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(54) **FLAVONOL EXPRESSING DOMESTICATED TOMATO AND METHOD OF PRODUCTION**

FLAVONOLEXPRIMIERENDE DOMESTIZIERTE TOMATE UND HERSTELLUNGSVERFAHREN
TOMATE CULTIVEE EXPRIMANT DES FLAVONOLS ET SON PROCÉDE DE PRODUCTION

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Description

[0001] This invention is in the field of tomato breeding, specifically relating to the production of domesticated tomato plants that accumulate flavonols in the fruit peel and flesh, thereby significantly increasing the total content of health beneficial flavonols in the tomato fruit.

[0002] Tomato (*Lycopersicon esculentum*) is one of the major vegetables in human diets. Use of traditional breeding techniques has incorporated numerous beneficial traits into the tomato, including such traits as extended shelf-life, disease resistance, and sugar content. There is also a significant interest in the industry to develop tomatoes that express compounds that can impart health benefits to humans. These health benefits can be delivered by development of dietary supplements containing the extracted components and by production of these components directly in a food source for human consumption.

[0003] Flavonoids, especially flavonols such as quercetin and kaempferol, are believed to impart a number of health benefits if ingested in sufficient quantities. Studies have shown that flavonoids possess antioxidant and anticancer activities (Rice-Evans et al., *Free Radical Research*, 22:375-383, 1995). Additional health benefits include anti-platelet aggregation (Rice-Evans et al., *Trends in Plant Science*, 2:152-159, 1997), decreased blood viscosity, reduction in the severity of inflammation and allergies (Cook et al., *Nutritional Biochemistry* 7:66-76, 1996), as well as other health beneficial effects (e.g. antiviral activity, antituberculosis activity). Therefore, there is significant interest in developing plants accumulating high levels of flavonoids, especially flavonols.

[0004] In the scientific article published by Yen et al. (Yen et al., *Theoretical and Applied Genetics*, Vol. 95, no.7, 1069-1079 (1997)) DNA markers which may be useful for gene isolation and marker-assisted selection have been described.

[0005] Further, quantitative estimates of conjugated flavonoid content were obtained by using HPLC to analyze the level of free flavonoids present in acid-hydrolyzed extracts from commercial fruits and vegetables (Crozier et al., *Journal of Agricultural and Food Chemistry*, vol.45 no.3, 590-595 (1997)).

[0006] Eshed et al. describes the diminishing additivity of QTL effects, which is amplified when more loci are involved (Eshed et al., *Genetics*, vol. 143, 1807-1817 (1996)). It is proposed that this mode of epistasis may be an important factor in phenotype canalization and in breeding.

[0007] Another research article published by Eshed et al. highlights the potential of wild germ plasm for yield improvement and the ability of nearly isogenic populations to achieve this goal (Eshed et al., *Theoretical and Applied Genetics*, Vol. 93, no. 5/6, 877-886 (1996)).

[0008] Willits et al. described a nontransgenic metabolic engineering approach to developing a high flavonoid content in tomato peel using a wild tomato species (*Lycopersicon pennellii* var. *puberulum*) and demonstrate the opportunities for restoring functional pathways using the genetic resources of wild species (Willits et al., *Journal of Agricultural and Food Chemistry*, vol. 53, no. 4, 1231-1236 (2005)).

[0009] Analyses of red fruits using HPLC coupled to high-resolution quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) showed a twofold difference between the fruit peel of light-hyperresponsive high-pigment (hp) tomato (*Lycopersicon esculentum*) mutant plant and wild type plant (Bino et al., *New Phytologist*, vol. 166, no. 2, 427-438 (2005)).

[0010] It has been further shown that in contrast to fresh tomatoes, most tomato-based products contained significant amounts of free flavonols (Stewart et al., *Journal of Agricultural and Food Chemistry*, vol. 48, 2663-2669 (2000)).

[0011] Transformation of tomato with the *Petunia* chA gene encoding chalcone isomerase resulted in transgenic tomato lines which produced an increase of up to 78 fold in fruit peel flavonols, mainly due to an accumulation of rutin (Muir et al., *Nature Biotechnology*, vol. 19, 470-474 (2001)).

[0012] To increase flavonoid levels in tomato, Bovy et al. expressed the maize transcription factor genes LC and C1 in the fruit of genetically modified tomato plants (Bovy et al., *The Plant Cell*, vol. 14, 2509-2526 (2002)).

[0013] By using genetic modification, it was possible to generate several tomato lines with significantly altered flavonoid content (Verhoeven et al., *J. of Exp. Botany*, vol. 53, no. 377, 2099-2106 (2002)).

[0014] The flavonol, quercetin, is biosynthesized from p-coumaroyl-CoA (C₆C₃) and malonyl-CoA (C₃). In a polyketide synthesis reaction (catalyzed by chalcone synthase) the phenylpropane unit acts as a starting compound for the successive addition of three acetate units (C₂ - from malonyl-CoA) to form, after cyclization, naringenin chalcone (C₆C₃C₆). The flavone naringenin is generated by formation of the heterocycle C-ring, a reaction that is catalyzed by chalcone isomerase. Quercetin is then synthesized by successive oxidation reactions of naringenin that yield a double bond between C-2 and C-3 and hydroxylation of C-3 and C-3'. These oxidation reactions are carried out by flavanone-3-hydroxylase, flavonol synthase and flavonol-3',5'-hydroxylase. The final products of the pathway, quercetin mono- and di-glycosides (isoquercitrin and rutin, respectively), are formed through the action of O-glycosyltransferases. The monoglucoside of the flavonol kaempferol, which is found only in trace amounts in tomato, is synthesized in the same way but with the omission of the hydroxylation in the C-3' position.

[0015] Studies have shown, and we have confirmed, that in domesticated tomato varieties the chalcone isomerase (*CHI*) gene is present but not expressed in the fruit peel. Additionally, none of the flavonol biosynthesis genes are

expressed in the flesh of the tomato fruit. Both results clearly explain the observed lack of flavonol accumulation in domesticated tomato fruits. Thus, it has been assumed in the art that obtaining high flavonol tomatoes cannot be accomplished by using traditional breeding techniques, as *CHI* expression would remain blocked and the fruit flesh would continue to accumulate insignificant levels of flavonols. Modern recombination technology, or genetic modification, is now being used to selectively modify the flavonoid pathway to produce plants with elevated levels of flavonols. However, to date, enhanced expression of flavonols is substantially limited to the tomato peel. See, for example, Muir, S.R. et al. Overexpression of petunia chalcone isomerase in tomato results in fruit containing levels of flavonols Nature Biotechnology, 19:470-474 (2001) (Muir et al. detected approximately 0.5 mg/kg fresh weight [0.0045 µg/mgdwt] of rutin in tomato flesh, which was at the limit of detection); WO 00/37652, *Flavonoid Biosynthetic Enzyme*; WO 99/37794, *Methods and Composition for Modulating Flavonoid Content*; WO 99/14351, *Isoflavone Biosynthetic Enzymes*; WO 00/53771, *Genetic Manipulation of Isoflavonoids*; and WO 00/04175 *Methods and Composition for Modulating Flavonoid Content*. Table 1 shows the flavonol accumulation in the fruit of transgenic and non-transgenic tomato plants as revealed by various research groups. The high levels of flavonol accumulation of the cherry tomato is due to the high proportion of peel to flesh in cherry tomatoes compared to standard sized tomatoes.

