Article

Transcriptome analysis discovered the secrets of betalain biosynthesis in the fruit of Red Pitayas (*Hylocereus costaricensis*)

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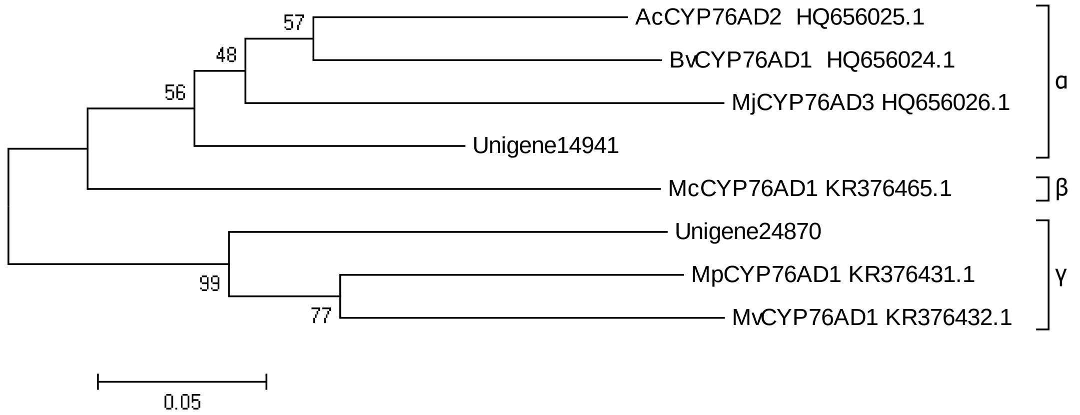


Figure S1. Phylogeny of the CYP76AD1 lineage, delimiting the CYP76AD1-α, -β and –γ clades. CYP76AD1 contained three subunit types, CYP76AD1-α recovered in *Amaranthus cruentus*, *Beta vulgaris* and *Mirabilis jalapa*, respectively. CYP76AD1-β recovered in *Mollugo cerviana*; CYP76AD1-γ recovered in *Mollugo pentaphylla* and *Mollugo verticillata* [34]. Unigene 14941 form a clade with CYP76AD1-α and Unigene24870 form a clade with CYP76AD1-γ.

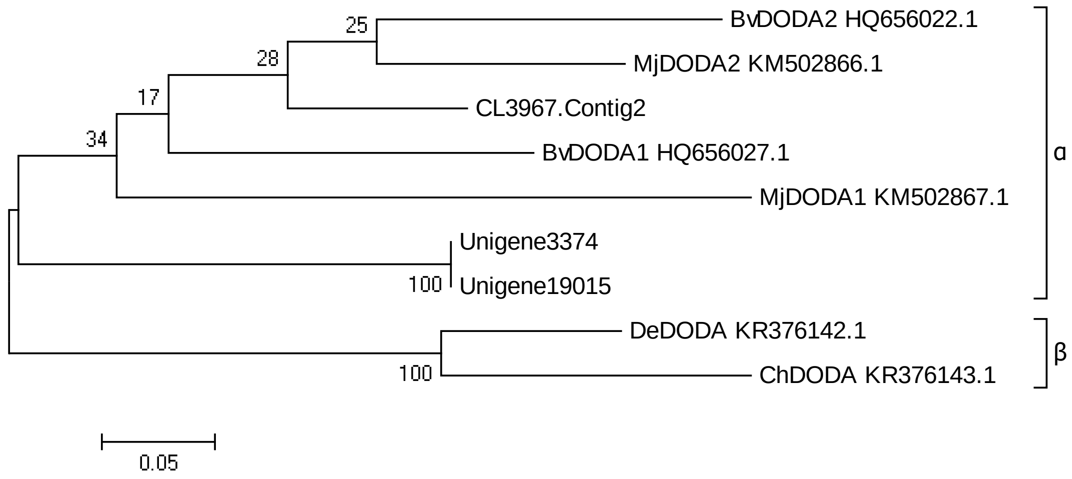


Figure S2. Phylogeny of the DODA lineage, delimiting the DODA-α, -β and –γ clades. DODA contained two subunit types, DODA–α recovered in *Beta vulgaris* and *Mirabilis jalapa*. DODA–β recovered in *Delosperma echinatum* and *Cypselea humifusa* [34]. CL3967, Unigene3374 and Unigene19015 form a clade with DODA –α.

