***De novo* transcriptome analysis and comparative expression profiling of genes associated with the taste-modifying protein neoculin in *Curculigo latifolia* and *Curculigo capitulata* fruits**

Satoshi Okubo

Nippon Shokubai Kabushiki Kaisha Suita Kenkyujo

Kaede Terauchi

University of Tokyo

Shinji Okada

University of Tokyo

Takao Yamaura

The Yamashina Botanical Research Institute, Nippon Shinyaku Co., Ltd.

Keiko Abe

University of Tokyo

Tomiko Asakura (✉ asakura@mail.ecc.u-tokyo.ac.jp)

University of Tokyo

Research article

Keywords: NGS, RNA-seq, neoculin, NBS, NAS, *Curculigo capitulata*, *Curculigo latifolia*, expression profile, gene duplication

Posted Date: July 14th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-41288/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Version of Record: A version of this preprint was published at BMC Genomics on May 13th, 2021. See the published version at <https://doi.org/10.1186/s12864-021-07674-3>.

Abstract

Background *Curculigo latifolia* is a perennial plant found in Southeast Asia whose fruits contain the taste-modifying protein neoculin, which binds to sweet receptors to make sour fruits taste sweet. Although neoculin is similar to *Galanthus nivalis* agglutinin (GNA), which contains mannose-binding sites in its sequence and 3D structure, neoculin lacks such sites and has no lectin activity. Whether the fruits of *C. latifolia* and other *Curculigo* plants contain neoculin and/or GNA family genes is unclear.

Results We performed *de novo* RNA-seq assembly of the fruits of *C. latifolia* and the related *C. capitulata* and analyzed the expression patterns of neoculin and neoculin-like genes in both species in detail. We assembled 85,697 transcripts from *C. latifolia* and 76,775 from *C. capitulata* using Trinity and annotated them using public databases. We identified 70,371 unigenes in *C. latifolia* and 63,704 in *C. capitulata*. In total, 38.6% of unigenes from *C. latifolia* and 42.6% from *C. capitulata* shared high similarity between the two species. We identified ten neoculin-related transcripts in *C. latifolia* and 15 in *C. capitulata*. Genes encoding both the basic and acidic subunits of neoculin were present in both plants. We aligned these 25 transcripts and generated a phylogenetic tree. Many orthologs in both species shared high similarity, despite the low number of common genes, suggesting that these genes likely existed before the two species diverged. The relative expression levels of these genes, as indicated by their transcript per million (TPM) values, differed considerably between the two species: the TPM values of neoculin genes were 60 times higher in *C. latifolia* than in *C. capitulata*, whereas those of GNA family members were 15,000 times lower in *C. latifolia* than in *C. capitulata*.

Conclusions The genetic diversity of the neoculin-related genes strongly suggests that neoculin genes underwent duplication during evolution. The marked differences in the expression profiles of neoculin-related genes between *C. latifolia* and *C. capitulata* could be due to mutations in regions involved in transcriptional regulation. Comprehensive analysis of the genes expressed in the fruits of these two *Curculigo* species helped elucidate the origin of neoculin at the molecular level.

Background

Curculigo latifolia (Hypoxidaceae family, formerly classified in the Liliaceae family) is a perennial plant found in Southeast Asia, especially the Malay peninsula [1, 2]. According to the Royal Botanic Gardens, Kew, there are 27 species of *Curculigo* [3]. The genetic diversity and morphology of *Curculigo* have long been of interest [4–7]. *C. latifolia* and *C. capitulata* were previously reclassified as members of the *Molineria* genus, but recent discussions have suggested that they should be returned to the *Curculigo* genus. Here, we use the traditional name, *Curculigo*.

C. latifolia and *C. capitulata* have a similar appearance (Fig. 1), but their flower and fruit morphology differ, and *C. capitulata* is more widely distributed than *C. latifolia*. Both species are diploids ($2n = 18$; $x = 9$) [8]. *C. latifolia* is self-incompatible [9], but *C. capitulata* plants from various botanical gardens in Japan have not been successfully crossed. The flowers, roots, stems, and leaves of *Curculigo* plants have traditionally been used as medicines [10–15]. Notably, unlike *C. capitulata*, *C. latifolia* fruits produce a taste-modifying protein, neoculin, that makes sour-tasting foods or water taste sweet [1, 16, 17, 18].

Neoculin itself has sweet taste and is 550 times sweeter than sucrose (w/w) [19, 20]. Furthermore, neoculin has taste-modifying activity that converts sourness to sweetness; for example, sour lemon taste is changed to sweet orange. Neoculin is received by the human sweet taste receptor T1R2-T1R3 [21]. Moreover, sweetness is induced by water in the presence of neoculin, and some organic acids taste sweet after tasting of neoculin [22]. Neoculin consists of two subunits that form a heterodimer: the neoculin basic subunit (NBS), also called curculin [16], and the neoculin acidic subunit (NAS) [18, 23]. NBS is 11 kDa and consists of 114 amino-acid residues [16, 24], while NAS is 13 kDa and consists of 113 amino-acid residues. These subunits share 77% amino-acid identity [18]. Several important amino-acid residues that are responsible for the taste-modifying properties of neoculin have been identified: His11 of NBS is responsible for pH-dependent taste-modifying activity [25], and Arg48, Tyr65, Val72, and Phe94 function in the binding and activation of human sweet taste receptors [26]. Changes in the 3D structures of these proteins at these amino-acid residues are thought to contribute to the taste-modifying properties of neoculin [27, 28].

Lectins are proteins that recognize and bind to specific carbohydrate structures [29, 30]. Plant lectins are classified into 12 families. The GNA (*Galanthus nivalis* agglutinin) family of lectins, which are present in bulbs such as snowdrops (*Galanthus nivalis*) and daffodils (*Narcissus pseudonarcissus*) [31], are thought to function as defense or storage proteins. Both the amino-acid sequences and 3D structures of neoculin NBS and NAS are similar to those of GNA family members [32, 33], but NBS and NAS lack the mannose-binding site (MBS) and do not have lectin activity [34–36]. GNA family members in plants such as snowdrop contain one disulfide bond, which functions in intra-subunit bonding, but neoculin exhibits both intra-subunit bonding and one inter-subunit bond between NBS and NAS [32].

The fruit of *C. latifolia* contains 1.3 mg neoculin per fruit [37] or 1.3 mg per 1 g pulp [38]. This level is thought to be considerably higher than the levels of all proteins in typical edible fruits [39]. Although the taste-modifying activity of neoculin is well known, its role in *C. latifolia* is unknown. In addition, as neoculin is not a lectin, it not clear which lectins are expressed in *C. latifolia* fruit, especially lectins of the GNA family. In addition, whether other *Curculigo* species have neoculin or neoculin-like proteins is unknown.

Here, we compared the gene expression patterns in the fruits of *C. latifolia* and *C. capitulata* by RNA sequencing (RNA-seq). The aim of this study was to comprehensively analyze the two species from the viewpoint of amino-acid sequences and gene expression levels to shed light on the origin of neoculin.

Results

Sequencing and de novo assembly

We sequenced cDNA libraries from *C. latifolia* and *C. capitulata* using the Illumina HiSeq 2500 system. To analyze the data, we filtered out raw reads with average quality values < 20, reads with < 50 nucleotides, and reads with ambiguous 'N' bases. After removing adapter elements and filtering, we obtained 44,396,896 reads from *C. latifolia* and 43,863,400 from *C. capitulata*. High-quality reads from *C. latifolia* and *C. capitulata* were assembled into 85,697 and 76,775 contigs with a mean length of 775.14 bp and 743.71 bp, respectively, using Trinity 2.11. The distributions of transcript lengths and transcripts per million (TPM) values are shown in Additional File 1 and Additional File 2. The N50 values of *C. latifolia* and *C. capitulata* transcripts were 1,324 and 1,205, respectively (Table 1). Unigene clustering using CD-Hit revealed 70,371 unigenes in *C. latifolia* and 63,704 in *C. capitulata* (Table 1).

Table 1
Overview of *de novo* RNA-seq assembly of *Curculigo latifolia* and *C. capitulata* fruits.

	<i>C. latifolia</i>	<i>C. capitulata</i>
High-quality reads	44,396,896	43,863,400
Total Trinity genes	69,446	63,951
Total Trinity unigenes	70,371	63,704
Total Trinity transcripts	85,697	76,775
GC (%)	44.0	45.6
N10	3,214	2,676
N20	2,460	2,103
N50	1,324	1,205
Total assembled bases	66,426,868	57,098,016

Sequence annotation

Low annotation rate of the transcripts: we aligned all transcripts with sequences in various protein databases including the nonredundant protein (NR) database of the National Center for Biotechnology Information (NCBI), RefSeq, UniProt/Swiss-Prot, and Clusters of Orthologous Groups of proteins (COG) and selected the top hits from these matches. Only 38,144 of 85,697 transcripts (44.5%) in *C. latifolia* and 40,322 of 76,775 transcripts (52.5%) in *C. capitulata* were annotated with a threshold of $1e^{-10}$ by performing BLASTx using the NR, RefSeq, UniProt, and COG databases. All annotations are listed in Additional File 3. The number of annotated transcripts for each database is listed in Table 2. The low annotation rate suggests that the two *Curculigo* species are significantly different from the model plants that supply much of the information in the public databases.

Table 2
Number of functional annotations of the transcripts of *Curculigo latifolia* and *C. capitulata* fruits.

Annotated database	<i>C. latifolia</i>	<i>C. capitulata</i>
COG ¹	11,875	12,448
RefSeq	37,922	39,369
Uniprot	36,783	38,901
NR ²	37,118	39,340
All four databases	38,145	40,324
¹ COG: Clusters Groups of proteins.		
² NR: nonredundant protein databases of the National Center for Biotechnology Information.		

