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Full Length Article

Comparative Transcriptomics-based Investigation of the Adaptive Evolution of the Red Pigment Radish (*Raphanus sativus*) Cultivar

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Abstract

The "red skin with red flesh" radish cultivar possesses a high concentration of red pigment, but the molecular mechanism of red pigment biosynthesis in this radish cultivar is unknown. The aims of this study were to investigate the phylogenetic relationship between six radish cultivars with different colours and to identify candidate genes involved in red pigment formation in the red pigment radish cultivar. We sequenced the transcriptome of six radish cultivars with different red pigment contents. We first examined their phylogenetic relationships and then performed the natural selection pressures tests on the basis of the ancestral lineage of the red pigment radish cultivar. Phylogenetic results indicated that red pigment radishes from two different cultivars were grouped into one clade and the white skin with white flesh cultivars was located at the basal clade. The molecular classifications of the radish cultivars are inconsistent with the traditional taxonomic classifications based on root skin and flesh colour. In addition, our molecular evolutionary analysis showed that around 105 genes were under selection along the ancestral linage of the red pigment radish cultivar. Further examinations showed that 12 genes may be involved in red pigment formation in the red pigment radish cultivar. Our study enriches the genetic resources available to radish cultivars, and it may open the door to investigations of the molecular evolution of the red pigment radish cultivar.

Keywords: Red pigment radish; Candidate gene; Adaptive evolution; Transcriptome

Introduction

Radish (*Raphanus sativus* L.), a member of the Brassicaceae family, is a common edible vegetable. China has a long history of radish cultivation, and it possesses the most radish genetic resources in the world (Wang *et al.*, 2015). Typically, Chinese radishes can be classified into five cultivars based on their flesh colour: "white skin with white flesh", "red skin with white flesh", "green skin with pinkish flesh", "red skin with pinkish flesh", and "red skin with red flesh" (Chen *et al.*, 2015). Among these cultivars, the "red skin with red flesh" cultivar (hereafter referred to as the "red pigment radish") is conspicuous for its high red pigment content. Previous research showed that the concentration of red pigment in the red pigment radish can reach 2.88% with an average concentration of 1.61% (Chen *et al.*, 2015). Thus, this cultivar has great potential in industry and as a commonly used root vegetable. Meanwhile, this radish cultivar may provide a basis to study the molecular mechanisms underlying the development of the phenotype (red pigment). Pigment is one type of water-soluble anthocyanin, and the accumulation of anthocyanin is a decisive factor for colour formation in many fruits (Khoo *et al.*, 2017). Moreover, red pigment formation has been studied in species like the red-fleshed apple and red-skinned pear cultivar. Results showed that the MYB (v-myb avian myeloblastosis viral oncogene homolog) transcription factor may contribute to the red pigment formation in the red-fleshed cultivar via the induction of anthocyanin accumulation (Espley *et al.*, 2007). Genes and transcription factors involved in the red-skin formation were also identified (Yang *et al.*, 2015). Given the importance of the red pigment radish cultivar, it is necessary to investigate the molecular mechanism of red pigment biosynthesis in this cultivar. However, genetic resources for studying the red pigment radish cultivar were scarce because less attention has been paid to the red pigment radish in research. Several efforts have been recently made towards generating the transcriptomic resources for radish (Wang *et al.*, 2013; Nie *et al.*, 2016; Yu *et al.*, 2016). However, none of these transcriptome studies were carried out on the red pigment radish cultivar. These issues make it difficult to study the molecular mechanism of red pigment biosynthesis in the red pigment radish cultivar. From this perspective, it is important to investigate genetic resources in the red pigment radish cultivar and to screen candidate genes responsible for red pigment formation in the red pigment radish group. Generally, if a gene is under selective pressure, it will be

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thought to be responsible for adaptative evolution (Hodgins *et al.*, 2015). The ω value is the ratio of the number of nonsynonymous substitutions per nonsynonymous site (dN) to the number of synonymous substitutions per synonymous site (dS). The ω value is understood to be a signal for selection pressure. The identification of genes under selection to investigate the molecular mechanisms of environmental adaptation has been widely employed in many non-model organism (Chen *et al.*, 2006; Koenig *et al.*, 2013; Hodgins *et al.*, 2015). Here, we try to reconstruct the relationship of the radish group and to identify candidate adaptive genes involved in the evolution of red pigment formation in the red pigment radish cultivar.

