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Utilization of the Genetic Resources of Wild Species To Create a Nontransgenic High Flavonoid Tomato

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Flavonoids represent a large and important group of plant natural products that are ubiquitous in the plant kingdom. Epidemiological studies have shown the health benefits of a diet high in flavonoids. However, the dietary intake of flavonoids in most western populations is limited, creating a need to find alternative food sources for these polyphenolic secondary metabolites. The domestication of many of our cultivated food crops has resulted in alterations in the biosynthetic pathways of many essential micronutrients and vitamins through inadvertent counterselection against nutritional traits in favor of agronomic ones. Flavonoids are nearly absent from fruits of cultivated tomato (*Lycopersicon esculentum* Mill.), a major vegetable in human diets. Previous attempts to restore the flavonoid pathway in tomato fruits have been limited to transgenic strategies, suggesting that the problem was intractable through traditional methods. Here, we describe for the first time a nontransgenic metabolic engineering approach to developing a high flavonoid tomato using a wild tomato species (*Lycopersicon pennellii* v. *puberulum*) and demonstrate the opportunities for restoring functional pathways using the genetic resources of wild species, resulting in production of healthier foods.

KEYWORDS: ***Lycopersicon pennellii*; *Lycopersicon esculentum*; tomato; flavonoid; natural products**

INTRODUCTION

There is increasing evidence that flavonoids, especially flavonols such as quercetin and kaempferol, impart a number of health benefits (*1*, *2*). A multitude of studies have shown that these secondary metabolites possess antioxidant and anticancer activities, prevent heart disease (*3*, *4*), have effects on antiplatelet aggregation (*5*), decrease blood viscosity, and reduce inflammatory responses and allergic reactions (*6*). Other effects include antiviral, antituberculosis, and antimalarial activities (*3*). Independent epidemiological studies have shown the health benefits of a diet high in flavonoids, but flavonoid intake in most western populations remains low due to eating habits (*7*). Therefore, there is increasing interest in developing alternative food sources rich in flavonoids. Tomatoes (*Lycopersicon esculentum* Mill.), including a wide variety of processed tomato food products (e.g., ketchup, pasta sauce, tomato puree, etc.), are one of the major vegetables in human diets and therefore an ideal candidate for improved flavonoid intake.

Flavonoids represent a large group of polyphenolic plant secondary metabolites that are ubiquitous in vascular plants (*8*). Quercetin, the main flavonol found in tomato leaves, is synthesized via the chalcone synthase (CHS) mediated condensation of phenylalanine-derived *ρ*-coumaroyl-CoA and 3 malonyl-CoA units, respectively (**Figure 1**), to yield naringenin chalcone after cyclization. Naringenin is generated by formation of the heterocyclic C ring through a reaction catalyzed by chalcone isomerase (CHI). Quercetin is synthesized from naringenin by successive enzymatic oxidations carried out by flavanone-3-hydroxylase (F3H), flavonol synthase (FLS), and flavonol-3′,5′-hydroxylase (**Figure 1**). Subsequent *O*-glucosylation reactions yield the flavonol mono- and diglycosides (isoquercitrin and rutin, respectively) that ultimately accumulate in plant tissues. 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 (**Figure 1**).

Quercetin accumulates to relatively high levels in tomato leaf tissue, whereas only small amounts are found in the fruits (*9*). Additional studies have shown that tomato fruit peel tissue in fact accumulates naringenin chalcone, the substrate for CHI (*9*), rather than the flavonol quercetin. When *Petunia hybrida CHI* was expressed in tomato behind a ripening specific promotor (E8), quercetin accumulated to high levels in the fruit peel but not in the flesh (*10*). These data, together with Northern blot analyses, demonstrate that unlike other steps in the flavonoid pathway the CHI step is blocked (**Figure 1**) in cultivated tomato fruit peel, whereas most of the pathway appears to be suppressed in fruit flesh. Here, we demonstrate that only a few wild

