RESEARCH PAPER

The multi-allelic \textit{APRR2} gene is associated with fruit pigment accumulation in melon and watermelon

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Abstract

Color and pigment contents are important aspects of fruit quality and consumer acceptance of cucurbit crops. Here, we describe the independent mapping and cloning of a common causative \textit{APRR2} gene regulating pigment accumulation in melon and watermelon. We initially show that the \textit{APRR2} transcription factor is causative for the qualitative difference between dark and light green rind in both crops. Further analyses establish the link between sequence or expression level variations in the \textit{CmAPRR2} gene and pigment content in the rind and flesh of mature melon fruits. A genome-wide association study (GWAS) of young fruit rind color in a panel composed of 177 diverse melon accessions did not result in any significant association, leading to an earlier assumption that multiple genes are involved in shaping the overall phenotypic variation in this trait. Through resequencing of 25 representative accessions and allelism tests between light rind accessions, we show that multiple independent single nucleotide polymorphisms in the \textit{CmAPRR2} gene are causative of the light rind phenotype. The multi-haplotypic nature of this gene explains the lack of detection power obtained through genotyping by sequencing-based GWAS and confirms the pivotal role of this gene in shaping fruit color variation in melon. This study demonstrates the power of combining bi- and multi-allelic designs with deep sequencing, to resolve lack of power due to high haplotypic diversity and low allele frequencies. Due to its central role and broad effect on pigment accumulation in fruits, the \textit{APRR2} gene is an attractive target for carotenoid bio-fortification of cucurbit crops.

Keywords: \textit{APRR2}, BSA-Seq, carotenoids, chlorophyll, fruit quality, GWAS, melon, QTL, RNA-Seq, watermelon.

Introduction

Flesh and rind pigmentation are key components affecting the nutritional value and consumer preference of the major cucurbit crops, melon and watermelon. Both crops exhibit extreme diversity in fruit traits, including size, shape, color, texture,
Regulation of rind color in cucurbits initiates early in fruit development and is expressed as green color intensity at the young fruit stage, reflecting chlorophyll concentrations (Tadmor et al., 2010). Most watermelons remain green at maturity, with chlorophyll being their main rind pigment, and therefore rind color variation in watermelon is mostly expressed as green pigment intensity in uniform or striped patterns (Gusmini and Wehner, 2005). Conversely, melon rind color transforms during development, leading to extensive variation in mature fruit pigment profiles that include different combinations of carotenoids, flavonoids, and chlorophylls (Burger et al., 2010; Tadmor et al., 2010). The genetic basis of this variation is only partly resolved. Several external fruit color quantitative trait loci (QTLs) have been mapped in populations derived from a cross between a Piel de Sapo line and PI161375 (Monforte et al., 2004). It has been previously reported that the mature yellow rind color of yellow casaba melon accessions (Cucumis melo var. inodorus) is caused by the accumulation of naringenin chalcone, a yellow flavonoid pigment (Tadmor et al., 2010). A Kelch domain-containing F-box protein-coding gene (CmKFB) on chromosome 10 was identified as causative for the naringenin chalcone accumulation in melon fruit rind (Feder et al., 2015). While it is logical to assume that young and mature fruit color intensity are correlated, and that common genetic factors may be involved, thus far such genes have not been reported in melon.

Three major flesh color categories are defined in melon: green, white, and orange, with β-carotene and chlorophyll being the predominant pigments of the orange and green phenotypes, respectively (Burger et al., 2010). The major locus qualitatively differentiating between orange and non-orange flesh is green flesh (gf), located on chromosome 9 (Cuevas et al., 2009). gf was recently shown to be the CmOr gene, which governs carotenoid accumulation and orange flesh color (Tzuri et al., 2015). A second qualitative flesh color locus, white flesh (uf), which is associated with the difference between white and green flesh, has been previously described and mapped to chromosome 8 (Clayberg, 1992; Monforte et al., 2004; Cuevas et al., 2009). Another layer of quantitative variation in flesh pigment content and color intensity exists within those color classes, as defined by several QTL mapping studies (Monforte et al., 2004; Cuevas et al., 2008, 2009; Paris et al., 2008; Harel-Beja et al., 2010; Diaz et al., 2011). These include the recent fine-mapping to a candidate causative gene level of flesh carotenoid QTLs using a recombinant inbred line (RIL) population (Galpaz et al., 2018). Thus far, however, causative genes governing this quantitative variation have not been shown.

In recent years, a few transcription factors involved in regulation and synchronization of chlorophyll and carotenoid accumulation were identified in plants. Among these are Golden2-like (GLK2) transcription factors, which regulate chloroplast development (Chen et al., 2016). Allelic or expression variation in the GLK2 gene was shown to be associated with levels of chlorophyll and carotenoids in Arabidopsis, tomato, and pepper (Waters et al., 2008, 2009; Powell et al., 2012; Brand et al., 2014). A related but distinct transcription factor gene, the ARABIDOPSIS PSEUDO-RESPONSE REGULATOR2-LIKE gene (APRR2) with phenotypic effects comparable with those of GLK2, was identified and shown to regulate pigment accumulation in tomato and pepper (Pan et al., 2013), and overexpression of the APRR2 gene in tomato increased the number of plastids and the color intensity. Recently, the APRR2 gene was also shown to be causative of the white immature rind color (w) in cucumber (Cucumis sativus) (Liu et al., 2016), a close relative of melon (Cucumis melo). The white rind phenotype of immature cucumbers in this study was shown to be associated with reduced chloroplast number and chlorophyll content.

In the current study, we used bi-parental populations to map and identify the APRR2 gene as a common causative regulator of pigment accumulation in both melon and watermelon. We show that the effect of this transcription factor on pigment accumulation is initially observed in the rind of young fruits (chlorophylls) and extends to rind and flesh of mature melon fruits (chlorophylls and carotenoids). Through further analysis of wider genetic variation in melon, we revealed a unique multi-allelic pattern that inhibited our ability to detect a significant signal through genotyping by sequencing (GBS)-based genome-wide association study (GWAS). By zooming in on this allelic series, we confirmed the central role of this gene in shaping the color variation of young fruit rind across melon diversity.

