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ORIGINAL PAPER

Flavonoid genes of pear (*Pyrus communis*)

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Abstract Pear (*Pyrus* sp.) is a major fruit crop of temperate regions with increasing extent of cultivation. Pear flavonoids contribute to its fruit color, pathogen defense, and are health beneficial ingredients of the fruits. Comparative Southern analyses with apple (*Malus x domestica*) cDNAs showed comparable genomic organization of flavonoid genes of both related genera. A homology-based cloning approach was used to obtain the cDNAs of most enzymes of the main flavonoid pathway of *Pyrus*: phenylalanine ammonia lyase, chalcone synthase, chalcone isomerase, flavanone 3β-hydroxylase, flavonol synthase, dihydroflavonol 4-reductase, leucoanthocyanidin reductase 1 and 2, anthocyanidin synthase, anthocyanidin reductase, and UDP-glucose : flavonoid 7-*O*-glucosyltransferase. The substrate specificities of the recombinant enzymes expressed in yeast were determined for physiological and non-physiological substrates and found to be in general agreement with the characteristic pear flavonoid metabolite pattern of mainly B-ring dihydroxylated anthocyanins, flavonols, catechins, and flavanones. Furthermore, significant differences in substrate specificities and gene copy numbers in comparison to *Malus* were identified. Cloning of the cDNAs and studying the enzymes of the *Pyrus* flavonoid pathway is an essential task toward a comprehensive knowledge of *Pyrus* polyphenol metabolism. It also elucidates evolutionary patterns of flavonoid/polyphenol pathways in the Rosaceae, which allocate several important crop plants.

Keywords Flavonoids · Pear (*Pyrus communis*) · Polyphenols · Rosaceae

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Introduction

Pears (*Pyrus communis*, European pear, *P. bretschneideri*, *P. ussuriensis*, Chinese pears, and *P. pyrifolia*, Asian pear or Nashi) are important pome fruits, since they are favorable foodstuff due to their delicious flavor and their manifold cultivars. World production of pear fruits is about 17 million tons per year. Currently, pear cultivation is continuously rising worldwide and drastically expanding in Asia (Fischer and Weber 2005). Despite this, they were only rarely investigated at the molecular level yet.

Flavonoids, polyphenols in general, are of great importance in plants. Apart from their many biological functions such as pollinator attraction, pollen fertility, UV protection, regulation of polar auxin transport, establishment of microbial symbioses, and pathogen defense, polyphenols contribute to or even determine additional features that are of special relevance in fruit crops (Schijlen et al. 2004). These include color and flavor, which both strongly influence fruit attractiveness of ripe fruits for herbivores. With respect to polyphenols, color is chiefly determined by anthocyanins, but also by flavonols, which act as their co-pigments. Fruit flavor is influenced by, e.g., bitter-masking flavanones (Ley et al. 2005) and astringent proanthocyanidins (polymeric flavan 3-ols), the latter are primarily

present in unripe fruits and contribute to herbivore deterrence (Harborne 1993). Furthermore, the contribution of secondary metabolites such as polyphenolic compounds to pathogen resistance is of special interest in fruit crops (Treutter 2005). Currently, the health-promoting effects of flavonoids in food are a highly debated topic (e.g., Punyasiri et al. 2004; Mendoza-Wilson and Glossmann-Mitnik 2006; Yilmaz 2006).

Pears contain a broad spectrum of phenolic compounds comprising different flavonoid classes [anthocyanins, flavonols, monomeric ('catechins'), and polymeric flavan 3-ols (proanthocyanidins, syn. condensed tannins), and flavanones], hydroxyphenolic acids (mostly hydroxycinnamic acids derived from caffeic acid and *p*-coumaric acid) and the *p*-hydroquinone-glucoside arbutin (Fig. 1) (Challice and Williams 1968; Macheix et al. 1990; Amiot et al. 1995; Andrade et al. 1998; Schieber et al. 2001; Petkou et al. 2002; Andreotti et al. 2006). In leaves arbutin, its derivatives, and hydroxycinnamic acids are dominating. In fruits hydroxycinnamic acids are the dominating polyphenols. Among flavonoids of leaves and fruits B-ring dihydroxylated flavonol derivates (quercetin and isorhamnetin) and monomeric and polymeric flavan 3-ols (epicatechin and proanthocyanidins) are dominant. These compounds contribute to color, fruit quality, and plant resistance. Cloning of pear flavonoid cDNAs was performed to provide the molecular biological information on this subject for fundamental knowledge and for practical application.

