Chapter 2

Review of Literature

2.1 Bioavailability of Vitamin A

Foods high in plants or animals are the main sources of retinyl esters and PVA, respectively. The quantity of consumed Pvac molecules that are absorbed, transported, stored, and used for physiological or developmental activities is known as the bioavailability of PVA (Rodriguez-Amaya, 2016). On the other hand, according to Kopec and Failla (2018), bio accessibility is the quantity of ingested PVA that is available for absorption. Food condition (raw or cooked), pVAC type, food processing method, food matrix type, food nutrient composition, Among the factors influencing pVAC bioavailability are food component interactions with other dietary components. (Tanumihardjo *et al.*, 2010; Rodriguez-Amaya, 2016; Schweiggert and Carle, 2017; Amah *et al.*, 2018) Further, the bioavailability could be affected by host body factors such as stage, nutrient condition, genotype, physiological condition (e.g. pregnancy, breastfeeding, and obesity), disease status (acute or chronic), and body secretions (HCl, gastric acid and bile juices) etc. Because pVACs are soluble in fat, fat promotes the excretion of bile salts and increases the production, solubilization, and bioavailability of carotenoid micelles (Bengtsson *et al.*, 2009). Additionally, only the type of pVAC, dietary fibers that include pVACs, pVAC-protein complexes, cell walls, and the amount of fat in the diet have an impact on the bio accessibility of pVACs. pVAC absorption is comparable to that of other fat-soluble substances. First, every single particulate matter (pVAC) is freed from the food matrix, and then it dissolves into oil droplets. These oil droplets are subsequently added to bile salt-containing micelles, which are created during the breakdown of fatty acids. The mucin layer subsequently allows these micelles to enter the gastrointestinal tract. While the fat-soluble pVACs are transported to the interior of the cell by interacting with brush border proteins, the bile salts dissociate and are reabsorbed in the ileum. Scavenger receptor class-B type-I (SR-B1), Niccol Pick C1Like1 (NPC1L1), and cluster determinant-36 (CD-36) all speed up the transport process (Harrison, 2012; Reboul, 2013; Bajka *et al.*, 2015; Reboul, 2019). The pages are converted to vitamin A once they are within the cells.

β-carotene is centrally cleaved by the enzyme β-carotene 15,15′-monooxygenase (CMO1) to yield two molecules of retinal. On the other hand, the β-carotene 9,10-dioxygenase (CDO) enzyme cleaves the pVACs eccentrically, resulting in the formation of apocarotenoids and a single retinal molecule (Raghuvanshi *et al.,* 2015). It has been reported that the absorption of β-carotene from green leafy vegetables is significantly higher (50 to 99%). Biofortified foods have been shown to have increased pVAC bio accessibility and bioavailability (La Frano *et al.,* 2014; Giuliano, 2017; Kopec and Failla, 2018). After processing, there is a 25–45% bio accessibility in cassava roots (Thakkar *et al.,* 2009). However, reduced bio accessibility (0.6-3%) was noted in potatoes (Failla *et al.,* 2009). In addition, 10–32% β-carotene bio accessibility was seen in bananas following boiling and *in vitro* digestion (Ekesa *et al.,* 2012). However, in animal experiments employing Mongolian gerbils, bioconversion of 28:1 was noted with processed banana flour (Arscott *et al.,* 2010). Furthermore, Bresnahan *et al.* (2012) found that cooking bananas increased their retinol bio-efficacy and delayed Mongolian gerbils' retinol depletion. Therefore, using the right cooking or processing techniques can increase the bioavailability of pVACs (Figure 2.1).

2.2 Vitamin A Recommended Dietary Allowance (RDA)

The term "recommended dietary allowance" (RDA) refers to the estimated daily intake of nutrients needed to meet the nutritional needs of 97-98% of healthy persons in a certain age or gender of the population. Retinol activity equivalents, or RAEs, are a measure of vitamin A's bioavailability from a particular diet that determines the recommended daily allowance (RDA) level. varying physiological groups, including newborns, preschoolers, kids, adults, pregnant women, nursing moms, and children, are recommended to receive varying amounts of RDA. Though variations in daily consumption could happen because of body demand or food accessibility, the average needs should be met eventually. Given the theoretical yield of two molecules of retinol resulting from the enzymatic cleavage of β-carotene, animal tests have only revealed a 50% conversion rate due to physiological inefficiencies. Green leafy vegetables, such as spinach, and animal sources, such as egg yolks and liver, can supply up to 30% and 70% of the daily requirement of vitamin A, respectively. The β- carotene to retinol conversion efficiency factor is 1:8, according to the ICMR 2010 report

(http://icmr.nic.in/fi nal/rda- 2010. pdf). Below is the vitamin A RDA table from the ICMR (2010) study (Table 2.1).

2.3 Carotenoids: Major Source of Vitamin A

The most prevalent pigment group in nature, carotenoids are secondary metabolites found in plants. All photosynthetic tissues include yellow to red lipid-soluble pigments that are incorporated in the membranes of chloroplasts and chromoplasts (Ruiz-Sola & Rodríguez-Concepcion, 2012). According to Johnson *et al.* (2018), these are divided into two classes: carotenes and xanthophylls. These give many flowers, fruits, and certain roots (carrots) their vibrant colors. Because these pigments are antioxidants, they protect photosynthetic organisms from the damaging effects of photooxidation. Certain carotenoid molecules are also found in the complexes that make up the photosynthetic antenna and reaction center. Moreover, these compounds function as a precursor to phytohormones including strigolactones and abscisic acid (ABA). (Nambara and Marion-Poll, 2005; Al-Babili and Bouwmeester, 2015). PVA-active carotenoids are an essential component of the human diet. The majority of the carotenoid that is consumed comes from agricultural and fruit plants, where it may be found in the roots, flowers, fruits, leaves, seeds, and shoots (Stahl and Sies, 2003).

