Horticultural Science Focus

The carrot genome provides insights into crop origins and a foundation for future crop improvement

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Figure 1. Carrot color conditioned by segregation at the *Y* locus. A. A carrot population segregating at the *Y* locus, in a y_2y_2 background. All these carrots accumulate alpha- and beta-carotene as their predominant carotenoids like typical orange carrots grown today. Like today's carrots, the genotype of the darker orange carrots (upper left row) is $yy\ y_2y_2$. The genotype of pale orange carrots (upper right row and lower row) is $Y\_\ y_2y_2$ and they typically contain only 10-25% as much alpha- and beta-carotene as orange carrots. B. Slices of yellow carrots (yyY_2Y_2), which contain lutein, and white carrots ($Y\_\ Y_2Y_2$), which contain, at most, trace amounts of carotenoids, from a population segregating at the *Y* locus, in a Y_2Y_2 background.

Introduction

Vavilov (1951) placed the center of origin of cultivated carrot in Central Asia, and an analysis of molecular diversity in wild and cultivated carrots from around the world demonstrated that wild carrots from Central Asia were more similar to cultivated carrots (Iorizzo et al., 2013), confirming Vavilov's conclusions. Carrots may have been cultivated as a root crop in the Roman Empire, with extensive cultivation first recorded around 900 AD in Central Asia – Afghanistan in particular (Stolarczyk and Janick, 2011; Banga, 1963). Color has played an important role in the history of carrot domestication. The first Central Asian carrots were yellow or purple, and in the early 1500s, orange carrots were noted in still life paintings and some written accounts in Europe. Central Asian carrots spread first to the west beginning in the 900s, through the Middle East, North Africa, and then Europe; and to the east to South and North Asia (Banga, 1963). Orange carrots are grown globally today but yellow, purple, red, and white carrot land races, and some modern cultivars, are grown on a more limited scale in several parts of the world.

Carrot is among the top 10 vegetable crops globally and is a rich source of vitamin A, thanks to the popularity of orange carrots, which contain vitamin A precursors α- and β-carotene (Simon et al., 2009). The lutein in yellow carrots, anthocyanins in purple carrots, and lycopene in red carrots are also well-documented phytochemicals (Arscott and Tanumihardjo, 2010). Carrot is a diploid outcrossing crop and all carrot cultivars were open pollinated before the discovery of the first cytoplasmic male sterility (CMS) in carrot by Welch and Grimball (1947). The discovery of CMS in carrot triggered an expanded effort in carrot breeding and genetics, with the development of the first hybrid carrot cultivars and the first genes named in the 1960s. By the mid-1980s about 20 simply-inherited traits had been reported. Carrot is typically categorized as a cool season crop and most production is in temperate climates, but subtropical cultivars have been developed and have expanded the climate range of carrot production, especially since the 1970s (Simon, 2000).

Laying the foundation for sequencing the carrot genome

The sequencing of the carrot genome was an effort that formally began in 2012

and culminated with the publication and release of the genome in 2016 (Iorizzo et al., 2016). Financial support from eight seed companies (Bejo, Carosem, Monsanto, Nunhems, Rijk Zwaan (RZ), Sumika, Takii, and Vilmorin) provided funds to complete the project, and RZ also provided the DH1 carrot, both BAC (bacterial artificial chromosome) libraries and BAC end sequences of the sequenced carrot.

Several important tools that contributed to the sequencing project were developed before the project began. Genetic linkage maps provided critical information to arrange and confirm the order of superscaffolds and pseudomolecules generated by the assembly of DNA sequence, and to independently confirm that order. Linkage


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maps were also essential to identify the candidate gene for the *Y* locus (Figure 1), which conditions carotenoid accumulation in the carrot tap root. These maps were generated from segregating populations developed from crosses made as early as 1989 to generate QTL maps for pigment accumulation and nematode resistance (Santos and Simon, 2002; Just et al., 2009) (Figure 2). Sequences for the carrot plastid (Ruhlman et al., 2006) and mitochondrial genomes (Robison and Wolyn, 2002; Iorizzo et al., 2012) had been published and contributed to confirmation of organellar genome sequence in this project. To verify the quality of the genome assembly, FISH (fluorescent in situ hybridization) (Iovene et al., 2008, 2011) and transcriptome sequence (Iorizzo et al., 2011) were used. Repetitive sequences account for a significant part of all eukaryotic genomes and previous research on MITEs (miniature inverted repeat transposable elements) (Grzebelus et al., 2006; Macko-Podgorni et al., 2013) provided a foundation to analyze their distribution in carrot and evolutionary trends.

