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Act. 523 RESPUESTA Folios: 60

Trámite : 011
Evento : 378
Actuación : 444

Referencia : Expediente No. **11.126.046**
Asunto : Solicitud de patente para: **COMPUESTO DERIVADO DE DELFINIDINA**
Solicitante : **SUNTORY HOLDINGS LIMITED**
Fecha de solicitud : 27 de Septiembre de 2011

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. **4525** notificado por fijación en lista el 12 de Marzo de 2013.

2. Mediante oficio No. **4525** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio se refieren la presencia de materia no considerada una invención, a excepciones de patentabilidad, a la presencia de usos o segundos usos, al título de la solicitud, y al nivel inventivo de las reivindicaciones 1 a 5 en vista de los documentos **D1**: Fukui Y. et Al, Tetrahedron 62, 9663-9670 y **D2**: CA2537065.

3. **Respuesta a las objeciones**

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

"EXCELENCIA LEGAL EN UN MUNDO SIN FRONTERAS"

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3.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 6 reivindicaciones, junto con un nuevo capítulo descriptivo.

El soporte de las modificaciones realizadas puede ser encontrado en el capítulo descriptivo como fue originalmente presentado, de la siguiente manera:

- (1) Se han incluido dos nuevas reivindicaciones 5 y 6 (antiguas 5-15), dicho soporte a dicha modificación se puede encontrar en los párrafos [0030] a [0054] de la descripción del inglés (ejemplos 1-4).

Las reivindicaciones 1 a 4 permanecen de manera similar, sin embargo las fórmula se han sustituido por unas más claras.

3.2 No es una invención

El examinador menciona que el objeto de las reivindicaciones 9 a 10 no se considera una invención, de acuerdo con el Art citado, puesto que es una parte de la planta tal como se encuentra en la naturaleza.

Con el fin de superar las objeciones planteadas, las reivindicaciones en mención han sido eliminadas.

3.3 Excepción a la Patentabilidad

El Examinador menciona que el objeto de las reivindicaciones 5 – 8 y 11 no es patentable de acuerdo con el Art citado, porque se trata de plantas.

Con el fin de superar esta objeción, las reivindicaciones en mención han sido eliminadas del presente capítulo reivindicatorio.

3.4 Reivindicaciones relativas a un uso

El Examinador describe que el método de las reivindicaciones 12 – 15, corresponde al uso del compuesto de fórmula (I), mencionado en la reivindicación 1 que es un pigmento de la rosa. Es evidente que la llamada etapa de “utilizar el compuesto para alterar el color de la planta” corresponde a la forma de usar los compuestos de fórmula (I), aunque en su preámbulo se reclame como método. Se le aclara al solicitante que un método cuya única etapa corresponde a utilizar o aplicar, dicha aplicación es un uso. Por lo tanto dicho método no es patentable de acuerdo con el artículo 14.

Con el fin de superar esta objeción realizada por el Examinador, la reivindicaciones 12 a 15 han sido eliminadas.

3.5 Modificación al Título de la Solicitud

El Examinador ha objetado el título de la solicitud mencionando que no corresponde con el objeto de la solicitud porque se debería mencionar el núcleo central de la estructura, que puede haber muchos compuestos contenidos en la rosa, por lo cual se sugiere al Solicitante hacer la corrección necesaria.

En cuanto a la objeción realizada el título se ha modificado como ha sugerido el Examinador a “COMPUESTO DERIVADO DE DELFINIDINA”.

3.6 Análisis de nivel inventivo

El Examinador refiere que los documentos D1 y D2, se consideran el estado del arte cercano a la invención definida en la reivindicación 1 a 4.

Con el fin de superar las objeciones a nivel inventivo, nos permitimos aclarar:

(1) Debe reconocerse la actividad inventiva de las invenciones de las reivindicaciones 1 a 4.

(2) Con respecto a las nuevas reivindicaciones 5 y 6 (es decir, un método para la preparación de los compuestos de las reivindicaciones 1-4), solicitamos muy respetuosamente el reconocimiento de la patentabilidad de dichas reivindicaciones, puesto que los compuestos de las reivindicaciones 1-4, son inventivos.

(3) En cuanto a la actividad inventiva de las invenciones de reivindicaciones 1-4, es reconocido generalmente en el arte que un flavonoide 3'-hidroxilasa (F3'5'H) pertenece a la familia del citocromo P450, se presume que el ancestro de este gen fue derivado antes de la derivatización en plantas superiores, y por eso P450 se localiza en el retículo endoplasmático. También generalmente se reconoce en el arte que un F3'5'H puede catalizar la hidroxilación en la posición 3" o en las posiciones 3' y 5' del flavanona, dihidroflavonol, flavonol y flavona.

También generalmente se reconoce en el arte que P450s se derivado de un eucariote, tales como animales, plantas, levaduras, etc. son - una proteína de membrana presente en retículo endoplasmático o en la mitocondria de las células.

(i) Sustrato de F3'5' H

En "Kaltenbach et al., The Plant Journal (1999) 19(2), 183-193" (Apéndice 2) describe que los F3'5' 'Hs revelan mayores actividades con naringenina y apigenina, pero también se obtuvieron significantes actividades con kaempferol y dihidrokaempferol (ver página 186, columna derecha, líneas 21-23, tabla 1, página 184, figura 1). En el Apéndice 2, se afirma "la dihidroquercetina es un intermediario en la síntesis de la antocianina y el co-pigmento, pero la hidroxilación -3' en la vía de la antocianina ocurre con

dihidrokaempferol, mientras que la hidroxilación -3' en la biosíntesis de co-pigmento ocurre con naringenina (ver pág. 187, columna derecha, líneas 12-18).

Además, Oslen et al., BMC Plant Biology 201, 10-21 (Apéndice 1) describe al Kaempferol como un sustrato de F3 '5' H además de Dihidrokaempferol y Dihidroquercetina (ver pág. 3, figura 1). Como puede verse en la figura 1, el sustrato principal de F3 '5' H es el Dihidrokaempferol (DHK) y Dihidroquercetina (DHQ) y el Dihidrokaempferol (el número de grupos hidroxilo del grupo fenilo unido al sistema de anillo de la cumarina es uno) es metabolizado a pelargonidina por la acción de la dihidroflavonol 4-reductasa (DFR) y la antocianidina sintasa (ANS), la Dihidroquercetina (el número de grupos hidroxilo del grupo fenilo unido al sistema de anillo de cumarina es dos) es metabolizado a cianidina por la acción de la DFR y la ANS y la Dihidromiricetina (DHM) (el número de grupos hidroxilo del grupo fenilo unido al sistema de anillo de cumarina es tres) es metabolizado a delfinidina por la acción de la DFR y la ANS.

(ii) Transporte de flavonoides en células

Zhao et al., 16 de diciembre de 2009, Trends in Plant Science, Vol. 15, N° 2 (Apéndice 3) describe ese complejo de la enzima biosintético del flavonoide que contiene F3'5'H que está unido a la membrana del retículo endoplasmático, los flavonoides son sometidos a modificaciones, tales como la glicosilación en el citoplasma y transferidos via vacuola, mediante una variedad de transportadores unidos a la membrana vacuolar (ver la página 76, Figura 2).

(iii) La rosacianina está presente en la vacuola

Como los pétalos de una planta de rosa se observan con un microscopio óptico, los pigmentos se pueden encontrar dentro de las vacuolas de la epidermis. Así, se reconoce que la rosacianina está presente en las vacuolas. También se considera que ya que la rosacianina es glicosilada, fue sintetizada fuera de las vacuolas y luego ser transferida a las vacuolas.

(iv) Ruta prevista de la síntesis de rosacianina

El documento D1 (Fukui et al., Tetraedron, Vol. 26, no. 41, 2006, p. 9661-9670) describe "las rosacianinas A1 (1) y A2 (2) tuvieron un cromóforo común, que contenía cianidina con un grupo de galol unido entre las posiciones 4 y 5 del grupo hidroxilo del núcleo flavilium y telimagrandina 1 y 2, respectivamente, en el C-3 del núcleo de cianidina" (ver página 9668, columna izquierda, líneas 15-19, página 9667, figura 6). D8 también describe "las rosacianinas se cree que son biosintetizadas por una cicloadición enzimática o por una adición nucleofílica y electrofílica de cianidina con una gran cantidad de telimagrandina 1 y 2, distribuidos en los pétalos de rosa" (ver página 9668, columna izquierda, líneas 40-44).

Como se describe en Katsumoto et al., Plant Cell Physiol. 48(11): 1589-1600 (2007) (Anexo 4), en la rosa recombinante, además del gen FS 'S' H, un iris DFR (que puede producir selectivamente la delfinidina de DHM) se introduce también al suprimir la expresión de un rosa DFR inherente en orden a sintetizar selectivamente la delfinidina de dihidroflavonol (ver la página 1590, Fig. 1, página 1593, Fig. 2, secuencia del gen de pSPB919, página 1592, columna derecha, líneas 13-27). Como resultado, no fue producida cianidina y así la rosa producida contiene delfinidina 100, y además tampoco rosacianina se produjo. De hecho, la rosacianina no está presente en la rosa recombinante y sólo rosadelfina es detectada. Partiendo de este hecho, puede decirse que la rosadelfina no puede ser producida de rosacianina como sustrato.

En el párrafo [0005] de la especificación de esta aplicación se describe como sigue:

"Puesto que las rosacianinas tienen una cadena principal cianidina en una porción de su estructura, existe la posibilidad de que se sinteticen basándose en cianidina, un precursor común con cianidina o un análogo de cianidina. Sin embargo, debido a que esto es sólo especulación, los tipos de sustratos realmente se utilizan como precursores y los tipos de vías, que se utilizan en la síntesis aún no han sido claramente determinadas"

Es decir, si bien fue encontrado antes de la fecha de prioridad de esta aplicación que rosacianina está presente en una rosa, su ruta de biosíntesis había estado totalmente incierta.

Por lo tanto a la luz de la experiencia técnica como se explicó anteriormente, la actividad inventiva de la invención de los compuestos reivindicadas, queda sustentada de la siguiente manera:

Como se mencionó anteriormente, el sustrato principal de F3 '5' H es el dihidrokaempferol (DHK) y la dihidroquercetina (DHQ) y el F3 '5' H está presente en la membrana de la vacuola. Por otra parte, la rosacianina está presente en la vacuola. Del hecho que las ubicaciones son diferentes entre sí, resulta claro que el F3 '5' H no puede actuar en rosacianina per se para agregar grupo hidroxilo adicional a los dos grupos hidroxilo en el grupo fenilo unido al sistema de anillo de cumarina de la rosacianina.

También como se indicó anteriormente, puesto que las rosacianinas tienen una cadena principal de cianidina como parte de su estructura, existe la posibilidad que se sinteticen partiendo de cianidina, un precursor común con cianidina o un análogo de la cianidina. Sin embargo, esto es sólo especulación. En la presente invención, primero se encontró que la expresión de F3 '5' H dio lugar a la síntesis de rosadelfina. Es decir, primero se aclaró que la rosacianina se sintetiza a partir de la cianidina, la cual es sintetizada por la ruta biosintética [DHK-(F3'H) DHQ-(DFR, ANS)-cianidina]. También podría presumirse que la rosadelfina no es sintetizada basada en rosacianina u otros análogos de la delfinidina, pero se sintetiza partiendo de delfinidina, que se ha reducido (via leucodelfinidina) por un Iris DFR con alta especificidad de sustrato y producido por ANS sin especificidad, vía [DHK-(FS'S' HJDHM (DFR específico, ANS) delfinidina]. Este es el primer hallazgo de que el novedoso pigmento (rosadelfina) de la presente invención está presente.

Puede decirse que la estructura química de rosadelfina se distingue de la rosacianina en el número de grupos hidroxilo en el grupo fenilo unido a los sistemas de anillo de cumarina. Sin embargo, en el proceso para producir un producto final de

rosadelfina, un gran número de enzimas está implicado. Por lo tanto, teniendo en cuenta la especificidad del sustrato a un número de enzimas implicadas, una persona experta en la materia no podría haber presumido con una expectativa razonable de éxito que toda la cantidad de enzimas actuarían en el proceso para la producción de rosacianina, y que también podrían actuar en el proceso para la producción de rosadelfina.

Por estas razones, en cuanto al punto de vista del examinador "el experto obviamente derivaría los compuestos de la presente reivindicación 1 incluyendo las enseñanzas del documento D1 en la divulgación de D2, con respecto a la cantidad de grupos hidroxilo hacia un color más azulado y por lo tanto la reivindicación 1 se considera obvia" muy respetuosamente se encuentra por fuera de los argumentos aquí presentados, que demuestran la altura inventiva de los compuesto de la presente solicitud.

En consecuencia, un técnico con conocimientos medios en la materia no estaba en condiciones de plantearse el problema, tampoco de resolverlo en la forma en que se reivindica y tampoco de prever el resultado, por lo que la presente solicitud posee la altura inventiva para que se le conceda el privilegio solicitado, a los compuestos de las reivindicaciones 1 a 4.

4. Modificación de la solicitud en trámite

Con el objeto de subsanar las objeciones presentadas por su Despacho y de acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar nuevo capítulo reivindicatorio modificado.

Sin embargo, la solicitante desearía señalar que cualquier materia divulgada borrada o no contenida en la presente serie de reivindicaciones no debe ser considerada como abandonada. Así mismo, la solicitante se reserva su derecho a incluir tal materia en futuras series de reivindicaciones.

5. De acuerdo con los argumentos y modificaciones presentados con este escrito, la presente solicitud cumple a cabalidad con los requerimientos exigidos en la Decisión 486 y las objeciones formuladas por el Examinador se ven superadas.

Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente. En este sentido, respetuosamente recordamos al Examinador que la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. **4525** toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

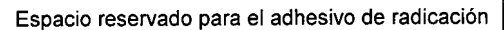
6. ANEXOS

- Nuevo pliego reivindicatorio conformado por 6 reivindicaciones
- Formulario de modificaciones en solicitud pendiente
- Identification and characterization of CYP75A31, a new flavonoid 3'5'-hydroxylase, isolated from solanum lycopersicum
- Flavonoid hydroxylase from Catharanthus roseus: cDNA, heterologous expression, enzyme properties and cell-type specific expression in plants
- The "ins" and "outs" of flavonoid transport
- Engineering of the Rose Flavonoid Biosynthetic Pathways Successfully Generated Blue-Hued Flowers Accumulating Delphinidin

Señor Director,


LUZ CLEMENCIA DE PAEZ
C.C. 35.456.344 de Usaquén
T.P.A. No. 23.555

COLO-17511-0841-004-2
SML\SML\ID-244345\WPC17511
No. M-2013-244345\SML



1. Identificación del Tramite

- ## 2. Datos del solicitante

☒

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro ☐ Pequeña ☐ Mediana ☐ Otra ☐

Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número _____

□

Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del apoderado:

Tarjeta profesional
T.P.A. 23.555

Dirección y domicilio		Ciudad
Carrera 4ª No. 72 –35		Bogotá
Dirección electrónica	No. Fax	Número telefónico
cavelier@cavelier.com	2 11 86 50	3 47 36 11
En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general		

4. Datos del trámite**Corrección:****Expediente No 11.126.046****Aparece****Debe ser****Modificación:****Modificación al capítulo reivindicatorio.**

Presento nuevo capítulo reivindicatorio compuesto por 6 reivindicaciones con el fin de superar las objeciones hechas por Su Despacho a la presente solicitud de patente y pido respetuosamente que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas.

Respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y además se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

**Modificación al capítulo descriptivo.****Modificación al resumen.****Modificación a los dibujos.****Modificación al Título.**

Solicito respetuosamente a su despacho tener en cuenta que el nuevo título de esta solicitud de patente ha quedado como sigue: **"COMPUESTO DERIVADO DE DELFINIDINA"**.

5. Anexos

Poder, si fuere el caso.



Documento que contiene las modificaciones o correcciones.

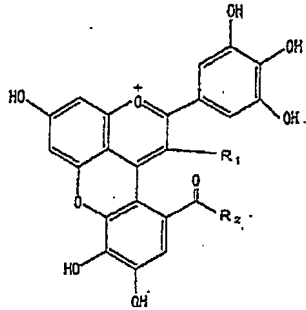


Copia de la solicitud y sus anexos en formato magnético.

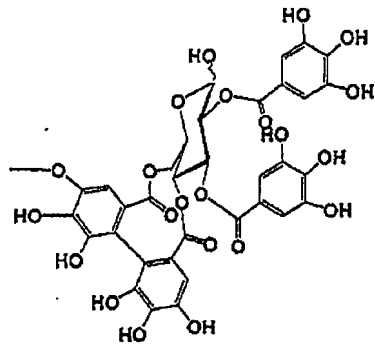
6. Firma	
Nombre y apellidos	Firma
LUZ CLEMENCIA DE PAEZ	<i>Luz Clemencia De Paez</i>
C.C. 35.456.344 DE Usaquén	Tarjeta Profesional 23.555

REIVINDICACIONES

1. Compuesto representado por la siguiente fórmula general (I):
[Fórmula Química 1]

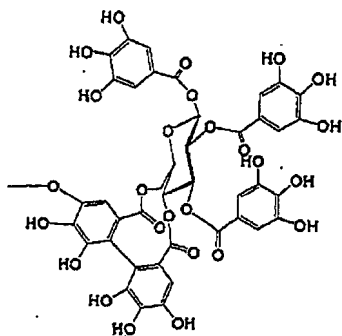


- en donde, R_1 representa el siguiente grupo:
[Fórmula Química 2]



- y R_2 representa -OH o R_1 y R_2 forman conjuntamente -O-, o R_1 representa el siguiente grupo:

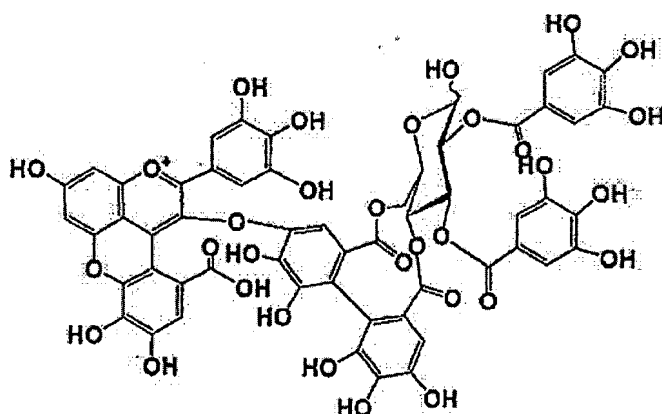
[Fórmula Química 3]



- y R_2 representa -OH, siempre y cuando la coordinación (línea ondulada) del grupo hidroxilo en la posición 1 de glucosa en R_1 exhiba tautomerismo entre la forma α y la forma β .

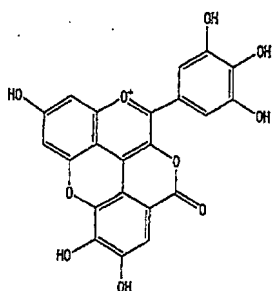
2. Un compuesto representado por la siguiente fórmula:

[Fórmula Química 4]



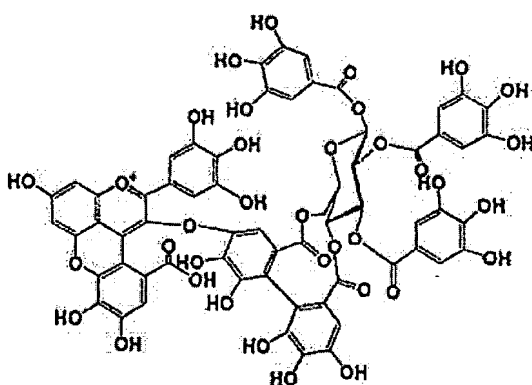
3. Un compuesto representado por la siguiente fórmula:

[Fórmula Química 5]



4. Un compuesto representado por la siguiente fórmula:

[Fórmula Química 6]



5. Un método para la preparación de un compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 4, comprendiendo el método las etapas de:

introducir de un gen flavonoide 3',5'-hidroxilasa derivado de un pensamiento a una planta que pertenece a la familia Rosaceae, género Rosa, *Rosa hybrida* a través de un *Agrobacterium* por el método de ingeniería genética, y

expresar el gen en los pétalos de la planta para producir el compuesto.

6. El método de acuerdo con la reivindicación 5, donde la *Rosa hybrida* es una variedad Lavande o WKS 82.

SML\SML\ID-244346\WPC17511

Appendix 1

Olsen et al., BMC Plant Biology 2010, 10:21

Olsen et al. BMC Plant Biology 2010, 10:21
<http://www.biomedcentral.com/1471-2229/10/21>



RESEARCH ARTICLE

Open Access

Identification and characterisation of CYP75A31, a new flavonoid 3'5'-hydroxylase, isolated from *Solanum lycopersicum*

Kristine M Olsen^{1*}, Alain Hehn², Hélène Jugdé², Rune Slimestad³, Romain Larbat², Frédéric Bourgaud², Cathrine Lillo¹

Abstract

Background: Understanding the regulation of the flavonoid pathway is important for maximising the nutritional value of crop plants and possibly enhancing their resistance towards pathogens. The flavonoid 3'5'-hydroxylase (F3'5'H) enzyme functions at an important branch point between flavonol and anthocyanin synthesis, as is evident from studies in petunia (*Petunia hybrida*), and potato (*Solanum tuberosum*). The present work involves the identification and characterisation of a F3'5'H gene from tomato (*Solanum lycopersicum*), and the examination of its putative role in flavonoid metabolism.

Results: The cloned and sequenced tomato F3'5'H gene was named CYP75A31. The gene was inserted into the pYeDP60 expression vector and the corresponding protein produced in yeast for functional characterisation. Several putative substrates for F3'5'H were tested *in vitro* using enzyme assays on microsome preparations. The results showed that two hydroxylation steps occurred. Expression of the CYP75A31 gene was also tested *in vivo*, in various parts of the vegetative tomato plant, along with other key genes of the flavonoid pathway using real-time PCR. A clear response to nitrogen depletion was shown for CYP75A31 and all other genes tested. The content of rutin and kaempferol-3-rutinoside was found to increase as a response to nitrogen depletion in most parts of the plant, however the growth conditions used in this study did not lead to accumulation of anthocyanins.

Conclusions: CYP75A31 (NCBI accession number GQ904194), encodes a flavonoid 3'5'-hydroxylase, which accepts flavones, flavanones, dihydroflavonols and flavonols as substrates. The expression of the CYP75A31 gene was found to increase in response to nitrogen deprivation, in accordance with other genes in the phenylpropanoid pathway, as expected for a gene involved in flavonoid metabolism.

Background

Flavonoids are plant secondary metabolites. They have a wide range of functions such as (a) providing pigmentation to flowers, fruits, and seeds in order to attract pollinators and seed dispersers, (b) protecting against ultraviolet light, (c) providing defence against phytopathogens (pathogenic microorganisms, insects, animals), (d) playing a role in plant fertility and germination of pollen and (e) acting as signal molecules in plant-microbe interactions [1,2]. Flavonoids receive a lot of attention due to their possible effects on human

health. Many flavonoids display antioxidant activity that confers beneficial effects on coronary heart disease, cancer, and allergies [3,4]. Reports also suggest that some of the biological effects of anthocyanins and flavonols may be related to their ability to modulate mammalian cell signalling pathways [5,6]. Enhancing the production of flavonoids in crop plants can therefore give an important boost to their nutritional value, which makes knowledge of expression and regulation of the flavonoid pathway important. Flavonoids constitute a relatively diverse family of aromatic molecules that are derived from phenylalanine and malonyl-coenzyme A. Most of the bright red and blue colours found in higher plants are due to anthocyanins. Anthocyanin biosynthesis has been studied extensively in several

* Correspondence: kristine.molsen@uis.no
¹University of Stavanger, Centre for Organelle Research, Faculty of Science and Technology, N-4036 Stavanger, Norway



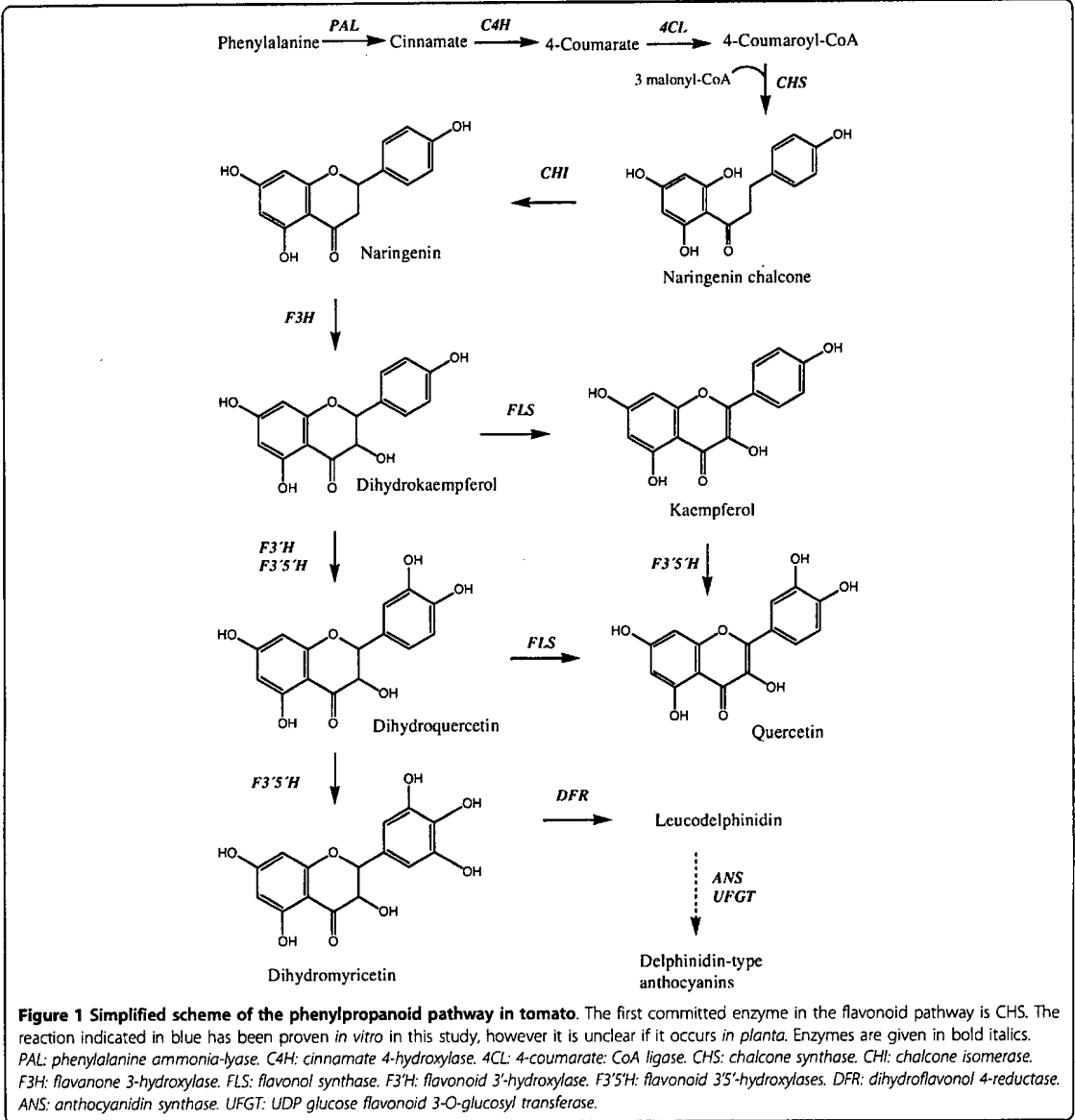
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plant species and detailed information on the pathway is available [7-9]. Information on substrate flow and regulation through the branch point between flavonol and anthocyanin synthesis is however not fully elucidated, and for tomato the enzymes acting in the branch point have not been extensively characterised. Experiments with expression of the snapdragon transcription factor genes *Delila*, a basic-helix-loop-helix (bHLH) transcription factor, and *Rosea1*, a R2R3 MYB-type transcription factor, showed that *F3'5'H* expression is necessary for activation of anthocyanin synthesis in tomatoes [10]. Introduction of these transcription factors under control of the fruit-specific E8 promoter increased the expression of most of the structural genes in the biosynthetic pathway in the tomato fruit, including *phenylalanine ammonia-lyase* (*PAL*), *chalcone isomerase* (*CHI*) and *F3'5'H*. *PAL* insures high flux into the phenylpropanoid pathway, whereas *CHI* and *F3'5'H* are essential for addressing the flux towards flavonoids in general and anthocyanin production specifically. The activity of *CHI* is normally low in the tomato skin, leading to accumulation of naringenin-chalcone in the skin of wild type tomatoes [11]. The cytochrome P450 dependent flavonoid hydroxylases introduce either one (flavonoid 3'-hydroxylase, *F3'H*) or two (*F3'5'H*) of the hydroxyl groups on the B ring of the flavonoid skeleton [7,12]. The *F3'5'H* belongs to the CYP75 superfamily of P450 enzymes [13,14]. These enzymes are anchored to the surface of the endoplasmic reticulum via their hydrophobic N- terminal end. Only plants that express the *F3'5'H* gene are capable of producing blue flowers, as these are dependent on 5'-hydroxylated anthocyanins. *F3'5'*-hydroxylases are previously known from other plants, such as *Petunia hybrida* (petunia), *Catharanthus roseus* (Madagascar periwinkle), *Vitis vinifera* (grape), *Campanula medium* (Canterbury bells), *Solanum tuberosum* (potato) and *Solanum melongena* (eggplant), among others. To be active P450 enzymes need to be coupled to an electron donor. This can either be a cytochrome P450 reductase or cytochrome *b₅*. The reductase will also be anchored to the surface of the endoplasmic reticulum via its N- or C-terminus [13]. Kaltenbach et al. [15] isolated the *F3'5'H* gene from *C. roseus* using heterologous screening with the CYP75 *Hf1* cDNA from *P. hybrida* [16]. Both the *C. roseus* gene, named CYP75A8, and the petunia *Hf1* were expressed in *E. coli* and found to accept flavones, flavanones, dihydroflavonols and flavonols as substrates, and both performed 3'- and 3'5'-hydroxylation. The genes encoding *F3'5'H* in grape have been shown to be expressed in different parts of the grape plant that accumulate flavonoids, especially in the skin of

ripening berries where the highest levels of anthocyanins are synthesized [17].