TABLE 1

Source	Tomato Variety	µg/mg dry weight (total fruit)	µg/mg dry weight (flesh)	µg/mg dry weight (peel)	mg flavonols per 57g tomato (estimate)
SYNGENTA Domesticated	ZTV 840	189	0.1	3.0	1.2
UNILEVER (<i>CHI</i> / Petunia) Muir, S., Collins, G., Robinson, S., Hughes, S., Bovy, A., DeVos, R., van Tunen, A., Verhoeyen, M. (2001) Nature Biotechnology, 19: 470-474	Transgenic	4,000		17 (used 2 mm thick peel)	25
	Nontransgenic			0.05 (used 2 mm thick peel)	
Non-Transgenic - Crozier, A., Lean, M., McDonald, Black, C. (1997) J. Agric. Food Chem, 45: 590-595	Different varieties				.6
Non-Transgenic - Crozier, A., Lean, M., McDonald, Black, C. (1997) J. Agric. Food Chem, 45: 590-595	Cherry tomatoes				2.3
Non-Transgenic-Stewart, A., Bozonnet, S. Mullen, W., Jenkins, G., Lean, M., Crozier, A. (2000) J. Agric. Food Chem., 49: 2663-2669	Different varieties		0.01	0.72	1.2

[0016] The present invention recognizes that there is a significant need to produce domesticated tomato plants, using traditional breeding techniques, that accumulate significant amounts of flavonols in the peel, as well as in the flesh, of the tomato fruit.

[0017] There is also a need for a method to identify tomato germplasm that expresses the *CHI* gene in the peel of the tomato fruit.

[0018] There is a further need to identify tomato germplasm that expresses the flavonols biosynthetic pathway in the flesh of the tomato fruit.

[0019] An additional need in the art is to identify tomato germplasm that expresses the *CHI* gene in the peel of the tomato fruit and the flavonol biosynthetic pathway in the flesh of the tomato fruit.

[0020] In one aspect tomato plants are described herein that accumulate high levels of flavonols in the fruit. In particular, tomato plants as described herein accumulate flavonols in the peel of the tomato fruit. Preferably, tomato plants, as described herein are generated using traditional breeding techniques, which accumulate flavonols in the flesh and peel

of the tomato fruit. Further described herein are the seeds and fruit, and parts thereof, of such plants.

[0021] The method as described herein further includes a method for assaying wild tomato plants for expression of *CHI* in the fruit peel and/or of one or more of the genes of the flavonol biosynthesis pathway in the fruit flesh. The method as described herein further includes selecting those plants that express *CHI* in the fruit peel and/or the genes of the flavonol biosynthesis pathway in the flesh, and introgressing, using traditional breeding techniques, the genetic factors responsible for such expression from those plants into a domesticated tomato plant.

[0022] Further described herein is a method of making a non-transgenic *L. esculentum* plant accumulating flavonols in the peel and flesh of the fruit of said plant comprising the steps of:

- a) crossing wild accessions of *Lycopersicon* that express *CHI* in the peel and that express the genes of the flavonol biosynthetic pathway in the flesh with a *L. esculentum* plant to produce a hybrid plant;
- b) harvesting fruit from the hybrid plant; and
- c) collectin seed from the fruit harvested in step b).

[0023] Also described herein is a method of making a non-transgenic *L. esculentum* plant including the step of screening *Lycopersicon* accessions for expression of *CHI* in the peel or for expression of the genes of the flavonol biosynthetic pathway in the flesh.

[0024] Further described herein is a method of making a non-transgenic *L. esculentum* plant wherein the *Lycopersicon* species are selected for crossing with a *L. esculentum* plant are *L. chilense* or *L. pennellii*, or any other wild tomato species that express the genes of the flavonol biosynthetic in the flesh and *CHI* in the peel of the fruit

[0025] Also described herein is a method of making a non-transgenic *L. esculentum* plant, wherein the accessions selected for crossing are LA1963, LA2884, and LA1926.

[0026] In one aspect a non-transgenic domesticated *L. esculentum* plant is described herein comprising a genetic factor that up-regulates the flavonol biosynthesis pathway in the fruit flesh of the plant.

[0027] In another aspect a non-transgenic domesticated *L. esculentum* plant is described herein comprising a genetic factor that restores *CHI* expression in the fruit peel of the plant

[0028] In one aspect inbred and hybrid *L. esculentum* plants are described developed using traditional breeding techniques comprising genetic factors that express the genes of the flavonol biosynthetic pathway in the flesh and *CHI* in the peel of the fruit, and further includes *L. esculentum* plants that are homozygous or heterozygous for these genetic factors.

[0029] The present invention thus relates to a non-transgenic domesticated *L. esculentum* plant growing fruits with a content of flavonols in the flesh of the fruit that is greater than 0.5 $\mu\text{g}/\text{mg}$ dwt and a content of flavonols in the peel of said fruit of at least 5 $\mu\text{g}/\text{mg}$ dwt due to up-regulated flavonol biosynthesis in the fruit flesh of said plant and restored *CHI* expression in the fruit peel of said plant, wherein said non-transgenic domesticated *L. esculentum* plant is obtainable by introgressing the *CHI* gene and the flavonol biosynthesis pathway genes *CHS*, *FSH* and *FLS* of *Lycopersicon* wild accessions LA1963, A2884 and LA1926 into a domesticated *L. esculentum* plant.

[0030] The invention further includes non-transgenic domesticated *L. esculentum* plants according to the invention that accumulate flavonol in the fruit flesh of the plant at levels greater than 1.0 $\mu\text{g}/\text{mg}$ dwt.

[0031] The invention further includes non-transgenic domesticated *L. esculentum* plants according to the invention that accumulate flavonol in the fruit flesh of the plant at levels greater than 1.5 $\mu\text{g}/\text{mg}$ dwt.