Goals and key aspects of the carrot genome sequencing project

A full genome sequence provides the ultimate foundation to study genetics, gene function, and evolution of a species. The primary goal of the carrot genome project was to generate a high quality genome assembly, including characterization of repetitive sequences and annotation of genes. The carrot sequenced in this project was DH1, a doubled haploid Nantes type orange carrot (Figure 3). The full genome sequence generated will serve as the standard of comparison for future studies evaluating the breadth of diversity of *Daucus carota*. To begin to assess that breadth of genetic diversity, we resequenced 35 diverse cultivated and wild carrots and four other *Daucus* species in this study, and evaluated their phylogenetic relationships. Gene function works in concert with a multitude of variables that impinge on that function, including development and numerous abiotic and biotic variables in the environment, to generate the phenotypic variation of biology and agriculture. To begin to assess gene function in the carrot genome, we evaluated the transcriptome of 20 diverse plant tissues of vegetative, flowering, and stressed plants. We also utilized the carrot genome sequence to better understand the function of the *Y* gene, which regulates high carotenoid accumulation in carrots that have either yellow or orange storage roots, relative to the white wild type. To study molecular plant evolution at the genome-wide level, full genome sequence data are necessary, and relatively few full plant genome sequences are published, particularly in the Euasterid II clade. We compared 12 plant genomes to carrot and evaluated evidence for genome duplication and evolutionary relationships in this study.

Assembly and characterization of the carrot genome

Sequenced Illumina libraries of the DH1 genome included 3 paired end libraries (170-800 nt insert size) and 5 mate-pair libraries (2-40 kb insert size). End sequences of BAC (bacterial artificial chromosome) libraries of the DH1 genome provided additional paired end sequences spanning 148±70 kb. These paired end sequences, ranging from 170 nt to >200 kb, combined with a newly developed consensus linkage map using marker data from QTL studies noted earlier, were used to generate the *de novo* assembly of the carrot genome. Several tests were made to evaluate the correctness of the assembly sequence, including an evaluation of the assembled sequence with an 8-kb 454 library, additional BAC paired end sequences not previously used, and a new GBS SNP genetic map, to evaluate the assembly on a larger scale. Gene sequence of expressed genes (ESTs) and ultra-conserved genes were used to evaluate the assembly on a smaller scale. Over 99.7% of unassembled sequence reads aligned to the assembly, over 94% of the 454 library, additional BAC ends, ESTs, and ultra-conserved genes aligned to the genome assembly. GBS markers also aligned with high collinearly, confirming the accuracy of the assembly. In addition, FISH experiments were carried out to evaluate the consistency and coverage of assembly, focusing on repetitive sequences in chromosome-specific regions, subtelomeric regions, telomeres, and centromeres. These experiments indicated that the carrot genome assembly extends into the telomeric and subtelomeric regions of the genome, confirming high coverage of the assembly. Based upon the assembly statistics, combined with corroborating studies to evaluate correctness of that assembly, the carrot genome assembly is among the most complete published, based upon both coverage and length of sequence contiguity. A total of 60 superscaffolds anchored with SNP markers mapped on the nine carrot chromosomes accounting for 85% of the carrot genome. One superscaffold on chromosome 4 spans 85% of that chromosome, and one recombination event occurs roughly every 388 kb.

Repetitive DNA sequences, including mobile elements (MEs) and large clusters of tandem repeats (TRs), typically account for a significant part of eukaryotic genomes, and carrot is no exception. MEs typically occupy 20-40% of plant genome assemblies and in carrot they account for 46% of the assembled genome. Most carrot MEs (98%) are transposable elements, and given their abundance, the distribution of carrot ME insertion sites was evaluated. Over half were found to be near (<2 kb), or in genes, distributed in a random pattern. Large clusters of TRs were observed in FISH studies in carrot telomeres, using a telomere-specific probe that hybridizes to all telomeres, and a centromere-specific probe that hybridizes to all centromeres in the carrot genome. A third probe was prepared to hybridize to a TR that forms a knob only on chromosome 1, called CL80. To expand our evaluation of repetitive DNA sequences beyond carrot to the other *Daucus* species, 106 paired end reads were also generated for *D. syrticus*, a species closely related to carrot, and to four more distantly related species (Arbizu et al., 2014a, b) – *D. aureus*, *D. guttatus*, *D. littoralis*, and *D. pusillus*. Interestingly, an evaluation of *D. syrticus* sequences revealed that the carrot centromeric repeat was highly abundant in this species; however, the CL80 repeat was not present. In two of the more distantly related species, *D. guttatus* and *D. littoralis*, FISH analysis revealed that the centromere repeat