Cloning of genes via sequence homology is a widely applied approach. Selected pear flavonoid genes [*flavanone 3β-hydroxylase* (*FHT*), *dihydroflavonol 4-reductase/flavanone 4-reductase* (*DFR/FNR*)] have previously been cloned for their involvement in inducible fire blight and scab resistance (Fischer et al. 2003; Halbwirth et al. 2006). This work now describes the cloning of pear main flavonoid cDNAs in general [*phenylalanine ammonia lyase* (*PAL*), *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavonol synthase* (*FLS*), *leucoanthocyanidin reductase* (*LAR1*, *LAR2*), *anthocyanidin synthase* (*ANS*), *anthocyanidin reductase* (*ANR*), and *UDP-glucose : flavonoid 7-O-glucosyltransferase* (*F7GT*)], which have been

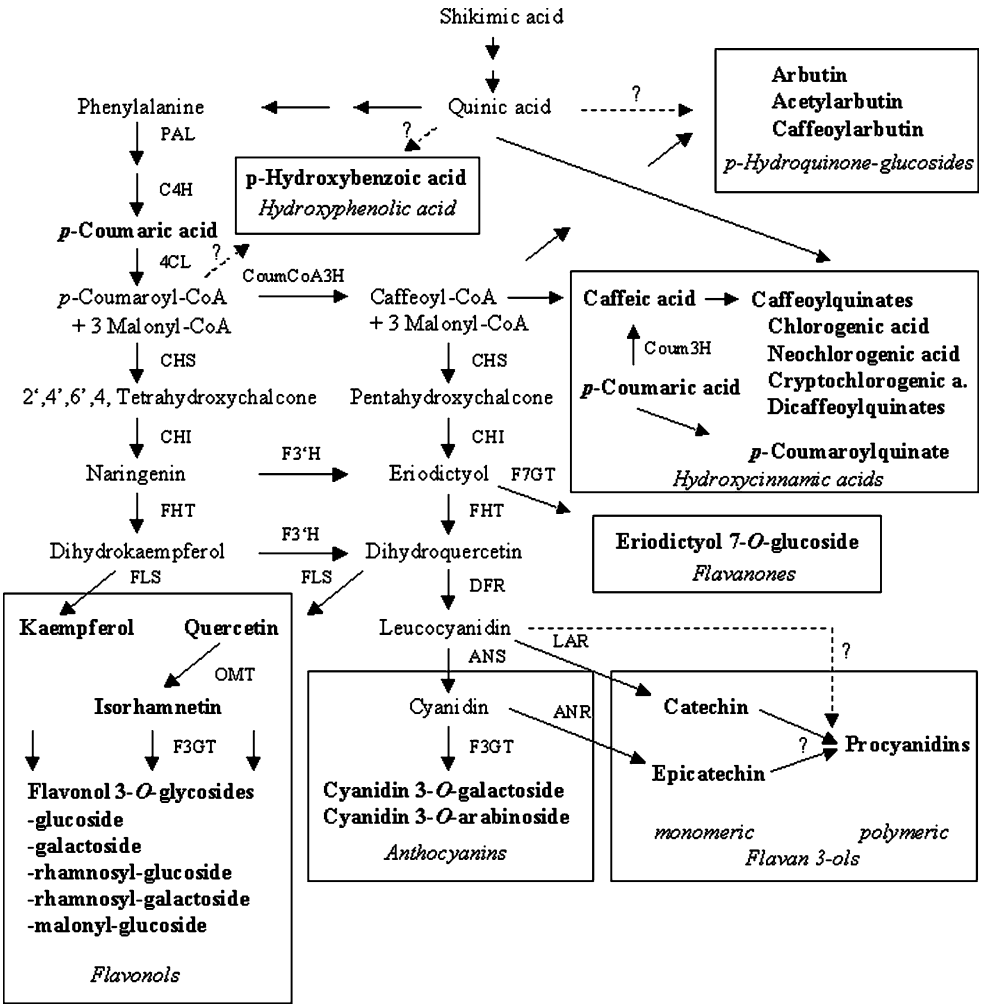

Fig. 1 Biosynthesis of the main constitutive flavonoids, phenols and polyphenols in European pear (*Pyrus communis*). End products are shown in *bold*. Beyond the specific flavonoid pattern, the occurrence of arbutin and the lack of dihydrochalcones (abundant in *Malus*) are *Pyrus* specific. Enzyme abbreviations: *ANR* anthocyanidin reductase, *ANS* anthocyanidin synthase, *C4H* cinnamate 4-hydroxylase, *CHI* chalcone isomerase, *CHS* chalcone synthase, *4CL* 4-coumarate : CoA ligase, *CoumCoA3H* *p*-coumaroyl-CoA 3-hydroxylase, *DFR* dihydroflavonol 4-reductase, *F3GT* UDP-glycosyl : flavonoid 3-*O*-glycosyltransferase, *F7GT* UDP-glucose : flavonoid 7-*O*-glucosyltransferase, *F3′H* flavonoid 3′-hydroxylase, *FHT* flavanone 3-hydroxylase (=F3H), *FLS* flavonol synthase, *FNR* flavanone 4-reductase, *LAR* leucoanthocyanidin reductase, *OMT* *O*-methyltransferase, *PAL* phenylalanine ammonia lyase

