Integrated omics analysis identified genes and their splice variants involved in fruit development and metabolites production in *Capsicum* species

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Abstract

To date, several transcriptomic studies during fruit development have been reported; however, no comprehensive integrated study on expression diversity, alternative splicing, and metabolomic profiling was reported in *Capsicum*. This study analyzed RNA-seq data and untargeted metabolomic profiling from early green (EG), mature green (MG), and breaker (Br) fruit stages from two *Capsicum* species, i.e., *C. annuum* (*Cann*) and *C. frutescens* (*Cfrut*) from Northeast India. A total of 117,416 and 96,802 alternatively spliced events (AltSpli-events) were identified from *Cann* and *Cfrut*, respectively. Among AltSpli-events, intron retention (IR; 32.2% *Cann* and 25.75% *Cfrut*) followed by alternative acceptor (AA; 15.4% *Cann* and 18.9% *Cfrut*) were the most abundant in *Capsicum*. Around 7600 genes expressed in at least one fruit stage of *Cann* and *Cfrut* were AltSpli. The study identified spliced variants of genes including transcription factors (TFs) potentially involved in fruit development/ripening (*Aux/IAA 16-like*, *ETR*, *SGR1*, *ARF*, *CaGLK2*, *ETR*, *CaAGL1*, *MADS-RIN*, *FUL1*, *SEPALLATA1*), carotenoid (*PDS*, *CA1*, *CCD4*, *NCED3*, xanthoxin dehydrogenase, *CaERF82*, *CahHLH100*, *CaMYB3R-1*, *SGR1*, *CaWRKY28*, *CaWRKY48*, *CaWRKY54*), and capsaicinoids or flavonoid biosynthesis (*CaMYB48*, *CaWRKY51*), which were significantly differentially spliced (DS) between consecutive *Capsicum* fruit stages. Also, this study observed that differentially expressed isoforms (DEiso) from 38 genes with differentially spliced events (DSE) were significantly enriched in various metabolic pathways such as starch and sucrose metabolism, amino acid metabolism, cysteine cutin suberin and wax biosynthesis, and carotenoid biosynthesis. Furthermore, the metabolomic profiling revealed that metabolites from aforementioned pathways such as carbohydrates (mainly sugars such as D-fructose, D-galactose, maltose, and sucrose), organic acids (carboxylic acids), and peptide groups significantly altered during fruit development. Taken together, our findings could help in alternative splicing-based targeted studies of candidate genes involved in fruit development and ripening in *Capsicum* crop.

Keywords *C. annuum* · *C. frutescens* · Alternative splicing · Isoforms · Metabolites · Fruit · Early green · Mature green · Breaker

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**Introduction**

Members of genus *Capsicum* from Solanaceae family, also known as chili or pepper, are widely grown due to the high economic importance and health benefits of its fruits (Sarpras et al. 2016). *Capsicum* fruits with ample amounts of several alkaloids, nutrients, vitamins, and pigments are beneficial for human health and usually consumed as spices or vegetables, either raw or in dried form (Aza-González et al. 2011; Bosland and Votava 2012; Kim et al. 2014). *Capsicum* has been widely studied for pungency, carotenoid content, fruit size and shape, resistance to diseases, etc. (Ramchiary et al. 2014). Recent reports on the phytochemicals and anti-oxidant properties of *Capsicum* fruits suggested the pharmacological and industrial importance of *Capsicum* (Baenas et al. 2019; Olatunji and Afolayan 2019; Sinisgalli et al. 2020; Sricharoen et al. 2019). Several phytochemicals such phenolics, flavonoids, carotenoids, capsaicinoids, and alkaloids naturally found in *Capsicum* can be used as multipurpose industry usage, viz., in increasing food shelf-life, improving the sensory properties and seasoning of foods, and coloring food, in cosmetic and pharma industry (Baenas et al. 2019). A wide range of physiological and morphological changes including shifts in transcriptional expression and biosynthesis of several metabolites take place during *Capsicum* fruit development (Ahmad et al. 2021; Sarpras et al. 2019). For instance, during the course of *Capsicum* fruit development, the differential expression of several genes such as *Psy*, *PDS*, *CA1*, *Ccs*, *NCED3*, *SGR1*, *CaERF66*, *CaERF82*, *CaERF97*, *CaERF107*, *CaDIV1*, *CaMYB3R-1*, *CabHLH09*, *CabHLH032*, *CabHLH095*, and *CabHLH100* is directly or indirectly involved in regulation of carotenoid biosynthesis (Berry et al. 2019; Islam et al. 2021; Liu et al. 2021, 2022; Luo et al. 2013; Song et al. 2020), while that in genes such as *CaMYB31*, *CaMYB48*, *CaDIV14*, *CcMYB108*, *CcMYB6*, *CaERF53*, *CaERF92*, *CaERF102*, *CaERF111*, *CabHLH007*, *CabHLH009*, *CabHLH086*, *CabHLH026*, and *CabHLH063* in capsaicinoid biosynthesis regulation (Arce-Rodríguez et al. 2021; Islam et al. 2021; Liu et al. 2021; Song et al. 2020) along with several auxin-responsive (ARF) genes such as *AUX1*, *AUX/IAA3*, ethylene receptor gene (ETR), *CAMADS-RIN*, *LeSPL-CNR*, *SEPALLATA 1* (*SEP1*), *CaAGL1*, etc. is involved in the regulation of fruit ripening and development in *Capsicum* (Dong et al. 2014; Dubey et al. 2019; Lai et al. 2005; Sung et al. 2001).

In plants, the expression of genes is regulated through various post-transcriptional mechanisms (PTM), which in turn regulate/affect the diverse developmental or physiological processes (Floris et al. 2009; Yan et al. 2021). Alternative splicing (AltSpI) is one of the indispensable PTM in the regulation of key biological processes such as fruit development and ripening (Yan et al. 2019; Wang et al. 2016; Sun and Xiao 2015) and biotic and abiotic stress-related responses (Floris et al. 2009; Gu et al. 2019; Liu et al. 2016; Martín et al. 2021; Li et al. 2020) through the alternative usage of exons/introns to attain expression of genes, thereby increasing the proteome diversity (Lareau et al. 2004). Several studies in plants have exerted the importance of distinct AltSpI of isofoms during different switching of developmental stages or at an individual stage. For instance, in tomatoes, around ~34% of auxin response factor (ARF) genes underwent splicing during the early fruit development (Zouine et al. 2014), and in Arabidopsis, the retention of intronic region in one of transcripts of *phytochrome interacting factor 6* (*PIF6*) was shown to be associated with seed dormancy, while other transcripts with spliced out of intronic region reduced seed dormancy (Penfield et al. 2010). Yan et al. (2021) reported splicing of cell-wall softening genes such as polygalacturonases (*PGs*) and galactanase (*TBG4*) is more in the ripe fruit stage than the immature stage in four different plant species (Yan et al. 2021), indicating the tissue-specific and conservation of AltSpI-events during the fruit development. Multiple spliced transcripts of a gene can be generated through the unification of multiple exons in many ways, generally known as AltSpI-events (AltSpI-events) which can be categorized into five categories: (i) intron retention (IR), retention of the intronic region in mature messenger RNA; (ii) skipped-exon (SE), skipping one or more alternative exons; (iii) mutually exclusive exons (MX), between two exons exclusion or splicing out either of the exon; (iv) alternative acceptor (AA); and (v) alternative donor (AD), includes enigmatic use of 3′ splice junction (SJ) or 3′ acceptor and 5′ SJ or donor site. The frequency and proportion of different AltSpI-events vary among most of the eukaryotic organisms (Kim et al. 2007; Martín et al. 2021). In human, SE is the most commonly found AltSpI-events and ~94% multi-exonic genes (MEG) undergo splicing (Chen et al. 2012; Wang et al. 2008). The IR is the most prevalently found AltSpI-event in most of the plant species (Filichkin et al. 2010; Jiang et al. 2017; Li et al. 2021; Sun and Xiao 2015; Thatcher et al. 2014; Wang et al. 2016), and the proportion of alternatively spliced MEG (AS-MEG) observed varies between ~25 and 65% depending on the coverage and depth of RNA sequencing data (Clark et al. 2019; Reddy et al. 2013; Sablok et al. 2017). In addition to this, AA and IR AltSpI-events were found to be the most conserved in monocots (Mei et al. 2017), between *Populus* and *Eucalyptus* (Xu et al. 2014), *Brassica* and *Arabidopsis* (Darracq and Adams 2013), and between...
fleshy fruits including papaya, melon, cucumber, and peach (Yan et al. 2021).