Figure 2. A carrot population segregating at both the Y_2 locus and one of the anthocyanin (*P*) loci. The *Y* locus is not segregating, *yy*. Carrots with orange color or absence of anthocyanins are homozygous recessive for the y_2y_2 or *pp* loci, respectively; yellow or purple carrots indicate the presence of at least one dominant Y_2 or *P* allele, respectively.

sequence found in carrot was absent, while the CL80 repeat was highly represented in subtelomeric and pericentromeric regions of most of these chromosomes. This redistribution and repurposing of repetitive sequences among *Daucus* species provides an interesting foundation for future research.

Characterization of the carrot genome identified 32,000 genes, based on comparison of genes annotated in the genomes of other plants, of which 98.7% were observed in the carrot transcriptome. Putative gene functions were assigned and aligned to catalogued plant proteins.

Carrot diversity

Genetic diversity in cultivated and wild carrots provides the heritable variation necessary to drive future crop improvement, and provides insights into crop domestication. For the carrot genome sequencing project, 35 diverse wild and cultivated carrots were resequenced, among which 1.3×10^6 SNPs were identified, and 49,365 SNPs were selected for phylogenetic and cluster analysis. As in previous studies with 3,202 SNPs (Iorizzo et al., in press), Central Asia and the Middle East were found to be primary centers of carrot domestication. The allelic constitution of eastern cultivated carrots (Asian carrots, so-named because they originated east of Central Asia) was more similar to that of Central Asia wild carrots than western cultivated carrots (European, North African, and Middle Eastern carrots), likely reflecting more intensive breeding in western cultivated carrots.

As was observed in previous studies, the genetic diversity of cultivated carrots, mostly open pollinated cultivars, was only slightly lower than that of wild carrots, suggesting a relatively limited bottleneck during carrot domestication. Comparing wild carrot with eastern cultivated accessions, five carrot chromosomes had genomic regions with high levels of population differentiation (F_{ST}), indicating that genomic regions on these chromosomes include genes for domestication traits.

Carrot genome evolution

Carrot is a member of the Euasterid II clade which also includes lettuce and sunflower, as well as other *Apiaceae* like celery, coriander and parsnip. To study the evolution of the carrot genome, the whole genome sequence of carrot and 12 other plants were compared, to identify orthologous genes – genes in different species that evolved from a common ancestral gene during speciation. Analysis of sequence divergence in ortholog sequences estimated the time frame in which Euasterids II diverged from Rosids, Ericales, and Euasterids I to be ~113, ~101, and ~90.5 million years ago, respectively, whereas carrot diverged from lettuce ~72 million years ago. Whole genome duplications, reflected in multiple blocks of syntenic genes, play a significant role in plant evolution. Two such duplications, or possibly a duplication and a triplication, occurred in the evolution of carrot. In addition to whole genome duplications, lineage-specific duplications expanded the representation of some gene families but not others. As a result, relative to other plants, the carrot genome, for example, contains few genes in the MADS transcription factor family, which plays an important role in the development of reproductive organs, and few genes in most classes of resistance genes involved in plant-pathogen introductions. In contrast, the carrot genome contains an expanded set of zinc-finger genes involved in several important biological processes including morphogenesis and symbiosis. Mechanisms by which plant genomes compensate for relative over- and under-represented gene families are not well understood so carrot may serve as a model for studying these mechanisms.

A candidate gene for carotenoid accumulation

While wild carrot tap roots accumulate little or no carotenoids, the first cultivated carrots were yellow, or purple and yellow, while most modern carrots are orange. The *Y* and *Y2* genes condition carotenoid accumulation, and the genotypes of white, yellow, and orange carrots are *Y_Y2_*, *y y Y2_*, and *y y y2 y2*, respectively (Buishand and Gabelman, 1979). The sequencing of the carrot genome provided an opportunity to identify candidate genes for the *Y* gene. Using two mapping populations segregating for the *Y* gene (*YyY2Y2* and *Yyy2y2*), fine-mapping identified a 75-kb region of chromosome 5 harboring the *Y* gene. None of the eight predicted genes in this region had a role in isoprenoid biosynthesis. Three predicted genes had synonymous SNPs but one gene, DCAR_032551, had a 212-nt insertion in its second exon in both mapping populations. That insertion creates a frame-shift mutation, which co-segregated with the *Y* allele phenotype, and with differential gene expression patterns. A survey of diverse wild carrots and white cultivated carrots revealed no samples with the *Y* inser-

tion, and all but two diverse orange carrots surveyed were homozygous for the insertion. Those two exceptional orange carrots were heterozygous for the insertion, and another nonsynonymous allele, a 1-nt insertion in the second exon that also causes a frameshift mutation, was noted. An evaluation of LD (linkage disequilibrium) in the genomic region bearing the *Y* gene found increased LD and reduced nucleotide diversity in *yy* carrots, indicating this region was under selection. The discovery of two different *y* alleles indicates that the *yy* high pigment phenotype was selected more than once.