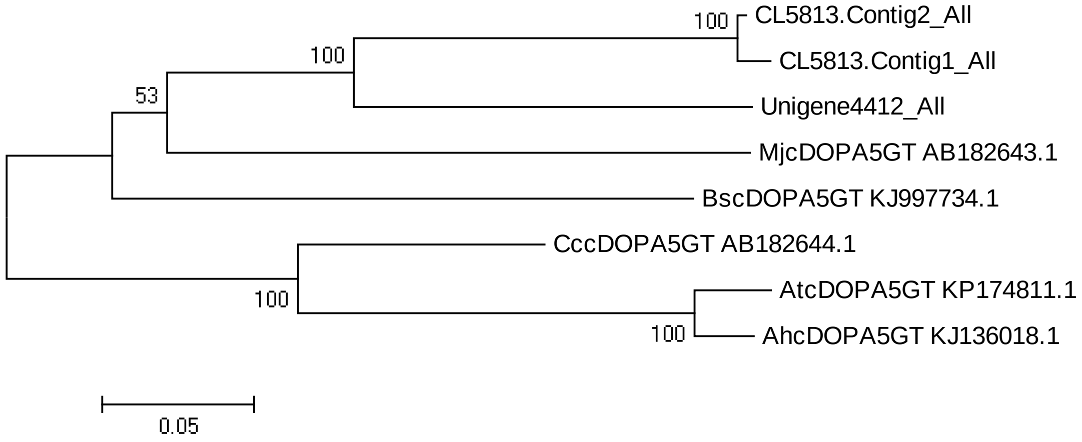


Figure S3. Phylogeny of the cDOPA5GT lineage. CL5813.Contig1, CL5813.Contig1 and Unigene4412 form a clade with cDOPA5GT in variety of species, which include *Mirabilis jalapa*, *Bougainvillea spectabilis*, *Celosia cristata*, *Amaranthus tricolor* and *Amaranthus hypochondriacus*.