Conservation among monocotyledons: we performed BLASTx search of the transcripts against the NR database, followed by Blast2GO [40]. We estimated the similarity of the two *Curculigo* species to various other species by counting the number of hits from each species from the results of BLAST searches (Fig. 2). The top six most highly homologous species were monocotyledons, like *Curculigo*, supporting the view that the list of the assembled *Curculigo* genes are highly similar to known monocotyledon genes. The top six homologous species were the same for *C. latifolia* and *C. capitulata* in terms of both species and rank.

Expression of functionally similar genes between two species: using the COG database, we classified 11,875 transcripts from *C. latifolia* and 12,448 from *C. capitulata* into functional categories (Fig. 3). No significant differences were observed between the two species. This result supports that these two species have functionally similar genes.

We analyzed the functions of the assembled transcripts via Gene Ontology (GO) analysis (Additional File 4). Again, no significant differences were observed between the two species. These results suggest that the repertoires of genes in the two species were similar to known genes.

Comparative analysis of genes expressed in *C. latifolia* and *C. capitulata* fruits

Using the unigene sequences, we analyzed the similarity of genes in *C. latifolia* and *C. capitulata*. We performed BLAST searches using each transcript from one species as the query sequence against all transcripts from the other species at E -values $1e^{-5}$ and selected the top hits. We defined unigenes with high similarity between the two species as 'common genes' and unigenes with low similarity between the species or present in only one species as 'unique genes'. In total, 38.6% of genes in *C. latifolia* and 42.6% of genes in *C. capitulata* were common genes (Fig. 4). The relatively small number of common genes between these species suggests that a long time has passed since their divergence. A long divergence time is consistent with the results of lineage analysis based on plastid DNA from Hypoxidaceae family members, which showed that the *Curculigo* genus belongs to a single clade, but *C. latifolia* and *C. capitulata* are not the most closely related species within this clade [5].

We investigated the proportions of annotated genes in these species using the COG, RefSeq, UniProt, and NR databases (shown in Table 2). Among the common genes, 17,260 and 17,139 genes were annotated (63.6% and 63.1% of common genes) in *C. latifolia* and *C. capitulata*, respectively. By contrast, there were 11,565 genes (26.8% of unique genes) annotated unique genes in *C. latifolia* and 14,718 (40.3% of unique genes) in *C. capitulata*. Thus, the annotation rate was higher for common genes than for unique genes despite the smaller number of common genes. Perhaps many of the genes common to both species are also common genes in the model plants that are mostly highly represented in the databases.

We then compared the expression profiles of 27,155 common genes in *C. latifolia* vs. *C. capitulata*. Although the sequences of the corresponding genes in *C. latifolia* and *C. capitulata* were similar, their expression profiles were not necessarily equivalent. Only 111 of the 27,155 common genes had TPM ratios ≥ 50 (Table 3). Of these 111 genes, five were neoculin-related genes, meaning that the expression profiles of neoculin-related genes significantly differ between the two species.

Table 3
Comparison of the expression profiles of *Curculigo latifolia* and *C. capitulata*.

<i>C. latifolia</i>			<i>C. capitulata</i>		
TRYNITY_ID	RefSeq	TPM	TRYNITY_ID	RefSeq	TPM
DN19492_c6_g1_i1	trans-resveratrol di-O-methyltransferase	36282	DN19332_c0_g2_i1	trans-resveratrol di-O-methyltransferase	277
DN20774_c6_g2_i5	trans-resveratrol di-O-methyltransferase	31648	DN20405_c1_g1_i2	trans-resveratrol di-O-methyltransferase	573
*DN22219_c0_g1_i1	mannose-specific lectin-like	7634	*DN16562_c0_g1_i1	mannose-specific lectin-like	80
DN22040_c0_g1_i1	chalcone synthase-like	6483	DN22230_c0_g1_i1	chalcone synthase-like	69
DN39489_c0_g1_i1	cinnamoyl-CoA reductase 1-like	4584	DN43958_c0_g1_i1	cinnamoyl-CoA reductase 1-like	37
DN17418_c0_g1_i1	benzyl alcohol O-benzoyltransferase	2848	DN20771_c2_g1_i3	benzyl alcohol O-benzoyltransferase	18
DN18625_c0_g1_i1	glutelin type-A 1-like	2641	DN18515_c0_g1_i1	glutelin type-A 1-like	35
DN20161_c0_g1_i1	probable polyamine oxidase 5	2333	DN20921_c0_g1_i1	probable polyamine oxidase 5	38
DN20171_c0_g1_i1	pyruvate decarboxylase 1 isoform X1	2140	DN19622_c0_g1_i1	pyruvate decarboxylase 1 isoform X1	30
DN19390_c0_g1_i1	benzyl alcohol O-benzoyltransferase-like	1721	DN20336_c0_g1_i1	benzyl alcohol O-benzoyltransferase-like	25
DN17288_c0_g1_i1	5-methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase 1	1527	DN20491_c0_g1_i4	5-methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase 2-like	19
DN22101_c0_g1_i1	cytochrome P450 71A1-like	1130	DN20591_c0_g1_i1	cytochrome P450 71A1-like	14
DN9054_c0_g2_i1	uncharacterized protein LOC105052971	891	DN20462_c0_g1_i1	uncharacterized protein LOC105052971	16
DN19899_c1_g1_i5	elongation factor 1-alpha-like	720	DN16211_c0_g1_i1	hypothetical protein CARUB_v100096370mg, partial	11
DN39417_c0_g1_i1	palmitoyl-acyl carrier protein thioesterase, chloroplastic-like	659	DN1125_c0_g1_i1	palmitoyl-acyl carrier protein thioesterase, chloroplastic-like	0.89
DN8999_c0_g1_i1	probable protein Pop3	657	DN3239_c0_g1_i1	probable protein Pop3	10
*DN16562_c0_g1_i1	mannose-specific lectin-like	652	*DN16324_c0_g1_i1	mannose-specific lectin-like	8
DN20784_c0_g1_i1	mannan endo-1,4-beta-mannosidase 5-like	477	DN20300_c0_g1_i1	mannan endo-1,4-beta-mannosidase 5-like	8
DN17063_c0_g1_i1	uncharacterized protein LOC103705182	457	DN15604_c0_g1_i1		7
DN9763_c0_g1_i1	4-hydroxyphenyl-pyruvate dioxygenase	441	DN17419_c0_g1_i2	4-hydroxyphenyl-pyruvate dioxygenase	6
DN15645_c0_g1_i1	hypothetical protein PHAVU_005G042200g	378	DN19503_c0_g1_i2	uncharacterized protein LOC103713005	4
DN39500_c0_g1_i1	uncharacterized protein C24B11.05-like isoform X2	323	DN15665_c0_g1_i2	uncharacterized protein C24B11.05-like isoform X2	6

*: neoculin-related transcripts (cf. Figure 5 and Additional File 5)

The common genes with TPM value ≥ 50 between the two species, except when the TPM values of both genes is < 100 . The genes were sorted based on the TPM value of *C. latifolia* along with the corresponding genes of *C. capitulata*. Note that there were no cases of genes that were highly expressed in both species. This pattern strongly suggests changes in the gene expression regulatory system due to divergence of two species.

<i>C. latifolia</i>			<i>C. capitulata</i>		
DN16206_c0_g1_i1	cytochrome P450 71A1-like	295	DN18399_c0_g1_i1	cytochrome P450 71A1-like	5
DN9770_c0_g1_i1	Os09g0480700, partial	278	DN11365_c0_g1_i1	Os09g0480700, partial	3
DN20943_c2_g1_i1	LOW QUALITY PROTEIN: ATP-citrate synthase beta chain protein 1-like	276	DN20189_c1_g1_i6	LOW QUALITY PROTEIN: ATP-citrate synthase beta chain protein 1-like	5
DN5031_c0_g1_i1		265	DN26197_c0_g1_i1		3
DN19581_c0_g1_i1	peroxidase 43	244	DN20763_c0_g1_i7	peroxidase 43	3
DN22200_c0_g1_i1		237	DN21279_c0_g3_i1		0
DN16082_c0_g1_i1	uncharacterized protein LOC105035694	230	DN20815_c0_g1_i2	uncharacterized protein LOC105035694	4
DN1821_c0_g1_i1	protein EARLY RESPONSIVE TO DEHYDRATION 15-like	213	DN5863_c0_g3_i1	protein EARLY RESPONSIVE TO DEHYDRATION 15-like	1
DN21840_c4_g7_i1		197	DN46444_c0_g1_i1		2
DN11489_c0_g1_i1		189	DN51079_c0_g1_i1		3
DN21813_c0_g1_i1	protein kinase APK1B, chloroplastic-like	184	DN20869_c0_g1_i9	protein kinase APK1B, chloroplastic-like	0.97
DN16611_c0_g1_i1		163	DN8161_c0_g1_i1		2
DN12355_c0_g1_i1	myb-related protein 306-like	160	DN7266_c0_g1_i1	myb-related protein 306-like	3
DN18378_c0_g1_i1	probable L-ascorbate peroxidase 4	158	DN17994_c1_2_i1	probable L-ascorbate peroxidase 4	2
DN21677_c0_g1_i1	S-adenosylmethionine decarboxylase proenzyme-like	149	DN15562_c0_g2_i1	S-adenosylmethionine decarboxylase proenzyme-like	0.92
DN14830_c0_g1_i1	NAC transcription factor 29-like	135	DN20428_c0_g1_i1	NAC transcription factor 29-like	0
DN14165_c0_g2_i1	probable peroxygenase 4	131	DN17339_c0_g1_i2	probable peroxygenase 4	2
DN21840_c4_g4_i2		130	DN11729_c0_g1_i1		2
DN39737_c0_g1_i1	Glutathione peroxidase 2	127	DN8347_c0_g1_i1	Glutathione peroxidase 2	2
DN4928_c0_g1_i1		124	DN44794_c0_g1_i1		1
DN20250_c0_g1_i1	protein NRT1/ PTR FAMILY 5.6-like	114	DN29979_c0_g1_i1	protein NRT1/ PTR FAMILY 5.6-like	2
DN15628_c0_g1_i1	formin-A-like	103	DN20575_c0_g1_i5	formin-A-like	0
DN21235_c2_g9_i1		101	DN9877_c0_g1_i1		1
*DN19752_c0_g1_i1	mannose-specific lectin 3-like	33	*DN18595_c0_g1_i1	mannose-specific lectin 3-like	2301

*: neoculin-related transcripts (cf. Figure 5 and Additional File 5)

The common genes with TPM value ≥ 50 between the two species, except when the TPM values of both genes is < 100 . The genes were sorted based on the TPM value of *C. latifolia* along with the corresponding genes of *C. capitulata*. Note that there were no cases of genes that were highly expressed in both species. This pattern strongly suggests changes in the gene expression regulatory system due to divergence of two species.

