

TIPS FOR TEXT FORMATTING

Please see a sample reprint at the end of this file

	INCORRECT	CORRECT
Title	FACTORS AFFECTING FRUIT ABORTION IN A GYNOECIOUS CUCUMBER CULTIVAR	Factors affecting fruit abortion in a gynoecious cucumber cultivar
Author(s) name	A. TAZUKE, P. BOONKORKAEW, S. HIKOSAKA AND N. SUGIYAM Or Tazuke A., Boonkorkaew P., Hikosaka S. and Sugiyama N.	A. Tazuke, P. Boonkorkaew, S. Hikosaka and N. Sugiyama Initials followed by surname and use “and” before last authors name if the number is more than one.
Main headings	ABSTRACT, INTRODUCTION, MATERIALS AND METHODS, RESULTS AND DISCUSSION, REFERENCES, ACKNOWLEDGEMENT	Abstract, Introduction, Materials and methods, Results and discussion, References, Acknowledgement Please use upper lower case
Sub headings	Evaluation of the initial period of slow growth: To evaluate the effect of the sum of RGRs, the sum of GRs, and the number of competing fruit on the period of initial slow growth,	Evaluation of the initial period of slow growth: To evaluate the effect of the sum of RGRs, the sum of GRs, and the number of competing fruit on the period of initial slow growth, Subheading should be bold followed with colon and then the text (not bold).
Paragraphs	To evaluate the effect of the sum of RGRs, the sum of GRs, and the number of competing fruit on the period of initial slow growth, Indent by using tab key or space bar not required	To evaluate the effect of the sum of RGRs, the sum of GRs, and the number of competing fruit on the period of initial slow growth, Start paragraph from first column

Use Times New Roman 12 points for text

You can follow the reprint at the end of the file for more clarity of the formatting style.

Please **don't** format the manuscript in **two columns** as given in reprint, formatting will be done by the press.

REFERENCE STYLE

(Use abbreviation list for titles of the journal)

References list should include only published, significant, and relevant sources accessible through a library or an information system. These include research papers, books, thesis, dissertations, proceedings, bulletins, reports, and published abstracts. Unpublished work, or information received personally should be noted parenthetically in the text [e.g., “(M.K. Singh, unpublished data)” or “(J.B. Howard, personal communication)”]. Manuscripts submitted to a publisher may not be used in literature citations unless the work has been accepted for publication, and the work may be cited as “(In press.)” at the end of the citation.

All citations mentioned in the text must be included in the references; also, all references listed in the References must be cited at least at one place in the text. Check the reference list against literature citations in the text before submitting the manuscript for publication. When two or more citations are listed in the text, list the citations chronologically first, then alphabetically, e.g., “(Stassen, 1996; Kader,

2001; Sharma, 2003, 2004).” Authors should ensure that each reference is complete, accurate, and traceable. Authors must see the original source and do not copy a reference from a previous list of citations, because the odds are that at least one error will be copied.

Format

Literature citations follow the last name(s) of the author(s) and the year of the publication cited in the text. References are arranged alphabetically (letter by letter not word by word) by last names of authors (then initials if last names are the same) and chronologically if duplicate author names appear.

Example

If a work has no author, give the name of the organization (committee, agency, etc.) responsible for the publication. If no authority is known, credit the work to the publisher, not to Anonymous. If an editor or editors is given, their names are followed by "(ed.)" or "(eds.)," respectively.

Following the name(s) of the author(s), give the year of publication (the copyright or publication date listed on the publication) followed by a period. If more than one work by the same author or set of authors is cited, list the publications in chronological order and, if the year is also identical, insert lowercase letters (in alphabetical sequence) after the date. All single-authored articles of a given individual precede multiple-authored articles of which that individual is senior author.

Titles should be lowercase except for the first word, proper names, or certain foreign-language conventions. Do not italicize titles except for words or phrases italicized in the title of the published work. Do not use quotation marks around titles. If an article, book, or chapter title has a subtitle (indicated by a dash, colon, semicolon, smaller type, or different typeface), place a colon before the subtitle and capitalize the first letter of the first word. Never abbreviate titles. Titles of non-English publications should retain their native spelling and diacritical marks. Languages that capitalize nouns (such as German) retain their capitalization, but the rest of the title should follow style in lowercase. Do not translate titles into English unless a translated copy was used. Titles that have been translated or transliterated into Roman characters should carry a parenthetical note [e.g., “(in Spanish)”] before the period ending the title.

Don’t abbreviate the titles with one-word names, e.g., *HortScience*, *Euphytica*, *Hilgardia*, *Nature*, *Phytopathology*, *Science*. Italicize publication titles. Capitalize the first letter of all words, but delete extraneous prepositions and articles. Abbreviate the roots of words when they stand alone or with a prefix, e.g., Anal. Biochem. (See “Abbreviations for Citation” for abbreviations of commonly used words in periodical titles.) Give the volume number in Arabic numerals, followed by the issue number (if available) in Arabic numerals in parentheses. Issue numbers are only necessary if the publication’s pages are renumbered from 1 with each issue within a volume. The pagination of the publication follows, connected to the volume number and/or issue number by a colon, and one space after “:” 56(6): 545–548. Give full pagination, e.g., use “1081–1082,” not “1081–2” or “1081–02.”