2.4 Biosynthesis of Carotenoids

In higher plants, carotenoids are synthesized and localized in the plastids. Isopentenyl Di phosphate (IPP) and dimethyl-allyl-diphosphate (DMAPP) are the main precursors for carotenoid biosynthesis. The mevalonate (MVA; cytosolic) and 2-C-methyl-D-erythritol 4-phosphate (MEP; plastidial) pathways, independently synthesize IPP and DMAPP. However, the plastidial MEP pathway is the major pathway for carotenoid biosynthesis (Figure 2.2).

2.5 The MEP Pathway

In plastids, IPP is synthesized in the MEP pathway from the condensation of pyruvate and glyceraldehyde 3-phosphate (GA3P). The 1-deoxyglucose 5-phosphate (DOXP) synthase (DXS) is the first enzyme of this pathway, which condenses pyruvate and GA3P to form DOXP (Lange *et al.,* 1998; Lois *et al.,* 1998). Further, DOXP

reductor isomerase (DXR) converts DOXP to methyl erythritol 4-phosphate (MEP) (Figure 2.2). Afterward, MEP is converted into 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP) by the series of enzymes, i.e. MEP-cytidylyl transferase (MCT), CDPME kinase (CMK) and ME-cPP synthase (MDS) sequentially. ME-cPP is further converted to (E)-4-hydroxy-3-methyl- but-2-enyl pyrophosphate (HMB-PP) and then to IPP by HMB-PP synthase (HDS) and HMB-PP reductase (HDR), respectively. Furthermore, IPP is isomerized to DMAPP by IPP isomerase (IDI) (Lange *et al.,* 1998; Rodriguez-Concepcion and Boronat, 2002).

2.5.1 Carotenoid Biosynthesis Pathway

IPP and DMAPP molecules from the MEP pathway are the primary substrates for carotenoid biosynthesis. Geranylgeranyl diphosphate (GGPP) synthase catalyzes the sequential addition of three IPP molecules to one DMAPP molecule resulting in GGPP formation (Lichtenthaler, 1999; Bouvier *et al.,* 2005; Rodriguez-Concepcion, 2010) (Figure 2.2). Further, PSY acts upon GGPP and forms phytoene, the first committed and rate-limiting step in the carotenoid biosynthesis pathway (Bouvier *et al.,* 2005; Sandmann *et al.,* 2006; Cazzonelli and Pogson, 2010). Subsequently, desaturation and isomerization of phytoene by two desaturases [phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS)] and two isomerases [ζ- carotene isomerase (Z-ISO) and carotenoid isomerase (CRTISO)] make the backbone of plant carotenoids, i.e. lycopene (Figure: -2.2). The rate of ζ-carotene formation is determined by phytoene desaturase (PDS) (Qin *et al.,* 2007). PDS activity entails plastid-targeted alternative oxidase (PTOX) and links carotenoid biosynthesis with the chloroplast electron transport chain (Yu *et al.,* 2007). CRTISO plays an important role in balancing *cis/trans* carotenoids (Yu *et al.,* 2011). *CRTISO* mutants showed an accumulation of *cis*-carotenoids in non-photosynthetic tissues (Park *et al.,* 2002). However, in the bacterial system, the above multi-step desaturation reaction is catalyzed by a single crtI enzyme (Mialoundama *et al.,* 2010). Carotenoid biosynthesis bifurcates after lycopene. At this point, cyclization of the ends of the lycopene by the enzymatic activity of lycopene-β-cyclase (LCYβ) and lycopene-ε-cyclase (LCYε) results into carotenes, i.e. α-carotene and β-carotene (Figure 2.2). LCYε regulates carotenoid flux in the β/ε branch and modulates the ratio of β- carotenoids to lutein (the most abundant carotenoid). In maize, LCYε is reported to be responsible for the deviation in lutein content (Harjes *et al.,*

2008; Howitt *et al.,* 2009). However, carotene β-ring hydroxylation by distinct classes of carotene hydroxylases eliminates the PVA potential of α-carotene and β-carotene. The hydroxylation of carotenes yields non-PVA pigments such as xanthophylls (lutein, zeaxanthin) (Bouvier *et al.,* 2005; Cunningham and Gantt, 1998; North *et al.,* 2007). Violaxanthin is formed from zeaxanthin through epoxidation. This reaction is reversible, and de-epoxidation can convert violaxanthin back to zeaxanthin. Moreover, these carotenoids and xanthophylls can be converted to a wide range of carotenoids and apo-carotenoid molecules present in plants and certain organisms (Walter and Strack, 2011).

2.5.2 Turnover and Degradation of Carotenoids

The carotenoid content at the sink level depends upon the storage ability of the plastids as well as on the balance between biosynthesis and their degradation rate. Carotenoid degradation can happen either due to enzymatic oxidation or photo-oxidation. Peroxidases and lipoxygenase oxidize the carotenoids unpacifically, while the oxygenase cleaves in a specific manner (Carail and Caris-Veyrat, 2006; Walter and Strack, 2011). In plants, carotenoid cleavage oxygenase (CCOs) are classified into two classes, i.e. carotenoid cleavage dioxygenases (CCDs) and 9-*cis*- epoxy carotenoid dioxygenases (NCEDs). They carry out the oxidative cleavage in a specific manner and produce apocarotenoids (Giuliano *et al.,* 2003; Bouvier *et al.,* 2005; Auldridge *et al.,* 2006).