The early fruit development in *Capsicum* involves active cell growth and an increase in cell diameter due to the high rate of the cell cycle, while later fruit stages mostly involve the expansion of cells (Tiwari et al. 2013). A comprehensive study of the expression diversity of genes and their transcripts is crucial for understanding their regulation during fruit development which in turn will help to dissect domestication processes and manipulation in breeding programs to improve quality and other fruit-related traits of *Capsicum* (Martínez et al. 2021). Till now, several transcriptomic studies related to *Capsicum* fruit development have transpired the expression dynamics genes/transcripts involved in the regulation of diverse processes including fruit shape, size, coloration, or pigmentation following ripening, softening of fruits, and synthesis and accumulation of metabolites during fruit development and ripening (Dubey et al. 2019; Jang et al. 2015; Martínez et al. 2021; Osorio et al. 2012; Razo-Mendivil et al. 2021). However, till now, no comprehensive study has been reported on alternative splicing and spliced transcript diversity during fruit development and ripening (Dubey et al. 2019; Razo-Mendivil et al. 2021). The present study therefore is aimed at understanding the regulation of diverse processes including fruit shape, size, coloration, or pigmentation following ripening, softening of fruits, and synthesis and accumulation of metabolites during fruit development and ripening (Dubey et al. 2019; Jang et al. 2015; Martínez et al. 2021; Osorio et al. 2012; Razo-Mendivil et al. 2021). However, till now, no comprehensive study has been reported on alternative splicing and spliced transcript diversity during *Capsicum* fruit development.

The present study therefore is aimed at understanding a landscape of AltSpli-events in *Capsicum annuum* (Cann) and *C. frutescens* (Cfrut) at three fruit developmental stages, i.e., early green (EG), mature green (MG), and breaker (Br).

**Materials and methods**

**Plant materials, growth conditions, and RNA extraction**

*Capsicum* genotypes belonging to *C. annuum* (Kosom moso; Acc-Ca18; low pungent with 36,000 Scoville heat unit [SHU] and purple fruit color before ripening) and *C. frutescens* (Kon jolokia; Acc-kok1; moderate pungent with 480,000 SHU) were grown in greenhouse of School of Life Sciences, Jawaharlal Nehru University, New Delhi, India, using standard growth conditions including temperature 27/19 °C (day/night) and 16 h of light per day. Fruit samples from three different developmental stages, i.e., early green (EG; 5–10 days post-anthesis; DPA), mature green (MG; 20–25 DPA), and breaker (Br; 30–34 DPA in *C. annuum*, 42–45 DPA in *C. frutescens*) stage, were collected separately in liquid nitrogen and stored at −80 °C until RNA and DNA extraction, as previously described by Islam et al. (2021). The total RNA using whole fruit from each developmental stage was extracted using the MN NucleoSpin RNA Plant kit (Takara, USA) following the manufacturers’ standard method as described previously by Dubey et al. (2019). The quality and integrity of extracted RNA from each sample were analyzed using an Agilent Bioanalyzer, and then RNA with RNA integrity number (RIN) > 8 was further used for RNA sequencing (RNA-seq). Distinct fruit samples with the different fruit developmental stages from the two *Capsicum* species are shown in Fig. 1A–B.

**RNA sequencing, reads alignment, and data processing**

For RNA-seq, an equal amount of total RNA (5–10 µg) from each sample with RIN > 8 was used for library preparation using Illumina’s TruSeq RNA sample Prep Kits (Illumina, USA) and sequenced using Illumina HiSeq X Ten paired-end platform with an average read length of 150 bp paired-end reads from each individual fruit developmental stage from the two *Capsicum* species (three biological replicates of each sample stage). The quality of raw RNA-seq reads was evaluated by FastQC (v0.11.5), and adapter sequences along with low-quality reads (phred score < 20) were removed using TrimGalore (v0.4.4). Genome sequences and genomic annotations for *C. annuum* (GCF_000710875.1_Pepper_Zunla_1_Ref_v1.0) (Qin et al. 2014) were obtained from NCBI. The genome sequence information of *C. frutescens* is still not available. Furthermore, the filtered good quality reads of each individual fruit stage from both *C. annuum* and *C. frutescens* were mapped to the *C. annuum* reference genomes using Tophat2 program (v2.1.1) following the parameters including (i) read mismatches = 5; (ii) read-gap-length = 3; (iii) read-edit-dist = 5; (iv) minimum and maximum intron-length = 30 and 500,000; (v) splice-mismatch = 0; (vi) library-type = fr-firststrand; and (vii) GTF = gtf annotation of know transcript of respective genome, as described earlier (Li et al. 2020; Sun and Xiao 2015; Wang et al. 2016) for splicing event identification. Then, the aligned reads were assembled into transcripts using Cufflink (v2.2.1) following the parameters such as –GTF-guide, –frag-bias-correct, –library-type = fr-firststrand, –min-isofrom-fracti0n = 0.5, and –small-anchor-fracti0n = 0.01. Furthermore, cufflink assembled transcripts from replicates at each developmental stage and all three developmental stages of a species were merged into single transcripts using cuffmerge program (v2.2.1) and were compared to reference annotation using cuffcompare (v2.2.1). The overall pipeline of sample preparation and detailed RNA-seq analysis is outlined in Fig. 1C. Cufflinks, cuffmerge, and cuffcompare are integral part of Tophat2 program (Trapnell et al. 2012).

**Differential isoform expression analysis**

To identify differential spliced (DS) transcripts and differentially expressed isoforms (DEiso) between any two consecutive fruit developmental stages, cuffdiff (v2.2.1) was implemented using parameters (i) –frag-bias-correct,
(ii) –multi-read-correct, (iii) –min-alignment-count 10, (iv) –library-type = fr-firststrand, and (v) false discovery rate (FDR) = 0.05 as described earlier (Sun and Xiao 2015). The expression difference of isoforms between two fruit stages with log2 fold change (lfc) > 2 and FDR adjusted \( p \) value < 0.01 was considered DEiso. Cuffdiff results were analyzed using cummeRbund R package (Goff et al. 2022) following Tuxedo pipeline (Trapnell et al. 2012). Heatmap of DEiso was represented using pheatmap package (Kolde 2019) in R. Cuffdiff, cufflinks, and cuffmerge are an integral part of Tophat2 program (Trapnell et al. 2012).

Identification and characterization of alternatively splicing events

To identify AltSpli-events from total fruit transcriptome of each Capsicum species, total merged transcript GTF generated from cuffmerge was used as input in the standalone Astalavista (v4.0) tool from Barna library (https://confluence.sammeth.net/). The AltSpli-events extracted using Astalavista were categorized into five different types, i.e., intron retention (IR), alternative acceptor (AA), alternative donor (AD), skipped-exon (SE), and mutually exclusive exons (MX), and others (others/mix) including the combination of more than one of the abovementioned five AltSpli-events. The event structure of most abundant AltSpli-events was named according to the previous study (Sammeth et al. 2008), and are denoted as follows: (i) IR: 0,1^2 or 0,1^2–3^4- or 1^2-,3^4- or 0,1^2–3^4–5^6- for one, two, or three retained introns, respectively; (ii) AA: 1-,2- for two alternative acceptor sites; (iii) AD: 1^,2^ for two alternative donor sites; (iv) SE: 0,1–2^ or 0,1–2^3–4^ for one or two skipped exons5 respectively; (v) MX: 1–2^,3–4^ for two mutually exclusive exons; (vi) others/mix: (AA + IR: 1^,2–3^4-); (SE + AA: 1–2^4-,3-); (SE + AD: 1^,2^3–4^); (SE + AA + IR: 1–2^3-,4-); (AD + AA: 1^4-,2^3- or 1^3-,2^4-).