Precautions while formatting references

Precaution	Incorrect	Correct
<p>Surname of the first author followed by initials.</p> <p>Initials of second author followed by surname.</p> <p>Each author name separated by “,”</p> <p>Use “and” to separate author name if authors more than two.</p>	<p>Goldberg, D., Cornat B. and Bar Y. 1991. The distribution of roots, water, and minerals as a result of trickle irrigation. <i>J. Amer. Soc. Hort. Sci.</i> 96: 645-648.</p>	<p>Goldberg, D., B. Cornat and Y. Bar, 1991. The distribution of roots, water, and minerals as a result of trickle irrigation. <i>J. Amer. Soc. Hort. Sci.</i>, 96: 645-648.</p>
<p>No space between initials</p>	<p>N. F. Weeden</p>	<p>N.F. Weeden</p>

In case of more than 2 authors comma after the surname of the last author	Goldberg, D., B. Cornat and Y. Bar. 1991.	Goldberg, D., B. Cornat and Y. Bar, 1991.
Please use comma after the name of the journal	Goldberg, D., B. Cornat and Y. Bar, 1991. The distribution of roots, water, and minerals as a result of trickle irrigation. <i>J. Amer. Soc. Hort. Sci.</i> 96: 645-648.	Goldberg, D., B. Cornat and Y. Bar, 1991. The distribution of roots, water, and minerals as a result of trickle irrigation. <i>J. Amer. Soc. Hort. Sci.</i> , 96: 645-648.
Space between colon and page number	<i>J. Amer. Soc. Hort. Sci.</i> , 96:645-648.	<i>J. Amer. Soc. Hort. Sci.</i> , 96: 645-648.
Use “-“ between page numbers not “—“	96: 645—648.	96: 645-648.
Use stop at the end of the reference	<i>J. Amer. Soc. Hort. Sci.</i> , 96: 645-648	<i>J. Amer. Soc. Hort. Sci.</i> , 96: 645-648.
Name of the journal in italics	<i>J. Amer. Soc. Hort. Sci.</i> , 96: 645-648	<i>J. Amer. Soc. Hort. Sci.</i> , 96: 645-648.

Electronic citations should include: (i) Name of author, editor, compiler, or translator of the source. (ii) Year of electronic publication; (iii) Title; (iv) Complete electronic address.

Specific examples of references

Commonly used references in Journal of Applied Horticulture.

PERIODICAL

Goldberg, D., B. Cornat and Y. Bar, 1991. The distribution of roots, water, and minerals as a result of trickle irrigation. *J. Amer. Soc. Hort. Sci.*, 96: 645–648.

ABSTRACT

Fu, J., N.F. Weeden, J.R. Kikkert, B. Wang and S.K. Brown, 1999. Development of a BAC library from Russian Seedling R12740-7A, an apple selection with two genes conferring resistance to scab. *Plant & Animal Genome VII* (Abstr. P108).

ABSTRACT FROM HORTICULTURAL ABSTRACTS

Guariento, M. 1977. Semi-forcing and protection of table grapes. *Vignevini* (1977) 4(6/7): 39-46. (Hort. Abstr., 48(3): 2195; 1978),

BOOK

Hartmann, H.T., D.E. Kester and F.T. Davies, Jr. 2002. *Plant Propagation: Principles and Practice*. Seventh Edition. Prentice-Hall, Englewood Cliffs, NJ.

BOOK CHAPTER

Janick, J., J.N. Cummins, S.K. Brown and M. Hemmat, 1996. Apples. p.1-77. In: *Fruit Breeding*, Volume I: Tree and Tropical Fruits, J. Janick and J.N. Moore (eds.). John Wiley & Sons, Inc.

BULLETIN

Brown, S.K., R.C. Lamb and D.E. Terry, 1986. Peach and nectarine varieties in New York state. *NY Food Life Sci. Bull.*, 117. Cornell Univ., Geneva, NY.

ELECTRONIC CITATION

WSU Apple Breeding Program, 2004, <<http://hort.tfrec.wsu.edu/breed.php>>

PROCEEDINGS

American Society for Horticultural Science. Tropical Region, 2000. Proc. XVIII Annu. Mtg., Miami, 25–30 Oct. 2000. (*Proc. Trop. Reg. Amer. Soc. Hort. Sci.*, 14).

PROCEEDINGS PAPER

Johnson, H.A., J.L. McLaughlin and J. Gordon, 1996. Monthly variations in biological activity of *Asimina triloba* (The North American pawpaw tree). *Progress in New Crops: Proceedings of the Third National New Crops Symposium* (J. Janick, ed.), John Wiley & Sons, New York, 1996, p. 609-614.

REPORTS

U.S. Department of Agriculture, 2006. *Agricultural statistics for 2005*. U.S. Dept. Agr., Washington, D.C.

THESIS OR DISSERTATION

Morse, S.I. 2000. *The Pennsylvania Horticultural Society and the Urban Landscape, 1827–1927*. Ph.D. Diss., Temple University, 2000. 188 pp.

Please follow following abbreviations for title of the journal

ABBREVIATIONS

The following list gives some of the more commonly used abbreviations in literature citations (note the words that are not abbreviated). When the proper abbreviation is in doubt, spell out the word; editors will abbreviate if appropriate. Generally, any word ending in “ology” is abbreviated “ol.” and any word ending in “culture” is abbreviated “cult.”

Word	Abbreviation	Word	Abbreviation
Abstract	Abstr.	Culture	Cult.
Academy	Acad.	Cytology, -ical	Cytol.
Acta	Acta	Department	Dept.
Advances	Adv.	Development	Dev.
Agriculture	Agr.	Digest	Dig.
Agronomy	Agron.	Disease	Dis.
America, -an	Amer.	Dissertation	Diss.
Analytical	Anal.	Distribution	Distrib.
Annals	Ann.	Division	Div.
Annual	Annu.	Ecology, -ical	Ecol.
Applied	Appl.	Economy	Econ.
Archives	Arch.	Education	Educ.
Associate(s), -ed	Assoc.	Encyclopedia	Encycl.
Association	Assn.	Engineers, -ring	Eng.
Australian	Austral.	Enology	Enol.
Austrian	Aust.	Entomology, -ical	Entomol.
Biochemistry	Biochem.	Environment	Environ.
Biology	Biol.	Experiment	Expt.
Biotechnology	Biotechnol.	Extension	Ext.
Botany	Bot.	Fertilizer	Fert.
Breeding	Breeding	Food	Fd.
British, Britain	Brit.	Forestry	For.
Bulletin	Bul.	Gazette	Gaz.
Bureau	Bur.	General	Gen.
Canada, -ian	Can.	Genetics	Genet.
Center	Ctr.	Government	Govt.
Chemical, -istry	Chem.	Handbook	Hdbk.
Circular	Circ.	Heredity	Hered.