<i>C. latifolia</i>			<i>C. capitulata</i>		
DN16463_c0_g2_i2	LOW QUALITY PROTEIN: S-norococlaurine synthase-like	16	DN6989_c0_g1_i1	LOW QUALITY PROTEIN: S-norococlaurine synthase-like	8393
DN32395_c0_g1_i1		14	DN4973_c0_g1_i1		8765
DN19456_c0_g1_i1	polyphenol oxidase, chloroplastic-like	13	DN20237_c3_g1_i1	polyphenol oxidase, chloroplastic-like	1496
DN14333_c0_g1_i1		12	DN13197_c0_g1_i1		42047
DN55067_c0_g1_i1	defensin Ec-AMP-D1 {ECO:0000303 PubMed:18625284}-like	9	DN39416_c0_g1_i1	defensin Ec-AMP-D1 {ECO:0000303 PubMed:18625284}-like	2475
DN5253_c0_g1_i1	Disease resistance-responsive (dirigent-like protein) family protein, putative	9	DN16870_c0_g2_i1	Disease resistance-responsive (dirigent-like protein) family protein, putative	547
DN1586_c0_g1_i1	glycine-rich protein-like isoform X1	8	DN39384_c0_g1_i1		2895
DN23556_c0_g1_i1	basic blue protein-like	5	DN14117_c0_g1_i1	basic blue protein-like	606
DN13618_c0_g1_i1	non-specific lipid-transfer protein 1-like	5	DN13976_c0_g1_i1	lipid transfer protein precursor	655
DN465_c0_g2_i1	microsomal glutathione S-transferase 3-like	5	DN4959_c0_g1_i1	microsomal glutathione S-transferase 3-like	246
DN21384_c3_g4_i1		5	DN17484_c0_g1_i1		424
DN9003_c0_g1_i1	dirigent protein 22-like isoform X1	5	DN19511_c0_g1_i1	dirigent protein 22-like	834
DN4015_c0_g1_i1	CASP-like protein 2A1	4	DN4840_c0_g1_i1	CASP-like protein 2A1	241
DN16618_c0_g1_i1	hypothetical protein SORBIDRAFT_05g026700	3	DN4999_c0_g1_i1	Bowman-Birk type trypsin inhibitor-like isoform X2	5459
DN4834_c0_g2_i1	xylem serine proteinase 1-like	3	DN9966_c0_g1_i1	subtilisin-like protease	232
DN6907_c0_g1_i1	serine/threonine-protein kinase CDL1-like	3	DN11871_c1_g1_i1	serine/threonine-protein kinase CDL1-like	183
DN17444_c0_g1_i1	cytochrome P450 CYP82D47-like	3	DN20684_c0_g1_i1	cytochrome P450 CYP82D47-like	182
DN40485_c0_g3_i1	non-specific lipid-transfer protein 1-like	3	DN39065_c0_g1_i1	non-specific lipid-transfer protein 1-like	1455
DN18380_c0_g1_i2	conserved hypothetical protein	3	DN39186_c0_g1_i1	conserved hypothetical protein	654
DN31252_c0_g1_i1		3	DN12650_c0_g1_i1	non-specific lipid-transfer protein-like	283
DN42464_c0_g2_i1	alpha carbonic anhydrase 8-like, partial	3	DN40148_c0_g1_i1	alpha carbonic anhydrase 7-like	235
DN13852_c0_g1_i1	endoglucanase 6	3	DN18579_c0_g1_i1	endoglucanase 19-like	707
DN39898_c0_g1_i1	oxygen-evolving enhancer protein 3 - 1, chloroplastic-like	2	DN12932_c0_g1_i1	oxygen-evolving enhancer protein 3 - 1, chloroplastic-like	160
DN6056_c0_g1_i1	Calvin cycle protein CP12-1, chloroplastic-like	2	DN12691_c0_g1_i1	calvin cycle protein CP12-1, chloroplastic	215
DN6093_c0_g1_i1		2	DN41578_c0_g1_i1		170

*: neoculin-related transcripts (cf. Figure 5 and Additional File 5)

The common genes with TPM value ≥ 50 between the two species, except when the TPM values of both genes is < 100 . The genes were sorted based on the TPM value of *C. latifolia* along with the corresponding genes of *C. capitulata*. Note that there were no cases of genes that were highly expressed in both species. This pattern strongly suggests changes in the gene expression regulatory system due to divergence of two species.

<i>C. latifolia</i>			<i>C. capitulata</i>		
DN17773_c0_g1_i1	uncharacterized protein LOC105056845	2	DN12165_c0_g1_i1	uncharacterized protein LOC105056845	249
DN24151_c0_g1_i1	ribonuclease 3-like	2	DN39292_c0_g1_i1	ribonuclease 3-like	389
DN8678_c0_g1_i1	uncharacterized protein LOC105056672	2	DN4730_c0_g1_i1	uncharacterized protein LOC105056672	116
DN19431_c2_g4_i1	polyubiquitin 4-like, partial	2	DN20039_c0_g8_i1	hypothetical protein PHAVU_003G1236000g, partial	2116
DN250_c1_g1_i1	probable glutathione S-transferase parA	2	DN16559_c0_g1_i1	probable glutathione S- transferase parA	419
DN10676_c0_g1_i1	probable linoleate 9S-lipoxygenase 5	2	DN17658_c0_g1_i1	probable linoleate 9S- lipoxygenase 5	1644
DN8975_c0_g1_i1	chitinase-like protein 1	2	DN16475_c1_g1_i1	chitinase-like protein 1	183
DN44393_c0_g1_i1	hypothetical protein POPTR_0004s03650g	2	DN18495_c2_g1_i1	conserved hypothetical protein	2751
DN759_c0_g1_i1	CAS1 domain-containing protein 1- like	2	DN21365_c0_g1_i1	CAS1 domain-containing protein 1-like isoform X2	183
DN30327_c0_g1_i1	conserved hypothetical protein	2	DN23037_c0_g1_i1	conserved hypothetical protein	134
DN5572_c0_g1_i1	short-chain type dehydrogenase/reductase-like	2	DN14194_c0_g1_i1	short-chain type dehydrogenase/reductase- like	205
DN45139_c0_g1_i1	putative germin-like protein 2 – 1	2	DN21890_c0_g1_i1	putative germin-like protein 2 – 1	111
DN22251_c0_g1_i1	xyloglucan endotransglucosylase/ hydrolase protein 9-like	2	DN40495_c0_g2_i1	LOW QUALITY PROTEIN: xyloglucan endotransglucosylase/ hydrolase protein 9-like	131
DN56341_c0_g1_i1	peroxidase 4-like	1	DN10149_c0_g1_i1	peptide-N4-(N-acetyl-beta- glucosaminy)asparagine amidase A-like	1301
DN56680_c0_g1_i1	peptide-N4-(N-acetyl-beta- glucosaminy)asparagine amidase A-like	1	DN19920_c0_g1_i1	peroxidase 4-like	583
*DN307_c0_g2_i1	mannose-specific lectin-like	1	*DN9931_c0_g1_i1	mannose-specific lectin-like	14867

*: neoculin-related transcripts (cf. Figure 5 and Additional File 5)

The common genes with TPM value ≥ 50 between the two species, except when the TPM values of both genes is < 100 . The genes were sorted based on the TPM value of *C. latifolia* along with the corresponding genes of *C. capitulata*. Note that there were no cases of genes that were highly expressed in both species. This pattern strongly suggests changes in the gene expression regulatory system due to divergence of two species.

