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GENOME WIDE IDENTIFICATION OF CCO GENE FAMILY IN BEETROOT (*BETA VULGARIS*) AND ITS COMPARISON WITH ARABIDOPSIS THALIANA

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ABSTRACT

The CCO (carotenoid cleavage oxygenase) gene family plays a significant role in development and growth of the plant with the production of several phytohormones specifically abscisic acid and strigolactones as well as essential for pigment production. These hormones play a crucial role in regulating plant responses to various stimulus. Beetroot (Beta vulgaris) is a famous root vegetable known for its paramount health benefits along with its use as a coloring agent. Genome wide analysis of beetroot CCO genes has not been reported. In this study, genome mining of beetroot identifies seven BvCCO gene family members (three NCED and four CCD genes) which belong to six subgroups. Most of them are present in chloroplast dispersed on different chromosomes. Hence, segmented duplication is observed predominantly. CRE (Cis regulatory element) analysis of BvCCO genes indicates that they might be related to hormone regulation, light and stress responsiveness as well as growth and development of beetroot plant. Gene expression of beetroot manifest two genes BvNCED5 and BvCCD8a and their orthologue in A. thaliana showed upregulated expression during drought stress. Furthermore, thorough genome wide study of CCO genes in B.vulgaris will provide an understanding of BvCCO genes which sets a base for further investigations in details of beetroot plant.

KEYWORDS: CCO gene family, Beetroot (Beta vulgaris), Phytohormones (abscisic acid, strigolactones), Genome-wide analysis, Drought stress response

1. INTRODUCTION

Carotenoids are important natural isoprenoid pigments [1]. Carotenoids are ubiquitly present in all kingdoms of life ranging from bacteria to higher plants and animals, which help in photosynthesis, signaling, electron transport, light absorption and antioxidant properties [2]. It is a fat-soluble pigment that imparts yellow, orange, and red colors to the fruits and flowers in different plants [3]. Structurally, carotenoid is a polymer made up of 40 carbons [4]. CCO (Carotenoid cleavage oxygenase) are proteins that catalyze cleavage of carotenoids at one or both ends to form apocarotenoids during their metabolic pathway and regulate plant activities against biotic and abiotic stresses [5] [6].

The CCOs can be divided into two forms, one named as CCD (Carotenoid cleavage dioxygenase) and the other is NCED (9-cis-epoxycarotenoid dioxygenase) depending on whether the substrates are epoxidated [7]. RPE65 (Retinal pigment epithelium membrane protein) is a conserved domain that is present in all CCO proteins [8]. In A.thaliana, CCO family comprises of nine members, out of which CCD subfamily is composed of four genes (AtCCD1, AtCCD4, AtCCD7, and AtCCD8) and the NCED subfamily contains five genes (AtNCED2, AtNCED3, AtNCED5, AtNCED6, and AtNCED9) [9]. In 2016, an addition to the CCO gene family has been discovered for the first time in tomato (Solanum lycopersicum) and named it CCD-like (CCDL) [10].

Studies have shown that among the CCD subfamily in arabidopsis, AtCCD1 and AtCCD4 are responsible for the production of pigments and aromatic volatiles such as α-ionone and β-ionone in the flowers and fruits. [11]. AtCCD7 and AtCCD8 are involved in the biosynthesis of aurolactones and strigolactones. They are involved in many processes like root growth, branching, flower development, regulation of senescence and morphogenesis during plant development and growth [12]. The NCED gene was first identified in the maize mutant Vp14 [13]. Consequently, Tamatim [14], cowpea [15] and rice [16] have been found to contain NCED genes. All members of NCED (9-Cis-epoxycarotenoid dioxygenase) subfamily are responsible for the production of abscisic acid (ABA) [17]. NCED is a rate limiting enzyme for ABA biosynthesis. Abscisic acid is also called as a stress hormone which plays an important role in plant’s growth and modulates the reactions of plants to different environmental stresses [18].

Strigolactones are phytohormones made by the roots of plants. It was first identified in cotton plant [19]. It possess three main functions: first as a germination stimulant for parasitic plant Striga commonly called witch weed [20]. Secondly, SLs promote the symbiotic association of host plant’s root with fungi specifically arbuscular mycorrhizae [21]. Thirdly, SLs serve as an inhibition hormone for secondary shoot growth of the plant [22]. Strigolactones regulate plant growth and development and involved in various biotic and abiotic stress responses by serving as a signal mediator and reducing their negative impact on beetroot crop productivity [23].

Beetroot (B.vulgaris) is an important sugar crop that contributes up to 30% of global sugar output [24]. Beetroot is widely grown across the globe, regularly consumed as part of a healthy diet and frequently utilized in food manufacture as the food coloring agent E162 [25]. Beetroot is a rich source of potentially bioactive elements such as phenolics, ascorbic acid, carotenoids, nitrates and a family of highly bioactive pigments known as betalains [26] [27]. Due to abiotic stresses, sugar beet production and food quality have become a great challenge. Systematic identification of plant stress resistant genes toward improving crop stress resilience and increasing yield is urgently needed. Since the CCO proteins are involved in many biological processes including plant growth and stress response, we hypothesize that some BvCCO protein encoding genes in sugar beet play important role in plant stress tolerance. In this study, we analyzed the sequences of the CCO family proteins in the sugar beet genome. The transcriptomic data analysis has identified two candidates (BvNCED5 and BvCCD8b) that display higher activity of stress tolerance, implying their involvement in plant growth and development as well as offering potential avenues for the development of drought-stress resistant beetroot varieties to enhance crop yield in drought conditions. Although further investigations are necessary to validate their roles in various physiological and biological processes.

2. MATERIALS AND METHODS

2.1. Database Search and Retrieval of Sequences

The amino acid (AA) sequence of the carotenoid cleavage oxygenase (CCO) protein was retrieved from NCBI database (accessed on 24 march 2023) and the RPE65 domain (PF03055) Pfam: RPE65 (genome.jp) was identified in these sequences. Using BLAST-P (Protein-basic local alignment search tool) program and the RPE65 domain (PF03055) as the target, CCO genes in B.vulgaris were found from Phytozomev13 (accessed on 24 March 2023) Phytozome (https://phytozome-next.jgi.doe.gov). The NCBI CDD (Conserved Domain Database) Conserved Domains Database (CDD) and Resources (nih.gov) (accessed on 24 march 2023) was used to verify the retrieved sequences of amino acid. The proteins lacking the conserved RPE65 domain (PF03055) were analyzed and excluded [28].