isolated via homology with apple sequences to elucidate gene functions, gene copy numbers, and gene relationships within the Maloideae. With the exception of *UDP-glycosyl : flavonoid 3-O-glycosyltransferase* (*F3GT*), which could not be obtained, the sequences of the most important pear flavonoid genes are now available. In future, these sequence data can serve as a base for the development of molecular markers for marker assisted selection or for metabolic engineering of pear. It is also demonstrated here that homologous pear genes generally can be obtained straightforward from the extensive sequence information available from apple.

Materials and methods

Comparative Southern blot analysis

The comparative Southern blot analysis for *Pyrus* and *Malus* flavonoid genes was performed with existent *Malus* cDNAs, which were derived from previous work (cited below). Genomic DNA was prepared from young leaves of *P. communis* cv. Pyrodwarf and of *M. x domestica* (syn. *Malus pumila*) cv. M9 using the DNeasy® Plant DNA Kit (Qiagen, Crawley, UK). In each case 5 µg DNA were digested with 50 U restriction enzyme (*Eco*RI, *Hin*dIII, *Sal*I, *Sac*I, and *Xba*I) in the respective buffer for 5 h at 37°C. The restricted DNA was ethanol precipitated, redissolved in 20 µl TE at 65°C and used for agarose gel electrophoresis (1% agarose, 1 mg ethidium bromide/l, TAE buffer, 30 V). The gel was soaked in 0.25 N HCl for 15 min afterwards, rinsed with water, washed twice in 0.5 N NaOH/1.5 M NaCl for 20 min, rinsed with water and washed two times in 1 M Tris pH 7.4/1.5 M NaCl for 20 min. Blotting transfer was done overnight with 0.4 N NaOH on Immobilon Nylon N+® membrane (Millipore, Bedford, MA, USA). After blotting, the membrane was briefly washed with 5 × SSPE, air-dried and baked for 30 min at 80°C. Hybridization probes were PCR amplified from plasmid inserts of full length *Malus* cDNAs [Table 1, Fischer et al. 2003 (*DFR*), Halbwirth et al. 2006 (*FHT* and *FLS*), Fischer et al. 2006 (*ANS*), Pfeiffer et al. 2006 (*LAR1, LAR2*, and *ANR*), this paper (*CHS* and *F7GT*)]. In case of *F3GT* a cDNA amplified from *Malus* cv. M9 relying on AF117267 (full size *F3GT*) was used. For *CHS* and *PAL*, fragments of cDNAs amplified from *Malus* cv. M9 were used relying on X68977 (*CHS*) and X68126 (*PAL*). Only for *CHI* the *Pyrus* cDNA described here was used as a probe.

Hybridizations were performed with ^{32}P-labeled DNA obtained with the Rediprime™ II kit (RPN 1633, Amersham, Freiburg, Germany) in the hybridization buffer (3 × SSPE/0.02% Ficoll/0.02% Polyvinylpyrrolidone/0.1% SDS/50 mg/l preboiled calf thymus DNA) at 63°C for at least 15 h. The blot was washed twice with 2 × SSPE/1% SDS and once with 2 × SSPE/0.1% SDS at 63°C for 5 min each. Bands were revealed by exposition with a phosphorimager (Fuji BAS 1000 Bio-Imaging Analyzer, screens: BAS-MS 2040, Fuji, Kanagawa, Japan).

Cloning of pear flavonoid cDNAs

For cloning of the pear flavonoid cDNAs (*PAL, CHI, FHT, FLS, DFR, LAR1, LAR2, ANS*, and *ANR*) total RNA was obtained from young leaves of the *P. communis* cv. 'Conference' and the cv. 'Pyrodwarf' using the RNeasy Plant Mini kit (Qiagen). For the cloning of *CHS* and *F7GT*, mRNA was isolated from young leaves of *P. communis* cv. 'Abbe Fetel' with the µMACS mRNA Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). Reverse transcription was performed with the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and the oligo(-dT) anchor primer GACCACGCGTATCGATGTCGAC$(T)_{16}$V. PCR conditions were 1.5 mM $MgCl_2$, 200 µM dNTPs, 500 nM each primer, with 10 ng cDNA and 1 U Taq polymerase in 1 × buffer (MBI). Cycling conditions were: 94°C for 1.5 min, 30 × (94°C for 30 s, 45-64°C for 1 min, 72°C for 2 min), 72°C for 7 min. For each cloning approach, three 5′ primers and three 3′ primers were derived from the non-coding regions of the *Malus* sequences listed in Table 2, where the primer combinations and annealing temperatures which were finally used for the cloning process are presented.