<i>C. latifolia</i>			<i>C. capitulata</i>		
DN48085_c0_g1_i1	probable indole-3-acetic acid-amido synthetase GH3.1	1	DN19080_c1_g1_i1	probable indole-3-acetic acid-amido synthetase GH3.1	107
DN46946_c0_g1_i1	chlorophyll a-b binding protein 7, chloroplastic-like	1	DN41884_c0_g1_i1	chlorophyll a-b binding protein, chloroplastic	141
DN4845_c0_g1_i1	chlorophyll a-b binding protein CP26, chloroplastic-like	1	DN10575_c0_g1_i1	chlorophyll a-b binding protein CP26, chloroplastic-like	353
DN23363_c0_g1_i1	uncharacterized protein LOC105056050	1	DN21609_c0_g1_i1	uncharacterized protein LOC105056050	1612
*DN30823_c0_g1_i1	mannose-specific lectin-like	1	*DN17363_c2_g1_i3	mannose-specific lectin-like	317
DN645_c0_g1_i1	putative lipid-transfer protein DIR1	1	DN12082_c0_g1_i1	putative lipid-transfer protein DIR1	108
DN50661_c0_g1_i1	oxygen-evolving enhancer protein 2, chloroplastic-like	1	DN14711_c0_g1_i1	oxygen-evolving enhancer protein 2, chloroplastic-like	133
DN16663_c0_g1_i2		1	DN20564_c0_g1_i1		642
DN41624_c0_g1_i1	isocitrate lyase	1	DN15046_c0_g1_i1	isocitrate lyase	116
DN33923_c0_g1_i1	galactinol synthase 2-like isoform X1	1	DN13705_c0_g1_i1	galactinol synthase 1-like	127
DN36400_c0_g1_i1	putative cell wall protein	0.98	DN26021_c0_g1_i1	putative cell wall protein	117
DN53880_c0_g1_i1	uncharacterized protein LOC105056050	0.93	DN5177_c0_g1_i1	proactivator polypeptide-like 1	644
DN6399_c0_g1_i1	auxin-induced protein 22D-like	0.93	DN10469_c0_g1_i1	auxin-induced protein 22D-like	171
DN10569_c0_g2_i1		0.91	DN16356_c0_g1_i1		1072
DN21646_c1_g1_i3	protein HOTHEAD-like	0.91	DN14207_c0_g1_i1	protein HOTHEAD-like	330
DN22097_c0_g1_i1		0.82	DN9693_c0_g2_i1		155
DN30250_c0_g1_i1	polygalacturonase inhibitor	0.64	DN17486_c1_g1_i2	Polygalacturonase inhibitor	171
DN50985_c0_g1_i1	putative phytosulfokines 6 isoform X1	0.47	DN22933_c0_g1_i1	putative phytosulfokines 6 isoform X2	136
DN39567_c0_g2_i1	profilin-1	0	DN15886_c0_g1_i1	profilin-1	615
DN5103_c0_g1_i1	trans-resveratrol di-O-methyltransferase-like	0	DN39904_c0_g1_i1	trans-resveratrol di-O-methyltransferase-like	430
DN24431_c0_g6_i1	60S ribosomal protein L24	0	DN1942_c0_g1_i1	60S ribosomal protein L24	278
DN3220_c0_g1_i1		0	DN1273_c0_g1_i1	chlorophyll a-b binding protein 6, chloroplastic	264
DN16735_c0_g2_i2	uncharacterized protein LOC105047938	0	DN39063_c0_g1_i1	uncharacterized protein LOC105047938	172
DN256_c0_g1_i2	Os06g0133500	0	DN16734_c1_g2_i1	Os06g0133500	151
*: neoculin-related transcripts (cf. Figure 5 and Additional File 5)					
The common genes with TPM value ≥ 50 between the two species, except when the TPM values of both genes is < 100 . The genes were sorted based on the TPM value of <i>C. latifolia</i> along with the corresponding genes of <i>C. capitulata</i> . Note that there were no cases of genes that were highly expressed in both species. This pattern strongly suggests changes in the gene expression regulatory system due to divergence of two species.					

Lectin genes expressed in *C. latifolia* and *C. capitulata* fruits

We previously demonstrated that *C. latifolia* fruits contain a taste-modifying protein comprising a heterodimer of NBS and NAS that is similar to lectins in the GNA family. We therefore determined the number of lectin genes expressed in the fruits of *C. latifolia* and *C.*

capitulata that were categorized into each of the 12 lectin families to better understand the general outline of the GNA family genes in these species. To determine the number of lectin genes, we performed tBLASTn searches against all transcripts in each species using the sequences of 12 representative lectins as queries [41] (Table 4). In both species, the largest lectin family was the GNA family, including the neoculin (*NBS* and *NAS*) genes. Ten of the 45 lectin genes in *C. latifolia* and 13 of the 49 lectin genes in *C. capitulata* were GNA family members. Thus, we analyzed the many GNA family genes in these species, including the neoculin genes, in more detail.

Table 4
The number of lectin genes predicted using tBLASTn in *Curculigo latifolia* and *C. capitulata* fruits.

Lectin domain	Model lectin	<i>C. latifolia</i>	<i>C. capitulata</i>
ABA domain	<i>Agaricus bisporus</i> agglutinin	0	0
Amaranthin domain	<i>Amaranthus caudatus</i> agglutinin	0	0
CRA domain	<i>Robinia pseudoacacia</i> chitinase-related agglutinin	3	4
Cyanovirin domain	<i>Nostoc ellipsosporum</i> agglutinin	0	0
EUL domain	<i>Euonymus europaeus</i> agglutinin	1	1
GNA domain	<i>Galanthus nivalis</i> agglutinin	10	13
Hevein domain	<i>Hevea brasiliensis</i> agglutinin	3	2
JRL domain	<i>Artocarpus integer</i> agglutinin	9	4
Legume domain	<i>Glycine max</i> agglutinin	8	16
LysM domain	<i>Brassica juncea</i> LysM domain	1	1
Nictaba domain	<i>Nicotiana tabacum</i> agglutinin	10	8
Ricin-B domain	<i>Ricinus communis</i> agglutinin	0	0
Total number of lectin genes		45	49

GNA family and neoculin-related transcripts

First, we constructed a phylogenetic tree using the deduced protein sequences of 17 transcripts of well-known GNA family members and 25 neoculin-related transcripts (10 transcripts from *C. latifolia* and 15 from *C. capitulata*; Fig. 5); the method used for sequence selection is shown in Additional File 5. The TPM values (calculated by RSEM) are listed after the transcript IDs. An alignment of all sequences is shown in Additional File 6. The *C. latifolia* transcript DN16562_c0_g1_i1 is a good match for NBS, and DN16562_c0_g1_i2 is a good match for NAS, except for one amino-acid substitution (Additional File 7); these transcripts are referred to as NBS and NAS hereafter. The neoculin-related transcripts formed groups different from that of the known GNA family members. Neoculin-like sequences formed one group that included NBS and NAS (named the 'neoculin group') as well as two other large groups (group 1 and group 2) (Fig. 5).

In each group, we detected neoculin-related orthologous transcripts with high similarity between *C. latifolia* and *C. capitulata*. The existence of many orthologs in each species, combined with the presence of relatively few common genes (comprising approximately 40% of all transcripts in both species; Fig. 4), is noteworthy. We inferred that these orthologs probably existed before the divergence of the two species, and their amino-acid differences probably arose afterward. Genetic diversity is beneficial for plants, including *Curculigo*, due to their lack of mobility. It would be interesting to determine whether *Curculigo* plants other than *C. latifolia* and *C. capitulata* contain neoculin-related genes, especially genes in the neoculin group.

Of the genes in the neoculin group, highly similar sequences such as those of the *NBS* and *NAS* genes were present in *C. latifolia*, and their orthologs were identified in *C. capitulata*. Notably, although *NBS* and *NAS* genes were highly expressed in *C. latifolia*, their orthologs were only weakly expressed in *C. capitulata* (DN16324_c0_g1_i1 and DN16324_c0_g1_i2). The TPM values of *NBS* and *NAS* genes in *C. latifolia* were 652 and 624, respectively: that is, the expression levels of *NBS* and *NAS* were approximately the same. This result is in agreement with the finding that these proteins form a heterodimer [18]. Although DN9931_c0_g1_i1 of *C. capitulata* was highly expressed, with a TPM value of 14,867 (the fifth highest expression level among *C. capitulata* transcripts), its ortholog (DN307_c0_g1_i1, D307_c0_g2_i1) was expressed at a very low level in *C. latifolia*. Curiously, both in the neoculin group and in groups 1 and 2, in cases where there were orthologs in both species, if a gene was highly expressed in one species, its ortholog was weakly expressed in the other species; there were no cases

of genes that were highly expressed in both species. The data shown in Table 3 also support this pattern. These results strongly suggest changes in the gene expression regulatory system due to divergence of two species.

Next, we aligned the deduced amino-acid sequences of the proteins in the neoculin group (Fig. 6a). We divided the sequences into nine regions, including the regions removed by mRNA processing and three MBS-like regions: N pro-sequence (N-Pro), N-terminal (N-term), MBS1, inter1, MBS2, inter2, MBS3, C-terminal (C-term), and C pro-sequence (C-Pro). In these regions, His11 of NBS, an essential residue for the pH-dependent taste-modifying activity of neoculin, is present in the N-term regions of NBS. DN16562_c0_g1_i1 of *C. latifolia* and DN16324_c0_g1_i1 of *C. capitulata*. By contrast, DN9931_c0_g1_i1 of *C. capitulata* and DN307_c0_g1_i1 and D307_c0_g2_i1 of *C. latifolia* (abbreviated 'DN9931') did not have His11, but rather Tyr11, like NAS. In addition, Cys77 and Cys109, which form an intermolecular disulfide bond between NBS and NAS, are present in the inter2 and C-term regions in both species. However, these residues were absent in DN9931. Thus, it is likely that DN9931 does not form dimers.

Four residues are responsible for the binding and activation of the human sweet receptor: Arg48, Tyr65, Val72, and Phe94 [26]. Although Tyr65 and Val72 were identified in DN9931, Leu48 and Val94 were not present in this protein. The lack of His11 and these four indispensable residues, as well as the lack of dimerization, indicate that DN9931 does not have a sweet taste or taste-modifying properties. Indeed, a preliminary test indicated that *C. capitulata* fruits did not have a sweet taste or taste-modifying properties despite the high expression level of DN9931_c0_g1_i1 (data not shown). Three sites similar to the MBS are present in the MBS1, MBS2, and MBS3 regions of this protein. Although the essential residues of the MBS were not conserved in NBS or NAS, all of them were conserved in DN9931_c0_g1_i1, marking DN9931_c0_g1_i1 as likely to be a lectin.