To detect the differentially spliced events (DSE) between consecutive fruit developmental stages, rMATs turbo (v.4.1.2) tool with parameter –libType fr-firststrand –nthread 8 –tstat 8 –cstat 0.0001 was implemented. Abundance of dinucleotides
at splicing sites were counted by comparing merged GTF files with the reference genome fasta file and extracted flanking sequences (24 bp) around splicing sites using in-house scripts coupled with bedtools and awk. The consensus logo for 5′ splicing donor site and 3′ acceptor site was constructed using WebLogo v.3 (http://weblogo.threeplusone.com/).

**Gene ontology and pathway enrichment analysis**

Gene ontology (GO) and KEGG pathway enrichment analysis of significant DEiso between any two fruit developmental stages was performed using R clusterProfiler (Yu et al. 2012) package. For enrichment analysis, SQLite-based custom annotation package named as “org.Cannuum.org.db” for C. annuum was prepared in R using AnnotationForge package (Carlson and Pages 2021). Using clusterProfiler, significant GO terms or KEGG pathway–associated DEiso were identified using enrichGO and enrichKEGG functions, respectively. GO terms or KEGG pathways with both p value and FDR <0.01 were considered significant and were represented using ggplot2 R package (Wickham 2009). GO terms were classified into major three categories, including cellular process (CC), biological processes (BP), and molecular functions (MF).

**Reverse transcription PCR (RT-PCR) validation of alternatively spliced events in fruit development**

In addition to the samples used in RNA-seq from Cann and Cfrut, the total RNA from each fruit developmental stage (EG, MG, and Br) belonging to C. chinense (Chin) was also extracted as described in the “Plant materials, growth conditions, and RNA extraction” section. RNA quality and quantity were checked using 1% agarose gel and NanoDrop 1000 (Thermo Scientific), respectively. Then, 1 μg of total RNA (equal quantity from each sample) was used to synthesize the cDNA using PrimeScript IV 1st strand cDNA Synthesis Mix (Takara, USA) following the manufacturer’s protocol. The cDNA from each sample was subjected to AltSpli-events validation using RT-PCR analysis with a thermal profile set with initial denaturation step (95 °C for 5 min), followed by 35-cycle amplification step involving 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 30 s (amplification step) followed by 10-min (72 °C) final amplification step, using AltSpli-events-specific primers. Details of AltSpli-events-specific primers are listed in Table S1. Actin was used as control and PCR reaction mixture without cDNA templates was used as a negative control.

**Untargeted GC–MS-based metabolomics analysis**

The global metabolome of EG, MG, and Br fruit developmental stages from both Cann and Cfrut (each stage with two biological replicates) was analyzed using gas chromatography coupled with mass spectrometry (GC–MS) following the method described by Lisec et al. (2006) with slight modification (Ahmad et al. 2021; Lisec et al. 2006). Briefly, 100 mg of fruit sample from each individual fruit stage was homogenized with mortar and pestle using liquid nitrogen and then 1400 μl of pure methanol (chilled) was added. For internal quantitative standard, 60 μl of ribitol (0.2 mg ml⁻¹) was added and kept for shaking for 10 min at 70 °C, followed by vortex at 11,000 g for 10 min. Furthermore, 750 μl of chloroform and 1500 μl of dH₂O were added and centrifuged for 15 min at 2200 g. Finally, the supernatant of each sample was dried in a vacuum concentrator without heating. For derivatization, 40 μl of methoxyamine hydrochloride (20 mg ml⁻¹) in pyridine was added in all samples, vortexed, and kept in a shaker for 2 h at 37 °C. For trimethylsilylation, 70 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as a silylating agent was added and then vortexed and incubated with shaking for 30 min at 37 °C. The metabolites were detected using reference mass spectral library (NIST14 and Wiley 8) and the relative quantification of each metabolite was calculated based on per area ratio of peak/analyte to internal standard ribitol using GC–MS post-run analysis program (Shimadzu Scientific Instruments). Pathway enrichment of metabolites was analyzed using MetaboAnalystR (Pang et al. 2021), and pathway number of metabolites > 3 and pathway impact > 0.2 along with p value <0.01 were considered significantly enriched. Metabolite differences between parents and hybrid with fold change >2 and p value <0.05 were considered significant.

**Result section**

**Transcriptome diversity during Capsicum fruit development**

For in-depth inquisition of AltSpli-events during fruit development, transcriptome diversity was analyzed from fruit samples at EG, MG, and Br developmental stage pertained from two Capsicum species, i.e., C. annuum (Cann) and C. frutescens (Cfrut). On average, 226.4 and 253 million raw RNA-seq reads collectively from three fruit stages were obtained from Cann and Cfrut, respectively. Around 90.7% of Cann followed by 85.9% of Cfrut paired-end reads with an average of 88.2% paired-end reads from all samples were uniquely aligned to the reference genome (Table S2). Of the total annotated genes (36,465 in Cann), around 84–85% of genes were detected to be multi-exonic (30,803 collectively from Cann and Cfrut) from all samples (Fig. 2A). Then based on the expression cutoff (FPKM >0.1), a total of 27,753 (76.1%) genes in Cann and 26,286 (72.1%) in Cfrut were expressed in at least one fruit developmental stage. However, at individual developmental stage, Br stage in both...
Cann and Cfrut showed 4–6% decrement in total expressed genes (Fig. 2B). Furthermore, the expressed genes from all samples were examined based on exon counts and it was observed that around 88.2 to 89% of total expressed genes are multi-exonic (MEG) from all samples (Fig. 2C). Also, approximately 31–31.63% (7664 in Cann and 7435 in Cfrut) of MEGs from all stages were observed to be alternatively spliced (AltSpli) (Fig. 2C–D).

**Alternative splicing landscape and diversity in fruits of two different Capsicum species**

Using the assembled transcripts from all three fruit developmental stages, a total of 117,416, 96,802, and 176,970 AltSpli-events were identified from Cann, Cfrut, and combined from both Cann and Cfrut, respectively (Table S3 and Table S4). Among the identified AltSpli-events, the majority of them belonged to IR (32.2% in Cann and 25.75% in Cfrut and 28.75% from combined), followed by AA (15.4% in Cann and 18.9% in Cfrut and 14.96 from combined) as the second highest AS-event in Capsicum fruits. The least number of AltSpli-events (0.81 to 1.13%) belonging to the exclusive nature of exons was under MX category (Fig. 3A–C). Also, the most notable top 15 AltSpli-events were compared between Capsicum species and noted that Cfrut comparatively have less number of AltSpli-events than Cann (Fig. S1).

Furthermore, AltSpli-events categories at each fruit developmental stage were compared and each category showed a more or less similar trend at intra- and inter-species levels across the three fruit developmental stages (Fig. S2). Likewise, most of the AltSpli-events from each category were homogeneously distributed across 12 different chromosomes; however, the least number of AltSpli-events was observed at chr5 and chr10 of Cann and chr7 and chr8 of Cfrut (Table S5). Additionally, around 28.4–31.5% of total AltSpli-events were unique to individual fruit stages, while only 4–7.3% were common between the three fruit stages of the Capsicum samples (Fig. S3A–B). Furthermore, a total of 45,517 AltSpli-events associated with the expressed MEGs (31.02–31.63%) were identified from all fruit stages (six samples) of both Cann and Cfrut (see Fig. 2C–D). Altogether, from all three fruit stages, on an average, around 1.84 and 1.94 transcripts per expressed MEG were produced in

![Fig. 2 Summary of expressed transcripts and events count at early green (EG), mature green (MG), and breaker (Br) fruit stage from C. annuum and C. frutescens. A Exonic structure based count information of genes in Capsicum genome, B total expressed genes (FPKM > 0.1), C multi-exonic genes (MEGs) count with and without events which are expressed in at least one of the fruit developmental stages, and D percent distribution of MEGs with alternative splicing events](image-url)
Cann and Cfrut respectively; however, fruit developmental stages in Cfrut showed slightly higher average number of transcripts per MEG than Cann stages (Fig. S3C). Besides, Br fruit stage in both Cann and Cfrut produced 2.09 and 2.19 transcripts per MEG, which is preferentially higher than EG and MG fruit stages (Fig. S3C).