All 5′ and 3′ primer combinations were tested for PCR amplification of expected DNA fragments at three to five different annealing temperatures in the range of 45-64°C. The optimal primers and conditions were chosen and the PCR was repeated with proof-reading polymerase (Taq/Pwo-polymerase Expand High fidelity PCR System®, Roche, Nutley, NJ, USA) with the optimal annealing temperature and other PCR conditions according to the manufacturer's instructions. The proof-reading PCR products were directly cloned into the yeast expression vector pYES2.1 (Topo TA Cloning Kit, Invitrogen), selected for sense insert orientation by PCR and commercially sequenced using vector primers.

Pyrus/*Malus* sequence comparisons

The *Pyrus* sequences obtained were compared with orthologous *Malus* sequences (Table 1) using *CLUSTALW Multiple Alignment* algorithm. Sequences from the *M. x domestica* cultivars 'Golden Delicious', 'Rewena', and the cv. 'M9' had been obtained previously making use of the

Table 1 Flavonoid genes cloned and reported from *Pyrus communis*, their cDNA sequence accessions, and the sources used for the *Malus*/*Pyrus* cDNA comparisons

Gene	*Pyrus* sequence	Source cultivar	*Malus* sequence	Source cultivar
PAL	DQ230992	Conference	CN903802, CN910163, EB124001, CN919710, CX025115, X68126 (ESTs)	Diverse
CHS	AY786998	Abbe fetel	AY786996	Rewena
CHI	EF446163	Conference	DT041465, CN900722 (ESTs)	Diverse
FHT	AY965342	Conference	AY965340	M9
FLS	DQ230993	Conference	AY965343	M9
DFR	AY227731	Conference	AY227728	M9
LAR1	DQ251190	Conference	DQ139836	Golden delicious
LAR2	DQ251191	Pyrodwarf	DQ139837	Golden delicious
ANS	DQ230994	Conference	DQ156905	M9
ANR	DQ251189	Conference	DQ139835	Golden delicious
F7GT	AY954922	Abbe fetel	AY786997	Rewena

Malus sequence accessions are the ones produced previously by the authors of this study and are from the *Malus* cultivars, which have been studied by them

Table 2 Apple (*Malus)* basic sequence information (Davies 1993; Honda et al. 2002; Kim et al. 2003; Takos et al. 2006) used for pear (*Pyrus*) flavonoid cDNA cloning, derived primer sequences and annealing temperatures (T_{ann}) finally used for obtaining the *Pyrus* cDNAs

	Malus sequence	5′ Primer	3′ Primer	T_{ann} (°C)
PAL	CN903802, X68126	GAGTTTTCAGTTTTTCGTAATTAACAT	GTTGAATGTGAAGGAATGCAGC	45
CHS	AB074485	CGATCGGATATACAGAAAAAATG	CCACAAGATATACCGGTGG	55
CHI	DT041465, CN900722	CAAACAATATATAAATTGTCAAATGGCT	GAGAGAGGAGAAATAAAACTTG	45
FHT	AF117270	GAACCAACAAATTCGACACC	GGAAGGAAAAGGTACAAGTAGC	45
FLS	AF119095	CGAACGTCCAAAGCCCTTC	CCCACTTCCACACCACTCAC	53
DFR	AF117268	CTTCGGTAAGCACATACACAC	GCAAACATTAATCACTCATC	45
LAR1	AY830131	TCTGTAGCAGGAARGAAGAGC	CRGTCYTGATTCKGATAGATTG	50
LAR2	AY830132	CTTTTCAAACTTGAGGGCA	CACCTCACATAATTTACCAGAC	50
ANS	AF117269	CGAGTAATATACTAGCTGAG	ATTAGGACGATAGTTCACAAC	50
ANR	AY830130	GACAGAGGAAGAGGAAACCATG	GCCAGACAATAGAGAGATAGC	50
F7GT	AY347843	GCAATCGCATA(A/C)GCCGCCATGG	AATCCGTCTAGGCGCCTGCCTAG	64

The untranslated 5′ sequence of the *CHS* was obtained by personal communication from C. Honda. The partial sequence of the *Malus F7GT* was completed by RACE with *Malus* cDNA first

sequence accessions listed in Table 2. The *Malus CHI* sequence was assembled from ESTs. Generally, all cDNAs of *Malus* and *Pyrus* were derived from young leaves.

Recombinant enzymes

For heterologous expression, the yeast expression vectors pYES2.1 harboring the RT-PCR-cloned flavonoid cDNAs in sense orientation were transformed into the yeast strain InvSc1 (Invitrogen) using the S.c. Easy Comp™ Transformation Kit (Invitrogen). The enzymes were prepared from galactose-induced yeast cultures as described by Urban et al. (1997). Transformation of yeast and preparation of yeast-derived enzymes was also done with empty pYES2-vector (pYES2 vector kit, Invitrogen) to provide a negative control for enzyme activities. Protein content was determined by a modified Lowry procedure (Sandermann and Strominger 1972) using BSA as a standard.