Apocarotenoids for example ABA, strigolactones, β-ionone and carlactone serve important regulatory/environment interaction functions or have agronomic importance in plants (Giuliano *et al.,* 2003; Nambara and Marion-Poll, 2005; Bouvier *et al.,* 2005; Van Norman and Sieburth, 2007; Xie *et al.,* 2010; Walter and Strack, 2011). Carotenoid turnover in photosynthetic tissue is affected by photo-oxidative degradation while in non-photosynthetic tissues, the CCOs play an important role. In various plants, CCO enzymes are further nominated based on their similarity to the Arabidopsis CCD family. In Arabidopsis seeds, Auldridge *et al.* (2006) reported enhanced and reduced content of carotenoids concerning *CCD1* mutant and overexpression line. However, in the leaf, the carotenoid content has not changed in either case. In other reports, CCD1/CCD4 are negatively associated with carotenoid content in strawberry fruit (Garcia-Limones *et al.,*2008), These CCDs and NCEDs were

noted to be substrate-specific and cleave at different sites CCDs and NCEDs were noted to be substrate-specific and cleave at different sites. CCD1 and CCD4 were observed to have wide substrate specificity, from phytoene to neoxanthin, and produce α-ionone, β-ionone, β-cycloidal, β-citraurin, bixin, crocin, and saffron (Ibdah *et al.,* 2006; Rubio *et al.,* 2008; Baldermann *et al.,* 2012; Ma *et al.,* 2013; Frusciante *et al.,* 2014; Zhang *et al.,* 2015). On the other hand, CCD7 and CCD8 lead to strigolactone synthesis from β-carotene via carlactones (Ruyter-Spira *et al.,* 2013). In contrast, NCDs specifically act upon violaxanthin and neoxanthin and form xanthoxin, which is further converted to ABA (Schwartz *et al.,* 1997). Frusciante *et al.* (2014) identified CCD2 from *Crocus sativus,* which is similar to CCD1 and acts on zeaxanthin. Even though numerous *CCDs* have been recognized and classified in vegetables, fruits, and flowers, however, their specific roles in carotenoid turnover remain to be elucidated.

2.5.3 Regulation of Carotenoid Flux: Role of Rate-Limiting Enzymes

In plants, carotenoids biosynthesis is dynamically regulated by distinct regulatory mechanisms in green tissues and fruits/flowers. Carotenoid biosynthesis is regulated depending upon the developmental requirement or in response to exterior environmental stimuli. Generally, carotenoids biosynthesis is regulated at both transcriptional as well as post-transcriptional levels. Transcriptional level regulation is affected by developmental signals, light response, ABA-mediated feedback, epigenetic regulation, and the circadian clock (Figure 2.3). At the post-transcriptional level, enzyme modulation, the channeling of metabolites, sequestration, storage, and turnover are the major factors.

Developmental cues: environmental and developmental signals together affect the carotenoids biosynthesis. During fruits ripening, the chloroplasts transform into chromoplast leading to chlorophyll degradation and accumulation of the carotenoids (Gillaspy, 1993; Giovannoni, 2001). This causes accumulation of *DXS* transcript followed by *PSY*, resulting in carotenoids accumulation. Differential expression of carotenogenic genes plays a key role in defining the content and type of definite carotenoids. As per the earlier reports, DXS, PSY, LCYβ, and LCYε have been evidenced as rate-limiting enzymes in different plant species (Cazzonelli and Pogson, 2010; Zhai *et al.,* 2016).

Substrates availability affects carotenogenesis: Plastidial MEP pathway provides a substrate for carotenoid biosynthesis. The initial steps of the MEP pathway are regulated by *DXS* and *DXR* and evidenced for limiting substrate availability. Expression analysis of *DXS* in tomato fruit showed a correlation between increased *PSY* mRNA transcripts and carotenoid accumulation (Lois *et al.,* 2000). In Arabidopsis seedlings, *DXS* and *DXR* overexpression resulted in >12% enhanced carotenoids while antisense silencing of *DXS*leads to a 13% reduction in the total carotenoid content (Estevez *et al.,* 2001; Carretero-Paulet *et al.,* 2006). Post-transcriptional regulation of *DXS* has also been demonstrated to regulate the accumulation of carotenoids (Cazzonelli and Pogson, 2010).

Phytoene biosynthesis is a bottleneck in carotenogenesis: Phytoene biosynthesis by PSY has been documented as an important regulatory step in carotenogenesis. Single or multiple homologs of *PSY* are present in different plant species (Li *et al.,* 2008; Welsch *et al.,* 2008; Chaudhary *et al.,* 2010; Howitt *et al.,* 2009). The activity of the multiple PSY enzymes appears redundant. However, they are reported to be expressed in a tissue-specific manner (Li *et al.,* 2008; Welsch *et al.,* 2008, Walter *et al.,* 2015). *PSY* expression is tightly coordinated and regulated via source and sink metabolites (Figure 2.3). Overexpression of *PSY* in Arabidopsis augmented carotenoid levels showed a simultaneous accumulation of high *DXS* transcripts in the seedlings, which revealed a feedback mechanism in the pathway commenced by *PSY* (Rodriguez *et al.,* 2009). However, in dark-grown seedlings, overexpression of *DXS* did not improve the carotenoid content (Rodriguez *et al.,* 2009). Besides, the maize *PSY* driven by a rice glutelin promoter significantly improved carotenoid (β-carotene) biosynthesis in the endosperm of transgenic rice, resulting in Golden Rice II with carotenoid content up to 37 µg/g (Paine *et al.,* 2005). In wheat and corn, overexpressing maize *PSY* homolog resulted in improvedβ-carotene content up to ~5µg/g dry weight (DW) and 169-fold, respectively (Cong *et al.,* 2009). Furthermore, Paul *et al.* (2017) affirmed enhanced β-CE up to 55 µg/g DW in Cavendish banana by overexpressing *PSY* (*MtPSY2a*) derived

β-Carotene content is affected by the activity of *LCYε* and *LCYβ*: Bifurcation after lycopene to ε- and β-carotenoids is a major regulatory point in modulating the proportion of lutein to the β-carotenoids (Cuttriss *et al.,* 2007; Cazzonelli *et al.,* 2010) (Figure 2.3). Flux from both branches can be regulated at the LCYε transcript level. In

maize, four natural *LCYε* polymorphisms explicated 58% of the disparity in lutein and β-carotenoids. It was reported that suppression of the *LCYε* gene in Arabidopsis altered the proportion of lutein to β-carotene (Harjes *et al.,* 2008).