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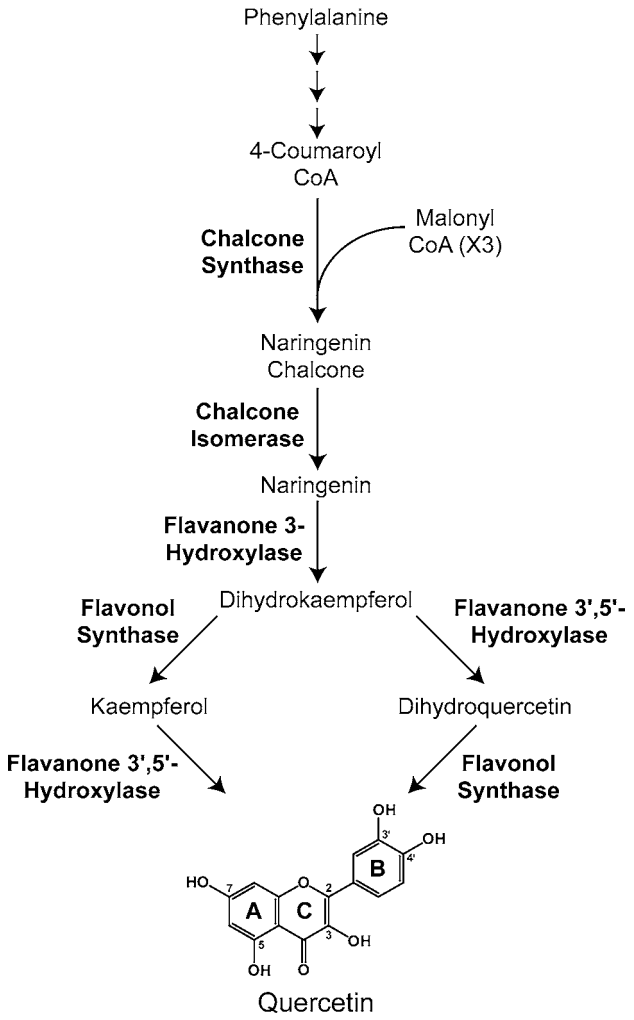

Figure 1. Biosynthetic pathway of quercetin and kaempferol, the main flavonoids produced in tomato. The first pathway specific step is catalyzed by CHS in which three acetate units (from malonyl-CoA) are successively added to 4-coumaroyl-CoA. After cyclization of the polyketide, naringenin chalcone is formed. In the next step, the heterocyclic C ring is closed by action of CHI yielding the flavanone naringenin. Quercetin and kaempferol are then synthesized by successive oxidizations of naringenin catalyzed by FLS, F3H, and flavanone 3′,5′-hydroxylase.

Lycopersicon accessions out of many studied express *CHI* in tomato fruit peel. A smaller subset of these accessions also expresses genes of the flavonol biosynthetic pathway, including *CHI*, in the fruit flesh. When one such accession of *Lycopersicon pennellii* v. *puberulum* (accession LA1926) was crossed with cultivated *L. esculentum*, high levels of quercetin accumulated in both the fruit flesh and the fruit peel. This discovery may be used to breed a high flavonoid tomato without genetic engineering and suggests that wild germplasm of other crops could be surveyed for the high flavonoid trait to create a platform for the production of additional high flavonoid vegetables.

MATERIALS AND METHODS

Plant Growth and Crosses. Seeds of wild *Lycopersicon* accessions were obtained from C. M. Rick (University of California, Davis). Plants were grown in soil-free Universal Mix (SunGrow, Pine Bluff Ark). *Lycopersicon* accessions LA2884 and LA1963 were grown in a growth chamber with a 12 h photoperiod at 26−28 °C and 50% relative humidity. The light intensity of the growth chamber was 200 μE m^{-2} s^{-1}. *L. esculentum* and the remaining *Lycopersicon* accessions (LA1330, LA1673, LA1286, LA1326, LA2727, LA0111, LA1292, LA1926, LA1261, LA0722, and LA 2184) were grown under greenhouse conditions with a 16/8 h photoperiod at 26−28 °C and 75−90% relative humidity. Supplemental lighting was turned on when the outdoor light intensity was less than 600 μmol m^{-2} s^{-1} in the winter months and less than 400 μmol m^{-2} s^{-1} during the rest of the year. Osmocote was used at 54 g per 2 gallon pot for all plants.

To hydridize *L. pennellii* v. *puberulum* (LA1926) with *L. esculentum*, pollen from *L. pennellii* was transferred to the pistils of emasculated flowers of *L. esculentum*. Pollen was collected by inserting the tips of flowers into small plastic tubes, vibrating the flowers with an electric toothbrush, and shaking the pollen into the tubes. Pollen collection was found to be the most successful early in the day when the relative humidity was at its lowest. The pollen was then transferred to the pistils of emasculated flowers of *L. esculentum*. The flowers of *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. 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 2 days.