[0032] The invention further includes non-transgenic domesticated *L. esculentum* plants according to the invention that accumulate flavonol in the fruit flesh of the plant at levels greater than 2.0 $\mu\text{g}/\text{mg}$ dwt.

[0033] The invention includes the *L. esculentum* plants according to the invention wherein the flavonol content in the fruit peel is at least 10 $\mu\text{g}/\text{mg}$ dwt.

[0034] The invention includes the *L. esculentum* plants according to the invention wherein the flavonol content in the fruit peel is at least 17 $\mu\text{g}/\text{mg}$ dwt.

[0035] The invention includes the fruit of the *L. esculentum* plants according to the invention that accumulate flavonol in the fruit flesh of the plant at levels greater than 0.5 $\mu\text{g}/\text{mg}$ dwt.

[0036] The invention includes seed of thy *L. esculentum* plant according to the invention that wherein said seed will grow into a plant accumulate flavonol in the fruit flesh of the plant at levels greater than 0.5 $\mu\text{g}/\text{mg}$ dwt.

[0037] The invention includes fruit of the *L. esculentum* plants according to the invention that accumulate flavonol in the fruit flesh of the plant at levels greater than 0.5 $\mu\text{g}/\text{mg}$ dwt.

[0038] Fruits containing the increased levels of flavonols of the present invention provide substantial health benefits, including the benefits of antioxidant activity, anticancer activities, anti-platelet aggregation, and decreased blood viscosity.

DESCRIPTION OF THE DRAWINGS

[0039]

FIG. 1 shows flavonol biosynthetic gene expression in the leaf and breaker fruit peel of *L. esculentum* by Northern blot analysis.

FIG. 2 shows flavonol biosynthetic gene expression in the peel and flesh of green (G), breaker (B), and red fruit (R) from three flavonol biosynthetic genes in *L. esculentum* breeding lines.

FIG. 3 shows flavonol biosynthetic gene expression in the peel and flesh of breaker fruit from five biosynthetic genes in *L. esculentum* v. *cerasiforme* (LA1286) and *L. pennellii* v. *puberulum* (LA1926).

FIG. 4 shows an HPLC analysis of flavonol content in the flesh and peel of the fruit of a plant resulting from a hybrid cross between *L. esculentum* and *L. pennellii* v. *puberulum*.

FIG. 5 shows accumulation of naringenin chalcone in the fruit peel in *L. esculentum* by HPLC analysis.

DETAILED DESCRIPTION OF THE INVENTION

[0040] "Domestication" or "domesticated" refers to a plant that has different genetic and structural characteristics from wild plants due to human selection.

[0041] "Wild" refers to a plant in or out of captivity that has not been subject to breeding to alter it from its native (wild) state.

[0042] "Accession" refers to a distinct, uniquely identified sample of seeds or plants that is maintained as part of a germplasm collection.

[0043] "germplasm" refers to seeds, plants, or plant parts that are useful in crop breeding, research, or conservation.

[0044] "Variety" refers to a plant type within a species that is distinguishable by one or more characters. When reproduced from seeds or by asexual means (e.g. cuttings) its distinguishing characters are retained.

[0045] "Introgression" refers to the insertion of the genes of one plant into the gene pool of another. This can occur when two plants interbreed to produce fertile hybrids. These can then back-cross with individuals of one of the parent plants.

[0046] "Species" refers to a taxonomic subdivision; a group or populations of organisms that actually or potentially interbreed and are reproductively isolated from other such groups. A particular species can contain many different accessions

[0047] Disclosed herein are methods of tomato breeding, including the production of a domesticated tomato that accumulates flavonols in the peel and flesh of the tomato fruits. Because the tomato peel is such a small fraction of the entire tomato, extremely large quantities of peel-localized flavonol must be produced to get a significant dose in each tomato. With entire fruit production flavonol levels are significantly increased. In another aspect a unique method is disclosed herein for enhancing accumulation of flavonol in tomato fruit that includes the step of identifying germplasm that expresses *CHI* in the fruit peel, and also includes identifying germplasm that expresses the genes of the flavonol biosynthetic pathway in the flesh. According to the method as described herein a *CHI* gene that expresses chalcone isomerase in tomato fruit peels has been identified in some wild *Lycopersicon* species. In another aspect, wild *Lycopersicon* species are identified that express the genes of the flavonol biosynthetic pathway in the flesh, i.e., in these species the flavonol biosynthetic pathway is upregulated in the flesh of the tomato fruit. In another aspect, wild *Lycopersicon* species are identified that express the *CHI* gene in the tomato fruit peel and that also express the genes of the flavonol biosynthetic pathway in the fruit flesh.

[0048] The method as disclosed herein further includes using traditional breeding techniques to generate F1 hybrids by crossing the domesticated tomato *L. esculentum* with *L. pennellii*, wherein the resulting F1 hybrid plants produce fruit that for the first time accumulate high levels of flavonols in both the tomato fruit flesh and peel.

Example I. Plant Growth and Tissue Harvest

[0049] Plants were grown in a non-soil potting mix in a greenhouse at 78 to 82°F, with 75 to 90% relative humidity, and a 16-hour photoperiod. Members of accessions LA1963, LA2884, LA1926, LA111, LA1292, and LA 1330 are self-incompatible and were pollinated by sib crossing.

[0050] Leaf tissue from each plant was harvested and frozen in liquid nitrogen. Tomato fruits were harvested at various stages of ripening: green mature, breaker, and ripe for red maturing lines; unripe and ripe for green maturing lines. Large tomatoes (*L. esculentum* and *L. esculentum* v. *cerasiforme*) were peeled with a knife. The peel tissue was frozen in liquid nitrogen. The seeds and locular gel were removed from the remaining tissue, and the combined pericarp and placental tissue were designated as flesh tissue and also frozen in liquid nitrogen. The peel tissue was removed from the small wild tomatoes by squeezing the tomatoes between forefinger and thumb. The internal tissues (seeds, locular gel, pericarp, and placental tissue) were collected together, designated as flesh tissue, and frozen in liquid nitrogen. The peel tissue was frozen in liquid nitrogen as well. Frozen tissue was stored at - 80°C.

Example II. Northern Blot Analysis

[0051] Expression levels of flavonol biosynthesis genes in *L. esculentum* were determined by Northern analysis. Leaf tissue was finely ground in a mortar and pestle under liquid nitrogen. Fruit tissue was finely ground in a coffee grinder under liquid nitrogen. Total RNA was isolated from ground tissue according to the protocol of Lagrimini et al. (1987). For Northern blot analysis, 10 µg of RNA was loaded onto formaldehyde agarose gels and electrophoresed at 240 volts for 45 minutes. RNA was then transferred onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech).