Light-triggered signaling: The light-triggered seedling de-etiolation is concomitant with the eruption of carotenoids production in chloroplasts to safeguard the photosynthesis apparatus and its development. During the de-etiolation process, MEP and carotenoid biosynthesis pathway genes get activated (Ghassemian *et al.,* 2006; Meier *et al.,* 2011). Further, during de-etiolation, carotenoids production is also conjoined by chlorophyll production and photosynthesis-related proteins (Welsch *et al.,* 2003; Wille *et al.,* 2004; Welsch *et al.,* 2007; Toledo-Ortiz *et al.,* 2010; Meier *et al.,* 2011). This coordination is due to the presence of a light-responsive element (ATCTA) in the promoter region (Welsch *et al.,* 2003; Botella-Pavía *et al.,* 2004). Even more, basic helix loop helix (bHLH) transcription factors related phytochrome-interacting factors (PIF) family play an important role in light-triggered de-etiolation. (Castillon *et al.,* 2007; Bae and Choi, 2008; Leivar and Quail, 2011). As per Shin *et al.* (2009), PIFs accumulate in de-etiolated seedlings and prevent photomorphogenic development in the dark. Upon light exposure, phytochromes get activated and interact with PIFs, instigating their phosphorylation, and degradation to continue the photomorphogenic growth (Bae and Choi, 2008; Leivar and Quail, 2011). Cazzonelli and Pogson (2010) reported that *PSY* is a major target of PIFs during de-etiolation for regulating carotenoids biosynthesis while PIFs represses *PSY* expression in completely de-etiolated plantlets (Toledo-Ortiz *et al.,* 2010) (Figure 2.3).

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Epigenetic regulation: Cazzonelli *et al.* (2010) reported the *CRTISO* gene being regulated at the epigenetic level by *CCR1/SDG8* (histone methyltransferase) hence, control its expression development and tissue-specific. However, no other gene was observed to be controlled at the epigenetic level.

CCOs play an important role in carotenoids sink: The type of carotenoids in the sink is determined by the rate of degradation by CCOs, which appears to have diverse substrate preferences (Garcia-Limones *et al.,* 2008; Gayen *et al.,* 2015; Qin *et al.,* 2016). Members of the CCO family are reported to be involved in the formation of phytohormones (ABA and strigolactone), apocarotenoids, flavor and aroma volatiles (Auldridge *et al.,* 2006) (Figure 2.3).

2.6 CRISPR-Mediated Gene Editing

Plant genome editing using CRISPR/Cas9 was first reported in 2013, when the approach was effectively used for both temporary expression and the recovery of stable transgenic lines. The effectiveness of this method was examined in a variety of crop species, including *Nicotiana benthamiana* and *Arabidopsis thaliana*, which are model plants (Li *et al.,* 2014), as well as *Oryza sativa* (Zhang *et al.,* 2014), Sorghum bicolor (Jiang *et al.,* 2013), *Triticum aestivum* (Wang *et al.,* 2018) and *Solanum lycopersicum* (Brooks *et al.*,2014). The number of stable transgenic lines, modifications reported for genes in primary transformants of crops increase CRISPR/Cas9 and is rapidly becoming the tool of choice for gene editing in plants, although further analysis is needed to determine collective efficacy of this method.

According to Jiang *et al*. (2013), *Streptococcus pyogenes'* CRISPR/Cas system is a powerful tool for modifying the genomes of both monocot and dicot plants. They have successfully produced a functioning GFP gene from a real-life GFP mutation in tobacco and Arabidopsis. According to their research, *Streptococcus pyogenes'* CRISPR/Cas system has promise as a genome editing tool for dicot crops.

The viability of using CRISPR/Cas9 genome editing tools to induce targeted changes in the TaMLO-A1 allele, which provides heritable broad-spectrum resistance to powdery mildew, in hexaploidy bread wheat was confirmed by *Wang et al*. (2013). The outcome demonstrated that it is possible to create targeted DNA insertion in bread wheat using the CRISPR/Cas9 system in polyploidy crops as well.

The use of the CRISPR/Cas9 system for targeted mutagenesis in liverwort (*Marchantia polymorpha* L.), a model species for examining the evolution of terrestrial plants, was investigated by Sugano *et al*. (2014). They created gRNA to interfere with the auxin response factor 1 (ARF1) gene. Cas9 expression under the direction of M. polymorpha EF1 or Cauliflower mosaic virus 35S produced the desired mutation. Through the asexual reproduction of T1 plants, they were able to produce auxin-resistant phenotypic mutants.

Liang *et al.* (2014) carried out comparative study to evaluate the efficiency of Transcription Activator Like Effector Nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) for genome editing. They have induced targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system using five TALENs targeting 4 genes, ZmPDS, ZmIPK1A, ZmIPK, ZmMRP4, and obtained targeting efficiencies of up to 23.1% in protoplasts, and found that about 13.3% to 39.1% of the transgenic plants were somatic mutations. In other case they constructed two gRNAs targeting the ZmIPK gene in maize protoplasts, at frequencies of 16.4% and 19.1%, respectively. They finally concluded that, the CRISPR/Cas system induced targeted mutations in Z. mays protoplasts with efficiencies (13.1%) in compare to those obtained with TALENs (9.1%).