Furthermore, we have also analyzed differential splicing events between two fruit stages using rMATs analysis (Table S6) and observed that among the identified events by rMATs, IR, followed by SE, was the most predominant splicing events (Fig. 3D). In addition, Br-vs-EG and MG-vs-EG fruit stage in Cann resulted in highest significant (4909 and 3369, respectively) DSE \((p \text{ value} < 0.001 \text{ and FDR } < 0.05)\), while MG-vs-EG in Cfrut showed the least (1552) number of significant DSE (Fig. 3D). In addition, a total 2418 significant DSE \((p \text{ value} < 0.001 \text{ and FDR } < 0.05)\) from all fruit tissues of both Capsicum species were also identified using cuffdiff (Table S7). Furthermore, around 1082 genes were commonly identified as DS using both analysis methods across fruit samples (Fig. S3D and Table S8), which were associated with high-confidence significant DSE \((p \text{ value} < 0.001 \text{ and FDR } < 0.05)\). Among these, genes with DSEs such as bZIP transcription factor 16 (CabZIP13; LOC107850501), cytochrome P450 71A3-like (LOC107862622), protein NRT1/Ptr FAMILY 8.1-like (LOC107877811), WD repeat-containing protein 75 (LOC107878901), riboflavin biosynthesis protein PYRR, chloroplastic (LOC107863918), cell differentiation protein RCD1 homolog (LOC107879031), G2/mitotic-specific cyclin-2-like (LOC107871998), cyclin-dependent kinase E-1 (LOC107867865), CaMYB4 (LOC107847790), CaMYB29 (LOC107843587),...
golden 2 like (CaGLK2; LOC107845460), auxin response factors (LOC107842580; LOC107850591), squamosa promoter-binding protein 1 (LeSPL-CNR; LOC107859362), and auxin-induced protein PCNT115 (LOC107864478) were observed to be involved in diverse key biological pathways including plant growth/development, fruit development/ripening, plant hormone signaling, phenylpropanoid pathway, carotenoid and flavonoid biosynthesis pathway, starch and sugar biosynthesis, and fatty-acid biosynthesis (Table S8).

Additionally, our analysis showed that the among the dinucleotides bases at 5′ splicing donor and 3′ acceptor sites, the canonical GT-AG had a genome-wide frequency of 93.38%, which is higher than the frequency (85.79%) observed in RNA-seq data (Fig. 3E), while among the non-canonical splicing sites, except AT-AG, the increased frequencies of the dinucleotide pairs such as CT-AC, GC-AG, GT-TT, and GT-AT AT-AC were observed in RNA-seq of diverse key biological pathways including plant growth/development, fruit development/ripening, plant hormone signaling, phenylpropanoid pathway, carotenoid and flavonoid biosynthesis pathway, starch and sugar biosynthesis, and fatty-acid biosynthesis (Table S8).

Expression diversity and differential splicing of plant hormone signaling pathway and fruit development/ripening-related genes

To understand plausible impact of splicing in fruit development/ripening regulation, we have analyzed significant differential expressed isoforms and their associated splicing events among fruit stages of both Cann and Cfrut. We observed that isoforms associated with a total of 89 genes in plant hormone signaling pathway were significant DE. Around 40.44% (36) of these genes were found to be alternatively spliced, of which 27.78% (10) were significantly (p value < 0.001 and FDR < 0.05) DSE among the fruit stages of Cann and Cfrut (Fig. S6). Genes under the plant hormone signaling pathway are involved in regulation of diverse functions during growth and development. We observed that genes such as auxin transporter-like protein 4 (AUX1; LOC107842765), auxin-responsive protein IAA29 (AUX/IAA29; LOC107872843), AUX/IAA8-like (LOC107867327), AUX/IAA13-like (LOC107864576), AUX/IAA16-like (LOC107841020), jasmonic acid-amido synthetase JAR1-like (GH3; LOC107845611), and auxin-induced protein 15A-like (SAUR; LOC107880012) related to plant growth and cell enlargement were alternatively spliced, while genes such as histidine kinase 4 (CRE1; LOC107870540), histidine-containing phosphotransfer protein 4-like (AHP; LOC10784840), two-component response regulator (A-ARR; LOC107874384, LOC107843885, LOC107848380), and shaggy-related protein kinase eta-like (BIN2; LOC107874384) related to cell division/elongation and shoot initiations were alternative spliced (Fig. S6). In addition, various other genes related to growth, seed dormancy, and senescence/stress response including DELLA protein GAI-like (DELLA; LOC107870479), transcription factor PIF4 (TF-PIF4; LOC107878077), probable protein phosphatase 2C 51 (PP2C 51; LOC107855301, LOC107855965), PP2C 16-like (AIP1; LOC107861707), abscisic acid-insensitive 5-like protein (ABI 5-like; LOC107840274, LOC107875685), jasmonic acid-amido synthetase JAR1-like (JAR1; LOC107839284), and protein TIFY 6B-like (JAZ; LOC107846940) were also observed with various splicing events. Additionally, IR events in genes such as AUX/IAA16-like, LOC107840274 (ABI 5-like), and LOC107848380 (A-ARR) were significant (p value < 0.001 and FDR < 0.05) DS across fruit tissues of both Cann and Cfrut. In Cfrut, AA events from AUX/IAA29 and JAZ were significantly DS at Br stage compared to both EG and MG stage, while two-component response regulator-like ARR2 (LOC10782899) with IR and AA events was significantly
down-regulated ($p$ value < 0.001 and FDR < 0.05) at MG and Br stages compared to EG in both Cann and Cfrut (Fig. S5, Table S6 and Table S10).

Furthermore, we have identified several fruit development/ripening-related genes such as ethylene receptor genes (ETR; LOC107852024, LOC107864062, LOC107873245, LOC107864066), ethylene insensitive 3-like (EIN3; LOC107874321), stay green 1 gene (SGR1; LOC107866321), polygalacturonase-2 (PG2; LOC107843830), axially regulator YABBY 5 like (YABBY5-like; LOC107848416), LeSP-LCR, peptide methionine sulfoxide reductase (PMRS or E4; LOC107862630), and E3 ubiquitin-protein ligase COP1-like (LOC107842184) associated with splicing events (Fig. 4A). Some of these genes showed significant differential splicing among fruit tissues. For instance, gene including ethylene receptor (ETR; LOC107852024) with AA event was significantly DS at Br fruit stage of Cann compared to EG and MG fruit stage. Also, genes involved in tissue softening and chlorophyll degradation during ripening such as polygalacturonase-2 with MX and SE event at Br-vs-MG and SGR1 gene with IR at both Br-vs-EG and Br-vs-MG stage comparison showed significant ($p$ value < 0.001 and FDR < 0.05) differential splicing. In Cfrut, AD event in axial regulator YABBY 5 like genes showed significant differential splicing at Br stage compared to both EG and MG fruit stages. Ripening-related gene LeSP-LCR showed IR event DS ($p$ value < 0.001 and FDR < 0.05) at Br stage compared to EG and MG stage of both Cann and Cfrut. Another gene peptide methionine sulfoxide reductase (LOC107862630), a homolog of tomato E4 fruit ripening gene, also showed AA event DS at MG and Br fruit stages compared to EG stage of Cann (Fig. 4A, Table S6 and Table S10).