Citriculture	Citricult.	Horticulture, -ae, -al	Hort.
Climatology, -ical	Climatol.	Indian	Indian
College	College	Industry, -ial	Ind.
Colloquium	Colloq.	Information	Info.
Commonwealth	Cmwlth.	Institute, -ion	Inst.
Communication	Commun.	International	Intl.
Conference	Conf.	Irrigation	Irr.
Congress	Congr.	Japanese	Jpn.
Contribution(s)	Contrib.	Journal	J.
Cooperative	Coop.	Laboratory, -ies	Lab.
Leaflet	Lflt.	Science(s)	Sci.
Letters	Lett.	Scientia	Scientia
Magazine	Mag.	Scientific	Scientific
Management	Mgt.	Series	Ser.
Market	Mkt.	Quarterly	Qrtly.
Marketing	Mktg.	Region	Reg.
Meeting	Mtg.	Regulator, -ion, -y	Regulat.
Meteorology, -ical	Meteorol.	Report(s)	Rpt.
Microscopy	Microsc.	Reporter	Rptr.
Molecular	Mol.	Research	Res.
Monograph	Monogr.	Resources	Resources
Mycology, -ical	Mycol.	Review(s), Revue(s)	Rev.
National	Natl.	Service	Serv.
Nematology, -ical	Nematol.	Society	Soc.
Netherlands	Neth.	Soil	Soil
New Zealand	N.Z.	Special	Spec.
Newsletter	Nwsl.	Standard	Std.
New York	NY	Station	Sta.
Nucleic	Nucl.	Statistics, -ical	Stat.
Nutrition, -al	Nutr.	Supplement(s)	Suppl.
Official	Offic.	Symposium	Symp.
Pathology, -ical	Pathol.	Technical, -que	Tech.
Photosynthesis	Photosyn.	Technology, -ical	Technol.
Physics, -ical	Phys.	Temperature	Temp.
Physiology, -ical, -ia	Physiol.	Thesis	Thesis
Phytology, -ical	Phytol.	Transactions	Trans.
Phytopathology, -ical	Phytopathol.	Tropical	Trop.
Planta	Planta	United States	U.S.
Plantae, -arum	Plant.	University	Univ.
Pomology, -ical	Pomol.	Variety, -ies	Var.
Proceedings	Proc.	Vegetable(s)	Veg.
Products	Prod.	Virology	Viol.
Progress	Prog.	Viticulture	Viticult.
Propagation	Prop.	Workshop	Wkshp.
Protection	Protection	Yearbook	Yrbk.
Publication(s)	Publ.		

UNITS AND ABBREVIATIONS

Please use metric measurements in SI units. If, non-SI units have to be used, the SI equivalents should be added in parentheses on first mention. Units should be spelled out except when preceded by a numeral, when they should be abbreviated g, mg, mL, d, h, ha *etc.* (not followed by full stops). Use the minus index (m^{-1} , L^{-1} , h^{-1}) except in such cases as 'per plant' or 'per pot'. Numbers up to ten should be given in words except when referring to measurements: give 11 and upwards as numerals, except at the beginning of a sentence. Fractions should be expressed as decimals. Use '%' not 'per cent' in the text. Dates should be as: 1 Jan, 2011.

GUIDE FOR FIGURES

- Please make graphs with a width of 3.5 or 7 inches according to the size to fit in the column(s)
- In place of colour, use black and white graphs and patterns in place of grey shades.
- Avoid 3D graphs until unless required for explaining 3 parameters.
- Use Arial font (8pt) for labeling the figure. Dont use bold letters
- Font size should be same in entire figure
- Apply DMRT for separation of means (example http://horticultureresearch.net/Graphs_DMRT_other_test.pdf)
- In bar graphs space between bars should be less than the bar width
- Export or print graphs directly to pdf from software it was made (eg. If made in excel print to from excel. Please do not scan for making pdf)
- Include all graphs in single pdf file

TABLE FORMAT SUITABLE FOR PRINTING

- Don't use space bar, tab or enter key for formatting table, rather use separate cell for different entry/text/value.
- Use Times New Roman font size 9 Pt.
- Avoid abbreviated column headings e.g. use September in place of Sept.
- Please don't use bold font for tables

Table 1. Relative humidity, relative sunshine duration, maximum, mean and minimum temperature in orchard from 15 September to 19 September

Parameter	15 September	16 September	17 September	18 September	19 September
Relative humidity (%)	70.0	66.0	45.0	46.0	51.0
Relative sunshine duration (%)	74.0	50.0	83.0	54.0	75.0
Maximum temperature (°C)	22.5	16.5	17.5	15.6	15.6
Mean temperature (°C)	12.4	7.9	10.7	10.3	9.6
Minimum temperature (°C)	-1.1	4	1.9	6.4	1.7

EXAMPLES OF TABLE **NOT SUITABLE** FOR PRINTING

- Don't use grid lines for entire table as used in following table. Only use lines as given in above table

Parameter	15 Sept.	16 Sept.	17 Sept.	18 Sept.	19 Sept.
Relative humidity (%)	70.0	66.0	45.0	46.0	51.0
Relative sunshine duration (%)	74.0	50.0	83.0	54.0	75.0
Maximum temperature (°C)	22.5	16.5	17.5	15.6	15.6
Mean temperature (°C)	12.4	7.9	10.7	10.3	9.6
Minimum temperature (°C)	-1.1	4	1.9	6.4	1.7

- Don't print several values of a column in a single cell. Use separate table row for each data record **not by using enter** or **using space** rather use **separate cell** of the table:

Table 1. Relative humidity, relative sunshine duration, maximum, mean and minimum temperature in orchard from 15 Sept. to 19 Sept. The application of 7 % lime sulphur was performed at 30 % bloom, on 16 Sept

Parameter	15 Sept.	16 Sept.	17 Sept.	18 Sept.	19 Sept.
Relative humidity (%)	70	66	45	46	51
Relative sunshine duration (%)	74	50	83	54	75
Maximum temperature (°C)	22.5	16.5	17.5	15.6	15.6
Mean temperature (°C)	12.4	7.9	10.7	10.3	9.6
Minimum temperature (°C)	-1.1	4.0	1.9	6.4	1.7