Based on this alignment, we investigated the amino-acid residue substitution in each region compared with each reference sequence: NBS or NAS (Additional File 8). The amino-acid substitution rate with reference to NBS is shown in the heatmap in Fig. 6b. Between the NBS series and the NAS series, 18–27% of substitutions occurred in the overall regions from the N-term region to C-term region (23%, 26 of 114 residues in NBS). The highest rate was 27% in the MBS2 region, followed by 24% in the inter2 and C-term regions. In the DN9931 series, the highest substitution rate was 53% in the C-term region, followed by the MBS3 region (44%) and inter2 region (43%). These results suggest that the region from inter2 to C-term is the primary region of sequence diversity in the neoculin group.

Biochemical analysis

We extracted proteins from *C. latifolia* and *C. capitulata* fruits and carried out SDS-PAGE, followed by Coomassie brilliant blue (CBB) staining and immunoblotting using anti-NBS and anti-NAS antibodies (Fig. 7). After CBB staining, we detected an 11-kDa band representing NBS and a 13-kDa band representing NAS from *C. latifolia* (Fig. 7a). For *C. capitulata*, we detected an 11-kDa band and many smaller bands. One of these smaller bands was probably DN9931_c0_g1_i1, which had a high TPM value. Immunoblotting confirmed the identity of the bands containing NBS and NAS in *C. latifolia* fruits. However, no bands were confirmed in *C. capitulata* fruits (Fig. 7b), perhaps because NBS and NAS are expressed at very low levels in this species, as reflected by their low TPM values (as described above). The amino-acid sequence of the C-term region, which is recognized by the antibody, was very different in DN9931_c0_g1_i1 compared to both NBS and NAS, which is consistent with the finding that the proteins detected by CBB staining were not detected by immunoblotting.

Discussion

C. latifolia and *C. capitulata* contain many neoculin-related genes, which are similar within and between species. This diversity of neoculin-related genes is thought to result from gene duplication, which is known to contribute to plant evolution [41–47]. Such gene duplication might place some genes under the same transcriptional regulation. The NBS and NAS genes are likely paralogs that arose due to tandem duplication. The characteristics of NBS and NAS genes in *C. latifolia* and *C. capitulata* are summarized in Table 5. Both *C. latifolia* and *C. capitulata* contained neoculin (NBS and NAS) transcripts, and the sequences of the DN9931 series transcripts matched those of active GNA family members. However, their expression levels in the two plant species were very different.

There was a notable difference in the expression level of these genes in *C. latifolia* and *C. capitulata*. *C. latifolia* fruit contains 1.3 mg neoculin per 1 g of pulp. Because neoculin is 550 times as sweet as sucrose [19, 20], 1 g of *C. latifolia* fruit pulp is estimated to be as sweet as 715 mg of sucrose. Therefore, *C. latifolia* fruits taste sweet. The TPM values of the neoculin genes in *C. capitulata* were only 1/60 those in *C. latifolia*. Therefore, *C. capitulata* fruits are estimated to contain approximately 22 ngg⁻¹ of neoculin and to be as sweet as 12 mg of sucrose. Based on this content, it is likely that *C. capitulata* fruits do not taste sweet, as confirmed in a preliminary test. Thus, neoculin levels, and therefore taste, differ greatly between these fruits. The taste of *C. latifolia* fruit could strongly influence its survival strategies. For example, the sweet taste of neoculin may facilitate the spreading of *C. latifolia* seeds by animals.

Table 5
Summary of neoculin group transcripts in fruits of two *Curculigo* species.

	Transcript ID	Reference transcript	No. of substitution (amino acid)	Heterodimerization (no. of Cys)	Lectin activity (no. of MBS*)	Taste modification	Expression (TPM)
C. latifolia	DN16562_c0_g1_i1 (NBS)	NBS	0	Yes (4)	No (0)	Yes	High (652)
	DN16562_c0_g1_i2 (NAS)	NAS	1	Yes (4)	No (0)	Yes	High (624)
	DN307_c0_g2_i1	DN9931_c0_g1_i1	1	Unknown (2)	Unknown (3)	Unknown	Very low (1.4)
	DN307_c0_g1_i1	DN9931_c0_g1_i1	1	Unknown (2)	Unknown (3)	Unknown	Very low (0.4)
C. capitulata	DN16324_c0_g1_i1	NBS	6	Probably Yes (4)	Probably No (0)	Probably Yes	Low (8.0)
	DN16324_c0_g1_i2	NAS	0	Probably Yes (4)	Probably No (0)	Probably Yes	low (10.8)
	DN9931_c0_g1_i1	DN9931_c0_g1_i1	-	Unknown (2)	Unknown (3)	Unknown (Fruits have no activity)	Very high (14867)
The number of amino-acid residues difference from the reference sequences, the existence of heterodimer, lection action, and taste modification, and expression level of the proteins from <i>C. latifolia</i> or <i>C. capitulata</i> fruits are summarized. As the reference sequences, amino-acid sequences of NBS, NAS, and DN9931_c0_g1_i1 of <i>C. capitulata</i> were used. MBS refers to the mannose-binding site.							

The structure of the taste-modifying protein miraculin is similar to those of the soybean Kunitz trypsin inhibitor and thaumatin, a sweet protein with an α -amylase or trypsin-inhibitor-like structure. Similarly, neoculin has a similar structure to lectin, a common molecular structure in plants [48–54]. Trypsin inhibitors, amylase inhibitors, and lectins are commonly expressed in fruits and seeds. The diversity of these proteins arose from gene duplications and mutations during evolution. It appears that during the process of evolution, neoculin, miraculin, and thaumatin happened to acquire sweetness or taste-modifying activity for humans.

Lectins are thought to play important protective and storage roles in common plants. Thus, the high expression levels of lectin genes in *C. capitulata* fruits are likely to reflect their important roles in this plant. Conversely, the low expression levels of neoculin genes in *C. capitulata* suggest that the compound is less useful in this plant. Similarly, in contrast to *C. capitulata*, active GNA family genes were barely expressed in *C. latifolia* fruits. Neoculin genes were highly expressed in *C. latifolia* but weakly expressed in *C. capitulata* despite the similar appearance of the two plants (Fig. 1). Perhaps these physiological differences are due to mutation of the cis-elements in these genes. Cis-elements, including promoters, enhancers, and silencers, are very important in regulation of gene expression [41, 55–58]. The different expression levels of related genes in *C. latifolia* vs. *C. capitulata* could be caused by mutations in their cis-elements. For example, the cis-elements of NBS and NAS genes may have mutated after the divergence of the two species, or perhaps these genes acquired mutations or lost cis-elements during the gene duplication that led to the divergence, leading to different expression patterns. Analyzing genomic information about these two species might help verify this notion.

Conclusions

RNA-seq analysis of *C. latifolia* and *C. capitulata* fruits revealed the presence of numerous neoculin-like genes. Among the various neoculin-related genes that arose through gene duplication, several mutations accumulated, resulting in the genes encoding NBS and NAS. These proteins form the heterodimeric protein neoculin, which has taste-modifying activity in humans. Our comprehensive investigation of the genes expressed in the fruits of these two *Curculigo* species helped uncover the origin of neoculin at the molecular level.

Methods

Plant materials

C. latifolia (voucher ID 26092) was obtained from the Research Center for Medicinal Plant Resources, National Institutes of Biomedical Innovation, Health, and Nutrition (originally from Indonesia). *C. capitulata* (voucher ID 31481) was obtained from The Naito Museum of Pharmaceutical Science and Industry. The plants were cultivated in a greenhouse at the Yamashina Botanical Research Institute. Photographs of the fruits of these plants are shown in Fig. 1.

Fruit setting

C. latifolia flowers were pollinated by hand in the morning on the first day of flowering. *C. capitulata* flowers were placed in 50 ppm of 1-naphthylacetic acid (NAA) in the mornings of both the first and second days of flowering. At ~ 60 days after flowering, mature fruits were harvested and immediately soaked in RNA later™ solution (Thermo Fisher Scientific, MA, USA). The fruits were stored at - 80 °C until use. The samples were ground into a powder in liquid nitrogen prior to RNA extraction. Total RNA was extracted from the frozen samples using the phenol-SDS method, and poly(A)⁺mRNA was purified using an mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK).

Sequencing

mRNA sequencing was performed by Hokkaido System Science Co., Ltd. (Hokkaido, Japan). A cDNA library was generated using a TruSeq RNA Sample Prep Kit v2 (Illumina, Inc., CA, USA) and sequenced on the Illumina HiSeq 2500 system (Paired-End, unstranded). The raw reads were cleaned using cutadapt1.1 [59] and trimmomatic0.32 [60]. We removed adapter sequences, low-quality sequences (reads with ambiguous 'N' bases), and reads with Q-value < 20 bases. Sequences smaller than 50 bases were eliminated. The high-quality reads were assembled into contigs using Trinity2.11 [61] with default options. We quantified transcript levels as transcripts per million (TPM) values using Bowtie1.12 [62] and RSEM (RNA-Seq by Expectation-Maximization) [63] in the Trinity package.

Sequence clustering

The assembled sequences were compared against the NCBI NR, prot-plant from RefSeq, and UniProt databases with an E-value < 1e⁻¹⁰. BLAST analysis was performed using BLAST version 2.2.31. CD-Hit (cd-hit-est) [64, 65] was used for clustering with the option of threshold (-c) 0.9 to obtain unigenes.

Comparison of gene expression in *C. capitulata* vs. *C. latifolia* fruits

To compare the transcripts in *C. capitulata* vs. *C. latifolia* fruits, a BLASTn search was performed with E-value < 1e⁻⁵ using each transcript from one species as the query sequence against all transcripts from the other species, and then the best hits were selected.