[0052] cDNA fragments for chalcone synthase-A (*CHS-A*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), and flavonol synthase (*FLS*) were isolated from *L. esculentum*, *L. pimpinellifolium* (LA1261), and *L. parviflorum* (LA1326). The fragments were obtained from leaf RNA by RT-PCR. All fragments were sequenced and compared across species. For each cDNA, the fragments were at least 98% identical across species. Therefore probes were prepared from cDNA fragments of *L. esculentum*.

[0053] Probes were labeled with ³²P and purified according to the instruction for the Rediprime II Random Prime Labeling System and ProbeQuant G50 Micro Columns (Amersham Pharmacia Biotech). Blots were hybridized overnight at 65°C in 1% BSA, 0.5M sodium phosphate buffer, 7% SDS, 1mM EDTA. Blots were washed 2 times at 65°C in 0.5% BSA, 40mM sodium phosphate buffer, 1% SDS, 1mM EDTA, and 2 times in 40mM sodium phosphate buffer, 1% SDS, 1mM EDTA. Blots were then exposed to X-ray film overnight at -80°C.

[0054] Total RNA was isolated from *L. esculentum* leaf, fruit peel, and fruit flesh (pericarp and placenta) tissue and hybridized with probes for chalcone synthase A (*CHS-A*), chalcone synthase B (*CHS-B*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), and flavonol synthase (*FLS*). All five genes were expressed in leaf tissue (FIG. 1), while none of the genes were expressed in fruit flesh (data not shown). In the fruit peel, all genes were expressed except for *CHI*. The absence of *CHI* expression in the fruit peel and the lack of expression of all genes in the fruit flesh explains the lack of flavonol accumulation in the fruit. The data showing insignificant flavonol accumulation in tomato fruit has led investigators in the art to conclude that all tomatoes were blocked at the *CHI* step, and that traditional breeding programs would not succeed in producing enhanced levels of flavonol in the tomato fruit.

[0055] The present invention, however, recognizes that the biosynthesis pathway is expressed in the fruit peel and flesh of some wild tomato species, and that screening tomato germplasm for expression of one or more of the flavonol biosynthetic genes in the flesh, and/or *CHI* expression in the fruit peel provides a unique means for identifying appropriate breeding candidates.

Example III. Northern blot analysis of *L. esculentum* breeding lines

[0056] Northern analysis was carried out on five different *L. esculentum* breeding lines. Total RNA was isolated from peel or flesh tissue of green, breaker, or red tomatoes and hybridized with probes for *CHS-A*, *CHI*, or *FLS*. As mentioned previously, little or no expression of any of the genes was detected in the fruit flesh tissue (Figure 2). For *CHS-A* and *FLS*, expression reached a peak in peel tissue of breaker tomatoes. No fruit-localized expression was detected for *CHI* in any of the analyzed lines. Flavonol analysis was carried out for an additional 17 lines, but no significant differences were detected (data not shown).

Example IV. Northern analysis and selection of wild *Lycopersicon* accessions

[0057] Northern analysis was also carried out on 13 wild *Lycopersicon* accessions, representing seven different species. The cultivated *L. esculentum* ZTV840 and the wild *Lycopersicon* accessions listed in Table 2 were grown and investigated for flavonol biosynthesis gene expression and flavonol accumulation.

[0058] As shown in Table 2 below and based on the Northern results, the wild accessions fell into two major classes: accessions with expression patterns similar to *L. esculentum* (LA1286, LA1673, LA722, LA1261, and LA2184) and accessions that exhibited *CHI* expression in the fruit peel (LA1963, LA2884, LA1926, LA111, LA1292, LA1330, LA1326, LA2727). Interestingly, three accessions (LA1963, LA2884, and LA1926) demonstrated expression of some or all of the flavonol biosynthetic genes in the tomato flesh tissue as well. Figure 3 compares the Northern results from *L. pennellii* v. *puberulum* (LA1926) with those from *L. esculentum* v. *cerasiforme* (LA1286). This result clearly demonstrates that *L. pennellii* v. *puberulum* (LA1926) shows strong expression of all investigated flavonol biosynthetic genes in both the fruit peel and flesh. Therefore *L. pennellii* v. *puberulum* (LA1926) was chosen as a crossing partner to introgress flavonol production into *L. esculentum*.

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Table 2: *Lycopersicon* accessions

		CHI		CHS, F3H, FLS	
		Peel	Flesh	Peel	Flesh
5	<i>L. esculentum</i> (domesticated)			X	
	<i>L. esculentum</i> v. <i>cerasiforme</i>	LA1286		X	
	<i>L. esculentum</i> v. <i>cerasiforme</i>	LA1673		X	
10	<i>L. pimpinellifolium</i>	LA722		X	
	<i>L. pimpinellifolium</i>	LA1261		X	
	<i>L. pimpinellifolium</i>	LA2184		X	
15	<i>L. chilense</i>	LA1963	X	X	X
	<i>L. chilense</i>	LA2884	X	X	X
	<i>L. pennellii</i> v. <i>puberulum</i>	LA1926	X	X	X
	<i>L. peruvianum</i>	LA0111	X		X
20	<i>L. peruvianum</i> f. <i>glandulosum</i>	LA1292	X		X
	<i>L. chmielewskii</i>	LA1330	X		X
	<i>L. parviflorum</i>	LA1326	X		X
25	<i>L. parviflorum</i>	LA2727	X		X

[0059] It is understood that the terms "germplasm" and "accessions" are somewhat interchangeable, and use of the term "accession" is not meant to exclude from the method of the invention use of wild *Lycopersicon* plants that are not uniquely identified or part of a germplasm collection.

Example V. Analysis of flavonol accumulation in wild tomato fruit

[0060] Flavonol analysis was carried out on the wild accessions in parallel with the Northern analysis (data not shown). However, only low concentrations of flavonols could be detected in the fruit, and if relied on by itself may lead an investigator to skip over the wild accessions that express the flavonol biosynthetic genes in the fruit flesh and peel as valuable breeding partners.