Members of accessions LA1963, LA2884, LA1926, LA0111, LA1292, and LA1330 are self-incompatible and were pollinated by sib crossing. The sib crossing was performed as described above except there was no need to emasculate the target flowers.

RNA Isolation and 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 (*11*) from ground tissue, and 10 μg of RNA was separated on formaldehyde agarose gels. Uniformity of RNA loading was based on ethidium bromide staining of agarose gels. RNA was then transferred onto Hybond-N^+ nylon membranes (Amersham Biosciences, Piscataway, NJ) and immobilized using UV cross-linking (UV Stratalinker, Stratagene, La Jolla, CA). The Northern blots were hybridized with ^{32}P-labeled DNA probes for *CHS-A*, *CHS-B*, *CHI*, *F3H*, and *FLS*.

Flavonoid Extraction and Analysis. All plant tissue was lyophilized with a Freeze-dry mobile 8SL Sentry prior to extraction. Tomato fruits were harvested and frozen at −20 °C. Prior to peeling, tomato fruits were allowed to thaw slightly to facilitate separation of the peel from the pericarp tissue. Dry peels were ca. 0.1 mm in thickness.

Flavonoid glycosides were extracted from lyophilized tomato fruit peel and flesh tissue of F1 hybrids. Approximately 100 mg of dry tissue was extracted overnight with a ratio of 80 μL of 80% methanol, pH 2.0 (HCl), per 1 mg dry weight (dwt) tissue. Aliquots of this methanol extract were used for high-performance liquid chromatography (HPLC) analysis of flavonoid glycosides. To hydrolyze the flavonoid glycosides, a 1 mL aliquot of the methanol extract was evaporated in vacuo at room temperature. The dried extracts were resuspended in 1 mL of 16% aqueous dimethyl sulfoxide (DMSO) to which was added Naringinase from *Penicillium decumbens* (Sigma-Aldrich), and flavonoid aglycones were produced after overnight incubation at 35 °C. Flavonoid aglycones were extracted with 50 μL of 0.1 N HCl and 700 μL of ethyl acetate. Samples were vortexed for 30 s followed by 10 min of sonication. Separation of organic and aqueous phases was accomplished with a 2 min, 15000*g* centrifugation step. After the organic phase containing flavonoids was evaporated in vacuo at room temperature, the dry aglycone samples were resuspended in 100% methanol and analyzed by HPLC.

Flavonoid samples were analyzed on an HPLC system consisting of a 2690 Alliance separations module (Waters, Milford, MA) and a 996 PDA detector. For all analyses, a 4.6 mm × 150 mm Xterra RP_{18} (5 μm) column protected by a C_{18} Nova-pack guard column kept at 50

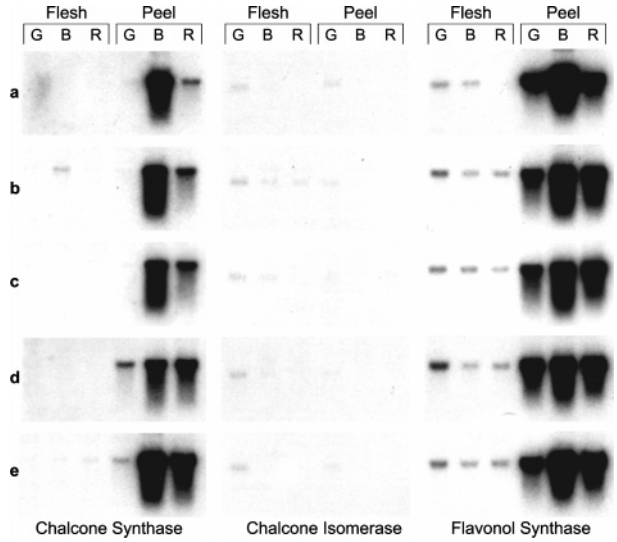

Figure 2. Northern analysis of *L. esculentum* breeding lines. Green (G), breaker (B), or red (R) tomatoes were harvested, and RNA was isolated from either peel or flesh tissue. Northern blots were hybridized with cDNA probes for *CHS*, *CHI*, and *FLS*. Five different breeding lines (a–e) were analyzed.

