en el próximo período académico (2006/2007).

Aplicabilidad

Centrando este análisis en el tema particular de Genómica aplicada en la Floricultura, es importante comenzar señalando que actualmente no existen estudios de este tipo en el país. Los estudios de genómica realizados en el país han abarcado otros temas como la fruticultura y la fitopatología.

En Floricultura, existen algunos proyectos de mejoramiento genético y aplicación de biotecnología para la obtención de nuevas variedades. Se ha explotado además el uso de especies nativas con potencial ornamental.

El desarrollo de este tipo de actividades de investigación centradas en genómica, han sido ampliamente estudiadas en el Reino Unido, lugar de realización de esta pasantía. Este tema es abordado transversalmente en varias especies. Particularmente en la Universidad de Cardiff, han concentrado el estudio en especies ornamentales como *Alstroemeria* y *Wallflower*. Además realizan estudios en tabaco y *Arabidopsis*. Otro centro de investigación ubicado en el Reino Unido es Warwick, en donde fue desarrollada parte de esta investigación. En este centro se desarrollan investigaciones en *Arabidopsis* y otras *Brassicaceae* de importancia hortícola, tales como repollo, brócoli, entre otras.

Considerando ahora la incorporación de los conocimientos adquiridos relativos al estudio de los procesos genéticos que influyen sobre la senescencia de una flor, es importante comenzar señalando que la vida en poscosecha es un gran factor diferenciador en la Floricultura, más aún comprendiendo el desafío que implica para la industria nacional la exportación de flores frescas hacia mercados lejanos, este tema es de suma relevancia para el sector floricultor nacional y debe ser abordado desde distintas áreas (fisiología, genética, manejo, etc.) y por los distintos agentes que están involucrados en su producción y comercialización.

Para esto, es necesario generar una sólida base que permita desarrollar soluciones definitivas a través del uso de la biotecnología, partiendo por entender los mecanismos genéticos que controlan la senescencia de flores y hojas en los cultivos ornamentales. Esta información permitirá definir de mejor forma las diferentes líneas de acción para abordar el problema, por ejemplo a través de programas de mejoramiento genético con selección asistida, apoyadas por herramientas biotecnológicas.

Detección de nuevas oportunidades y aspectos que quedan por abordar.

Los conocimientos adquiridos durante esta actividad, permitirán formular proyectos I+D que permitirán aplicar las tecnologías y experiencias adquiridas en el medio nacional. Aún cuando este tipo de actividades ha sido muy poco desarrollado en el país, deberían presentarse como una interesante alternativa para solucionar los problemas que afecten a la industria nacional.

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3. ASPECTOS RELACIONADOS CON LA ORGANIZACIÓN Y EJECUCIÓN DE LA PROPUESTA

Programa de actividades

Fecha	Actividad	Objetivo	Lugar
30-01-06 / 07-02-06	Extracción de ARN desde hojas y pétalos de Wallflower (Erysimum linifolium).	Obtener el ARN de distintos estados de desarrollo para luego obtener cDNA a través de RT-PCR.	Cardiff University, School of Biosciences
07-02-06 / 14-02-06	Obtención del cDNA.	Obtener el cDNA de distintos estados de desarrollo de pétalos y hojas, para identificar la presecia de genes específicos.	Cardiff University, School of Biosciences
14-02-06 / 21-02-06	Optimización de protocolos de PCR.	Desarrollar un protocolo de PCR que permita realizar una óptima amplificación.	Cardiff University, School of Biosciences
21-02-06 / 02-03-06	Amplificación de algunas secuencias con partidores específicos.	Identificar la presencia en el genoma Wallflower de algunos genes descritos previamente en Arabidopsis.	Cardiff University, School of Biosciences
02-03-06 / 13-03-06	Desarrollo de Semi-quantitative RT-PCR.	Conocer en detalle los patrones de expresión de 3 genes seleccionados, durante la senescencia de Wallflower.	Cardiff University, School of Biosciences
13-03-06 / 18-03-06	Secuenciación de 10 genes que presenten patrones de expresión interesantes.	Conocer la función de genes específicos, durante la senescencia de Wallflower.	Cardiff University, School of Biosciences
18-03-06 / 23-03-06	Secuenciación de 96 genes que presenten patrones de expresión interesantes.	Conocer la función de genes específicos, durante la senescencia de Wallflower.	Warwick University
23-03-06 / 31-03-06	Comparación de los datos analizados con los obtenidos previamente en Arabidopsis.	Establecer diferencias especie-específicas entre Arabidopsis Wallflower.	Cardiff University, School of Biosciences
31-03-06 / 10-05-06	Preparación de informe y análisis de datos.	Reunir y analizar toda la información obtenida durante la pasantía de investigación.	Universidad de Chile, Facultad de Cs. Agronómicas
10/05/06	Charla de difusión.	Difundir la información y la experiencia adquirida durante la pasantía de investigación.	Universidad de Chile, Facultad de Cs. Agronómicas

Contactos Establecidos

Institución Empresa Organización	Persona de Contacto	Cargo	Fono/Fax	Dirección	E-mail
Cardiff University School of Biosciences	Dr. Hilary Rogers	Senior Lecture	+44 (0)29 20 876352/5880	Cardiff School of Biosciences, Main Building, Museum Avenue. Cardiff CF10 3TL UK	RogersHJ@cardiff.ac.uk
Cardiff University School of Biosciences	Dr. Dennis Francis	Senior Lecture	+44 (0)29 20 875086		FrancisD@cardiff.ac.uk
School of Biological Sciences Royal Holloway, University of London	Dr. Anthony Stead	Senior Lecture	+44 (0)17 84 443761	Egham, Surrey, TW20 0EX United Kingdom	a.stead@rhul.ac.uk
Warwick HRI, University of Warwick	Dr. Vicky Buchanan-Wollaston	Investigador	+44 (0) 24 76 575136	Wellesbourne CV35 9EF Warwick, UK	vicky.b-wollaston@warwick.ac.uk
ENEA C.R. Trisaia BIOTEC-GEN	PhD. Carlo Rosati	Investigador	+39 0835 974.354	S.S. 106, Km 419+500 I-75026 Rotondella (MT) Italy	carlo.rosati@trisaia.enea.it

Material Recopilado

Tipo de Material	Nº Correlativo (si es necesario)	Caracterización (título)
Artículo	Anexo 4	"Programmed cell death (PCD) processes begin extremely early in *Alstroemeria* petal senescence"
Artículo	Anexo 5	"Gene expresion patterns to define stages of post-harvest senescence in Alstroemeria petals"

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4. PROGRAMA DE DIFUSIÓN EJECUTADO

Material entregado en las actividades de difusión

Tipo de material	Nombre o Identificación	Preparado por	Cantidad
Carpeta	Informe de resultados	Danilo Aros O.	48
Carpeta	Diapositivas impresas	Danilo Aros O.	48

Participantes en actividades de difusión

Nombre	
Apellido Paterno	
Apellido Materno	
RUT Personal	
Dirección, Comuna y Región	
Fono y Fax	
E-mail	
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	
Rubro, área o sector a la cual se vincula o en la que trabaja	

LISTA DE ASISTENCIA

"Comparación de la expresión de genes durante la senescencia de hojas y pétalos de Erysimum linifolium" - 10 de Mayo de 2006

NOMBRE	INSTITUCIÓN	CORREO ELECTRÓNICO	FIRMA
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LISTA DE ASISTENCIA

"Comparación de la expresión de genes durante la senescencia de hojas y pétalos de Erysimum linifolium" - 10 de Mayo de 2006

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LISTA DE ASISTENCIA

"Comparación de la expresión de genes durante la senescencia de hojas y pétalos de Erysimum linifolium" - 10 de Mayo de 2006

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LISTA DE ASISTENCIA

"Comparación de la expresión de genes durante la senescencia de hojas y pétalos de Erysimum linifoilum" - 10 de Mayo de 2006

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LISTA DE ASISTENCIA

"Comparación de la expresión de genes durante la senescencia de hojas y pétalos de Erysimum linifoilum" - 10 de Mayo de 2006

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5. EVALUACIÓN DE LA PROPUESTA

Organización durante la actividad (indicar con cruces)[1]

Ítem	Bueno	Regular	Malo
Recepción en país o región de destino según lo programado	X		
Cumplimiento de reserva en hoteles	N/A	N/A	N/A
Cumplimiento del programa y horarios según lo establecido por la entidad organizadora	X		
Facilidad en el acceso al transporte	X		
Estimación de los costos programados para toda la actividad	X		

Evaluación de la actividad de formación

a) Efectividad de la convocatoria

La difusión de la convocatoria fue realizada a través de e-mail, página web, afiches (Anexo 6) y contactos personales. La efectividad de esta convocatoria ha sido buena, considerando la asistencia de 46 personas al evento.

Posiblemente, el bajo nivel de convocatoria por parte de personas externas al ámbito científico, se deba principalmente al carácter específico del tema de la charla. Si bien es cierto el tema de genómica ha sido identificado como un tema de amplio interés para el sector científico del país, este tema no ha sido abordado en particular desde el punto de vista de la floricultura. Por otra parte, este tema resulta ser complejo y poco aplicable para la industria, compuesta principalmente por agricultores, exportadores y comerciantes.

b) Grado de participación de los asistentes (interés, nivel de consultas, dudas, etc)

En general se mostró buena disposición por parte de los asistentes, colaborando con comentarios y sugerencias. Además se plantearan dudas interesantes que permitieron establecer algún tipo de discusión respecto al tema relacionado con aplicaciones biotecnológicas y mejoramiento genético en el sector floricultor.

[1] En caso de existir un ítem Malo o Regular, señalar los problemas enfrentados durante el desarrollo de la actividad de formación, la forma como fueron abordados y las sugerencias que puedan aportar a mejorar.

c) Nivel de conocimientos adquiridos en función de lo esperado (se debe indicar si la actividad contaba con algún mecanismo para medir este punto)

Los conocimientos adquiridos durante esta actividad, han permitido al participante tener una visión distinta para abordar el tema de la Floricultura. Las aplicaciones biotecnológicas en este tema requieren aún de mucho desarrollo en el país, pero se considera que mediante actividades como éstas, el avance será sostenido y pronto se conseguirán interesantes resultados.

d) Calidad de material recibido durante la actividad de formación

N/A

e) Nivel de adecuación y facilidad de acceso a infraestructura/equipamiento necesario para el logro de los objetivos de la actividad de formación.

No hubo problemas para el acceso a laboratorios equipados con lo necesario para cumplir con las actividades planteadas.

f) Indique las materias que fueron más interesantes, más desarrolladas a lo largo de la actividad de formación y las que generan mayor interés desde el punto de vista de la realidad en la cual se desenvuelve el participante.

El punto más interesante ha sido sin duda la experiencia obtenida del método con el cual se desarrollan las investigaciones en este tipo de países. El trabajo se realiza con disciplina y rigurosidad.

g) Problemas presentados y sugerencias para mejorarlos en el futuro

No hubo problemas y los objetivos planteados fueron cumplidos

Aspectos relacionados con la postulación al programa de formación o promoción

a) Apoyo de la Entidad Patrocinante (cuando corresponda)

_X_ bueno ____ regular ____ malo

Justificar: Se cumplió sin problemas con los ítems comprometidos en el proyecto

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b) Información recibida por parte de FIA para realizar la postulación

_X_ amplia y detallada ____ aceptable ____ deficiente

Justificar: Los formularios han sido muy claros y no ha habido problema en completarlos. Además, el contacto con la gente de FIA fue siempre fluido y cordial para resolver dudas.

c) Sistema de postulación al Programa de Formación o Promoción (según corresponda)

_X_ adecuado ____ aceptable ____ deficiente

Justificar:

d) Apoyo de FIA en la realización de los trámites de viaje (pasajes, seguros, otros) (sólo cuando corresponda)

_X_ bueno ____ regular ____ malo

Justificar: Los pasajes aéreos y el seguro han sido entregados a tiempo, sin inconvenientes y en las fechas programadas

e) Recomendaciones (señalar aquellas recomendaciones que puedan aportar a mejorar los aspectos administrativos antes indicados)

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ANEXO 1

Cardiff School of Biosciences
Head of School Professor J L Harwood PhD DSc

Ysgol y Biowyddorau, Caerdydd
Pennaeth yr Ysgol Yr Athro J L Harwood PhD DSc

CARDIFF UNIVERSITY
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www.cardiff.ac.uk/biosi/

Prifysgol Caerdydd
Prif Adeilad
Plas y Parc
Caerdydd CF10 3TL
Cymru, Y Deyrnas Gyfunol

30th March 2006

Re: Danilo Aros
"Comparacion de la expresion de genes durante la senescencia de hojas y petalos de Erysimum linifolium", codigo: FIA-FP-V-2005-1-a-097

Danilo spent 2 months in my laboratory (30/01/06 to 30/03/06) working on a project characterising gene expression during petal and leaf senescence in wallflowers. He obtained excellent results for the data on gene expression and has succeeded in his aim of preparing approximately 200 clones for sequencing and data analysis. His time in Cardiff has therefore been very productive and he has mastered a number of techniques that were new to him and which hopefully will be useful in his future career. His data will contribute to a publication in a major plant science journal which we plan to submit in the next few months.

I was very happy with Danilo's visit and would welcome the opportunity to repeat the experience. I am hopeful that this visit may lead to a long-term collaboration between Cardiff University and the University of Chile.

Yours faithfully,

Dr. Hilary J Rogers
(Senior Lecturer)

ANEXO 2

Asunto: Re: FW: Funding Opp: The British Council -Italy, British-Italian Partnership Programme
De: "Hilary Rogers" <rogershj@Cardiff.ac.uk>
Fecha: Mie, 7 de Junio de 2006, 9:27 am
Para: "Stead A" <A.Stead@rhul.ac.uk> (más)
Prioridad: Normal
Opciones: Ver encabezado completo | Vista preliminar

Thanks Tony,
I wonder if we could try to dress up the scent as evolutionary/developmental biology? I guess it certainly is developmental?
Carlo/Danilo what do you think?? Carlo should we try for it?
Hilary

Dr. Hilary J Rogers
School of Biosciences,
Main Building
Cardiff University
PO Box 915
Cardiff,
CF10 3TL
Tel: 02920 87 6352/5880
Fax: 02920 874305

>>> "Stead A" <A.Stead@rhul.ac.uk> 07/06/2006 13:22:07 >>>
Don't know if you see these? Busy marking now!

Tony

-----Original Message-----
From: Bramley P
To: Biol Sci - Academic Staff
Sent: 6/7/2006 9:05 AM
Subject: FW: Funding Opp: The British Council -Italy, British-Italian Partnership Programme

FYI. Some relevant areas for us here.

Peter

Prof Peter M Bramley

Director of Research

Head of Centre of Chemical and Bioanalytical Sciences

School of Biological Sciences

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Egham, Surrey

TW20 0EX

Tel +44(0)1784 443555

Fax +44(0)1784 414224

Web site: http://www.rhul.ac.uk/Biological-Sciences/bramley/

-----Original Message-----
From: Makwana Urvi
Sent: 06 June 2006 17:32
To: Bramley P; Gamble Clive; Fowler M; Saunders J; Rastle Kathy
Cc: Patel Hitesh; Boon Lotte
Subject: Funding Opp: The British Council -Italy, British-Italian Partnership Programme

Dear All

I would be grateful if you could circulate the following opportunity within your department. Many thanks!

Kind regards

Urvi

R&E

Extn 4924

The British Council - Italy

British-Italian Partnership Programme

Closing Date: 28 July 2006

Established in 1990, this programme is designed to increase collaboration between research groups in Italy and the United Kingdom. It is jointly financed and managed by the British Council and the Italian Ministry for Education, Universities and Research (MIUR) in collaboration with the Conferenza dei Rettori delle Universita' Italiane

(CRUI). Its aim is to promote the development of British-Italian scientific cooperation between universities and public sector research institutes in Italy and the UK in priority areas identified annually.