Example VI. Hybridization of *L. esculentum* and *L. pennellii* v. *puberulum* - production of domesticated tomato plants

[0061] Pollen was collected from *L. pennellii* v. *puberulum* (LA1926), by inserting the tips of flowers into a small plastic tube, vibrating the flowers with an electric toothbrush, and shaking the pollen into the tube. Pollen collection was found to be the most successful early in the day when the relative humidity is at its low. The pollen was then transferred to the pistils of emasculated flowers of *L. esculentum* (ZTV840). The flowers of the *L. esculentum* were emasculated in order to prevent self-pollination. Floral buds were emasculated if they were large enough to be manipulated and the corolla tips had not separated. The fusion of the corolla and androecium of the flower forms a cap that covers the pistil or pollen recipient site. This cap was removed with forceps by inserting one tip between the style and anther and the other tip between the anther and calyx. Then by pulling slowly away from the floral axis, the cap was removed leaving only the thin style and pistil. The styles were then dipped into the plastic tube containing the pollen collected from the *L. pennellii* flowers, coating the pistils with the pollen. The emasculated flowers were then tagged, and all other naturally set fruit were removed from the calyx to ensure maximum fruit set from the controlled matings.

[0062] Fruits from crosses of *L. esculentum* and *L. pennellii* v. *puberulum* were harvested, and the seeds were separated from the locular gel. The seeds were washed with water and then dried at room temperature for two days.

[0063] Seeds were germinated in MetroMix 360. Young leaf tissue was used to prepare DNA by a standard DNA miniprep protocol. PCR primers were designed to anneal to the start and stop codons of the *CHI* coding region: forward primer, 5'-AAG GAT CCA TGG CGG TTG TTA CAA AAT TGC-3' (SEQ ID NO. 1) and reverse primer, 5'-AAA AGC TTA CTG GAC TGA CGA TAG CTC ACT TC-3' (SEQ ID NO. 2). PCR amplification of *CHI* from *L. esculentum* and *L. pennellii* v. *puberulum* results in different size products due to variations in intron structure between the two species. Therefore amplification with these primers provides a marker that distinguishes homozygous parents from heterozygous F1 plants.

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Ten putative F1 hybrids were screened using this PCR assay and were determined to be heterozygous at the *CHI* locus, which confirms the hybrid nature of these ten plants.

[0064] The F1 hybrids of the invention can be used to produce domesticated tomato plants that accumulate flavonols in the fruit flesh and peel. Traditional tomato breeding techniques are used. The particular breeding method is not critical to the invention, so long as the genetic factors responsible for *CHI* expression in the fruit peel and for upregulation of the biosynthesis pathway in the fruit flesh are stably incorporated into the genome of the tomato variety. Descriptions of traditional breeding techniques that are commonly used for different traits and crops can be found in one of several reference books (e.g., Allard, R. W., (1960) Principles of Plant Breeding; Simmonds, N. W., (1979) Principles of Crop Improvement; Sneep, J. et al., (1979) Plant Breeding Perspectives; Fehr, (1987) Principles of Cultivar Development—Theory and Technique).

Example VII. HPLC Determination of Flavonol Accumulation

[0065] Flavonol glycosides were extracted from lyophilized tomato fruit peel and flesh tissue of F1 hybrids. Approximately 100 mg of dry tissue was extracted overnight at a ratio of 80 μ l of 80% methanol pH 2.0 (HCl) per 1 mgdwt tissue. Aliquots of this methanol extract were used for HPLC analysis of flavonol glycosides. For enzyme hydrolysis of glycosides, 1 ml of methanol extract was evaporated *in vacuo* at room temperature. Dried extracts were resuspended in 1 ml of 16% aqueous DMSO. Naringenase from *Penicillium decumbens* (Sigma-Aldrich) was added and incubated overnight at 35°C. Hydrolysis reactions were extracted with 50 μ l HCl and 700 μ l of ethyl acetate. Samples were vortexed for 30 seconds followed by 10 minutes of sonication. Separation of organic and aqueous phases was accomplished with a 2 minute, 15,000 rfc centrifugation step. The organic phase containing the aglycones was evaporated *in vacuo* at room temperature. Dried samples were resuspended in 100% methanol and used for HPLC analysis of flavonol aglycones.

[0066] Flavonol samples were analyzed on an HPLC system consisting of a 2690 Alliance separations module (Waters, Milford, Massachusetts) and a 996 PDA detector. For all analysis a 4.6 mm x 150 mm Xterra RP₁₈ (5 μ m) column protected by a C₁₈ Nova-pack guard column kept at 50°C was used. Flavonoid standards were purchased from Indofine (Somerville, New Jersey). The standards were dissolved in either methanol or DMSO and kept refrigerated at 4°C. HPLC grade acetonitrile from Fisher Scientific (Fair Lawn, New Jersey) and water from a Milli-Q Water System from Millipore (Bedford, Massachusetts) were used as mobile phase. In all separations 1 ml of trifluoroacetic acid from Pierce (Rockford, Illinois) was added to 1 liter of the water used as mobile phase. For flavonol glycoside analysis, the gradient was 10-55% acetonitrile in 12 minutes at a flow-rate of 1.5 ml/min. For aglycone analysis, the gradient was 20-40% acetonitrile in 20 minutes at a flowrate of 1.5 ml/min. Quantification was performed using peak area of external standard mixtures with known concentrations ranging from 100 ng to 4000 ng of standard compound per injection.

[0067] FIG. 4 shows a HPLC analysis of the flavonol content in the flesh and peel of the fruit of a plant resulting from a hybrid cross between *L. esculentum* and *L. pennellii v. puberulum*. A control HPLC analysis of *L. esculentum* fruits shows the expected accumulation of naringenin chalcone, with no accumulation of flavonols (FIG. 5). Clearly, the introduction of the genetic factors from *L. pennellii* into the hybrid plant results in the expression of the flavonol biosynthetic genes and subsequent production of flavonols and flavonol glucosides in the peel and also in the flesh. Analysis of the HPLC data is shown in Table 3 below, where the accumulation of flavonols in both the peel and flesh is significantly enhanced compared to a standard domesticated tomato, *L. esculentum*.

TABLE 3

	Flavonol Content μ g/mgdwt		Estimated Total Flavonol Content (mg) in a 57g tomato
	Peel	Flesh	
<i>L. esculentum</i>	3	0.1	1.2
<i>L. esculentum</i> X <i>L. pennellii v. puberulum</i>	17	2	12

[0068] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the invention, as limited only by the scope of the appended claims.