Enzyme assays

Enzyme assays were carried out according to Halbwirth et al. (2002) (CHS, FHT, FLS, and DFR/FNR), Pfeiffer et al. (2006) (ANR and LAR), S. Martens (unpublished, ANS), and Stich et al. (1997) (F7GT). In brief, [^{14}C]-labeled substrates were incubated under conditions described with the respective recombinant enzyme. Products were extracted with ethylacetate and subjected to thin layer chromatography (TLC). Scanning radiography of TLC plates was used for qualitative and quantitative analysis of enzyme reaction products. Only anthocyanidins for ANR reaction were used in unlabeled form, epicatechin products being detected with dimethylaminocinnamicaldehyde (DMACA). [^{14}C]-labeled flavonoids were synthesized via [2-^{14}C]-malonyl-coenzyme A (2 GBq/mmol), which was purchased from Amersham International. Phenylalanine for the PAL assays was also [^{14}C]-labeled (127 pmol/µl,

5.25 kBq/pmol; Sigma, St. Louis, MO, USA). *p*-Coumaroyl-CoA (Coum-CoA) and caffeoyl-CoA (Caff-CoA) were obtained according to Stockigt and Zenk (1976). [^{14}C]-Naringenin (NAR), [^{14}C]-eriodictyol (ERI), [^{14}C]-dihydrokaempferol (DHK), and [^{14}C]-dihydroquercetin (DHQ) were synthesized as described previously (Halbwirth et al. 2006). Unlabeled naringenin, eriodictyol, apigenin, luteolin, kaempferol (Km), quercetin (Qu), pelargonidin, cyanidin, and delphinidin for use as reference substances were purchased from Extrasynthesis (Genay, Lyon-Massieux, France). Enzymatic formation of the flavonoid 7-*O*-glucosides by F7GT was confirmed with HPLC and TLC with authentic standards according to Stich et al. (1997). CHI activity was assayed (pH 8) photometrically by observing the isomerization of naringenin chalcone at 385 nm with controls for yeast enzymes (empty expression vector) and the slower non-enzymatic isomerization to racemic naringenin.

Results

Comparative Southern blot analysis of *Malus* and *Pyrus* genomic DNA hybridized with cDNAs of *Malus* flavonoid genes revealed related band patterns (Fig. 2), and, hence, correspondingly similar gene copy numbers for some, but not all flavonoid gene classes (Table 3, columns 1–3). Especially for UDP-glycosyl : flavonoid- glycosyltransferases (*FGT*s) significant discrepancies were observed. Even so, this result indicated suitability of a homology-based cloning approach for most *Pyrus* flavonoid genes.

Cloning of the *Pyrus* flavonoid cDNAs was performed by deriving for each target sequence three 5′ primers and three 3′ primers from the non-coding 5′ and 3′ regions of available corresponding *Malus* flavonoid cDNA sequences (Table 2). Full size coding sequences of *PAL*, *CHS*, *CHI*, *FLS*, *LAR1*, *LAR2*, *ANS*, *ANR*, and *F7GT* (Table 3) were successfully amplified with at least one primer combination in each case, *DFR/FNR* (Fischer et al. 2003) and *FHT* (Halbwirth et al. 2006) had been obtained previously. All sequences have been submitted to GenBank (Table 1). The cDNA of *LAR2* could not be obtained from the cv. 'Conference' by this approach, but only from the cv. 'Pyrodwarf'.

The sequences of the *Pyrus* cDNAs obtained were different from those of *Malus*, both on nucleotide and amino acid level (Table 3, columns 4–6), being represented on the amino acid level by substitutions and also by insertions/deletions in many cases. The amplified 11 cDNAs, each representing the complete open reading frame, were directly cloned into a yeast expression vector. The recombinant enzymes were used to determine the function of the respective sequences (examples *CHS, FHT, DFR,* and *FLS* are shown in Fig. 3) and to analyze the substrate specificities of the *Pyrus* flavonoid enzymes (Table 3, column 7). For all assays, yeast enzyme preparations from an empty pYES2 line were used as a negative control. Product identification was done by co-chromatography with authentic standards.