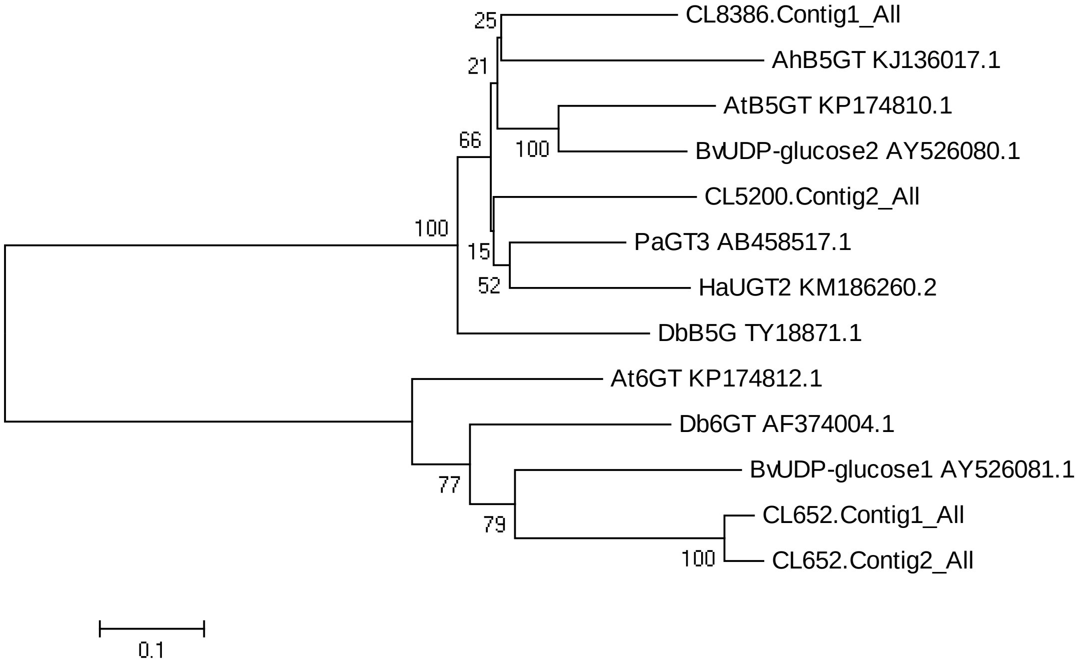


Figure S4. Phylogeny of the 5GT and 6GT lineage. CL5200.Contig2 and CL8386.Contig1 form a clade with 5GT in *Amaranthus hypochondriacus, Amaranthus tricolor, Beta vulgaris ,Phytolacca Americana, Haloloxylon ammodedron, Dorotheanthus bellidiformis*. While CL652.Contig1 and CL652.Contig2 form a clade with 6GT in *Amaranthus tricolor, Dorotheanthus bellidiformis, Beta vulgaris*.