SEQUENCE LISTING

[0069]

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<120> FLAVONOL EXPRESSING DOMESTICATED TOMATO AND METHOD OF PRODUCTION

5 <130> 70058WO-PCT

<150> US 60/389,061

<151> 2002-06-14

10 <160> 2

<170> Patent In version 3.1

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Claims

1. A non-transgenic domesticated *L. esculentum* plant
growing fruits with a content of flavonols in the flesh of the fruit that is greater than 0.5 $\mu\text{g}/\text{mg}$ dwt and a content of
45 flavonols in the peel of said fruit of at least 5 $\mu\text{g}/\text{mg}$ dwt due to up-regulated flavonol biosynthesis in the fruit flesh
of said plant and restored *CHI* expression in the fruit peel of said plant, wherein said non-transgenic domesticated
L. esculentum plant is obtainable by introgressing the *CHI* gene and the the flavonol biosynthesis pathway genes
CHS, FSH and FLS of *Lycopersicon* wild accessions LA1963, LA2884 and LA1926 into a domesticated *L. esculentum*
50 plant.
2. The non-transgenic domesticated *L. esculentum* plant according to claim 1, wherein said content of flavonols is
greater than 1.0 $\mu\text{g}/\text{mg}$ dwt.
3. The non-transgenic domesticated *L. esculentum* plant according to claim 1, wherein said content of flavonols is
55 greater than 1.5 $\mu\text{g}/\text{mg}$ dwt.
4. The *L. esculentum* plant according to claim 1, wherein said content of flavonols is greater than 2 $\mu\text{g}/\text{mg}$ dwt.

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5. The *L. esculentum* plant according to claims 1,2, 3, and 4 which exhibits a content of flavonols in the peel of said fruit of at least 10 µg/mg dwt.
- 5 6. The *L. esculentum* plant according to claims 1,2,3, and 4 which exhibits a content of flavonols in the peel of said fruit of at least 17 µg/mg dwt.
7. Seed of said *L. esculentum* plant of claim 1, wherein said seed will grow into a plant having a content of flavonols in the flesh of the fruit of said plant that is greater than 0.5 µg/mg dwt.
- 10 8. Fruit of a *L. esculentum* plant of claim 1 that accumulate flavonol in the fruit flesh of the plant at levels greater than 0.5 µg/mg dwt.

Patentansprüche

- 15 1. Nichttransgene domestizierte *L. esculentum*-Pflanze, die aufgrund einer hinaufregulierten Flavonolbiosynthese im Fruchtfleisch dieser Pflanze und restaurierter *CHI*-Expression in der Fruchtschale dieser Pflanze Früchte mit einem Flavonolgehalt im Fruchtfleisch von mehr als 0,5 µg/mg TG und einem Flavonolgehalt in der Schale dieser Frucht von mindestens 5 µg/mg TG erzeugt, wobei die
- 20 nichttransgene domestizierte *L. esculentum*-Pflanze durch Introgression des *CHI*-Gens und der Flavonolbiosyntheseweggene *CHS*, *FSH* und *FLS* der *Lycopersicon*-Wildaccessionen LA1963, LA2884 und LA1926 in eine domestizierte *L. esculentum*-Pflanze erhältlich ist.
- 25 2. Nichttransgene domestizierte *L. esculentum*-Pflanze nach Anspruch 1, wobei der Flavonolgehalt mehr als 1,0 µg/mg TG beträgt.
3. Nichttransgene domestizierte *L. esculentum*-Pflanze nach Anspruch 1, wobei der Flavonolgehalt mehr als 1,5 µg/mg TG beträgt.
- 30 4. *L. esculentum*-Pflanze nach Anspruch 1, wobei der Flavonolgehalt mehr als 2 µg/mg TG beträgt.
5. *L. esculentum*-Pflanze nach den Ansprüchen 1, 2, 3 und 4, die einen Flavonolgehalt in der Schale der Frucht von mindestens 10 µg/mg TG aufweist.
- 35 6. *L. esculentum*-Pflanze nach den Ansprüchen 1, 2, 3 und 4, die einen Flavonolgehalt in der Schale der Frucht von mindestens 17 µg/mg TG aufweist.
7. Samen der *L. esculentum*-Pflanze nach Anspruch 1, wobei der Samen zu einer Pflanze mit einem Flavonolgehalt im Fruchtfleisch der Pflanze von mehr als 0,5 µg/mg TG heranwachsen wird.
- 40 8. Früchte einer *L. esculentum*-Pflanze nach Anspruch 1, die Flavonol im Fruchtfleisch der Pflanze in Mengen von mehr als 0,5 µg/mg TG akkumulieren.

Revendications

- 45 1. Plante de *L. esculentum* domestiquée non transgénique développant des fruits ayant une teneur en flavonols dans la chair du fruit qui est supérieure à 0,5 µg/mg de poids sec et une teneur en flavonols dans la peau dudit fruit d'au moins 5 µg/mg de poids sec en raison d'une biosynthèse de flavonol régulée à la hausse dans la chair du fruit de
- 50 ladite plante et une expression de *CHI* restaurée dans la peau du fruit de ladite plante, ladite plante de *L. esculentum* domestiquée non transgénique pouvant être obtenue par introgression du gène *CHI* et des gènes de la voie de biosynthèse de flavonol *CHS*, *FSH* et *FLS* des numéros d'accession de *Lycopersicon* sauvage LA1963, LA2884 et LA1926 dans une plante de *L. esculentum* domestiquée.
- 55 2. Plante de *L. esculentum* domestiquée non transgénique selon la revendication 1, dans laquelle ladite teneur en flavonols est supérieure à 1,0 µg/mg de poids sec.
3. Plante de *L. esculentum* domestiquée non transgénique selon la revendication 1, dans laquelle ladite teneur en

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flavonols est supérieure à 1,5 µg/mg de poids sec.

4. Plante de *L. esculentum* selon la revendication 1, dans laquelle ladite teneur en flavonols est supérieure à 2 µg/mg de poids sec.

5

5. Plante de *L. esculentum* selon les revendications 1, 2, 3, et 4, et qui présente une teneur en flavonols dans la peau dudit fruit d'au moins 10 µg/mg de poids sec.

10

6. Plante de *L. esculentum* selon les revendications 1, 2, 3, et 4, et qui présente une teneur en flavonols dans la peau dudit fruit d'au moins 17 µg/mg de poids sec.

7. Graine de ladite plante de *L. esculentum* de la revendication 1, ladite graine croissant en une plante ayant une teneur en flavonols dans la chair du fruit de ladite plante qui est supérieure à 0,5 µg/mg de poids sec.

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8. Fruit d'une plante de *L. esculentum* de la revendication 1, qui accumule du flavonol dans la chair du fruit de la plante à des taux supérieurs à 0,5 µg/mg de poids sec.