Materials and methods

Plant materials and field trials

The germplasm used in this study included four sets. (i) TAD×DUL RILs (F1)—bi-parental segregating population derived from the cross of the dark rind line, ‘Dulce’ (DUL; C. melo var. reticulatus) with the light rind line, ‘Tam Dew’ (TAD; C. melo var. inodorus) (Tzuri et al., 2015). One hundred and sixty-four F1 RILs were developed through single-seed-descent. All RILs, F1, and the parental lines were grown in a randomized block design (RCBD) in an open field at Newe Ya’ar Research Center, in the spring–summer seasons of 2016 and 2017. Each line was represented by two replicates of five plants per plot. (ii) NA×DUL F3 and F3:4—an F3 population from this cross was grown in two repetitions in a greenhouse at Beth Elzari, Israel in 2013 as previously described (Ríos et al., 2017). This population is derived from the cross between the light rind line ‘Noy-Amid’ (NA; C. melo var. inodorus) and the common dark rind parent, DUL. One hundred and fourteen F3 families from F3 genotyped plants alongside the parental lines and their F1 were grown in an RCBD in an open-field trial at Newe Ya’ar Research Center in the spring–summer season of 2017 in two replicates of six plants per plot. (iii) Melo180 GWAS panel—a Newe-Ya’ar melon collection used in this study comprised 177 diverse accessions that represent the two melon subspecies (sp. agrestis and sp. melo) and 11 taxonomic groups. Each accession was represented by three plots of five plants each in an RCBD in the open field at Newe-Ya’ar in summer 2015 (Gar et al., 2017). (iv) NY0016×EMB F3 family—f the mapping the light rind trait in watermelon, the light rind inbred accession NY0016 was crossed with the canary yellow accession Early Moon Beam (EMB) to produce 87 F2 families (Brandham et al., 2017). During the summer of 2016 and 2017, 10 plants per F3 family and two plots of 10 plants from the parents and F1 were sown in the open field at Newe-Ya’ar. All the populations used in this study were grown under standard horticultural conditions in open fields at Newe Ya’ar Research Center, northern Israel (32°43′05.4″N, 35°10′47.7″E), soil type was grumusol, and the plants were drip-irrigated and drip-fertilized.

Fruit color phenotyping

In the melon populations, fruit images were taken with a digital camera on developing fruits of all accessions throughout the season, from anthesis...
to harvest. Rind color of young fruits was scored in the field at 10–15 d after anthesis and confirmed based on fruit images from the same developmental stage. Mature rind and flesh color were measured on ripe fruits, which were harvested based on abscission in climacteric fruits, or days after anthesis, rind color, and TSS (total soluble solids) in non-climacteric fruits. Five mature fruits per plot were photographed externally then cut along the longitudinal section and scanned for internal imaging, using a standard document scanner (Canon, Lide120) as described previously (Gur et al., 2017). Scanned images were analyzed using the Tomato Analyzer software (Rodríguez et al., 2010) for color (L, A, B, Chroma, and Hue) and morphological features. Rind and flesh tissues were sampled into 50 ml tubes from at least three fruits per plot, immediately frozen in liquid nitrogen, and then stored at –80 °C for further analyses. For the watermelon mapping experiment (NY0016×EMB F2:3), 10 F3 individuals per F2:3 family were harvested at maturity (~70 d post-sowing), imaged, and phenotyped for rind color as above.

Quantification of carotenoids and chlorophyll
Carotenoids were extracted from 0.5 mg ground tissue samples in a mixture of hexane:acetone:ethanol (2:1:1, v/v/v) as described previously (Tadmor et al., 2005), and separated using a Waters 2695 HPLC apparatus equipped with a Waters 996 PDA detector (Milford, MA, USA). Carotenoids were identified by their characteristic absorption spectra, distinctive retention time, and comparison with authentic standards. Quantification was performed by integrating the peak areas with standard curves of authentic standards with the Waters millennium chromatography software. Lutein and β-carotene were relatively quantified at 450 nm and 270 nm, respectively, by integrating their peak areas and calculating their percentage from total integrated peak areas. Tissues for chlorophyll determination were sampled as explained for carotenoid analysis. Chlorophyll extraction was performed in dimmed light to avoid possible photodegradation of chlorophyll. Chlorophyll was extracted by adding 5 ml of DMSO to 0.5 g, vortexing, and incubating in the dark at room temperature for 24 h. The extract was analyzed for absorbance in the wavelengths of 663 nm and 645 nm using a Cary50 Bio spectrophotometer (Varian). Chlorophyll concentration was calculated as described by Tadmor et al., (2010).

Genotyping
DNA isolations were performed using the GenElite™ Plant Genomic Miniprep Kit (Sigma–Aldrich, St. Louis, MO, USA). DNA quality and quantification were determined using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) spectrophotometer, electrophoresis on an agarose gel (1.0%), and Qubit® dsDNA BR Assay Kit (Life Technologies, Eugene, OR, USA).