Reduced ethylene production in transgenic carnations transformed with ACC oxidase cDNA in sense orientation

Takayuki Inokuma¹, Tomoyuki Kinouchi¹ and Shigeru Satoh^{1, 2*}

¹Graduate School of Agricultural Sciences, Tohoku University, Sendai 981-8555, Japan. ² Present address: Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto 606-8522, Japan. *E-mail: ssatoh@kpu.ac.jp

Abstract

'Lillipot' carnation, which is usually cultivated as a potted ornamental, was transformed with a cDNA for carnation 1-aminocyclopropane-1-carboxylate (ACC) oxidase. Two lines, which harbor an *sACO* transgene, had a vase life of cut flowers more than twice longer than that of the non-transformed (NT) control. Flowers of the long vase life lines senesced with discoloring and browning in petal margins, which is typical to ethylene-independent senescence in carnation flowers. They produced negligible amount of ethylene for the first 8 day, whereas flowers of the NT control showed a climacteric ethylene production with a maximum on day 3. Transcripts for *DC-ACSI* and *DC-ACOI* were absent in petals of the long vase life flowers undergoing senescence. The present study revealed that transformation with *sACO* transgene may be useful to generate potted carnation plants with a long display time.

Key words: ACC oxidase gene (*DC-ACOI*), *Dianthus caryophyllus*, ethylene biosynthesis, flower senescence, potted carnation

Introduction

Carnations are used as ornamentals as potted plants and as cut flowers. Ethylene is a primary plant hormone involved in the senescence of cut carnation flowers (Reid and Wu, 1992). It is synthesized in a large amount, mostly from the petals, at a later stage of flower senescence (Borochoy and Woodson, 1989; Abeles *et al.*, 1992; Reid and Wu, 1992; Woodson *et al.*, 1992). Increased ethylene production accelerates wilting of the petals. Inhibition of the synthesis or action of ethylene delays the onset of senescence and extends the vase life of flowers.

In senescing carnation flowers, ethylene is first produced in the gynoecium, and the ethylene evolved acts on petals and induces autocatalytic ethylene production in the petals. This results in petal wilting (Jones and Woodson, 1997; Shibuya *et al.*, 2000; ten Have and Woltering, 1997). Ethylene is synthesized through the pathway: L-methionine → S-adenosyl-L-methionine → ACC → ethylene. ACC synthase and ACC oxidase catalyze the last two reactions (Kende, 1993; Yang and Hoffman, 1984). So far, three genes encoding ACC synthase (*DC-ACSI*, *DC-ACS2* and *DC-ACS3*) and one gene encoding ACC oxidase (*DC-ACOI*) have been identified in carnations (Henskens *et al.*, 1994; Jones and Woodson, 1999; Park *et al.*, 1992; Wang and Woodson, 1991). Out of these genes, *DC-ACSI* and *DC-ACOI* have been shown to play a pivotal role in ethylene production in both the gynoecium and petals of senescing carnation flowers (Nukui *et al.*, 2004; Satoh and Waki, 2006).

Currently, prevention of senescence of carnation flower is being attained by treatment of the flower with chemical preservatives which inhibit the synthesis or action of ethylene (Veen, 1979; Midoh *et al.*, 1996). Another option for preventing senescence is the generation of transgenic flowers with suppressed production or action of ethylene. So far, the lines transformed with cDNAs for carnation 1-aminocyclopropane-1-carboxylate (ACC) oxidase (*DC-ACOI*) and ACC synthase (*DC-ACSI*) in sense or antisense

orientation (Savin *et al.*, 1995; Kosugi *et al.*, 2000, 2002; Iwazaki *et al.*, 2004) and a line harboring an *Arabidopsis thaliana etr1-1* allele capable of rendering ethylene insensitivity (Bovy *et al.*, 1999) have been generated. Cut flowers of the transgenic lines have a prolonged vase life compared with those of non-transgenic plants.

The preservatives described above are considered to be not applicable to potted plants, since they are usually administered to cut flowers through vascular transport by immersing the cut stem end in solutions containing the preservative. Therefore, the generation of transgenic plants is a promising way to retard senescence of flowers of potted carnations, *i.e.*, to lengthen their display time. Kinouchi *et al.* (2006) recently generated potted carnation plants transformed with cDNAs for carnation ACC synthase (*DC-ACSI*, *s/aACS* transgenes) or ACC oxidase (*DC-ACOI*, *s/aACO* transgenes) in sense or antisense orientation or mutated carnation ethylene receptor cDNA (*DC-ERS2'*) by *Agrobacterium*-mediated gene transfer. They partly characterized the transformants by investigating the conversion of exogenously-applied ACC to ethylene in leaflet segments. A performance test of the transformants as potted plants remains to be carried out. However, we should first know the synthesis and action of ethylene in flowers in each transformant to select the best line for the large scale performance test. Therefore, in this study, we cultivated several lines of the transformants on soil until flowering, and characterized their senescence, ethylene production and gene expression in cut flowers.

Materials and methods

Plant materials: Plantlets of the transgenic lines of carnation (*Dianthus caryophyllus* L. cv. Lillipot), generated previously (Kinouchi *et al.*, 2006), and the NT control were grown *in vitro* to about 5 cm in height, were transplanted into a commercial horticulture soil in a plastic container, under conditions described previously (Iwazaki *et al.*, 2004) in a containment green house

at Tohoku University. Out of 39 transgenic carnation lines generated previously (Kinouchi *et al.*, 2006), 6 transgenic and the non-transformed (NT) control lines were used since these lines flowered one year after transplanting to soil. Three transgenic lines studied were pMLH-sACO-2, -3 and -12, which were transformed with carnation *DC-ACO1* cDNA in sense orientation (*sACO* transgene) in a pMLH2113 vector. Two other transgenic lines, pIG-sACS-1 and pIG-sACO-1, were transformed with carnation *DC-ACSI* and *DC-ACO1*, respectively, in sense orientation (*sACS* and *sACO* transgenes) using the pIG121 vector. Finally, pIG-DC-ERS2'-2 was transformed with a mutated carnation ethylene receptor cDNA (*DC-ERS2'*) in the pIG121 vector. Flowering started around one year after transplanting. Flowers were harvested during the following 5-6 months. Only the first and second flowers opening on each stem were used.