The themes for 2006-2007 are:

* FOOD, NUTRITION AND HEALTH - Both the UK and Italy are committed to remedy the negative effects of poor dietary and unhealthy lifestyles, especially among the very young and elderly

Proposals may address issues related to:

- Nutrition, obesity and diabetes
- Food science and technology
- The role of physical education and sport in education

* ECOLOGY AND EVOLUTIONARY BIOLOGY - understanding biological systems at the individual, population and community levels of organisation utilising both plant, animal and microbial systems

Proposals may include strands of one or more of the following research areas:

- Molecular evolution/ecology
- Evolutionary genetics
- Evolutionary and developmental biology
- Ecosystem ecology

* MEDICAL IMAGING - the development of new algorithms and mathematical tools for the advanced processing of medical and biological images

Proposals may reflect one or both complementary levels of research:

- The fundamental and mathematical aspects of imaging
- Application-oriented projects in collaboration with researchers in medicine and biology

* NANOTECHNOLOGY - nanotechnology is recognised as a vital and innovative area impinging on our everyday lives and capable of creating new products and industries

Projects should support research into the development and production of biomedical products based on advances in nanotechnology.

Selection Criteria: The key objective of this programme is to focus on new or recently initiated collaborative links offering international experience to young researchers. Projects must clearly indicate how the collaboration will continue if the time-span envisioned is more than the one year for which funding will be given. The grant must be used primarily for travel and subsistence and cannot be used to cover bench fees, running costs, equipment, consumables or other similar expenses. The amount of each award will be based on the breakdown of expenses indicated in the proposal.

Further details and an application form:
http://www.britishcouncil.org/italy-science-partnership-programme.htm
<http://www.britishcouncil.org/italy-science-partnership-programme.htm>

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ANEXO 3

REGISTRY, CARDIFF UNIVERSITY

To: Mr Danilo Fernando Aros Orellana
Las Diademas # 18.745
Maipu
Santiago
Chile

Application No: 0617502
Please quote this in all correspondence with the University.

Cardiff University is pleased to offer you admission to the scheme of study detailed below.

Scheme: MASTER OF PHILOSOPHY (BIOLOGICAL SCIENCE) (DRFBSCPL1)
School: Cardiff School of Biosciences
Attendance: Full-Time Duration: 1 year
Commencing: 01 October 2006
Tuition Fee: £10,620 per annum. Fee Status: Overseas Student

Please note that the fee amount quoted above is that for academic year 2005/6. Tuition fees are subject to annual review. You will receive confirmation of the actual rate in your enrolment information.

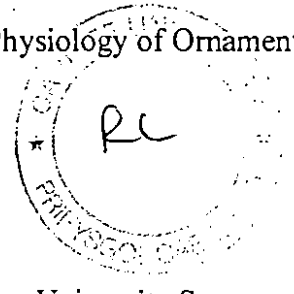
Conditions of Offer/Comments:

This offer is unconditional.

Please note that you should ensure that you have sufficient funds to pay your tuition fees and living expenses for the duration of your studies.

Proposed Research Topic: Gene Expression and Post-Harvest Physiology of Ornamental Alstroemeria Species.

Names(s) of Proposed Supervisor(s): Dr Hilary Rogers



University Stamp

This offer is made by Cardiff University on the understanding that in accepting it you agree to observe its Charters, Statutes, Ordinances and Regulations and such other rules and regulations as the University makes for its students from time to time.

Further information concerning study as a research student at the University may be obtained at www.Cardiff.ac.uk.

In order to ensure a professional and responsive service to all of our postgraduate students, the postgraduate team at Cardiff University Registry communicate via email wherever possible. This means that all postgraduate students must check their official university email account regularly, or ensure that this email account is autoforwarded if they use an alternative email provider.

Registry
Director Professor A Cryer BSc PhD (Sheff)

Y Gofrestrfa
Cyfarwyddwr Yr Athro A Cryer BSc PhD (Sheff)

Cardiff University
McKenzie House
30 - 36 Newport Road
PO Box 927
Cardiff CF24 0DE
Tel *Ffôn* +44(0)29 2087 4404
Fax *Ffacs* +44(0)29 2087 4130
www.cardiff.ac.uk
Prifysgol Caerdydd
Tŷ McKenzie
30 - 36 Newport Road
Blwch SP 927
Caerdydd CF24 0DE

Dear Applicant,

I am pleased to be able to offer you a place at Cardiff University for the programme of study detailed overleaf.

I should be grateful if you would confirm, by completing the attached decision slip, whether you will be taking up this offer of a place. A pre-addressed envelope is also enclosed for your convenience.

If your offer is subject to any conditions, you should inform me, in writing, as soon as you have satisfied the conditions. Please enclose **copies** of any supporting documentation. **Original certificates should not be sent at this stage**, although you will need to produce them after you have enrolled at Cardiff.

I hope very much that you will decide to accept this offer and look forward to welcoming you to our academic community at Cardiff. Cardiff University and the University of Wales College of Medicine merged in August 2004, building on our existing strong partnership to create an even more broadly based university which is already known throughout the world for the quality of its teaching and research.

The merger is supported by a multi-million pound programme of additional investment and will directly benefit students by increasing teaching, learning, research and support capabilities, as well as providing substantial academic, economic and healthcare benefits to society. The merged university is known as Cardiff University.

Full information about merger can be found at www.cardiff-uwcm.ac.uk

If you have any questions regarding your offer, please let me know.

Yours sincerely

RPE

Mr Rhodri Evans
Senior Assistant Registrar

Living Expenses

In addition to the payment of fees, you will require extra money to cover your living expenses.

For session 2006/2007, minimum basic living expenses for an overseas student (excluding fees) are estimated to be £605 per month. Please note that this is an **estimated** figure for regular expenditure only and will vary according to personal circumstances. Overseas students are strongly advised to have an additional fund for use in emergencies.

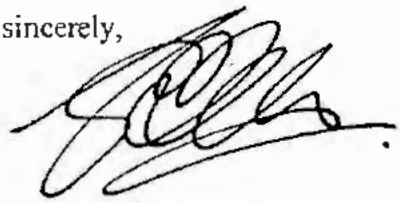
If you are married, you will have to double the living cost if you intend to bring your spouse with you.

Finance Arrangements

Please arrange, before leaving your own country, for the transfer of currency to pay at least one term's living expenses as well as fees in full. Foreign exchange clearance may take a considerable time **AND YOU SHOULD THEREFORE APPLY TO THE RELEVANT AUTHORITIES IN YOUR COUNTRY WITHOUT DELAY.** Cheques for living expenses must not be made payable to the University but to yourself, and you should open a bank account in the United Kingdom as soon as possible. Neither the UK Government nor the University will be able to help you financially should your arrangements prove inadequate.

You will see from the above information that you will need a sum of money for the payment of fees and an additional sum for living expenses (food, accommodation, travel etc). Cardiff University will assume that, when you complete and return the slip accompanying your Offer of Admission, you have understood the above financial advice and that you will arrange to arrive at the University, on enrolment day, with sufficient money in sterling to pay tuition fees and to meet your living expenses.

Yours sincerely,



Dr Annabelle Willox
Assistant Registrar

Contact Details: Registry
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30-36 Newport Road
Cardiff, CF24 0DE.
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Registry
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Tŷ McKenzie
30 - 36 Newport Road
Blwch SP 927
Caerdydd CF24 0DE

date as postmark

Dear Applicant,

Financial Advice for Overseas Applicants

This letter is sent to you as part of the University's offer of admission. Its purpose is to explain to you the amount of money you will need, in £ sterling, both to pay fees and to meet living expenses.

Tuition Fees

Your Offer letter contains a statement about the tuition fees applicable for your scheme of study. Please be aware that, if your Offer letter is dated before May 2006, then the fees quoted relate to entry in 2005/6 and are indicative only. You can expect the fees for 2006/7 to be increased by an amount roughly in line with the level of UK price inflation.

The actual amount you will be required to pay will be confirmed in the Enrolment Information booklet that should be despatched from July 2006 onwards.

You can also check on the University's web site from April/May onwards:-

www.cardiff.ac.uk/courses/fees

Paying in Instalments

Please note that postgraduate self-supporting students are allowed to pay fees by three equal instalments, the first of which must be paid on or before enrolment. You should, therefore, arrange for the required amount to be available at that time. Foreign exchange clearance may take a considerable time **AND YOU SHOULD THEREFORE APPLY TO THE RELEVANT AUTHORITIES IN YOUR COUNTRY WITHOUT DELAY.** If you are sponsored by an overseas body, you should inform your sponsors of this fact and arrange for a cheque, made payable to Cardiff University, to be forwarded to the Finance Section, Cardiff University, PO Box 497, Cardiff CF10 3XR, by the required date.

Students who are unable to fulfil their financial obligations on the day of enrolment may, at the discretion of the University, be offered 'temporary enrolment' only.

GOBIERNO DE CHILE
FUNDACIÓN PARA LA
INNOVACIÓN AGRARIA

ANEXO 4

Plant Biotechnology Journal (2004) 2, pp. 155–168 Doi: 10.1111/j.1467-7652.2004.00059.x

Gene expression patterns to define stages of post-harvest senescence in *Alstroemeria* petals

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Received 25 September 2003;
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Summary

Petal senescence in many species is regulated by ethylene but some flowers, such as those on the monocotyledonous plant *Alstroemeria*, var. Rebecca are ethylene insensitive. Changes in gene expression during the post-harvest senescence of *Alstroemeria* flowers were investigated using several different techniques. Suppressive subtractive hybridization (SSH) was used to obtain cDNA libraries enriched for genes expressed at selected stages of petal senescence. Sequencing of the EST clones obtained resulted in over 1000 sequences that represent approximately 500 different genes. Analysis of the potential functions of these genes provides a snapshot of the processes that are taking place during petal development. Both cell wall related genes and genes involved in metabolism were present at a higher proportion in the earlier stages. Genes encoding metal binding proteins (mostly metallothionein-like) were the major component of senescence enhanced libraries. This limited the diversity of genes identified showing differential expression at the later stages. Changes in the expression of all genes were analysed using microarray hybridization, and genes showing either up or down-regulation were identified. The expression pattern of a selection of genes was confirmed using Northern hybridization. Northern hybridization confirmed the up-regulation of metallothioneins after floral opening, however, this was not detected by the microarray analysis, indicating the importance of using a combination of methods to investigate gene expression patterns. Considerably more genes were up-regulated than down-regulated. This may reflect the need during *Alstroemeria* petal senescence for the expression of a whole new set of genes involved with degradation and mobilization. The potential uses of expression profiling to improve floral quality in breeding programmes or as a diagnostic tool are discussed.

Keywords: senescence, petal, *Alstroemeria*, EST, microarray, gene expression.

Introduction

The primary function of large and colourful floral structures (the corolla) on plants is to attract insects or other pollinators. Once pollination has occurred, the role of the flower is over and the corolla is rapidly lost from the plant. The final stages of floral development can take a number of different forms (van Doorn, 2001). The petals of some flowers abscise from the plant with no obvious signs of deterioration, indicating that little remobilization has occurred. The petals of other flowers show extensive wilting (turgor loss, increased translucency and slow desiccation) before they are abscised from the plant. This could indicate that degradation and remobilization of its cellular constituents has taken place. The majority of large monocotyledonous flowers, such as those of the Liliales order including those of *Alstroemeria*, show this type of senescence.

Alstroemeria is a member of the *Alstroemeria*ceae family and is an important cut flower in Northern Europe. It has a relatively long vase life, taking around 10 days under optimal conditions after harvest to reach full senescence. A time frame for floral senescence including the measurement of biochemical and physiological parameters such as protein degradation has been developed and described previously (Leverentz *et al.*, 2002; Wagstaff *et al.*, 2001, 2003), and

gene expression patterns for a small number of genes during senescence have been analysed (Wagstaff *et al.*, 2002).

Molecular studies of leaf senescence have shown that gene expression patterns change dramatically as senescence progresses, with a large number of genes either showing reduced or increased expression (Buchanan-Wollaston *et al.*, 2003 for review). Novel gene expression is essential for senescence to occur. Many different biochemical events occur during leaf senescence, macromolecules are degraded and soluble nutrients are remobilized. Thus, the purpose of leaf senescence is one of redistribution of resources.

The study of gene expression changes during flower senescence has not been extensive, although a number of senescence-enhanced genes have been isolated from a variety of different flowers (Rubinstein, 2000). Many of these encode potential catabolic enzymes such as proteases and nucleases, and genes encoding enzymes related to ethylene biosynthesis have also been identified. A more global overview of genes expressed in petals was obtained by DNA sequence analysis of nearly 2000 individual cDNA clones from RNA isolated from rose petals at four different stages of development including senescent petals (Channeliere *et al.*, 2002). A few of these genes were shown to exhibit enhanced expression in senescing petals. In many species, the time to petal senescence is regulated by ethylene, and a manipulation of the levels of ethylene biosynthesis has been effective in delaying the rate of senescence in several flower types such as the carnation (Michael *et al.*, 1993) and *Torenia* (Aida *et al.*, 1998). However, flowers of some species, such as many of the monocotylenonous plants including *Alstroemeria*, are not dependent on ethylene for senescence, even though the final abscission of the wilted petal may be ethylene-dependent.

Genes that show enhanced expression in senescing monocotyledon flowers have been identified in a number of different ways including differential display (in daylily, Panavas *et al.*, 1999) and subtractive hybridization (in daffodil, Hunter *et al.*, 2002). In each case a small number of genes was identified.

We have used a suppressive subtractive hybridization technique to produce several cDNA libraries enriched for genes expressed at particular stages of *Alstroemeria* petal senescence. Genes showing both up and down-regulation during senescence have been identified, and microarrays were used to determine the overall changes in gene expression patterns that occur during post-harvest senescence. This is a novel approach to the analysis of flower senescence; it has identified extensive collections of genes that reflect the complex processes occurring during petal senescence. A selection of these genes may represent useful markers to determine the stage of flower senescence in this and other floral crops.

Results

Construction of subtracted libraries and gene identification

Eight different stages of *Alstroemeria* flower development and senescence have been identified by clearly defined visible changes (Figure 1). Subtracted libraries were constructed

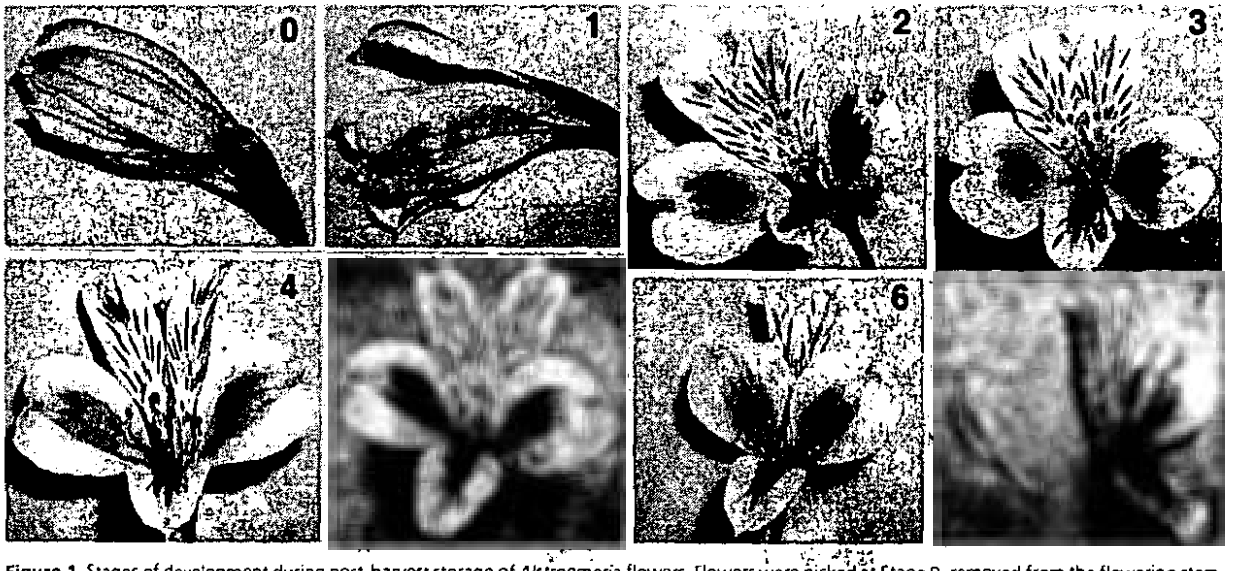

Figure 1 Stages of development during post-harvest storage of *Alstroemeria* flowers. Flowers were picked at Stage 0, removed from the flowering stem and placed in water. Visible signs were used to designate the stage of the flowers. Under good conditions (21 °C, 14 h day, 20.25 μmol.photons/m^2/s, 60% r.h.) the flowers took 10 days to reach Stage 7.

to clone and identify genes showing differential expression between these stages. Initially, four libraries were made and these were enriched for: (1) genes expressed in Stage 0 and not in Stage 2, (2) genes expressed at Stage 2 and not at Stage 0, (3) genes expressed in Stage 2 and not in Stages 4 + 5 combined, and (4) genes expressed in Stages 4 + 5 and not in Stage 2. Over 80% of the clones in library (4) represented the same gene family (see below) and so a fifth library was constructed that was enriched for genes expressed in Stage 3 and not in Stage 2.