GBS analysis, SNP calling, and map construction

**TAD×DUL RILs**
DNA from 164 F2 individuals was processed by Novogene (Novogene Bioinformatics Institute, Beijing, China) for GBS analysis. An aliquot of 0.3–0.6 μg of genomic DNA from each sample was digested with the ApeKI restriction enzyme, based on the in silico evaluation results, and the obtained fragments were ligated with two barcoded adaptors at each end of the digested fragment. Following several rounds of PCR amplification, all the samples were pooled and size-selected for the required fragments to complete the library construction. Samples were diluted to 1 ng μl–1 and the insert size was assessed using the Agilent® 2100 bioanalyzer; quantitative PCR was performed to detect the effective concentration of each library. Libraries with a concentration >2 nM were sequenced on an Illumina HiSeq 2000/2500 platform as 144 bp, paired-end reads and mapped to the *C. melo* reference genome DHL92 v3.5.1 (Garcia-Mas et al., 2012; available at http://melonomics.cragenomica.es/files/Genome/Melon_genome_v3.5.1/). Over 570 million reads were produced covering nearly 21% of the genome across >35 million tags at an average read depth of nine reads per site. Single nucleotide polymorphism (SNP) calling was carried out using Broad Institute’s genome analysis toolkit (GATK) (McKenna et al., 2010), resulting in 1 205 528 raw SNPs. Sites with a depth of less than three reads per site or >50% missing data were filtered out using TASSEL v.5.2.43 (Bradbury et al., 2007). Data were then imputed using the full-sib families linkage disequilibrium (LD) algorithm (Swarts et al., 2014) followed by the removal of individuals with excessive heterozygosity. The genotypic data set was phased to ABH format consisting of 89 343 SNPs across 146 lines. Binning was performed using SNPhitner (Gonda et al., 2019) with a minimum ratio between cross-points set at 0.001 and minimum bin size of 1000 bp. Bin statistics and genetic distance were calculated using an in-house script developed in python, based on the Kosambi mapping function (Kosambi, 1943). The final set included 2853 recombination bins across 146 lines. Evaluation of genotypic data quality was done by accurately mapping fresh color to a 55 kb interval spanning the previously published CmOr gene (Melo3C05449) (Tzuri et al., 2015).

**NY0016×EMB F2:3**
DNA from 140 F3 individuals was processed by NRGene LTD (Nes Zyyona, Israel) for restriction site-associated DNA sequencing (RAD-seq) (Rios et al., 2017). SNP calling was performed following similar methods, and the initial marker set included 43 975 SNPs across 140 individuals with an average depth of 16 reads per site. Further filtering and imputation were performed as described above, and the final set for binning was composed of 19 015 SNPs across 134 individuals. Binning and genetic map construction were carried out using the same parameters as those used for the TAD×DUL population, yielding 1321 bins across 134 individuals.

**GWAS180**
Genotyping of this diversity panel was performed using GBS, as described by Gur et al. (2017). The final SNP set included 23 931 informative SNPs [at a minor allele frequency (MAF) of >5% across 177 accessions.

**NY0016×EMB F2:3**
The watermelon mapping population NY0016×EMB was genotyped by GBS. Library construction, sequencing, and SNP calling were performed at the Genomic Diversity Facility at Cornell University (Ithaca, NY, USA) as described by Branham et al. (2017). Sequences in this project were aligned to the Charleston Gray genome, version 1 (available at http://cucurbitgenomics.org/organism/4).

**Bulk segregant analysis by sequencing (BSA-Seq) of the watermelon population**
DNA samples from 35 F3 plants (from the NY0016×EMB cross) homozygote for the rind color trait (based on the F1 family’s phenotypes) were prepared in two bulks (light rind, 19 F3 samples; and dark rind, 16 F3 samples). These samples, as well as DNA samples of the parental lines (EMB and NY0016), were used for whole-genome resequencing (WGS) performed at the DNA Services Center at the University of Illinois, Urbana-Champaign. Six shotgun genomic libraries were prepared with the Hyper Library construction kit from Kapa Biosoysms (Roche) with no PCR amplification. The libraries were quantitated by qPCR and sequenced on one lane for 151 cycles from each end of the fragments on a HiSeq 4000 using a HiSeq 4000 sequencing kit version1. Fastq files were generated and demultiplexed with the bc12fastq v2.17.1.14 Conversion Software (Illumina). Average output per library was 44 million reads of 150 bp. All raw reads were mapped to the Charleston Gray reference genome using the Burrows–Wheeler Aligner (BWA), producing analysis-ready BAM files for variant discovery with Broad Institute’s GATK. Homozygous SNPs between the two parents were extracted from the vcf file that was further filtered to a total depth of >20 reads per site. The read depth information for the homozygous SNPs in the ‘light’ and ‘dark’ pools was obtained to calculate the SNP-index (Takagi et al., 2013). For each site, we then calculated for each bulk the ratio of the number of ‘reference’ reads to the total number of reads, which represented the SNP index of that site. The difference between the SNP-index of two pools was

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**McKenna et al., 2010**
calculated as ΔSNP-index. The sliding window method was used to perform the whole-genome scan and identify the trait locus confidence interval on chromosome 9.

**WGS of 25 representative diverse melon accessions**
DNA of the 25 core accessions was shipped to the Genomic Diversity Facility at Cornell University (Ithaca, NY) for WGS to an estimated 30× depth.

**Validation of rare alleles at the APRR2 genes**
The causative variants at the different APRR2 alleles in melon and watermelon, which were discovered based on next-generation sequencing (NGS) of genomic DNA, were confirmed on the parental lines and relevant segregants through Sanger sequencing of genomic DNA in melon and cDNA from mRNA that was extracted from fruits in watermelon.

**qRT-PCR analysis**
Rind samples were peeled from fruits harvested throughout development, from anthesis to maturity, and immediately frozen in liquid nitrogen. Three fruits were sampled from each genotype at each developmental stage. A 100–150 mg aliquot of frozen rind tissue per sample was used for RNA extraction using a Plant/Fungi Total RNA Purification Kit (NORGEN Biotek Corp., Canada). First-strand cDNA was synthesized using a cDNA Reverse Transcription Kit (Applied Biosystems, USA). The 10 μl qPCR volume included 1 μl of cDNA template, 0.2 μl of each primer (10 μM), 5 μl of Fast SYBR Green Master Mix (Applied Biosystems, USA), and RNase-free water to a final volume of 10 μl. qRT-PCR, with an annealing temperature of 60 °C, was performed in triplicate on a 96-well plate in the Step-One Plus Real-Time PCR system (Applied Biosystems, USA). The melon Cymopolin A gene (Melo3C013375) was used as a control to normalize the qRT-PCR values across different samples. Primers are listed in Supplementary Table S5 at *JXB* online.