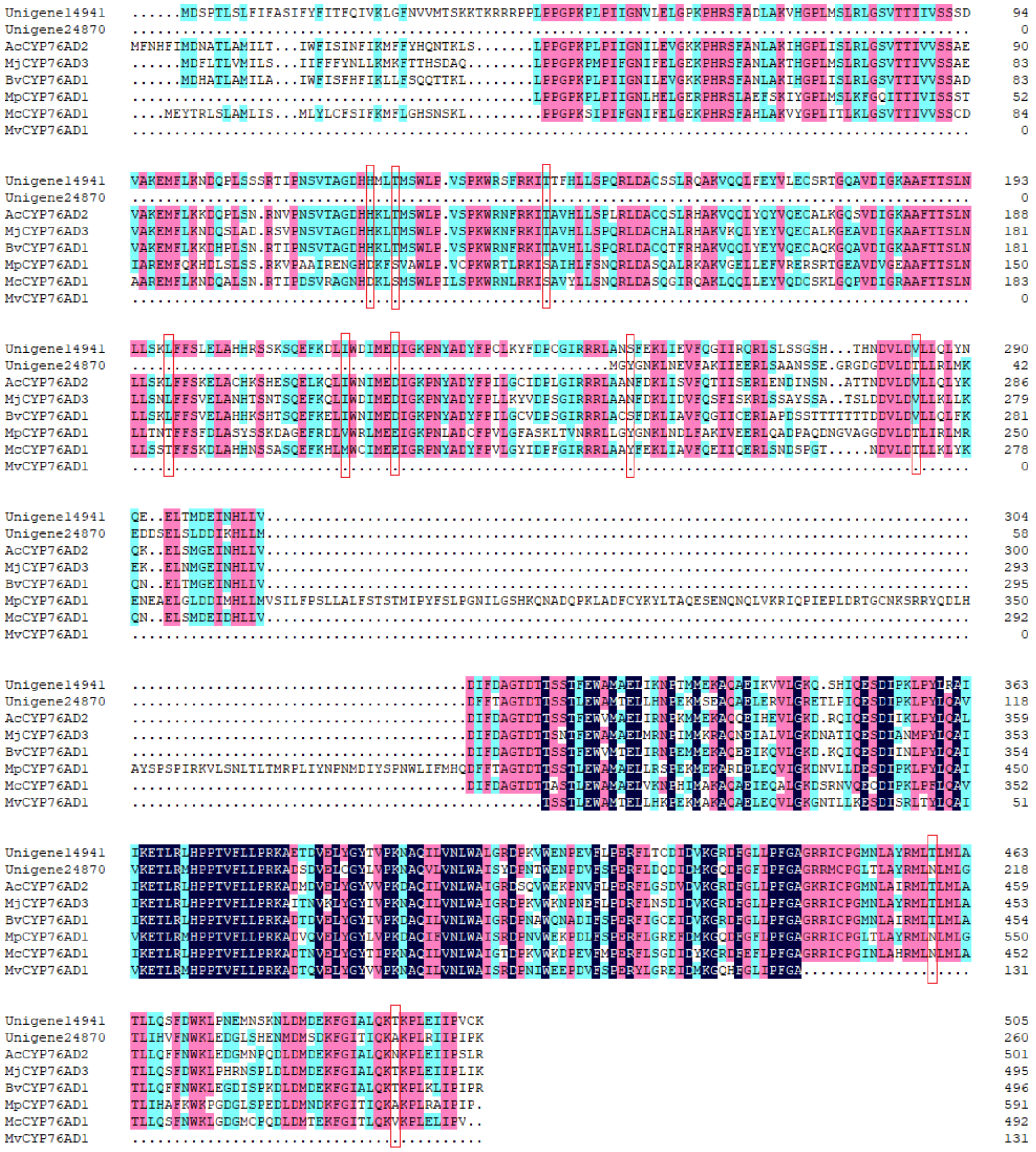
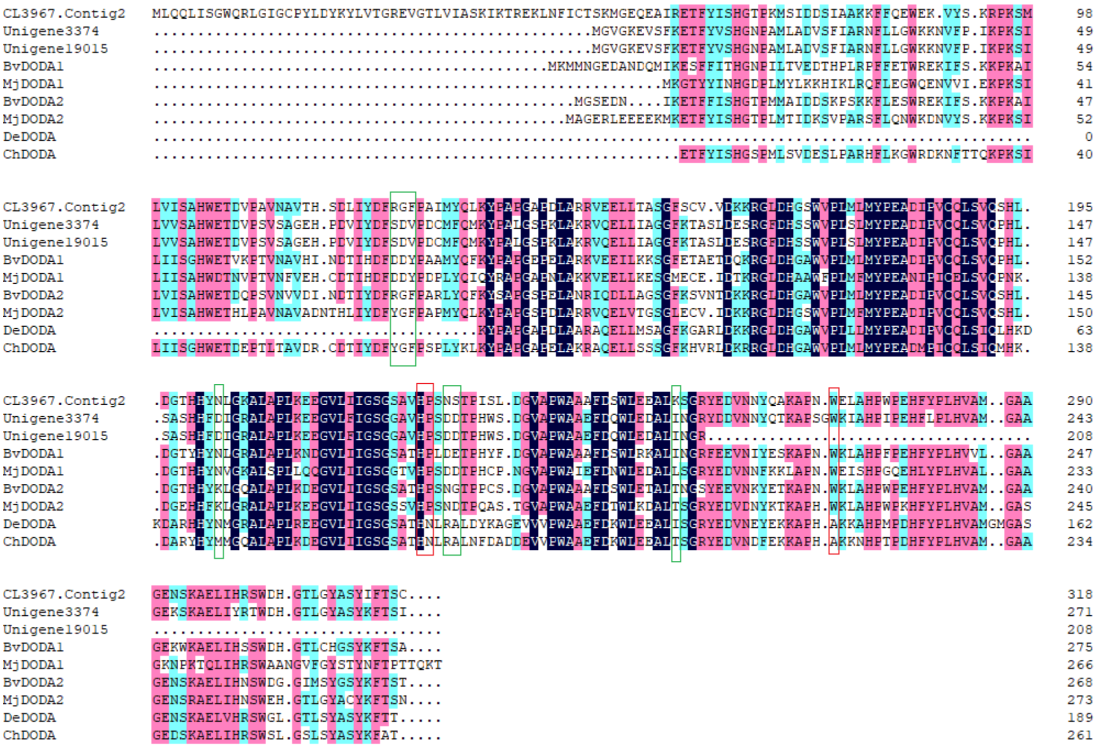


Figure S5. Amino acid sequences alignment of CYP76AD1, red in-line indicates the isomer-invariant residue. Based on Brockington's classification system, for CYP76AD1, several residues were also found to be diagnostic for the CYP76AD1-α clade, including histidine at site 111, threonine at sites 114 and 131, leucine at site 186, isoleucine at site 207, aspartic acid at site 213, asparagine at site 241 and valine at site 275, threonine at site 450, threonine at the site 486. While several residues were found to be diagnostic for the CYP76AD1-β clade, including aspartic acid at site 111, serine at sites 114 and 131, threonine at site 186, methionine at site 207, glutamic acid at site 213, tyrosine at site 241 and threonine at site 275, asparagine at site 450, valine at the site 486 [34]. CYP76AD1-β is closest to CYP76AD1-γ. Several residues were found to be diagnostic for the CYP76AD1-γ clade, including aspartic acid at site 111, serine at sites 114 and 131, threonine at site 186, glutamic acid at site 213, tyrosine at site 241 and threonine at site 275, asparagine at site 450, valine at the site 486. Residues are numbered on the basis of B. vulgaris CYP76AD1-α. Unigene 14941 is closest to CYP76AD1-α and Unigene24870 is closest to CYP76AD1-γ