Several genes in the flavonoid pathway display differences in substrate specificity or preference in various plant species. *Petunia* dihydroflavonol 4-reductase (*DFR*), for instance, does not utilize dihydrokaempferol [18]. *Arabidopsis* *DFR* converts dihydroquercetin into leuco-cyanidin, but will use dihydrokaempferol when dihydroquercetin is not available, e.g. in plants lacking functional *F3'H* enzyme [19]. This is because the plants lacking *F3'H* activity cannot produce dihydroquercetin (fig 1). So far there is not much information on *F3'5'H* substrate specificity. Available data [15,20] generally confirm the same substrates, without reporting negative results for other substrates tested. However, Tanaka et al. [20] reported that the petunia *Hf2* cDNA expressed in a yeast system did not accept apigenin as substrate. Kaltenbach et al. did, however, show that the petunia *Hf1* can accept apigenin as substrate, when expressed in an *E. coli* system [15].

F3'5'H competes with flavonol synthase (*FLS*) for the substrates dihydrokaempferol and dihydroquercetin (Figure 1). The preferred substrate for *DFR* in the tomato plant is dihydromyricetin [21], which can be produced from dihydrokaempferol and dihydroquercetin by *F3'5'H*. This is the first step in the branch leading to anthocyanins (delphinidin type), which are normally only found in the vegetative tissues of tomato. According to Bovy et al. [21] tomato *FLS* prefers dihydroquercetin and dihydrokaempferol as substrates, and does not use dihydromyricetin, hence *DFR* and *FLS* do not compete for the same substrate. Nevertheless *FLS* can still deplete the flow of substrate towards *DFR* by using dihydrokaempferol and dihydroquercetin as they precede dihydromyricetin in the synthesis pathway. *F3'H* might also compete with *FLS* and *F3'5'H* for dihydrokaempferol, although it is unclear, as the enzyme has not been characterised from tomato so far. The activities of *FLS*, *F3'5'H*, *DFR*, and possibly *F3'H*, hence regulate the distribution between flavonols and anthocyanins in tomato plants. As a consequence, *F3'5'H* can be a bottleneck in this system as *DFR* relies on its activity to proceed the synthesis towards anthocyanins. Bovy et al. [11] has shown that silencing of the *FLS* gene leads to more anthocyanins in vegetative tomato tissue. Introduction of an *FLS* RNAi construct into tomato plants led to decreased levels of quercetin-3-rutinoside (rutin) in tomato peel, and to accumulation of anthocyanins in leaves, stems and flower buds. This indicates that less competition from flavonol synthesis will enhance the flux towards anthocyanins by allowing more substrate for *DFR*. In this study we cloned, sequenced and characterised the *F3'5'H* enzyme, which produces substrate for



DFR in tomato. Accumulation of flavonoids, and distribution of products through the different branches of the flavonoid pathway, has previously been shown to be influenced by nitrogen supply [22,23]. An agricultural plant like tomato is typically given nitrogen through fertilization; hence the level of nitrogen available to the plant can be monitored. It is, therefore, important to elucidate the effects nitrogen has on expression of genes and accumulation of compounds, such as flavonoids. Extensive knowledge on the branch-point enzyme

F3'S'H is crucial for understanding the distribution of flow through the flavonoid pathway, potentially enabling manipulation of desired end-product accumulation in fruits and vegetables in response to growth conditions.

Results

Sequence analysis

The *CYP75A31* gene was isolated using sequence homology with a potato *F3'S'H* and 3' RACE to identify the 3' end of the gene. A tomato EST sequence found in

the TIGR database was assumed to be the 5' end of the gene (accession number DB723744), and primers based on these sequences led to isolation of the cDNA and DNA sequences for *CYP75A31*. The 3133 bp gene sequence (Figure 2) consists of three exons (gray), which is consistent with what is previously reported for potato, petunia and soybean [24,25]. A Blast search (NCBI) performed with the coding sequence revealed 94% identity to a *S. tuberosum*, 88% identity to a *S. melongena* and 84% identity to a *P. hybrida* *F3'5'H* sequence.

Phylogenetic analysis

The phylogenetic tree (Figure 3) was made using protein sequences from several plant *F3'5'H* enzymes retrieved from the NCBI web page. The tree clearly visualises that *CYP75A31* is most closely related to the *F3'5'H* enzymes of the *Solanum* species potato and eggplant.

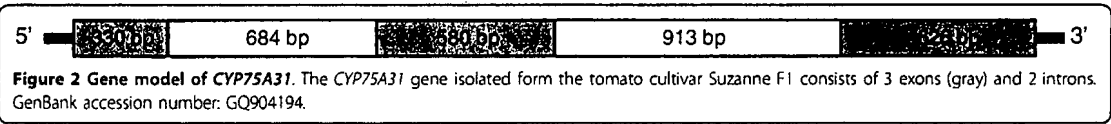
CYP75A31 Substrate Specificity

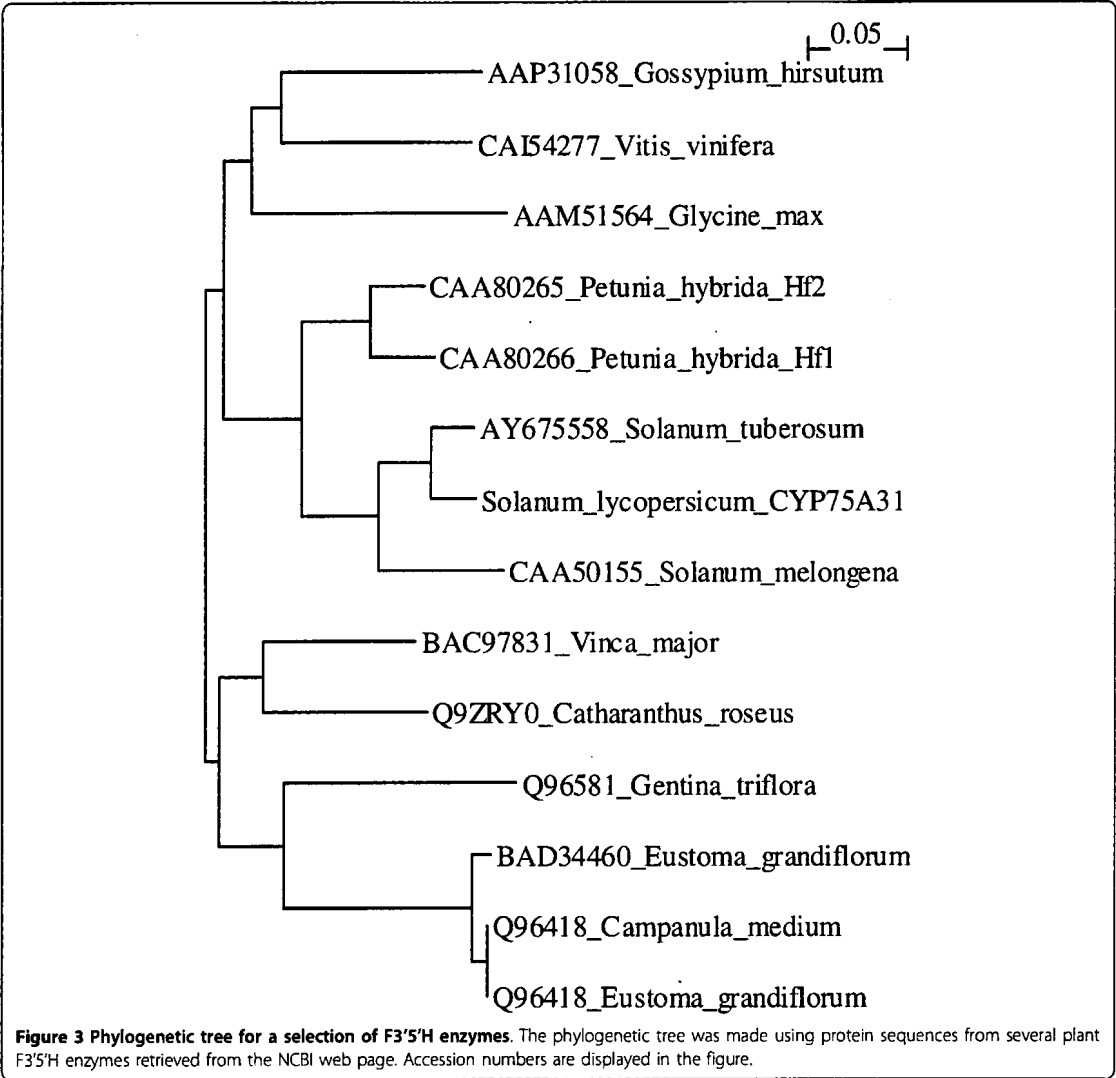
The coding sequence of the *CYP75A31* gene was transformed into yeast for heterologous expression. Enzyme assays were run on isolated microsome fractions, substrates and products were analysed by HPLC and MS. The substrates found to be metabolized by *CYP75A31* are listed in table 1. Luteolin (5,7,3',4'-tetrahydroxyflavone) gave tricetin (5,7,3',4',5'-pentahydroxyflavone) as the only product. Naringenin (5,7,4'-trihydroxyflavanone) gave rise to two peaks in the HPLC-spectrum identified as eriodictyol (5,7,3',4'-tetrahydroxyflavanone), and 5,7,3',4',5'-pentahydroxyflavanone. As expected, eriodictyol as substrate gave only one product, 5,7,3',4',5'-pentahydroxyflavanone. Dihydrokaempferol (3,5,7,4'-tetrahydroxyflavanone) gave two peaks, dihydroquercetin (3,5,7,3',4'-pentahydroxyflavanone), and dihydromyricetin (3,5,7,3',4',5'-hexahydroxyflavanone). Dihydroquercetin as substrate gave one product, as expected, identified as dihydromyricetin. Kaempferol (3,5,7,4'-tetrahydroxyflavone) resulted in two peaks, identified as quercetin (3,5,7,3',4'-pentahydroxyflavone) and myricetin (3,5,7,3',4',5'-hexahydroxyflavone). Quercetin as substrate gave myricetin as the only product, and liquiritigenin (7,4'-dihydroxyflavanone) gave two products: butin (7,3',4'-trihydroxyflavanone) and 7,3',4',5'-tetrahydroxyflavanone. Neither the control reactions without NADPH, nor assays with microsomes isolated from yeast transformed with *pYEDP60* vector lacking an insertion, showed any product formation.

Gene expression

Tomato plants were grown on rock-wool with complete nutrient supply under continuous light. The rock-wool

was rinsed with water to remove previous nutrient solution, and plants were randomly divided in two batches. One batch continued with complete nutrient solution, whereas the second batch received nutrient solution with no nitrogen. Samples were harvested before change of nutrients (day 0) and again after three days. Gene expression was measured by real-time PCR, using the shoot top (young tissue, e.g. shoot apex with primordia and developing leaves, including first unfolded still small leaf) on day 0 as calibrator. Relative expression of all genes is hence given as a fold change related to the shoot top sample taken on day 0. Expression of the *F3'5'H* gene, six other structural genes of the phenylpropanoid pathway and transcription factors *anthocyanin 1* (*ANT1*) and *SIJAF13* (which is a putative homolog to the petunia *JAF13* gene [26]) was tested by real-time PCR. All nine genes showed a general increase in response to nitrogen deprivation (Figure 4a-i). Averaged over all parts of the plant the expression of *chalcone synthase 2* (*CHS2*), *F3'H*, *PALS*, *FLS*, *F3'5'H*, *DFR*, *SIJAF13* and *ANT1* on day 3 was 22.0, 19.6, 16.2, 15.7, 13.3, 8.9, 8.9 and 8.0 fold higher, respectively, in nitrogen deprived plants as compared to plants given full nutrient solution. At day 3, *flavanone 3-hydroxylase* (*F3H*) (Figure 4c) showed detectable expression only for nitrogen deprived plants, which overall was 20 fold higher than on day 0. *F3H* is the only gene with no detectable transcripts in plants receiving nitrogen on day 3; the reason for this is unknown. All of the genes, with the exception of *F3'H* (Figure 4d), showed highest expression in nitrogen depleted leaflets (from 5th leaf from the hypocotyl) on day 3. For *F3'H* the highest expression was found in nitrogen depleted petioles (from 5th leaf from the hypocotyl). The nitrogen effect in leaflets was especially high for *F3'5'H* (Figure 4e). *PALS* (Figure 4a) showed a clear increase in response to nitrogen deprivation, also in roots. *SIJAF13* (Figure 4i) showed a clear nitrogen effect in all plant parts tested, as did *ANT1* (Figure 4h). Expression of *CHS2* (Figure 4b) displayed a convincing nitrogen effect in shoot top, petiole, leaflets and stalk (of the whole plant). *DFR* (Figure 4g) was expressed in much the same way as *CHS2* but showed a slightly higher increase in relative expression in the leaflets, and lower in the shoot top of nitrogen deprived plants. Expression of *FLS* (Figure 4f) was clearly elevated in all parts of nitrogen deprived plants while the level remained relatively stable in plants receiving nitrogen.





Phenolic content

Measurements of phenolic content were conducted on the same samples as the expression analysis. Rutin was detected in all samples, except roots at day 0. In all parts of the plant the content had increased from day 0 to day 3 and was clearly higher in nitrogen deprived plants (Figure 5a). The overall content of rutin in nitrogen deprived plants on day 3 was 1.9 times higher than in nitrogen replete plants. Kaempferol-3-rutinoside was not detected in samples from stalk or root, and only in nitrogen deprived leaflets. In the shoot top and petiole there was a clear increase from day 0 to day 3, especially in nitrogen depleted plants (fig 5b). The overall content of kaempferol-3-rutinoside in nitrogen deprived plants

on day 3 was 2.3 times higher than in nitrogen replete plants. Anthocyanins were not detectable in any samples under the growth conditions used.

Discussion

When starting the in vitro enzyme assays, substrates were chosen based on previous findings on accepted substrates for F3'5'H enzymes from other plants. Substrates were also chosen based on structural similarity to these compounds. With the exception of liquiritigenin, substrates found to be metabolized by CYP75A31 were also found to be metabolized by CYP75A8, which was previously isolated from *C. roseus* [15]. The Kaltenbach group also tested a petunia F3'5'H in the *E. coli*

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Table 1 List of accepted substrates for CYP75A31

Substrate	Product of 3'-hydroxylation	Product of 5'-hydroxylation	Class
Luteolin (20.3) [286]	-	Tricetin (18.2) [302]	Flavone
Naringenin (21.2) [272]	Eriodictyol (19.1) [288]	5,7,3',4',5'-pentahydroxyflavanone (16.3)	Flavanone
Eriodictyol (18.9) [288]	-	5,7,3',4',5'-pentahydroxyflavanone (16.2) [304]	Flavanone
Dihydrokaempferol (17.0) [288]	Dihydroquercetin (15.0) [304]	Dihydromyricetin (12.4) [320]	Dihydroflavonol
Dihydroquercetin (15.0) [302]	-	Dihydromyricetin (12.4) [318]	Dihydroflavonol
Kaempferol (22.4) [286]	Quercetin (20.1) [302]	Myricetin (17.1) [318]	Flavonol
Quercetin (20.0) [302]	-	Myricetin (17.0) [318]	Flavonol
Liquiritigenin (19.0) [256]	Butin (17.03) [272]	7,3',4',5'-tetrahydroxyflavanone (14.4)	Flavanone

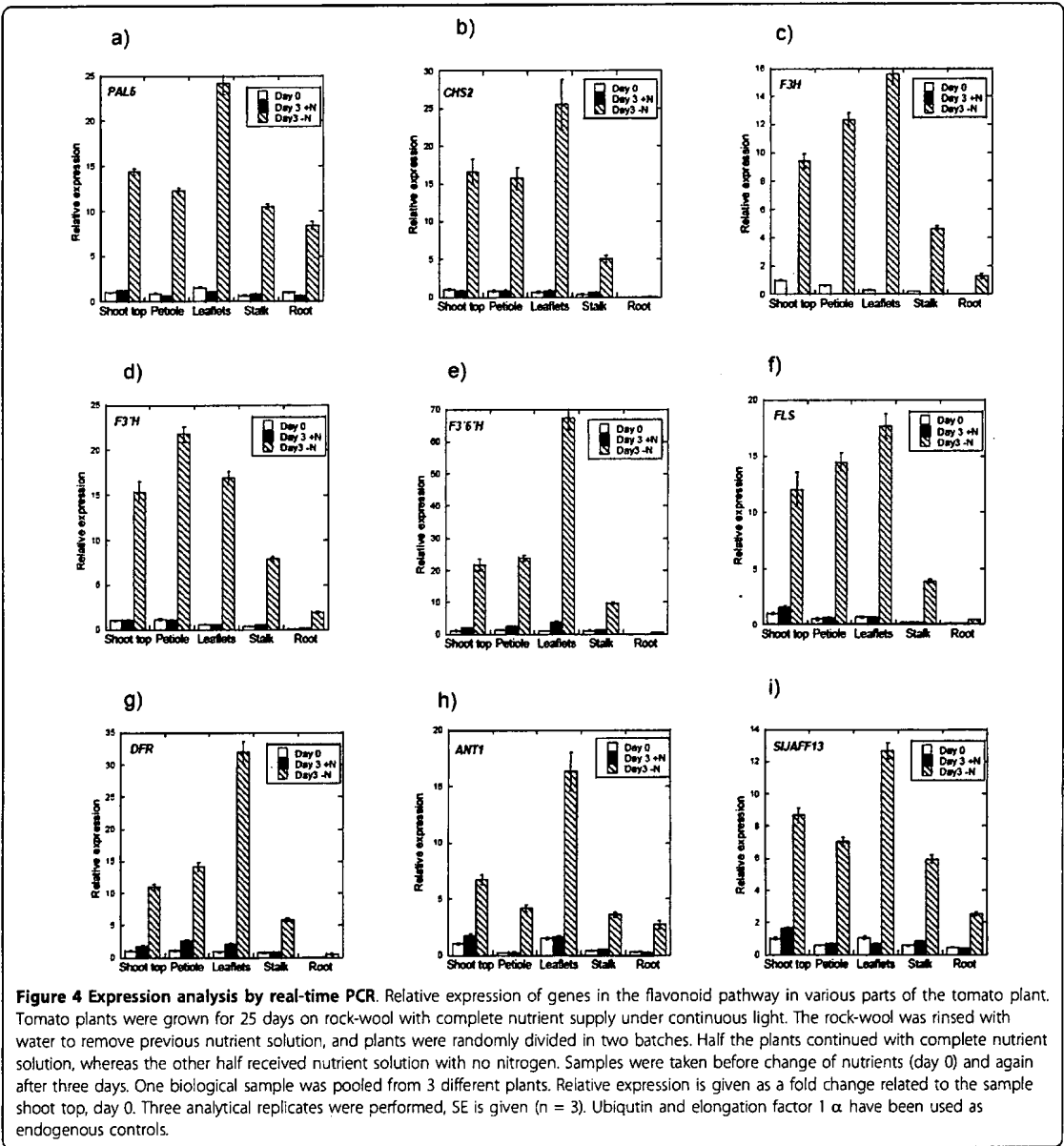
Enzyme assays were run on microsome preparations of yeast transformed with the CYP75A31 gene. Product formation was analysed by HPLC and MS. HPLC retention times in minutes are given in parenthesis. Masses in g/mol, as determined by MS, are given in brackets.

expression system used for CYP75A8, and found that the petunia F3'5'H accepted the same substrates. Whereas the *C. roseus* F3'5'H had highest activity with apigenin, the petunia F3'5'H had highest activity with naringenin [15]. For the CYP75A31 enzyme there was a clear preference for naringenin and liquiritigenin, as these substrates were metabolised also in dilute microsome preparations. In the present study, CYP75A8 was also expressed in the same yeast (expression) system as CYP75A31. K_m for naringenin was measured to 1.20 μ M for CYP75A31, and 0.83 μ M for CYP75A8. Kaltenbach et al. [15] reported an apparent K_m of 7 μ M for naringenin when expressing CYP75A8 in the *E. coli* expression system. The rate of hydroxylation performed by a F3'5'H enzyme is dependent on the reductase used in the expression system. De Vetten et al. [27] has shown that a cytochrome b_5 is required for full activity of F3'5'H in petunia. The gene encoding a cytochrome b_5 was inactivated by targeted transposon mutagenesis, which resulted in reduced F3'5'H activity and reduced accumulation of 5'-substituted anthocyanins, leading to an alteration in flower colour. Our expression studies utilized the Arabidopsis ATR1 reductase, whereas in the expression studies performed by Kaltenbach et al. [15], a *C. roseus* P450 reductase was used in the *E. coli* expression system. The use of different expression systems, and reductases, may explain the difference in K_m values obtained for the *C. roseus* CYP75A8 enzyme in the two studies [28].

Liquiritigenin has to our knowledge not been shown to be metabolized by a F3'5'H enzyme previously. Liquiritigenin in plants is mostly associated with the legumes, which have a CHI capable of isomerising 6'-hydroxy- and 6'-deoxychalcones to 5-hydroxy- and 5-

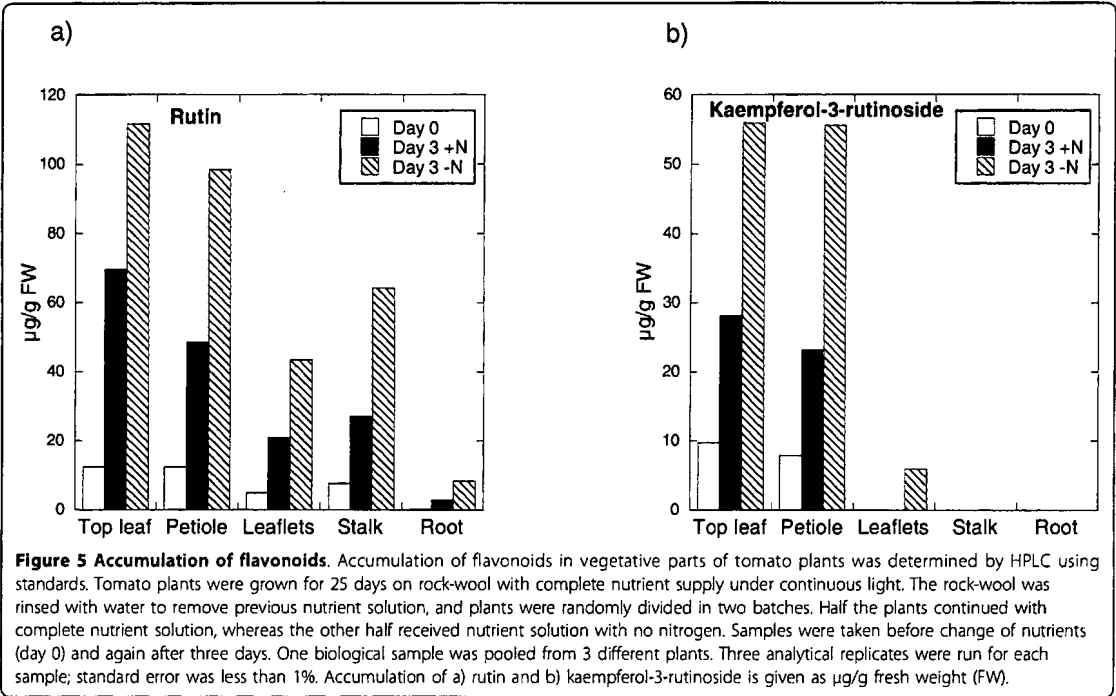
deoxyflavanones respectively. Joungh et al. [29] reported that the tobacco CHI is able to isomerise the 6'-deoxychalcone isoliquiritigenin to the 5-deoxyflavanone, liquiritigenin, in transgenic tobacco over-expressing a *Pueraria montana* chalcone reductase gene. Tanaka et al. [20] showed that the F3'5'H from *Gentiana triflora* catalysed the hydroxylation of naringenin to eriodictyol, eriodictyol to 5, 7, 3', 4', 5'-pentahydroxyflavanone, dihydrokaempferol to dihydroquercetin, dihydroquercetin to dihydromyricetin and apigenin to luteolin when expressed in *S. cerevisiae* under the control of a glyceraldehyde-3-phosphate dehydrogenase promoter. The reaction rates and substrate preferences recorded in bacteria or yeast expression systems do not necessarily represent the actual rate or preference *in planta*. As demonstrated in this study, the tomato F3'5'H is capable of metabolizing liquiritigenin, although to our knowledge liquiritigenin has never been found in tomato plants.