DNA sequence analysis followed by database searches for each subtracted clone identified genes with a wide range of different potential functions. For each library, between 200 and 400 clones were sequenced and, once poor and short sequences and sequences of structural RNA had been removed, a total of 991 EST sequences were generated. In order to identify ESTs that represented the same gene, all the sequences obtained were entered into the alignment program SEQMAN (DNASTAR) and common sequences were grouped together as contigs. This resulted in 500 different groups, each representing a separate *Alstroemeria* gene (Supplementary Table S1A). Of the 991 EST clones characterized in this way, 297 were singletons, i.e. a single representative of a particular gene. All the other sequences occurred at least twice, with the most common gene occurring 21 times. The redundancy of the EST collection was calculated to be 70% (number of ESTs in clusters/total number of ESTs, Sterky *et al.*, 1998). Therefore any new sequence has a 70% chance of already being represented in the collection. The contigs represented by two or more sequences were given an ALF TC number (*Alstroemeria* Flower Tentative Contigs). Singletons were given an ALF number. There was likely to be some redundancy in the TC and EST sets, because sequences must have a matching overlap at a certain percentage identity (95%) to be combined, and different parts of the same gene may be represented separately. The potential function of each gene was identified by database searching with the TC or single EST sequence, using BLASTN and TBLASTX databases. For each cloned *Alstroemeria* gene, its likely function and the most similar rice gene or *Arabidopsis* gene are shown in Supplementary Table S1A. Rice and *Arabidopsis* were used to identify the closest matched gene, since the genomes of these two plants are almost completely sequenced and annotated. Each of the sequences has been submitted as an EST sequence to GENBANK and the GENBANK codes of the ESTs found in each contig are shown in Supplementary Table S1B.

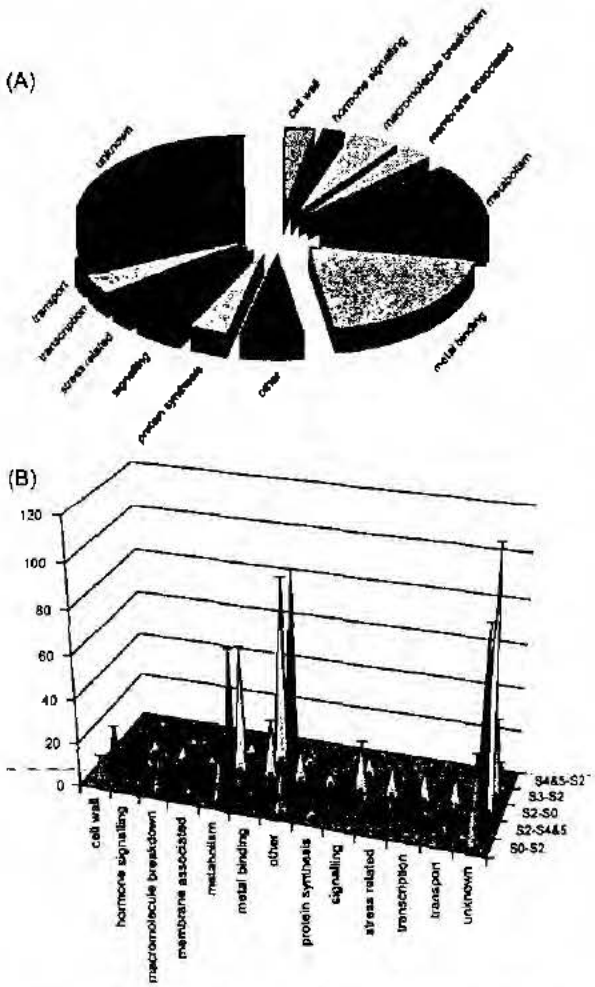

Figure 2 Putative function of genes represented by EST clones. The designated function for the 1007 EST sequences identified in each library is shown in Table S2. (A) Pie chart showing the proportion of genes with different putative functions in the whole collection of 1007 EST clones. (B) Graph showing the relative proportion of each functional class in the different subtracted libraries.

Differential gene expression determined by subtractive hybridization

Five different subtracted libraries were generated as described above. Up to 400 clones were sequenced from each library and genes encoding a wide range of different functions were obtained (Supplementary Table S1). The genes identified were assigned to potential functional groups according to their sequence similarity and an overall summary of these functions in the EST collection is shown in Figure 2A. A high proportion (29%), of the clones sequenced represented genes with no matches in the database or which showed a similarity to genes of unknown function. Metal binding proteins (mostly metallothionein-like proteins) were the most highly represented class of genes with known potential function (19%).

A comparison of the potential functions of the genes identified in each of the subtracted libraries could give an

indication of the processes taking place in the petals through post-harvest development. Each subtracted library contained clones representing a different collection of genes, and these genes are listed in Supplementary Table S2. The five different subtracted libraries showed a considerable variation in the range of genes they represented and the potential function of each gene was used to classify them into groups. The relative proportions of different gene functions in each library are illustrated in Figure 2B, and show a considerable variation between the libraries. This graph shows the relative proportions of each functional class of gene in the five different libraries and may be a reflection of the changes that are taking place as the petals are undergoing post-harvest senescence. The libraries are ordered on this graph to show the progression of senescence, the first two (S0–S2 and S2–S4 & 5) should be enriched for genes that are decreasing in expression levels as senescence progresses. The other three libraries (S2–S0, S3–S2 and S4 & 5–S2) should be enriched for genes that are increasing in expression as senescence progresses.

From the graph it appears that there are considerable differences in the transcripts that are present at different stages. Cell wall related genes are only present in the first two libraries and also genes involved in metabolism are present at a higher proportion in the earlier stages. As has been described above, genes encoding metal binding proteins (mostly metallothionein-like) were the major component of the subtracted libraries that were constructed to identify senescence enhanced genes, i.e. 4/5–2 and 3–2, and also there were a significant number of metallothionein clones in the library 2–0. However, metallothionein genes were not represented at all in the libraries enriched for genes showing decreasing expression during senescence (i.e. S0–2 and S2–4/5). This indicates that the metallothionein transcripts were subtracted in these libraries and suggests that the subtraction procedure was successful.

Metallothionein genes

Grouping the EST sequences together illustrated the extent of the redundancy in the EST collection. Many genes were represented only once or twice while the largest group consisted of 21 different sequences. The SEQMAN assembly was carried out with a high stringency (95% sequence identity) resulting in separation of different members of the same gene family. For example, the alignment of the many metallothionein sequences showed that there were at least 38 different genes represented, many of which were present multiple times in the EST collection (Supplementary Table S1). The protein sequences for 17 of these different groups of metallothionein genes (those that were each represented four times or more in the EST collection and therefore had a reliable consensus sequence) were predicted from the consensus DNA sequence and compared in an alignment programme using CLUSTAL W, to determine the differences between them (Figure 3A). Two main classes of protein were apparent from this alignment (Figure 3B). The majority of genes appeared to encode a protein of around 60 amino acids, and this class can be divided into two subclasses, 1A and 1B, as shown in Figure 3A,B. These proteins had between 100 and 90% identity (Class 1A) and 75–67% identity (class 1B) to the ALF 1 protein. The second class of metallothionein (Class 2) was illustrated by two of the TC sequences (ALF TC41 and ALF TC42, each derived from six individual EST clones); these appeared to encode a much smaller protein of around 40 amino acids and showed around 30% identity to the ALF1 protein.

Many different metallothionein genes have previously been identified from a variety of plant species. The *Alstroemeria* genes in class 1A and 1B showed the closest similarity to the metallothionein 1 genes from *Fritillaria agrestis*, another member of the Liliales order (Figure 3C). The most similar match to this protein in *Arabidopsis* was shown by the Met3 gene (At3g15353), a single copy gene in the *Arabidopsis* genome (Figure 3D). The *Alstroemeria* Class 2 metallothionein proteins are closely related to the C terminal part of a metallothionein protein (79 amino acids) from *Typha latifolia* (common cattail) but are most similar in size to the Mt1a protein from *Arabidopsis* (44 amino acids). The C terminals of these proteins showed similarity, but the N terminal half of the proteins were very different. All the putative metallothionein proteins have characteristic conserved cysteine residues and these are shown in Figure 3A and C.

Gene expression patterns from microarray analysis

All the EST clones generated in the subtractive library screening (1532 clones) were used to generate a microarray. Each slide carried two replicates of an array that contained three copies of each probe. Therefore, each DNA fragment was present six times on a slide and each hybridization was carried out four times with reciprocal labelling. Thus the final data points are the result of hybridization to 24 copies of each gene.

Microarrays were hybridized with labelled cDNA made from RNA from four different stages of petal development (Stages 0, 2, 3 and 4/5 mixed together). Some of the genes placed on the microarray were not sequenced until after the hybridization had indicated that they showed potentially interesting expression patterns. For the GENESPRING analysis, data was only included from clones that had been shown by sequence analysis to contain potential gene transcripts. Unsequenced clones, clones

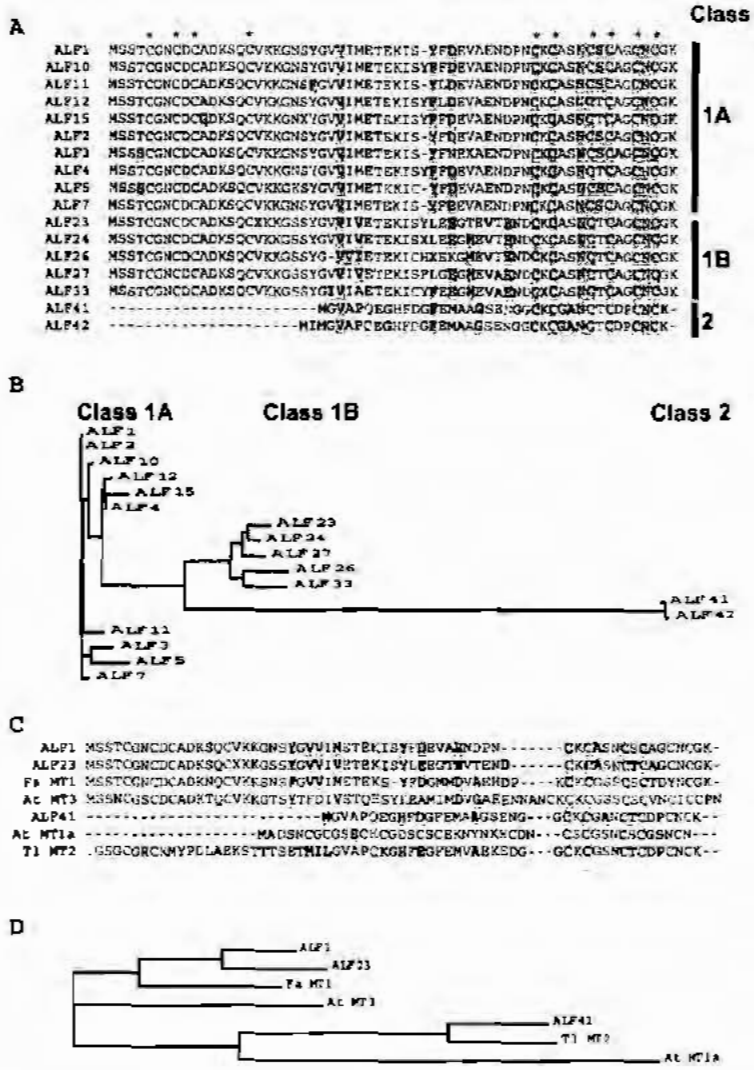

Figure 3 Analysis of metallothionein proteins. (A) Multiple alignment (CLUSTAL W) of the predicted proteins from 17 of the metallothionein contigs identified from the *Alstroemeria* cDNA libraries. Each contig is the result of four or more individual cDNA clones. Conserved cysteine residues are labelled with '*'. (B) Phylogenetic tree from (A) indicating the relationship between the different metallothionein proteins and showing the three different classes identified. (C) Multiple alignment (CLUSTAL W) of representatives of the three different *Alstroemeria* metallothionein genes (ALF1, ALF23 and ALF41) with the most similar genes from other organisms. Fa MT1 (AAB95219 from *Fritillaria agrestis*), At MT3 (AA86723 from *Arabidopsis*), Tl MT2 (AAL09705 from *Typha latifolia*), At MT1a (P43392 from *Arabidopsis*). (D) Phylogenetic tree showing the relationship of the genes aligned in (C).

containing no inserts and clones representing untranslated RNA were excluded from the analysis. A representative clone for each contig was then selected at random and data for 500 cDNA clones, each representing a different gene, was analysed.

From the 500 cDNA clones used for the analysis, 251 showed an expression pattern that was significantly up or down-regulated ($P < 0.05$) for at least one point over the time course. Normalized data indicated the relative expression level of each gene in comparison to all the other genes at that stage of development. The *t*-test *P*-value showed how significantly this normalized figure differed from the average (i.e. a value of 1). The expression level of each gene at each time point (*y*-axis value) was calculated taking account of both the total expression of all genes at that time point to standardize each hybridization, and also the changes in expression level of each gene across the four time points. This allowed the changes in expression levels of each gene over time to be assessed.

The changes in expression of this group of genes is shown in Figure 4A (GENESPRING graphs). Only genes that showed at least a 2.5-fold change in normalized expression level between Stages 0 and 4/5 were then selected for further analysis. From this group, two separate clusters were identified, those showing high expression at Stage 0, decreasing at later stages and those showing low expression levels at Stage 0 increasing at Stages 3 or 4/5 (Figure 4B,C).