°C was used. Flavonoid standards were purchased from Indofine (Somerville, NJ). The standards were dissolved in either methanol or DMSO and kept refrigerated at 4 °C. HPLC grade acetonitrile from Fisher Scientific (Fair Lawn, NJ) and water from a Milli-Q Water System from Millipore (Bedford, MA) were used as the mobile phase. In all separations, 1 mL of trifluoroacetic acid from Pierce (Rockford, IL) was added to 1 L of the water used as the mobile phase. For flavonoid glycoside analysis, the gradient was 10−55% acetonitrile in 12 min at a flow rate of 1.5 mL min^{-1}. For aglycone analysis, the gradient was 20−40% acetonitrile in 20 min at a flow rate of 1.5 mL min^{-1}. Quantification was performed using peak area of external standard mixtures with known concentrations ranging from 100 to 4000 ng of standard compound per injection.

RESULTS

Analysis of Flavonoid Biosynthetic Gene Expression in *L. esculentum*. To clarify the molecular basis for the low levels of flavonoid accumulation (*9*) in the tomato fruit peel and flesh (placenta, pericarp, and seeds), flavonoid biosynthetic gene expression was investigated in several breeding lines of *L. esculentum*. Tomato fruits were harvested at the green, breaker, and ripe stages, and RNA was isolated from the peel and flesh. Northern blots were probed with cDNAs from the flavonoid biosynthetic enzymes, *CHS*, *CHI*, and *FLS* (**Figure 2**). While all three genes were expressed in leaves (data not shown), tomato flesh, which accumulates very low levels of flavonoids, did not express any of these genes. In the peel, the expression of *CHS* and *FLS* peaked at the breaker stage, while no expression was seen for *CHI* in the peel at any stage. This result is in agreement with the previously observed patterns of *CHS*, *CHI*, and *FLS* gene expression that results in the accumulation of the naringenin chalcone intermediate rather than quercetin (**Figure 1**) in the fruit peel (*10*). Flavonoid analysis of the *L. esculentum* cultivars described in **Figure 2** as well as 17 other lines (data not shown) showed that they all make small amounts of naringenin chalcone rather than quercetin, with no significant differences between cultivars. Because analysis of a wide range of other *L. esculentum* cultivars (*12*−*14*) also displayed a similar gene expression pattern as described in **Figure 2**, this strongly suggests that cultivated *L. esculentum* germplasm may not be useful to produce a high flavonoid tomato (*14*). It is interesting that transformation of cultivated tomato with a petunia *CHI* gene expressed behind the E8 promotor complemented the lack of *CHI* expression and resulted in the accumulation of quercetin rather than naringenin chalcone within tomato peels (*10*).

Table 1. Interpretation of Northern Data for Flavonoid Biosynthetic Gene Expression in Wild *Lycopersicon* Accessions

		CHI expression		*CHS* and *FLS* expression	
Lycopersicon species	accession	peel	flesh	peel	flesh
L. esculentum (domesticated)	NA[c]	○[d]	○	●[e]	○
L. esculentum v. *cerasiforme*	LA1673[a]	○	○	●	○
L. esculentum v. *cerasiforme*	LA1286	○	○	●	○
L. pimpinellifolium	LA1261	○	○	●	○
L. pimpinellifolium	LA0722[b]	○	○	●	○
L. pimpinellifolium	LA2184[a]	○	○	●	○
L. chmielewskii	LA1330[b]	●	○	●	ND[f]
L. parviflorum	LA1326[b]	●	○	●	ND
L. parviflorum	LA2727[a]	●	ND	●	ND
L. peruvianum	LA0111	●	○	●	○
L. peruvianum f. *glandulosum*	LA1292[b]	●	ND	●	ND
L. chilense	LA2884	●	●	●	●
L. chilense	LA1963	●	●	●	●
L. pennellii v. *puberulum*	LA1926	●	●	●	●

[a] Peel and flesh Northern data not shown for LA1673, LA2184, and LA2727. [b] Flesh Northern data not shown for LA722, LA1330, LA1326, and LA1292. [c] NA stands for not applicable. [d] Indicates no observed expression. [e] ● indicates observed expression of genes in the particular tissue. [f] ND stands for not determined due to insufficient recovery of RNA.