Materials and Methods

Plant Material

Six inbred lines of different colours from various cultivars were used: Radish 1 (red skin with red flesh) and Radish 2 (red skin with pink coloured flesh) belonging to the Guanguan cultivar; Radish 3 (red skin with red flesh), Radish 4 (red skin with pink coloured flesh), and Radish 5 (red skin with white flesh) belonging to the Redheart1 cultivar; and Radish 6 (white skin with white flesh) from the White1 cultivar (Fig. 1). The seedlings were cultured in an experimental farm at Yangtze Normal University in Fuling District Chongqing City, China. For sequencing analysis, taproots were sampled at mature stages, and the skin and flesh were manually combined, collected (not separated) and cut into small cubes at a mature stage prior to the experiment. All samples were washed with RNase-free water to exclude impurities and stored at -80ºC immediately prior to RNA extraction.

RNA (ribonucleic acid) Extraction and Sequencing

Total RNA from each sample was extracted using the traditional TRIzol method (Wang *et al.*, 2012). RNA samples that passed quality assurance were used to prepare sequencing libraries. Equal amounts of RNA from each sample were used for cDNA preparation, poly-A-enriched mRNA was fragmented to approximately 200 bp for the library preparation, and sequencing was conducted using an Illumina 2000 instrument.

De novo Assembly of the Transcriptome

Before assembling the transcriptome, the sequence quality of each sample was assessed using FastQC version 10.1 (www.bioinformatics.bbsrc.ac.uk/projects/fastqc), and samples with low quality, such as sequences with a quality score less than five, sequences with ambiguous sequences (N), and sequences with adaptor sequences and primer sequences, were discarded. *De novo* assembly of clean reads in each sample was performed using Trinity (v2.1.0) (Haas *et al.*, 2013) with default settings. As *de novo* transcriptome assembly involves many isoforms of a transcript, the longest isoform was extracted and defined as a unigene.

Identification of Orthologous Genes

To find one-to-one orthologs among these species, we adopted the BBH (bidirectional-best blast hits) method. The genome of *Arabidopsis thaliana*, which was downloaded from the *A. thaliana* TAIR10 database (20101214), was used as the reference. The genome of *Raphanus raphanistrum* radish was also used; the genome was downloaded from the website http://radish.plantbiology.msu.edu/index.php/Genome_sequence/analysis. Unigenes of each species were used as queries to search against reference species sequences (BLASTX) or as targets for the reference species (TBLASTN) with an E-value < 10–5. If all 7 species had sequences corresponding to the same gene of the reference genome (*A. thaliana*), then one ortholog cluster was generated. Ortholog sequences were excluded if they contained stop codons and were shorter than 120 bp. After 1:1:1 orthologous identification, orthologous sequences in each ortholog cluster were aligned using the Probabilistic Alignment Kit (PRANK) software at the codon level with default options (Loytynoja and Goldman, 2005). To find the conserved regions for these orthologs, we used the Gblocks program with default settings (Castresana, 2000). The workflow for this study is shown in Fig. 2.

Phylogenetic Reconstruction

To reconstruct the phylogenetic relationships of radish group, selected conserved regions of orthologs were concatenated to a gene matrix. The concatenated 2,690,124 nucleotide sequences from 4488 orthologs were used to build a maximum likelihood (ML) tree in Phyml (Version PhyML-3.1) (Guindon *et al.*, 2010). The best-fit GTR nucleotide substitution model was GTR which was derived from jModelTest (version 2.1.4) (Darriba *et al.*, 2012). The ML gene tree was constructed with 100 bootstrap replications.