Expression analysis showed that all the major genes of the flavonoid pathway tested, including F3'5'H, had a clear increase in expression as a result of three days of nitrogen deprivation (Figure 4). Despite what seemed to be a general up-regulation of the flavonoid pathway in this study, the growth conditions applied had not resulted in accumulation of anthocyanins at the time of sampling. At the time of sampling, the increase in gene expression was more prominent than the increase in level of rutin and kaempferol-3-rutinoside. As gene expression increases prior to accumulation of product this implies that accumulation of rutin and kaempferol-3-rutinoside had not yet reached the maximum. Similar studies (unpublished results) conducted on nitrogen deprived tomato plants have shown that also anthocyanins will appear over time. Possibly the concentrations



of dihydrokaempferol and/or dihydroquercetin have to exceed a threshold level for F3'5'H to metabolise what FLS does not have capacity for. Similar studies [30] showed far higher levels of flavonol-derivatives than in the present study at the time of anthocyanin accumulation, which might indicate that FLS does not have the capacity to metabolise all the dihydrokaempferol/dihydroquercetin as the flow through the pathway escalates. The increase in transcripts of F3'H in all parts of the

nitrogen deprived plants, indicates increased production of the F3'H enzyme, which hydroxylates dihydrokaempferol to dihydroquercetin. The action of this enzyme, (together with F3'5'H), might explain why the content of rutin is much higher than kaempferol-3-rutinoside, since they have dihydroquercetin and dihydrokaempferol as precursors respectively. It should be mentioned that although the F3'H tested here was a clear orthologue to the petunia F3'H, the tomato F3'H has not yet been



cloned and characterised, hence its function still needs to be established. This is especially relevant considering that the F3'5'H present in tomato is also capable of catalysing the 3'-hydroxylation.

A similar study [30] showed accumulation of anthocyanins in leaves of nitrogen deprived tomato plants. In this study the nitrogen deprivation lasted a minimum of four days, and flavonoid content continued to increase from the fourth to the eighth day of nitrogen deprivation.

Consistent with the increase in rutin and kaempferol-3-rutinoside, the enzyme responsible for increasing flux into the phenylpropanoid pathway, *PAL5* increased in expression as a response to nitrogen deprivation. The MYB-type transcription factor *ANT1*, and the putative bHLH transcription factor *SIJAF13*, also increased in all parts of nitrogen deprived plants. This is consistent with the general increase in all the flavonoid structural genes tested, and the increase in flavonoid content.

Conclusions

The sequenced gene, *CYP75A31*, encodes a flavonoid 3'5'-hydroxylase which accepts luteolin, naringenin, eriodictyol, dihydrokaempferol, dihydroquercetin, kaempferol, quercetin and liquiritigenin as substrates. The ability to do 3'- and especially 5'-hydroxylation of intermediates in the flavonoid pathway places *CYP75A31* at

an important branch point in the regulation between flavonol and anthocyanin synthesis. Expression of the *CYP75A31* gene increased in response to nitrogen deprivation, in accordance with other genes in the phenylpropanoid pathway, which is an expected response to abiotic stress in plants.

Methods

Plant Material

Suzanne F1 seeds were sown on rock wool and given Hoagland nutrient solution containing 15 mM NO₃⁻ [31]. RNA and DNA used to identify coding sequence and introns of the *F3'5'H* gene was isolated from plants grown in a 12 h light/dark regimen. Expression and metabolite analysis were performed on plants grown in continuous light, and given complete Hoagland solution before shifted to a nitrogen deprived regimen where KNO₃ was replaced by KCl and Ca(NO₃)₂·4H₂O was replaced by CaCl₂.

Identifying the F3'5'H gene

RNA was isolated from leaves of the cherry tomato Suzanne F1 using the RNeasy Plant Mini Kit (Qiagen, USA). To identify the 3' end of the *F3'5'H* gene the GeneRacer™ Kit (Invitrogen, USA) was used. The gene specific left primer used for the 3' end had the sequence ACAAGGATGGGAATAGTGATGGT and was based on a *F3'5'H* sequence for *Solanum tuberosum* (accession

number: AY675558). The cDNA amplified was sequenced, and a nucleotide BLAST against the GeneBank (NCBI) showed close similarity to other *F3'5'H* sequences. An EST sequence was found in the TIGR database (accession number DB723744) which was assumed to be the 5' end of the gene. Based on the obtained sequences for 3' and 5' ends, new primers covering the entire gene were made. The 3' sequence was used to make the primer 75ALerevECO (GGAATTCTCAGCAACGATAAACGTCCAAAGATAG) with an additional *EcoRI* site for the 3' end of the gene. The 5' end primer, 75ALedirBAM (GGGATCCATGGCGTTACGTATTAATGAGTTATTT), includes an additional *BamHI* site.

cDNA for cloning was made using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The ORF of *CYP75A31* was amplified by PCR introducing *BamHI/EcoRI* restriction sites upstream of the start ATG and downstream to the stop codon TGA using Platinum™ *Taq* DNA Polymerase High Fidelity (Invitrogen). PCR program was as follows: 95°C for 5 min, followed by 5 cycles of 95°C for 1 min, 40°C for 1 min and 72°C for 1.5 min. Then 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1.5 min. At the end there was an extra 5 min elongation at 72°C before cooling to 4°C. The product was ligated into a TOPO vector using the pCR® 8/GW/TOPO™ TA Cloning™ Kit (Invitrogen) as recommended. The ligated vector was transformed into OneShot™ Chemically Competent *E. coli* (Invitrogen) and grown on LB-media containing spectinomycin. Several individual colonies were picked and grown to amplify and isolate the plasmids for sequencing. The obtained sequences were subjected to a BLAST search, and were shown to display significant similarities to *F3'5'H* genes isolated from other species.

Expression Constructs

CYP75A31 was cut from the TOPO vector using *BamHI* and *EcoRI*, then ligated into the *pYeDP60* vector [32] for expression in yeast.

Yeast Expression and microsome preparation

The yeast strain *Saccharomyces cerevisiae* WAT11, engineered to over-express the P450 reductase isoform ATR1 from *Arabidopsis thaliana* when induced with galactose [32], was used for the expression. Transformation with the *pYeDP60* expression construct was performed as previously described by Gietz et al. [33]. Propagation of yeast cells and preparation of microsomes was done as described by Pompon et al. [32] with some modifications. Liquid SGlu, 50 ml, was inoculated by a single colony from a SGLu plate and grown at 30°C for 48 h. The culture was then transferred to 200 ml YPGlu medium, containing 20 g/l glucose, and grown at 30°C for 24 h. The yeast cells were spun down (2000 × g, 3 min) and re-suspended in YPGal medium

containing 20 g/l galactose for induction of microsomes at 16°C for 24 h. Microsomes were isolated in the following way: The yeast culture was centrifuged (2 000 × g, 10 min) and the pellet re-suspended in 50 ml TEK (100 mM KCl in 50 mM Tris-HCl with 1 mM EDTA), centrifuged at 6 100 × g for 3 min and the pellet re-suspended in 2 ml extraction buffer (20 mM β-mercaptoethanol, 1% BSA and 0.6 M sorbitol in 50 mM Tris-HCl with 1 mM EDTA). Glass beads were added, and the suspension was shaken in an automatic shaker (Retsch MM200 Mixer Mill, Krackeler Scientific, USA) 4 × 2 min at a vibration frequency of 30. Between two shaking cycles the suspension was placed on ice for 3 min. Portions of 10 ml extraction buffer was added to the beads 4 times, shaken and decanted to retrieve the microsomes. Extraction buffer was centrifuged for 15 min at 6 100 × g, the supernatant was filtered, and MgCl₂ added to a final concentration of 50 mM in order to precipitate the microsomes [34]. The suspension was placed on ice for approximately 1 h before centrifugation at 12 500 × g for 20 min. The pellet was dissolved in 1.0 to 1.5 ml TEG (30% glycerol in 50 mM Tris-HCl with 1 mM EDTA) and homogenized using a Teflon pestle. Work was carried out on ice, all buffers/solutions and centrifuge were pre-cooled to 4°C.

CYP75A31 Enzyme assays

Several compounds were tested as potential substrates for *CYP75A31*. Microsomes isolated from yeast *CYP75A31* transformants were incubated in 0.1 M sodium phosphate buffer, pH 7.0 containing 1.0 mM NADPH, or without NADPH (as a negative control). The assay mixture was equilibrated for 2 min at 27°C prior to starting the reaction by addition of microsomes. Concentration of substrate in the assays ranged between 20 to 100 μM. Total volume of assay was 200 μl. After 10 to 30 min the reaction was stopped by adding 75 μl of acetonitrile/concentrated HCl (99:1). Precipitated proteins were removed by a 10 min centrifugation (9300 × g); the supernatant was used directly for HPLC and MS analysis to assess product formation and substrate consumption. To validate that hydroxylations occurred due to *CYP75A31* activity, assays were run with a microsome preparation made from WAT11 transformed with the *pYeDP60* vector without any insertions.

Real-Time PCR

Plants were sown on rock-wool and grown at 22°C for 25 days with full Hoagland nutrient solution, in continuous light (approximately 200 μmolm⁻²s⁻¹ PAR). The rock-wool was rinsed thoroughly with tap water to remove nutrients, before adding nutrient solution deprived of nitrogen (referred to as day 0). The following samples were taken from three plants and pooled to one sample (for each part of the plant): shoot top (young tissue, e.g. shoot apex with primordia and

developing leaves, including first unfolded still small leaf), petiole (from the 5th leaf from the hypocotyl), leaflets (from the 5th leaf from the hypocotyl), stem (the whole stem of the plant) and roots (efforts were made to retrieve as much of the root as possible, but some finer parts were lost in the rock wool). The tissues were snap frozen in liquid nitrogen and stored at -80°C before ground into powder in liquid nitrogen (samples for RNA and phenolic analysis were taken from the same powder). Samples were pooled from three plants receiving nitrogen and three plants deprived of nitrogen at day three. Total RNA was isolated using RNeasy® Plant Mini Kit (Qiagen). RNA was quantified by spectrophotometer and cDNA synthesised using the High Capacity cDNA Archive Kit (Applied Biosystems, USA) (concentration of RNA in the reaction tube was 10 µg mL⁻¹). Real-time PCR reactions were assayed using an ABI 7300 Fast Real-Time PCR System (Applied Biosystems) with Sybr-Green for detection. The reaction volume was 20 µL containing 10 µL qPCR Master Mix (PrimerDesign, UK), 0.3 µM primer (forward and reverse) and 1 µL cDNA. Standard cycling conditions (2 min at 50°C, 10 min at 95°C and 40 cycles altering between 15 s at 95°C and 1 min at 60°C) were used for product formation. Forward and reverse primers were as follows (with RTPrimerDB <http://www.rtpimerdb.org> identification number given in brackets when available); PAL5-F, 5'-TTTCTCCATTACAAATCAAACCA-3' and PAL5-R, 5'-TTCACCTTCATCCAAATGACTCC-3', CHS2 LOC778295 (7794); DFR LOC544150 (7795); FLS-F, 5'-TAAGATTTGGCCTCCTCCTG-3' and FLS-R, 5'-ACCAAGCCCAAGTGATAAGC-3'; F3H-F, 5'-AGTGGTGAATTGCAATAGCAGTAG-3' and F3H-R, 5'-TTTCCTCCTGTACATTTCTGCAA-3'; F3'H-F, 5'-GAGGAGTTCAAGTTAATGGTGGT-3' and F3'H-R, 5'-ACTCGCTTTTCCTTGTGTTCTT-3'; ANT1 (7793); JAF13-F, 5'-AGGAGAGTTCAGGAGCTGGAG-3'; JAF13-R, 5'-GCCTTCCTTTTGTTCGGTAG-3' [30] and; F3'5'H-F, 5'-TCCCTCAACGCCACTAAATC-3' and F3'5'H-R, 5'-TTTTCCCGCTAAGGAACC-3'. Gene expression for each sample was calculated on three analytical replicates normalized using the geometric average of the reference genes ubiquitin and elongation factor 1α [35] in the qBaseplus software [36], using the shoot top harvested at day 0 as calibrator. Thus, relative quantity of any gene is given as fold change relative to day 0.

Flavonoid standards

Naringenin, dihydroquercetin, kaempferol and quercetin were obtained from Sigma-Aldrich (USA). Liquiritigenin was obtained from Extrasynthèse (France). Luteolin, eriodictyol and dihydrokaempferol were obtained from TransMIT (Germany).

HPLC and MS analysis

Analysis of enzyme substrates and products

The flavonoids were analysed on a HPLC system (LC 20AD, Shimadzu Corporation, Japan) equipped with a C18 LichroCART 125-4 column (Merck, Germany) connected to a diode array detector (SPD M20A, Shimadzu Corporation). Substrates and products separations were done using a solvent system of (A) 0.1% (v/v) acetic acid in water and (B) methanol:acetonitrile (1:1). The column was equilibrated in solvent A at a flow rate of 0.9 ml/min for 5 min, and the elution was performed using a linear gradient of solvent B from 0 to 67% for 25 min, followed by 100% B for an additional 5 min. Detection was made on a wavelength range of 220-400 nm. Injection volume was 50 µL.

Mass spectrometric analyses

The HPLC-MS system comprised the binary solvent delivery pump (Surveyor MS, ThermoFinnigan, USA) connected to a diode array detector (Surveyor PDA plus, ThermoFinnigan) and a linear ion trap mass spectrometer (LTQ-MS, ThermoFinnigan). Products separation was done as described in the above paragraph. LTQ equipped with an atmospheric pressure ionization interface operating in ESI mode. Data were processed using LCQuan software (version 2.0). Computer was controlled by Xcalibur 1.4 software. The operational parameters of the mass spectrometer were as shown below. The spray voltage was 5 kV and the temperature of the heated capillary was set at 200°C. The flow rates of sheath gas, auxiliary gas, and sweep gas were set (in arbitrary units min⁻¹) to 50, 10, and 10, respectively. Capillary voltage was +20/-20V (positive/negative polarity), tube lens was +65/-65V (positive/negative polarity) and the front lens was +5/-5V (positive/negative polarity).

Characterisation of product formation

The products eriodictyol, dihydroquercetin and quercetin were identified using HPLC-standards, and MS (table 1). Triacetin, 5,7,3',4',5'-pentahydroxyflavanone, dihydromyricetin and myricetin were identified by MS (table 1). Absorbance maximum for substrates and products are given in Additional file 1. Structure for substrates and products are given in Additional file 2.

Analysis of flavonoids in vegetative parts of the tomato plant

Samples of approximately 100 mg were extracted in 1 ml of 1% (v/v) trifluoroacetic acid (TFA) in methanol, and analyzed by use of a liquid chromatograph (Agilent 1100-system, Agilent Technologies, Norway) supplied with a photodiode array detector. Separation was achieved on an Eclipse XDB-C8 (4.6 × 150 mm, 5 µm) column (Agilent Technologies) by use of a binary solvent system consisting of (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile. The gradient (%B in A)

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was linear from 5 to 10 in 5 min, from 10 to 25 for the next 5 min, from 25 to 85 in 6 min, from 85 to 5 in 2 min, and finally recondition of the column by 5% in 2 min. The flow rate was 0.8 ml/min, 10 µl samples were injected on the column, and separation took place at 30° C. Detection was made over the interval 230-600 nm in steps of 2 nm in order to obtain full absorbance spectrum of the compounds of interest. Peak characterization was done in accordance to previous results [37,38]. Quantitative levels of the rutinoides of kaempferol and quercetin, the major flavonoids in tomato seedlings, were calculated as peak areas obtained at 370 nm compared to the responses of authentic samples (rutin and kaempferol-3-rutinoides, provided by PlantChem, Norway). All results were corrected against the exact weight of the sample. One biological sample, pooled from three individual plants, was analyzed. Three analytical replicates were done for each sample; standard error was less than 1%.

Phylogenetic analysis

Protein sequences of previously published F3'5'H enzymes were obtained from the NCBI home page (accession numbers are given in the phylogenetic tree, figure 3). The phylogenetic analysis was done using the default settings of ClustalX (1.83).

List of abbreviations

4CL: 4-coumarate: CoA ligase; ANS: anthocyanidin synthase; ANT1: anthocyanin 1; bHLH: basic-helix-loop-helix; C4H: cinnamate 4-hydroxylase; CHI: chalcone isomerase; CHS2: chalcone synthase 2; DFR: dihydroflavonol 4-reductase; F3H: flavanone 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; F3'5'H: flavonoid 3'5'-hydroxylase; FLS: flavonol synthase; PAL5: phenylalanine ammonia-lyase 5; TFA: trifluoroacetic acid; UFGT: UDP glucose flavonoid 3-O-glucosyl transferase.

Additional file 1: Absorption maximum for substrates and products.
HPLC absorption maximum for substrates and products used.
Click here for file
[<http://www.biomedcentral.com/content/supplementary/1471-2229-10-21-S1.PDF>]

Additional file 2: Structures of substrates and products. Structures for substrates and products.
Click here for file
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Author details

¹University of Stavanger, Centre for Organelle Research, Faculty of Science and Technology, N-4036 Stavanger, Norway. ²UMR 1121 Nancy Université (INPL)-INRA Agronomie et Environnement (Nancy/Colmar), IFR110, 2, av. de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy, France. ³PlantChem, Særaheim Research Center, N-4353 Klepp stasjon, Norway.

Authors' contributions

KMO performed the cloning and expression studies, HPLC analysis on enzyme assays, real-time PCR analysis, and drafted the manuscript. AH provided guidance and help in cloning and expression studies. HJ did expression studies and measured K_m. RS performed HPLC analysis on enzyme assays, and measured phenolic content in tomato plant samples. RL gave guidance in HPLC, and did MS analysis. FB defined the project presented and provided general advice. CL provided general advice, especially on writing of article. All authors read and approved the final manuscript.

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Appendix 2

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Flavonoid hydroxylase from *Catharanthus roseus*: cDNA, heterologous expression, enzyme properties and cell-type specific expression in plants

Melanie Kaltenbach¹, Gudrun Schröder¹,
Elmon Schmelzer², Verena Lutz^{1,*} and
Joachim Schröder^{1,2}

¹Institut für Biologie II, Universität Freiburg,
Schänzlestrasse 1, D-79104 Freiburg, Germany, and
²Max-Planck-Institut für Züchtungsforschung, Abteilung
Biochemie, Carl-von-Linné-Weg 10, D-50829 Köln,
Germany

Summary

We investigated the P450 dependent flavonoid hydroxylase from the ornamental plant *Catharanthus roseus*. cDNAs were obtained by heterologous screening with the CYP75 Hf1 cDNA from *Petunia hybrida*. The *C. roseus* protein shared 68–78% identity with other CYP75s, and genomic blots suggested one or two genes. The protein was expressed in *Escherichia coli* as translational fusion with the P450 reductase from *C. roseus*. Enzyme assays showed that it was a flavonoid 3',5'-hydroxylase, but 3'-hydroxylated products were also detected. The substrate specificity was investigated with the *C. roseus* enzyme and a fusion protein of the *Petunia hybrida* CYP75 with the *C. roseus* P450 reductase. Both enzymes accepted flavanones as well as flavones, dihydroflavonols and flavonols, and both performed 3'- as well as 3',5'-hydroxylation. Kinetics with *C. roseus* cultures on the level of enzyme activity, protein and RNA showed that the F3',5'H was present in dark-grown cells and was induced by irradiation. The same results were obtained for cinnamic acid 4-hydroxylase and flavanone 3 β -hydroxylase. In contrast, CHS expression was strictly dependent on light, although CHS is necessary in the synthesis of the F3',5'H substrates. Immunohistochemical localization of F3',5'H had not been performed before. A comparison of CHS and F3',5'H in cotyledons and flower buds from *C. roseus* identified CHS expression preferentially in the epidermis, while

F3',5'H was only detected in the phloem. The cell-type specific expression suggests that intercellular transport may play an important role in the compartmentation of the pathways to the different flavonoids.

Introduction

Flavonoids have been of interest for many years and their roles and the biosynthetic enzymes have been the subject of many investigations. Of particular interest is their contribution to flower colour (Biolley *et al.*, 1994; Holton and Cornish, 1995; Mol *et al.*, 1998). A major factor is the cytochrome P450 dependent flavonoid hydroxylases (EC 1.14.13.21) which introduce either one (F3'H) (Forkmann *et al.*, 1980) or two (F3',5'H) (Stotz and Forkmann, 1982) of the hydroxyl groups which strongly influence flower colours. Plants containing F3',5'H may also contain F3'H, as shown in some cases by genetics, e.g. with *Petunia hybrida* (Wiering and De Vlaming, 1984), and it is difficult to exclude the presence of F3'H by *in vitro* experiments. It is not understood whether the sequential hydroxylations performed by F3',5'H involve release and re-binding of the 3'-hydroxylated first product, or whether they are coupled without release of the intermediate. Although most enzymes were tested with only a few substrates, the overall picture suggested that both F3'H and F3',5'H hydroxylated several classes of flavonoid aglycones (Figure 1). This was one of the main reasons for the proposal that intermediates in flavonoid biosynthesis can be synthesized by multiple paths (grid-type pathway) (Stafford, 1990).

Sequences for F3'H are not yet available but, as shown by heterologous expression, cDNAs from *Petunia hybrida* (Holton *et al.*, 1993; Toguri *et al.*, 1993) and *Gentiana triflora* (Tanaka *et al.*, 1996) code for F3',5'H. The proteins belong to the CYP75 family in the superfamily of P450 enzymes (Nelson *et al.*, 1996). The other CYP75 family members have not been functionally confirmed after heterologous expression, i.e. from *Solanum melongena* (Toguri *et al.*, 1993), *Eustoma grandiflorum* and *russellianum* (Nielsen and Podivinsky, 1997), and *Campanula medium* (GenBank accession number D14590).

Many of the enzymes involved in flavonoid biosynthesis have been studied in great detail. The P450 dependent flavonoid hydroxylases are one of the few exceptions, and

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*For correspondence (fax +49 761 203 2601; e-mail jschro@ruf.uni-freiburg.de).

¹Present address: Klinische Forschergruppe Prof. Schmitt, Frauenklinik des Klinikums, Ismaningerstraße 22, 81675 München, Germany.

Abbreviations: C4H, cinnamic acid 4-hydroxylase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3',5'H, flavonoid 3',5'-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FHT, flavanone 3 β -hydroxylase; P450, cytochrome P450-dependent enzyme; P450Red, cytochrome P450 reductase; PAL, phenylalanine ammonia-lyase.

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this led us to investigate the enzyme in *C. roseus*. We describe cDNAs for F3'5'H, the functional identification, and a detailed enzymatic characterization of the F3'5'Hs from *C. roseus* and *P. hybrida* after heterologous expression. We also investigated the regulation in cell cultures in comparison with several other enzymes involved in flavonoid biosynthesis. These studies revealed that chalcone synthase (CHS) expression was strictly dependent on light, while F3'5'H expression was not, although CHS is necessary for the synthesis of the F3'5'H substrates. One of the possible explanations was a different cell-type specific expression. Immunohistochemical localization experiments with plant tissues indeed showed that the predominant expression of F3'5'H and CHS was not in the same cells.

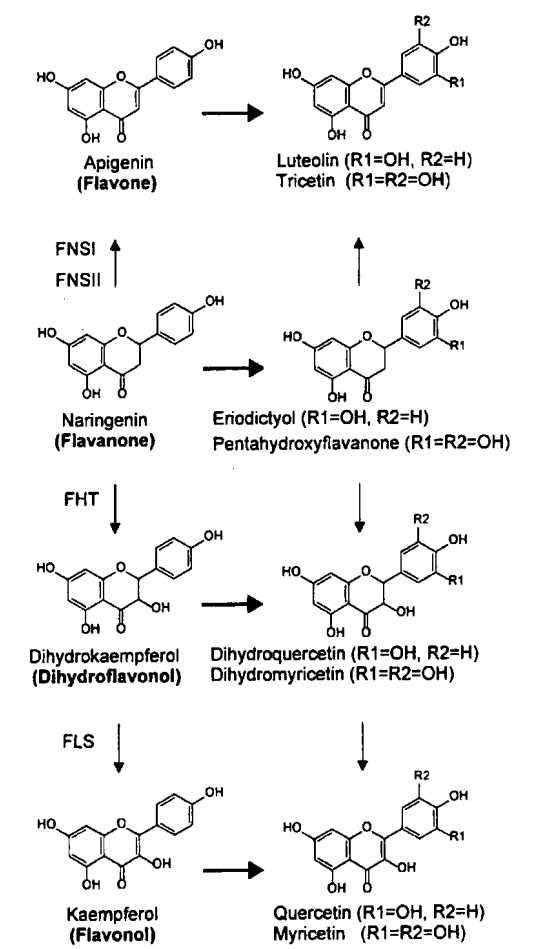


Figure 1. Overview of substrates and products of cytochrome P450-dependent flavonoid hydroxylase reactions (bold arrows). Other enzymes: FHT, flavanone 3β-hydroxylase; FLS, flavonol synthase; FNSI and FNSII, flavone synthases.