Northern Screen of Wild *Lycopersicon* Accessions. The expression pattern of the flavonoid biosynthetic pathway in the cultivated tomato fruit peel suggests that the lack of flavonoid accumulation may be due to a mutation conditioning the loss of *CHI* expression but not the rest of the pathway. Such a mutation raises the possibility that *Lycopersicon* germplasm may contain alleles that could restore the pathway, leading to flavonoid accumulation in fruits. The lack of *CHI* expression in the fruit peels of all investigated cultivated tomato varieties prompted the screening of wild germplasm for this trait. Because *CHI* is expressed in tomato leaves, the fruit of wild tomato lines were analyzed by Northern analysis for *CHI* expression, rather than using a screen based on either polymerase chain reaction (PCR) or RT-PCR of seedlings.

Thirteen *Lycopersicon* accessions representing seven different species were cultivated under greenhouse conditions (**Table 1**). Ripe fruits from different accessions were harvested, and RNA was isolated from peel and flesh fractions. Because of the small size of the wild tomatoes and the low yield of RNA obtained from flesh tissue, it was not possible to obtain enough flesh RNA from all accessions. Northern analysis was performed using *CHS*, *CHI*, and *FLS* probes as described for the cultivated lines (**Figure 3**, **Table 1**). Several accessions (LA1673, LA1286, LA1261, LA0722, and LA2184) displayed an expression pattern similar to that of *L. esculentum*, with no *CHI* expression in the peel and no expression of any of the three investigated pathway genes in the flesh. Others (LA1330, LA1326, and LA0111) expressed varying levels of *CHI* in the fruit peel but lacked expression of at least one pathway gene in the flesh. Although it was not possible to recover sufficient RNA from fruit flesh from two other accessions (LA2727 and LA1292), the data from very close relatives (LA1326 and LA0111) suggest that they fall into this class. Three other accessions (LA2884, LA1963, and LA1926) not only expressed *CHI* in the peel but also expressed *CHS*, *CHI*, and *FLS* in the flesh, where LA1926 (*L. pennellii* v. *puberulum*) had the strongest expression of the three genes in both peel and flesh (**Figure 3**).

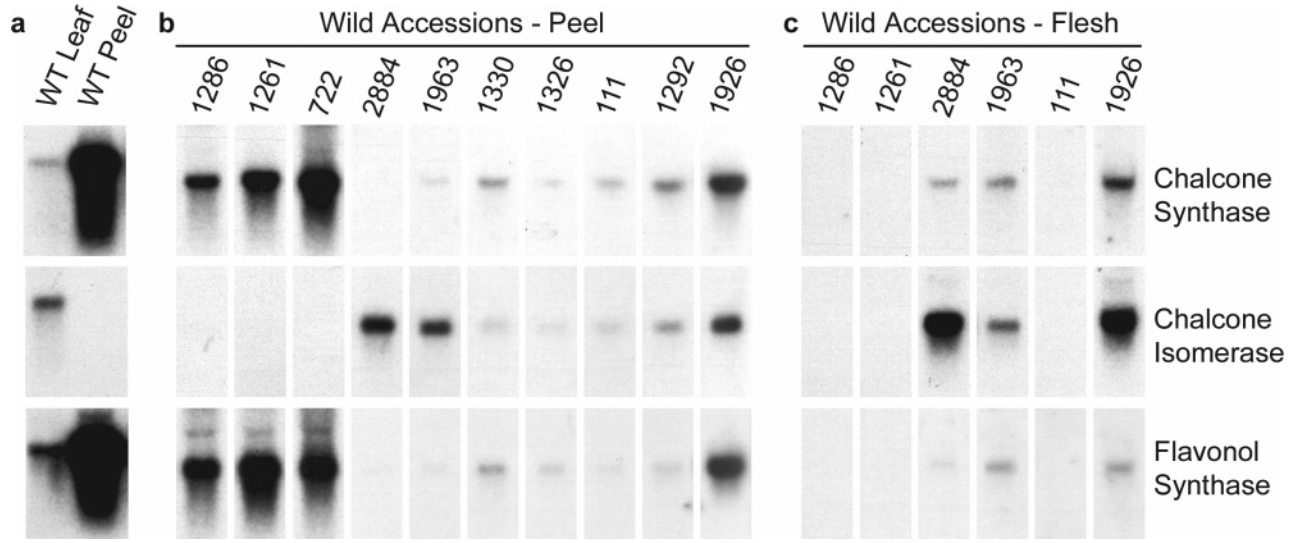

Figure 3. Northern screen of wild *Lycopersicon* accessions. (**a**) Wild-type leaf and fruit peel RNA were analyzed with cDNA probes for *CHS*, *CHI*, and *FLS*. Peel tissue (**b**) and flesh tissue (**c**) RNA were analyzed with the same probes. The accession numbers are shown above the blots.