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CL3967.Contig2   MLQQLISGWQRLGIGCPYLDYKYLVTGREVGTLVIASKIKTREKLNFICTSKMGEQEAIRETFYISHGTPKMSIDDSIAAKKFFQEWEK.VYS.KRPKSM    98
Unigene3374      ..................................................MGVGKEVSFKETFYVSHGNPAMLADVSFIARNFLLGWKKNVFP.IKPKSI    49
Unigene19015     ..................................................MGVGKEVSFKETFYVSHGNPAMLADVSFIARNFLLGWKKNVFP.IKPKSI    49
BvDODA1          .............................................MKMMNGEDANDQMIKESFFITHGNPILTVEDTHPLRPFFETWREKIFS.KKPKAI    54
MjDODA1          ..........................................................MKGTYYINHGDPLMYLKKHIKLRQFLEGWQENVVI.EKPKSI    41
BvDODA2          ................................................MGSEDN....IKETFFISHGTPMMAIDDSKPSKKFLESWREKIFS.KKPKAI    47
MjDODA2          ...............................................MAGERLEEEEKMKETFYISHGTPLMTIDKSVPARSFLQNWKDNVYS.KKPKSI    52
DeDODA           ....................................................................................................     0
ChDODA           ............................................................ETFYISHGSPMLSVDESLPARHFLKGWRDKNFTTQKPKSI    40

CL3967.Contig2   LVISAHWETDVPAVNAVTH.SDLIYDFRGFPAIMYQLKYPAPGAPDLARRVEELLTASGFSCV.VDKKRGLDHGSWVPLMLMYPEADIPVCQLSVQSHL.   195
Unigene3374      LVVSAHWETDVPSVSAGEH.PDVIYDFSDVPDCMFQMKYPALGSPKLAKRVQELLIAGGFKTASLDESRGFDHSSWVPLSLMYPEADIPVCQLSVQPHL.   147
Unigene19015     LVVSAHWETDVPSVSAGEH.PDVIYDFSDVPDCMFQMKYPALGSPKLAKRVQELLIAGGFKTASLDESRGFDHSSWVPLSLMYPEADIPVCQLSVQPHL.   147
BvDODA1          LIISGHWETVKPTVNAVHI.NDTIHDFDDYPAAMYQFKYPAPGEPELARKVEEILKKSGFETAETDQKRGLDHGAWVPLMLMYPEADIPVCQLSVQPHL.   152
MjDODA1          LIISAHWDTNVPTVNFVEH.CDTIHDFDDYPDPLYQIQYRAPGAPNLAKKVEELLKESGMECE.IDTKRGLDHAAWFPLMFMYPEANIPICELSVQPNK.   138
BvDODA2          LVISAHWETDQPSVNVVDI.NDTIYDFRGFPARLYQFKYSAPGSPELANRIQDLLAGSGFKSVNTDKKRGLDHGAWVPLMLMYPEADIPVCQLSVQSHL.   145
MjDODA2          LVISAHWETHLPAVNAVADNTHLIYDFYGFPAPMYQLKYPAPGSPDLARRVQELVTGSGLECV.IDKKRGLDHGSWVPLMFMYPEADIPVCQLSVQSHL.   150
DeDODA           .....................................KYPAPGAPDLAARAQELLMSAGFKGARLDKKRGLDHGAWVPLLLMYPEADIPVCQLSIQLHKD    63
ChDODA           LIISGHWETDEPTLTAVDR.CDTIYDFYGFPSPLYKLKYPAPGAPELAKRAQELLSSSGFKHVRLDKRRGLDHGAWVPLMLMYPEADMPICQLSIQMHK.   138