To confirm the expression patterns of these selected sets of genes, the experiment was repeated by hybridizing array slides carrying the same cDNA clones, with RNA isolated from a different batch of petals harvested at a different time. At least two slides were hybridized with each RNA sample (i.e. 12 data points, $n = 4$) and the data analysed. The expression patterns of genes

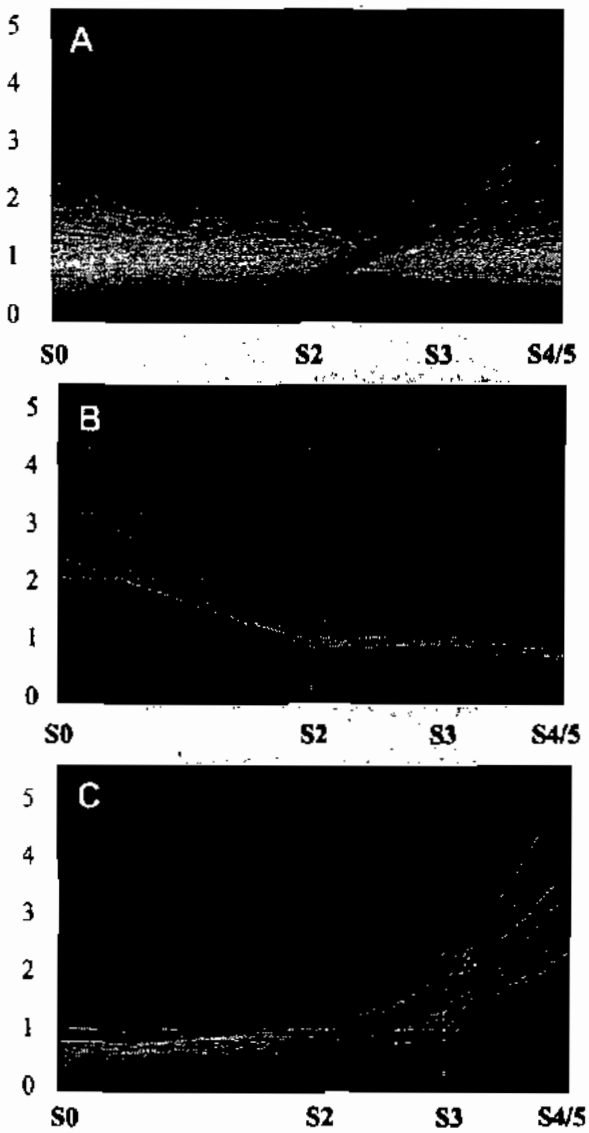

Figure 4 Gene expression during petal senescence. Graphs to show the relative changes in expression of each gene in the different stages of petal senescence. Imagene data files were analysed using GeneSpring version 5.1 (Silicon Genetics). Background values were subtracted from each spot value and then the data was normalized in the following order: measurements less than 0.0 were set to 0.0, whole chip data was normalized to the 50th percentile and data for each gene was normalized to the median. The data shown is the averaged values for eight array hybridizations with each array carrying three copies of each gene. (A) Expression data for 500 EST clones (for genes appearing more than once in the EST collection, a representative cDNA was selected at random from each contig). (B) Cluster of genes showing down-regulation as senescence progresses. (C) Cluster of genes showing up-regulation as senescence progresses.

Table 1 Potential functions of genes showing up or down regulation during post-harvest senescence in *Alstroemeria* petals

Potential role	Number	Examples
Up-regulated genes		
DNA binding/ transcription	5	DNA binding protein PF1
		zinc finger
		RNA polymerase
		DEAD/DEAH box helicase
Signalling	9	protein kinase Xa21 receptor type
		Ras-related GTP-binding protein
		leucine rich repeat protein
		phosphoinositide kinase
		shaggy-related protein kinase
		phosphoinositide phosphatase
		ADP-ribosylation factor-like protein
Transport	6	sugar transporter
		potassium transporter
		ABC transporter family
		Clathrin heavy chain
Protein degradation	4	ubiquitin-conjugating enzyme
		aspartic proteinase
		ATP-dependent Clp proteinase
		collagenase
Carbohydrate metabolism	10	Endoxyloglucan transferase
		cell wall invertase
		xylose isomerase
		sucrose synthase
		triosephosphate isomerase
		Cer1 cuticle biosynthesis
		short-chain dehydrogenase/reductase
Defence/stress related	4	chitinase II
		glutathione peroxidase
		S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase
		ABA-responsive protein
Protein synthesis	5	ribosomal proteins
Secondary product synthesis	3	terpene synthase
		dihydroflavonol 4-reductase
Electron transport	3	NADH dehydrogenase 49 kDa chloroplast
		NADH dehydrogenase mitochondrial
Other	17	cytosolic glutamine synthetase
		cytochrome P450
Unknown/no match	27	
Down-regulated genes		
Signalling	2	receptor protein kinase
		gibberellin induced protein
Amino acid biosynthesis	2	isopropylmalate dehydrogenase, tryptophan synthase
Photosynthesis	2	PSII cytochrome b559, cytochrome b/f
Energy production	1	2-oxoglutarate dehydrogenase
Lipid synthesis	2	sterol desaturase
		fatty acyl coA reductase
Other	1	Cytochrome P450
Unknown/no match	7	

in the two clusters identified as described above were analysed in the new experiment, and in general the patterns of expression were highly comparable. However, a few of the genes did not show a significant change in expression pattern in the repeat

experiment and these were removed from the list. Supplementary Table S3 shows the resulting lists of genes that were significantly altered in expression in both experiments and presents the normalized expression level for each gene at the different time points. From this data, the relative expression change between Stages 0 and 4/5 can be calculated.

The data in Supplementary Table S3 is summarized in Table 1, which shows the potential functions of the genes that are present in the up and down-regulated gene lists. There are considerably more genes represented in the up-regulated list than in the list of down-regulated genes. This may be a reflection of the changes in metabolic activity in the petals. In the early stages, 0 and 2, the petals are already fully formed and are not undergoing dramatic metabolic changes, while in later stages a whole new set of genes involved with the degradation and mobilization typical of senescing tissues is expressed (Table 1).

Northern hybridization

Microarray experiments are extremely useful for showing the relative changes in expression levels of a large number of different genes during development. However, the data from microarrays can be subject to considerable experimental variation, and conclusions from these experiments should be tested using techniques such as Northern hybridization to confirm expression patterns for a select number of genes. Northern hybridization analysis was carried out with a selection of genes from Supplementary Table S3.

A range of genes was selected from the lists shown in Supplementary Table S3 and these showed the expected expression pattern in Northern hybridization experiments (Figure 5). The *MtN19*-like gene, represented by ALF TC139, was identified in the list of genes showing highest expression in Stages 3 and 4/5. The Northern hybridization results support this and show that the expression of this gene continues to increase at later stages of development (Figure 5B). Similarly, the *Cer1* gene, represented by ALF TC46, appeared in the list showing significant increased expression in Stages 3 and 4/5. The Northern hybridization mirrored this expression pattern exactly (Figure 5C). However, unlike ALF TC139, the expression of this gene then decreased at later stages of senescence. The S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase like gene, represented by the contig ALF TC141, was also present in the list of genes showing significantly higher expression in Stage 4/5. The Northern blotting data also supported this observation and showed that the expression of this gene peaked at Stage 5 and then started to fall in later developmental stages (Figure 5D). The 2-oxoglutarate dehydrogenase gene was present in the list of genes showing high expression at Stage 0 with reduced expression later in development. The Northern hybridization results from this gene confirmed this expression (Figure 5E).

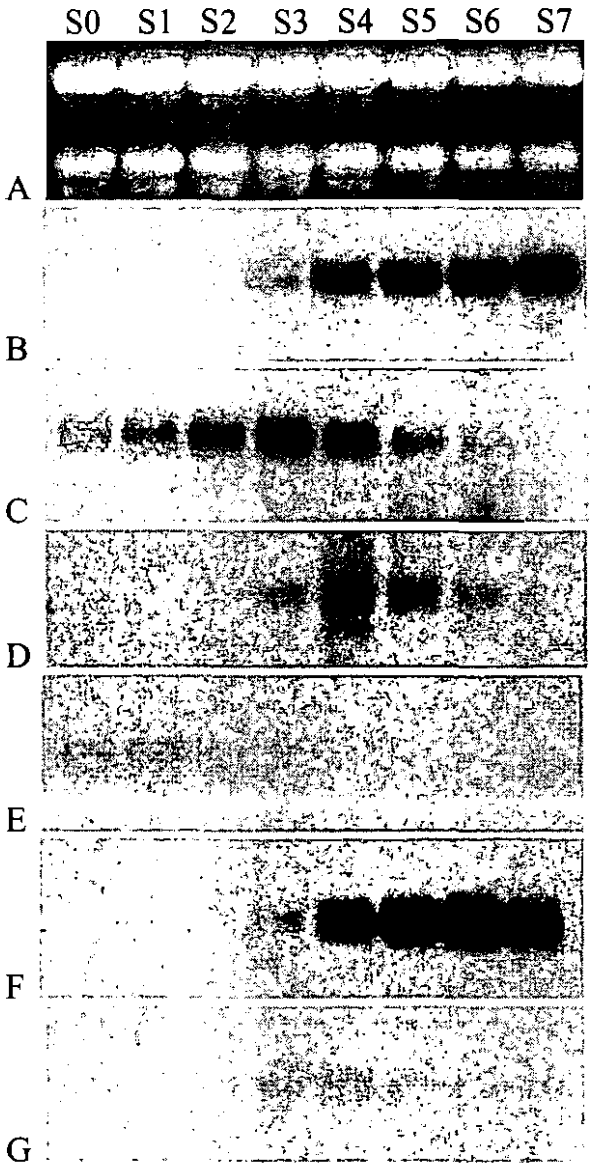

Figure 5 Northern hybridization analysis with selected Alstroemeria EST clones. RNA (10 μg) was separated on a denaturing gel, blotted to nylon membrane and hybridized with ^{32}P labelled EST cDNA fragments. (Contig number; EST GenBank accession number). (A) Ethidium bromide stained RNA gel. (B) MtN19 (ALF TC139; CF569317). (C) Cer1 (ALF TC46; CF569316). (D) SAM.SA carboxymethyl transferase (ALF TC141; CF569321). (E) 2-oxoglutarate dehydrogenase (ALF TC324; CF569319). (F) MT1 (ALF TC1; CF569320). (G) MT42 (ALF TC42; CF569318).

Interestingly, the expression pattern found for the *MET1* gene (ALF TC1) was very different to that seen on the microarrays. This gene and most of the other *MET* contigs were not

represented in the senescence enhanced gene list in spite of the fact that the subtraction data suggested that members of this gene family were very highly expressed in the later stages of development. The Northern data confirms the subtraction results – the expression levels detected on the Northern blot were very high in late developmental stages; the autoradiograph shown in Figure 5 was the result of a film exposure of only 3 h. This shows very high levels of transcript at stages 3, 4, 5, 6 and 7 (Figure 5F). A longer exposure showed that some transcript was detectable at Stage 2, a very small amount at Stage 1, but none at all was detected at Stage 0 (data not shown). Although the probe was made using the ALF TC 1 cDNA, it is likely that the hybridization seen is also to many of the other *MET* contigs of Class 1A and 1B, which were very similar in sequence. The DNA sequence of the Class 2 represented by ALF TC 42 was divergent enough to make cross-hybridization unlikely. The expression pattern of this gene was completely different, with detectable transcript at all stages increasing to Stage 4 and then decreasing (Figure 5G). The expression level of this gene was also quite high, but weaker than that of ALF TC1 (the autoradiograph shown in Figure 5G was also the result of a 3 h film exposure).

Discussion

Characterization of subtracted libraries

The suppressive subtractive hybridization technique (Diatchenko *et al.*, 1996) has been used extensively for the identification of differentially expressed genes in many different organisms. The technique was developed to enrich for differentially expressed genes and at the same time normalize the relative abundance of the different messages in the target population allowing more of the rare transcripts to be cloned. A reported potential drawback of this method is the presence of background clones representing non-differentially expressed genes that can make up a large proportion of the library (Rebrikov *et al.*, 2000). Five subtracted libraries were constructed using RNA isolated from different stages of petal senescence. From these libraries good sequences were obtained for between 100 and 320 different clones (Supplementary Table S2). Analysis of the DNA sequences of the collections of genes in each library revealed a wide range of different potential gene functions. The most obvious difference between the libraries was the proportion of clones representing a metallothionein-like protein which was often identified in the libraries that were enriched for senescence enhanced genes (Stages 2–0, 4/5–2 and 3–2) but very rarely in the other two libraries that were potentially enriched for genes decreasing in expression during senescence (Stages 0–2 and 2–4/5). This comparison indicated that the subtraction procedure had at least been successful for this gene. However, the very high proportion of metallothionein-like genes (83% and 55%, respectively) in the senescence-enhanced libraries Stages 4/5–2 and 3–2 indicated that the suppression of abundant transcripts for this class of gene was not successful. In fact, this proportion of metallothionein clones exceeds that seen in unsubtracted (and unsuppressed) cDNA libraries made from RNA from these developmental stages where metallothioneins make up about 30% of the identified genes (C. Wagstaff, unpublished results). It therefore appears that the very high level of metallothionein transcripts present in the older tissues have been preferentially amplified and cloned in this SSH procedure. This has presumably prevented the detection of the many other transcripts that are present in this tissue in these libraries, as shown by the microarray and Northern hybridization analyses.

The cDNA cloning method has, however, provided an extensive collection of genes that are transcribed during post-harvest petal senescence and the analysis of the potential functions of these can give an indication of the processes that are occurring. The content of the first three libraries, that do not contain many metallothionein transcripts, provides a snapshot of genes that are transcribed in the petals at Stages 0 and 2 (Figure 2B). Overall, the genes identified in the different libraries reflect similar functional classifications to those found in other developmental sequencing projects in plants such as *Lotus japonicus* (Asamizu *et al.*, 2000) and *Citrus sinensis* (Bausher *et al.*, 2003). For example, genes relating to cell wall synthesis, protein synthesis, metabolism and signalling were most abundant in the younger developmental stages. Channeliere *et al.* (2002) carried out a similar analysis on rose petals; they cloned and sequenced 1794 ESTs and showed a range of potential functions comparable to those found in the *Alstroemeria* petals.

Analysis of the microarray data

More information about the genes expressed at the different developmental stages can be obtained from the microarray data, which provides an expression pattern for each gene during development. Many of the genes on the array did not show significant changes in expression across the four time points, indicating that they were expressed at a relatively constant level in all petal stages. Two clusters were identified containing genes either strongly down-regulated during senescence or showing significantly increased expression

between Stage 0 and Stages 3 and 4/5. (Figure 4, Tables S3 and 1). Analysis of the functions of these genes can give an idea of the processes that are occurring at the different stages.

The small number of genes that were only expressed during the early stages, and were down-regulated as senescence commenced, mainly encoded genes involved in lipid and amino acid biosynthesis as well as genes involved in the TCA cycle and in photosynthesis. The presence of these genes indicated that biosynthetic processes are occurring in the young petal and energy is being produced via the TCA cycle and photosynthesis. Petals in Stage 0 are quite green and this is lost in the later stages (Figure 1). The rapid reduction in expression levels of 2-oxoglutarate dehydrogenase, a TCA cycle enzyme, implied that the production of energy via this pathway only occurred in young petals.

Many more genes showed increased expression during the later stages of senescence (Table 1). This indicated that novel pathways are induced during senescence, and the enhanced expression of a wide range of transcription factors and other signalling factors supports the evidence from many plant systems showing that senescence is an active process requiring new transcription and translational events in order to recycle components to other parts of the plant (Thomas *et al.*, 2003). Many of the genes identified as being senescence-enhanced in *Alstroemeria* petals have previously been implicated in senescence in other plants and tissues. Protein degradation is an important process that occurs during senescence, and the enhanced expression of genes such as the ubiquitin conjugating enzyme, the aspartic protease and the Clp proteinase show that this process is occurring in the petal tissues. Similar genes have all been shown to have senescence-enhanced expression in other plant systems (Buchanan-Wollaston *et al.*, 2003) and an aspartic protease was identified in senescing daylily petals (Panavas *et al.*, 1999). Cytosolic glutamine synthetase has a role in the mobilization of nitrogen from senescing leaves (Kamachi *et al.*, 1992) and the presence of this gene in Stage 4/5 *Alstroemeria* petals indicates that nitrogen mobilization is occurring from the petals, presumably to the developing gynoecium (Nichols and Ho, 1975; Nichols, 1976). Genes involved in carbohydrate metabolism such as endoxyloglucan transferase and sucrose synthase have also been identified as showing enhanced expression in senescing *Arabidopsis* leaves (Park *et al.*, 1998; V.B.-W., unpublished data). These genes may have a role in mobilizing C from cell wall components and converting it to sucrose for transport. The senescence-enhanced expression of a sugar transporter has also been reported previously (Quirino *et al.*, 2001).