Identification of Candidate Genes

For each ortholog, evolution analysis for natural selection detection was conducted using codeml in the PAML package (Yang, 2007). The branch-site model, branch model and clade model were used in our study. In these models, specific branches will be considered as foreground branches and the remaining branches are considered as background branches. However, there were significant differences among these three models. The branch model was used to detect the ω (nonsynonymous to synonymous substitution rates) difference between foreground and background, an alternative two ratio model which assumes two different ω values in the background branch and

foreground branch was compared to a one ratio model that considers a uniform ω ratio across all species branches. In the branch site model, the LRT test was employed to test positive selection signal on the foreground branch. In the Clade model C, the alternative clade model C was compared to the null model M1a (nearly neutral) to detect divergent selection.

GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway Enrichment Analysis

To better understand the genes defined using the three models in PAML and meeting the criterion (uncorrected *P* value < 0.05), these genes were used to conduct both GO and KEGG pathway enrichment analyses. Namely, GO enrichment analysis was performed using DAVID (Huang *et al.*, 2009a). The gene IDs for genes identified were converted to Uniprot accession numbers, and the *A. thaliana* gene sets were defined as the background. Modified Fisher's exact tests were used to correct for the significance of functional enrichment. The GO terms were divided into three GO domains: MF (molecular function), CC (cellular component), and BP (biological process). If the modified Fisher exact test *P* value of one term was less than 0.05, then this term will be considered significantly enriched. KEGG pathway enrichment analysis was also conducted using KOBAS 3.0 (Xie *et al.*, 2011). The selected genes identified along the ancestral linage of red pigment radish using the branch site model, branch model and clade model C were tested using KEGG pathway enrichment analysis. Similar to the method used in GO enrichment analysis, all *A. thaliana* genes were defined as the background. The Benjamini-Hochberg method was used for statistical FDR (false discovery rate) correction to identify significantly enriched pathways.

Results

De Novo Assembly and One-to-one Ortholog Identification

In this study, total RNA from the roots of 6 radishes (Table 1) was used to perform sequencing and *de novo* transcriptome assembly. We combined our transcriptome data with the publicly available *R. raphanistrum* genome sequences. One-to-one orthologs were identified using reciprocal BLAST searches. Briefly, the coding sequences of each species were used as queries to search against *A. thaliana* sequences (BLASTX) or used as targets for *A. thaliana* protein sequences (TBLASTN). The best hits from each BLAST search that had an E-value < 1e-5 were used in further analysis. After identifying orthologs, multiple fasta files for each ortholog were extracted using a Perl script. A total of 5401 pairs of ortholog clusters were gained using this method, and 4488 orthologs remained after identifying the conserved region and removing short orthologs.

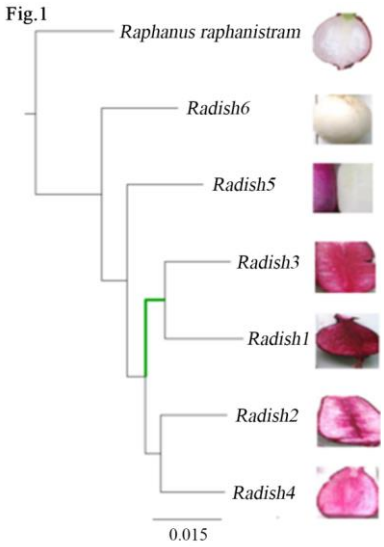

Fig. 1: Phylogenetic relationship of radish group generated in this study. The green line represents the lineage where positive selection was detected. All nodes underwent 100 bootstrap replicates, and *A. thaliana* was used as the outgroup. Scale bar indicates 0.015 estimated substitutions per site. Radish1, Red skin with red flesh of Guanguan cultivar; Radish 2, Red skin with pink coloured flesh of Guanguan cultivar; Radish 3, Red skin with red flesh of Redheart1 cultivar; Radish 4, Red skin with pink coloured flesh of Redheart1 cultivar; Radish 5, Red skin with white flesh of Redheart1 cultivar; Radish 6, white skin with white flesh of White1 cultivar

The maximum likelihood (ML) method implemented in Phyml software was employed to reconstruct the phylogenetic relationship of radish cultivars used in this study. Results from 2,690,124 concatenated nucleotides from 4488 orthologs showed a close relationship between the red pigment radishes from Guanguan cultivar and from the Redheart1 cultivar. Radishes with red skin and pink coloured flesh from these two cultivars grouped together and comprise the sister group to the red pigment radishes; white skin with white flesh radishes of White1 cultivar occupied the basal position within the radish cultivar group and the wild radish (*R. raphanistrum*) occupied the basal position within the whole radish group. All nodes recovered support of 100 bootstraps (Fig. 1). The radish phylogeny of the selected species was constructed using *A. thaliana* as the outgroup.