CL3967.Contig2   .DGTHHYNLGKALAPLKEEGVLIIGSGSAVHPSNSTPISL.DGVAPWAAAFDSWLEEALKSGRYEDVNNYQAKAPN.WELAHPWPEHFYPLHVAM..GAA   290
Unigene3374      .SASHHFDIGRALAPLKEEGVLFIGSGGAVHPSDDTPHWS.DGVAPWAAEFDQWLEDALINGRYDDVNNYQTKAPSGWKIAHPIPEHFLPLHVAM..GAA   243
Unigene19015     .SASHHFDIGRALAPLKEEGVLFIGSGGAVHPSDDTPHWS.DGVAPWAAEFDQWLEDALINGR.....................................   208
BvDODA1          .DGTYHYNLGRALAPLKNDGVLIIGSGSATHPLDETPHYF.DGVAPWAAAFDSWLRKALINGRFEEVNIYESKAPN.WKLAHPFPEHFYPLHVVL..GAA   247
MjDODA1          .DGTHHYNVGKALSPLLQQGVLIIGSGGTVHPSDDTPHCP.NGVAPWAIEFDNWLEDALLSGRYEDVNNFKKLAPN.WEISHPGQEHLYPLHVAL..GAA   233
BvDODA2          .DGTHHYKLGQALAPLKDEGVLIIGSGSATHPSNGTPPCS.DGVAPWAAAFDSWLETALTNGSYEEVNKYETKAPN.WKLAHPWPEHFYPLHVAM..GAA   240
MjDODA2          .DGEHHFKLGRALAPLKEEGVLIIGSGSSVHPSNDTPQAS.TGVAPWAAEFDTWLKDALTSGRYEDVDNYKTKAPH.WKLAHPWPKHFYPLHVAM..GAS   245
DeDODA           KDARHHYNMGRALAPLREEGVLIIGSGSATHNLRALDYKAGEVVVPWAAEFDKWLEEALISGRYEDVNEYEKKAPH.AKKAHPMPDHFYPLHVAMGMGAS   162
ChDODA           .DARYHYMMGQALAPLKDEGVLIIGSGSATHNLRALNFDADDEVVPWAAEFDKWLEEALTSGRYEDVNDFEKKAPH.AKKNHPTPDHFYPLHVAM..GAA   234

CL3967.Contig2   GENSKAELIHRSWDH.GTLGYASYIFTSC....                                                                     318
Unigene3374      GEKSKAELIYRTWDH.GTLGYASYKFTSI....                                                                     271
Unigene19015     .................................                                                                     208
BvDODA1          GEKWKAELIHSSWDH.GTLCHGSYKFTSA....                                                                     275
MjDODA1          GKNPKTQLIHRSWAANGVFGYSTYNFTPTTQKT                                                                     266
BvDODA2          GENSKAELIHNSWDG.GIMSYGSYKFTST....                                                                     268
MjDODA2          GENSRAELIHNSWEH.GTLGYACYKFTSN....                                                                     273
DeDODA           GENSKAELVHRSWGL.GTLSYASYKFTT.....                                                                     189
ChDODA           GEDSKAELIHRSWSL.GSLSYASYKFAT.....                                                                     261
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Figure S6. Amino acid sequences alignment of DODA, the red box line indicates the conserved residues of each isomer, and the green box line indicates the conserved residues to classify DODA1 and DODA2. Based on Brockington's classification system, two residues were diagnostic for the DODA-a clade, including proline at site 176 and asparagine at site 220. Two residues were diagnostic for the DODA-a clade, including proline at site 176 and asparagine at site 220. Two residues are diagnostic for the DODA-β clade, including asparagine at site 176 and alanine at site 220 [34]. CL3967, Unigene3374 and Unigene19015 are closest to DODA –α. Seven residues that are diagnostic for the DODA1 clade, including aspartic acid, aspartic acid and Tyrosine at site 74, 75 and 76, respectively, Asparagine at site 152, aspartic acid at site 178, glutamic acid at site 179, Isoleucine at site 203 [35]. CL3967 belongs to DODA2, while Unigene3374 and Unigene19015 are DODA1-like. Although Unigene19015 and Unigene3374 have the same amino acid sequences, Unigene19015 has the early termination at site 208. Residues are numbered on the basis of *B. vulgaris* DODA2.