2.2. Determination of Physio-Chemical Properties and Subcellular Localization

The ProtParam tool ProtParam tool (https://web.expasy.org/protparam/) (accessed on 10 April 2023) was used to predict the length of the carotenoid cleavage oxygenase (CCO) proteins as well as the molecular weight and isoelectric point (pI) value of beetroot. From phytozome Phytozome (https://phytozome-next.jgi.doe.gov the information of gene IDs, chromosomal location, direction and sequences of protein was retrieved. The subcellular localizations of BvCCO genes were identified with the help of WoLFPSORT program (accessed on 18 April 2023). (https://wolfpsort.hgc.jp)

2.3. Phylogenetic Analysis

Phylogenetic analysis was performed between the amino acid sequences of CCO proteins from B.vulgaris, A.thaliana, Cucurbita pepo. and Cucurbita maxima. The phylogenetic analysis was conducted using the MEGA software (Molecular Evolutionary Genetic study) (accessed on 18 April 2023) Home (megasoftware.net). Alignment of these amino acid sequences of CCO proteins was carried out using MUSCLE method. The Neighbor-Joining (NJ) method in MEGA 11 software was employed to construct a phylogenetic tree based on the alignment of these protein sequences with a bootstrapping value of 1000 replications. The iTOL website iTOL: Interactive Tree Of Life (embl.de) was used to visualize the obtained phylogenetic tree.

2.4. Gene Structure, Cis regulatory Analysis and Motif analysis

The Gene Structure Display Server (GSDS v2.0) Gene Structure Display Server 2.0 (gao-lab.org) (accessed on 8 May2023) was used to depict the intron-exon structure of the CCO genes. For this analysis, Genomic and CDS sequences were taken from phytozome. For cis regulatory analysis, the cis elements associated with these genes were identified using the PlantCare database (accessed on 18 May 2023) PlantCARE, a database of plant promoters and their cis-acting regulatory elements (ugent.be). MEME program (Multiple EM for motif elicitation) (accessed on 18 April 2023) MEME - Submission form (meme-suite.org) was used to detect motifs. The discovered motifs were shown using the TBtools.

2.5. Gene Duplication and Synteny Analysis

The divergence time of the CCO genes were figured out using Ka/Ks values with the help of TB tools. Default parameters were used as directed by the program. The molecular evolution rate of each paralogous gene pair was identified by determining the Ka/Ks ratio. The Divergence time (DT) was estimated by using the equation $T = Ks/2r$ in which “r” represents the neutral substitution rate and is equal to 1.5×10^-8. With the default settings, MCScanX v1.0 (Multiple Collinearity Scan toolkit) was used to analyze gene duplication events. In order to execute synteny and dual synteny, three crops i.e, arabidopsis, beetroot and spinach were used respectively. Their maps were generated using Advanced Circos in TbTools.

2.6. Chromosomal mapping and Protein-Protein interaction

Starting and ending positions of carotenoid cleavage oxygenase (CCO) were taken from the phytozome v13. The gene's chromosomal mapping was displayed using the TBtools. For protein-protein interaction analysis, String database STRING: functional protein association networks (string-db.org) accessed on 11 September 2023 was used.

2.7. Geo ontology analysis

To confirm the activities of CCO genes, gene ontology term enrichment analysis was carried out using GO annotations. To determine the molecular functions and biological processes, UniProt website UniProt (accessed on 11 September 2023) was used. To better understand CCO gene function in beetroot, an internet application named ShinyGo v0.741 was employed.

2.8. Expression Analysis

The expression levels of all CCO genes were examined by using previously generated high through put RNA sequencing data with GEO accession (GSE205413). The stages of plant development and their reactions to diverse biotic and abiotic stimuli were identified using the NCBI Gene Expression Omnibus (GEO) database accessed on 11 August 2023 Home - GEO - NCBI (nih.gov).

2.9. Micro RNA analysis

The micro-RNA (miRNA) sequences of BvCCO genes in beetroot were obtained from NCBI GenBank4 Overview (nih.gov). For the identification of mature RNAs targeting BvCCO genes, CDS sequences were used in psRNATarget website accessed on 24 July 2023 psRNATarget: A Plant Small RNA Target Analysis Server (2017 Update) (zhaolab.org).

3. RESULTS

3.1. Identification of CCO gene in Beta vulgaris

all, of which 3 NCED genes and 4 CCD genes, were discovered in the B.vulgaris genome. The BvCCO genes that code for the protein, range in size from 581 to 644 (Table 1),

Table 1. Overview of Identified BvCCO Genes in Beta vulgaris Genome

NCED gene	**Source Accession No.**			**Chr#**	**Chromosome Location**		**Direction**	**pI**	**Mw**	**AA No.**	**AI**	**Gravy**
Name	**Phytozome**	**Ensemble**	**Rename**		**Start**	**End**						
NCED1	Bevul.9G022500.1	BVRB_9g204380	BvNCED1	9	3793979	3796164	Forward	6.60	71993.16	644	80.78	-0.369
NCED2	Bevul.5G098700.1	BVRB_5g107230	BvCCD4a	5	20329662	20331477	Forward	6.85	67138.82	604	78.59	-0.253
NCED3	Bevul.1G139500.1	BVRB_1g018290	BvCCD8a	1	53745922	53752621	Reverse	8.66	70105.46	614	82.88	-0.287
NCED4	Bevul.8G090500.1	BVRB_004050	BvNCED4	8	26368457	26370266	Reverse	6.27	67154.41	602	77.76	-0.334
NCED5	Bevul.4G090100.1	BVRB_4g094240	BvNCED5	4	22009183	22010977	Forward	6.23	66538.90	597	80.84	-0.330
NCED6	Bevul.2G190200.1	BVRB_2g029320	BvCCD1a	2	50156072	50167412	Reverse	6.01	65414.03	581	80.84	-0.312
NCED7	Bevul.2G158700.1	BVRB_2g032350	BvCCD8b	2	45306235	45314076	Reverse	8.49	67494.93	606	77.43	-0.346

AA and molecular weight from 65414.03 to 71993.16 kD, with BvNCED6 being the smallest and BvNCED1 being the longest protein. Proteins that have been identified contain pI values between 6.01 and 8.49.

Subcellular localization of these seven BvCCO genes revealed that the majority of the genes (BvNCED1, BvNCED4 and BvCCD4a) were located in chloroplast. Some were in cytoplasm (BvNCED5 and BvCCD1a), mitochondria (BvCCD8a) and nucleus. Least of them were present in plasmid, E.R (BvCCD8b), vacuoles and extra cellular structures etc. (Figure 1 A). In this way, it is found that chloroplast has 42.8%, cytoplasm has 28.5% and endoplamic reticulum has 14.2% of the total BvCCO genes.