**Fig. 4** Expression diversity of alternatively spliced isoforms related to A fruit development/ripening, MADS-box transcription factors (TFs), ethylene response factors (ERF), response regulators, and B bHLH, bZIPs, MYB, and WRKY TFs family members across fruit developmental stages of both C. annuum and C. frutescens (note: isoforms with significant differential splicing events between any two consecutive fruit stages were marked with asterisk sign)
Expression and differential splicing of various transcription factors genes during *Capsicum* fruit development

Earlier different transcription factors (TFs) were reported to have diverse role in plant growth and development including fruit ripening. We have analyzed the expression pattern of several TF genes from *MADS-box* TF family (Dong et al. 2014; Dubey et al. 2019; Sung et al. 2001), etylene response factors (ERF) TF family (Song et al. 2020), basic helix–loop–helix (bHLH) TF family (Liu et al. 2021), basic leucine zipper domain (bZIP) TF family (Gai et al. 2020), myeloblastosis (MYB) (Arce-Rodriguez et al. 2021; Islam et al. 2021), and WRKY TF family (Cheng et al. 2016) and their associated splicing events. We identified that isoforms from a total of 34 *MADS-box*, 4 *ARR*, 9 *ERF*, 19 *bHLH*, 10 *bZIPs*, 19 *MYBs*, and 19 WRKY TFs were DE among the *Capsicum* fruit developmental stages and were alternatively spliced (Fig. 4B). From *MADS-box* TF genes, *floral homeotic protein AGAMOUS-like* (CaAGL1; LOC107878477) which is involved in carpel expansion and fruit ripening was DS with AD event between Br-vs-EG and Br-vs-MG stages, while IR events from *agamous-like MADS-box protein AGL104* (AGL104; LOC107867200) was DS between Br-vs-MG stages of *Cann*. In *Cfrut*, SE event from *MADS-box* transcription factor 6-like (LOC107862765) was DS between Br-vs-EG stages. *MADS-box* TFs which are involved in *RIN* mediated fruit ripening including *FULLI* (LOC107853404) with SE event and *SEPALLATA 1* (LOC107860422) with AA events were also observed to be DS (p value < 0.001 and FDR < 0.05) in both *Cann* and *Cfrut* between MG-vs-EG and Br-vs-MG stages, respectively. The *FULLI* and *SEPALLATA 1* were highly expressed at Br and EG fruit stages, respectively, and both showed opposite expression pattern with *Capsicum* fruit development (Fig. 4B, Table S6 and Table S10). Isoforms from *ERF* TF family including CaERF82 (involved in carotenoid biosynthesis) and *AP2-like ERF TOE3* (LOC107868117) showed very high tissue-specific expression at Br stage (twofold higher in *Cann* than *Cfrut*) and were significantly (p value < 0.001 and FDR < 0.05) alternatively spliced with AA/AD and AA events at Br than EG and MG in *Cann* and *Cfrut*, respectively, while members from *bHLH* TF family including *CabHLH003* (AD/IR event), *CabHLH046* (IR event), and *CabHLH060* (IR event) showed EG fruit-specific expression and were significantly DS (p value < 0.001 and FDR < 0.05) in both *Cann* and *Cfrut*. Also, *CabHLH100* (involved in carotenoid biosynthesis) and *CabHLH_LOC107861840* showed increasing and decreasing expression trends with fruit maturation, respectively, in both *Capsicum* species and were significantly DS with IR at MG and IR/AD Br stage, respectively compared to EG and MG fruit stages (Fig. 4B, Table S6 and Table S10). In continuation, *bZIP* TFs such as *CabZIP7* (AD event in *Cfrut*), *CabZIP25* (IR event in *Cann*), and *CabZIP23* (AA event in *Cann* and *Cfrut*) showed increasing expression with fruit maturation and were significantly DS at Br stage compared to EG and MG fruit stages, while *CabZIP10* and *CabZIP13* (with SE and IR event in *Cann*, respectively) showed higher expression at both MG and Br fruit stages compared to EG and both were significantly DS (Fig. 4B). Among the *MYB* TFs, *CaDIVI* showed significantly increasing expression with fruit maturation (but relatively 10–12 fold higher at Br stage than EG and MG) and was DS with AA events at Br fruit stage of both *Cann* and *Cfrut*. Likewise, one isoform of *CaGLK2* showed species-specific expression, while other transcripts showed higher expression at MG and Br fruit stage relative to EG fruit of both *Cann* and *Cfrut*. In addition, with SE or MX events, these isoforms of *CaGLK2* also showed significant (p value < 0.001 and FDR < 0.05) differential splicing. Furthermore, both *CaMYB3R-1* and *CaMYB3R-5* (with AA event in *Cfrut*) showed moderate expression across all tissues and were DS between Br-vs-EG stages, while *CaMYB48* (with AD event) showed relatively higher expression and significant differential splicing at both MG and Br stage than EG of *Cann* (Fig. 4B, Table S6 and Table S10). Among the significant DE WRKY TFs, most of the members such as *CaWRKY28* (with IR event), *CaWRKY48* (with IR event), *CaWRKY54* (with AA event), and *CaWRKY_LOC107856725* (with IR/AA events) showed Br fruit stage-specific higher expression and were DS in both *Capsicum* species, while *CaWRKY51* with AA events significantly DS between Br-vs-EG stages showed decreasing expression trend with fruit maturation and was higher at EG fruit stages of both *Capsicum* species (Fig. 4B, Table S6 and Table S10).

Expression diversity and differential splicing status of metabolite biosynthesis pathway genes

In phenylpropanoid biosynthesis pathway, transcripts from a total of 77 genes were significant DE among fruit tissues of *Capsicum* and *Cfrut* (Fig. 5A). Around 31.16% (24) genes including 4-coumarate-CoA ligase-like 6 (*4CL-like 6*; LOC107869755), 4CL-2-like (*LOC107877487*), agmatine coumaroyltransferase-2-like (*ACT-2-like*; LOC107875625), caffeoyl-CoA O-methyltransferase 6 (*CCoAOMT6*; LOC107840262), *CcoAOMT-like* (*LOC107860279*), cinnamoyl-CoA reductase 1-like (*CCR1*; LOC107861980, LOC107861981), caffeic acid 3-O-methyltransferase (*COMT*; LOC107862989, LOC107862991), COMT-like (*LOC107875336*), probable cinnamyl alcohol dehydrogenase 6 (*CAD6*; LOC107858423), peroxidase gene (*POD*; LOC107840010, LOC107851630, LOC107859813), beta-glucosidase 40 (*BGL40*; LOC107876837), *BGL42* (*LOC107851487*), *BGL11-like* (*LOC107854417*), and *BGL18-like*...
were alternatively spliced (Fig. 5A). In addition, lignin biosynthesis-related gene CCR1 (with IR event) showed significant (p value < 0.01) highest expression at MG than decreased at Br stage of Cfrut, while Cann showed opposite expression with IR event significant (p value < 0.001 and FDR < 0.05) DS between MG-vs-EG stages of Cann. Likewise, high expression of BGL 42 at MG stage in both Capsicum species and differential splicing between Br-vs-MG stages of Cann was observed. Also, increasing expression trend with fruit maturation was observed for BGL 11-like gene which is associated with fruit softening/ripening. However, Br stage of Cfrut showed threefold higher expression than Cann Br stage and was significantly (p value < 0.001 and FDR < 0.05) DS with AD event between Br-vs-MG fruit stage of Cann, while 4CL-like 6 (with AA events) showed decrease in expression pattern with fruit maturation (but was comparatively higher at Cfrut MG than Cann). From flavonoid biosynthesis pathway, transcripts from a total of 25 genes were significant DE, and out of that, 9 genes (36%) were alternatively spliced. These genes were probable chalcone-flavanone isomerase 3 (CHI3; LOC107871144), naringenin,2-oxoglutarate 3-dioxygenase (F3H; LOC107859880), flavonoid 3′-mono-oxygenase (F3′H; LOC107862334), dihydroflavonol-4-reductase (DFR; LOC107860031), agmatine coumaroyltransferase-2-like (ACT2-like; LOC107875625), cytochrome P450 9A2-like (LOC107844023, LOC107844024), caffeoyl-CoA

Fig. 5 Heatmap representing significant differentially expressed isoforms in A phenylpropanoid biosynthesis pathway, B flavonoid biosynthesis, and C carotenoid biosynthesis across the fruit developmental stages of both C. annuum and C. frutescens. (Note: EG, MG, and Br represent early green, mature green, and breaker fruit stages of Capsicum species. Alternatively spliced isoforms are marked with asterisk symbol.)
O-methyltransferase 6 (CCoAOMT6; LOC107840262), and CcoAOMT-like (LOC107860279) (Fig. 5B), while from carotenoid biosynthesis pathway, transcripts from 25 different genes were significantly DE among tissues. Of these, 15 genes including 15-cis-zeta-carotene isomerase (LOC107850257), zeta-carotene desaturase (ZDS; LOC107839468), 15-cis-phytoene desaturase (PDS; LOC107861625), beta-carotene hydroxylase 1 (CA1; LOC107863219), zeaxanthin epoxidase (ZEP; LOC107860302), probable carotenoid cleavage dioxygenase 4 (CCD4; LOC107848895), 9-cis-epoxy-carotenoid dioxygenase NCED3 (NCED3; LOC107846808), xanthoxin dehydrogenase-like (LOC107855067, LOC107855068), abscisic-aldehyde oxidase-like (LOC107847367), benzaldehyde dehydrogenase (NAD(+) like (LOC107847514), abscisic acid 8-hydroxylase 4-like (CYP707A70; LOC107867833), abscisic acid 8-hydroxylase 1 (LOC107870293), beta-carotene isomerase D27 (LOC107863814), and CCD7 (LOC107844820) were alternatively spliced (Fig. 5C).