**Data analysis**

**Trait mapping**
Genome-wide linkage analysis for young fruit rind color in the bi-parental populations was performed in R/qtl (Broman et al., 2003) using the Composite Interval Mapping (CIM) function and confirmed using single marker analyses in TASSEL utilizing a generalized linear model (GLM) and in the JMP V13.1 software package (SAS Institute, Cary, NC, USA). A GWAS of the melon diversity collection was performed by a mixed linear model (MLM) analysis in TASSEL, using both the population structure (Q matrix) and relatedness (kinship (k) matrix) as covariates to control for population structure. Multiple comparison corrections to significance thresholds were performed using the FDR (false discovery rate) approach (Benjamini and Hochberg, 1995). All further statistical analyses (correlations and ANOVA) were performed using the JMPV13.1 software package.

**Population structure, kinship, and LD analysis**
Relatedness between the melon accessions in the diverse collection was estimated in TASSEL software v5.2.43 using the pairwise kinship matrix (k matrix) through the Centered IBS method. LD between intrachromosomal pairs of sites was done on chromosome 4 using the full matrix option in TASSEL.

**Sequence analyses**
Sequence alignments and comparison of APRR2 alleles were performed using the BioEdit software package (Hall, 1999) and the integrative genomics viewer (IGV) package (Robinson et al., 2011). Comparative analysis of haplotype diversity across 2200 genes on melon chromosome 4 was performed following these steps: (i) a vcf file containing ~4 000 000 high quality SNPs across the core set of 25 melon lines (MAF >0.1 and <10% missing data per SNP) was created based on alignments to the melon genome version 3.5.1; (ii) the corresponding gene annotations file was used to create a subset of exonic SNPs on all annotated genes on chromosome 4; and (iii) the number of exonic SNP haplotypes per gene was calculated.

**Results**

**GWAS of young fruit rind color in melon**
Most melons can be visually classified into two distinct young fruit [~10 days post-anthesis (DPA)] rind colors; dark or light green, reflecting qualitative variation in chlorophyll content. Light immature rind color was previously reported to display a recessive single-gene inheritance in a bi-parental segregating population (Burger et al., 2006a). In the current study, young fruit rind color was visually scored on a previously described diverse melon collection composed of 177 accessions (Gur et al., 2017). The collection was genotyped genome-wide with 23 931 informative, GBS-derived SNP markers, and was shown to be an effective resource for mapping simple traits in melon (Gur et al., 2017). Here, we used a subset composed of 120 accessions with a clearly defined dark or light rind phenotype (see the example in Fig. 1A) for GWAS. Accessions with prominent non-uniform rind color (stripes or dots) were excluded from this analysis. We also excluded Charentais lines, as their dominant grayish light rind is exceptional and phenotypically distinct from the common light rind in other melon types. While the dark and light phenotypes were distributed uniformly across the genetic variation and were represented in balanced proportions across this set (41% and 59%, respectively; Fig. 1B), a genome-wide population structure-corrected analysis did not result in any significant marker–trait association. This result has led to the assumption that while this highly heritable trait may show simple inheritance in a specific bi-allelic cross, it is possibly more complex and explained by multiple loci across a multi-allelic diverse collection.

**Mapping and identification of the young fruit light rind gene in melon**
In order to dissect this trait further using a simpler genetic design, we analyzed two segregating bi-parental populations: the first is composed of 164 RILs (F₃) from a cross between a light rind honeydew parent (TAD) and a dark rind reticulatus parent (DUL; Fig. 1B). The second population is composed of 114 F₃:₄ families derived from a cross of DUL with another light rind accession, a yellow casaba inodorus melon (NA; Fig. 1B). These segregating populations were visually phenotyped for young fruit rind color over two seasons, and a consistent single gene (Mendelian) ratio was observed in dark/light phenotypes. The populations were then genotyped through GBS, and 89 343 (TAD×DUL RILs) and 43 975 (NA×DUL F₃:₄) informative SNP markers were identified and used for mapping. Whole-genome linkage analysis using the four data sets (two populations over two growing seasons) resulted in the identification of a single highly significant consistent trait locus on chromosome 4 (Fig. 1C). The common confidence interval for this trait locus spans a 290 kb region (chromosome 4: 640–930 kb; Fig. 1D) on the melon reference...
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Garcia-Mas et al., 2012; http://cucurbitgenomics.org/organism/3), as confirmed also through substitution mapping using recombinants within this interval in the TAD×DUL RILs population (Fig. 2A). Annotation of the genomic sequence at this interval revealed 33 putative genes (Supplementary Table S1) including a strong candidate, Melo3C003375, which is annotated as an **ARABIDOPSIS PSEUDO-RESPONSE REGULATOR2-LIKE (APRR2)** gene, the melon homolog of a recently reported causative gene of the recessive white rind (w) mutation in cucumber (Liu et al., 2016).

We then compared the CmAPRR2 gene (Melo3C003375) sequence between the parental lines of the mapping populations. Genomic and mRNA sequencing revealed multiple polymorphisms, including two different exonic polymorphisms causing independent stop codons in each of the light rind parents compared with the common dark parent (DUL). G to T substitution in exon 8 in TAD compared with DUL led to a premature stop codon and a predicted aberrant protein of 292 amino acids compared with the normal 527 amino acid protein of DUL (Fig. 2B, C; Supplementary Fig. S1). A 13 bp insertion in exon 9 of NA result in a frameshift leading to a different premature stop codon in this line and a predicted protein of 430 amino acids (Fig. 2C; Supplementary Fig. S1).

Furthermore, we crossed TAD and NA with each other and with DUL (as a reference testcross) and phenotyped the F1s for young fruit rind color. Both testcrosses with DUL resulted, as expected, in a dark rind in the F1. However, the F1 of TAD×NA had a clear light rind and confirmed the allelic nature of these recessive phenotypes (Fig. 3A). This further corroborates that these independent predicted causative mutations in the CmAPRR2 gene are indeed allelic.