Results and Discussion

CYP75 cDNA and genomic blot

The CYP75 from *C. roseus* was obtained by heterologous screening with the *P. hybrida* cDNA. The first three cDNAs were from the same gene but were of different length (1.4, 0.9, 0.6 kbp). All contained the 5'-end of the coding region for a putative flavonoid hydroxylase but lacked various lengths from the 3'-end of the mRNA. Rescreening of the libraries with a homologous probe resulted in the identification of 17 additional cDNAs of various sizes. The overlapping sequences indicated that the cultures expressed a single CYP75 gene under the conditions tested. None of the clones contained the entire protein coding region, and the complete cDNA was constructed from two clones. The predicted polypeptide (named CYP75A8) contained 512 amino acids, had a calculated mass of 57.805 kDa, and shared 68–78% identity with the other proteins of the CYP75 family. Figure 2 shows the comparison between *C. roseus* and *P. hybrida*. The latter was chosen because its enzyme properties were also investigated in this work. The genomic blot with the complete cDNA (Figure 3) revealed a simple pattern of strongly hybridizing fragments, suggesting that *C. roseus*

a) <i>C. roseus</i>	MALEKLVLPDFLAAISILILVQKFIQIVFLRSSS
b) <i>P. hybrida</i>	-----MMLLTTELGAATSIPLIAHIIISTLISKTT
a)	RIRLPPGPKGWPIIGALPYLGTMPHSILANMAKRYGPIINYLKLGTNG
b)	GRH.....R....V.....L..A....VS..K.....A.....V..C..
a)	MVVASTPDVAKAFLRLTDMNFSNRPIDAGATHLAYNAQDMVFAHYGP
b)	.A.....A.....K...I.....FN.....
a)	KWKLLRKLSNLHMLGGKALENWSNVRATELGYMLQAMYESSRKGETV
b)	R.....A.....N...H...KS.SDM..E.QR..
a)	VVPEMLTYAMANNIGQVILSRVVFVTKSLESNEFKDMVVELMTTAGY
b)	.A.....F.....M..K....D.GV.....I....
a)	FNIGDFIPSIAMMDLQGIERGMRKLHKKFDPALLTKMLEHKSSSHKR
b)	CL.....KR.....FD...ATTYE..
a)	KEKPDFLDYVLANRDNSEGERLTTNIKALLNLFTAGTDTSSSVIE
b)	.G.....V..ME.G.....S.....A...
a)	WAISEMLKNPTILKRAQEEMDQVIGNRRLMESDIPKLPYLQAIKCE
b)	..LA..M...A...K..A.....L.....N...R....
a)	TFRKHPSTPLNLPRIAQKDCQVNGYYIPKGTSLSVNIWAIGRDPNVW
b)	SNEP..I.D.....N.....Q...
a)	ENPLEFNPDRFLSGMKAKIEPRGNDPELIPFGAGRRICAGTRMGIVL
b)	E.....R.S..D.....M
a)	VEYILGTLVHSPDWKLPFDDINELNMDESFGALQKAVPLVAMVSPR
b)	S-EVI.....E.A.....E..T...
a)	LPINAYSP
b)	.QLDV..V..

Figure 2. Comparison of the flavonoid hydroxylases from *C. roseus* (a) and *P. hybrida* Hf1 (b). Only the first is printed fully; dots in (b) indicate sequence identity.

contained either two closely related genes or one gene with additional restriction sites in introns.

Heterologous expression in E. coli: the CYP75 from C. roseus is a F3'5'H

The function was investigated after heterologous expression, applying a previously established strategy which uses translational fusions of the P450s with the P450 reductase (P450Red) from *C. roseus* (Hotze *et al.*, 1995). Figure 4, an immunoblot of the membrane fraction with antiserum against the P450Red, shows that the bacteria synthesized a protein of the predicted size (lane Cr, 129 kDa). The smaller protein (115 kDa) could be explained by incomplete synthesis or partial degradation. The fusion protein was tested with [¹⁴C]naringenin as substrate, and Figure 5a shows a representative example of the analysis after TLC separation of substrate and products. The enzyme converted naringenin into both eriodictyol and

pentahydroxyflavanone (3' and 3'5'-hydroxylation, Figure 1). The control (microsomes from the same cells, but not induced for protein expression) showed that the activity was dependent on the presence of the fusion protein (Figure 5b). More detailed experiments indicated that the ratio of 3',4',5'-(pentahydroxyflavanone) and 3',4'-hydroxylated products (eriodictyol) was dependent on the assay conditions. The lowest ratios were obtained with short incubations and low protein concentrations. The highest ratios (about 2:1) were detected after long incubations and with high protein concentrations, i.e. when all of the naringenin was converted into products, as in the example shown in Figure 5a.

The fusion protein had an apparent *K_m* of 7 μM for naringenin, and the variation of substrate concentrations had no drastic effect on the product ratio. It is not excluded that the *K_m* was influenced by the translational fusion to the P450Red. Data for other F3'5'H are not available. The F3'Hs in microsomes from *Petroselinum crispum* (Hagmann *et al.*, 1983), *Zea mays* (Larson and Bussard, 1986) and *Citrus sinensis* (Doostdar *et al.*, 1995) had apparent *K_m*s in a similar range (0.5–24 μM).

The results indicated that the CYP75 cloned from *C. roseus* is a F3'5'H. Although the flavonoids in this plant have not been investigated in detail, the conclusion is consistent with reports that *C. roseus* contains 3',4' 5'-hydroxylated anthocyanins as the major pigments in cell cultures exposed to light (Carew and Krueger, 1976; Knobloch *et al.*, 1982) and in the flowers (Milo *et al.*, 1985). The release of 3',4'-hydroxylated products was unlikely to be the result of heterologous expression because the same was observed with microsomes isolated from plants containing F3'5'H, but not F3'H (Menting *et al.*, 1994).

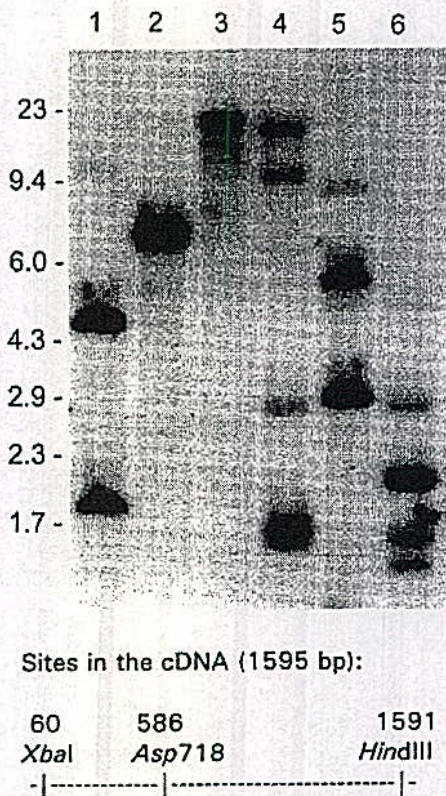


Figure 3. Genomic Southern blot of *C. roseus* with the complete cDNA. Restriction digests: 1, *Xba*I; 2, *Eco*RI; 3, *Bam*HI; 4, *Asp*718; 5, *Hind*III; 6, *Hind*III plus *Xba*I. Each lane contained 30 μg DNA. The numbers at the left indicate the DNA size markers (kbp). Bottom: scheme of the cDNA with the restriction sites used in the genomic blot.

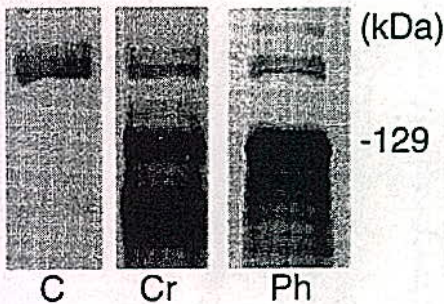


Figure 4. Immunoblots of fusion proteins expressed in *E. coli*. The proteins were displayed with the antiserum against the P450Red from *C. roseus* which is common to both proteins. C, control (plasmid for the *C. roseus* protein, but no induction for protein expression); Cr and Ph, *C. roseus* and *P. hybrida* fusion proteins (induction of protein expression for 8 h). Protein per lane (membrane fractions): C and Cr, 75 μg; Ph, 120 μg. The size of the fusion proteins is indicated (129 kDa). The large cross-reacting proteins are from *E. coli* (Hotze *et al.*, 1995).

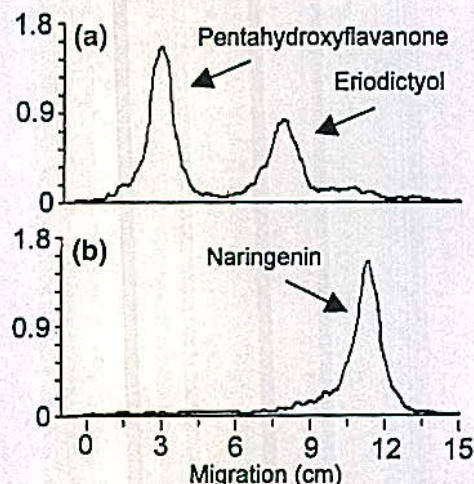


Figure 5. TLC analysis of the products synthesized from [^{14}C]naringenin by the *C. roseus* fusion protein expressed in *E. coli*. Substrate and products were separated by TLC, and the radioactive spots were quantified with a TLC scanner. (a) induction of protein expression for 8h; (b) no induction. The incubations contained 0.2mg protein from the membrane fractions. The numbers at the ordinate represent $\text{dpm} \times 10^{-3}$.

Two other cloned CYP75 have been functionally identified before, and yeast expression of the hydroxylases was used in both cases. The specific activity of the *Gentiana triflora* enzyme (Tanaka *et al.*, 1996) was about 500-fold lower than that of the *C. roseus* fusion protein. That enzyme accepted the 3',4'-hydroxylated substrates eriodictyol and dihydroquercetin (see overview in Figure 1), and it performed only one hydroxylation with naringenin or dihydrokaempferol. Our observations with the *C. roseus* enzyme suggest that 3',5' hydroxylation was not detectable with the *G. triflora* protein because of its low activity. The *P. hybrida* enzyme synthesized pentahydroxyflavanone from naringenin, and it converted dihydroquercetin into dihydromyricetin (5'-hydroxylation) (Holton *et al.*, 1993).

Heterologous expression of plant P450s: *E. coli* as an alternative to yeast

A previous study with cinnamic acid 4-hydroxylase (C4H) (Hotze *et al.*, 1995) and the present work suggest that *E. coli* can be used as a host for the functional expression of plant P450. We extended these experiments to the F3'5'H of *P. hybrida* in order to test further the usefulness of the strategy and to obtain the possibility to compare the properties of the two F3'5'Hs. The expression of the *P. hybrida* F3'5'H fusion protein (Figure 4, lane Ph) was about twofold lower than that of the *C. roseus* CYP75 fusion, as judged from immunoblots with antiserum against the

Table 1. Substrate specificities of the F3'5'Hs from *C. roseus* and *P. hybrida*

Substrate	Activity (%) <i>C. roseus</i>	<i>P. hybrida</i>
4'-Hydroxylated		
Naringenin	94	100
Apigenin	100	88
Kaempferol	63	71
Dihydrokaempferol	27	35
3',4'-Hydroxylated		
Eriodictyol	31	50
Luteolin	40	50
Quercetin	23	41
Dihydroquercetin	13	8

Assay conditions: 50 μg *C. roseus* or 200 μg *P. hybrida* F3'5'H fusion protein, 16 μM substrate, incubation for 40 min at 30°C; analysis of substrate and products by HPLC. The highest activities (100%) were: *C. roseus*, 4.67 pkat mg^{-1} ; *P. hybrida*, 1.32 pkat mg^{-1} . The activities with the 4'-hydroxylated substrates were calculated as the sum of the 3',4'- and 3',4' 5'-hydroxylated products.

P450Red which is common to both proteins. The amount of bands smaller than the expected protein size was lower than with the *C. roseus* fusion protein. Assays with [^{14}C]naringenin showed that the enzyme was active and that it also produced both eriodictyol and pentahydroxyflavanone, but the specific activity was only about 50% when compared with the *C. roseus* fusion protein. One of the reasons may be that the coupling between the *P. hybrida* CYP75 and the *C. roseus* P450Red was not as efficient as with the two proteins from the same plant. The data showed that the expression of translational fusions of P450 and P450/Red in *E. coli* is a useful alternative to yeast expression for the functional characterization of P450 activities.

The F3'5'Hs from *C. roseus* and *P. hybrida*: substrate acceptance and competition

We analyzed all four substance classes reported for enzymes from different plants (Biolley *et al.*, 1994; Forkmann and Heller, 1999; Holton *et al.*, 1993; Menting *et al.*, 1994; Stotz and Forkmann, 1982; Tanaka *et al.*, 1996). Both proteins revealed highest activities with naringenin and apigenin, but significant activities were also obtained with kaempferol and dihydrokaempferol (Table 1). The products were in all cases both the 3',4'- and 3',4' 5'-hydroxylated forms, and the ratios were comparable to those observed with [^{14}C]naringenin. The 3',4'-hydroxylated products were also investigated as substrates because previous studies with eriodictyol and dihydroquercetin had shown that they were accepted (Holton

Table 2. Inhibition of naringenin hydroxylation by other substrates

Substrate added	Inhibition of [¹⁴ C]naringenin use (%)	
	<i>C. roseus</i>	<i>P. hybrida</i>
4'-Hydroxylated		
Kaempferol	100	72
Apigenin	51	40
Dihydrokaempferol	35	12
3',4'-Hydroxylated		
Quercetin	71	26
Luteolin	11	4
Dihydroquercetin	5	0
Eriodictyol	0	2

The unlabelled flavonoids were added at 4 µM final concentration. Assay conditions: 50 µg *C. roseus* or 100 µg *P. hybrida* fusion protein, 1.6 µM [¹⁴C]naringenin, incubation at 30°C for 5 min (*C. roseus*) or 10 min (*P. hybrida*). The assay conditions were chosen so less than 50% of the [¹⁴C]naringenin was used during the incubations. The products from naringenin were quantified after TLC analysis. Specific activities without added flavonoid: *C. roseus*, 2.5 pkat mg⁻¹; *P. hybrida*, 0.6 pkat mg⁻¹.

et al., 1993; Menting *et al.*, 1994; Stotz and Forkmann, 1982; Tanaka *et al.*, 1996). All served as substrates for the 5'-hydroxylation, although with lower efficiency (Table 1), and the activities roughly reflected those of the corresponding 4'-hydroxylated substrates.

The data suggested competition if more than one substrate is present. We therefore investigated the effect of other substrates on the use of [¹⁴C]naringenin. Table 2 shows that kaempferol reduced the use of [¹⁴C]naringenin very efficiently, while apigenin as well as dihydrokaempferol had less drastic effects. Among the 3',4'-hydroxylated substrates, only quercetin revealed a significant inhibition, with a significant difference between the *C. roseus* and the *P. hybrida* enzyme.

The addition of eriodictyol was of particular interest because it is not only a substrate (Table 1) but also the intermediate in the reaction sequence from naringenin to the 3',4',5'-hydroxylated product. Table 2 shows that a 2.5-fold excess had no significant effect on the use of [¹⁴C]naringenin, and additional experiments revealed that a 10-fold excess was necessary to reduce the reaction with [¹⁴C]naringenin by 50%. The ratio of [¹⁴C]pentahydroxyflavanone and released [¹⁴C]eriodictyol (about 1:2) was not changed significantly by the addition of unlabelled eriodictyol. Taking into account that these assays were terminated before 50% of the [¹⁴C]naringenin were converted into products, this indicated that released or added eriodictyol was only accepted for a 5'-hydroxylation after most of the naringenin was used up. Such use of released eriodictyol is a likely explanation for the finding

that the ratio of 3',4',5'- and 3',4'-hydroxylated products was increased in extended incubations. It is not known whether the F3'H function and the use of eriodictyol are significant *in vivo*.

The substrates used *in vivo* are usually not precisely identified (Biolley *et al.*, 1994). The broad substrate acceptance is therefore consistent with the possibility that multiple paths lead to the same intermediates and that competition could occur if the substrates are in the same cell. However, precursor feeding studies with petals from *Rhododendron simsii* suggested that pathway compartmentation may serve to avoid competition. Dihydroquercetin is an intermediate in both anthocyanin and copigment synthesis, but the 3'-hydroxylation in the anthocyanin pathway occurs with dihydrokaempferol, while 3'-hydroxylation in the co-pigment biosynthesis takes place with naringenin (De Cooman *et al.*, 1993). However, the mechanisms were not investigated.

Regulation in cultured cells

The regulation of F3'5'H was investigated on three levels (enzyme activity, protein amount and RNA), and other enzymes (C4H, CHS and FHT) were included in the analysis to obtain a more comprehensive picture. Phenylalanine ammonia-lyase (PAL) and P450Red were investigated only on the protein and the RNA levels, respectively. We used cultures grown for 7 days after dilution into fresh MX medium. The dark-grown cells contained F3'5'H, C4H, and FHT activities (1.4, 26 and 7 pkat mg⁻¹, respectively), but CHS was not detectable. No significant changes were observed with any of the enzymes in the subsequent 36 h if the cultures remained in the dark. Irradiation induced all four enzyme activities, and the increases corresponded to increases in immunodetectable protein and in transient increases in the mRNA (Figure 6). PAL protein showed a slight increase, and the mRNA for P450Red revealed a transient increase which followed the C4H induction, but not the F3'5'H kinetics.

Similar kinetics were observed after irradiation accompanied by a nutritional down-shift (transfer of the cells into a 8% sucrose solution), but the increases were lower than in MX medium (CHS, F3'5'H and FHT, about 20% lower; C4H about 40% lower). The nutritional down-shift itself led to increases in the activities of C4H (threefold) and F3'5'H (twofold), and these were accompanied by transient increases of the transcripts. No significant change was observed with FHT under these conditions. CHS activity, protein or transcripts were never detected without irradiation of the cultures.

It was unexpected that F3'5'H was present in cultures maintained in the dark, while the expression of CHS, the enzyme essential for the formation of the F3'5'H substrates, was strictly dependent on light. Interestingly, a

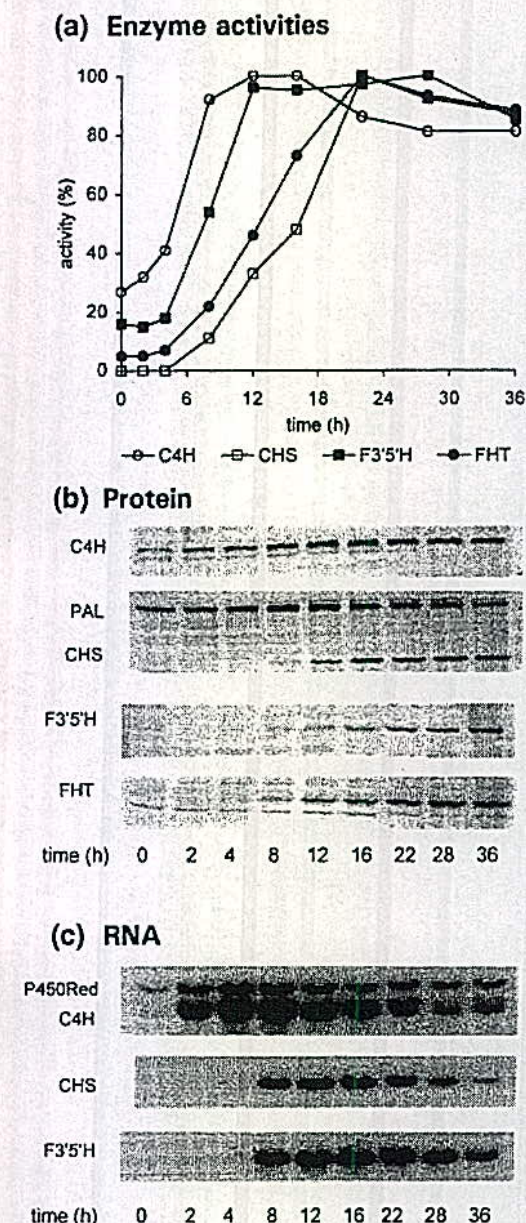


Figure 6. Light induction of enzymes in suspension-cultured cells in MX medium.
(a) Enzyme activities. The data were normalized to the maximal values (pkat mg⁻¹): C4H, 105; CHS, 1.4; FHT, 227; F3'5'H, 9.3 (sum of 3',4'- and 3',4',5'-hydroxylated products obtained with [¹⁴C]naringenin).
(b) Protein immunoblots. Application per lane: C4H, 75 µg; PAL and CHS (assayed in the same blot), 50 µg; F3'5'H, 120 µg; FHT, 75 µg.
(c) RNA. All blots were performed with 16 µg total RNA per lane.

closer inspection revealed that the suspension culture was not homogeneous in response to irradiation. It consisted

mostly of clumps of 10–30 cells, and cells kept in the dark were light yellow. After about 30 h of continuous irradiation the culture revealed the red colour characteristic of the anthocyanins in *C. roseus* (Knobloch *et al.*, 1982). However, a microscopic inspection showed that only about 30–50% of the cells were coloured, and often two neighbours joined by large areas of common cell wall were strikingly different: one contained anthocyanins, while the other retained the colour of dark-grown cells. The simplest explanation was that only the anthocyanin-accumulating cells expressed the biosynthetic enzymes. However, several studies suggested that the sites of accumulation are not necessarily the sites of biosynthesis and that transport of non-coloured intermediates between cells may be involved (Claudot *et al.*, 1997; Hrazdina *et al.*, 1982; Knogge and Weissenböck, 1986; Reinold and Hahlbrock, 1997). It is therefore possible that the different regulation of CHS and F3'5'H reflected the expression in different cells and a heterogeneity in the differentiation state of the cells in culture.

Predominant expression of F3'5'H and CHS is in different cell-types

We used immunohistochemical staining of plant tissues to investigate whether the two enzymes are expressed in the same cells. For these experiments, parallel sections were incubated with the specific antisera as well as with the respective pre-immune sera as controls. Figure 7(a) shows representative examples with cross-sections of cotyledons. CHS was detected exclusively in the upper (adaxial) epidermis. However, with the F3'5'H antiserum only background signals were obtained in these cells. Remarkably, very intense staining was observed in a different tissue adaxial to the xylem representing the internal phloem. Both enzymes were present at the same locations in cotyledons of dark grown seedlings; irradiation led to an increase of CHS, but not to a significant change of F3'5'H (not shown).

A similar cell-type specific expression of the two enzymes was found with longitudinal sections of flower buds (Figure 7b). CHS was most abundant in the adaxial epidermal cell layer which later forms the upper epidermis of petals, whereas F3'5'H was present in the internal phloem. Other experiments showed that CHS and F3'5'H were no longer detectable in fully opened flowers. Differential expression was also observed in leaves, where F3'5'H was again clearly visible in the internal phloem while signals for CHS were only at background level (not shown). Due to the limitations in the sensitivity of the technique, very low expression of the enzymes would not be detected. It is therefore not excluded that the epidermal cells contained very low levels of F3'5'H, and the same applied for the presence of CHS in other cell-types.

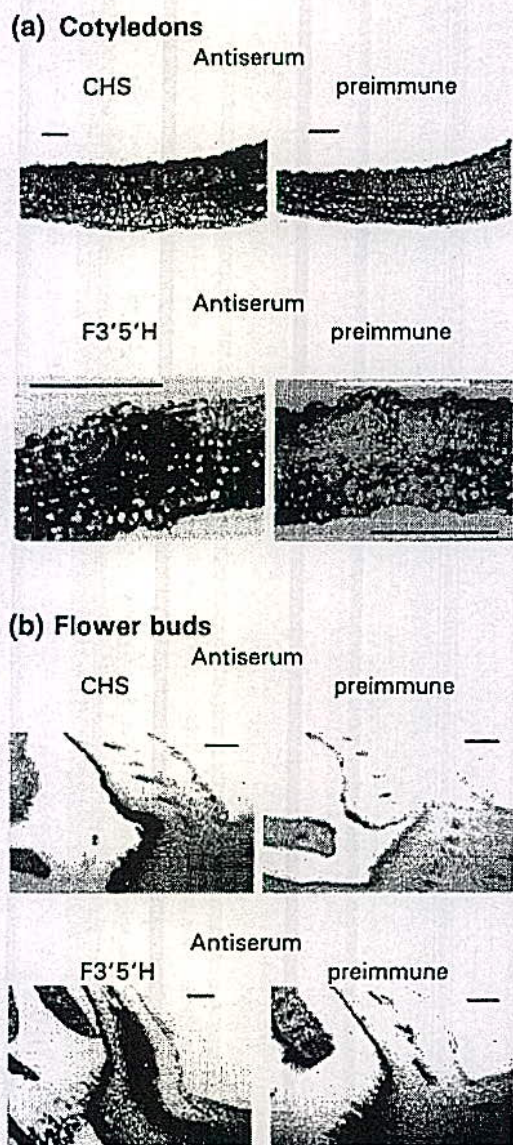


Figure 7. Immunohistochemical analysis of plant tissues with CHS and F3'5'H antisera (left side) and with pre-immune antisera (right side). (a) Cotyledons. CHS, seedlings grown for 6 days in the dark and irradiated for the last 30 h; F3'5'H, seedlings kept for 6 days in the dark. (b) Longitudinal section through a flower bud. The bars represent 0.4 mm.