Molecular Evolution and Enrichment Analysis

We tested if each gene was under selection pressure along the ancestral linage of the red pigment radish. In the ancestral red pigment radish linage, 58, 68 and 105 genes were under positive selection, fast evolving genes and under divergent selection, respectively (likelihood ratio test: uncorrected *P* value < 0.05) (Supplementary Table S1–3). For the GO enrichment analysis, all three gene sets (56, 68

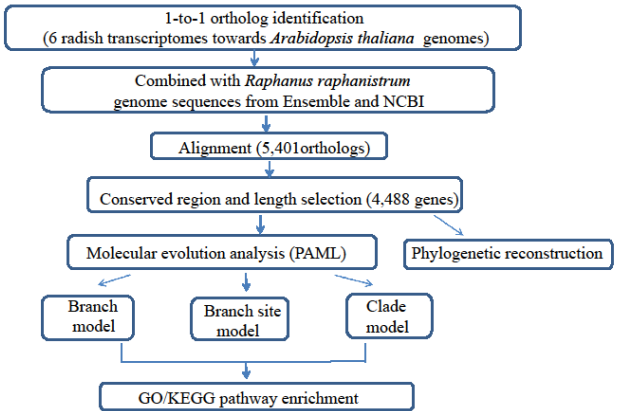

Fig. 2: Overview of workflow and analysis protocols used in this study. The protocol mainly includes ortholog identification, alignment, selection analyses and enrichment analysis steps

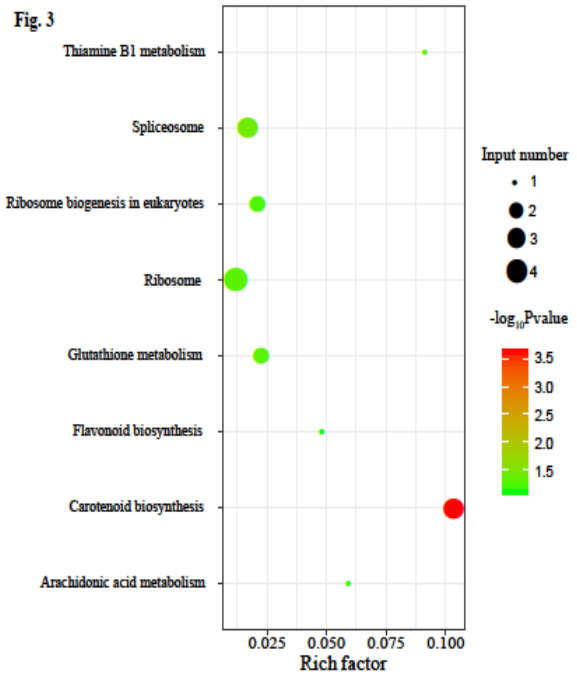

Fig. 3: Scatterplots of enriched KEGG pathways for genes (uncorrected) under selection identified using Clade model C along the ancestral linage of red pigment radish. Rich factor is the ratio of the number of genes under selection to the number of total genes in a specific pathway. The dot size was scaled to the number of selective genes, and the colour of the dots represented -log10 (*P* value) value, green to red corresponding to a larger trend of the -log10 (*P* value) value

and 105) recovered using the branch site model, branch model, and clade model were used to perform GO enrichment analysis (likelihood ratio test: uncorrected $P < 0.05$). GO terms were divided into three GO domains: MF (molecular function), CC (cellular component) and BP (biological process) for simplicity. Key enriched terms consisted of "fruit development" and "seed development" (Supplementary Table S4–6). As for KEGG pathway enrichment analysis, gene sets (56, 68 and 105) (likelihood ratio test: uncorrected $P < 0.05$) recovered from the branch model, branch site model and clade model C along the ancestral linage of red pigment radish were used to perform KEGG pathway enrichment analysis (Supplementary Table S7–9). As the gene sets from the branch model and branch site model did not show interesting enrichment results (Supplementary Table S7–8), we were mainly interested in the genes from the clade model. Several enriched pathways were shown in Fig. 3. Enriched pathways included "carotenoid biosynthesis," "flavonoid biosynthesis," and "glutathione metabolism." These pathways may be involved in red pigment biosynthesis processes in the red pigment radish cultivar.