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FIG. 1

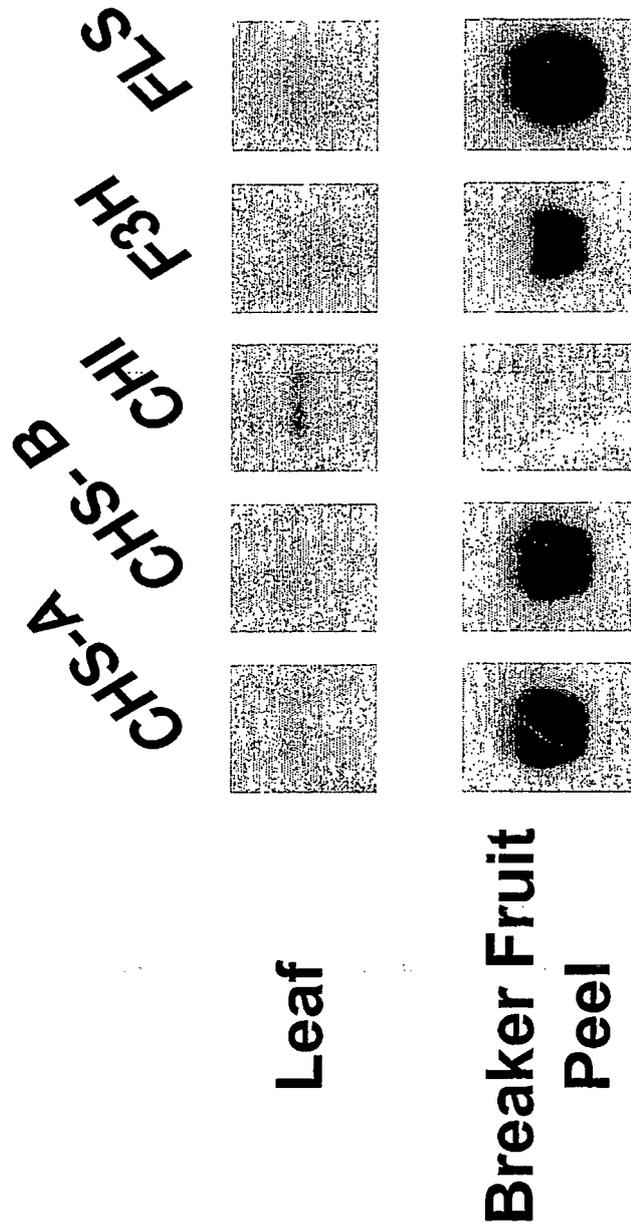
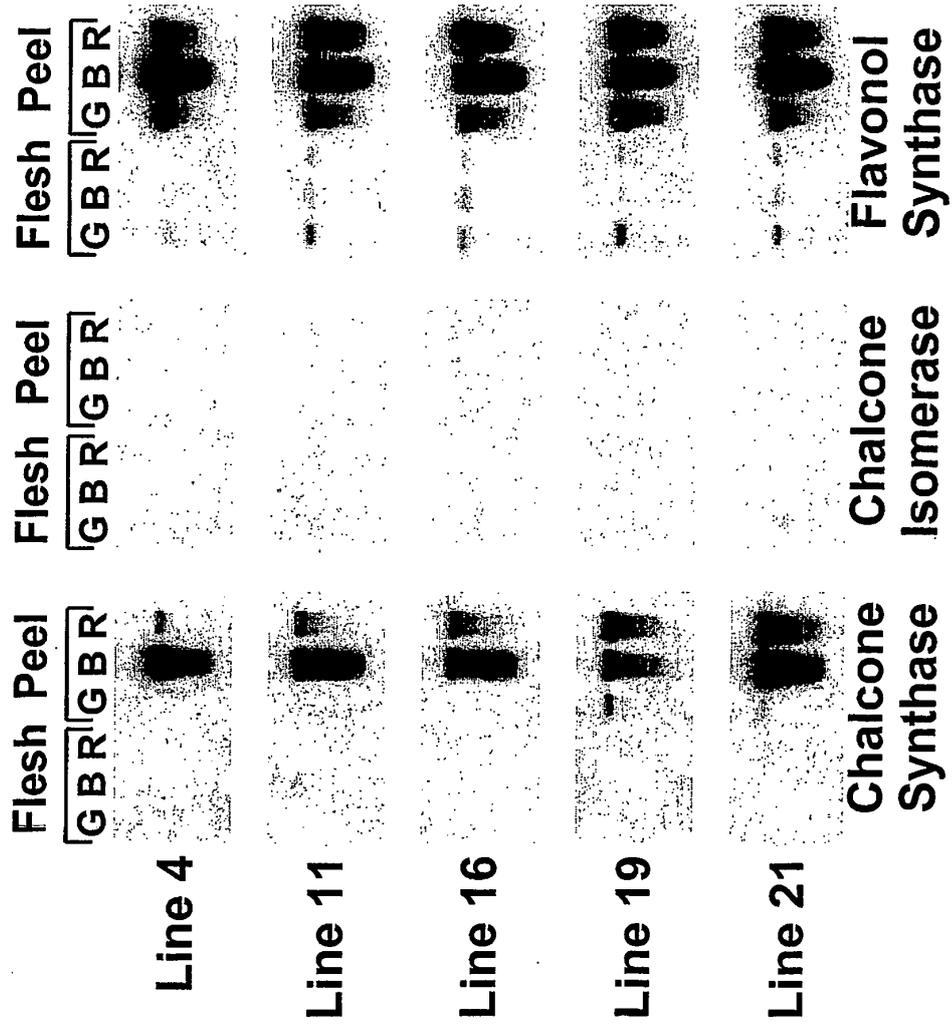