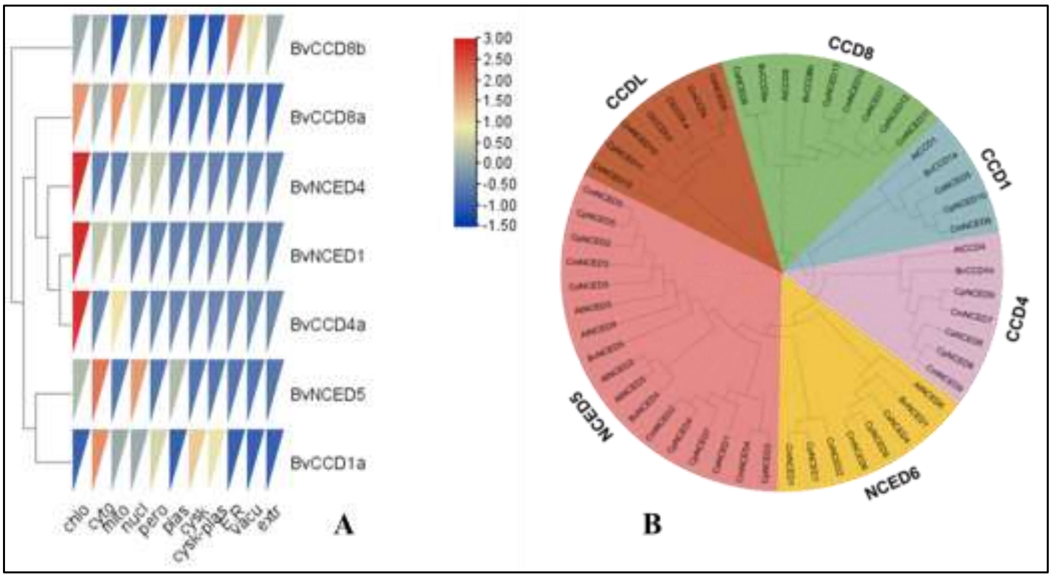

Figure 1: Heat map shows the localization of seven BvCCO genes in cellular structures including chloroplast, cytoplasm, mitochondria, nucleus, peroxisome, endoplasmic reticulum, vacuole and extracellular structures. The scale of availability of genes in a particular cellular region is given on the right. Red color represents the highest functional presence, white color represents the moderate availability while the blue colored region indicates absence of gene (A). Phylogenetic relationship among the CCO genes of B.vulgaris, A. thaliana, Cucumis sativus, Cucurbita maxima, Cucurbita pepo and Citrullus lanatus was studied. The evolutionary history was inferred using the NJ method with 1000 bootstrap replications in MEGA11 (B).

3.2.Phylogenetic Analysis

A phylogenetic tree was made using the reference sequences of 7 BvCCO genes from B.vulgaris, 10 genes from Cucumis sativus, 8 genes from A. thaliana, 12 genes from Cucurbita maxima, 13 genes from Cucurbita pepo and 3 genes from Citrullus lanatus. A total of 53 CCO's in all were used for the tree. According to the phylogenic study, BvCCO genes comprises of four CCD (BvCCD1a, BvCCD4a, BvCCD8a and BvCCD8b) and three NCED (BvNCED1, BvNCED4 and BvNCED5) genes. On the basis of presence of arabidopsis gene in each clade, the tree was categorized into groups. The phylogenetic analysis revealed that BvCCO genes fall into six distinct subgroups: CCD1, CCD4, CCD8, NCED5, NCED6 and CCDL. Among the discovered subgroups, NCED5 stands out as the biggest group with 17 genes, followed by CCD8 in second place with 9 genes. NCED6 is in third place containing 8 genes, followed by CCDL and CCD4 with 7 genes each. CCD1, which has just 5 genes, is the smallest group. Notably, no beetroot gene was discovered in the CCDL subgroup. The results demonstrate that 1 gene of beetroot was present in CCD1 clade, 2 genes were present in CDD8 clade, 1 gene was present in CCD4 clade and CCD6 clade also contain 1 gene while 2 genes of beta vulgaris was present in NCED5. A distinct clade comprising CCDL genes was obtained from a different crop (Figure 1 B). As these genes were absent in arabidopsis, their unique characteristics are unknown till now.

3.3.Gene structure and motif analysis

The intron exon analysis predicted the BvCCO gene structure, which shows that four out of seven genes BvNCED1, BvNCED4, BvNCED5 and BvCCD4a contain 1 exon and 0 intron. Two genes BvCCD8a and BvCCD8b contain 7 exons and 6 introns. Only a single and last gene BvCCD1a contains 14 exons and 13 introns. This analysis shows that the genes having the same number of introns and exons share common functions and common ancestors as compared to genes with different numbers of intron and exons which show functional variability as shown in figure 2.

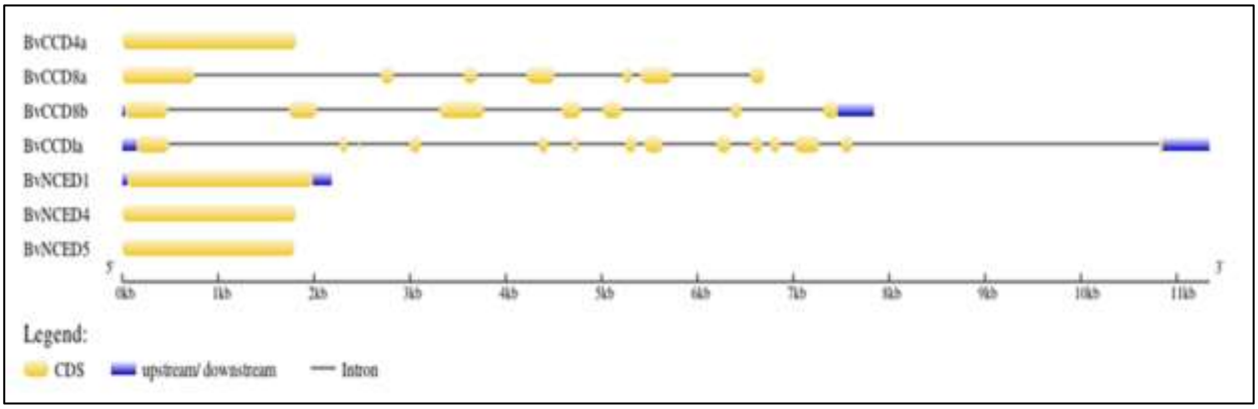

Figure 2: This graph illustrates the structure of seven BvCCO genes where yellow segments indicate exon regions that encode proteins and the black lines represent the introns, which are non-coding regions. The blue segments indicate upstream or downstream values.