The RIN gene, which controls fruit ripening, was the target of Ito *et al*. (2015), who showed how the CRISPR/Cas9 system can be used to efficiently mutate the tomato genome. They targeted three regions of the RIN gene, which resulted in mutations that

either included a single base insertion or the deletion of more than three bases in putatively regenerated plants. The RIN protein-deficient mutants produced incomplete ripening fruits with red color pigmentation that was significantly lower than that of the wild type, while heterologous mutants expressing the remaining wild type gene reached full ripening red color, confirming the crucial role of RIN in ripening. Multiple mutations generated at three independent target sites were inherited in the T1 progeny, confirming the applicability of the CRISPR/Cas9 system in tomatoes.

By focusing on multicopy genes, Lawrenson *et al*. (2015) examined the application and target specificity of RNA-guided Cas9 genome editing in Brassica oleracea and barley (Hordeum vulgare). Two copies of HvPM19 were targeted in barley, and in the first generation of 23% and 10% of the lines, respectively, Cas9-induced mutations were observed. 10% of screened first-generation plants in B. oleracea have Cas9-induced mutations because of targeting BolC.GA4.a. Stable Cas9-induced mutations are passed on to T2 plants in both barley and B. oleracea, regardless of the T-DNA construct.

Using a plant codon-optimized Cas9 gene, Ma *et al*. (2015a) examined the resilience of the CRISPR/Cas9 vector system for easy and highly effective multiplex genome editing in monocot and dicot plants. In Arabidopsis, they achieved consistent biallelic, heterozygous, homozygous, and chimeric mutations, whereas in rice, they used this genome editing assembly to edit 46 target sites with an average rate of 85.4% mutation, primarily in biallelic and homozygous condition. From their study they concluded that, targeted mutations in both rice and *Arabidopsis* were heritable. Targeting of multiple genes resulted in loss-of-function mutations in T0 rice and T1 *Arabidopsis* plants shows that CRISPR/Cas9 is a versatile toolbox for studying functions of multiple genes and gene families in plants for basic research and genetic improvement.

Michno *et al.* (2015) targeted two genes in soybean Glutamine Synthase (GS1) gene and chalcone-flavanone isomerase gene (CHI20). To test these targets, they have synthesized a codon-optimized Cas9 (GmCas9) based on the preferred codon usage of soybean. They have transfected plants with hairy root transformation. Further they validated the codon optimized GmCas9 in Medicago truncatula to target GUS gene. Finally, they concluded that, modified Cas9 enzyme was shown to be successful in

mutation of targeted genes in somatic cells of both the legume species, soybean and *Medicago truncatula*.

Xu *et al.* (2016) studied the CRISPR/Cas9-mediated genome editing of four different genes (OsAOX1a, OsAOX1b and OsAOX1c) in rice, they were oxidases with broad substrate specificity, oxidizing aromatic azaheterocycles. Further they studied the inheritance pattern of biallelic mutation and found higher percentage of putative biallelic mutations in T0 generations. Their results also indicated that the progeny genotypes of biallelic T0 lines are frequently difficult to predict and that the transmission of mutations largely does not conform to classical genetic laws, Which suggests that the mutations in T0 transgenic rice are mainly somatic mutations. further, they have followed the inheritance pattern of T1 plants and found that regardless of the presence of the CRISPR/Cas9 transgene, the mutations in T1 lines were stably transmitted to later generations, indicating a standard germline transmission pattern.

Zhang *et al.* (2015) demonstrated the CRISPR/Cas9 system in petunia (Petunia hybrid), an important model ornamental plant for research. They have targeted Phytoene desaturase (PDS) gene. It was reported that transgenic shoot lines with albino phenotype account for 55.6% to 87.5% of the total regenerated T0 Basta resistant lines. A homozygous deletion close to 1 kb in length generated and identified in the first generation.

Iaffaldano *et al.* (2016) demonstrated the potential of CRISPR/Cas9 in Taraxacum koksaghyz plants which produces high molecular weight rubber in its roots. They have targeted fructan: fructan 1-fructosyltransferase (1-FFT) gene which is involved in inulin biosynthesis, as inulin is an expected antagonist of rubber production. T.koksaghyz plantlets were inoculated with Agrobacterium rhizogenes harboring a plasmid encoding a Cas9 and sgRNA targeting TK 1-FFT. Mutagenesis was affirmed by observing a loss of restriction sites within 1-FFT, followed by sequencing of 11 hairy root among them 10 showed the presence of genome editing with mutation rates as high as 88.9% suggesting a high efficiency mutagenesis induced by CRISPR/Cas9 via A. rhizogene mediated transformation.

Nishitani *et al.* (2016) described induced targeted gene mutation in the endogenous phytoene desaturase (PDS) gene of apple using the CRISPR/Cas9 system.

They designed four guide RNAs (gRNAs) and transformed with Cas9 separately in apple. Complete and partial albino phenotypes were observed in 31.8% of regenerated plantlets for one gRNA and bi-allelic mutations in apple PDS were confirmed by DNA sequencing.

Ren *et al.* (2016) reported targeted genome editing in Chardonnay (Vitis vinifera L.) suspension cells and plants via the CRISPR/Cas9 system. They have designed two single guide RNAs (sgRNAs) to target distinct sites of the L-idonate dehydrogenase gene (IdnDH). CEL I endonuclease assay and sequencing results revealed the expected indel mutations at the target site. Mutation frequency of 100% was observed in the transgenic cell mass (CM) as well as corresponding regenerated plants with expression of sgRNA/Cas9. The majority of the detected mutations in transgenic CM were 1-bp insertions, followed by 1- to 3-nucleotide deletions.