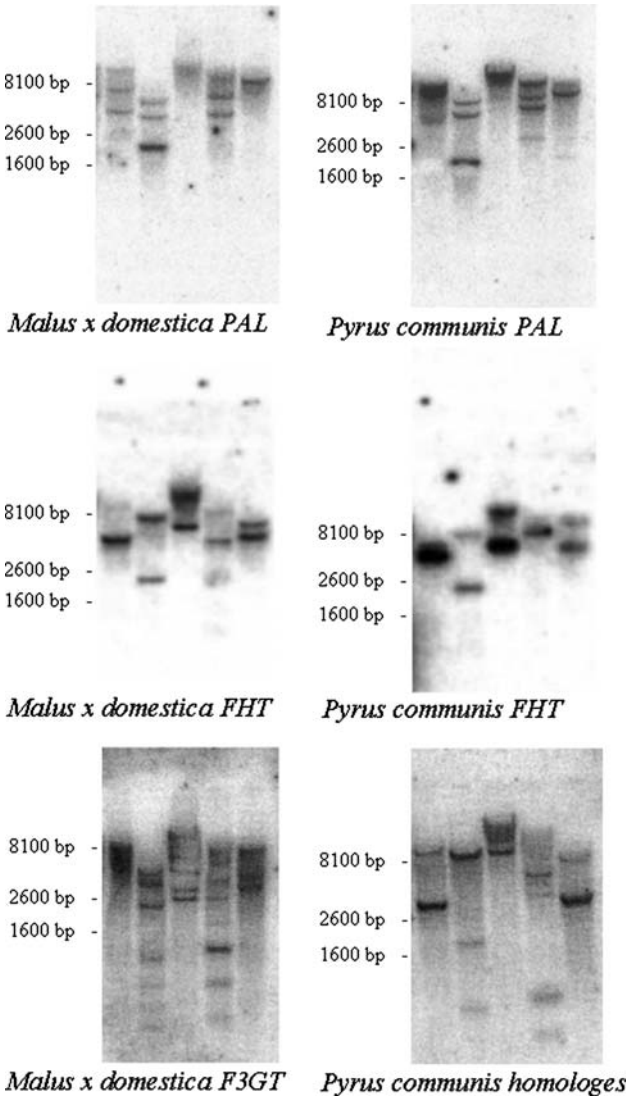

Fig. 2 Comparative Southern blot analysis of Malus and Pyrus genomic DNA with Malus probes: examples shown for *PAL*, *FHT*, and *F3GT* cDNA hybridizations. Lanes for each blot from left to right: restriction enzymes *Eco*RI, *Hind*III, *Sal*I, *Sac*I, and *Xba*I

Comparison of substrate specificities of recombinant *Pyrus* enzymes with known metabolic end product analyses of *Pyrus* fruits and leaves (cited above) allows to deduce the metabolic route realized in *Pyrus* tissues (Fig. 1). PAL converts phenylalanine to cinnamic acid. After 4-hydroxylation to *p*-coumaric acid and CoA conjugation, the product *p*-coumaroyl-CoA together with 3 malonyl-CoA is converted to the flavanone naringenin by the enzyme pair

Table 3 Comparison of *Pyrus* and *Malus* flavonoid cDNAs/genes with respect to gene copy numbers by Southern analysis, number of variable positions of the coding sequences (*S* substitutions, *D* deletions, *I* insertions, *Pyrus*, and *Malus* sequences given in Table 1 were compared), and enzyme substrate acceptance

Gene	Gene copy number		cds (*Pyrus*) length bp/aa	Differing nucleotides *Pyrus*/*Malus*	Differing amino acids *Pyrus*/*Malus*	Effective substrates of *Pyrus* enzymes (bold: natural substrates)
	Malus	*Pyrus*				
PAL	3–4	3–4	2,163/720	S 225	S 38	**Phe**
CHS	4–5	4–5	1,176/391	S 32	S 2	**Coum-CoA/Caff-CoA + Mal-CoA**
CHI	4–7	4–7	660/219	S 40/D 42	S 14/D 14	**NAR-Chalcone**
FHT	2	2	1,095/364	S 22/D 3	S 7/D 1	**ERI**, **NAR**
FLS	2–3	3–4	1,014/337	S 24	S 6	**DHK**, **DHQ**
DFR/FNR	3–5	2–3	1,044/347	S 21/D 6/I 3	S 2/D 2/I 1	**DHQ**, DHK, ERI, DHF, GAR
LAR1	n.d.	2	1,059/352	S 32/D 6	S 8/D 2	**LCy**, LPg, LDp
LAR2	n.d.		1,059/352	S 30/D 9	S 11/D 3	**LCy,** LPg, LDp
ANS	1	1–2	1,074/357	S 15	S 4	**LCy**
ANR	n.d.	2–3	1,020/339	S 21	S 7	**Cy**, Pg, Dp
F3GT	4–5	2–3	–	–	–	–
F7GT	~9	~4	1,446/481	S 44	S 23	NAR, **ERI**, Km, Qu **+UDP-Gluc**