The analysis of motifs in seven BvCCO genes showed the presence of 20 motifs in total. Among these, motif-1, motif-3, motif-7, and motif-10 were found to be conserved in all BvCCO genes, while the rest displayed variations. Motif-4 and motif-6 were conserved in six BvCCO genes, except for the BvCCD8b gene. Similarly, motif-5 was present in all BvCCO genes, except for the BvCCD8a gene (Figure 3 A). The RPE65 domain: [FAPVPEQAVKHYLPVAGTIPDCINGVYLRNGANPLFQPTAGHHLFDGDGMVHAVSINHG-SASYACRFTQTQRLVQERRLGRPVFPKAIGELHGHSGIARLLLFYARGVFGLVDHKKGTGVANAGLVYFNDRLLAMSEDDLPYHVRITPSGDLQTVGRYDFDKQLHSTMIAHPKVDPISKELYALSYDVVRK-PYLKYFRFSPNGIKSKDVEIPLETPTMMHDFAITENFVVIPDQQVVFKLQEMVKGGSPVIYDKNKKSRFGILPKNATDSKDLIWVESPDTFCFHLWNAWEEPETEEVVVIGSCMTPPDSIFNECEEN-LKSVLSEIRLNLRTGKSTRRAIIKEEKEQVNLEAGMVNKNRVGRKSRYAYLAIAEPWPKVSGFAKVDLVTGEVKKHIYGGRKFGGEPFFLPKEENSKEEDEGYILAFVHDERTWKSEVQIVNAKDLKVEATIKLPSRVPYGFHGTFVQ] was present in all *BvCCO* proteins.

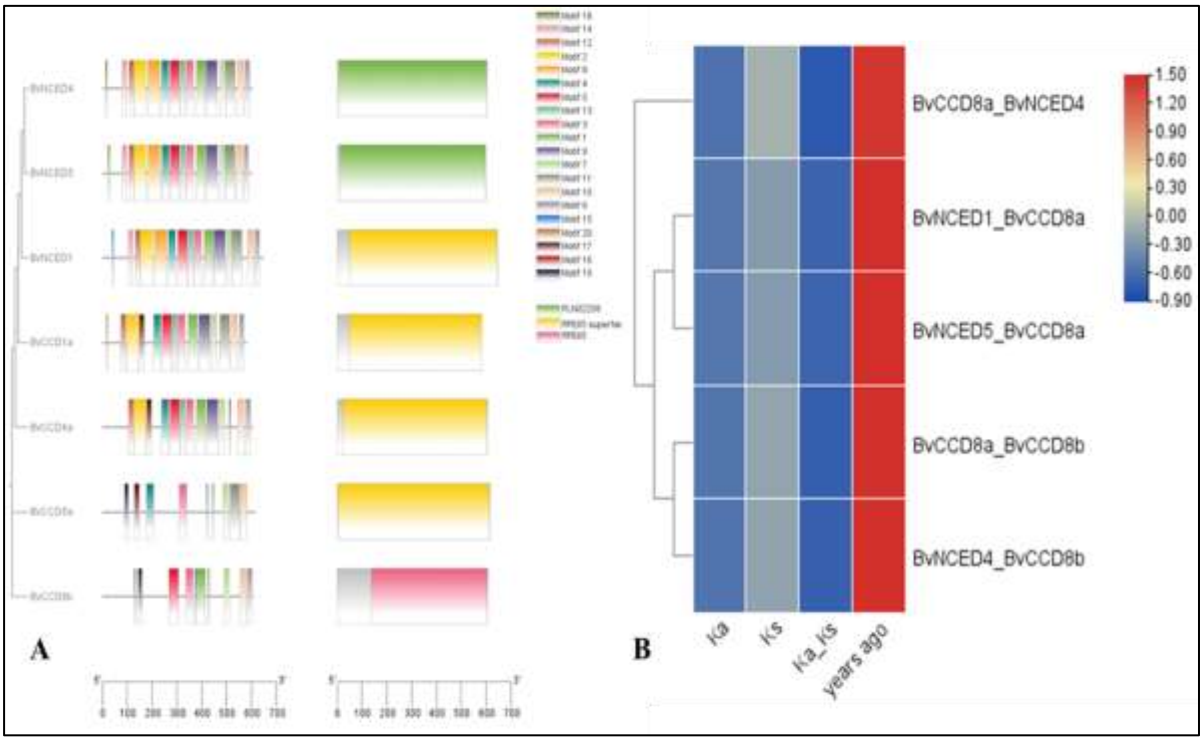

Figure 3: The Graphical representation of 16 motifs distributed along seven BvCCO genes with conserved domain present in all. The motifs were found in beetroot by using MEME and inter-linking it with a phylogenetic tree to gain a good understanding of their association (A). The bars depict motifs, where various color codes correspond to distinct motif categories. Heat map illustrating the time of duplication of five paralogous pairs of BvCCO genes by measuring the ka (non-synonymous substitution) over ks (synonymous susubstitution) mutations ratio ka/ks (B).

3.4.Gene duplication and gene mapping of CCO gene

TBtools was used to determine the gene duplication of CCO gene family. The Ka/Ks ratio showed variation, with the highest value of 0.420091663 observed in BvNCED5_BvCCD8a and the lowest value of 0.195156213 observed in BvCCD8a_BvNCED4. It was supposed that these paralogous pairs: BvNCED1_BvCCD8a have been duplicated around 68587326.2 million years ago (Mya), BvCCD8a_BvNCED4 about 147722046.3 Mya, BvCCD8a_BvNCED8b about 91440672.77 Mya, BvNCED4_BvCCD8b about 84142545.57 Mya, and BvNCED5_BvCCD8a about 65759102.4 Mya. All five paralogous pairs exhibited Ka/Ks ratios greater than 0.05 and less than 1, indicating a notable divergence imposed by the purifying selection procedure (Figure 3 B).

The chromosomal localization analysis showed that BvCCO genes were intended to be spread across many chromosomes based on their chromosomal placement. BvNCED1, BvCCD4a, BvCCD8a, BvNCED4, BvNCED5, BvCCD1a, and BvCCD8b were present on chromosomes 9, 5, 1, 8, 4, 2 and 2 respectively (Figure 4 A). Genes located on same chromosome as in the case of chr no 2, two genes were present which suggests the chance of tendom duplication while the genes on different chromosome as in all other five genes suggest segmented duplication which was dominant here in beetroot.