Analysis of vase life of cut flowers: Three to ten flowers, depending on the line, of each of the transgenic and NT control lines were harvested at the full opening stage (day 0; their outermost petals were at right angles to the stem of flower). Stems were trimmed to 0.5 cm in length, and placed with their cut end in distilled water in 5-ml plastic vials. The flowers were left at 23°C under a 16-h photoperiod using white fluorescent light (20-30 $\mu\text{mol m}^{-2} \text{sec}^{-1}$). The water was replaced daily. Senescing flowers were observed and photographed daily to record in-rolling and subsequent wilting of petals, the desiccation, and discoloration of the petal margins. Vase life in days is expressed as the mean \pm SE of given numbers of flowers.

Assay of ethylene production: Ethylene production from carnation flowers was monitored daily by enclosing individual flowers in plastic vials in 140-mL glass containers (1 flower per container) for 1 h at 23°C. A 1-mL gas sample was taken with a hypodermic syringe from inside the container through a rubber septum of a sampling port on the container and injected into a gas chromatograph (Shimadzu GC-14A, Kyoto, Japan), equipped with an alumina column and a flame ionization detector to determine ethylene content.

Treatment with exogenous ethylene of flowers of the transgenic lines: Two to five cut flowers each of the respective transgenic lines and the NT control were enclosed in a 60 L glass chamber and exposed to ethylene at 10 $\mu\text{l L}^{-1}$ for 16 h at 23°C under white fluorescent light (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). After the treatment, the flowers were held in open air for 1 h to let exogenous ethylene diffuse. They were subsequently encapsulated and their ethylene production was determined by gas-chromatography. The petals and gynoecia were immediately excised and prepared for total RNA extraction.

Northern blot analysis: Total RNA was isolated by the SDS-phenol method (Palmiter, 1974) from the petals and with RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) from gynoecium of cut flowers of the respective transgenic lines and the NT control line at given time after the full opening of flowers (day 0). Pistils and petals were detached from one to three flowers, depending on lines, at the given time and combined to make one sample each. Also, total RNA was isolated from the gynoecium and petals of the flowers treated with ethylene as described above. Ten μg of total RNA was denatured, separated on a 1.0% agarose gel, transferred onto nylon membranes (Hybond N⁺, Amersham Pharmacia Biotech, Tokyo, Japan) and hybridized with the DNA

probes for *DC-ACSI* and *DC-ACO1* transcripts. The DNA probe for *DC-ACSI* transcript was 560 bp which corresponded to the position 1 bp to 560 bp of the coding region of *DC-ACSI* cDNA (GenBank Accession No. M66619), and that for *DC-ACO1* transcript 560 bp corresponded to the position 261 bp to 820 bp of the coding region of *DC-ACO1* cDNA (GenBank Accession No. M62380). The DNA probes were labeled with HRP and hybridized with the blot by using ECL Direct™ (Amersham Pharmacia Biotech) according to the manufacturer's instruction. Hybridization signals were detected by exposure to X-ray film (RX-U, Fuji Photo Film, Tokyo, Japan).

Results

Vase-life and senescence profile of the transgenic flowers: Cut flowers of each of the transgenic lines had vase-life of various length varying from 3.0 ± 0.4 to 7.6 ± 0.4 days, whereas that of the NT control line was 2.8 ± 0.2 days (Table 1). Vase lives of the pMLH-sACO-2 and -12 lines were 7.6 ± 0.4 and 6.3 ± 0.5 days, respectively (significantly different from the NT control, at $P=0.001$ by *t* test). Other transgenic lines had vase lives that were not significantly different from that of the NT control, except for the pIG-DC-ERS2'-2 line. We did not investigate further the pIG-DC-ERS2'-2 line.

Flowers of the NT control remained turgid until day 3, showed in-rolling of petals on day 4, and completely wilted thereafter. On contrast, flowers of the pMLH-sACO-2 and -12 lines remained turgid without petal in-rolling until day 6 or more, but eventually began to show desiccation and discoloration in the rim of petals. Petal in-rolling at the onset of wilting is a well-known characteristic of ethylene-dependent senescence of carnation flowers. Desiccation, discoloration, and browning of the rim of petals are characteristics of ethylene-independent senescence of carnation flowers. These findings suggested little or no function of ethylene during the senescence of petals of the pMLH-sACO-2 and -12 flowers. In the following experiments, we characterized ethylene production, expression of genes for ethylene biosynthesis and response to exogenous ethylene of flowers of the pMLH-sACO-2 and -12 lines by comparison with those of the NT control.

Ethylene production of the transgenic flowers: Flowers of the NT control showed a climacteric rise in ethylene production, attaining a maximal rate on day 3 (Fig. 1). Flowers of the pMLH-sACO-2 and -12 lines produced very small amounts of ethylene during senescence period of 8 days. Their maximum ethylene production rates were around 10% that of the NT control. The

Table 1. Senescence of flowers of the NT control and transgenic carnations

Lines	Number of flowers tested	Vase life ^a (days)	Senescence pattern ^b
NT control	6	2.8 \pm 0.2	W
pMLH-sACO-2	7	7.6 \pm 0.4	D
pMLH-sACO-3	6	3.2 \pm 0.4	W
pMLH-sACO-12	4	6.3 \pm 0.5	D
pIG-aACS-1	10	3.0 \pm 0.4	W
pIG-aACO-1	3	3.3 \pm 0.9	W
pIG-DC-ERS2'-2	4	4.5 \pm 1.0	W

^a Each value is the mean \pm SE.

^b W, in-rolling and wilting of the petals; D, desiccation, discoloration and necrosis of the petals.

lack of petal in-rolling and prolonged vase-life in flowers of the pMLH-sACO-2 and -12 lines coincided with a marked reduction in ethylene production.