The localization of F3'5'H to the phloem was unexpected, and therefore we rechecked the specificity of the antiserum in immunoblots with microsomes from the tissue culture and from cotyledons. Both revealed no significant reaction with the F3'5'H pre-immune serum (not shown). Figure 8 summarizes the results with the F3'5'H antiserum. It

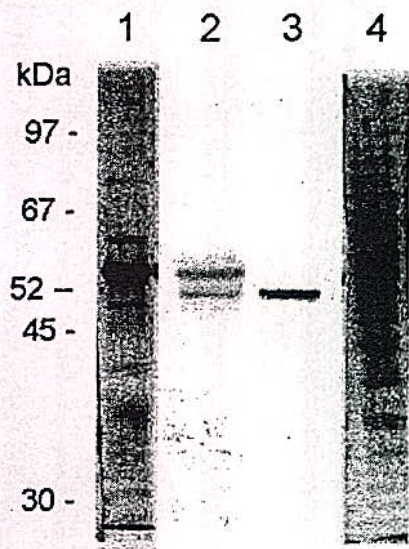


Figure 8. Total proteins and immunoreaction with F3'5'H antiserum in microsomes from cotyledons and the cell culture. The proteins were separated by SDS PAGE. Lanes: 1, cotyledons, stain with Ponceau S; 2, cotyledons, reaction with F3'5'H antiserum; 3, cell culture, reaction with F3'5'H antiserum; 4, cell culture, stain with Ponceau S. Each lane contained 75 µg protein. The microsomes were isolated after irradiation for 10 h (cotyledons) and 22 h (cell culture).

recognized a single protein band in microsomes from irradiated cell cultures (lane 3). Its size (52 kDa) was smaller than predicted from the sequence (57.8 kDa), but that is typical for these hydrophobic proteins (Nebert and Gonzalez, 1987). As expected, F3'5'H was present in microsomes from irradiated cotyledons, but a broad band of a larger polypeptide was also stained (lane 2). A general protein stain with Ponceau S prior to the immunoreaction showed that it was precisely at the position of a protein which represented more than 50% of the total protein (lane 1). The same result was obtained with microsomes from etiolated seedlings (not shown). The abundance of the protein suggested that it represented the large subunit of Rubisco, and this is in agreement with its absence in the cultured cells (lane 4) which do not form functional chloroplasts. Attempts to remove the protein by repeated washings of the microsomes (resuspension and centrifugation) failed, even under such drastic conditions as with 0.1 M sodium carbonate at pH 11 (Fujiki *et al.*, 1982).

A likely explanation for the reaction of the F3'5'H antiserum at the position of Rubisco is that the large amount of protein concentrated on one small area of the blotting membrane led to unspecific adsorption of the antibodies. The immunohistochemical experiments revealed no indication for a similar artefact because a strong

reaction with the antiserum was observed only in the phloem (Figure 7) which contains few if any functional chloroplasts, but not in the mesophyll cells which are the dominant sites of photosynthesis. It is not rigorously excluded, however, that the unspecific adsorption to Rubisco in the immunoblots masked a cross-reaction of the F3'5'H antibodies with an unknown P450 which migrated precisely to that position. This possibility cannot be discounted although no other known plant P450 shares such high identity with F3'5'H that a strong cross-reaction would be expected, even with a polyclonal antiserum. The antiserum did not detect such protein in the cell cultures (Figure 8, lane 3), but it could be argued that it is not expressed in these cells. It is therefore not only excluded that the immunohistochemical staining identified F3'5'H, but also a weak cross-reaction with another P450. However, this would not invalidate the conclusion that the expression of CHS is in different cell types because the immunostaining with the F3'5'H antiserum revealed no significant reaction in the epidermis, the predominant site of CHS expression (Figure 7).

The predominant expression of CHS and F3'5'H in different cell types is consistent with the proposal that intercellular transport may play an important role in the compartmentation of the pathways to the different flavonoids. The localization of CHS in the epidermis has been described for several other plants (Beerhues *et al.*, 1988; Haussühl *et al.*, 1996; Hrazdina *et al.*, 1982; Jähne *et al.*, 1993; Kobayashi *et al.*, 1998; Schmelzer *et al.*, 1988; Zobel and Hrazdina, 1995), and our data are consistent with those results. To our knowledge there are no previous investigations with F3'5'H. It is of particular interest that we localized this enzyme in the internal phloem, a tissue that is characteristic for several dicotyledonous families including the Apocynaceae (*C. roseus*). It is believed that the internal phloem serves distinct transport functions, e.g. in potato for transport of carbohydrates from the leaves into developing tubers (Burton, 1989). Other enzymes have been found to be specifically expressed in this tissue as, for example, glutamine synthetase (Pereira *et al.*, 1996) or tyrosine/dihydroxyphenylalanine decarboxylase (Facchini *et al.*, 1998).

Conclusion

It was proposed that phenylpropanoid metabolism and possibly the entire pathway from phenylalanine to the various flavonoids is channeled in a multi-enzyme complex (Hrazdina and Jensen, 1992). The proposal for the later reactions is difficult to reconcile with the finding that CHS and F3'5'H are expressed in different cell types. It was also recently suggested that CHS, chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) are involved in protein-protein interactions (cited in Graham, 1998). The

association of CHS and CHI is plausible because CHI is necessary to obtain the correct flavanone isomer for the later reactions. It is thought, however, that the hydroxylations performed by F3'5'H (or F3'H) precede the DFR reaction (see for example Biolley *et al.*, 1994; Holton and Cornish, 1995; Shirley, 1996), and it would be interesting to see whether F3'5'H (or F3'H) are part of such a complex. We have no information at present on the nature of the transported substance(s), but the broad activities of F3'5'H (and F3'H) with various substrate classes, the versatility of the metabolic grid, and the possibility of glycosylated forms (see Forkmann and Heller, 1999; for a recent review) suggest several alternatives. The cell type specific expression may be relevant to the efforts to modify flower colours by the introduction of the flavonoid hydroxylases.

Experimental procedures

Cell culture and induction

The cell suspension culture of Madagascar periwinkle (*Catharanthus roseus* L.G. Don, line CP3a), its maintenance on MX growth medium in continuous dark with subcultures every week, and the nutritional down-shift by transfer into 8% aqueous sucrose have been described previously (Vetter *et al.*, 1992). The induction by light was performed with a light field from six white light lamps (Philips 40W/18) and four UV-A fluorescent lights (Osram, 40W/73). It was positioned 45 cm above the culture flasks (8 W m⁻²). The induction experiments were carried out 7 days after dilution into fresh medium.

Plant material and irradiation

Seeds from *C. roseus* (var. Little Linda, Samenhandlung G. Vath, Freiburg, Germany) were germinated for 6 days at 26°C in the dark. The irradiation was performed with fluorescent lamps (Philips, 20W, F20 T12CW and Osram 20W/73) positioned 20 cm above the seedlings (6 W m⁻²).

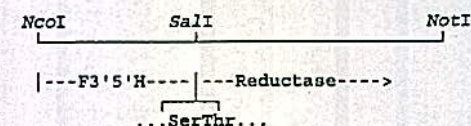
F3'5'H and CHS cDNAs from *C. roseus*

Poly(A)-rich RNA was isolated from cell cultures induced by a nutritional down-shift (transfer into 8% aqueous sucrose) and irradiation for 1.5 or 3 h. The construction of the cDNA libraries in phage NM1149 and the screening techniques have been described previously (Vetter *et al.*, 1992). The screening for CYP75 cDNAs was performed with the F3'5'H (*Hf1*) cDNA from *Petunia hybrida* (Holton *et al.*, 1993). The first CHS sequences were obtained by polymerase chain reaction with oligonucleotides from conserved regions, and clones representing the complete cDNA were obtained from the cDNA library.

Blots with RNA and genomic DNA

The isolation of RNA and DNA and the hybridizations with the cDNAs were carried out as described by Sambrook *et al.* (1989). The *C. roseus* cDNAs for C4H and P450Red have been published previously (Hotze *et al.*, 1995). The CHS probe was a fragment of 645 bp which covered the region from the active site cysteine to

F3'5'H/P450 reductase fusion proteins



C. roseus

a) N-terminus

Original: ATGGCTCTAGAAAACTGGTCCTTTTGGATTCC
MetAlaLeuGluLysLeuValLeuPheAspPhe

Modified: 5' GCTCTAGCCCATGGCGGTCCTTTTGGATTCC 3'
MetAla.....
-NcoI-

b) C-terminus

ATCAATGCTTATTCTCCTTGAGAGCGGCCGCG
Original: TAGTTACGAATAAGAGGAAGCTCTCGCCGCGC
IleAsnAlaTyrSerPro-p-

Modified: 3' GAATAAGAGGACCGAGCTGCGGCGC 5'
.....GlySerThr
-SalI-

P. hybrida

a) N-terminus

Original: ATGATGCTACTTACTGAGCTTGGTGACGA
MetMetLeuLeuThrGluLeuGlyAlaAla

Modified: 5' GCTACCCATGGCGCTTGGTGC 3'
MetAla.....
-NcoI-

b) C-terminus

CAATTGGATGTTTATGTACCATAGCTATAG
Original: GTTAACCTACAAATACATGGTATCGATATC
GlnLeuAspValTyrValPro-a-

Modified: 3' CCTACAAATACATGGCAGCTGTATC 5'
.....ProSerThr
-SalI-

Figure 9. Expression of translational fusions between P450 and P450 reductase in *E. coli*.

Principle of the fusion proteins and details of the cDNA modifications performed to create a suitable N-terminal membrane anchor and a SalI-linker between the F3'5'Hs and the P450 reductase.

the conserved FGFG-motif close to the end of the protein in the cDNA from *C. roseus*.

Heterologous expression of F3'5'Hs

We employed a previously described strategy which uses translational fusions between P450 and P450 reductase (Hotze *et al.*, 1995) to express the CYP75 proteins from *C. roseus* and *P. hybrida* (Hf1) in *E. coli*. The P450 cDNAs were modified in two steps: (i) introduction of an NcoI site at the N-terminal and simultaneous creation of a membrane anchor suitable for expression of the protein in the bacteria (Barnes *et al.*, 1991); and (ii) introduction of a SalI site which eliminates the stop codon and provides a Ser-Thr linker to the P450 reductase (details shown in Figure 9). The modifications were confirmed by sequence analysis. The engineered cDNAs were then used to replace the C4H NcoI/SalI fragment in the previous C4H/P450Red construct (Hotze *et al.*, 1995) by the F3'5'H cDNAs. The fusion proteins

(129 kDa) were expressed in the *E. coli* strain RM82 containing plasmid pUBS520 (Schenk *et al.*, 1995). The protein induction, the preparation of the membrane fraction, and the identification of the fusion proteins by immunoblots with antiserum against *C. roseus* P450Red have been described previously (Hotze *et al.*, 1995).

Immunoblots

Immunoblots were performed with the proteins separated by SDS PAGE and transferred to nitrocellulose sheets (Vetter *et al.*, 1992). The antisera against PAL (Schröder *et al.*, 1976) and C4H (Koopmann and Hahlbrock, 1997) from *P. crispum*, FHT from *P. hybrida* (Britsch, 1990), and P450Red from *C. roseus* (Hotze *et al.*, 1995) have been described. CHS was detected with a mono-specific antiserum (S. Raiber and J. Schröder, unpublished results) raised in rabbits against the *Pinus sylvestris* CHS which is 82% identical to the *C. roseus* protein. The antiserum showed no cross-reaction with other proteins in immunoblots with *C. roseus* extracts. The staining reactions were performed with a secondary antibody coupled to alkaline phosphatase (Sigma Biochemicals). The stain for total proteins was performed with Ponceau S (Serva, Germany).

Antiserum against the CYP75 from *C. roseus*

A cDNA fragment encoding the complete F3'5'H (except for the two N-terminal amino acids) was fused in frame with the sequence encoding the 42 kDa maltose-binding-protein in vector pMal-C2 (Maina *et al.*, 1988). The fusion protein (100 kDa) was purified by affinity chromatography on an amylose resin column (protocol from New England Biolabs) followed by preparative SDS PAGE. The use of a protein expressed in *E. coli* excluded contamination of the antigen with other plant proteins. The antisera were raised in rabbits (Eurogentec, 4102 Seraing, Belgium). They reacted with a single protein of the F3'5'H size in immunoblots. The simultaneously produced antibodies against the maltose-binding-protein reveal no cross-reaction with proteins in *C. roseus* (Schulze-Muth *et al.*, 1996).

Extracts from cell cultures and plants

The frozen cells or tissues were homogenized in buffer A (0.1 M Tris-HCl, pH 7.5, 10 mM KCl, 10 mM MgCl₂, 10 mM EDTA, 5 mM DTT, 20 mM sodium ascorbate, 0.5 M sucrose) and centrifuged for 10 min at 15000 g and 4°C to remove insoluble material. The pellet was discarded, and the supernatant was centrifuged again for 15 min at 350 000 g. The supernatant was used for assaying CHS and FHT. The pellet was resuspended in buffer B (50 mM potassium phosphate, pH 7.5, 3 mM DTT, 20% glycerol) and used for measuring the C4H and F3'5'H activities. Protein concentrations were determined with published procedures (Shakir *et al.*, 1994).

Enzyme assays

The F3'5'H incubations (50 µl) contained 1.6 µM [¹⁴C]naringenin (11 × 10³ dpm) (TLC analysis) or 16 µM unlabelled flavonoids (HPLC analysis), 50 mM sodium phosphate (pH 7.5), a NADPH-regenerating system (10 mM glucose-6-phosphate, 17.5 mU glucose-6-phosphate dehydrogenase), 5 mM sodium ascorbate, 2 mM

DTT, and protein. The reactions were started by the addition of NADPH to a final concentration of 0.5 mM. The incubations were at 30°C and were stopped by extraction of the substrates and products with ethyl acetate (details are given in the text or in the figure legends). The quantitative evaluation of radioactive substrates and products by TLC in the solvent chloroform/glacial acetic acid/water (50:45:5, v/v/v) has been described previously (Vetter *et al.*, 1992). Non-radioactive substrates and products were quantified by HPLC (Shimadzu CBM-10AV, LC-10AD, with an UV detector SPD-10AV, and a 125 × 3 mm Chromasil 100 C₁₈ column). The separation was achieved with a gradient increasing from 20% to 99% methanol in 5 min and maintaining the final methanol concentration for a further 4 min. All solvents contained 1% glacial acetic acid, and the flow rate was 0.5 ml min⁻¹. Retention times and the quantification were calibrated with reference substances (Roth, Karlsruhe, Germany), and the detection wavelength was chosen according to the optima of the substances (flavanones 289 nm, flavonols 370 nm, dihydroflavonols 295 nm, flavones 340 nm).

The assays for C4H (Hotze *et al.*, 1995), CHS (Tropf *et al.*, 1995) and FHT (Britsch, 1990) have been described previously.

Immunohistochemistry

The preparation of the tissues and the staining reactions were performed with published techniques (Reinold and Hahlbrock, 1997; Schmelzer *et al.*, 1988). Briefly, tissue samples were fixed with 1% glutaraldehyde and embedded in paraffin. Semi-thin sections were prepared and stained by indirect immunohistochemistry using specific antisera and alkaline phosphatase-linked secondary antibodies.

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The 'ins' and 'outs' of flavonoid transport

Jian Zhao and Richard A. Dixon

Plant Biology Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA

The sites of plant flavonoid biosynthesis, storage and final function often differ at the subcellular, cell, and even tissue and organ levels. Efficient transport systems for flavonoids across endomembranes and the plasma membrane are therefore required. However, a clear picture of the dynamic trafficking of flavonoids is only now beginning to emerge and appears to have many players. Here, we review current hypotheses for flavonoid transport, discuss whether these are mutually exclusive, highlight the importance of flavonoid efflux from vacuoles to the cytosol and consider future efforts to catch flavonoids 'in the act' of moving within and between cells. An improved understanding of transport mechanisms will facilitate the successful metabolic engineering of flavonoids for plant protection and human health.

The pieces of the flavonoid transport puzzle

Flavonoids represent one of the largest classes of plant secondary metabolites, and are involved in a multitude of physiological functions, including UV protection, insect attraction, pathogen defense, symbiosis and variation of flower color [1]. Flavonoids have been associated with many favorable agronomic traits and health benefits to humans, and their metabolic engineering is therefore an important target for plant biotechnology [2,3]. Pathways, enzymes and regulatory mechanisms of flavonoid biosynthesis have been well studied [4], yet flavonoid location and trafficking *in vivo* are still not clearly established.

There appear to be tight links between the biosynthesis, transport and cellular deposition of flavonoid compounds. Successful metabolic engineering of flavonoid production, therefore, requires a full consideration of regulatory mechanisms not only for biosynthesis, but also for transport and storage of the end products in cells and tissues. In this respect, conclusions from earlier microscopy observations of flavonoid-containing vesicles have yet to gain molecular genetic support and membrane transporter-mediated flavonoid transport has been confirmed in only a few cases [5]. Furthermore, transport across the vacuolar membrane is not unidirectional for flavonoid storage, as plant cells have mechanisms for re-mobilization of vacuolar stores of flavonoid compounds under certain physiological conditions. Parallel pathways appear to exist for the transport and storage of flavonoids at the tissue, cellular, subcellular and molecular levels, with their relative importance and modes of regulation still poorly understood. The field is however, attracting increasing attention and is set to see major advances with the application of increasingly sophisticated

genetic, biochemical and cell biological approaches. Here, we summarize recent efforts made to identify flavonoid transport mechanisms, and outline future research targets aimed at piecing together the complex puzzle linking

Glossary

ABC transporter: a large superfamily of ATP-binding cassette (ABC) proteins. They usually contain a nucleotide-binding domain and a transmembrane domain for mediating MgATP-energized transmembrane transport and/or regulation of other transporters [41]. Different subfamilies of ABC transporters have essential and diverse roles in the transport of metal ions and primary and secondary metabolites across membranes.

Anthocyanic vacuolar inclusions (AVIs): intravacuolar bodies of varying sizes containing concentrated anthocyanins. AVIs are observable in petal cells of carnation (*Dianthus caryophyllus*) and lisianthus (*Eustoma grandiflorum*), and in other anthocyanin-accumulating cells in grapevine, sweet potato (*Ipomoea batatas*), and maize. AVIs do not have membrane boundaries, but contain membrane lipids and a protein matrix bound to anthocyanins, particularly acylated anthocyanins [19].

Anthocyanoplast: initially used to describe cytoplasmic membrane-bound vesicles containing high levels of anthocyanins and regarded as anthocyanin biosynthetic sites. However, later studies confused them with anthocyanic vacuolar inclusion (AVIs), which are more widely observed inside the vacuoles of many plant species. Anthocyanoplasts are found exclusively in the cytoplasm in grapevine cells, and in protoplasts prepared from red radish (*Raphanus sativus*) seedlings.

MATE transporter: multidrug and toxic compound extrusion (MATE) family transporters. They use H⁺/Na⁺ gradients across membranes as a force to drive waste or toxic compounds out of the cytoplasm. MATE transporters perform conserved and basic transport functions in most prokaryotes and eukaryotes.

Pre-vacuolar compartment (PVC): an endocytic multivesicular compartment involved in ER-Golgi-vacuole vesicle trafficking. It carries proteins and other metabolites to fuse to the large central vacuole. The PVC has SNAREs or vacuolar-sorting receptors to accept precursor vesicles and then fuse to the vacuole. A specific type of PVC in *Isatis* epidermal cells contains anthocyanin and was proposed to transport anthocyanins into the central vacuole [19].

Protein storage vacuole (PSV): a type of vacuole and compound organelle formed during plant seed development and maturation and containing large amounts of storage proteins. PSVs contain vacuolar-sorting receptors to recognize cargo molecules. A PSV marker co-localizes with anthocyanins, leading to the suggestion that anthocyanins are transported directly via ER-derived vesicle trafficking in a Golgi-independent manner [22].

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptors. These are small but abundant integral membrane proteins that mediate vesicle fusion and reside on the surface of the transport vesicle (v-SNAREs) and target membrane (t-SNAREs). They have a cytosolic domain called a SNARE motif, which assembles with another SNARE motif into parallel four-helix bundles within SNARE complexes and brings the transmembrane anchors and the two membrane vesicles into close proximity.

Tapetosomes: abundant organelles in the tapetum cells of anthers during the active stage of pollen maturation in Brassicaceae species. Tapetosomes originate from massive ER cisternae, which release lipid droplets that are fused into large vesicles. These lipid vesicles further fuse with ER-derived vesicles containing flavonoids. Upon tapetum cell death, tapetosomes release alkanes and oleosins in lipid droplets, along with flavonoids, to the pollen surface.

trans-Golgi network (TGN): a dynamic series of membrane compartments at the trans-face of the Golgi stacks. The TGN mainly processes and sorts various proteins and glycolipids at the interface of the biosynthetic and endosomal pathways. The generation and maintenance of apical and basolateral membranes relies on sorting events that occur in the TGN.

Vacuolar sorting receptors (VSRs): integral membrane proteins responsible for the proper targeting of cargo proteins to their destination compartments. VSRs are localized to ER, PVC, TGN and Golgi apparatus.

Corresponding author: Dixon, R.A. (radixon@noble.org).

flavonoid biosynthesis, transport and storage processes in plant cells.

Subcellular sites of flavonoid biosynthesis

The enzymes of flavonoid biosynthesis are believed to be localized on the cytosolic side of the endoplasmic reticulum (ER), organized into a multi-enzyme complex centered around the ER-associated cytochrome P450 enzymes cinnamate 4-hydroxylase, flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase [6,7]. The enzyme complexes might be brought together by protein-protein interactions on the surface of the ER [6].

Some of the enzymes of the pathway have also been shown to co-localize to the tonoplast and nucleus [6-8], consistent with the idea that flavonoid biosynthetic enzyme complexes might be dynamic to facilitate channeling or shifting of end-product biosynthesis to meet physiological requirements under different conditions [6,7]. Such enzyme complexes associated with the ER could also facilitate the transport of flavonoid products through membrane trafficking.

Subcellular distribution of flavonoids

Consistent with their diverse physiological functions, including UV protectants and modifiers of auxin transport [1,4,9], flavonoids are found in most plant cell compartments, including the cytosol, vacuole, ER, chloroplast, nucleus and small vesicles, as well as the extracellular space. Most conjugated flavonoids, such as anthocyanins and flavonol and flavone glycosides, are found primarily in the vacuole [1,4,10], where vacuolar pH and the presence of co-pigments determine anthocyanin-mediated flower coloration. The vacuole is also believed to be the site for the biosynthesis of some flavonoid derivatives. For example, catechin and epicatechin monomer units are glycosylated and then transported into the vacuole for polymerization into proanthocyanidins (PAs) in the endothelial layer of the seed coat in many species, and are finally exported to the apoplast [10]. Mutation of genes involved in PA biosynthesis or transport leads to a *transparent testa (tt)* phenotype. In common snapdragon (*Antirrhinum majus*) flowers, yellow aurone pigments are produced from chalcones by aureusidin synthase, a vacuolar polyphenol oxidase [11]. Increasing evidence suggests that the vacuole also acts as an important source and/or sink, regulating the homeostasis of cytosolic metal ions, metabolite precursor pools and secondary metabolites. This role requires mechanisms for both vacuolar influx and efflux.

Quercetin and kaempferol glycosides have been detected in chloroplasts, which are capable of flavonoid biosynthesis [12]. Some flavonoids and their biosynthetic enzymes are also present in the nucleus [8]. UV protection is probably a major function of flavonoids in both these compartments. In the model legume barrel medic (*Medicago truncatula*), the isoflavonoids coumestrol and 4',7-dihydroxyflavone accumulate to higher levels in the nucleus than in the cytoplasm [13], and 4',7-dihydroxyflavone might act as a signal for jasmonate-induced mobilization of vacuolar isoflavonoid glucosides for phytoalexin biosynthesis [14]. However, it is still unclear whether

flavonoids are transported into the nucleus or if the nucleus is a site for their synthesis.

Some flavonoid conjugates and aglycones are secreted into the apoplastic space [15]. Flavone and flavonol aglycones have been detected in root exudates from numerous species, and flavones and isoflavones are secreted by legume roots into the rhizosphere, either to attract mutualistic microorganisms, such as nitrogen-fixing bacteria, or to provide defense against pathogens [16]. Flavonoids are also present on pollen surfaces [17].

Flavonoid transport

The wide distribution of flavonoids in plant cells and their probable biosynthetic sites on the cytosolic face of the ER imply that plants have efficient flavonoid transport systems with which to deliver these metabolites across various membrane-limited compartments. However, the transport mechanisms involved in trafficking of most primary and secondary metabolites are still poorly understood [5]. Two major hypotheses have been proposed for flavonoid transport: membrane vesicle-mediated transport (Figure 1) and membrane transporter-mediated transport (Figure 2). These might not be mutually exclusive.

Vesicle trafficking-mediated flavonoid transport

The concept of vesicle-mediated flavonoid transport originated from microscopy observations. Anthocyanoplasts (see Glossary; Figure 1) were first assumed to be transport vesicles or sites of anthocyanin biosynthesis [5,18-20]. These cytoplasmic anthocyanin bodies are covered by a membrane and originate from a large number of smaller vesicle-like structures that gradually fuse together. Similar cellular structures, anthocyanic vacuolar inclusions (AVIs), are present in the vacuoles of many species and also show dynamic movement [18,19,21] (Figure 1). Although associated with specific proteins and membranous substances, AVIs are not surrounded by a membrane [18,19], and are more likely to be storage complexes than to be involved in anthocyanin transport. However, cytoplasmic vesicle-like structures containing anthocyanins (similar to anthocyanoplasts) have been observed to associate with AVIs after they have fused into the vacuole [19]. These might be transported into the central vacuole through fusion of prevacuolar compartments (PVCs) and then the central vacuole (Figure 1). In addition, anthocyanin-containing vesicle-like structures can co-localize with protein storage vacuoles (PSVs) and transport anthocyanins in a *trans*-Golgi network (TGN)-independent ER-to-PVC vesicle trafficking pathway [22] (Figure 1).

The tapetosomes in tapetal cells of *Arabidopsis thaliana* anthers are specialized bodies that are structurally distinct from anthocyanoplasts. They contain ER-derived flavonoids, which are released to the pollen surface upon tapetum cell death, supporting the idea that flavonoids can be transported from the ER via cytoplasmic vesicles [17]. The ER-associated biosynthetic enzyme complex might facilitate the loading of flavonoids into the ER-derived membrane vesicles.