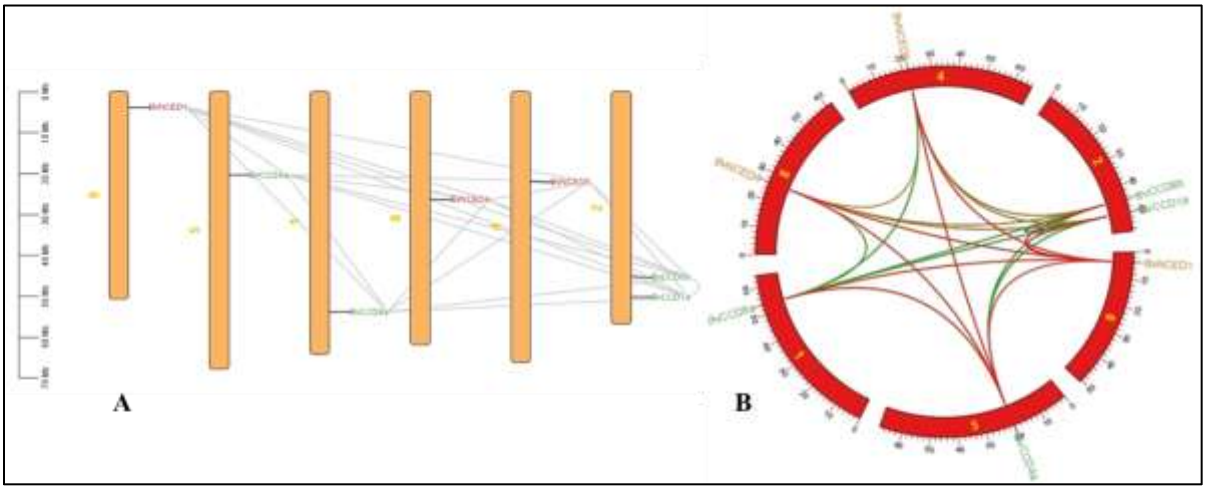

Figure 6: Bars represent chromosomes with their numbers on left side. The presence of gene on specific chromosome no is shown by joining lines with the gene name on the right side of bar. Analysis revealed that each chromosome contains one gene except chromosome no 2 which contains two genes (A). Genome wide synteny analysis of BvCCO indicating the duplicate counter parts having conserved genomic sequences with their paralogues. The level of similarity is depicted by red, green and brown lines (B).

Further syntenic analysis showed that all the BvCCO genes have paralogous counterparts which means they have conserved genomic regions which can lead to duplication and demonstrate structural similarities. None of the BvCCO gene was singleton. Dual syntenic research revealed that 8 genes are duplicated between arabidopsis and beetroot while, 5 duplicated genes were found between spinach and beetroot as displayed in figure 4B.

*3.5.*Interaction analysis of BvCCO proteins and Geo ontology

String database STRING: functional protein association networks (string-db.org) was used for the prediction of protein-protein interacting network. BvCCD8a protein was found to interact with multiple other proteins such as BvNCED1, BvCCD4a, BvNCED4, BvNCED5, BvCCD1a and BvCCD8b (Figure 5A). The biological, cellular and molecular functions of all BvCCO proteins were provided by geo ontology. GO functional annotation and enrichment analysis showed that all genes were highly enriched in biological activity such as carotene catabolic process.

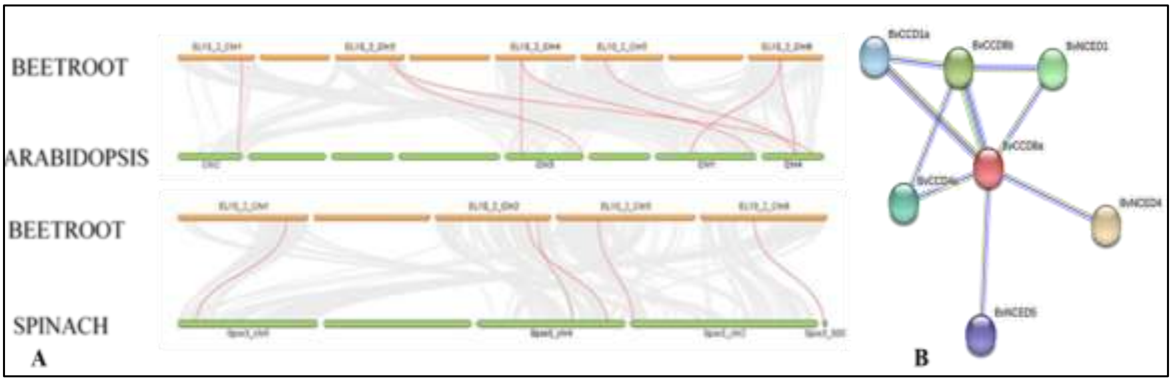

Figure 5: Dual syntenic analysis of beetroot with arabidopsis and beetroot with spinach reveals the dominance of segmental duplication over tendom duplication. Orange segments represent beetroot chromosomes and green segments represent arabidopsis and spinach chromosomes. Red lines depicting the duplication and similarity between them (A). Graph showing interaction network between BvCCO proteins. Analysis revealed that BvCCD8a interacts with all other proteins. BvCCD1a, BvCCD8b, BvNCED1 and BvCCD4a are also bound to one another (B).

*36.*MicroRNA target site analysis

A total of 151 miRNAs were discovered, all of which target seven specific genes: BvNCED1, BvCCD4a, BvCCD8a, BvNCED4, BvNCED5, BvCCD1a, and BvCCD8b. Among these genes, BvNCED1 was targeted by 15 mature miRNAs, BvCCD4a by 11 mature miRNAs, BvCCD8a by 12 mature miRNAs, BvNCED4 and BvNCED5 by 8 and 13 mature miRNAs respectively, while BvCCD1a and BvCCD8b were targeted by 13 and 79 mature miRNAs respectively. Consequently, BvCCD8b was the gene targeted by the most miRNAs, while BvNCED4 had the lowest number of miRNAs targeting it (Figure 5B).