Caff-CoA caffeoyl-CoA, *Coum-CoA p*-coumaroyl-CoA, *Cy* Cyanidin, *DHF* dihydrofisetin, *DHK* dihydrokaempferol, *DHQ* dihydroquercetin, *Dp* Delphinidin, *ERI* eriodictyol, *GAR* garbanzol, *Km* kaempferol, *LCy* leucocyanidin, *LDp* leucodelphinidin, *LPg* leucopelargonidin, *Mal-CoA* malonyl-CoA, *NAR* naringenin, *Phe* Phenylalanin, *Pg* Pelargonidin, *Qu* quercetin, and *UDP-Gluc* UDP-glucose

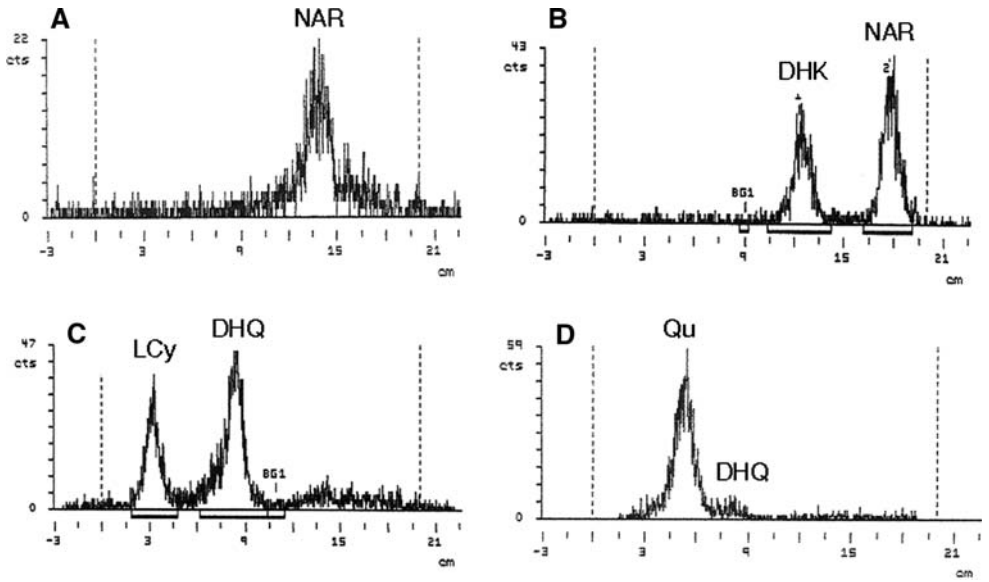

Fig. 3 Examples of gene-function verifications with selected recombinant *Pyrus* enzymes: Radioscans of TLCs on cellulose with solvent system *CAW* (chloroform/glacial acetic acid/water 10/9/1) from incubation of: **a** [^{14}C]-malonyl CoA and *p*-coumaroyl-CoA with enzyme preparations from yeast expressing the *CHS* cDNA, **b** [^{14}C]-NAR in the presence of 2-oxoglutarate, ascorbate and Fe^{2+} with enzyme preparations from yeast expressing the *FHT* cDNA, **c** [^{14}C]-DHQ in the presence of NADPH with enzyme preparations from yeast expressing the *DFR* cDNA, **d** [^{14}C]-DHQ in the presence of 2-oxoglutarate, ascorbate, and Fe^{2+} with enzyme preparations from yeast expressing the *FLS* cDNA. Abbreviations: *NAR* naringenin, *DHK* dihydrokaempferol, *DHQ* dihydroquercetin, *LCy* leucocyanidin, *Qu* quercetin

CHS and CHI. Alternatively, in some plants the dihydroxylated caffeoyl-CoA is produced from *p*-coumaric acid/*p*-coumaroyl-CoA. Also caffeoyl-CoA may thus be used to form a flavanone by CHS/CHI, resulting in the B-ring dihydroxylated eriodictyol, as *Pyrus* CHS possesses both substrate specificities. However, in most plant species with dihydroxylated flavonoids, like *Malus*, second B-ring hydroxylation is performed by a flavonoid 3′hydroxylase acting on flavanones (or dihydroflavonols), but this enzyme is not demonstrated for *Pyrus* yet. The major physiological

substrate of FHT in *Pyrus* is eriodictyol which is 3-hydroxylated to dihydroquercetin, the last common precursor of flavan 3-ols (catechins), flavonols and anthocyanins. Eriodictyol is also the natural substrate of the F7GT, which catalyzes the formation of eriodictyol 7-*O*-glucoside. Despite possessing F7GT activity, its sequence identity to F5GTs is also high (data not shown), but it does not possess a F5GT activity. Dihydroquercetin is oxidized by FLS to the flavonol quercetin. Only minor amounts of the B-ring monohydroxylated flavonol kaempferol are formed from dihydrokaempferol as another substrate for FLS. Kaempferol is the only B-ring monohydroxylated flavonoid end product in *Pyrus*. Dihydroquercetin is also substrate for DFR producing leucocyanidin. (The side activity of DFR as FNR converting eriodictyol to luteoforol is only observed in *Pyrus* tissue with artificially blocked FHT activity (Fischer et al. 2003; Halbwirth et al. 2006)). Leucocyanidin is either converted by LAR 1 or LAR 2 to catechin, or by ANS to form cyanidin. The latter is reduced by ANR to epicatechin, or glycosylated to build various cyanidin 3-*O*-glycosides.