Fig. 2



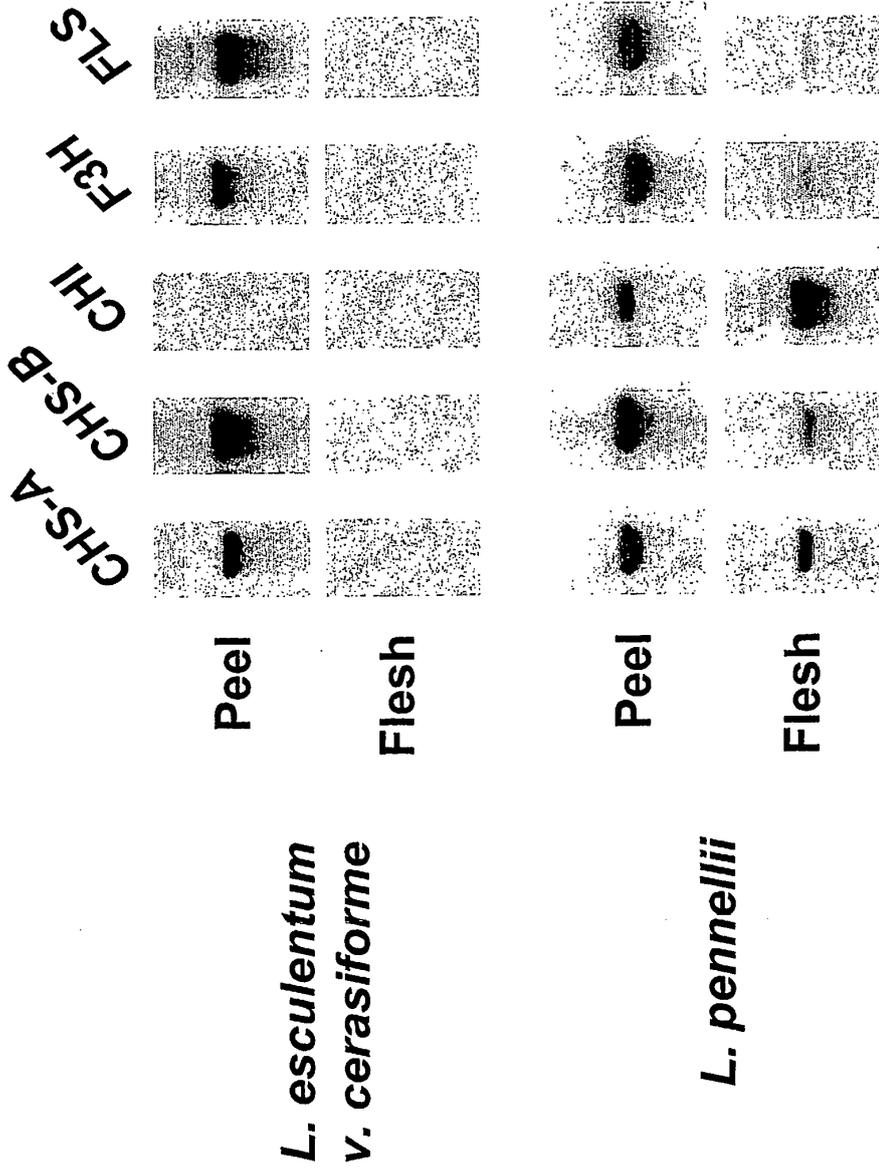


FIG. 3

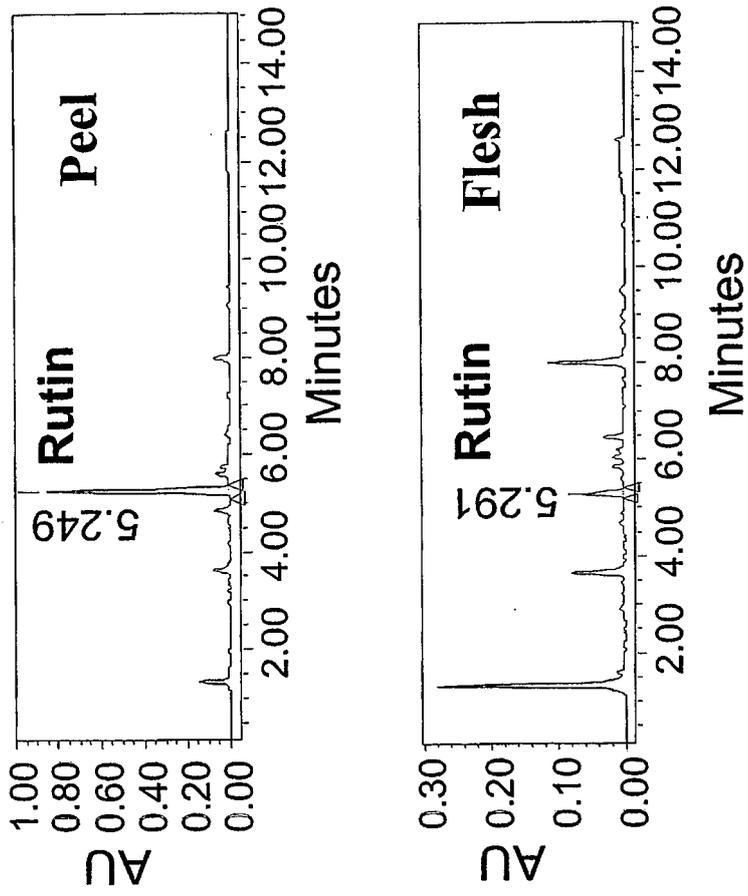


FIG. 4

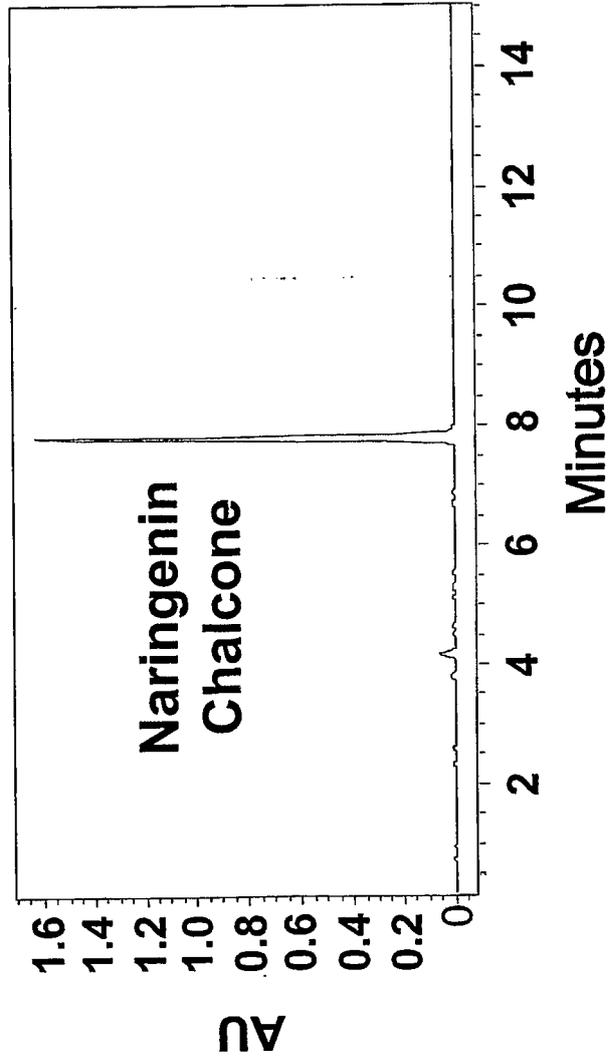


FIG. 5

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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