### Expression pattern of the CmAPRR2 gene in melon fruit

Fruits from the light (TAD) and dark (DUL) parental lines were sampled during development from anthesis to maturity, and mRNA levels of the CmAPRR2 gene were analyzed by
We show here that *CmAPRR2* has a higher expression level in fruit compared with leaves (Fig. 2D), as also shown in cucumber (Liu et al., 2016) and pepper (Brand et al., 2014), and in agreement with the Melonet-DB gene expression atlas (Yano et al., 2018). In accordance with these studies, we also show that the *CmAPRR2* peak expression in fruit rind occurs at ~15 DPA, before the initiation of ripening and color change. Comparison between the parental lines of the mapping population also showed significantly lower levels of *CmAPRR2* expression in light rind fruits throughout fruit development. Both light and dark lines have reduced *CmAPRR2* expression levels at the mature fruit stage and were not significantly different from each other at that stage (Fig. 2D).

Multiple independent predicted causative mutations in the *CmAPRR2* gene across melon diversity

In light of the conflict between the clear identification of a single causative gene through linkage mapping in two different bi-parental crosses on the one hand, and the absence of any significant genome-wide signal from the GWAS analysis on the other hand, we re-sequenced and compared the genomic sequence of the *CmAPRR2* gene across a core panel of 25 diverse melon lines. This core panel was selected to represent the different groups and overall diversity in our collection as described previously (Fig. 3B; Gur et al., 2017). Nineteen lines from the panel, showing a clear dark or light young fruit rind phenotype, were used for the sequence comparison.
Seventeen SNPs and InDels within exons in the *CmAPRR2* gene were identified across this panel (Fig. 2B). Eight of these SNPs were either synonymous or did not show a distinct allelic state between dark and light accessions. The remaining nine polymorphisms are independently inherited (not in LD with each other) and display low frequency alleles (0.05–0.15) which are unique to the light rind accessions (Fig. 2E). Four of these polymorphisms (2, 6, 13, and 14) are SNPs that change a single amino acid. Three are InDels (4, 7, and 12) that cause frameshifs leading to major modification in predicted protein sequence, and the remaining two (9 and 11) are the causative polymorphisms described above, leading to premature stop codons as in TAD and NA. These five major polymorphisms (4, 7, 9, 11, and 12) explain the light rind phenotype in eight of the 11 light rind accessions in the core panel. The non-synonymous polymorphisms 2, 6, and 13 are potentially causative of the light rind phenotype in accessions BAHC and QME. The only light rind accession that could not be explained by non-synonymous variation within the *CmAPRR2* coding sequence is SAS. However, the low mRNA expression of *CmAPRR2* in young fruit (5 DPA) rinds of SAS, which was similar to the expression level in TAD, and significantly lower compared with DUL (Fig. 2D), suggests expression level variation as a possible causative element for the light rind phenotype in this line. To test whether all these ‘light’ accessions are indeed allelic and caused by different mutations in the *CmAPRR2* gene, we performed allelism tests where all the ‘light’ accessions (*n* = 11) were intercrossed and the resulting 55 F$_1$ hybrids were phenotypically evaluated for young fruit rind color. As a reference, these ‘light’ accessions were crossed with two ‘dark’ testers (DUL and Ananas Yqne’am; AY). Figure 3C shows that all 55 ‘light’×‘light’ F$_1$ hybrids displayed light immature fruit rinds, while all 22 ‘light’×‘dark’ testcrosses displayed dark rinds. These results confirm the allelism between the 11 ‘light’ lines, including the light rind phenotype of SAS. Combined interpretation of the sequence variation and allelism tests across this representative core panel indicates that most of the young fruit rind color variation can be explained by multiple independent polymorphisms related to the *CmAPRR2* gene. Extrapolation of the results from this core set suggests that each of these causative variants is most probably also present at low allele frequency, across the wider diversity panel, leading to the non-significant associations observed in our GBS-based GWAS experiment. It is worth noting that these independent mutations are not in LD with each other, leading to the high haplotype diversity in this gene. In a comparative analysis of haplotype diversity based on exonic SNPs across 2200 genes on melon chromosome 4, we found that *CmAPRR2* is indeed
the second most diverse gene, irrespective of the number of SNPs and transcript length (see Supplementary Fig. S2 and the Materials and methods). Analysis of the LD pattern in the genomic region surrounding the \textit{CmAPRR2} locus confirmed the low LD between SNPs in this region (Supplementary Fig. S3). The fact that these allelic polymorphisms are not in LD with each other allows us to aggregate them into a theoretical unified functional polymorphism, resulting in increased frequency of the aberrant \textit{CmAPRR2} allele (0.52; Fig. 2E right column). This analysis, in turn, produces a significant association between the \textit{CmAPRR2} gene and the light rind trait (Fig. 2F).