Wang *et al.* (2016a) used CRISPR/Cas9 genome editing to improve disease resistance in rice against blast by targeting the OsERF922 gene. The rice ERF transcription factor OsERF922 negatively regulates resistance to Magnaporthe oryzae and salt tolerance. Twenty-one C-ERF922 induced mutant plants (42.0%) were identified from fifty T0 transgenic plants. Sequencing revealed that putative plants re-generated were found to incorporate various insertion or deletion (InDel) mutations at the target site. They reported that all of the C-ERF922-induced allele mutations were transmitted to subsequent generations. They examined six T2 homozygous mutant lines for a blast resistance phenotype and agronomic traits, the results revealed that the number of blast lesions formed following pathogen infection was significantly decreased in all six mutant lines compared with wild-type plants at both the seedling and tillering stages.

Wang *et al.* (2016b) described the CRISPR/Cas9 mediated efficient editing of representative SNF (symbiotic nitrogen fixation) related genes in the legume model plants Lotus japonicas via Agrobacterium mediated hairy root transformation. They have targeted SYMRK (symbiosis receptor like kinase) loci and achieved ∼35% mutagenic efficiency in twenty T0 transgenic plants. Sequencing results showed that among the edited plants two of them contain biallelic homozygous mutations with two bp deletions adjacent the PAM region.

Chen *et al.* (2017) obtained the targeted mutagenesis in upland cotton (*Gossypium hirsutum* L.) using the CRISPR/Cas9 genome editing system. They have designed two guide RNAs to target distinct sites of the cotton *Cloroplastos alterados* 1 (GhCLA1) and vacuolar H+-pyrophosphatase (GhVP) genes. Mutations in these two genes were detected in cotton protoplasts. Results revealed that, most of the mutations were nucleotide substitutions with one nucleotide insertion and one substitution found in GhCLA1 and one deletion found in GhVP in cotton protoplasts.

Nekrasov *et al.* (2017) generated a non-transgenic tomato resistant to the powdery mildew fungal pathogen using the CRISPR/Cas9 technology. Whole genome sequencing showed that edited plants do not carry any foreign DNA sequences but only carries a deletion that is indistinguishable from naturally occurring mutations and it was conform that CRISPR/Cas9 is a highly precise tool which has very minimal off-target mutations.

Tian *et al.* (2017) reported the genome editing in watermelon by CRISPR/Cas9 system. ClPDS phytoene desaturase in watermelon was selected as the target gene because its mutant bears evident albino phenotype. They obtained insertions or deletions at the expected position, through protoplast transfection. All edited watermelon plants harboured ClPDS mutations showed complete or mosaic albino phenotype indicating that CRISPR/Cas9 system proved to be efficient genome editing tool.

To perform targeted editing of stress-responsive transcription factor genes, wheat Dehydration Responsive Element Binding protein 2 (TaDREB2) and wheat Ethylene Responsive Factor 3 (TaERF3), Kim *et al.* (2018) investigated the CRISPR/Cas9 genome editing method in wheat protoplast. Through the temporary production of Cas9 protein and short guide RNA in wheat protoplasts, targeted genome editing of TaDREB2 and TaERF3 was accomplished. By using the T7 endonuclease assay, restriction enzyme digestion assay, and sequencing, the efficacy of mutagenesis in wheat protoplast was verified. According to the results, the CRISPR/Cas9 genome editing system is simple to set up on wheat protoplasts and offers a great deal of potential for precisely modifying the wheat genome for agricultural enhancement.

Li *et al.* (2018a) used a bidirectional strategy to promote the biosynthesis of lycopene, while inhibiting the conversion from lycopene to β- and α-carotene. The accumulation of lycopene was promoted by knocking down genes associated with the carotenoid metabolic pathway. For their study they have selected five genes for editing in genome by CRISPR/Cas9 system using *Agrobacterium tumefaciens* mediated transformation. The lycopene content in tomato fruit subjected to genome editing was successfully increased to about 5.1-fold and it was reported that the homozygous mutations were stably transmitted to subsequent generations.

Tomlinson *et al.* (2018) studied the single guide RNAs mediated targeted mutations to the one of domain of PROCERA DELLA proteins which are nuclear localized, negative growth regulators. The tomato PROCERA gene encodes a DELLA protein and loss-of-function mutations derepress growth. Mutations in the PROCERA DELLA domain resulted in several loss-of-function mutations and a dominant dwarf mutation that carries a deletion of one amino acid in the DELLA domain. The mutated allele retains partial responsiveness to exogenously applied gibberellin. Heterozygotes show an intermediate phenotype at the seedling stage, but adult heterozygotes are as dwarfed as homozygotes.

2.7 Genetic improvement of groundnut through transgenesis

Illingsworth (1968) used de-embryonated cotyledons to effectively establish a groundnut *in vitro* regeneration system for the first time. Transgenic legume development was initially challenging since the cells of these plants are resistant to transformation mediated by *A. tumefaciens* (Lacorte *et al.,* 1997). Lacorte et al. (1991) investigated the sensitivity of the cells of several cultivars of groundnut to wild type strains of A. tumefaciens. There were no reports of regenerated transgenic plants, and they could only grow tumors from co-cultivated explants. Mansur *et al*. (1993) investigated the variables influencing groundnut genetic transformation.