Identification of lectin gene transcripts in *C. capitulata* and *C. latifolia* fruits

A tBLASTn search (E-value < 1e⁻⁴; other options set to the default) was performed against all transcripts in *C. capitulata* and *C. latifolia* fruits with the following protein sequences as the queries, which represent each plant lectin family [41]: *Agaricus bisporus* agglutinin (UniProtKB/Swiss-Prot: Q00022.3—ABA), *Amaranthus caudatus* agglutinin (GenBank: AAL05954.1—amaranthin), *Robinia pseudoacacia* chitinase-related agglutinin (GenBank: ABL98074.1—CRA), *Nostoc ellipsosporum* agglutinin (UniProtKB/Swiss-Prot: P81180.2—cyanovirin), *Euonymus europaeus* agglutinin (GenBank: ABW73993.1—EUL), *Galanthus nivalis* agglutinin (UniProtKB/Swiss-Prot: P30617.1—GNA), *Hevea brasiliensis* agglutinin (GenBank: ABW34946.1—hevein), *Artocarpus integer* agglutinin (GenBank: AAA32680.1—JRL), *Glycine max* agglutinin (UniProtKB/Swiss-Prot: P05046.1—legume lectin), *Brassica juncea* LysM domain (GenBank: BAN83772.1—LysM), *Nicotiana tabacum* agglutinin (GenBank: AAK84134.1—Nictaba), and the lectin chain of *Ricinus communis* agglutinin (GenBank: PDB: 2AAL_B—ricin B). The top hits were selected.

Phylogenetic analysis of GNA family proteins

The sequences of 17 well-known GNA family proteins and species of origin were selected according to Shimizu-Ibuka *et al.* [36]. The ASA, *Allium sativum* (garlic) (1BWU); GNA, *Galanthus nivalis* (snowdrop) (1MSA); and NPL, and *Narcissus pseudonarcissus* (wild daffodil) (1NPL) sequences were obtained from the Protein Data Bank. Others sequences were selected from GenBank as follows: PRA, *Polygonatum roseum* (AY899824); PMA, *Polygonatum multiflorum* (Solomon's seal) (U44775); CMA, *Clivia miniata* (kaffir lily) (L16512); ZCA, *Zephyranthes candida* (autumn zephyr lily) (AF527385); AAA, *Allium ascalonicum* (shallot) (L12172); ACA, *Allium cepa* (onion) (AY376826); AUA, *Allium ursinum* (ramsons) (U68531); THC, *Tulipa* hybrid cultivar (tulip) (U23043); ZOA, *Zingiber officinale* (ginger) (AY657021); ACO, *Ananas comosus* (pineapple) (AY098512); AKA, *Amorphophallus konjac* (konjac) (AY191004); DPA, *Dioscorea polystachya* (yam tuber) (AB178475); CHC, *Cymbidium* hybrid cultivar (cymbidium) (U02516); and EHA, *Epipactis helleborine* (broad-leaved helleborine) (U02515). These 17 sequences were aligned using ClustalW [66].

Biochemical analysis

SDS-PAGE was carried out using fruit extracts from *C. latifolia* and *C. capitulata*. The proteins were confirmed by Coomassie brilliant blue (CBB) staining. Protein gel blotting was carried out using anti-NAS or anti-NBS antibodies [38, 67], which were targeted to the C-terminus of NAS or NBS, respectively. Preparation and purification of fruit extracts were performed as described previously [18, 38]. Each 0.1 g pulp sample was treated with 0.5 mL of 0.5 M NaCl to obtain an extract, which was combined with the appropriate volume of buffer containing 2-mercaptoethanol for SDS-PAGE. 5 µL and 20 µL of fruit extract from *C. latifolia* and *C. capitulata* was subjected to SDS-PAGE and CBB staining and 20 µL and 10 µL was subjected to immunoblotting, respectively.

List of abbreviations

AAA, *Allium ascalonicum* agglutinin; ACA, *Allium cepa* agglutinin; ACO, *Ananas comosus* lectin; AKA, *Amorphophallus konjac* agglutinin; ASA, *Allium sativum* agglutinin; AUA, *Allium ursinum* agglutinin; CHC, *Cymbidium* hybrid cultivar agglutinin; CMA, *Clivia miniata* agglutinin; COG, Cluster of Orthologous Groups; DPA, *Dioscorea polystachya* agglutinin; EHA, *Epipactis helleborine* agglutinin; GNA, *Galanthus nivalis* agglutinin; GO, Gene Ontology; NAA, 1-naphthylacetic acid; NAS, neoculin acidic subunit; NCBI, the National Center for Biotechnology Information; NBS, neoculin basic subunit; NGS, next generation sequencing; NPL, *Narcissus pseudonarcissus* lectin; PMA, *Polygonatum multiflorum* agglutinin; PRA, *Polygonatum roseum* agglutinin; THC, *Tulipa* hybrid cultivar lectin; ZOA, *Zingiber officinale* agglutinin; TPM, transcripts per million; ZCA, *Zephyranthes candida* agglutinin.

List Of Abbreviations

AAA, *Allium ascalonicum* agglutinin; ACA, *Allium cepa* agglutinin; ACO, *Ananas comosus* lectin; AKA, *Amorphophallus konjac* agglutinin; ASA, *Allium sativum* agglutinin; AUA, *Allium ursinum* agglutinin; CHC, *Cymbidium* hybrid cultivar agglutinin; CMA, *Clivia miniata* agglutinin; COG, Cluster of Orthologous Groups; DPA, *Dioscorea polystachya* agglutinin; EHA, *Epipactis helleborine* agglutinin; GNA, *Galanthus nivalis* agglutinin; GO, Gene Ontology; NAA, 1-naphthylacetic acid; NAS, neoculin acidic subunit; NCBI, the National Center for Biotechnology Information; NBS, neoculin basic subunit; NGS, next generation sequencing; NPL, *Narcissus pseudonarcissus* lectin; PMA, *Polygonatum multiflorum* agglutinin; PRA, *Polygonatum roseum* agglutinin; THC, *Tulipa* hybrid cultivar lectin; ZOA, *Zingiber officinale* agglutinin; TPM, transcripts per million; ZCA, *Zephyranthes candida* agglutinin.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The raw data and processed data from this study have been uploaded to the NCBI Gene Expression Omnibus (GSE151377) and are available in the NCBI database under accession number PRJNA635640, <https://www.ncbi.nlm.nih.gov/bioproject/635640>

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Cross-ministerial Strategic Innovation Promotion Program (Grant No. 14532924; K.A.), a Grant-in-Aid for Scientific Research B (Grant No. 19300248; T.A.) from the Society for the Promotion of Science in Japan and Adaptable and Seamless Technology transfer Program through Target-driven R&D (A-STEP) from Japan Science and Technology Agency to T.A. (Grant No.JPMJTR194F).

Authors' contributions

TA* conceived the study and participated in the design of all experiments. S Okubo, KT, S Okada, TY, and KA analyzed and interpreted data. S Okubo and TY cultivated plants and performed sample preparation. S Okubo and S Okada performed biological experiments. S Okubo and KT wrote the manuscript. KA discussed the experiments and manuscript. All authors read and approved the final manuscript.

Acknowledgements

We are grateful to the late Dr. Kosaburo Nishi of Research Center for Medicinal Plant Resources (Tsukuba Division), National Institutes of Biomedical Innovation, Health and Nutrition, for kindly providing the *Curculigo latifolia* plants. We also thank Hiroshi Morita, the Director of The Naito Museum of Pharmaceutical Science and Industry, for kindly providing the *Curculigo capitulata* plants. Computations were partially performed on the NIG supercomputer.

Authors' information

Satoshi Okubo and Kaede Terauchi contributed equally to this work.

References

1. Burkill IH. A dictionary of the economic products of the Malay Peninsula. London: Crown Agents for the Colonies; 1966. pp. 713-4
2. Perry LM. Medicinal plants of East and Southeast Asia. Cambridge: MIT Press; 1895. p. 12.
3. Plants of the World Online. <http://www.plantsoftheworldonline.org/>. Accessed May 17 2020.
4. Kocyan A. The discovery of polyandry in *Curculigo* (Hypoxidaceae): implications for androecium evolution of asparagoid monocotyledons. *Ann Bot*. 2007;100(2):241-8.
5. Kocyan A, Snijman DA, Forest F, Devey DS, Freudenstein JV, Wiland-Szymańska J, et al. Molecular phylogenetics of Hypoxidaceae-evidence from plastid DNA data and inferences on morphology and biogeography. *Mol Phylogenet Evol*. 2011;60(1):122-36.
6. Liu KW, Xie GC, Chen LJ, Xiao XJ, Zheng YY, Cai J, et al. *Sinocurculigo*, a new genus of Hypoxidaceae from China based on molecular and morphological evidence. *PLoS One*. 2012;7(6):e38880.
7. Ranjbarfard A, Saleh G, Abdullah NAP, Kashiani P. Genetic diversity of *lemba* (*Curculigo latifolia*) populations in Peninsular Malaysia using ISSR molecular markers. *Australian Journal of Crop Science*. 2014;8(1):9-17.
8. Eksomtramage L, Kwandarm M, Purintavaragul C. Karyotype of some Thai Hypoxidaceae species. *Songklanakarin J Sci Technol*. 2013;35(4):379-82.
9. Okubo S, Yamada M, Yamaura T, Akita T. Effects of the pistil size and self-incompatibility on fruit production in *Curculigo latifolia* (Liliaceae). *J Japan Soc Hort Sci*. 2010;79(4):354-9.
10. Asif M. A review on phytochemical and ethnopharmacological activities of *Curculigo orchioides*. *Mahidol University Journal of Pharmaceutical Sciences*. 2012;39(3-4):1-10.
11. Babaei N, Abdullah NAP, Saleh G, Abdullah TL. An efficient in vitro plantlet regeneration from shoot tip cultures of *Curculigo latifolia*, a medicinal plant. *ScientificWorldJournal*. 2014;2014:275028.
12. Ishak NA, Ismail M, Hamid M, Ahmad Z, Abd Ghafar SA. Antidiabetic and hypolipidemic activities of *Curculigo latifolia* fruit: root extract in high fat fed diet and low dose STZ induced diabetic rats. *Evid Based Complement Alternat Med*. 2013;2013:601838.
13. Li S, Yu JH, Fan YY, Liu QF, Li ZC, Xie ZX, et al. Structural elucidation and total synthesis of three 9-torlignans from *Curculigo capitulata*. *J Org Chem*. 2019;84(9):5195-202.
14. Nie Y, Dong X, He Y, Yuan T, Han T, Rahman K, et al. Medicinal plants of genus *Curculigo*: traditional uses and a phytochemical and ethnopharmacological review. *J Ethnopharmacol*. 2013;147(3):547-63.
15. Wang KJ, Zhu CC, Di L, Li N, Zhao YX. New norlignan derivatives from *Curculigo capitulata*. *Fitoterapia*. 2010;81(7):869-72.
16. Yamashita H, Theerasilp S, Aiuchi T, Nakaya K, Nakamura Y, Kurihara Y. Purification and complete amino acid sequence of a new type of sweet protein taste-modifying activity, curculin. *J Biol Chem*. 1990;265(26):15770-5.
17. Nakajima K, Asakura T, Oike H, Morita Y, Shimizu-Ibuka A, Misaka T, et al. Neoculin, a taste-modifying protein, is recognized by human sweet taste receptor. *Neuroreport*. 2006;17(12):1241-4.
18. Shirasuka Y, Nakajima K, Asakura T, Yamashita H, Yamamoto A, Hata S, et al. Neoculin as a new taste-modifying protein occurring in the fruit of *Curculigo latifolia*. *Biosci Biotechnol Biochem*. 2004;68(6):1403-7.
19. Kant R. Sweet proteins-potential replacement for artificial low calorie sweeteners. *Nutr J*. 2005;4:5.