\section*{Mapping and identification of the light rind color gene in watermelon}

In parallel with melon, we also studied the genetics of rind color in watermelon. The main difference is that in watermelon, due to its non-climacteric fruit ripening, chlorophylls are the main rind pigments also during fruit maturity, and therefore light and dark green were visually scored on mature fruits. Our light rind source in this study was an heirloom accession named NY0016 (Tadmor \textit{et al.}, 2005) that was crossed on different lines, varying in their rind color, to produce F1s and F2s. All the F1 hybrids had dark rind fruits (irrespective of the stripe pattern in the ‘dark’ parents), proving the recessive nature of the light rind phenotype of NY0016. All F2 populations were phenotyped for rind color, and a consistent 3:1 Mendelian ratio was observed for dark and light rinds, respectively (Fig. 4A; Supplementary Table S2). The cross between the light rind accession (NY0016) and a striped (dark) parent (EMB) was selected for linkage mapping, and this population was advanced to F3 to perform F2:3 analysis (Fig. 4B). GBS of the F2 was selected for linkage mapping, and this population was admixed accession (NY0016) and a striped (dark) parent (EMB) 4A; Supplementary Table S2). The cross between the light rind phenotype of NY0016. All F2 populations were observed for dark and light rinds, respectively (Fig. 4A; Supplementary Table S2). The cross between the light rind accession (NY0016) and a striped (dark) parent (EMB) was selected for linkage mapping, and this population was advanced to F3 to perform F2:3 analysis (Fig. 4B). GBS of the F2 population (n=87) resulted in a final high-quality set of 3160 filtered SNPs (Branham \textit{et al.}, 2017) that was used for genetic mapping of the light rind phenotype. Seven to ten fruits per F3 family were visually scored, and each family was classified into a defined category: fixed for light rind, fixed for dark rind, or segregating for rind color (Supplementary Fig. S4). The observed 1:2:1 frequencies of light, segregating, and dark across the F3 families supported a single gene inheritance for this trait (Fig. 4B). Whole-genome linkage analysis resulted in the identification of a single significant trait locus on chromosome 9 (R²=0.62, P=2.9×10⁻¹⁸; Fig. 4C). The confidence interval for this locus spanned 1.7 Mb with 80 predicted genes on the watermelon reference genome (Charleston Gray, http://cucurbitgenomics.org/organism/4). In order to narrow down this genomic interval, we performed mapping by sequencing of DNA bulks (BSA-Seq) from 35 selected F3 segregants that were homozygous for light or dark rind (on the basis of fixed F3 phenotypes). The ~30× WGS resulted in the identification of 400 000 high-quality SNPs differentiating between parental lines. Comparison of allele frequencies between the light and dark bulks across the 400 000 SNPs (ΔSNP-index analysis) confirmed the trait locus on chromosome 9 and allowed us to narrow down the genomic confidence interval to a 900 kb region with 30 predicted genes (Fig. 4D; Supplementary Table S3). Review of the list of genes within the confidence interval revealed a strong candidate, CICG09G012330, the watermelon homolog \textit{ClAPRR2} gene, highly similar to the causative melon (Mel3C003375) and cucumber (Cs3G004140.3; Liu \textit{et al.}, 2016) genes. Comparison of the genomic sequence of CICG09G012330 between the mapping population parental lines revealed several SNPs, none of them within exons. The only putative causative SNP at that point was at the intron 6–exon 7 junction (Fig. 4E). Parent and segregant mRNA sequence comparison revealed an alternative splicing in the intron 6–exon 7 junction, leading to a 16 bp deletion at the mRNA of the light rind parent and corresponding segregants carrying the ‘light’ allele. This 16 bp deletion, which created a frameshift leading to a premature stop codon and a predicted aberrant protein, is most probably causative for the light rind phenotype of NY0016 (Fig. 4F, G).

\section*{Allelic variation in the \textit{CmAPRR2} gene is associated with mature fruit rind and flesh pigmentation in melon}

Earlier analyses of rinds from TAD, DUL, and selected F3 families from their cross demonstrated the correlation between young fruit chlorophyll content and mature fruit carotenoids (Supplementary Fig. S5). To test whether the \textit{CmAPRR2} gene is also associated with mature fruit rind pigmentation in melon, we analyzed mature fruits from the TAD×DUL RIL population for rind color and carotenoid content. We harvested 10 mature fruits per line, external images of the fruits were taken for color scoring, and the rinds were sampled for carotenoid profiling. Color intensity variation at the young (green) stage in this population is qualitative and could be visually classified into two distinct classes (dark or light green) on a single-fruit basis. However, at the mature stage, any effect of the \textit{CmAPRR2} gene on rind color is visually of a quantitative nature (Fig. 5A), as also shown in tomato (Pan \textit{et al.}, 2013). Rind netting, which segregates in this population, further masked rind color and complicated visual scoring and carotenoid quantification. For the parental lines, TAD, with the light green rind at the young fruit stage, has a cream-yellowish rind at maturity, and DUL, with the dark green rind at the young stage, has an orange rind masked by dense rind netting (Fig. 2D). Visual observation of standardized external images of selected mildly netted mature fruits, representing both alleles in the \textit{CmAPRR2} gene, suggested a possible effect of this gene on rind color intensity, such that on average the ‘dark’ allele is associated with a deeper orange color (Fig. 5A). We confirmed this effect through analysis of the carotenoid content in rinds of 50 selected mildly netted segregants (25 RILs carrying each allele in the \textit{CmAPRR2} gene). The ‘light’ allele was significantly associated with a >10-fold reduction in total carotenoids in the rind, which is consistent with the reduced chlorophyll levels observed in young fruits of this group (Fig. 5B; Supplementary Fig. S5). Allelic variation in this gene explains 37% of the variation in lutein content (P=2×10⁻⁶), 34% of the variation in β-carotene content (P=8×10⁻⁶), and 37% of total carotenoids (P=3×10⁻⁶) in fruit rind in this population.

To examine whether the effect of the \textit{CmAPRR2} gene also extends to mature fruit flesh color, we phenotyped the TAD×DUL RILs for flesh color intensity using longitudinal
fruit section scanning and quantitative image analysis (n=145 lines×10 fruits per line). This population is segregating for the main flesh color gene in melon, CmOr, discriminating between orange and non-orange flesh (Tzuri et al., 2015), and it is therefore composed of orange flesh lines (48%) and green flesh lines (49%); the remaining 3% of the lines are segregating due to residual heterozygosity (Fig. 5C). In order to test the association of the CmAPRR2 gene with flesh color, we analyzed the orange and green fruits separately. A significant association between the CmAPRR2 allelic segregation and color intensity was found in the orange-flesh group, which accumulated β-carotene as the main flesh pigment (R²=0.25, P=4.7×10⁻⁵; Fig. 5D, E) and, as expected, the ‘dark’ allele was associated with stronger pigmentation and higher predicted β-carotene content (R²=0.62, P=0.0053; see the Materials and methods and Supplementary Fig. S6). Significant association of this gene with flesh color was also found in the green flesh group, which accumulated chlorophyll as the main flesh pigment (R²=0.31, P=2×10⁻⁶) as the ‘dark’ allele was significantly associated with higher green Chroma (Fig. 5F, G) reflecting higher chlorophyll content.