To produce a few transformed lines (1%) that produced transgenic plants at low frequencies, including chimeric or transformants resulting from a few transformation events, the biolistic gene delivery method for transforming embryogenic calli and embryo axes is labor intensive, requires an optimized protocol, and involves bombarding a large number of explants (Ozias-Akins *et al.,* 1993). Three fundamental

requirements must be met for effective genetic transformation in groundnuts: an appropriate regeneration system, particularly the kind of explants and their capacity to regenerate; an effective gene transfer technique; and a gene construct that can provide transgene expression and stable transformation (Livinstone and Birch, 1995).

According to earlier reports, only 0.2 to 9% of transgenic plants were produced (Eapen and George, 1994; Cheng *et al.,* 1996; Fang *et al.,* 1996). This was primarily because it was difficult to transform groundnut cells frequently, produce shoots from transformed cells, and get transgenic plantlets to root. The identification of competent cells in various explant types was aided by anatomical analysis of the explants co-cultured with *A. tumefaciens*. It was discovered that, in leaf explants, both vascular and parenchymatic cells underwent transformation and direct organogenesis via callus, whereas only parenchymatic cells adjacent to the inoculation site underwent transformation in cotyledons. In light of these experiments and findings in groundnut, according to Freitas *et al*. (1997), various cell types also affected regeneration efficiency and susceptibility to *Agrobacterium* infection, in addition to the kind of explant. According to reports, *Agrobacterium* successfully transformed groundnuts utilizing hypocotyl, cotyledon, and cotyledonary node explants. Venkatachalam *et al*. (1998) investigated the variables influencing the regeneration of the transgenic plant. They noted that pre-cultivating the explants before co-cultivation is one of the crucial elements that contribute to the high transformation rates in groundnuts. Numerous bacterial and plant-related parameters, including virulence, age, physiological state, co-cultivation conditions, transforming vector, bacterial strain-genotype compatibility, and explant type, affect the effectiveness of transformation (Freitas *et al.,* 1997). When compared to co-cultivation with Agrobacterium without pre-culturing, pre-culturing decreases the hypersensitive reaction. For effective transformation, it was determined that the explants should be pre-cultured in the regeneration medium for two days (d) before co-cultivation.

Using cotyledon explants through somatic embryogenesis and direct organogenesis, transgenic groundnuts have also been created (Venkatachalam *et al.,* 2000; Sharma and Anjaiah, 2000). In a few instances, only transgenic calli were created because of the low peanut cell regeneration efficiency. Up till now, many explant forms have been utilized to create transgenic plants with varying concentrations and

combinations of plant growth regulators. Regeneration media containing silver nitrate ($AgNO_3$) has been utilized to guide the development of altered somatic embryos into whole plants (Ozias-Akins *et al.,* 1993).

To create a viable methodology that could be rapid and independent of both tissue culture and cultivar, transgenic groundnuts were created utilizing a non-tissue culture- based approach (McKently *et al.,* 1995; Rohini and Rao, 2000). The labor-intensive nature of these techniques, the need for vir gene induction treatments (e.g., tobacco leaf extracts to increase the transformation efficiency of *Agrobacterium*), and the number of treatments needed to produce a relatively small number of independent transformants limit their applicability.

From split cotyledon explants of mature seeds, Sharma and Anjaiah (2000) obtained over 90% regenerated adventitious shoot buds and a significant percentage (55%) of transgenic plants. They came to the conclusion that this system could be an excellent mean for producing a large number of transgenic groundnuts in a relatively short amount of time.

Several researchers conducted several investigations to define an appropriate technique and assess the transformation efficiency of groundnuts utilizing just reporter and/or selectable marker genes. Acetosyringone can be utilized to stimulate the expression of *vir* genes in co-cultivation media (Tiwari *et al.,* 2008).

The development of transgenic groundnuts using seedlings and an in-planta transformation process opened the door for the investigation of non-tissue culture-based transgenic research methods (Sundaresha *et al.,* 2010). *Agrobacterium rhizogenes*-mediated genetic transformation was used by Akasaka *et al.* (1998) to integrate Ri T-DNA into the genome of transgenic hairy root cells. Transgenic hairy roots have formed root nodules. Lacorte et al. created a transgenic groundnut with enhanced nutritional value (1997). Most of the aforementioned research was done to create an appropriate technique for the high frequency of *Agrobacterium*-mediated genetic transformation in groundnuts. To introduce a foreign gene or genes into groundnuts, direct gene transfer techniques including electroporation and biolistic gene guns have also been employed. Additionally, certain transgenic groundnuts with enhanced agronomic characteristics have been created in India and other countries (Table; Figure 2.2).

Table 2.1: Recommended Dietary Allowance of Vitamin A (ICMR, 2010)

Group	Age/Category	Retinol (RE)	β-carotene (µg/day)
Adult Man/Woman		600	4800
Pregnant Woman		800	6400
Lactating Mother		950	7600
Infants	0-6 month	350	-
	6-12 months		2100
Children	1-6 years	400	3200
	7-9 years	600	4800
Teenagers	10-17 years	600	4800

Table 2.2: List of National and International Studied on genetic transformation of groundnuts.