3.7.Cis element analysis

Cis regulatory elements analysis was done to evaluate the promoter region's elements which serve as the binding sites for transcription factor in order to start transcription. The recruitment of transcription factors (TFs) to cis-regulatory regions allows them to influence the transcriptional regulation of gene expression. It is essential to comprehend the roles of cis elements for the purpose of clarifying the mechanisms of cell's response to their environment and participation in organism development. Total of 19 cis regulatory elements were discovered in beetroot named as AAGAA-motif, AT~TATA-box,

Box 4, CAAT-box, DRE, DRE core, ERE, F-box, GA-motif, MYB, MYB-like sequence, MYC, STRE, TATA, TATA-box, TATC-box, TC-rich repeats, W box and WRE3. All these cis regulatory elements were present in one out of seven BvCCO genes (Figure 6A). BvNCED1 was the one to have all these. F-box and AAGAA-motif are developmental cis elements in which AAGAA-motif promoter has function related to pathogenesis. W-box and WRE 3 is a biotic stress-responsive cis regulatory element which interact with WRKY transcription factors thereby playing a role in defense against biotic stresses and acting as a responsive element to wounds. TATA-box, CAAT-box, AT~TATA box and TATA are promoter related cis regulatory elements. AT–TATA box are promoter binding sites. TATA-box and CAAT-box play a role in initiating transcription by acting as binding sites for transcription factors. TATA element aids in the binding of TATA-box with the initiation site. GA-motif and Box4 are light responsive cis regulatory elements. BOX4 covered a spacious region responsible for sustaining the rate at which transcription of light-controlled genes occur. Cis regulatory elements like DRE core, DRE1, STRE, TC rich repeats, MYB, MYC and MYB like site were identified in beetroot with their function of abiotic stress responsiveness. Hormone-related cis regulatory element includes ERE, TATC-box. ERE is also referred to as an ethylene-responsive element and an abscisic acid-responsive element.   TATC-box functions as gibberellin-responsive elements [29].

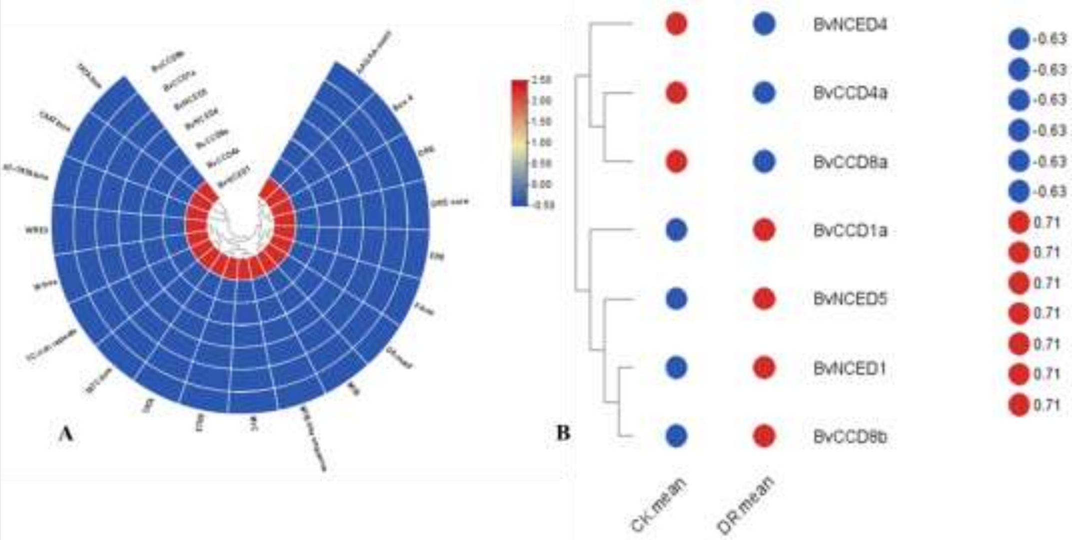

Figure 6. Graph showing all the cis regulatory elements on promoter site and their presence in BvCCO genes. Red color indicates the highest functional intensity as compared to white which shows moderate intensity while blue indicates low or zero intensity (A). Graphical representation of BvCCO genes expression in drought stress. The scale of expression is given on right side. Red dots are high expression indicators while blue dots indicate minimum expression (B).

3.8.Gene expression analysis

In this study, RNAs extracted from sugar beet leaves subjected to water control treatment was examined, aiming to identify their potential role in regulating drought responses. This gene expression analysis of all seven BvCCO genes showed that three genes BvCCD4a, BvNCED5 and BvCCD8b are significantly expressed during drought condition (Figure 6B). Out of these, only BvCCD4a was down regulated in their expression while the other BvNCED5 and BvCCD8b shown up regulation during stress. The number of folds these genes were up regulated and were 5 and 2 times their actual values respectively.

4. DISCUSSION

Beetroot is an important crop that makes upto 30 percent of total sugar output in Pakistan. It aids in the prevention of hypertension and helps manage conditions associated with poor nitrate availability and oxidative stressors [26]. Its use as a coloring agent is high on demand as it contains belatanin pigments. Carotenoids are isoprenoid compounds causing red, yellow or orange colors to the plants [3].To enhance the pigmentation of belatanin in beetroot, carotenoids coding genes CCO (carotenoid cleavage oxygenase) conferred to play a crucial role. BvCCO is responsible for enhancing beta carotene catabolic processes.

BvCCO genes were further categorized into two subgroups CCD (carotenoid cleavage dioxygenase) and NCED (9-cis epoxy carotenoid dioxygenase). CCD was responsible for pigment production as well as biosynthesis of phytohormones e.g, strigolactones thus enhancing root development. It helps in plant development and responsiveness against stress. NCED was a rate limiting enzymes for ABA production as it is the protein that encode to produce ABA and also responsible for hormonal crosstalk. [30] [31]

Absicisic acid is a stress hormone as it plays a significant role in plants resistance against abiotic stresses and help in growth as well as development with a good yield [18]. Abscisic acid (ABA) is synthesized from carotenoids in plastid and cytoplasm. [32]. ABA can be synthesized both directly and indirectly in plants. The Indirect ABA biosynthesis is done by C40 pathway. [33]. In this pathway, zeaxanthin (carotenoid) is catalyzed by ZEP (zeaxanthin epoxidase) enzyme and converted into violaxanthin which then converts into neoxanthin. 9-cis-epoxycarotenoids are formed by the rearrangement of trans-violaxanthin. Neoxanthin and violaxanthin are cleaved to produce xanthoxin by catalytic action of NCED enzyme (9-cis-epoxycarotenoid dioxygenase) [34]. Conversion of xanthoxin into abscisic acid (ABA) is done by two enzymes, ABA2 and AAO3 (aba-aldehyde oxidase) [35]. Finally, ABA is converted into phaseic acid (PA) through the enzyme ABA-8′-hydroxylase. [36]