Expression level of the CmAPRR2 gene is associated with mature flesh color in melon

In order to test whether the expression level of the CmAPRR2 gene is associated with mature fruit pigmentation, we analyzed RNA-Seq data and mature fruit flesh carotenoids on a different RIL population derived from a cross between DUL and an Indian phut snapmelon (Momordica group), PI414723 (hereafter called 414) (Galpaz et al., 2018). While 414 has a spotted rind (and not a clear light or dark phenotype), we assume based on testcrosses with some of the light rind accessions that, like DUL, it also carries a ‘dark’ allele of the CmAPRR2 gene. This assumption is supported by the fact that it does not show any of the predicted ‘light’ non-synonymous polymorphisms found at the CmAPRR2 gene (Fig. 2B, E). DUL and 414 are genetically and phenotypically distant and differ in their mature fruit flesh color and carotenoid content (Fig. 6A). DUL has dark orange flesh while 414 has light (salmon-colored) orange flesh and, accordingly, the RIL population segregates for these traits (Harel-Beja et al., 2010; Galpaz et al., 2018). RNA-Seq was previously performed on mature fruit flesh of 96 RILs from this
cross (Freilich et al., 2015) and allowed us to execute a genome-wide eQTL analysis for the CmAPRR2 gene (Melo3C003375). A single, highly significant, cis-eQTL was mapped to chromosome 4 and defined by a 270 kb interval flanking this locus (Galpaz et al., 2018; Fig. 6B, C). This result confirmed the heritable variation in the CmAPRR2 expression level in this population and that the expression of this gene is mostly regulated by cis-acting sequence variants. We then tested the correlation between the CmAPRR2 expression level and flesh β-carotene content across the 96 RILs, and found a significant positive correlation ($R=0.38$, $P=0.0008$; Fig. 6D). Since both parental lines of this population carry the ‘dark’ allele based on the coding sequence of the CmAPRR2 gene, this result provides a quantitative support for the possible relationship between the expression level of this gene and pigment accumulation in melon. It is important to note that this population is segregating at additional QTLs that affect carotenoid content in mature fruit flesh, including a major QTL on chromosome 8, which was recently mapped to a candidate gene level (Diaz et al., 2011; Galpaz et al., 2018). This variation further masked the specific effect of the CmAPRR2 expression level on flesh carotenoid content. We also assume that the observed correlation is an underestimation, as gene expression in this experiment was measured on mature fruits, whereas the peak of expression of the CmAPRR2 gene is much earlier, before fruit ripening (~15 DPA).

**Expression of CmAPRR2 in melon is correlated with plastid development-related genes**

To characterize co-expression patterns associated with the CmAPRR2 gene, we calculated the correlations between the expression of Melo3C003375 and all annotated melon genes ($n=27,557$), using RNA-Seq data from mature fruits of the 414×DUL RIL population ($n=96$) (Freilich et al., 2015). Fourteen thousand genes expressed in mature fruit flesh were used for this correlation analysis. Gene Ontology (GO) enrichment analysis was performed on 200 genes that had the strongest correlations with Melo3C003375 ($R>0.43$, FDR-adjusted $P<0.001$). The four most significant functional groups that were enriched are related to photosynthesis, light reaction, and plastid organization, and the 15 most enriched components are related to plastids and chloroplasts (Supplementary Table S4). These results support the predicted involvement of the CmAPRR2 gene in the regulation of chloroplast and chromoplast development.
APRR2 is associated with fruit pigmentation in melon and watermelon

Discussion

Color variation in immature fruit rind in melon

External fruit color is an important attribute in melons as it is a key factor defining consumers’ preference. Melon rind color transforms during fruit development and ripening, mainly by shifting from the green rind of immature fruits, where chlorophyll is the main pigment, to variable rind colors composed of different combinations of chlorophylls, carotenoids, and flavonoids (Tadmor et al., 2010). Inheritance of external color of immature fruit has been previously described in two different studies: the white color of immature fruits was reported by Kubicki (1962) to be dominant to green immature fruits and controlled by a single gene named Wi (Dogimont, 2011). Burger et al. (2006a) described a recessive gene for light immature exterior color in a cross between an American muskmelon (Reticulatus group) and an American honeydew-type melon (Inodorous group). Recently, a major gene for external color of immature fruit was mapped in a cross between ‘Védrantais’, a Charentais line from the Cantalupensis group, and ‘Piel de Sapo’, from the Inodorous group (Pereira et al., 2018). The dominant light rind from the ‘Védrantais’ parent, that most likely correspond to the Wi gene, was mapped to an ~1.6 Mb interval on chromosome 7 (Pereira et al., 2018). These results confirm our observation that the dominant light grayish rind of Charentais accessions is phenotypically and genetically distinct and controlled by a different gene from the one we identified in the current study, which correspond to the recessive gene described by Burger et al. (2006a).

APRR2-like transcription factors are key regulators of fruit pigmentation

The results of the current study support the pivotal role of APRR2-like genes in regulation of pigmentation in fruits. Pan et al. (2013) showed that overexpression of an APRR2 gene in tomato resulted in increased chlorophyll content in immature
fruits and a higher level of carotenoids in ripe tomatoes. Both effects resulted from an increase in plastid number. They also provided evidence for association between a null mutation in an APR2 gene and external fruit color intensity in green peppers. These results are complementary to reports on the role of a related transcription factors group, GLKs, which were shown to be associated with levels of chlorophyll and carotenoids in Arabidopsis, tomato, and pepper (Waters et al., 2008, 2009; Powell et al., 2012; Brand et al., 2014). Recently, Liu et al. (2016) reported that an APR2 gene is causative for the white rind (w) mutation in cucumber, expressed as reduced chloroplast density and chlorophyll content in young cucumber fruits. In all these crop plant species (tomato, pepper, and cucumber), there is also a correlation between expression levels of either APR2 or GLK genes and pigment intensity. In the current study, we performed high-resolution NGS-based mapping in segregating populations and found that null mutations in the *CmAPRR2* and *CiAPRR2* genes are associated with light rind color in melon and watermelon, respectively. We also showed that expression of the *CmAPRR2* gene is correlated with pigment intensity in melon (Figs 2D, 6D) and that, as in pepper (Brand et al., 2014) and cucumber (Liu et al., 2016), these transcription factors show their strongest expression in fruit and reach their peak expression at ~10–20 DPA and before fruit ripening. Our results expand the extent of experimental data that demonstrate the conserved function of APR2-like genes in regulating fruit pigmentation as shown by the comparable expression profiles and analogous phenotypes associated with variation in these genes.