Candidate Gene Identification

Based on enrichment results and previous studies, several candidate genes were shown to be involved in red pigment formation during the evolution of radishes with red skin and red flesh (Table 2). These genes were involved in many processes, including transcription—*KUA1* (KUODA1), *bHLH155* (proneural basic helix-loop-helix 155), ethylene-responsive transcription factor *ERF012*—carotenoid accumulation—*CCD4*, *BETA-OHASE 1* and *NCED3*—and GST (glutathione S-transferase) superfamily genes like *AXX17_At2g26980* and *AXX17_At4g13080*.

Discussion

A total of 12 genes were identified as candidate genes in adaptation to red pigment formation. Among the 12 red pigment cultivar genes, *KUA1*, a transcript factor, which regulates anthocyanin production in *Arabidopsis* (Nguyen and Lee, 2016), can play a role in regulating red pigment biosynthesis. At5g15710 (F-box/kelch-repeat protein At5g15710) encodes the F-box/kelch-repeat protein, which may affect the accumulation of flavonoid/anthocyanin pigments in *Arabidopsis* (Zhang *et al.*, 2013), so it might play a role in anthocyanin regulation. Previous research showed that transcription factors like those belonging to the BHLH family and MYB families play a role in regulating structural genes of chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavonoid 3-hydroxylase (*F3H*) and dihydroflavonol 4-reductase (*DFR*) in anthocyanin biosynthesis (Davies *et al.*, 2012). Here, we found that *BHLH155*, a member of the BHLH family, was a fast-evolving gene in the red pigment radish lineage (Table 2), suggesting that this gene may contribute to the adaptive evolution of the red pigment radish. UDP-glucosyltransferases are a big protein family in plants, and they contribute to the biosynthesis of flavonoids, phenylpropanoids, terpenoids and in the regulation of plant hormones (Liu *et al.*, 2015). Among fast-evolving genes, we found *UGT71C5* (UDP-glycosyltransferase 71C5), a

Table 1: Those *Raphanus* samples analyzed in this study

Sample	Cultivar	Pigment characteristics	Red pigment content (‰)
Raphanus raphanistrum	wild radish	Purple skin with white flesh	unexamined
Radish1	Guanguan	Red skin with red flesh	25.46
Radish2	Guanguan	Red skin with pinkly color flesh	12.34
Radish3	Redheart1	Red skin with red flesh	28.75
Radish4	Redheart1	Red skin with pinkly color flesh	15.72
Radish5	Redheart1	Red skin with white flesh	2.13
Radish6	White1	white skin with white flesh	0

Table 2: Candidate genes involved in the red pigment formation of red skin with red flesh cultivar

	Gene name	*P* value	Swissprot annotation
Branch	*KUA1*	0.041	Transcription factor KUA1
	At5g15710	0.0032	F-box/kelch-repeat protein At5g15710
	BHLH155	0.010	Transcription factor bHLH155
	ERF012	2.86E-08	Ethylene-responsive transcription factor ERF012
	UGT71C5	0.0029	UDP-glycosyltransferase 71C5
Branch Site	*ERF012*	2.05E-11	Ethylene-responsive transcription factor ERF012
	ERF4	0.0015	Ethylene-responsive transcription factor 4
Clade model	*CCD4*	0.0021	Probable carotenoid cleavage dioxygenase
	BETA-OHASE 1	0.0015	Beta-carotene 3-hydroxylase 1, chloroplastic
	NCED3	0.00020	9-cis-epoxycarotenoid dioxygenase
	AXX17_At2g26980	2.15E-14	GSTF9
	AXX17_At4g13080	1.03E-05	Glutathione peroxidase