Strigolactones are phytohormones made by the roots of plants. It was first identified in cotton plant. [19]. Strigolactones regulate plant growth and development and response to various biotic and abiotic stress responses as it is a signalling hormone.[23]. SLs have three main functions: first as a germination stimulant for parasitic plant Striga commonly witchweed. [20]. Secondly, SLs promote the symbiotic association of host plants root with fungi specifically arbuscular mycorrhizae [21]. Thirdly, SL serves as an inhibition hormone for secondary shoots of the plant. [22]. Strigolactones are derived from carotenoids which are basically Isoprenoids. SLs are produced by carlactone which have alike biological activities. Biosynthesis of carlactone takes place in chloroplast [37] . First step was the isomerization of 9trans β-carotene (carotenoid) by DWARF27 isomerase and an iron cofactor into 9cis β-carotene. CCD7 (carotenoid cleavage dioxygenase 7) catalyze the cleavage and produce 9 cis configured aldehyde and 9 β-ionine. An enzyme called CCD8 then convert it into 9-cis β-apo-10'-carotenal which subsequently goes through further rearrangements by adding three oxygens to form carlactone which is a precursor of SL. [37]. MAX1 protein convert carlactone into 5- deoxystrigol by adding 2 oxygen atoms. 5-deoxystrigol is the simplest strigolactone. [38]

In the present study, a total of seven BvCCO genes were identified in beetroot. Out of these, 3 genes are NCED (BvNCED1, BvNCED4 and BvNCED5) and 4 are CCD (BvCCD4a, BvCCD8a, BvCCD8b and BvCCD1a). Their localization analysis showed their respective positions. It was observed that most genes were found in chloroplast. Some were in cytoplasm, mitochondria and nucleus. A very few were present in plasmid, E.R, vacuoles and extra cellular structures etc. An overview of genetic structure revealed that BvCCO genes show functional variability as they differ in intron and exon numbers. Putative miRNA study showed miRNAs which targeted BvCCO genes. It was found that 79 miRNAs targeted BvCCD8b, 13 miRNAs targeted BvCCD1a and BvNCED5 each. BvCCD4a, BvCCD8a and BvNCED1 were targeted by 11, 12 and 15 miRNAs respectively. Least miRNA targeted BvNCED4. Promoter analysis found many developments and hormone related cis regulatory elements were present as well as biotic and abiotic stress particularly light specific cis regulatory elements were observed on promoter site. It means BvCCO genes have a great approach in mitigating stresses and involves in growth and development of plant. Protein interaction study showed that BvCCD8a interacts with all other BvCCD and BvNCED genes. BvCCO are enriched with carotene catabolic process and mostly active in chloroplast.Based on their transcriptomic analysis, gene expression of BvCCO genes were observed under stress conditions. It was found that three genes BvCCD4a, BvNCED5 and BvCCD8a showed significant expression when subjected to drought stress. Out of these BvNCED5 up-regulated 5 folds from its actual expression values and BvCCD8a showed 2 folds increase in expression. Their arabidopsis orthologues (AtNCED5 and AtCCD8) have functions related to beta carotene catalytic activity suggesting their similar roles under drought stress. BvCCD8a participates in the synthesis of phytohormones, specifically strigolactones, which play a pivotal role in the development and growth of plants by facilitating enhanced branching.

5. CONCLUSION

In the present study, seven BvCCO genes in beetroot were identified and described. Based on the thorough analysis of amino acid sequences, these BvCCO genes were categorized into 3 BvCCD genes and 4 BvNCED genes. These genes underwent comprehensive investigation including their structure analysis, cis elemental analysis, chromosomal and subcellular localizations, protein interactions, transcriptomic or expression profiling and evolutionary analysis. Positional study confirmed the presence of most BvCCO genes in chloroplast. On the basis of structure, they contain 1 to 14 exons with introns up to 13. Duplication was observed in all BvCCO genes, and its analysis showed the dominance of segmented duplication. Beetroot found 8 and 5 duplicates with arabidopsis and spinach respectively. Time of duplication of specific paralogous pairs serves to diversify the future evolutionary research of beetroot. Based on promoter analysis, BvCCO genes have cis elements associated with development, hormonal responses, as well as responses to biotic and abiotic stresses. This indicated their potential involvement in growth regulation and their ability to confer tolerance against adverse conditions. Based on gene expression profiles, two genes BvNCED5 and BvCCD8a showed their involvement in drought stress tolerance by up regulating their expression and laid the groundwork for further elucidating their functions in stress regulation, facilitating beetroot's growth under challenging conditions. BvCCO genes in beetroot are fully understood through an in-silico genome wide analysis, but additional studies are needed to confirm these gene’s functional roles in relation to various physiological and biological processes.