Transcript levels for *DC-ACS1* and *DC-ACO1* in the gynoecium and petals of the transgenic flowers: As described in introduction, *DC-ACS1* and *DC-ACO1* play a pivotal role in ethylene production in both the gynoecium and petals of senescing carnation flowers (Nukui *et al.*, 2004; Satoh and Waki, 2006). We examined the transcript levels for *DC-ACS1* and *DC-ACO1* in the gynoecium and petals of the transgenic flowers undergoing senescence.

On day 0 (at the time of full opening of flowers), *DC-ACS1* and *DC-ACO1* transcripts were absent in both the gynoecium and petals of carnation flowers (Fig. 2). With the NT control flowers, *DC-ACS1* and *DC-ACO1* transcripts accumulated abundantly in the gynoecium on day 2 (the day before petal in-rolling), and significantly in the petals on day 3 when a maximum ethylene production from flowers occurred (Fig. 1). In the pMLH-sACO-2 and -12 lines, tissue sampling was conducted on days 0, 3 and 6. In gynoecia of the pMLH-sACO-2 line, *DC-ACS1* and *DC-ACO1* transcripts accumulated, although to small amounts in the latter, on day 3. These transcripts diminished on day 6. Both transcripts were absent in the petals on days 3 and 6. Similarly, with pMLH-sACO-12 line, *DC-ACS1* and *DC-ACO1* transcripts accumulated abundantly in the gynoecium on day 3 and diminished on day 6, but they were absent in the petals on both days. Absence of *DC-ACS1* and *DC-ACO1* transcripts in the petals of pMLH-sACO-2 and -12 lines coincided with the negligible amount of ethylene production from flowers of these lines (Fig. 1).

Responses to exogenous ethylene of flowers of the transgenic lines: In carnation flowers, the expression of *DC-ACS1* and *DC-ACO1* genes in petals can be induced by exogenously applied ethylene and by ethylene produced endogenously from the gynoecia (Shibuya *et al.*, 2000). The response of the transgenic lines and the NT control to exogenously applied ethylene was investigated after treatment with ethylene at $10 \mu\text{l L}^{-1}$ for 16 h. Ethylene evolution was determined from flowers at the beginning

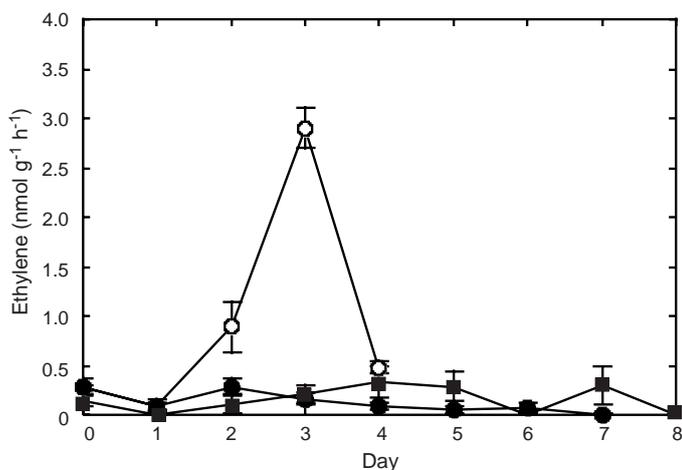


Fig. 1. Ethylene production from cut carnation flowers during the senescence period in the NT control and the transgenic lines. Flowers (numbers shown in Table 1) of three lines were harvested at full opening stage (day 0) and their ethylene production was monitored daily. Data are shown by the mean \pm SE. ○, NT control; ●, pMLH-sACO-2; ■, pMLH-sACO-12.

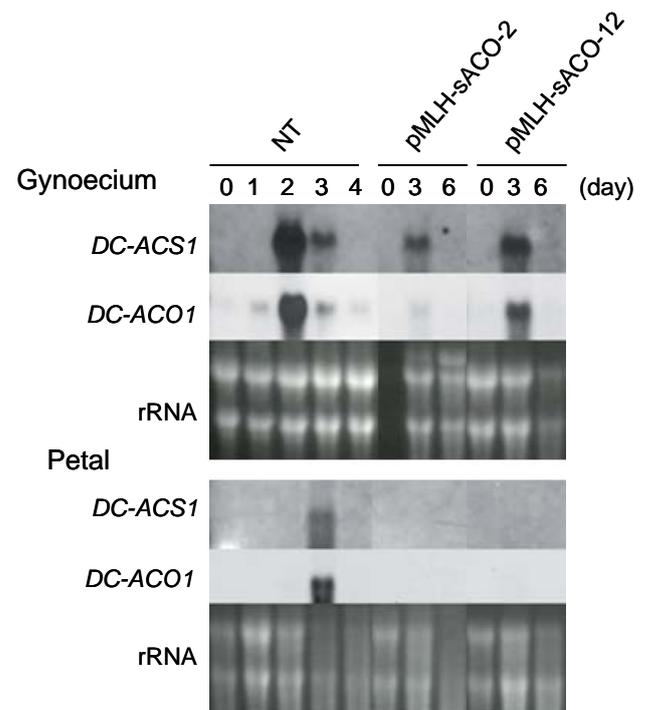


Fig. 2. RNA gel blot analysis of *DC-ACO1* and *DC-ACS1* transcripts in the gynoecium and petals of the NT control and in two transgenic lines during natural senescence. Gynoecium and petals were isolated from cut flowers at given days after full opening of flowers; days 0, 1, 2, 3, and 4 for the NT control, but days 0, 3 and 6 for pMLH-sACO-2 and -12 transgenic lines which did not show wilting. Ten μg of total RNAs isolated from respective flower tissues were separated on an agarose gel and hybridized to DIG-labeled *DC-ACS1* and *DC-ACO1* probes. Equal loading of total RNAs was checked by ribosomal RNAs visualized by ethidium bromide staining of the agarose gel. No data for the gynoecium of pMLH-sACO-2 on day 0.