Discussion

Flavonoid enzymes

Differences in the substrate acceptance of recombinant DFR enzymes from *Pyrus* and *Malus* were observed although only a few exchanges at the amino acid sequence existed (Fischer et al. 2003). When more than one cultivar from each genus was considered, as for *DFR* (Fischer et al. 2003) and *FHT* (Halbwirth et al. 2006), some seemingly genus specific amino acid substitutions could be identified in addition to the sequence variation observed between cultivars of either genus. Also for gene copy numbers variation was found between *Pyrus* and *Malus*, most obvious for either *F3GT* or *F7GT*, which each show quite different gene copy numbers and band patterns in Southern analyses probed with *Malus* cDNAs. Furthermore, only *F3GT* cDNA could not be cloned by the approach described, even if no alternative approaches were undertaken in comparison to the other cloning tasks. In contrast, the distantly related *F7GT* could be cloned, relying on a *Malus F7GT* sequence. Correspondingly to the differences at the gene level, glycosylation patterns of flavonoids of *Pyrus* and *Malus* are divergent.

Pyrus polyphenols

Beyond flavonoids, there are considerable differences in the polyphenol composition of *Pyrus* compared to *Malus*. Most important, pears contain the *p*-hydrochinone arbutin as a glucoside, but lack the dihydrochalcone phloretin of *Malus*; many anthocyanins and flavonol glycosides differ, too. These differences were also exploited for chemotaxonomic differentiation (Challice 1981) and are practically applied for juice authenticity control (Schieber et al. 2001). The presence of different polyphenols indicates that, in addition, several *Pyrus*-specific polyphenol enzymes or enzyme functions have to exist.

Pyrus gene cloning

The applicability of a homology cloning approach for *Pyrus* was indicated by the positive result of the Southern analyses with existent *Malus* cDNAs. The cloning approach solely relied on sequence similarities in transcribed, but non-coding parts (5′ leader and 3′ trailer) of known *Malus* sequences. When the various 5′ and 3′ primer combinations were tested for each gene, mostly 50% of the RT-PCR reactions resulted at least in low amounts of RT-PCR products of the expected size. Homology approaches relying on *Malus* sequences will thus be valuable for cloning other *Pyrus* genes. Considering the current number of accessions of the genera *Pyrus*, *Malus*, *Prunus, Fragaria*, and *Rosa* in GenBank (Table 4), it is obvious that *Malus* sequences are the best source of sequence information for *Pyrus* gene cloning, especially by assembling *Malus* expressed sequence tags (ESTs) (Newcomb et al. 2006) as demonstrated in this work for *PAL* and *CHI*. Even if less related, *Prunus* sequences might become a valuable source of information as well.

Marker development/transgenic plants

Genetic markers for marker-assisted selection are especially valuable with respect to the long generation time of *Pyrus*, demanding highly efficient selection measures. Recently, synteny between *Malus* and *Pyrus* chromosomes was reviewed (Arus et al. 2006), and simple sequence repetition (SSR) markers have been reported to be transferable from *Malus* to *Pyrus* (Yamamoto et al. 2001; Pierantoni et al. 2004). The *Pyrus* cDNA sequences of

Table 4 Current numbers of sequence accessions for the genera *Pyrus*, *Malus*, *Prunus, Fragaria*, and *Rosa* in GenBank (27/11/2006)

Genus	Subfamily	Core nucleotides	Expressed sequence tags	Genome survey sequences
Pyrus	Maloideae	663	1,592	0
Malus	Maloideae	2,061	260,318	20
Prunus	Prunoideae	4,058	92,122	188
Fragaria	Rosoideae	1,379	19,562	18
Rosa	Rosoideae	6,953	41,614	2

flavonoid genes obtained can serve as a base to develop non-anonymous markers for relevant alleles of the structural flavonoid genes in breeding for color, for improved flavonoid content with respect to pathogen resistance or for health-promoting fruit components.

In addition, the cloned cDNAs of *Pyrus ANR*, *LAR1*, and *LAR2* allow metabolic engineering aimed at plants with higher contents of catechins and proanthocyanidins (Xie et al. 2006), making use of *Pyrus* cDNAs rather than foreign sequences for overexpression or silencing strategies. It was reported that public acceptance of transgenic crops is better when genes of the same species are used (Fischer and Weber 2005).

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