Table S1. Summary of transcriptome sequencing data in *Hylocereus undatus* and *Hylocereus costaricensis*.

Sample	Total Raw Reads(M)	Total Clean Reads(M)	Total Clean Bases (Gb)	Clean Reads Q20 (%)	Clean Reads Q30 (%)	Clean Reads Ratio(%)
red1	52.87	52.84	7.93	98.55	95.76	99.95
red2	52.74	52.73	7.91	98.56	95.79	99.97
white1	36.38	36.36	5.45	98.43	95.55	99.95
white3	44.67	44.64	6.7	98.26	95.26	99.92

Table S2. Summary statistics of functional annotation for unigenes.

Sample	Total Number	Total Length	Mean Length	N50	N70	N90	GC(%)
red1	35203	32005035	909	1459	881	379	43.67
red2	39033	38336515	982	1592	993	410	43.52
white1	44167	51429992	1164	1856	1238	519	43.09
white3	58225	71951833	1235	1945	1341	572	42.71
All-Unigene	79049	1.05E+08	1333	2163	1482	620	42.55

Table S3. Differential expression analysis of Unigenes related to betalains and anthocyanins biosynthesis.

Pathway	Gene	KEGG ID (EC: NO)	Reference genes Length (bp)	GeneID	Length (bp)	Red-Expression	White-Expression	RED/WHITE	Up/Down-Regulation	Padj	Pvalue
Betalain	P450-CYP76AD	-	1488	Unigene14941	2129	2473.5980	2.0093	1231.1048	Up	0.0000	0.0000
				Unigene24870	1123	5.3334	8.1054	0.6580	Down	0.7751	0.5993
				Sum		2478.9314	10.1146	245.0834	Up		
	DODA	K15777 (1.13.11.-)	810	Unigene3374	1284	880.8943	1.7528	502.5507	Up	0.0000	0.0000
				Unigene19015	1592	39.1489	0.3090	126.6955	Up	0.0000	0.0000
				CL3967.Contig1	1533	25.4439	18.6377	1.3652	Up	0.7004	0.4929
				CL3967.Contig2	2037	215.9282	16.8624	12.8053	Up	0.0000	0.0000
				CL3967.Contig3	1549	85.2638	168.6405	0.5056	Down	0.0028	0.0004
				CL3967.Contig4	2429	164.5340	0.3386	485.9392	Up	0.0000	0.0000
				CL3967.Contig5	2933	22.3344	32.8289	0.6803	Down	0.5632	0.2873
				CL3967.Contig6	1471	115.9562	0.3205	361.7658	Up	0.0000	0.0000
				Sum		1549.5036	239.6904	6.4646	Up		
	cDOPA5GT	K15776 (2.4.1.-)	1500	CL5813.Contig1	1725	122.4929	4.9142	24.9263	Up	0.0000	0.0000
				CL5813.Contig2	2147	47.0533	80.1905	0.5868	Down	0.1166	0.0338
				Unigene4412	1420	36.8831	15.8661	2.3246	Up	0.1255	0.0370
				Sum		206.4292	100.9707	2.0444	Up		
	5GT	K15774	1467	CL8386.Contig1	1740	76.8512	105.1601	0.7308	Down	0.6697	0.4178