member of the UDP-glucosyltransferase family. In our study, the pathway "carotenoid biosynthesis" was found to be enriched and genes annotated to that pathway included *CCD4* (carotenoid cleavage dioxygenase), *BETA-OHASE 1* (beta-carotene 3-hydroxylase 1, chloroplastic), and *NCED3*. *CCD4*, was originally found in Chrysanthemum (*Chrysanthemum morifolium)* (Ohmiya *et al*., 2006); RNA interference (RNAi) against the CCD4 gene showed that suppression of this gene may result in colour variation, demonstrating that CCD4 is involved in controlling the red colour (Huang *et al.*, 2009b). *NCED3* (9-cis-epoxycarotenoid dioxygenase 3) catalyses the first step of abscisic-acid biosynthesis from carotenoids (Ruggiero *et al.*, 2004) and *BETA-OHASE 1* plays a role in xanthophyll biosynthesis (Fiore *et al.*, 2006). Previous research showed that flavonoid/anthocyanin biosynthesis could be affected by carotenoid accumulation (Cao *et al.*, 2015) and this showed that these three genes could have some relationship to pigment formation. Another enriched pathway was "flavonoid biosynthesis" and *AXX17_At4g13080* (glutathione peroxidase) is a member of this pathway. *AXX17_At2g26980* (glutathione S-transferase 9) encodes the protein GSTF9 (glutathione S-transferase). GST proteins are known to act as carrier proteins in flavonoid transport (Grewal *et al.*, 2012). Except for the genes mentioned above, two ethylene-responsive transcription factors, *ERF012* and *ERF4* (ethylene-responsive transcription factor 4), were also identified. An earlier study showed that ethylene appears to be a key factor regulating anthocyanin biosynthesis and colour development in the "Pink Lady" apple (Whale and Singh, 2007), and flavonoid biosynthesis is regulated by endogenous factors like plant hormones of ethylene (Nguyen and Lee, 2016). These genes are valuable resources for red pigment formation in the red pigment radish. Further studies of gene expression profiles and genes functions are important and will help a lot in understanding the mechanisms of red pigmentation in radish species. Here, the value of this study is mainly to identify possible candidate genes for future study.

The phylogenetic relationship was reconstructed using concatenated nucleotide sequences of different radish cultivars with the maximum likelihood method. The results showed that radish cultivars with red pigment grouped together, and two radish cultivars with red skin and pinkly colour flesh also grouped into a clade. These results indicated that molecular classifications of radish cultivars were consistent with the traditional taxonomic classifications based on root skin and flesh colour and novel EST SSR markers (Jiang *et al.*, 2012), but it was not consistent with previous analyses of genetic markers (Liu *et al.*, 2008; Wang *et al.*, 2015). These inconsistencies may derive from the sample size and methodological differences. Here, a relatively smaller sample size and the different tree reconstruction method were compared to the previous study. Therefore, more samples are needed to further confirm the phylogenetic relationship of radish cultivars.

Here, complete transcriptomes of six different colour radish cultivars were obtained using Illumina Solexa sequencing. Molecular evolution analysis showed that a total of 58, 68 and 105 genes were identified under selection using the branch model, branch site model and clade model C, respectively. However, we only focused on coding regions of genes under selection. The transcript level and regulated non-coding RNAs, including long ncRNAs (long-noncoding RNAs) and siRNAs (small interfering RNA) might also be considered. In addition, selection cases are

always episodic at a special period and do not occur across the whole period of evolution. Therefore, it could not detect adaptation signals across the whole period of evolution. Therefore, some new methods must be developed and employed to detect more selection signals. Moreover, we found that most candidate genes related to red pigment formation were not annotated in public databases like the NR (NCBI non-redundant protein) and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) databases. These specific genes may be very helpful for understanding the evolution and origin of red pigment radish cultivars in the future.

Conclusion

Around 105 genes were found to be under selection along the ancestral linage of the red pigment radish cultivar. Among 105 identified genes involved in ancestral linage of the red pigment radish, 12 candidate genes were shown to be associated with the red pigment formation. Our study enriches the genetic resources available for radish cultivars, and it may also open the door for investigating the molecular evolution of the red pigment radish group.

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