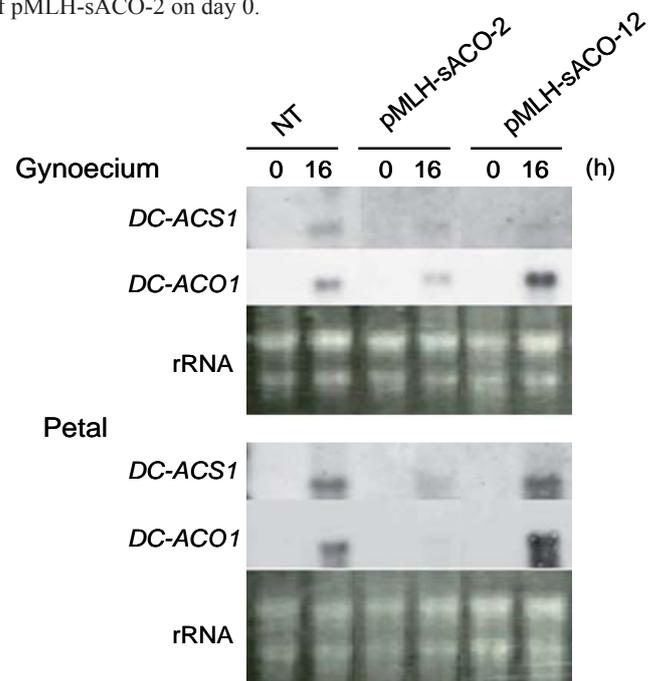


Fig. 3. RNA gel blot analysis of *DC-ACS1* and *DC-ACO1* transcripts in gynoecium and petals of the NT control and two transgenic flowers before and after ethylene treatment. Cut carnation flowers of respective lines were treated with $10 \mu\text{l L}^{-1}$ ethylene for 16 h. Ethylene production from flowers was measured before and after the ethylene treatment by enclosing them for 1 h and measuring ethylene produced. Numbers of flowers used were 2, 5 and 3 for the NT, pMLH-sACO-2 and -12 in this order. After ethylene assay, the flowers were subjected to analysis of amounts of *DC-ACS1* and *DC-ACO1* transcripts as described in the legend to Fig. 2.

and end of ethylene treatment. We also determined transcript levels for *DC-ACSI* and *DC-ACO1* in the gynoecia and petals. Treatment with exogenous ethylene for 16 h caused petals of the transgenic lines and the NT control to wilt, indicating that flowers of the transgenic lines were responsive to ethylene.

At the beginning of experiment, ethylene production from flowers was not detected or negligible ($< 0.2 \text{ nmol g}^{-1} \text{ h}^{-1}$). In flowers of the NT control treated with ethylene for 16 h, ethylene production was $9.38 \pm 0.27 \text{ nmol g}^{-1} \text{ h}^{-1}$. Ethylene evolution from the transgenic lines was less than that of the NT control; 1.96 ± 0.54 and $4.46 \pm 1.34 \text{ nmol g}^{-1} \text{ h}^{-1}$ for the pMLH-sACO-2 and -12 lines, respectively. Exogenous ethylene treatment caused an accumulation of *DC-ACSI* and *DC-ACO1* transcripts in the gynoecium and petals of the NT control and the transgenic lines, although the level of *DC-ACSI* and *DC-ACO1* transcripts in pMLH-sACO-2 line was lower than that in the NT control (Fig. 3).

Discussion

In this study we used six transgenic lines, which flowered a year after transplanting and cultivation, in soil, out of 39 transgenic lines generated previously (Kinouchi *et al.*, 2006). In the six transgenic lines, two lines transformed with pMLH2113-Hm/*sACO* construct, pMLH-sACO-2 and -12 lines, had a vase life more than twice longer than that of the NT control. The *sACO* transgene in the pMLH2113 vector efficiently suppressed ethylene production, which resulted in longer-lasting flowers.

Flowers of the pMLH-sACO-2 and -12 lines did not show petal in-rolling and wilting which are typical for ethylene-dependent senescence in carnation petals. Instead, the flowers showed browning and drying in the rim of petals, which spread out to all the portion of the petals, and eventually the flowers faded out at the late stage of vase life, which was about twice that of the NT control flowers. These are typical of ethylene-independent senescence of carnation flowers.

DC-ACSI and *DC-ACO1* transcripts were absent in the petals of both pMLH-sACO-2 and -12 flowers undergoing natural senescence (Fig. 2). This explained the reduced ethylene production in flowers of the two lines (Fig. 1). Previously, Kosugi *et al.* (2002) suggested that the *sACO* transgene integrated into carnation inhibited ethylene production in the flowers by cosuppression of expression of endogenous *DC-ACO1* gene in flower tissues. This seems also true in the present *sACO* transgenes with a long vase life. Kosugi *et al.* (2002) showed that the integrated *sACO* transgene might act first in the gynoecium, inhibiting the expression of *DC-ACO1* and suppressing ethylene production in the gynoecium and, subsequently, the expression of *DC-ACO1* and *DC-ACSI* in the petals. This was also found as an explanation for the absence of *DC-ACSI* transcript in the petals of both pMLH-sACO-2 and -12 flowers undergoing natural senescence.

The accumulation of *DC-ACSI* and *DC-ACO1* transcripts in flower tissues of the transgenic carnations (pMLH-sACO-2 and -12 lines) after treatment with exogenous ethylene ($10 \mu\text{L L}^{-1}$ for 16 h), indicated that the integrated *sACO* transgene did not impair their responsiveness to ethylene. The accumulation of *DC-ACO1* transcripts in the petals of the pMLH-sACO-2 and -12 flowers

after exogenous ethylene treatment indicated that exogenous ethylene treatment overcame the effect of the integrated *sACO* transgene in these two lines. Kinouchi *et al.* (2006) tested *in vivo* ACC oxidase activity using leaflet segments of the transgenic plants harboring *sACO* transgene. The *in vivo* ACC oxidase activities of the pMLH-sACO-2 and -12 lines were similar to and lower than the NT control, respectively.