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7. REFERENCES

1. Kloer, D.Schulz, G., Structural and biological aspects of carotenoid cleavage. Cellular and Molecular Life Sciences CMLS, 2006. 63: p. 2291-2303.
2. Zia-Ul-Haq, M., Historical and introductory aspects of carotenoids. Carotenoids: structure and function in the human body, 2021: p. 1-42.
3. Langi, P.; Kiokias, S.; Varzakas, T.Proestos, C., Carotenoids: From plants to food and feed industries. Microbial carotenoids: Methods and protocols, 2018: p. 57-71.
4. Zhou, Q.; Li, Q.; Li, P.; Zhang, S.; Liu, C.; Jin, J..;Yang, Y., Carotenoid cleavage dioxygenases: identification, expression, and evolutionary analysis of this gene family in tobacco. International Journal of Molecular Sciences, 2019. 20(22): p. 5796.
5. González-Verdejo, C. I.; Obrero, Á.; Román, B.Gómez, P., Expression profile of carotenoid cleavage dioxygenase genes in summer squash (Cucurbita pepo L.). Plant foods for human nutrition, 2015. 70(2): p. 200-206.
6. Moreno, J. C.; Mi, J.; Alagoz, Y.Al-Babili, S., Plant apocarotenoids: from retrograde signaling to interspecific communication. The Plant Journal, 2021. 105(2): p. 351-375.
7. Su, W.; Zhang, C.; Feng, J.; Feng, A.; You, C.; Ren, Y..;Xu, L., Genome-wide identification, characterization and expression analysis of the carotenoid cleavage oxygenase (CCO) gene family in Saccharum. Plant Physiology and Biochemistry, 2021. 162: p. 196-210.
8. Kim, Y.; Hwang, I.; Jung, H.-J.; Park, J.-I.; Kang, J.-G.Nou, I.-S., Genome-wide classification and abiotic stress-responsive expression profiling of carotenoid oxygenase genes in Brassica rapa and Brassica oleracea. Journal of plant growth regulation, 2016. 35: p. 202-214.
9. Iuchi, S.; Kobayashi, M.; Taji, T.; Naramoto, M.; Seki, M.; Kato, T..;Shinozaki, K., Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. The Plant Journal, 2001. 27(4): p. 325-333.
10. Wei, Y.; Wan, H.; Wu, Z.; Wang, R.; Ruan, M.; Ye, Q..;Yang, Y., A comprehensive analysis of carotenoid cleavage dioxygenases genes in Solanum lycopersicum. Plant molecular biology reporter, 2016. 34: p. 512-523.
11. Serra, S., Recent advances in the synthesis of carotenoid-derived flavours and fragrances. Molecules, 2015. 20(7): p. 12817-12840.
12. Gomez-Roldan, V.; Fermas, S.; Brewer, P. B.; Puech-Pagès, V.; Dun, E. A.; Pillot, J.-P..;Portais, J.-C., Strigolactone inhibition of shoot branching. Nature, 2008. 455(7210): p. 189-194.
13. Tan, B. C.; Schwartz, S. H.; Zeevaart, J. A.Mccarty, D. R., Genetic control of abscisic acid biosynthesis in maize. Proceedings of the National Academy of Sciences, 1997. 94(22): p. 12235-12240.
14. Burbidge, A.; Grieve, T. M.; Jackson, A.; Thompson, A.; Mccarty, D. R.Taylor, I. B., Characterization of the ABA-deficient tomato mutant notabilis and its relationship with maize Vp14. The Plant Journal, 1999. 17(4): p. 427-431.
15. Iuchi, S.; Kobayashi, M.; Yamaguchi-Shinozaki, K.Shinozaki, K., A stress-inducible gene for 9-cis-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. Plant Physiology, 2000. 123(2): p. 553-562.
16. Zhu, G.; Xu, W.; Wu, D.Zhang, Q., Effect of ascorbic acid biosynthesis gene NCED6 on glucose-induced delay of seed germination of Arabidopsis thaliana. Plant Physiology Communications, 2009. 46(2): p. 139-142.
17. Frey, A.; Effroy, D.; Lefebvre, V.; Seo, M.; Perreau, F.; Berger, A..;Marion-Poll, A., Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. The Plant Journal, 2012. 70(3): p. 501-512.
18. Seo, M.Koshiba, T., Complex regulation of ABA biosynthesis in plants. Trends in plant science, 2002. 7(1): p. 41-48.
19. Tsuchiya, Y.Mccourt, P., Strigolactones: a new hormone with a past. Current Opinion in Plant Biology, 2009. 12(5): p. 556-561.
20. Xie, X.; Yoneyama, K.Yoneyama, K., The strigolactone story. Annual review of phytopathology, 2010. 48: p. 93-117.
21. Smith, S. M., Q&A: What are strigolactones and why are they important to plants and soil microbes? BMC Biology, 2014. 12(1): p. 19.
22. Brewer, P. B.; Koltai, H.Beveridge, C. A., Diverse roles of strigolactones in plant development. Molecular plant, 2013. 6(1): p. 18-28.
23. López-Ráez, J. A.; Shirasu, K.Foo, E., Strigolactones in plant interactions with beneficial and detrimental organisms: the Yin and Yang. Trends in plant science, 2017. 22(6): p. 527-537.
24. Zhang, Y.-F.; Li, G.-L.; Wang, X.-F.; Sun, Y.-Q.Zhang, S.-Y., Transcriptomic profiling of taproot growth and sucrose accumulation in sugar beet (Beta vulgaris L.) at different developmental stages. PLoS One, 2017. 12(4): p. e0175454.
25. Zielińska-Przyjemska, M.; Olejnik, A.; Dobrowolska-Zachwieja, A.Grajek, W., In vitro effects of beetroot juice and chips on oxidative metabolism and apoptosis in neutrophils from obese individuals. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 2009. 23(1): p. 49-55.

26. Clifford, T.; Howatson, G.; West, D. J.Stevenson, E. J., The potential benefits of red beetroot supplementation in health and disease. Nutrients, 2015. 7(4): p. 2801-2822.
27. Lee, C.-H.; Wettasinghe, M.; Bolling, B. W.; Ji, L.-L.Parkin, K. L., Betalains, phase II enzyme-inducing components from red beetroot (Beta vulgaris L.) extracts. Nutrition and cancer, 2005. 53(1): p. 91-103.
28. Ferro, C. G.; Zerbini, F. M.; Navas-Castillo, J.Fiallo-Olive, E., Revealing the Complexity of Sweepovirus-Deltasatellite-Plant Host Interactions: Expanded Natural and Experimental Helper Virus Range and Effect Dependence on Virus-Host Combination. Microorganisms, 2021. 9(5).
29. Maqsood, H.; Munir, F.; Amir, R.Gul, A., Genome-wide identification, comprehensive characterization of transcription factors, cis-regulatory elements, protein homology, and protein interaction network of DREB gene family in Solanum lycopersicum. Frontiers in Plant Science, 2022. 13: p. 1031679.
30. Ren, H.; Fan, Y.; Gao, Z.; Wei, K.; Li, G.; Liu, J..;Jia, W., Roles of a sustained activation of NCED3 and the synergistic regulation of ABA biosynthesis and catabolism in ABA signal production in Arabidopsis. Chinese Science Bulletin, 2007. 52: p. 484-491.
31. Parwez, R.; Aftab, T.; Gill, S. S.Naeem, M., Abscisic acid signaling and crosstalk with phytohormones in regulation of environmental stress responses. Environmental and Experimental Botany, 2022. 199: p. 104885.
32. Xu, Z.-Y.; Kim, D. H.Hwang, I., ABA homeostasis and signaling involving multiple subcellular compartments and multiple receptors. Plant cell reports, 2013. 32: p. 807-813.
33. Nambara, E.Marion-Poll, A., Abscisic acid biosynthesis and catabolism. Annu. Rev. Plant Biol., 2005. 56: p. 165-185.
34. Schwartz, S. H.; Tan, B. C.; Gage, D. A.; Zeevaart, J. A.Mccarty, D. R., Specific oxidative cleavage of carotenoids by VP14 of maize. Science, 1997. 276(5320): p. 1872-1874.
35. Seo, M.; Aoki, H.; Koiwai, H.; Kamiya, Y.; Nambara, E.Koshiba, T., Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. Plant and cell physiology, 2004. 45(11): p. 1694-1703.
36. Kushiro, T.; Okamoto, M.; Nakabayashi, K.; Yamagishi, K.; Kitamura, S.; Asami, T..;Nambara, E., The Arabidopsis cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. The EMBO journal, 2004. 23(7): p. 1647-1656.
37. Alder, A.; Jamil, M.; Marzorati, M.; Bruno, M.; Vermathen, M.; Bigler, P..;Al-Babili, S., The path from β-carotene to carlactone, a strigolactone-like plant hormone. Science, 2012. 335(6074): p. 1348-1351.
38. Seto, Y.Yamaguchi, S., Strigolactone biosynthesis and perception. Current Opinion in Plant Biology, 2014. 21: p. 1-6.