Among these carotenoid biosynthesis genes, PDS showed significant DE and differential spliced AA events (p value < 0.001 and FDR < 0.05) at Br compared to EG and MG fruit stages, while xanthoxin dehydrogenase gene LOC107855068 showed decreased expression with from EG and MG to Br stages in both Capsicum species with significant differential IR event (p value < 0.001 and FDR < 0.05) between MG-vs-EG and Br-vs-EG stage comparison of Cfrut. The spliced variants of CA1 also showed a similar expression trend to PDS in both the Capsicum species. Similar to PDS and CA1, the expression of benzaldehyde dehydrogenase (NAD(+) like (LOC107847514) gene increased with fruit maturation, and with SE event, it showed significant differential splicing (p value < 0.001 and FDR < 0.05) pattern between Br-vs-EG fruit stages of Cann. In addition, the spliced variant of ZEP with IR and SE events showed contrastingly opposite expression between both Cann and Cfrut, and was decreased in Cann with fruit developmental stages. Also, the expression of ZEP at MG of Cann and Br of Cfrut showed significant low (−1.4 log2FC) and high expression difference (p value < 0.01 and FDR < 0.05) compared to EG fruit stage (Table S6, Table S10, and Fig. 5C).

Gene ontology (GO) terms and pathways significantly associated with differentially expressed isoforms

To provide insight into the biological or functional knowledge, DEiso from each fruit stage comparison (MG-vs-EG; Br-vs-EG; and Br-vs-MG) were classified and categorized into different GO terms and KEGG pathways (Table S11). It was observed that GO terms related to heme-binding, iron-ion-binding, hydrolase activity, transferase activity, and mono-oxygenase activity were most significantly (p value < 0.001 and FDR < 0.01) enriched under MF categories across the fruit developmental stages of both Capsicum species, while GO terms related to cell-wall, cell-surface, extracellular region, and monolayer-surrounded lipid storage body were significantly (p value < 0.001 and FDR < 0.05) enriched under CC category, and GO terms including proteolysis, carbohydrate metabolic process, auxin-activated signaling pathway, lipid-biosynthesis process, and cell-wall organization or biogenesis were significantly (p value < 0.001 and FDR < 0.05) enriched under BP category (Fig. S7A).

Furthermore, isoforms DE at different consecutive fruit developmental stages were enriched across different developmental/signaling pathways (Table S12). The pathways related to plant hormone signal transduction, MAPK signaling in plants, phenylpropanoid biosynthesis, cutin, suberin, and wax biosynthesis were significantly enriched during Capsicum fruit development (Fig. S7B). The Br stage represents the early onset of fruit ripening, which is quite different from EG and MG stage. The Br stage when compared with EG and MG showed plant hormone signal transduction pathway as the most significantly enriched pathway including in both Capsicum species (Fig. S7B). The MAPK signaling and flavonoid biosynthesis pathways were enriched between MG and Br stages of Cann (Fig. S7B), while pathways such as phenylpropanoid and carotenoid biosynthesis were significantly enriched in Cfrut during fruit development/ripening (Fig. S7B). Moreover, the expression of transcripts/isoforms DE across fruit developmental stages was also deciphered for plant hormone signal transduction pathway (Fig. S6), phenylpropanoid biosynthesis (Fig. 5A), flavonoid biosynthesis (Fig. 5B), and carotenoid biosynthesis (Fig. 5C) pathways. This provided pan-view of expression regulation of transcripts across three fruit developmental stages involved in regulation of diverse biological functions during development/ripening of Capsicum fruits.

RT-PCR expression analysis of spliced variants

In Capsicum, we observed that several transcripts/isoforms related to genes involved in regulation of different biological processes during fruit development were AltSpli through one or more types of splicing events (Table 1). Therefore, using RT-PCR analysis, we have validated different AltSpli events associated with 7 genes significantly expressed during Capsicum fruit development/ripening (Fig. 6). The expression of AltSpli isoforms at EG, MG, and Br fruit stages was identified and it was observed that AltSpli isoforms with specific events showed tissue- or species-specific expression patterns in Capsicum. For instance, CA1 from the carotenoid biosynthesis pathway, with IR event, was expressed at EG stage specific to Cann, while at MG and Br stages, specific to both Cfrut and Chin. Similar to this, EG and Br
Table 1  Fruit development/ripening-related genes significantly expressed and undergone alternative splicing during *Capsicum* fruit development and their functional summary based on earlier reports

| Gene description | Expression in *Capsicum* fruit (in this study) | Probable function | References |
|------------------|-----------------------------------------------|-------------------|------------|
| Cyclin D3;1 (CycD3;1) | High expression in early green stage of fruit | Transduced the signals leading to fruit growth by cell divisions | Kvarnheden et al. (2000) |
| Cyclin-dependent kinase B2-1 (CDKB2;1) | High expression in early green stage of fruit | Cell cycle progression/regulation in early fruit | Chevalier (2008); Czerednik et al. (2012) |
| NAC transcription factors (NAC-NAM-2; NAC29) | Increased with fruit development | Plant growth, fruit ripening/softening/pigmentation | Forlani et al. (2021); Jia et al. (2021) |
| Purple acid phosphatase (EC 3.1.3.2) | Increased expression at mature green and breaker fruit stage | Seed development and seed phytate accumulation | Lott et al. (2000); Bhadouria et al. (2017) |
| Putative indole-3-acetic acid-amido synthetase GH3.8 | High expression at breaker fruit stage | Prevents free accumulation of IAA, disease resistance | Xinhua et al. (2008); Gan et al. (2019) |
| Sucrose synthase (EC 2.4.1.13) | Increased with fruit development/maturation | Fruit ripening, catalyze the cleavage of sucrose in growing fruit | Stein and Granot (2019); D’Aoust et al. (1999) |
| 4-Coumarate–CoA ligase (EC 6.2.1.12) (4CL) | Decreased with fruit development | Expressed in flower and early fruit, declined with fruit maturation | Arce-Rodríguez et al. (2021); Cao et al. (2016); Islam et al. (2021) |
| YABBY transcription factors | Decreased expression from early green to breaker stage in *C. annum* and *C. frutescens* | Flower, fruit, and leaf development; fruit size/shape | Cong et al. (2008); Han et al. (2015) |
| HD-Zip HOX27 | Highest expression in mature green fruit | Plant growth and development, mediates ethylene biosynthesis during fruit ripening | Gu et al. (2019) |
| Auxin response factor | Highest expression in green fruit (at early green and mature green stage) | Regulates cell division in early fruit development and delay fruit ripening | Kumar et al. (2011); Li et al. (2016); Liu et al. (2018) |
| WRKY transcription factor 6 | Increased with fruit development | Plant development and stress responses; fruit development; fruit ripening in capsicum | Cheng et al. (2016); Wang et al. (2017); Zheng et al. (2019) |
| Protein STAY-GREEN LIKE, chloroplastic | High expression at breaker fruit stage | Retaining as well as in degradation chlorophyll | Barry et al. (2008); Eftutti et al. (2005) |
| Protein NRT1/PTR FAMILY 2.11-like | Increased expression from early to mature green but decreased at breaker fruit stage | Transports the plant hormones auxin, abscisic acid (ABA), and gibberellin (GA) and secondary metabolites | Chiba et al. (2015) |
| Vacuolar-processing enzyme (VPE) | Increased with fruit development | Sugar accumulation in fruits | Ariizumi et al. (2011) |
| Xyloglucan endotransglucosylase/hydrolase (EC 2.4.1.207) | Increased with fruit development | Fruit development/ripening | Saladé, M., et al. (2006); Morales-Quintana et al. (2020) |
| Ethylene receptor (ETR) | High expression at breaker fruit stage | Fruit ripening | Hou et al. (2018); Razo-Mendivil et al. (2021) |
| Peroxygenase 5 | High expression at mature green and breaker stage | Quercetin and octadecenoid acid conversion during fruit ripening and flavonoid biosynthesis | Aghofack-Ngueze et al. (2011) |
stage-specific AltSpI isoform with IR events was observed for *CaWRKY34* gene. Furthermore, species-specific AltSpI isoforms related to *ETR* (*ethylene receptor*) which involved in regulation of fruit ripening/development had a transcript with SE event only expressed during *Chin* fruit development. Opposite to *ETR*, auxin-responsive protein (*AUX/IAA13*-like) which is from plant hormone signaling pathway and is involved in cell enlargement/plant growth had one AltSpI isoform (with AA and AD events, respectively) specifically expressed in *Cann* and *Cfrut* at the EG and MG fruit development stage (Fig. 6).