20. Yamashita H, Akabane T, Kurihara Y. Activity and stability of a new sweet protein with taste-modifying action, curculin. *Chem Senses*. 1995;20(2):239-43.
21. Koizumi A, Nakajima K, Asakura T, Morita Y, Ito K, Shimizu-Ibuka A, et al. Taste-modifying sweet protein, neoculin, is received at human T1R3 amino terminal domain. *Biochem Biophys Res Commun*. 2007;358(2):585-9.
22. Nakajima K, Koizumi A, Iizuka K, Ito K, Morita Y, Koizumi T, et al. Non-acidic compounds induce the intense sweet taste of neoculin, a taste-modifying protein. *Biosci Biotechnol Biochem*. 2011;75(8):1600-2.
23. Suzuki M, Kurimoto E, Nirasawa S, Masuda Y, Hori K, Kurihara Y, et al. Recombinant curculin heterodimer exhibits taste-modifying and sweet-tasting activities. *FEBS Lett*. 2004;573(1-3):135-8.
24. Abe K, Yamashita H, Arai S, Kurihara Y. Molecular cloning of curculin, a novel taste-modifying protein with a sweet taste. *Biochim Biophys Acta*. 1992;1130(2):232-4.
25. Nakajima K, Yokoyama K, Koizumi T, Koizumi A, Asakura T, Terada T, et al. Identification and modulation of the key amino acid residue responsible for the pH sensitivity of neoculin, a taste-modifying protein. *PLoS One*. 2011;6(4):e19448.
26. Koizumi T, Terada T, Nakajima K, Kojima M, Koshihara S, Matsumura Y, et al. Identification of key neoculin residues responsible for the binding and activation of the sweet taste receptor. *Sci Rep*. 2015;5:12947.
27. Morita Y, Nakajima K, Iizuka K, Terada T, Shimizu-Ibuka A, Ito K, et al. pH-dependent structural change in neoculin with special reference to its taste-modifying activity. *Biosci Biotechnol Biochem*. 2009;73(11):2552-5.
28. Ohkubo T, Tamiya M, Abe K, Ishiguro M. Structural basis of pH dependence of neoculin, a sweet taste-modifying protein. *PLoS One*. 2015;10(5):e0126921.
29. Van Damme EJM, Peumans WJ, Barre A, Rougé P. Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. *Critical Reviews in Plant Sciences*. 1998;17:575-692.
30. Van Damme EJM, Lannoo N, Peumans WJ. Plant lectins. *Advances in Botanical Research*: Elsevier; 2008. pp. 107-209.
31. Barre A, Van Damme EJM, Peumans WJ, Rougé P. Structure-function relationship of monocot mannose-binding lectins. *Plant Physiol*. 1996;112(4):1531-40.
32. Shimizu-Ibuka A, Morita Y, Terada T, Asakura T, Nakajima K, Iwata S, et al. Crystal structure of neoculin: insights into its sweetness and taste-modifying activity. *J Mol Biol*. 2006;359(1):148-58.
33. Kurimoto E, Suzuki M, Amemiya E, Yamaguchi Y, Nirasawa S, Shimba N, et al. Curculin exhibits sweet-tasting and taste-modifying activities through its distinct molecular surfaces. *J Biol Chem*. 2007;282(46):33252-6.
34. Barre A, Van Damme EJM, Peumans WJ, Rougé P. Curculin, a sweet-tasting and taste-modifying protein, is a non-functional mannose-binding lectin. *Plant Mol Biol*. 1997;33(4):691-8.
35. Harada S, Otani H, Maeda S, Kai Y, Kasai N, Kurihara Y. Crystallization and preliminary X-ray diffraction studies of curculin. A new type of sweet protein having taste-modifying action. *J Mol Biol*. 1994;238(2):286-7.
36. Shimizu-Ibuka A, Nakai Y, Nakamori K, Morita Y, Nakajima K, Kadota K, et al. Biochemical and genomic analysis of neoculin compared to monocot mannose-binding lectins. *J Agric Food Chem*. 2008;56(13):5338-44.
37. Nakajo S, Akabane T, Nakaya K, Nakamura Y, Kurihara Y. An enzyme immunoassay and immunoblot analysis for curculin, a new type of taste-modifying protein: cross-reactivity of curculin and miraculin to both antibodies. *Biochim Biophys Acta*. 1992;1118(3):293-7.
38. Okubo S, Asakura T, Okubo K, Abe K, Misaka T, Akita T. Neoculin, a taste-modifying sweet protein, accumulates in ripening fruits of cultivated *Curculigo latifolia*. *J Plant Physiol*. 2008;165(18):1964-9.
39. Standard tables of food composition in Japan - 2015 - (Seventh revised version) 2015. MEXT. https://www.mext.go.jp/en/policy/science_technology/policy/title01/detail01/1374030.htm. Accessed May 17 2020.
40. Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, et al. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res*. 2008;36(10):3420-35.
41. De Schutter K, Tsaneva M, Kulkarni SR, Rougé P, Vandepoele K, Van Damme EJM. Evolutionary relationships and expression analysis of EUL domain proteins in rice (*Oryza sativa*). *Rice (N Y)*. 2017;10(1):26.
42. Cannon SB, Mitra A, Baumgarten A, Young ND, May G. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biol*. 2004;4:10.
43. Copley SD. Evolution of new enzymes by gene duplication and divergence. *FEBS J*. 2020;287(7):1262-83.
44. Dang L, Van Damme EJM. Genome-wide identification and domain organization of lectin domains in cucumber. *Plant Physiol Biochem*. 2016;108:165-76.

45. Fukushima K, Fang X, Alvarez-Ponce D, Cai H, Carretero-Paulet L, Chen C, et al. Genome of the pitcher plant *Cephalotus* reveals genetic changes associated with carnivory. *Nat Ecol Evol.* 2017;1(3):59.
46. Panchy N, Lehti-Shiu M, Shiu SH. Evolution of gene duplication in plants. *Plant Physiol.* 2016;171(4):2294-316.
47. Yan J, Li G, Guo X, Li Y, Cao X. Genome-wide classification, evolutionary analysis and gene expression patterns of the kinome in *Gossypium*. *PLoS One.* 2018;13(5):e0197392.
48. de Vos AM, Hatada M, van der Wel H, Krabbendam H, Peerdeman AF, Kim SH. Three-dimensional structure of thaumatin I, an intensely sweet protein. *Proc Natl Acad Sci U S A.* 1985;82(5):1406-9.
49. Kurihara Y. Characteristics of antisweet substances, sweet proteins, and sweetness-inducing proteins. *Crit Rev Food Sci Nutr.* 1992;32(3):231-52.
50. Liu JJ, Sturrock R, Ekramoddoullah AK. The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. *Plant Cell Rep.* 2010;29(5):419-36.
51. Petre B, Major I, Rouhier N, Duplessis S. Genome-wide analysis of eukaryote thaumatin-like proteins (TLPs) with an emphasis on poplar. *BMC Plant Biol.* 2011;11:33.
52. Selvakumar P, Gahloth D, Tomar PP, Sharma N, Sharma AK. Molecular evolution of miraculin-like proteins in soybean Kunitz superfamily. *J Mol Evol.* 2011;73(5-6):369-79.
53. Theerasilp S, Hitotsuya H, Nakajo S, Nakaya K, Nakamura Y, Kurihara Y. Complete amino acid sequence and structure characterization of the taste-modifying protein, miraculin. *J Biol Chem.* 1989;264(12):6655-9.
54. Witty M, Higginboyham JD. Thaumatin. Florida, USA: CRC Press, Inc.; 1994. pp. 20-35.
55. Jiang SY, Ma Z, Ramachandran S. Evolutionary history and stress regulation of the lectin superfamily in higher plants. *BMC Evol Biol.* 2010;10:79.
56. Lambin J, Asci SD, Dubiel M, Tsaneva M, Verbeke I, Wytynck P, et al. OsEUL lectin gene expression in rice: stress regulation, subcellular localization and tissue specificity. *Front Plant Sci.* 2020;11:185.
57. Li XQ. Developmental and environmental variation in genomes. *Heredity (Edinb).* 2009;102(4):323-9.
58. Wittkopp PJ, Kalay G. Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. *Nat Rev Genet.* 2011;13(1):59-69.
59. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal.* 2011;17(1).
60. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014;30(15):2114-20.
61. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Trinity: reconstructing a full-length transcriptome without a genome from RNA-seq data. *Nat Biotechnol.* 2013;29(7):644-52.
62. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 2009;10(3):R25.
63. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-seq data with or without a reference genome. *BMC Bioinformatics.* 2011;12:323.
64. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics.* 2012;28(23):3150-2.
65. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics.* 2006;22(13):1658-9.
66. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics.* 2007;23(21):2947-8.
67. Nakajima K, Asakura T, Maruyama J, Morita Y, Oike H, Shimizu-Ibuka A, et al. Extracellular production of neoculin, a sweet-tasting heterodimeric protein with taste-modifying activity, by *Aspergillus oryzae*. *Appl Environ Microbiol.* 2006;72(5):3716-23.