Stress related genes such as chitinase and glutathione peroxidase are senescence-enhanced in other systems (Hanfrey *et al.*, 1996; Page *et al.*, 2001). The gene encoding S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, which catalyses the methyl esterification of salicylic acid, producing methylsalicylate, has been implicated in defence responses in *Brassica* (Zheng *et al.*, 2001). Moreover, methylsalicylate is a component of the scent of flowers such as *Clarkia breweri* and the activity of the enzyme encoded by this gene is enhanced in mature flowers of this plant, declining several days after anthesis (Dudareva *et al.*, 1998). The gene encoding terpene synthase may also have a role in scent production (Dudareva *et al.*, 2003). The role of these enzymes in the unscented *Alstroemeria* flowers is not clear, but the coordinated expression of both genes during flower development may indicate the presence of a scent production pathway in *Alstroemeria*. Although commercial hybrid *Alstroemerias* such as var. Rebecca used in the work are not scented, native Brazilian cultivars are; it is therefore likely that at least some of the scent producing metabolic pathway is present. In fact, the failure of *Alstroemeria* flowers to be scented could be related to the mutation of a single gene. Thus the potential for generating scented *Alstroemeria* flowers, either by conventional breeding or through genetic manipulation, would appear possible.

The Northern hybridization results generally confirmed the expression patterns seen in the microarrays. A notable exception to this was the result for the metallothionein genes which showed little change in expression on the microarrays, but were strongly up-regulated according to the Northern hybridization. The reasons for this discrepancy are not obvious. There may be some difference in the stringency of hybridization in the two different systems resulting in more background hybridization on the microarray, or it is possible that the large numbers of different metallothioneins in the subtracted libraries, and consequently the many copies on the microarrays, reduced the hybridization intensity to each individual spot. However, the similarity in sequences between the genes would result in hybridization of the labelled probe to all the similar transcripts in the Northern hybridization, indicating a high expression level.

Potential role of metallothioneins

By far the largest group of genes (19%) found in the libraries encoded a Type 3 metallothionein-like protein defined by Cobbett and Goldsbrough (2002). The Northern hybridization experiment showed that an extremely high level of this transcript was present in the older petal stages. The large number (> 20) of different members of this gene family in *Alstroemeria* may be a reflection of the duplication in the

genome in this species. Genes encoding metallothionein-like proteins have also been found in large numbers in the EST collection from rose petals (10.7%) although these were mainly of Type 2 (Channeliere *et al.*, 2002). Of 401 cDNA sequenced as showing differential expression during strawberry fruit development, only one clone was identified as a Type 3 metallothionein and was represented 27 times (Aharoni *et al.*, 2000). A Type 3 metallothionein was also the most abundant transcript found in a recent SAGE analysis of rice leaves (2.83%) and accounted for 3.17% of ESTs in a TIGR rice EST collection (Gibbings *et al.*, 2003). Thus the high proportion of these genes represented in our libraries is not unusual, although the levels are higher than in other systems.

Metallothioneins generally contain two cysteine-rich domains, which are able to bind to a variety of metals through mercaptide bonds, and plant metallothioneins can be divided into four types based on amino acid sequence and distinct arrangements of the cysteine residues (Cobbett and Goldsbrough, 2002). Both Class 1A and Class 1B *Alstroemeria* metallothioneins are members of the Type 3 family of metallothioneins. The *Alstroemeria* Class 2 metallothionein-like genes cannot be classified, since they do not possess the N-terminal cysteine rich region found in all other plant metallothioneins. Thus, they either represent a novel class of metallothionein or may represent a related gene.

The function of metallothioneins in plants is not clear-cut. The isolation of metallothionein proteins from plants has proven difficult due to their instability in the presence of oxygen, and thus their function has largely been inferred from gene expression. Type 3 metallothionein transcripts are highly expressed in ripening fleshy fruits, e.g. in banana (Clendennen and May, 1997), apple (Reid and Ross, 1997) and kiwi (Ledger and Gardner, 1994), and in plants producing non-fleshy fruits, like *Arabidopsis*, they are also expressed at high levels in senescing leaves (Cobbett and Goldsbrough, 2002), however, no direct link to metal binding has been demonstrated for this type of metallothionein. This is the first report of an abundance of Type 3 metallothioneins in senescent petals, and provides an interesting link between processes occurring in fruit ripening and *Alstroemeria* petal senescence. Metallothionein genes have been reported in petals from other species, e.g. daffodil (Hunter *et al.*, 2002) and rose (Channeliere *et al.*, 2002), however, in both cases the metallothioneins reported were of Type 2. Increased expression of metallothioneins during organ senescence has been reported in *Brassica napus* leaves (Buchanan-Wollaston, 1994), elder leaves (Coupe *et al.*, 1995) and rice leaves (Hsieh *et al.*, 1995), however, again these were not Type 3 metallothioneins. Plant senescence is known to generate an increase in free radicals from membrane degradation (Voisine *et al.*, 1993), and may also generate an increase in free metals such as copper from pigment complexes. Interestingly, metallothioneins are up-regulated in the DAF2 strain of *C. elegans* that has an increased life-span (Murphy *et al.*, 2003), where it may be playing a role in preventing or repairing oxidative damage and thereby increasing longevity. In addition, a metallothionein transcript was the most up-regulated transcript in mouse brain tissue that had suffered stroke damage (Trendelenberg *et al.*, 2002). Therefore, there may be a common protective role for metallothioneins in stress responses in both animals and plants.

Conclusions

The application of a suppressive subtractive hybridization technique for the cloning of petal transcripts, combined with the use of microarrays to show the expression pattern for each gene has proved a powerful method for the identification of differentially expressed transcripts. However, the very high abundance of the metallothionein transcripts caused a serious problem in the use of the SSH procedure, since few other transcripts were identified in the senescence enhanced libraries. Moreover, the metallothionein genes did not appear to be senescence enhanced in the microarray analysis, even though the Northern analysis showed that this gene family was very strongly up-regulated during petal senescence. This indicates that none of the methods are without potential pitfalls and shows that the application of a combination of techniques is essential to obtain informative results.

It is clear from the gene expression analysis in this paper that many senescence-related processes are taking place during the post-harvest senescence of *Alstroemeria* petals. It is likely that macromolecule degradation and mobilization are occurring in these organs, since genes related to these functions are expressed. Ultrastructural analysis of developing *Alstroemeria* petals has shown that many structural changes occur before flower opening (Wagstaff *et al.*, 2003) and many degradative processes may be underway, even by the Stage 0 defined in this paper. These findings are consistent with those shown in other floral systems such as *Iris* (van der Kop *et al.*, 2003), although in others such as *Sandersonia* ultrastructural changes occurred only after flower opening (O'Donoghue *et al.*, 2002). Therefore, future work will include the analysis of gene expression patterns in younger bud stages.

As in medical diagnosis, where expression profiling is proving a powerful tool in identifying marker genes to distinguish, for example, between ovarian and colon cancers (Nishizuka

et al., 2003), we believe that these techniques offer the opportunity to identify genes that are indicative of floral quality. A comparison of the expression profiles of floral genes from a range of genotypes with different post-harvest performance could identify biochemical processes causatively linked to ethylene-independent senescence and thus provide targets for chemical or genetic manipulation. Furthermore, based on the results of the present study, it is now possible to design a microarray that would be informative regarding the ageing of flowers even where visible symptoms are not apparent. A similar approach, using expression profiling, is being proposed in several areas of medicine, for example for the detection of micrometastatic breast cancer (Baker *et al.*, 2003). A diagnostic chip for floral quality could be used to identify cultivars with improved post-harvest performance or to identify flowers in which the potential vase life has been reduced due to poor handling practices. This could provide retailers with the opportunity to adjust their vase life guarantees to the consumer to reflect the quality of material whilst helping wholesalers and growers obtain premium prices for better quality produce.

Experimental procedures

Plant material

Alstroemeria flowers (cv. Rebecca) were harvested at Stage 0 of bud development (see Figure 1) from Oak Tree Nursery, Egham, UK and transported dry back to the laboratory. Stems were rehydrated in water for *c.* 30 min before individual cymes were isolated and maintained in vials of dH_2O. The upper two petals of each developmental stage from bud opening to senescence were harvested, immediately frozen in liquid nitrogen and powdered in a mortar and pestle. Ground, frozen tissue was stored at –80 °C until required for RNA extraction.

RNA isolation

RNA was extracted from 0.5 g aliquots of frozen, ground petal tissue using 5 mL Trireagent (Sigma) according to the manufacturer's protocol but with the addition of two phenol : chloroform : isoamylalcohol separations after resuspension of the first pellet in water. The final aqueous layer was ethanol precipitated overnight at –80 °C and after centrifugation the final pellet was resuspended in RNAse-free water. Total RNA was further purified using an RNAeasy purification column (Qiagen) followed by DNAse treatment with RQ1 DNAse (Promega).

Subtracted library construction

Five subtracted libraries were made using RNA from the following stages of petal development: Stage 0 vs. 2; Stage 2 vs. 0; Stage 3 vs. 2; Stage 4 & 5 vs. 2 and Stage 2 vs. 4 & 5.

First strand cDNA was synthesized from 3 μg total RNA using the Smart cDNA synthesis Kit (Clontech). Synthesis of second strand cDNA by LD PCR was optimized to ensure that the ds cDNA was in the exponential phase of amplification. For all templates, amplification was carried out over 17 cycles. PCR-Select cDNA subtraction (Clontech) was performed using Smart ds cDNA according to the manufacturer's protocol, except for the final amplification step, which was carried out over 11 cycles. A subtraction efficiency test was performed for each subtraction as described in the Clontech handbook. The subtracted cDNAs were cloned into the pGEM-T-vector (Promega) and the library was then transformed into *E. coli* JM109 (Promega).

Colony PCR was performed on between 192 and 480 colonies from each subtracted library in order to amplify the inserts for sequencing using M13 forward and reverse primers. DNA was amplified using standard procedures (35 cycles, annealing at 55 °C). PCR products were purified using Millipore MANU 03050 plates and then sequenced using BigDye version 2 (Applied Biosystems) and analysed on an Applied Biosystems 373 sequencer. Database searches were carried out using the BLAST network service (NCBI). EST sequences were trimmed and assigned to contigs using SeqMan (DNASTAR). Alignments of protein sequences were carried out using the VectorNTI ALIGNX programme.

Arrays

Purified PCR products were dried down under vacuum and resuspended in 50% DMSO to give a final DNA concentration of approximately 0.2 μg/μL. Microarrays were printed on CMT-GAPS coated slides (Corning) using a BioRobotics Microgrid II robot. Each slide carried two replicates of an array which itself contained three copies of each target DNA. Slides were baked for 4 h at 80 °C and then stored with dessicant at room temperature.

Probes

Probes were prepared in duplicate with Cy3 and Cy5 reciprocal labelling. Total RNA was treated with RQ1 DNase (Promega), purified with an RNeasy column, vacuum dried and resuspended in ddH_2O to a concentration of 3.33 μg/μL. For each probe, 20 μg total RNA was reverse transcribed to

give either Cy3-dUTP or Cy5-dUTP labelled first-strand cDNA. Briefly, 2 μg oligo $pd(T)_{12-18}$ (Invitrogen) was annealed to the RNA by heating to 70 °C for 10 min and then cooled on ice for 1 min. A master mix containing (final concentrations in a total volume of 19 μL) 1× First Strand buffer (Invitrogen); 1 mM DTT (Invitrogen); 1 mM each dATP, dCTP and dGTP (Invitrogen); 0.2 mM dTTP (Invitrogen); 3 nmols Cy3- or Cy5-dUTP (Amersham Biosciences) and 50 Units SuperScript II reverse transcriptase (Invitrogen) was added and the probe incubated at 42 °C for 1 h. A further 1 μL (50 Units) of SuperScript II was then added and the sample incubated at 42 °C for an additional 1 h. The reaction was stopped using 1.5 μL 20 mM EDTA, and the template RNA degraded by the addition of 1.5 μL 500 mM NaOH and heating to 70 °C for 10 min. Samples were neutralized by adding 1.5 μL 500 mM HCl. The Cy3 and Cy5 labelled probes were then combined and purified using a QiaQuick PCR clean-up column (Qiagen), vacuum dried and re-dissolved in 20 μL ddH_2O.

Microarray slides were pre-hybridized in 5× SSC, 0.1% SDS and 1% BSA for 45 min at 42 °C in a Coplin jar and then washed in ddH_2O for 1 min, rinsed with isopropanol and air-dried.

For hybridization, 2 μL yeast tRNA (4 μg/μL) (Invitrogen) and 1 μL polyA (8 μg/μL) (Amersham Biosciences) were added to the purified probe and denatured at 95 °C for 3 min. The probe (23 μL) was then mixed with 23 μL 2× hybridization buffer containing 50% formamide, 10× SSC and 0.2% SDS pre-warmed to 42 °C and applied to the microarray under a HS-60 22 × 60 mm cover slip (Hybrislip, Grace Biolabs). The array was placed in a hybridization chamber (Corning) and incubated for 16–20 h at 42 °C. After hybridization, the slides were washed by agitation successively in 1× SSC/0.2% SDS for 2 × 4 min; 0.1× SSC/0.2% SDS for 1 × 4 min and 0.1% SDS for 4 × 4 min. The arrays were then dried by centrifugation at room temperature and immediately scanned.

Slide analysis

The slides were scanned using an Affymetrix 428 array scanner at 532 nm (Cy3) and 635 nm (Cy5). Scanned data was quantified using IMAGENE version 4.2 software (BioDiscovery) using the following settings: Spot quality labelling (flags) was defined for empty spots with a signal strength threshold of 1.68 and for poor spots with a threshold of 0.27. Background measurements were taken for each spot and were set to 4.0 pixels for the background buffer and 3.0 pixels for the background width. Signal intensity range was set between 19 and 90% and the background intensity range was set from 4 to 90%.

Imagene data files for signal median, signal standard deviation, background mean and flags were analysed using GENESPRING version 5.1 (Silicon Genetics). Background values were subtracted from each spot value and the data was then normalized according to the standard 1 colour scenario in the following order; measurements less than 0.0 were set to 0.0, whole chip data was normalized to the 50th percentile and data for each gene was normalized to the median. Thus the total fluorescence for each slide was used to normalize the data to allow comparisons to be made between slides.

Each slide carried three replicates of each gene contained within two separate arrays. The scores for the three replicates within each array were immediately averaged in GENESPRING, and the two arrays on each slide were analysed as separate replicates. Therefore if four slides were analysed per treatment, the data obtained was the result of eight replicate arrays. Therefore $n = 8$ in the statistical analysis carried out using GENESPRING.

Genes were identified that showed a significantly different ($P < 0.05$) expression from the majority in at least one stage of development ($n = 8$). From this list of genes, cluster analysis (K-means) was performed to identify two lists of genes that showed either decreasing expression levels over time (higher expression in Stage 0), or increased expression over time (higher expression at Stages 3 and 4/5)

Northern hybridization

This was carried out as described by Buchanan-Wollaston and Ainsworth (1997). DNA probes were PCR amplified EST products labelled with ^{32}P dCTP using the RediPrime random priming labelling kit (Amersham BioSciences).

Acknowledgements

We would like to thank the UK Department of Environment, Food and Rural Affairs (DEFRA) for providing the funding for this work.

Supplementary material

The following supplementary tables are available from http://www.blackwellpublishing.com/products/journals/suppmat/PBI/PBI_059/PBI_059sm.htm. **Table S1A** Alstroemeria Contig information. **Table S1B** GENBANK accession numbers for ESTs contributing to each contig. **Table S2** Genes represented in the different subtracted libraries. **Table S3** Genes showing altered expression during petal senescence.