These results suggested that the integrated *sACO* transgene exerted its effect in the leaflet segments of pMLH-sACO-12 line, but not pMLH-sACO-2 line. In this study, however, *DC-ACO1* transcript accumulated in low amounts in the gynoecium and was absent in the petals of pMLH-sACO-2 flowers. It was present abundantly in the gynoecium but also absent in the petals of pMLH-sACO-12 flowers on day 3. These differences suggest different action (expression) of the integrated *sACO* transgene between the leaflet and flower tissues, and also a difference between the two transgenic lines.

Acknowledgements

We thank Sakata Seed Corp., Kakegawa Research Center, Kakegawa, Japan, for the use of 'Lillipot' carnation.

References

- Abeles, F.B., P.W. Morgan and M.E. Saltveit, Jr., 1992. *Ethylene in Plant Biology*. 2nd ed. Academic Press, San Diego, CA.
- Bovy, A.G., G.C. Angenent, H.J.M. Dons and A.C. van Altvorst, 1999. Heterologous expression of the *Arabidopsis etr1-1* allele inhibits the senescence of carnation flowers. *Mol. Breed.*, 5: 301-308.
- Borochoy, A. and W.R. Woodson, 1989. Physiology and biochemistry of flower petal senescence. *Hort. Rev.*, 11: 15-43.
- Henskens, J.A.M., G.J.A. Rouwendal, A. ten Have and E.J. Woltering, 1994. Molecular cloning of two different ACC synthase PCR fragments in carnation flowers and organ-specific expression of the corresponding genes. *Plant Mol. Biol.*, 26: 453-458.
- Iwazaki, Y., Y. Kosugi, K. Waki, T. Yoshioka and S. Satoh, 2004. Generation and ethylene production of transgenic carnations harboring ACC synthase cDNA in sense or antisense orientation. *J. Appl. Hort.*, 6: 67-71.
- Jones, M.L. and W.R. Woodson, 1997. Pollination-induced ethylene in carnation. Role of styler ethylene in collolla senescence. *Plant Physiol.*, 115: 205-212.
- Jones, M.L. and W.R. Woodson, 1999. Differential expression of three members of the 1-aminocyclopropane-1-carboxylate synthase gene family in carnation. *Plant Physiol.*, 119: 755-764.
- Kende, H. 1993. Ethylene biosynthesis. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 44: 283-307.
- Kinouchi, T., R. Endo, A. Yamashita and S. Satoh, 2006. Transformation of carnation with genes related to ethylene production and perception: towards generation of potted carnations with a longer display time. *Plant Cell Tiss. Organ Cult.*, 86: 27-35.
- Kosugi, Y., K. Shibuya, N. Tsuruno, Y. Iwazaki, A. Mochizuki, T. Yoshioka, T. Hashiba and S. Satoh, 2000. Expression of genes responsible for ethylene production and wilting are differently regulated in carnation (*Dianthus caryophyllus* L.) petals. *Plant Sci.*, 158: 139-145.
- Kosugi, Y., K. Waki, Y. Iwazaki, A. Mochizuki, N. Tsuruno, A. Mochizuki, T. Yoshioka, T. Hashiba and S. Satoh, 2002. Senescence and gene expression of transgenic non-ethylene-producing carnation flowers. *J. Japan. Soc. Hort. Sci.*, 71: 638-642.

- Midoh, N., Y. Saijou, K. Matsumoto and M. Iwata, 1996. Effects of 1,1-dimethyl-4-(phenylsulfonyl)semicarbazide (DPSS) on carnation flower longevity. *Plant Growth Regul.*, 20: 195-199.
- Nukui, H., S. Kudo, A. Yamashita and S. Satoh, 2004. Repressed ethylene production in the gynoecium of long-lasting flowers of the carnation 'White Candle': role of the gynoecium in carnation flower senescence. *J. Exp. Bot.*, 55: 641-650.
- Palmiter, R.D. 1974. Magnesium precipitation of ribonucleoprotein complexes: Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. *Biochemistry*, 13: 3606.
- Park, K.Y., A. Drory and W.R. Woodson, 1992. Molecular cloning of an 1-aminocyclopropane-1-carboxylate synthase from senescing carnation flower petals. *Plant Mol. Biol.*, 18: 377-386.
- Reid, M.S. and M.J. Wu, 1992. Ethylene and flower senescence. *Plant Growth Regul.*, 11: 37-43.
- Satoh, S. and K. Waki, 2006. Repressed expression of *DC-ACS1* gene in a transgenic carnation supports the role of its expression in the gynoecium for the onset of ethylene production in senescing flower. *J. Japan. Soc. Hort. Sci.*, 75: 173-177.
- Savin, K.W., S.C. Baudinette, M.W. Graham, M.Z. Michael, G.D. Nugent, C-Y. Lu, S.F. Chandler and D. Cornish, 1995. Antisense ACC oxidase RNA delays carnation petal senescence. *HortScience*, 30: 970-972.
- Shibuya, K., T. Yoshioka, T. Hashiba and S. Satoh, 2000. Role of the gynoecium in natural senescence of carnation (*Dianthus caryophyllus* L.) flowers. *J. Exp. Bot.*, 51: 2067-2073.
- ten Have, A. and E.J. Woltering, 1997. Ethylene biosynthetic genes are differentially expressed during carnation (*Dianthus caryophyllus* L.) flower senescence. *Plant Mol. Biol.*, 34: 89-97.
- Veen, H. 1979. Effects of silver on ethylene synthesis and action in cut carnations. *Planta*, 145: 467-470.
- Wang, H. and W.R. Woodson, 1991. A flower senescence-related mRNA from carnation shares sequence similarity with fruit ripening-related mRNAs involved in ethylene biosynthesis. *Plant Physiol.*, 96: 1000-1001.
- Woodson, W.R., K.Y. Park, A. Drory, P.B. Larsen and H. Wang, 1992. Expression of ethylene biosynthetic pathway transcripts in senescing carnation flowers. *Plant Physiol.*, 99: 526-532.
- Yang, S.F. and N.E. Hoffman, 1984. Ethylene biosynthesis and its regulation in higher plants. *Ann. Rev. Plant Physiol.*, 35: 155-189.