Location	Total Reports	For Development of Transformation Protocol	For Conferring Economically Important Character
Within India	19	6	13
Outside India	34	17	17
Total	**53**	**23**	**30**

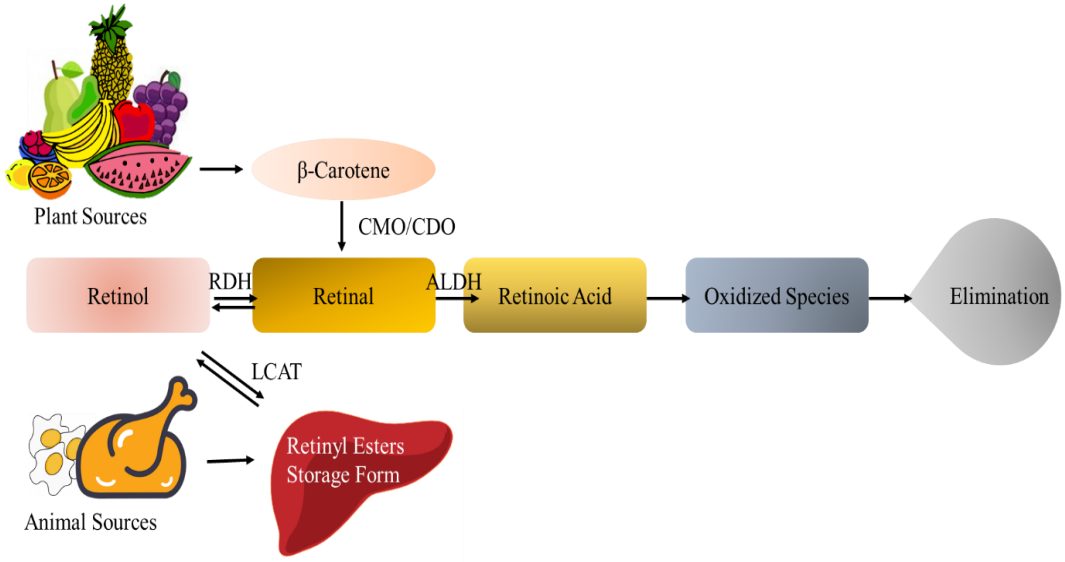

Figure 2.1: Representation of steps/enzymes involved in the formation of different forms of vitamin A in the body. CMO/CDO: β-Carotene 15-15'-monooxygenase/dioxygenase; RDH: retinol dehydrogenase; LCAT: lecithin-retinol acyltransferase; ALDH: aldehyde dehydrogenase.

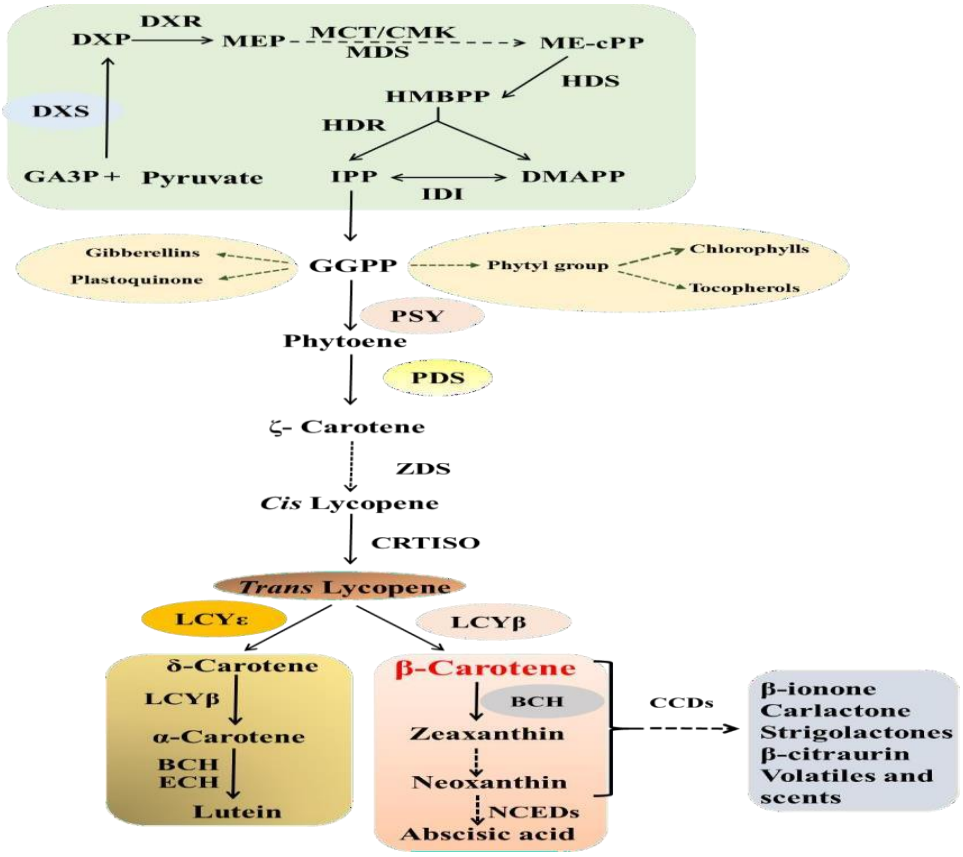

Figure: 2.2 Carotenoid biosynthesis pathway. Methylerythritol 4-phosphate (MEP) and carotenoid biosynthesis pathway. Enzymatic reactions are represented by arrows. Enzymes/products: GA3P, D-glyceraldehyde 3-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXP, 1-deoxy-D-xylulose 5-phosphate; ME-cPP, 2-*C*-methyl-D- erythritol 2,4-cyclodiphosphate; HMBPP,(E)-4-Hydroxy-3-methyl-but-2-enylpyrophosphate, DXP reduction-isomerase; MEP, methylerythritol phosphate; MCT, MEP cytidylyl transferase; CMK, CDPME kinase; MDS, ME- cPP synthase; HDS, HMBPP synthase; HDR, HMBPP reductase; IPP, isopentenyl diphosphate; IDI, IPP isomerase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYβ, lycopene β- cyclase; LCYε, lycopene ε-cyclase; CRTISO, carotenoid isomerase; BCH, β-ring hydroxylase, ECH, ε-ring hydroxylase; CCD, carotenoid cleavage dioxygenase. NCED,9-*cis*epoxy carotenoid dioxygenase.