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ANEXO 5

Programmed cell death (PCD) processes begin extremely early in *Alstroemeria* petal senescence

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Received: *26 March 2003*
Accepted: *5 June 2003*

doi: 10.1046/j.1469-8137.2003.00853.x

Summary

- In the Liliaceous species *Alstroemeria*, petal senescence is characterized by wilting and inrolling, terminating in abscission 8–10 d after flower opening.
- In many species, flower development and senescence involves programmed cell death (PCD). PCD in *Alstroemeria* petals was investigated by light (LM) and transmission electron microscopy (TEM) (to study nuclear degradation and cellular integrity), DNA laddering and the expression programme of the *DAD-1* gene.
- TEM showed nuclear and cellular degradation commenced before the flowers were fully open and that epidermal cells remained intact whilst the mesophyll cells degenerated completely. DNA laddering increased throughout petal development. Expression of the *ALSDAD-1* partial cDNA was shown to be downregulated after flower opening.
- We conclude that some PCD processes are started extremely early and proceed throughout flower opening and senescence, whereas others occur more rapidly between stages 4–6 (i.e. postanthesis). The spatial distribution of PCD across the petals is discussed. Several molecular and physiological markers of PCD are present during *Alstroemeria* petal senescence.

Key words: *Alstroemeria*, *DAD-1* expression, DNA laddering, petal, programmed cell death (PCD), senescence, ultrastructure.

Introduction

The concept of apoptosis (or cell death) originates from the Greek term describing the falling of leaves and petals during natural senescence. Senescence can be thought of as the terminal-phase in the development of leaves and flowers, and thereby involves processes such as protein remobilization and protoplasmic elimination, which are characteristic of apoptosis and PCD. Modern research often uses apoptosis and PCD interchangeably, even in animal systems. However, caution needs to be exercised with the use of these terms. PCD has been defined in animal systems (Ellis *et al.*, 1991) as a type of cell death that is a normal part of an organism's life cycle, is initiated by specific physiological signals, and requires *de novo* gene transcription. Apoptosis was originally used to define particular ultrastructural and biochemical characteristics of cells undergoing PCD, including chromatin condensation, membrane blebbing and DNA laddering (Kerr *et al.*, 1972). However, the notion that all PCD manifests apoptotic features has been challenged (Schwartz *et al.*, 1993); some animal cells appear to undergo physiologically programmed PCD without any of the apoptotic hallmarks. In plants, PCD has been recognized as part of a number of developmental processes including anther development, abscission of plant organs, megasporogenesis in angiosperms, sex determination, hypersensitive responses, tracheary element formation, destruction of the suspensor, and formation of aerenchyma (Jones & Dangl, 1996; Ranganath & Nagashree, 2000). In some of these cell types apoptotic characteristics can be detected, in others however, not all of the recognized signs of apoptosis are

observed, leading some to question the similarities between senescence and PCD (Thomas *et al.*, 2003).

The structural changes that occur during PCD can be followed by electron microscopy. In the early 1970s Matile & Winkenbach (1971) suggested that in *Ipomoea* petals the vacuole acts as an autophage. Invaginations of the tonoplast surround parts of the cytoplasm that are ultimately pinched off, resulting in lysosomal-like compartments within the vacuole. The membrane surrounding these vesicles decays and the cytoplasmic material then appears to be degraded by vacuolar enzymes. Such cytoplasmic degradation is concomitant with the visible collapse of the corolla but structural changes reminiscent of cell death have been described even before flower opening in *Ipomoea* (Phillips & Kende, 1980). In *Sandersonia* there is an increase in the air spaces between the mesophyll cells; this may be the result of tepal expansion without cell division or cell expansion. The mesophyll cells degrade further as the flower senesces such that the only identifiable cells other than epidermal cells are associated with vascular traces (O'Donoghue *et al.*, 2002).

One of the easiest PCD markers to follow at a subcellular level is that of DNA laddering, which in animal cells is the result of the activation of a DNA ladder nuclease or caspase-activated DNase (CAD). However, several authors have questioned the correlation between laddering and apoptosis (Hengartner, 2000), and it has been shown that DNA fragmentation is not an absolute requirement for PCD (Vaux & Korsmeyer, 1999). Evidence for PCD in plants has been based extensively on the presence of DNA laddering, which has been observed in numerous systems. These include carpel cells of *Pisum sativum* (Orzáez & Granell, 1997), cowpea cells infected by fungi (Ryerson & Heath, 1996), during somatic embryogenesis of Norway spruce (Havel & Durzan, 1996), in developing anthers (Wang *et al.*, 1999), in senescing tomato cells (Wang *et al.*, 1996) and in senescing *Petunia* petals (Xu & Hanson, 2000).

Despite some similarities between animal and plant PCD at the cellular level, very few homologues of animal PCD-related genes have been identified in plants. One of the few is *DAD-1* (*defender against apoptotic death*) that has been detected in *Arabidopsis* leaves (Gallois *et al.*, 1997), citrus leaves (Moriguchi *et al.*, 2000) and pea petals where, in all three, levels decline during senescence (Orzáez & Granell, 1997). A direct role in PCD was ascribed to this gene following the isolation of a hamster cell line in which a single amino acid change in the DAD-1 protein caused the cells to become apoptotic (Nakashima *et al.*, 1993). In mammals *DAD-1* encodes a subunit of oligosaccharyltransferase, an enzyme involved in *N*-linked glycosylation (Kelleher & Gilmore, 1997). Yeast and mammals with a disrupted *DAD-1* gene show premature entry into PCD and express abnormal *N*-glycosylated proteins, although this has not yet been proven in plant systems. It has thus been suggested that *DAD-1* may in fact not be a part of the PCD machinery, but rather an essential gene whose disruption triggers cells to enter PCD. Whether the plant DAD-1 protein performs the same function is not known. This gene, however, remains a useful marker as its expression is closely correlated with entry into cell death in several plant systems studied, although not in all (Danon *et al.*, 2000).

The triggering and progression of PCD in plants can be usefully studied using petal senescence as a model system as this is a carefully programmed process; within a given species, it is possible to predict exactly when a bud will open and how rapidly the petals will senesce (Molisch, 1938). However, both the trigger and the co-ordination vary in different plant species. In one group of species the production of a burst of ethylene co-ordinates petal senescence, itself sometimes triggered by pollination (Stead, 1992). However, in another group, ethylene does not appear to co-ordinate senescence, even though it is almost always associated with the final event of petal abscission (Stead & van Doorn, 1994). Thus in these species, although a programme clearly exists, both the nature of the trigger and the co-ordination of cellular events is as yet unclear. *Alstroemeria* belongs to this latter group. Recent studies (Leverentz *et al.*, 2002) indicate that unlike ethylene-insensitive species such as daylily and gladioli (Woltering & van Doorn, 1988; Peary & Prince, 1990; Rubinstein, 2000), lipoxygenase (responsible for oxidation of polyunsaturated fatty acids containing a 1,4 pentadiene moiety) does not appear to play a major role in bringing about petal senescence in *Alstroemeria*. Petal senescence in *Alstroemeria* is, however, associated with an increase in proteolytic activity and a dramatic depletion of complex lipids, suggesting active remobilization of nutrients from the floral tissues (Leverentz *et al.*, 2002; Wagstaff *et al.*, 2001, 2002).

In this paper cytological and molecular markers are used to chart the progression of PCD in *Alstroemeria* petals and thus this paper comprises the first integrated study of plant PCD using molecular, structural and physiological information. These results and observations are presented and used, together with information on this system from previous work (Leverentz *et al.*, 2002; Wagstaff *et al.*, 2002), to present a model for *Alstroemeria* senescence in which a coincidence between changes in membrane integrity and PCD markers is revealed.

Materials and Methods

Plant material

Seven stages (S = stage) of *Alstroemeria peruviensis* var. Samora petal development and senescence were used as described in Table 1 and Wagstaff *et al.* (2001, 2002). Flowers were removed from the plant 2 d before flower and transported back to the laboratory dry. Individual cymes were then removed from each inflorescence and placed into dH_2O. Petals from each stage were used for RNA extraction.

Table 1 Definition of floral stages during flower opening and senescence of *Alstroemeria*

Stage	Day relative to flower opening	Description of floral features
1	−2	Outer sepals pigmented. Tips of sepals loosening
2	0	Sepals reflexed. No anthesis
3	+2	Upper three anthers bent upwards and anthesed
4	+4	Lower three anthers bent upwards and anthesed
5	+6	Separation and reflexing of stigmatic lobes
6	+8	Discolouration of petals. Translucence around margin of sepals. Reproductive organs lying on lower petal
7	+10	Abscission of petals and sepals when lightly tapped

Cell/nuclear area

The top third of each petal and a quadrant from the top of each sepal (from several replicate flowers) was fixed in 3 : 1 ethanol : acetic acid and stored at 4°C. Fixed samples were rinsed in water then hydrolysed with 5 M HCl for 30 min. Following a wash in distilled water for 30 min on ice, samples were Feulgen stained for 1.5 h then rinsed in 45% acetic acid. Cells and nuclei were observed under a light microscope (Olympus BH-2) and images captured using a Fujitsu HC 300Z digital camera. Cell and nuclear areas were measured using SigmaScan (Jandel Scientific, San Rafael, CA, USA). Ten cells nearest the outer margins were examined and in each case cell size and their associated nuclei were measured for each developmental stage.

Electron microscopy

Tissue pieces (approximately 1 × 1 mm^2) from either the edge or the middle of the petal lamina (avoiding the vascular tissue) were fixed in 3% glutaraldehyde: 4% formaldehyde in 0.1 M PIPES buffer (pH 7.6) for 2 h. Post fixation was performed in 1% aqueous osmium tetroxide for 2 h. The material was then dehydrated in a graded series of ethanol and embedded in Spurr resin (at 60°C).

Ultrathin (50–100 nm) cross sections of the petal material were obtained using a glass knife. Sections were collected on standard 200 mesh copper grids and positively stained firstly in uranyl acetate (alc) for 20 min followed by lead citrate solution for 5 min. Micrographs were taken with an H600 Hitachi TEM microscope at 75 kV.

DNA-laddering

Genomic DNA was extracted from 2 g (f. wt) of petals at each stage of development, essentially according to Doyle & Doyle (1987). EDTA was present in the extraction buffer at a final concentration of 25 mM. DNA (30 µg) was run on a 1.5% agarose gel and transferred to nylon membrane by capillary blotting. Pre-hybridization and hybridization were performed at 60°C in a solution containing 5× Denharts, 6× SSC, 0.1% SDS, 5% PEG, 0.1% tetrasodium pyrophosphate and 100 µg l^{-1} denatured herring sperm DNA. Random primed probes were prepared as described in Feinberg & Vogelstein (1983) using 5 µg genomic DNA digested with *Sau*3A.

Cloning of *DAD-1* partial cDNA from *Alstroemeria* petals

RNA extraction and cDNA synthesis were performed as described in Wagstaff *et al.* (2002). Degenerate primers to *DAD-1* (DAD1F: GGGTCRTTYCCHTTYAAC and DAD1R: CAYAGRACGAAAWCWGCAAA) were designed from a comparison of conserved regions of *DAD-1* plant genes. Partial cDNAs were amplified from *Alstroemeria* cDNA using 0.625 units of Qiagen Taq polymerase, Qiagen buffer, 125 ng of cDNA from petals of flowers at stage 1, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 µg of each PCR primer. Reactions were cycled in a Hybaid OMNE machine for 35 cycles of 94°C 1 min, 56°C 1 min, and 72°C 1 min. PCR products were cloned into pGEMEasy T (Promega) and sequenced using an ABI377 automated sequencer.

Semi-quantitative RT-PCR

Specific *Alstroemeria* PCR primers were designed from the partial *ALSDAD-1* cDNA clone (DADAF: GGGTCGTTT-CCATTCAAC and DADAR: CATAGGACGAAATCTG-CAA). An initial PCR reaction was conducted using the *Alstroemeria* specific primers on 125 ng of the cloned partial cDNA to estimate the optimal cycle number for exponential amplification. PCR conditions were as described above, except that an annealing temperature of 54°C was used. Tubes were removed from the thermocycler at 16, 18, 20, 22, 24, 26, 28 and 30 cycles and the products were analysed on a 1.5% agarose gel. A total of 28 cycles produced a barely visible band and was subsequently used for semi-quantitative RT-PCR in which 125 ng of cDNA from the seven defined stages of *Alstroemeria* petals was used as a template under the conditions as described earlier in this section. To test for contamination of the cDNA with genomic DNA, primers spanning a conserved intron in the β-tubulin gene were designed by comparison of available monocot β-tubulin sequences (TUBGENF: GAATGCHGAYGAGTGYATG

and TUBGENR: CGGCGCRAABCCSACCAT). Using these primers with *Alstroemeria* genomic DNA template yields a PCR product of approximately 450 bp compared to the 231 bp from cDNA.

Quantification of expression of *ALSDAD-1*

Quantity One image analysis (Bio-Rad, Hemel Hempstead, UK) was used to quantify the signal from exposure of the radioactive blot to phosphorimager film. The signal was normalized to the data obtained previously of ubiquitin expression from the same batch of cDNA (Wagstaff *et al.*, 2002).

Results

Cell size and cell : nuclear size ratios in petals and sepals

Measurements of cell width and length (Fig. 1a) showed that the change in cell area was due almost entirely to elongation of the cells in one axis. Measurements of cell area showed a gradual increase in size from stage 1 to stage 7 in both sepals and petals (Fig. 1b) with an overall increase of 2.8-fold in petals and 2-fold in sepals. Conversely, nuclear area decreased with developmental stage with an overall decrease of 1.7-fold for petals and 1.8-fold in sepals. Thus the ratio of cell area to nuclear area showed a marked negative correlation with increasing petal age.

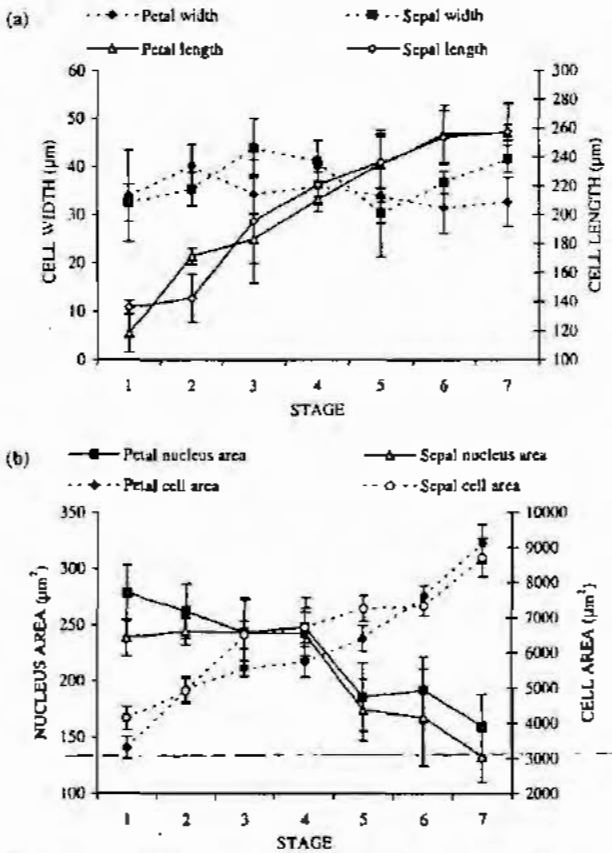


Fig. 1 Whole mount light microscopy measurements of cell and nuclear area. (a) Measurements of cell width and length of epidermal cells near the petal and sepal margins of *Alstroemeria* flowers. The length was taken as the longest side of these rectangular shaped cells. (b) Cell and nuclear area of epidermal cells as described in (a) above. All measurements were taken from captured images using SigmaScan image analysis software with 10 replicates.