How might the *Y* gene function?

In an analysis of the carrot transcriptome, 925 genes were co-expressed with the candidate for the *Y* gene, DCAR_032551, and gene expression in the isoprenoid pathway was especially enriched. Earlier research had also demonstrated that genes in this pathway are expressed in orange, yellow, and white carrot storage roots (e.g. Just et al., 2007). The functioning of the isoprenoid pathway in orange roots may, on first consideration, be surprising, because carotenoids have no known function in roots. However, downstream products of the pathway beyond carotenoids, such as abscisic acid and strigolactones, are critical for root growth and response to abiotic stress. With that in mind, it is the accumulation of carotenoids in yellow and orange carrot storage roots that is unusual, rather than carotenoid biosynthesis itself. While analysis of expression confirmed overexpression of several genes in the MEP (methylerythritol phosphate) and carotenoid pathways in *yy* plants, genes in the monoterpenoid biosynthetic portion of pathway, in contrast, were generally underrepresented in *yy* orange and yellow carrots. Volatile monoterpenoids account for a significant portion of the flavor of raw carrots, so it might be speculated that yellow and orange carrots are less flavorful than white carrots, but that has not been observed (Surles et al., 2004), suggesting additional regulation of monoterpenoid biosynthesis.

While the DCAR_032551 gene is not a biosynthetic gene in the isoprenoid pathway, it does have a homolog in the *Arabidopsis* genome, *pel* (PSEUDO-ETIOLATION IN LIGHT). Mutants of the *pel* gene have a defective response to light in leaves and stems (Ichikawa et al., 2006), but without a known function. Aspects of the *Y* gene are reminiscent of the *cop/det* photomorphogenic repressor genes of Arabidopsis, in which seedlings grown in the dark lack the typical characteristics of etiolation (elongated hypocotyls and cotyledons; chlorosis). Instead, they resemble light-grown seedlings in overall morphology, plastid differentiation, and carotenoid accumulation, including a transcriptome pattern of light-regulated genes comparable to that for seedlings grown in light (Lau and Deng, 2012). Given the similarity between the COP/DET mutant phenotype, referred to as de-etiolated, the similar transcriptome pattern observed with the *Y* gene, and physiological studies in carrot demonstrating ectopic chloroplast differentiation in roots exposed to light (Fuentes et al., 2012), the *Y* gene function resembles de-etiolation in the carrot tap root. The extent to which this similarity holds up will be revealed as the function of the *pel* gene in Arabidopsis and the *Y* gene in carrot are studied in more detail.

Figure 3. DH1, the carrot used to sequence the genome.

Applications of the carrot genome

The sequencing of the carrot genome provides a foundation for additional studies in plant evolution, especially as more Euasterid II plant genomes are sequenced, and as comparative genomic and FISH studies involving tandem repeats are pursued. The carrot genome sequence has already served as a valuable resource in clarifying the phylogeny of the genus *Daucus* and close relatives (Arbizu et al., 2014a, b). Similarly, insights into the phenotypic signatures of carrot domestication can be associated with selection for specific genes with access to the genome sequence. Several immediate applications are already being pursued with the development of molecular markers closer to, or in, genes under selection in breeding programs for more simply inherited traits, or in applying GWAS (genome-wide association study) approaches to select complex traits in carrot. The identification of candidate genes will provide a deeper understanding of traits. A more extensive catalog and intensive understanding of the carrot transcriptome is also already growing, given the release of the carrot genome. In 2008, only 600 DNA nuclear sequences were at NCBI for *Daucus*, and 5350 for all *Apiaceae*; whereas in 2014, those numbers had risen to 30,855 sequences for *Daucus*, and 54,000 for all *Apiaceae* (Iorizzo et al., in press). The carrot genome sequencing project, in combination with additional genomic analysis in other *Apiaceae*, has increased those numbers to 92,674 and 121,068 today. Whole genome sequencing and resequencing projects will certainly continue to enrich the genomic resources available to plant scientists working with the *Apiaceae*, and with other plants.

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