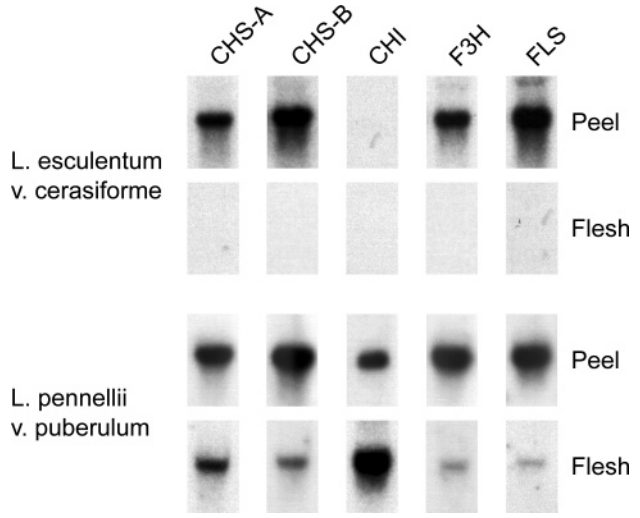

Figure 4. Comparison of flavonoid biosynthetic gene expression in *L. esculentum* v. *cerasiforme* (LA1286) and *L. pennellii* v. *puberulum* (LA1926). Total RNA from fruit peel and flesh was analyzed from the wild accessions using probes for *CHS-A*, *CHS-B*, *CHI*, *F3H*, and *FLS*. *L. pennellii* v. *puberulum* demonstrated strong expression of all investigated biosynthetic genes in the peel and the flesh.

Figure 4 compares the expression patterns of accessions LA1286 (*L. esculentum* v. *cerasiforme*) and LA1926 (*L. pennellii* v. *puberulum*). *L. esculentum* v. *cerasiforme* has an expression pattern that is identical to *L. esculentum*, with no expression of *CHI* in the fruit peel and no expression of any of the investigated flavonoid biosynthetic genes in the fruit flesh. Because *L. pennellii* (LA1926) expressed high levels of all five biosynthetic genes (*CHS-A*, *CHS-B*, *CHI*, *F3H*, and *FLS*) in both fruit peel and flesh, it was chosen as a candidate for breeding of tomato lines that accumulate high levels of flavonoids throughout the tomato fruit.

Interspecific Hybridization of *L. pennellii* and *L. esculentum*. An interspecific cross was made between the wild accession *L. pennellii* v. *puberulum* (female) and an Elite Syngenta cultivar of *L. esculentum* (male). The sterile F1 progeny from this cross were verified to be heterozygous at the *CHI* locus using a PCR marker (data not shown). Flavonoid glycosides or aglycones were analyzed by HPLC from the fruit peel and flesh tissue of the F1 hybrid and the parent lines.