Additional Files

Additional File 1. Supplemental Figure 1. Length distribution of the assembled transcripts (>1 TPM) from *Curculigo latifolia* (purple) and *C. capitulata* (orange) fruits.

Additional File 2. Supplemental Figure 2. Distribution of transcripts per million (TPM) values of the assembled transcripts from *Curculigo latifolia* (purple) and *C. capitulata* (orange) fruits.

Average, Median and Mode of *C. latifolia* were 11.7, 1.6 and 2, respectively, and those of *C. capitulata*, 13.0, 1.9 and 2, respectively.

Additional File 3. Supplemental annotation. Transcripts from *Curculigo latifolia* and *C. capitulata* fruits were annotated by BLASTx (E-value $< 1e^{-10}$) against the NCBI NR, RefSeq, UniProt, and COG databases. Expression values (TPM), unigenes clustered by CD-Hit, and the unique or common genes from *C. capitulata* or *C. latifolia* were also included.

Additional File 4. Supplemental Figure 3. GO annotation of transcripts from *Curculigo latifolia* (purple) and *C. capitulata* (orange) fruits.

In total, 25,372 (*C. latifolia*) and 27,484 (*C. capitulata*) transcripts were classified by GO term. No significant differences were observed between the two species.

Additional File 5. Supplemental Table 1. Selection of sequences for phylogenetic analysis using BLAST search of transcripts from *Curculigo latifolia* and *C. capitulata* fruits.

The query sequences were the amino-acid sequences (AA) of GNA (UniProtKB/Swiss-Prot: P30617.1) and the nucleotide sequences (nucl) and AA of NBS (GenBank: X64110.1, GenBank: CAA45476.1) and NAS (GenBank: AB167079.1, GenBank: BAD29946.1). Each top hit was selected. The contigs that were selected for each query and used as neoculin-related sequences in phylogenetic analysis (Figure 5) are indicated by checkmarks (✓).

Additional File 6. Supplemental Figure 4. Amino-acid sequence alignment of 10 *Curculigo latifolia* transcripts, 15 *C. capitulata* transcripts, and 17 well-known GNA family members used in the phylogenetic analysis shown in Figure 5.

Additional File 7. Supplemental Figure 5. Comparison of the amino-acid sequences of neoculin (NBS and NAS) in public databases and the *Curculigo latifolia* proteins identified in the present study.

Amino-acid residues that differ between NBS and NAS are shown in blue for NBS residues and red for NAS residues. Asn2 of DN16562_c0_g1_i2 was the only residue that differed from NAS, which has Ser at position 2 (GenBank: BAD29946.1). These *de novo* assemblies were good matches with the known sequences (NBS and NAS). This level of matching supports the accuracy of the assembly.

Additional File 8. Supplemental Table 2. Numbers of amino-acid residue substitutions in neoculin group proteins.

The values show the number of substituted amino-acid residues in each region. The transcripts were divided into nine regions, including N-Pro, N-term, MBS1, inter1, MBS2, inter2, MBS3, C-term and C-Pro, the regions removed by processing, the N- or C-terminal regions, the mannose-binding sites MBS 1 to 3, and the regions between the MBSs. "A" indicates residues of NAS different from those of NBS. "B" indicates residues of NBS different from those of NAS. "C" indicates residues different from those of both NBS and NAS. "D" indicates the residues only present in DN9931_c0_g1_i1.

Figures

Figure 1

Photographs of *Curculigo latifolia* and *C. capitulata*. *Curculigo latifolia* (a–c) and *C. capitulata* (d–f) in the greenhouse at the Yamashina Botanical Research Institute. (b and e) Inflorescences; (c and f) fruits.

Figure 2

The assembled *Curculigo latifolia* and *C. capitulata* are highly similar to known monocotyledon genes. The percentage of species in *Curculigo latifolia* (outer circle) and *C. capitulata* (inner circle) was obtained from the results of BLAST search against the NR database. The top six most highly homologous species were monocotyledons, like *Curculigo*.

Figure 3

Curculigo latifolia and *C. capitulata* have functionally similar genes. Functional classification of transcripts of these two species were performed using the COG database. In total, 11,875 (*C. latifolia*) and 12,448 (*C. capitulata*) transcripts were grouped into 26 COG categories (A to Z). No significant differences were observed between the two species.

Figure 4

Majority of unigenes of *Curculigo latifolia* and *C. capitulata* are unique genes with low similarity. The number of unigenes based on the sequence similarity between *Curculigo latifolia* and *C. capitulata* fruits were investigated. The number of high similar unigenes, common genes, (Clat-comm: common genes of *C. latifolia*, and Ccap-comm: common genes of *C. capitulata*) and unigenes with low similarity, unique genes, (Clat-uniq: unique genes of *C. latifolia*, and Ccap-uniq unique genes of *C. capitulata*).

Figure 5

Phylogenetic analysis of neoculin-related and GNA family genes. NGS transcript IDs for *C. latifolia* and *C. capitulata* are shown in purple and orange, respectively. DN16562_c0_g1_i1 and DN16562_c0_g1_i2 of *C. latifolia* correspond to NBS and NAS, respectively (see Additional File 7). The transcript per million (TPM) values are listed to the right of the transcript IDs. Transcripts from the two species with highly similar sequences are shown in pairs. Transcripts sharing high similarity with NBS and NAS are referred to as the neoculin group (surrounded by a red frame). Groups of other highly similar transcripts are shown in groups 1 and 2. Arrows to the right of the TPM value highlight the ortholog pairs with extremely different TPM values. The sequences and species of origin of the selected GNA family members are as follows: ASA, *Allium sativum* (1BWU); GNA, *Galanthus nivalis* (1MSA); and NPL, *Narcissus pseudonarcissus* (1NPL) were extracted from Protein Data Bank entries. Others sequences were selected from GenBank entries: PRA, *Polygonatum roseum* (AY899824); PMA, *Polygonatum multiflorum* (U44775); CMA, *Clivia miniata* (L16512); ZCA, *Zephyranthes candida* (AF527385); AAA, *Allium ascalonicum* (L12172); ACA, *Allium cepa* (AY376826); AUA, *Allium ursinum* (U68531); THC, *Tulipa hybrid cultivar* (U23043); ZOA, *Zingiber officinale* (AY657021); ACO, *Ananas comosus* (AY098512); AKA, *Amorphophallus konjac* (AY191004); DPA, *Dioscorea polystachya* (AB178475); CHC, *Cymbidium hybrid cultivar* (U02516); and EHA, *Epipactis helleborine* (U02515).

Figure 6

Comparison of the amino-acid sequences of neoculin group members. (a) Amino-acid sequence alignment of neoculin group members from *Curculigo latifolia* and *C. capitulata* fruits. In each alignment, the residues that are consistent with only NBS or only NAS are shown in blue and red, respectively. The residues that are not consistent with NBS or NAS are shown in pink, and those that are consistent with only DN9931_c0_g1_i1 (Ser17) are shown in light green. His11 and cysteine residues are highlighted in dark red and dark green, respectively. Arg48, Tyr65, Val72, and Phe94 are highlighted in pale green. Mannose-binding sites (MBS, QxDxNxVxY) are indicated by a dagger (†), and conserved residues are highlighted in yellow. MBS residues that are conserved in all sequences are indicated by a double dagger (‡). MBS residues of DN307_c0_g2_i1, DN307_c0_g1_i1, and DN9931_c0_g1_i1 are shown in boxes. The transcripts were divided into nine regions, N-Pro, N-term, MBS1, inter1, MBS2, inter2, MBS3, C-term, and C-Pro, based on the regions removed by mRNA processing, the N- or C-terminal regions, the regions of MBS 1 to 3, and the regions between the MBSs. (b) Amino-acid residue substitutions in neoculin group proteins. The region from inter2 to C-term is the primary region of sequence diversity in the neoculin group. The values shown in heatmap are amino-acid substitution rates (%) of neoculin group. The NBA sequence was used as the reference sequence.

Figure 7

Biochemical analysis of *Curculigo latifolia* and *C. capitulata* fruits. Extracts from one *C. latifolia* fruit and four *C. capitulata* fruits were subjected to SDS-PAGE. (a) For CBB staining, 5 μ L of *C. latifolia* extract and 20 μ L of *C. capitulata* extract were applied to each well. (b) For immunoblotting, 10 μ L of *C. latifolia* extract and 20 μ L of *C. capitulata* extract were applied to each well. "*" indicates the band thought to be non-glycosylated NAS [38](Okubo et al., 2008).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AdditionalFile8.pdf](#)
- [AdditionalFile7.pdf](#)
- [AdditionalFile6.pdf](#)
- [AdditionalFile5.pdf](#)
- [AdditionalFile4.pdf](#)
- [AdditionalFile3.xlsx](#)
- [AdditionalFile2.pdf](#)
- [AdditionalFile1.pdf](#)