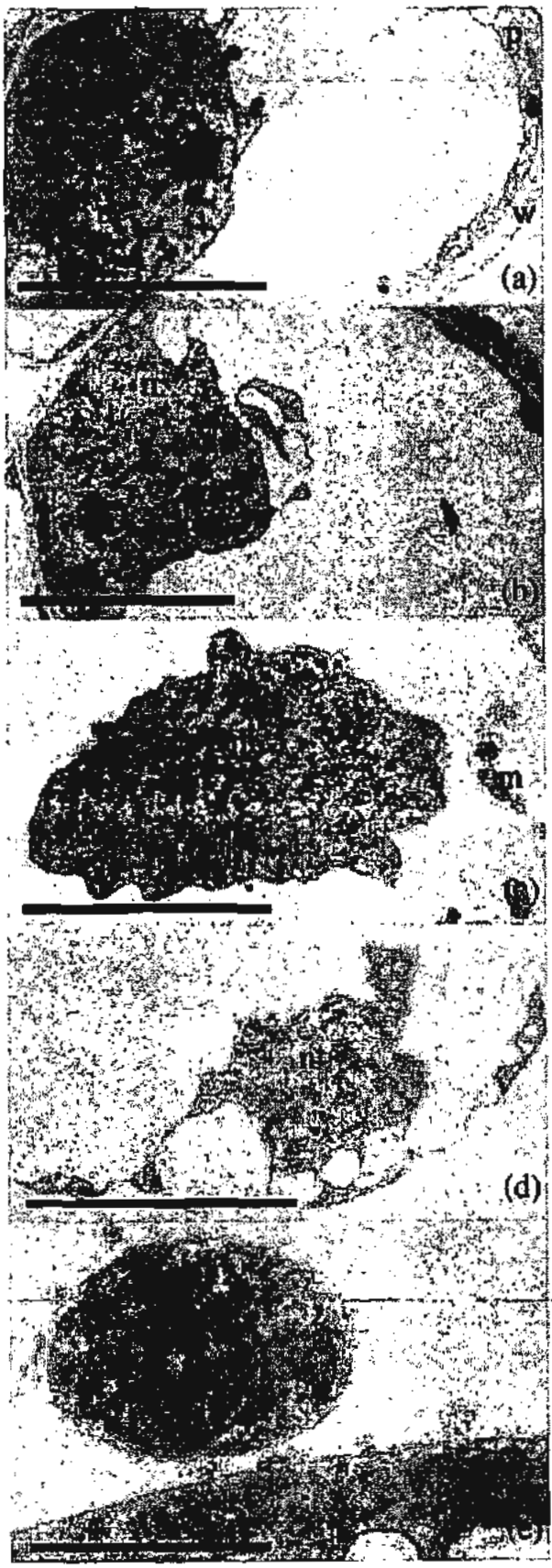
Electron microscopy

Transmission electron microscopy of epidermal cells from *Alstroemeria* petals showed increased nuclear shrinkage with increasing age of the petals (Fig. 2a–e). The reduction in nuclear size is noticeable from just before flower opening (< S1; Fig. 2a) and continues until the oldest stage (S6; Fig. 2e) by which time the nucleus appears to be the only remaining recognizable structure within the cell. The other noticeable change is associated with the cell wall. In young buds the wall is smooth (Fig. 2a) but when fully open the wall is ridged due to outgrowths of the wall (Fig. 2b), there is little further increase in the cell wall thickness, even in the oldest flowers (Fig. 2e). When the flower has just opened (S2) the epidermal cells contained a large vacuole with only a peripheral cytoplasm (Fig. 3) and the nucleus was surrounded by cytoplasm (Fig. 2b). Both the adaxial and abaxial petal surfaces had a ridged cellulose cell wall (Figs 2b and 3) that was absent in the petals of young flower buds (Fig. 2a). Even as the flower opened the integrity of the mesophyll cells at the petal and sepal margins appeared limited since, unlike the epidermal cells, the cytoplasm appeared disrupted with only the small plastids being identifiable (Fig. 3). Furthermore, much cellular material appeared to have accumulated in the intercellular spaces suggesting that the mesophyll cells had become disrupted. The cell walls of both adaxial and abaxial surfaces showed the characteristic ridges seen in many petals. Little or no content remained in the mesophyll cells 4 d after opening, and in places the cell wall appeared to be broken (Fig. 4). Although the epidermal cells remained intact their contents appeared to be reduced with only a very thin cytoplasmic layer in which numerous small lipid-containing plastids were visible.

By stage 5 (6 d after flower opening) many mesophyll cells and some epidermal cells had completely collapsed and lacked any cytoplasm (Fig. 5). This process appeared to continue with one epidermal surface collapsing whist the other remained intact (Fig. 6; Stage 6, 8 d after flower opening) but by this time there was little evidence of the previous mesophyll cell layer other than a series of collapsed cell walls.

DNA laddering

Clear evidence of DNA laddering was obtained, even by visualization with ethidium bromide, but was clearer after the



Southern blotting (Fig. 7). Laddering was present to some extent from 2 d post-flower opening (stage 3), but increased markedly during the final stages of senescence.

Isolation and characterization of a partial *Alstroemeria* DAD-1 cDNA and its expression during petal senescence Degenerate primers were designed by comparison to plant *DAD-1* genes in the databases and used to isolate a partial *DAD-1* cDNA from *Alstroemeria* petal tissue (*ALSDAD-1*). Comparison of the sequence to *DAD-1* genes from other species confirms the putative identity of this cDNA (the nucleotide sequence reported here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under Accession number AJ514409). All the plant *DAD-1* genes show very close homology at the amino acid level.

Semi quantitative RT-PCR was used to investigate the expression programme of *ALSDAD-1* in petals. Early in petal development there was significant expression that then declined soon after flower opening (Fig. 8a). RT-PCR using β-tubulin primers spanning an intron only showed amplification of the correct sized product from the cDNA template (Fig. 8b), with no product of the size expected from the genomic DNA template. Normalization of the *ALSDAD-1* gene expression relative to that of ubiquitin showed that the maximum expression of the *ALSDAD-1* cDNA is more than 7-fold that of the minimum (Fig. 8c).

Discussion

Two of the hallmarks of PCD in animal systems are DNA laddering and nuclear shrinkage (Kerr *et al.*, 1972). Both of these processes are evident in *Alstroemeria* petals. By light microscopy nuclear area declined by nearly 50% of the maximum nuclear area seen at stage 1, however, the decline was continuous suggesting that the maximum nuclear area might be before flower opening. This was confirmed using slightly younger flowers and TEM. Both techniques revealed that the most rapid decline in nuclear area was between stages 4 and 5, i.e. concomitant with the first externally observed signs of petal deterioration. The degeneration of the nucleus occurs against a background of gradually increasing cell size, indeed cell size increased throughout the life of the petal, a situation that has been reported for other flowers (Phillips & Kende, 1980).

In *Alstroemeria* many indicators of PCD appear to start very early, indicating that some cell death is occurring from

Fig. 2 Transmission electron micrographs of nuclei in epidermal cells of *Alstroemeria* petal margins. (a) Tight green bud (< stage 1). Magnification 3.5K, scale bar, 10 μm; (b) Stage 2. Magnification 3K, scale bar, 10 μm; (c) Stage 4. Magnification 7K, scale bar, 5 μm; (d) Stage 5. Magnification 7.6K, scale bar, 5 μm; (e) Stage 6. Magnification 17K, scale bar, 2 μm. Nuclei (n), outer cell wall (w), plastids (p) and mitochondria (m) are labelled.

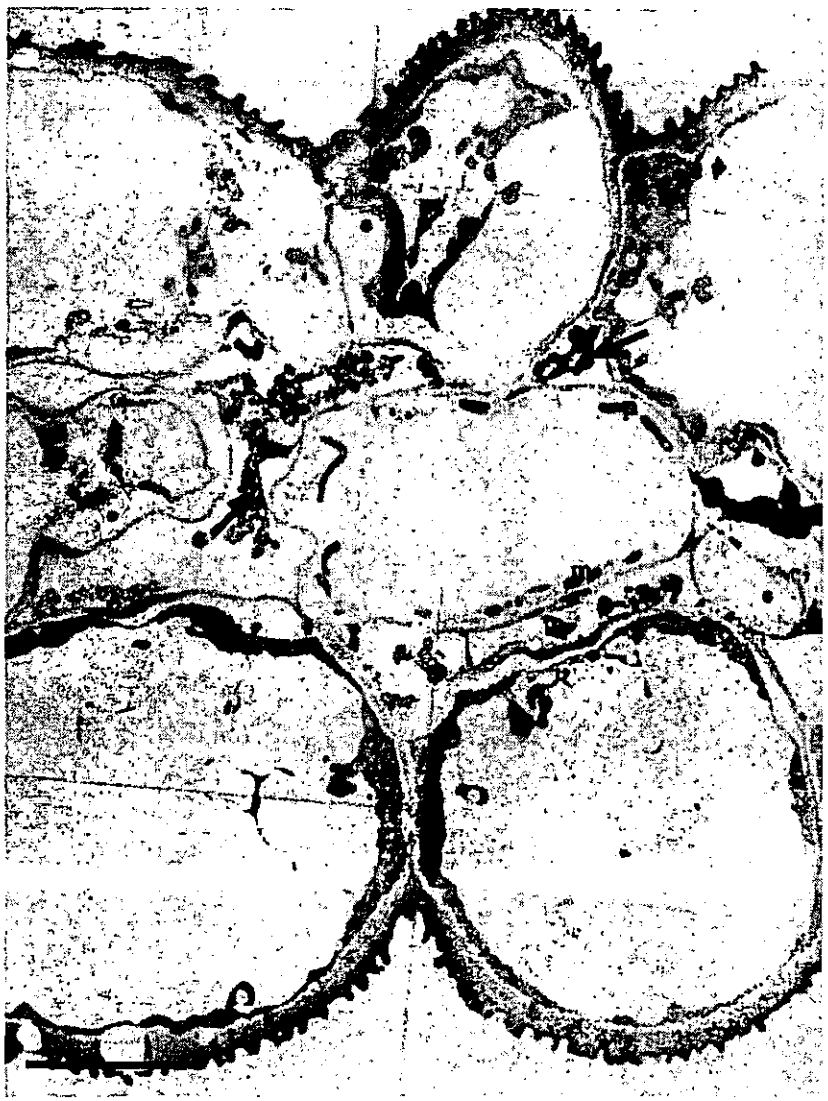


Fig. 3 Transmission electron micrograph montage across petals of *Alstroemeria* near the petal margins at stage 2 of development. The two epidermal layers are clearly visible and mesophyll cells can be identified in between. Epidermal cells have ridged outer cell walls (w); cytoplasm of mesophyll cells is not electron dense and some cytoplasmic contents (arrow) appear to lie in the intercellular spaces. Within the mesophyll cells plastids (p), some of which contain starch, and mitochondria (m) can be identified. Magnification 3K, scale bar, 10 μm.

the earliest stages of flower development. This includes the gradual increase in the expression of a cysteine protease (Wagstaff *et al.*, 2002) starting from the earliest tissues examined (stage 1) and the sharp decline in total LOX activity and lipid content (Leverentz *et al.*, 2002), again starting early in floral development (stage 1 and stage 2, respectively). It also supports the structural data since the reduction in nuclear size occurs from a very early stage of flower development (before Stage 1). However, in addition to an early start of some PCD-associated processes, another feature of petal senescence in this species is that several of these processes appear to accelerate at the time at which the first visible signs of senescence are detectable. Total protease activity (Wagstaff *et al.*, 2002), electrolyte leakage (Leverentz *et al.*, 2002) and DNA laddering all rise sharply around stages 4–5. *DAD-1* expression, used as another marker of PCD in this system, also declines 3-fold between stages 4 and 5. Although DNA laddering has not always been found in systems otherwise showing signs of PCD (Buckner *et al.*, 2000; Herbert *et al.*, 2001), it has been detected in many other plant PCD systems (Jones & Dangl, 1996; De Jong *et al.*, 2000; Ranganath & Nagashree, 2000). A fall in *DAD-1* expression has been associated with petal senescence in peas (Orzáez & Granell, 1997), leaf senescence in citrus (Moriguchi *et al.*, 2000) and with silique maturation in *Arabidopsis* (Gallois *et al.*, 1997). In the majority of theses cases the association of *DAD-1* expression with senescence has been correlative, rather than causative, although a plant *DAD-1* gene was shown to rescue a hamster cell line lacking the mammalian equivalent from entering into cell death (Gallois *et al.*, 1997). In *Alstroemeria* petals, expression was very similar to that in pea petals, being high in young petals and declining in older petals. Put together, these data suggest to us that some key events in *Alstroemeria* petal senescence are occurring at around stage 4–5.

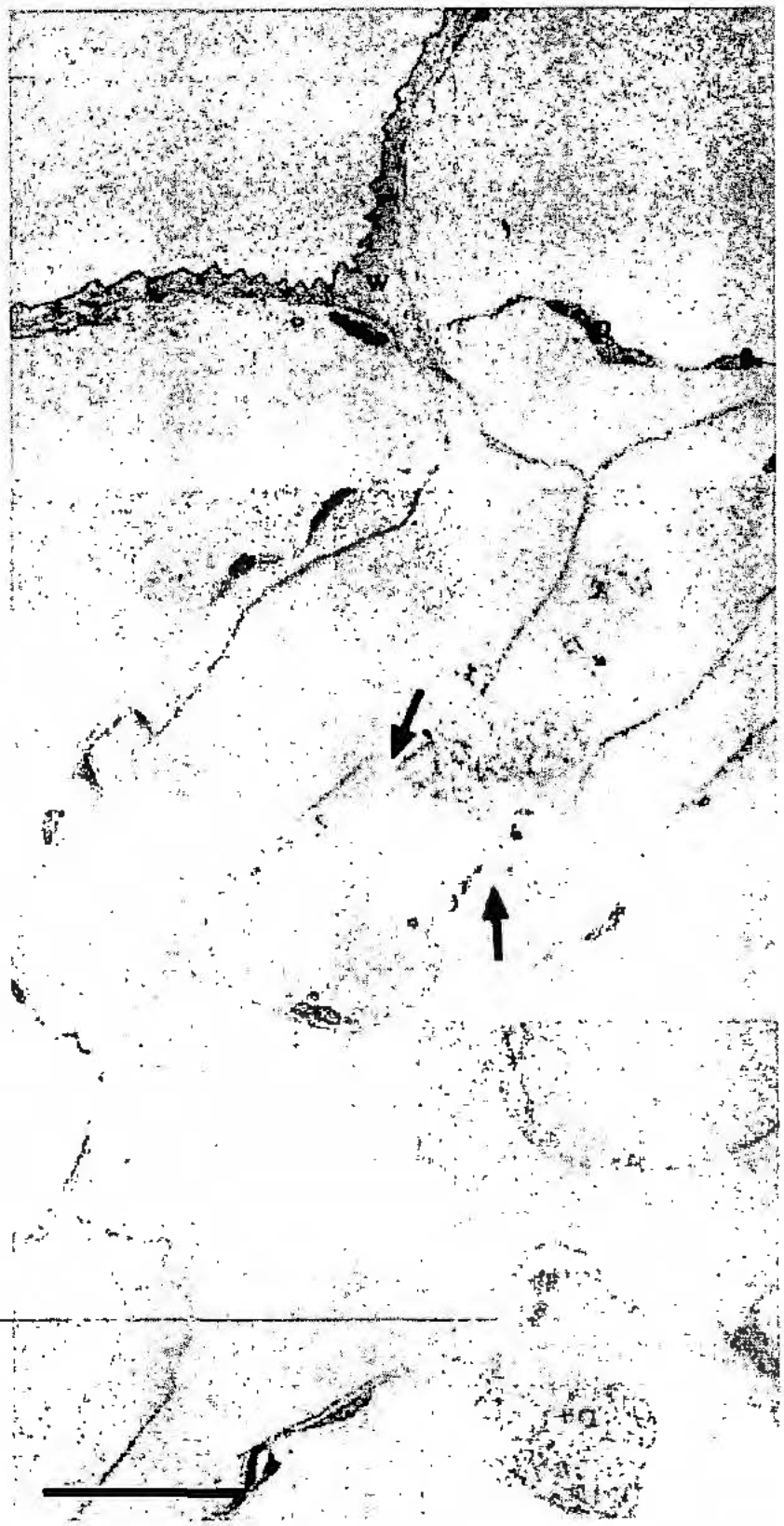


Fig. 4 Transmission electron micrograph montage across petals of *Alstroemeria* near the petal margins at stage 4 of development. Mesophyll cells contain little cytoplasm and the cell wall (w) appears disrupted in places (arrows); epidermal cells contain plastids with numerous lipid droplets (p). Magnification 3K, scale bar, 10 μm.