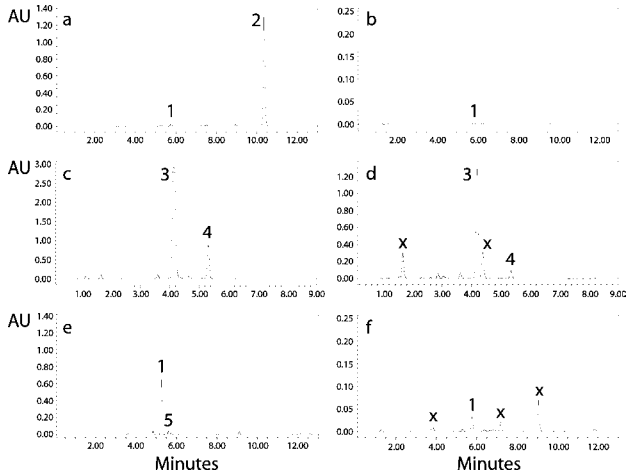

Figure 5. Flavonoid analysis of the domesticated tomato *L. esculentum*, the wild accession *L. pennellii* (LA1926), and the F1 cross of both by HPLC. Peel (**a**) and flesh (**b**) of domesticated *L. esculentum* (Syngenta Elite germplasm) showed the accumulation of low levels of quercetin diglycoside (peak 1, rutin) and high levels of naringenin chalcone (peak 2) in the peel and very low levels of quercetin diglycoside accumulation in the flesh. Peel (**c**) and flesh (**d**) of the wild tomato accession *L. pennellii* showed a low level accumulation of quercetin (peak 3) and kaempferol (peak 4) in the peel and in the flesh. Peel (**e**) and flesh (**f**) of the *L. esculentum* × *L. pennellii* cross demonstrated a high level of flavonol accumulation (95% quercetin diglycoside, 5% quercetin monoglycoside) in the peel and also in the flesh. Flavonoids were extracted as glucosides for **a**, **b**, **e**, and **f**. Because of interference with other glycosides, the flavonoids of the wild accession *L. pennellii* (**c**, **d**) were analyzed as aglycones. Peaks: 1, quercetin diglycoside (rutin); 2, naringenin chalcone; 3, quercetin; 4, kaempferol; 5, quercetin monoglucoside; and *x*, compounds not identified as flavonoids.

Figure 5a,**b** demonstrates that fruit tissue from *L. esculentum* accumulates very low levels of quercetin diglycoside (peak 1, rutin), with 3 μg/mg dwt in the peel and 0.1 μg/mg dwt in the flesh (**Table 2**). The peel tissue accumulates significant amounts of naringenin chalcone (peak 2, **Figure 5a**), which is consistent with a block at CHI. *L. pennellii* v. *puberulum* accumulates slightly higher levels of quercetin (peak 3) and kaempferol (peak 4), with 4.7 μg/mg dwt in the peel and 1.2 μg/mg dwt in the flesh (**Figure 5c**,**d** and **Table 2**) and no accumulation of

Table 2. Flavonoid Accumulation in *Lycopersicon* Species

Lycopersicon species	quercetin diglycoside accumulation μg/mg dwt peel	quercetin diglycoside accumulation μg/mg dwt flesh	estimated total flavonoid mg/tomato[a,b]
L. esculentum	3	0.1	1.6
L. pennellii v. *puberulum*	4.7	1.2	9.7
L. esculentum × *L. pennellii*	17	2	18.9

[a] Estimates of total flavonoid content were based on a 57 g (kiwi size) tomato and showed a 11.8-fold increase in the F1 cross as compared to the domesticated line. [b] Other examples of high quercetin sources (normalized to 57 g fresh weight): apple, 2–4 mg (*15*); yellow onion, 17–51 mg (*16*); and lettuce, 8–26 mg (*17*).

naringenin chalcone. The F1 progeny of the cross between *L. esculentum* and *L. pennellii* v. *puberulum* accumulated high levels of quercetin diglycoside (peak 1) and monoglycoside (peak 5), with 17 μg/mg dwt in the peel and 2 μg/mg dwt in the flesh (**Figure 5e**,**f** and **Table 2**). As found for *L. pennellii* v. *puberulum*, naringenin chalcone was not detected in the F1 lines. On the basis of these numbers, fruits from the F1 hybrids showed an 11.8-fold increase in flavonoid levels over *L. esculentum*, yielding a tomato with an estimated 19 mg flavonoids per 57 g fruit (**Table 2**). These results demonstrate the transfer of the genetic elements responsible for these traits into domesticated tomato, resulting in high flavonoid accumulation throughout the fruit.

It turned out to be very difficult to produce tomatoes from these crosses, and the fruit was invariably seedless. For this reason, it was not possible to further analyze progeny from the F1 hybrid. To accomplish this, a breeding program to remove interspecific breeding barriers between *L. pennellii* and *L. esculentum* is required in order to obtain fertile hybrids for subsequent analysis of the high flavonoid trait in the next generation.