**Metabolite diversity during fruit development**

Using the GC–MS analysis, around 84–85 metabolites from groups including carbohydrates, organic acids, nucleic acids, peptides, and lipids were identified in fruit samples (EG, MG, and Br) of both *C. annuum* and *C. frutescens* (Table S13A–B). Out of which, a total of 59 metabolites were common between fruit stages of both the *Capsicum* species, and changes in the metabolic content between any two fruit developmental stages were analyzed in terms of log2 ratio of the normalized area or log2 fold change (Table S13C). During fruit development, the most significantly altered metabolites between the fruit stages were from carbohydrate, organic acids (carboxylic acids), and peptides group (Fig. 7A). For instance, among the carbohydrates, both fructose and sucrose showed a significant increase (*p* value < 0.001) content in both MG and Br fruit stage compared to EG; however, the content of fructose was overall higher in *Cann* than *Cfrut*. Maltose showed significant increase (lfc > 3.7, *p* value < 0.001) in its content at Br fruit stage than both EG and MG fruit stages of *Cann*, while both MG and Br stages in *Cfrut* showed overall decrease (lfc < −0.46, non-significant) in its content compared to EG fruit stage. Furthermore, metabolites such as octanoic acid and quininic acid from carboxylic group showed significant higher content (lfc > 0.4; *p* value < 0.001) in both MG and Br stages compared to EG stage of *Cann*; however, contrasting to this, significant opposite trend (i.e., lower content than EG) was observed in *Cfrut*. Additionally, metabolites belonging to peptides (amino acids) group such asparagine at Br stage showed significantly higher (lfc > 1.5, *p* value < 0.001) content than EG and MG fruit stage of *Cann*, while, in *Cfrut*, its content was significantly higher (lfc > 1.3; *p* value < 0.001) in both MG and Br than EG fruit stage. Similar to asparagine, L-norvaline also showed similar trend (*p* value < 0.001 and *p* value < 0.05) in *Cann*, and contrasting to this, *Cfrut* showed significant decrease (opposite to *Cann* trend) in its content at Br stage than the both EG and MG stages. Moreover, several pathways including D-glutamine and D-glutamate metabolism, galactose metabolism, aminoacyl-tRNA biosynthesis, amino acids (arginine, valine, leucine, and isoleucine) biosynthesis, and starch and sucrose metabolism were significantly enriched (*p* value < 0.01) during *Capsicum* fruit development (Fig. 7B).
Correlation between metabolites and spliced transcript expressions

To understand the plausible role of alternative splicing on metabolites during fruit development in Capsicum, we have investigated the expression of significant DEiso enriched in various pathways (Fig. S7B and Table S12). We observed that around 40.8% (151) of total genes from significant DEiso enriched in various biological pathways underwent splicing, and of that, 25% (38) genes were significantly DS (p value < 0.01) between two consecutive fruit developmental stages (Table S14). Interestingly, these genes were significantly enriched in pathways related to starch and sucrose metabolism; cutin, suberin, and wax biosynthesis; amino acid metabolism; carotenoid and phenylpropanoid biosynthesis; and plant hormone signaling. In addition to this, our enrichment analysis showed that these pathways were also significantly enriched with metabolites identified in fruit samples. For instance, metabolites such as D-fructose, D-galactose, maltose, and sucrose from starch and sucrose metabolism pathway were significantly different (p value < 0.001) between two fruit stages, and content of sucrose and D-fructose was significantly increasing with fruit development in both Capsicum species, while genes from sucrose and starch biosynthesis pathways including glucose-1-phosphate adenylyltransferase large subunit 1 (LOC107840421), beta-fructofuranosidase, insoluble isoenzyme 1-like (LOC10784263), alpha,1,4 glucan phosphorylase L-2 isozyme (LOC107847630), 1,4-alpha glucan-branching enzyme-like (LOC107867385), and probable alpha, alpha-trehalose-phosphate synthase (LOC107877529) were significantly DS during fruit development (Table S14).

Fig. 7 Metabolite profiling and enrichment analysis. A Heatmap showing difference in metabolite content commonly identified across fruit tissues of both C. annuum and C. frutescens and B their pathway enrichment.
Discussion

In plants, the detection of AltSp1i-events has been improved with the improvement of quality and sequencing depth of high throughput RNA-seq data (A. thaliana, tomato, maize, rice paper, etc.). In the current study, we have identified the landscape of AltSp1i-events in the two Capsicum species and compared the event distribution between EG, MG, and Br stages of Capsicum fruit development. Earlier, it was reported that nearly 22,427 (64%) of total annotated Capsicum genes were expressed in different Capsicum accessions at the different fruit developmental stages (Martinez et al. 2021). Similar to earlier, this study observed that approximately 68.7–76.1% of total annotated genes were expressed in at least one fruit developmental stage (Fig. 2A–B). With fruit growth and advanced stage, overall decreased expressions of genes were observed in Capsicum (Martinez-Lopez et al. 2014). Br stage in both Cann and Cfrut showed 4–6% of global total annotated genes were expressed in at least one fruit and was higher than flower and seedling (Sun and Xiao 2014; Luo et al. 2013), while silencing of SGR1 and involved in carotenoid/lycopene accumulation (Balazadeh 2014; Dubey et al. 2019; Lai et al. 2015; Luo et al. 2013; Razoo-Mendivil et al. 2021). Also, it was found that SGR1 on interacting with light harvest complex II and chlorophyll catabolic enzyme acts as anti-chlorophyll and involved in carotenoid/lycopene accumulation (Balazadeh 2014; Luo et al. 2013), while silencing of SGR1 in tomato prevented the degradation of chlorophyll (Hu et al. 2011). In this study, it was observed that transcripts from ARF, AUX/IAA, and LeSPL-CNR genes are expressed during early and mature green fruit stages, while ETR and SGR1 are expressed at breaker fruit stages, and these genes were significantly alternatively spliced among fruit stages (Fig. S6, Fig. 4A, and Table S6), which indicated potential role of alternative splicing in expression regulation during Capsicum fruit development/ripening.