Early studies suggested that prevacuole-like vesicle structures containing PAs can fuse with the central vacuole and might represent a PA transport pathway

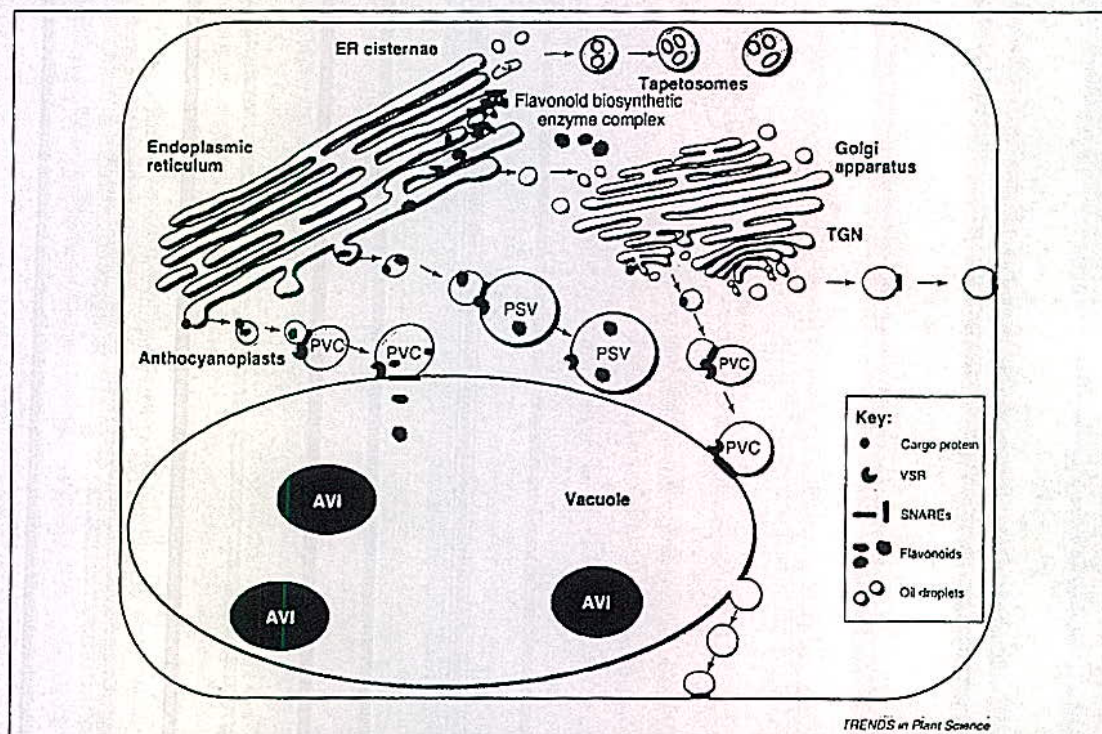


Figure 1. Proposed models for vesicle-mediated transport of flavonoids. Anthocyanoplasts have been proposed to transport anthocyanins to the central vacuole, probably by fusion into PVCs, in grapevine, red cabbage and lisianthus epidermal cells. In *Arabidopsis* leaves, a TGN-independent, ER-derived vesicle-mediated flavonoid transport directly from ER to PSVs has been proposed. In anther tapetum cells of Brassica and *Arabidopsis*, cytoplasmic vesicles called tapetosomes transport ER-derived flavonoids. The general protein secretory pathway involves trafficking of transport vesicles from the ER to the cis-face of the Golgi apparatus, followed by release through TGN vesicles to destinations such as the vacuole and plasma membrane. These processes involve recognition of cargo molecules by vacuolar sorting receptor (VSR)-mediated docking, and SNARE-mediated vesicle fusion. However, so far there is no genetic evidence linking this general secretory pathway to flavonoid transport. AVIs of various shapes are often found inside the vacuole without a membrane boundary; these contain membranous substances and proteins, and thus might be anthocyanin storage bodies.

(reviewed in Ref. [23]). More recently, it has been shown that *Arabidopsis* mutants with reduced PA production (*tt* mutants) also exhibit morphological defects in the central vacuole of the seed coat endothelium cells. PAs had a patchy, filamentous distribution pattern in seed of the *tds4* (leucoanthocyanidin dioxygenase) mutant, and PA storage was confined to prevacuole-like small vesicles, with no central vacuole present [24]. Similar phenotypes are observed following mutation of the *Arabidopsis* *TT19* glutathione *S*-transferase (GST) [25] and the *AHA10* P-type H^+ -ATPase and *TT12* proton/flavonoid antiporter [26]. Therefore, either PA accumulation and vacuole biogenesis appear to be closely related, or PA precursors are transported through ER-derived vesicles to the vacuole. However, mutation of the tonoplast-localized *H*-ATPase *ph5* did not lead to abnormal vacuoles in petunia (*Petunia hybrida*), even though *PH5* is an ortholog of *AHA10* and is also involved in vacuolar PA accumulation in the seed coat [27].

In sorghum (*Sorghum bicolor*), colored 3-deoxyanthocyanidin phytoalexins are formed in response to fungal infection. Vesicle-like red-pigmented inclusion bodies rapidly appear in leaf epidermal cells and move to challenge sites, where they release the antimicrobial flavonoids into the cytoplasm [28]. Similar to anthocyanoplasts, these

vesicles mediate a directional transport of flavonoids. Such vesicles have not yet been observed in dicots, however.

Despite the above observations, direct genetic, molecular, or biochemical evidence supporting vesicle-mediated flavonoid transport is still lacking. Many fundamental questions concerning the origin, structure, composition and behavior of anthocyanin-containing vesicles remain. If vesicle-like anthocyanin bodies are destined for the central vacuole or other membranes, they should contain coat or cargo proteins on their membranes to direct them and to be recognized by vacuolar sorting receptors (VSRs) or other protein components for vesicle trafficking (Figure 1). Currently known proteins include small GTPases, VSRs and soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), which are required for budding, targeting, docking, fusion and recycling of vesicles or trafficking proteins [29–31] (Figure 1). However, such proteins have yet to be associated with either flavonoid transport or accumulation.

The elusive role of glutathione *S*-transferases in flavonoid transport

Several studies point to the involvement of GSTs in anthocyanin accumulation [25,32–34]. Initially, it was

proposed that GST is required for GS-X pump type ABC transporter-mediated vacuolar sequestration of glutathione-conjugated anthocyanins, based on the observation that a multidrug resistance-associated protein (MRP)-type ABC transporter can transport glutathione-conjugates into the vacuole [32]. A similar mechanism was proposed for transport of the isoflavonoid-derived phytoalexin medicarpin [35]. However, biochemical and genetic cross-complementation studies show that the GST protein itself, rather than GST activity, is required for anthocyanin sequestration [33,36]. There are, to the best of our knowledge, no reports of naturally occurring anthocyanin-glutathione conjugates from plants, but GST can bind to anthocyanin or flavonol to form a GST-anthocyanin or GST-flavonol complex, suggesting that GSTs protect flavonoids from oxidation and/or guide them to the central vacuole [37]. It is likely that GST is one of the proteins in the flavonoid-protein complexes related to flavonoid transport. This is further supported by the observation that the *Arabidopsis tt19* GST mutant is defective in ER-tapetosome-pollen surface transport of flavonoids, in addition to exhibiting reduced PA accumulation [17,25]. Association of a GST-flavonol complex with membranes has also been demonstrated [38], providing a further piece to be fitted into the GST-mediated flavonoid transport puzzle.

Because of their close biosynthetic relatedness, anthocyanins and PAs could share similar transport mechanisms. The maize (*Zea mays*) *BZ2* GST complements the petunia *AN9*-depleted anthocyanin phenotype [33], whereas petunia *AN9* complements the anthocyanin-deficient phenotype of *Arabidopsis tt19* mutants, but not the seed coat PA-deficient phenotype [25]. It has therefore been proposed that *AN9* is only involved in anthocyanin binding and transport, whereas *TT19* is involved in both anthocyanin and PA transport [25].

ATP binding cassette (ABC) transporters

An increasing body of genetic, biochemical and molecular biological evidence implicates ABC and multidrug and toxic compound extrusion (MATE) transporters in the transport of plant secondary metabolites, including flavonoids [39–41]. However, a full understanding of the molecular mechanisms of flavonoid transport by membrane transporters is only just beginning to emerge.

The ABC transporter family is both large and ubiquitous. The unique molecular structures of these proteins enable them to transport a range of substrates across membranes using the energy from ATP hydrolysis [42] (Figure 2). The *Arabidopsis*, rice (*Oryza sativa*), and *M. truncatula* genomes each have >120 putative ABC transporters, but only a few have been characterized. These show diverse transport activities toward metal ions, auxin, malate, defensive secondary metabolites, or xenobiotics [41]; specific examples include AtWBC12 (cutin and wax secretion) [43], AtPDR8 (pleiotropic drug resistance, exporting toxic materials to inhibit pathogen penetration) [44], AtMRP1 and 2 (glutathione conjugated and non-conjugated chlorophyll metabolites) [45,46], tobacco plasma membrane PDR-type ABC transporter (secretion of the antifungal terpenoid sclareol) [47] and Japanese gold-

thread (*Coptis japonica*) CjMDR1 (berberine alkaloid import) [48].

To date, only MRP-type ABC transporters have been implicated in flavonoid transport. Suppression of the maize tonoplast-localized ABC-type transporter ZmMRP3 results in reduced anthocyanin accumulation [39], although anthocyanin transport activity of ZmMRP3 has yet to be shown. It will be interesting to know if it can transport GST-anthocyanin conjugates. Plasma membrane ABC transporter-mediated secretion of the isoflavone genistein has been reported in soybean roots, but the gene and protein responsible are yet to be identified [49]. On the basis of inhibitor studies, MRP-type ABC transporters have also been implicated in the long-distance transport of flavonoids such as naringenin, dihydrokaempferol and dihydroquercetin via unidirectional cell-cell movement between root and shoot. This work requires additional experimental validation, but provides a first indication that ABC transporters participate in flavonoid distribution at the cell-type, tissue and organ levels [50].

MATE transporters

MATE transporters comprise a large family of transporters that are widely distributed in all living organisms, from prokaryotes to eukaryotes [51]. As secondary transporters, MATE transporters use electrochemical gradients of protons or sodium ions that are created by proton pumps, such as plasma membrane P-type ATPases, vacuolar V-type ATPases, or V-pyrophosphatase [52]. Plant MATE transporters are involved in the detoxification of xenobiotics and in the transport of a wide range of metabolites, such as cations, organic acids and secondary metabolites [53]; plant genomes therefore encode large MATE transporter families with ~60 members [53,54].

Several *Arabidopsis* MATE transporters have been characterized. These include AtALF5 (exports toxic cations such as tetramethylammonium) [55] and AtDTX1 (confers norfloxacin, berberine and cadmium tolerance when expressed in *Escherichia coli*) [56]. The plasma membrane MATE transporter SbMATE from sorghum [57] exports citrate and confers aluminum tolerance. A berberine/proton antiporter located on the vacuolar membrane has been characterized from Japanese goldthread [58], and JAT1 is a jasmonate-inducible alkaloid transporter that can transport nicotine into the vacuole in the aerial parts of tobacco [59,60].

PA deficiency in seeds of the *Arabidopsis tt12* mutant is caused by loss of function of a MATE transporter, TT12 [61], which mediates vacuolar sequestration of cyanidin 3-O-glucoside and is localized to the tonoplast [40]. It is now known that TT12 and its ortholog MATE1 from *M. truncatula* expressed in yeast preferentially transport epicatechin 3'-O-glucoside, which is proposed to be a precursor for the biosynthesis of PAs [54] (Figure 2). This explains the *tt12* seed phenotype, and similar PA transport mechanisms could be applicable in other species. Recently, two MATE-type transporters from grapevine (*Vitis vinifera*), AM1 and AM3, were characterized as vacuolar transporters of acylated anthocyanins [62], and the tomato (*Solanum lycopersicon*) MATE transporter LaMTP77 might have a similar function [63].

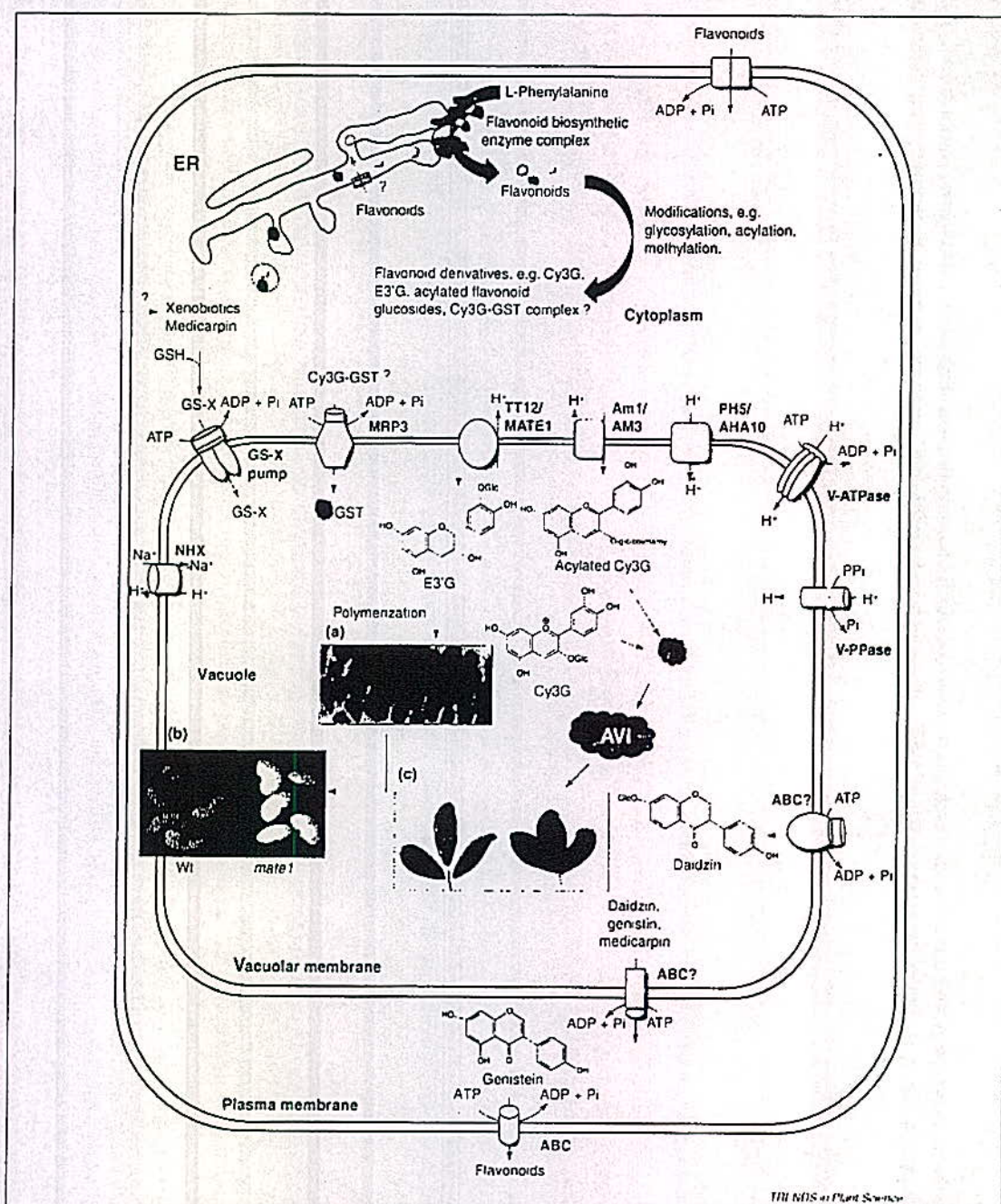


Figure 2. Schemes for membrane transporter-mediated flavonoid transport. Flavonoids are synthesized on the cytosolic face of the ER membranes by the action of biosynthetic enzyme complexes. Flavonoid aglycones are then subjected to various modifications, such as glycosylation and acylation. The vacuolar membrane MATE transporters TT12 from *Arabidopsis* and MATE1 from *Medicago truncatula* preferentially transport epicatechin 3'-O-glucoside (E3'G) into the vacuole for PA biosynthesis. The grapevine MATE transporters AM1 and AM3 specifically transport p-coumaroyl modified anthocyanins, such as cyanidin 3-O-glucoside (Cy3G), into the vacuole. MATE transporters are driven by a proton gradient that is established by the action of a V-ATPase (such as the petunia vacuolar H⁺-ATPase PH5 and possibly *Arabidopsis* AHA10) or a V-PPase. By contrast, the acidic vacuolar lumen can be neutralized by an H⁺/Na⁺ exchanger (NHX). The maize vacuolar ABC transporter MRP3 is involved in sequestration of anthocyanins into the vacuole, but its exact substrate is not yet known; it might transport an anthocyanin-GST complex. Foreign flavonoids can also be detoxified by GSH conjugation and the conjugates then transported into the vacuole by the MRP-type GS-X pump, as is the isoflavonoid phytoalexin medicarpin. Isoflavonoid glucosides such as daidzin and genistein can be transported into the vacuole by an ABC transporter, and the isoflavone aglycone genistein is transported into

Role of H⁺-ATPase in flavonoid transport

Given that MATE transporters are secondary transporters that use an electrochemical gradient (usually H⁺ in plants) as the driving force to transport other substrates across membranes, their function and activity largely depend on various types of H⁺-ATPases. P-type H⁺-ATPase can provide and maintain the H⁺ gradient across the plasma membrane, whereas V-type H⁺-ATPase or vacuolar pyrophosphatase (V-PPase) proton pumps provide and maintain H⁺-gradients across the vacuolar membrane [27,52] (Figure 2). Mutation of these essential enzymes should result in *tt* phenotypes or flower color changes (because anthocyanin content/composition and vacuolar pH largely determine flower color). Indeed, knockout of the P-type H⁺-ATPase AHA10 from *Arabidopsis* results in a *tt* seed phenotype and reduces PA content [26]. Mutation of the vacuolar membrane-localized H⁺-ATPase PH5 from petunia alters the pH of the vacuolar lumen and thereby changes flower color and reduces seed PA content [27]. Mutations in the petunia *ANI* and *PH4* genes also affect the pH of petal homogenates, alter flower color and reduce anthocyanin or PA production [64], further confirming that destroying the proton gradient across the tonoplast can affect MATE transporter activity for vacuolar sequestration of anthocyanins and PAs [27]. Seed coat endothelial cells in the *Arabidopsis aha10* and *tt12* mutants contain many small vacuoles instead of one large central vacuole [26,40]. Although PH5 is localized to the tonoplast [27], the exact localization of AHA10 is unclear; it was originally predicted to be a plasma membrane protein [26], but this appears inconsistent with its knock-out phenotype.

Induction of anthocyanin biosynthesis in *Medicago* spp. expressing the LAP1 MYB transcription factor is associated with the coordinated up-regulation of anthocyanin modification enzymes (glycosyltransferases and acyl transferases), a MATE-like antiporter, a Na⁺/H⁺ antiporter, and two putative ABC transporters [34]. Some or all of these molecules might be associated with vacuolar sequestration of anthocyanins.

Chemical requirements for directional flavonoid transport

Flavonoids are often subjected to multiple modifications upon biosynthesis, such as glycosylation, hydroxylation, acylation (including aromatic acylation and acylation of the attached sugar residue) and methylation. These modifications are important for increasing their solubility or stability and reducing toxicity; downregulation of cyanidin 3-*O*-glucosyltransferases, such as maize BZ1, *Arabidopsis* ANL1 or *Medicago* UGT78G1 strongly decreases anthocyanin accumulation [22,34]. Acylation of anthocyanin or apigenin 7-*O*-(6-*O*-malonylglucoside) is a prerequisite for their uptake [62], but glycosylation alone is sufficient to enable the vacuolar uptake of many flavonoids [40,54,65–69].

Some MATE transporters exhibit relatively strict substrate specificity. For example, both TT12 and MATE1 transport epicatechin 3'-*O*-glucoside with higher affinity than cyanidin 3-*O*-glucoside in yeast, and neither transports catechin 3-*O*-glucoside, flavonol glucosides such as quercetin-3-*O*-glucoside, or flavonoid aglycones [40,54]. Cyanidin 3-*O*-glucoside requires acylation before it can be transported into the vacuole by grapevine AM1 and AM3 [62]. Although flavonoid aglycones might not be suitable substrates for MATE transporters, a MATE transporter from tobacco (*Nicotiana tabacum*) can transport non-modified nicotine directly into the vacuole [59,60]; other MATE transporters, such as AtDTX1, and ABC transporters such as CjMDR1, show a relatively broad substrate spectrum [48,56].

It is not understood how plants choose between ATP-hydrolysis-dependent or H⁺/Na⁺-gradient dependent mechanisms for transport of native metabolites or xenobiotics. The conjugation ligands, glucose or glutathione, appear to determine the transport mechanism for vacuolar uptake of herbicides [68]. The same herbicide, if conjugated with glucose, can be taken up by a proton-gradient dependent antiporter, whereas its glutathione conjugate is taken up by an ATP-energized ABC transporter. The native flavonoids saponarin (apigenin 6-*C*-glucosyl-7-*O*-glucoside) and isovitexin (apigenin 6-*C*-glucoside) are transported into the vacuole by a secondary energized proton antiporter in barley (*Hordeum vulgare*) [65,67]. However, *Arabidopsis* takes up saponarin, a foreign compound, into the vacuole using an ABC transporter [65,67]. In Japanese goldthread and meadow rue (*Thalictrum minus*) cell cultures, both ABC transporters and proton antiporters mediate the transport of berberine into the cell or vacuole, as well as out of the cell [48,58,70].

Efflux of vacuolar flavonoids

Import and export of natural products across the tonoplast or plasma membrane can be carried out by different mechanisms. For example, the import of natural products or xenobiotics into the vacuole usually occurs with conjugated forms, whereas export of the same compound does not seem to require conjugation.

How vacuolar uptake and efflux of the same flavonoid compound are controlled is a particularly interesting question, and is well exemplified by the case of the isoflavone conjugates in legume cell cultures. Chickpea (*Cicer arietinum*) cell suspension cultures constitutively accumulate isoflavone malonyl glucosides in the central vacuoles [71,72]. It is assumed that glycosylation is essential for vacuolar uptake, and that malonylation of the sugar group somehow acts to maintain the conjugate in the vacuole. Treatment of the cultures with a fungal elicitor preparation resulted in accumulation of pterocarpan phytoalexins derived from the isoflavonoid pathway; these were synthesized *de novo*, and did not utilize vacuolar stores of isoflavonoid pathway intermediates [71]. However, if the

the soybean spoplast by another ABC-type transporter. Inset pictures show (a) PA (blue staining) in the vacuoles in a cross-section of the *Medicago* seed coat; (b) *Medicago* MATE1 mutant (*mte1*) seeds with a TRANSPARENT TESTA phenotype as compared with wild-type (wt); and (c) leaves of nontransgenic alfalfa (green) and transgenic alfalfa expressing the MtLAP1 MYB transcription factor (purple), thereby overproducing a range of anthocyanins, including Cy3G. See main text for further details.

cells were treated with a fungal elicitor plus an inhibitor of phenylalanine ammonia-lyase, the first enzyme of the phenylpropanoid/(iso)flavonoid pathway, pterocarpan phytoalexin was still formed, but was derived from the mobilization of vacuolar isoflavone intermediates. The conversion of isoflavone aglycones to pterocarpan is believed to occur in the cytoplasm. Thus, when cells sense a requirement for isoflavone precursors that cannot be provided through *de novo* synthesis, vacuolar efflux is activated. This is associated with expression of glucosyl hydrolase genes [13]. The localization of these enzymes is yet to be definitively determined, so it is unclear whether a conjugate or free aglycone is the substrate for the vacuolar efflux transporter. Neither is the nature of the efflux transporter known, although isoflavone efflux across the plasma membrane occurs via an ABC-type transporter in soybean [49] (Figure 2), and methyl jasmonate-induced accumulation of medicarpin in cell suspensions of *M. truncatula* in the absence of *de novo* synthesis is accompanied by parallel depletion of vacuolar isoflavone glycoside pools and upregulation of several ABC transporter genes [13]. It also remains to be determined how the cell senses flux through the isoflavonoid pathway to determine whether to activate influx or efflux systems. Similar sensing mechanisms are likely to operate to determine the directionality of transport of other flavonoid compounds.

Vesicle-mediated efflux of vacuolar metabolites, including flavonoids, has also been proposed [5]. It has also been suggested that SNARE protein-mediated exocytosis pathways, or nondirectional secretion of cargo containing antimicrobials, are involved in the efflux of antimicrobial flavonoids into the apoplastic space, but there is as yet no direct evidence in support of this hypothesis [73].

Co-migration of flavonoids and their transporters with trafficking vesicles?

There are many pieces to the puzzle of flavonoid transport. As described above, credible evidence exists for at least three mechanisms (GST-linked, vesicle trafficking and MATE transporters, with the least equivocal evidence being available for the latter) for vacuolar uptake of flavonoids. Knockout of proteins involved in transport of PAs, such as MATE1, TT12, TT19, AHA10, or PH5, still results in a detectable level of PAs in the seed coat [25–27,40,54], suggesting that redundant mechanisms are involved in PA sequestration into the vacuole.

Can these three apparently independent mechanisms occur in parallel in a single cell type, or can the data be viewed in a more reductionist manner? Some commentators have questioned the tonoplastic localization of the MATE transporters and P-type H⁺-ATPase involved in anthocyanin/PA trafficking, citing technical difficulties with cell-type specific localization of GFP-labeled transporters [74] and the possibility for dynamic movement (cycling) and/or multiple localizations of these transporters [75,76]. In the case of PAs, no enzyme has yet been implicated in their oligomerization, and chemical studies suggest non-enzymatic oligomerization at low pH. Could this low pH be provided by a vesicle-localized ATPase, such that the PAs are already undergoing oligomerization in vesicles before delivery to the vacuole? Do MATE trans-

porters function to load flavonoid conjugates into vesicles rather than directly into the vacuole? Furthermore, do these flavonoid transporters or transport-related membrane proteins co-migrate with flavonoids in trafficking vesicles?

The defective tapetosome-mediated flavonoid transport to the pollen surface in *Arabidopsis tt12* and *tt19* mutants suggests that TT12 and/or TT19 are involved in the loading of flavonoids into ER-derived tapetosomes [17]. Membrane transporters, including tonoplast targeted transporters, are themselves believed to be targeted to their destination membranes via vesicle trafficking after their synthesis on the rough ER [29,30,75,77]. The vacuolar membrane transporters contain vacuolar-sorting determinants in their sequences. Therefore, they could also carry out their transport functions on ER-derived vesicles during vesicle trafficking, if ATPase provided a proton gradient to these vesicles. Therefore, H⁺-ATPases such as AHA10/PH5 and MATE transporters such as TT12/MATE1 might be functionally coupled during vesicle trafficking to the vacuole, together with flavonoids. However, this interesting hypothesis requires experimental support.

Completing the puzzle

Most of our current knowledge of flavonoid transport is related to vacuolar sequestration. Mechanisms for efflux of flavonoids from the vacuole and plant cell, uptake into the plant cell, or transport into the nucleus, chloroplast, or subcellular vesicle compartments are poorly understood, although such transport might have essential roles in plant development, growth, reproduction and defense. The question of how cells determine whether a compound is destined for influx (to the vacuole) or efflux cannot be conclusively answered until more is known about efflux transporters for secondary metabolites. In one probable model, metabolite levels in specific cell compartments are first sensed, and this activates a signal transduction pathway to regulate target transporter gene expression. Many transporter genes, such as those encoding ABC transporters, are highly regulated by various stress signals [13,44,47].