The molecular nature of the changes in gene expression between *L. esculentum* and *L. pennellii* is unclear; however, they are likely due to different genetic factors. The lack of *CHI* expression in the fruit peel could be due either to a mutation in a fruit specific element (leaf expression is normal) of the *CHI* promoter or a mutation in a transcription factor responsible for *CHI* expression in the peel. To distinguish between these two possibilities, a genomic fragment containing the *CHI* gene and the corresponding 5′- and 3′-regions was PCR-amplified and cloned from *L. parviflorum* (LA1326), another accession that expresses *CHI* in the fruit peel. When transformed into *L. esculentum*, this genomic fragment was able to restore the flavonoid pathway, resulting in high flavonoid accumulation in the peel (data not shown). This result suggests that the *L. esculentum CHI* gene is not expressed in fruit peels because of a fruit specific promoter mutation. However, a *CHI* promoter mutation does not readily explain the lack of expression of the biosynthetic pathway in the flesh tissue. Rather, it is more likely that a transcription factor(s) is expressed in *L. pennellii* v. *puberulum* flesh tissue that is able to induce the biosynthetic pathway.

DISCUSSION

The detailed molecular analysis of several lines of cultivated tomato described in this study confirms the suggestion (*10*) that flavonoids do not accumulate because *CHI* is not expressed in the fruit peels of *L. esculentum* lines, even though *CHS* and *FLS* are expressed at high levels. Molecular analyses of fruit flesh from *L. esculentum* lines suggest that they do not express most of the flavonoid pathway, which leads to the characteristic low flavonoid phenotype of cultivated tomato. This pattern of expression of the flavonoid pathway results in fruit peels that accumulate the intermediate, naringenin chalcone (*9*), due to a lack of expression of the *CHI* gene (*10*). The expression of the entire flavonoid biosynthesis pathway in tomato peel suggests that the default state for this tissue is to produce these phenolic compounds. On the basis of Northern data for several *L. esculentum* lines, including *L. esculentum* v. *cerasiforme* (**Figures 2** and **4**), combined with the fact that there are no known high flavonoid cultivated tomato lines, it is likely that the mutation leading to the lack of *CHI* expression in the peel occurred relatively early in or before the domestication of tomato.

This information led to the present screen for wild *Lycopersicon* accessions that might express *CHI* in the fruit. The results show that wild accessions can be divided into three basic expression patterns: no expression of *CHI* in the peel and no flavonoid biosynthetic gene expression in the flesh, expression of *CHI* in the peel and no biosynthetic gene expression in the flesh, and expression of *CHI* in the peel and of the entire pathway in the flesh (**Figure 3**). Species with the latter expression pattern were the most promising candidates for the breeding of tomato lines accumulating high levels of flavonoids throughout the fruit (**Figure 3**; accession LA1926).

The most promising accession, *L. pennellii* v. *puberulum* (LA1926), had high level expression of the pathway genes in the peel and flesh tissue but showed only modest levels of flavonoid accumulation as compared to tomato lines transformed with the *CHI* gene (*10*). This result clearly demonstrates the value of an RNA-based approach to screen potential germplasm, as the flavonoid levels alone will not identify appropriate germplasm for the high flavonoid trait. Although it is not clear why *L. pennellii* v. *puberulum* accumulates low levels of flavonoids, progeny from the interspecific cross accumulated high levels of flavonoid in the fruit peel and flesh (**Figure 5** and **Table 2**). At least one genetic factor responsible for the lack of *CHI* expression in the peel appears to be a mutation in a fruit specific element of the promoter, whereas induction of the pathway in the flesh certainly involves additional contributing regulatory factors. Further investigation will be necessary to understand the nature of increased gene expression in the flesh tissue. Segregation analysis of fertile hybrids will reveal whether regulation of flavonoid biosynthesis is mono- or polygenic. Analysis of *L. pennellii* introgression lines could also help to identify the genomic regions that encode the regulatory factor(s) involved in producing the phenotype (*18*−*20*).

To date, transgenic high flavonoid tomato lines have been created by overexpressing a heterologous *CHI* gene (*10*) or the maize transcription factors *LC* and *C1* (*21*, *22*). In the first strategy, high flavonoid lines were produced, but accumulation was limited to the peel. In the second strategy, flavonoids were produced throughout the fruit, but the induction of tomato genes by the maize transcription factor was apparently not uniform and led to altered patterns of flavonoid accumulation. The breeding approach outlined in this report resulted in more uniform expression of flavonoid pathway genes and high level accumulation of quercetin in both peel and flesh.

The strategy presented in this report may create new opportunities for the nontransgenic manipulation of metabolic pathways. Here, we show that in the case of tomato, utilization of the genetic diversity available in wild germplasm resources (*23*) can be a remarkably powerful approach for specifically altering metabolic biochemistry to produce a healthier food.

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