*CmAPRR2* is associated with pigment accumulation across fruit developmental stages and tissues

Variation in ripe fruit color is substantially wider compared with the variation in the immature stage. While in the immature stage, color variation mostly reflects chlorophyll concentrations, in the mature stage, biosynthetic pathways of additional pigments (i.e. carotenoids and flavonoids) are involved, leading to extended complexity of the genetic architecture. This complexity is also expressed by the independent genetic control of flesh and rind colors in melon, as best described in the genetic architecture of rind and flesh color QTLs that naturally segregate in both chlorophyll and carotenoid concentrations (Fig. 5C–G). We also showed here, using a different segregating population (414×DUL RILs) that was previously subjected to mature fruit RNA-Seq and carotenoid analysis (Freilich et al., 2015; Galpaz et al., 2018), that the cis-regulated *CmAPRR2* expression variation is correlated with flesh β-carotene content in mature fruits (Fig. 6). Since both parental lines of this population carry a predicted ‘dark’ allele based on the coding sequence of the *CmAPRR2* gene, we assume that this experiment provided another piece of evidence for the relationship between the expression level of *CmAPRR2* and flesh pigment content. The proposed involvement of this transcription factor in the segmentation and detection will not necessarily overlap across different mapping populations. The same can apply to simple traits, where independent mutations in different genes, which are involved in a common biological process, lead to the same discrete phenotype. While bi-parental populations will comprise only part of the picture in such cases, diverse collections or multi-parental segregating populations are more effective in comprehensively characterizing this architecture. In the current study, we tried to genetically characterize the light immature rind phenotype in melon using a diverse collection, assuming that it is under a simple genetic control as previously described (Burger et al., 2006a). In GWAS, lack of detection power can result from low heritability, low frequency of the phenotype under investigation, strong confounding effect of population structure, or insufficient markers density (Korte and Farlow, 2013). While none of these factors seemed to apply in our case (Gur et al., 2017; Fig. 1), we did not obtain any significant GWA signal, which led to the intuitive assumption that multiple genes are associated with the light immature rind phenotype in our collection. The identification of two independent allele nonsense mutations in the *CmAPRR2* gene, through linkage analyses (Figs 1, 2), indicated that we might be looking at a different scenario. Through complementary resequencing of a diverse core panel and comprehensive allelism testing (Figs 2, 3), we were able to demonstrate that this trait is a unique case of simple genetic architecture. On the functional level, it seems to be controlled by a single gene that segregates in a Mendelian manner in bi-parental crosses, but the multi-allelic pattern at the *CmAPRR2* gene drove reduced power through the GWAS, which masked this simplicity and created the observed contradiction between the different mapping strategies. These results provide a thought-provoking example of another possible inherent complexity that can arise in GWAS—indeed low-frequency causative variants within a common gene. In the current scenario, even whole-genome deep resequencing of the GWAS panel, which would target each of the variants in the *CmAPRR2* gene, would
not necessarily resolve the lack of detection power, as each of these independent variants remains at low frequency. Genetic mapping studies are rapidly shifting towards sequencing-based genotyping and, in most cases, marker density is no longer a bottleneck in GWAS (Yano et al., 2016; Misra et al., 2017).

A key challenge remains in prioritizing GWAS signals and improving weak signals obtained from low-frequency causal variants (Lee and Lee, 2018). The availability of whole-genome assemblies and corresponding protein-coding gene annotations, alongside additional layers of information, such as expression profiles from RNA-Seq experiments, now facilitate the integration of multiple data layers to improve GWAS results (Shim et al., 2017; Lee and Lee, 2018; Schaefer et al., 2018). Our example of the CmAPRR2 gene suggests that adding functional annotation prediction to GWAS SNPs and treating predicted genes as integral functional units could potentially be used as an informative layer that can boost the signal of causative weak associations.

In summary, we have identified the CmAPRR2 gene as a common regulator of fruit pigmentation in melon and watermelon. The conserved and broad effect of this gene across species, fruit tissues, developmental stages, and different types of pigment accumulated suggests its potential as a useful target for carotenoid bio-fortification of cucurbits and other fruits.

Supplementary data
Supplementary data are available at JXB online.

Fig. S1. cDNA sequence comparison between the mapping population parental lines DUL, TAD, and NA.

Fig. S2. Number of annotated transcript haplotypes versus number of SNPs per base pair (a) and transcript length (b) across 2200 genes on chromosome 4.

Fig. S3. CmAPRR2 gene is located in a low LD region.

Fig. S4. Segregation and scoring of rind color of 87 F2 families from the NY0016×EMB cross.

Fig. S5. Analysis of rind chlorophyll and carotenoids during fruit development on TAD, DUL, and selected F2 families from their cross.

Fig. S6. Prediction of flesh β-carotene based on fruit-section image analyses in the TAD×DUL RILs.

Table S1. Melon young fruit light rind QTL interval: annotations and positions of genes.

Table S2. Segregation of light rind in four F2 watermelon populations.

Table S3. Light rind QTL interval in watermelon: annotations and positions of genes.

Table S4. Gene Ontology (GO) enrichment analysis for 200 genes correlated with expression of the APRR2 gene (Melo3C003375) in melon fruit.

Table S5. List of primers used for RT-qPCR.

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