Although many questions still remain, recently developed genomics tools promise more rapid progress than seen to date. New informatics methods are facilitating prediction of transporter functions from genome sequence and microarray co-expression data [78], the discovery of the transcription factors that activate whole pathways of flavonoid biosynthesis and transport [79,80] provides an additional tool for candidate gene discovery, mutant resources are becoming available in multiple genetically tractable model systems (e.g. Ref. [81]), and combined biochemical, cell biological and genetic analysis of plant transporters is becoming routine and provides more significant insights into their functions [36,50]. However, the major challenges are likely to be in the area of cell biology, where real-time whole-cell imaging techniques will be needed to catch flavonoids 'in the act'.

Increasing the biosynthesis of flavonoids in plants is often limited by complex and yet poorly understood feedback inhibition effects. Membrane transporters might have important roles in regulating metabolic flux. Therefore, the

study of flavonoid transport will not only fill a major gap in current understanding of the complete picture of flavonoid metabolism, but will also benefit the metabolic engineering of useful flavonoid compounds in crops for improving agronomic traits and the nutritional quality of foods.

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Appendix 4

Katsumoto et al., Plant Cell Physiol. 48(11): 1589-1600 (2007)

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Engineering of the Rose Flavonoid Biosynthetic Pathway Successfully Generated Blue-Hued Flowers Accumulating Delphinidin

Yukihisa Katsumoto¹, Masako Fukuchi-Mizutani¹, Yuko Fukui¹, Filippa Brugliera², Timothy A. Holton², Mirko Karan², Noriko Nakamura¹, Keiko Yonekura-Sakakibara^{1,3}, Junichi Togami¹, Alix Pigeaire², Guo-Qing Tao², Narendra S. Nehra², Chin-Yi Lu², Barry K. Dyson², Shinzo Tsuda¹, Toshihiko Ashikari^{1,2}, Takaaki Kusumi¹, John G. Mason² and Yoshikazu Tanaka^{1,*}

¹ Suntory Limited Research Center, Institute for Plant Science, 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka, 618-8503 Japan
² Florigene Pty. Ltd, 1 Park Drive, Bundoora, VIC 3083, Australia

Flower color is mainly determined by anthocyanins. *Rosa hybrida* lacks violet to blue flower varieties due to the absence of delphinidin-based anthocyanins, usually the major constituents of violet and blue flowers, because roses do not possess flavonoid 3',5'-hydroxylase (F3'5'H), a key enzyme for delphinidin biosynthesis. Other factors such as the presence of co-pigments and the vacuolar pH also affect flower color. We analyzed the flavonoid composition of hundreds of rose cultivars and measured the pH of their petal juice in order to select hosts of genetic transformation that would be suitable for the exclusive accumulation of delphinidin and the resulting color change toward blue. Expression of the *viola F3'5'H* gene in some of the selected cultivars resulted in the accumulation of a high percentage of delphinidin (up to 95%) and a novel bluish flower color. For more exclusive and dominant accumulation of delphinidin irrespective of the hosts, we down-regulated the endogenous dihydroflavonol 4-reductase (*DFR*) gene and overexpressed the *Iris x hollandica DFR* gene in addition to the *viola F3'5'H* gene in a rose cultivar. The resultant roses exclusively accumulated delphinidin in the petals, and the flowers had blue hues not achieved by hybridization breeding. Moreover, the ability for exclusive accumulation of delphinidin was inherited by the next generations.

Keywords: Anthocyanin — Delphinidin — Flavonoid — Flower color — Metabolic engineering — *Rosa hybrida*.

Abbreviations: AT, hydroxycinnamoyl CoA:anthocyanin hydroxycinnamoyl transferase; CaMV, cauliflower mosaic virus; DFR, dihydroflavonol 4-reductase; F3'5'H, flavonoid 3',5'-hydroxylase; NMR, nuclear magnetic resonance; NPTII, neomycin phosphotransferase; RHSCC, Royal Horticultural Society Color Chart; RNAi, RNA interference; siRNA, small interfering RNA; TFA, trifluoroacetic acid; TOF-MS, time-of-flight-mass spectrometry.

Introduction

Flower color plays important roles in plant sexual hybridization by attracting pollinators, such as insects

and birds. Flower color is also attractive to people and is an important characteristic of flowers from a floricultural viewpoint. Hybridization breeding has partially achieved the diversification of flower color of floricultural crops.

Plant species have adopted versatile and ingenious ways to exhibit flower color (Grotewold 2006, Tanaka and Brugliera 2006). Such ingenuity has been studied and understood in terms of chemistry, biochemistry and molecular biology (Forkmann and Heller 1999, Goto 1987, Tanaka and Brugliera 2006). Among various pigments in petals, anthocyanins, a class of flavonoids, are major constituents of flower color from orange/red to violet/blue. Their chemical structures primarily determine their color, i.e. the number of hydroxy groups on the B-ring and/or aromatic acyl moieties modifying anthocyanins increase, causing a bathochromic shift to blue (Honda and Saito 2002). The majority of violet/blue flowers contain delphinidin-based anthocyanins modified with one or more aromatic acyl moieties (Honda and Saito 2002). Anthocyanins change their color depending on the pH of the vacuole in which anthocyanins localize; their color is bluer in weakly acidic or neutral pH, and redder in acidic pH. Co-pigments, usually flavones and flavonols, cause a bathochromic shift of anthocyanins when they stack with anthocyanins (Goto and Kondo 1991). The formation of a complex with metal ions can give a blue color (Kondo et al. 1992, Yoshida et al. 2003, Shiono et al. 2005, Shoji et al. 2007).

Among the factors influencing flower color, flavonoid/anthocyanin biosynthesis has been the most extensively studied. The pathway leading to anthocyanidin 3-glucoside is generally conserved among higher plant species (Fig. 1) (Forkmann and Heller 1999, Grotewold 2006, Tanaka and Brugliera 2006). Each plant species usually accumulates limited kinds of anthocyanins and exhibits limited flower color by the expression of a specific set of biosynthetic genes, the substrate specificity of key enzymes and/or the temporal and spatial regulation of the biosynthetic genes. Therefore, it is rare for a single species to have the entire

³Present address: RIKEN Plant Science Center, 1-7-22 Suicho-cho, Tsurumi-ku, Yokohama City, Kanagawa, 230-0045 Japan.
^{*}Corresponding author. E-mail, Yoshikazu_Tanaka@suntory.co.jp; Fax, +81-75-962-3791.

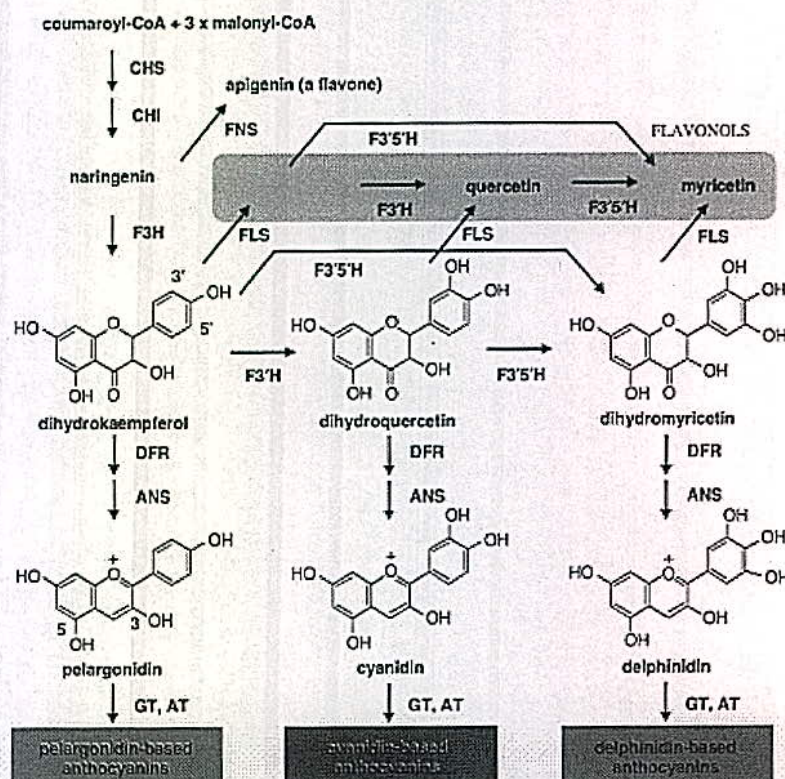


Fig. 1 Generalized flavonoid biosynthetic pathway relevant to flower color. Native rose petals only accumulate pelargonidin and cyanidin-based anthocyanins, mainly pelargonidin and cyanidin 3,5-diglucoside. Lack of delphinidin-based anthocyanins, which is attributed to deficiency of *F3'5'H*, has hampered the generation of rose flowers having blue and violet hues. The expression of a heterologous *F3'5'H* gene in rose is expected to generate delphinidin and, thus, a novel flower color with a blue hue. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; GT, anthocyanidin glucosyltransferase; AT, anthocyanin acyltransferase.

spectrum of flower color, although floricultural breeders have always sought novel flower colors (Tanaka et al. 2005, Tanaka and Brugliera 2006). For example, roses, carnations and chrysanthemum, which are the most important floricultural crops, do not accumulate delphinidin-based anthocyanins. Thus, they lack violet/blue varieties. This is attributed to their deficiency of flavonoid 3',5'-hydroxylase (*F3'5'H*), a key enzyme in the synthesis of delphinidin (Fig. 1) (Holton and Tanaka 1994).

On the other hand, petunia and cymbidium lack brick red/orange varieties due to the lack of pelargonidin-based anthocyanins because their dihydroflavonol 4-reductases (DFRs) do not utilize dihydrokaempferol as a substrate (Forkmann and Heller 1999, Johnson et al. 1999). Some species that usually accumulate delphinidin-based anthocyanins and do not accumulate pelargonidin-based

anthocyanins in their petals, such as iris and gentian, are likely to have DFRs with a similar substrate specificity to that of petunia DFR. Rose DFR can utilize dihydromyricetin as a substrate on the basis of feeding of dihydromyricetin to rose petals (Holton and Tanaka 1994).

Advances in molecular biology and plant transformation technology have made possible the engineering of an anthocyanin biosynthetic pathway and, thus, flower color by the overexpression of heterologous flavonoid biosynthetic genes and/or the down-regulation of endogenous genes in transgenic plants, including petunia, torenia and carnation, as reviewed by Tanaka (2006). In carnation, the overexpression of the *F3'5'H* gene alone was insufficient to convert the metabolic flux fully toward delphinidin biosynthesis. White carnation cultivars that specifically lacked the *DFR* gene were transformed with the petunia or

viola *F3'5'H* gene in combination with the petunia *DFR* gene. As a result, violet carnations accumulating delphinidin-based anthocyanins were almost exclusively generated (Holton 1996, Fukui et al. 2003). These results indicate that it is possible to generate a novel flower color by changing the structure of anthocyanin, more specifically the number of hydroxyl groups on the B-ring, with genetic modification of the pathway. They also indicate that the selection of cultivars that have proper genetic background and flavonoid compositions and/or the artificial down-regulation of a competing endogenous pathway is necessary to obtain a desirable phenotype (Tanaka 2006, Tanaka and Brugliera 2006).

Roses are the most important flowers in today's global flower market and have been the center of attraction for consumers and breeders for hundreds of years. Modern roses (*Rosa hybrida*) have resulted from extensive hybridization of wild rose species and have various flower colors, except those in the violet to blue range (Vries et al. 1974, Holton and Tanaka 1994). Rose breeders have long endeavored to create blue roses, but their efforts have so far only led to pink and pale mauve-colored flowers. This is not surprising because rose petals contain pelargonidin/cyanidin 3-glucoside or 3,5-diglucoside (Biolley and Jay 1993, Mikanagi et al. 2000) and carotenoids (Vries et al. 1974). Some roses produce only small amounts of acylated anthocyanins, and roses do not produce flavones (Mikanagi et al. 2000), which are stronger co-pigments than flavonol (Yabuya et al. 1997). The vacuolar pH of rose petal epidermal cells is low (from pH 3.69 to 5.78) (Biolley and Jay 1993). Therefore, roses fundamentally lack the components required to yield violet/blue flowers.

More recently, some rose cultivars have been shown to contain a small amount of blue pigments (rosacyanin) that are derivatives of cyanidin (Fukui et al. 2006). Blue coloration in the cultivar 'Rhapsody in Blue' is caused by the accumulation of cyanin (cyanidin 3,5-*O*-di-glucoside) in anthocyanic vacuolar inclusions (AVIs) (Gonnet 2003). However, the molecular mechanisms of rosacyanin and AVIs is not yet understood, and their utilization to engineer blue/violet roses is not currently possible.

Although there may be multiple ways to create blue roses, we have chosen delphinidin production in rose petals because delphinidin biosynthesis is regulated by a single enzyme, *F3'5'H*, and its overexpression is unlikely to have a detrimental effect on plants. Delphinidin production presents a major breakthrough in the achievement of blue roses (Holton and Tanaka 1994). The expression of *viola F3'5'H* genes produced delphinidin (Brugliera et al. 2004). However, the presence of pelargonidin and cyanidin-based anthocyanins derived from the endogenous pathway prevented sufficient coloration toward blue. Unlike carnations, we were not able to obtain white rose cultivars that specifically lacked *DFR* activity.

Here, we analyzed the flavonoid composition of many rose cultivars in order to select cultivars which will exhibit a blue hue when delphinidin is accumulated. The overexpression of a *viola F3'5'H* gene resulted in the efficient accumulation of delphinidin and color changes with a novel blue hue in a few of the selected cultivars. Furthermore, the down-regulation of the rose *DFR* gene and overexpression of the iris *DFR* gene, as well as the overexpression of the *viola F3'5'H* gene, resulted in more efficient and exclusive delphinidin production and a bluer flower color. It is noteworthy that exclusive delphinidin production is heritable by the progeny, which should change rose breeding as far as flower color is concerned.

Results

Cultivar screening for engineering a flavonoid pathway

Hundreds of rose cultivars that claimed to be 'blue' and/or have names associated with 'blue' have been commercialized. Their flower color ranges are relatively blue among roses and in fact range from pink/red purple to gray/mauve. Some of these cultivars were subjected to flavonoid analysis and pH measurements, and the results are shown in Table 1. Their petals contained mainly cyanidin as an anthocyanidin, but not delphinidin. They contained a large amount of flavonols. Their bluish color may be due to a high flavonol/anthocyanidin ratio, and flavonols may function as co-pigments. Their higher pH than that of red roses may also contribute to their flower color (Table 1). These results are consistent with a previous report (Biolley and Jay 1993). Table 1 includes some red cultivars for comparison, which contain cyanidin and/or pelargonidin. They tend to have fewer flavonols and a lower pH. We recently discovered that some of these roses contain small amounts of novel cyanidin derivatives (rosacyanins) exhibiting a blue color (Fukui et al. 2006), but the degree of their contribution to flower color is not clear.

The criteria for the selection of possible host cultivars to achieve flower color modification toward blue by delphinidin production were: (i) they accumulated flavonols that were expected to be co-pigments; (ii) they had a higher vacuolar pH; (iii) ideally, they did not have *F3'H* activity; and (iv) they accumulated pelargonidin rather than cyanidin.

In order to choose cultivars to meet these criteria, several hundreds of rose flower cultivars were screened initially by visual observation: pink to mauve roses were mainly selected. Dark color cultivars and red cultivars were not selected, since they did not contain much flavonol and their pH tends to be low. Yellow and white cultivars were not selected either, as they are not able to accumulate anthocyanins in the petals. The bluish rose cultivars shown in Table 1 were not subjected to further study because of their low transformation frequencies or their low

Table 1 Flavonoid composition of commercial bluish rose varieties

Cultivar	Anthocyanidin (mg g ⁻¹) ^a			Flavonol (mg g ⁻¹) ^a		pH
	cya	pel	peo	Q	K	
‘Blue’ varieties						
Blue Bajou	0.048			3.956	0.379	5.11
Blue Heaven	0.008			1.504	0.569	4.90
Blue Moon	0.049			1.341	0.119	5.25
Delilah	0.066			0.611	0.277	5.35
Grey Pearl	0.036			3.708	0.417	5.12
Lavande	0.109			1.020	0.195	4.70
Madam Violet	0.060			1.780	0.150	4.70
Ondina	0.019			2.056	1.043	4.99
Rhapsody in Blue	1.851	0.011	0.005	3.456	1.622	5.67
Seiryu	0.015			3.030	1.300	5.13
Shocking Blue	0.196			1.198	0.194	5.40
Sterling Silver	0.035			1.495	0.363	5.71
Vol de Nuit	0.317	0.003		2.661	0.316	4.90
Watarase	0.056			3.048	0.124	5.07
Pink-red varieties, etc.						
Black Baccara	4.043	0.007		0.705	0.119	4.58
First Red	1.959	0.291		0.086	0.340	4.70
Jacaranda	0.315	0.000		1.238	0.468	4.80
Kardinal	1.898	0.125		0.156	0.296	4.40
Kiss	0.006	0.038		0.028	1.381	4.02
Lambada	0.036	0.164		0.014	0.466	4.40
Medeo	0.004	0.004		0.028	2.323	4.40
Rote Rose	3.480	0.034		0.238	0.285	4.37
Sonia	0.060	0.300		0.006	0.650	4.85

cya, cyanidin; pel, pelargonidin; peo, peonidin; Q, quercetin; K, kaempferol.
^amg g⁻¹ fresh petals.

anthocyanin contents to achieve clear color change. The petals of 169 selected cultivars were harvested and subjected to flavonoid analysis and pH measurements. Some of the results are shown in Table 2 (see controls).

Only pelargonidin and cyanidin were detected as anthocyanidins, and kaempferol and quercetin as flavonols, which is consistent with previous reports (Biolley and Jay 1993, Mikanagi et al. 2000). The absence of delphinidin and myricetin confirmed the deficiency of F3'5'H activity in the petals of these cultivars. The absence of flavones, which are stronger co-pigments, was also confirmed. The cultivars WKS77, WKS82, WKS100, WKS116, WKS124 and WKS140 were selected for genetic transformation.

Cloning of flavonoid biosynthetic genes and construction of binary vectors

The aromatic acylation of anthocyanin shifts its color toward blue by 3–4 nm (Fujiwara et al. 1998). For

co-expression with the F3'5'H gene in rose, the cDNA of hydroxycinnamoyl CoA:anthocyanin 5-hydroxycinnamoyl transferase (SAT) was obtained from the *Torenia hybrida* petal cDNA library. Torenia SAT exhibited 37 and 35% amino acid sequence identity to gentian SAT (Fujiwara et al. 1998) and perilla 3AT (Yonekura-Sakakibara et al. 2000), respectively. Its function was confirmed by expressing the cDNA in *Escherichia coli* and yeast as previously described (Fujiwara et al. 1998, Yonekura-Sakakibara et al. 2000). The enzyme catalyzed the transfer of the coumaroyl or caffeoyl moiety to 5-glucose of anthocyanidin 3,5-diglucosides (data not shown).

The substrate specificity of the DFR often determines which anthocyanidins a plant accumulates (Forkmann and Heller 1999). The DFR of some species, such as iris and gentian, which mainly accumulate delphinidin and lack pelargonidin, is expected to have a similar substrate to petunia DFR, which efficiently reduces dihydromyricetin. Iris DFR cDNA was isolated from the *Iris × hollandica* petal cDNA library. The iris DFR exhibited reasonable amino acid sequence identity to the DFRs of other plants (61, 58 and 56% to cymbidium, rose and petunia DFR, respectively) and had a specific feature, namely that it did not utilize dihydrokaempferol as a substrate (Johnson et al. 1999). Such substrate specificity is suitable for the conversion of the metabolic flux toward delphinidin biosynthesis when it is co-expressed with the F3'5'H gene.

The binary vectors used in this study (pSPB130, pSFL207, pSFL236 and pSPB919) are shown in Fig. 2. The vector pSPB130 is designed for the constitutive overexpression of the *viola* F3'5'H BP40 gene and the *torenia* SAT gene in rose. The binary vector pSFL207 is used for the constitutive overexpression of the *viola* F3'5'H gene alone, and pSFL236 is for the co-expression of the *viola* F3'5'H and the iris DFR genes. The vector pSPB919 is to down-regulate the endogenous rose DFR gene using RNA interference (RNAi) and to overexpress the iris DFR and the *viola* F3'5'H genes.

Generation of transgenic roses and their phenotype

Embryogenic calli were induced from the selected cultivars and subjected to transformation with *Agrobacterium tumefaciens* containing pSPB130 to assess how efficiently these cultivars can accumulate delphinidin and the effect of delphinidin on flower color. At the same time, the effect of 5-acylation catalyzed by *torenia* SAT on flower color could be evaluated. Transgenic roses were obtained, and flowers with altered color were subjected to flavonoid analysis. The results are summarized in Table 2. The production of delphinidin and myricetin indicates that the introduced F3'5'H gene functioned in transgenic roses. The delphinidin contents (percentage of the total anthocyanidins and amount) and the changes in flower color varied with the transgenic hosts and events.

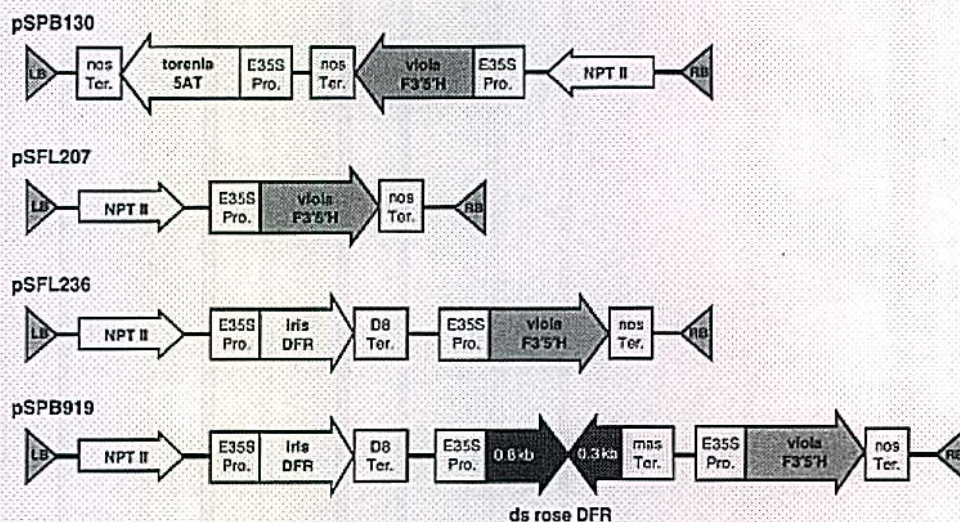


Fig. 2 Schematic representation of binary vectors constructed for color modification. Only some T-DNA regions are shown. The directions of the cDNA sense strand are shown by arrows. All of them have the *nptII* gene as the selectable marker for plant transformation. E35S Pro., enhanced CaMV 35S promoter; mas Ter., terminator region from manopine synthase; nos Ter., nopaline synthase gene terminator; D8 Ter., terminator region from a petunia phospholipid transfer protein gene (D8) (Holton 1996); F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; 5AT, anthocyanin 5-acyltransferase.

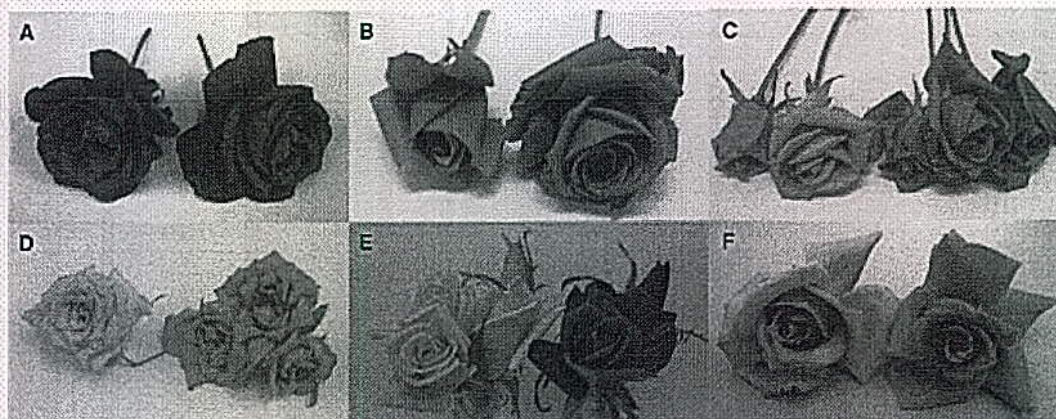


Fig. 3 Flower color changes by delphinidin production. The rose cultivars WKS77, WKS82, WKS100, WKS116, WKS124 and WKS140 were transformed with pSPB130, and their flower color changed (left, host; right, a transformant). A flower of the line exhibiting the most significant color change is shown. (A) WKS77, (B) WKS82, (C) WKS100, (D) WKS116, (E) WKS124, (F) WKS140.