Recently, immature fruit-specific expression of plastid development–related gene PRR2 was reported in Capsicum (Jeong et al. 2020). The same study observed the patches with ivory to pale green color in immature green fruit and ivory to light yellow color in mature green fruits in PRR2-silenced plants (Jeong et al. 2020). In corroboration with earlier findings, this study observed that spliced variants of PRR2 (LOC107872899) with IR and AA events showed EG fruit-specific expression in
both Cann and Cfrut and might regulate the fruit color in Capsicum (Fig. 4A). Also, members of different TF families showed their importance in the regulation of several biological functions including plant growth and development (Brand et al. 2014; Sun et al. 2020; Sung et al. 2001). Previous increased expression of TFs such as CaERF82 (Song et al. 2020) and CabHLH100 (Liu et al. 2021) with fruit development/ripening and their role potential role in carotenoid biosynthesis was reported in Capsicum. In accordance with earlier findings, this study observed significant DS variants of these genes with similar increasing expression trend during Capsicum fruit development, and were highly expressed at Br fruit stage of both Capsicum species (Fig. 4). Also, CaMYBs such CaMYB31 and CaMYB48 were found to be involved in regulation/accumulation of capsaiacinoids in C. annuum (Arce-Rodríguez et al. 2021; Sun et al. 2020), while CaMYB homologs in C. chinense such as CcDIV1, CcMYB4, CcMYB52, CcMYB86, and CcMYB108 were found to be co-expressed with capsaiacinoid biosynthesis pathway genes (Islam et al. 2021). Additionally, other MYBs such as CaMYB3R-1 and CaMYB3R-5 were reported to involve cell cycle process, CaGLK in chloroplast development and plant defense, while CaDIV1 in regulation of capsanthin/capsorubin synthase (Ccs) gene (Arce-Rodríguez et al. 2021). This study observed that the differential expression of TFs such CaERF82, CabHLH100, CaMYB48, CaMYB3R-1, CaMYB3R-5, CabDIV1, and CaGLK2 is due to DS variants which regulate capsaiacinoid/carotenoid biosynthesis in Capsicum (Fig. 4B and Table S6). Furthermore, the study also reports that DS variants of CaWRKY28, CaWRKY48, CaWRKY54, and CaWRKY LOC1078536825 showed breaker fruit-specific expression signature and their expression trend was similar to phytoene synthase (Psy), PDS, CAI, and Ccs genes, which suggested their plausible role in accumulation of capsanthin/capsorubin and β-carotene in Capsicum. Also, similar to the expression of chalcone synthase 2 (CHS), the spliced variants of CaWRKY51 showed decreased expression with fruit development and therefore might be involved in capsaiacinoid or anthocyanin/flavonoid biosynthesis, which further need to be validated (Fig. 4B, Table S6, and Table S10).

Earlier, in Capsicum, it was reported that ethylene and abscisic acid (ABA) levels are regulated by 1-aminocyclopropane-1-carboxylate oxidase 3 (ACO3; LOC107853805) and NCED1 or NCED3 (Hou et al. 2018). In this study, opposite expression trend of both ACO3 and NCED was observed during Capsicum fruit development (Table S10), which is in agreement with earlier results. The dynamic expression of NCED and ABA hydroxylase (CYP707A) also regulates ABA synthesis, while expression of Psy, Ccs, POD (peroxidase P7), PAL (phenylalanine ammonia-lyase), CAI, CCD4, 4CL, CCRI, and CHS genes also regulated dynamically across phenylpropanoid, carotenoid, and flavonoid biosynthesis during fruit development/ripening (Arce-Rodriguez et al. 2021; Leng et al. 2013; Li et al. 2018). In 2014, using the virus-induced gene silencing, the role of Ccs, Psy, Lcyb, and Crtz was established in capsanthin biosynthesis and regulation of fruit color in Capsicum (Tian et al. 2014). Our results also endorsed that these aforementioned genes are differentially regulated during Capsicum fruit development (Figs. 4–5 and Fig. S6). However, DE transcripts of genes such as Psy, Ccs, and CAI of carotenoid biosynthesis showed ~4–27 times higher expression at Br stage of Cann than Cfrut (Fig. 5C and Table S10). Moreover, it was reported that PDS positively regulates fruit ripening in tomato, and silencing of it led to yellow color fruit development (Naing et al. 2019). Another study reported that the content of carotenoids in tomato is associated with ZDS, PDS, and CtrlSO genes (Fantini et al. 2013). This study observed that one of the spliced variants of PDS, ZDS, and 15-cis-zeta-carotene showed increased expression with fruit maturation and was highly expressed at Br fruit stages of both Capsicum species suggesting the potential role of spliced variants in the regulation of fruit coloration in Capsicum (Fig. 5C). Besides this, apparent tissue- or species-specific expression of significant AltSpli DEiso was observed during Capsicum fruit development (Fig. 6). Previously, it was reported that several members of WRKY including their spliced variants are involved in plant growth, in fruit development/ripening, and in biotic and abiotic stress responses (Mandadi and Scholthof 2015; Staiger and Brown 2013; Wang et al. 2014; Zheng et al. 2019), and earlier increased expression of CaWRKY34 from MG to Br fruit stage was reported in Capsicum (Cheng et al. 2016). Here, in Capsicum, stage-specific expression of AltSpli isoform from genes including CaWRKY34 and 4CL suggested that AltSpli might modulate development/ripening of Capsicum fruits. In plants, regulation of auxins is mediated by auxin-responsive proteins AUX/IAA13-like which along with ARF are involved in plant growth and cell enlargement (Kumar et al. 2011; Li et al. 2016b; Liu et al. 2018; Razo-Mendivil et al. 2021). In Arabidopsis, Ghelli et al. (2018) showed that tissue-specific AltSpli isoforms of ARF was involved in flower development (Ghelli et al. 2018). ETR is mainly involved in fruit ripening and is expressed at the breaker to ripe fruit stage (Dubey et al. 2019). In Capsicum, isoforms of Aux/IAA13-like, ARF, ETR, and several other fruit development/ripening-related genes showed alternative expression regulation at the fruit developmental stage or species-specific (Fig. 6 and Table 1), suggesting that AltSpli is involved in proteome diversity during fruit development.
Conclusion

Understanding the genes and spliced variants in regulation of economically important traits is required. Therefore, in the current study using the fruit RNA-seq data from EG, MG, and Br fruit developmental stages from the two Capsicum species (Cann and Cfrut), a comprehensive landscape of Altspli-events was investigated in Capsicum, which was not documented earlier. In this study, around 32–64% of MEGs involved in different signaling pathways during fruit development were identified to be associated with 45,517 and 49,949 Altspli-events. Also, it was observed that Altspli-events may regulate developmental stage-specific or species-specific proteome diversity during development and ripening of Capsicum fruits. For example, Altspli isoforms of genes related to fruit development/ripening such as Aux/IAA 16-like, CycD3;1, ARF, CA1, ETR, SGR1, GLK2, MADS-RIN, FUL1, and SEPAL-LATA1; carotenoid biosynthesis/accumulation-related genes such as PDS, CA1, CDD4, NCED3, xanthoxin dehydrogenase, CaERF82, CabHLL100, CaMYB3R-1, SGR1, CaWRKY28, CaWRKY48, and CaWRKY54; and capsaicinoid biosynthesis (CaMYB48, CaWRKY51) were differentially expressed and significantly differentially spliced among the developmental stages during Capsicum fruit development. Therefore, the catalog of Altspli-events and splicing associated with development and ripening-related genes identified in this may provide invaluable information about the interplay or switching of Altspli during Capsicum fruit development. Genes that are involved in various fruit metabolite biosynthesis pathways and other fruit traits with Altspli may further be studied/validated and may be manipulated in Capsicum improvement programs.

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Author contribution NR conceived and designed the research. AR, IA, KI, JM, AK, and VJ conducted field and lab experiments. AR and IA analyzed and interpreted results data. All the authors read, revised, and approved the final manuscript.

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Data availability The RNA-seq data used in this study can be accessed at Sequence Read Archive on NCBI using SRA accessions SRR12963501, SRR12963503, and SRR12963504 for early green (EG); SRR12963495-SRR12963497 for mature green (MG); and SRR12963498-SRR12963500 for breaker (Br) fruit tissues from Capsicum annuum, while SRR12963487, SRR12963512, and SRR12963511 for EG; SRR12963505-SRR12963507 for MG; and SRR12963508-SRR12963510 for Br fruit tissues from Capsicum frutescens.

Declarations

Ethics approval and consent to participate Not applicable.

Human and animal ethics Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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