		(2.4.1.-)		CL5200.Contig2	1449	58.4911	5.2783	11.0814	Up	0.0005	0.0001
				Sum		135.3423	110.4384	1.2255	Up		
	6GT	K15775 (2.4.1.-)	1443	CL652.Contig1	1556	21.3411	82.7718	0.2578	Down	0.0082	0.0014
				CL652.Contig2	1717	124.7915	7.2972	17.1013	Up	0.0000	0.0000
				CL652.Contig3	482	2.7056	0.7965	3.3969	Up	0.6264	0.3450
				CL652.Contig4	518	6.3597	0.5264	12.0815	Up	0.1567	0.0489
				Sum		155.1979	91.3919	1.6982	Up		
Anthocyanin	C4H	K00487 （1.14.13.11）	1620	CL5457.Contig1	2030	426.3982	2021.5383	0.2109	Down	0.0000	0.0000
				Sum		426.3982	2021.5383	0.2109	Down		
	CHS	K00660 （2.3.1.74）	669	CL2124.Contig1	887	37.7740	1.9125	19.7511	Up	0.0000	0.0000
				CL2124.Contig2	685	0.6861	17.9800	0.0382	Down	0.0028	0.0004
				CL2124.Contig3	5300	72.6080	135.0453	0.5377	Down	0.2138	0.0733
				Unigene4340	2166	66.9432	30.6317	2.1854	Up	0.0772	0.0204
				Unigene6998	477	2.6340	3.1328	0.8408	Down	0.9191	0.8421
				Sum		180.6453	188.7024	0.9573	Down		
	CHI	K01859 (5.5.1.6)	702	Unigene36137	871	10.4987	0.3050	34.4220	Up	0.0108	0.0020
				Unigene6320	1151	40.9843	12.3565	3.3168	Up	0.0166	0.0033
				Sum		51.4830	12.6615	4.0661	Up		
	F3H	K00475 (1.14.11.9)	1131	Unigene4833	1587	73.6927	261.8570	0.2814	Down	0.0000	0.0000
				Sum		73.6927	261.8570	0.2814	Down		
	F3'H	K05280 （1.14.13.21）	1608	Unigene14914	1721	9.8985	34.6906	0.2853	Down	0.0383	0.0087
				Sum		9.8985	34.6906	0.2853	Down		
	F3'5'H	K13083 （1.14.13.88）	1599								
	DFR	K13082(1.1.1.	1170								

		234/1.1.1.219)									
	ANS	K05277 （1.14.11.19）	462								
	FLS	K05278 (1.14.20.6)	1038	Unigene2990	1267	119.9337	237.2475	0.5055	Down	0.1009	0.0283
				sum		119.9337	237.2475	0.5055	Down		
	ANR	K08695 （1.3.1.77）	1041	CL2486.Contig2	1423	21.3233	214.0709	0.0996	Down	0.0000	0.0000
				CL2486.Contig1	1834	2.8514	16.5003	0.1728	Down	0.2554	0.0927
				Sum		24.1747	230.5712	0.1048	Down		
	LAR	K13081 （1.17.1.3）	1077	CL54.Contig4	1634	0.8029	10.1991	0.0787	Down	0.1611	0.0508
				CL54.Contig3	1509	1.8325	221.8759	0.0083	Down	0.0000	0.0000
				CL54.Contig2	1396	0.9132	12.4012	0.0736	Down	0.1462	0.0449
				CL54.Contig1	1271	1.2597	4.6547	0.2706	Down	0.5422	0.2701
				Sum		4.8083	249.1308	0.0193	Down		
Transcription factors	MYB	K09422	738	Unigene16444	2411	183.3128	748.0341	0.2451	Down	0.0000	0.0000
				Unigene14050	1143	19.2694	29.8014	0.6466	Down	0.4937	0.2335
				CL7.Contig2	2865	146.6169	0.3296	444.8723	Up	0.0000	0.0000
				Unigene16202	1048	13.6855	9.5435	1.4340	Up	0.7004	0.4846
				CL3418.Contig4	1381	0.2092	0.6780	0.3086	Down	0.6320	0.3541
				CL3418.Contig2	1341	86.4349	0.3112	277.7249	Up	0.0000	0.0000
				Unigene11816	1438	66.9351	97.7102	0.6850	Down	0.6829	0.4510
				Unigene5220	1183	17.2148	51.6077	0.3336	Down	0.0523	0.0127
				CL3418.Contig1	1530	2.0733	385.1637	0.0054	Down	0.0000	0.0000
				Unigene1252	1028	3.0084	12.0591	0.2495	Down	0.1403	0.0426
				Unigene17810	1302	29.2391	24.0746	1.2145	Up	0.7857	0.6122

				Unigene19345	1117	5.8893	37.4391	0.1573	Down	0.0101	0.0018
				Unigene14477	2650	184.2094	295.5953	0.6232	Down	0.2142	0.0735
				Sum		763.2877	1712.165	0.4458	Down		