The accumulation of delphinidin conferred flower color changes. Some of them, such as WKS82, WKS100, WKS116 and WKS140 transformants (Fig. 3B, C, D, F), exhibited a novel blue hue that has not been achieved in hybridization breeding. First, WKS82/130-4-1 and WKS82/

130-9-1 were selected from these transgenic roses on the basis of flower color and performance in a greenhouse, and were subjected to a field trial. Transgenic WKS77 and WKS124 also changed their flower color with delphinidin production. The amount of anthocyanins increased, and the

Table 2 Flavonoid composition of Keisei rose varieties transformed with pSPB130

Host	Vector		Anthocyanidin (mg g ⁻¹) ^a			del (%)	acyl (%)	Flavonol (mg g ⁻¹) ^a			pH
			del	cya	pel			M	Q	K	
WKS77	Control		0.000	1.103	0.005	0.0%	0.0%	0.000	1.513	0.303	5.05
	pSPB130	Average (22)	0.600	1.343	0.007	32.4%	10.2%				
		Best line	1.068	0.814	0.004	56.6%	26.1%	0.604	0.503	0.126	
WKS82	Control		0.000	0.124	0.000	0.0%	0.0%	0.000	1.598	0.081	5.46
	pSPB130	Average (52)	0.055	0.038	0.000	54.4%	9.0%				
		Best line	0.139	0.008	0.000	94.5%	22.7%	1.929	0.436	0.036	
WKS100	Control		0.000	0.066	0.000	0.0%	0.0%	0.000	1.248	0.368	5.34
	pSPB130	Average (71)	0.044	0.044	0.000	45.6%	11.2%				
		Best line	0.152	0.015	0.000	91.2%	39.5%	1.320	0.448	0.120	
WKS116	Control		0.000	0.017	0.000	0.0%	0.0%	0.000	1.725	0.636	5.29
	pSPB130	Average (40)	0.040	0.012	0.000	73.2%	8.7%				
		Best line	0.051	0.003	0.000	94.9%	39.3%	1.226	0.245	0.096	
WKS124	Control		0.000	0.022	0.045	0.0%	0.0%	0.000	0.192	2.011	4.85
	pSPB130	Average (16)	0.728	0.098	0.021	82.3 %	24.6%				
		Best line	1.017	0.058	0.000	94.6%	44.2%	1.161	0.140	0.262	
WKS140	Control		0.000	0.075	0.000	0.0%	0.0%	0.000	2.412	0.271	5.25
	pSPB130	Average (49)	0.077	0.063	0.000	47.3%	11.4%				
		Best line	0.114	0.007	0.000	94.1%	33.6%	0.689	0.136	0.031	

del, delphinidin; cya, cyanidin; pel, pelargonidin; del (%), delphinidin/total anthocyanidin; acyl (%), acylated anthocyanin/total anthocyanin; M, myricetin; Q, quercetin; K, kaempferol; best line, a line producing the highest percentage of delphinidin and exhibiting clear color change.

^amg g⁻¹ fresh petals.

flower color became darker. The color of the flowers remained magenta, probably because the pH of the petals was lower than that of other cultivars.

These results indicated that delphinidin production confers a blue hue to the flowers. They also revealed that both the delphinidin contents and the resultant color depend on the host cultivars. A dominant production of delphinidin is not always easy to achieve by overexpression of the *F3'S'H* gene even in the selected cultivars.

Only some anthocyanins (up to 44%) were revealed to be aromatically acylated by the introduced *torenia SAT* gene. The effects of anthocyanin acylation on flower color were not visually observable when the anthocyanins were partly acylated because the 5-acylation of anthocyanin shifts only 4 nm to a longer wavelength (Fujiwara et al. 1998).

Transgenic plants can be used as breeding material, since the transgene is expected to be heritable by progeny. Dominant production of delphinidin irrespective of the cultivars is a desirable characteristic to incorporate into rose breeding. However, since the delphinidin content depends on cultivars to a large extent, the incorporation of the overexpression of the *viola F3'S'H* gene alone would not generate roses containing exclusively delphinidin. This prompted us to design another binary vector (pSPB919) in order to down-regulate an endogenous pathway that potentially competes against the introduced *F3'S'H* gene.

Exclusive delphinidin accumulation by functional replacement of rose *DFR* with *iris DFR* in vivo

A commercial mauve cultivar, 'Lavande', was used to evaluate the efficacy of the binary vectors, pSFL207, pSFL236 and pSPB919 (Fig. 2), because Lavande exhibits the highest transformation frequency and the earliest flowering. The results of the transformation and anthocyanidin and flower color of the resultant transgenic flowers are summarized in Table 3 and Fig. 4. The transgenic rose flowers exhibited various degrees of flower color change, and the relationship between the hue and the delphinidin content (%) derived from pSFL207, pSFL236 and pSPB919 is summarized in Fig. 4. A higher delphinidin content yielded a bluer flower color, as shown in Fig. 4. Despite the fact the overexpression of the *viola F3'S'H* gene (pSFL207) successfully yielded up to 44% of delphinidin in the petals, the amount of cyanidin still surpassed that of delphinidin, about two-thirds of the transgenic plants produced <20% delphinidin and the change in flower color was not clear. Additional expression of the *iris DFR* gene (pSFL236) increased the delphinidin contents (Table 3, Fig. 4), indicating that the *iris DFR* gene was useful to change the metabolic flux toward delphinidin. The variability of the delphinidin content is large for pSFL236 plants and ranges from 0 to 75% (Fig. 4).

Table 3 Summary of the flavonoid composition in transgenic Lavande

Vector		Anthocyanidin (mg g ⁻¹) ^a		del (%)	Flavonol (mg g ⁻¹) ^a		
		del	cya		M	Q	K
Control		0.000	0.109	0.0%	0.000	1.020	0.195
pSFL207	Average (75)	0.009	0.106	9.3%			
	Best line	0.052	0.066	44.2%	0.216	0.312	0.010
pSFL236	Average (145)	0.048	0.150	24.1%			
	Best line	0.141	0.046	75.3%	0.159	0.387	0.013
pSPB919	Average (48)	0.045	0.019	78.7%			
	Best line	0.101	0.002	98.2%	0.420	0.135	0.008

del, delphinidin; cya, cyanidin; del (%), delphinidin/total anthocyanidin; M, myricetin; Q, quercetin; K, kaempferol; best line, a line producing the highest percentage of delphinidin.
^amg g⁻¹ fresh petals.

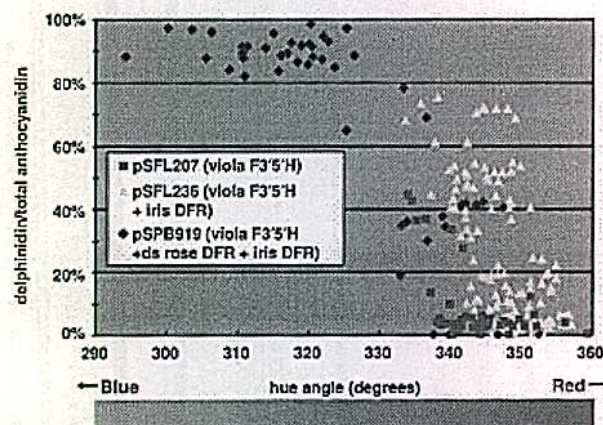


Fig. 4 Correlation of delphinidin content and petal colors in transgenic Lavande. The percentage of delphinidin in the petals of individual transgenic plants was plotted against the flower color represented by the hue value in degrees (hue angle). Pure red and blue have hue values of 360 and 270° in the hue angle, respectively. The higher the percentage of delphinidin was, the bluer the flower color became. The color gradation bar approximately indicates the corresponding petal color of transgenic Lavande petals.

On the other hand, more than two-thirds of the transgenic rose plants harboring pSPB919 produced >80% delphinidin. These lines contained exclusively delphinidin, which indicates that the successful conversion of cyanidin synthesis to delphinidin synthesis was achieved (Table 3). Some of them (LA/919-4-10, etc.) exhibited the bluest hue (Fig. 5), and the Royal Horticultural Society Color Chart (RHSCC) number of their petals was 85b (Violet group), while that of the control Lavande flower was 186c (Greyed-purple group). It is noteworthy that the transgenic plants harboring pSPB919 exhibited a higher delphinidin content and showed much less variation in delphinidin content than those harboring pSFL207 and pSFL236. The results indicate that the rose *DFR* gene was down-regulated and the iris *DFR* gene was expressed. In other words, the rose *DFR* was functionally

replaced with the iris *DFR* in vivo. Myricetin was detected in the transgenic plants (Table 3), which confirmed that the introduced *F3'5'H* gene functioned.

Some of the transgenic roses derived from pSPB919 exhibited a paler flower color and contained less anthocyanidin (Fig. 5A, center). This is probably due to suppression of the endogenous *DFR* gene and inefficient complementation by the introduced iris *DFR* gene.

Analysis of anthocyanins

The anthocyanins in LA/919-4-10 petals were analyzed in detail. The ratio of anthocyanin in petals is 90.0% delphinidin 3,5-diglucoside and 10.0% delphinidin 3-glucoside. The identities of these pigments were confirmed with co-chromatography with authentic compounds using

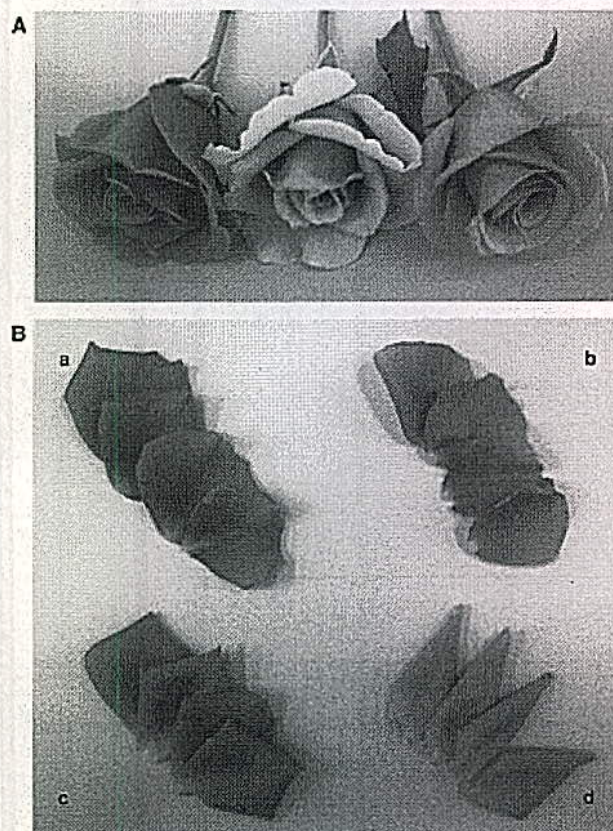


Fig. 5 Flower and petal color comparison. The pink-flowered rose cultivar Lavande (A, left and B, a) was transformed with pSPB919. The resultant transgenic plants including LA/919-4-10 produced violet-colored transgenic flowers (A, right and B, b) containing 98% delphinidin. The flower color is evidently bluer than that of the hosts, the commercial varieties Madam Violet (B, c) and Seiryu (B, d). Madam Violet and Seiryu are regarded as the bluest of the current rose varieties. Some of the transgenic roses derived from pSPB919 exhibited paler flower color and contained decreased amounts of anthocyanidin (A, center).

HPLC (data not shown). Their structures were further confirmed by time-of flight-mass spectrometry (TOF-MS) and nuclear magnetic resonance (NMR). The host Lavande accumulates 96.0% cyanidin 3,5-diglucoside and 4.0% cyanidin 3-glucoside. The presence of 3-monoglucoside and 3,5-diglucoside in *R. hybrida* and wild roses has been previously reported (Biolley and Jay 1993, Mikanagi et al. 2000), although it was recently proposed that the 5-glucosylation of anthocyanins proceeds to 3-glucosylation in *R. hybrida* (Ogata et al. 2005).

Molecular analysis of the transgenic rose LA/919-4-10

The results of the Northern analysis of LA/919-4-10 are shown in Fig. 6A. The transcripts of the *viola F3'S'H* and *iris DFR* genes were observed to have intact sizes, while

only smaller size bands of the rose *DFR* mRNA were detected. Small interfering RNA (siRNA) of about 23 bp was detected when using the rose *DFR* cDNA as the probe. These results reveal that the rose endogenous *DFR* mRNA was successfully degraded by the introduced RNAi cassette of the rose *DFR* (Fig. 6B). Southern analysis of LA/919-4-10 with the *viola F3'S'H*, *iris DFR*, rose *DFR* and *NPTII* genes indicates that the plant contains single-copy T-DNA (data not shown), which prompted us to breed this rose as the parent.

Hybridization breeding

In order to evaluate the efficacy of the redirection of the pathway from cyanidin to delphinidin, the pollen of LA/919-4-10 was hybridized with a seed parent

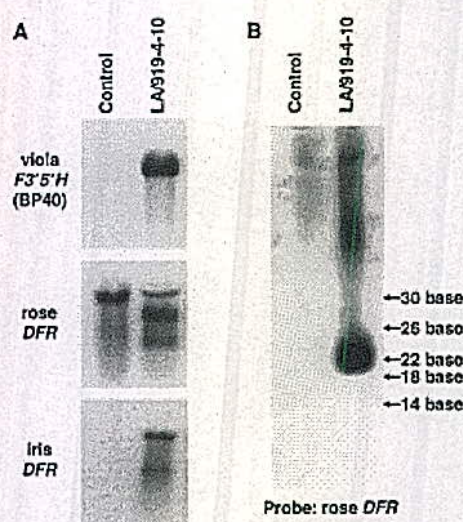


Fig. 6 Northern blot analysis of LA919-4-10. The expected sizes of the transcripts of *viola F3'5'H BP40* (1.8 kb) and *iris DFR* (1.7 kb) genes were observed, while only the smaller size was detected for *rose DFR* mRNA (A). A *rose DFR* probe detected about 23 bp of the small sized RNA, which was supposed to be a degraded endogenous *rose DFR* transcript with RNAi (B).

'Black Baccara', a dark-red variety that accumulates a large amount of cyanidin (Table 1). About 50% of the seeds germinated normally in the kanamycin media, and delphinidin was detected in all of the kanamycin-resistant F_1 progeny. The delphinidin content was 99.9% at maximum (average: 98.7%) in these progeny. Some delphinidin-producing F_1 plants were backcrossed to Black Baccara. The anthocyanidin analysis of the F_2 progeny revealed that the flowers also contained delphinidin almost exclusively (Fig. 7). The results indicate that the character of exclusive accumulation of delphinidin is heritable as a single locus. Although the flower color varied from red-purple to deep magenta depending on the genetic background, probably their pH and the amount of flavonols, the introduced transgenes were able to convert the metabolic flux completely toward delphinidin. Complete modification of the metabolic flux toward delphinidin should confer the ability to produce delphinidin exclusively in the progeny.

Discussion

In this study, we selected rose cultivars that are suitable for delphinidin production and color change toward blue. Constitutive expression of the *viola F3'5'H* gene successfully resulted in delphinidin accumulation and color changes with a blue hue that has commercial value as far as novel

flower color is concerned. More efficient and dominant modification of the flavonoid pathway toward delphinidin was achieved by functional replacement of DFRs, i.e. the down-regulation of the endogenous *DFR* gene and overexpression of the *iris DFR* gene in vivo, in addition to the overexpression of the *viola F3'5'H* gene. The ability for the exclusive accumulation of delphinidin was heritable by progeny, indicating that the flavonoid pathway was consistently modified toward delphinidin production.

Overexpression of the *F3'5'H* gene in selected cultivars

The biggest advantage of genetic engineering in plant breeding is that a novel gene from a heterologous source can be utilized to improve a plant, which liberates plant breeding from genetic constraints. However, the overexpression of a heterologous gene alone does not always efficiently yield a desired compound, as reported above. In order to achieve the accumulation of a desirable amount of a compound, it is necessary to select host cultivars that have the least competing reaction against an introduced gene, as in the case of the blue carnation (Holton 1996). In the case of color modification toward blue, higher amounts of flavonols and higher vacuolar pH are also desirable. Even when delphinidin accumulated in the selected cultivars, the flower color varied depending on the cultivars (Table 2, Fig. 3). Since cultivars such as WKS82, WKS100, WKS116 and WKS140, which give a blue hue, contain large amounts of flavonols and have a higher pH, flavonols may contribute to the blue coloration by co-pigmentation.

The delphinidin content (%) and flower color varied depending on the cultivar, and, even in selected cultivars, it depended on the transgenic events, as shown in Table 2 and Fig. 4 (pSFL207 and pSFL236 plants). Such variations of phenotypes have often been observed in transgenic plants (Filipecki and Malepszy 2006). The generation of many independent transgenic plants in various host cultivars and selection of lines having desirable phenotypes among them is a practical procedure to obtain transgenic plants that have commercial value.

Functional replacement of the *rose DFR* with the *iris DFR* resulted in the cultivar-independent exclusive accumulation of delphinidin

The exclusive production of delphinidin and the most significant color change toward blue were achieved with pSPB919 in Lavande. We succeeded in the replacement of the function of *rose DFR* genes with an *iris DFR* gene in transgenic roses by using RNAi. We previously showed that RNAi gave more efficient gene suppression than antisense or sense suppression (Nakamura et al. 2006). The degradation of *rose DFR* transcripts and overexpression of the *iris DFR* were confirmed by Northern analysis.

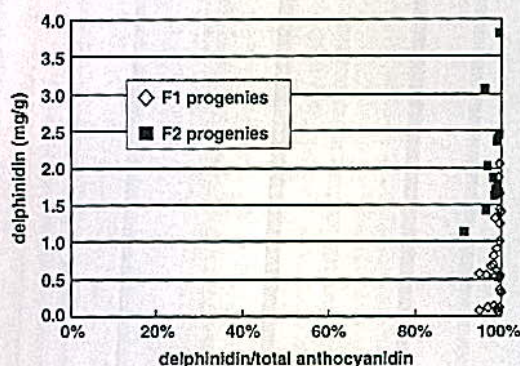


Fig. 7 Delphinidin contents of the transgenic progeny. The accumulation of delphinidin was confirmed in all of the Km^R progeny of LA/919-4-10. The flowers of the F₁ and F₂ progeny contained exclusively delphinidin.

The replacement was efficient; a higher frequency and less deviated delphinidin accumulation and color change were observed.

Moreover, the flowers of the F₁ and F₂ progeny of LA/919-4-10 exclusively contained delphinidin. Such a dominant character conferred by the transgenes is very useful for the incorporation of a novel ability (delphinidin production) in rose breeding, which has suffered from the limited ability to synthesize only pelargonidin and cyanidin as anthocyanidins. The ability to synthesize delphinidin achieved in this study will change rose breeding and increase rose varieties as far as flower color is concerned. The fact that the flower color of the progeny is not as blue as that of LA/919-4-10 indicates that other factors, such as the presence of a suitable amount of flavonols (co-pigments) and a higher vacuolar pH, are necessary to achieve blue color in addition to delphinidin production. Since hybridization breeding can generate host rose cultivars with various genetic backgrounds, delphinidin production in these backgrounds will yield various novel color tones, in addition to blue, in their flowers.

Replacement of the enzymatic function achieved in this study should be useful to engineer various plant metabolic pathways not limited to the flavonoid or other secondary metabolic pathways. Although replacement of an endogenous gene by homologous recombination would be desirable, homologous recombination in plants has only been reported in a few model plants (Terada et al. 2007). Many commercially important plants have high ploidy. For example, cultivated roses and chrysanthemums are tetraploids and hexaploids. The functional replacement depending on post-transcriptional gene silencing shown in this study is practical in plants having high ploidy.

Toward bluer/future perspectives

The production of delphinidin-based anthocyanins in heterologous plants has been the preferable strategy to generate flowers with a blue hue, which is also achieved in rose in this study. In addition to delphinidin production, blue flowers generate their flower color through the combination of various strategies, such as highly modified anthocyanins, a stable complex with co-pigments, a higher vacuolar pH and metal ions, as reported above. The reconstitution of these factors in roses should generate bluer color. However, this is a very challenging task. One reason is that many genes have to be properly expressed in roses, and the genes involved in vacuolar pH regulation and metal ion uptake have not been well characterized.

Therefore, a feasible strategy toward bluer follows. The co-existence of delphinidin-based anthocyanins and a flavone compound gave bluer color in a transgenic carnation (Fukui et al. 2003) than delphinidin alone. In this case, the flavone was derived from a host carnation cultivar. The co-expression of a flavone synthase gene (Akashi et al. 1999) in roses may result in a bluer color than that obtained in this study. The multiple aromatic acylation of anthocyanins generates a stable blue color (Honda and Saito 2002). We demonstrated that the rose can synthesize aromatically acylated anthocyanins by the expression of *torenia* SAT. *Gentian* 5.3'AT (Mizutani et al. 2006) and *Clitoria ternatea* AT (Noda et al. 2006), which can add multiple acyl moieties to anthocyanins, are useful molecular tools. The efficient acylation of anthocyanins may not be easy, since the acylation by *torenia* SAT remained at <40%.

Although more effort will be required to achieve sky-blue roses, the exclusive delphinidin production and the resultant color changes presented in this study represent a historic milestone in rose breeding.

Materials and Methods

Plant materials and plant tissue culture

Flowers of rose cultivars were screened and harvested in Keisei Rose Nurseries (Sawara, Japan) in 1997–2000. Stem internodes were harvested from rose plants grown in a greenhouse, and the surface was sterilized by immersing them in a 5% (v/v) sodium hypochlorite solution for 5 min and then rinsing them with sterilized water. The explants (1 cm in length) were prepared from the stem internodes and cultured at 25 °C under 16 h/8 h conditions in a medium consisting of Murashige and Skoog mineral salts and vitamins, 30 g l⁻¹ sucrose, 12 g l⁻¹ agar and 1 mg l⁻¹ 6-benzylaminopurine (BA). Rose transformation was described previously (Firoozabady et al. 1994) in a study using *Agrobacterium tumefaciens* Agl0 (Lazo et al. 1991). The acclimated transgenic plants were potted and then grown in contained greenhouses until blooming.

Molecular procedures

Most molecular procedures, including cDNA library construction, screening of the library, binary vector construction

and Southern/Northern analysis, were described previously (Fukuchi-Mizutani 2003). The detection of siRNA was carried out as described previously using digoxigenin-labeled rose DFR cDNA as a probe, as previously described (Goto et al. 2003).

The procedures involving binary vectors for rose transformation were also described previously (Fukuchi-Mizutani et al. 2003). In brief, pBin19 and pBinPLUS (van Engelen et al. 1995), which contain neomycin phosphotransferase II (NPTII) as the selection marker, were used to construct a binary vector, and the enhanced cauliflower mosaic virus (CaMV) 35S promoter derived from pE2113 (Mitsuhara et al. 1996) was used to regulate the transgenes. Torenia anthocyanin 5-acyltransferase cDNA (database accession No. AB332099) was obtained from its petal cDNA library (Suzuki 2000) as described previously (Yonekura-Sakakibara et al. 2000). *Iris x hollandica* DFR cDNA (AB332098) was cloned from its petal cDNA (Imayama et al. 2004) as described before (Tanaka et al. 1995). *Viola* spp. contains two *F3'5'H* genes (Brugliera et al. 2004), and one of them (clone BP40) was used in this study (AB332097).

Measurement of flower color and petal pH

The flower petal color was evaluated using colorimetric values in a CIE L*a*b* system measured with a CM-2022 spectrophotometer (Minolta Co., Ltd, Tokyo, Japan), and the data were calculated under a 10° observer and D65 illuminants as described before (Fukui et al. 2003). Color data were analyzed using SpectraMagic™ color-control software (Minolta). The RHSCC number which was closest to the flower color was selected by Color Classification System version 2.1.1 (The Japan Research Institute Co., Ltd; Shibano et al. 2002), which is based on the color value (CIE L*a*b* color system) obtained by visual discrimination and measurement with the device mentioned above. This system can be used for objective selection of the closest RHSCC number by color data. RHSCC is a standard reference for plant color identification in the horticultural industry.

Fresh rose petals (2g) were frozen at -80°C and homogenized. The pH of the pressed juice was measured with an F-22 pH meter with a 6069-10C electrode (Horiba Ltd, Kyoto, Japan).

Analysis of anthocyanins

Rose anthocyanins were extracted with 50% (v/v) acetonitrile containing trifluoroacetic acid (TFA) as previously reported (Fukui et al. 2003). HPLC was conducted using an RSpak DE-413L (25 cm × 4.6 mm, Shoko Co., Ltd, Tokyo, Japan) column and a flow rate of solvent of 0.6 ml min⁻¹; the solvent system used was as follows: after a 15 min linear gradient elution from 10 to 50% of acetonitrile containing 0.5% TFA in H₂O, 10 min of isocratic elution of 50% of acetonitrile containing 0.5% TFA in H₂O was carried out, and detection was conducted with a photodiode array detector SPD-M10A (Shimadzu Co., Ltd, Kyoto, Japan) that can measure absorbance at 600–250 nm. The retention times of aromatically acylated anthocyanins were around 19 min in this analysis. The anthocyanin spectra were measured with a photodiode array. Aromatically acylated anthocyanins can be identified because they exhibit a longer wave shift and a peak at 280 nm. TOF-MS and NMR analyses were described before (Fukui et al. 2003).

Analysis of anthocyanidins

Petal extracts prepared as described previously (Fukui et al. 2003) were dissolved in 6 N HCl (0.2 ml) and kept at 100°C for 20 min. The hydrolyzed anthocyanidins were extracted with 0.2 ml

of 1-pentanol. HPLC was performed using an ODS-A312 (15 cm × 6 mm, YMC Co., Ltd, Kyoto, Japan) column, a flow rate of solvent of 1 ml min⁻¹, and detection at an absorbance of 600–400 nm on a SPD-M10A photodiode array detector (Shimadzu Co., Ltd). The solvent system used was as follows: acetic acid:methanol:water = 15:20:65. Under these HPLC conditions, the retention time and λ_{max} of delphinidin were 4.0 min and 534 nm, respectively, and these values were compared with those of authentic delphinidin chloride (Funakoshi Co., Ltd, Tokyo, Japan).

Analysis of flavonols

Rose hosts and some of the transgenic rose flowers were subjected to flavonol analysis. Petal extracts were hydrolyzed in 0.2 ml of 0.1 M potassium phosphate buffer (pH 4.5) with 6 U of β -glucosidase (Shin-nihon Chemical Co., Ltd, Japan) and 1 U of naringinase (Sigma Chemical Co., St Louis MO, USA) under incubation for 16 h at 30°C. Acetonitrile (0.2 ml, 90%) was added to the reaction mixture, which was then subjected to HPLC analysis. HPLC was conducted using a Develosil C30-UG-5 (15 cm × 4.6 mm, YMC Co., Ltd) column and a flow rate of solvent of 0.6 ml min⁻¹; the solvent system used was as follows: after a 10 min linear gradient elution using 18–63% acetonitrile containing 0.1% TFA in H₂O, 5 min of isocratic elution of 63% acetonitrile containing 0.1% TFA in H₂O was carried out with absorbance at 400–250 nm detected using the photodiode array detector SPD-M10A (Shimadzu Co., Ltd).

Rose hybridization

Hybridization breeding was carried out using a transformant (LA/919-4-10) as a pollen parent and a dark-red rose variety, 'Black Baccara' (Meilland International), as the seed parent. Fruits were collected on the 100th day after pollination. Seed germination was accomplished by peeling the achenes and embedding them in moistened filter paper in plastic dishes. The water used for seed germination was sterilized water containing 1.0 ml l⁻¹ PPM™ (Plant Preservative Mixture; Plant Cell Technology, Inc., Washington, DC, USA) and 50 mg l⁻¹ kanamycin, and seedlings were raised by potting only normally budded plants with green cotyledons.

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