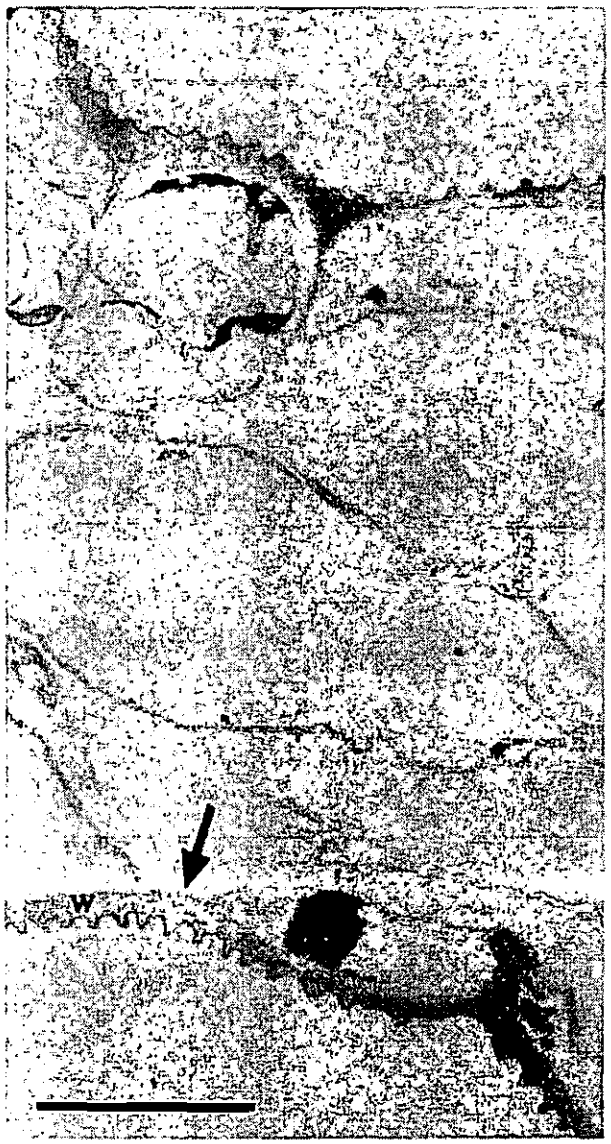


Fig. 5 Transmission electron micrograph montage across petals of *Alstroemeria* near the petal margins at stage 5 of development. Upper epidermal cells appear turgid although cytoplasmic contents are minimal, lower epidermal cells appear squashed with their inner and outer cell walls (w) pressed together in places (arrow). Mesophyll cells are similarly disrupted. Magnification 3K, scale bar, 10 μm.

An important aspect of petal senescence is the spatial distribution of cell death across the petals. Inrolling and wilting in *Alstroemeria* clearly starts in the petal margins, and further studies will need to address whether cell death starts in the margins of the petals, and moves inwards as senescence progresses. Ultrastructural evidence to date suggests that this is likely to be the case with the cells surrounding the vascular tissue being the very last to degrade (data not shown). The very marked loss of contents and collapse of the mesophyll layers of *Alstroemeria* petals occurs against a background of marked epidermal cell expansion. Expansion occurs in just one plane when the petals are viewed from the surface, although evidence from the ultramicrographs suggests that there may be some loss of depth in the epidermal cells during the latter stages of senescence. Previous studies of this system (Wagstaff *et al.*, 2001) have shown that f. wt declines from S4; the collapse of the mesophyll cells at this stage may contribute to the f. wt loss. Thus some cells within the petal remain fully functional and gene transcription and translation are occurring until the latest stages of petal senescence. Although we do not know from which cells transcription and translation occurs, it shows that at least some cells are active (Wagstaff *et al.*, 2002), even when other cells are at an advanced state of senescence. This is in accordance with studies of the ultrastructure and water relations of senescing *Iris* petals (Celikel & van Doorn, 1995; Bailly *et al.*, 2001) and collapsing cells of senescing *Sandersonia* petals (O'Donoghue *et al.*, 2002) and serves to illustrate the importance of examining tissues on a cell by cell basis, as well as looking at the more global picture. The increases seen in DNA laddering and the decline in *ALSDAD-1* gene expression are a reflection of the average state of the whole petal, whereas individual cells, possibly even neighbouring cells, may be in different physiological states.

Bringing together the biochemical, ultrastructural and molecular data for this system (from the data presented in this paper and previous publications) suggests a pattern of sharp acceleration of cell death against a background of increasing nutrient degradation (Fig. 9). Some processes appear to occur gradually, e.g. nuclear condensation and lipid peroxidation, whereas other events, e.g. upregulation of protease activity, loss of membrane integrity as measured by increased conductivity, and DNA laddering are temporally more precise in their occurrence and coincide with a specific stage in the senescence programme when the first visible signs of petal deterioration become apparent. It is as yet unknown in species such as *Alstroemeria*, where ethylene does not seem to act as a co-ordinator, what the trigger for petal senescence might be, or how its progress might be regulated. We propose a model for *Alstroemeria* petal senescence in which the trigger is in fact a threshold effect of one or more of the gradual biochemical processes, e.g. nuclear shrinkage or lipid breakdown. This then initiates the rapid onset of more catastrophic processes, e.g. loss of membrane integrity, DNA laddering, mesophyll collapse resulting in PCD.

This study has illustrated how early some PCD processes start during senescence. Structural degradation occurs to both cell membranes and nuclei before the flower is fully open. The implication of this is that biochemical pathways must be actively degrading these cell components in order to bring about these structural changes, and this has indeed been found for many of the processes discussed in this paper. Bringing together this large body of data in one system allows us to

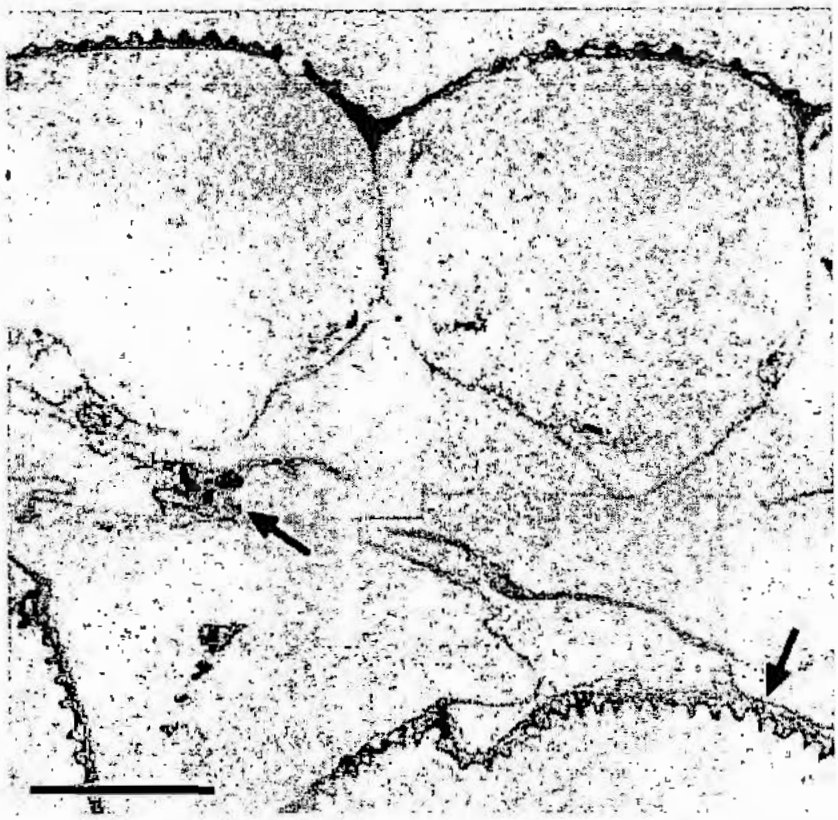


Fig. 6 Transmission electron micrograph montage across petals of *Alstroemeria* near the petal margins at stage 6 of development. Upper epidermal cells appear turgid although cytoplasmic contents are almost absent, lower epidermal cells appear squashed with their inner and outer cell walls (w) pressed together in places (open arrow). Mesophyll cells completely disrupted (solid arrow). Magnification 3K, scale bar, 10 μm.

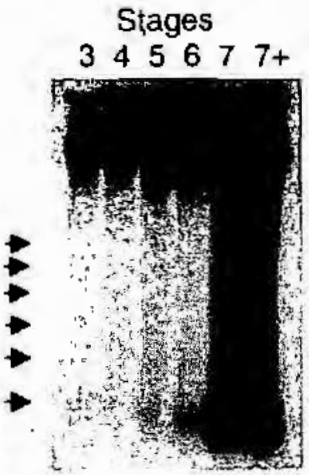


Fig. 7 Southern blot of DNA from *Alstroemeria* petals spanning the stages of development and senescence (Stage 7 + indicates petals which had just abscised). DNA was extracted as described in materials and methods and 30 μg run per lane on a 1.5% agarose gel before transfer to nylon membrane by capillary blotting. The laddering signal was enhanced by hybridization with a probe made from genomic DNA digested with the *Sau*3A restriction enzyme. Arrows indicate the DNA ladder.

reveal a novel pattern of floral senescence. Thus PCD during petal senescence is not simply a gradual running down of resources, and therefore 'death by starvation' for the cell. A number of catastrophic events occur (DNA laddering, electrolyte leakage, increased protease activity) later in senescence that may be triggered by a threshold effect arising from the more gradual biochemical processes. Further studies using microarrays are in progress to sample gene expression across the transcriptome. This broader approach will help to identify some of the early changes in gene expression associated with senescence in this system and therefore point to the biochemical pathways that are of the earliest significance in senescence of *Alstroemeria* petals.

Acknowledgements

The authors would like to thank Gareth Lewis for sequencing and Lyndon Tuck for plant maintenance. The data for epidermal cell and nuclear area was obtained in the laboratory of Dr Dennis Francis (Cardiff) and we are grateful to him and Mike O'Reilly for advising Arfhan Rafiq. Electron microscopy was performed in the EM Unit at Royal Holloway and we are indebted to the staff there for their help. The authors also wish to thank Oak Tree Nurseries, Egham for provision of the bulk of the floral material. The work was funded by (MAFF) DEFRA.

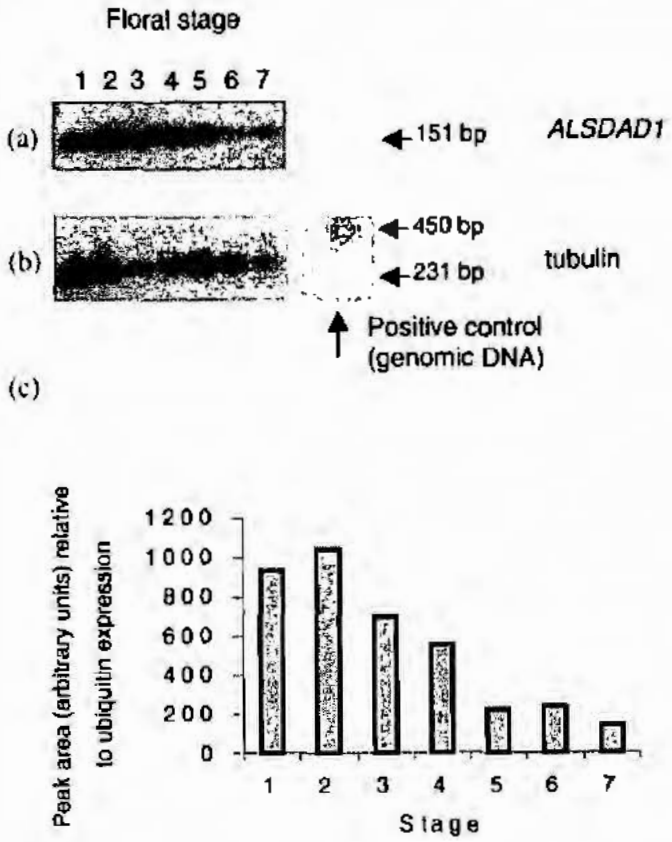


Fig. 8 RT-PCR of *ALSDAD-1* gene expression during development and senescence. The optimal number of cycles (28) was determined in a previous experiment as described in materials and methods such that the amount of PCR product was within the linear phase of amplification and had not reached threshold levels. The products were then transferred to nylon membrane by capillary blotting and hybridized to a probe made from the PCR product of an equivalent reaction and radioactively labelled with ^{32}P. (a) Expression of *ALSDAD-1*. (b) Expression of tubulin using a conserved primer set that amplifies a region from this gene that spans an intron. Genomic DNA was used as a template for the positive control to illustrate the different sized products that would be expected if any contaminating genomic DNA was present in the cDNA used to determine the gene expression of *ALSDAD-1* above. (c) Graph quantifying *ALSDAD-1* expression normalized to ubiquitin expression.

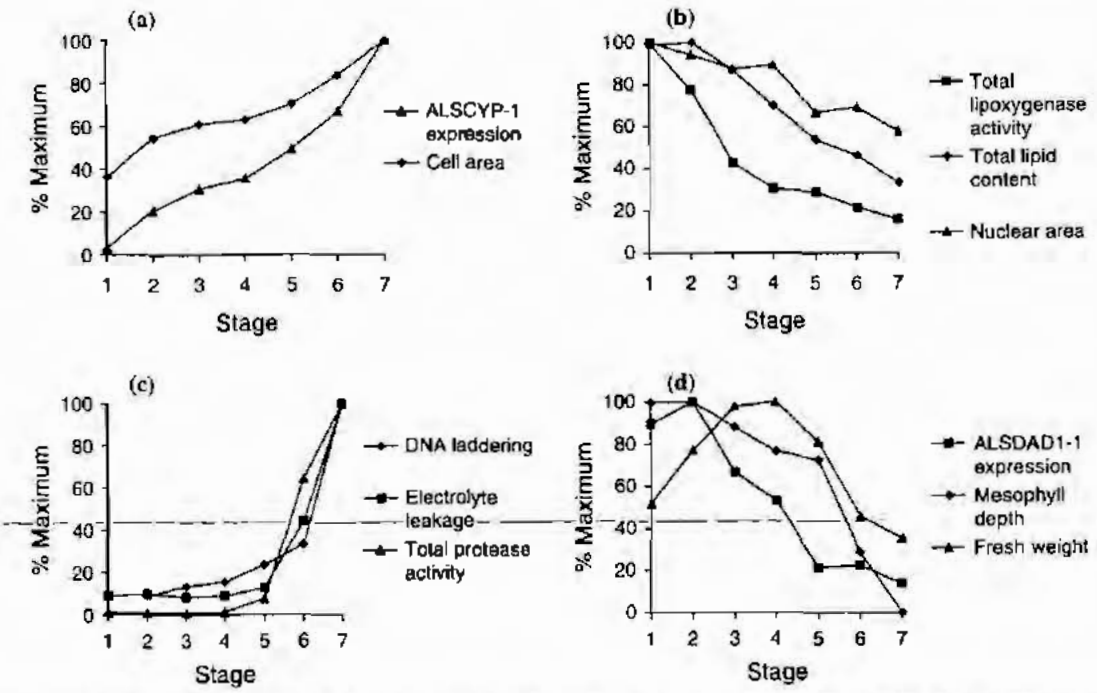


Fig. 9 Summary of programmed cell death in *Alstroemeria*. (a) Events that show a steady increase from the first stage examined. (b) Events that show a decline from the first stage examined. (c) Events showing a sharp acceleration around stage 5 (first visible sign of senescence). (d) Events that show a sharp decrease between stages 4 and 5. Data in part derived from previous published studies by the same group on *Alstroemeria* senescence (Wagstaff *et al.*, 2001, 2002; Leverentz *et al.*, 2002).

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